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Supplemental Data

Mutations in *CAPN1* Cause Autosomal-Recessive Hereditary Spastic Paraplegia

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Supplemental Note: Case Reports

Family A

Individual V-2

Individual V-2 presented with onset of progressive walking difficulties at the age of 20. She was initially examined by us at the age of 31 years, at the time complaining of gait difficulty and urinary frequency and incontinence. At the time of examination, the individual had spastic gait typical to HSP, however she could walk without aid. Muscle strength was 4/5 in the lower limbs, and tendon reflexes were brisk in the four limbs with bilateral Babinski sign. There was no muscle wasting, there was no sensory deficit nor were there cerebellar or extrapyramidal signs. Motor coordination and cranial nerves examinations were normal. Eye movement and fundus examinations were normal. She had moderate bilateral pes valgus with hallux valgus. Electroneuromyography (ENMG) examination was normal. The individual also suffered from mild dysarthria.

Family B.

Individual IV-1

Individual IV-1 presented with onset of spastic paraplegia at the age of 35. He experienced lower limbs weakness that predominated on the left side and he also complained of dysarthria. No balance problems were reported. He was examined at the age of 47, and according to the individual he could walk more than 5000 meters, but had difficulties in running and going down the stairs. At the time of examination, Individual IV-1 had spastic gait, paralytic dysarthria with akinetic face, proximal reduced strength in lower limbs (4/5) and brisk tendon reflexes with bilateral

Babinski sign. There was no muscle wasting. He had bilateral and distal superficial hypoesthesia in both upper and lower limbs and reduced vibration sensation in lower limbs. The upper limb coordination and cranial nerves examination were normal. Eye movement and fundus examination were normal. He had no skeletal deformities. The ENMG examination showed a moderate sensory axonal neuropathy predominating in the lower limbs, sympathetic skin reflex was normal in upper limbs and abolished in lower limbs (Table S1).

Individual IV-2

Individual IV-2 presented with onset of spastic paraplegia at the age of 36 years with lower limbs weakness and ataxic gait. She was examined at the age of 44, and at the time of examination she needed cane support for walking, that she started using at the age of 40. At examination, the individual had reduced strength in lower limbs (4/5), without muscle wasting, and with brisk tendon reflexes in all limbs with bilateral Babinski sign. She had no sensory deficit, however she had moderate upper limbs dysmetria, as well as dysarthria and moderate facial hypokinesia. The cranial nerves examination was normal, with normal eye movement and fundus examination. Cognition was normal and there were no skeletal deformities with the exception of pes cavus. Her ENMG examination showed a moderate sensory axonal neuropathy predominating in the lower limbs, sympathetic skin reflex was normal in upper limbs and abolished in lower limbs (Table S1).

Individual IV-4

Individual IV-4 presented with onset of spastic paraplegia at the age of 22 years, with lower limbs stiffness at walking that progressed to spastic gait and weakness. He was examined at the age of

42 years old. At the time of the examination, he had spastic gait, however he reported that he could walk 1000 meters unaided. The lower limbs strength was 4/5 in proximal muscles and 5/5 in distal muscles, without muscle wasting. Tendon reflexes were brisk in all limbs with bilateral Babinski sign. He had no sensory deficit, and motor coordination and cranial nerve examinations were all normal. He had dysarthria and a subtle bilateral pes varus. Spinal cord and cerebral MRI were normal. Eye movement and fundus examinations were normal.

Blood tests were performed with the following results: normal sedimentation rate, complete blood count identified a moderate leukopenia (3900/mm^3), normal serum electrolytes and hepatic tests (bilirubin, transaminases, gamma-GT), moderate hyperlipidemia (Cholesterol - 2.73 g/l, triglycerides - 2.14 g/l), normal vitamin B12, B9 and vitamin E levels, moderately reduced vitamin B1 level (86 nmol/l), normal long chain fatty acids and phytanic acid levels. Immunoelectrophoresis of proteins and ponderal levels of immunoglobulins were normal.

Individual IV-5

Individual IV-5 presented with onset of spastic paraplegia at the age of 39 years. He was examined one year later at the age of 40. At the time of examination the individual had clear spastic gait with ataxia, however he could walk without aid. He had moderate dysarthria, normal strength in all muscles, and brisk tendon reflexes in all limbs. There was no muscle wasting in lower limbs. He had normal plantar cutaneous reflexes and normal sensory examination. He had a discrete upper limbs dysmetria and bilateral nystagmus. Eye movement and fundus examinations were normal. He had no skeletal deformities.

Individual IV-9

Individual IV-9 presented with onset of spastic paraplegia at the age of 24 with lower limbs stiffness when walking. At the time of examination, she was 30 years old. She had moderate lower limbs spasticity without any motor or sensory deficit, without muscle wasting. Tendon reflexes were brisk in all limbs with bilateral Babinski sign. There was no cerebellar or extrapyramidal signs. Eye movement and fundus examinations were normal. She had moderate bilateral pes cavus. The ENMG examination was normal but sympathetic skin reflex was normal in upper limbs and abolished in lower limbs (Table S1).

Family C

Individual IV-7

Individual IV-7 presented with onset of spastic paraplegia at the age of 33, with spastic gait and discoordination. His disease was slowly progressive, with little follow up. He was examined at the age of 35, and noted to have spastic gate, discoordination, bilateral Babinski sign, and ankle clonus. Cranial nerves examination was normal, including eye movement and fundus examination. Reported results from MRI were described as mild atrophy of cervical spinal cord. Individual IV-7 was lost of further follow-up.

Individual IV-13

Individual IV-13 presented with initial symptoms of spastic paraplegia at the age of 19, with mild lower limbs discoordination and weakness. At the first neurological examination, at the age of 22 years, she had normal motor examination with the exception of bilateral weakness (4/5) of the iliopsoas muscles. There was no muscle wasting. Sensory examination was normal. The gait was waddling, and she could not perform heel walk, and was unable to rise from a crouched position. Funduscopic examination and cranial nerves examination including eye movements were normal. Ten months prior to the first examination, she had CSF examination, myelogram and head CT, all of which were within the normal limit except for a slightly prominent sulci observed on the CT. At the time of recruitment, she was 31 years old, and presented with lower limbs spasticity, ataxic gait, poor balance and frequent tripping. Reflexes of the upper limbs were brisk and the lower limbs were spastic, with positive bilateral Babinski sign. Strength was 4/5 in both legs, and sensation was normal in both upper and lower limbs. She complained on urinary urgency and frequency, with stress incontinence, which deteriorated in the 3 years prior to the exam, and she also had pes cavus.

Supplemental figures and legends

Figure S1 - *clp-1*(RNAi) produces motor phenotypes and axonal degeneration in *C. elegans*

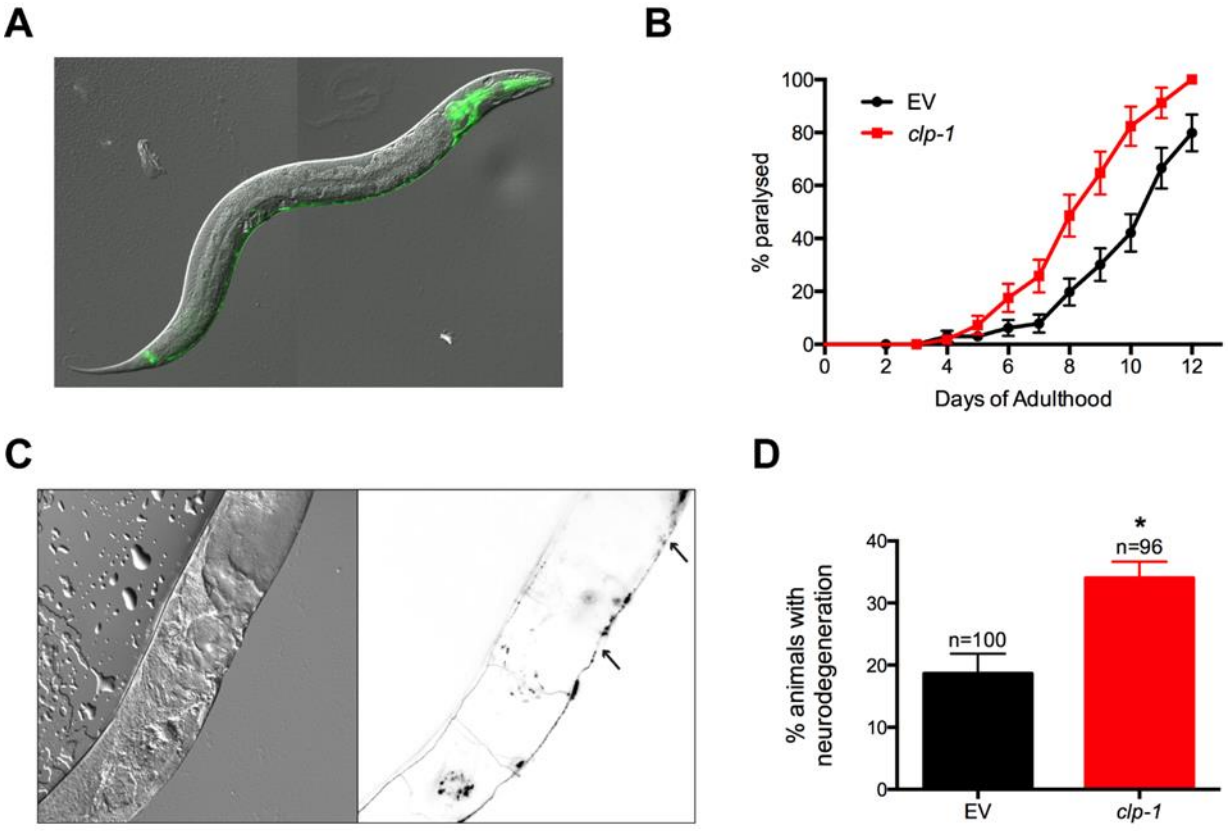

Figure S1 legend

clp-1*(RNAi) produces motor phenotypes and axonal degeneration in *C. elegans

The *C. elegans clp-1* encodes an orthologue of human calpain, and to investigate the contribution of *clp-1* to neuronal function, RNAi was used to knockdown the expression of endogenous *clp-1* in *C. elegans*. The *C. elegans strains* - XE137518 wpIs36 [unc-47p::mCherry] I; wpSi1 [unc-47p::rde-1::SL2::sid-1 + Cbr-unc-119(+)] II; eri-1(mg366) IV; rde-1(ne219) V; lin-15B(n744) X] were used for RNA interference (RNAi) assays. GABAergic neurons were visualized through and RNAi sensitivity only in GABAergic motor neurons was conveyed via wpSi1. Visualization of clp-1 expression was done using the transgenic GFP reporter strain BC1465819 dpy-5(e907) I;

sEx14658 [rCes C06G4.2b::GFP + pCeh361]. *RNAi procedures* - (RNAi)-treated strains were fed E. coli (HT115) containing an empty vector (EV) or clp-1(RNAi). RNAi clones were from the ORFeome RNAi library (Open Biosystems). RNAi experiments were performed at 20°C. Worms were grown on Nematode Growth Media enriched with 1 mM isopropyl-ß-D-thiogalactopyranoside. RNAi neurodegeneration tests were performed using the strain XE1375. Eggs were placed on plates with RNAi until day 5 of adulthood. Worms were transferred on fresh plates every 2 days. *Neurodegeneration assays* - For scoring of neuronal processes for gaps or breakage, XE1375 transgenic animals were selected at day 5 of adulthood for visualization of motor neuron processes in vivo. Animals were immobilized in 60% glycerol and mounted on slides with 2% agarose pads. mCherry was visualized at 585 nm using a Zeiss Axio Imager M2 microscope, using the Zen Pro 2012 software. Approximately 100 worms were scored per condition and each experiment was performed in triplicates. The mean and standard error of the mean were calculated for each trial and 2-tailed t tests were used for statistical analysis. Prism 5 (GraphPad Software) was used for all statistical analyses.

A. *clp-1*::GFP reporter shows expression in the pharynx, nerve ring, and along the ventral nerve cord. **B.** RNAi knockdown of the expression of endogenous *clp-1* in *C. elegans*. Worms treated with *clp-1*(RNAi) had motility defects, culminating in an age-dependent paralysis phenotype, which occurred at a higher frequency than control worms treated with empty vector (EV). RNAi *clp-1*(RNAi) (*clp-1*, n=62) enhances paralysis in a strain sensitized to RNAi only within the nervous system compared to empty vector (EV) controls (EV, n=72). Error bars represent SEM, ***P<0.001, log-rank (Mantel-Cox)-test. **C.** Visualization of neurodegeneration observed as gaps or breaks along neuronal processes (arrows) in a worm (*left*, differential interference contrast, *right*, inverted image from mCherry-fluorescence) in the nervous system exposed to *clp-1*(RNAi).

clp-1 (RNAi) treatment resulted in degeneration, observed as gaps or breaks along axonal processes and this occurred at a greater rate than compared to EV RNAi control worms **D.** Quantification of neurodegeneration in worms treated with EV (n=100) or *clp-1*(RNAi) (n=96) during development and examined at day 5 of adulthood. *P<0.05, unpaired t-test. Error bars represent SEM. Overall, these data suggest that *clp-1* protects the nervous system against dysfunction and degeneration.

Figure S2 - Pan neuronal expression of *calpain B* RNAi leads to defects in negative geotaxis in *Drosophila*.

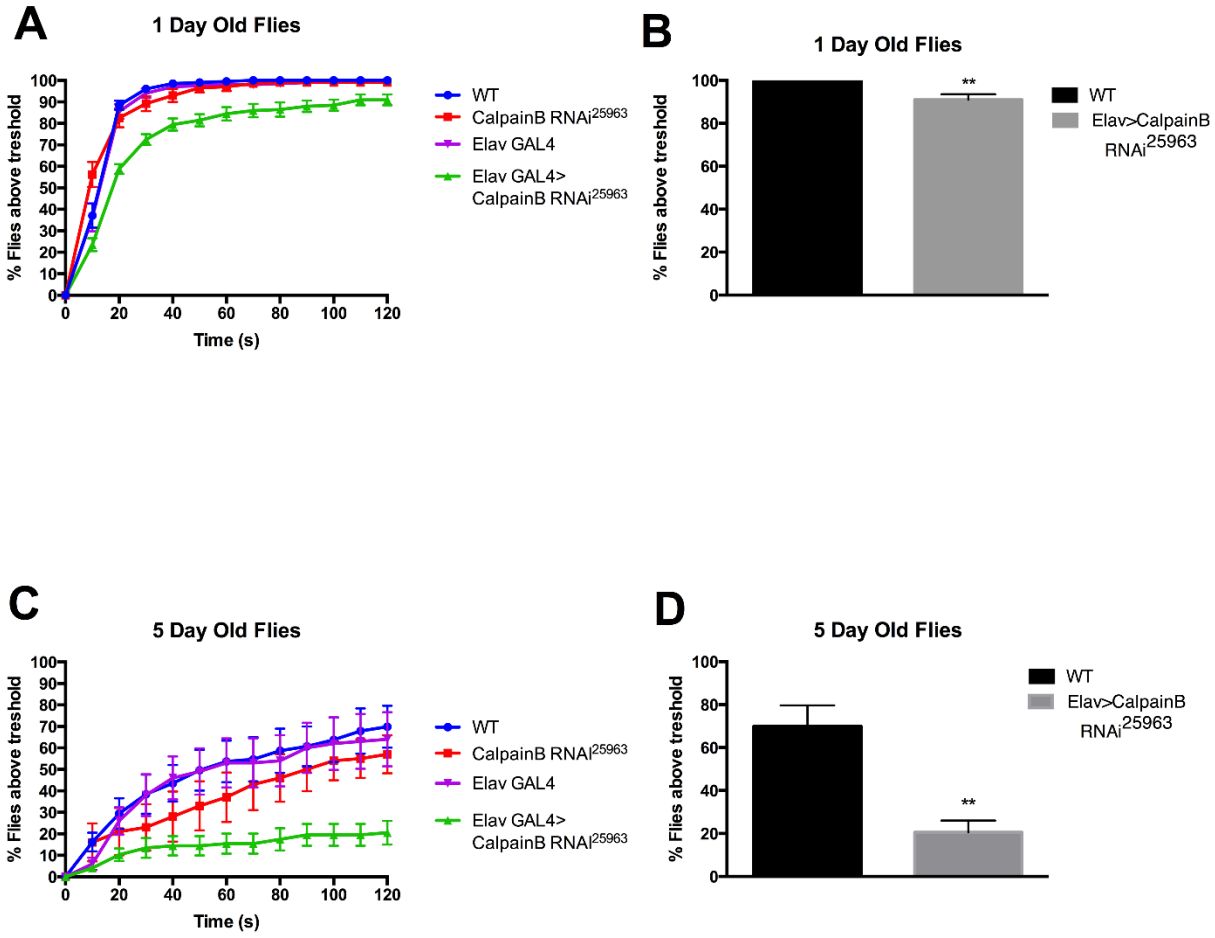

Figure S2 legend

Drosophila contains 3 paralogues of *CAPN1*. *Calpain B* has the highest homology with 45% blast identity versus 40% for *calpain A* and 28% for *calpain C* (Geneseer). Transgenic Drosophila containing the RNAi construct 25963 against Calpain B were obtained from the Vienna Drosophila Resource Center (VDRC). Pan-neuronal expression of the RNAi transgene was obtained by crossing the RNAi stocks to Elav-GAL4 homozygous flies. The flies were raised at 25°C. One day-old or 5 day-old flies were used for the climbing assay. Briefly, groups of 20 homogenous flies are tested at the time for their ability to climb in a glass cylinder above the 190mL line of a 250mL cylinder. The flies were filmed and then the percentage of flies that climbed above the

target line was plotted every 10 seconds, and results were plotted using Prism 5 (GraphPad Software). SigmaPlot 11.0 integrated with SigmaStat 3.1 was used to assess data groupings for significance. Statistical analyses used one-way ANOVA and Chi-square.

A. *Drosophila* transgenic for both the pan-neuronal driver ElavGAL4 and the responder UAS-calpain B RNAi present significant defect in their ability to climb above a reference target line when compared to the appropriate genetic controls (Elav GAL4 or UAS CalpainB RNAi. **B.** Climbing ability at 120 second for WT and Elav>CalpainB RNAi 25963 is significantly different (N=10, p=0.0064 t-test). **C.** Similarly, flies aged for 5 days show a more severe defect in climbing when compared to the same genetic controls. **D.** This defect is significant (N=5, p=0.0063 t-test).

Figure S3 - Pan neuronal expression of calpain B RNAi leads to defects in neuromuscular junction neurons.

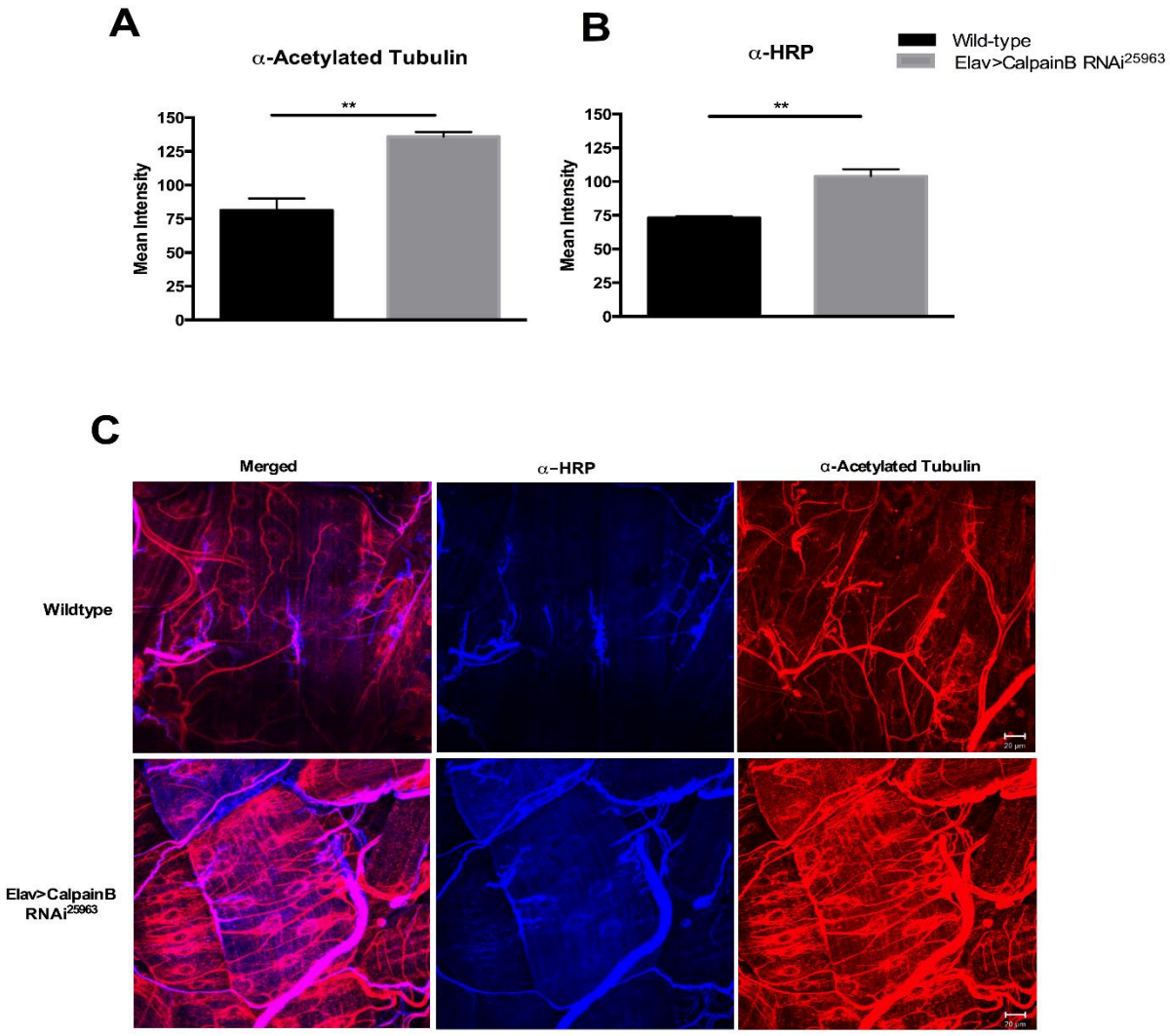

Figure S3 legend

Defects in axons were observed in transgenic flies expressing calpain B with the pan-neuronal driver Elav. Axons appeared of larger diameter and with increased level of acetylated tubulin. Increased acetylated tubulin is associated with hyperstabilization of microtubules and has been associated with *SPAST* mutations previously. Drosophila confocal imaging of the 3rd instar

neuromuscular junction was performed on third instar larvae. Antibodies against acetylated tubulin (Sigma T7451-200UL) and HRP (ThermoFisher PA1-26409) were utilized at a concentration of 1:1000. JacksonImmuno secondary antibodies Cy3 anti-mouse (115-165-003) and Cy5 anti-rabbit (JacksonIummuno 711-175-152) were purchased from Cedarlane and utilized at a concentration of 1:200.

Confocal imaging was performed using Zeiss LSM 700. Graph were made using data from mean intensity of the region of interest with Prism 5 (GraphPad Software). Image analysis was completed using ImageJ.

A. We observed a significant increase in the level of acetylated tubulin in the axons and muscles of the neuromuscular junction of larva expressing pan neuronally Calpain B RNAi. (N=3, P=0.0045, t-test). **B.** In addition, we observed that axons were larger on HRP immunohistochemistry suggesting larger fibers (N=3, P= 0.0039, t-test) **C.** Representative immunohistochemistry for the wild-type and pan-neuronal expressing Calpain RNAi larva. Interestingly, the acetylated tubulin were elevated and covered the entire diameter of large fibers compared to controls.

Figure S4 - Clusters of acetylated tubulin are present in capn1a morphants zebrafish

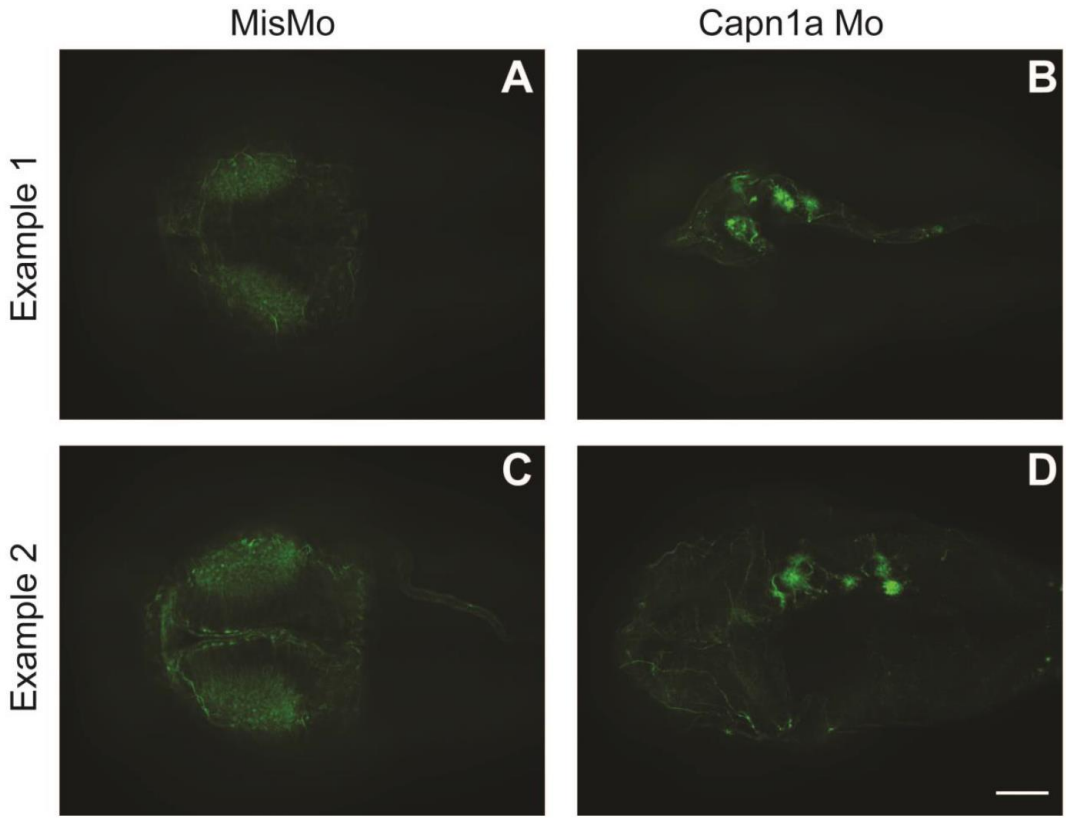

Figure S4 legend

Single slice images of acetylated tubulin staining in the brain of MisMo (**A, C**) and *capn1a* Mo (**B, D**) injected embryos. Images from 2 different embryos are shown for each condition, in order to show the variable clusters of acetylated tubulin observed in capn 1a morphants (**B, D**). Slices from the approximate same region of the brain are shown for the mismatch morphants (**A, C**). Scale bar is 60 μm.

Figure S5. *Capn1a* morpholino induces morphological defects and abnormal development in *Danio rerio*

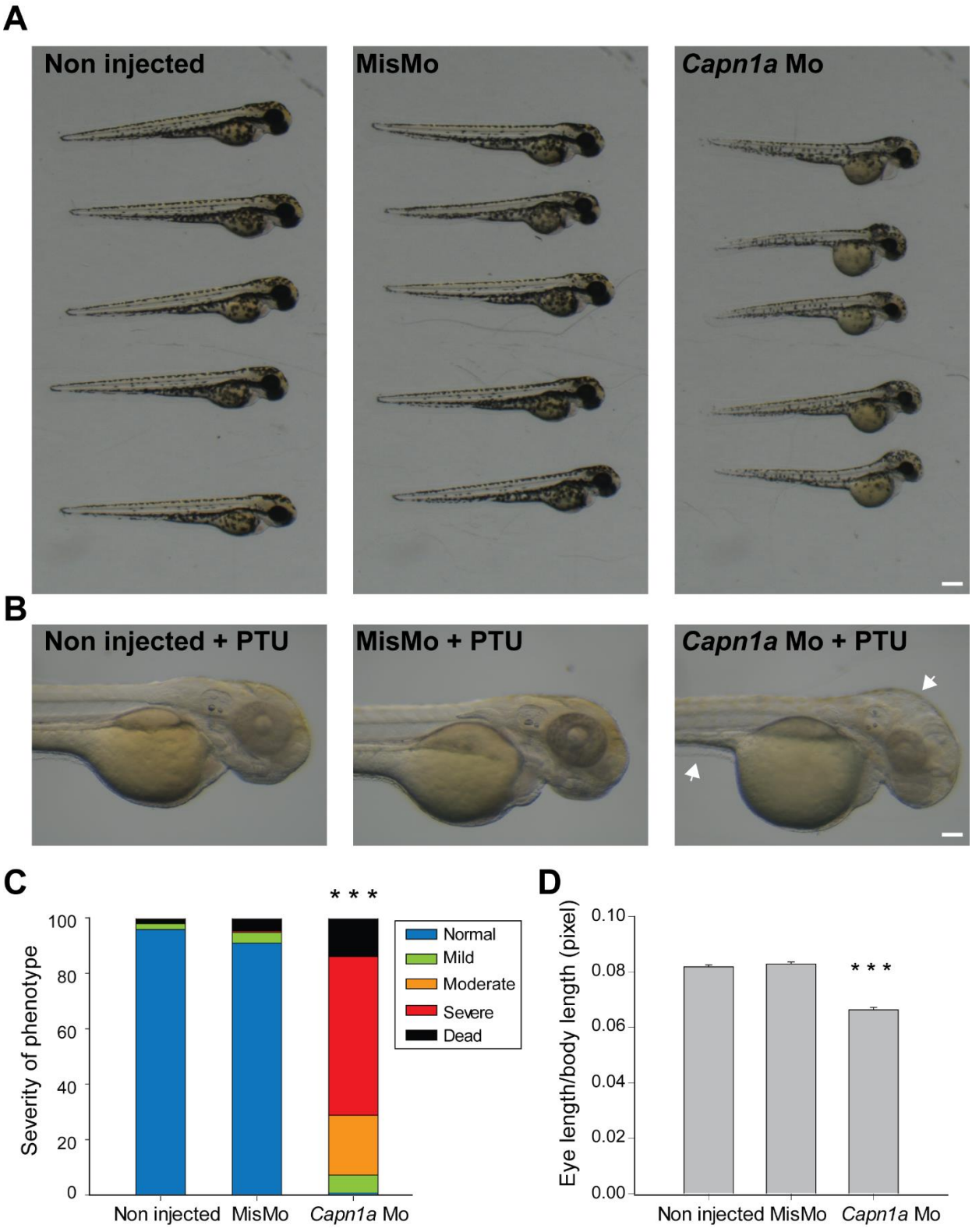

Figure S5 legend

Knockdown of zebrafish calpain1a and calpain 1b was achieved using morpholino oligonucleotides targeting the translation initiation site (*capn1a* Mo 5'-GAGATCCCGCCGTATGAGAACATGC-3'; *capn1b* Mo 5'-TCGACACACCTCCAATTGAAAACAT-3' Gene Tools, LLC). As an injection control, a mismatched *capn1a* morpholino with a five nucleotides substitution was used (MisMo 5'-GAcATCCaGCCcTATGAcAACATcC-3'). The morpholinos were diluted in deionized water with 0.05% Fast Green vital dye (Sigma) and 5 ng per embryo was pulse-injected into 1-4 cell stage embryos using a Picospritzer III pressure ejector. For this study, either AB-Tu wild-type or Islet1:GFP transgenic embryos were used. Embryos were kept until 5 days post-fertilization and the phenotype was quantified as follows: normal - no abnormal features or developmental delay; mild - absence of swim bladder; moderate - absence of swim bladder and presence of inflated pericardium; severe - absence of swim bladder, severely inflated pericardium with fluid accumulation around the yolk sac and extension. Eye phenotype assessment was performed using ImageJ 1.43m (NIH); the eye length was measured in the longitudinal plan, and divided by the length of the body, measured from the tip of the tail, until the frontal-most part of the head. Six hours post-fertilization embryos were treated with 0.2mM 1-phenyl 2-thiourea (PTU) until imaged, in order to prevent pigmentation from appearing. Two days post fertilization (dpf) embryos were anesthetized in 0.04% tricaine (Sigma) and mounted in 3% methylcellulose on a depression slide. The hindbrain of these embryos was visualized with a 10× water-immersion lens. Acetylated tubulin staining was performed as previously published. Briefly, 2 dpf embryos previously treated with PTU were fixed in Dent's fixative (80% methanol, 20% DMSO) overnight at 4 °C and washed 3X 10 minutes in 1% PBST. Embryos were blocked for one hour at room temperature in PBST

with 2% BSA and 2% normal goat serum, followed by an overnight incubation at 4°C with anti-acetylated tubulin monoclonal antibody (Sigma) in block (1:500). The next day, the embryos were washed 3X 10 minutes in PBST, then blocked for 1 hour, followed by a 4 hours incubation with a goat anti-mouse secondary antibody conjugated with Alex Fluor 488 (1:1,000). Embryos were then washed in PBST overnight, cleared in 80% glycerol and deyolked before being mounted either dorsal up, or on the side and imaged with a 10X air objective. Embryos were imaged with a Quorum Technologies spinning disk confocal microscope with a CSU10B (Yokogawa) spinning head mounted on an Olympus BX61W1 fluorescence microscope and connected to a Hamamatsu ORCA-ER camera and acquired using Volocity software (Improvision). The images were then processed using ImageJ 1.43m (NIH). Z-projections of confocal images are shown, except when otherwise specified.

A. Morphological features of 2 days post fertilization (dpf) embryos either not injected, injected with a mismatch morpholino (MisMo), or with a morpholino against *calpain 1a* (*Capn1a* Mo). Five representative embryos are shown for each condition. Scale bar is 250 μm. **B.** Non injected, MisMo injected and capn1a Mo injected embryos were treated with 1-phenyl 2-thiourea (PTU) to prevent pigmentation from developing. Arrows indicate defects in the head (hydrocephalus) and yolk sac extension. Scale bar is 50 μm. **C.** Quantification of severity of phenotype displayed at 5 dpf by non-injected (n=189), MisMo injected (n=209) and *capn1a* Mo (n=236) larvae. ***$p<0.001$, chi-square test. **C.** Ratio of the eye longitudinal length with the body length of 2 dpf non injected (0.0818, n=91), MisMo injected (0.0829, n=60) and *capn1a* Mo (0.0663, n=79) embryos. ***$p<0.001$, one-way ANOVA. Error bars represent standard error.

Figure S6 - Western-blot analysis of calpain 1 expression levels in zebrafish

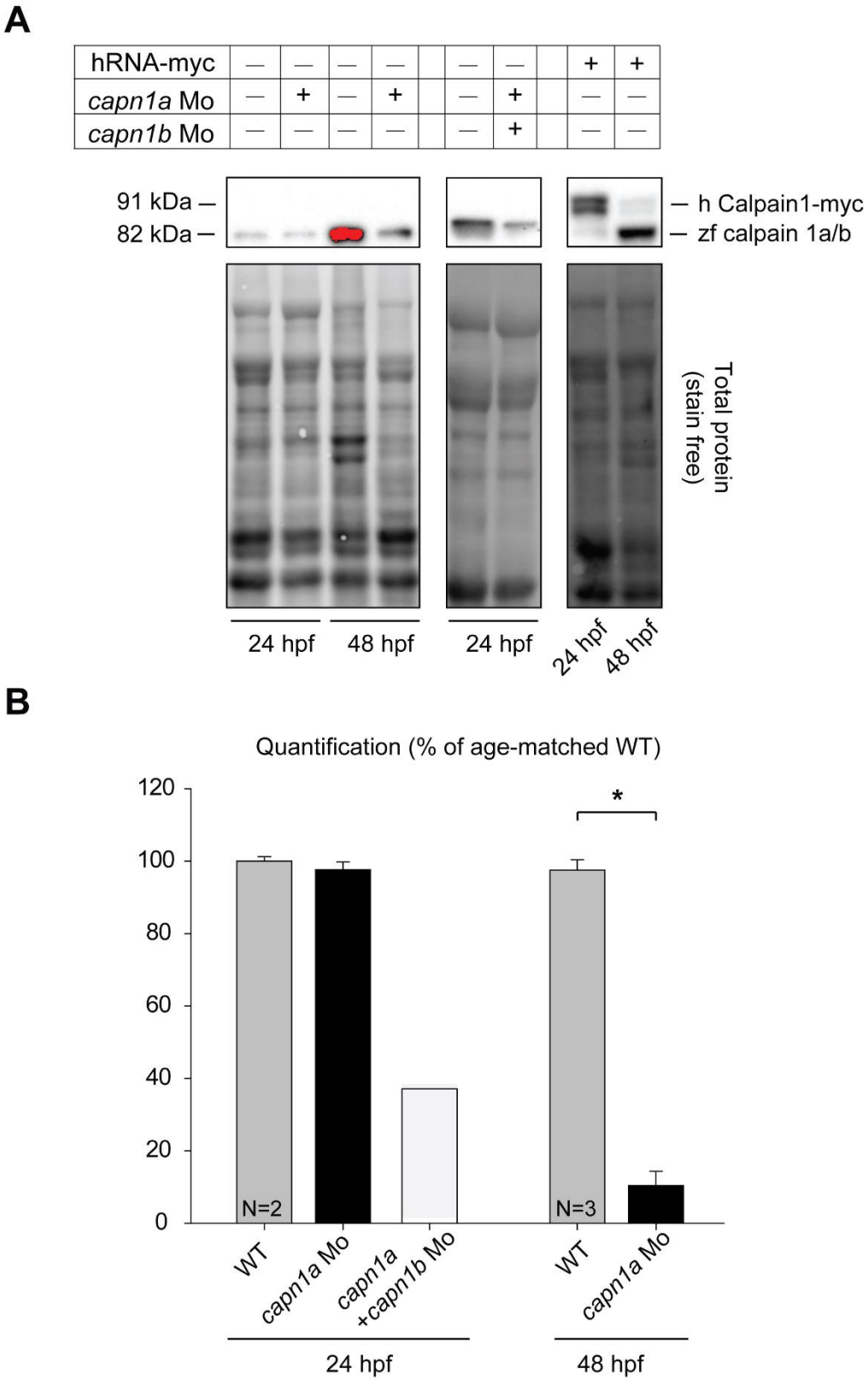

Figure S6 legend

About 30 embryos at 24 and 48 hours post-fertilization were lysed in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1 % Triton X-100, 0.1 % SDS, 1 % Na deoxycholate, 0.1 % protease inhibitor), maintained on ice and homogenized with a hand-held pestle. The lysates were centrifuged for 10 minutes at 10,000 rpm at 4°C and the supernatant was collected. Protein concentration was established using Bio-Rad DC Protein Assay and 60 µg of proteins were loaded in 2X Lämmli buffer after boiling the samples for 5 minutes at 95°C. TGX Stain-Free FastCast acrylamide Kit (Bio-Rad) was used to make 10% Tris-glycine. After electrophoresis, the gels were activated by exposure to UV for one minute, and transferred to either regular or low-fluorescence PVDF membrane (Bio-Rad). After transfer, total proteins on membranes were detected by UV. Membranes were then blocked for one hour using 5 % fat free milk in PBST. The primary antibody (Calpain 1, 1:1000, Novus Biological NB120-3589) was incubated overnight at 4°C in 5 % fat free milk in PBST. After washing 3x 5 minutes in PBST, the membrane was incubated with an anti-mouse IgG, HRP conjugated (1:5000, Jackson Immuno) in 5 % fat free milk in PBST for one hour at room temperature, and then washed 3x 5 minutes. Membranes were exposed to Clarity enhanced chemiluminescence (ECL, Bio-Rad) for 5 minutes at room temperature and visualized using a ChemiDoc MP (Bio-Rad). Detection and quantification of band intensities was conducted using Image Lab 5.2 software (Bio-Rad). Bands were normalized to total protein by dividing the intensity of the band by the intensity of the total proteins from the same samples on the same blot, and the ratio was expressed as percentage of the age-matched wild-type control. Student's t-test was used to compare samples with their age-matched control. A value of $p < 0.05$ was considered statistically significant. **A.** Western blot analysis of calpain 1 in zebrafish at 24 hours post-fertilization (hpf) and 48 hpf without or with capn1a Mo knock down (first panel, the 48 hpf wild-type condition is saturated on purpose. This exposure time is not used for quantification); without

or with a double knock down of capn1a and capn1b (second panel); and following injection of 500 pg of human CAPN1 mRNA with 6x myc-tags, at 24 and 48 hpf (third panel, hRNA-myc). Total protein visualized by the stain-free technology is shown in the lower panel. **B.** Quantification of the western blots shown in A. The results are expressed as mean +/- SEM, * $p<0.05$. h: human; zf: zebrafish; hpf: hours post-fertilization; WT: wild-type.

Supplemental tables

Table S1 – primers used for amplification and sequencing of *CAPN1* mutations

Variant	Forward	Reverse	Tm (c)	Length (bp)
p.Pro136Argfs*40	AACCCCAGATTCTCCCTAGC	GCAGGTATTGTGGGTCATCC	61	273
c.1605+5G>A	CTGGAGTCTGGGTCTGGG	ACTCAGGGGACAGGACACC	61	527
p.Gln527*	CTGGAGTCTGGGTCTGGG	ACTCAGGGGACAGGACACC	61	527
p.Arg295Pro	AAGAGAATTGAATTGCTTGAACC	CGAGCAGAAATGTCGGG	61	316

c, Celsius; bp, base-pares

Table S2 – Genetic data on exome sequencing results

Family	Family A	Family B	Family C
Number of individuals sequenced	3	4	2
Average coverage	133X	130X	121X
All variants	215843	250171	168177
Coding/splicing [a]	37430	39414	28903
NS/SS/Indels [b]	16637	5400	15161
MAF 1KG [c]	2425	1841	2275
MAF EVS [d]	538	465	1815
Absent from In-house database	158	276	377
Common to affected	27	25	133
Genes with homozygous/compound heterozygous mutations	2	1	3
Deleterious [e]	1	1	1

[a] Filtering out variants that are not in the coding sequences or in the 6 base-pairs before and after the exon.

[b] Filtering out synonymous variants and including only non-synonymous/stop (NS), splice site (SS) and insertions and deletions (Indels).

[c] Including variants that had minor allele frequency (MAF) < 0.005 in the 1000 genome project (1KG) database.

[d] Including variants that had minor allele frequency (MAF) < 0.005 in the exome variant server (EVS) database.

[e] Predicted to be deleterious and conserved by SIFT, PolyPhen 2, MutationTaster, PhyloP and GERP++.

Table S3 - Nerve conduction study data

	Family B Individual IV-1	Family B Individual IV-2	Family B Individual IV-9	Family A Individual V-2	Normal limits
Median nerve	L	L	L	NA	
DML (ms)	3,5	3,6	3,1		< 4
CMAP amplitude (mV)	9,4	9,1	9,6		>4,8
MNCV m/s	60	62	58		> 47
FWL (ms)	28,8	23,3	23,7		<33
Ulnar nerve	R	R	R	NA	
DML (ms)	3,1	2,4	2,8		<3,2
CMAP amplitude (mV)	8,1	11,5	11,2		>4,8
MNCV m/s	58	63	60		> 47
FWL (ms)	28,6	25	25,8		<33
Peroneal nerve	L	L	R	R	
DML (ms)	6,3	5,6	4,9	3,8	<6,5
CMAP amplitude (mV)	2,5	2,8	6,9	1,9	>2
MNCV m/s	43	49	50	48,5	>40
FWL (ms)	52,7	47,7	46,9	40,6	<52
Tibial nerve	R	R	NA	NA	
DML (ms)	5	3,6			<6
CMAP amplitude (mV)	4,3	5,3			>3,5
MNCV m/s	46	50			>40
FWL (ms)	50,5	45,2			<52
Median nerve	R	L	L	NA	
SNAP amplitude (uV)	26,3	31	30		>12
SCV (m/s)	NA	47	50		>40
Ulnar nerve	R	R	R	NA	
SNAP amplitude (uV)	11,8	12	16		>8
SCV (m/s)	NA	55	NA		>40
Sural nerve	R L	R L	R L	R L	
SNAP amplitude (uV)	5,6 3,8	5,3 5,4	26 26	16 21	>10
SCV (m/s)	38 39	40 41	32 33	45 36	>35
Upper limbs	L	L R	L R	NA	
Latency (s)	1,7	1,39 1,35	1,23 1,38		Positive
Lower limbs	L	L R	L R	NA	
Latency (s)	NO	NO NO	NO NO		Positive

DML: distal motor latency; CMAP: compound muscle action potential; MNCV: motor nerve conduction velocity; FWL: F wave latency; SNAP: sensory nerve action potential; SCV: sensory conduction velocity; NA: not available; NO: not obtained; R: right; L: left.