Research paper

Amyotrophic lateral sclerosis-associated mutant profilin 1 increases dendritic arborisation and spine formation in primary hippocampal neurons

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HIGHLIGHTS

• Mutant PFN1C71G expression in cultured hippocampal neurons promotes dendrite extension and branching.
• PFN1C71G increases dendritic spine density in cultured hippocampal neurons.
• PFN1C71G forms cytoplasmic inclusions in hippocampal neurons.

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease and familial ALS accounts for 10% of cases. The identification of familial ALS mutations in the actin-binding protein profilin 1 directly implicates actin dynamics and regulation in the pathogenesis of ALS. The mechanism by which these mutations cause ALS is unknown. In this study we show that expression of the ALS-associated actin-binding deficient mutant of PFN1 (PFN1C71G) results in increased dendritic arborisation and spine formation, and cytoplasmic inclusions in cultured mouse hippocampal neurons.

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1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a rapidly progressive neurodegenerative disease affecting both upper and lower motor neurons [16]. Treatments are limited, with the average life span post-diagnosis being 3 years [30]. Although familial forms of the disease only account for approximately 10% of all cases [28], various histopathological hallmarks, such as TDP-43 inclusions, are conserved across familial and sporadic cases [24,33]. Our knowledge of mechanisms in the pathogenesis of ALS are incomplete, which is reflected in the lack of treatment options for the disease.

Pathogenic mutations in the profilin 1 (PFN1) gene were identified in familial ALS [34]. Although it is estimated that PFN1 is only responsible for less than 1% of familial ALS cases [18,28,32], identifying how mutant PFN1 causes ALS will further our understanding of the complexities of the disease and may aid in the development of treatments. Although cytoskeletal dysfunction has previously been suggested to be associated with ALS [3,33], the identification of PFN1 mutations directly implicates the actin cytoskeleton in the pathogenesis of the disease.

PFN1 is a 12–15 kDa ubiquitous actin-binding protein [21]. Its involvement in actin filament polymerization is well characterized and it carries out both an inhibitory and a promoting role in actin filament extension [10]. The actin cytoskeleton is essential for a variety of cellular functions, which include cell division, morphogenesis and motility. Dysfunction in the actin cytoskeleton has been extensively studied in neuronal function and development as well as in neurodegenerative diseases (reviewed in [4,12]).

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Limited functional analysis has been carried out on the ALS-associated PFN1 mutations. It is known that mutant PFN1 has a reduced ability to bind to actin, which results in reduced axonal outgrowth in primary mouse motor neurons, and have an impaired function in stress granules in yeast \[13,34\]. Due to the role that PFN1 plays in actin filament elongation, the hypothesis emerged that ALS-associated PFN1 mutations impact the dynamics of the actin cytoskeleton, which affects neuronal morphology and function; and that these effects contribute to neuronal dysfunction in ALS not only in motor neurons but also central nervous system (CNS) neurons of the hippocampal region.

Here, we studied the impact of the most common ALS-associated PFN1 mutation (C71G) \[34\] on neuronal morphogenesis in mouse primary hippocampal neurons, showing that the expression of PFN1\(^{C71G}\) leads to a specific increase in the length of dendrite extension and arborisation while having no obvious effect of axonal growth.

2. Materials and methods

2.1. Plasmids and cloning

Human PFN1 was amplified from human embryonic kidney (HEK293T) cell cDNA with flanking restriction enzyme sites and an N-terminal V5 epitope tag. The amplified PFN1 insert was ligated into the T-overhangs within the multiple cloning site of the pGEM-T easy vector (Promega), where site directed mutagenesis was performed as previously described (Quickchange Multi SDM kit, Agilent) \[19\] to introduce the C71G mutation. Human and mouse sequences of PFN1 at the C71 site are identical. The inserts were confirmed by sequencing (Macrogen) and sub-cloned into a pLVX-IRES-puro (Clontech) expression vector. mNeonGreen vector was provided by Allele Biotechnology & Pharmaceuticals \[31\].

2.2. Primary neuronal cell culture

All cell culture experiments in this study were carried out with mouse primary hippocampal neurons. Hippocampal neurons were prepared from E16.5 embryos from C57 black 6 mice as described previously \[11\]. In brief, hippocampi were dissected out using ultra-fine micro-dissecting scissors (Fine science tools), enzymatically digested for 20 min at 37 °C with 1x trypsin (Sigma–Aldrich) and triturated using fine-polished glass Pasteur pipettes. Cells were plated in DMEM with 10% FBS at a density of 3.7 × 10⁴ cells/cm² on poly-D-lysine (Sigma–Aldrich) coated glass coverslips (12 mm, #1.0 thickness, glass; Menzel-Glaser, Germany) in 24-well plates (Corning, USA). After 2 h, the medium was changed to Neurobasal medium (Life Technologies) supplemented with 2% (v/v) B27 (Life Technologies) and 0.25% (v/v) Glutamax (Invitrogen). The cells were maintained at 37 °C and 5% CO₂ in a humidified atmosphere.

2.3. Transfection

Primary mouse hippocampal neurons were transfected with V5-PFN1\(^{wt}\) or V5-PFN1\(^{C71G}\) in pLVX-IRES-puro and a control pmaxGFP (Lonza, Switzerland) construct using Lipofectamine® LTX (Life Technologies) at 3 days in vitro (DIV). Before transfection, 500 µL of conditioned medium were set aside. The lipid complex was prepared by adding 200 ng of plasmid and 0.4 µL of Lipofectamine® LTX to 100 µL of Neurobasal medium and briefly vortexing. The complex was incubated at RT for 25 min then 100 µL were added to the culture. Medium change with conditioned medium was completed after 60 min incubation (Table 1).

Double-transfections with mNeonGreen protein as transfection reporter and either no pLVX, V5-PFN1\(^{wt}\) or V5-PFN1\(^{C71G}\) in pLVX-IRES-puro were performed in a similar manner except 66.7 ng of each plasmid were added.

2.4. Antibodies

Antibodies used for immunocytochemistry were as follows: mouse anti-V5 (1:200, life sciences), chicken anti-β3 tubulin (1:500, Millipore), rabbit anti-TDP 43 (1:400, Proteintech), donkey anti-mouse Alexa Fluor-488 (1:500, Invitrogen), donkey anti-rabbit Alexa Fluor-488 (1:500, Invitrogen), donkey anti-mouse Alexa Fluor-555 (1:500, Invitrogen), goat anti-chicken Alexa Fluor-555 (1:500, Invitrogen) and donkey anti-mouse Alexa Fluor-647 (1:500, Invitrogen).

2.5. Immunocytochemistry

Cells were fixed for 20 min with 4% paraformaldehyde (ProSciTech, Australia) at 5 DIV for the single transfection or at 19 DIV for the double-transfection. Fixed cells were permeabilized with 0.1% Triton X-100 for 5 min, and blocked in 2% PBS diluted in PBS for 30 min. Cells were incubated for 1 h at RT with primary antibodies diluted in blocking solution (PBS with 2% FBS) followed by 40 min incubation with secondary antibodies, diluted in blocking solution. The coverslips were mounted onto glass slides (Livingstone, Australia) with Prolong Gold antifade mounting medium with DAPI (Life Technologies).

2.6. Imaging

Stained coverslips were imaged on a BX51 microscope (Olympus, Japan) with a 40× and 100× objective with the Neurolucida (MBF Bioscience, USA) program. Morphometric analysis of neurons was performed on Neurolucida with AutoNeuron interactive and manual neuron tracing. Five neurons per condition across 3 independent sets were analysed. The majority of the neurons in the culture (>90%) and the cells that were analysed in this study, were multipolar with 3–8 dendrites. The data were exported to Excel (Microsoft, USA) using Neurolucida Explorer.

Immunofluorescence images of dendritic spines were acquired with a FluoView FV1000 confocal microscope (Olympus, USA) using the UplanSApo 60 × 1.35 NA oil immersion objective. Z-stacks were taken at 0.4 µm intervals with four images taken per cell. The number of spines in 20 µm long segments of dendrites at a distance of 50 µm from the cell body was analysed. Spine number was analysed using Image J (Version 1.47).

2.7. Statistical analysis

Data was processed in Excel and analyzed in GraphPad Prism (v.6.00, GraphPad Software, USA). Outliers were removed using ROUT analysis. One way ANOVA with multiple comparisons were used on parametric data. All values are presented as mean ± standard error.

3. Results

3.1. PFN1\(^{C71G}\) increases dendritic arborisation of hippocampal neurons

To investigate the effects of PFN1 on neuronal development, we first generated expression constructs encoding V5-tagged wild-type (wt) and C71G mutant human PFN1. Hippocampal neurons were transfected with V5-PFN1\(^{wt}\), V5-PFN1\(^{C71G}\) or a GFP control vector at 3 DIV, fixed at 5 DIV and then immuno-stained with antibodies directed against the V5-tag and β3-tubulin (Fig 1A-F). V5-PFN1\(^{wt}\) and V5-PFN1\(^{C71G}\) localised to both the axonal and
Table 1
Morphological changes in V5-PFN1<sup>C71G</sup> and V5-PFN1<sup>wt</sup> expressing hippocampal neurons.

<table>
<thead>
<tr>
<th></th>
<th>V5-PFN1&lt;sup&gt;C71G&lt;/sup&gt;</th>
<th>V5-PFN1&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>Control</th>
<th>p-Value compared to control</th>
<th>V5-PFN1&lt;sup&gt;C71G&lt;/sup&gt;</th>
<th>V5-PFN1&lt;sup&gt;wt&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dendritic length [μm]</td>
<td>199.3 ± 32.9</td>
<td>113.6 ± 4.0</td>
<td>81.9 ± 15</td>
<td>0.013&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.503</td>
<td></td>
</tr>
<tr>
<td>Number of branches per dendrite</td>
<td>5.04 ± 0.67</td>
<td>1.85 ± 0.38</td>
<td>1.68 ± 0.11</td>
<td>0.001&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.647</td>
<td></td>
</tr>
<tr>
<td>Axonal length [μm]</td>
<td>1206 ± 196.50</td>
<td>873.3 ± 88.65</td>
<td>1200 ± 50.27</td>
<td>0.999</td>
<td>0.201</td>
<td></td>
</tr>
<tr>
<td>Number of branches per axon</td>
<td>25.17 ± 3.38</td>
<td>17.33 ± 2.46</td>
<td>25.38 ± 3.14</td>
<td>0.999</td>
<td>0.423</td>
<td></td>
</tr>
<tr>
<td>Number of spines per 20 μm dendrite segment</td>
<td>13.11 ± 0.22</td>
<td>9.98 ± 0.55</td>
<td>9.02 ± 0.09</td>
<td>0.002&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.317</td>
<td></td>
</tr>
<tr>
<td>Percent of V5-positive aggregates</td>
<td>3.47 ± 1.5</td>
<td>0</td>
<td>N/A</td>
<td>p-Value, V5-PFN1&lt;sup&gt;C71G&lt;/sup&gt; compared to V5-PFN1&lt;sup&gt;wt&lt;/sup&gt;</td>
<td>0.034&lt;sup&gt;†&lt;/sup&gt;</td>
<td></td>
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</tbody>
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<sup>†</sup> p ≤ 0.05.
<sup>**</sup> p ≤ 0.01.
<sup>***</sup> p ≤ 0.001.

Fig. 1. The C71G mutation in PFN1 promotes dendritic arborisation. Hippocampal neurons expressing GFP control, V5-PFN1<sup>wt</sup> or V5-PFN1<sup>C71G</sup> (A–F) were analysed morphometrically at 5 days in vitro and dendritic and axonal extension and branching were quantified (G–J). Note the significant increase in dendritic branching and mean dendrite length in neurons expressing PFN1<sup>C71G</sup> as compared to mNeonGreen control and PFN1<sup>wt</sup> expressing neurons. * p ≤ 0.05. Scale bars = 20 μm.

3.2. PFN1<sup>C71G</sup> increases dendritic spine density in hippocampal neurons

As PFN1<sup>C71G</sup> had a dendrite-specific effect on hippocampal morphology in developing neurons, we next looked at the effect of V5-PFN1<sup>wt</sup> and V5-PFN1<sup>C71G</sup> expression in long-term cultures of hippocampal neurons. Neurons were transfected at 3 DIV with mNeonGreen with or without co-transfection of V5-PFN1<sup>wt</sup> or V5-PFN1<sup>C71G</sup>. mNeonGreen was used to visualise the entire morphology of transfected neurons, including the dendritic spine compartments. Cells were fixed at 19 DIV and immuno-stained with an antibody directed against the V5-tag (Fig 2A–F). Spine morphology was predominantly mature with no overt differences in the proportion of mature and filopodial spines between the different conditions. Quantification of dendritic spines showed significantly higher numbers of dendritic spines in hippocampal neurons, expressing V5-PFN1<sup>C71G</sup> (13.1 ± 0.2 spines per 20 μm of dendrite segment) as compared to neurons expressing V5-PFN1<sup>wt</sup> (10 ± 0.6 spines per 20 μm of dendrite segment) or control neurons (9 ± 0.1 spines per 20 μm of dendrite segment) (Fig 2G). No statistically significant difference was found between cells expressing V5-PFN1<sup>wt</sup> and control neurons, indicating that the effect on spine density is linked to the C71G mutation in PFN1.

3.3. PFN1<sup>C71G</sup> expression induces PFN1 aggregation in hippocampal neurons

Insoluble protein aggregates are a hallmark of neurodegenerative diseases. Wu et al. showed aggregates of PFN1<sup>C71G</sup> in N2a neuroblastoma cells and primary motor neurons treated with MG132 [34]. To determine whether the PFN1 mutations could cause cytoplasmic aggregates in CNS neurons, hippocampal neurons were transfected at 3 DIV, fixed at 4 or 5 DIV and then stained with V5 and β3-tubulin antibodies. Cells transfected with V5-PFN1<sup>C71G</sup> showed sporadic aggregation (3.5 ± 1.5% of all transfected cells analysed; n = 214) (Fig. 3). Staining with antibodies specific for TDP-43...
Fig. 2. The C71G mutation in PFN1 leads to increased dendritic spine density. Hippocampal neurons expressing mNeeonGreen alone, or together with V5-PFN1wt or V5-PFN1C71G (A–F) were analysed at 19 days in vitro for dendritic spine density. Dendritic spine density is significantly increased in neurons expressing as compared to GFP control and PFN1wt expressing neurons (G). Higher magnification of dendrites visualized with mNeeonGreen (B, D, F) * p ≤ 0.05. Scale bars = 20 μm.

show no TDP-43 immunoreactivity of the aggregates or cytoplasmic localization of TDP43 (Fig. S1). No aggregates were found in neurons transfected with V5-PFN1wt (n = 468) (Fig. 3).

4. Discussion

Pathogenic mutations in PFN1 were identified in familial ALS in 2012 [34]. Wu and colleagues have found that mutant PFN1C71G has reduced actin-binding in co-immunoprecipitation experiments [34]. A subsequent study [7] used an actin polymerisation assay to show that the mutant PFN1 had the same inhibitory effect as PFN1wt in high concentrations, which highlights the need for further studies into the impact of the disease-associated PFN1 mutation and the functional consequence on actin dynamics. Mutant PFN1C71G has also an increased susceptibility for aggregation [32]. The impact of PFN1 mutations on different CNS neurons, however, remains
unclear. In the current study we analysed the effect of the most common mutation in PFN1, C71G, on neuronal structure in mouse primary neurons from the hippocampal region. The hippocampal formation is impacted at late stages in ALS pathology [1,5–7,24].

We show that expression of PFN1C71G results in a significant increase of dendrite length and branching. We observed no changes in axon length or branching. Hence, expression of PFN1C71G has a compartment specific effect on neuronal morphology. Neurite outgrowth is highly dependent on the regulation of the dynamics of the actin cytoskeleton and actin-associated proteins can regulate axonal and dendritic outgrowth in a compartment specific manner (for a review, see [15]). Therefore, it is not surprising that inhibition of profilin/actin interaction and thereby modulation of actin dynamics impacts on neurite formation. However, our results are in contrast to the findings by Wu et al., who showed attenuated axonal length in cultured motor neurons in response to PFN1C71G expression [34]. Furthermore, no phenotypic changes of the somato-dendritic compartment were reported in this study [34]. A possible explanation for the lack of a somato-dendritic phenotype in Wu’s study is the early time point of analysis (3DIV) at which dendrites are poorly developed. Alternatively, the differences in phenotype reported may represent a neuronal cell type specific effect of mutations in PFN1. An in vivo study in SOD1G85R mice by [14], showed an increase in dendritic branching in lower motor neurons, demonstrating that mutations in other familial ALS-associated genes can also result in a somato-dendritic phenotype.

Concomitant with the increased dendritic arborisation, we found an increase in the density of dendritic spines in mature cultures of hippocampal neurons. Proper control of actin filament dynamics is critical for dendritic spine stability and function [17]. Previous work has implicated function of the related profilin 2 in the stabilisation of dendritic spines in hippocampal neurons [2]. Accordingly, using a vector-based RNAi approach for knockdown of PFN2a in hippocampal slice cultures showed reduced dendrite complexity and spine numbers of CA1 neurons [23], which was compensated by PFN1 overexpression. However, spine morphology and synaptic plasticity were not affected in a study using profilin 1 deficient mice. These data suggest a functional role of profilin 1 at the synapse with partial overlap in the function of profilin 1 and profilin 2. While profilin 1 primary function is to promote actin filament assembly, it can inhibit the activity of the actin nucleating complex Arp2/3 [29]. The ability of profilin1 to inhibit Arp2/3 depends on its ability to bind to actin monomers. PFN1 interacts with the proline-rich motifs of Ena/VASP and formin and the C71G mutation in the actin-binding region of PFN1 is not expected to affect these interactions. Therefore, an increase in Arp2/3 activity could account for the significant increase in dendritic arborisation and spine density observed in hippocampal neurons expressing PFN1C71G. Without the inhibition of Arp2/3, dendritic arborisation could increase by de novo branch formation along the shaft of the developing dendrites. Also, Arp2/3 is an integral component of branched actin filament populations in the dendritic spine compartment, in particular near the post-synaptic density [20], which could account for the increased spine density in cells expressing PFN1C71G. Changes in the spine density, and therefore synaptic connectivity, could lead to altered excitability of neurons. While hippocampal excitability has not been specifically studied in ALS, altered cortical and motor neuron excitability has been reported in mouse models of ALS and human ALS cases [22,25].

In the current study, we show for the first time PFN1C71G aggregates in cultured hippocampal neurons (Fig. 3). Protein aggregations are a histopathological hallmark of neurodegenerative disease and protein aggregation in motor neurons is found in several mouse models of ALS [9,33]. Although ALS is characterised by a loss of upper and lower motor neurons, extra-motor neuron areas are being increasingly implicated in ALS. Hippocampus-associated learning and microcircuitry deficits have been found in pre-symptomatic SOD1 mouse models of ALS [26]; and diffusion tensor MRIs on ALS patients has revealed damage to hippocampal microstructure [5]. An association of a reduction of hippocampal grey matter and memory performance in non-FTD ALS patients implicates the hippocampus in cognitive dysfunction in late stages of disease [27]. Furthermore, as the disease progresses, pathology becomes widespread involving regions such as the cerebellum and hippocampus [1,6]. For example, phosphorylated TDP-43-positive lesions have been found in the hippocampus of late stages of ALS [8]. The presence of PFN1C71G positive aggregations in cultured hippocampal neurons is therefore of particular interest and will need to be further investigated in an in vivo model of ALS.

Taken together, our study has shown new data of an ALS-associated PFN1 mutation in hippocampal neurons. ALS is a complex neurodegenerative disease and a more complete picture of the pathomechanisms involved will advance the search for much needed therapies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2015.09.034.


