DYNC1H1 mutation alters transport kinetics and ERK1/2-cFos signalling in a mouse model of distal spinal muscular atrophy

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Mutations in the gene encoding the heavy chain subunit (DYNC1H1) of cytoplasmic dynein cause spinal muscular atrophy with lower extremity predominance, Charcot–Marie–Tooth disease and intellectual disability. We used the legs at odd angles (Loa) (DYNC1H1⁵⁸⁰Y⁵⁸⁰) mouse model for spinal muscular atrophy with lower extremity predominance and a combination of live-cell imaging and biochemical assays to show that the velocity of dynein-dependent microtubule minus-end (towards the nucleus) movement of EGF and BDNF induced signalling endosomes is significantly reduced in Loa embryonic fibroblasts and motor neurons. At the same time, the number of the plus-end (towards the cell periphery) moving endosomes is increased in the mutant cells. As a result, the extracellular signal-regulated kinases (ERK) 1/2 activation and c-Fos expression are altered in both mutant cell types, but the motor neurons exhibit a strikingly abnormal ERK1/2 and c-Fos response to serum-starvation induced stress. These data highlight the cell-type specific ERK1/2 response as a possible contributory factor in the neuropathological nature of Dync1h1 mutations, despite generic aberrant kinetics in both cell types, providing an explanation for how mutations in the ubiquitously expressed DYNC1H1 cause neuron-specific disease.

Keywords: cytoplasmic dynein; endosomes; Loa; motor neurons; ERK 1/2

Abbreviations: ERK1/2 = extracellular signal-regulated kinases 1/2; Loa = Legs at odd angles; MAP kinase = mitogen activated protein kinase; MEF = mouse embryonic fibroblast

Introduction

Cytoplasmic dynein is a 1.2 MDa complex responsible for the majority of minus-end (also referred to as retrograde in axons) directed microtubule-dependent trafficking within cells. At the core of the complex is a DYNC1H1 heavy chain homodimer. The C-terminus of each dynein heavy chain is the site of ATP hydrolysis, essential for powering the complex along microtubules, which also bind at this end. Amino acids 446–800 in the N-terminus (residues 1 to ~1400), associate with dynein intermediate and light intermediate chains (Tynan et al., 2000), plus accessory and regulatory proteins including dynactin (reviewed in Vallee
et al., 2012; Carter, 2013; Kikkawa, 2013; Schiavo et al., 2013). These components are required for dynein function and/or help confer specificity for cargo, including organelles such as endosomes, which have vital roles in responding to external stimuli, environmental fluctuations, stressors and implementing general housekeeping functions.

Mutations within dynein heavy chain are causative for spinal muscular atrophy with lower extremity predominance (SMA-LED), Charcot–Marie–Tooth disease type 2 (CMT2), microcephaly, and intellectual disability (reviewed in Schiavo et al., 2013). Moreover, mutations in the p150 subunit of dynactin have been linked to motor neuron disease (Puls et al., 2003, 2005; Munch et al., 2004). The severity of these diseases highlights the importance of cytoplasmic dynein in cellular function; however, the overlapping symptoms of many of the aforementioned diseases poses the question of why there is a disproportionate impact on neuronal cells. To address this question we compared two distinct cell types: mouse embryonic fibroblasts (MEFs); and motor neurons, both derived from the legs at odd angles (Loa) mouse.

Loa mice carry a F580Y mutation within the dynein heavy chain. DynCh1 c^Loa/Loa^ (from here on referred to as ^Loa/Loa^) mice have severe loss of spinal anterior horn motor neurons (Hafezparast et al., 2003), impairment of cortical lamination (Ori-McKenney and Vallee, 2011) and die perinatally, whereas DynCh1^+/Loa^ (referred to as ^+/Loa^) manifest an age-related neuromuscular deficit and sensory neuropathy (Hafezparast et al., 2003; Chen et al., 2007; Ilieva et al., 2008; Wiggins et al., 2012). At the molecular level, the Loa mutation has been shown to adversely affect dynein complex assembly (Deng et al., 2010) and processivity (Ori-McKenney et al., 2010).

Motor neurons rely on trophic signalling mediated by TrkB (tropomyosin-related kinase B)—a member of the receptor tyrosine kinase (RTK) superfamily—for survival. Upon binding of brain-derived neurotrophic factor (BDNF) to TrkB, the receptor is activated by dimerization and autophosphorylation. The catalytically active BDNF-TrkB complexes are then endocytosed and the progression of these ‘signalling endosomes’ along the endocytic pathway for signalling in the soma, followed by degradation in the lysosomes requires the activity of cytoplasmic dynein (Yano et al., 2001; Bhattacharyya et al., 2002; Heerssen et al., 2004). Similar to the BDNF-TrkB trafficking in motor neurons, activation of epidermal growth factor receptor (EGFR) by EGF triggers the endocytosis of EGF-EGFR complexes and their targeting to late endosomes/lysosomes in a dynein-dependent process in MEFs (Bonfaccino and Traub, 2003).

The function of the signalling endosomes is particularly important in neuronal cells in which receptor-ligand complexes undergo long range translocation from nerve termini to the cell body using the dynein-dependent retrograde transport machinery. Activation of the BDNF or EGFR receptors initiates a transient signalling cascade involving Ras and Raf upstream of extracellular signal-regulated kinases (ERK) 1/2. On the other hand, sustained activation of ERK1/2 involves CT10 regulator of kinase (CRK) adaptor proteins and the small GTP protein RAP1 (Nguyen et al., 1993). BDNF also stimulates additional intracellular signalling cascades including phospholipase Cγ (PLCγ) and phosphoinositide 3-kinase (PI3K) (reviewed in Harrington and Ginty, 2013).

Moreover, transient and sustained activation of ERK1/2 in neuronal cells have been shown to alter their morphological characteristics and cellular differentiation (Tanaka et al., 1993; Ji et al., 2010). Downstream of ERK1/2, transcription of the immediate early gene c-Fos can be promoted through ELK1. c-Fos dimerizes with c-Jun to form the AP1 complex to initiate transcription of genes important for cell survival, growth, proliferation and differentiation. Owing to the location of the Loa mutation, it would be reasonable to predict that a delayed degradation of the signal due to an impairment of receptor-ligand complex trafficking to the lysosomes would likely lead to prolonged activation of ERK1/2 and an increase in immediate early gene expression.

Thus, to explore the cell-type specific effects of the Loa mutation, we specifically looked at the endocytic trafficking of BDNF and EGF and their associated ERK1/2 signalling (Burke et al., 2001; Howe et al., 2001; Howe and Mobsley, 2004; Driskell et al., 2007; Taub et al., 2007; Schuster et al., 2011). Our data show generic aberrations in transport kinetics in two distinct cell types MEFs and motor neurons, both of which harbour the Loa mutation. Endosomes in Loa cells display reduced minus-end directed velocity and increased plus-end directed movements. The Loa phenotype is associated with increased numbers of side-steps that are both slower and smaller than in wild-type cells. We also show cell-specific alterations in ERK1/2 and the immediate early gene c-Fos expression. Specifically, Loa/Loa MEFs are not affected by serum deprivation, as measured by ERK1/2 levels in comparison with wild-type cells, and in response to EGF stimulation ERK1/2 activation is sustained. In contrast, Loa motor neurons are markedly more affected by nutrient deprivation than their wild-type controls, as shown by increased levels of phosphorylated ERK1/2 (pERK1/2) and c-Fos. We suggest that these cell-type specific responses are contributing mechanisms resulting in the DYNCH1 neuropathogenesis that arise from mutations in this ubiquitously expressed protein.

Materials and methods

Cell culture

MEFs were cultured in Dulbecco’s modified Eagles medium supplemented with 15% HyClone™ foetal bovine serum (Fisher), 1% penicillin/streptomycin and 1% l-glutamine. They were cultured at 37°C with 3% O2 and 5% CO2. Starvation media consisted of the same without the addition of serum. EGF stimulation (20ng/ml) was applied as a continuous stimulation for biochemical analysis. A 10-min pulse of EGF-Alexa Fluor® 546 (Molecular Probes) was used for live-cell imaging, or EGF-Alexa Fluor® 555 (Invitrogen) for fixed-cell imaging.

Embryonic Day 13 motor neurons were dissected and cultured based on an established protocol (Camu and Henderson, 1994) with minor modifications; motor neuron culture media (Neurobasal™ media plus B27 supplement, 0.25% glutamine, 0.1% β-mercaptoethanol, 2% horse serum, 0.1% fungizone, 1% penicillin/streptomycin) were supplemented with 0.1ng/ml GDNF (R&D systems), 0.5ng/ml CNTF and 0.1ng/ml BDNF (Invitrogen). Cells were incubated at 37°C in 5% CO2. During 2-h serum starvation, cells were incubated in motor neuron culture medium lacking horse serum.
For live-cell imaging motor neurons or MEFs were rinsed and replenished with warmed motor neuron or MEF starvation medium, respectively; however, the Neurobasal® medium or Dulbecco’s modified Eagles medium was replaced in both instances with Leibovitz L-15 CO₂-independent medium. Imaging was undertaken at 37°C. BDNF at a concentration of 5 ng/ml was used for motor neuron stimulation and this was applied either as a 10-min ‘pulse’ for live-cell imaging or left as ‘continuous’ stimulation for cell signalling assays. pHrodo™ and this was applied either as a 10-min ‘pulse’ for live-cell imaging a concentration of 5 ng/ml was used for motor neuron stimulation to be included in the analysis. Live cell images were captured/C213 were mounted using ProLong™/C11 albumin and then stained with anti-

with 0.1% Triton™ X-100, blocked for 60 min in 2% bovine serum

ANOVA analysis. Results

Aberrant transport kinetics in Loa/Loa mouse embryonic fibroblasts

To examine the impact of the Loa mutation on growth factor transport and degradation, we serum starved wild-type and Loa/Loa MEFs for 2 h before stimulation with 20 ng/ml EGF-Alexa Fluor® 555 for 10 min. MEFs were then rinsed and fixed for imaging after 0-, 10-, 30- and 60-min chase. To ensure differences were not a reflection of cell size (Rishal et al., 2012), the analysed areas of 37 cells from each genotype were compared. No significant difference was found (wild-type = 2057 ± 89.3 μm² and Loa/Loa = 1992 ± 86.7 μm²) (Fig. 1C).

At 0-min chase both wild-type and Loa/Loa had similar numbers of EGF-containing endosomes: 746.9 ± 103.8 and 781.9 ± 74.9, respectively (Fig. 1A and B). However by 10-min chase there were significantly more EGF-AlexaFluor® 555 containing vesicles remaining in Loa/Loa when compared to wild-type cells (wild-type = 454.6 ± 34.6, Loa/Loa = 753.5 ± 71.2, P = 0.0014).

By 30-min a distinct reduction in particle numbers in both genotypes was apparent, although, quantification remained higher in Loa/Loa than in wild-type (289.5 ± 29.3 and 231.529.3 ± 26.9, respectively). The 60-min chase highlighted significance once again with wild-type particles having reduced to 108 ± 10.1 and Loa/Loa to only 148.1 ± 9.9 (P = 0.0152).

Due to the prominent difference in EGF-containing vesicles at 10 min we sought to distinguish between delayed convergence of early endosomes and a delay in EGF degradation. We then plotted the probability distributions of EGF-positive vesicles as a function of the area of EGF-containing organelles. In wild-type MEFs endosome size was greater than in Loa/Loa at both 0- and 30-min chase; however, by 60-min this difference was less marked (Fig. 1D). As early endosomes increase in size during fusion events leading to their maturation, these data suggest that the Loa mutation delays the progression of endocytosed EGF along the endocytic pathway.

To gain further insight into endosomal movement, live-cell imaging was carried out. We used an assay similar to that shown in Fig. 1, but instead of fixing the cells, they were rinsed, left in CO₂-independent media at 37°C and chased for 10 and 30 min (Fig. 2A–D). Minus-end directed transport velocity is conventionally measured as positive movements towards the nucleus whereas plus-end directed transport velocity is represented as negative.

Initial observations of the minus-end directed movements identified a distinct lack of fast carriers moving at velocities above 2 μm/s in Loa/Loa, and 60% less moving at velocities above 1 μm/s at both time points (Fig. 2C). In both genotypes, the majority of displacements occurred at speeds below 0.25 μm/s.

Image acquisition and analysis

For EGF particle assays, fixed-cell images were obtained using FITC, Cy5 and DAPI filters. Deconvolved images were analysed with the aid of standard ImageJ programs. Probability distribution frequencies were plotted by measuring EGF-containing vesicle size in pixels and converting this to μm² from the pixels:microns microscopy image scale. Live-cell images were collected at 10 min and 30 min post-removal of growth factor and tracks required an overall net retrograde migration to be included in the analysis. Live cell images were captured every 2 s for 1 min duration in MEFs and every 3 s for 2 min in motor neurons, using a Delta Vision microscope (Applied Precision). The ImageJ ‘Manual Tracking’ plugin (Fabrice P. Cordelieres) was used to gather track information. Both live cell imaging and fixed cell assays were analysed with the assistance of Excel and GraphPad Prism using the Mann-Whitney U-test. Kymographs were produced using software from the MacBiophotonics ImageJ for microscopy users.
Figure 1  EGF-Alexa Fluor® 555 processing is altered in Loa MEFs. (A) Representative images of EGF-Alexa Fluor® 555 in wild-type and Loa/Loa MEFs after a 10-min pulse. Images taken at 0-, 10-, 30- and 60-min chase show increased numbers of EGF-containing vesicles in Loa/Loa compared with wild-type MEFs over time. Scale bar = 20 μm. (B) Quantification of the number of EGF-Alexa Fluor® 555 vesicles remaining in MEFs at each chase time (asterisks represent P-values of $P = 0.001$ at 10 min and 0.015 at 60 min, $n = 15$). (C) Average area analysed for vesicle counting was the same in wild-type and Loa/Loa MEFs, $n = 37$. (D) Histograms depicting a probability distribution frequency (PDF) of area of vesicles containing labelled EGF. After EGF stimulation, Loa/Loa MEFs show a delay in forming large vesicles.
Figure 2  Endosome trafficking is aberrant in Loa. (A) Representative tracks from wild-type and Loa/Loa MEFs. Arrowheads and asterisks indicate the tracked endosomes and nuclei, respectively. As shown in the magnified insets, the Loa/Loa path seems shorter and more ambiguous than in wild-type. (B) Representative track displacements in wild-type and Loa/Loa at 10- and 30-min chase. (C) The velocity of dynein-dependent minus-end directed transport is reduced in Loa/Loa at 10 (P < 0.0001) and 30 min (P < 0.007) chase. Plus-end directed transport velocity is not significantly different across the genotypes. (D) Overall track length is reduced in Loa/Loa compared with wild-type at 10- and 30-min chase (P = 0.002 at 10 min and P = 0.044 at 30 min). Data analysed are from sample sizes of n = 349 movements for wild-type from 13 tracks (four cells) and 419 movements from 15 tracks (five cells), for Loa/Loa, at 10 min. At 30 min; n = 232 movements for wild-type from eight tracks (five cells) and 337 movements for Loa/Loa from 12 tracks (six cells). (E) Sideward velocity and distance travelled are reduced in Loa/Loa compared with wild-type (P = 0.0003 for both assays). For sideward speed and distance analysis, measurements from 10-min and 30-min chase were pooled together. Asterisks represent the indicated P-values.
This was not surprising as many endosomes remain static for several seconds before longer movements were made.

Analysis of dynein-dependent minus-end directed transport velocities highlighted a significant shift towards slower velocity in Loa/Loa at 10- and 30-min chase (Fig. 2C). The minus-end directed movements were significantly slower in Loa/Loa compared with that in wild-type MEFs at both 10 min (medians of 0.11 μm/s and 0.17 μm/s, respectively; $P < 0.0001$) and 30 min (medians of 0.07 μm/s and 0.11 μm/s, respectively; $P < 0.007$). Plus-end directed transport velocities were not significantly different across the time points or genotypes (Fig. 2C).

To determine whether the altered dynamics of Loa/Loa velocities compared with wild-type was sufficient to affect the overall displacement, all tracks (76% of total tracks measured) that remained in focus for at least 96% of frames were analysed. Displacement in Loa/Loa was significantly reduced when compared to wild-type at 10-min chase ($P = 0.002$) (Fig. 2D). Track length ranged from 9.2 μm to 15.8 μm (median 13.4 μm) in wild-type and 0.90 μm to 12.2 μm (median 4.4 μm) in Loa/Loa. This trend remained at 30 min, with wild-type total displacement ranging from 5.1 μm to 42.3 μm (median 8.2 μm) and 2.2 μm to 9.2 μm (median 5.3 μm) in Loa/Loa.

In addition, we found movements that were neither minus-end nor plus-end directed, but sideward relative to the main travelling direction of the endosome. Approximately 5% of wild-type movements across both chase points were classed as side steps, whereas in Loa/Loa this number was increased to ~7%. When measuring the velocity and distance of these sideward movements it was found that in Loa/Loa the velocity was significantly slower [means of 0.45 (±0.1 SEM) μm/s and 0.21 (±0.02 SEM) μm/s for wild-type and Loa/Loa, respectively, $P = 0.0003$] and movement was over shorter distances [0.90 (±0.2 SEM) μm and 0.42 (±0.5 SEM) μm for wild-type and Loa/Loa, respectively ($P = 0.0003$)] (Fig. 2E). These might represent the off-axis movements of dynein, observed by several studies (Reck-Peterson et al., 2006; DeWitt et al., 2012; Qiu et al., 2012), in which dynein uses its flexibility to step on neighbouring protofilaments and microtubules for navigating through a crowded cytoplasm. The observed slower sideward speed and smaller distance travelled by dynein in Loa MEFs might reflect a reduced flexibility of dynein to reach neighbouring microtubules, caused by impaired dynein complex assembly and binding to dynactin (Deng et al., 2010).

Together these data show aberrant transport kinetics in Loa/Loa MEFs, which may delay maturation of early endosomes into late endosomes and multivesicular bodies.

**Transport kinetics are impaired in +/Loa motor neurons**

As cytoplasmic dynein mutations have been linked to several neurological diseases (Puls et al., 2003, 2005; Munch et al., 2004; Schiavo et al., 2013), it was important to assess transport parameters in neuronal cells. Although deficits in both motor and sensory neurons have been identified in the Loa mouse, we chose to examine intracellular transport kinetics within motor neurons, to advance our earlier work (Hafezparast et al., 2003), which identified impairments in axonal retrograde transport in this cell type.

Embryonic motor neurons were dissected at embryonic Day 13 and maintained in culture for 1 week. The motor neurons isolated from Loa embryos looked indistinguishable from those derived from embryos expressing green fluorescent protein (GFP) under control of the motor neuron specific transcription factor, homeobox gene 9 (Hb9). Only cells with large soma, single axon and complex dendritic arborization were analysed.

Because of the dominant nature of dynein mutations in human pathologies (Schiavo et al., 2013), we decided that +/Loa motor neurons would be more representative of human disease than Loa/Loa and thus carried out live-cell imaging comparisons between wild-type and +/Loa.

For live-cell tracking in motor neuron axons, and in the absence of a fluorophore-conjugated BDNF, we starved the motor neurons for 2 h and stimulated them with native BDNF to induce activation of its receptor TrkB and increase the BDNF-TrkB-containing endosomal pool. We then used the pH-sensitive fluorescent dye pHrodo™TM to detect the endosomes and analyse the global axonal retrograde transport of endocytic and phagocytic organelles in these cells. The majority of tracking was from DIC (differential interference contrast) images of axons in which vesicles >500 nm were clearly visible, many co-located with pHrodo™ fluorescent.

The range of velocities observed in motor neuron tracking experiments were similar to those seen in MEFs, from ~1.36 μm/s (plus-end directed, or anterograde) to ~2 μm/s (minus-end directed or retrograde) and were in agreement with those observed in our previous study (Kieran et al., 2005). There were also fewer endosomes moving at speeds in excess of 1 μm/s in +/Loa compared to wild-type at both 10- and 30-min chase (30% less at 10 min and 70% less at 30 min) (Fig. 3A and B).

Minus-end directed movements identified a trend for slower velocities in +/Loa at 10 min, which reached significance at 30-min chase ($P = 0.01$) (Fig. 3B). Moreover, the velocity of minus-end directed movements decreased in +/Loa between 10 and 30-min chase ($P = 0.0001$). Interestingly, although plus-end directed velocity was not significantly different between the genotypes at 10-min chase, there was an increased propensity towards movements in this direction in +/Loa compared to wild-type motor neurons, which had a distinct lack of such movements during the 30-min chase (Fig. 3B). Upper and lower ranges of track displacement in +/Loa compared to wild-type were distinctly different at 10 min (20.4 μm to 61.0 μm in wild-type and 4.2 μm to 55.4 μm in +/Loa) and at 30-min chase (14.1 μm to 42.2 μm for wild-type and 7.6 μm to 33 μm for +/Loa) (Fig. 3C). These differences, however, were not statistically significant ($P = 0.2$ and 0.9, respectively).

Taken together, these data show that the Loa mutation causes a significant impairment to endosomal axonal transport, determining not only a reduction in minus-end directed velocity and overall track length, but also a tendency towards increased plus-end movements.
Prolonged ERK1/2 activation and increased c-Fos in Loa/Loa mouse embryonic fibroblasts

We next investigated the effect of the delayed trafficking of the EGF-containing signalling endosomes on ERK1/2, a well-established downstream effector of EGF-initiated signalling. Wild-type and Loa/Loa MEFs were serum starved for 2 h before stimulation with 20 ng/ml EGF. Cells were lysed at 10, 30, 60, 120, and 180 min after stimulation.

A peak of phosphorylated ERK1/2 (pERK1/2) was apparent at 10 min post-stimulation. This activation was transient and by 60 min the wild-type levels of pERK1/2 were nearing those of the serum-starved control (Fig. 4A and B). In contrast, pERK1/2 activation was found to be more sustained in Loa/Loa such that at 30 min post-stimulation there was an increase in pERK1/2. At 60, 120, and 180 min after stimulation, the fold increases were 1.4, 1.2, and 5.3, respectively, when compared to wild-type (Fig. 4A and B). Two-way ANOVA analysis of these
data revealed that EGF had a significant effect over all the time points for both genotypes $F(5,24) = 61.11$, mean square $= 19.35$, $P < 0.0001$, and although there was a trend in higher levels of pERK1/2 in Loa/Loa MEFs, its overall effect did not reach significance $F(1,24) = 0.13$, mean square $= 0.04$, $P = 0.73$. There was no significant interaction between the genotypes and time $F(5,24) = 2.12$, mean square $= 0.67$, $P = 0.1$, indicating that the pattern of response to EGF is similar in Loa and wild-type MEFs over the observed time points. Mitogens are known to induce a biphasic activation of pERK1/2 (reviewed in Meloche and Pouyssegur, 2007) and the second peak at 120-min time point is likely to represent this phenomenon (Fig. 4B).

It was important to clarify that the tendency towards increased levels of pERK in mutant MEFs was not due to prolonged serum starvation, as in some cell types, ERK1/2 is known to become active in response to serum deprivation to induced autophagy for cell survival. To test this, MEFs were serum-starved for up to 5 h and pERK1/2 levels were examined. pERK1/2 was not detectable in the serum starved MEFs when compared to the untreated control cells (Fig. 4C).

Prolonged activation of ERK1/2 led us to hypothesize that the expression of immediate early genes could be significantly increased as a result. As pERK1/2, through ELK1, activates the transcription of c-Fos we chose to compare the levels of c-Fos in wild-type and Loa/Loa MEFs stimulated with EGF. The identity of the c-Fos bands on western blots was determined by $\lambda$-protein phosphatase treatment and c-Fos blocking peptide (Supplementary Fig. 1).

Although low in abundance, we found 0.9-fold less c-Fos in Loa/Loa compared with wild-type after serum starvation (Fig. 4A and Supplementary Fig. 2). However, after EGF stimulation c-Fos levels were increased in Loa/Loa on average by 1.5-, 1.6-, 1.6-, 1.1- and 2.1-fold at 10, 30, 60, 120, and 180 min, respectively.

As expected levels of stable phosphorylated c-Fos (pc-Fos) were found to be low after serum starvation of the MEFs. After stimulation with 20ng/ml EGF a delay of at least 30 min was observed before the Ras/Raf/ERK pathway resulted in a significant increase in c-Fos phosphorylation (Fig. 4B); however, the c-Fos phosphorylation profile in Loa/Loa was similar to wild-type (Fig. 4B).

Moreover, we did not observe any difference in proliferation rates between Loa/Loa and wild-type MEFs (Supplementary Fig. 3).

In summary, these data reveal that the F580Y mutation in Dynct1h1 results in a trend towards prolonged activation of ERK1/2 in response to EGF stimulation in Loa MEFs. Moreover, this prolonged activation is not a result of serum starvation, but likely due to delayed maturation and trafficking of EGF-containing endosomes towards the lysosome. These changes are, however, not dramatic enough to have a significant impact on transcriptional and phosphorylation profiles of c-Fos or the proliferation response in Loa MEFs.

**Aberrant ERK1/2 activation and increased c-Fos in +/Loa motor neurons**

As BDNF-stimulated Loa motor neurons showed impaired transport kinetics similar to EGF-stimulated MEFs, we asked whether the ERK1/2 signalling in mutant motor neurons would display the same pattern as in the corresponding MEFs. Thus, after 2 h serum starvation and subsequent stimulation with 5 ng/ml BDNF, motor neuron extracts were collected from wild-type and +/Loa at 10 and 30 min post-stimulation (Fig. 5A). pERK1/2 levels were found to be similar between wild-type and +/Loa in untreated cells (Fig. 5A and B). After 2 h starvation, however, there was a trend for pERK1/2 levels in +/Loa to be higher than those of wild-type. After BDNF stimulation, there is a slight decline in pERK1/2 levels in the wild-type over the time course of the experiment. BDNF stimulation also results in increased pERK1/2 levels in +/Loa motor neurons, which are higher than in wild-type neurons (Fig. 5A and B). This trend remains the same at longer BDNF stimulation times (Fig. 5C), indicating that BDNF induces sustained activation of ERK1/2 in +/Loa motor neurons over time.

When looking at c-Fos activation after serum starvation and BDNF stimulation, we observed increased pc-Fos levels in starved and BDNF stimulated motor neurons in both genotypes (Fig. 5D and E). Although the increase in pc-Fos after serum starvation coincided with increased pERK1/2, we questioned whether the rise in pERK1/2 in +/Loa was sufficient to produce the dramatic pc-Fos peak observed. As nutrient deprivation is a major stress on cells, we examined whether the activation of c-Fos in motor neurons during starvation is induced by ERK1/2 and/or the stress activated protein kinases JNK and p38. To this end we treated wild-type motor neurons with U0126 (a highly selective inhibitor of MEK1 and MEK2 upstream of ERK1/2) and SP600125 (a selective inhibitor of JNK). As shown in Fig. 5F, ERK1/2 inhibition attenuates c-Fos activation during serum starvation and after stimulation with BDNF, relative to the cells with no inhibitors at each time point, whereas JNK inhibition appears to have the opposite effect, leading to increased pc-Fos levels. Analysis of p38 levels showed a slight increase in active p38 dimers in both genotypes, which declined after BDNF stimulation before increasing again after 8 h, likely as a result of depletion of BDNF (Supplementary Fig. 4). Collectively, these data show that in motor neurons, starvation induces activation of ERK1/2 and c-Fos to a greater extent in +/Loa than in wild-type. Moreover, BDNF-induced activation of ERK1/2 and c-Fos is also greater in +/Loa motor neurons when compared with wild-type.

**Discussion**

We began by identifying altered EGF trafficking in MEFs isolated from wild-type and Loa/Loa embryos. We show increased numbers of EGF vesicles in Loa/Loa with the greatest difference at 10-min chase (Fig. 1A and B). Moreover, we show a reduction in size of EGF-containing vesicles, which is apparent immediately following the pulse with EGF (Fig. 1D). During this time frame clathrin-coated vesicles, after uncoating, fuse to early endosomes that then mature to late endosomes. These data therefore suggest delayed endosomal maturation in the mutant cells. This observation is intriguing as we showed previously that the Loa mutation impairs the interactions of dynein heavy chain with dynein intermediate and light intermediate chains, and consequently p150 subunit of dynactin (Deng et al., 2010). As dynein intermediate...
**Figure 5** ERK1/2 and c-Fos are activated by serum starvation in motor neurons. (A and B) pERK1/2 levels in +/-Loa motor neurons are higher than wild-type after serum starvation. +/-Loa does respond to BDNF activation, but not to the same extent as wild-type. (C) Western blot analysis shows higher levels of phospho-ERK1/2 in +/-Loa than in the wild-type motor neurons following starvation. BDNF stimulation, further increases active ERK1/2 in +/-Loa motor neurons than the wild-type. A marked increase of p-ERK1/2 is observed at 1 h after stimulation with BDNF, which declines over time. (D) pc-Fos is dramatically induced by serum deprivation, followed by a decline in response to BDNF stimulation in +/-Loa motor neurons. (E) Quantification of pc-Fos in D. (F) ERK1/2 inhibition attenuates c-Fos induction during starvation and after stimulating with BDNF. Western blot shows levels of pc-Fos in wild-type motor neurons at different time points: with no inhibition, JNK inhibitor SP600125, or U0126 MEK/ERK1/2 inhibitor. XRCC1 was used as a loading control and its signals were used to normalize ERK1/2 and c-Fos levels.
and light intermediate chains, and dynactin are regulators of cargo attachment, this finding may be indicative of impaired cargo docking to the dynein complex, delaying subsequent transport along microtubules. At later time points increased numbers of EGF-containing vesicles are found in Loa/Loa compared to wild-type MEFs, suggesting a delay in EGF-EGFR trafficking to the lysosomes.

Our live cell data show reduced minus-end transport velocity in Loa MEFs and motor neurons and an increased likelihood of plus-end directed steps. This finding bears the question of how a mutation in the cargo binding domain of dynein may affect the speed of the cargo. The answer to this question could lie in our previous finding that the Loa mutation impairs the interaction of dynein with dynactin (Deng et al., 2010). It is also known that the number of simultaneously engaged motors affects the cargo’s average speed by influencing its run length and duration of the pauses (Ori-McKenney et al., 2010; and reviewed in Xu and Gross, 2012). Thus the reduced velocity of the signalling endosomes in Loa cells could be the result of recruitment of fewer dynein motors to the endosomes at any given time. Moreover, cooperation between cytoplasmic dynein and its opposing motor kinesin have been established including the suggestion that one cannot function in the absence of the other (Ally et al., 2009). However, this is not always the case (Flores-Rodriguez et al., 2011) and thus it is plausible that aberrant binding of endocytic cargo to dynein in Loa may result in a tug-of-war between kinesin and mutant dynein being balanced more favourably towards the plus-end directed movement of kinesin than it is towards the minus-end directed dynein movement.

A consequence of the impaired trafficking of signalling endosomes harbouring EGF in the Loa MEFs is a trend in higher levels of pERK1/2 without significant impact on its downstream effector c-Fos. In motor neurons, however, serum starvation induced activation of ERK1/2 in both genotypes. This is an intriguing observation as it demonstrates that the response of motor neurons to serum withdrawal is completely different to that of the MEFs. In contrast to MEFs, serum deprivation led to a striking activation of ERK1/2 and induction of active c-Fos particularly in Loa motor neurons (Fig. 5A–E). Moreover, the elevated pc-Fos is mainly a response to ERK1/2 activation and not the stress activated protein kinases JNK or p38, as unlike the ERK1/2 inhibitor U0126, JNK inhibitor SP600125 did not reduce pc-Fos in serum-starved motor neurons. These data indicate that firstly, the post-mitotic motor neurons are specifically equipped with a stress response machinery which activates the ERK1/2 MAP kinase pathway and their downstream effectors such as c-Fos, possibly as a protective mechanism against cellular stress; secondly, this response is exacerbated in +/Loa motor neurons. Moreover, on the addition of BDNF, the pERK1/2 and p-c-Fos levels remain higher in +/Loa motor neurons.

Almeida et al. (2005) have shown that BDNF induces transient ERK1/2 activity to promote survival in response to glutamate toxicity. In contrast, persistent ERK1/2 activity caused by glutamate-induced oxidative stress causes cell death in cortical neurons (Stanciu et al., 2000; Luo and DeFranco, 2006). It is therefore possible that the tendency of +/Loa motor neurons to increased and persistent levels of pERK1/2, caused by impaired trafficking and delayed transport of signalling endosomes towards lysosomes, contribute to neuronal cell death by making them more susceptible to physiological insults such as oxidative stress.

In a recent study, Mitchell et al. (2012) have demonstrated that in cortical neurons, BDNF stimulation of ERK1/2 leads to phosphorylation of dynein intermediate chains and recruitment of cytoplasmic dynein to signalling endosomes for retrograde transport. Intriguingly, we have previously shown that the amount of phosphorylated dynein intermediate chains is reduced in brain tissues of Loa/Loa mice (Deng et al., 2010). Thus, it is plausible that the increased binding affinity of dynein heavy chain to the intermediate chains (Deng et al., 2010) could lead to conformational changes in the intermediate chains, which may impede their phosphorylation following ERK1/2 activation by BDNF. Consequently, this would lead to impaired dynein-mediated transport of endosomes to lysosomes for degradation and consequently further accumulation of active ERK1/2 as reported here.

We have identified aberrant transport kinetics in Loa, which are not cell-type specific. However the effect on cell signalling is substantially different between mitotic MEFs and terminally-differentiated motor neurons. This work shows for the first time a direct comparison between two very different cells types and identifies underlying signalling events that might contribute to the neurological phenotypes of Dync1h1 mutations.

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Supplementary material

Supplementary material is available at Brain online.

References


