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Cortical astroglia undergo transcriptomic dysregulation in the G93A SOD1 ALS mouse model

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Abstract

Astroglia are the most abundant glia cell in the central nervous system, playing essential roles in maintaining homeostasis. Key functions of astroglia include, but are not limited to, neurotransmitter recycling, ion buffering, immune modulation, neurotrophin secretion, neuronal synaptogenesis and elimination, and blood-brain-barrier maintenance. In neurological diseases, it is well appreciated that astroglia play crucial roles in the disease pathogenesis. In amyotrophic lateral sclerosis (ALS), a motor neuron degenerative disease, astroglia in the spinal cord and cortex downregulate essential transporters, among other proteins, that exacerbate disease progression. Spinal cord astroglia undergo dramatic transcriptome dysregulation. However, in the cortex, it has not been well studied what effects glia, especially astroglia, have on upper motor neurons in the pathology of ALS. To begin to shed light on the involvement and dysregulation that astroglia undergo in ALS, we isolated pure grey-matter cortical astroglia and subjected them to microarray analysis. We uncovered a vast number of genes that show dysregulation at end-stage in the ALS mouse model, *G93A SOD1*. Many of these genes play essential roles in ion homeostasis and the Wnt-signaling pathway. Several of these dysregulated genes are common in ALS spinal cord astroglia, while many of them are unique. This database serves as an approach for understanding the significance of dysfunctional genes and pathways in cortical astroglia in the context of motor neuron disease, as well as determining regional astroglia heterogeneity, and providing insight into ALS pathogenesis.

Keywords

ALS; SOD1; astroglia; transcriptome; neurodegeneration

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INTRODUCTION:

Astroglia are essential for maintaining nervous system homeostasis (Zhang & Barres, 2010). They are a tremendously diverse glial cell type that performs a vast array of functions, including and not limited to: neurotransmitter recycling, ion homeostasis, neuronal spine formation and elimination, immune-modulation, neurotrophin release, and maintenance of the blood-brain-barrier (Miller & Rothstein, 2016). Astroglia in different anatomical regions exhibit highly different molecular profiles. Importantly, these molecular profiles also have been shown to change dramatically in neurological disease (Miller, Zhang, Glatzer, & Rothstein, 2016). In motor neuron diseases, such as amyotrophic lateral sclerosis (ALS), astroglia downregulate essential ion and neurotransmitter transporters such as the synaptically-localized glutamate-transporter I, Glt1, and potassium ion channel, Kcnj10 (Kaiser et al., 2006; Rothstein, Van Kammen, Levey, Martin, & Kuncl, 1995). The downregulation of these key membrane proteins is apparent in areas of motor neuron death, such as the lower motor neurons in the spinal cord and the upper motor neurons in the cortex.

From ongoing studies of ALS, it is now appreciated that this is a non-cell autonomous disease, where glia and neurons both play critical roles in disease progression (Yamanaka et al., 2008). However, one area of interest has been attempting to understand why certain astroglia populations are affected while others are not. Further, within affected cell populations, we know that their dysfunction varies greatly based on their neuroanatomical localization. To shed light on this, our group and others have recently shown that spinal cord astroglia in the ALS mouse and human model, *G93A SOD1*, undergo substantial transcriptomic changes before and during ALS disease progression *in vivo* that are detrimental to motor neurons (Haidet-Phillips A.M. & Frakes A., 2011; Miller et al., 2016). Many of the genes that become dysregulated are involved in ion homeostasis and immune modulation. These findings are supported by observations that in the ventral horn of the lumbar spinal cord and the motor cortex, areas where motor neurons degenerate, Kcnj10 levels are dramatically reduced (Kaiser et al., 2006).

In the cortex of the *G93A SOD1* mouse model there is an extensive degree of cortical pathology (Fogarty, Noakes, & Bellingham, 2015). For instance, dendritic spine simplification is observed, whereby dendritic spines undergo regression. In addition, neurons in the motor cortex become hyperexcitable, a potential consequence of abnormal ion balance from neighboring astroglia (Fogarty, Mu, Noakes, Lavidis, & Bellingham, 2016). Furthermore, the *G93A SOD1* mouse model also experiences a loss of cortical thickness (Fogarty et al., 2016). Whether this cortical loss is due to the degeneration of neurons or glia still remains unknown. Thus, further studies evaluating the cortex of the *G93A SOD1* mouse model are imperative and may provide us with cellular and molecular information on ALS pathology.

To expand our knowledge of the *G93A SOD1* mouse model cortex, we focused on astroglia dysregulation. This study focused on the whole cortex due to methodological limitations on isolating solely the motor cortex and the requirement for an abundant amount of astroglia. We used fluorescence-assisted cell sorting (FACS) to isolate pure cortical astroglia by

generating a double transgenic mouse model, *ALDH1L1-eGFP/G93A SOD1* (Philips & Rothstein, 2015; Yang et al., 2011). This mouse model allows us to label all astroglia with eGFP in order to isolate astroglia using FACS and subject these cells to transcriptomic analyses. For this study, we chose to explore the transcriptome of mice aged to P120 and showed complete hind leg paralysis. This time point we refer to as end-stage. The end-stage phenotype was selected because this is when we hypothesize astroglia dysfunction would be at its highest level.

After successfully isolating pure cortical astroglia and performing microarray analyses we uncovered over 1000 genes that are dysregulated in *G93A SOD1* mice compared to their control littermates. Several of the most upregulated genes are involved in the Wnt-signaling pathway, a pathway shown to be highly affected in neurodegenerative diseases (De Ferrari et al., 2003). This was supported by our pathway analytics showing that the Wnt/ β -catenin pathway was affected. In addition, our analyses showed that immune signaling and cholesterol biosynthesis were also greatly dysregulated. Some of the most downregulated genes were involved in metal homeostasis and neuronal maintenance, which is reflected in previous experiments (Angel et al., 2002). To date, this is the first study to thoroughly elucidate the transcriptomic changes that occur at end-stage in cortical astroglia of an ALS mouse model. It is important to note that due to the large degree of astroglia heterogeneity, this database can also serve as a resource to identify those regional differences among astroglia (i.e. cortical vs spinal cord). In closing, our study provides a database for neuroscientists to explore potential candidate genes involved in the astroglia-based pathogenesis of ALS, to exploit regional transcriptomic differences in astroglia populations, and it may provide molecular markers to further understand the functional consequences that diseased astroglia may have on their neuronal neighbors.

RESULTS:

Cortical G93A SOD1 Astroglia Display a Dramatic Transcriptomic Change

To better understand the role of cortical astroglia in ALS pathogenesis, we chose to use the *G93A SOD1* mouse which has been widely studied and used as an ALS model (Philips & Rothstein, 2015). However, to allow us to isolate pure grey-matter astroglia we also used the *ALDH1L1-eGFP* mouse model (Yang et al., 2011). The *ALDH1L1-eGFP* mouse model labels all astroglia with eGFP fluorescence, and thus we used this fluorescence as a tool to isolate the astroglia via fluorescence-assisted cell sorting (FACS) in the double transgenic mice.

We crossed the *ALDH1L1-eGFP* mice with *G93A SOD1* mice and aged the progeny to P120. We selected only mice with the same phenotype at P120. This age is accompanied by complete hindleg paralysis and termed “end-stage” for this ALS mouse model. At P120, we isolated pure cortical astroglia from both the diseased *ALDH1L1-eGFP/SOD1 G93A* mice and their control littermates, using a widely used method (Foo, 2013). After isolating pure astroglia, we explored their transcriptome by subjecting purified astroglia to microarray analysis.

Our results displayed a dramatic change in the transcriptome of diseased astroglia compared to their wild-type controls. In aggregate, we found a total of 105 genes above 2 standard deviations that were upregulated in cortical *G93A SOD1* astroglia, and 140 genes below 2 standard deviations (Supp. Fig. 1). When displaying the differentially expressed genes by standard deviation, the graph distribution represents a bell-curve (Supp. Fig. 1). Notably, a large number of genes both upregulated and downregulated were below a p-value of 0.05 as displayed by the volcano plot and identified in red (Fig. 1).

When exploring the top upregulated genes, Coiled-Coil Domain Containing 85B (*Ccdc85b*) was the most upregulated gene. *Ccdc85b* plays a role as a transcriptional repressor that may inhibit the action of β -catenin (Table 1) (Iwai et al., 2008). This is in support of ingenuity pathway analysis (Qiagen, IPA) that shows the Wnt/ β -catenin signaling pathway as being one of the most upregulated pathways in cortical *G93A SOD1* astroglia (Table 2). In addition, several other genes involved in the Wnt pathway were also among the top ten upregulated genes (Table 1). The Wnt signaling pathway has been implicated in various neurodegenerative disorders, including ALS (Arrazola, Silva-Alvarez, & Inestrosa, 2015; Chen et al., 2012). Other genes that the microarray analytics found to be significantly upregulated includes genes involved with neuron-specific transcription, dendritic spine formation, and adaptor proteins involved in multiple signaling pathways (Table 1).

Not surprisingly, we found that in the top ten most downregulated genes, those involved in ion homeostasis were dysregulated, including *Metal Regulatory Transcription Factor 1*, *Mtf1* (Table 3). This finding supports past literature showing that dysregulation of ion balance in the central nervous system is a common theme in various neurological disorders (Angel et al., 2002; Saini, Georgiev, & Schaffner, 2011). In addition to genes involved in ion homeostasis, our microarray analytics also show that genes involved in neuronal differentiation and neurogenesis were downregulated (Table 3). Overall STRING map was generated to illustrate all of the top downregulated genes within 2 standard deviations (Fig. 5).

Astroglia are essential for the biosynthesis of crucial lipids in the central nervous system (Pfrieger & Ungerer, 2011). In our pathway analysis, we discovered that the canonical cholesterol biosynthesis pathway was significantly downregulated (Table 2). This was in addition to observing that mineralocorticoid and glucocorticoid pathways were significantly downregulated in the *G93A SOD1* cortical astroglia (Table 2).

Next, we aimed to determine the distribution of the genes most dysregulated. When evaluating both up- and downregulated genes, we found that most genes were localized to the cytoplasm (Fig. 2). Secondly, we found that the next subcellular localization of differentially expressed genes was concentrated in the nucleus (Fig. 2). This is in support of our top gene lists that show several transcription factors and regulators as being dysregulated in *G93A SOD1* cortical astroglia compared to control (Tables 1, 3). Not surprisingly, and potentially of great biological interest, is the multitude of plasma membrane proteins we observed to be differentially expressed in our studies (Fig. 2).

Lastly, we generated a STRING map to evaluate interacting proteins to some of the most upregulated genes (Fig. 3,4). The Wnt signaling pathway was found to be upregulated in our analytics (Table 1). When evaluating proteins in the Wnt pathway associated with *Csnk1g1*, we found that in agreement with *Csnk1g1* upregulation, we also found several other members of this pathway to be upregulated (Fig. 3). One downregulated gene, *Smo*, is found to be involved in the Sonic Hedgehog signaling pathway, thus this downregulation in cortical *G93A SOD1* astroglia is unsurprising. Overall top upregulated genes above 2 standard deviations is illustrated using a STRING map (Fig. 4).

DISCUSSION:

Selection of the G93A SOD1 Mouse Model

In this study we chose to use the *G93A SOD1* mouse model because to date, compared to other ALS mouse models, it recapitulates many of the clinical and neuropathological features of ALS pathology. The *SOD1* gene, with over a hundred mutations identified, make up about 20% familial ALS (fALS) and 2–7% of sporadic ALS (sALS) cases. Recently however, mutations found in the *C9ORF72* gene have been found in about 40% of fALS and 10% of sALS cases (Nardo et al., 2016). There have been a few groups who have generated transgenic models of *C9ORF72* but they are not readily available and do not display the same overall phenotypes of ALS pathology (Koppers et al., 2015; Y. Liu et al., 2016). Therefore, the *G93A SOD1* mouse model is still the best ALS mouse model available.

Regional Differences in the Transcriptomic Changes of G93A SOD1 Astroglia

Astroglia are a highly diverse cell type. Current work shows that astroglia populations in different anatomical regions have major differences in their molecular profiles (John Lin et al., 2017; Tsai et al., 2012). These differences begin in development and have been shown to remain in adulthood. This is most likely due to an interplay among different neuronal populations and other cell types (Oberheim, Goldman, & Nedergaard, 2012). Astroglia that interact with these vastly different cell types, even at times when those cells are in other states (i.e. polarized microglia vs un-polarized), must perform unique functions in order to maintain homeostasis in their physiological niches.

Previously our group performed microarray analytics on spinal cord astroglia and uncovered markers and pathways unique to these astroglia in the *G93A SOD1* mouse model (Miller et al., 2016). When comparing our findings on the transcriptomic changes in cortical *G93A SOD1* astroglia vs spinal cord, we found that there are major differences in the most dysregulated genes; however, there also remain some similarities, such as genes involved in ion balance (i.e. transporters, channels) (Table 4).

Prior work performed by other groups studying the transcriptome changes in astroglia have largely focused on *in vitro* cultures (Hemali P. Phatnani & Richard M. Myers, 2013). One major caveat to this approach is making the assumption that all astroglia are homogeneous in their cultures. We show in this study and in other work that astroglia are indeed very heterogeneous (Miller & Rothstein, 2016). Thus, future studies should continue to address this issue and not categorize all astroglia into a homogeneous cell group.

With our study, we were able to isolate pure populations of astroglia in the cortex of control and end-stage *G93A SOD1* mice. This allows us to find unique genes and pathways differentially affected solely in cortical astroglia. Investigators can now use our extensive study to find novel genes and pathways that may be implicated in ALS pathology.

End-Stage Cortical G93A SOD1 Astroglia Display Abnormal Pathway Regulation

In our analyses, we used ingenuity pathway analysis (Qiagen, IPA) to illustrate canonical pathways that may be dysregulated in end-stage ALS mice compared to their control littermates. In doing so, we found that the Wnt signaling pathway, toll-like receptor signaling, and cell cycle regulation were significantly upregulated. In contrast, we found that pathways involved in cholesterol, mineralocorticoid, and glucocorticoid biosynthesis was significantly downregulated.

The Wnt/ β -catenin signaling pathway has been implicated in various neurological and degenerative disorders, and this pathway is known to be upregulated in neurodegeneration. Upregulation of the Wnt signaling pathway has been suggested to play a role in anti-apoptosis and mitochondrial support, where the Wnt pathway can prevent further mitochondrial damage (Arrazola et al., 2015). Other work in Alzheimer's disease has shown that in disease, β -catenin, is mislocalized leading to increased toxicity (De Ferrari et al., 2003). However, this increase in Wnt signaling may be compensating for this loss and providing a cellular attempt to restore endogenous levels of β -catenin, a key component of the Wnt signaling pathway.

In ALS glia, the Wnt signaling pathway regulates the proliferation of astroglia (Chen et al., 2012). This may be an attempt to replenish diseased reactive glia with non-reactive astroglia. In Alzheimer's disease, there is a loss of astroglia (Smale G., 1995). However, in ALS this still remains a subject of debate with little evidence of actual loss of astroglia. Given the multitude of studies showing the involvement of the Wnt signaling pathway in ALS pathology, future work should continue to address this issue.

Another pathway that was significantly upregulated is the Toll-like receptor (TLR) signaling pathway. The TLR pathway is becoming recognized as significant in various neurological disorders, such as multiple sclerosis and Alzheimer's disease (Okun et al., 2009). Activation of TLRs leads to the production of pro-inflammatory cytokines in astroglia (Okun et al., 2009). Furthermore, it is known that TLRs may also be involved in neuronal plasticity. With this new evidence that cortical *G93A SOD1* astroglia display a significant increase in TLRs we provide further support for a cortical astroglial response to ALS pathogenesis in the *G93A SOD1* mouse model. Together, these transcriptome changes support the idea that there is still a strong astroglial response despite a lack of *glial fibrillary acidic protein* (*Gfap*) upregulation in the *G93A SOD1* mouse cortex.

Similar to studies on spinal cord glia that suggest a role in proliferation and dysregulation of the cell cycle, we also note an upregulation of these pathways in the cortex of the *G93A SOD1* mice (Chen et al., 2012). In neurodegeneration, it is known that astroglia also degenerate and thus proliferation appears to be an attempt to restore glia cell numbers. In the *G93A SOD1* mouse model, the cortex loses cortical thickness (Fogarty et al., 2016). Perhaps

this loss of thickness is contributed by the loss of glia cell types or is a consequence of altered astroglia profiles that eventually lead to altered dendrites, neuronal morphology, and components of the neuropil. Further work is needed to fully understand this phenomenon and studies should focus not solely on the spinal cord but also the glia in the cortex.

Astroglia are the major contributor to central nervous system cholesterol (Allaman, Belanger, & Magistretti, 2011). Dysregulation of cholesterol has implicated in various neurodegenerative diseases such as ALS, Alzheimer's disease, Parkinson's disease, and Huntington's disease (Vance, 2012). Cholesterol plays essential roles in neuronal synaptogenesis (Pfrieger, 2010). In our study, we found that cortical ALS astroglia downregulate cholesterol biosynthesis. This finding may help explain the neuronal dendritic spine loss observed in the *G93A SOD1* cortex. Furthermore, cholesterol levels need to be kept in tight regulation, where imbalance in either direction leads to neuronal deficits (Valenza et al., 2015; Vance, 2012).

Genetic Markers that are Dysregulated in End-Stage SOD1 G93A Cortical Astroglia

Coiled-coiled domain containing 85B (CCDC85B) was one of the most upregulated genes in end-stage astroglia. *Ccdc85B* is a nuclear transcriptional repressor and can function as a transcriptional regulator of β -catenin in a p53-dependent manner (Du, Wang, Hirohashi, & Greene, 2006; Iwai et al., 2008). Iwai et al showed that p53-stimulated *Ccdc85B* activity could downregulate canonical Wnt signaling by enhancing degradation of β -catenin. Additionally, *Ccdc85B* was shown to reduce levels of β -catenin by competitively interacting with T-cell factor 4 (TCF4), leading to inhibition of cellular proliferation. Given its role as a modulator of β -catenin activity, it is not surprising that *CCDC85B* is also important for neurodevelopment, as knockdown experiments of *CCDC85B* in zebrafish resulted in an open-neural tube phenotype similar to *N-cadherin* mutants, and previous work has implicated *CCDC85B* in murine models of hydrocephalus and Down Syndrome (S. S. Li et al., 2016; Markham et al., 2014; Mori et al., 2012).

In the cerebral cortex, *CCDC85B* mRNA is expressed moderately in astrocytes, neurons, oligodendrocytes, and highly expressed in microglia (Zhang et al., 2014). While the exact role of *Ccdc85B* in glial cells (and specifically in astrocytes) is unclear, reduced activity of β -catenin and aberrant Wnt signaling have previously been associated with multiple neurodegenerative diseases (Kahn, 2014; Libro, Bramanti, & Mazzon, 2016), (previously discussed). The upregulation of *Ccdc85B* found in our study could represent a protective mechanism to counter altered Wnt signaling and aberrant glial proliferation in ALS, as increased expression β -catenin and cyclin D1 have been associated with activation of glial proliferation in the spinal cord of *G93A SOD1* mice (Chen et al., 2012).

Interestingly, p53 (shown to induce expression of *CCDC85B* in Iwai et al) can be alternatively spliced or use alternative promoters to generate multiple isoforms, which can function as either promoters or inhibitors of cellular senescence (Fujita et al., 2009). Senescent cells can secrete cytokines to induce inflammation, and senescent and senescent-like astrocytes, as well as altered p53 isoform expression has recently been found in cortical tissue from ALS patients (Turnquist et al., 2016). While it is unknown whether these different p53 isoforms can also upregulate *Ccdc85B*, it is tempting to hypothesize that in

end-stage disease, cellular senescence may also function as a protective measure in response to aberrant activation of proliferative pathways (such as Wnt signaling), which may in turn exacerbate disease even further.

Another gene that we found to be significantly upregulated was *Protein Phosphatase 2 Catalytic Subunit Alpha (PPP2CA)*. *PPP2CA* encodes the catalytic subunit of protein phosphatase 2A (PP2A), one of the four major Ser/Thr phosphatases, with diverse cellular functions including its function as a tumor suppressor by regulating diverse signaling molecules such as Akt, p53, and β -catenin (Janssens & Goris, 2001; Sangodkar et al., 2016; Seshacharyulu, Pandey, Datta, & Batra, 2013). Different subunits of PP2A can bind to various components of the Wnt signaling pathway, and its role in regulating β -catenin levels in particular can be mixed depending on the level of the Wnt pathway that PP2A acts on (Persad et al., 2016; Ratcliffe et al., 2000; N. Yu et al., 2015).

In the cerebral cortex, the isoform *PPP2CA* is highly expressed in newly-formed and myelinating oligodendrocytes and is highly expressed in microglia and astrocytes (Zhang et al., 2014). Given that PP2A is a major phosphatase in the CNS, it is not surprising that dysfunction of this enzyme has been implicated in a range of neurodegenerative conditions, including ALS. In Alzheimer's Disease (AD), deficient PP2A activity, altered subunit expression, and aberrant post-translational modifications have been implicated with hyperphosphorylated tau, synapse loss and amyloidogenesis (C. Liu & Gotz, 2013; X. P. Liu et al., 2012; Sontag & Sontag, 2014). Interestingly a study examining the tg2576 mouse model of AD found that while overall cortical PP2A levels were decreased, levels of PP2A in GFAP-positive reactive astrocytes were found to be increased as compared to GFAP-positive astrocytes in the cortex of wild-type mice (X. P. Liu et al., 2012). Additionally, an RNAi screen in astrocytes revealed that PP2A could mediate dephosphorylation of NF- κ B and TRAF2 complexes, thus attenuating chemokine and cytokine signaling in astrocytes (S. Li, Wang, Berman, Zhang, & Dorf, 2006). Similar to AD, inhibition of PP2A signaling is associated with ALS disease pathogenesis in the spinal cord (Wang et al., 2014).

Furthermore, metabolic dysfunction in the spinal cord of *SOD1 G93A* mice has shown to be in part mediated by PP2A, via mutant TDP43-induced inhibition of AMPK phosphorylation (Perera et al., 2014). Thus, it is likely that upregulation of PP2A, and in particular *PPP2CA*, can serve diverse functions in the end-stage of ALS *G93A SOD1* mice in the cortex by mediating various cellular pathways. Additionally, the upregulation of this gene may serve as a negative regulator of aberrant proliferative pathways which become activated in the disease context.

Lastly, we found levels of *GRB2 Associated Binding Protein 2 (Gab2)* to be highly elevated in end-stage *G93A SOD1* astrocytes. GAB2 is a scaffolding protein that mediates interaction between receptor tyrosine kinase (RTK)s and intracellular signaling molecules such as ERK, AKT, and PI3K, through its association with Grb2. In the CNS, *GAB2* mRNA expression is low across most CNS cell types, with the exception of microglia, but its expression appears to increase with age in older astrocyte populations when compared to younger ones (Orre et al., 2014; Zhang et al., 2014). Interestingly, mutations in *GAB2* have been associated with increased risk for late-onset Alzheimer's Disease and cancer (Adams, Aydin, & Celebi, 2012; Bertram & Tanzi, 2009; Han, Huang, Gao, & Huang, 2017; Zou et al., 2013). In

normal physiological conditions, Gab2 is protective, playing roles in many signaling pathways and increased Gab2 levels have been shown to be associated with reduced senile plaques and neurofibrillary tangles in Alzheimer's disease. Though there is little research specifically related to GAB2's role in either astrocytes, or in ALS disease conditions, upregulation of this gene may again be a protective response in end-stage astrocytes in order to correct dysfunctional signaling networks.

When evaluating the most downregulated genes from our study, we found that *Von Willebrand Factor C-Domain Containing Protein 2-Like (Vwc2l; Brorin-like)* was significantly downregulated in end-stage cortical astroglia as compared to controls. Vwc2l is primarily expressed in neurons, and its expression increases with age in astrocytes (Orre et al., 2014; Zhang et al., 2014). Vwc2l is similar to the protein Brorin, a unique member of the Chordin family and a neural-specific antagonist of BMP signaling, which was shown to promote neurogenesis at the expense of astrogenesis in mouse neural precursor cells (Koike et al., 2007). This is fitting with its role as an antagonist of BMP, as BMP functions to switch neural precursor cell fate from neurogenesis to astrogenesis. Vwc2l (Brorin-like) was shown to function in a similar way to Brorin and was likewise able to antagonize BMPs and promote neurogenesis in mouse neural precursor cells (NPCs) (Miwa et al., 2009). Like Brorin, Vwc2l treatment on mouse NPCs did not promote astrogenesis, and inhibition of Vwc2l led to impaired neurogenesis in zebrafish embryos. While Vwc2l expression retains a similar profile to that of Brorin, it is more widely expressed in the adult mouse brain in the hippocampus, thalamus, and hypothalamus.

Interestingly, *brorin* knock-down in zebrafish embryos leads to abnormal development of the forebrain (Miyake et al., 2017). Furthermore, the same study showed that *brorin* knock-down prevented the development of GABAergic interneurons, oligodendrocytes, and impaired axon guidance in the forebrain. Instead, *brorin* knock-down enhanced the development of astrocytes, as measured by glutamine synthetase expression. The latter result suggests that *Brorin* normally functions to suppress astrogenesis during forebrain development, and downregulation of brorin may play a key role in astrocyte proliferation. In our study, downregulation of Vwc2l in end-stage cortical *G93A SOD1* astroglia may represent a switch from a quiescent astrocyte state to reactive astrogliosis, either as a result or mediating factor in the pathogenesis of ALS. This idea is supported by our finding that the cyclins and cell cycle regulation pathway was upregulated in *SOD1* cortical astroglia. Additionally, the expression pattern of Vwc2l in the hippocampus of the adult mammalian brain may suggest that Vwc2l downregulation could play a role in other neurodegenerative diseases, like AD.

Ion and metal homeostasis is widely accepted to be altered in neurodegenerative disorders (Angel et al., 2002; Saini et al., 2011). In our study, we uncovered *Metal Regulatory Transcription Factor 1 (Mtf-1)* as one of the most downregulated genes in end-stage astroglia. In normal physiologic conditions, Mtf-1 induces the expression of metallothioneins in a zinc-dependent manner, and functions to bind heavy metal ions and activate transcription of genes with metal responsive elements in order to prevent cellular toxicity (Bahadorani, Mukai, Egli, & Hilliker, 2010; Grzywacz et al., 2015). While Mtf-1 retains high affinity for zinc ions in humans, it also can regulate expression of genes related

to copper homeostasis and copper sensing (Bellingham, Coleman, Masters, Camakaris, & Hill, 2009; Grzywacz et al., 2015).

Despite its low expression in the CNS under normal physiological conditions, Mtf-1 has been implicated in a number of neurodegenerative diseases and neuropathological conditions. In human fibroblasts depleted of a major copper efflux protein, siRNA knock-down of *MTF-1* was able to suppress mutant prion gene expression and deplete prion protein levels (Bellingham et al., 2009). In a model of temporal lobe epilepsy, Mtf-1 was demonstrated to bind to metal-responsive elements in the voltage-gated calcium channel 3.2 promoter, leading to increased expression of this channel and downstream enhanced excitability in the hippocampus, which was not observed in the presence of a dominant-negative form of Mtf-1 (van Loo et al., 2015). In contrast to these studies, Mtf-1 over-expression in a *Drosophila* model of Friedrich's ataxia suppressed motor impairment, while Mtf-1 loss of function enhanced it (Soriano et al., 2016). Additionally, in a *Drosophila* model of Parkinson's disease (Parkin mutant), the overexpression of Mtf1 ameliorated disease pathogenesis, including motoric functions and lifespan (Saini et al., 2011). Importantly, Mtf1 restored oxidative stress levels and mitochondrial structural abnormalities. The Mtf1 mutant was, however, embryonic lethal, further supporting the essential cellular roles of Mtf1 in metal homeostasis. Lastly, in another *Drosophila* model, a null mutation in *MTF-1* led to a shortened lifespan of adult flies fed a normal diet but not a high-zinc diet and, conversely, *MTF-1* over-expression resulted in decreased lifespan for flies fed a high-zinc diet (Bahadorani et al., 2010). This study suggests that at least in *D. melanogaster*, the levels of zinc and *MTF-1* must both be considered when examining either a protective (resistance to heavy metals) or pathogenic (enhanced sensitivity to heavy metals) role of *MTF-1*.

Interestingly, prolonged heavy metal ion exposure has previously been associated with the development of sporadic ALS, and susceptibility to heavy metal ion exposure may be mediated by genetic variants that impair metal detoxification (Armon, Kurland, Daube, & O'Brien, 1991; Chancellor, Slattery, Fraser, & Warlow, 1993; Morahan, Yu, Trent, & Pamphlett, 2007; Roelofs-Iverson, Mulder, Elveback, Kurland, & Molgaard, 1984). At least one single-nucleotide polymorphism in *MTF-1* is found in some female sALS patients but not male sALS patients, though it was not associated with increased susceptibility to heavy metal ion exposure nor change in amino acid sequence of Mtf-1, leaving the functional consequence of this polymorphism unclear (Morahan et al., 2007). In our study, decreased expression of *MTF-1* in astroglia may lead to altered metal ion homeostasis, leaving diseased astroglia at a disadvantage to metabolically support neuronal neighbors, and may thus contribute to some of the neuronal deficits such as hyperexcitability in this mouse model. Future studies could explore Mtf1 for potential therapeutic relevance.

Lastly, we also found decreased expression of genes associated with other neurological diseases. *Ganglioside-Induced Differentiation-Associated Protein 1* (*Gdap1*) is a mitochondrial fission factor, and loss-of-function mutations in this gene leading to impaired redox homeostasis are associated with demyelinating (type 4A), axonal recessive (type 2), or mixed forms of Charcot-Marie-Tooth Disease, an umbrella name for a group of hereditary motor and sensory neuropathies (Cassereau, Chevrollier, Bonneau, et al., 2011; Cassereau,

Chevrollier, Gueguen, et al., 2011; Niemann et al., 2014). *Ganglioside-Induced Differentiation-Associated Protein 1 Like 1 (Gdap1l1)* is similar in sequence to *Gdap1*, and is highly expressed in neurons, but not astrocytes, leaving the functional consequence of its downregulation in *G93A SOD1* unclear (Zhang et al., 2014). Impaired mitochondrial function due to the *SOD1* mutation may exacerbate downregulation of other genes involved in redox and ion homeostasis, such as *Gdap1l1*.

We also saw significant down-regulation of *nucleolar protein 56 (Nop56)*, a gene which has previously been implicated in the pathogenesis of spinocerebellar ataxia type 36 (SCA36), a late-onset and slowly-progressing form of the disease with motor neuron involvement (Arias, Quintans, Garcia-Murias, & Sobrido, 1993; Garcia-Murias et al., 2012; Kobayashi et al., 2011). In SCA36, RNA foci form as a result of a hexanucleotide repeat expansion in intron 1 of the *NOP56* gene (Figley, Thomas, & Gitler, 2014; Loureiro, Oliveira, & Silveira, 2016). While most people have under 14 copies of hexanucleotide repeats in this region, affected individuals can have more than 650 repeats (Garcia-Murias et al., 2012; Kobayashi et al., 2011). *Nop56* is moderately-highly expressed in most CNS cell types, including astrocytes and neurons, and intranuclear RNA foci have been found in the frontal cortex and spinal motor neurons of affected patients (W. Liu et al., 2014; Zhang et al., 2014). The potential role of *Nop56* in ALS pathogenesis is unclear, as a large sample of sporadic ALS patients (n = 352) were found to have a similar number of repeats in this gene as healthy control subjects, all within the healthy range, suggesting that variation in this region does not contribute to sporadic ALS pathogenesis (Figley et al., 2014). Interestingly though, another study using the *G93A SOD1* mouse model also found progressive reduction of *Nop56* in the lumbar and cervical spinal cord of early-symptomatic to end-stage disease by immunohistochemistry, although they did not find reduction of *Nop56* protein in the primary motor cortex (Miyazaki et al., 2013). Given that this last study only examined gross anatomy at the protein level, it is still unclear what role *Nop56* downregulation specifically in cortical astrocytes may play in ALS pathogenesis.

Differences and similarities between *G93A SOD1* astroglia from the cortex and spinal cord

In our previous work, we examined the transcriptome of early (P60) and mid-stage (P90) astroglia in the spinal cord of the *G93A SOD1* mouse (Miller et al., 2016). In this paper, we extend our studies into the cortex at end-stage (P120) disease. Despite regional heterogeneity between astrocytes in the cortex and spinal cord, these two studies together illustrate a dynamic picture of the transcriptome of astroglia as the *G93A SOD1* mouse progresses from pre-onset, to onset of motor symptoms, to full-blown symptoms in end-stage disease. Importantly, we have uncovered similar genetic dysregulation between disease states. We have illustrated top dysregulated genes both downregulated and upregulated that are shared between the spinal cord and cortical *G93A SOD1* astroglia (Table 4). For example, cellular ion homeostasis is disrupted at all time points: *KCNK1* and *SPARCL1* (potassium channel and calcium ion binding proteins, respectively) are upregulated in early to mid-stage spinal cord astrocytes, while end-stage astrocytes in the cortex show downregulation of *MTF-1*, a gene crucial in inducing genes involved in metal ion homeostasis. This is consistent with many studies showing that ion and metal homeostasis is altered in neurodegenerative diseases (Angel et al., 2002; Saini et al., 2011). Additionally, the change in dysregulated

genes may reflect a changing microenvironment and failure of astrocytes to buffer ion dyshomeostasis and/or to provide support to neighboring neurons over disease course.

Interestingly, genes involved in pathways governing metabolic conditions and cell fate decisions such as the PI3K/AKT/mTOR and Wnt signaling pathways were dysregulated at multiple time points. While calcium transport and glutamate receptor signaling pathways were upregulated in early-disease in the spinal cord, we did not see this same dysregulation in end-stage disease in the cortex, and instead saw upregulation of pathways involved in cellular proliferation, such as the Wnt signaling pathway and (likely as a result of increased Wnt signaling) cyclins and cell-cycle regulation. This latter result is in concordance with prior work showing that the Wnt signaling pathway regulates the proliferative state of ALS astroglia, and that canonical Wnt molecules are upregulated at end-stage GFAP-positive astrocytes in the spinal cord of *G93A SOD1* mice, and down-regulated at mid-disease (Chen et al., 2012; L. Yu et al., 2013). Given the interconnectedness of these pathways, a likely imbalance in one, for example, decreased expression of lysosomal proteinases and inhibition of autophagy by enhanced mTOR signaling at mid-disease, may lead to differential expression of Wnt signaling components such as upregulation of *Dvl* and decreased activity of GSK3 and increased canonical Wnt signaling by end-stage disease (Hermida, Dinesh Kumar, & Leslie, 2017). Additionally, aberrant synaptic activity throughout the disease course may contribute to astrocytic dysfunction, as synaptic activity in neurons can lead to differential effects on Akt, the Wnt pathway, mTOR, and GSK3, mediated by voltage-dependent calcium channels (Hermida et al., 2017; Ma, Tzavaras, Tsokas, Landau, & Blitzer, 2011). Given that astrocytes are a crucial component of neuronal synapses, and that we have previously shown at least one mediator of excitatory synaptic formation, *SPARCL1*, to be upregulated in early disease states, taken with our findings of ion dysregulation at all disease states, it is not inconceivable that aberrant synaptic activity could also underlie our findings of dysregulation of major signaling pathways. Because this study is unique in its examination of cortical astrocytes at end-stage disease, and given the discrepancies in the molecular profiles of cortical and spinal cord astrocytes, further investigation is necessary to determine if the transcriptome changes in early and mid-disease in the spinal cord also occur in the cortex.

CONCLUSIONS:

SOD1 G93A Cortical Astroglia Transcriptome Database as a Source for Therapeutic Investigations and Conclusions.

In this study, we isolated pure cortical astroglia from the *SOD1 G93A* mice, an ALS motor neuron disease model, and performed microarray analyses to gain knowledge about some of the altered pathways and genes in diseased astroglia. This is the first study of its kind to focus solely on cortical ALS astroglia transcriptomic changes from an adult rodent model. This complements our previous study that focused on spinal cord ALS astroglia. However, with the growing knowledge that astroglia in distinct neuroanatomical regions are vastly different, we can now use this extensive transcriptome database to compare the major dysregulated genes and pathways between the cortex and spinal cord astroglia. Unsurprisingly, we found expression of genes that make up several pathways to be similar

between cortical and spinal cord diseased astroglia, but we also uncovered a large number of unique pathways and genes.

During our investigations using pathway analysis software and statistical approaches to determine the most up- or down-regulated genes and pathways, we found some common candidates that are shared with other neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease. Some of these pathways included the Wnt/Beta-catenin signaling pathway, ion homeostasis, and cell cycle regulation. Additionally, we found genes heavily involved in these pathways that provide us with clues into the pathology of ALS such as glial proliferation markers and regulators of metal homeostasis.

We believe that this database of cortical astroglia in the ALS *SOD1 G93A* mouse model will be useful for future investigations focused on regional heterogeneity in disease (i.e. spinal cord vs cortex) and should aid understanding of the cortical pathology of motor neuron diseases such as ALS.

MATERIALS AND METHODS:

Animal Models:

BAC-ALDH1L1-eGFP and the G93A SOD1 (Jackson Labs) mice were used for in vivo experiments. The care and treatment of animals is in accordance with the NIH Guide for the Care and Use of Laboratory Animals, the Guidelines for the Use of Animals in Neuroscience Research, and the Johns Hopkins University IACUC (protocol M14M089). Mice were housed at standard temperature (21C) and in light-controlled environment with ad libitum access to the food and water. BAC-ALDH1L1-eGFP mice were crossed with G93A SOD1 mice to generate double transgenic mice. Littermates were used as control and for comparison between different astrocyte populations. No more than five mice were kept in a cage, in accordance with Johns Hopkins IACUC. Mice were then sacrificed at a designated time point post breeding and aging.

Fluorescence-activated cell sorting (FACS):

Cortical astrocytes from BAC-ALDH1L1-eGFP and BAC-ALDH1L1-eGFP/G93A SOD1 double transgenic mice were analyzed by FACS. Mice were anesthetized with an intraperitoneal injection of ketamine xylazine. The entire cortex was immediately dissected and dissociated as previously described¹⁴. Single cells were then sorted by using MoFlo high-speed cell sorter and gated based on eGFP fluorescence. Three to four mice were used for each group.

Microarray:

Microarray procedure and analyses were performed as previously published. Briefly, total RNA was isolated from FACS sorted populations using the RNA isolation kit (Qiagen) and the concentration was determined with Nanodrop (Thermo Fisher Scientific) and Bioanalyzer (Agilent Technologies). Only samples with a RNA integrity (RIN) score greater than 5 were used. Total RNA was lineally amplified and labeled in a Nugene protocol.

Sample labeling and hybridization with Mouse Exon 1.0 ST chips (Affymetrix) were performed in the Johns Hopkins University microarray facility following manufacturer's protocol. After hybridization, hybridization signals were acquired and normalized with the use of Partek Genomics Suite Software (Partek). Differential gene expression between the different conditions was assessed by statistical linear model analysis using the Partek, Tibco Spotfire, and Prism 7 software packages. The moderated t-statistic p-values derived from the Partek analysis above were further adjusted for multiple testing by Benjamini and Hochberg's method to control false discovery rate (FDR). The FDR cutoff of <10% was used to obtain the list of differentially expressed genes. In total a minimum of three mice were used for these experiments. (Note: Data deposition is submitted to GEO by the following accession number: GSE111031).

Pathway Analysis:

Gene ontology and pathway analysis were performed from data obtained from Partek and Spotfire post statistical analyses using the Ingenuity Pathway Analysis software package.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SOD1_1Way-P120 vs Control Volcano Plot

Figure 1:

Volcano plot represents a large number of significantly significant differentially expressed genes. A) Genes below a p-value of 0.05 were labeled in red and genes not significant are labeled in blue. The y-axis represents the negative Log10 of cortical SOD1 astroglia vs control p-value. The x-axis is represented by the fold-change of cortical SOD1 astroglia vs control.

Figure 2:

Subcellular localization shows preferences in cortical G93A SOD1 end-stage astroglia dysregulated genes. A) Genes above a standard deviation of at least 2, p -value < 0.05 , were compartmentalized based on their cellular localization. B) Genes below a standard deviation of at least 2, p -value < 0.05 , were compartmentalized based on their cellular localization. Ingenuity pathway analysis was used to identify the localization of candidate genes and displayed with a graph generated with Prism 7. Genes have a standard deviation of at least ± 2 , and a p -value < 0.05 .

Figure 3: STRING map showing interconnecting proteins dysregulated in the Wnt signaling pathway in G93A SOD1 cortical astroglia. A) STRING map showing proteins that interact with Csnk1g1. B) Table showing fold-change values for the STRING map proteins in G93A SOD1 cortical astroglia vs control.

Figure 4:
STRING map showing interconnecting proteins that are upregulated. A) STRING map showing the most upregulated genes at least 2 standard deviations above.

Figure 5:
 STRING map showing interconnecting proteins that are downregulated. A) STRING map showing the most downregulated genes at least 2 standard deviations below.

Table 1:

Top upregulated genes in end-stage P120 ALS cortical grey-matter astroglia.

Gene	FC (SOD1 vs Control)	Function
Ccdc85b	5.70	Transcriptional repressor; may inhibit CTNNB1
Ppp2ca	4.04	Serine/Threonine phosphatase; implicated in negative control of cell growth and division
Gab2	3.40	Adaptor protein involved in regulating multiple signaling pathways
Slc30a7	3.22	Zinc transporter that facilitates zinc transport from the cytoplasm to the Golgi apparatus; role in zinc homeostasis
Elp4	2.68	Subunit of RNA polymerase II elongator complex; may play a role in chromatin remodeling
Phi21a	2.57	Component of the BHC complex, that acts to repress transcription of neuron-specific genes in non-neuronal cells
Il1rap11	2.56	Plays a role in presynaptic and postsynaptic differentiation and dendritic spine formation
Csnk1g1	2.37	Serine/Threonine-protein kinase; participates in Wnt signaling
Sfrp2	2.36	Modulator of Wnt signaling by interactions with Wnts
Zip236	2.33	Zinc finger protein involved in nucleic acid binding

Microarray results were subjected to two-way ANOVA and statistical analyses. Top candidates were selected based on their enrichment in the end-stage P120 ALS cortical grey-matter astroglia compared to littermate controls. Functional annotation was obtained from [GeneCards.org](#). A complete list of enriched genes can be found in Supplementary Table 1.

Table 2:
Top differentially regulated canonical pathways at end-stage P120 SOD1 cortical astroglia.

Canonical Pathway	p-value	Regulation
Wnt/ β -catenin Signaling	1.78e-05	Upregulated in SOD1 cortical astroglia
Toll-like Receptor Signaling	8.99e-03	Upregulated in SOD1 cortical astroglia
Cyclins and Cell Cycle Regulation	9.94e-03	Upregulated in SOD1 cortical astroglia
Mineralocorticoid Biosynthesis	2.46e-02	Downregulated in SOD1 cortical astroglia
Glucocorticoid Biosynthesis	2.68e-02	Downregulated in SOD1 cortical astroglia
Cholesterol Biosynthesis I	2.90e-02	Downregulated in SOD1 cortical astroglia

Microarray results were subjected to two-way ANOVA and statistical analyses. Post-analyses, all genes up- or down-regulated that were at least +/- 2 standard deviations with a p-value of 0.05 were subjected to ingenuity pathway analysis. The top canonical pathways affected are listed in the table.

Table 3:

Top downregulated genes in end-stage P120 ALS cortical grey-matter astroglia.

Gene	FC (SOD1 vs Control)	Function
Zfp513	-3.48	Transcriptional regulator involved in retinal development and maintenance
Vwc2l	-3.27	Plays a role in neurogenesis
Gdap1l1	-2.98	After neurite differentiation, this gene becomes upregulated
Mtf1	-2.89	Transcription factor involved in inducing expression of genes involved in metal homeostasis
Nop56	-2.65	Nucleolar protein involved in pre-rRNA processing
Man2a1	-2.63	Catalyzes the first step in the biosynthesis of complex N-glycans
Tnfrsf13	-2.48	Tumor necrosis factor ligand family; may induce apoptosis through interactions with other tumor necrosis factor receptors
Eprs	-2.43	Catalyzes the attachment of cognate amino acid to the corresponding tRNA
Erv3	-2.40	Retroviral envelope protein that may mediate recognition and membrane fusion during infection
Nvl	-2.19	Participates in the assembly of telomerase holoenzyme; may play a role in 60S ribosomal subunit biogenesis

Microarray results were subjected to two-way ANOVA and statistical analyses. Top candidates were selected based on their downregulation in the end-stage P120 ALS cortical grey-matter astroglia compared to littermate controls. Functional annotation was obtained from [Genecards.org](#). A complete list of enriched genes can be found in Supplementary Table 1.

Table 4:

Top differentially regulated genes shared between Spinal Cord and Cortical G93A SOD1 astroglia.

Region	Spinal Cord	Spinal Cord	Cortex
Age	P60	P90	P120
Downregulated genes	Zfp513		
	GM2518		
	Man2a1		
	Tnfsf13		
Upregulated genes	Mip		
	Tmem60		
	Mettl16		
	Tle1		