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MOTOR NEURONS WITH DIFFERENTIAL VULNERABILITY TO DEGENERATION SHOW DISTINCT PROTEIN SIGNATURES IN HEALTH AND ALS

L. COMLEY,[a†] I. ALLODI,[a†] S. NICHTERWITZ,[a†] M. NIZZARDO,[b] C. SIMONE,[b] S. CORTI[b] AND E. HEDLUND[a*]

[a] *Department of Neuroscience, Karolinska Institutet, Retzius v. 8, 171 77 Stockholm, Sweden*

[b] *Dino Ferrari Center, Neuroscience Section, Department of Pathophysiology and Transplantation, University of Milan, Neurology Unit, Istituto Di Ricovero e Cura a Carattere Scientifico Foundation Ca' Granda Ospedale Maggiore Policlinico, Milan 20122, Italy*

Abstract—The lethal disease amyotrophic lateral sclerosis (ALS) is characterized by the loss of somatic motor neurons. However, not all motor neurons are equally vulnerable to disease; certain groups are spared, including those in the oculomotor nucleus controlling eye movement. The reasons for this differential vulnerability remain unknown. Here we have identified a protein signature for resistant oculomotor motor neurons and vulnerable hypoglossal and spinal motor neurons in mouse and man and in health and ALS with the aim of understanding motor neuron resistance. Several proteins with implications for motor neuron resistance, including $GABA_A$ receptor α1, guanylate cyclase soluble subunit alpha-3 and parvalbumin were persistently expressed in oculomotor neurons in man and mouse. Vulnerable motor neurons displayed higher protein levels of dynein, peripherin and $GABA_A$ receptor α2, which play roles in retrograde transport and excitability, respectively. These were dynamically regulated during disease and thus could place motor neurons at an increased risk. From our analysis is it evident that oculomotor motor neurons have a distinct protein signature compared to vulnerable motor neurons in brain stem and spinal cord, which could in part explain their resistance to degeneration in ALS. Our comparison of human and mouse shows the relative conservation of signals across species and infers that transgenic $SOD1^{G93A}$ mice could be used to predict mechanisms of neuronal vulnerability in man.

Key words: oculomotor, motor neuron, amyotrophic lateral sclerosis, neurodegeneration, selective vulnerability.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal disease characterized by a progressive loss of somatic motor neurons in the spinal cord and brain stem. However, certain groups of somatic motor neurons, including those in the oculomotor nucleus (CNIII), which control eye movements, and those in Onuf's nucleus, that control pelvic muscles, are generally spared (Gizzi et al., 1992; Kubota et al., 2000; Nimchinsky et al., 2000; Haenggeli and Kato, 2002; Tjust et al., 2012). The reasons for this differential vulnerability among motor neurons remain unknown.

ALS can be inherited (familial, fALS, ∼10%) due to mutations identified in a number of genes, the two most common of which are superoxide dismutase 1 (SOD1) and C9ORF72 (chromosome 9 open reading frame 72) (Al-Chalabi et al., 2012), but the majority of ALS cases appear sporadic (sALS, ∼90%). The pathology and pattern of selective motor neuron vulnerability is similar in fALS and sALS (Shaw et al., 1997). This indicates that differential vulnerability is independent of the cause of disease. Therefore elucidation of the mechanisms of selective vulnerability in mouse models of fALS could be applicable also to sALS. Importantly, *in vivo* and *in vitro* models of fALS indicate that factors intrinsic to motor neurons are crucial for initiation of degeneration (Boillee et al., 2006; Jaarsma et al., 2008; Huang et al., 2012; Kiskinis et al., 2014), while astrocytes and microglia drive disease progression (Boillee et al., 2006; Yamanaka et al., 2008; Das and Svendsen, 2014). It therefore seems that differential expression of factors intrinsic to motor neurons render these cells more or less vulnerable to toxic events (Saxena et al., 2009; Hedlund et al., 2010; Kaplan et al., 2014). Thus, identification of cell intrinsic mechanisms of protection and vulnerability may lead to therapies preventing the progressive loss of motor neurons. Toward this goal, we previously analyzed the global gene expression profiles of vulnerable spinal and hypoglossal motor neurons and spared oculomotor and trochlear neuron groups in the normal rat and identified multiple intrinsic factors that may underlie their differential vulnerability (Hedlund et al., 2010).

To further our understanding of motor neuron vulnerability and resistance, we selected six genes that

*Corresponding author. Tel: +46-852487884.
E-mail address: eva.hedlund@ki.se (E. Hedlund).
† These authors contributed equally to this work.

Abbreviations: ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; ChAT, choline acetyltransferase; CNIII, oculomotor nucleus; CNXII, hypoglossal nucleus; Gabra1, $GABA_A$ receptor α1; Gabra2, $GABA_A$ receptor α2; Gucy1a3, guanylate cyclase soluble subunit alpha-3; NBB, Netherlands Brain Bank; ND, non-demented; NDRI, National Disease Research Interchange; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SC, spinal cord; SOD1, super oxide dismutase 1.

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Table 1. Candidate genes selected for across species analysis in health and ALS based on differential mRNA expression

Gene name	Known biological functions	Motor neurons with highest mRNA level*
		Control rat
Gucy1a3	Main receptor for nitric oxide (Zabel et al., 1998), potentially implicated in modulating Fas ligand activation (Hedlund et al., 2010)	CNIII
Parvalbumin	Calcium-binding protein with motor neuron protective effects (Van Den Bosch et al., 2002; Dekkers et al., 2004)	CNIII
Gabra1	Inhibitory synaptic transmission, neuronal excitability (Lorenzo et al., 2006; Brockington et al., 2013)	CNIII
Gabra2	Inhibitory synaptic transmission, neuronal excitability (Lorenzo et al., 2006; Brockington et al., 2013)	SC
Peripherin	Intermediate neurofilament, over-expression of which causes ALS-like MN loss (Beaulieu et al., 1999)	CNXII and SC
Dynein	Retrograde transport protein, mutations in which are linked to motor neuron degeneration (LaMonte et al., 2002; Hafezparast et al., 2003)	CNXII and SC

CNIII = oculomotor nucleus, CNXII = hypoglossal nucleus, SC = spinal cord.
* Based on our previously published gene array study (Hedlund et al., 2010).

differed in their mRNA expression between resistant and susceptible motor neurons in the healthy rodent (Hedlund et al., 2010), and analyzed the corresponding protein levels in health and ALS, in man and mouse. Specifically, the proteins analyzed in this study were guanylate cyclase soluble subunit alpha-3 (Gucy1a3), parvalbumin, $GABA_A$ receptor α1 (Gabra1), $GABA_A$ receptor α2 (Gabra2), peripherin and dynein, which all have previous implications for motor neuron disease (summarized in Table 1). Our initial analysis was performed in early symptomatic mutant $SOD1^{G93A}$ fALS mice and wild-type litter-mates. In order to elucidate if genes showed similar expression patterns and regulation in human disease and thus could be inferred to play a role in protection and vulnerability we subsequently analyzed post mortem tissues from non-demented (ND) controls and sporadic ALS patients. Our analysis showed that resistant oculomotor motor neurons have a distinct protein signature, which is relatively conserved between man and mouse. This unique profile could in part explain their preservation in ALS when other motor neurons degenerate.

EXPERIMENTAL PROCEDURES

Ethics statement

All the work involving animal or human subjects/tissues has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and with national legislation as well as our institutional guidelines. Ethical approval for the use of the human samples analyzed in this publication was obtained by Dr. Hedlund from the regional ethical review board in Stockholm, Sweden (Regionala Etikprövningsnämnden, Stockholm, EPN, http://www.epn.se/sv/stockholm/om-naemnden/), approval number 2012/111-31/1. All human tissues were obtained from the Netherlands Brain Bank (NBB, www.brainbank.nl) or the National Disease Research Interchange (NDRI, www.ndriresource.org) with the written informed consent from the donors or the next of kin. All procedures involving animals were approved by the Swedish animal ethics review board (Stockholms Norra Djurförsöksetiska nämnd, http://www.jordbruksverket.se/amnesomraden/djur/olikaslagsdjur/forsoksdjur/etiskprovning.html) with ethical approval numbers N352/11, N264/12 and N82/13 (Hedlund).

Mouse tissue processing

Analysis was performed on tissues from 112-day-old (P112) early-symptomatic $SOD1^{G93A}$ fALS mice, which display hindlimb tremors and partial denervation of motor end-plates (Fischer et al., 2004; Schaefer et al., 2005). P112 $SOD1^{G93A}$ mice on a C57Bl/6J background (Gurney, 1994) and age-matched wild-type litter-mates from the same colony, were anesthetized with avertin (tribromoethanol, Sigma) and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains and spinal cords were dissected and postfixed (for 3 and 1 h respectively). All tissues were cryoprotected in sucrose and sectioned (30 μm).

Immunofluorescence analysis of mouse tissues

Tissues were blocked in 10% donkey serum (Jackson Laboratories, West Grove, PA, USA) and 0.1% Triton-X100 (Sigma-Aldrich AB, Stockholm, Sweden) in PBS for 1 h prior to incubation with primary antibodies (Table 2) overnight at 4 °C. This was followed by incubation with Alexafluor secondary antibodies (1:500) for 1 h, counter staining with either Hoechst 33342 or Neurotrace 435 (1:200, Invitrogen) and coverslipping for confocal analysis (Zeiss lsm700). Negative controls were performed for all stainings by omitting either primary or secondary antibodies.

Human tissue processing

Human brain and spinal cord tissues from ALS patients and ND controls were retrieved from the Netherlands Brain Bank (NBB, http://www.brainbank.nl) and the National Disease Research Interchange (NDRI, http://