Loss and gain of FUS function impair neuromuscular synaptic transmission in a genetic model of ALS

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Abstract

Amyotrophic Lateral Sclerosis (ALS) presents clinically in adulthood and is characterized by the loss of motoneurons in the spinal cord and cerebral cortex. Animal models of the disease suggest that significant neuronal abnormalities exist during preclinical stages of the disease. Mutations in the gene fused in sarcoma (FUS) are associated with ALS and cause impairment in motor function in animal models. However, the mechanism of neuromuscular dysfunction underlying pathophysiological deficits causing impairment in locomotor function resulting from mutant FUS expression is unknown. To characterize the cellular pathophysiological defect, we expressed the wild type human gene (wtFUS) or the ALS-associated mutation R521H (mutFUS) gene in zebrafish larvae and characterized their motor (swimming) activity and function of their neuromuscular junctions (NMJs). Additionally, we tested knockdown of zebrafish fus with an antisense morpholino oligonucleotide (fus AMO). Expression of either mutFUS or knockdown of fus resulted in impaired motor activity and reduced NMJ synaptic fidelity with reduced quantal transmission. Primary motoneurons expressing mutFUS were found to be more excitable. These impairments in neuronal function could be partially restored in fus AMO larvae also expressing wtFUS (fus AMO + wtFUS) but not mutFUS (fus AMO + mutFUS). These results show that both a loss and gain of FUS function result in defective presynaptic function at the NMJ.
Introduction

ALS presents clinically in adulthood and is characterized by the loss of both upper and lower motoneurons. The progressive paralysis associated with the disease often culminates with complications associated with respiratory function and death usually occurs within 2-5 years after diagnosis. Mutations in \textit{FUS} account for \(~1\%\) and \(~4\%\) of sporadic and familial forms of ALS cases respectively (1-3). The majority of ALS-causing mutations are located in the C-terminus encoding a nuclear localization signal or the glycine rich region (1-2). FUS shares some common structural similarities with the Transactive Response DNA/RNA-Binding Protein (TDP-43), which is also linked to ALS (4-5). The pathophysiological consequences of mutations in \textit{FUS} remain unknown, but understanding them is vital for clarifying the mechanisms of neurodegeneration and the development of treatments for the disease.

Several lines of evidence, based upon animal models of ALS, implicate early and selective changes at neuromuscular junctions (NMJ) prior to clinical presentation of the disease. Transgenic mice over expressing human mutant (G93A) Cu-Zn superoxide dismutase 1 (SOD\textsuperscript{G93A}) display early loss of muscle innervation long before these mice develop clinical symptoms of muscle weakness and before spinal motoneurons die (for review see: (6-7)). In contrast to models of SOD1, less is known about the pathophysiological defects that arise in other genes associated with ALS. However, recent progress has been made in studies of TDP-43 using \textit{Drosophila} and zebrafish (\textit{Danio rerio}), which revealed significant synaptic defects at the NMJ (8-9). In comparison to TDP-43 much less is known about the synaptic pathophysiology related to FUS though reduced tetanic force production has been reported in a mouse model expressing human wild type FUS (10) and mutant FUS expression in \textit{Caenorhabditis elegans}.
has been reported to cause a progressive motor dysfunction phenotype with reduced lifespan, suggesting an impairment in the neuronal or muscular systems (11).

To investigate the mechanism by which altered FUS expression impairs synaptic function at the NMJ, we utilized a larval zebrafish preparation (12-13) to investigate NMJ dysfunction. Here we characterized functional abnormalities arising at the larval NMJ following expression of wild type or mutant human FUS and also examined NMJ function in zebrafish upon knockdown of fus using antisense morpholino oligonucleotides.

**Results**

*MutFUS expression or knockdown of fus impairs locomotor function.*

Larval zebrafish expressing missense mutations such as the ALS-causing R521H mutation of human FUS or following knockdown of zebrafish fus with an AMO (but not with a 5 base pair mismatch AMO) were shown previously to have impairments in locomotor activity with abnormal motor axon projections at 48 hpf (12), but otherwise have normal gross morphology (Figure 1 A). To further our understanding of motor dysfunction in these larvae we performed high speed video analyses of touch-evoked locomotor behaviour (Supplementary video 1). Following touch, wild type larvae exhibited robust escape responses, characterized by swimming away from the tactile stimuli for a few seconds. Larvae expressing the human FUS\textsuperscript{R521H} missense mutation (mutFUS) and larvae injected with the AMO against zebrafish fus (fus AMO) responded to touch, eliciting a bout of swimming but fatigued quickly, displaying shorter swim duration when compared to wild type larvae ($P < 0.05$; Figure 1 B and C). Furthermore, mutFUS and fus AMO animals swam shorter distances ($P < 0.05$; Figure 1D) and
had reduced maximum swim velocities ($P < 0.05$; Figure 1 E) compared to wild type and larvae expressing the wild type human $FUS$ (wt$FUS$). Expression of wt$FUS$ in larvae injected with the $fus$ AMO ($fus$ AMO + wt$FUS$) recovered both the swim distance (Figure 1 D) and the maximum swim velocity (Figure 1E) during touch-evoked swim responses. Expression of mut$FUS$ did not recuperate poor swim performance in $fus$ AMO injected larvae ($fus$ AMO + mut$FUS$; Figure 1 D, E).

**NMAD-induced cell death.**

Exposure of larvae expressing mutant $TARDBP$ to the excitatory neurotransmitter NMDA resulted in increased cell death in the spinal cord (8). We examined if a similar phenomenon occurred consequent to expression of mut$FUS$ or $fus$ knockdown. Larvae not exposed to NMDA displayed no differences in the number of dead cells detected by acridine orange (AO) staining ($P = 0.723$; Supplementary Figure 1 A, B) amongst treatment groups (wild type, wt$FUS$, mut$FUS$, $fus$ AMO, $fus$ AMO + wt$FUS$, $fus$ AMO + mut$FUS$). However, following exposure to NMDA, $fus$ AMO + mut$FUS$ expressing larvae displayed significantly more AO positive cells than the number found in wild type, wt$FUS$ and in rescued $fus$ AMO +wt$FUS$ larvae ($P < 0.05$; Supplementary Figure 1 C, D). No differences were found in mut$FUS$ larvae when compared to wild type or wt$FUS$ larvae; however, we did find a statistical difference between this group and the rescued $fus$ AMO +wt$FUS$ group ($P < 0.05$; Supplementary Figure 1 D). These results indicate that under extreme conditions (loss of normal function with gain of toxic function, which may be a closer disease parallel) a stressor such as NMDA can induce cell death in the spinal cord.
**MutFUS expression or knockdown of fus does not alter muscle electrophysiological properties.**

Patch-clamp recordings of fast-twitch muscle cells did not yield any differences in electrophysiological properties (Table 1). Across treatment groups fast-twitch muscles maintained similar membrane potentials, cell capacitances and membrane resistivity. Furthermore, the frequency at which fast-twitch muscle cells entered tetanus was also not found to be different. Thus muscle cells did not appear to be affected by fus loss and/or toxic gain of function.

**Increased motoneuron excitability following mutFUS expression.**

We next sought to determine if motoneurons showed altered properties upon altered FUS expression. Each hemisomite has three primary motoneurons, one of which is the caudal primary (CaP) motoneuron located just below the dura. These neurons possess large perikarya and send axonal projections, via the ventral root, to ventral trunk muscles (14). Whole-cell recordings of CaP motoneurons did not show any differences in membrane potential, cell capacitance, input resistance, or action potential threshold across treatment groups (Table 1). However, larvae expressing mutFUS and in the fus AMO + mutFUS group displayed reduced rheobase current when compared to other experimental groups ($P < 0.05$; Table 1). Furthermore, CaP motoneurons from these two groups, along with fus AMO + wtFUS, generated higher frequencies of action potentials following current injection ($P < 0.05$, Table 1) indicating that these motoneurons were more excitable.
Impaired NMJ synaptic transmission following mutFUS expression or fus knockdown.

To probe neuromuscular connectivity, we obtained whole cell recordings from polyinnervated muscle fibers in restrained larvae during episodes of locomotor activity. These recordings indicated a severe reduction in synaptic activity in fus AMO and mutFUS larvae (Figure 2 A). To resolve NMJ activity in single motor units, we performed paired CaP motoneuron-fast twitch muscle cell patch-clamp recordings to assess synaptic transmission (Supplementary video 2). Muscle endplate currents (EPCs) were generated by stimulating the CaP motoneuron at 10 Hz or 30 Hz for 10 s. As previously reported (8, 15), the fidelity of NMJ synaptic transmission in wild type larvae was high (Figure 2 B, C, D). However, larvae expressing mutFUS or injected with the fus AMO had reduced synaptic transmission success (Figure 2 B, C, D). Furthermore, mean EPC amplitude was reduced in these larvae (Figure 2 E) and post stimulation release, observed nearly all the time in wild type recordings (Figure 2 C, arrow), was absent in all mutFUS and fus AMO recordings. Strikingly, impaired synaptic transmission success and attenuated EPC amplitude following 10 and 30 Hz CaP stimulation in fus AMO-treated larvae could be rescued by expression of wtFUS (fus AMP + wtFUS) but not mutFUS (fus AMP + mutFUS) (Figure 2 D, E). These data suggest that strong deficits in both pre- and post-synaptic NMJ function occur following expression of mutFUS or knockdown of zebrafish fus.

Abnormal NMJ structure following mutFUS expression or fus knockdown.

We have previously demonstrated over-branching of ventral motoneuron axons following knockdown of zebrafish fus or mutFUS expression (12). Here we performed double labeling of
synaptotagmin 2 (ZNP-1 antibody, a presynaptic marker) and alpha bungarotoxin (alphaBTX, which binds irreversibly to acetylcholine receptors) to examine structural components of the NMJ. Normal NMJs have a tight juxtaposition of these pre- and postsynaptic components (16) and this was observed in wild type zebrafish and fish expressing wtFUS, wherein these animals displayed few orphaned ZNP-1 puncta (Figure 3 A and B) and few orphaned alphaBTX clusters (Figure 3 A and C). Conversely, zebrafish expressing mutFUS or zebrafish with knockdown of fus (fus AMO) displayed a higher number of both orphaned ZNP-1 puncta ($P < 0.050$) and orphaned alphaBTX clusters ($P < 0.050$) when compared to wild type larvae and larvae expressing wtFUS (Figure 3 A, B and C). Rescue of this phenotype was observed after co-expression of wtFUS but not mutFUS (Figure 3 A, B and C). These data demonstrate that mutant FUS expression or knockdown of zebrafish fus resulted in aberrant NMJs.

**Reduced frequency of quantal events in mutFUS.**

Although we observed structural abnormalities at the NMJ of zebrafish expressing mutFUS and in larvae with knockdown levels of fus, this does not necessarily demonstrate a specific functional deficit at individual endings. To more accurately quantify function of the NMJs we examined miniature endplate currents (mEPCs). The mEPCs occur spontaneously at healthy synapses and correspond to the unitary (quantal) event during synaptic transmission (Figure 4 A). The frequency of mEPCs in zebrafish expressing mutFUS and fus AMO larvae was reduced when compared to wild type zebrafish ($P < 0.04$; Figure 4 B), suggesting a reduction in the number of functional presynaptic endings, consistent with NMJ morphology. Expression of wtFUS in fus AMO-treated larvae (fus AMO + wtFUS) restored mEPC frequency
to that of wild type mEPC frequency ($P = 0.93$). We also observed that expression of mutFUS in fus AMO larvae (fus AMO + mutFUS) also had a slight restorative effect on mEPC frequency when compared to wild type mEPC frequency ($P = 0.23$). Examination of other mEPC parameters did not reveal any major significant differences across treatment groups. Thus we did not find any significant differences in mean mEPC amplitude across treatments ($P = 0.76$; Figure 4 D) or mEPC decay constant ($P = 0.58$; Figure 2 E). We did find a slight but significant increase in mEPC rise time specifically in fus AMO + wtFUS larvae ($P < 0.05$; Figure 4 C).

We next estimated the quantal content (as EPC/mEPC amplitude) in paired CaP motoneuron – fast-twitch muscle recordings and found that at 10 Hz stimulation, mutFUS expressing larvae had a significantly reduced estimated quantal content when compared to both wild type larvae and wtFUS larvae ($P < 0.001$; Figure 4 F) but this was not statistically apparent at 30 Hz stimulation ($P = 0.09$). Estimates in larvae with knockdown levels of fus displayed significantly reduced quantal content at both 10 and 30 Hz CaP stimulations ($P < 0.001$; Figure 4 F). Expression of wtFUS but not mutFUS in larvae with knockdown levels of fus restored estimated quantal content (Figure 4 F). This data suggest a significant presynaptic defect occurs at the NMJ of larvae either expressing mutFUS or following knockdown of zebrafish fus.

Discussion

Although our understanding of the genetic underpinnings of ALS have advanced in recent years, our understanding of the pathophysiological mechanisms of neuronal dysfunction associated with neurodegeneration is incomplete. In this study we present results which implicates both a gain of toxic function following expression of mutant (but not wild type)
human FUS and a loss of function following antisense morpholino knockdown of zebrafish fus generate locomotor impairment by reducing presynaptic function at the NMJ. This builds upon previous descriptions of locomotor impairments in zebrafish (12), Drosophila (17) and mouse (10) models of FUS. We further demonstrated that expressing wtFUS in larvae with knockdown levels of fus rescues the impairment in locomotion.

We believe that the locomotor impairments in fus AMO or mutFUS larvae arose not as a result of altered fast-twitch muscles, which were normal in physiological function, but as a result of deficits at the NMJ. We observed reduced synaptic transmission success, reduced EPC amplitude and reduced frequency of quantal transmission across the NMJ in larvae with either knockdown levels of fus or following expression of mutFUS. Poor fidelity of synaptic transmission at the NMJ has been reported previously in zebrafish larvae following expression of mutant TDP-43 (8) and in mice expressing mutant SOD1\textsuperscript{G93A} (18). We also observed an increase in orphaned terminals and, a reduction in the frequency of mEPCs, which has been observed in tissue from ALS patients (19) and in models of SOD1 and TDP-43 (8-9, 20). Both of these observations (reduced EPC fidelity and mEPC frequency) are indicative of deficits in presynaptic function. As the amplitude and time course of mEPCs were unaffected in mutFUS and fus knockdown, acetylcholine, when released, appears to act with normal effectiveness. The reduction in evoked EPC amplitude is therefore indicative of reduced presynaptic release. Interestingly, previous work has demonstrated that depletion of Fus with antisense oligonucleotides in the mouse results in the concomitant down regulation of several genes involved in synaptic transmission (21). Together these results suggest that defects in NMJ transmission may be due to misprocessing of genes necessary for proper presynaptic function.
Along with defects associated with peripheral motor control, central abnormalities within the spinal cord were also observed in larvae expressing mut\textit{FUS}. In response to current injection, CaP motoneurons expressing mut\textit{FUS} generated higher frequencies of action potentials and these neurons displayed a lower rheobase current, indicating that they were indeed more excitable. Interestingly, reduced rheobase current in CaP motoneurons was not observed in \textit{fus} AMO-treated zebrafish and AP frequencies during current injection, though significantly higher than the control group, were not as high as larvae expressing mut\textit{FUS}. This might suggest that the pathological underpinnings of \textit{fus} AMO and mut\textit{FUS} expression may differ. We have reported similar findings in larvae expressing mutant TDP-43 (8) and in models of mutant SOD1, increased excitability driven by augmented persistent inward currents of Na\textsuperscript{+} and Ca\textsuperscript{2+} and reduced glycinergic inhibitory input have been reported by other groups (22-25). A widely held theory for motoneuron death in ALS is a result of glutamatergic excitotoxicity (26-29). Neurons with increased excitability would be at a greater risk of damage following glutamatergic neuronal depolarization and calcium ion influx. In this study we observed an increase in spinal cell death in larvae exposed to NMDA, which depolarizes larval motoneurons (30), with knockdown levels of \textit{fus} and also expressing mut\textit{FUS} and as reported previously in larvae expressing mutant TDP-43 (8).

Although ALS manifests clinically in adulthood, inconspicuous changes in neuronal function may occur at earlier stages. Support for the importance of pre-clinical stages of the disease have been largely learned from mouse and zebrafish models of SOD1, which revealed early abnormalities both peripherally and centrally in the nervous system (for review see: (6-7, 29, 31-34)). Mice expressing mutant SOD1 have a significant proportion (40%) of denervated endplates with a selective loss of fast-fatigable NMJ synapse at 47-50 days (35-36) while
impairments in motor function, loss of ventral root axons and loss of alpha motoneuron cell bodies was not observed until days 78, 80 and 100 respectively (36). Furthermore, a recent report utilizing a zebrafish model of SOD1 described an early stressed-induced reduction in glycinergic interneuron synaptic connectivity with motoneurons (25). The loss of inhibitory synaptic connections in the adult spinal cord may contribute to motoneuron stress at symptomatic stages of the disease (25). These reports and the data presented here describing both a gain and loss of FUS function conferring a pathophysiological NMJ impairment must of course be interpreted cautiously. Our experiments were performed at stages when the larvae are undergoing rapid development and it is unclear if and how developmental processes interact with degenerative pathways, particularly in the context of late onset disorders. Furthermore, expression of either wild type or mutant FUS and knockdown of zebrafish fus were transient as such some caution is warranted in our interpretation of the results. Nevertheless, the data presented here emphasizes that early changes occur at the NMJ and the importance of developing therapeutic strategies during pre-clinical stages of FUS-related ALS cases.

Materials and Methods

Zebrafish lines

Wild type zebrafish (D. rerio) were bred and maintained according to standard procedures (37). All experiments were performed in compliance with the guidelines of the Canadian Council for Animal Care and conducted at the Université de Montréal. All experiments were performed on sexually undifferentiated zebrafish larvae 50-54 hours post fertilization (hpf).
Preparation and Expression of FUS

Human FUS cDNA was obtained from Open Biosystems. R521H mutation was introduced using site-directed mutagenesis in the appropriate vector using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) as previously described for TARDBP and FUS (12, 38). cDNA constructs encoding N-FLAG and C-Myc were incorporated and subcloned into a pCS2+ plasmid vectors which were subsequently used to generate mRNA. An antisense morpholino oligonucleotide (AMO) was designed to bind and inhibit specifically the 1st ATG site of the zebrafish fus gene with the following sequence (GGCCATAATCATTTGACGCCATGTT) and reduced fus expression by 60 % as previously described (12). A BLAST search did not recognize any other sequences in the zebrafish or human genomes. Working concentrations for FUS mRNA and fus AMO was 25 ng/µl and 0.2 mM respectively, and injection volume was 3.5 - 4.0 nl made into the 1-2 cell stage blastulae as previously described (38).

Free-swimming restrained tail-beat behaviour

Assessment of zebrafish locomotor patterns was performed at room temperature (22-25 °C) as described previously (8). Larvae were placed (obliquely with the head towards the upper right quadrant) in the middle of a circular arena (150 mm diameter), filled with aquarium water. Burst swimming was initiated by a single touch to the tail and locomotor activity was recorded from above digitally at 30 Hz (Grasshopper 2 camera, Point Grey Research). Swim duration, swim distance and maximum swim velocity were quantified offline using the manual tracking plug-in for ImageJ.
**Muscle whole cell voltage-clamp recordings**

All chemicals were obtained from Sigma-Aldrich (unless otherwise stated) and dissolved in Evan’s solution (see below), using a minimum amount of DMSO if required (0.1%) and bath applied to semi-intact preparations. As described previously (39), zebrafish were anaesthetized in 0.04% tricaine (Sigma) dissolved in modified Evans solution containing, in mM: 134 NaCl, 2.9 KCl, 2.1 CaCl₂, 1.2 MgCl₂, 10 HEPES, 10 glucose, adjusted to 290 mOsm and pH 7.8. The zebrafish were then pinned with fine (0.001 in.) tungsten wires through their notochords to a Sylgard-lined dish. The outer layer of skin between the pins was removed using a fine glass electrode and forceps exposing the musculature. The preparation was then visualized by oblique illumination (Olympus BX61W1).

Standard whole-cell voltage clamp recordings were obtained from fast twitch (embryonic white) muscle cells (39). In these recordings 20 µM N-benzyl-p-toluenesulfonamide, an inhibitor of myosin ATPase, dissolved in 0.1% DMSO was added to the saline to minimize muscle contractions. 3-4 MΩ glass electrodes were pulled from thin-walled Kimax-51 borosilicate glass (Kimble Chase) and filled with the following intracellular solution (in mM: 130 CsCl, 2 MgCl₂, 10 HEPES, and 10 EGTA adjusted to pH 7.2, 290 mOsm.). Cells were held near their resting potential at -65mV and series resistant was < 8 MΩ and compensated to 70-90 %. In some experiments 1 µM TTX was perfused over the preparation to isolate spontaneous (quantal) miniature endplate currents (mEPC). All electrophysiological data were sampled at 40 kHz using an Axopatch 200B amplifier (Molecular Devices) and digitized using a Digidata 1440A (Molecular Devices) and stored on a computer for later analysis using pCLAMP 10 software (Molecular Devices). Small, slow, mEPCs were excluded from the analyses as these are known to be due to electrical coupling with adjoining muscle cells (40).
Paired motoneuron/muscle recordings

Paired motoneuron/muscle recordings were performed following procedures previously described (15). Briefly, paired motor motoneuron/muscle recordings were obtained by perfusing collagenase (1 mg/ml) over the preparation for 10 min. This allowed for partial digestion before red and white muscles cells overlying the spinal cord were removed by aspiration to expose the spinal cord while leaving the ventral root and deeper muscle cells intact. Somites 13-16 were selected for recording. 7-9 MΩ patch-clamp electrodes were filled with the following intracellular solution in mM: 116 K-gluconate; 16 KCl; 2 MgCl₂; 10 HEPES; 10 EGTA adjusted to pH 7.2, 290 mOsm. The caudal and primary (CaP) motoneuron was selected because of its size and projection pattern to intact ventral regions of the trunk musculature. Motoneuron action potentials were elicited by a train of 200 pA, 2 ms current steps into the motoneuron soma in current clamp mode at 10 Hz or 30 Hz for 10 seconds. Muscle recordings were the same as described above except 1 μM QX-314 was added to the patch pipette solution to block voltage gated sodium currents and muscle contractions.

Acridine orange staining

Zebrafish were incubated in 1 μg/ml acridine orange (AO) for 30 min then repeatedly washed in aquarium water (8). Larvae were anesthetized in tricaine before being visualized under a 10X water immersion lens mounted on a Quorum Technologies spinning disk confocal microscope with CSU10B (Yokogawa) spinning head mounted on an Olympus BX61W1 fluorescence microscope and connected to a Hamamatsu ORCA-ER camera. Spinal cords were
examined under a 470-490 nM excitation filter and images were acquired using Volocity software (Improvision). The number of neurons per 5 somites was counted per embryo from a set of stacked Z-series images. AO stained spinal cord images are presented in pseudocolour.

**Immunohistochemistry**

Animals were fixed in 4 % paraformaldehyde overnight at 4 ºC. After fixation the larvae were rinsed several times (1 hr) with PBS and then incubated in PBS containing 1 mg/ml of collagenase (20 min) to remove skin. The collagenase was washed off with PBS (1 hr) and the larvae were incubated in PBST (PBS with Triton X-100) containing 10 mg/ml of sulforhodamine conjugated alpha-bungarotoxin (30 min). The larvae were then rinsed several times with PBST (30 min) and then incubated in freshly prepared block solution containing primary antibody ZNP-1 (1:100, DSHB) overnight at 4 ºC. Following this, larvae were incubated in block solution containing a secondary antibody (Alexa fluor 488, 1:1000, Invitrogen) for 6 hrs at 4 ºC before being mountain on a glass slide in 70 % glycerol. The neuromuscular junctions were visualized using a Quorum Technologies spinning disk confocal microscope with CSU10B (Yokogawa) spinning head mounted on an Olympus BX61W1 fluorescence microscope and connected to a Hamamatsu ORCA-ER camera. Images were acquired using Volocity software (Improvision).

**Statistical analysis**

SigmaPlot 11.0 integrated with SigmaStat 3.1 was used to assess data groupings for significance. Statistical analyses used one-way repeated measures ANOVA, followed by a post-
hoc Tukey multiple comparison test. For non-parametric tests a Kruskal-Wallis one way ANOVA on ranks was performed. Significance was assessed at P < 0.05. Asterisks, daggers and double bars represent statistical differences from wild type, wtFUS and the fus AMO + wtFUS larval groups respectively.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest with the material published within this manuscript.
References


Legends to Figures

Figure 1. Knockdown of larval zebrafish fus expression or expressing mutFUS impaired locomotor function. (A), representative pictures of larvae from each treatment group. Note the lack of gross morphological defects; scale represents 1 mm. Swim behaviour was reliably evoked by a light touch to the tail. (B), Examples of 10 superimposed locomotor path traces from each treatment group. Mean swim duration (C), swim distance (D) and maximum swim velocity (E). Animals expressing mutFUS or animals with knockdown levels of fus displayed impairments in locomotor behaviour. Locomotor function in fus AMO larvae could be recovered if co-injected with wtFUS but not mutFUS mRNA. Asterisks, daggers and double bars represent statistical differences from wild type, wtFUS and the fus AMO + wtFUS larval groups respectively.

Figure 2. Expression of mutFUS or knockdown of zebrafish fus reduced the fidelity of NMJ synaptic transmission and attenuated EPC amplitude. (A), Example whole-cell voltage clamp recordings (bottom trace) of central pattern generator-driven activity following touch (top trace) from polyinnervated fast-twitch muscles. Note the reduced EPC amplitude from mutFUS and fus AMO larvae. (B), to examine NMJ synaptic connectivity in single motor units paired (primary motoneuron/fast-twitch muscle) were performed. Traces of paired recordings in wild type, mutFUS and fus AMO larvae showing whole cell current-clamp traces of action potentials (AP) generated in the CaP motoneuron (top traces) and corresponding EPCs recorded in fast-twitch muscle cells under whole cell voltage-clamp (bottom trace) during a 10 s train of depolarizing (AP generating) current steps delivered at 10 Hz and in C at 30 Hz. Insets correspond to enlarged example traces of EPCs denoted by the black dash above EPCs in B & C. The X’s indicate NMJ
synaptic transmission failure and the arrow indicates post-stimulus EPCs. Both mutFUS and fus AMO displayed a significant reduction in the success of synaptic transmission (D) and attenuated EPC amplitude (E) at both 10 and 30 Hz stimulation frequencies. Recovery of synaptic transmission and EPC amplitude could be recovered in fus AMO larvae expressing wtFUS (fus AMO + wtFUS) but not mutFUS (fus AMO + mutFUS). Numbers in parenthesis represent sample sizes. Asterisks, daggers and double bars represent statistical differences from wild type, wtFUS and the fus AMO + wtFUS larval groups respectively.

Figure 3. Zebrafish expressing mutFUS or with knockdown levels of zebrafish fus displayed orphaned presynaptic endings and acetylcholine receptor clusters. (A), Representative images of one ventral root projection double labeled for ZNP-1 (presynaptic marker, i) and alphaBTX (postsynaptic ,ii). Wild type and wtFUS larvae showed extensive co-localization of both ZNP-1 and alphaBTX (iii, merged). However, we observed an increase in the number of orphaned ZNP-1(arrows) and alphaBTX labeling (arrowheads) in mutFUS and fus AMO larvae (B) and (C). Fus AMO larvae also expressing wtFUS (fus AMO + wtFUS) but not mutFUS (fus AMO + mutFUS) significantly reduced the incidence of orphaned ZNP-1 puncta and alphaBTX clusters. Numbers in parenthesis represent sample sizes. Asterisks, daggers and double bars represent statistical differences from wild type, wtFUS and the fus AMO + wtFUS larval groups respectively. Scale bars in Ai represent 25 µm and insets represent 10 µm.
Figure 4. Both mutFUS and fus AMO zebrafish displayed reduced frequencies of miniature endplate currents (mEPCs) and attenuated quantal content in paired recordings. (A), Representative mEPC traces. (B). mutFUS and fus AMO larvae displayed mEPCs at reduced frequencies when compared to wild type larvae indicating that the numbers of functional synapses are reduced or a proportion are non-functional. Recovery following fus knockdown was achieved with expression with both wtFUS (fus AMO + wtFUS) and mutFUS (fus AMO + mutFUS). The kinetics of mEPC were largely unaffected by expression of mutFUS nor for fus AMO knockdown. (C), Rise time. (D), Amplitude. (E), Decay constant. (F), Quantal content measured from paired recordings at 10 and 30 Hz were calculated by dividing the amplitude of individual EPC by the mean mEPC amplitude. Fish expressing mutFUS and fus AMO displayed significantly reduced quantal content from each AP. Restoration of quantal content could be recovered in fus AMO larvae also expressing wtFUS (fus AMO + wtFUS) but not mutFUS (fus AMO + mutFUS). Numbers in parenthesis represent sample sizes. Asterisks, daggers and double bars represent statistical differences from wild type, wtFUS and the fus AMO + wtFUS larval groups respectively.
Abbreviations

ALS: amyotrophic lateral sclerosis.
AO: acridine orange
AP: action potential
CaP: caudal and primary motoneuron
EPC: endplate current
fus AMO: fus antisense morpholino oligonucleotide
FUS: human fused in sarcoma
fus: zebrafish fused in sarcoma
mutFUS: mutant (R521H) human FUS
mEPC miniature endplate current
NMJ: neuromuscular junction
SOD1: Cu-Zn superoxide dismutase 1
wtFUS: wild type human FUS
## Table 1. Properties of fast twitch muscle and primary motoneurons in larval zebrafish.

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<td>(primary motoneuron)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Vm (mV)</td>
<td>-63.1 ± 0.7 (15)</td>
<td>-63.9 ± 0.8 (13)</td>
<td>-61.4 ± 1.5 (14)</td>
<td>-62.4 ± 1.5 (11)</td>
<td>-66.0 ± 0.8 (16)</td>
<td>-65.3 ± 0.8 (10)</td>
</tr>
<tr>
<td>Cm (pF)</td>
<td>7.6 ± 0.3 (15)</td>
<td>8.9 ± 1.0 (13)</td>
<td>7.6 ± 0.7 (14)</td>
<td>7.6 ± 0.5 (11)</td>
<td>7.5 ± 0.1 (16)</td>
<td>7.5 ± 0.2 (10)</td>
</tr>
<tr>
<td>Rm (MO)</td>
<td>291.7 ± 18.5 (15)</td>
<td>250.1 ± 41.4 (13)</td>
<td>297.1 ± 39.6 (14)</td>
<td>266.0 ± 10.0 (11)</td>
<td>250.5 ± 43.7 (16)</td>
<td>252.6 ± 73.8 (10)</td>
</tr>
<tr>
<td>Rheobase (pA)</td>
<td>67.2 ± 4.0 (10)</td>
<td>69.7 ± 6.4 (10)</td>
<td>52.2 ± 4.8 (11)*</td>
<td>63.6 ± 6.8 (8)</td>
<td>61.0 ± 7.6 (3)</td>
<td>54.2 ± 3.1 (10)*</td>
</tr>
<tr>
<td>AP frequency (Hz) @ 20 pA injection</td>
<td>0 (10)</td>
<td>0 (8)</td>
<td>0 (11)</td>
<td>0 (8)</td>
<td>0 (8)</td>
<td>0 (10)</td>
</tr>
<tr>
<td>AP frequency (Hz) @ 40 pA injection</td>
<td>0 (10)</td>
<td>0 (8)</td>
<td>12.9 ± 8.1 (11)*</td>
<td>0 (8)</td>
<td>10.3 ± 7.0 (8)*</td>
<td>17.0 ± 17.0 (10)*</td>
</tr>
<tr>
<td>AP frequency (Hz) @ 60 pA injection</td>
<td>3.6 ± 3.6 (10)</td>
<td>8.4 ± 8.4 (8)</td>
<td>76.6 ± 13.8 (11)*</td>
<td>33.63 ± 16.6 (8)*</td>
<td>29.3 ± 13.4 (8)*</td>
<td>78.0 ± 9.0 (10)*</td>
</tr>
<tr>
<td>AP frequency (Hz) @ 80 pA injection</td>
<td>58.8 ± 14.0 (10)</td>
<td>65.3 ± 19.5 (8)</td>
<td>79.9 ± 17.0 (11)</td>
<td>61.5 ± 23.2 (8)</td>
<td>56.2 ± 15.0 (8)</td>
<td>97.7 ± 14.5 (10)*</td>
</tr>
<tr>
<td>Threshold (mV)</td>
<td>-33.5 ± 0.9 (10)</td>
<td>-35.6 ± 1.3 (8)</td>
<td>-35.1 ± 1.3 (11)</td>
<td>-34.4 ± 2.0 (8)</td>
<td>-31.6 ± 0.9 (3)</td>
<td>-36.7 ± 2.0 (10)</td>
</tr>
</tbody>
</table>

* Represent statistical differences from wild type larvae ($P < 0.05$).
Figure 3

A

Wild type

wtFUS

mutFUS

fus AMO

fus AMO + wtFUS

fus AMO + mutFUS

B

C

Orphaned Zn1-puncta/somite

Orphaned alphaBTX clusters/somite
Figure 4