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Selective loss of alpha motor neurons with sparing of gamma motor neurons and spinal cord cholinergic neurons in a mouse model of spinal muscular atrophy

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Keywords: Spinal Muscular Atrophy, SMA, alpha motor neuron, gamma motor neuron, cholinergic, spinal cord

Running title: Gamma motor neurons in SMA
SUMMARY

Spinal muscular atrophy (SMA) is a neuromuscular disease characterised primarily by loss of lower motor neurons from the ventral grey horn of the spinal cord and proximal muscle atrophy. Recent experiments utilising mouse models of SMA have demonstrated that not all motor neurons are equally susceptible to the disease, revealing that other populations of neurons can also be affected. Here, we have extended investigations of selective vulnerability of neuronal populations in the spinal cord of SMA mice to include comparative assessments of alpha motor neuron (α-MN) and gamma motor neuron (γ-MN) pools, as well as other populations of cholinergic neurons. Immunohistochemical analyses of late-symptomatic SMA mouse spinal cord revealed that numbers of α-MNs were significantly reduced at all levels of the spinal cord compared to controls, whereas numbers of γ-MNs remained stable. Likewise, the average size of α-MN cell soma was decreased in SMA mice with no change occurring in γ-MNs. Evaluation of other pools of spinal cord cholinergic neurons revealed that pre-ganglionic sympathetic neurons, central canal cluster interneurons, partition interneurons and preganglionic autonomic dorsal commissural nucleus neuron numbers all remained unaffected in SMA mice. Taken together, these findings indicate that α-MNs are uniquely vulnerable amongst cholinergic neuron populations in the SMA mouse spinal cord, with γ-MNs and other cholinergic neuronal populations being largely spared.

Keywords: Spinal Muscular Atrophy, SMA, alpha motor neuron, gamma motor neuron, cholinergic neuron, spinal cord
INTRODUCTION

Spinal muscular atrophy (SMA) is a neuromuscular disorder characterised by loss of lower motor neurons from the ventral grey horn of the spinal cord and wasting of proximal skeletal musculature. With no approved treatment options currently available, and an incidence of 1 in 6,000-10,000 births, in its most severe forms SMA is a leading genetic cause of infant death (Lunn and Wang, 2008).

SMA is an autosomal recessive disease caused by low levels of the survival motor neuron (SMN) protein resulting from mutations or deletions in its coding gene (SMN1) (Lefebvre et al., 1995). SMN protein is ubiquitously expressed in all cells and tissues of the body (Battaglia et al., 1997). Complete loss of SMN protein is not compatible with cell viability in any tissues or organs (Hamilton and Gillingwater, 2013, Shababi et al., 2014), likely due to its important contribution to pathways regulating RNA processing (Pellizzoni et al., 1998, Paushkin et al., 2002, Fallini et al., 2012) and ubiquitin homeostasis (Wishart et al., 2014, Powis et al., 2014, Groen & Gillingwater, 2015), amongst others. However, a small amount of full-length SMN protein can be produced from a duplicate SMN2 gene in human patients, but alternative spicing of SMN2 mRNA results in the majority of transcript generated from the SMN2 locus lacking exon 7, leading to rapid degradation of the resulting protein (Lefebvre et al., 1995, Lorson et al., 1999, Monani et al., 1999). The severity of SMA is therefore dependent on levels of full-length SMN protein, with SMN2 copy number inversely correlated with disease severity (Feldkotter et al., 2002, Wirth et al., 2006). As a result, patients are categorised in to one of four main disease sub-types depending upon their clinical presentation, with Type I SMA being the most severe (mortality is expected...
before the age of 2) and Type IV the least severe (with muscle weakness present but a full lifespan predicted) (Lunn and Wang, 2008).

A detailed understanding of the genetic defects underlying SMA has facilitated the successful creation of multiple different animal models to help explore cellular and molecular aspects of disease pathogenesis. A range of SMA mouse models have been generated, all of which replicate the seminal patient phenotype of lower motor neuron loss from the spinal cord (Park et al., 2010, Sleigh et al., 2011). However, just as in human patients, closer examination of motor neuron pools in SMA mice has revealed that not all motor neurons are equally affected by the disease, with some appearing to possess disease-resistant characteristics (Murray et al., 2008, Ling et al., 2012, Thomson et al., 2012). Moreover, recent studies have suggested that neurons forming synaptic connections onto motor neurons can also be directly affected in SMA (Ling et al., 2010, Mentis et al., 2011).

At present it remains unclear why some neurons are vulnerable in SMA whereas others remain largely resistant, although (in contrast to other forms of motor neuron disease such as amyotrophic lateral sclerosis) it doesn’t appear to be determined by the underlying morphological characteristics of each motor neuron (Thomson et al., 2012). A more detailed understanding of the nature of vulnerable and disease-resistant neuron pools in the spinal cord is therefore required. For example, within the mammalian spinal cord lower motor neuron pools in the ventral grey horn contain both alpha motor neurons (α-MNs), which innervate extrafusal muscle fibres responsible for skeletal muscle contraction, and gamma motor neurons (γ-MNs), which innervate the intrafusal fibres of muscle spindles and
regulate their sensitivity to stretch (Kanning et al., 2010). And yet, it remains to be
determined whether α-MNs and γ-MNs are equally affected in SMA.

The study of different lower motor neuron subpopulations has been limited by the absence
of distinguishing molecular markers. Recently, however, a range of molecular differences
between α- and γ-MNs have been identified in both the postnatal (Friese et al., 2009,
Shneider et al., 2009, Misawa et al., 2012, Edwards et al., 2013) and prenatal (Ashrafi et al.,
2012) rodent spinal cord. This has led to the development of a robust
immunohistochemistry protocol where α-MNs can be identified by immunoreactivity to
choline acetyltransferase (ChAT) and strong neuronal nuclei (NeuN) expression, whereas γ-
MNs were found to show strong ChAT immunoreactivity but no NeuN labelling (Friese et al.,
2009, Shneider et al., 2009). Here, we have adapted this protocol for use on spinal cords of
neonatal SMA mice (modelling severe forms of the disease), allowing us to quantify and
compare α- and γ-MN vulnerability in vivo.
MATERIALS AND METHODS

Animals and tissue preparation

The ‘Taiwanese’ mouse model of severe SMA (Smn<sup>−/−</sup>; SMN2<sup>tg/0</sup>) (Hsieh-Li et al., 2000) was used for all experiments, utilising the breeding strategy developed by (Riessland et al., 2010) with phenotypically normal littermates (Smn<sup>+/−</sup>; SMN2<sup>tg/0</sup>) used as controls. Late-symptomatic mice at postnatal day 9 (P9) were chosen for use at a time point where motor neuron loss has been previously been reported (Riessland et al., 2010). All experiments were performed under appropriate licenses from the UK Home Office and were approved by an internal ethical review committee at the University of Edinburgh. The number of animals used for each experiment is detailed in the figure legends.

Mice were culled by Schedule 1 methods. Whole spinal columns were removed and a 23 gauge needle attached to a 5ml syringe filled with phosphate buffered saline (PBS) (Sigma-Aldrich) was inserted into the sacral end of the column. Gentle pressure was then applied to the syringe to flush out the spinal cord from the cervical end of the column. Isolated spinal cords were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 4 hours before being transferred to 30% sucrose (Sigma-Aldrich) in PBS overnight at 4°C for cryoprotection. The cervical enlargement, lumbar enlargement and thoracic region were then dissected and embedded separately in optimal cutting temperature compound (OCT) (CellPath), frozen on dry ice and stored at -80°C until further use. Using a cryostat (Leica, CM3050S), spinal cord sections were cut at 25um thickness and every 4<sup>th</sup> section collected onto Poly-L-lysine coated slides (Thermo Scientific). Excess OCT around sections was removed using PBS and a narrow paintbrush. Slides were left to dry before being processed for immunohistochemistry.
**Immunohistochemistry**

Using a Shandon Sequenza Immunostaining Centre (Thermo-Scientific) sections were permeabilised in 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 20 minutes followed by application of blocking solution (4% bovine serum albumin (Sigma-Aldrich), 0.3% Triton X-100 in PBS) for 30 min at room temperature. Goat anti-choline acetyltransferase (ChAT) primary antibody (1:100 diluted in PBS; Merck Millipore; AB144P) was then applied and left to incubate for 48 hrs at 4°C. Slides were washed in PBS (3 x 5 min) and Alexa Flour 594 donkey anti-goat IgG (1:500, Life Technologies, A-11058) secondary antibody applied for 2 hrs at room temperature. Following PBS washes, blocking solution was reapplied followed by mouse anti-neuronal nuclei (NeuN) primary antibody, clone A60 (1:100, Merk Millipore; MAB377) for 2hrs at room temperature. After PBS washes, Alexa Flour 488 donkey anti-mouse IgG (1:500, Life Technologies, A21202) secondary antibody was applied for 2hrs at room temperature. Following PBS washes, DAPI nuclei stain (1:1,000, Life Technologies, D1306) was applied for 10 minutes. After final PBS washes, coverslips were placed on slides using Mowoil mounting media.

**Microscopy and image analysis**

Spinal cord sections were imaged using a Leica DMIRB microscope equipped with a Retiga 2000R camera and QCapturePro-6 software for epi-fluorescence imaging (Objective: HC PL APO 20X with Numerical Apperture 0.7, OIL immersion objective). Images taken from red and green channels were merged using FIJI software (Schindelin et al., 2012), allowing neurons from different cholinergic pools to be counted and cell body areas to be measured by manually tracing around the cell soma using the 'freehand selections' tool in the FIJI.
software. To be included in these analyses, each cell had to be identifiable by the presence of a clear nucleus. In the ventral horn, α-MNs were defined as having ChAT+/NeuN+ expression whereas γ-MNs possessed ChAT+/NeuN- labelling (Friese et al., 2009, Shneider et al., 2009). All cell body counts and measurements were performed by an investigator who was blind to the identity of the tissue.

For motor neuron cell body size distribution analyses, measurements were taken and then rounded into 50μm² bins. Other cholinergic neuron cell body populations analysed included: central canal cluster interneurons, partition cell interneurons, preganglionic sympathetic neurons in the intermediolateral nucleus (IML; lateral grey horn), and preganglionic autonomic dorsal commissural nucleus, as previously defined by rodent spinal cord atlases (Barber et al., 1984, Watson et al., 2009, Sengul et al., 2012). Example images were taken using a Nikon A1R confocal system combined with Ti:E inverted microscope (Objective: x10, x40 and x60 Plan Apo OIL, NA=1.4).

**Data collection and statistical analysis**

Data were analysed using Microsoft Excel 2010 and Graph Pad Prism 6 software. All data are reported as mean ± standard error mean (SEM). Data from control and SMA mice were compared using an unpaired, two tailed Student’s t-test. Statistical significance was considered to be $P < 0.05$ for all analyses. Figures were created using Adobe Photoshop 6 software.
RESULTS

First, we wanted to establish whether the immunohistochemical labelling protocol previously used to distinguish α-MNs (ChAT+/NeuN+ cells with soma located in the ventral grey horn) from γ-MNs (ChAT+/NeuN- cells with soma located in the ventral grey horn) (Friese et al., 2009, Shneider et al., 2009) could be employed successfully in spinal cord preparations from neonatal SMA mice and littermate controls. These initial experiments confirmed that we could reliably distinguish α-MNs from γ-MNs in the ventral grey horn from all regions of the spinal cord (cervical, thoracic and lumbar) (Fig. 1), with no discernable differences observed in the quality of labelling between mice of different genotypes (data not shown). In addition, this technique allowed us to identify other populations of cholinergic neurons in the spinal cord with cell bodies outside the ventral grey horn, including: pre-ganglionic sympathetic neurons, central canal cluster interneurons, partition interneurons and preganglionic autonomic dorsal commissural nucleus neurons (Fig. 1).

High power micrographs of the ventral grey horn were subsequently obtained from late-symptomatic (P9) SMA mice and control littermates in order to reliably identify/distinguish and count numbers of α-MNs and γ-MNs (Fig. 2A-B). Initial counts of the total number of lower motor neurons (α-MNs and γ-MNs combined; identified by ChAT immunoreactivity) in cervical, thoracic and lumbar regions of the spinal cord in control littermate mice provided base-line data for subsequent comparisons (Fig. 2C), confirming the presence of higher overall numbers of MNs in the cervical and lumbar enlargements compared to the thoracic region, as expected (Fig. 2C). The relative proportions of α-MNs and γ-MNs remained
relatively consistent between different regions of the spinal cord, with around 80% of all
MNs being α-MNs and the remaining 20% being γ-MNs (Fig. 2D).

Subsequent counts of α-MNs and γ-MNs across all three distinct anatomical regions of the
spinal cord confirmed a significant loss of α-MNs from all regions in SMA mice (Fig. 2E-G).
For example, in lumbar regions of spinal cord there were ~20% fewer α-MNs on average per
ventral grey horn in littermate controls compared to SMA mice (P<0.05; 23.5 α-MNs in
littermate controls versus 19.5 α-MNs in SMA mice; Fig. 2G). In striking contrast, numbers of
γ-MNs remained consistent between SMA mice and littermate controls, across all regions of
the spinal cord (Fig. 2E-G). For example, in lumbar spinal cord - where there was a 20% loss
of α-MNs - the average number of γ-MNs per ventral grey horn in SMA mice was 6.4
compared to 6.3 in littermate controls (P>0.05; Fig. 2F).

Whilst our initial experimental findings suggested that α-MNs were being preferentially lost
in SMA mice, whereas γ-MNs remained unaffected, it remained possible that more subtle
pathological changes were still occurring across both α-MN and γ-MN populations that were
not being detected by a simple count of cell numbers. To examine this possibility we next
measured cell soma size of α-MNs and γ-MNs in SMA mouse spinal cord, as reduced soma
size is a well-characterised morphological correlate of motor neuron pathology in vivo
(D’errico et al., 2013). Measurements of cell soma size for α-MNs revealed a significant
reduction in SMA mice compared to control littermates across all regions of the spinal cord
(Fig. 3). In contrast, the size of γ-MN cell soma remained unchanged in SMA mice (Fig. 3).
Taken together, these data show that, within lower motor neuron pools, α-MNs are
selectively targeted whereas γ-MNs remain unaffected in SMA.
Given the ability to identify other populations of cholinergic neurons within the spinal cord using our immunohistochemical labelling protocol (Fig. 1 & Fig. 4A-B), we were also able to test whether a cholinergic neuronal identity correlated with overall increased susceptibility to degeneration in our SMA mouse model. We therefore quantified numbers of preganglionic sympathetic neurons, central canal cluster interneurons, partition interneurons and preganglionic autonomic dorsal commissural nucleus neurons in the spinal cord of SMA mice and littermate controls.

Preganglionic sympathetic neurons resident in the intermediolateral nucleus (lateral grey horn) project to locations outside of the spinal cord in order to reach their respective postganglionic neuron targets (e.g. the paravertebral ganglia of the sympathetic trunk). Likewise, the dorsal commissural nucleus (also known as the central autonomic nucleus, a midline preganglionic autonomic cell column in the spinal cord of rodents) contains the cell bodies of sympathetic preganglionic neurons providing central control to pelvic adrenergic neurons (Hancock & Peveto, 1979). Central canal cluster neurons represent a small group of cholinergic interneurons that can influence motor network activity. Similarly, partition cells are known to generate synaptic inputs on to motor neurons in order to regulate their excitability (Miles et al., 2007, Stepien et al., 2010, Bertrand and Cazalets, 2011; Witts et al., 2014).

There was no significant change in the number of any of these other cholinergic neuron subtypes in SMA mouse spinal cord, suggesting that the cholinergic identity of α-MNs is unlikely to contribute to their increased susceptibility in SMA (Fig. 4C-F). Thus, of all the cholinergic
neuronal populations examined in SMA mouse spinal cord at a late-symptomatic time-point,

neuronal loss was only observed in α-MNs.
DISCUSSION

The current study provides experimental evidence supporting the selective vulnerability of α-MNs in SMA mice, whilst demonstrating that γ-MNs and other populations of cholinergic neurons in the spinal cord are largely spared in the disease, even at late-symptomatic time-points. These findings are consistent with previous reports in the literature suggesting that muscle spindles on intrafusal muscle fibres innervated by γ-MNs remain intact in mouse models of severe SMA (Mentis et al., 2011) (Kariya et al., 2008). However, these findings contradict reports from human SMA patients, where changes in muscle spindles have previously been identified, particularly in less severe forms of the disease (Hausmanowa-Petrusewicz et al., 1968, Marshall and Duchen, 1975). Given the use of mice modelling severe forms of SMA in the current study and previous studies of intrafusal muscle fibres (Mentis et al., 2011) (Kariya et al., 2008), it is possible that the differences observed between mouse and human data could have arisen from comparisons between severe and less severe forms of SMA. Further investigations of γ-MNs, and other populations of cholinergic neurons in the spinal cord, may therefore be warranted in mice modelling less severe forms of the disease where α-MN pathology is still known to occur (Ruiz and Tabares, 2014).

The observation that γ-MNs were spared in SMA mice correlates with similar findings from studies of other forms of motor neuron disease, such as adult-onset amyotrophic lateral sclerosis (ALS). For example, whilst early patient studies hinted at a loss of both α-MNs and γ-MNs at the disease end-stage of ALS (Saito et al., 1978, Swash and Fox, 1974), a more recent study in a mouse model of ALS, using staining techniques that accurately differentiate
between the two neuronal populations, indicated that there was a selective loss of α-MNs and sparing of γ-MNs (Mohajeri et al., 1998).

The reasons why γ-MNs are spared in SMA, but α-MNs are severely affected remain unclear. One possibility is that γ-MNs are intrinsically more resistant to stressor stimuli. Evidence in support of this hypothesis comes from the finding that γ-MNs are more resistant to age-associated pathology than α-MNs, at least in rodent models (Hashizume et al., 1988). Another possibility is that γ-MNs are more resistant due to reduced metabolic requirements resulting from their smaller motor unit size relative to α-MNs. γ-MNs are known to have less dendrite branching, lower synaptic dendrite densities, smaller membrane input resistances, slower axon conductivity and shallower synaptic folds at the neuromuscular junction compared to α-MNs (Kanning et al., 2010). However, motor unit size was not found to correlate with differing susceptibility between pools of α-MNs in SMA mice (Thomson et al., 2012). Alternatively, it is possible that the differing muscle fibre targets of α-MNs and γ-MNs (extrafusal and intrafusal fibres respectively) influence their relative susceptibility. It is known that intrinsic molecular defects occur in skeletal muscle fibres from SMA mice (Mutsaers et al., 2011), but potential differences between extrafusal and intrafusal fibres have yet to be directly investigated. Similarly, it is known that muscle spindles are a source of neurotrophic factors, such as GDNF, that can influence γ-MN survival (Whitehead et al., 2005, Friese et al., 2009).

The finding that other populations of cholinergic neurons in the spinal cord were unaffected in SMA mice provides further insights into the selective vulnerability of different neuronal populations during the disease. Partition cells provide inputs to motor neurons and regulate
their excitability (Miles et al., 2007, Stepien et al., 2010, Bertrand and Cazalets, 2011), but they remained unchanged in SMA mice, suggesting that partition cells are unlikely to be contributing to the deafferentation of lower motor neurons previously reported (Mentis et al., 2011). Similarly, the finding that numbers of preganglionic sympathetic neurons in the lateral grey horn and numbers of preganglionic autonomic neurons in the dorsal commissural nucleus (also known as the central autonomic nucleus) remained unchanged in SMA mice suggests that the autonomic dysfunction previously reported in SMA patients (Hachiya et al., 2005, Arai et al., 2005, Rudnik-Schoneborn et al., 2008) and also SMA mice (Heier et al., 2010) does not result from direct targeting of cholinergic autonomic neuronal populations in the spinal cord.
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FIGURE LEGENDS

Figure 1. Immunohistochemical labelling of cholinergic neuronal populations in the spinal cord of neonatal mice. Representative confocal micrographs showing a transverse section through the upper lumbar spinal cord (L1-L2; containing both the dorsal commissural nucleus and the intermediolateral nucleus, not present at all levels of the spinal cord) in a postnatal day 9 littermate control mouse, immunohistochemically labelled for NeuN (green) and ChAT (red). Nuclei were labelled with DAPI (blue). In the ChAT section note the presence of motor neurons in the ventral grey horn (white arrow), preganglionic sympathetic neurons in the intermediolateral nucleus (black arrow), partition cells (grey arrow), central canal cluster cells (yellow arrow) and preganglionic autonomic neurons in the dorsal commissural nucleus (blue arrow). Scale bar = 250µm.

Figure 2. Selective loss of α-MNs but sparing of γ-MNs in late-symptomatic SMA mouse spinal cord. A-B: Representative low (A) and high (B) power confocal micrographs of the ventral grey horn of the postnatal day 9 mouse spinal cord immunohistochemically labelled for NeuN (green) and ChAT (red) (nuclei were labelled with DAPI [blue]). Black arrows indicate α-MNs (ChAT+/NeuN+ cells) and white arrows indicate γ-MNs (ChAT+/NeuN- cells). Scale bars = 50µm (A) and 25µm (B). C-D: Bar charts (mean ± SEM) showing the number of total motor neurons (C) and percentage of α-MNs and γ-MNs (D) in the cervical, thoracic and lumbar regions of littermate control mouse spinal cord at P9 (N=4 mice, mean number of motor neurons counted per control spinal cord region: cervical n=1146 ± 216; thoracic n=639 ± 84; lumbar n=1085 ± 297). E-G: Bar charts showing the number of α-MNs and γ-MNs in the cervical, thoracic and lumbar regions of spinal cord from littermate control and late-symptomatic SMA mice at P9 (ns=not significant; *P<0.05, **P<0.01; unpaired two-
tailed Student’s t-test. N=4 mice per genotype, except for SMA cervical measurements where N=3).

Figure 3. Reduction in the size of the cell soma of α-MNs but not γ-MNs in late-symptomatic SMA mouse spinal cord. A-C: Bar charts (mean ± SEM) showing the mean cell soma size for α-MNs and γ-MNs in the cervical, thoracic and lumbar regions of spinal cord from littermate control and late-symptomatic SMA mice at P9 (ns=not significant, *P<0.05, unpaired two-tailed Student’s t-test. N=4 mice per genotype, except for SMA cervical measurements where N=3). D-I: XY graphs showing mean α-MN (D, F, H) and γ-MN (E, G, I) cell body size distributions in control (black line) and SMA (grey line) mice at cervical (D&E), thoracic (F&G) and lumbar (H&I) spinal cord levels. α-MNs and γ-MN soma measurements were rounded into 50μm² bins and expressed as a percentage of total control motor neuron size distribution measurements. Error bars = SEM.

Figure 4. Sparing of other cholinergic neuronal populations in the spinal cord from late-symptomatic SMA mice. A-B: Representative confocal micrographs showing cholinergic neuronal populations surrounding the central canal (A) and the in the intermediolateral nucleus (B) in a transverse section through the upper lumbar spinal cord (L1-L2) in a postnatal day 9 littermate control mouse, immunohistochemically labelled for NeuN (green) and ChAT (red). Nuclei were labelled with DAPI (blue). Scale bars = 50um. A: In the ‘merged’ panel examples of partition cells (black arrows), central canal cluster cells (white arrows) and preganglionic autonomic neuron in dorsal commissural nucleus (grey arrows) are indicated. C-D: Bar charts (mean ± SEM) showing the number of ChAT+ partition cells (C) and central canal cluster cells per transverse spinal cord section in the cervical, thoracic and
lumbar regions in littermate control and late-symptomatic SMA mice at P9. E-F: Bar charts (mean ± SEM) showing the number of ChAT+ preganglionic autonomic neurons in dorsal commissural nucleus (E; taken from L1/L2 spinal cord only) and preganglionic sympathetic neurons in intermediolateral nucleus (F; taken from thoracic and upper lumbar levels of spinal cord) in P9 littermate control and late-symptomatic SMA mice. (ns=not significant, unpaired two-tailed Student’s t-test. N=4 mice per genotype, except for SMA cervical measurements where N=3).
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