

Role of Riluzole in Amyotrophic Lateral Sclerosis

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Previous studies have demonstrated that there were global losses of Na,K-ATPase activity in mutant Amyotrophic Lateral Sclerosis mice (ALS) G93A. The purpose of this study was to investigate the difference in Na,K-ATPase activity between ALS-like symptomatic L126Z-SOD1 mutant mice and wild-type mice in the brainstem, forebrain, and spinal cord. Furthermore, the study investigated the effect of riluzole on the Na,K-ATPase activity of L126Z-SOD1 mutant mice tissue. Tissue slices from L126Z-SOD1 mutant mice brainstem, forebrain, and spinal cord slices were incubated with agonists and antagonists in a physiological buffer. Na,K-ATPase activity was determined by assaying hydrolysis of adenosine triphosphate (ATP) in suspended permeabilized tissue slices. L126Z-SOD1 mutant mice tissues were examined for differences in Na,K-ATPase activity across three different tissue types. L126Z-SOD1 spinal cord tissue was found to have significantly lower Na,K-ATPase activity when compared to the wild-type spinal cord tissue. L126Z-SOD1 brainstem tissue was not significantly different in Na,K-ATPase activity when compared to its respective wild-type tissue. L126Z-SOD1 forebrain tissue was not significantly different in Na,K-ATPase activity when compared to its respective wild-type tissue. With the application of riluzole, the Na,K-ATPase activity in L126Z-SOD1 spinal cord, brainstem, and forebrain tissue decreased when compared to their respective untreated controls. The observed difference in Na,K-ATPase activity in L126Z-SOD1 spinal cord tissue posits that the L126Z-SOD1 mutation in mice does not exhibit global Na,K-ATPase losses in all tissue types as previously reported in the G93A mutation. Furthermore, decreases in Na,K-ATPase activity after the application of riluzole indicates that riluzole effectively decreased Na,K-ATPase activity in brainstem, forebrain, and spinal cord tissue in L126Z-SOD1 mutant mice.

INTRODUCTION

Presentation of Symptoms in Patients with ALS

ALS, colloquially known as Lou Gehrig's disease, is one of the most common forms of adult onset neurodegenerative disorders. ALS typically presents between 50 and 60 years of age, and is highly fatal. ALS presents itself in two particular types, familial ALS (FALS) and sporadic ALS (SALS), and is indistinguishable from a pathophysiological standpoint (Rosen et al., 1993). The actual cause of ALS disease is unknown currently, but many different factors have been implicated.

Common symptoms of ALS are found in the early stages of the disease and include degeneration of the upper motor neurons in the motor cortex of the brain, as well as the lower motor neurons in the spinal cord and brainstem. The degradation of these motor neurons leads to fasciculations, muscular atrophy in the upper and lower body, and hyperreflexia; however, the leading cause of death in ALS patients is respiratory failure. The pathology of ALS is characterized from the aforementioned motor neuron loss, as well as abnormal mitochondria and insoluble neurofilament aggregates. These aggregates are found throughout the progression of the disease; the steady-state levels of the protein are lower than the normal SOD1

expression. Studies have shown that the ventral horn of the spinal cord showed accumulation of dead motor neuron cells and increased immunoreactivity to L126Z-SOD1 (Wang et al., 2005; Jaarsma et al., 2008; Johnston et al., 2000). The progression of the disease proceeds quickly, with death resulting from two to three years after early diagnosis (Barber et al., 2010).

The causes of ALS have not been elicited, but studies have pointed to the first mutated gene identified in ALS, the copper/zinc superoxide dismutase (Cu/Zn SOD1) (Rosen et al., 1993), Cu/Zn SOD1 is an enzyme found in cytosolic and mitochondrial intermembrane space tasked with the destruction of free radicals by conversion into hydrogen peroxide and molecular oxygen (Rosen et al., 2003; Olanow, 1993). Superoxide, a reactive oxygen species, reacts with nitric oxide (NO) produced by nitric oxide synthase (NOS) leading to the production of peroxynitrite (ONOO⁻); ONOO⁻ has been shown to cause intercellular DNA, protein, and lipid damage (Barber et al., 2010). While other genes have been identified to be mutated in ALS, such as TAR DNA-binding protein, a majority of studies conducted on ALS have focused on Cu/Zn SOD1 (Barber et al., 2010). Mice that have mutant SOD1 have demonstrated ALS-like muscular degeneration symptoms (Jaarsma et al., 2008). These studies posit that the biological mechanisms that may be responsible for

ALS, such as oxidative stress and glutamate excitotoxicity on motor neuron cells, and their contribution to the pathophysiology of ALS. However, it has been shown that a gain of toxic function of mutant Cu/Zn SOD1 that might present a possible reason for ALS occurrence (Barber et al., 2010; Kuo et al., 2003). There are many different types of mutations in the Cu/Zn SOD1 enzyme. The one examined in this study was L126Z (leucine at codon 126 changed to a stop codon), which presented ALS-like symptoms in mice models when compared to wild-types (Wang et al., 2005). The L126Z-SOD1 mutation codes for a truncation due to early termination during the translation of exon 5 (Zu et al., 1997; Wang et al., 2005). Pathophysiologically, L126Z-SOD1 presents characteristic detergent-insoluble aggregates in the spinal cord tissue of mice, but the effect of the L126Z-SOD1 mutant in humans has not been investigated (Wang et al., 2005).

Therapeutic Intervention in Response to ALS

Riluzole is the only FDA-approved pharmaceutical treatment for FALS and SALS; riluzole demonstrates neuroprotective properties that extend the average lifespan of an ALS patient by three months. Riluzole's mechanism has not been fully elicited, but studies have posited riluzole acts by indirect antagonistic effects on glutamate receptors, thereby lowering the glutamate excitotoxicity demonstrated by motor neurons in the spinal cord and cortical areas; however, there has been conflicting data presented regarding this. Furthermore, it is posited that riluzole also affects the voltage-gated Na⁺ channels by blocking their activity (Herbert et al., 1994; Cheah et al., 2010).

The Na/K ATPase pump is a major part of the neuronal system, responsible for 50% of the energy that is consumed by the CNS (Ames et al., 2000). The pump consists of a transmembrane ion transport enzyme that moves 2 Na⁺ ions out of the cell and 3 K⁺ into the cell. The Na,K-ATPase pump is the mechanism by which the resting potential of neurons is established, after an action potential has fired. A previous study by Ellis et al. found that there are global losses in Na,K-ATPase activity in G93A-SOD1 mutant mice across all three subunits, even in areas of the mice brain that were not previously shown to be affected by ALS. This demonstrated a truly global loss of Na,K-ATPase activity across the brain (Ellis et al., 2003). Consequently, in our investigation, we posit that similar losses in Na,K-ATPase activity will be found globally in the brain, rather than simply in the spinal cord, where one would expect to see the most amount of loss in this neuromuscular degenerative disorder.

In this study, we examined the mutation L126Z-SOD1 in ALS-symptomatic mice and investigated the difference in Na,K-ATPase activity between the brainstem, spinal cord, and forebrain of the mice to determine if there would be global losses in the Na,K-ATPase activity; furthermore, we

investigated the effects that Riluzole would have on this particular SOD1 mutation across the brainstem, spinal cord and forebrain of L126Z-SOD1 mice. Comparing the data gathered to that of Ellis et al. will allow for a more complete understanding of Na,K-ATPase loss in the L126Z-SOD1 mutation of ALS, as well as an insight into understanding the mechanism behind riluzole and its interaction with ALS. We hypothesized that we would see global Na,K-ATPase losses across all tissue types, as well as a decrease in Na,K-ATPase activity across the three tissue types after the application of riluzole.

METHOD

Tissue

Tissue samples from L126Z mice were obtained from Dr. David Borchelt (Santa Fe Health Alzheimer's Disease Research Center, McKnight Brain Institute, University of Florida, Gainesville, FL). The tissue slices were sliced into .4 mm X .4 mm X 1mm sizes on a Brinkmann chopper and cooled to 4°C and suspended (25-30 mg/ml) in microdissection buffer containing in (mM): 137 NaCl, 5 KCl, .8 MgSO₄, .25 CaCl₂, 1.0 MgCl₂, 10 HEPES, and 2 NaOH with the pH adjusted to 7.4 at 34°C.

Procedure

One mM riluzole was added to tubes that contained 1 mL aliquots of slice suspensions. Tubes were incubated for 15 minutes at 34°C with rocking and then frozen at -80°C. Tubes were thawed for usage in ATPase assays. The activity was determined by the colorimetric ATPase assay: ATP was hydrolyzed, and the released Pi was measured by forming a complex with molybdate. The pelleted tissue slices were resuspended and refrozen for at least 20 min at -80°C in 1 ml of resuspension buffer. Tubes were thawed on ice water. Aliquots of tissue slices (10–15 µg; 7.5–10 µl) were added to 300 µl of ATPase buffer containing in (mM): 3 ATP, 140 NaCl, 20 KCl, 3 MgCl₂, and 30 histidine, pH 7.2, with or without 3 mM ouabain. Treated tissue slices were homogenized with a ground glass homogenizer. Na,K-ATPase activity was calculated from the difference between the slopes in the time course of absorption change at 700 nm in the absence and the presence of 3 mM ouabain.

Protein concentrations for each sample were also calculated to standardize the Na,K-ATPase concentration values obtained; the incubated sample was homogenized using a glass homogenizer from Fisher. The homogenized tissue samples were then subject to standard Lowry Protein assay protocol, and the protein concentration was calculated from difference between the slopes in the time course of absorption change at 750 nm. With these numbers, the Na,K-ATPase concentration was determined.

Statistical Analysis

After determining the concentrations of ouabain-sensitive Na,K-ATPase activity in the three tissue types of L126Z-SOD1 mice, statistical significance tests were performed by using one-way analysis of variance, followed by Fisher’s protected least significant difference (PLSD).

RESULTS

In this study, we investigated the differences in Na,K-ATPase activity between the wildtype and the L126Z-

SOD1 mutant mice across all three tissue types; we also investigated the effect of riluzole on three different areas of neuronal tissue in L126Z-SOD1 mice compared against control mice.

In Figure 1 we compared the wild-type Na,K-ATPase activity to L126Z-SOD1 mutant mice activity in spinal cord tissue. This was done to determine if a difference existed between the level of Na,K-ATPase activity between the two types of tissue types. There was a significant difference ($p < .01$) in the ouabain-sensitive Na,K-ATPase activity between the L126Z-SOD1 tissue and the wild-type tissue.

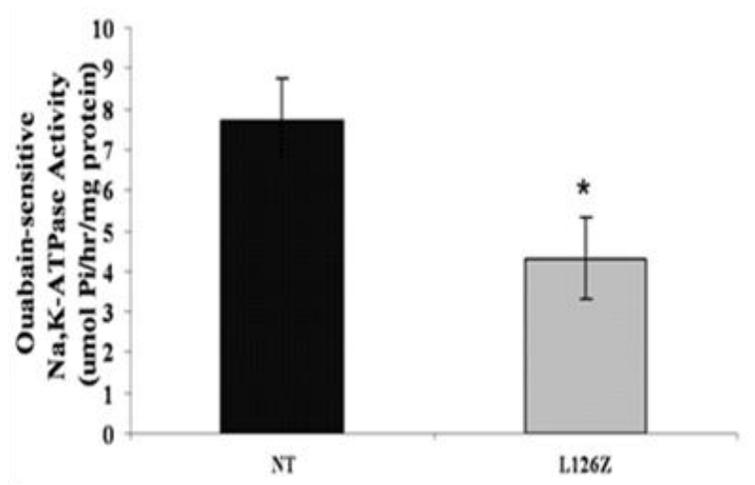


Figure 1. Na,K-ATPase in transgenic L126Z-SOD1 in spinal cord tissue

In Figure 2 we compared the wild-type Na,K-ATPase activity to L126Z-SOD1 mutant mice activity in the brainstem. There was no significant difference in the

ouabain-sensitive Na,K-ATPase activity between the L126Z-SOD1 tissue and the wild-type tissue.

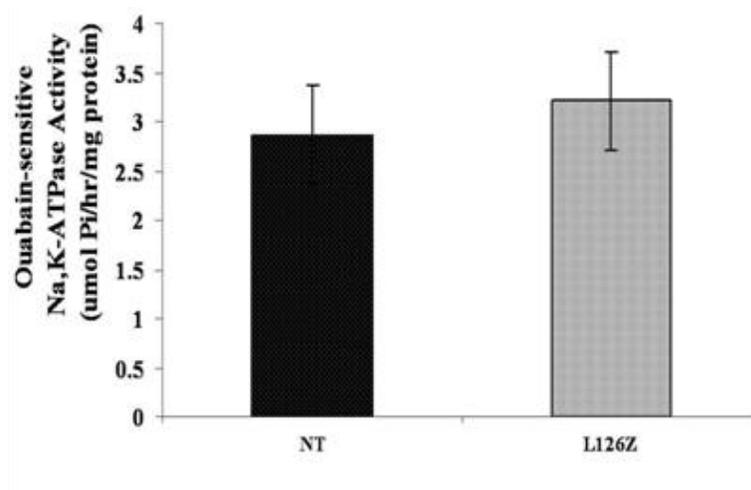


Figure 2. Na,K-ATPase activity in transgenic L126Z-SOD1 mice brainstem tissue

In Figure 3 we took tissue slices from the spinal cord tissue of the L126Z-SOD1 mutant to determine whether there was an effect by Riluzole on Na,K-ATPase activity. The tissue slices were exposed to a 1mM riluzole. Riluzole caused a decrease in Na,K-ATPase activity in the L126Z-SOD1 tissue when compared against the L126Z-SOD1

untreated tissue as well as in the wild-type tissue when compared to the wild-type untreated tissue. This decrease was significant ($p < .05$) in both L126Z-SOD1 and in the wild-type tissue when compared against their respective untreated tissues.

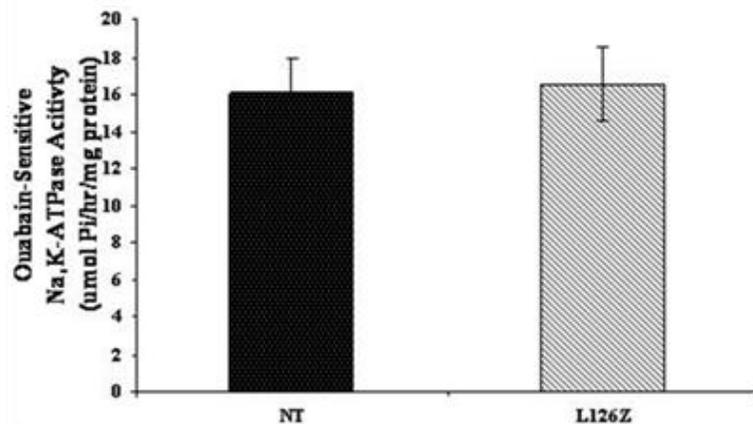


Figure 3. Na,K-ATPase activity in transgenic L126Z-SOD1 mice forebrain tissue

In Figure 4 we took tissue slices from the spinal cord tissue of the L126Z-SOD1 mutant to determine whether there was an effect by Riluzole on Na,K-ATPase activity. The tissue slices were exposed to a 1mM riluzole. Riluzole caused a decrease in Na,K-ATPase activity in the L126Z-SOD1 tissue when compared against the L126Z-SOD1

untreated tissue as well as in the wild-type tissue when compared to the wild-type untreated tissue. This decrease was significant ($p < .05$) in both L126Z-SOD1 and in the wild-type tissue when compared against their respective untreated tissues.

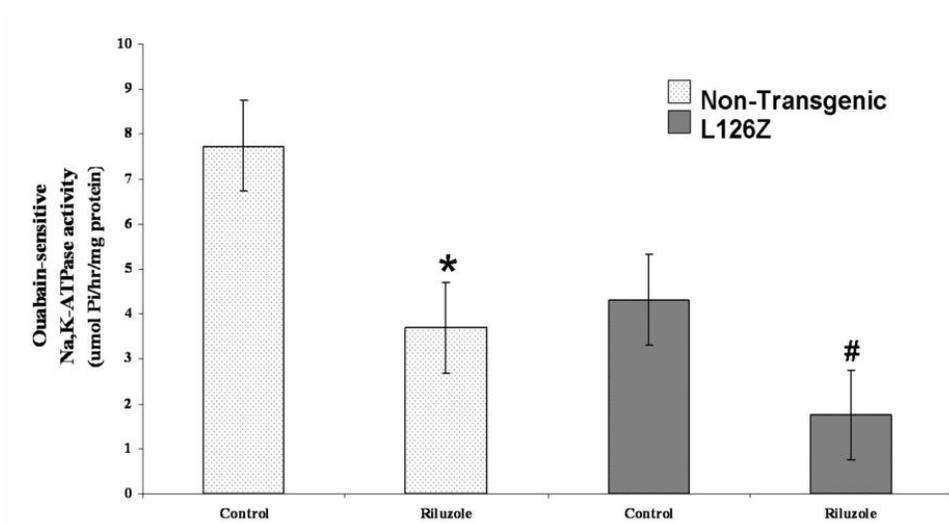


Figure 4. Riluzole Na,K-ATPase inhibits activity in spinal cord tissue of transgenic and control mice

In Figure 5 we took tissue slices from the brainstem tissue of the L126Z-SOD1 mutant to determine whether there was an effect by Riluzole on Na,K-ATPase activity. The tissue slices were exposed to a 1mM riluzole. Riluzole caused a decrease in Na,K-ATPase activity in the L126Z-SOD1 tissue when compared against the L126Z-SOD1

untreated tissue as well as in the wild-type tissue when compared to the wild-type untreated tissue. This decrease was significant ($p < .05$) in both L126Z-SOD1 and in the wild-type tissue when compared against their respective untreated tissues.

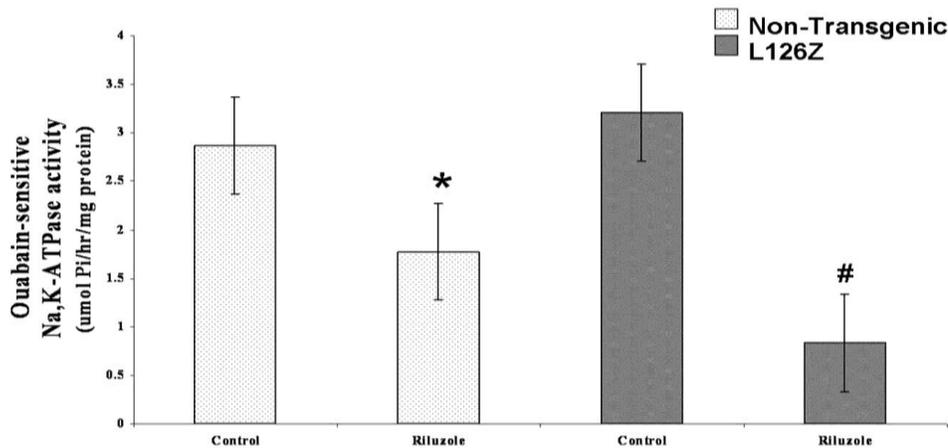


Figure 5. Riluzole inhibits Na,K-ATPase activity in brainstem tissue of transgenic and control mice

In Figure 6 we took tissue slices from the forebrain tissue of the L126Z-SOD1 mutant to determine whether there was an effect by Riluzole on Na,K-ATPase activity. The tissue slices were exposed to a 1mM riluzole. Riluzole caused a decrease in Na,K-ATPase activity in the L126Z-SOD1 tissue when compared against the L126Z-SOD1

untreated tissue as well as in the wild-type tissue when compared to the wild-type untreated tissue. This decrease was significant ($p < .05$) in both L126Z-SOD1 and in the wild-type tissue when compared against their respective untreated tissues.

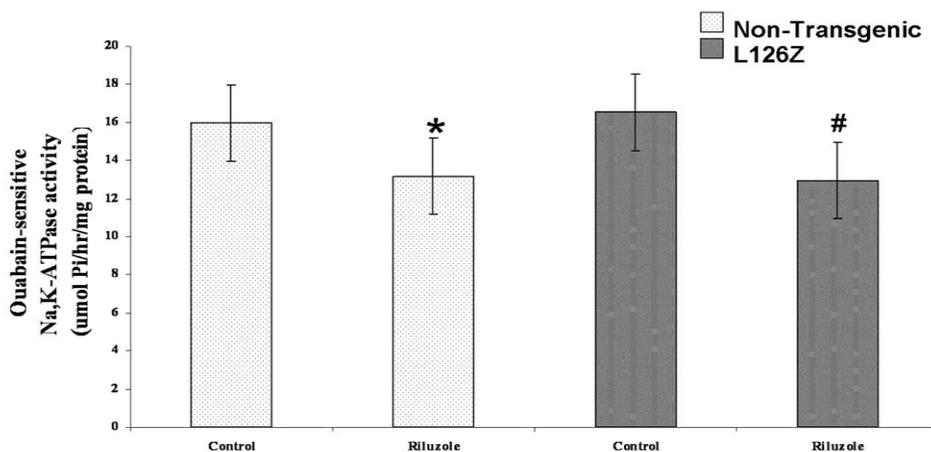


Figure 5. Riluzole inhibits Na,K-ATPase activity in forebrain tissue of transgenic and control mice

DISCUSSION

In this study, we investigated the difference in Na,K-ATPase activity between the L126Z-SOD1 mutant mice that express ALS-like symptoms, and the wild-type across 3 different tissue types. Our results clearly demonstrate that there is a loss in Na,K-ATPase activity between the L126Z-SOD1 mutant and the wild-type in the spinal cord only, and that there was no significant difference observed between the Na,K-ATPase activities for brainstem and forebrain tissue of L126Z-SOD1 mice, when compared to their respective wild-type tissues. It is possible that in the L126Z-SOD1 mutation of the original SOD1 enzyme, there is a key structural change at the 126th codon that causes Na,K-ATPase activity loss selectively at sites directly damaged by ALS-like symptoms in L126Z-SOD1 mutant mice (Zu et al., 1997). Studies have shown that this might be implicated with the loss of the original dismutase activity of the normal SOD1 enzyme (Zu et al., 1997). It is possible that this damage caused by the L126Z-SOD1 mutation comes from the opening of an active site channel, leading to an increase in the accessibility of metal ions to the enzyme active site; it is postulated that these could cause an increase in the catalysis of harmful reactive oxygen species such as peroxynitrite (ONOO⁻), leading to increased motor neuron death (Zu et al., 1997).

The biological phenomenon of neuron signaling is highly dependent on the level of activity of the Na,K-ATPase pump; it is responsible for mechanisms such as restoration of the action potential for signal propagation. However, previous research by Ellis et al. has shown a global loss in Na,K-ATPase activity in neuronal tissue from mice expressing the G93A-SOD1 mutation. This loss of Na,K-ATPase function may be due to the production of reactive oxygen species such as nitric oxide. This can lead to a raising of the intracellular Na⁺ levels within the neuronal cells, causing neuronal depolarization and release of glutamate; this prevents the cell from successfully restoring the membrane potential (due to the aforementioned inactivation of the Na,K-ATPase pump). This inability to restore the resting potential of the neuron leads to glutamate excitotoxicity and the emergence of the recognized pathophysiological symptoms of ALS.

Furthermore, we investigated the effect that riluzole would have on spinal cord, brainstem and forebrain tissue types. Here, we found that riluzole caused a significant

decrease in the Na,K-ATPase activity of both the L126Z-SOD1 mutant and the wild-type tissue, in all three tissue types taken. This could have been due to the fact that riluzole might exert its neuroprotective effects through several interdependent pathways, such as the Ca⁺⁺ and Na⁺ signaling pathways. Ca⁺⁺ mediates important intracellular signaling mechanisms that are associated with apoptotic-like signaling, through molecules such as Ca⁺⁺-dependent phospholipases and proteases (Cheah et al., 2010). Consequently, the application of riluzole would lead to decreased Ca⁺⁺ signaling, due to decreased Na⁺ activity. Since Na⁺ activity is directly related to glutamate release in the motor neuron, this would lead to lessened motor neuronal death by rescuing the excitotoxic environment that ALS-affected motor neurons are in. Riluzole would play a central role in blocking Ca⁺⁺ and Na⁺ signaling channels, which would eventually lead to the interruption of glutamatergic transmission. This would be a downstream effect of riluzole, which reversibly blocks Na⁺ channels by specifically targeting the α -subunit of the Na,K-ATPase pump (Herbert et al., 1994). These two postulated signaling mechanisms in the motor neurons are highly interconnected, and play a critical role in the neuroprotective effects of riluzole by dampening the vulnerability to hyperexcitability by glutamate in ALS (Cheah et al., 2010).

Further research must be conducted, based on the findings of this study; while we investigated the effects of riluzole on different tissue types from L126Z-SOD1 mutant mice, investigations should also be conducted on the other mutation types that are found within ALS such as the G93A, G37R and H46R/H48Q mutations. The Na,K-ATPase pump is regulated by nitric oxide (NO) signaling via the soluble guanylate cyclase pathway and cyclic guanosine monophosphate (cGMP). A study could potentially examine the role of NO and the effect that an NO donor such as sodium nitroprusside (SNP) in the mutation studied here would have on Na,K-ATPase activity in L126Z-SOD1 and other ALS mutations. These avenues of research, when placed in juxtaposition with this study and the previous research conducted by Ellis et al. will broaden the current viewpoint on ALS, as well as provide an insight into the intracellular signaling that lead to the emergence of this disease.

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