The microtubule-severing protein Spastin is essential for axon outgrowth in the zebrafish embryo

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ABSTRACT

Hereditary spastic paraplegia (HSP) is a collection of neurological disorders characterised by developmental failure or degeneration of motor axons in the corticospinal tract and progressive lower limb spasticity. SPG4 mutations are the most common cause of autosomal dominant HSP and spastin (the SPG4 gene product) is a microtubule severing protein that shares homology with katanin, the microtubule severing activity of which promotes axon growth in cultured neurons. Given the sequence and functional similarity between spastin and katanin, we hypothesised that spastin promotes the dynamic disassembly and remodelling of microtubules required for robust, properly directed motor axon outgrowth. To investigate this hypothesis, we cloned the zebrafish spg4 orthologue and used morpholino antisense oligonucleotides directed against the translation start site and the intron 7-8 splice donor site to knock down spastin function in the developing zebrafish embryo. Reduced spg4 function caused dramatic defects in motor axon outgrowth without affecting the events driving the initial specification of motor neurones. Other neuronal subtypes also exhibited a requirement for spg4 function, since spg4 knock down caused both widespread defects in neuronal connectivity and extensive CNS-specific apoptosis. Our results reveal a critical requirement for spastin to promote axonal outgrowth during embryonic development, and they validate the zebrafish embryo as a novel model system in which to dissect the pathogenetic mechanisms underlying HSP. Taken together with other recent studies, our findings suggest that axon outgrowth defects may be a common feature of childhood SPG3A and SPG4 cases.
Introduction

The hereditary spastic paraplegias (HSP) are a collection of neurodegenerative diseases which are characterised by progressive lower limb spasticity, and caused by developmental failure or degeneration of motor axons in the corticospinal tract (1, 2). More than 20 genetic loci have been identified, several of which encode proteins involved in intracellular transport and trafficking (1, 2). Mutations in the *SPG4* gene are the most common cause of autosomal dominant HSP and spastin (the *SPG4* gene product) is an AAA protein (ATPases associated with diverse cellular activities) that is most closely related to the microtubule-severing protein katanin. Like katanin, recombinant spastin possesses ATPase activity and severs microtubules in vitro (3-5).

The vast majority of *SPG4* mutations are either nonsense, frameshift or splice site mutations (these are distributed throughout the gene), or missense mutations in the AAA cassette, all of which act dominantly and are likely to cause loss of function of the mutant allele. The sole exception is the S44L mutation, which exhibits a recessive mode of inheritance (6). The age of onset and rate of symptom progression vary widely among and within families with *SPG4* mutations (7), but independent inheritance of an AAA domain mutation with an S44L or P45Q allele is associated with severe infantile HSP (8, 9). Two recent studies have demonstrated that *Drosophila* Spastin regulates synaptic microtubule stability, thereby modulating glutamatergic synapse structure and function (10, 11). Taken together, these studies lend support to the idea that haploinsufficiency for *SPG4* impairs neuronal microtubule dynamics and neuronal development or maintenance. Another recent study in *Drosophila* suggests that certain spastin point mutants can act in a dominant-negative manner (12).
Dynamic remodelling of microtubule structure is essential for axon extension, arborisation and growth cone motility (13, 14), and enzymes that break down microtubules perform critical roles in these processes. For example, the atypical kinesin KIF2A depolymerises microtubules at the growth cone edge and plays an important role in regulating collateral branch extension (15). In isolated primary neurones, the microtubule-severing activity of katanin regulates axon growth by untethering microtubules from the centrosome and controlling their number and length in the neuronal cytoplasm (16, 17). This severing activity generates short microtubules that can be efficiently transported and remodelled, thereby facilitating axon growth.

Given the sequence and functional similarity between spastin and katanin, and the apparent requirement for \textit{SPG4} function in motor neurones, we hypothesized that spastin promotes motor axon outgrowth and target innervation via a mechanism that facilitates dynamic remodelling of the microtubule network. We have tested this hypothesis by cloning the zebrafish \textit{spg4} orthologue and microinjecting \textit{spg4}-specific and missense control morpholino antisense oligonucleotides (MO) into one-cell zebrafish embryos to specifically knock down Spastin function. Our results show that reduced Spastin function severely impairs motor axon outgrowth and they establish a clear role for \textit{spg4} in promoting the remodelling of microtubule structure that is essential for axon growth and arborisation. These findings provide new and important insights into the nature of the molecular defects causing the most common form of HSP.
Results

Identification of the zebrafish spastin orthologue

A full-length zebrafish spg4 EST clone (fo75d06.y1) was identified in the IMAGE collection using BLAST searches, and its complete DNA sequence was determined (GenBank accession number AY304504). The zebrafish genome appears to encode a single spastin orthologue, which is 60.9% identical to human spastin. Supplementary Fig. 1 shows an alignment of human, mouse, chicken, Xenopus, zebrafish and Drosophila spastin proteins. The MIT (microtubule interacting and trafficking molecule) and AAA domains (residues 116-194 and 377-562 respectively of human spastin) are highly conserved across all species. Zebrafish spg4 was mapped 13cR from marker z25625 on linkage group 1 with LOD score 9.003 using radiation hybrid instant mapping (see http://zfrhmaps.tch.harvard.edu/ZonRHmapper/instantMapping.htm). This map position has been confirmed by whole genome shotgun sequencing, which has designated spg4 as Ensembl gene ENSDARG00000024933. Like the human gene, zebrafish spg4 contains 17 exons and all the positions of the exon-intron boundaries are conserved. RNA in situ hybridisation demonstrated that zebrafish spg4 is maternally expressed and transcripts are distributed at low levels throughout the embryo up to 24 hours post-fertilization (hpf; Supplementary Fig. 2). RT-PCR confirmed spg4 transcripts were present in 1-2 cell, 1000 cell, 75% epiboly, 10 somite and 26 somite embryos at low levels (data not shown). Over-expression of human spastin has been shown to cause disassembly of the microtubule network in cultured cells (3, 18, 19) and we confirmed that the zebrafish spastin orthologue had the same effect when over-expressed in HEK293 cells (Supplementary Fig. 3).
Branchiomotor neurones of spg4-deficient embryos exhibit reduced axonal outgrowth and aberrant positioning of neuronal cell bodies

The dominant mode of inheritance of SPG4 HSP may result from haploinsufficiency of wild-type SPG4 in heterozygous individuals, as a consequence of loss-of-function mutations in SPG4. Independent inheritance of an AAA domain mutation with an S44L or P45Q allele is associated with severe infantile HSP (8, 9), consistent with neurodevelopmental defects. The related microtubule-severing protein katanin has been shown to regulate axon outgrowth in cultured neurones (16, 17). Therefore, in order to determine whether reduced levels of spastin could affect axonal outgrowth by branchiomotor neurones, we microinjected a morpholino designed to specifically inhibit translation of spg4 mRNA (spg4atg1) into Islet1:GFP transgenic embryos, in which GFP expression is targeted to branchiomotor neurones (20). Injected embryos were fixed at 30 and 36 hpf, and GFP-expressing branchiomotor neurones were visualised by confocal microscopy. At 30 hpf, the nV (trigeminal) and nVII (facial) motor neurones of spg4 morphant embryos (Fig. 1A) were much shorter than those of embryos injected with a control morpholino (Fig. 1B). By 36 hpf, axonal projections from nV and nVII motor neurones were fewer, shorter and more disordered than those of embryos injected with the control morpholino (Fig. 1C,D). Aberrant positioning of branchiomotor neurone cell bodies was also observed in spg4-deficient embryos, suggesting that spg4 function may also be required for correct migration of some neuronal populations during development.

Spastin is required for spinal motor neurone axon outgrowth

In order to determine whether reduced levels of spastin could also affect growth of spinal motor neurone axons, embryos were microinjected at the one-cell stage with
either spg4-specific (spg4atg1) or a mismatch control (spg4CoMO) morpholino and analysed by immunostaining with the monoclonal antibody znp-1, which reveals the overall morphology of differentiating spinal motor neurones. Injected embryos were also immunostained with monoclonal antibody 39.4D5, which recognises the Islet-1 transcription factor in the nuclei of motor neurones and Rohon-Beard sensory neurones and is an early marker of their specification. Immunostaining for Islet1 revealed that both motor neurones and Rohon-Beard cells were properly specified in spg4-deficient embryos (Fig. 2C,D). However, analysis with znp-1 revealed that inhibition of spg4 dramatically impaired outgrowth of motor axons from the spinal cord (Fig. 2A,B). Immunostaining with znp-1 also revealed reduced axonal staining in the spinal cord (Fig. 2A,B), suggesting a possible requirement for spg4 function in spinal interneurones. Similarly, longitudinal fascicles in the hindbrain were disordered and less intensely stained in spg4 morphants compared to control embryos (data not shown).

One possible consequence of the impaired axonal outgrowth observed in spg4-deficient embryos, is that neurones could fail to find their trophic targets and undergo programmed cell death. We therefore reasoned that significant levels of apoptosis might accompany axon growth defects caused by reduced spg4 function, and so spg4atg1- and control-injected embryos were analysed by the TUNEL method. A substantial amount of apoptosis was detected in the CNS of spg4 morphant embryos in comparison to that observed in control-injected embryos (Fig. 2E,F; Table 1; Supplementary Fig. S4). Consistent with this observation, the number of Islet1-positive spinal motor neurones was approximately 20% less in spg4 morphant embryos than in controls (Table 2). Moreover, the apoptosis-inducing effect of spg4...
knock down was limited to the developing CNS, demonstrating that spastin activity is specifically required to maintain the viability of neural cells.

To confirm that the observed effects were specifically caused by reduced spg4 activity, zebrafish embryos were microinjected with a second spg4-specific morpholino targeting the intron 7-8 splice donor site (spg4exon7). As with spg4atg1-injected embryos, znp-1 immunostaining revealed fine, truncated spinal motor neurone axons in spg4exon7-injected embryos (Fig. 3B). Translation blocking morpholinos target both maternal and zygotic transcripts, whereas splice blocking morpholinos target only zygotic mRNA. Correspondingly, a larger dose of the spg4exon7 morpholino (1.2 pmol) was required to produce a phenotype comparable to that obtained with 0.6 pmol of spg4atg1 (Table 3). Spg4exon7 morpholino-injected embryos also exhibited CNS-specific apoptosis (data not shown).

spg4 transcripts are distributed throughout the embryo at low levels (Supplementary Fig. 2) and we were unable to detect endogenous spastin in embryo extracts by immunoblotting with a well characterised anti-human spastin antibody (21). This antibody detects zebrafish spastin when over-expressed in transfected mammalian cells, suggesting that very low levels of spastin protein are present in the early embryo (MB and JDW unpublished). RT-PCR was therefore used to assess the degree of spastin knock down resulting from microinjection of the spg4exon7 morpholino. This morpholino was predicted to cause skipping of exon 7 so PCR primers were designed to amplify across exons 5-9. RT-PCR analysis using cDNA prepared from control embryos yielded a single product of the predicted size (466 b.p.; Fig. 3C), while cDNA from embryos injected with spg4exon7 yielded additional products at 372 b.p. (predicted size if exon 7 skipped) and around 1386 b.p. (predicted size after inclusion of intron 7-8). Embryos injected with 0.6 pmol of spg4exon7
morpholino retained a significant proportion of the correctly processed message and did not show severe axon outgrowth defects (Fig. 3A). In contrast, embryos injected with 1.2 pmol of spg4exon7 morpholino showed only a trace of products derived from the normal transcript and exhibited pronounced axon outgrowth defects (Fig. 3B).

To further assess whether the observed effects of the morpholinos were specifically caused by targeted knock down of the same spg4 transcripts, spg4atg1 and spg4exon7 morpholinos were co-injected at doses that did not markedly perturb motor axon outgrowth when either morpholino was injected singly (Table 4, Figure 4). Thus, injection of 0.3pmol spg4atg1 inhibited motor axon outgrowth in only 13% (n=196) of embryos, and injection of 0.9pmol spg4exon7 inhibited motor axon outgrowth in only 21% (n=200) of embryos, whereas co-injection of both morpholinos together caused a substantial inhibition of motor axon outgrowth in 46% (n=176) of injected embryos (Table 4; Figure 4). Embryos co-injected with low doses of both morpholinos also exhibited CNS-specific apoptosis (data not shown).

**Motility defects in spg4 morphant embryos**

The severe neurodevelopmental defects in spg4 morphant embryos were correlated with a loss of transparency of hindbrain tissue at 24 hpf that coincided with the appearance of CNS-specific cell death by TUNEL labelling (Fig. 2F; Table 1). To determine whether morphant embryos exhibited motor impairment at later stages of development, morpholino-injected embryos were allowed to develop to 5 days post-fertilisation and motility defects were scored (Table 5). At 5 days, 68% of surviving spg4atg1 (0.6 pmol) and 58% of spg4exon7 (1.2 pmol)-injected embryos were immotile and failed to hatch, compared to 7% of CoMO (1.2 pmol)-injected embryos. Less severely affected embryos (11% of spg4atg1-injected and 27% of spg4exon7-
injected embryos) did hatch and showed reduced motility or impaired swimming at 5 days post-fertilisation. Representative bright field images of a normal control-injected and impaired spg4 morphant embryo at 5 days are shown in Fig. 5.

**Reduced spg4 function disrupts axonal microtubule networks**

To provide further insight into how reduced spg4 function causes axon growth defects, axonal microtubule networks were compared in spg4 morphant and control morpholino-injected embryos. Embryos were fixed under conditions designed to preserve microtubule integrity and stained with an anti-acetylated-tubulin monoclonal antibody. Confocal z-series of images taken laterally through the trunk were then projected to visualise the microtubule network in spinal neurones. In control morpholino-injected embryos, microtubule staining demonstrated that spinal motor neurones followed a curvilinear path along the centre of each somite, projecting into an extensive network of dorsal connections with spinal interneurons, and terminating ventrally on muscle fibres (Fig. 6A). In contrast, however, staining for microtubules in the trunk of spg4 morphant embryos demonstrated that spinal motor neurone axons were shorter than those of control embryos and they did not appear to form proper connections with spinal interneurones (arrows in 6B). Moreover, spastin-deficient embryos exhibited a complete absence of longitudinal fascicles (arrowheads in 6A) in the spinal cord and thickened bundles of axonal microtubules were apparent in the spinal cord (arrowhead in 6B). Consequently, connectivity between motor neurone and interneurone axons, as well as between interneurone axons within the spinal cord, appears to be considerably reduced by loss of Spastin function (Fig. 6B).

**Effect of Spastin-deficiency on neuromuscular synapse formation**
The substantial inhibition of spinal motor and branchiomotor axon outgrowth that was observed in spg4 morphant embryos suggested that neuromuscular synapse formation might also be affected in spg4 morphant embryos. To address this possibility, spg4 and control morpholino-injected embryos were fixed at 48 hpf and double-labelled for confocal microscopy with znp-1 and FITC-conjugated α-bungarotoxin, to identify the axons of spinal motor neurones and the clusters of post-synaptic acetylcholine receptors (AChR) in target muscle cells, respectively (Fig. 7). In control-injected embryos, each znp-1-positive spinal motor axon fascicle could be clearly distinguished at 48 hpf with an extensive set of collateral branches. Moreover, znp-1 staining strongly co-localised with the clusters of post-synaptic acetylcholine receptors, apart from in the nascent, distal portions of the collateral axonal branches, which were znp-1-positive (red) but not associated with AChR (green). By contrast, in spastin-deficient embryos, znp-1 staining was greatly reduced in the spinal cord and ventrally descending motor fibres at 48 hpf, which was consistent with the weak znp-1 staining also observed in spg4 morphant embryos at 28 hpf (Fig. 2B). In spite of the reduction in motor axon outgrowth, muscle fibres of spastin-deficient embryos still stained strongly with FITC-α-bungarotoxin, and much of this staining was clustered into discrete domains within muscle cells. The positions of these patches of clustered AChRs coincided with areas of weak znp-1 positivity, emphasising the presynaptic nature of the defect in motor axon outgrowth.

Discussion

Analysis of spastin function in vitro (3, 4) and in cultured cells (18, 19) reveals that spastin, like katanin, is a microtubule severing enzyme. Studies of spastin function in the Drosophila larva have confirmed these observations and also demonstrated that
spastin regulates synaptic microtubule stability and thus modulates glutamatergic synapse structure and function (10, 11). In light of the fact that the closely related microtubule severing enzyme katanin regulates axonal growth (16, 17), we reasoned that in addition to modulating the structure and function of the neuromuscular junction, spastin may also be important for axonal morphogenesis and function in vertebrates. We therefore identified the zebrafish spg4 orthologue and used morpholino antisense oligonucleotides to knock down spastin function in the developing zebrafish embryo.

Consistent with our hypothesis, reduced spastin activity severely impaired outgrowth of spinal and branchiomotor neurone axons (Figs. 1-4). Other neuronal populations were clearly affected, since significant levels of cell death were observed throughout the CNS and the axonal networks were disorganized and incomplete throughout the spinal cord (Fig. 6). Whilst the TUNEL assay revealed a substantial amount of apoptosis in the CNS, Islet1 immunostaining showed that spinal motor neurones were relatively spared from cell death, although there was an approximately 20% reduction in the overall number of spinal motor neurones. We infer from this that other cell types, such as interneurones and neural progenitors also succumb to apoptosis as a result of reduced Spastin function. Neural cell apoptosis in spg4 morphants could be due to a direct requirement for Spastin to maintain neuronal viability, or a consequence of impaired axon outgrowth and failure to find trophic targets. Similarly correlated effects on retinal axon outgrowth and neural retina survival were observed in Cdhl knock down zebrafish embryos (22). SPG4 mutations normally cause pure HSP (spastic paraplegia with no additional symptoms), but a number of families with complicated HSP, where lower limb spasticity is accompanied by other neurological symptoms, such as cerebellar ataxia, epilepsy and
cognitive decline, have also been documented (23-25). Spastin may therefore be important for the functioning of multiple neuronal subtypes, consistent with our observation of an apparent requirement for spastin function in neurones of non-motor identity.

Dynamic microtubules are required for many aspects of axonal development such as extension, guidance, growth cone motility, and branching (14, 26, 27). Microtubule severing is prominent at the neuronal centrosome (28), axonal branch points (29) and in growth cones (30), suggesting that microtubule severing is fundamental to many aspects of axonal morphogenesis. Our data show that spastin is required for motor axon morphogenesis and function, and they imply that microtubule severing may be a limiting factor in production of the dynamic microtubules that drive axon outgrowth. In neurons, katanin is required to release microtubules from the centrosome and it may also play a role in regulating the length of tubulin oligomers so that they can be efficiently transported into axonal processes (16). Given that spastin has been reported to interact with a centrosomal protein (31), it is possible that spastin acts in a similar manner to katanin. However, spastin has also been reported to localise to distal axons of immortalised motor neurons (31), which suggests that spg4 knock down could directly inhibit growth cone motility. Therefore impaired growth cone motility may also contribute to the axon outgrowth defects observed in spastin-deficient embryos.

Our results demonstrate that reduced spg4 function in the zebrafish embryo severely impairs early neural development. By contrast, mutation of SPG4 in humans typically causes adult onset neurodegenerative disease, with the age of onset being highly variable both among and within families (7). However, the vast majority of HSP-inducing SPG4 mutations are dominant and experimental evidence supports the
view that haploinsufficiency is a likely pathogenetic mechanism (32). Consequently, further analysis of the spastin-deficient phenotype we describe here at the molecular level should provide additional insights into the nature of the defects in HSP.

Interestingly, the S44L mutation has been reported to act in an autosomal recessive fashion (6) and co-inheritance of this allele or an allele with a mutation of the adjacent residue (P45Q) with a missense or splicing mutation in the AAA domain of the other SPG4 allele leads to a striking decrease in the age of onset. All such compound heterozygotes reported to date had infantile or childhood onset spastic paraplegia (8, 9), consistent with a neurodevelopmental defect. RT-PCR analysis of zebrafish embryos injected with a splice-blocking morpholino demonstrated that near total spg4 knock down was required to obtain a severe axon outgrowth phenotype (Fig. 3C). We therefore suggest that S44L and P45Q alleles partially reduce spastin activity or levels and more than a 50% decrease in spastin activity leads to developmental defects in axon outgrowth, thereby causing infantile disease, whereas a 50% decrease in spastin activity is sufficient for relatively normal CNS development but specifically impairs motor axon maintenance, leading to adult onset axonal degeneration. However, such a model does not take into account potential dominant-negative effects on axonal transport that are associated with some missense mutations (19), or the contributions of other genetic modifiers.

HSP is considered to be a dying back neuropathy characterised by distal degeneration of long tracts in the spinal cord with preservation of cell bodies. Consistent with this, axonal loss has been documented in two mouse models of HSP and found to correlate with defective axonal transport (33, 34). We have demonstrated that the microtubule-severing protein spastin is critically required to promote axon morphogenesis and suggest that this may be relevant to infantile HSP cases. No
pathological data is available from infantile human cases to support or refute this hypothesis. Interestingly, the \textit{SPG3A} gene product atlastin, which interacts with spastin \cite{35}, has recently been shown to promote axon elongation during neuronal development \cite{36} and \textit{SPG3A} mutations are the most common cause of autosomal dominant HSP before 10 years of age \cite{37}. Taking these studies into consideration with our own findings, axon outgrowth defects could be a common feature of childhood HSP and spastin and atlastin may act in a common pathway to regulate axon outgrowth. One challenge that remains is to understand why spastin mutations cause adult onset neurodegeneration in the majority of cases. A number of HSP genes are implicated in intracellular transport and trafficking, so possibilities are that impairment of microtubule dynamics via \textit{SPG4} haploinsufficiency could inhibit axonal transport processes \cite{38}, or that mutant spastin dominantly interferes with transport \cite{19}.

The zebrafish embryo has the potential to become an important model organism for the development of models of neurodegenerative disease as it is amenable to both large-scale reverse genetic screens \cite{39-41} and high throughput therapeutic drug screening \cite{42}. Our results validate the zebrafish embryo as a tractable model system in which the pathogenetic mechanisms underlying HSP can be dissected using a molecular genetic approach. Further studies towards identification and characterisation of genetic mutants for \textit{spg4} and orthologues of other spastic paraplegia genes in the zebrafish may therefore have important implications for identifying novel therapies for HSP.
Materials and methods

Zebrafish stocks

WIK, AB and Islet1:GFP transgenic fish (20) maintained at the University of Sheffield were used for this study.

spg4 cloning & RH mapping

BLAST searches were used to identify zebrafish EST clones fo75d06.y1 (2382 b.p. full length cDNA clone) and fj29a10.y1 (partial cDNA clone encoding nucleotides 1329-2380 of fo75d06.y1), which were obtained from RZPD (Berlin, Germany). An additional 1.6 kb clone encoding nucleotides 78-1678 of fo75d06.y1 was generated for sequencing by RT-PCR (35 cycles) with Platinum Pfx DNA polymerase (Invitrogen, Paisley, UK) using the following primers: ZFSPG4F1: ACTCCCGGCCCGTTATGGTATGG and ZFSPG4R1: CTCCGGCTTCAGCTCTCGTATCG. The PCR product was TA cloned and the cloned PCR product and both EST clones fully sequenced on both strands. Sequence data was collated using SeqMan software.

Zebrafish spg4 was radiation hybrid (RH) mapped by PCR amplification of a 256 b.p exon 1 fragment (nt. 78-333) from RH panel DNAs provided by Dr. H. Roehl (University of Sheffield) using the following primers: ZFSPGFEX1F: ACTCCCGGCCCGTTATGGTATGG and ZFSPG4EX1R: CCGTCGGGGCCGCACTCTTT. Data was analysed using “Instant Mapping” at http://zfrhmaps.tch.harvard.edu/ZonRHmapper/instantMapping.htm
RNA in situ hybridisation

Digoxigenin-labelled antisense and sense spg4 probes were prepared from clone fj29a10.y1 linearised with SalI and NotI respectively using SP6 and T7 RNA polymerases according to the manufacturer (Roche, Lewes, UK). Whole-mount in situ hybridisation on a staged series of AB embryos was performed using standard procedures (43).

Mammalian cell culture, spastin transfection and immunocytochemistry

Zebrafish spg4 was FLAG epitope tagged by subcloning a 2.4 kb EcoRI-KpnI fragment derived from fo75d06.y1 into pFLAG-CMV-6a (Sigma, St. Louis, MO). HEK293 cells were cultured in DMEM supplemented with 10% FBS and antibiotics and transfected using Lipofectamine PLUS according to the manufacturers instructions (Invitrogen). Cells were fixed with 4% paraformaldehyde and double-stained for immunofluorescence microscopy with a rabbit polyclonal anti-FLAG antibody (Sigma, St. Louis, MO) and a mouse monoclonal anti-α-tubulin (DM1A; Sigma, St. Louis, MO).

Microinjection of morpholinos

Morpholino antisense oligonucleotides (spg4atg1: 5’-AATTCATTCACCCTTCTCGGGCTCT-3’, and spg4exon7: 5’-GATGTGAAAACAGACCTCTGGACGT-3’ were designed by Gene Tools, LLC (Philomath, OR) to block translation and correct splicing of spg4 mRNA respectively. Spg4atg1 targets the first AUG codon in the spg4 message. Morpholinos that target between the 5’ cap and the initiating AUG block translation so this morpholino should effectively block translation from both the first and second AUG codons. Control
morpholinos used were an identical morpholino to spg4atg1, but with five mismatched nucleotides (spg4CoMO: 5’-AATTAATTCTCCCTTGCGCGCTAT-3’; mismatches underlined) and an irrelevant morpholino (CoMO: 5’-CCTCTACCTCAGTTACAATTATA-3’). One-cell zebrafish embryos were microinjected with morpholinos resuspended in water with phenol red. Embryos were maintained at 28ºC in E3 medium and dechorionated with fine forceps prior to fixation.

**RT-PCR**

Total RNA was isolated from zebrafish embryos at 28 h.p.f. using TRIZOL reagent (Invitrogen) and treated with RQ1 RNase-free DNase (Promega). First strand cDNA was synthesised using SuperScript II (Invitrogen) and a region of spg4 spanning exons 5-9 (nucleotides 741-1206) amplified using the following primers: 5’-GGCCCAAAAATCCTCCCAAATCTA-3’ and 5’-GTGGCAGCGCTGATGTTGAAGAAA-3’.

**Immunostaining of embryos**

Embryos were fixed with 4% paraformaldehyde at 4ºC overnight and immunohistochemistry performed using standard procedures (44). 39.4D5 (anti-Islet1) and znp-1 (both from Developmental Studies Hybridoma Bank, University of Iowa, IA) were both used at a 1/2000 dilution. Primary antibody binding was visualised using a VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA). Islet1:GFP transgenic embryos were fixed for 2 hours to preserve GFP fluorescence.
To visualise motor axons and AChR (45), embryos (48 hpf) were fixed with
4% paraformaldehyde for 4 hours, treated with 0.1 % collagenase (Sigma C-9891) for
45 minutes, incubated with 1/500 znp1 overnight followed by 1/200 Cy3-conjugated
sheep anti-mouse secondary antibody for 4 hours (Sigma C-2181), then incubated
with 10 μg/ml FITC-α-bungarotoxin for 20 minutes.

To preserve microtubule integrity for immunofluorescence microscopy,
embryos were fixed for 2 hours at room temperature with MSB fix (3.9 %
paraformaldehyde, 0.1 % glutaraldehyde, 80 mM PIPES, 5 mM EGTA, 1 mM
MgCl2, 0.2 % Triton X-100), and microtubules visualised using the 6-11B-1 anti-
acetylated tubulin monoclonal antibody (Sigma, St. Louis, MO) at a 1/1000 dilution
and a Cy3-conjugated secondary antibody as above.

**TUNEL staining**

TUNEL labelling was performed using a Serologicals Corporation Apoptag kit and
labelled cells visualised with an anti-digoxigenin-alkaline phosphatase conjugated
antibody (Roche Diagnostics).

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References


Figure Legends

Figure 1. Branchiomotor neurones of spg4-deficient Islet1:GFP transgenic embryos exhibit reduced axonal outgrowth and aberrant positioning of neuronal cell bodies. Spg4atg1 morpholino-injected embryos (91%; n=148) exhibited truncated and more disordered nV (trigeminal) and nVII (facial; arrows) motor neurone axonal projections (A,C), in comparison to control morpholino-injected embryos (B,D), which exhibited normal axon outgrowth and patterning (100%, n = 140). A and B, embryos at 30 hpf; C and D, embryos at 36 hpf.

Figure 2. Morpholino-mediated knock down of spg4 function with a translation blocking morpholino (spg4atg1) inhibits spinal motor neurone axon outgrowth. Spastin (B,D,F) and control (A,C,E) morphant embryos (28 hpf) were immunostained with znp-1 (A,B) and anti-Islet1 (C,D) antibodies. Reduced spg4 function dramatically impaired outgrowth of motor axons from the spinal cord and promoted aberrant branching (arrowhead) (B), of correctly specified spinal motor neurones (D). TUNEL staining (E,F) revealed that axon growth defects in spg4 morphant embryos were accompanied by significant CNS-specific cell death.

Figure 3. A splice blocking morpholino (spg4exon7) inhibits spinal motor neurone axon outgrowth. Embryos were injected with 0.6 (A) or 1.2 (B) pmol of spg4exon7 morpholino and immunostained with znp-1, with the higher dose causing axon outgrowth defects similar to those obtained with the translation blocking morpholino. RT-PCR analysis (C) suggests that most (>90%) spg4 transcripts are incorrectly spliced at the higher dose of spg4exon7 morpholino.
Figure 4. Immunostaining with znp-1 demonstrates that co-injection of low doses of spg4atg1 and spg4exon7 morpholinos substantially increases the frequency of embryos exhibiting reduced motor axon outgrowth. Embryos injected with 0.3 pmol sp4atg1 (A) or 0.9 pmol spg4exon7 (B) mostly exhibit no or mild impairment of motor axon outgrowth, whereas a substantially increased frequency of co-injected embryos (C) exhibit reduced motor axon outgrowth compared to uninjected controls (D). See Table 4 for quantification.

Figure 5. Spg4 morphant embryos are morphologically abnormal and show impaired motility. A normal control morpholino-injected (A) and an impaired spg4atg1-injected embryo (B) at 5 days post-fertilisation are shown. Panel A is a composite made from separate images of the head and trunk of the same embryo.

Figure 6. Spg4 knock down severely disrupts axonal networks. Confocal imaging of axonal microtubules with an anti-acetylated tubulin antibody reveals an extremely disordered and incomplete axonal network in the trunk of spg4 morphant embryos. spg4 morphant embryos (B) display short, thickened and malformed microtubules (white arrowhead) and a highly disordered axonal network, in comparison to embryos injected with control morpholino (A), where an extensive network of fine, properly elongated axons, some of which are clearly bundled together in fascicles, is visible. Microtubule staining reveals that motor axons are disconnected from spinal cord neurones in spg4-morphant embryos (arrows in B), in contrast to the situation in control embryos (A). Spastin-deficient embryos also exhibit a complete absence of
longitudinal, fasciculated axonal microtubule staining in the spinal cord (arrowheads in A).

**Figure 7.** Effect of Spastin-deficiency on neuromuscular synapse formation. Double labelling of *spg4* morphant and control-injected embryos, for acetylcholine receptors with FITC-α-bungarotoxin (green, C,F) and spinal motor neurones with znp-1 (red, B,E) at 48 hpf. Motor axon outgrowth, arborisation and extensive neuromuscular synapse formation, indicated by colocalization of motor axons and AChRs in A, is evident throughout each somitic hemisegment of control-injected embryos (A-C). Distal tips of some growing collateral axons that have not yet recruited AChRs are visible (arrowhead in A). In *spg4* morphant embryos, axon outgrowth is much reduced and the degree of colocalisation is greatly diminished (D-F), apart from a small area in the mid-region of some somites (arrows in D).
Table 1. Reduced spastin activity induces apoptosis in the developing CNS. Embryos were treated as indicated, fixed at 28 hpf and stained using the TUNEL method. Data pooled from three independent experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Embryos showing CNS apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>0% (n=56)</td>
</tr>
<tr>
<td>0.6 pmol spg4atg1</td>
<td>87% (n=67)</td>
</tr>
<tr>
<td>0.6 pmol spg4CoMO</td>
<td>2% (n=57)</td>
</tr>
</tbody>
</table>
Table 2. Quantification of spinal motor neurone numbers in morpholino-injected and uninjected embryos. Embryos were treated as indicated, fixed at 28 hpf and immunostained for Islet1. The total number of Islet1-positive nuclei in the spinal cord spanning 10 anterior somites on both sides of the spinal cord was counted using a compound microscope. Values given are mean cell counts per side (±s.e.m.) where n=6-8 individual embryos in each group. The difference between spg4atg1 injected embryos and the controls groups was statistically significant using one-way ANOVA (p<0.0001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spinal motor neurone count</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6 pmol spg4atg1</td>
<td>89.4±1.7</td>
</tr>
<tr>
<td>0.6 pmol spg4CoMO</td>
<td>111±3.2</td>
</tr>
<tr>
<td>Uninjected</td>
<td>111±3.8</td>
</tr>
</tbody>
</table>
Table 3. Quantification of spinal motor neurone axon outgrowth defects. Embryos were microinjected with morpholinos at the doses indicated, fixed at 28 h.p.f. and spinal motor neurone axons immunostained using znp-1. Embryos with truncated axons or with fine, weakly staining fascicles were scored as showing reduced axon outgrowth. Data pooled from 4 (spg4exon7), 5 (spg4CoMO) and 7 (spg4atg1 and uninjected) independent sets of injections respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Embryos showing reduced spinal motor neurone axon outgrowth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>5% (n=142)</td>
</tr>
<tr>
<td>0.6 pmol spg4CoMO</td>
<td>19% (n=69)</td>
</tr>
<tr>
<td>1.2 pmol spg4CoMO</td>
<td>9% (n=35)</td>
</tr>
<tr>
<td>0.6 pmol spg4atg1</td>
<td>91% (n=113)</td>
</tr>
<tr>
<td>1.2 pmol spg4exon7</td>
<td>87% (n=54)</td>
</tr>
</tbody>
</table>
Table 4. Co-injection of spg4atg1 and spg4exon7 morpholinos increases the frequency of embryos exhibiting markedly reduced motor axon outgrowth (see Fig. 4C for example). Data pooled from 6 independent experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Embryos showing markedly reduced motor axon outgrowth</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 pmol spg4atg1</td>
<td>13% (n=196)</td>
</tr>
<tr>
<td>0.9 pmol spg4exon7</td>
<td>21% (n=200)</td>
</tr>
<tr>
<td>0.3 pmol spg4atg1 + 0.9 pmol spg4exon7</td>
<td>46% (n=176)</td>
</tr>
<tr>
<td>1.2 pmol CoMO</td>
<td>14% (n=167)</td>
</tr>
<tr>
<td>Uninjected</td>
<td>3% (n=194)</td>
</tr>
</tbody>
</table>
Table 5. Motility defects in spg4 morphant embryos at 5 d.p.f. Embryos were scored as immotile if they failed to hatch from the chorion or failed to respond when touched, and impaired if they only twitched or showed abnormal swimming behaviour when touched with a mounted needle. Embryos that failed to survive to 5 days were not included in the analysis (34% of 0.6 pmol spg4atg1 and 48% of 1.2 pmol spg4exon7 injected embryos died compared to 24% of 1.2 pmol CoMO injected and 14% of uninjected embryos). Data pooled from 4 independent experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal</th>
<th>Impaired</th>
<th>Immotile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 pmol spg4atg1 (n=144)</td>
<td>74%</td>
<td>13%</td>
<td>13%</td>
</tr>
<tr>
<td>0.6 pmol spg4atg1 (n=95)</td>
<td>21%</td>
<td>11%</td>
<td>68%</td>
</tr>
<tr>
<td>1.2 pmol spg4exon7 (n=75)</td>
<td>35%</td>
<td>27%</td>
<td>58%</td>
</tr>
<tr>
<td>1.2 pmol CoMO (n=134)</td>
<td>89%</td>
<td>4%</td>
<td>7%</td>
</tr>
<tr>
<td>Uninjected (n=110)</td>
<td>98%</td>
<td>1%</td>
<td>1%</td>
</tr>
</tbody>
</table>
Fig. 4
Fig. 6