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Genetic ablation of phospholipase C delta 1 increases survival in SOD1^{G93A} mice

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a devastating progressive neurodegenerative disease, resulting in selective motor neuron degeneration and paralysis. Patients die approximately 3–5 years after diagnosis. Disease pathophysiology is multifactorial, including excitotoxicity, but is not yet fully understood. Genetic analysis has proven fruitful in the past to further understand genes modulating the disease and increase knowledge of disease mechanisms. Here, we revisit a previously performed microsatellite analysis in ALS and focus on another hit, *PLCD1*, encoding phospholipase C delta 1 (PLCδ1), to investigate its role in ALS. PLCδ1 may contribute to excitotoxicity as it increases inositol 1,4,5-trisphosphate (IP_3) formation, which releases calcium from the endoplasmic reticulum through IP_3 receptors. We find that expression of PLCδ1 is increased in ALS mouse spinal cord and in neurons from ALS mice. Furthermore, genetic ablation of this protein in ALS mice significantly increases survival, but does not affect astrogliosis, microgliosis, aggregation or the amount of motor neurons at end stage compared to ALS mice with PLCδ1. Interestingly, genetic ablation of PLCδ1 prevents nuclear shrinkage of motor neurons in ALS mice at end stage. These results indicate that PLCD1 contributes to ALS and that PLCδ1 may be a new target for future studies.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a devastating progressive neurodegenerative disease, which involves the loss of motor neurons and denervation of muscle fibres, resulting in muscle weakness and paralysis. The disease has an annual incidence of 2.7 cases per 100,000 people in Europe (Logroscino et al., 2010) and most patients succumb to the disease within 3 to 5 years of diagnosis. A family history of ALS is present in approximately 10% of cases. Family-based studies have led to the identification of disease-causing mutations in the genes

Abbreviations: ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; C9orf72, chromosome 9 open reading frame 72; DPP6, dipeptidyl-peptidase 6; ELP3, elongator protein 3; ER, endoplasmic reticulum; FUS/TLS, fused in sarcoma/translocated in liposarcoma; GFAP, glial fibrillary acidic protein; ITPR2, inositol 1,4,5-trisphosphate receptor 2; IP_3, inositol 1,4,5-trisphosphate; POAG, primary open-angle glaucoma; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PFN1, profilin 1; PLCD1, phosphoinositide phospholipase C; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SNP, single nucleotide polymorphism; SOD1, superoxide dismutase 1; TARDBP, TAR-DNA binding protein 43; UBQLN2, ubiquilin 2; UNC13a, unc-13 homolog A.

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encoding superoxide dismutase 1 (*SOD1*) (Rosen et al., 1993), TAR-DNA binding protein 43 (*TARDBP*) (Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008), fused in sarcoma/translocated in liposarcoma (*FUS/TLS*) (Kwiatkowski et al., 2009; Vance et al., 2009), chromosome 9 open reading frame 72 (*C9orf72*) (Dejesus-Hernandez et al., 2011; Renton et al., 2011), ubiquilin 2 (*UBQLN2*) and profilin 1 (*PFN1*) (reviewed in Andersen and Al-Chalabi, 2011). Disease-associated gene variants have also been identified in genes encoding elongator protein 3 (*ELP3*) (Simpson et al., 2009), inositol 1,4,5-trisphosphate receptor 2 (*ITPR2*) (van Es et al., 2007), dipeptidyl-peptidase 6 (*DPP6*) (van Es et al., 2008) and unc-13 homolog A (*UNC13a*) (van Es et al., 2009), although the exact role of these genes in ALS is not fully clear.

As disease is indistinguishable between familial and sporadic ALS, it is likely that there are common disease mechanisms involved. Such mechanisms include aggregation, inflammation and mitochondrial dysfunction (reviewed in Bruijn et al., 2004). Additionally, there is evidence for the involvement of excitotoxicity as illustrated by the therapeutic effect of riluzole, currently the only disease-modifying drug available (Miller et al., 2012). Excitotoxicity is the glutamatergic overstimulation of motor neurons leading to neurodegeneration by excessive cytosolic calcium. Calcium influx can originate from the extracellular space through stimulation of calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Cid et al., 2003; Van Damme et al., 2003a). Additionally, calcium can also originate from intracellular stores such as the endoplasmic reticulum (ER) through activation of inositol 1,4,5-trisphosphate (IP_3) receptors located in the ER membrane and that allow calcium to flow from the ER lumen into the cytosol after binding of IP_3. This process may be detrimental in ALS (Staats et al., 2012a).

We have previously reported the results of a microsatellite based genome-wide association study using 1884 markers, in which two pairs of markers, each about 1 Mb apart, were considered appropriate for follow-up. At least one marker of each pair was ranked in the top four results, and all markers were in the top 12 (Simpson et al., 2009). In conjunction with a parallel mutagenesis study in *Drosophila* the study of the first set of markers identified *ELP3* variants on chromosome 8 as associated with ALS (Simpson et al., 2009).

We have now studied the second pair of markers, which suggest *PLCD1* as a candidate gene that modulates ALS. In addition we have assessed the potential role of its gene product in ALS mice by genetic ablation.

Material and methods

Genetic association

Details of the microsatellite association study methods, patient populations and results are given elsewhere (Simpson et al., 2009). In brief, 1884 polymorphic microsatellite markers were typed in three populations comprising 1483 individuals using standard PCR of pooled DNA using fluorescently labelled oligonucleotides followed by analysis of fluorescence intensity patterns after electrophoresis using an Applied Biosystems ABI3100 or 3130XL Genotyper (UK and Belgium) or LiCor Genotyping system (USA) and associated software. Allele image patterns were converted to estimates of allele frequency using an automated method (Simpson et al., 2009). Analysis of frequency estimates was by meta-regression, followed by microsatellite genotyping of individual DNA samples for confirmation of findings, prioritised for adjacent markers showing association in the top 1% of results. SNPs in and around candidate genes were genotyped as part of genome-wide association studies (Shatunov et al., 2010; van Es et al., 2007, 2008, 2009; Wain et al., 2009) and non-genotyped SNP alleles were imputed using MaCH and minimac (Scott et al., 2007) with 1000 genomes V3.20101123. Build 37 (Hg19) of the human genome was used for mapping.

Human material

All experiments on human DNA were approved by the Ethical Committee of University Hospital Leuven and the London–Camberwell St. Giles Research Ethics Committee. We collected samples after we obtained informed consent from all human subjects.

Animal generation and housing

Mice overexpressing human $SOD1^{WT}$ and $SOD1^{G93A}$ were purchased from The Jackson Laboratories (Bar Harbor, USA) and maintained on a C57BL/6 background. PLCδ1 knockout mice were obtained on a mixed 129/Ola × C57BL/6 background and subsequently backcrossed for another 3 generations prior to intercrossing females with $SOD1^{G93A}$ males on a C57BL/6 background to obtain experimental mice. Heterozygous $PLC\delta1^{+/-}$ mice do not phenotypically differ from wild-type ($PLC\delta1^{+/+}$) mice and in line with a previous study (Hirata et al., 2011) we used these mice as controls. Chow and water were provided *ad libitum* and mice were housed in the conventional animal facility of KU Leuven under standard conditions according to the guidelines of the KU Leuven. All animal experiments were performed with the approval of the Animal Ethical Committee of KU Leuven (020/2010).

Behavioural testing

The hanging wire test was used to determine disease onset by assessing the ability of the mice to hold their own weight for 60 s. In brief, the mice are placed on a wire grid and turned over while hanging upside–down. When a mouse fails (drops from the grid before 60 s) and in consecutive trials cannot hold its own weight for 60 s, it is defined as symptomatic. Disease onset determined by the hanging wire test was used to calculate disease duration. Additionally, mice were weighed every 5 days and relative weight was obtained by normalising the absolute weight to the average weight of each mouse between day 90 and 105. For weight curves, mice no longer surviving were assessed as 0 g. End stage was defined as the age at which mice could no longer right themselves within 30 s when placed on their back. End stage is used as a measurement of survival and is the moment when mice are euthanized to prevent further suffering.

Laser capture microscopy

Mice spinal cords were snap-frozen in Tissue-Tec (Sakura Finetek Europe, Alphen aan de Rijn, The Netherlands) to make cryostat sections of 20-μm thickness. Then, cresyl violet–stained motor neurons, located in the ventral horn of the lumbar spinal cord, were collected on membrane slides 1.0 PEN (Carl Zeiss AG, Oberkochen, Germany), using dissection by a laser-capture microscope (Carl Zeiss AG) and capturing in Adhesive Cap 500 opaque (Carl Zeiss AG). Only motor neurons in which the nucleus was visible and with soma area > 250 μm^2, were collected. We dissected at least 1500 motor neurons for each animal.

Quantitative PCR

Isolation of mRNA occurred by the TriPure (Roche, Basel, Switzerland) method and the RNeasy kit (Qiagen, Venlo, The Netherlands). Reverse transcriptase PCR used random hexamers (Life Technologies, Carlsbad, USA) and Moloney Murine Leukaemia Virus Reverse Transcriptase (MMLV RT; Invitrogen, Carlsbad, USA). Quantitative PCR was performed with the StepOnePlus (Life Technologies) and TaqMan Universal PCR Master Mix (Life Technologies). Gene expression assays were purchased from Life Technologies and IDT DNA (Coralville, USA).