An ALS-Associated Mutant SOD1 Rapidly Suppresses KCNT1 (Slack) Na⁺-Activated K⁺ Channels in *Aplysia* Neurons

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Mutations that alter levels of Slack (KCNT1) Na⁺-activated K⁺ current produce devastating effects on neuronal development and neuronal function. We now find that Slack currents are rapidly suppressed by oligomers of mutant human Cu/Zn superoxide dismutase 1 (SOD1), which are associated with motor neuron toxicity in an inherited form of amyotrophic lateral sclerosis (ALS). We recorded from bag cell neurons of *Aplysia californica*, a model system to study neuronal excitability. We found that injection of fluorescent wild-type SOD1 (wt SOD1YFP) or monomeric mutant G85R SOD1YFP had no effect on net ionic currents measured under voltage clamp. In contrast, outward potassium currents were significantly reduced by microinjection of mutant G85R SOD1YFP that had been preincubated at 37°C or of cross-linked dimers of G85R SOD1YFP. Reduction of potassium current was also seen with multimeric G85R SOD1YFP of ~300 kDa or >300 kDa that had been cross-linked. In current clamp recordings, microinjection of cross-linked 300 kDa increased excitability by depolarizing the resting membrane potential, and decreasing the latency of action potentials triggered by depolarization. The effect of cross-linked 300 kDa on potassium current was reduced by removing Na⁺ from the bath solution, or by knocking down levels of Slack using siRNA. It was also prevented by pharmacological inhibition of ASK1 (apoptosis signal-regulating kinase 1) or of c-Jun N-terminal kinase, but not by an inhibitor of p38 mitogen-activated protein kinase. These results suggest that soluble mutant SOD1 oligomers rapidly trigger a kinase pathway that regulates the activity of Na⁺-activated K⁺ channels in neurons.

Key words: ALS; SOD; sodium-activated potassium channel

Significance Statement

Slack Na⁺-activated K⁺ channels (KCNT1, K_{Na1.1}) regulate neuronal excitability but are also linked to cytoplasmic signaling pathways that control neuronal protein translation. Mutations that alter the amplitude of these currents have devastating effects on neuronal development and function. We find that injection of oligomers of mutant superoxide dismutase 1 (SOD1) into the cytoplasm of invertebrate neurons rapidly suppresses these Na⁺-activated K⁺ currents and that this effect is mediated by a MAP kinase cascade, including ASK1 and c-Jun N-terminal kinase. Because amyotrophic lateral sclerosis is a fatal adult-onset neurodegenerative disease produced by mutations in SOD1 that cause the enzyme to form toxic oligomers, our findings suggest that suppression of Slack channels may be an early step in the progression of the disease.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disease that prominently features degeneration of

motor neurons, and results in paralysis within 2–5 years of diagnosis (Peviani et al., 2014). Mutations in Cu, Zn-superoxide dismutase (SOD1) account for ~2% of ALS cases, associated with autosomal dominant inheritance (Rosen, 1993; Saccon et al., 2013). This form of ALS has been modeled in transgenic mice carrying multiple copies of mutant human ALS-associated SOD1 genes (Gurney et al., 1994). Both *in vitro* studies and examination of transgenic mice have enabled study of the physiology and toxicity of mutant SOD1. Toxicity likely relates to misfolding and oligomerization/aggregation of misfolded species (Bruijn et al., 2004; Lindberg et al., 2005; Valentine et al., 2005; Hart, 2006). The proximate targets of the misfolded SOD1 species that mediate toxicity are not precisely known, but effects have been reported on mitochondria, ER, and axonal trafficking (Pasinelli and Brown, 2006; Matus et al., 2013).

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Figure 1. Effects of microinjection of wt SOD1YFP and different forms of G85R SOD1YFP on outward currents in *Aplysia* neurons. Currents were evoked from -60 mV to potentials between -80 mV and 70 mV in 10 mV increments. Peak currents were measured at 70 mV at 5 min intervals. Difference currents were obtained by subtracting peak currents between control and 10 min after injection of G85R SOD1YFP. Normalized group data in time course plots are expressed as mean $\pm$ SEM. **A, B**, Injection of wild-type SOD1YFP (**A**) or of G85R SOD1YFP (**B**) (Figure legend continues.)

Reports have suggested that altered excitability might be another form of toxicity produced by mutations or misfolding of SOD1. This has been described for motor neurons recorded in neonatal slice preparations from SOD1 mutant transgenic mice (Kuo et al., 2005; van Zundert et al., 2008; Meehan et al., 2010; Delestrée et al., 2014; Hadzipasic et al., 2014), as well as in motor neurons derived from iPS cells of ALS patients (Wainger et al., 2014). Some studies have pointed to an increase in a persistent voltage-dependent Na⁺ current as a cause of increased excitability, although changes in outward currents have also been reported (Kuo et al., 2005; Wainger et al., 2014). In neurons, however, the persistent Na⁺ current rapidly and selectively activates Na⁺-activated K⁺ channels (K_{Na} channels) (Hage and Salkoff, 2012). These are encoded by the *Slack* (K_{Na}1.1, Slo2.2, KCNT1) and *Slick* (K_{Na}1.2, Slo2.1, KCNT2) genes (Kaczmarek, 2013). The activation of these channels produces an outward current that directly opposes the inward current that flows through Na⁺ channels, and the resultant persistent current is determined by the balance of Na⁺ and K_{Na} currents (Hage and Salkoff, 2012). Human genetic mutations in *KCNT1* cause early-onset epilepsies but also severely impact neuronal development and intellectual function, likely because these channels interact directly with proteins that regulate activity-dependent translation of mRNAs (Brown et al., 2010; Barcia et al., 2012; Heron et al., 2012; Kim and Kaczmarek, 2014).

In this study, we have used neurons of the marine invertebrate *Aplysia* to investigate how SOD1 proteins influence neuronal excitability, and specifically whether misfolded mutant G85R SOD1 proteins have an acute effect on K_{Na} currents. *Aplysia* neurons have been widely used as a model system to study the regulation of intrinsic excitability, as well as changes in synaptic structure during learning and memory (Kandel et al., 2014). A group of neurons in the abdominal ganglion of these animals, termed the bag cell neurons, offer the major experimental advantage that enzymes and other proteins can be directly injected into their large cell bodies with no disruption of cytoplasmic signaling pathways (Kaczmarek et al., 1980), an approach not readily feasible with mammalian neurons. Moreover, K_{Na} channels in these neurons are encoded primarily by the *Slack* gene, and both the gene and the characteristics of the channels are highly conserved from invertebrates to mammals (Zhang et al., 2012). We find that oligomers of the ALS mutant SOD1 rapidly suppress Slack K_{Na} currents and that this effect is mediated by a MAP kinase cascade including the apoptosis signaling regulating kinase ASK1 and c-Jun N-terminal kinase (JNK).

Materials and Methods

Animals, culture, and recordings. Adult *Aplysia californica* weighing 150–200 g were obtained from Marine Specimens Unlimited or Marinus. These animals are hermaphrodites. Primary cultures, current-clamp and voltage-clamp recordings, and injections were made using bag cell neurons as described previously (Zhang et al., 2012). In all cases, currents

were compared before and after injections to provide an internal control and to analyze difference currents in each cell.

Protein preparations. Both G85R SOD1YFPHis and wt SOD1YFPHis were produced in *Escherichia coli* BL21/DE3. To obtain stable mutant G85R SOD1YFP oligomers, G85R SOD1YFP in PBS was incubated at 37°C for 24 h and then cross-linked with 1 mM disuccinimidyl suberate for 1 h at room temperature. The cross-linking reaction was quenched by 100 mM glycine, pH 8.0, for 20 min at room temperature, followed by gel filtration and isolation of dimer, 300 kDa and >300 kDa. Specific size fractions were then concentrated with an Ultra-4 (Amicon) (Song et al., 2013).

siRNA interference. Predesigned silencer select siRNAs (*Aplysia* Slack and scrambled siRNA) were purchased from Ambion. For each gene siRNA, we targeted two different sites and then mixed the two different siRNA products for treatment of neurons as described previously (Zhang et al., 2012).

Results

Outward potassium currents are reduced by cross-linked G85R SOD1YFP

To test the effects of mutant SOD1 on excitability, we first recorded outward currents from isolated cultured bag cell neurons of *Aplysia* using the sharp single-electrode voltage-clamp technique. After the recordings were stable, intracellular injections were made using a single pulse of pressure (14 psi, 100 ms duration) applied to the intracellular electrodes, which contained 200 μM SOD1YFP proteins in 0.5 M K⁺ acetate. Recordings were continued for at least 15 min, and the amplitude of currents was evaluated at 5 min intervals. Intracellular injection of wt SOD1YFP or of monomers of the ALS mutant G85R SOD1YFP had no effect on the outward current (Fig. 1*A,B*; *n* = 6,6). In contrast, injections of G85R SOD1YFP that had been induced to form oligomers by preincubation at 37°C for 24 h resulted in a 20%–30% reduction in outward current (Fig. 1*C*; *n* = 6). A similar reduction was seen when 37°C treated material was cross-linked, purified by gel filtration, and cross-linked dimers of G85R SOD1YFP were injected (Fig. 1*D*; *n* = 6). An even greater reduction in current was produced after injection of cross-linked G85R SOD1YFP oligomers of 300 kDa (Fig. 1*E*; *n* = 6) or cross-linked G85R SOD1YFP oligomers of greater size (>300 kDa, Fig. 1*F*; *n* = 6). In all cases, suppression of current was complete by 10 min after injection (Fig. 1*A–F*).

To characterize further the currents sensitive to oligomers of G85R SOD1YFP, we calculated difference currents obtained by subtracting currents after injection from controls (Fig. 1*A–F*) and calculated their voltage dependence (Fig. 1*H,I*). Although difference currents sometimes had a component of inward current at the onset of depolarization, particularly at negative potentials (e.g., Fig. 1*D,F*), this was not consistent from experiment to experiment. Moreover, the greatest difference currents were recorded at the most positive potentials (70 mV), where the contribution of Na⁺ and Ca²⁺ currents would be minimal. The voltage dependence of the difference currents matched those of K⁺ currents, as shown for injections of 300 kDa and >300 kDa G85R SOD1YFP oligomers (Fig. 1*H,I*).

Outward currents suppressed by mutant SOD1 oligomers are Na⁺-dependent K⁺ currents

We next tested the Na⁺ dependence of the outward current that is suppressed by SOD1 oligomers. For this and future experiments, we used injections of cross-linked 300 kDa G85R SOD1YFP (“300 kDa”), which in control neurons produces a 38 ± 4% reduction in total outward current (Fig. 1*G*). When external Na⁺ was replaced by *N*-methyl D-glucamine, injections of

(Figure legend continued.) monomers (*B*) had no effect on outward current. *C–F*, Injection of G85R SOD1YFP incubated at 37°C for 24 h (*C*), cross-linked G85R SOD1YFP dimers (*D*), cross-linked 300 kDa (*E*), or cross-linked oligomers with a size >300 kDa (*F*) reduced outward current. *G*, Group data for effects of wild-type and different species of G85R SOD1YFP on outward currents. Data (mean ± SEM) were normalized to the mean current preinjection. Statistics for *p* values represent a one-way ANOVA; *n* = 6 for each group. *H, I*, Current–voltage relationships for controls (preinjection) and 10 min after injection of cross-linked 300 kDa (*H*) or cross-linked oligomers with a size >300 kDa (*I*), and for the difference currents measured by subtracting peak currents 10 min after injection from controls.

Figure 2. Removal of Na^+ from the external solution or treatment with Slack siRNA reduces effect of cross-linked 300 kDa on outward currents. Recordings were performed as in Figure 1. Data are mean $\pm$ SEM. **A**, Cross-linked 300 kDa has no effect on bag cell neuron outward current in a 0-Na^+ external solution ($n = 6$). **B**, Microinjection of cross-linked 300 kDa had little effect on outward currents in Slack siRNA-treated neurons ($n = 6$). **C**, Microinjection of cross-linked 300 kDa reduced outward current after treatment of neurons with scrambled siRNA ($n = 6$). Bottom, Mean time courses and current–voltage relations.

300 kDa produced an overall decrease of $<10\%$ in outward current (Fig. 2A; $n = 6$).

The finding that suppression of K^+ current depends on external Na^+ suggests that the current regulated by SOD1 oligomers may be a Na^+ -dependent K^+ current (K_{Na} current). In *Aplysia* neurons, as in mammalian neurons, the Slack ($\text{K}_{\text{Na}}1.1$) subunit is a major component of K_{Na} currents, and downregulation of Slack using siRNA technique suppresses this component of current (Zhang et al., 2012). To test the effects of mutant SOD1 oligomers in neurons deficient in Slack channels, we divided bag cell neu-

rons isolated from the same animal into two groups. In the first group, $1\text{ }\mu\text{M}$ Slack siRNA was injected into each neuron, whereas the second group was injected with control scrambled siRNA. Three days after injection of the siRNA, outward currents were recorded in both groups of cells before and after injection of 300 kDa. This procedure has been shown to reduce levels of Slack protein by $>60\%$ in Slack siRNA-treated neurons (Zhang et al., 2012). Injections produced only a small decrease in outward current in neurons pretreated with Slack siRNA (Fig. 2B; decrease $17 \pm 2\%$, $n = 6$, $p < 0.05$). In contrast, in neurons pretreated with

Figure 3. Injection of cross-linked 300 kDa enhances *Aplysia* bag cell neuron excitability. **A**, Left, Superimposed traces of action potentials evoked by a train of 10 depolarizing current pulses (0.6 nA, 2 Hz) before and after injection of cross-linked 300 kDa. Right, Graph represents the mean time course of changes in bag cell neuron resting membrane potential after injection of cross-linked 300 kDa. Data are mean $\pm$ SEM; $n = 6$. Injection of cross-linked 300 kDa depolarized the resting membrane potential. **B**, Same as for **A** but with recordings performed in 0-Na⁺ external solution. **C**, Same as for **A** but with neurons treated with Slack siRNA. **D**, Same as for **A** but with neurons treated with scrambled siRNA. Small peaks in voltage traces that coincide with the end of a current pulse represent passive return to the resting potential.

the control scrambled siRNA, injections of 300 kDa produced suppression of current comparable with that in untreated neurons (Fig. 2C; decrease $34 \pm 3\%$, $n = 6$, $p < 0.01$).

Microinjection of mutant SOD1 oligomers causes depolarization and increases excitability

We next tested the effect of 300 kDa on excitability by recording the response to depolarizing current pulses in the current-clamp

recording mode. A train of ten 100 ms suprathreshold depolarizing current pulses (0.6 nA, 2 Hz) was applied at 5 min intervals before and after injections. Such repetitive stimulation typically triggers 1–3 action potentials in response to each current pulse (in Fig. 3A, left [1 or 2], C [2], D, left [2 or 3]). These action potentials increase in height and width with successive pulses in the train, an effect termed frequency-dependent spike broadening (Fig. 3) (Kaczmarek and Strumwasser, 1981).

Figure 4. ASK1 inhibitor and JNK inhibitor prevent effects of cross-linked 300 kDa on outward currents. Recordings performed as in Figure 1. **A**, Cross-linked 300 kDa had no effect on outward current in bag cell neurons pretreated with ASK1 inhibitor NQDI-1. **B**, Injection of cross-linked 300 kDa reduced outward current in the presence of the p38 inhibitor MW069. **C**, Injection of cross-linked 300 kDa had no effect on outward currents after treatment with JNK inhibitor II SP600125. **D**, Treatment with ASK1 inhibitor NQDI-1 abolished the (Figure legend continues.)

In untreated neurons, injection of 300 kDa resulted in depolarization of the resting membrane potential by 13.25 ± 1.8 mV within 10 min (Fig. 3A; $n = 6$). The mean number of action potentials per pulse evoked by the stimulus train was increased from 1.3 ± 0.1 to 1.7 ± 0.2 (Fig. 3A). The action potentials of bag cell neurons have both an Na^+ and Ca^{2+} component, and Ca^{2+} action potentials can be evoked in the absence of external Na^+ (Kaczmarek et al., 1982). Consistent with the voltage-clamp experiments, injection of 300 kDa into neurons in a Na^+ -free medium had little or no effect on resting membrane potential and did not alter the number or pattern of Ca^{2+} action potentials evoked by the stimulus (Fig. 3B).

We also compared current-clamp responses of neurons pre-injected with Slack siRNA with those treated with scrambled siRNA. Little change in resting membrane potential was observed in the Slack siRNA-treated neurons after injection of 300 kDa (decrease 3.75 ± 1.8 mV, $n = 6$), and no change in firing pattern was observed following injection (Fig. 3C). In contrast, the depolarization of neurons treated with scrambled siRNA was comparable with that in untreated cells (decrease 12.48 ± 1.5 mV, $n = 6$, $p < 0.001$), and the mean number of action potentials evoked per pulse was increased from 1.4 ± 0.1 to 1.8 ± 0.3 after injection of 300 kDa (Fig. 3D).

Effects of 300 kDa mutant SOD1 oligomers are blocked by inhibition of ASK1 or JNK kinase

The effects of mutant G85R SOD1YFP on axoplasmic transport in squid axoplasm have been shown to be mediated by the apoptosis signaling regulating kinase ASK1 (MAPKKK) and its downstream kinase p38 MAPK (Song et al., 2013). We therefore tested the effect of 300 kDa injection on neurons pretreated with $0.5 \mu\text{M}$ ASK1 inhibitor NQDI-1 for 30 min. In voltage-clamp experiments, the suppression of outward current by 300 kDa was greatly reduced by NQDI-1 (Fig. 4A; decrease $2 \pm 1\%$, $n = 6$). Surprisingly, however, no inhibition of the effects of 300 kDa was detected on pretreatment with $5 \mu\text{M}$ p38 MAPK inhibitor MW069 (Fig. 4B; decrease $36 \pm 2\%$, $n = 6$, $p < 0.001$).

The activation of the MAPKKK ASK1 triggers the downstream activation of at least two MAP kinases: p38 MAPK and JNK (Shiizaki et al., 2013). Because the p38 MAPK inhibitor failed to prevent suppression of K^+ current by 300 kDa, we tested the effects of a JNK inhibitor (SP600125). As with the ASK1 inhibitor, pretreatment with $1 \mu\text{M}$ SP600125 for 30 min suppressed the actions of 300 kDa (Fig. 4C; decrease = $3 \pm 5\%$, $n = 6$).

Finally, we tested whether the ASK1 inhibitor NQDI-1 prevents the effects of mutant SOD1 oligomers on excitability measured in current clamp. NQDI-1 prevented the actions of 300 kDa on both the depolarization of resting membrane potential and the increase in action potentials evoked by a stimulus train (Fig. 4D). In addition, we measured the latency from the onset of the first depolarizing current pulses of the stimulus train to the peak of the first action potential. In untreated neurons, as well as in neurons pretreated with scrambled siRNA, this latency was reduced by ~ 15 – 20 ms after injection of 300 kDa. In neurons pretreated with NQDI-1, as well as in neurons in 0-Na^+ medium

or neurons treated with Slack siRNA, however, no significant change in latency was detected after injection (Fig. 4E).

Discussion

We have found that injection into isolated *Aplysia* neurons of oligomeric forms of a mutant G85R SOD1 associated with ALS in both humans and transgenic mice reduces net outward K^+ current and increases excitability. This effect could be largely suppressed either by omitting Na^+ from the external medium or by reducing the expression of Slack K_{Na} channels. Downregulation of Slack with siRNA reduced a major part, but not all, of the effects of G85R SOD1 oligomers on outward current. This could result from incomplete suppression of Slack expression or because other channels are also affected. Nevertheless, the degree of Slack suppression by siRNA was sufficient to eliminate any significant effects on excitability. The effect of the oligomers on K^+ currents could be blocked by inhibitors of MAPKKK, ASK1, and its downstream MAPK, JNK.

Na^+ activated K^+ channels may regulate more than neuronal excitability. Slack and the closely related Slick ($\text{K}_{\text{Na}}1.2$) channels are broadly distributed in the CNS and highly expressed in a variety of motor neurons (Kaczmarek, 2013). Slack channels co-immunoprecipitate with neuronal mRNAs, and their cytoplasmic C terminus binds proteins that regulate mRNA translation, suggesting that channel activity may regulate protein synthesis (Brown et al., 2010; Zhang et al., 2012). Moreover, activation of Slack channels causes the dissociation of Phactr-1, a protein that interacts with phosphatases and with the actin cytoskeleton (Fleming et al., 2016). Human gain-of-function mutations in the Slack gene, *KCNT1*, result in increased K_{Na} currents and severe intellectual dysfunction (Kim and Kaczmarek, 2014). So far, however, no currently characterized human mutations produce decreases in K_{Na} current. Thus, postdevelopmental suppression of Slack activity by mutant SOD1 oligomers may have cellular consequences unrelated to effects on firing patterns.

Recordings of motor neurons in animal models that express ALS-associated mutations in SOD1 have demonstrated an increase in an inward current that is blocked by the Na^+ channel blocker TTX and that appears to be a persistent Na^+ current (I_{NaP}) (Kuo et al., 2005; van Zundert et al., 2008; Meehan et al., 2010; Delestrée et al., 2014), in addition to changes in some other currents (Zona et al., 2006; Delestrée et al., 2014). TTX, however, also completely eliminates K_{Na} currents that are functionally coupled to the I_{NaP} channels (Hage and Salkoff, 2012). The observed increase in I_{NaP} in the animal models of ALS may therefore represent loss of K_{Na} current rather than, or in addition to, increased Na^+ current.

The acute effects of mutant SOD1 on K_{Na} currents we have described are very rapid and are likely to lead to longer-lasting changes in neuronal function and excitability. Moreover, activation of the ASK1/JNK pathway may also regulate other aspects of neuronal function (Kawarazaki et al., 2014; Fujisawa et al., 2016). Activation of ASK1 by G85R SOD1 oligomers reduces anterograde vesicular transport in squid axoplasm, an effect mediated by the downstream MAPK p38 (Song et al., 2013). Ultimately, however, the effects of mutant SOD1 oligomers lead to the demise of motor neurons in mammals. For example, a study of transgenic mice with the G85R SOD1 mutation found that a subset of rapidly firing neurons are selectively lost in these animals (Hadzipasic et al., 2014). The extent to which long-term suppression of Slack channels contributes to this pathology remains to be determined. If this is the case, however, increasing the expression of Slack channels may be able to reverse at least some

(Figure legend continued.) effects of cross-linked 300 kDa on excitability. Left, Superimposed traces of action potentials evoked by a train of 10 depolarizing current pulses (0.6 nA, 2 Hz) before and after injection of cross-linked 300 kDa in the presence of NQDI-1. Right, Mean time course of changes in bag cell neuron resting membrane potential after injection (mean $\pm$ SEM; $n = 6$). E, Summary of the effects of injection of mutant cross-linked 300 kDa on action potential latency. Data are mean $\pm$ SEM. *** $p < 0.001$ (one-way ANOVA).

of the deleterious effects of mutant SOD1 oligomers, and pharmacological agents that activate Slack channels may be therapeutically useful.

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