Mutant SOD1-expressing astrocytes release toxic factors that trigger motor neuron death by inducing hyper-excitability

Abbreviated title: Hyper-excitability kills motoneurons in ALS model

Authors: Elsa Fritz¹,²†, Pamela Izaurieta¹,²†, Alexandra Weiss³, Franco R. Mir⁴, Patricio Rojas⁵, David Gonzalez¹, Fabiola Rojas¹,², Robert H. Brown Jr², Rodolfo Madrid⁵ and Brigitte van Zundert¹*

Author addresses:
¹Center for Biomedical Research, Faculty of Biological Sciences and Faculty of Medicine, Universidad Andres Bello, Avenida República 217, Santiago, Chile. ²Faculty of Biological Science, University of Concepción, Concepción, Chile. ³Department of Neurology, University of Massachusetts Medical Center, Worcester, Massachusetts, USA. ⁴Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC-CONICET, Córdoba, Argentina. ⁵Department of Biology, Faculty of Chemistry and Biology, University of Santiago de Chile, Chile. †E.F. and P.I. contributed equally to this work.

* Corresponding author:
Center for Biomedical Research, Faculty of Biological Sciences and Faculty of Medicine, Universidad Andres Bello, Avenida Republica 217, Santiago, Chile.
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Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating paralytic disorder caused by dysfunction and degeneration of motor neurons starting in adulthood. Recent studies using cell or animal models document that astrocytes expressing disease-causing mutations of human superoxide dismutase 1 (hSOD1) contribute to the pathogenesis of ALS by releasing a neurotoxic factor(s). Neither the mechanism by how this neurotoxic factor induces motor neuron death nor its cellular site of action has been elucidated. Here we show that acute exposure of primary wild-type spinal cord cultures to conditioned medium derived from astrocytes expressing mutant SOD1 (ACM-hSOD1<sub>G93A</sub>) increases persistent sodium currents (PC<sub>Na</sub>), repetitive firing and intracellular calcium transients, leading to specific motor neuron death days later. In contrast to TTX, which paradoxically increased twofold the amplitude of calcium transients and killed motor neurons, reduction of hyper-excitability by other specific (mexiletine) and non-specific (spermidine and riluzole) blockers of Na<sub>v</sub> channels restored basal calcium transients and prevented motor neuron death induced by ACM-hSOD1<sub>G93A</sub>. These findings suggest that riluzole, the only FDA-approved drug with known benefits for ALS patients, acts by inhibiting hyper-excitability. Together, our data document that a critical element mediating the non-cell autonomous toxicity of ACM-hSOD1<sub>G93A</sub> on motor neurons is increased excitability, an observation with direct implications for therapy of ALS.
Introduction

ALS is a fatal, adult-onset paralytic disorder caused by degeneration of cranial and spinal motor neurons. Much of our understanding of this disease comes from studies of a subgroup of familial (FALS) cases that arises from mutations in the gene encoding for SOD1 (Rosen et al., 1993; Beckman et al., 2001; Valentine et al., 2005). Recent studies also raise the hypothesis that misfolded wild-type SOD1 is pathogenic in many individuals with sporadic ALS (Bosco et al., 2010; Haidet-Phillips et al., 2011). The precise mechanisms whereby mutant SOD1 or misfolded WT SOD1 are toxic to motor neurons are not defined. Also the mechanisms of the beneficial actions of riluzole, the only FDA-approved ALS drug, have not yet been established (Bellingham, 2011).

In vivo and in vitro studies using mutant hSOD1 transgenic mice reveal many pathogenic changes in affected motor neurons, including hyper-excitability, disturbed calcium homeostasis, mitochondrial dysfunction, SOD1 aggregation, cytoskeletal disruption, activation of cell death signals and oxidative stress (Cleveland and Rothstein, 2001; Pasinelli and Brown, 2006; Bento-Abreu et al., 2010; van Zundert et al., 2012). It is not clear yet whether these abnormalities are part of a primary or secondary event, or the result of compensatory mechanisms.

To get insights in these issues, we previously investigated disturbances of physiological functions in motor neurons in acute slice preparations from the brainstem of neonatal hSOD1\textsuperscript{G93A} mice (postnatal days [P4-10]), 2–3 months prior to the appearance of motor neuron degeneration and clinical symptoms, and delineated functional alterations in motor neuron, including increases in repetitive firing, synaptic transmission and persistent sodium inwards currents (PC\textsubscript{Na}) mediated by voltage-sensitive sodium (Na\textsubscript{v}) channels (van Zundert et al., 2008). To date, these are the earliest abnormalities documented for the hSOD1\textsuperscript{G93A} mice (van Zundert et al., 2012). Our data, together with other findings on spinal and cortical neurons in cultures and slice preparations from mutant SOD-linked transgenic
ALS mouse models, indicate that electrophysiological abnormalities in motor neurons are a very early event in ALS (Kuo et al., 2005; Bories et al., 2007; Pambo-Pambo et al., 2009; Quinlan et al., 2011), possibly because of disturbance of a Naᵥ channel activity causing an increment in PCNa (Kuo et al., 2005; Pieri et al., 2009; van Zundert et al., 2008; ElBasiouny et al., 2010; Quinlan et al., 2011; van Zundert et al., 2012).

Many elegant studies have shown that ALS is at least partially a non-cell autonomous disease and that non-motor neuron cells such as astrocytes expressing mutant hSOD1 contribute to the pathogenesis of ALS (reviewed in Ilieva et al., 2009). In in vitro models of ALS, for example, extensive death of primary spinal cord motor neurons and embryonic stem-cell-derived motor neurons is induced when cells are cultured on astrocytes expressing hSOD1G93A or when exposed to ACM-hSODG93A (Nagai et al., 2007; Di Giorgio et al., 2007). However, the primary target of the toxic factor is not known. In the present study, using this ACM-hSODG93A in vitro model system we have elucidated a critical role for hyper-excitability, mediated at least in part through increased Naᵥ channel activity, in inducing motor neuron death.
MATERIALS AND METHODS

Animals

Care and use of rodents was in accordance with the US National Institute of Health guidelines, and was approved by the Institutional Animal Care and Use Committees of Andres Bello University, University of Concepción, and University of Massachusetts. Hemizygous transgenic B6SJL mice carrying a high copy number of mutant human SOD1 (hSOD1\textsuperscript{G93A}), or WT human SOD1 (hSOD1\textsuperscript{WT}), were originally obtained from Jackson Laboratories (Bar Harbor, USA). Non-transgenic littermates (mSOD1\textsuperscript{WT}) and transgenic mice over-expressing the gene for hSOD1\textsuperscript{WT} were used as controls. Transgenes were identified by polymerase chain reaction (Rosen et al., 1993; van Zundert et al., 2008). The hSOD1\textsuperscript{G93A} mice, but not the hSOD1\textsuperscript{WT} mice, develop signs of neuromuscular deficits (tremor of the legs and loss of extension reflex of the hind paws) and have an average lifespan of 19-21 weeks (Gurney et al., 1994; Scott et al., 2008; Del Signore et al., 2009).

Conditioned media preparation

ACM was prepared similarly as described (Nagai et al., 2007). Cultures of astrocytes were prepared from P1-2 wild-type mice and from transgenic mice expressing either hSOD1\textsuperscript{G93A} or hSOD1\textsuperscript{WT}. Cultures were maintained in DMEM (Hyclone SH30081.02) containing 10% FBS (Hyclone SH30071.03; lot ATC31648) and 1% penicillin-streptomycin (Invitrogen 15070-063) at 37°C 5% CO\textsubscript{2}. Cultures reached confluence after approximately 14 days and contained >95% GFAP\textsuperscript{+} astrocytes. Residual microglia cells were removed by shaking in an orbital shaker (200 r.p.m. in the incubator) for 6 hours. Then, media was replaced by spinal culture media (see below). After 7 days (21 DIV), ACM was collected, centrifuged (500g for 10 min) and stored at -80°C. Before use, the ACM was supplemented with 4.5 mg ml\textsuperscript{–1} D-glucose (final concentration) and penicillin/streptomycin and filtered. We also added a chick hindlimb muscle extract (Sepulveda et al., 2010). For all experiments (including the motor neuron
survival analysis, patch-clamp recordings and calcium imaging), we used ACM that was
diluted 8-fold (12.5%); at this concentration ACM-hSOD1^{G93A} strongly reduced motor neuron
survival, whereas ACM-hSOD1^{WT} was not toxic. The ACM was applied to ventral spinal cord
cultures derived from rats because better quality motor neurons can be obtained from these
rodents relative to mice spinal cord cultures. The use of co-cultures mixing different species
(rat, mice, human) does no induce apparent side effects (e.g. Pehar et al., 2004; Nagai et al.,
2007; Di Giorgio et al., 2007).

Primary neuronal cultures

Pregnant Sprague-Dawley rats were deeply anesthetized with CO₂ and primary spinal
cultures were prepared from E14 pups (Sepulveda et al., 2010). Briefly, whole spinal cords
were excised and placed into ice-cold HBSS (Invitrogen 14185-052) containing 50 µg/ml
penicillin/streptomycin (Invitrogen 15070-063). The dorsal part of the spinal cord was
removed using a small razor blade, and the ventral cord was minced and incubated with pre-
warmed HBSS containing 0.25% trypsin (Invitrogen 15090-046) in an incubator for 20
minutes at 37°C. After enzymatic treatment, the cells were transferred to a 15 ml tube
containing neuronal growth media (70% MEM (Invitrogen 11090-073), 25% Neurobasal
media (Invitrogen 21103-049), 1% N2 supplement (Invitrogen 17502-048), 1% L-glutamine
(Invitrogen 25030-081), 1% penicillin-streptomycin (Invitrogen 15070-063), 2% horse serum
(Hyclone SH30074.03; lot AQH24495) and 1 mM pyruvate (Sigma). Cells were precipitated,
transferred to a new 15 ml tube containing 2 ml growth media, re-suspended by mechanical
agitation through fire-polished glass Pasteur pipettes of different tip diameters, and counted;
1×10⁶ cells were plated on freshly prepared poly-L-lysine-coated 6-well plates (1 mg/ml;
30,000-70,000 MW; Sigma P2636) or 6 mm #00 cover slips (Menzel-Gläser) for calcium
imaging experiments in growth media; they were cultured for 3 days at 37°C under 5% CO₂,
and supplemented with 45 µg/ml E18 chick leg extract; media was refreshed every 3 days.
Riluzole (Sigma; 100 μM) was dissolved in distilled water (plus 10% Tween20), while spermidine (Sigma), mexilitine (Sigma) and TTX (Alomone) were dissolved in water and stored at 100 mg/ml at -20°C.

Cell labeling and counting
Primary spinal cultures were fixed at 7 DIV with 4 % paraformaldehyde, and immunostained with an antibody against MAP2 (1:400; Santa Cruz Biotechnology) to visualize all neurons (interneurons as well as motor neurons); immunostaining with the SMI-32 antibody (1:1,000, Sternberger Monoclonals) revealed the presence of unphosphorylated neurofilament-H, expressed specifically in motor neurons in spinal cord cultures (Urushitani et al., 2006; Nagai et al., 2007); previously we found that our wild-type primary spinal cultures typically contain at least 8-10% motor neurons until 12 DIV (Sepulveda et al., 2010). Fluorescent neurons were visualized with epifluorescent illumination on an Olympus IX81 microscope, or on a Nikon C1 confocal microscope on which stacks of 0.50 µm optical sections were acquired through entire neurons. Labeling patterns were documented with a 20x objective and a Q-Imaging Micropublisher 3.3 Real-Time Viewing camera; MAP2- and SMI-32-positive neurons were counted offline within 20 randomly chosen fields, and the percentage of SMI-32-positive motor neurons within the total number of MAP2-positive cells was calculated. Each condition was replicated in at least 3 independent cultures, and in duplicate.

Electrophysiology
Whole cell patch-clamp recordings were taken from primary spinal neurons and analyzed as previously described (van Zundert et al., 2008; Sepulveda et al., 2010). For recording action potential (AP) firing and voltage-sensitive persistent sodium currents (PC_{Na}) cells were maintained in a solution containing: 150 mM NaCl, 5.4 mM KCl, 2.0 mM CaCl\textsubscript{2}, 2.0 mM MgCl\textsubscript{2}, 10 mM HEPES (pH 7.4), and 10 mM glucose. After formation of a high resistance
seal and break-in (>800 MΩ), whole-cell voltage signals were recorded with an Axopatch 200B amplifier (Molecular Devices). Pipette and whole-cell capacitance, and series resistance were compensated using amplifier circuitry. Signals were low pass-filtered (5 kHz) and digitized (5-40 kHz) on a PC using PClamp 9.2 software. The passive membrane properties were measured from those cells from which we recorded APs. The resting membrane potential was corrected for a calculated junction potential of 13.7 mV (JPCalc, Clampfit). For recording APs, a K gluconate-based internal solution was used, containing (in mM) of: 17.5 KCl, 122.5 K gluconate, 9 NaCl, 1 MgCl₂, 10 HEPES, 0.2 EGTA, 3 MgATP, 0.3 GTP-Tris. APs were evoked by injection of rectangular depolarizing current pulses (10-40 pA for 300 msec). Cells were discarded if V rest was >-40 mV or if a train of APs could not generated in response to any depolarizing current steps. For recording PCNa, a Cs gluconate-based internal solution was used, containing (in mM): 122.5 Cs gluconate, 17.5 CsCl, 8 NaCl, 10 HEPES, 0.2 EGTA, 2 MgATP, 0.3 GTP-Na. In some experiments, we added CdCl₂ (100 μM to the bath) to block voltage-gated calcium channels, and TEA-Cl (5 mM to pipette and 10 mM to bath) and 4-aminopyridine (1 mM to the bath) to block potassium channels (Fleidervish et al., 2008). PCNa was recorded by applying a slow voltage ramp from -60 mV to +10 mV and back (14 mV/s) (Powers and Binder, 2003). Cell-attached recordings were performed simultaneously with calcium imaging at 34°C, as previously described in (Madrid et al., 2006), using 7–8 MΩ patch pipettes fabricated from GC150F-7.5 borosilicate glass capillaries (Harvard Apparatus Ltd).

Calcium imaging

Calcium imaging studies were carried out under controlled temperature 34 ± 1°C (Madrid et al., 2006) as previously described with some modification. Cultured spinal neurons were incubated with 5 μM fura-2AM (Invitrogen) dissolved in standard extracellular solution and 0.02% Pluronic (Invitrogen) for 45 min at 37°C. Fluorescence measurements were made with
a Nikon Ti inverted microscope fitted with a 12-bit cooled CCD camera (Orca C8484-03G02, Hamamatsu). Fura-2 was excited at 340 nm and 380 nm with a Polychrome V monochromator (Till Photonics) and the emitted fluorescence was filtered with a 510 nm longpass filter. 340/380 nm ratios (at 1 Hz) were displayed online with HCImage software v2.2.1 (Hamamatsu). Bath temperature was sampled simultaneously with intracellular calcium recordings with a BAT-12 microprobe thermometer, supplemented with an IT-18 T-thermocouple (Physitemp Instruments), using Axoscope 10.3 Software (Molecular Devices). Cover slip pieces (6 mm in diameter / 55 to 80 µm in thickness (#00) from Menzel-Gläzer) with cultured neurons were placed in a microchamber and continuously perfused (∼1 ml/min) with solutions warmed at 34 ± 1 ºC. The temperature was adjusted with a water-cooled computer-controlled Peltier device, with the outlet close to the imaging field, and controlled by a feedback device.

Pharmacological treatments in culture
Riluzole (Sigma) was dissolved in distilled water (plus 10% Tween20) at 100 µM, and added to cultures to final concentrations ranging from 25 nM to 500 nM. Spermidine (Sigma) was dissolved in water at 100 mg/ml and added to cultures to a final concentration of 10 µM. A 1 mM stock solution of TTX (Alomone) was prepared, citrate-buffered, and added to cultures to final concentrations ranging from 1-500 nM. Mexiletine (Tocris) was dissolved in water to 100 mM and used at final concentrations ranging from 5 to 25 nM.

Data Analysis
ANOVA, followed by post hoc Tukey tests, was used to detect significant changes. Student's t tests were used to compare the response of two populations to individual treatments. Non-parametric Kolmogorov-Smirnov test was used to test the significance of the cumulative
distributions of the calcium transient amplitudes. Unless otherwise stated, in all figures, error bars represent the mean ± s.e.m.; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
RESULTS

Chronic and short-term treatment of primary spinal cultures with ACM-hSOD1^G93A triggers death of motor neurons

In vitro, chronic exposure of primary mouse spinal cord neurons to undiluted astrocyte conditioned media (ACM) from transgenic rodents expressing mutant human SOD1, including the G93A allele (ACM-hSOD1^G93A), causes extensive (~60%) and specific death of motor neurons (Nagai et al., 2007). The identity of the offending, soluble toxic factors is not known. We have used this model to gain insight into the nature of the toxic soluble factors in ACM-hSOD1^G93A and into its primary targets. ACM-hSOD1^G93A prepared using astrocytes derived from hSOD1^G93A mice was added at an 8-fold dilution (see Materials and Methods) to wild-type primary rat spinal cultures at 3 DIV for either 4 days or 30 minutes; its effects on neuron survival were assessed at 7 DIV (Fig. 1A). To define the presence of both interneurons and motor neurons an antibody against microtubule-associated protein 2 (MAP2) was used (Fig. 1B,C; red). The SMI-32 antibody, which recognizes unphosphorylated neurofilament-H, was used to specifically identify motor neurons in spinal cord cultures (Fig. 1B,C; green arrow), as previously described (Urushitani et al., 2006; Nagai et al., 2007; Sepulveda et al., 2010).

Consistent with previous studies (Nagai et al., 2007), chronic 4 day exposure (from 3 to 7 DIV) spinal cultures to ACM-hSOD1^G93A induced ~50% motor neuron death (Fig. 1C,D). In addition, we found that a 30 minutes exposure to 3 DIV spinal cultures to ACM-hSOD1^G93A was sufficient to cause a similar extensive death of motor neurons analyzed at 7 DIV (Fig. 1C,D). We also obtained control ACM from astrocytes harvested from transgenic mice that carry the non-pathological human wild-type SOD1 gene (ACM-hSOD1^WT) or from non-transgenic mice (ACM-mSOD1^WT). Neither of these media caused motor neuron death (Fig. 1D and data not shown). The finding that ACM-hSOD1^WT was not toxic indicates that the
factor inducing motor neuron death is specifically attributable to the hSOD1\textsuperscript{G93A} mutation, rather than to the overexpression of the human SOD1 protein.

**ACM-hSOD1\textsuperscript{G93A} rapidly increases PC_{Na}, neuronal excitability and intracellular calcium dynamics**

We next sought to identify potential targets of the soluble toxic factors in the ACM-hSOD1\textsuperscript{G93A}. We had previously shown in acute slice preparations from neonatal hSOD1\textsuperscript{G93A} mice that mutant motor neurons displayed increases in repetitive firing, synaptic transmission and PC_{Na} (van Zundert et al., 2008). Based on these findings, we hypothesized that ACM-hSOD1\textsuperscript{G93A} application enhances the Na\textsubscript{v} channel permeability and thereby increase PC_{Na}, excitability and synaptic transmission, leading to sustained and toxic levels of sodium and calcium influxes that instigate motor neuron death. To get insights in this hypothesis, we first exposed 5-7 DIV spinal neurons to ACM-hSOD1\textsuperscript{G93A} and analyzed functional changes by whole-cell patch recordings and calcium imaging.

To analyze the effect of mutant SOD1 on the PC_{Na}, we treated primary spinal cultures with ACM-hSOD1\textsuperscript{G93A} for 30-90 minutes, and selectively recorded this “non-inactivating” current, independent of the transient sodium current (T_{Na}), by applying a slow voltage ramp from -60 mV to +10 mV and back (14 mV/s) (Fig. 2A; lower figure) as described previously (Powers and Binder, 2003; Kuo et al., 2005; Theiss et al., 2007; van Zundert et al., 2008). Representative sample traces reveal that, as compared to control, untreated cells (Fig. 2A; green line), application of ACM-hSOD1\textsuperscript{G93A} generated a marked inward current, which peaked at around -40 mV (Fig. 2A; red line, arrowhead). Typically, much of the inward persistent current was eliminated by application of the Na\textsubscript{v} channel blocker tetrodotoxin (TTX; 1 µM) (Fig. 2A; black lines), indicating that the persistent inward current derives predominantly from Na\textsubscript{v} channels (PC_{Na}) rather than voltage-sensitive calcium (Ca\textsubscript{v}) channels in these young spinal cultures.
We next asked whether the ACM-hSOD1\textsuperscript{G93A}-induced PC\textsubscript{Na} can be detected in all neurons (including interneurons) or is exclusive to motor neurons. As one approach to this problem we examined the relationship between the maximum PC\textsubscript{Na} (quantified by measuring the peak of the amplitude of the inward current after subtraction of the TTX-insensitive current) and membrane capacitance (C\textsubscript{m}), as a surrogate marker for neuronal size. We observed that neurons with a C\textsubscript{m} of >20 pF predominantly displayed a large soma (>20 μm diameter) and expressed 5 or more primary dendrites; these parameters are typical for motor neurons (Fig. 2B, right image). On the contrary, neurons with a C\textsubscript{m} of <15 pF are likely to be interneurons as they are smaller (<20 μm diameter) and contain typically 3-4 primary branches (Fig. 2B, left image). The plots in Figure 2C show that for control neurons (dotted line), as well as for neurons treated with ACM-hSOD1\textsuperscript{G93A} (solid line), the magnitude of the sodium inward current is dependent on the size of the neuron: the larger the neuron, the larger the PC\textsubscript{Na}. After normalizing the inward current for the area of the cell membrane, the mean peak amplitude of the PC\textsubscript{Na} in primary spinal cultures treated with ACM-hSOD1\textsuperscript{G93A} (2.8±0.3 pA/pF) was twice the size compared to that for control neurons (1.5±0.2 pA/pF, t-test, P<0.01) (Fig. 2D).

To analyze the effect of mutant SOD1 on neuronal excitability, we performed current-clamp recordings to examine spiking frequency in response to depolarizing current steps. Remarkably, within minutes of ACM-hSOD1\textsuperscript{G93A} application, the patched neuron started to fire action potentials (APs) at significantly higher rates (Fig. 3A; arrowheads). After 30 min of ACM-hSOD1\textsuperscript{G93A} application, the average neuronal firing rate increased by up to 151±17% (t-test, P<0.05 relative to ACM-hSOD1\textsuperscript{WT} at min 30) (Fig. 3B). In contrast, application of ACM-hSOD1\textsuperscript{WT} did not change AP firing rates during the same exposure (97±11% relative to min 0) (Fig. 3B). Analyses of both passive and active properties of spinal cord neurons before and after 30 min of bath application of ACM-hSOD1\textsuperscript{G93A} did not reveal other significant differences (data not shown).
We also performed some experiments to get insights to whether in addition to the non-cell autonomous effects mediated by ACM-hSOD1\textsuperscript{G93A} exogenous introduction of mutant SOD1 into neurons can induce cell-autonomous effects. For this, VSCNs (4-5 DIV) were transfected using a CaPO\textsubscript{4} transfection protocol (Sepulveda et al., 2010) with either a plasmid encoding for hSOD1\textsuperscript{G93A-GFP} or hSOD1\textsuperscript{WT-GFP} (Turner et al., 2005). Twenty-four hours after exogenous expression, voltage steps from -70 to -40 mV were applied to transfected neurons to generate Na\textsubscript{v} channel dependent currents; because the transfection rate is very low (<5%) and VSCNs contain 90-92% interneurons and only 8-10% motor neurons, our analyses were performed on healthy looking transfected interneurons. Note, that electrophysiological recordings displayed in Figures 2 and 3 also include predominantly interneurons. Because transfected neurons with hSOD1\textsuperscript{G93A-GFP} (but hSOD1\textsuperscript{WT-GFP}) did not display resting membrane potentials more negative than -40 mV, required to detect reliable APs, we were unable to determine whether the excitability of neurons expressing mutant SOD1 was increased. Next we applied voltage steps from -70 to -40 mV to generate Na\textsubscript{v} channel-dependent currents. We found that hSOD1\textsuperscript{G93A-GFP}-transfected neurons displayed approximately a threefold increase in the maximum Na\textsubscript{v} channel-mediated current compared to hSOD1\textsuperscript{WT-GFP}-expressing neurons (-3906 ±1220 pA in hSOD1\textsuperscript{G93A-GFP} (n=3) vs -1101 ±219 pA, hSOD1\textsuperscript{WT-GFP} (n=3); p < 0.001). These results indicate that mutant SOD1\textsuperscript{G93A} also mediates cell-autonomous effects through regulation of Na\textsubscript{v} channels.

Next we evaluated whether hSOD1\textsuperscript{G93A-GFP} transfected neurons displayed DNA damage (Hoechst and DAPI staining), membrane permeabilization (incorporation of rhodamine-conjugated dextran amine Mini Ruby) and caspase-3 activation (immunocytochemistry with a caspase-3 phosphorylation antibody). We exogenously introduced either hSOD1\textsuperscript{G93A-GFP} or hSOD1\textsuperscript{WT-GFP} into 4 DIV VSCNs and analyzed these parameters 1-4 days later. ACM-hSOD1\textsuperscript{G93A} and H\textsubscript{2}O\textsubscript{2} (as positive control) were also applied to the cultures. In contrast to H\textsubscript{2}O\textsubscript{2} application, neither addition of ACM-hSOD1\textsuperscript{G93A} nor expression of hSOD1\textsuperscript{G93A-GFP} (or hSOD1\textsuperscript{WT-GFP}) resulted in DNA damage,
membrane permeabilization, or caspase-3 activation of interneurons (data not shown). These results are in agreement with our findings that ACM-hSOD1<sup>G93A</sup> application does not affect interneuron cell survival (Figure 1) and indicate that although mutant SOD1<sup>G93A</sup> mediates cell autonomous and cell non-autonomous effects through regulation of Na<sub>v</sub> channels, the activation of these channels is insufficient to cause detectable pathology and cell death in interneurons.

To determine whether hyper-excitability induced by ACM-hSOD1<sup>G93A</sup> also affects calcium homeostasis, we recorded intracellular calcium transients ([Ca<sup>2+</sup>]) in spinal neurons in culture using Fura-2. In about 43% of the neurons we observed spontaneous oscillations in the [Ca<sup>2+</sup>], as shown in the representative example traces (Fig. 4A). These calcium transients showed an averaged amplitude of 0.05 (ΔF<sub>340/380</sub>) and a basal mean frequency of 0.06 Hz, often in bursts. The smallest value of ΔF<sub>340/380</sub> considered as a calcium transient was 0.01. Under control conditions or after administration of ACM-mSOD1<sup>WT</sup> the frequency of calcium transients declined until ~30% of the initial value at minute 30 (Fig. 4A,B). By contrast, 30 minutes application of ACM-hSOD1<sup>G93A</sup> markedly increased the frequency of the calcium transients (124±8% of control at min 0, ANOVA, P<0.001 relative to control and ACM-hSOD1<sup>G93A</sup> at min 30) (Fig. 4A,B). Short-term application of ACM-hSOD1<sup>G93A</sup> also significantly (p<0.05) reduced the mean amplitude of the calcium transients (Fig. 4C).

In order to study the correlation of calcium transients with the electrical excitability of cultured neurons in non-invasive conditions, we performed simultaneous recordings of the intracellular calcium concentration and action potential firing from the soma, using the cell-attached mode of the patch-clamp technique (Fig. 5). As we show in Fig. 5A-B, spontaneous action potential firing in these neurons appeared in bursts (see Fig. 5D), which are coincident with calcium transients (Fig. 5A-B). The mean firing frequency during a calcium transient was 5.6±0.3 impulses/s (Fig. 5C) quantified in 198 transients from four independent experiments. Inter-transient periods were in parallel with an almost complete absence of action potential...
firing (Fig. 5C). These results are consistent with the idea that calcium transients are directly related to spontaneous action potential firing of cultured spinal cord neurons. Accordingly, the increase of the frequency of calcium transients induced by ACM-hSOD1G93A could be due to an increase in neuronal excitability.

Effects of Na\textsubscript{v} channel blockers on functional alterations and motor neuron death induced by ACM-hSOD1G93A.

Because neuronal excitability regulates synaptic transmission and fluxes of sodium and calcium, we tested if it would be possible to rescue motor neuron death induced by ACM-hSOD1G93A by pharmacological blockage of Na\textsubscript{v} channels to counterbalance the increased excitability evoked by this toxic media. Toward that end, we tested TTX, mexiletine, spermidine and riluzole, reagents well documented to block Na\textsubscript{v} channel permeability hereby reducing PC\textsubscript{Na} and the frequency of APs (Kuo et al., 2005; Theiss et al., 2007; Fleidervish et al., 2008; Olschewiski et al., 2009; Bellingham, 2011), as we also confirmed in the present study (see Fig. 6D for effects of drugs on excitability). Multiple doses of each Na\textsubscript{v} channel blocker on the survival of motor neurons were assessed under control conditions and after co-application with ACM-SOD1G93A (data not shown); for each drug the maximum effect in rescuing motor neuron survival is displayed in Figure 6 (Fig. 6A-C).

We first analyzed TTX, a classic neuronal Na\textsubscript{v} channel pore blocker that binds the “neurotoxin receptor at site 1” with IC\textsubscript{50}’s in the nM range (reviewed in Catterall et al., 2005). We found that chronic application of TTX at a wide range of concentrations (1 nM to 500 nM) was unable to significantly prevent ACM-hSOD1G93A-induced motor neuron death; under the best circumstances, in the presence of ACM-SOD1G93A and 1 nM TTX motor neuron survival was only 75±2% (Fig. 6B; P<0.05 relative to control (indicated with *) and P>0.05 relative to ACM-SOD1G93A). Notably, however, we found that comparable administration of TTX to control spinal cord cultures also significantly killed motor neurons (Fig. 6C; P<0.01 relative to
control). In our view, this initially surprising result reflects a paradoxical effect of TTX on enhancing drastically the amplitude of calcium transients (see below) and possible by increasing neuronal excitability by homeostatic mechanisms (Desai et al., 1999; Fishbein and Segal, 2007; Schonfeld-Dado et al., 2009; Koch et al., 2010). We next tested mexiletine, an orally active lidocaine analog, which is a local anesthetic and antiarrhythmic drug that targets the “local anesthetic receptor site” of Na\(_{\nu}\) channels with strong use- or activity-dependent properties and with wide ranges of IC\(_{50}\) (Ragsdale et al., 1994; Catterall et al., 2005; Olschewiski et al., 2009). Importantly, we found that chronic co-application of ACM-SOD\(_1^{G93A}\) with 25 nM mexiletine prevented motor neuron death (88±2% survival, ANOVA, P<0.01 relative to ACM-SOD1\(^{G93A}\) (indicated with ##) and P>0.05 relative to control) (Fig. 6B). We also observed that chronic application of this specific Na\(_{\nu}\) channel blocker to control spinal cord cultures did not cause significant motor neuron cell death (Fig. 6C). The beneficial effects of mexiletine as compared to TTX might be a consequence of the strong use-dependent properties of mexiletine. Thus, antiarrhythmics such as mexiletine, unlike TTX, are very suitable for clinical use because they are able to curtail high-frequency firing of excitable neurons at concentrations that have little effects on basal activity. Consequently, as can be observed in the representative traces of Fig. 6D, although low doses of mexiletine (5 nM) reduce the number of spikes within the AP train, substantially increasing concentrations by 5 (25 nM) to 20 (100 nM) times causes only a gradual suppression in the neuronal activity. Even at very high doses of mexiletine (500 nM), APs are still generated and within a train pattern. This is very different from the influence of TTX (Fig. 6D), for which a 10-fold increase in concentration (from 1 to 10 nM) largely suppresses spiking and dissembles the train pattern. Slightly higher doses of TTX (≥25 nM) even suppress the initial firing altogether (Fig. 6D; arrow).

Next we analyzed the effects of the polyamine spermidine, which is known to affect the gating of varies ion channels and serves as an endogenous, activity-dependent Na\(_{\nu}\),
channel blocker; its affinity is in the µM range, but depends on the membrane potential (Williams, 1997; Fleidervish et al., 2008). We found that 10 µM spermidine totally prevented motor neuron death induced by ACM-SOD1^{G93A} (101±3% survival, ANOVA, P<0.001 relative to ACM-SOD1^{G93A} and P>0.05 relative to control) (Fig. 6B). In addition, large increases in spermidine concentrations (e.g. 25 times from 10 to 250 µM) eliminated only few APs without altering spiking patterns until very high doses (1 mM - 2.5 mM) (Fig. 6D).

Finally, we tested riluzole, which over a large dose range (<1 µM to 1 mM) has multiple effects including inhibition of neurotransmitter release and potentiation of glutamate transporters ( reviewed in Bellingham, 2011). Riluzole also suppresses neuronal excitability at low concentrations from 100 nM-5.0 µM by affecting Na, channels (Kuo et al., 2005; Theiss et al., 2007; Bellingham, 2011). Interestingly, we found that chronic co-application of ACM-SOD1^{G93A} with 100 nM riluzole was able to completely prevent motor neuron death (94±7% survival, ANOVA, P<0.001 relative to ACM-SOD1^{G93A} and P>0.05 relative to control) (Fig. 6B). As with the other reagents, the dose of riluzole that prevented ACM-hSOD1^{G93A}-induced on motor neuron survival caused only a slight reduction in spiking frequency (Fig. 6D). However, riluzole appears not to be an optimal therapeutic drug, as it causes death of control neurons (Fig. 6C), possibly due to the fact that this drug not only affects PC Na but also many other targets that influence neuronal activity, including calcium-dependent K+ currents, voltage-gated K+ currents, voltage-gated Ca^{2+} currents, etc (Bellingham, 2011). We also found that riluzole, at increasing concentrations, rapidly suppresses the spiking and train pattern, while it leaves the initial AP intact (Fig. 6D). Together, our data suggest that the favorable effects of mexiletine, spermidine and riluzole on motor neuron survival are principally the result of counterbalancing the increases in neuronal excitability induced by ACM-hSOD^{G93A}.

We next analyzed the effects of Na, channel blockers on the altered calcium homeostasis induced by ACM-hSOD1^{G93A} (Fig. 7A), using the same concentrations that had
been used to test cell survival. Application of mexiletine, spermidine or riluzole to cultures incubated for the first 30 minutes with ACM-hSOD1<sup>G93A</sup> markedly decreased both the average frequency and the amplitude of the intracellular transients within minutes (Fig. 7B,C). By contrast, application of TTX differentially affected the [Ca<sup>2+</sup>]i dynamics. Like the other Na<sup>+</sup> blocking compounds, TTX significantly reduced the mean frequency of intracellular calcium transients (Fig. 7B); however, it also strongly increased (by 225%) the mean amplitude of the remaining transients (Fig. 7C). As discussed below, this effect of TTX, which seems paradoxical by comparison with the effect of mexiletine, spermidine and riluzole may account for its toxicity to control neurons and its inability to prevent ACM-SOD1<sup>G93A</sup>-induced motor neuron death (see Fig. 6).
Discussion

Many studies report that ALS is at least partially a non-cell autonomous disease and that astrocytes participate in the death of motor neurons (Clement et al., 2003; Nagai et al., 2007; Di Giorgio et al., 2007; Cassina et al., 2008; Yamanaka et al., 2008; Ilieva et al., 2009; Haidet-Phillips et al., 2011). In an unique established *in vitro* model of ALS, for example, extensive death of primary motor neurons is induced when spinal cultures are exposed to conditioned media derived from astrocyte expressing SOD$^{G93A}$ (ACM-hSOD$^{G93A}$) (Nagai et al., 2007). However, the primary target of the toxic factors is unknown. In the present study, we have used the *in vitro* system reported by Nagai et al. (2007) to demonstrate that even short-term (30 min) exposures of wild-type spinal cord neurons to the ACM-hSOD$^ {G93A}$ suffices to trigger death of motor neurons. Moreover, acute exposure to the ACM-hSOD$^{G93A}$ rapidly increases levels of a PC$_{Na}$, excitability and calcium transients of spinal neurons. Although the PC$_{Na}$ is only a small fraction (1-5%) of the total sodium current, this “non-inactivating” component can have important effects on cell and network behavior. Because it can be activated close to the cell's resting potential, small increases in this current can enhance intrinsic excitability, alter spike initiation, amplify and prolong firing rate, and influence release of neurotransmitters (Crill et al., 1996; Goldin, 2003; ElBasiouny et al., 2010; van Zundert et al., 2012). Persistent increases in this sodium current may thus lead to excessive influxes of sodium and calcium ions. Because motor neurons have a limited cytosolic calcium buffering capacity, excessive uptake of calcium by mitochondria could be an initial step in a cascade of events that impair mitochondrial function and ultimately lead to motor neuron degeneration and death (Lewinski and Keller, 2005; Bento-Abreu et al., 2010; van Zundert et al., 2012). Our finding that bigger ventral spinal cord neurons have a larger PC$_{Na}$ is likely an additional factor that can explain the selective vulnerability of motor neurons this ALS model system. Based on the above data and previously reported experimental evidence obtained in neonatal hSOD$^{G93A}$ mice and cultures (Kuo et al., 2005; van Zundert et
al., 2008; Pieri et al., 2009; ElBasiouny et al., 2010; Quinlan et al., 2011; Schuster et al., 2011), it seems likely that an important mechanism by which mutant SOD1 induces motor neuron death is through the activation of PC$_{Na}$ hereby increasing excitability which in turn causes toxic levels of cytosolic calcium (van Zundert et al., 2012). This hypothesis is supported by the observation that exposure of spinal cultures to riluzole, mexiletine, and spermidine, three reagents that reduce PC$_{Na}$ and hyper-excitability, restored calcium homeostasis and prevented cell death induced by ACM-hSOD1$^{G93A}$. Together, based on our findings we support the view that induction of Na$_v$ channel-mediated hyper-excitability by mutant SOD1$^{G93A}$ is a central factor initiating motor neuron death in ALS.

Despite our findings with the Na$_v$ channel blockers mexiletine, riluzole, and spermidine, we were intrigued to observe that TTX (1 nM) was the only compound unable to effectively prevent motor neuron death when chronically co-applied with ACM-hSOD1$^{G93A}$. The exact structural basis for how these different reagents block Na$_v$ channels is currently unknown; however, this should change rapidly with the recent establishment of the first crystal structure of a bacterial Na$_v$ channel obtained at 2.7Å resolution (Payandeh et al., 2011). Of course it is very plausible that the beneficial effects of riluzole and spermidine are completely independent of the suppression of Na$_v$ channels and involves other ion channels/receptors. For example, it is well known that riluzole can inhibit multiple voltage-gated calcium and potassium channels and potentiates the activity of glutamate transporters (Bellingham, 2011). The polyamine spermidine also is able to block voltage-gated calcium and potassium channels and modulates several glutamate receptor types (Williams, 1997). However, these additional targets of riluzole and spermidine do not explain why the specific Na$_v$ channel blocker mexiletine is able to prevent the ACM-hSOD1$^{G93A}$-induced motor neuron death. Accordingly, in our view, it remains possible that the primary neuroprotective effect of mexiletine, spermidine and riluzole in this in vitro system is mediated by the reduction in excessive Na$_v$ channel current.
If this is the case, the issue that arises is the apparent discrepancy between the effects of mexiletine and TTX: why does the latter Na\textsubscript{v} blocker not rescue motor neuron death induced by ACM-hSOD\textsuperscript{G93A}? Two considerations may explain this observation. First, although short-term co-application of TTX (1 nM) with ACM-hSOD\textsuperscript{G93A} significantly decreased the frequency of calcium transients, paradoxically the amplitude of the remaining events was strongly increased. Second, many studies have shown that while TTX at high concentrations (1 µM) can suppress neuronal network activity it induces neuronal death, presumably by induction of intrinsic and synaptic homeostatic plasticity processes. For example, in neuronal cultures TTX actually increased intrinsic excitability (Desai et al., 1999; Koch et al., 2010), enhanced AMPA receptor-mediated postsynaptic activity (Fishbein and Segal, 2007), and reduced GluR2 expression, a subunit critical to block calcium entry through AMPA receptors (Schonfeld-Dado et al., 2009). These results suggest that events or pathways that are components of homeostatic plasticity, and compensates for the TTX-induced reduction in neuronal activity, can lead indirectly to injurious calcium influxes (Fishbein and Segal, 2007; Schonfeld-Dado et al., 2009; Turrigiano, 2011). It is plausible that in our culture system similar processes are induced by the chronic application of low doses of TTX, leading to specific death of vulnerable cells like motor neurons, which have a very limited calcium-buffering capacity. Notably, while 1 nM TTX killed untreated motor neurons, TTX doses of ≥500 nM were required to affect the survival of non-motor neurons within the same culture (data not shown).

How do cytotoxic factors of the ACM-hSOD\textsuperscript{G93A} enhance the persistent sodium current of Na\textsubscript{v} channels? The amplitude of the PC\textsubscript{Na} is influenced by many factors, including the type of alpha subunit underlying the currents (PC\textsubscript{Na} is greater for Na\textsubscript{v1.1} and Na\textsubscript{v1.6} as compared to Na\textsubscript{v1.2} and Na\textsubscript{v1.3}), the presence of Na\textsubscript{v} channel beta subunits, exposures to different reactive oxygen species, the activity of critical protein kinases (e.g. PKC) and levels of external potassium (Goldin, 1999; Somjen and Muller, 2000; Hammarström and Gage,
2000; Franceschetti et al., 2000; Kassmann et al., 2008; Aman et al., 2009; van Zundert et al., 2012). In our experiments, the PC_{Na} are not modified by 30 mM KCl (data not shown), suggesting that potassium fluxes are not the source of heightened Na_{v} channel permeability. Additional experiments, out of the scope of this work, will be required to define the specific factors within the ACM-hSOD1^{G93A} that affect the PC_{Na}.

As for the understanding of the molecular and cellular basis of the astrocytic non-cell autonomous toxicity, evidence exists showing that mutant SOD1-expressing astrocytes release factors such as glutamate, cytokines, chemokines and death-receptor activated components that affect motor neuron survival (Hensley et al., 2006; Pehar et al., 2006; Milanese et al., 2010; Aebischer et al., 2011). Evaluation of the ACM generated by the laboratory of Przedborski (which is similar to our media) discarded, however, that many of these molecules are likely to be involved in the toxicity of motor neuron death induced by mutant hSOD^{G93A}-expressing astrocytes (see Supplementary Fig. 5 Nagai et al., 2006). Precise identification of the neurotoxic factor(s) in the ACM-hSOD1^{G93A}, however, is likely to be challenging; recent quantitative mass spectrometry studies for example reveal that 92 out of 516 proteins in ACM-hSOD^{G93A} are enriched >1.5-fold relative to those in cell lysate (Greco et al., 2010). Furthermore, analyses of microarray gene expression showed multiple genes involved in transcription, signalling, cell proliferation, extracellular matrix synthesis, response to stress, and steroid and lipid metabolism are expressed differentially in hSOD^{G93A} versus wild-type astrocytes (Vargas et al., 2008).

Together, the results reported here provide the first evidence indicating that the neurotoxicity of medium conditioned astrocytes expressing ALS-linked mutated SOD^{G93A} is closely associated with its induction of motor neuron hyper-excitability. Our findings that mexiletine and spermidine (in addition to riluzole) completely reverse ACM-hSOD1^{G93A}-induced motor neuron death in this ALS model system suggest that these agents are plausible candidate therapies for ALS patients. Finally, because patients with sporadic and
familial ALS display similar pathology, have comparable clinical symptoms, and experience a
modest but beneficial response to riluzole, it is conceivable that reduction of hyper-excitability
by pharmacological agents will similarly prove beneficial in both forms of ALS.
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Figure 1. Long-term as short-term treatment of primary spinal cultures with ACM-hSOD1<sup>G93A</sup> equally trigger cell death of motor neurons. A, Flow diagram of experiment. Media was conditioned for 7 days by astrocytes derived from transgenic mice overexpressing hSOD1<sup>G93A</sup> (ACM-hSOD1<sup>G93A</sup>). Primary rat spinal cord cultures (3 DIV) were exposed to ACM-hSOD1<sup>G93A</sup> for 4 days or for 30 minutes; all were fixed at 7 DIV to assay cell survival with immunocytochemistry. B, Fixed 7 DIV primary spinal cultures were double-labeled with anti-MAP2 antibody (red) to visualize interneurons (arrowhead) and motor neurons (arrow), and with the SMI-32 antibody (green) to identify motor neurons (arrow). Scale bar: 25 µm C, Spinal cultures were treated with control media for 4 days (upper pictures) or ACM-hSOD1<sup>G93A</sup> for either 4 days (middle pictures) or 30 minutes (lower pictures), fixed at 7 DIV and labeled with MAP2 and SMI32. Note that a single, short-term 30 minutes exposure of spinal cord neurons to ACM-hSOD1<sup>G93A</sup> is as effective in triggering motor neuron cell death as chronic application. Interneurons are spared in both conditions. Scale bar: 200 µm. D, Graph of the ratio of SMI-32<sup>+</sup>/MAP2<sup>+</sup> neurons showing the percentage of surviving motor neurons at 7 DIV after treatment with control media or ACM-hSOD1<sup>G93A</sup> applied for 4 days or 30 minutes. E, Percentage of surviving motor neurons at 7 DIV after 4 days or 30 minutes application of 3 DIV cultures of media that was conditioned by astrocytes derived from transgenic mice overexpressing hSOD1<sup>WT</sup> (ACM-hSOD1<sup>WT</sup>). Values represent means ± s.e.m from at least 3 independent experiments performed in duplicate, analyzed by one-way ANOVA followed by a *Tukey post hoc test.* ***P<0.001 relative to control media at 7 DIV (white bar).
**Figure 2.** ACM-hSOD1\(^{G93A}\) increases \(\text{PC}_{\text{Na}}\).  
A, Representative persistent inward current traces generated by a slow triangular voltage-clamp command (14 mV/s) from a holding potential of -60 to +10 mV (4 sec) and back (4 sec) (bottom graph) in the absence (colored traces) or presence (black traces) of the \(\text{Na}^+\) channel blocker TTX (1 \(\mu\)M); recordings from a 6 DIV control spinal neuron (upper traces) and a sister neuron incubated with ACM-hSOD1\(^{G93A}\) for \(\geq\) 30 minutes (middle traces). As can be observed from the merge (lower traces), \(\text{PC}_{\text{Na}}\) (arrow; TTX-sensitive inward current activated at voltages starting at around -40 mV) is significantly larger in neurons treated with ACM-hSOD1\(^{G93A}\) (red traces) relative to the control cell (green traces).  
B, Typical patched interneuron with cell membrane area (\(C_m\)) of \(\sim\)10 pF (left photo) and typical patched motor neuron with \(C_m\) of \(\sim\)20 pF (right photo).  
C, \(\text{PC}_{\text{Na}}\) of individual spinal cord neurons (5-7 DIV) were generated under control conditions (dotted line) or after application of ACM-hSOD1\(^{G93A}\) for 30-90 minutes (solid line) by a slow voltage-clamp command (as in Figure 2A) and plotted against its \(C_m\). The best-fitted slopes (S) highlight that \(\text{PC}_{\text{Na}}\) is dependent on the size neurons.  
D, Averaged mean peak \(\text{PC}_{\text{Na}}\) amplitude normalized to membrane capacitance (pA/pF) for 5-7 DIV control neurons and those incubated with ACM-hSOD1\(^{G93A}\). Values represent means \(\pm\) s.e.m from at least 9 neurons, analyzed by t-test. **\(P<0.01\) relative to control.

**Figure 3.** ACM-hSOD1\(^{G93A}\) rapidly increases neuronal excitability and \(\text{PC}_{\text{Na}}\).  
A, Whole-cell current-clamp recordings of control primary spinal neuron in the presence of ACM-hSOD1\(^{G93A}\). Action potentials (APs) are evoked by injection of rectangular depolarizing current pulses (bottom graph; 10, 20 and 40 pA for 300 msec). Representative membrane potential traces show that bath application of ACM-hSOD1\(^{G93A}\) to 5-7 DIV primary spinal neurons rapidly increases the firing frequency (arrowheads).  
B, Average change in AP firing
frequency produced by 30 minutes application of ACM-hSOD1\textsuperscript{G93A} (●) or ACM-hSOD1\textsuperscript{WT} (○).

Values represent means ± s.e.m from at least 5 neurons/time point, analyzed by t-test.

*P<0.05 compared to ACM-hSOD1\textsuperscript{WT} at same time point.

**Figure 4. ACM-hSOD1\textsuperscript{G93A} rapidly increases the frequency of calcium transients in cultured spinal neurons.** A, Representative ratiometric [Ca\textsuperscript{2+}]\textsubscript{i} transient traces of two independent spinal neurons (5-7 DIV) measured before (min 0; left graphs) and 30 min after application of either control media or ACM-hSOD1\textsuperscript{G93A} (min 30; right graphs). Parallel lines correspond to 30 minutes incubation periods. B-C, Mean fraction of calcium transient frequencies (B) and amplitudes (C) at 30 minutes after treatment with the different media, relative to control activity measured at min 0. Values represent means ± s.e.m from 23-45 neurons/condition, analyzed by one-way ANOVA followed by a Tukey post hoc test. *P<0.05 and ***P<0.001 relative to control media and ACM-mSOD1\textsuperscript{WT} at minute 30. D, Line series graphs of calcium transients of individual neurons at 0 and 30 min under control conditions (left graph) or after treatment with ACM-mSOD1\textsuperscript{WT} (middle graph) or ACM-hSOD1\textsuperscript{G93A} (right graph).

**Figure 5. Calcium transients in cultured spinal cord neurons correlate with action potential firing.** A, Simultaneous recording of spontaneous action potential firing in cell-attached mode (top trace) and calcium transients (bottom trace) in a spinal cord neuron. B, Expanded time scale for the same neuron in A. Note the coincidence of calcium transients with bursts of action potential firing. C, Bar graph showing the mean frequency (impulses/s) of action potential firing from 198 transients recorded in four cultured spinal cord neurons. Note that during inter-transient (inter-t.) periods, action potential firing is virtually absent. Values represent means ± s.e.m. t-test, ***P<0.001. D, Action currents in an expanded time scale from the last burst in B (dotted box).
**Figure 6. Na\textsubscript{v} channel blockers prevent ACM-hSOD1\textsuperscript{G93A}-induced motor neuron death at doses that slightly reduce excitability.**

**A**, Flow diagram of experiment. Media (ACM-hSOD1\textsuperscript{G93A} or control media) was applied chronically at 3 DIV (as in Fig. 1) alone or together with the Na\textsubscript{v} blockers TTX (1 nM), mexiletine (25 nM), spermidine (10 µM) or riluzole (100 nM) and cell survival was assayed at 7 DIV. **B**, Percentage of surviving motor neurons at 7 DIV treated with the different Na\textsubscript{v} blockers, relative to sister neurons treated with control media (white bar; indicated with *) or to ACM-hSOD1\textsuperscript{G93A} alone (first black bar; indicated with #). Note that the drugs mexiletine, spermidine and riluzole prevented motor neuron death induced by ACM-hSOD1\textsuperscript{G93A}. TTX trends towards prevention but is unable to significantly reduce ACM-hSOD1\textsuperscript{G93A}-mediated motor neuron death. **C**, Effects of the same doses of Na\textsubscript{v} channel blockers, co-applied with control media, on motor neuron survival. Note that the drugs did not improve survival of control neurons. Values represent means ± s.e.m. from at least 3 independent experiments, analyzed by one-way ANOVA followed by a Tukey post hoc test. *P<0.05, **P<0.01, ***P<0.001 relative to survival with control media at 7 DIV; ##P<0.01 and ###P<0.001 compared to survival with ACM-hSOD1\textsuperscript{G93A} at 7 DIV. **D**, Representative membrane potential traces (rectangular current pulse of 100 pA for 500 msec) of control 5-7 DIV primary spinal neurons treated with increasing concentrations of TTX, mexiletine, spermidine or riluzole; the boxes highlight the doses used for the above motor neuron survival studies. The number stated above the last AP in each trace indicates the number of spikes in the train. Note that at the doses at which mexiletine, spermidine and riluzole were able to prevent the ACM-hSOD1\textsuperscript{G93A}-induced cell death, these compounds only eliminated a few spikes within the train of APs. At higher doses, spike suppression was strong and drugs killed motor neurons (data not shown). Also, while even high concentrations of mexiletine and spermidine do not suppress the initial firing, increasing
doses of riluzole and TTX abruptly reduce the firing pattern and rate and ultimately abolish the first AP (arrows).

**Figure 7. Acute application of Na\textsubscript{v} channel blockers has differential effects on calcium dynamics modified by ACM-hSOD1\textsuperscript{G93A}.** A, Flow diagram of experiment. ACM-hSOD1\textsuperscript{G93A} was applied at 5-7 DIV spinal cultures for 30 min (as in Fig. 3) to measure calcium transients ("ACM - Na\textsubscript{v} blockers"). Thereafter, Na\textsubscript{v} channel blockers TTX (1 nM), mexiletine (25 nM), spermidine (10 µM) or riluzole (100 nM) were applied to measure calcium transients of the same neurons 7 min later ("ACM + Na\textsubscript{v} blockers"); Na\textsubscript{v} channel blockers were used at the same concentrations as in Figure 4. B-C, Bar graphs showing the mean fraction of calcium transient frequencies (B) and amplitudes (C) after the ACM-hSOD1\textsuperscript{G93A} and drug treatments ("ACM + Na\textsubscript{v} blockers") relative to ACM-hSOD1\textsuperscript{G93A} alone ("ACM - Na\textsubscript{v} blockers"). Note that all Na\textsubscript{v} channel blockers markedly decreased mean frequency (to 20-30%). However, whereas mexiletine, spermidine and riluzole significantly decreased the mean amplitude of the calcium transients (to 60%), TTX increases this parameter by \( \sim 225\% \). Values represent means ± s.e.m from 17-19 neurons/condition, analyzed by one-way ANOVA followed by a Tukey post hoc test. *\( \text{P}<0.05 \), ***\( \text{P}<0.001 \) relative to 30 minutes ACM-hSOD1\textsuperscript{G93A} (black bars).
Figure 1
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Figure 5
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Figure 6
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A. ACM-hSOD1<sup>G93A</sup> (0-37 min) → + Nav blockers (30-37 min): TTX (1 nM) Mexiletine (25 nM) Spermidine (10 μM) Riluzole (100 nM) → Calcium imaging: "ACM + Nav blockers"

B. Frequency of calcium transients (% of ACM - Nav blocker)

- ACM - Nav blockers
- ACM + Nav blockers

C. Amplitude of calcium transients (% of ACM - Nav blocker)

- ACM - Nav blockers
- ACM + Nav blockers

Figure 7
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