INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is characterized by a progressive degeneration of motor neurons. Familial ALS accounts for 10% of all cases and about 20% of the familial cases, mutations of the superoxide dismutase 1 (SOD1) gene have been identified (1). Transgenic mice overexpressing human mutant SOD1<sup>G93A</sup> develop a progressive motor neuron disorder and provide a good model for familial ALS (2).

Glutamate-induced, AMPA receptor-mediated excitotoxicity contributes to the selective motor neuron degeneration in ALS. AMPA receptor antagonists prolong survival of ALS mice (3, 4) and motor neurons are known to be particularly vulnerable to Ca<sup>2+</sup> influx through AMPA receptors (5–8). The Ca<sup>2+</sup> permeability of the AMPA receptor is determined by the presence of the GluR2 subunit in the receptor complex. Receptors containing at least one GluR2 subunit have a very low relative Ca<sup>2+</sup> permeability compared to GluR2-lacking receptor channels (9). The low permeability to Ca<sup>2+</sup> is attributable to the presence of a positively charged arginine at position 586 (Q/R site) instead of the genetically encoded neutral glutamine. This arginine residue at the Q/R site is introduced by editing of the GluR2 pre-mRNA (10). The editing efficiency at the Q/R site is virtually 100% under normal conditions (11, 12), but incomplete editing has been reported to occur in motor neurons of ALS patients (13). Apart from the Ca<sup>2+</sup> permeability, other functional properties of AMPA receptors (e.g., sensitivity to channel block by external polyamines and current rectification) also depend on the presence of GluR2. GluR2-lacking channels are easily blocked by external polyamines (14, 15) and display a strong inward rectification (9, 16, 17).

As Ca<sup>2+</sup> influx through AMPA receptors underlies the selective vulnerability of cultured motor neurons, these cells are expected to have a low GluR2 expression. Although this was observed in a number of studies (18–22), it was contradicted by other reports (11, 12, 23–26). Two recent studies using a laser microdissection technique to isolate motor neurons from human spinal cords suggest that human motor neurons indeed have a constitutively low GluR2 expression (27, 28). We previously showed that low GluR2 expression determines the selective vulnerability of cultured motor neurons to excessive AMPA receptor stimulation (29), but the relevance of low GluR2 expression for motor neuron degeneration in vivo remains unknown. GluR2 knockout mice have been generated, but they were reported to have normal brain morphology and no overt motor neuron degeneration (30). This suggests that a low GluR2 level itself is not sufficient to cause ALS, but rather is a modifier of motor neuron degeneration in ALS. Therefore, we studied the impact of GluR2 deficiency on AMPA receptor-mediated motor neuron death and on the motor neuron degeneration in mutant SOD1<sup>G93A</sup> mice.
MATERIALS AND METHODS

Motor Neuron Cultures

Motor neurons were cultured as previously described (6, 7), with minor modifications. Ventral spinal cords were dissected from 13-day-old mouse embryos obtained by mating of a GluR2+/− male and female and dissociated. Genotype of embryos was established by PCR (31). A motor neuron-enriched neuronal population was purified from the ventral spinal cord by centrifugation on a 6.3% Optiprep (Axis-Shield, Oslo, Norway) cushion and was cultured on a pre-established glial feeder layer of the same genotype. The culture medium consisted of L15 supplemented with sodium bicarbonate (0.2%), glucose (3.6 mg/ml), progesterone (20 nM), insulin (5 μg/ml), putrescine (0.1 mM), conalbumin (0.1 mg/ml), sodium selenite (30 nM), penicillin (100 IU/ml), streptomycin (100 μg/ml), chick embryo extract (5%), and horse serum (2%). As previously described, more than 80% of the cells in culture are motor neurons as shown by immunostaining with the motor neuron marker peripherin (32). Cultures were kept in a 7% CO2 humidified incubator at 37°C. Neurons were used for experiments after 1 week in culture.

Electrophysiology

The gramicidin perforated patch clamp technique was used for electrophysiological recordings, as previously described (6). Pipettes were back-filled with pipette solution containing 50 to 75 μg/ml gramicidin, after tip-filling with gramicidin-free solution. Gramicidin was freshly dissolved in DMSO (50 μg/μl) for each experiment. Pipettes had a resistance of 2–4 MΩ when filled with intracellular solution. After seal formation, the progress of perforation was followed by evaluating the decrease in series resistance. Cells were accepted for study if series resistance (R_s) dropped below a resistance of 2–4 MΩ and remained stable during the experiment. Cells were held at a membrane potential of –60 mV and I-V relationships were generated using voltage ramps from –100 mV to +50 mV. AMPA receptor currents were elicited by 100 μM kainate (KA). Signals were recorded using a L/M-EP/C7 List-Medical amplifier, filtered at 3 kHz, sampled at 2 kHz and analyzed offline (Digidata 1200, pClamp8, Axon Instruments, Union City, CA). The normal pipette solution consisted of 125 mM CsCl, 1.2 mM MgCl₂, 10 mM Hepes, 2 mM Na₂ATP, and 1 mM EGTA, pH adjusted to 7.3 with CsOH. The standard extracellular solution contained 110 mM NaCl, 30 mM TEACl, 5.9 mM KCl, 3.2 mM CaCl₂, 1.2 mM MgCl₂, 11.6 mM Heps, 11.5 mM glucose, pH 7.3 with NaOH. TEACl was added to this solution to avoid KA-induced inhibition of voltage-gated K⁺ channels (32). The Na⁺-rich extracellular solution contained 105 mM NaCl, 30 mM TEACl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 15.8 mM glucose, pH adjusted to 7.3 with NaOH. The Ca²⁺-rich extracellular solution consisted of 30 mM CaCl₂, 30 mM TEACl, 75 mM N-methyl-D-glucamine, 5 mM HEPES, 5 mM glucose, pH adjusted to 7.3 with HCl. All experiments were carried out in the presence of 500 nM tetrodotoxin (TTX), 10 μM MK-801, and 100 μM Cd²⁺ to block voltage-gated Na⁺ channels, NMDA receptors, and voltage-operated Ca²⁺ channels, respectively. 1-naphthyl acetyl spermine (NAS) was applied as a selective antagonist of GluR2-lacking AMPA receptors (33). To estimate GluR2 expression at the functional level, 3 GluR2-dependent properties of AMPA receptor currents were determined. The Ca²⁺ permeability ratios, P_Ca/P Na, were determined from the reversal potentials obtained in the Na⁺-rich (VrevNa) and the Ca²⁺-rich solution (VrevCa), according to the equation (34): P_Ca/P Na = 0.25 a Na/a Ca [exp(2VrevCa – VrevNa)/R] + [exp[(VrevCa – VrevNa) F/R T]] where a Na and a Ca are the ion activities of Na⁺ and Ca²⁺ in the extracellular solution and F, R, T have their conventional meaning. Activity coefficients were estimated by interpolation of tabulated values (0.75 for NaCl, 0.55 for CaCl₂). NAS-sensitivity was determined as the percentage of AMPA receptor current inhibition induced by 100 μM NAS at –60 mV. The rectification of AMPA receptor currents was quantified using the following expression (35): Rectification index = [Isto/(40-Erev)]/[Isto/(-60-Erev)].

For measurements of AMPA receptor current amplitudes, whole cell recordings were performed using the same pipette solution without gramicidin and the current amplitude elicited by 20 μM KA at -60 mV was normalized to the membrane capacitance (current density).

Ca²⁺ Measurements

Motor neurons cultured on chambered coverglasses were loaded with the low-affinity Ca²⁺ indicator Indo-1FF (AM, 5 μM) in the presence of 0.08% pluronic acid. Ca²⁺ changes were monitored using a laser scanning Bio-Rad MRC-1024 confocal system built around an inverted microscope (Bio-Rad, Hercules, CA). The source of UV light was an Enterprise Coherent laser (Laser Innovations, Suffolk, UK). Excitation wavelength was 361 nm and emitted fluorescence at 405 and 460 nm was collected every 0.8 seconds. Signals were obtained from rectangular areas covering the soma and proximal neurites of motor neurons. Cells were continuously superfused with the same Krebs solution as used for cell death experiments. All Ca²⁺ measurements were performed in the presence of the NMDA receptor antagonist MK-801 (10 μM) and the inhibitor of voltage-gated Ca²⁺ channels, verapamil (100 μM). [Ca²⁺], was determined by the equation: [Ca²⁺] = K_D(R – R_min)/(R_max – R), R being the observed 405/460 fluorescence ratio. The K_D was used was 21 μM (36). Using the low affinity dye Indo-1FF there was no difference between R determined in cells bathing in a Ca²⁺-free solution or in the normal extracellular solution. Therefore, R determined at the beginning of each experiment in the normal extracellular solution was used as R_min, R_max was determined at the end of each experiment during exposure to 5 μM ionomycin.

Toxicity Experiments

Motor neuron cultures on day 8 in culture were exposed to AMPA receptor agonists for 30 minutes at 37°C in a modified Krebs solution (in mM: 122.3 NaCl, 5.9 KCl, 10 CaCl₂, 1.2 MgCl₂, 11.6 Heps, and 11.5 glucose, pH 7.3 with NaOH). MK-801 (10 μM) was added during all exposures. The survival of motor neurons was quantified by counting motor neurons (with neurites more than 2 times the soma diameter, approximately 200–300 per dish) in a marked...
area of 1 cm² under phase contrast before and 24 hours after the exposure, with the observer blinded to the treatment protocol. For counting experiments, “n” refers to the number of cultures assessed.

**Breeding and Evaluation of Mice**

GluR2+/− mutant SOD1G93A males were mated with GluR2+/- females to obtain double transgenic mice. TgN(SOD1-G93A)G1H mice and heterozygous GluR2 knockout in C57/Bl6J were obtained from The Jackson Laboratories (Bar Harbor, ME). Mutant SOD1G93A mice were crossed to C57/Bl6J for more than 10 generations prior to the onset of crossbreeding with GluR2 knockout mice. This was done to eliminate variability caused by mixture of different backgrounds. The genotype was established by PCR (31) and littermates were used for further analyses. The grip strength of the forelimbs was measured using a Grip Strength Meter (Columbus Instruments, Columbus, OH). The maximal force (in g) of 6 attempts was averaged, and normalized to the body weight. A rotarod (SciPro, Inc., North Tonawanda, NY) rotating at 15 rpm was used to evaluate motor performance. Each mouse was given 5 trials of maximally 180 seconds and the average time on the rotarod was used as a measure for the motor performance. For measurements of spontaneous over-night activity, mice were placed in activity cages with free access to a treadmill (37). Disease onset was defined as the onset of clinical signs in hind limbs (tremor and paresis). The maximal strength of the forelimbs was measured using a Grip Strength Meter (Columbus Instruments, Columbus, OH). The maximal force (in g) of 6 attempts was averaged, and normalized to the body weight. A rotarod (SciPro, Inc., North Tonawanda, NY) rotating at 15 rpm was used to evaluate motor performance. Each mouse was given 5 trials of maximally 180 seconds and the average time on the rotarod was used as a measure for the motor performance. For measurements of spontaneous overnight activity, mice were placed in activity cages with free access to a treadmill (37). Disease onset was defined as the onset of clinical signs in hind limbs (tremor and paresis). When mice could no longer roll over within 10 seconds after being pushed on their back, they were killed and this time point was considered as the time of death.

**Histological Analysis of Spinal Cords**

Double transgenic mice of 115 days were killed with CO₂ immediately followed by transcardiac perfusion with PBS and PBS with 4% paraformaldehyde. Spinal cords were dissected, fixed in 4% paraformaldehyde, and embedded in paraffin. Sections of 7-µm thickness were made of the lumbar spinal cord, deparaffinized, and stained with H&E. In electronic pictures of every tenth slide at 20× magnification, the area of normal appearing neurons with nucleoli in the ventral horn was calculated using Lucia Image (version 4.60, Laboratory Imaging, Prague, Czech Republic) and the number of neurons in different size groups was determined.

**Materials and Statistics**

Media and additives were obtained from Gibco BRL (Grand Island, NY); TTX was from Calbiochem (San Diego, CA); MK-801 was from Tocris Cookson (Bristol, UK); Indo-1FF AM was from Tef Labs (Austin, TX); and gramicidin was from Fluka (Seelse, Germany). All other chemicals were from Sigma (St. Louis, MO). Student t-tests were used to calculate significance. If more than 2 groups were compared, an ANOVA with Bonferroni correction was used. For the GluR2-dependent properties a multiple way ANOVA was used. A logrank test was used to analyze the disease onset and survival data.

**RESULTS**

**GluR2 Deficiency Aggravates AMPA Receptor-Mediated Excitotoxicity in Cultured Motor Neurons**

To study the effects of GluR2 deficiency on AMPA receptors present in motor neurons we measured 3 properties that depend on the relative abundance of GluR2 in AMPA receptors and AMPA receptor current densities. As expected, the AMPA receptor currents in GluR2+/- neurons displayed a high degree of inhibition by the external polyanine 1-naphthyl acetyl spermine (NAS), a strong inward rectification and a high relative Ca²⁺ permeability (P_Ca/P_Na, Fig. 1A–G). The relative Ca²⁺ permeability, rectification index, and sensitivity to NAS of AMPA receptors correlated with GluR2 gene levels (Fig. 1E–G). To estimate the number of AMPA receptors present in the cell membrane, the AMPA receptor current density at −60 mV was measured. The AMPA receptor current density did not vary with GluR2 expression levels (Fig. 1H), suggesting that GluR2 deficiency did not significantly alter the number of AMPA receptors in the cell membrane.

In order to evaluate the effects of GluR2 deficiency on intracellular Ca²⁺ levels during AMPA receptor stimulation, Ca²⁺ measurements with the low affinity dye Indo-1FF were performed. GluR2-deficient motor neurons had higher elevations of the intracellular Ca²⁺ concentration upon AMPA receptor stimulation (Fig. 2A). Likewise, the vulnerability to AMPA receptor-mediated excitotoxicity increased with decreasing GluR2 gene levels (Fig. 2B). The AMPA receptor-mediated cell death in GluR2-deficient motor neurons was abolished by NAS (Fig. 2B), confirming the importance of Ca²⁺ influx through AMPA receptors for this type of motor neuron death.

**GluR2 Deficiency Accelerates Motor Neuron Degeneration in Mutant SOD1G93A Mice**

Having established that cultured GluR2-deficient motor neurons are more prone to AMPA receptor-mediated excitotoxicity, we delineated the role of GluR2 in an in vivo model of ALS. For this purpose, we deleted GluR2 subunit expression in mutant SOD1G93A mice by crossbreeding GluR2-deficient mice with mutant SOD1G93A mice.

It was previously reported that single transgenic GluR2−/− display no motor neuron phenotype and have normal brain morphology, but have some behavioral abnormalities (such as decreased object exploration, rearing, grooming, inability to walk on rotating rod, decreased eye closure reflex, and impaired learning), and that GluR2−/− pups suffer from postnatal developed growth retardation (30, 38). In the original study, no apparent change in lifespan was observed up to 12 months of age (30).

We also found that GluR2−/− mice failed to walk on a rotarod (Fig. 3A). In addition, measurements of grip strength in fore limbs were slightly lower in GluR2−/− mice (Fig. 3B), most likely due to a reduced ability of GluR2−/− to grip the bar, rather than a reduction in muscle force. During handling, GluR2−/− mice had abnormal posturing of both forelimbs and hind limbs (Fig. 3C), which slowly disappeared when they were put back on a flat surface. During clinical...
testing, GluR2−/− mice clearly had a reduced object exploration, but when left alone in their cage they had a normal locomotion. However, the spontaneous activity during the night, measured in activity cages, was clearly reduced in GluR2−/− mice (Fig. 3D). These behavioral abnormalities (decreased object exploration, abnormal posturing of limbs during manipulations, inability to walk on a rotarod, and decreased spontaneous activity during the night) were present from early ages on and not progressive. Histologic analysis of the lumbar spinal cord at 6 months of age was performed in search for motor neuron degeneration in GluR2−/− mice (Fig. 3E). Quantification of the number of neurons in the ventral horn did not reveal any loss of motor neurons in GluR2−/− mice (Fig. 3F).

Double transgenic GluR2−/− mutant SOD1G93A mice had identical behavioral abnormalities from early ages on and did not learn to walk on a rotarod (Fig. 4D). Double transgenic GluR2−/− mutant SOD1G93A motor neurons had an identical high relative Ca2+ permeability (3.13 ± 0.45, n = 5) and the editing efficiency of GluR2 in double transgenic GluR2−/− mutant SOD1G93A animals remained virtually 100% (data not shown). The onset of mutant SOD1-induced motor neuron disease in double transgenic animals (defined as the onset of

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**FIGURE 1.** Electrophysiological properties of GluR2-deficient motor neurons. (A–D) AMPA receptor currents induced by 100 μM kainate (KA). I-V relation of AMPA receptor current in the absence and presence of 100 μM 1-naphthyl acetyl spermine (NAS) in a GluR2+/+ (A) and GluR2−/− neuron (B). I-V relation of AMPA receptor current in the Na+ rich and Ca2+ rich solution in a GluR2+/+ (C) GluR2−/− neuron (D). (E–G) GluR2-dependent properties of AMPA receptors in GluR2-deficient motor neurons. Average Pca/Pna ratios (E), NAS sensitivity (F), and rectification index (G) in GluR2+/+, GluR2−/−, and GluR2−/− neurons (n = 5–10 per group, p < 0.001 with multiple way ANOVA, significant difference between 3 groups: p = 0.02 for GluR2+/+ vs. GluR2−/− and p < 0.001 for other comparisons). (H) Density of AMPA receptor currents (20 μM KA at –60 mV) in GluR2-deficient motor neurons (n = 10 per group, p = 0.60).
SOD1G93A mice, respectively, which is a 15.1% reduction in 2q2005 American Association of Neuropathologists, Inc.
motor neurons (n = 3–6 per group). GluR2 expression in different neuronal cell types. On the other hand, confirms that these properties similarly depend on GluR2 (39). This, together with an extremely high NAS-sensitivity, cultured CA1 neurons (30) and cortical pyramidal neurons rectification, in agreement with previous observations in cultured motor neurons is one of the factors underlying the selective motor neuron death and to clarify the role of GluR2 deficiency in mutant SOD1G93A mice. GluR2 deficiency proved to be reduced (13, 43). GluR2 subunits translated from unedited GluR2 mRNA do not reduce the Ca2+ permeability of the AMPA receptor. This suggests that motor neurons from ALS patients in the final stages of the disease contain AMPA receptors with a higher relative Ca2+ influx accompanied by an increased vulnerability to AMPA receptor-mediated excitotoxicity. GluR2-deficient motor neurons could still be rescued from excitotoxic cell death by NAS, a selective antagonist of Ca2+-permeable AMPA receptors. These data confirm that the motor neuron vulnerability is entirely dependent on Ca2+ influx through AMPA receptors in this model, which is in its turn determined by the relative abundance of the GluR2 subunit. For cortical neurons this inverse correlation between GluR2 expression levels and neuronal vulnerability was not observed (39), suggesting that Ca2+ influx through AMPA receptors is not equally toxic to different neuronal cell types. This difference highlights the particular sensitivity of motor neurons to Ca2+ influx through AMPA receptors. Apart from low GluR2 levels, a low Ca2+ buffering capacity (36, 41) and Ca2+ -induced mitochondrial dysfunction (42) are thought to underlie the selective vulnerability of motor neurons.

Whether Ca2+ influx through AMPA receptors and low GluR2 expression play pivotal roles in the motor neuron degeneration in vivo remains unknown. However, several observations support the importance of AMPA receptors and GluR2 in the pathogenesis of ALS. In autopsy material from the ventral spinal cord of ALS patients, the editing efficiency of GluR2 at the Q/R site proved to be reduced (13, 43). GluR2 subunits translated from unedited GluR2 mRNA do not reduce the Ca2+ permeability of the AMPA receptor. This suggests that motor neurons from ALS patients in the final stages of the disease contain AMPA receptors with a higher relative Ca2+ permeability compared to AMPA receptors from normal individuals, but whether this is pathogenetically relevant is unknown. Mice overexpressing GluR2 with an asparagine (GluR2-N) at the Q/R site were shown to develop a motor paresis in the hind limbs by clinical testing) was significantly earlier in GluR2−/− mutant SOD1G93A mice (99.3 ± 2.1 days, 113.0 ± 2.1 days, and 117.3 ± 1.8 days, for GluR2−/−, GluR2+/−, and GluR2+/+ mutant SOD1G93A mice respectively, Figure 4A). This is a reduction of 15.4% of the time without symptoms. The grip strength in the forelimbs decreased more rapidly over time in GluR2−/− mutant SOD1G93A mice (Fig. 4C) and GluR2−/− mutant SOD1G93A mice had a clearly shortened lifespan (Fig. 4B). The average survival was 116.5 ± 0.6, 132.6 ± 2.8, and 137.1 ± 2.6 days for GluR2−/−, GluR2+/−, and GluR2+/+ mutant SOD1G93A mice, respectively, which is a 15.1% reduction in lifespan of GluR2−/− mutant SOD1G93A mice compared to GluR2+/+ mutant SOD1G93A mice.

To assess the extent of motor neuron degeneration, a histologic examination of the lumbar spinal cord was performed. In sections of the lumbar spinal cord, the number of preserved neurons in the ventral horn was determined. At 115 days, a much more pronounced degeneration of large neurons in the ventral horn from GluR2−/− mutant SOD1G93A mice was noted (Fig. 4E, F), whereas small neurons were preserved.

DISCUSSION

Excessive stimulation of AMPA receptors contributes to the selective motor neuron degeneration in ALS (3, 4) and motor neurons are known to be selectively vulnerable to AMPA receptor stimulation (5, 7, 8). A low expression of GluR2 in motor neurons is one of the factors underlying the selective vulnerability of motor neurons to AMPA receptor stimulation (29). In this study, we used GluR2-deficient mice to study the importance of GluR2 in AMPA receptor-mediated motor neuron death and to clarify the role of GluR2 in an in vivo model of ALS (mutant SOD1G93A mice). GluR2 deficiency aggravated Ca2+ influx and AMPA receptor-mediated toxicity in cultured motor neurons and accelerated motor neuron degeneration in mutant SOD1G93A mice.

AMPA receptors in GluR2-deficient motor neurons displayed a high relative Ca2+ permeability and strong inward rectification, in agreement with previous observations in cultured CA1 neurons (30) and cortical pyramidal neurons (39). This, together with an extremely high NAS-sensitivity, confirms that these properties similarly depend on GluR2 expression in different neuronal cell types. On the other hand, the AMPA receptor current density, which reflects the number of functional AMPA receptors present, appears to be regulated differently in different cell types. In cortical neurons, GluR2-deficiency resulted in larger AMPA receptor currents (39), whereas in hippocampal neurons smaller currents were observed (30), possibly due to a reduced efficiency of GluR2-lacking AMPA receptors to reach the plasma membrane (40). In motor neurons, AMPA receptor current density remained constant, suggesting that GluR2 deficiency did not dramatically affect the total number of AMPA receptors.

The elevation of intracellular Ca2+ concentration during AMPA receptor stimulation was estimated in Ca2+ measurements. GluR2−/− neurons had a higher amount of Ca2+ influx accompanied by an increased vulnerability to AMPA receptor-mediated excitotoxicity. GluR2-deficient motor neurons could still be rescued from excitotoxic cell death by NAS, a selective antagonist of Ca2+-permeable AMPA receptors. These data confirm that the motor neuron vulnerability is entirely dependent on Ca2+ influx through AMPA receptors in this model, which is in its turn determined by the relative abundance of the GluR2 subunit. For cortical neurons this inverse correlation between GluR2 expression levels and neuronal vulnerability was not observed (39), suggesting that Ca2+ influx through AMPA receptors is not equally toxic to different neuronal cell types. This difference highlights the particular sensitivity of motor neurons to Ca2+ influx through AMPA receptors. Apart from low GluR2 levels, a low Ca2+ buffering capacity (36, 41) and Ca2+ -induced mitochondrial dysfunction (42) are thought to underlie the selective vulnerability of motor neurons.

Whether Ca2+ influx through AMPA receptors and low GluR2 expression play pivotal roles in the motor neuron degeneration in vivo remains unknown. However, several observations support the importance of AMPA receptors and GluR2 in the pathogenesis of ALS. In autopsy material from the ventral spinal cord of ALS patients, the editing efficiency of GluR2 at the Q/R site proved to be reduced (13, 43). GluR2 subunits translated from unedited GluR2 mRNA do not reduce the Ca2+ permeability of the AMPA receptor. This suggests that motor neurons from ALS patients in the final stages of the disease contain AMPA receptors with a higher relative Ca2+ permeability compared to AMPA receptors from normal individuals, but whether this is pathogenetically relevant is unknown. Mice overexpressing GluR2 with an asparagine (GluR2-N) at the Q/R site were shown to develop a motor...
neuron syndrome (44, 45). GluR2-N protein expression exceeded the wild-type GluR2 expression in these mice, and there was 2-fold increase in the Ca\(^{2+}\) permeability of AMPA receptors. The fact that these mice suffered from motor neuron degeneration indicates that overexpression of AMPA receptors with an increased relative Ca\(^{2+}\) permeability is sufficient to cause motor neuron loss. By contrast, GluR2-deficient mice, in which all AMPA receptors display a high Ca\(^{2+}\) permeability, do not develop a motor neuron syndrome (30). However, in these mice, no overexpression of Ca\(^{2+}\)-permeable AMPA receptors is obtained. Several factors can account for the lack of motor neuron pathology in these mice: reduced efficacy of AMPA receptor assembly and surface expression in the absence of GluR2, differences in gating kinetics and single channel conductance, and differences in the localization of AMPA receptors and their ability to interact with intracellular proteins. Furthermore, recovery from desensitization was significantly slower in GluR2\(^{-/-}\) cortical pyramidal neurons (46). These adaptations can limit the total Ca\(^{2+}\) influx during physiological AMPA receptor stimulation and protect neurons from GluR2\(^{-/-}\) mice. In addition to the previously reported minor phenotype of GluR2\(^{-/-}\) mice (reduced object exploration, reduced self-directed behavior, and inability to walk on a rotarod) (30, 38), we observed a stress-induced abnormal posturing of limbs which interfered with grip strength testing. These symptoms were not progressive and we found no motor neuron loss in the spinal cord. The origin of these abnormalities remains unknown.

In order to clarify the role of GluR2 in motor neuron degeneration, we crossbred GluR2-deficient mice with mutant

**FIGURE 3.** Phenotype of single transgenic GluR2-deficient mice. (A) Average time on rotaord of GluR2\(^{+/+}\), GluR2\(^{+/−}\), and GluR2\(^{-/-}\) mice (n = 4–7 per group). (B) Grip strength of fore limbs normalized to body weight of GluR2\(^{+/+}\), GluR2\(^{+/−}\), and GluR2\(^{-/-}\) mice (n = 4–7 per group). (C) Abnormal posturing of limbs in GluR2\(^{-/-}\) mouse when lifted by the tail. (D) Total distance walked in treadmill overnight in GluR2\(^{+/+}\), GluR2\(^{+/−}\), and GluR2\(^{-/-}\) mice (n = 8–9 per group, p = 0.018, *, significantly different from GluR2\(^{+/+}\), p < 0.05). (E, F) Histological analysis of lumbar spinal cords in GluR2\(^{+/+}\), GluR2\(^{+/−}\), and GluR2\(^{-/-}\) mice at 6 months of age. Hematoxylin and eosin staining of the ventral horn from spinal cord section of GluR2\(^{+/+}\) and GluR2\(^{-/-}\) ([E], scale bar = 20 \(\mu\)m), and histogram of the number of neurons of different size in the ventral horn ([F], n = 3 per group, p > 0.50).
SOD1G93A mice. The increase in Ca$^{2+}$ permeability observed in GluR2+/- motor neurons appeared not to be sufficient to worsen the disease course of mutant SOD1G93A mice. A possible explanation for this lack of dose-response effect on survival of mutant SOD1G93A mice is the nonlinear relationship between relative GluR2 expression and relative Ca$^{2+}$ permeability (11, 34). This may also explain why heterozygous (GluR2+/-) neurons often behaved not significantly different from GluR2+/+ neurons, but clearly different from GluR2--/- neurons. Therefore, it is possible that the increase in Ca$^{2+}$ permeability of AMPA receptors in GluR2+/-- mice is insufficient to worsen the motor neuron disease. In GluR2--/- mutant SOD1G93A mice however, the onset of clinical symptoms occurred 18 days earlier (15.4%) and survival was shortened by 20.6 days (15.1%). This suggests that GluR2 deficiency mainly affects disease onset. The shortened survival was accompanied by a much more pronounced degree of motor neuron degeneration in the lumbar spinal cord. This is the largest negative effect on survival of ALS mice reported to our knowledge, indicating that GluR2 deficiency contributes significantly to motor neuron degeneration in vivo. This idea is in agreement with a very recent study in which crossbreeding of GluR2 overexpressing mice with mutant SOD1G93A mice resulted in a 14.3% increase of survival (47).

FIGURE 4. GluR2 deficiency accelerates motor neuron degeneration in mutant SOD1G93A mice. (A) Probability of disease onset in GluR2+/+, GluR2+/-, and GluR2--/- mutant SOD1G93A mice (n = 4–16 per group, p = 0.003, GluR2+/+ significantly different from GluR2--/-, p < 0.0001, GluR2+/+ not significantly different from GluR2+/-). (B) Probability of survival in GluR2+/+, GluR2+/-, and GluR2--/ mutant SOD1G93A mice (n = 4–16 per group, p < 0.0001, GluR2+/+ significantly different from GluR2--/-, p < 0.0001, GluR2+/+ not significantly different from GluR2+/-). (C) Grip strength of forelimbs normalized to body weight (in g/g) of GluR2+/+, GluR2+/-, and GluR2--/ mutant SOD1G93A mice (n = 4–16 per group, p = 0.018). (D) Average time GluR2+/+, GluR2+/-, and GluR2--/ mutant SOD1G93A mice (n = 4–16 per group) were able to stay on a rotarod. (E, F) Histologic analysis of lumbar spinal cords in GluR2+/+, GluR2+/-, and GluR2--/ mutant SOD1G93A mice. Hematoxylin and eosin staining of the ventral horn from spinal cord section of GluR2+/+ and GluR2--/ mutant SOD1G93A mouse (E), and histogram of the number of neurons of different size in the ventral horn ([F], n = 3–4 per group; *, different from GluR2+/+, p < 0.05; #, different from GluR2+/-, p < 0.05).
Future strategies that increase GluR2 expression in motor neurons and/or reduce Ca\(^{2+}\) influx through AMPA receptors will hopefully lead to better treatments of ALS patients.

**REFERENCES**


