Axotomy-induced target disconnection promotes an additional death mechanism involved in motoneuron degeneration in ALS transgenic mice

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Abstract

The target disconnection theory of amyotrophic lateral sclerosis (ALS) pathogenesis suggests disease onset is initiated by a peripheral pathological event resulting in neuromuscular junction loss and motoneuron (MN) degeneration. Pre-symptomatic mSOD1G93A mouse facial MN (FMN) are more susceptible to axotomy-induced cell death than wild-type (WT) FMN, which suggests additional CNS pathology. We have previously determined that the mSOD1 molecular response to facial nerve axotomy is phenotypically regenerative and indistinguishable from WT, whereas the surrounding microenvironment shows significant dysregulation in the mSOD1 facial nucleus. To elucidate the mechanisms underlying the enhanced mSOD1 FMN loss after axotomy, we superimposed the facial nerve axotomy model on pre-symptomatic mSOD1 mice and investigated gene expression for death receptor pathways after target disconnection by axotomy vs. disease progression. We determined that the TNFR1 death receptor pathway is involved in axotomy-induced FMN death in WT, and partially responsible for the mSOD1 FMN death. In contrast, an inherent mSOD1 CNS pathology resulted in a suppressed glial reaction and an upregulation in the

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Conflict of Interest Statement
The authors declare that they have no conflict of interest.

Role of Authors
All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: MMH, NAM, VMS, KJJ. Acquisition of data: MMH, RJB, TDA. Analysis and interpretation of data: MMH, NAM, VMS, KJJ. Drafting of the manuscript: MMH. Critical revision of the manuscript for important intellectual content: MMH, VMS, KJJ. Statistical analysis: MMH, RJB. Obtained funding: VMS, KJJ. Administrative, technical, and material support: MMH, NAM, RJB, TDA. Study supervision: KJJ.
Fas death pathway after target disconnection. We propose that the dysregulated mSOD1 glia fail to provide support to injured MN, leading to Fas-induced FMN death. Finally, we demonstrated that during disease progression, the mSOD1 facial nucleus displays target disconnection-induced gene expression changes that mirror those induced by axotomy. This validates the use of axotomy as an investigative tool in understanding the role of peripheral target disconnection in the pathogenesis of ALS.

**Indexing Terms**

motoneuron; MN; facial nerve axotomy; SOD1; ALS; gene expression

**INTRODUCTION**

Amyotrophic lateral sclerosis (ALS) is the most common adult motoneuron (MN) degenerative disease, with a mean survival of only three to five years after symptom onset. ALS affects voluntary muscle movement and ultimately leads to respiratory failure among other pulmonary complications (Wijesekera and Leigh, 2009; Naganska and Matyja, 2011; Brooks et al., 2000). Clinical diagnostic criteria of ALS often include signs of both upper and lower MN deficits in the absence of evidence for other neurological conditions (Rowland and Shneider, 2001; Kraemer et al., 2010). Genetic studies have determined that a small portion of cases are inherited (fALS); whereas 90% of cases are sporadic (sALS; Mulder et al., 1986). Mutations in the gene encoding the enzyme Cu/Zn superoxide dismutase 1 (SOD1) make up nearly 20% of fALS cases and 7% of sALS cases (Rosen et al., 1993; Saeed et al., 2009; Andersen, 2006).

The first and most widely studied ALS mouse model involves overexpression of the human mutant SOD1 gene with a Gly93→Ala (mSOD1G93A) substitution within exon 4 (Gurney et al., 1994). Additional SOD1 mutations have been used on various background strains of mice. In the present study we utilized the B6/SJL hybrid mSOD1G93A mouse, which displays pre-symptomatic, symptomatic, and end-stage ALS-like disease phases. In the B6/SJL mSOD1G93A mouse, the first symptoms appear at approximately 90 days of age and consist of a slight tremor in the hindlimbs. Proximal muscle weakness and atrophy begin to develop by 120 days of age and the animal reaches end-stage by 136 days of age, which is marked by severe paralysis. MN cell death accompanies the progression of symptoms and continues into end-stage during which the MN loss reaches approximately 50% in the ventral horn of the spinal cord (Chiu et al., 1995). Brainstem nuclei, such as the hypoglossal, trigeminal and facial motor nuclei, are also affected, but display significant MN loss during the late symptomatic stage or at end-stage (Haenggeli and Kato, 2002; Niessen et al., 2006; Chiu et al., 1995; Nimchinsky et al., 2000).

ALS is likely a multifactorial disease involving several cell types that directly or indirectly affect MN functionality and survival (Bilsland et al., 2010, Liscic and Breljak, 2011, Philips and Robberecht, 2011). The initial pathology of the disease appears to be a pre-symptomatic denervation of muscle endplates around 47 days of age (Fischer et al., 2004). Decreases in muscle mass and fiber diameter follow, and are considered a result of the loss of functional
motor units (Marcuzzo et al., 2011). While compensatory axonal sprouting is evident during the pre-symptomatic stage, it appears to be transient as neuromuscular junction (NMJ) loss continues (Schaefer et al., 2005). By the time the mSOD1 mouse reaches the symptomatic stage, significant MN loss has already occurred (Chiu et al., 1995). These findings support the target disconnection or “die-back” theory of ALS pathogenesis, in which disease onset is initiated by an unknown peripheral pathological event resulting in functional NMJ loss and, although some MN attempt to reconnect to the lost targets, they are relatively unsuccessful and the result is MN degeneration (Fischer et al., 2004; Dadon-Nachum et al., 2011).

Pre-symptomatic mSOD1 facial MN (FMN) are considerably more susceptible to axotomy-induced cell death than wild-type (WT) FMN (Mariotti et al., 2002; Ikeda et al., 2005), which suggests that an additional CNS pathology, beyond the initiating axonal die-back events, may contribute to differential axotomy-induced target deprivation responses. To explore the differences in responses to axotomy by mSOD1 and WT mice, we superimposed the facial nerve axotomy model on pre-symptomatic mSOD1 mice, analyzed gene expression changes within the facial motor nucleus and found, surprisingly, that mSOD1 FMN display a WT-like phenotypic regenerative response to injury (Mesnard et al., 2011). However, we did discover aberrant non-neuronal responses to axotomy in the brainstem of mSOD1 mice (Mesnard et al., 2011) that are consistent with recent data implicating the microenvironment surrounding MN cell bodies as causative in MN cell death (Clement et al., 2003; Sargsyan et al., 2005; Pramatarova et al., 2001; Ilieva et al., 2009).

The goal of the current study was to elucidate the molecular mechanism(s) underlying the enhanced mSOD1 FMN loss after target deprivation by axotomy. We hypothesized that MN death in WT and mSOD1 could be explained by one of two scenarios: 1) a common death pathway, under differential regulation, or 2) completely distinct death pathways resulting in different rates of cell death. We determined that the Tumor Necrosis Factor Receptor 1 (TNFR1) death receptor pathway is likely involved in axotomy-induced FMN death in the WT, and partially responsible for mSOD1 mouse FMN death, as indicated by mRNA expression of TNFR1 pathway components. In contrast, inherent pre-symptomatic mSOD1 mouse pathology also involves the upregulation of mRNA for molecules involved in the Fas death receptor (Fas) pathway. We propose that, after facial nerve transection in the mSOD1 mouse, a dysregulated glial response, representing an aspect of the primary mSOD1 pathology, likely plays a role in upregulating the MN-specific Fas death pathway and inducing rapid MN death. Finally, we demonstrated that, as the mSOD1 mouse progresses through the pre-symptomatic and symptomatic stages of disease, the facial motor nucleus reveals target disconnection-induced gene expression changes that resemble those induced by axotomy. The current study validates the use of axotomy in the mSOD1 mouse model as a tool for investigating mSOD1 pathologies.

MATERIALS AND METHODS

Animals and surgical procedures

For all experiments in this study, mice were purchased from Jackson Labs (Bar Harbor, ME) at seven weeks of age. The mice utilized in the present study were on two different background strains; C57BL/6 WT control mice (B6; #000664) and B6/SJL transgenic
mSOD\textsuperscript{G93A} (B6SJL-Tg(SOD1-G93A)\textsubscript{Gur}; #002726) mice. The WT mouse, on the B6 background, was selected as it has been used extensively in peripheral nerve injury studies by our laboratory and others, and the cellular and molecular responses are well documented (Canh et al., 2006; Beahrs et al., 2010; Mesnard et al., 2010; Xin et al., 2011). The background strain of the mSOD\textsuperscript{G93A} mouse was on a hybrid, non-uniform background consisting of a mixture of SJL and B6. This particular ALS mouse model was the first developed and subsequently the most widely used and well-characterized, particularly with respect to documenting pathological changes during disease progression (Gurney et al., 1994; Dadon-Nachum et al., 2011). We have previously determined that there are no strain differences between WT B6 and WT B6/SJL with respect to their responses to axotomy, including gene expression and MN survival numbers (Mesnard et al., 2011).

All animal procedures were performed in accordance with institutional and National Institutes of Health guidelines on the care and use of laboratory animals for research purposes and approved by the Institutional Animal Care and Use Committee (IACUC). Mice were housed under a 12 hour light/dark cycle in autoclaved microisolator cages and provided autoclaved pellets and autoclaved drinking water ad libitum. The facility that housed the mice was equipped with a laminar flow system in order to maintain a pathogen-free environment. Mice were permitted to acclimate to their environment for one week; therefore, surgical procedures were performed at 56 days of age. Female mice were used for all experiments because of their reduced aggressive behavior, relative to males, during group housing.

Prior to all surgical procedures, mice were fully anesthetized with 3% isoflurane inhalation and maintained at 2% isoflurane throughout the procedure. The right facial nerve was exposed at the level of the stylomastoid foramen and completely transected proximal to the bifurcation of the posterior and anterior auricular branches, as described previously (Serpe et al., 1999). Successful transections were verified by complete, unilateral loss of vibrissae movement and eye blink reflex on the ipsilateral side. The left facial nerve remained intact and served as an internal control.

The left facial motor nucleus served two purposes within this study. First, it was used as an uninjured internal control for comparison purposes to the right, axotomized facial motor nucleus, to calculate percent FMN survival and percent change in mRNA expression following facial nerve axotomy. Second, the left uninjured control nucleus was used to investigate disease-induced changes in mSOD1 facial motor nuclei with age. Age-related changes in FMN numbers and relative mRNA expression, within the left uninjured mSOD1 facial motor nucleus, were measured and compared to that of WT left uninjured facial nuclei. Therefore, axotomy-induced changes and disease-induced changes can be separately acquired from the same groups of mice. Each group of mice within the various time courses was identified not only by days-post axotomy (dpa), referring to the percent change in axotomized nucleus compared to control nucleus; but also days of age, referring to the age of the uninjured, contralateral control nucleus.
Behavioral Assessment

WT and mSOD1 mice (n = 6 and 9, respectively) were evaluated for motor deficiency three times per week, beginning at 79 days of age and ending on the day of euthanasia (112 days of age). Mice were individually placed within a clean, transparent, acrylic open field box (dimensions: 46cm × 33cm × 19cm) for 2 minutes and were directly observed for motor deficiencies as well as video recorded for later analysis. Mouse strain and identification information was coded by an uninvolved investigator throughout the duration of behavioral assessments and the order that the mice were tested was rotated every testing day. The behavioral scoring system used was based on a combination of systems used routinely within the literature (Knippenberg et al., 2010; Tada et al., 2011; Marcuzzo et al., 2011; Irwin, 1968). The following 7-point scale was used to evaluate motor symptoms present: 6 = normal posture, gait and forward motion; 5 = abnormal posture or trunk position during forward motion, such as a hunched appearance or sloped rump; 4 = waddling or lateral wobbling movement of the pelvis during forward motion; 3 = dysmetria or incoordinate movements with a distinct jerky, oscillating component during forward motion; 2 = at least one knee contacts ground during forward motion; 1 = inability to properly place at least one hind foot flat during forward motion, often manifests as knuckle walking; 0 = at least one hindlimb is paralyzed, inability to move the hindlimb results in dragging limb during forward movement. Open field video recordings were scored by a second investigator for verification purposes. Mean scores ± standard error of the mean (SEM) were calculated for WT and mSOD1 mice and statistical analysis to determine symptom onset was accomplished by using a two-way repeated measures analysis of variance (ANOVA), followed by the Student-Newman-Keuls post hoc multiple comparison test, with significance at $P < 0.05$ (SigmaPlot, version 12.3).

To date, it is estimated that the mSOD1 mouse strains provide a model for only 20% of fALS cases and 7% of sALS cases (Rosen et al., 1993; Saeed et al., 2009; Andersen, 2006). While the disease pathology and symptomatic phenotypes are relatively consistent between the mSOD1 mouse models and the ALS patient population (Shibata, 2001), we must keep in mind that, while the overall disease-related processes may be similar, specific disease-related mechanisms will undoubtedly be distinctly different. Findings generated from studies using mSOD1 mouse models will need to be further investigated within the ALS patient population and, therefore, results presented within this publication should be interpreted with caution.

Facial motoneuron (FMN) counts

WT and mSOD1 mice (n = 4–6/timepoint/group) received a right facial nerve axotomy at 56 days of age and survived either 28 or 56 dpa (84 and 112 days of age, respectively). Mice were euthanized by CO$_2$ asphyxiation in an isolated chamber followed by cervical dislocation. The brains were removed, flash-frozen and cryosectioned throughout the facial nucleus at 25µm, as previously described (Mesnard et al., 2010). The slide-mounted tissue sections were fixed in 4% paraformaldehyde in 1mM PBS for 15 minutes then processed for routine histology (Mesnard et al., 2010). All slides were coded by an uninvolved investigator prior to analysis.
A Leica DMRB light microscope equipped with a digital camera (Microfire Optronics #S97808) and the Neurolucida Tracing System (version 8.23) was used to collect representative photomicrographs of thionin-stained control and axotomized facial motor nuclei. The photomicrographs were transferred to Adobe illustrator for figure development. Slight adjustments in contrast and brightness were made for comparison purposes. FMN displaying a clear nucleus and nucleolus were morphologically identified and demarcated with a symbol for counting purposes using Neurolucida. All sections throughout the facial nucleus for each animal were counted in order to determine the relative number of FMN within the control nucleus compared to the axotomized nucleus for two timepoints, 28 and 56 dpa (84 and 112 days of age, respectively). For the study of disease progression, the average number of FMN per section was calculated using the total number of FMN in the uninjured control nucleus divided by the total number of sections counted for that nucleus. For the axotomy experiments, the mean percent FMN survival was calculated using the total number of FMN in the axotomized nucleus divided by the total number of FMN in the uninjured control nucleus, multiplied by 100%. The Abercrombie correction factor, \[ N = \frac{n \times T}{T + D} \], was used; where \( N \) is the actual number of cells, \( n \) is the number of nuclear profiles, \( T \) is the section thickness (25\( \mu \)m), and \( D \) is the average diameter of nuclei (18.7\( \mu \)m; Coggeshall, 1992). Statistical analysis was accomplished by using a one-way ANOVA, followed by the Student-Newman-Keuls post hoc multiple comparison test, with significance at \( P < 0.05 \) (SigmaPlot, version 12.3).

**Laser microdissection**

WT and mSOD1 mice (n = 5–9/timepoint/group) received a right facial nerve axotomy at 56 days of age and were euthanized by CO\(_2\) asphyxiation followed by cervical dislocation at 3, 7, 14, 28 or 56 dpa (corresponding to 59, 63, 70, 84, or 112 days of age). No injury groups were included (n = 5) as a 0 dpa timepoint for visualizing and comparing mRNA expression changes after axotomy, across time for individual genes. Brains were immediately removed, rapidly frozen, cryosectioned at 25\( \mu \)m throughout the rostrocaudal extent of the facial motor nucleus and thaw-mounted onto glass polyethylene membrane-slides (Nuhsbaum #11505158), as previously described (Mesnard et al., 2011). Prior to laser microdissection, slides were permitted to come up to −20°C, from −80°C. Great care was taken throughout the procedure to minimize contamination or RNA degradation. Sides were individually fixed and stained for histological identification. Fixing and staining was performed as previously described (Mesnard et al., 2011). The control and axotomized facial motor nuclei were laser microdissected (Leica AS LMD) and collected into 65\( \mu \)l of extraction buffer (PicoPure RNA Isolation Kit, Invitrogen, #KIT0204), which had been added to the collection tube caps. Pooled samples, from axotomized and control facial nuclei were separately collected for each mouse, and stored at −80°C until RNA extraction was performed.

**Semiquantitative real-time qPCR**

Total cellular RNA was isolated from laser microdissected samples using the PicoPure RNA Isolation Kit (Invitrogen, #KIT0204) including a DNase treatment step (Qiagen, #79254). Total RNA quantification was determined using a NanoDrop 2000 spectrophotometer and concentrations were standardized for reverse-transcription. Complementary DNA was
obtained using Superscript First Strand Synthesis System (Invitrogen, #11904-018) according to the manufacturer’s instructions.

Polymerase chain reaction (PCR) primer sets used were designed from published mouse sequences using Oligo Primer Analysis software (version 6.54, Molecular Biology Insights) and selected for duplex stability, internal stability, and low complementarity. Primer sets consisted 16–25 nucleotides each and produced amplicon lengths between 78 and 121 base-pairs (Table 1). All designed primer sets were custom ordered (Bio-Synthesis, Inc.). Genes were selected and grouped for analysis: TNFR1 death receptor pathway genes (Tnfa/Tnfr1/Tradd/Sodd/Caspase-8/Caspase-3), Fas death receptor pathway genes (Fas/Fadd/Daxx/Ask1/Nnos), regenerative and pro-survival genes (Gap-43/βII-tubulin/Cx3cr1/Tnfr2/Traf2/Pac1-r) and glial-specific genes (Gfap/Cd68; Table 1). The primer set for the gene target, neuronal nitric oxide synthase (Nnos), did not yield acceptable results during primer design but was commercially available. Therefore, Nnos and the housekeeping gene, glyceraldehydes 3-phosphate dehydrogenase (Gapdh), were purchased from SABiosciences/Qiagen (#PPM05114E, #PPM02946E). The annealing temperature (T_A) for each primer set was experimentally determined using uninjured whole mouse brain cDNA as the template, over a temperature gradient based on the T_A estimated by the design software or indicated within the product information sheet for those primers obtained from SABiosciences/Qiagen (Table 1).

Semi-quantitative real-time reverse transcription-PCR (qPCR) was performed using the iCycler iQ detection system (Bio-Rad; Sharma et al., 2010; Mesnard et al., 2010). A reaction volume of 25µl contained 1× SYBR Green PCR Master Mix (Applied Biosystems #4312704), 1µl fluorescein (Bio-Rad, #1708780), 200nM forward and reverse primers and 1µl of cDNA. The qPCR cycle parameters included an initial 10 minute, 95°C denaturing step, followed by 40 cycles of three repeated, 30 second steps of denaturing at 95°C, annealing at the predetermined T_A for the respective primer set, and extension at 65°C. Immediately after conclusion of the amplification protocol, a melt curve analysis was performed to verify the specificity of the amplified product. The melting temperature (T_m) for each primer set was compared to the experimentally determined value (Table 1).

All cDNA samples were run in triplicate and from which an average threshold cycle (C_T) was calculated. Relative mRNA expression levels were analyzed using the comparative C_T method in which the C_T value for a gene of interest is normalized to the C_T value for the endogenous housekeeping gene, Gapdh, resulting in the C_T difference or the ΔC_T. Gapdh, as an endogenous housekeeping gene, has been well-established as an internal standard for the facial nerve axotomy model (Raivich et al., 1998; Livak and Schmittgen, 2001; Li et al., 2000; Wainwright et al., 2009). For consistency in normalization, primers sets for genes of interest were normalized to a Gapdh primer set purchased from the same company (SABiosciences/Qiagen or Bio-Synthesis, Inc.). The ΔC_T was calculated for both facial nuclei (right/axotomized and left/uninjured control). Two different analyses were performed utilizing ΔC_T: one to calculate axotomy-induced percent change mRNA expression, relative to the uninjured control nucleus, and a second utilizing only the left, uninjured control nucleus, to assess changes in relative mRNA expression with age/disease.
**Axotomy-induced percent change mRNA expression**

WT and mSOD1 axotomy-induced percent change in mRNA expression for a gene of interest, relative to control nucleus (0, 3, 7, 14, 28 and 56 dpa), was calculated by subtracting the axotomized ΔC_T, right facial nucleus, from the uninjured control ΔC_T, left facial nucleus, yielding the ΔΔC_T. The ΔΔC_T was linearized and used to calculate the percent change in mRNA expression, \((2^{-\Delta\Delta C_T} - 1) \times 100\) (Mesnard et al., 2010). Previously, we have established axotomy-induced percent change in mRNA expression for the following genes, βII-tubulin, Gap-43, Caspase-8, Tnfsf and Gfap, at 0, 3, 7, 14 and 28 dpa, in WT and mSOD1 (Mesnard et al., 2011). These data were replicated in the current study along with the additional, extended timepoint, 56 dpa. For the purposes of visualizing the entire axotomy-induced molecular response over time, the replicated data was presented within the figures and included in the statistical analyses.

**Disease-induced change in relative quantity mRNA expression**

Relative quantity mRNA expression in the WT and mSOD1 facial motor nucleus was determined using only the left, uninjured control facial nuclei at three ages; corresponding to the pre-symptomatic stage (70 days and 84 days) and an age well within the symptomatic stage of disease (112 days). Aged-matched WT groups (70, 84 and 112 days of age) were also included in this analysis to serve as baseline comparison. The ΔC_T, for a gene of interest, was converted to a linear form using \(2^{-\Delta C_T}\) (Livak and Schmittgen, 2001, Schmittgen and Livak, 2008). No statistical differences were determined between WT at 70, 84 and 112 days of age (one-way ANOVA; SigmaPlot, version 12.3). Therefore, WT data was averaged to produce a single, WT baseline measurement of relative quantity mRNA expression for a gene of interest. Averaged WT baseline mRNA expression was compared to mSOD1 mRNA expression at 70, 84 and 112 days of age. As the mSOD1 mice aged, changes in relative quantity mRNA expression within the uninjured facial nucleus, compared to WT baseline expression, were attributed to disease progression.

**Statistical analysis of mRNA expression**

Prior to statistical analysis, raw data for both axotomy-induced percent change mRNA expression and control nucleus relative quantity mRNA expression, for each individual gene, was assessed for normality (Kolmogorov-Smirnov statistic with a Lilliefors’ significance level and Shapiro-Wilk statistic; SPSS, version 20). Those that did not approximate normal by either test were transformed by using square root, natural or Log_{10}-Transformed data for each individual gene (all three transformation methods) were assessed for normality as described for the raw data. In cases where more than one transformation method resulted in normalized data, normal and detrended Q-Q plots were examined. The transformation method that best yielded a straight line on normal Q-Q plots and approximately equal spread on detrended Q-Q plots was selected (SPSS, version 20). Data presented in figures is raw, untransformed data, while data used for statistical analyses was transformed as necessary.

Statistical analysis for axotomy-induced percent change mRNA expression was accomplished by first using a two-way ANOVA (group × time), followed by the Student-Newman-Keuls post hoc multiple comparison test, with significance at \(P < 0.05\) (SigmaPlot,
version 12.3). To analyze the timepoints near and within the symptomatic stage of disease, WT and mSOD1 group comparisons were made at each delayed timepoint (28 and 56 dpa) using a Student’s t-test adjusted for multiple comparisons, followed by the Student-Newman-Keuls post hoc multiple comparison test (SigmaPlot, version 12.3). This subsequent analysis was performed in order to assess statistical differences between WT and mSOD1 facial nuclei after axotomy that may also include a third affect, the effect of disease that may not be present during the four earlier timepoints (0, 3, 7 and 14 dpa). Comparisons were restricted to individual genes and not made between genes.

Relative quantity mRNA expression changes in mSOD1 uninjured control nucleus with age, relative to WT baseline expression, were accomplished by using a one-way ANOVA, followed by Dunnett’s post hoc multiple comparison test, with significance at $P < 0.05$ (SigmaPlot, version 12.3). Comparisons were not made between mSOD1 groups and individual genes were analyzed separately.

**Results**

**Symptom onset occurs by 98 days of age in mSOD1 mice**

The age of symptom onset was determined in order to define pre-symptomatic and symptomatic disease stages among the group of mSOD1 mice used in the present study. Statistical analysis revealed an overall main effect between WT and mSOD1 groups ($F_{1,182} = 20.78; P < 0.05$), a main effect of age ($F_{14,182} = 14.79; P < 0.05$) as well as an interaction between group × age ($F_{14,182} = 14.79; P < 0.05$; Fig. 1). Behavioral assessment scores were significantly different between WT (6.0 ± 0.0) and mSOD1 mice (5.1 ± 0.3) as early as 98 days of age (Fig. 1). Therefore, we conclude that symptom onset occurred by 98 days of age in the mSOD1 group.

**Disease-induced facial motoneuron (FMN) loss occurs by 112 days of age in mSOD1 mice**

To determine the time course of FMN loss in mSOD1 mice during disease progression and in the absence of axotomy, facial motor nuclei were examined at different days of age in mSOD1 mice and compared to aged-matched WT mice. The photomicrographs in Fig. 2 and 3 are representative sections from uninjured control and axotomized facial motor nuclei of WT and mSOD1 mice at 84 and 112 days of age (28 and 56 dpa). FMN numbers were not statistically different between uninjured control facial nuclei of WT and pre-symptomatic mSOD1 mice at 84 days of age (80 ± 2.9 and 81 ± 2.8, respectively), consistent with previous findings (Fig. 2A, 2C and 4A; Mesnard et al., 2011). FMN numbers were significantly decreased in mSOD1 control nucleus (69 ± 1.8; $P < 0.05$; Fig.3C and 4A) at 112 days of age, which is within the symptomatic stage of disease, relative to WT (80 ± 2.5; Fig.3A and 4A). A one-way ANOVA revealed a main effect between groups ($F_{3,16} = 5.57; P = < 0.05$; Fig. 4A). Thus, disease-induced cell loss in the facial nucleus occurred between 84 and 112 days of age, likely sometime during the symptomatic stage.

**Effects of facial nerve axotomy and disease progression on FMN numbers**

To compare the effects of axotomy on FMN loss in mSOD1 and WT mice, WT and pre-symptomatic mSOD1 mice received a right facial nerve axotomy at 56 days of age and FMN
survival levels were assessed at 28 and 56 dpa (84 and 112 days of age) and expressed as percent change of axotomy/control (Ax/C). Overall, a main effect between groups was detected \((F_{3,16} = 12.52; P < 0.05; \text{Fig. 4B})\). At 28 dpa, FMN levels were significantly reduced in the mSOD1 mice, relative to WT control (63 ± 4.2% and 84 ± 2.2%, respectively; \(P < 0.05; \text{Fig. 2 and 4B}\)), consistent with previous findings (Mesnard et al., 2011). By 56 dpa, there was a significant decrease in FMN survival levels in WT mice, relative to 28 dpa (55 ± 4.1% and 84 ± 2.2%, respectively; \(P < 0.05; \text{Fig. 2A–B, 3A–B and 4B}\)). In contrast, FMN survival levels in the mSOD1 mice at 56 dpa were not significantly different from those at 28 dpa (56 ± 4.2% and 63 ± 4.2%, respectively; \(\text{Fig. 2C–D, 3C–D and 4B}\)). Thus, axotomy-induced FMN loss occurred more rapidly in the mSOD1 mouse than in the WT, suggesting a differential response to target deprivation in the mSOD1 mouse. Interestingly, however, MN loss induced by axotomy in the WT, while delayed, eventually reached comparable levels to that of the mSOD1.

**TNFR1 death-inducing signaling pathway gene expression following axotomy in WT and mSOD1 facial motor nuclei**

To elucidate the molecular mechanisms underlying rapid mSOD1 FMN loss after target deprivation, we investigated gene expression changes in WT and mSOD1 facial motor nuclei for two distinct death receptor pathways.

The death receptor, TNFR1, is known to initiate apoptotic signaling cascade in response to TNFα binding (Chen and Goeddel, 2002). Fig. 5A depicts the classic TNFR1 death-inducing signaling cascade, of which six molecules were selected and included in the analysis of mRNA expression changes after axotomy (adapted from Grivennikov et al., 2006; Harrington, 2000). Tnfa mRNA expression has been previously characterized by our laboratory in WT and mSOD1 facial nuclei up to 28 dpa (Mesnard et al., 2011). In WT facial motor nuclei, Tnfa mRNA expression was shown to be undetectable within the uninjured control nucleus, but transiently induced after axotomy. In this study, Tnfa mRNA expression at 56 dpa in both control and axotomized WT facial motor nuclei remained undetectable (data not shown). Therefore, average percent change of mRNA expression \(\pm\) SEM for Tnfa was only calculated and analyzed in the mSOD1 mouse and found to be significantly upregulated at 7, 14 and 28 dpa in mSOD1 (371 ± 104%, 366 ± 91% and 350 ± 72%, respectively; \(P < 0.05\)), relative to no injury or 0 dpa (Fig. 5B). The mRNA expression at 3 and 56 dpa (123 ± 42% and 127 ± 21%, respectively), was not statistically different than no injury (Fig. 5B). A one-way ANOVA for mSOD1 Tnfa mRNA expression across post-operative time revealed a main effect between timepoints \((F_{5,28} = 6.81; P < 0.05; \text{Fig. 5B})\).

Tnfr1 mRNA expression was evaluated in both mSOD1 and WT after facial nerve axotomy. A main effect of post-operative time was observed \((F_{5,60} = 25.49; P < 0.05)\), however, no differences between groups \((F_{1,60} = 0.03; P = 0.865)\) or interactions between groups \(\times\) post-operative time \((F_{5,60} = 0.78; P = 0.57)\) were detected (Fig. 5C). Axotomy resulted in a similar upregulation of Tnfr1 mRNA at 3, 7, 14, 28 and 56 dpa in both WT (127 ± 24%, 137 ± 10%, 163 ± 9%, 76 ± 15% and 62 ± 14%, respectively; \(P < 0.05\)) and mSOD1 (110 ± 15%, 131 ± 12%, 160 ± 29%, 111 ± 18% and 44 ± 7%, respectively; \(P < 0.05\)), relative to no injury or 0 dpa (Fig. 5C).
After ligand binding, the silencer of death domains (SODD) dissociates from the cytoplasmic death domain of TNFR1 (Fig. 5A). It has been suggested that SODD plays an important regulatory role in TNFR1 signaling (Harrington, 2000; Takada et al., 2003). A main effect of post-operative time was observed for Sodd mRNA expression after axotomy ($F_{5,60} = 22.54; P < 0.05$; Fig. 5D). Both WT and mSOD1 axotomized facial nuclei demonstrated a significant down regulation at 3, 7 and 14 dpa (WT: $-29 \pm 5\%, -29 \pm 6\%$ and $-24 \pm 4\%$; mSOD1: $-24 \pm 5\%, -35 \pm 3\%$ and $-25 \pm 4\%$, respectively; $P < 0.05$), relative to no injury or 0 dpa (Fig. 5D). By 28 dpa, the level of Sodd mRNA expression in the axotomized facial nucleus was no different than baseline or the no injury groups (WT: $-16 \pm 5\%$; mSOD1: $-2 \pm 11\%$; Fig. 5D). While there was an upregulation in Sodd mRNA expression at 56 dpa, no statistically significant difference was observed compared to no injury in WT or mSOD1 ($10 \pm 7\%, 17 \pm 6\%$, respectively; $P < 0.05$), overall there was no main effect of group ($F_{1,60} = 0.75; P = 0.39$) or effect of group × post-operative time ($F_{5,60} = 0.74; P = 0.59$) observed.

After TNFR1 activation, the adaptor protein, TNFR1-association death domain (TRADD), binds to the cytoplasmic death domain of TNFR1, previously occupied by SODD, and is responsible for initiating the apoptotic signaling cascade through formation of the death inducing signaling complex (DISC; Fig. 5A). Axotomy did not alter mRNA expression levels of Tradd to a great degree. No main effects of post-operative time ($F_{5,59} = 1.97; P = 0.096$), group ($F_{1,59} = 0.00; P = 0.954$) or interaction between group × post-operative time ($F_{5,59} = 2.06; P = 0.083$) were identified (Fig. 5E). mSOD1 axotomized facial nuclei did not reveal any significant differences relative to no injury at 3, 7, 14, 28 and 56 dpa ($17 \pm 12\%, -3 \pm 2\%, -1 \pm 9\%, -4 \pm 9\%$ and $7 \pm 5\%$, respectively; Fig. 5E). Tradd mRNA expression was also unaffected by axotomy in WT at 3, 7, 14, 28 and 56 dpa ($-9 \pm 12\%, 1 \pm 5\%, 5 \pm 5\%, -5 \pm 7\%$ and $26 \pm 8\%$, respectively), relative to 0 dpa (Fig. 5E).

Caspase-8 is an initiator Caspase, recruited to the DISC where it autoactivates via proteolytic cleavage forming an active Caspase-8 dimer (Grivennikov et al., 2006). Caspase-8 mRNA expression was previously evaluated for axotomy-induced expression up to 28 days following facial nerve axotomy (Mesnard et al., 2011). In the current study, the extended time course did not reveal a main effect of group ($F_{1,62} = 0.49; P = 0.488$), or an interaction between group × post-operative time ($F_{5,62} = 0.25; P = 0.94$), but did reveal a main effect of post-operative time ($F_{5,62} = 21.13; P < 0.05$; Fig. 5F). Axotomy resulted in a similar upregulation of Caspase-8 mRNA at 3, 7, 14, 28 and 56 dpa in both WT ($320 \pm 54\%, 305 \pm 51\%, 299 \pm 64\%, 259 \pm 98\%$ and $112 \pm 21\%$, respectively; $P < 0.05$) and mSOD1 ($321 \pm 41\%, 343 \pm 55\%, 253 \pm 95\%$, $194 \pm 42\%$ and $95 \pm 26\%$, respectively; $P < 0.05$), relative to no injury or 0 dpa (Fig. 5F).

The final molecule in the classical TNFR1 death-inducing signaling cascade, depicted in Fig. 5A, is the effector Caspase, Caspase-3 (Grivennikov et al., 2006). Consistent with the literature (Vanderluit et al., 2000), axotomy increased mRNA expression, for Caspase-3, with a main effect of post-operative time ($F_{5,60} = 73.04; P < 0.05$; Fig. 5G). Upregulation of Caspase-3 mRNA was detected at 3, 7, 14 and 28 dpa in both WT ($320 \pm 54\%, 305 \pm 51\%, 299 \pm 64\%, 259 \pm 98\%$ and $112 \pm 21\%$, respectively; $P < 0.05$) and mSOD1 ($322 \pm 35\%, 343 \pm 55\%, 253 \pm 95\%, 194 \pm 42\%$ and $95 \pm 26\%$, respectively; $P < 0.05$), relative to no injury or 0 dpa.
There was no upregulation at 56 dpa in WT or mSOD1 (68 ± 12% and 39 ± 12%, respectively), relative to no injury, and also no main effect of groups (F_{1,60} = 2.59; P = 0.113) or interaction between groups × post-operative time (F_{5,60} = 1.27; P = 0.287). However, the 28 day timepoint revealed that average percent change in Caspase-8 mRNA expression was significantly increased in mSOD1 compared to WT (P = < 0.05; Fig. 5G).

In conclusion, axotomy-induced gene expression changes for the TNFR1 death pathway were similar in both WT and mSOD1 facial nuclei, suggesting that this death pathway plays a role in FMN death after axotomy.

Fas death-inducing signaling pathway gene expression following axotomy in WT and mSOD1 facial motor nuclei

Another death receptor and member of the TNFR superfamily, Fas, was also examined for mRNA expression of molecules involved in its signaling pathway, particularly because of its potential role in mSOD1 disease progression (Raoul et al., 2002, Raoul et al., 2006, Locatelli et al., 2007). To determine the average percent change of mRNA expression for the Fas signaling pathway, Fas and signaling intermediates were examined following facial nerve axotomy at no injury (0), 3, 7, 14, 28 and 56 dpa in WT and mSOD1.

Fas-induced cell death in MN is thought to occur through two distinct pathways downstream of Fas (Raoul et al., 2002). These two separate pathways are depicted in Fig. 6A (adapted from Raoul et al., 2002; Raoul et al., 2006). The pathway involving DISC formation and subsequent Caspase activation shares many similarities to the TNFR1 cell death pathway with regard to molecules and events (Grivennikov et al., 2006). However, the presence of a second coexisting signaling pathway leading to increased transcription of Nnos appears to be unique to MN (Raoul et al., 2002; Raoul et al., 2006). To further investigate these downstream signaling pathways, genes for several of the molecules involved were selected for axotomy-induced mRNA expression.

Investigation of Fas mRNA expression after axotomy resulted in a main effect of groups (F_{1,60} = 10.94; P = < 0.05), a main effect of post-operative time (F_{5,60} = 4.08; P = < 0.05) as well as a statistical interaction between groups × post-operative time (F_{5,60} = 3.07; P = < 0.05; Fig. 6B). The axotomized facial nuclei of WT revealed no differences compared to baseline or no injury in Fas expression at 3, 7, 14, 28 and 56 dpa (21 ± 15%, 26 ± 15%, 36 ± 20%, 29 ± 16% and 12 ± 13%, respectively; Fig. 6B). No significant Fas mRNA upregulation was observed in axotomized mSOD1 facial motor nuclei at early timepoints; specifically 3, 7 and 14 days after axotomy (29 ± 19%, 21 ± 16% and 83 ± 36%, respectively; Fig. 6B). However, by 28 and 56 dpa, Fas mRNA expression was significantly increased in mSOD1 (201 ± 49% and 116 ± 23%, respectively; P = < 0.05), relative to no injury or 0 dpa (Fig. 6B). The upregulation of Fas mRNA in the mSOD1 axotomized facial nucleus resulted in a statistical difference compared to WT at 28 and 56 dpa (P = < 0.05; Fig. 6B).

Following Fas activation, DISC is formed while the adapter protein, Fas-associated death domain (FADD), interacts directly with the receptor (Fig. 6A). No significant differences were detected for Fadd mRNA expression at 3, 7, 14, 28 and 56 dpa in WT mice (28 ± 7%,
4 ± 5%, 6 ± 14%, 22 ± 9% and 32 ± 12%, respectively), relative to no injury or 0 dpa (Fig. 6C). Fadd mRNA expression did not differ from no injury in mSOD1 facial nuclei at early timepoints 3, 7 and 14 dpa (20 ± 15%, 1 ± 6% and 6 ± 14%, respectively), but was significantly upregulated at 28 and 56 dpa (72 ± 17% and 87 ± 10%, respectively; \( P = < 0.05 \); Fig. 6C). Overall, a main effect of post-operative time was observed (\( F_{5,58} = 9.70; P = < 0.05 \)) as well as a statistical interaction between groups×post-operative time (\( F_{5,58} = 2.60; P = < 0.05 \); Fig. 6C). While no overall effect was observed between group for axotomy-induced Fadd mRNA expression (\( F_{1,58} = 3.77; P = 0.057 \)), there was a statistical difference between WT and mSOD1 at 28 and 56 dpa (\( P = < 0.05 \); Fig. 6C).

The second downstream pathway of Fas, thought to be coactivated in MN cell death, utilizes a key mediator, death associated protein-6 (Daxx), responsible for recruitment of consecutive signaling molecules (Fig. 6A). WT and mSOD1 axotomized facial nuclei did not reveal any significant differences in Daxx mRNA expression, relative to no injury or baseline, at 3, 28 and 56 dpa (WT: 7 ± 8%, 8 ± 13% and 4 ± 12%; mSOD1: 5 ± 5%, 26 ± 12% and 11 ± 5%, respectively), although at 7 and 14 dpa there was a significant upregulation (WT: 39 ± 12% and 38 ± 9%; mSOD1: 38 ± 18% and 19 ± 7%, respectively; \( P = < 0.05 \); Fig. 6D). Axotomy revealed a main effect of post-operative time (\( F_{5,59} = 4.31; P = < 0.05 \)) with respect to Daxx mRNA expression, but not for group (\( F_{1,59} = 0.05; P = 0.83 \)) or group×post-operative time (\( F_{5,59} = 0.97; P = 0.44 \); Fig. 6D).

The adapter protein Daxx is responsible for recruitment of apoptosis signal-regulating kinase-1 (ASK1) following Fas activation (Raoul et al., 2002; Fig. 6A). The upregulation in Ask1 mRNA following axotomy led to a main effect of post-operative time (\( F_{5,59} = 3.27; P = < 0.05 \)), although, there was no overall effect of group (\( F_{1,59} = 1.53; P = 0.221 \)) or interaction between group×post-operative time (\( F_{5,59} = 1.45; P = 0.22 \); Fig. 6E). Axotomy resulted in upregulation of Ask1 mRNA throughout the time course of 3, 7, 14 and 56 dpa in both WT (27 ± 12%, 39 ± 12%, 41 ± 8% and 10 ± 11%, respectively) and mSOD1 (32 ± 15%, 31 ± 6%, 33 ± 12% and 45 ± 9%, respectively); however, no statistical differences were observed at 28 dpa in WT and mSOD1 (7 ± 6% and 23 ± 16%, respectively), relative to no injury (Fig. 6E).

ASK1 phosphorylates p38, which leads to increased transcription of Nnos and subsequent increased production of nitric oxide (NO) that can spontaneously react with superoxide anion to form the damaging chemical, peroxynitrite (Raoul et al., 2002; Fig. 6A). Evaluation of Nnos mRNA expression after axotomy revealed a main effect of postoperative time (\( F_{5,60} = 5.62; P = < 0.05 \)) as well as group (\( F_{1,60} = 14.56; P = < 0.05 \); Fig. 6F). However, because no interaction between group×post-operative time (\( F_{5,60} = 1.60; P = 0.175 \)) was observed, mRNA expression for Nnos at 3, 7, 14, 28 and 56 dpa in both WT (−32 ± 13%, −6 ± 23%, −2 ± 20%, 24 ± 20% and 16 ± 23%, respectively) and mSOD1 (15 ± 28%, −11 ± 9%, 76 ± 32%, 138 ± 45% and 185 ± 50%, respectively), was not identified as statistically different compared to no injury (Fig. 6F). Additional evaluation of later timepoints, near and within the mSOD1 symptomatic stage of disease, revealed a significant difference between WT and mSOD1 at 56 dpa (\( P = < 0.05 \)) and therefore also provides evidence for an upregulation in Nnos mRNA in the mSOD1 facial nucleus after axotomy (Fig. 6F).
Taken together, the axotomy-induced upregulation in mRNA expression for the Fas pathway during the delayed timepoints in the mSOD1 facial nucleus, and the absence of this upregulation in the WT, suggests that the Fas pathway may play a role in the pathological mSOD1 FMN cell death after axotomy.

**Regenerative and pro-survival gene expression following axotomy in WT and mSOD1 facial motor nuclei**

Our laboratory previously revealed that following axotomy, both WT and mSOD1 FMN mount a regenerative response by upregulating MN-specific regenerative genes such as βII-tubulin and growth associated protein-43 (Gap-43) within the first four weeks after axotomy (Mesnard et al., 2011). To further investigate this regenerative response, we extended the time course for βII-tubulin and Gap-43, analysis and also assessed additional pro-survival genes, such as Cx3cr1, Tnfr2, Traf2 and Pac1-r.

Differential regulation of mRNA expression and protein synthesis occur after peripheral nerve injury and play a role in transitioning the neuron from a signaling mode to a regenerative growth mode (Hoffman and Cleveland, 1988; Lieberman, 1971; Fu and Gordon, 1997). The extended time course did not reveal any differences between groups after axotomy for Gap-43 (F1,59 = 2.75; P = 0.102) or an interaction between group×post-operative time (F5,59 = 0.98; P = 0.435; Fig. 7A). However, analysis did reveal a main effect of post-operative time (F5,59 = 191.89; P < 0.05; Fig. 7A). In the axotomized facial nuclei, a significant upregulation of Gap-43 occurred at 3, 7, 14, 28 and 56 dpa in WT (1222 ± 69%, 1256 ± 91%, 1202 ± 61%, 518 ± 122% and 123 ± 18%, respectively; P < 0.05) and in mSOD1 (1432 ± 200%, 1531 ± 92%, 1407 ± 102%, 510 ± 81% and 96 ± 13%, respectively; P < 0.05), relative to baseline or no injury (Fig. 7A).

β-Tubulin is a protein component of microtubules. Specifically, the isotype βII-Tubulin has been shown to be particularly important in MN axonal elongation during regeneration after injury (Hoffman and Cleveland, 1988; Moskowitz and Oblinger, 1995; Jones and Oblinger, 1994; Jones et al., 1999; Tetzlaff et al., 1991). The extended time course for βII-tubulin did not reveal any differences between groups (F1,58 = 0.05; P = 0.822) or an interaction between group×post-operative time (F5,58 = 1.05; P = 0.398), but did reveal a main effect of only post-operative time (F5,58 = 62.71; P = < 0.05; Fig. 7B). Axotomy resulted in an upregulation of βII-tubulin mRNA at 3, 7, 14, 28 and 56 dpa in both WT (89 ± 7%, 188 ± 26%, 204 ± 17%, 105 ± 29% and 34 ± 4%, respectively; P = < 0.05) and mSOD1 (131 ± 18%, 196 ± 22%, 228 ± 28%, 114 ± 38% and 21 ± 7%, respectively; P = < 0.05), relative to no injury or 0 dpa (Fig. 7B).

It has been well-established that fractalkine (CX3CL1) and its receptor (CX3CR1) are important for signaling after neuronal injury and mediating neuroprotection (Chapman et al., 2000; Cardona et al., 2006). Analysis of the average percent Cx3cr1 mRNA expression after axotomy revealed a main effect of post-operative time (F5,59 = 61.77; P = < 0.05; Fig. 7C). The facial nuclei of both groups underwent a significant increase in Cx3cr1 mRNA expression at 3, 7, 14, 28 and 56 dpa (WT: 527 ± 32%, 514 ± 47%, 529 ± 83%, 334 ± 82% and 153 ± 34%; mSOD1: 540 ± 129%, 638 ± 60%, 462 ± 81%, 366 ± 61% and 71 ± 15%, respectively; P = < 0.05), relative to no injury or 0 dpa (Fig. 7C). There was no observed
overall main effect of groups ($F_{1,59} = 0.11; P = 0.74$) and no interaction between group × post-operative time ($F_{5,59} = 1.27; P = 0.29$; Fig. 7C).

TNF receptor 2 (TNFR2) lacks a cytoplasmic death domain and receptor activation results in cell survival and neuroprotection (Marchetti et al., 2004; Cabal-Hierro and Lazo, 2012). TNFR2 is proposed to be localized primarily to microglia, within the CNS, where it promotes induction of anti-inflammatory pathways (Veroni et al., 2010). Axotomy-induced upregulation of $Tnfr2$ mRNA was seen in WT at 3, 7, 14, 28 and 56 dpa ($529 \pm 48\%$, $600 \pm 150\%$, $466 \pm 93\%$, $231 \pm 61\%$ and $144 \pm 21\%$, respectively; $P = < 0.05$), relative to no injury (Fig. 7D). While a main effect of post-operative time was observed ($F_{5,60} = 67.68; P = < 0.05$), a main effect of groups was not ($F_{1,60} = 0.82; P = 0.37$), as well as no interaction between group × time ($F_{5,60} = 2.01; P = 0.09$; Fig. 7D).

TNF receptor-associated factor 2 (TRAF2) is a key mediator in signaling of TNFR2 and is responsible for the subsequent cell proliferation and survival signals. In addition, TRAF2 can bind to TRADD, a downstream signaling molecule of TNFR1, and can ultimately result in transcription of anti-apoptotic genes via NF-κB (Cabal-Hierro and Lazo, 2012). Therefore in addition to TNFR2, TRAF2 can also act as a mediator of TNFR1 signaling. Following axotomy, WT and mSOD1 facial nuclei revealed an initial down regulation in $Traf2$ mRNA expression followed by a return to baseline expression. This resulted in a main effect of post-operative time ($F_{5,60} = 7.54; P = < 0.05$; Fig. 7E). $Traf2$ mRNA expression was significantly downregulated at 3 and 7 dpa (WT: $−30 \pm 9\%$ and $−30 \pm 7\%$; mSOD1: $−17 \pm 5\%$ and $−20 \pm 6\%$, respectively; $P = < 0.05$), but no differences, with respect to the no injury group, were seen throughout the remainder of the time course at 14, 28 and 56 dpa (WT: $−4 \pm 7\%$, $−12 \pm 11\%$ and $13 \pm 15\%$; mSOD1: $−8 \pm 8\%$, $33 \pm 22\%$ and $35 \pm 11\%$, respectively; Fig. 7E). There was an overall effect of group following axotomy ($F_{1,60} = 6.50; P = < 0.05$), however, no interaction between group × post-operative time ($F_{5,60} = 1.27; P = 0.29$; Fig. 7E).

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic and multifunctional peptide known to promote neurite outgrowth and cell survival (Somogyvari-Vigh and Reglodi, 2004; Reglodi et al., 2011). PACAP 1-Receptor (PAC1-R) is highly specific for PACAP and its downstream signaling pathway has been linked to cell survival and anti-inflammatory effects such as inhibition of apoptosis and attenuation of TNFa (Vaudry et al., 2000). A main effect of post-operative time was observed for $Pac1-r$ mRNA expression after axotomy ($F_{5,59} = 77.24; P = < 0.05$; Fig. 7F). Both groups experienced a significant down regulation at 3, 7 and 14 dpa (WT: $−46 \pm 6\%$, $−52 \pm 2\%$ and $−34 \pm 5\%$; mSOD1: $−43 \pm 4\%$, $−52 \pm 2\%$ and $−24 \pm 5\%$, respectively; $P = < 0.05$), relative to no injury or 0 dpa (Fig. 7F). This down regulation after facial nerve axotomy is consistent with the literature regarding $Pac1-r$ mRNA expression (Zhou et al., 1999). By 28 dpa, the level of $Pac1-r$ mRNA expression in the axotomized facial nucleus is no different than baseline (WT: $2 \pm 7\%$; mSOD1: $29 \pm 14\%$; Fig. 7F). This increase in $Pac1-r$ mRNA continues until 56 dpa, where it is significantly increased from baseline or no injury in both WT and

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mSOD1 (26 ± 11% and 53 ± 10%, respectively; \( P = < 0.05 \); Fig. 7F). Although no differences between groups were seen among later individual timepoints (28 and 56 dpa), there was a main effect of group overall (\( F_{1,59} = 6.83; P = < 0.05 \)); however, no interaction between group \( \times \) postoperative time (\( F_{5,59} = 0.95; P = 0.46 \); Fig. 7F) was observed.

These results reveal that axotomy-induced expression of regenerative and prosurvival genes are similar, temporally and in magnitude, for both WT and mSOD1 mice, supporting our previous findings (Mesnard et al., 2011) and extending them to suggest that differential expression of death receptor genes is likely responsible for the rapid FMN cell loss in the mSOD1 following axotomy.

Glial-specific gene expression following axotomy in WT and mSOD1 facial motor nuclei

Previously our laboratory identified a dysregulation in the axotomy-induced upregulation of *glial fibrillary acidic protein (Gfap)* mRNA in mSOD1 facial nucleus in comparison to WT. This significantly reduced *Gfap* mRNA expression persisted until the last timepoint analyzed, 28 dpa (Mesnard et al., 2011). GFAP is the major protein constituent of glial filaments and is the cell-specific intermediate filament in astrocytes. It is well documented that facial nerve axotomy elicits a significant increase in *Gfap* mRNA expression by resident reactive astrocytes as early as 24 hours after peripheral nerve injury (Tetzlaff et al., 1988; Jones et al., 1997). To further investigate the axotomy-induced astrocyte reaction in the facial nuclei of WT and mSOD1 mice, the time course following axotomy was extended to include 56 dpa for *Gfap*. Extension of the time course revealed a main effect, of *Gfap* mRNA expression, between WT and mSOD1 groups after axotomy (\( F_{1,59} = 28.49; P = < 0.05 \)), a main effect of post-operative time (\( F_{5,59} = 36.49; P = < 0.05 \)) as well as a statistical interaction between group \( \times \) time (\( F_{5,59} = 2.67; P = < 0.05 \); Fig. 8A). Axotomy resulted in an upregulation of *Gfap* mRNA at 3, 7, 14, 28 and 56 dpa in both WT (605 ± 84%, 559 ± 81%, 1181 ± 201%, 806 ± 228% and 455 ± 119%, respectively; \( P = < 0.05 \)) and mSOD1 (213 ± 70%, 209 ± 80%, 442 ± 99%, 712 ± 158% and 161 ± 33%, respectively; \( P = < 0.05 \)), relative to no injury or 0 dpa (Fig. 8A). The average percent change mRNA expression was significantly increased in WT, compared to mSOD1 for 3, 7, 14 and 56 dpa (\( P = < 0.05 \)), however no difference in expression was observed between groups at 28 dpa (Fig. 8A).

In a variety of axonal-lesion models, endogenous microglial cells become activated and rapidly proliferate between one-and-three days post-injury (Dissing-Olesen et al., 2007; Hailer et al., 1999). To evaluate the microglia response, mRNA expression for the glycoprotein, Cluster of Differentiation 68 (CD68) was used as it is a well-accepted marker for microglia within the CNS (Graeber et al., 1990; Lemstra et al., 2007; Holness and Simmons, 1993). Although CD68 is both a monocyte and macrophage specific marker, numerous studies have shown that following nerve injury paradigms that leave the blood brain barrier (BBB) intact, proliferating CD68-positive cells are unambiguously microglia cells and are not infiltrating blood macrophages (Streit et al., 1988; Graeber et al., 1988; Streit and Kreutzberg, 1988; Graeber et al., 1990).

*Cd68* mRNA expression was significantly upregulated in the axotomized WT facial nucleus relative to no injury at 3, 7, 14, 28 and 56 dpa (654 ± 88%, 687 ± 97%, 813 ± 135%, 599 ± 130% and 385 ± 94%, respectively; \( P = < 0.05 \); Fig. 8B). *Cd68* expression in mSOD1...
axotomized facial nuclei also revealed a statistically significant upregulation in mRNA percent change compared to baseline at 3, 7, 14, 28 and 56 dpa (201 ± 42%, 356 ± 69%, 418 ± 125%, 645 ± 99% and 119 ± 34%, respectively; *P* < 0.05; Fig. 8B), however, as was seen with *Gfap*, the level of *Cd68* mRNA expression was decreased compared to WT at the following timepoints: 3, 7, 14 and 56 dpa (*P* < 0.05; Fig. 8B). Overall there was a main effect between WT and mSOD1 groups following axotomy (*F*₁,₆₀ = 23.56; *P* < 0.05), a main effect of post-operative time (*F*₅,₆₀ = 26.12; *P* < 0.05) as well as a statistical interaction between group × post-operative time (*F*₅,₆₀ = 3.0; *P* < 0.05; Fig. 8B).

These results support our previous findings of a dysregulated mSOD1 astrocytic response, in comparison to WT (Mesnard et al., 2011), and also extend these findings to reveal a comparable, dysregulation in the mSOD1 microglial response to axotomy.

**Disease-induced TNFR1 death-inducing signaling pathway gene expression in facial motor nuclei**

To determine if the target deprivation produced by axotomy and disease induce similar molecular changes in facial motor nuclei, relative mRNA expression was assessed within the uninjured control facial nuclei in mSOD1 mice at 70, 84 and 112 days of age. As indicated, no statistical differences were identified between the different WT age groups for all genes assessed (individually) therefore, all WT values were averaged together (per gene) as WT baseline and used for comparison purposes to mSOD1 values. We observed significant FMN loss by 112 days of age (symptomatic stage of disease; Fig. 1, 2 and 4A), suggesting that disease-induced target disconnection, with respect to mSOD1 FMN, is well underway throughout the early symptomatic stage and possibly during the pre-symptomatic stage. We hypothesized in the current study that, prior to and/or during significant disease-induced FMN cell death, a measurable molecular response within the facial nucleus will be detectable, indicative of the ongoing target disconnection and this mRNA response will resemble the axotomy-induced mRNA response. Results of all genes analyzed in the mSOD1 facial nucleus at 112 days of age (symptomatic stage of disease; Fig. 1), compared to averaged WT baseline expression, are summarized in Table 2 and in addition, for comparison purposes, a summary of mSOD1 axotomy-induced mRNA expression at delayed timepoints (28 and/or 56 dpa) relative to no injury is also provided.

*Tnfα* mRNA expression was below detectable levels in the WT uninjured control facial nucleus, therefore, relative expression levels were considered to be zero (Fig. 9A). However, in mSOD1 facial nuclei, relative *Tnfα* mRNA expression increased with age at 70, 84 and 112 days of age (0.00004 ± 0.00001, 0.00005 ± 0.00002 and 0.00007 ± 0.00001, respectively). For the reason that there is no measurable *TnfFα* mRNA expression within the WT control facial nucleus, expression in the mSOD1 facial nucleus is considered to be significantly upregulated at all ages assessed (Fig. 9A).

*Tnfr1* mRNA expression in mSOD1 facial nuclei at 70 days of age (0.0168 ± 0.0017) was not different than averaged WT baseline expression (0.0146 ± 0.0005; Fig. 9B). However, by 84 and at 112 days of age (0.0196 ± 0.0013 and 0.0269 ± 0.0016, respectively), *Tnfr1* mRNA expression was significantly increased in the mSOD1 facial nucleus compared to...
WT baseline ($P = < 0.05$; Fig. 9B). Not surprisingly, a main effect between mSOD1 groups and WT Tnfr1 mRNA expression was observed ($F_{3,35} = 22.435; P < 0.05$; Fig. 9B).

mSOD1 facial nuclei revealed no increase or decrease in Sodd mRNA expression levels at 70, 84 and 112 days of age ($0.01550 \pm 0.00098$, $0.01450 \pm 0.00135$ and $0.01470 \pm 0.00037$, respectively), compared to averaged WT baseline ($0.01360 \pm 0.00038$; Fig. 9C). No overall effect between mSOD1 groups and WT was observed ($F_{3,35} = 1.615; P = 0.203$) for Sodd mRNA expression (Fig. 9C).

The mRNA expression levels for the adapter protein, TRADD, revealed no differences seen between averaged WT baseline ($0.00271 \pm 0.00013$) and mSOD1 expression at 70, 84 and 112 days of age ($0.00278 \pm 0.00015$, $0.00334 \pm 0.00035$ and $0.00328 \pm 0.00016$, respectively; Fig. 9D). While individual comparisons did not reveal differences between WT and mSOD1, a main effect between mSOD1 groups and WT was observed ($F_{3,34} = 3.15; P < 0.05$), due to the overall increased expression of Tradd in the mSOD1 facial nucleus (Fig. 9D).

Relative mRNA expression for the initiator Caspase, Caspase-8, revealed a main effect between mSOD1 groups and averaged WT baseline ($F_{3,35} = 15.017; P < 0.05$; Fig. 9E). Caspase-8 mRNA was significantly upregulated in mSOD1 facial nuclei at 112 days of age ($0.00074 \pm 0.00027; P = < 0.05$), compared to averaged WT baseline expression ($0.00029 \pm 0.00002$; Fig. 9E). However, pre-symptomatic Caspase-8 mRNA expression at 70 and 84 days of age in mSOD1 facial nuclei ($0.00041 \pm 0.00016$ and $0.00041 \pm 0.00005$, respectively) was not found to be statistically significant relative to WT baseline (Fig. 9E).

Caspase-3 mRNA expression levels in mSOD1 facial nuclei at 70 and 84 days of age ($0.00177 \pm 0.00012$ and $0.00164 \pm 0.00010$, respectively) were not statistically different compared to mRNA baseline levels in WT ($0.00158 \pm 0.00008$; Fig. 9F). However, Caspase-3 mRNA was significantly increased at 112 days of age in mSOD1 facial nucleus ($0.00215 \pm 0.00150; P = < 0.05$), compared to averaged WT (Fig. 9F). Overall, there was a main effect between mSOD1 age groups and averaged WT baseline Caspase-3 mRNA expression ($F_{3,35} = 5.198; P = < 0.05$; Fig. 9F).

Thus, these results reveal that TNFR1-associated genes are regulated similarly following axotomy and during disease progression in the mSOD1 facial nucleus.

**Disease-induced Fas signaling pathway gene expression in facial motor nuclei**

Averaged relative Fas mRNA expression in WT facial nucleus ($0.00089 \pm 0.00007$) was no different than mSOD1 expression at 70 and 84 days of age ($0.00088 \pm 0.00010$ and $0.00098 \pm 0.00012$, respectively; Fig. 10A). However, by 112 days of age, Fas mRNA expression in mSOD1 facial nucleus was significantly increased ($0.00184 \pm 0.00023; P = < 0.05$), compared to WT baseline and this difference is reflected by the finding of an overall main effect of group ($F_{3,35} = 9.92; P = < 0.05$; Fig. 10A).

No increase in Fadd mRNA expression was observed in mSOD1 facial nuclei at 70, 84 or 112 days of age ($0.00155 \pm 0.00017$, $0.00139 \pm 0.00013$ and $0.00132 \pm 0.00012$,
respectively), relative to averaged WT baseline Fadd expression (0.00124 ± 0.00005; Fig. 10B). Consequently, no main effect of group was observed (F\textsubscript{3,35} = 1.443; P = 0.247; Fig. 10B).

Relative mRNA expression for the adapter protein, Daxx, also revealed no main effect between mSOD1 age groups and averaged WT baseline (F\textsubscript{3,34} = 1.147; P = 0.344; Fig. 10C). Therefore, no differences in Daxx mRNA expression levels were observed between mSOD1 facial nuclei at 70, 84 and 112 days of age (0.00385 ± 0.00033, 0.00398 ± 0.00033 and 0.00374 ± 0.00021, respectively) compared to WT (0.00345 ± 0.00011; Fig. 10C).

Compared to averaged WT baseline Ask1 mRNA expression (0.00467 ± 0.00010), within the control facial nucleus, mSOD1 expression was significantly increased at all assessed ages, 70, 84 and 112 days of age (0.00599 ± 0.00035, 0.00627 ± 0.00051 and 0.00567 ± 0.00041, respectively; P = < 0.05; Fig. 10D). Therefore, a main effect of group was detected (F\textsubscript{3,35} = 7.086; P = < 0.05; Fig. 10D).

The final gene assessed regarding the Fas death receptor signaling pathway was Nnos. There were no statistical differences observed between Nnos mRNA expression in the facial nuclei of mSOD1 at 70, 84 and 112 days of age (0.00049 ± 0.00010, 0.00052 ± 0.00009 and 0.00041 ± 0.00003; Fig. 10E). Consequently, no main effect of between mSOD1 groups and WT baseline was observed (F\textsubscript{3,35} = 0.567; P = 0.641; Fig. 10E).

Collectively, these data indicate that Fas-associated death pathway genes are upregulated in response to both disease and axotomy in the mSOD1 facial motor nuclei.

Disease-induced expression of regenerative and pro-survival genes in facial motor nuclei

\textit{Gap-43} mRNA expression in mSOD1 facial nuclei at 70 and 84 days of age (0.02250 ± 0.00094 and 0.02610 ± 0.00182, respectively) was no different than averaged WT baseline expression (0.02110 ± 0.00095; Fig. 11A). However, by 112 days of age (0.03870 ± 0.00669), \textit{Gap-43} mRNA expression was significantly upregulated in mSOD1 facial nucleus compared to averaged WT (P = < 0.05; Fig. 11A). This upregulation in mSOD1 \textit{Gap-43} mRNA also resulted in a main effect of group (F\textsubscript{3,34} = 9.632; P = < 0.05; Fig. 11A).

The second MN-specific regenerative gene assessed, βII-tubulin, revealed no main effect of group (F\textsubscript{3,34} = 1.794; P = 0.167) for relative mRNA expression levels between mSOD1 or WT (Fig. 11B). In addition, no statistical differences between βII-tubulin mRNA expression in the facial nuclei of mSOD1 at 70, 84 and 112 days of age (0.1080 ± 0.0086, 0.1130 ± 0.0048 and 0.1130 ± 0.0103, respectively) were revealed in comparison to averaged WT expression (0.0958 ± 0.0041; Fig. 11B).

Compared to averaged WT Cx3cr1 baseline mRNA expression (0.00614 ± 0.00014), within the facial nucleus, mSOD1 expression was significantly increased at 70, 84 and 112 days of age (0.01040 ± 0.00169, 0.01010 ± 0.00070 and 0.01980 ± 0.00194, respectively; P = < 0.05; Fig. 11C). Not surprisingly, an overall main effect of group was detected (F\textsubscript{3,34} = 46.417; P = < 0.05; Fig. 11C).
Analysis of Tnfr2 mRNA expression in the mSOD1 facial nucleus revealed no differences at 70 and 84 days of age (0.00071 ± 0.00008 and 0.00069 ± 0.00014, respectively), but increased expression was detected at 112 days of age (0.00131 ± 0.00024; P = < 0.05), relative to averaged WT expression (0.00079 ± 0.00005; Fig. 11D). This upregulation in Tnfr2 mRNA in the mSOD1 facial nucleus at 112 days of age, resulted in an overall main effect between groups (F_{3,35} = 4.744; P = < 0.05; Fig. 11D).

The key mediator of activated TNFR2 signaling, TRAF2, revealed no main effect in mRNA expression between mSOD1 groups and averaged WT baseline (F_{3,35} = 1.626; P = 0.201; Fig. 11E). No individual differences were detected between mRNA expression in the facial nuclei of mSOD1 at 70, 84 and 112 days of age (0.00542 ± 0.00044, 0.00549 ± 0.00049 and 0.00528 ± 0.00024, respectively), compared to relative Traf2 expression in averaged WT facial motor nuclei (0.00476 ± 0.00017; Fig. 11E).

mRNA expression in the uninjured facial motor nucleus for the pro-survival receptor, PAC1-R, was assessed at 70, 84 and 112 days of age in mSOD1 mice (0.04100 ± 0.00318, 0.03910 ± 0.00170 and 0.04140 ± 0.00204, respectively) and compared to averaged WT baseline Pac1-r expression (0.04140 ± 0.00120; Fig. 11F). No statistical differences were detected and no main effect between groups was observed (F_{3,34} = 0.246; P = 0.864; Fig. 11F).

These results reveal an upregulation in several regenerative and pro-survival genes during disease progression in the mSOD1 uninjured control facial nucleus as was seen following axotomy. Identification of Gap-43, Cx3cr1 and Tnfr2 (Fig. 11) as indicators of disease-induced target disconnection in the facial motor nucleus of symptomatic SOD1 mice is relatively unique, as most genes used to measure disease onset, in general, are markers for cell death or inflammation (Malaspina and de Belleroche, 2004; Yoshihara et al., 2002).

These findings suggest that significant changes in expression of regenerative and pro-survival genes can be used as reliable indicators of the disease process in the mSOD1 mouse.

**Disease-induced glial-specific gene expression following in facial motor nuclei**

The astrocyte-specific marker, GFAP was assessed for disease-induced mRNA expression. Compared to averaged WT Gfap baseline mRNA expression (0.0365 ± 0.0020), within the facial nucleus, mSOD1 expression was significantly increased at 70, 84 and 112 days of age (0.1880 ± 0.0542, 0.1920 ± 0.0359 and 0.7730 ± 0.1390, respectively; P = < 0.05; Fig. 12A). These differences in Gfap mRNA expression between mSOD1 groups and WT baseline resulted in the observation of an overall effect of group (F_{3,34} = 70.714; P = < 0.05; Fig. 12A).

Lastly, Cd68 mRNA expression was measured as a marker for the microglial response to disease. A main effect of group was revealed between mSOD1 age groups and averaged WT baseline Cd68 mRNA expression (F_{3,35} = 33.356; P = < 0.05; Fig. 12B). Individually, mSOD1 Cd68 mRNA expression was significantly increased at 70, 84 and 112 days of age.
Therefore, these results identify a glial response to disease-induced target disconnection within the facial nucleus during the pre-symptomatic stage (Fig. 1), as early as 70 days of age.

Discussion

Two pathologies exist within the mSOD1 mouse model of ALS

Recent research utilizing the mSOD1 mouse model has provided evidence for the existence of two pathologies, peripheral target disconnection and MN cell death evoked by disease-related axonal die-back (Fischer et al., 2004; Dadon-Nachum et al., 2011), and enhanced MN cell death resulting from axonal injury after peripheral nerve transection (Mariotti et al., 2002; Ikeda et al., 2005; Mesnard et al., 2011). Specifically, we and others have determined that pre-symptomatic mSOD1 FMN show greater sensitivity than WT to axotomy, despite the fact that disease-related MN degeneration has not yet occurred (Mariotti et al., 2002; Ikeda et al., 2005; Mesnard et al., 2011). In support of this additional, mSOD1 CNS pathology, it has been shown that ALS patients with ulnar nerve entrapment experience more rapid axonal loss (Chaudhry and Clawson, 1997). Analysis of FMN numbers within the pre-symptomatic stage revealed no significant mSOD1 FMN loss, however, significant cell loss occurred by end-stage disease (Chiu et al., 1995; Haenggeli and Kato, 2002). The objective of this study was to investigate the underlying mechanism(s) of mSOD1 MN death after target disconnection, using the well-established rodent facial nerve injury model (Moran and Graeber, 2004; Lieberman, 1971; Graeber et al., 1988; Blinzinger and Kreutzberg, 1968). We have previously determined that the molecular regenerative response to axotomy by mSOD1 mouse FMN is phenotypically indistinguishable from WT, whereas, the surrounding microenvironment shows significant molecular dysregulation in the mSOD1 facial nucleus (Mariotti et al., 2002; Mesnard et al., 2011). Thus, our previous work (Mesnard et al., 2011), as well as results from the current study, suggest that the absence of a regenerative/survival MN response is not causative in the rapid mSOD1 FMN cell death. We hypothesized that MN death in WT and mSOD1 could be explained by one of two scenarios: 1) a common death pathway, under differential regulation, or 2) completely distinct death pathways resulting in different rates of cell death.

Axotomy induces expression of distinct death receptor pathways in WT vs. mSOD1 facial motor nuclei

Examination of the TNFR1 death pathway revealed that axotomy-induced mRNA upregulation for components of that pathway is comparable, temporally and in magnitude, between WT and mSOD1 mouse facial nuclei. These results indicate that selective activation of the TNFR1 death pathway, as indicated by the axotomy-induced upregulation of mRNA, probably does not account for the pathological, rapid FMN death in the mSOD1 facial nucleus. While the TNFR1 pathway is thought to be ubiquitously expressed, and the present study assessed mRNA expression representative of the whole facial motor nucleus, deletion of TNF receptors has been shown to specifically prevent MN death following

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axotomy in WT (Raivich et al., 2002), suggesting that the TNFR1 death pathway is directly responsible for the MN death after axotomy and is not acting on other cell types.

The Fas receptor pathway, the second cell death pathway analyzed in the current study, is particularly relevant to MN cell death due to the recent discovery of a novel secondary pathway downstream of Fas activation that is MN-specific (Raoul et al., 2002). This MN-specific pathway involves the adapter protein Daxx, recruitment of ASK1, phosphorylation of p38 and increased transcription of Nnos (Raoul et al., 2006). The enzyme nNOS produces NO that can spontaneously react with superoxide anion to form the toxic oxidant peroxynitrite (Martin et al., 2005; Martin et al., 2005; Brown and Bal-Price, 2003). Fas-induced MN death requires co-activation of both the traditional downstream and secondary MN-specific pathway, suggesting that Fas activation is highly destructive to MN and an additional pathway is necessary to regulate and protect against Fas-induced cell death (Raoul et al., 2006). We examined the regulation, by axotomy, of several molecules involved in both the traditional and secondary Fas pathways. Although relatively no changes in mRNA expression for Fas pathway components were detected in WT or mSOD1 facial nuclei during the initial post-axotomy stages, striking differences were identified at later timepoints. mRNA expression for molecules representing both downstream Fas pathways were significantly upregulated within the axotomized mSOD1 facial motor nucleus. Taken together, these data reveal that axotomy-induced Fas pathway mRNA expression is differentially regulated in WT and mSOD1 mice, and that this increased expression temporally coincides with the enhanced mSOD1 FMN cell death. In addition, mRNA upregulation for components representing the novel secondary downstream pathway is suggestive of MN-specific expression (Raoul et al., 2006; Raoul et al., 2002).

The results indicate that distinct death pathways are likely responsible for the axotomy-induced MN death in WT and mSOD1 facial motor nuclei and provide an explanation for the differential rates of cell death. While TNFR1 appears to be responsible for axotomy-induced FMN cell death in the WT, a combination of TNFR1 and Fas receptor pathways appears to be involved in the mSOD1 FMN death. Further examination of these death receptor pathways, with respect to axotomy-induced protein levels, cell-type localization and receptor pathway activation, will need to be completed for confirmation purposes.

The glial response to axotomy is dysregulated in the mSOD1 facial motor nucleus

It appears that the mSOD1 CNS pathology, responsible for the rapid MN cell death, is passive until axonal target disconnection is initiated by a peripheral nerve injury (Mariotti et al., 2002; Ikeda et al., 2005; Mesnard et al., 2011). In our attempt to identify potential mechanisms or components of the mSOD1 CNS pathology, leading to Fas mRNA upregulation and subsequent FMN death; we focused our investigation on local CNS processes that also remain passive until peripheral nerve injury occurs. Specifically, we investigated mRNA expression for genes known to play an important role in the reaction to peripheral nerve injury. We have previously determined that the regenerative response to axotomy by mSOD1 mouse FMN is phenotypically indistinguishable from WT (Mesnard et al., 2011). In the present study, we expanded this investigation by analyzing additional pro-survival genes and reached the same conclusion.
Another important local CNS response to peripheral nerve transection is the reaction of resident astrocytes and microglia. Previously, using Gfap mRNA expression profiles, we have shown that astrocytic activation in the mSOD1 axotomized facial motor nuclei, is significantly reduced compared to WT (Mesnard et al., 2011). Extension of the post-axotomy time course and analysis of Cldn68 mRNA expression, accomplished in the current study, reveals further evidence for both microglial and astrocytic dysregulation after axotomy in the mSOD1 facial motor nuclei relative to WT. The essential roles that microglia and astrocytes play in protecting and supporting injured neurons is well documented (Faulkner et al., 2004; Banati and Graeber, 1994). While the differential glial response to axotomy in WT and mSOD1 mice must be interpreted with caution, because only a single marker was chosen for each cell-type, alterations in both astrocytic and microglial gene expression together suggest pro-survival glial responses in WT mice and that they may be dysregulated in the mSOD1.

**Target disconnection by axotomy provides a standardized experimental paradigm when superimposed upon the mSOD1 mouse**

Superimposition of axotomy on the mSOD1 transgenic mouse model provides an experimental paradigm in which to explore ALS disease pathogenesis and progression because complete target disconnection in the mSOD1 through axotomy can be accomplished in a temporally controlled manner and compared to WT. By profiling the changes in mRNA expression within the uninjured, control mSOD1 facial motor nucleus as the mouse ages and progresses throughout the pre-symptomatic and symptomatic stages of disease, we were able to confirm that the mSOD1 responses to target disconnection by axotomy resemble the responses to target disconnection that occur during disease.

Increased glial reactivity in mSOD1 mice and ALS patients has been well documented within the literature (Malaspina and de Belleroche, 2004; Turner et al., 2004; Aronica et al., 2001). Even during the mSOD1 pre-symptomatic stage, when disease-induced target disconnection has been initiated (Fischer et al., 2004) but MN cell loss has not yet reached statistical significance (Chiu et al., 1995), mSOD1 spinal cord glia undergo similar reactive responses to those identified after axotomy (Yoshihara et al., 2002; Chen et al., 2004; Malaspina and de Belleroche, 2004; Yamanaka et al., 2008). Neuroregenerative and pro-survival responses have also been demonstrated, such as increased GAP-43 protein and/or Gap-43 mRNA expression in spinal cord tissue of mSOD1 mice (Perrin et al., 2005; Lobsiger et al., 2007) and fALS and sALS patients (Parhad et al., 1992; Ueki et al., 1993; Kage et al., 1998). With respect to the TNFR1 and Fas death receptor pathways, detection of upregulated mRNA, protein and evidence of active TNFR1 and Fas pathways within the spinal cord of mSOD1 mice, as well as ALS patient CSF and post-mortem CNS tissue, are consistent with our results following axotomy (Perrin et al., 2005; Hu et al., 2003; Yi et al., 2000; Raoul et al., 2006; Hensley et al., 2002; Chen et al., 2004; Satoh et al., 2005). Therefore, the literature provides further support that our mSOD1 axotomy-induced responses resemble responses to disease progression. In addition, treatments against Fas-(Locatelli et al., 2007; Facchinetti et al., 1999) and TNFR1-induced (Tweedie et al., 2007) cell death in mSOD1 mice have resulted in increased MN survival, delayed symptom onset.
and increased survival of mice by approximately 1–3 weeks, confirming their role in disease-induced MN degeneration.

While a majority of the facial nerve axotomy-induced mRNA expression has also been identified in mSOD1 spinal cord and ALS postmortem tissue, disease-related gene expression changes have not been investigated in the facial motor nucleus of mSOD1 mice or ALS patients. As a secondary objective of this study, we confirmed that disease-induced target disconnection recapitulates axotomy in terms of gene expression changes examined. At 112 days of age, within the symptomatic stage of disease, there is a significant decrease in mSOD1 FMN numbers compared to aged-matched WT. To our knowledge, this is the earliest documentation of disease-induced FMN cell loss in the mSOD1G93A mouse. Others have assessed FMN numbers within the pre-symptomatic stage of disease, at 69 and 90 days of age, and determined that significant cell loss has not yet occurred. However, significantly reduced MN numbers within the mSOD1 facial motor nuclei were confirmed at end-stage disease (Chiu et al., 1995; Haenggeli and Kato, 2002). Therefore, according to the die-back theory, disease-induced target disconnection of FMN occurs prior to 112 days of age, which is supported by MRI changes indicative of the disease process within the facial nucleus of mSOD1 mice as early as 70 days of age (Marcuzzo et al., 2011). We determined that a majority of the genes analyzed followed an axotomy-like expression pattern during disease progression, and this validates facial nerve axotomy as a sensitive and specific tool to investigate mSOD1 pathology (Table 2). With respect to two genes that did not reveal an upregulation with disease (βII-tubulin and Fadd), as was shown following axotomy, we cannot exclude the possibility that the axotomy-like response of these genes does exist within the mSOD1 facial nuclei as disease progression proceeds, but we were unable to capture this among the three ages analyzed in the present study. Because disease-induced target disconnection occurs slowly over time, it is difficult to predict when sufficient numbers of FMN may be disconnected by disease at any given point to result in measurable changes in some genes. This highlights the strengths of utilizing the axotomy model in standardizing the “onset” of the target disconnection as well as in generating measurable and robust mRNA responses in comparison to the nature of disease-induced target disconnection.

Target disconnection in the mSOD1 mouse reveals a dysregulated glial response and expression of distinct death receptor pathways

Application of our results to current understanding of mSOD1 disease progression suggests that an initial pathological event within the periphery disrupts functional NMJ (Fischer et al., 2004), resulting in a regenerative MN response (Schaefer et al., 2005), in conjunction with a pre-symptomatic CNS glial response (Yoshihara et al., 2002; Malaspina and de Belleruche, 2004) that we have identified as dysregulated or suppressed. The target disconnection process also results in a characteristic death pathway response within the mSOD1 CNS that includes both TNFR1 and Fas (Raoul et al., 2006; Hensley et al., 2002). While the TNFR1-induced MN death appears to be a standard WT response to target disconnection, we hypothesize that the Fas-induced MN death is a direct result of the secondary mSOD1 CNS pathology and leads to rapid FMN cell death. We have determined that Fas death pathway mRNA expression significantly increases in mSOD1 facial motor nuclei after axonal...
disconnection by means of axotomy or disease and these findings support the theory that a mSOD1 CNS pathology exists and mediates cell death following target disconnection. Our previous work, as well as data presented within the current study, indicates that the glial response to axotomy is significantly reduced in mSOD1 facial nucleus and we speculate that this dysregulated response to axotomy is a component of the mSOD1 CNS pathology.

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Figure 1.
Longitudinal analysis of motor deficits in mSOD1 mice. mSOD1 and WT mice were evaluated for signs of motor deficiency within an open field testing box using a 7-point scoring system described in materials and methods. Behavioral assessment began at 79 days of age and continued 3 times per week until 112 days of age. Data are presented as mean score ± SEM. Two-way repeated measures ANOVA (group × age) with Student-Newman-Keuls multiple comparison post hoc test: * represents a significant difference between WT and mSOD1, at $P < 0.05$. 
Figure 2.
Representative photomicrographs of thionin-stained uninjured, control and axotomized facial motor nuclei at 28 days following axotomy (84 days of age). Control (A, C) and axotomized (B, D) facial motor nuclei of WT (A, B) and mSOD1 (C, D) mice. Scale bar = 100µm in A.
Figure 3.
Representative photomicrographs of thionin-stained uninjured, control and axotomized facial motor nuclei at 56 days following axotomy (112 days of age). Control (A, C) and axotomized (B, D) facial motor nuclei of WT (A, B) and mSOD1 (C, D) mice. Scale bar = 100μm in A.
Figure 4.
Assessment of facial motoneuron (FMN) levels in the facial motor nuclei of WT and mSOD1 mice at 28 and 56 days post-facial nerve axotomy (dpa; 84 and 112 days of age). A: Average number of FMN per section ± SEM in the uninjured, control facial nuclei of WT and mSOD1 at 84 and 112 days of age. B: Average percent survival ± SEM of FMN from axotomized facial nuclei (Ax) relative to the uninjured, control nuclei (C) of WT and mSOD1 at 28 and 56 dpa (84 and 112 days of age). One-way ANOVA with Student-
Newman-Keuls multiple comparison post hoc test: * represents a significant difference compared with other timepoints or groups, at $P < 0.05$. 
Figure 5.
mRNA expression of genes involved in the TNFR1 death receptor pathway in WT and mSOD1 facial motor nuclei in response to facial nerve axotomy. A: Schematic of TNFR1 death-inducing signaling cascade (adapted from Grivenikov et al., 2006 and Harrington, 2000). B–G: Average percent of mRNA expression ± SEM in the axotomized facial nuclei (Ax) relative to the non-axotomized control nuclei (C). Time course of average mRNA expression ± SEM includes no injury (0), 3, 7, 14, 28, and 56 days post-axotomy (dpa) for Tnfa (only mSOD1 shown; B), Tnfr1 (C), Sodd (D), Tradd (E), Caspase-8 (F) and
Caspase-3 (G). Two-way ANOVA (group × time) and Student’s t-test adjusted for multiple comparisons (between groups at 28 and 56 dpa), both analyses utilizing Student-Newman-Keuls multiple comparison post hoc test: * represents a significant difference between WT and mSOD1, at $P < 0.05$. 
Figure 6.
mRNA expression of genes involved in the Fas death receptor pathway in WT and mSOD1 facial motor nuclei in response to facial nerve axotomy. A: Schematic of Fas death-inducing signaling cascade (adapted from Raoul et al., 2002 and Raoul et al., 2006). B–F: Average percent of mRNA expression ± SEM in the axotomized facial nuclei (Ax) relative to the non-axotomized control nuclei (C). Time course of mRNA expression includes no injury (0), 3, 7, 14, 28, and 56 days post-axotomy (dpa) for Fas (B), Fadd (C), Daxx (D), Ask1 (E) and Nnos (F). Two-way ANOVA (group × time) and Student’s t-test adjusted for multiple
comparisons (between groups at 28 and 56 dpa), both analyses utilizing Student-Newman-Keuls multiple comparison post hoc test: * represents a significant difference between WT and mSOD1, at $P < 0.05$. 
Figure 7.
mRNA expression of genes involved in pro-survival and neuroregeneration in WT and mSOD1 facial motor nuclei in response to facial nerve axotomy. A–F: Average percent change of mRNA expression ± SEM in the axotomized facial nuclei (Ax) relative to the non-axotomized control nuclei (C). Time course of mRNA expression includes no injury (0), 3, 7, 14, 28, and 56 days post-axotomy (dpa) for Gap-43 (A), βII-tubulin (B), Cx3cr1 (C), Tnfr2 (D), Traf2 (E) and Pac1-r (F). Two-way ANOVA (group × time) and Student’s t-
test adjusted for multiple comparisons (between groups at 28 and 56 dpa), both analyses utilizing Student-Newman-Keuls multiple comparison post hoc test.
Figure 8.
mRNA expression of glial-specific genes in WT and mSOD1 facial motor nuclei in response to facial nerve axotomy. A–B: Average percent of mRNA expression ± SEM in the axotomized facial nuclei (Ax) relative to the non-axotomized control nuclei (C). Time course of mRNA expression includes no injury (0), 3, 7, 14, 28, and 56 days post-axotomy (dpa) for Gfap (A) and Cd68 (B). Two-way ANOVA (group × time) and Student’s t-test adjusted for multiple comparisons (between groups at 28 and 56 dpa), both analyses
utilizing Student-Newman-Keuls multiple comparison post hoc test: * represents a significant difference between WT and mSOD1, at $P < 0.05$. 
Figure 9.
Relative mRNA expression of genes involved in the TNFR1 death receptor pathway in uninjured, control facial motor nuclei of WT and mSOD1 mice. WT mRNA expression was averaged as baseline expression as it did not differ with age (70, 84 and 112 days of age), while mSOD1 mRNA expression is displayed with increasing age. A–F: Average mRNA expression ± SEM of for Tnfa (A), Tnfr1 (B), Sodd (C), Tradd (D), Caspase-8 (E) and Caspase-3 (F) in the facial nuclei of WT (average across all ages) and mSOD1 mice at 70,
84 and 112 days of age. One-way ANOVA with Dunnett’s multiple comparison post hoc test; * represents a significant difference between WT (averaged) and mSOD1, at $P < 0.05$. 
Figure 10.
Relative mRNA expression of genes involved in the Fas death receptor pathway in uninjured, control facial motor nuclei of WT and mSOD1 mice. WT mRNA expression was averaged as baseline expression as it did not differ with age (70, 84 and 112 days of age), while mSOD1 mRNA expression is displayed with increasing age. A–E: Average mRNA expression ± SEM of for *Fas* (A), *Fadd* (B), *Daxx* (C), *Ask1* (D) and *Nnos* (E) in the facial nuclei of WT (average across all ages) and mSOD1 mice at 70, 84 and 112 days of age.
One-way ANOVA with Dunnett’s multiple comparison post hoc test: * represents a significant difference between WT (averaged) and mSOD1, at $P < 0.05$. 
Figure 11.
Relative mRNA expression of genes involved in pro-survival and neuroregeneration in uninjured, control facial motor nuclei of WT and mSOD1 mice. WT mRNA expression was averaged as baseline expression as it did not differ with age (70, 84 and 112 days of age), while mSOD1 mRNA expression is displayed with increasing age. A–F: Average mRNA expression ± SEM of for Gap-43 (A), βII-tubulin (B), Cx3cr1 (C), Tnfr2 (D), Traf2 (E) and Pac1-r (F) in the facial nuclei of WT (average across all ages) and mSOD1 mice at 70, 84
and 112 days of age. One-way ANOVA with Dunnett’s multiple comparison post hoc test: * represents a significant difference between WT (averaged) and mSOD1, at $P < 0.05$. 

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Figure 12.
Relative mRNA expression of glial-specific genes in uninjured, control facial motor nuclei of WT and mSOD1 mice. WT mRNA expression was averaged as baseline expression as it did not differ with age (70, 84 and 112 days of age), while mSOD1 mRNA expression is displayed with increasing age. A–B: Average mRNA expression ± SEM of for Gfap (A) and Cd68 (B) in the facial nuclei of WT (average across all ages) and mSOD1 mice at 70, 84 and 112 days of age.
112 days of age. One-way ANOVA with Dunnett’s multiple comparison post hoc test: * represents a significant difference between WT (averaged) and mSOD1, at $P < 0.05$. 
## qPCR primers designed from published murine complementary DNA sequences

<table>
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<th>Gene</th>
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<th>Accession Number</th>
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<th>$T_a$ (°C)</th>
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| Ask1     | F 5'-CATTTGAGCTCAGGCCACACT-3'  
  R 5'-GGCTACAGCTCACCCACC-3' | BC133697 | 55 | 82 | 120 |
| βIII-tubulin | F 5'-TGGAAGGCGAATCCT-3'  
  R 5'-GAATGTCGCCCGAGAC-3' | NM009450 | 54 | 84 | 79 |
| Caspase-3 | F 5'-TGGAAAGGGAGCATCCT-3'  
  R 5'-CAGGTATCCCTGCGCAAGG-3' | BC038825 | 55 | 85 | 109 |
| Caspase-8 | F 5'-CGCCAGATTGAAGTCTT-3'  
  R 5'-CGCCAGTACGAGGATCTAAG-3' | AF067834 | 53 | 81 | 123 |
| Cd68     | F 5'-CCCATAATTCAATCCGAATCC-3'  
  R 5'-GATCCGTCACAACACTCC-3' | BC021637 | 51 | 80 | 110 |
| Cx3cr1   | F 5'-CTACCTCCGACATCCCAACGACT-3'  
  R 5'-AGGGAAGGAAAGCCAAAGGAACAC-3' | AF074912 | 55 | 84 | 98 |
| Daxx     | F 5'-TGCAACACATCCTCAGGGTTC-3'  
  R 5'-GTTCAGAGCTGTAACTGAC-3' | BC128373 | 55 | 83 | 80 |
| Fadd     | F 5'-TGCTCAGGCAAGAGTGAAT-3'  
  R 5'-TTCTCAGATTCTCTTCCAGACTTTCA-3' | BC021400 | 55 | 82 | 97 |
| Fas      | F 5'-ACCGAAAGAAGATGCTGCT-3'  
  sR 5'-GGAAATCTGCCGAGTTATTAAC-3' | BC061160 | 53 | 79 | 121 |
| Gap-43   | F 5'-CTCAAGGAAAGTGCCCCACAG-3'  
  R 5'-CAGGTGCGGGAGAACGCG-3' | BC028288 | 54 | 84 | 102 |
| Gapdh    | F 5'-GAACATCATCCCTCAGTACA-3'  
  R 5'-CCAGTGAGCTCCTCAGCT-3' | M32599 | 50–57 | 81 | 78 |
| Gapdh2   | F unknown  
  R unknown | NM008084.2 | 55 | 85 | 140 |
| Gfap     | F 5'-AGACAACTTGGCTGATAT-3'  
  R 5'-TCTCTCCCTCCAGCGAT-3' | AF32062 | 55 | 84 | 93 |
| Nnas2    | F unknown  
  R unknown | NM008712.2 | 55 | 86 | 167 |
| Pac1-r   | F 5'-GGCTCTCGTGCTTCACTCT-3'  
  R 5'-CCGGTCTCCGAGACATGTA-3' | BC067039 | 55 | 82 | 118 |
| Sodd     | F 5'-CAGTGCAGAATGTCCTTCCAGGT-3'  
  R 5'-GACAGCAGCTACAGACAC-3' | AF328263 | 53 | 81 | 115 |
| Tnks     | F 5'-ATTACCTGAGCCCTCAGATG-3'  
  R 5'-AGGAGGCGCTGAGATATCC-3' | X02611 | 53 | 82 | 102 |
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<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Accession Number</th>
<th>$T_a$(°C)$^1$</th>
<th>$T_m$(°C)$^1$</th>
<th>Amplicon Length (bp)</th>
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$^1$The optimal annealing temperature ($T_a$) and the peak melt curve temperature ($T_m$) were determined experimentally.

$^2$Primer sets for Nnos and one of the Gapdh sets were purchased from SuperArray Biosciences.

Forward Primer (F), Reverse Primer (R).
Summary: mSOD1 axotomy-induced vs. disease-induced mRNA expression in facial motor nucleus

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<tr>
<th>Gene</th>
<th>Axotomy</th>
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<th>Gene</th>
<th>Axotomy</th>
<th>Disease</th>
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<td>↑</td>
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<td>Cd68</td>
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<td>Daxx</td>
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<td>no Δ</td>
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† (axotomy-induced): mSOD1 mRNA expression is significantly upregulated in the axotomized facial motor nucleus (Ax) at 28 and/or 56 days post-axotomy (dpa), relative to no injury or 0 dpa.
† (disease-induced): mSOD1 mRNA expression is significantly increased in uninjured, control facial motor nucleus (C) at 112 days of age, relative to averaged WT baseline expression.
no Δ (axotomy-induced): no statistical difference in mRNA expression in the mSOD1 axotomized facial nucleus at 28 and/or 56 dpa, relative to no injury or 0 dpa.
no Δ (disease-induced): no difference in mSOD1 mRNA expression in uninjured, control facial motor nucleus (C) at 112 days of age, relative to averaged WT baseline expression.

* mRNA expression is upregulated after axotomy (delayed timepoints) in mSOD1 facial nuclei, but no upregulation was seen during disease progression at 112 days of age.