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Early Pathogenesis in the Adult-Onset Neurodegenerative Disease Amyotrophic Lateral Sclerosis

Brigitte van Zundert[1,*], **Pamela Izaurieta**[1,2], **Elsa Fritz**[1,2], and **Francisco J. Alvarez**[3]

[1]Faculty of Biological Sciences and Faculty of Medicine, Center for Biomedical Research, Universidad Andres Bello, AvenidaRepublica217, Santiago, Chile

[2]Faculty of Biological Science, University of Concepción, Concepción, Chile

[3]Department of Physiology, Emory University, Atlanta, Georgia 30322

Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating paralytic disorder caused by dysfunction and degeneration of motor neurons starting in adulthood. Most of our knowledge about the pathophysiological mechanisms of ALS comes from transgenic mice models that emulate a subgroup of familial ALS cases (FALS), with mutations in the gene encoding superoxide dismutase *(SOD1)*. In the more than 15 years since these mice were generated, a large number of abnormal cellular mechanisms underlying motor neuron degeneration have been identified, but to date this effort has led to few improvements in therapy, and no cure. Here, we consider that this surfeit of mechanisms is best interpreted by current insights that suggest a very early initiation of pathology in motor neurons, followed by a diversity of secondary cascades and compensatory mechanisms that mask symptoms for decades, until trauma and/or aging overloads their protective function. This view thus posits that *adultonset ALS* is the consequence of processes initiated during *early development*. In fact, motor neurons in neonatal mutant SOD mice display important alterations in their intrinsic electrical properties, synaptic inputs and morphology that are accompanied by subtle behavioral abnormalities. We consider evidence that human mutant SOD1 protein in neonatal $hSOD1^{G93A}$ mice instigates motor neuron degeneration by increasing persistent sodium currents and excitability, in turn altering synaptic circuits that control excessive motor neuron firing and leads to excitotoxicity. We also discuss how therapies that are aimed at suppressing abnormal neuronal activity might effectively mitigate or prevent the onset of irreversible neuronal damage in adulthood.

Keywords

ALS; SYNAPSE; EXCITOTOXICITY; PATHOLOGY; COMPENSATION; SODIUM CHANNELS

Patients with amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and other neurodegenerative disorders typically start to display clinical symptoms during adulthood. Groundbreaking genetic studies in humans, rodents, *Caenorhabditis elegans*, *Drosophila*, and yeast have identified mutations in genes such as SOD1, FUS/TLS and TDP-43 (for ALS), APP and presenilin (for AD),

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[*]Correspondence to: Dr. Brigitte van Zundert, Faculty of Biological Sciences and Faculty of Medicine, Center for Biomedical Research, Universidad Andres Bello, Avenida Republica 217, Santiago, Chile. bvanzundert@unab.cl.

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parkin and alpha-synuclein (for PD), and huntingtin (for HD), as being responsible for causing these diseases in adults, even though the mutation is present throughout life. Despite remarkable advances in understanding the diseases, no mechanism-based cures are currently available. This unfortunate situation is mainly due to the fact that the primary target(s) of the mutant proteins are not known, in part because early and ubiquitous expression of the aberrant genes in the developing CNS leads to many secondary effects and triggers compensatory mechanisms that mask the primary pathological event. Thus, although the primary disease process might be present throughout life, onset of symptoms coincides with the saturation of protection and compensation mechanisms, either by accumulation of dysfunction or via other pathogenic events such as trauma and environmental factors, which may be aggravated by aging [DeKosky and Marek, 2003; Palop et al., 2006]. To enable identification of mutant gene-driven mechanisms that trigger the cascade of events that culminate in degeneration and death later in life, a first step is to establish exactly when the neuropathology is initiated. The fact that the expression of the disease-causative proteins starts during embryonic stages raises the intriguing possibility that pathological changes in patients with *adult-onset* neurodegenerative disorders are triggered much *earlier*—in humans, such alterations may occur decades, and in mouse models months—prior to the manifestation of symptoms.

Recent support for this contention derives from findings that alterations are found in many CNS regions in pre-symptomatic transgenic mouse models (and even in pre-symptomatic human patients) of ALS (see below), AD [Santos et al., 2010], PK [Obeso et al., 2010], and HD [Raymond et al., 2011]. Based on evidence that is discussed more fully below, we propose a model (Fig. 1) in which mutant SOD1 initiates synaptic pathogenesis very early in development, causing alterations in neural circuits and network activity, and resulting in a vicious cycle that leads to neurological impairment. The onset of symptoms of ALS in humans may take years and even decades following these early events, and may become apparent only when compensatory mechanisms breaks down. Thus, it is imperative that the primary target(s) of disease-causing proteins be identified, which can then be used to establish pre-symptomatic diagnostic tools, and to develop novel therapies that can effectively prevent the onset of irreversible neuronal damage.

SOD1 MUTATIONS AND FAMILIAL ALS

ALS is a fatal paralytic disorder caused by the progressive dysfunction and degeneration of cranial, brainstem, and spinal cord motor neurons in adulthood that leads to death by respiratory failure within 3–5 years of diagnosis. At least 15 ALS-associated gene loci have been identified, although most of our knowledge of this disease is based on studies of a subgroup of familial (FALS) cases, all of which have their origin in mutations in the gene encoding superoxide dismutase (SOD1) [Pasinelli and Brown, 2006; Bento-Abreu et al., 2010]. SOD1 pathology, however, might be more widespread in ALS: recent studies in sporadic ALS (SALS) patients suggest that misfolded wild-type SOD1 is present in many cases and can acquire toxic properties, hereby inducing pathogenic mechanisms similar to mutant SOD1 [Bosco et al., 2010; Haidet-Phillips et al., 2011; Guareschi et al., 2012]. These findings, together with reports showing that different mutant SOD1 induce multiple misfolded conformations [Prudencio and Borchelt, 2011], might explain why more than 150 mutations (http://alsod.iop.kcl.ac.uk/), dispersed throughout the SOD1 sequence, all lead to the ALS phenotype.

SOD1 is a cytosolic metalloenzyme of 153 amino acids ubiquitously expressed in all mammalian cells. It catalyzes the dismutation (conversion) of toxic superoxide anion $\left(O_2^-\right.$ (to hydrogen peroxide (H_2O_2); this latter molecule is further detoxified to water and oxygen by glutathione peroxidase and catalase [Beckman et al., 2001]. Dominant inheritance of

mutant SOD1, lack of symptoms in SOD1 knockout mice, and absence of a correlation between the enzymatic activity of the different mutant proteins and motor neuron toxicity, together indicate that mutations in this protein cause ALS through a gain-of-toxic-function [Cleveland and Rothstein, 2001; Pasinelli and Brown, 2006]. The discovery of the gene mutations has also led to the generation of transgenic mice that overexpress mutant SOD1 protein, and consequently develop a motor neuron disease that closely resembles human ALS. In 1994, Gurney and colleagues generated the first transgenic ALS mouse model by expressing high levels of human SOD1 that contained a substitution of glycine to alanine at amino acid position 93 ($hSOD1^{G93A}$) driven by its endogenous human *SOD1* promoter; these mice become paralyzed in one or more limbs and die of respiratory failure at 4–5 months of age [Gurney et al., 1994; Bellingham, 2011], despite the fact that the mutation has little effect on SOD's enzyme activity. In the more than 15 years since the development of the Gurney model, the high expressor mutant $hSOD1^{G93A}$ transgenic mouse has been extensively studied—but disappointingly, we do not yet understand the exact molecular mechanisms that underlie mutant SOD1-mediated motor neuron degeneration. As a result, current therapies (e.g., riluzole; see below) rely more on alleviating symptoms rather than on modifying or curing the disease.

SOD1 AND THE HYPOTHESIS OF GLUTAMATE-INDUCED EXCITOTOXICITY

Understanding the cellular pathophysiological processes that underlie motor neuron dysfunction in ALS is an essential prerequisite for developing mechanism-based therapies for ALS. In vivo and in vitro studies with mutant hSOD1 transgenic mice reveal potential pathological changes in motor neurons that include excitotoxicity, hyperexcitability, disturbed calcium homeostasis, mitochondrial dysfunction, SOD1 aggregation, cytoskeletal disruption, deficits in axonal transport, activation of cell death signals, and oxidative stress [Cleveland and Rothstein, 2001; Pasinelli and Brown, 2006; Ilieva et al., 2009; Grosskreutz et al., 2010]. Whether these pathological events reflect the activity of a single unifying signaling cascade, or of multiple pathways that act in parallel to compromise the viability of motor neurons, is not yet clear. Based on decades of research, however, we can assert that glutamate-induced excitotoxicity is likely a node of convergence for multiple signaling cascades. The increased glutamate-induced excitotoxicity observed in ALS patients and in mouse models of ALS may be the result of increased glutamate release because enhanced Na_v channel activity (hyperexcitability), decreased uptake of extrasynaptic glutamate by reduced EAAT2/GLT1 transporter activity/number, and/or alterations in synaptic glutamate receptor number and Ca^{2+} permeability (Fig. 2 and Supplementary information for references indicated in this figure). In turn, glutamate excitotoxicity increases the Ca^{2+} load of motor neurons and interneurons. The Ca^{2+} buffering capacity of motor neurons is limited, and relies heavily on mitochondrial uptake; thus excess Ca^{2+} might further alter the function of mitochondria in motor neurons, which could already be affected by abnormal accumulations of mutated SOD1 aggregates. Mitochondrial dysfunction is likely a following central event causing energetic and metabolic failures that trigger cellular stress responses, affect motor neuron axon function (including transmission at the neuromuscular junction) and, eventually lead to apoptosis cascades and cell death (Fig. 2) [for reviews see Bento-Abreu et al., 2010; Grosskreutz et al., 2010; Carrì and Cozzolino, 2011].

EARLY PATHOGENIC PROCESSES IN MOTOR NEURONS AND DISEASE PROGRESSION

Many alterations in pre-synaptic neurons, post-synaptic neurons, or glial cells that might contribute to glutamate-induced excitotoxicity in ALS have been described (Fig. 2). A fundamental question concerns the manner in which mutated SOD1 induces glutamate

excitotoxicity: whether this is an early event associated with primary pathology (Fig. 1), or whether it is a late event linked to disease onset?

To reveal when the earliest deficits occur in ALS mouse models, studies must focus on early pre-symptomatic stages, and use techniques that are sensitive enough to determine subtle changes in individual neuronal subtypes. Using single cell-based techniques such as electrophysiological recordings, electron microscopy, immunohistochemical techniques, and functional as well as structural studies of motor units, many abnormalities in vulnerable motor neurons are detected in ALS mouse models weeks and even months before disease onset, this being defined by overt weakness and motor neuron death [Chiu et al., 1995]. In fact, and as indicated in the established time-line shown in Figure 3, in the high-expresser $hSOD1^{G93A}$ mouse (in contrast to wild-type and high-expresser $hSOD1^{WT}$ mouse) alterations in electrophysiological properties and synaptic circuits are detected already during the first days after birth. For example, use of acute slice preparations made from brains of P4–P10 mice reveals that hypoglossal brainstem motor neurons of the $hSOD1^{G93A}$ model display increased repetitive firing and persistent inwards currents (PICs) mediated by voltage-sensitive Na^+ channels (PC_{Na}) [van Zundert et al., 2008]. Analysis of young motor neurons in acute spinal cord slice preparations of $hSOD1^{G93A}$ mice also shows an enhanced PC_{Na} from P0 through P12; moreover, from P6 through 12, the PC_{Na} is accompanied by significantly larger PICs that are mediated by Ca^{2+} (PC_{Ca}), which are likely regulated by L-type $Ca_{v1.3}$ channels [Quinlan et al., 2011]. In addition, brainstem neurons from P4–10 $hSOD1^{G93A}$ mice display increases in spontaneous synaptic activity, mediated via excitatory glutamatergic AMPA/kainate receptors, and also via inhibitory $GABA_A$/glycine receptors, suggesting an augmented activity in networks that drive motor neurons, which can result in enhanced and disorganized motor neuron bursting activity [Jiang et al., 2009]. Brainstem motor neurons in neonatal $hSOD1^{G93A}$ mice display also NMDA receptor currents with fast decay times, typically seen when receptor subunit composition changes from NR2B-rich (large calcium influx) to NR2A-rich (reduced calcium influx) [van Zundert et al., 2008]; such a subunit change could occur as a consequence of early activity-driven maturation, and might slow down activity-induced toxicity. Altered expression of NMDA receptor subunits can also influence the dendritic architecture of neurons [Sepulveda et al., 2010]; in support of this idea, precocious dendritic remodeling has been reported for hypoglossal motor neurons in the $hSOD1^{G93A}$ mice [van Zundert et al., 2008]. Subtle neuromotor abnormalities are also detected in these mice, likely as a consequence of neuronal circuitry alterations: forelimb placing and righting responses are transiently delayed between P2 and P4, but then recover [van Zundert et al., 2008]. Additional behavioral tests of labyrinth function and vibrissa sensibility were normal, highlighting that only specific motor functions are impaired in ALS.

Further evidence for abnormalities during early postnatal development in ALS is reported for the $hSOD1^{G85R}$ and the low-expressor $hSOD1^{G93A}$ mouse models, in which alterations in neuronal excitability, reflex responses, and dendritic branching of lumbar motor neurons were also observed [Amendola et al., 2004; Durand et al., 2006; Bories et al., 2007; Amendola and Durand, 2008; Pambo-Pambo et al., 2009; Filipchuk and Durand, 2012]. Note, however, that despite similar alterations in dendritic branching the reported alterations on neuronal excitability in the two ALS model systems are not identical: neonatal motor neurons expressing $hSOD1^{G85R}$ show *hypo*-excitability [Bories et al., 2007; Pambo-Pambo et al., 2009], whereas those expressing $SOD1^{G93A}$ (either low- or high-expressors, in slice preparations or cultures) display *hyper*-excitability [Kuo et al., 2005; van Zundert et al., 2008; Pambo-Pambo et al., 2009; Pieri et al., 2009]. Many factors (e.g., type of SOD1 mutant, different time course of the disease in each model, type of recordings, or type of neuronal preparations studied) may contribute to the discrepancies between the excitability changes in the different SOD1 models: here we underscore, however, the results of recent

computer modeling studies, which suggest that alterations in morphology (increased dendrite surface) and membrane biophysical properties (decrease in membrane resistivity, R_m) of hSOD1^{G85R} mice motor neurons are both necessary to explain decreased input resistances (R_i) and their hypo-excitability, offsetting the presence of increased PICs [Amendola and Durand, 2008; Elbasiouny et al., 2010a]. In contrast, neonatal hSOD1^{G93A} motor neurons display small changes, if any, in R_i [van Zundert et al., 2008; Quinlan et al., 2011]. A possible lack of changes in R_m and the fact that most dendrite surface is added at some distance from the cell body (where R_i is measured), suggest that the increase in firing response to somatic injected current in hSOD1^{G93A} motor neurons (hyper-excitability) is dominated by an increase of PC_{Na} [van Zundert et al., 2008]. An important question that has not yet been addressed is whether the functional and structural alterations that lead to changes in excitability are causal or adaptive in nature. In the context of the first possibility, note that most of the electrical and morphological alterations observed following the expression of mutant SOD1 are normally seen in more mature wild-type motor neurons, raising the possibility that pathology could be, at least in part, the result of accelerated aging [see also Discussion in Quinlan et al., 2011]. Independent of the motor neuron type of excitability, however, the observed abnormalities in function, neuronal structure, and behavior in the different transgenic SOD1 mouse models collectively argue that the pathological synaptic circuit alterations in the *adult-onset* disease ALS are actually initiated very *early during development.*

Early maturation of other pathways has also been documented in the ALS mouse models. Thus, ER stress-management pathways are upregulated as early as P12 in the more vulnerable SOD1^{G93A} motor neuron subpopulations [Saxena et al., 2009], and coincide with the earliest ultrastructural evidence of mitochondria pathology [Bendotti et al., 2001]; both changes are evident just after the detected alterations in electrophysiological properties. In addition, upregulation of unfolded protein response (UPR)-related genes and proteins initiates at P32 and peaks at P38 [Saxena et al., 2009]. The ER stress responses are likely responsible for the formation of aggregates of mutant SOD1 observed starting to be detectable at P30 in spinal motor neurons [Johnston et al., 2000]. Soon after, SOD1^{G93A} spinal motor neurons display DNA damage (P49) [Martin et al., 2007] and start to express apoptotic markers (P60) [Li et al., 2000]. In parallel, functional and structural changes in motor units are reported from P40 to P50 in mSOD1^{G93A} mice [Frey et al., 2000; Hegedus et al., 2007], with fast motor units exhibiting pathological changes earlier than slow motor units. Collateral sprouts from slow motor axons transiently reoccupy vacated neuromuscular junctions, providing some compensation that may allow the animal to maintain muscle strength until clear signs of disease progression are evident in the majority of motor units [Frey et al., 2000; Hegedus et al., 2007]. Finally, at ∼P90, limb weakness can be clearly demonstrated by the loss of grip strength in the hanging wire test [Chiu et al., 1995].

In summary, the earliest changes in the hSOD1^{G93A} model are characterized by hyper-activity and alterations in protein metabolism. Both precede the expression of functional deficits in the motor unit, which are the immediate cause of paresis. This pathology is eventually followed by induction of apoptosis/necrosis pathways and motor neuron cell death. Whether the early processes are independent, synergistic or interact in complex ways is not yet fully understood. In addition, it is important to dissect which processes are cell-autonomous and which mechanisms receive contribution from other cells. In particular, increases in motor neuron activity can be strongly influences by other cells. First, glial cells are important modulators of synaptic function and deficits in glial-mediated glutamate clearance might be a major contributor to disease [Bento-Abreu et al., 2010]. Second, the pre-motor interneuronal networks show alterations that could modify the balance of excitation and inhibition resulting in an excess of excitatory drive. It is likely that neurons other than motor neurons are also hyper-excitable. In fact, electrophysiological recordings

from P10 to P12 interneurons of the superior colliculus (SC) from neonatal hSOD1 ^{G93A} mice showed increases in PC_{Na} similar to vulnerable brainstem motor neurons [van Zundert et al., 2008]. Cortical circuit hyper-excitability is also present in ALS patients, even before the onset of motor symptoms, and in pre-symptomatic animal models [Vucic et al., 2008; Kiernan and Petri, 2012; and Fig. 2 for additional reference]. Similarly, excessive network excitability has been observed using in vitro spinal cord preparations [Jiang et al., 2009] and decreasing excitatory synaptic activity in VGLUT2 mutants has been shown to be partially protective of motor neuron degeneration [Wootz et al., 2010].

Excessive excitatory drive might be exacerbated by decreases in inhibition and there are some suggestions that motor neuron vulnerability is correlated with the composition and strength of inhibitory synapses [Lorenzo et al., 2006]. In particular, the recurrent inhibitory feedback circuit mediated by Renshaw cells and that controls the level of motor neuron firing is altered just before disease onset [F.J.A. and B.v.Z. unpublished work]. In this circuit, motor axon intraspinal collaterals establish synapses that excite interneurons known as Renshaw cells and these in turn inhibit the same motor neurons through a feedback loop [Alvarez and Fyffe, 2007]. Degenerative changes of motor axon synapses on Renshaw cells starting at 60–80 days in the hSOD1 ^{G93A} model results in the break-down of this circuit resulting in the loss of an important inhibitory control for motor neuron excessive firing [F.J.A. and B.v.Z. unpublished work]. Thus, motor axon synaptic pathology closely correlates temporally both centrally (inside the spinal cord) and in the periphery (at the neuromuscular junction) and its timing is suggestive that it is a contributor to the initiation of the last phase of motor neuron degeneration and eventual death. In addition, there might be more subtle changes that directly decrease inhibitory glycinergic (but not GABAergic) synaptic strength in general, even from the earliest stages of synaptogenesis [Chang and Martin, 2011], and there is also evidence that premotor inhibitory interneurons show stress markers and synaptic deficits around disease onset [Hossaini et al., 2011]. The loss of inhibition from feedback control circuits, and possibly also other inhibitory inputs, reduces the capacity of spinal circuits to counteract excessive motor neuron hyper-excitability.

POSSIBLE CELLULAR AND MOLECULAR MECHANISMS THAT UNDERLIE EARLY HYPER-EXCITABILITY IN ALS

We argue that very early increases in Na^+ and Ca^{2+}-mediated PICs could play a pivotal role in initiating the cascade of events responsible for hyper-excitability leading to glutamate excitotoxicity.

WHAT IS THE PHYSIOLOGICAL FUNCTION OF PICs?

Although PICs are only a small fraction of the total current (e.g., 1– 5% in the case of PC_{Na}), they can profoundly affect neuron and network behavior. The prevailing view is that because PC_{Na} is generated by the same Na_v channels that produce the typical fast transient sodium current (T_{Na}), and because PC_{Na} can be activated close to the cell's resting potential, small increases in this current can enhance intrinsic excitability, alter spike initiation, and amplify the firing rate [Crill, 1996; Goldin, 2003; Elbasiouny et al., 2010b]. In addition, the excitatory synaptic inputs received by a given neuron can be greatly amplified and prolonged by PC_{Na} and PC_{Ca}, thereby impacting the neuron's output by increasing its firing rates in response to synaptic modulation [Elbasiouny et al., 2010b].

HOW CAN INCREASED PICs DURING NEONATAL DEVELOPMENT BE PATHOLOGICAL IN ALS?

The increased PC_{Na} and PC_{Ca} detected in motor neurons and interneurons of the spinal cord and brainstem in slice preparations made from neonatal mSOD1^{G93A} mice is accompanied

by alterations in the excitability, synaptic inputs, and morphology of the neurons [van Zundert et al., 2008; Quinlan et al., 2011; Fig. 3]. Importantly, these abnormal functional and structural alterations are also linked to minor behavioral symptoms in mSOD1^{G93A} neonates [van Zundert et al., 2008]. Based on these findings, and on the fact that PICs largely influence synaptic inputs and firing rates, we hypothesize that, during neonatal development of mutant SOD1 mice, increases in Na^+ and Ca^{2+} PICs in motor neurons as well as in non-motor neurons significantly enhance intrinsic and extrinsic (network) excitability, amplify and prolong synaptic inputs, and increase firing rates (Fig. 4). It is also interesting that recurrent inhibition through Renshaw cells is a major modulator of PIC-mediated amplification of synaptic inputs and firing [Bui et al., 2008]. Therefore, the failure of this circuit before disease onset would exacerbate pathology resulting from excessive PIC activity already established during early development. The post-synaptic and pre-synaptic membrane depolarization induced by influxes through Na_v channels (PC_{Na}) and $Ca_{v1.3}$ channels (PC_{Ca}), will also cause sustained Ca^{2+} (and Na^+) influxes through other receptors/channels, such as NMDA- and AMPA-receptors and other Ca_v channels, hereby directly contributing to excitotoxicity. Because motor neurons express low levels of Ca^{2+}-binding proteins (calbindin-D28K, parvalbumin), Ca^{2+} influx in these cells would have to be mainly buffered by mitochondria: while these organelles do have a limited capacity for buffering of Ca^{2+} that aids in maintaining mitochondrial function, prolonged Ca^{2+} overload would structurally and functionally damage mitochondria, and lead to a deficit in energy production and apoptotic and/or necrotic cell death cascades. Failure to control hyper-excitability and excitotoxicity in the hSOD1^{G93A} mice leads ultimately to motor neuron degeneration and death 4–4.5 months later.

HOW WOULD mSOD1 INFLUENCE THE FUNCTION OF Na_V CHANNELS AND PC_{Na}?

Regulation of PC_{Na} has been well studied: it is widely believed that this current can be modulated by several mechanisms, including the alpha and beta subunits of Na_v channels (PC_{Na} is greater for $Na_{v1.1}$ and $Na_{v1.6}$ as compared to $Na_{v1.2}$ and $Na_{v1.3}$), several protein kinases, and different types of reactive oxygen and nitrogen species (ROS/ RNS) [Franceschetti et al., 2000; Hammarström and Gage, 2000; Goldin, 2003; Kassmann et al., 2008; Nani et al., 2010]. Of particular interest to ALS are biochemical studies that indicate that mutant SOD1, either alone or by binding to Rac1/NADPH-oxidase (Nox), can paradoxically generate O_2^-; together with nitric oxide (NO), the O_2^- can produce peroxynitrite ($ONOO^-$) [Harraz et al., 2008; see also Beckman et al., 2001 and Cleveland and Rothstein, 2001 for discussion on several hypotheses concerning the production of different ROS/RNS by mutant SOD1]. Evidence for the harmful production of Nox-derived O_2^- was obtained in vivo in both human SALS and the SOD1 ^{G93A} transgenic mouse model [Wu et al., 2006; Harraz et al., 2008]. Deleting the Nox protein gp91^{phox} increased the lifespan and reduced neurodegeneration in SOD1^{G93A} transgenic mice [Wu et al., 2006]. Moreover, treatment of hSOD ^{G93A} transgenic mice with the Nox inhibitor apocynin (starting at P14) markedly slows disease progression (from approximately 100 to 200 days), and resulted in a large increase in life span (from 125 to 238 days) [Harraz et al., 2008].

In vitro studies also show that SOD1^{G93A}-expressing astrocytes and microglial cells can produce toxic ROS/RNS that kills wild-type motor neurons [Cassina et al., 2008; Harraz et al., 2008]. Similar results are obtained when microglial cells are incubated with extracellular SOD1^{G93A} protein [Zhao et al., 2010]. These findings are in line with the fact that ALS is at least partially a non-cell autonomous disease, and that expression of mutant SOD1 in cultured astrocytes can induce the release of toxic factors [Di Giorgio et al., 2007; Nagai et al., 2007; Haidet-Phillips et al., 2011; reviewed in Ilieva et al., 2009]. It might be interesting to undertake an electrophysiological analysis aimed at determining which specific ROS/

RNS is released by ALS astrocytes and capable of activating Na_v channels, and thereby increase PC_{Na} and motor neuron excitability.

Undoubtedly, the hSOD1 ^{G93A} -generated ROS/RNS would have many targets besides Na_v channels. Several studies provide compelling evidence that a number of proteins, and even RNA, are modified by ROS/RNS in ALS patients and in transgenic mouse models [Wu et al., 2006; Chang et al., 2008]. However, when compared to the functional alterations in the Na_v channels, the protein and RNA modifications may cause less immediate damage. For example, ROS/RNS can actually decrease the function of many Ca^{2+} permeable channels/receptors, including the NMDA receptors [Bodhinathan et al., 2010], AMPA receptors [Plested and Mayer, 2009] and L-type Ca_v channels [Li et al., 2007]—this is accomplished either directly by altering their redox state, or indirectly by changing the oxidative state of their signaling or scaffolding proteins, which therefore could "protect" neurons (at least temporally) from toxic Ca^{2+} influxes.

CONSEQUENCES OF EARLY ABNORMAL NEURONAL ACTIVITY ON THERAPEUTIC PROSPECTS

Findings on the outcome of abnormal neuronal activity that are reviewed below increase our understanding about the molecular-pathophysiological underpinnings of ALS. Here, we present these observations within the framework of two questions that are fundamental for the development of meaningful therapies: (i) Is abnormal neuronal activity also a key event in SALS? and (ii) Why are Na_v channel inhibitors not more effective in delaying onset of ALS symptoms and in enhancing motor neuron survival?

CAN FAMILIAL AND SPORADIC ALS BOTH BE TRIGGERED BY A COMMON PATHOGENIC MECHANISM(S) THAT INVOLVES ABNORMAL NEURONAL ACTIVITY?

Patients with FALS and SALS present symptoms that are indistinguishable: we thus argue that disease progression in both types of patients has a common pathogenic mechanism. The most widely accepted argument that supports the contribution of glutamate excitotoxicity to the pathogenesis of ALS is based on the beneficial effects of riluzole: the drug extends the survival of patients with SALS and FALS by ∼3 months [Bellingham, 2011]. At the clinical level, mounting evidence also suggests that motor neuron hyperexcitability is a common pathophysiological feature in both types of ALS, and that such increased activity precedes clinical onset in FALS patients [Vucic et al., 2008; Kiernan and Petri, 2012 and see references listed in Fig. 2]. At the molecular level, recent studies with the use of a conformation-specific antibody (C4F6), which was generated specifically against the hSOD1 ^{G93A} mutant protein, is of particular interest: while the C4F6 antibody does not react with unmodified endogenous wild-type SOD1, it does recognize oxidized wild-type SOD1 (SOD1ox) [Urushitani et al., 2007; Bosco et al., 2010]. Furthermore, positive C4F6 staining on spinal cord tissue is observed in almost 50% of SALS cases tested [Bosco et al., 2010] and wild-type SOD1 is oxidized in SALS patients [Guareschi et al., 2012]. Together, these findings indicate that wild-type SOD1 can mimic the structural features of FALS SOD1 mutants, and can become pathogenic in SALS via non-heritable, post-translational modifications. We thus conclude that the SOD1-dependent pathogenic mechanisms—and also the comparable clinical symptoms (including hyperexcitability) shared by patients with sporadic as well as familial ALS—argue in favor of the possibility that suppression of abnormal neuronal activity early in life will prove beneficial in both forms of ALS.

WHY ARE Na_V INHIBITORS NOT MORE EFFECTIVE IN DELAYING ONSET AND SURVIVAL?

Riluzole is thought to exert its beneficial effects by reducing glutamatergic synaptic activity, although it is precise mechanism(s)-of-action remain unclear because this drug can affect

multiple targets [Bellingham, 2011]. Nevertheless, clinically relevant concentrations (1–2 μM in plasma) of riluzole are most effective in blocking Na_v channels, thereby suppressing PC_{Na} and neuronal excitability [Kuo et al., 2005; Theiss et al., 2007; Bellingham, 2011; Schuster et al., 2012]. Surprisingly, however, riluzole treatment starting at P30–P50 delays death in transgenic $mSOD1^{G93A}$ ALS mice by only 9–14 days [Bellingham, 2011]. This modest delay in survival after riluzole treatment is disappointing from a therapeutic and conceptual point of view, especially when considering that riluzole did not significantly affect disease onset. These studies need, however, to be considered in view that electrophysiological abnormalities that are likely targeted by riluzole are initiated much earlier (P4–P10) than the start of the drug treatment in these studies (P30–P50). Increased excitability and neuronal activity during neonatal development might induce a pathophysiological cascade of events that cannot be reversed by later riluzole treatment.

In addition to the timing of treatment, another concern is that the precise mechanisms, whereby mutant SOD1 is toxic to motor neurons are not fully defined and it is possible that other processes induced at early stages independently contribute to the eventual demise of motor neurons. For example, future studies should elucidate how early abnormal protein metabolism is related to hyper-excitability and whether these two early alterations constitute independent parallel pathways of cellular pathology with additive properties. One possibility is that a constellation of early changes is induced and each one has the capacity of causing motor dysfunction by itself given enough time. This might explain why most treatments targeting just a single mechanism (as riluzole) achieve at most a slight slowing of the pathology.

CONCLUSION

Detailed analyses of the $mSOD1^{G93A}$ mouse model during various stages of development have gradually revealed that pathologies in motor neurons and interneurons can be detected from the first postnatal days, much before the onset of behavioral symptoms. It is not clear whether the Na_v channel-mediated abnormal neuronal activity and circuit alterations observed in neonatal mice are the first pathogenic processes or whether in fact the disease starts even earlier, during embryonic stages, when the expression of SOD1 begins. Identification of the primary pathogenic event in SOD1-ALS mice is imperative for the development of rational, mechanism-based therapies, and will likely yield important insights about the underlying mechanisms for disease onset in ALS that is induced by other mutations (e.g., TDP43 and FUS/TLS) as well as in sporadic ALS. Without such insights, even the development of symptomatic treatments for ALS patients is a challenge, because we do not know how secondary events and compensatory mechanisms are defining the course of pathogenic processes. Results of longitudinal studies on disease pathogenesis should also lead to the identification of specific biomarkers of temporal disease progress, which could result in more precise interventional treatments for specific disease stages. Longitudinal studies also have the potential to uncover the compensatory mechanisms that the body uses to mitigate the effects of degeneration of the vulnerable motor neurons. Such novel therapies should prolong the intrinsic compensatory mechanisms and significantly delay the suffering of ALS patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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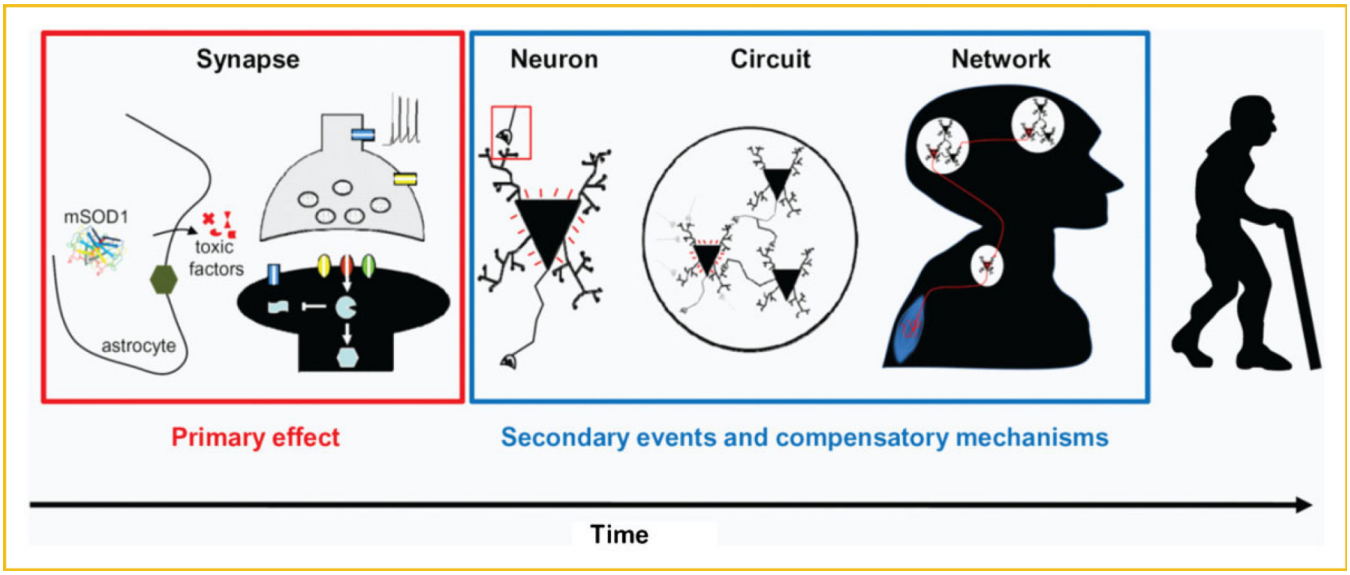

Fig. 1.

Model of how mutant SOD1 may underlie the etiology of ALS. Subtle synaptic dysfunction may be initiated by mutant SOD1 during development: either at early postnatal stages, or even during embryogenesis, when expression of wild-type and mutant SOD1 is first detected. Although this primary event causes little pathogenesis by itself, it may initiate a diversity of secondary cascades and compensatory mechanisms, which will influence the development and functioning of neuronal circuits and networks. Gradually (and in a vicious cycle) neuronal function is lost; however, during this early period—which can span a period of months in mouse models and decades in humans— symptoms have not yet manifested. Onset of disease may become apparent when compensatory mechanisms saturate and/or break down, either by accumulation of dysfunction or via other pathogenic events, such as trauma and environmental factors that may be aggravated by age. The mechanism(s) by which mutant SOD1 causes synaptic dysfunction is unknown, however, it could relate to the fact that ALS is at least partially a non-cell autonomous disease, and that expression of mutant SOD1 in astrocytes can induce the release of toxic factors (see text for more details).

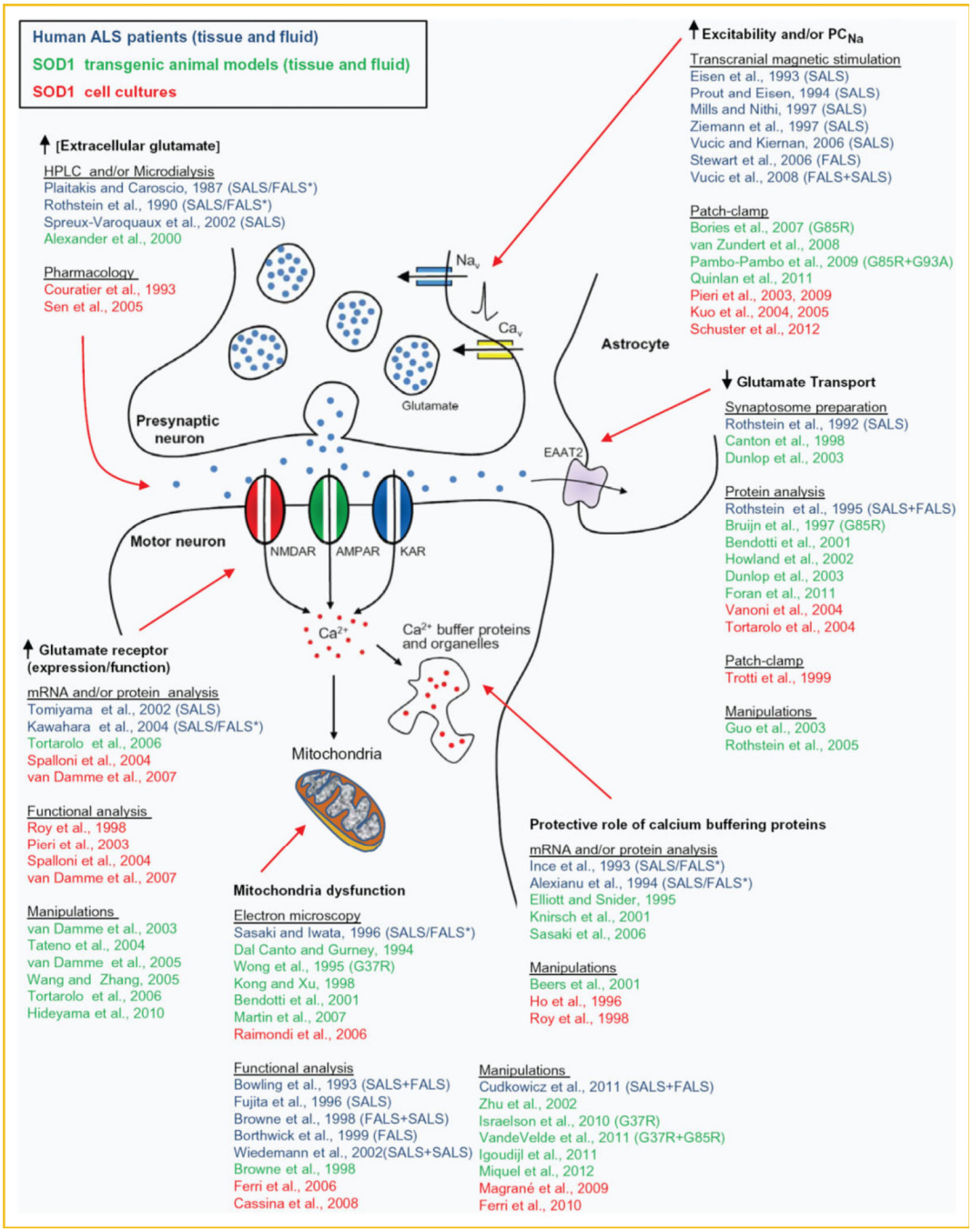

Fig. 2.

Evidence for the hypothesis that glutamate-induced excitotoxicity underlies the pathology in ALS. Schematic diagram to show how a variety of alterations in pre-synaptic neurons, post-synaptic neurons or glial cells might result in excessive Ca^{2+} entry into motor neurons. Citations listed on the diagram refer to reports that document the cellular pathophysiological processes underlying glutamate-induced excitotoxicity by analyzing fluid and tissue samples from either ALS patients (in blue) or SOD1 ALS mice models (in green). Results from cell culture-based SOD1 ALS models are also shown (in red). Studies are from high-expressor SOD1 ^{G93A} unless stated.* indicates that the SOD mutation was not identified in the FAIS

patients. We have tried to acknowledge as many original studies as possible and apologize for those we have omitted. Reference list is shown in the supplementary data.

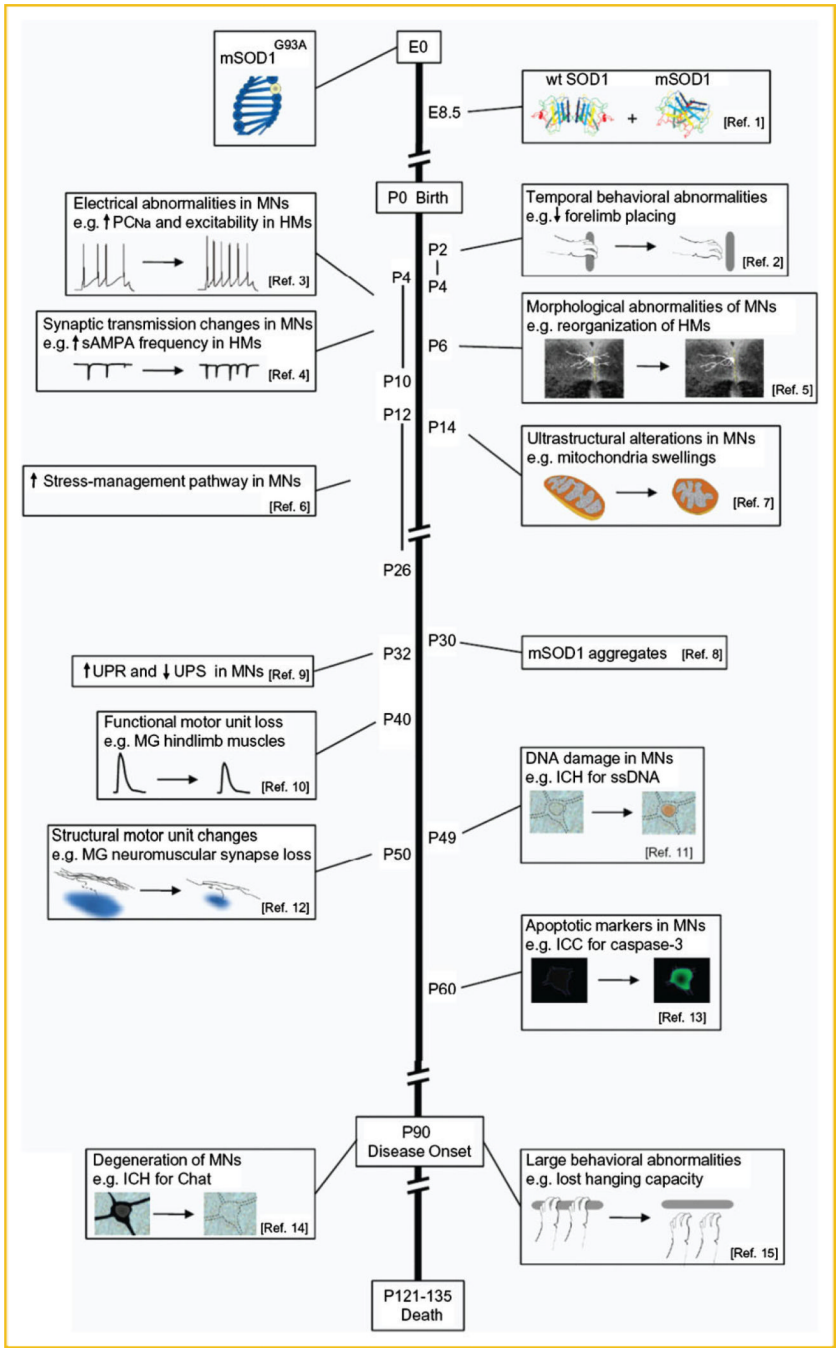

Fig. 3.

Time-line of pathological and clinical changes in the high-expressor hSOD1^{G93A} line of transgenic mice. The most important and earliest abnormalities reported for the hSOD1^{G93A} transgenic mouse model are shown. Months before motor neurons degenerate and clinical symptoms appear (P90), widespread and early onset of pathological abnormalities are detected in this ALS mouse model. See references stated here and text for additional information. (*Ref. 1* SOD1 expression is detected from embryonic (E) day 8.5 (E8.5) [Yon et al., 2008]). (*Ref. 2* Temporal behavioral abnormalities during early postnatal (P) development: P2–4 mSOD1^{G93A} mice show reduced forelimb placing and righting capacities [van Zundert et al., 2008]. Similar reversible sensorimotor alterations are

observed for neonatal (P1–P7) hSOD1^{G85R} mice [Amendola et al., 2004]). (*Ref. 3* Electrical abnormalities in hSOD1^{G93A} motor neurons (MNs) at P4–P10; hypoglossal motor neurons (HMs) display increased excitability and PC_{Na} [van Zundert et al., 2008]. hSOD1^{G93A} spinal cord motor neurons also possess enhanced PC_{Na} (P0–P12)and PC_{Ca}(P6–P12) [Quinlan et al., 2011]. In addition, early (P6–P10) abnormal changes in electrical properties, including in excitability, are detected in the low-expressor hSOD1^{G93A} transgenic line and the hSOD1^{G85R} mutant mice [Bories et al., 2007; Pambo-Pambo et al., 2009]). (*Ref. 4* Synaptic transmission mediated by AMPA, NMDA and glycine-receptors is altered for HMs in P4–10 hSOD1^{G93A} mice [van Zundert et al., 2008]). (*Ref. 5* Morphological abnormalities of motor neurons of hSOD1^{G93A} mice at P6; precocious remodeling of HMs [van Zundert et al., 2008]. Early (P6–P10) abnormal dendritic branching is also observed for hSOD1^{G85R} transgenic mice [Amendola and Durand, 2008]). (*Ref. 6* Genes involved in stress-related pathways are transiently upregulated between P12 and P26, in vulnerable hSOD1^{G93A} spinal motor neurons [Saxena et al., 2009]). (*Ref. 7* Ultrastructural alterations of motor neurons in mSOD1^{G93A} mice, starting at P14 (2 weeks); mitochondria swellings and small vacuoles are present in distal dendrites and in the cell bodies of spinal cord motor neurons [Bendotti et al., 2001]). (*Ref. 8* mutant SOD1 aggregates in spinal motor neurons of hSOD1^{G93A} mice at P30 [Johnston et al., 2000]). (*Ref. 9* Genes involved in UPR and in the ubiquitin proteasome system (UPS) are up- and down-regulated, respectively, in vulnerable P32 spinal motor neurons of hSOD1^{G93A} mice [Saxena et al., 2009]). (*Ref. 10* Functional loss of motor unit for fast-twitch hind-limb muscles (medial gastrocnemius [MG]) is detected starting at P40in hSOD1^{G93A} mice [Hegedusetal., 2007]). (*Ref. 11* DNA damage (e.g., immunohistochemistry (ICH) with antibodies recognizing single-stranded breaks) is detected in motor neurons of hSOD1^{G93A} mice, starting at P49 (7 weeks) [Martin et al., 2007]). (*Ref. 12* Structural changes in motor units of hSOD1^{G93A} mice starting at P50; loss of MG neuromuscular synapses and prominent vacuolation in nerve terminals [Frey et al., 2000]). *[Ref. 13* Markers of apoptosis (including caspases 1 and 3) in motor neurons are detected as of P60 in spinal cord motor neurons of hSOD1^{G93A} mice [Li et al., 2000]. See Martin et al., [2007] for discussion on apoptotic-necrotic hybrid forms of motor neuron death in ALS]. (*Ref. 14* Degeneration of motor neurons (e.g., those that display choline acetyltransferas (Chat)-positive IRR)of hSOD1^{G93A} mice starts at ∼P90 [Chiu et al., 1995]). (*Ref. 15* Clinical symptoms (e.g., small tremors, weight lose) [Chiu et al., 1995] and more prominent behavioral abnormalities (e.g., decline in hanging test and rotarod performance) of hSOD1^{G93A} mice starts at ∼P90). See also additional reviews for deficits during development in other mutant SOD1 mice, including the low-expressor hSOD1^{G93A}, hSOD1^{G85R}, and hSOD1^{G127X} mice [Durand et al., 2006; Elbasiouny et al., 2010b; Quinlan, 2011].

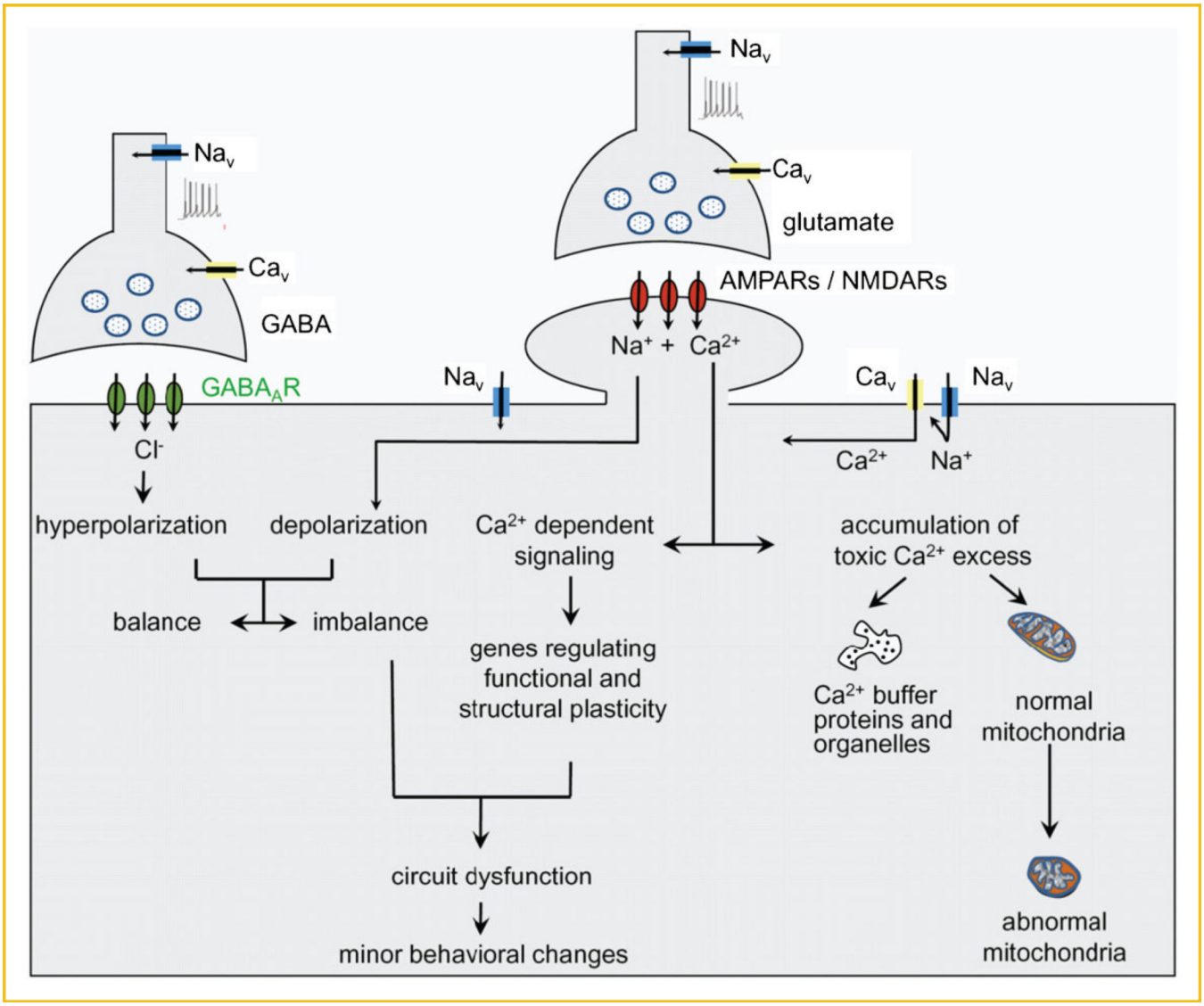

Fig. 4.

Model of how PC_{Na} might induce synaptic dysfunction in pre-symptomatic neonatal/juvenile $hSOD1^{G93A}$ transgenic mice. Based on the alterations in electrophysiological properties and synaptic circuits that are detected in motor neurons and interneurons of $hSOD1^{G93A}$ mice during the first days after birth, we propose a model in which small increases in PC_{Na} mediated by Na_v channels and PC_{Ca} mediated by Ca_v channels (likely $Ca_{v1.3}$) may significantly enhance neuronal excitability and increase synaptic transmission, leading to sustained toxic influxes of and Ca^{2+} (and Na^+) through NMDA- and AMPA-receptors, and through Ca_v channels. In motor neurons, the limited expression of Ca^{2+} buffering proteins obligates the mitochondria to perform the buffering task, leading to mitochondrial dysfunction and damage. Excess Ca^{2+} also induces expression of plasticity-related genes that may, together with the imbalance between hyper-polarization and depolarization, disrupt local circuitry and networks, induce minor behavioral changes. The underlying mechanism(s) responsible for the increased PC_{Na} has not been determined, but could be related to the ROS/RNS produced by astrocytes that express mutations in SOD1 (not shown).