Senataxin modulates neurite growth through fibroblast growth factor 8 signalling

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Senataxin is encoded by the SETX gene and is mainly involved in two different neurodegenerative diseases, the dominant juvenile form of amyotrophic lateral sclerosis type 4 and a recessive form of ataxia with oculomotor apraxia type 2. Based on protein homology, senataxin is predicted to be a putative DNA/RNA helicase, while senataxin interactors from patients’ lymphoblast cell lines suggest a possible involvement of the protein in different aspects of RNA metabolism. Except for an increased sensitivity to oxidative DNA damaging agents shown by some ataxia with neuropathy patients’ cell lines, no data are available about possible functional consequences of dominant SETX mutations and no studies address the function of senataxin in neurons. To start elucidating the physiological role of senataxin in neurons and how disease-causing mutations in this protein lead to neurodegeneration, we analysed the effect of senataxin on neuronal differentiation in primary hippocampal neurons and retinoic acid-treated P19 cells by modulating the expression levels of wild-type senataxin and three different dominant mutant forms of the protein. Wild-type senataxin overexpression was required and sufficient to trigger neuritogenesis and protect cells from apoptosis during differentiation. These actions were reversed by silencing of senataxin. In contrast, overexpression of the dominant mutant forms did not affect the regular differentiation process in primary hippocampal neurons. Analysis of the cellular pathways leading to neuritogenesis and cytoprotection revealed a role of senataxin in modulating the expression levels and signalling activity of fibroblast growth factor 8. Silencing of senataxin reduced, while overexpression enhanced, fibroblast growth factor 8 expression levels and the phosphorylation of related target kinases and effector proteins. The effects of senataxin overexpression were prevented when fibroblast growth factor 8 signalling was inhibited, while exogenous fibroblast growth factor 8 reversed the effects of senataxin silencing. Overall, these results reveal a key role of senataxin in neuronal differentiation through the fibroblast growth factor 8 signalling and provide initial molecular bases to explain the
neurodegeneration associated with loss-of-function mutations in senataxin found in recessive ataxia. The lack of effect on neuritogenesis observed with the overexpression of the dominant mutant forms of senataxin apparently excludes a dominant negative effect of these mutants while favouring haploinsufficiency as the pathogenic mechanism implicated in the amyotrophic lateral sclerosis 4-related degenerative condition. Alternatively, a different protein function, other than the one involved in neuritogenesis, may be implicated in these dominant degenerative processes.

Keywords: senataxin; ALS4; AOA2; FGF8; neurite growth

Abbreviations: ALS4 = amyotrophic lateral sclerosis type 4; AOA2 = ataxia with oculomotor apraxia type 2; DAPI = 4',6-diamidino-2-phenylindole; FGF = fibroblast growth factor; GFP = green fluorescent protein; GSK3β = glycogen synthase kinase 3β; MAP = microtubule-associated protein; PI3K = phosphoinositide 3-kinase

Introduction

Senataxin is a large protein of 2677 amino acids encoded by SETX, a gene involved in two different forms of neurodegenerative diseases depending on the mutation type. Homozygous or compound heterozygous SETX mutations likely leading to loss of protein function, are associated with a recessive form of ataxia with axonal neuropathy (AOA2) and elevated serum levels of α-fetoprotein (Moreira et al., 2004). Onset of ataxia occurs between 10 and 22 years of age and almost 60% of patients subsequently develop oculomotor apraxia. Disease progression slowly leads to a severe disability (Fogel et al., 2007).

Heterozygous SETX mutations (Chen et al., 2004; Hirano et al., 2010) were found in a rare dominant form of amyotrophic lateral sclerosis with juvenile onset [amyotrophic lateral sclerosis type 4 (ALS4)]. This is a slowly progressive form of amyotrophic lateral sclerosis, characterized by limb weakness, severe muscle wasting and pyramidal signs associated with degeneration of motoneurons in the brain and spinal cord (Rabin et al., 1999; De Jonghe et al., 2002; Chen et al., 2004). Unlike the adult-onset forms of amyotrophic lateral sclerosis, and despite the long disease duration, respiratory and bulbar involvement does not occur in ALS4 (Rabin et al., 1999; De Jonghe et al., 2002; Chen et al., 2004). Families showing different phenotypes including hereditary motor neuropathy, or combining features of either AOA2 or ataxia–tremor and motoneuron diseases were also reported (Schöls et al., 2008, Hirano et al., 2010), thus widening the phenotypic spectrum associated with SETX mutations.

Senataxin protein contains a typical superfamily I DNA/RNA helicase domain (residues 1931–2456) with an ATP/GTP-binding motif A (P-loop) (residues 1963–1970) essential for DNA unwinding in other systems (Chen et al., 2004). The helicase domain is conserved in the budding yeast Sen1p (Chen et al., 2006) and is highly homologous to the helicase domains of the human RENT1 and IGHMBP2 proteins (46 and 42% of identity, respectively), thus suggesting a putative DNA/RNA helicase role for the protein (Chen et al., 2004). The N-terminus region of senataxin (residues 64–593) is evolutionarily conserved in a zebrafish protein (XP_690945) and in Saccharomyces pombe protein Sen1p2 (Chen et al., 2006). The function of this region is still unknown although it is predicted to be a protein interaction domain (Chen et al., 2006). Indeed, by co-immunoprecipitation assay from control and AOA2 lymphoblast cell lines, several senataxin-interacting proteins were identified, all of them involved in transcriptional regulation and pre-messenger RNA processing (Suraweera et al., 2009). The physiological and pathological roles of senataxin are still largely unknown. Loss of senataxin, by either AOA2 mutations or silencing with RNA interference, induced abnormalities in transcription termination and messenger RNA processing (Suraweera et al., 2009). Consistent with this observation, senataxin shows mainly, although not exclusively, a nuclear localization (Moreira et al., 2004; Chen et al., 2006; Suraweera et al., 2007).

Senataxin was also demonstrated to be involved in response to oxidative DNA damage (Suraweera et al., 2007; Airoldi et al., 2010). It was also hypothesized that misregulation of transcription and aberrant pre-messenger RNA processing in AOA2 contribute to the oxidative stress and neurodegeneration observed in this syndrome (Suraweera et al., 2009). All evidence reported above, however, was obtained using non-neuronal cells (Suraweera et al., 2007, 2009) or a neuroblastoma cell line (Airoldi et al., 2010). The physiological role of senataxin in neurons and the pathogenic mechanism(s) leading to degenerative conditions involving mainly the motoneurones (ALS4) or extending also to the peripheral nervous systems (AOA2) still need to be identified. Senataxin expression data in mouse brain indicate a general neuronal expression pattern with the highest levels in cerebellum and hippocampus (Chen et al., 2006).

Here, we analysed the effect of senataxin on neuronal differentiation in primary hippocampal neurons and in P19 cells treated with retinoic acid, by modulating the expression levels of either wild-type or three ALS4-related mutant forms of senataxin (setxT3I, setxL389S and setxR2136H; Chen et al., 2004). Two of these mutations fall within the N-terminus of the protein, either outside (T3I) or inside the conserved protein interacting region (L3865), while the third one lies in the helicase domain (R2136H). We found that overexpression of wild-type senataxin was sufficient to trigger neuronal differentiation acting both as a cytoprotective agent and by promoting neuritogenesis. Senataxin acted by activating the signalling pathways dependent on fibroblast growth factor 8 (FGF8), one of the 22 fibroblast growth factors involved in the regulation of neurogenesis, neuronal differentiation, survival and synaptic plasticity, both during development and in adulthood, through activation of FGF receptors (FGFR1−4) (Mason, 2007). In contrast, overexpression of the mutant forms of senataxin (setxT3I, setxL389S and setxR2136H) did not affect the regular differentiation process in primary hippocampal neurons.
These results provide an initial molecular basis to explain how senataxin acts in neuronal cell physiology and suggest that different mechanisms of neurodegeneration exist, possibly related to different protein functions, linked to either recessive or dominant senataxin mutations.

Materials and methods

Expression constructs and generation of stable clones

A full-length human SETX clone (DKFZp686E1810Q2), including 186bp 5′-UTR and 362 bp 3′-UTR, was obtained from RZPD German resource centre for genome research and subcloned into the pcDNA3.1/CTGFP expression vector (Invitrogen Life Science) in frame with the green fluorescent protein (GFP) reporter gene, as described (Airoldi et al., 2010). The point mutations c.8C > T (p.T3I), c.1166T > C (p.L389S) and c.6407G > A (p.R2136H) were obtained by mutagenesis of the wild-type vector pcDNA3.1/CTGFPsetxwt using a Quikchange® Site-directed Mutagenesis Kit (Stratagene). Mutant forms were sequence verified. The plasmids for RNA interference studies were purchased from SABiosciences Corporation. A set of four different short hairpin RNAs was tested (Cat. N. KM28910 with neomycin selection or with the GFP reporter gene) and the most efficient (short hairpin RNA-1) was chosen. The short hairpin RNA specificity was determined by analysis of senataxin expression levels in P19 cells transiently transfected with the short hairpin RNAs or with the scrambled control sequence by quantitative real-time polymerase chain reaction as described (Airoldi et al., 2010).

For neuronal differentiation experiments, P19 cells were stably transfected with pcDNA3.1/CTGFP vector alone, pcDNA3.1/CTGFPsetxwt or setx short hairpin RNA-1 plasmids with neomycin selection gene. Stable transfectants were obtained after selection in 500 μg/ml G418 (Invitrogen Life Science) and senataxin levels were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blot. The expression levels of endogenous Setx in the short hairpin RNA-transfected clones were also determined by quantitative real-time polymerase chain reaction. For each vector, three different clones with comparable senataxin levels were used for each experiment, with similar results. Results shown for each vector are an average of the data from the three clones, whereas limited to immunofluorescence images, we always reported representative pictures; these were all taken from the same clone.

Cell cultures and P19 cell neuronal differentiation

Human neuroblastoma (SKNBE) cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen Life Science) supplemented with 20% foetal bovine serum (Hyclone), 100 U/ml penicillin/streptomycin and 2 mM l-glutamine (Invitrogen Life Science). The mouse embryocarcinoma P19 cells (Bain et al., 1994) were grown in the same medium supplemented with 10% foetal bovine serum. P19 cells can be induced to differentiate into neurons and glial cells in the presence of retinoic acid and neuronal differentiation requires both the formation of cellular aggregates and the treatment with retinoic acid (Jones-Villeneuve et al., 1982). P19 cells were differentiated with 5 μM all trans-retinoic acid (Sigma-Aldrich) for 4 days in bacteriological non-adhesive plates. Aggregates were then dissociated with trypsin, suspended in neurobasal medium with N2 supplement (Invitrogen Life Science) and 0.5 mM l-glutamine and plated onto 6-well plates coated with 100 μg/ml poly-l-lysine (Sigma-Aldrich), 1 x 10⁶ cells/well. Cells were cultured for a total of 14 days. Each day, duplicate samples were taken for RNA or protein extraction. For the rescue of setx shRNA clones neuronal differentiation, cells were incubated with 500 ng/ml FGFB (Sigma-Aldrich) for 2 days in the presence of retinoic acid. Treatment with the glycogen synthase kinase 3β (GSK3β) inhibitor SB216768 (Sigma-Aldrich) was for 24 h with 10, 20 or 50 μM as described (Owen and Gordon-Weeks, 2003).

Hippocampal cultures and morphometric analysis

Hippocampal primary cultures were prepared from CD1 mice as described (Martel et al., 2008). Experiments were in accordance with the standard ethical guidelines (National Institute of Health and European Community Guidelines on the Care and Use of Laboratory Animals) and were approved by the local ethics committee. Briefly, hippocampi were dissected from embryos at embryonic Day 18 in Hank’s balanced salt solution (Invitrogen Life Science) and mechanically dissociated. Cells were then plated onto poly-l-lysine coated coverslips (100 μg/ml) at 1 x 10⁶ cells/cm² in Dulbecco’s modified Eagle’s medium (50%, Invitrogen Life Science) and HAM’s F-12 nutrient medium (50%, Invitrogen Life Science), supplemented with 5 mM HEPES buffer, 0.6% glucose, 20 μg/ml insulin (Sigma-Aldrich) and N2 supplement. Cultures used were analysed by immunofluorescence staining and were >90% positive for microtubule-associated protein 2 (MAP2), MAP1B and neurofilament markers. For morphometric analyses, cells were seeded at 0.25 x 10⁵ cells/cm² and transfected 5 h after plating with the vector alone, setxwt, setxT3I, setxL389S, setxR2136H or setx short hairpin RNA vectors, all with the GFP reporter gene, using Lipofectamine 2000 (Invitrogen Life Science). To analyse the effect of senataxin on neurite formation, two different short hairpin RNA vectors (short hairpin RNA-1 and short hairpin RNA-2), showing similar silencing efficiency in transient transfection, were used. Seventy-two hours after transfection, neurons were processed for immunofluorescence as described (Vantaggiato et al., 2009). Morphometric analyses were performed on GFP-positive cells in three reproducible experiments for a total of 120 hippocampal neurons or P19-derived neurons for each transfected construct. Images were acquired with a Leica DMIRE2 microscope at x40 magnification and 1024 x 1024 pixel resolution and neurite length measured with the ImageJ programme. For the analyses of neuronal structure and morphology, neurons were transfected with the same vectors 1 week after plating and processed for immunofluorescence 72 h later. To analyse the effect of FGFR8 on neurite formation, FGFR8 (20 ng/ml) and the FGFR1 inhibitor SU5402 (20 μM, Calbiochem, Merck Chemicals Ltd) were added at the moment of transfection and maintained for 72 h.

For morphometric analyses in the P19 cell line, cells were induced to differentiate in the presence of retinoic acid for 4 days, aggregates were then dissociated and cells were transfected 5 h after plating with the vector alone, setxwt, setxT3I, setxL389S, setxR2136H or setx short hairpin RNA-1 and short hairpin RNA-2 vectors. Cells were processed for immunofluorescence 72 h later. Images were acquired with a Leica DMIRE2 microscope at x40 magnification and 1024 x 1024 pixel resolution.
Protein and messenger RNA levels analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blot were performed as described (Panzeri et al., 2006). Relevant antigens were revealed using antibodies against tubulin βIII (Chemicon International, Inc), senataxin, nibrin, GAP43, β-actin (all from Santa Cruz Biotechnology, Inc), GSK3β, phospho-Ser9-GSK3β, extracellular signal-regulated kinase-1/2 mitogen-activated protein kinase, phospho-extracellular signal-regulated kinase-1/2 mitogen-activated protein kinase, Akt, phospho-Akt, Smad1, phospho-Ser206-Smad1 (all from Cell Signalling Technology, Inc.) and phospho-MAP1B activated protein kinase, Akt, phospho-Akt, phospho-Ser9-GSK3β, GSK3β, phospho-MAP1B (SMI-31), Ab24573, Abcam) (Goold and Gordon-Weeks, 2005).

For nuclear and for cytosolic fraction analysis, SKNBE and hippocampal cells were washed in phosphate-buffered saline and suspended in 10 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol, 10 mM NaF, 0.1 mM EDTA, 10 mM HEPES, pH 7.9, supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and incubated in ice for 20 min. Cell lysis was carried out by addition of 0.6% Nonidet P40, samples were then centrifuged at 3000 rpm for 5 min and the supernatant containing the cytoplasmic fraction removed. Nuclear pellets were suspended in Tris–HCl pH 6.8, 2.5% sodium dodecyl sulphate and centrifuged at 12,000 rpm for 5 min.

RNA was prepared using TRIzol® (Invitrogen Life Science) and 1 µg/sample was reverse transcribed into complementary DNA using the Superscript® First Strand Synthesis System for RT-PCR kit (Invitrogen Life Science) and random hexamers. The expression levels of Setx, Mash1, Wnt1, Oct3/4, N-cadherin, FGF8, FGF1, FG2, FG4, FGF5, FGRF1, neural cell adhesion molecule, Pax2, Lmx1b, Map1b and Gap43 were analysed by quantitative real-time polymerase chain reaction on an ABI PRISM® 7900HT Fast Real-Time PCR Systems (Applied Biosystems) using specific gene expression primer pairs. The expression of the human non-small nuclear ribonucleoprotein particles or nuclear speckle marker (Ab11826), RNA polymerase II, lamp1 as lysosomal marker, 160 + 200 kDa phosphorylated neurofilaments (phospho-NF) (Abcam), eukaryotic translation initiation factor eIF4G2 (DAP5, Santa Cruz Biotechnology), phospho-MAP1B (SMI-31), Ab24573, Abcam) (Goold and Gordon-Weeks, 2005).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with phosphate-buffered saline containing 0.1% saponin and 1% bovine serum albumin for 30 min. Samples were then incubated for 2 h with primary antibodies against GFP (Chemicon International), fibrillarin as nucleolus marker, SC35 as marker for a phospho-epitope of the human non-small nuclear ribonucleoprotein particles or nuclear speckle marker (Ab11826), RNA polymerase II, lamp1 as lysosomal marker, 160 + 200 kDa phosphorylated neurofilaments (phospho-NF) (Abcam), eukaryotic translation initiation factor eIF4G2 (DAP5, Santa Cruz Biotechnology), phospho-MAP1B (SMI-31), senataxin, phospho-Ser9-GSK3β and phospho-Akt and revealed using the secondary antibodies AlexaFluor-488 and 546 (Invitrogen Life Science). Senataxin localization was also analysed with a specific antibody obtained from Eurogentec (Seraing) and produced against the N-terminus of the protein (anti-senataxin pepN) (Airoldi et al., 2010). Growth cones in undifferentiated clones were stained using AlexaFluor-546 Phalloidin (Invitrogen Life Science) to label F-actin (Schindelholz and Reber, 1999). For the staining of mitochondria, cells were transfected with the mito-DSRed vector (Cipolat et al., 2004). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Images were acquired using a Leica TCS SP2 AOB5 confocal laser scanning microscope with a ×63 oil immersion lens at 1024 × 1024 pixel resolution. For quantitation of senataxin, phospho-MAP1B, phospho-Ser9-GSK3β, phospho-Akt, lamp1 and mito-DSRed fluorescence intensity, images were acquired at the same laser attenuation (Oon et al., 2006). Fluorescence intensity was measured using the ImageJ programme as the average pixel intensity within a box of defined size drawn on the neurite shaft, in the growth cone or in the cell body.

Cell death analysis

Control, setxwt and setx shRNA P19 stable clones were induced to differentiate with and without retinoic acid and cell death was analysed 24 and 48 h later using propidium iodide and DAPI (Hamada-Kanazawa et al., 2004). Cell aggregates were incubated with 3 µM propidium iodide (Sigma-Aldrich) and 1 µg/ml DAPI for 15 min at room temperature to stain nuclei of dead and both dead and living cells, respectively, and photographed using a Leica DMIRE2 microscope at ×20 magnification and 1300 × 1030 pixel resolution. Apoptosis was detected in the same cells treated with or without retinoic acid for 24 and 48 h using a Caspase 3 Colorimetric Activity Assay kit (Chemicon International), according to the manufacturer’s instructions. To correlate FGF8 levels with setx shRNA cell death, cells were induced to differentiate in the presence of retinoic acid and 20, 100 or 500 ng/ml FGF8β. Twenty-four hours later cells were collected and caspase 3 activity was analysed.

Statistical analysis

The results are expressed as means ± standard error of the mean (SEM); n represents the number of individual experiments. Statistical analysis was carried out using the Student’s t-test for unpaired variables (two-tailed), double or triple asterisks refer to statistical probabilities (P < 0.01 and < 0.001, respectively), measured in the various experimental conditions. P < 0.05 was considered statistically significant.

Results

Intracellular localization of senataxin in primary neuronal cells

We analysed the subcellular localization of endogenous senataxin in hippocampal neurons, in differentiated mouse P19 cells and in the human neuroblastoma cell line SKNBE. In all cell types, senataxin localized in the nucleus and in the cytoplasm; in hippocampal and P19-derived neurons it was detected in the perinuclear region as well as in the axon and growth cone (Fig. 1 and Supplementary Fig. 1). Nuclear and cytosolic localization was confirmed by fractionation analysis in SKNBE and hippocampal cells (Fig. 1C). Interestingly, the distribution of senataxin between the cytosol and the nucleus differed among the cells within the same population, with a staining that was mainly cytosolic or mainly nuclear or occurring in both regions (Fig. 1D). This different localization is consistent with the possible dual role of senataxin in the nucleus as a helicase and in the cytosol in the regulation of transcription and RNA metabolism (Suraweera et al., 2009). In the nucleus senataxin co-localized with the RNA polymerase II marker and not with the nucleolus marker fibrillarin, as reported for fibroblasts and...
Figure 1  Intracellular localization of senataxin in hippocampal neurons and SKNBE cells. (A) Hippocampal cells were fixed and immunostained with anti-senataxin (green) and fibrillarin, RNA polymerase II or DAP5 (red) antibodies. Panels on the right are a higher magnification of the cell body indicated in the square. Yellow in the merge images indicates co-localization of senataxin with RNA polymerase II and DAP5. Scale bar = 100 μm. (B) SKNBE cells were fixed and immunostained with anti-senataxin (green) and anti-SC35 (red) antibodies. The antibodies available for SC35 detect only the protein of human origin; thus the data are shown in SKNBE cells. Yellow in the merge images indicates co-localization of senataxin with SC35. Scale bar = 10 μm. (C) Senataxin expression levels in SKNBE and
HeLa cells (Suraweera et al., 2007) (Fig. 1A and Supplementary Fig. 1). Moreover, we found that in the nucleus, senataxin also co-localized with the splicing complex marker SC35, indicating that the protein, like its human homologue IGHMBP2 (Molnar et al., 1997), is also present in the spliceosomes (Fig. 1B). Due to the overlapping localization of IGHMBP2 and senataxin observed in the perinuclear region, in the axons and in the growth cone, we also checked whether senataxin localizes to ribosomes, as demonstrated for IGHMBP2 (Guenther et al., 2009). We found that the protein co-localized with the ribosomal marker DAP5 (Fig. 1A). No localization was detected with the endoplasmic reticulum (Supplementary Fig. 1). All the subcellular localization data were confirmed in SKNBE cells using an additional antibody to detect endogenous senataxin (anti-senataxin pepN) (Airoldi et al., 2010) (Supplementary Fig. 2A). The specificity of the immunofluorescence signal was also confirmed by the loss of senataxin staining in setx short hairpin RNA-transfected hippocampal cells (Supplementary Fig. 2B). SKNBE cells were also transiently transfected with setxwt and the mutant forms setxT3I, setxL389S and setxR2136H and immunostained with an anti-GFP antibody to detect exogenous senataxin (Supplementary Fig. 3). Exogenous wild-type senataxin showed the same localization of the endogenous protein. Moreover, the mutations T3I, L389S and R2136H did not alter the localization of the protein (Supplementary Fig. 3).

**Senataxin is required for axonal growth in primary hippocampal neurons**

We tested the effects of senataxin overexpression or depletion on early neuronal differentiation using primary hippocampal neurons, an established model of differentiation. Embryonic hippocampal neurons were dissociated, plated and transfected immediately with the vector alone, setxwt, the mutant forms setxT3I, setxL389S and setxR2136H or two different setx short hairpin RNA plasmids (short hairpin RNA-1 and short hairpin RNA-2), showing a similar silencing efficiency (Fig. 2A), all with the GFP reporter gene (Riano et al., 2009; Vantaggiato et al., 2009). Morphometric analyses were performed on GFP-positive cells at Stage 3 of *in vitro* development. Neurons transfected with control vector showed the characteristic morphology of hippocampal neurons at this stage (Fig. 2A) (Dotti et al., 1988). Overexpression of wild-type senataxin and protein depletion affected neurite development and length, since silencing of senataxin determined a significant reduction in neurite length compared with the control and its overexpression increased neurite length (Fig. 2A). The expression of the mutant forms setxT3I, setxL389S and setxR2136H did not alter neurite length compared with the control, indicating that these mutations have no effects on this process. To discriminate whether senataxin acted on neuritogenesis only or also on the maintenance of neuronal structure and morphology, we examined the effect of senataxin silencing or overexpression, using the wild-type and the mutant forms on fully differentiated neurons. No differences in neurofilament staining or in the distribution and density of mitochondria and lysosomes in the cell body, along the axons and in the growth cones, were detected, indicating that senataxin affects neuritogenesis, but not the maintenance of neurite structure and neuron morphology (Supplementary Figs 4 and 5).

The same analysis was carried out on mouse embryonic carcinoma P19 cell line (Jones-Villeneuve et al., 1982). P19 cells were induced to differentiate for 4 days with retinoic acid, transfected with the vector alone, setxwt, the mutant forms setxT3I, setxL389S and setxR2136H or the two setx short hairpin RNA plasmids after plating (Day 4) and processed 72 h later. Senataxin was expressed in undifferentiated P19 cells and its expression levels were not affected by retinoic acid during neuronal differentiation (not shown). Senataxin affected neurite development and length as in hippocampal neurons (Fig. 2B). These findings indicate that P19 cells are a suitable model for the study of the effects of senataxin on neuronal differentiation.

**Senataxin regulates cell death during retinoic acid-induced neuronal differentiation**

We investigated the effects of senataxin overexpression and depletion on neuronal differentiation. To this purpose P19 cells were stably transfected with wild-type senataxin or with a specific short hairpin RNA to silence the gene, or with the vector alone as control. The senataxin silenced-P19 stable clones (setx shRNA) selected for the experiments showed a reduction of 90% in Setx expression levels (Fig. 3A). Three clones for each vector with comparable senataxin expression levels were selected for the experiments (Fig. 3B and C). The effect of senataxin on neuronal differentiation was analysed from its early phases, which, in P19 cells, coincide with cellular aggregation. Whereas all clones formed aggregates similar in number and size, on Day 4 no live setx shRNA cells could be collected and dissociated from aggregates. We therefore investigated whether neuronal differentiation was impaired in setx shRNA stable clones. We analysed in setxwt, setx shRNA and control cells the expression levels of the stem cell marker Oct3/4 and of the retinoic acid-induced genes Mash1, Wnt1 and N-cadherin. In control cells retinoic acid treatment increased Wnt1, Mash1 and N-cadherin expression levels and decreased Oct3/4 expression (Fig. 4A), as described (Bain et al., 1994). In setxwt cells, the expression levels of Wnt1.
Figure 2. Effect of senataxin on neuronal differentiation. (A) Effect of senataxin on neurite formation in hippocampal primary neurons. Hippocampal neurons were transfected after plating with setxwt, setxT3I, setxL389S, setxR2136H, setx short hairpin RNA-1 (shRNA1), setx short hairpin RNA-2 (shRNA2) or with the vector alone (ctr) tagged with green fluorescent protein (GFP), fixed 72 h later and stained with anti-GFP green and anti-senataxin antibodies. A representative image for each vector is shown. Scale bar = 100 μm. Morphometric analyses were performed on GFP-positive cells in three reproducible experiments for a total of 120 hippocampal neurons for each transfected construct. Neurite length is shown in the graphs (**P < 0.01). Senataxin fluorescence intensity in GFP-positive cells is shown (continued)
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with a peak on Day 1 (Fig. 5A), confirming previous data (Wang et al., 2007). Retinoic acid-induced FGF8 expression levels in control, setxwt and setx shRNA cells (Fig. 5B). Significantly higher compared with control and in setx shRNA cells, FGF8 expression levels were decreased.

We quantified the increased cell death observed in setx shRNA stable clones using propidium iodide and DAPI to stain dead and both dead and living cells, respectively, 24 and 48 h after retinoic acid induction. Setx shRNA aggregates presented higher levels of dead, propidium iodide-positive cells compared with the control after 24 h, and showed only few living cells after 48 h (Fig. 4B and data not shown). Retinoic acid induces apoptosis through caspase 3 activation (Miho et al., 1999); we therefore analysed the levels of caspase 3 activity in setx shRNA clones during retinoic acid treatment. Twenty-four hours after retinoic acid induction, control cells presented a small increase in caspase 3 activity compared with untreated cells; caspase 3 activity was instead significantly increased (3-fold over the control) in setx shRNA cells and, consistently, it was decreased in setxwt cells (Fig. 4C). These data indicate that neither senataxin overexpression nor depletion affect the early events in neuronal differentiation; however, they do affect retinoic acid-induced apoptosis.

Senataxin knockdown affects FGF8 expression levels

Neuronal differentiation of P19 cells requires both the treatment with retinoic acid and the formation of cellular aggregates (Jones-Villeneuve et al., 1982), the latter inducing a rapid and transient increase in FGF8 levels, a key factor that promotes neuronal differentiation and survival (Wang et al., 2006). We decided to test if senataxin affects the FGF8 signalling pathway during P19 stable clone neuronal differentiation. We analysed the expression levels of FGF8 and its receptor FGFR1, the most abundant isoform in the nervous system (Ford-Perriss et al., 2001). In control cells, aggregation in the presence of retinoic acid-induced FGF8 expression with a peak on Day 1 (Fig. 5A), confirming previous data (Wang et al., 2006). In setxwt cells, FGF8 expression levels were significantly higher compared with control and in setx shRNA cells, FGF8 expression was prevented. No differences were detected in FGFR1 expression levels in control, setxwt and setx shRNA cells (Fig. 5B). The expression of the neurogenic factor FGF2 and the other FGF family members, FGF1, FGF4 and FGF5, were not affected by senataxin (Fig. 5B and data not shown) (Wang et al., 2006). Setx shRNA cells did not show aggregation defects (Fig. 4B) and no differences in neural cell adhesion molecule expression levels were detected during retinoic acid treatment (Fig. 5C), excluding the absence of FGF8 induction derived from effects of senataxin on cellular aggregation. Moreover, setx shRNA cells did not show alteration in Pax2 and Lmx1b expression levels, the two known genes necessary and sufficient for FGF8 induction during midbrain and cerebellum development (Guo et al., 2007) (Fig. 5D). This excludes the possibility that senataxin modifies FGF8 expression levels by affecting the expression of these two upstream factors. Whether overexpression and depletion of senataxin affect FGF8 expression levels directly or through as yet unidentified factors remains to be determined.

Senataxin regulates cell death during neuronal differentiation of P19 cells through FGF8-activated signalling pathways

Among the effects of FGF8 ultimately promoting neuronal differentiation is the inhibition of cell death induced by retinoic acid and bone morphogenetic protein 4, a protein involved in the regulation of differentiation and apoptosis (Massagué et al., 1998). This effect is dependent on the activation by FGF8 of phosphoinositide 3 kinase (PI3K)-Akt and Ras-mitogen-activated protein kinase pathways with the inhibition of retinoic acid and bone morphogenetic protein 4-induced caspases and DNA fragmentation (Wang et al., 2006). Akt activation determines the phosphorylation and inactivation of caspase 9 and the pro-apoptotic factor Bad (Datta et al., 1997; Cardone et al., 1998). It also inhibits GSK3β, which plays a critical role in the induction of apoptosis, by phosphorylating it at Ser9 (Beurel and Jope, 2006). Moreover, extracellular signal-regulated kinase-1/2 mitogen-activated protein kinase (ERK1/2 MAPK) phosphorylates at Ser206 the bone morphogenetic protein 4-activated Smad1 protein, blocking its nuclear translocation and inhibiting the expression of bone morphogenetic protein target genes (Pera et al., 2003). To investigate whether FGF8 was responsible for the effects of senataxin on retinoic acid-induced cell death, we analysed its downstream signalling pathways investigating the phosphorylation and activation of Akt, GSK3β, ERK1/2 MAPK and Smad1 during neuronal differentiation. Phosphorylation of Akt, GSK3β at Ser9, ERK1/2 MAPK and Smad1 on Ser206 was reduced in setx shRNA cells and enhanced in setxwt cells (Fig. 6A). To correlate FGF8 levels directly with setx shRNA cells death, setx shRNA stable clones were differentiated in the presence of retinoic acid and increasing concentrations of FGF8b (Alam et al., 2009). Twenty-four hours later cells were collected and caspase 3 activity was analysed. FGF8b

Figure 2 Continued

in the small graph (right) (**P < 0.01; ***P < 0.001). (B) Effect of senataxin on neurite formation in P19 cells. P19 cells were induced to differentiate with retinoic acid and on Day 4 were transfected with setxwt, setxT3I, setxL389S, setxR2136H, setx shRNA1, setx shRNA2 or with the vector alone. GFP-positive cells were photographed 72 h after transfection. Scale bar = 100 μm. Morphometric analyses were performed on GFP-positive cells in three reproducible experiments for a total of 120 P19-derived neurons for each transfected construct. Neurite length is shown in the graphs (**P < 0.01).
Figure 3 Senataxin expression levels in P19 clones. (A) Expression level of Setx in setx shRNA clones. RNA was extracted from P19 clones stably transfected with setx short hairpin RNA-1. Setx expression levels were analysed by real-time polymerase chain reaction and compared with Setx levels in P19 transfected with the scrambled sequence. P19 cells stably transfected with the vector alone were used as endogenous controls (ctr). Results are expressed as mean ± SEM of three independent experiments (***P < 0.001). The graph shows the residual Setx expression in the three setx shRNA clones (clones 17, 18 and 32) used in the experiments. (B) Expression level of senataxin in ctr, setxwt and setx shRNA clones. Total extracts prepared from P19 cells stably transfected with pcDNA3.1/CTGFP vector, pcDNA3.1/CTGFPsetxwt and setx short hairpin RNA1 plasmid were run on 6 and 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis gels and probed with anti-senataxin and anti-actin antibodies. The gel shows senataxin expression levels in the three control (ctr, clones 7, 62 and 63), setxwt (clones 40, 67 and 69) and setx shRNA (17, 18 and 32) stable clones used in the experiments. (C) Immunofluorescence analyses of senataxin levels in control and setxwt stable clones. Control and setxwt stable clones and P19 cells transfected with setx short hairpin RNA-1 were fixed and immunostained with anti-green fluorescent protein (anti-GFP) and anti-senataxin antibodies. A representative image of the three clones for each vector is shown.
Figure 4  Effect of senataxin on P19 cells neuronal differentiation. (A) Neuronal markers expression levels. Setxwt, setx shRNA and control P19 stable clones were induced to differentiate for 4 days with retinoic acid (RA) and RNA was extracted every day and used to analyse Oct3/4, Mash1, Wnt1 and n-cadherin expression levels by real-time polymerase chain reaction. Undifferentiated P19 cells stably transfected with the vector alone were used as endogenous controls (und, arbitrarily set at 1) and indicated only in top left graph. The data are the mean ± SEM of three independent experiments each one performed in duplicate using three independent clones for each genotype. (B) Senataxin overexpression and silencing affect cell death during neuronal differentiation. Setxwt, setx shRNA and control P19 stable clones were induced to differentiate in the presence of retinoic acid and 48 h later aggregates were stained with 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide to detect dead and living and only dead cells, respectively. Scale bar = 50 μm. (C) Senataxin overexpression and silencing affect retinoic acid-induced apoptosis. Setxwt, setx shRNA and control P19 stable clones were induced to differentiate with and without retinoic acid. Aggregates were collected, dissociated and tested for caspase 3 activity, 24 and 48 h later. Results shown are the mean ± SEM of three independent experiments each one performed in duplicate, using three independent clones for each vector (***P < 0.001).
treatment induced a concentration-dependent reduction of caspase 3 activity in retinoic acid-treated cells compared with untreated cells (Fig. 6B). Moreover, setx shRNA cells treated with retinoic acid and 500 ng/ml FGF8b progressed through neuronal differentiation (Fig. 6C), while retinoic acid treatment alone induced cell death, thereby blocking neuronal differentiation (Fig. 4).

Senataxin regulates neurite outgrowth in hippocampal cells through FGF8-activated signalling pathways

FGF8 promotes neurite outgrowth regulating microtubule dynamics through the activation of PI3K-Akt and Ras-mitogen-activated protein kinase pathways (Tanaka et al., 2001). In particular, Akt has a positive influence on neurite elongation, calibre and branching in primary neuronal cells (Read and Gorman, 2009) and in NGF-stimulated PC12 cells (Kimura et al., 1994; Jackson et al., 1996). PI3K-Akt and Ras-mitogen-activated protein kinase pathways stimulate axon growth by regulating microtubule dynamics through the modulation of GSK3β activity. Akt inhibits GSK3β by phosphorylating it at Ser9 and this reduces the phosphorylation of GSK3β-primed substrates, such as the microtubule-associated protein APC, MAP2 and tau, increasing microtubules stability in growing axons (Wagner et al., 1996; Read and Gorman, 2009). The mitogen-activated protein kinase pathway stimulates axon growth by increasing the GSK3β-dependent phosphorylation of

Figure 5 Senataxin overexpression or depletion affects fibroblast growth factor 8 (FGF8) expression levels during P19 neuronal differentiation. Setxwt, setx shRNA and control P19 stable clones were induced to differentiate for 4 days with retinoic acid and RNA was extracted every day and used to analyse gene expression by real-time polymerase chain reaction. Undifferentiated P19 cells stably transfected with the vector alone were used as endogenous controls (arbitrarily set at 1). Results shown are the mean ± SEM of three independent experiments each one performed in duplicate using three independent clones for each vector. (A) FGF8 expression levels (**P < 0.01; ***P < 0.001). (B) FGF receptor 1 (FGFR1) and FGF2 expression levels. (C) Neural cell adhesion molecule (NCAM) expression levels. (D) Pax2 and Lmx1b expression levels.
Figure 6 Fibroblast growth factor 8 (FGF8) signalling affects retinoic acid (RA)-induced apoptosis and neuronal differentiation. (A) Senataxin overexpression or depletion affects FGF8 signalling during neuronal differentiation. Total extracts were prepared from control, setxwt and setx shRNA stable clones 22, 24 and 26 h after retinoic acid treatment, run on a 10% sodium dodecyl sulphate polyacrylamide electrophoresis gel and probed with anti phospho-Akt (p-Akt), Akt, phospho-Ser9-GSK3β (p-GSK3β), glycogen synthase kinase 3β (GSK3β), phospho-extracellular signal-regulated kinase-1/2 (p-ERK), extracellular signal-regulated kinase-1/2, phospho-Ser206-Smad1 (p-Smad1) and Smad1 antibodies. Shown is a representative blot out of three reproducible ones. Phosphorylation levels of Akt, GSK3β, extracellular signal-regulated kinase-1/2 and Smad1 were quantified from the gels above and normalized on total Akt, GSK3β, extracellular signal-regulated kinase-1/2 and Smad1. The graphs show the mean ± SEM of three independent experiments each one performed using three independent clones for each vector (***P < 0.001; **P < 0.01). (B) Effect of FGF8 on retinoic acid-induced setx shRNA cells death. Setx shRNA stable clones were induced to differentiate in the presence of retinoic acid and of increasing concentrations of FGF8b.
the microtubule-associated protein MAP1B (Goold and Gordon-Weeks, 2005).

We investigated whether FGF8 signalling was responsible for the effect of senataxin overexpression and silencing on neurite formation in hippocampal neuronal cells (Fig. 2). We therefore analysed the activation of FGF8 signalling in setxwt and setx short hairpin RNA-transfected neurons, determining the phosphorylation levels and localization of Akt, GSK3β and MAP1B in axons and growth cones (Bush et al., 1996; Zhou et al., 2004; Ooms et al., 2006). Hippocampal neurons transfected with setxwt presented higher levels of phosphorylated MAP1B in axons and growth cones compared with the control, while setx short hairpin RNA-transfected cells presented lower levels of phospho-MAP1B (Fig. 7A). Moreover, setxwt cells showed increased levels of phosphorylated Akt and GSK3β at the growth cone, while in setx short hairpin RNA-transfected neurons the amount of phosphorylated proteins at the growth cone decreased (Fig. 7B and Supplementary Fig. 6). The same phosphorylation profile and protein localization at the growth cone were observed in P19 transfected cells (Supplementary Fig. 7). These data suggest that senataxin regulates neurite extension through FGF8 activation of Akt/GSK3β and mitogen-activated protein kinase pathways.

Rescue of the effect of senataxin silencing on neurite outgrowth through FGF8

We investigated the cause-effect relationship between senataxin and FGF8 on neurite extension by modulating FGF8 signalling in hippocampal neurons transfected with setxwt, setx short hairpin RNA or the control vector. We inhibited FGF8 signalling using the FGFR1 inhibitor SU5402 (Wang et al., 2001) and increased FGF8 signalling by incubating neurons with 20 ng/ml FGF8b (Tanaka et al., 2001). After 72 h, neurons were fixed and morphometric analyses were performed on GFP-positive cells. The two treatments had opposite effects on control cells confirming the prominent role of FGF8 in neurite formation and elongation (Fig. 8).

Interestingly, in control cells, FGF8b induced the same increase in neurite length observed in setxwt cells, while inhibition of FGFR1 decreased neurite length to the values obtained with the silencing of setx. Setxwt neurons treated with SU5402 presented a significant reduction in neurite extension compared with untreated setxwt cells and the treatment with FGF8 did not induce an additional increase in neurite length. Senataxin-silenced neurons treated with FGF8b presented a significant increase in neurite length (Fig. 8), while the inhibitor SU5402 did not alter the length of neurite. These results indicate a direct correlation between senataxin levels, FGF8 expression and neurite length and demonstrate that the regulation of FGF8 expression levels is the mechanism by which senataxin modulates neurite extension.

Senataxin is sufficient to promote neurite outgrowth in the absence of neuronal commitment

We investigated whether senataxin promoted neuritogenesis directly in the absence of neurogenic cues, or whether it could only act in cells already committed to differentiate, as retinoic acid-treated-P19 cells or hippocampal neurons. Undifferentiated setxwt P19 stable clones, in which neuronal differentiation was not induced, presented neurite-like structures positive to phalloidin staining of F-actin, the supporting cytoskeletal element of the growth cone (Fig. 9A). No sprouting was detected in setx shRNA and control cells. Undifferentiated setxwt cells expressed the neuronal markers GAP43 and tubulin (III) and higher phospho-MAP1B levels compared with controls (Fig. 9B). In addition, FGF8 was increased in setxwt cells, and decreased in setx shRNA cells (Fig. 9C). Moreover, Akt, GSK3β-Ser9 and ERK1/2 MAPK were more phosphorylated in undifferentiated setxwt cells (Fig. 9D), while only basal protein phosphorylation was detected in setx shRNA and control cells. No differences were detected in the expression of FGFR2, FGFR1, Pax2 and Lmx1b (data not shown). The effect of senataxin overexpression on neurite formation in undifferentiated setxwt clones was reversed by incubating the cells with the GSK3β inhibitor SB216763 for 24 h (Fig. 9E). The treatment of setxwt cells with the inhibitor decreased not only the phosphorylation of GSK3β at Ser9 in a concentration-dependent way, but also MAP1B phosphorylation levels, determining the loss of neurite-like structures. This confirms that the neuronal phenotype of setxwt stable clones is determined by the increased FGF8 signalling through GSK3β.

These data indicate that senataxin overexpression is sufficient to trigger FGF8 expression and activate Akt/GSK3β and ERK1/2 MAPK pathways, leading to neurite formation also in the absence of neuronal commitment. We then assessed whether the independent action of senataxin on neurites synergizes with differentiating stimuli. Retinoic acid activated the neurogenic transcription factors cascade to similar degrees in setxwt and control cells (Fig. 4A); however, differentiated cells overexpressing senataxin presented higher expression levels of the neurite markers GAP43 and MAP1B (Fig. 9F). These data indicate that senataxin overexpression alone is able to induce neurite formation per se and that this process is synergic with a differentiating stimulus.
Figure 7 Senataxin affects fibroblast growth factor 8 (FGF8) signalling in hippocampal neurons. (A) Hippocampal neurons were transfected 5 h after plating with setxwt, setx short hairpin RNA and control vector tagged with green fluorescent protein (GFP), fixed 72 h later and stained with anti-GFP and anti phospho-microtubule-associated protein (MAP)1B (SMI-31) antibodies. The localization and the phosphorylation levels of MAP1B were analysed in GFP-positive cells at the same laser attenuation. The small panels on the right show a higher magnification of the growth cones indicated in the square. Scale bar = 100 μm. The fluorescence intensity of phospho-MAP1B at the growth cone was normalized on the one detected at the shaft of the same neurite and shown in the graph. The data are the mean ± SEM of three independent experiments for a total of 50 neurites for each vector (***P < 0.001). (B) Hippocampal neurons were treated as described in A and stained with anti-GFP and anti-phospho-Akt (p-Akt) antibodies. The localization and the phosphorylation (continued)
Discussion

The involvement of senataxin in two different neurodegenerative diseases, a juvenile form of amyotrophic lateral sclerosis, ALS4, and a recessive form of ataxia with neuropathy, AOA2, suggests a double pathophysiological role of the protein, played both in the central and peripheral nervous systems. In spite of this, the function of senataxin is still largely unknown. More importantly, the role of the protein in neurons and the effects of its mutations leading to different neurodegenerative conditions such as AOA2 or ALS4, had never been investigated. To address these issues we carried out a series of morpho-functional studies on different types of neuronal cells, by analysing the effects of senataxin silencing and overexpression of either the wild-type or three ALS4-related mutant forms of the protein. The findings presented here indicate a role of senataxin in neuritogenesis and cytoprotection during neuronal differentiation and place the protein within the context of FGF8-dependent signalling, a key pathway in neuronal development.

Our data on protein subcellular localization already provide some clues about possible pathogenetic mechanism(s) in which senataxin might be involved. We found a double subcellular localization of senataxin in the nucleus and in the cytoplasm. We also detected neurons with an exclusive localization of senataxin either nuclear or cytoplasmic or in which both areas stained positively for the protein, thus indicating that senataxin can shuttle from the nucleus to the cytoplasm.

In the nucleus, senataxin co-localized with the nucleoplasmic marker RNA polymerase II and not with fibrillarin (nucleolus) as observed in other cell types (Suraweera et al., 2007) and with the splicing complex marker SC35, indicating that senataxin is also present in the spliceosome. This suggests a possible involvement of the protein in pre-messenger RNA splicing, analogously to the human SETX homologue, IGHMBP2, whose helicase domain shows 42% of identity with that of senataxin (Chen et al., 2004). Overlap between IGHMBP2 and SETX localization extends also to the cytoplasm. Indeed, analogously to IGHMBP2 (Guenther et al., 2009), we found that senataxin co-localized with the ribosomal marker DAP5, thus predicting the involvement of the protein in the regulation of translation of specific messenger RNAs, as already demonstrated for IGHMBP2 (Guenther et al., 2009).

Overall, the localization data here reported support a possible helicase function for senataxin in neurons. Based on the functional heterogeneity associated with the helicase domain (DNA repair, replication, recombination and transcription, RNA processing, transcript stability and translation initiation, see Tanner and Linden, 2001), the dual nuclear and cytoplasmic localization is not unexpected and needs to be functionally dissected. In line with this view and consistent with our data, a role for senataxin in transcriptional regulation was recently suggested based on the nature of the different senataxin interactors identified in non-neuronal cells, all involved in different facets of RNA metabolism, pre-messenger RNA processing, splicing and RNA transcription termination (Suraweera et al., 2009).

Therefore, both the structural similarity and the overlapping localization of the two proteins support the hypothesis that different aspects of RNA metabolism might be impaired in SETX-related motoneuron degeneration, as it was demonstrated for IGHMBP2 in distal spinal muscular atrophy 1 (Guenther et al., 2009; Suraweera et al., 2009).

To elucidate the function of senataxin in neurons and correlate it with the SETX-related diseases, we first analysed its role in physiological conditions in primary hippocampal neurons and found that the protein acts as a crucial determinant of neurite outgrowth. Wild-type senataxin overexpression increased, while its silencing inhibited neurite outgrowth showing its obligatory role for this process. In contrast, in T3I, L389S and R2136H mutants, senataxin overexpression in neurons did not show any effect on this process, with neuronal phenotypes indistinguishable from those observed in the controls. Based on these data, the lack of effects observed, compared with the wild-type-protein overexpression results, excludes a dominant negative effect of these ALS4-related mutations rather favouring a pathogenic model of haploinsufficiency. Alternatively, a different protein function other than the one involved in neuritogenesis may be implicated in these dominant degenerative processes.

Replication of the overexpression and silencing experiments in P19 cells undergoing retinoic acid-induced neuronal...
Figure 9 Senataxin overexpression affects neurite outgrowth in undifferentiated stable clones. (A) Undifferentiated setxwt stable clones presented neurite-like structures. Undifferentiated control, setxwt and setx shRNA P19 stable clones were stained with phalloidin to label F-actin in the growth cone. Images were acquired at the same laser attenuation. Shown is a representative image for each vector. Scale bar = 100 μm. (B) Undifferentiated setxwt clones express neuronal proteins. Total extracts were prepared from undifferentiated setxwt, setx shRNA and control stable clones, run on 6 and 10% sodium dodecyl sulphate polyacrylamide electrophoresis gels and probed with anti-GAP43, phospho-microtubule-associated protein (MAP)1B, tubulin βIII (Tub βIII), senataxin and actin antibodies. (C) Fibroblast growth factor 8 (FGF8) expression levels increase in undifferentiated setxwt clones. RNA was extracted from undifferentiated setxwt, setx shRNA and control cells of B and used to analyse FGF8 levels by real-time polymerase chain reaction. Undifferentiated P19 cells stably transfected with the vector alone were used as endogenous controls (ctr, arbitrarily set at 1). Results shown are the mean ± SEM of three independent experiments each one performed in duplicate, using three independent clones for each vector (***P < 0.001). (D) FGF8 signalling is increased in undifferentiated setxwt stable clones. Total extracts from B were run on 10% sodium dodecyl sulphate polyacrylamide electrophoresis gels and probed with anti-phospho-Akt (p-Akt), Akt, phospho-Ser9-GSK3B (p-GSK3B), GSK3B, phospho-extracellular signal-regulated kinase-1/2 (p-ERK) and extracellular signal-regulated kinase1/2 antibodies. Shown is a representative blot out of three reproducible ones. (E) Undifferentiated setxwt stable clones were incubated with 10, 20 and 50 μM glycogen synthase kinase 3β (GSK3β) inhibitor SB216763 for 24 h. After incubation cells were stained with phalloidin and photographed. Shown is a representative image of setxwt cells untreated or incubated with 50 μM SB216763. Scale bar = 100 μm. Total extract prepared from the same cells were run on 6 and 10% sodium dodecyl sulphate polyacrylamide electrophoresis gels and probed with anti-phospho-Ser9-GSK3B (p-GSK3B),
differentiation yielded similar results; this prompted us to use these
cells to clarify the mechanism of action of senataxin taking advan-
tage of their well-characterized pattern of neuronal differentiation
(Bain et al., 1994).

The use of these cells revealed an additional role of senataxin
important for neuronal differentiation, i.e. inhibition of apoptosis.
Silencing of senataxin promoted cell death at Day 1 of P19 neur-
onal differentiation with increased caspase 3 activity. Wild-type
senataxin overexpression was instead cytoprotective and led to
normal neuronal differentiation. The key step in neuronal differen-
tiation that we found to be controlled by senataxin is FGF8
expression, with direct consequences on its related signalling. No
expression of FGF8 and neuritogenesis were detected in P19 cells
clones in which senataxin had been silenced, whereas FGF8 levels
were significantly increased in differentiating P19 cells overex-
pressing the protein. The link between senataxin levels and
FGF8 expression that we have demonstrated here is selective for
this growth factor without affecting expression of neural cell
adhesion molecule (mediating cellular aggregation in P19 cells)
or Pax2 and Lmx1b (two upstream FGF8 activator genes) (Guo et al.,
2007).

We analysed how changes in FGF8 mediate the cytoprotective
and differentiating effects of senataxin and found that silencing of
senataxin reduced, while overexpression enhanced, the phosphor-
ylation of several FGF8 target proteins including Akt, GSK3β
(at Ser9), ERK1/2 MAPK, MAP1B and Smad1 (on Ser206). The morphological correlation between FGF8-dependent signals and
senataxin is also strengthened by biochemical and functional
evidence in the literature. FGF8 acts as a neurogenic survival
factor by enhancing the transcription of basic helix-loop-helix
neurogenic factors and by inhibiting pro-apoptotic events such as
caspase 3 activation and DNA fragmentation induced by retin-
oidal acid and bone morphogenetic protein 4, through the activation
of PI3K-Akt and Ras-mitogen-activated protein kinase pathways
(Tanaka et al., 2001; Mason, 2007). In particular, ERK1/2 MAPK
inhibit apoptosis induced by retinoic acid and bone morphogenetic
protein through the regulation of Smad1 activity and the inhibition
of bone morphogenetic protein target genes expression (Pera et al.,
2003). In addition, extracellular signal-regulated kinase-1/2
induce phosphorylation of the microtubule-associated protein
MAP1B, whose phosphorylation levels increase during neurite
extension (Fischer and Romano-Clarke, 1990) and are associated
with axon elongation (Goold and Gordon-Weeks, 2005).
Phosphorylated MAP1B proteins are located mainly in the axon
with highest levels at the growth cone, while the unphosphory-
lated forms are present in the cell body and dendrites (Bush et al.,
1996; Goold and Gordon-Weeks, 2001). Our results, showing
opposite changes induced by senataxin overexpression and silen-
cing on phosphorylation of ERK1/2 MAPK and MAP1B, are con-
sistent with this picture.

A second important FGF8-dependent pathway we found to be
affected by senataxin expression levels is the one triggered by Akt
phosphorylation. Akt is a known pro-survival factor (Datta et al.,
1997; Cardone et al., 1998) and is also involved in neurite exten-
sion by stimulating early stages of dendrite formation and axon
growth (Kimura et al., 1994; Jackson et al., 1996; Lim and
Walikonis, 2008; Tucker et al., 2008; Read and Gorman, 2009).
Among the key players involved in this process downstream to Akt
is GSK3β. GSK3β is inhibited by Akt phosphorylation at Ser9, with
ensuing reduced phosphorylation of GSK3β-primed substrates,
such as MAP2 and tau, and increased microtubule stability in
growing axons (Wagner et al., 1996; Read and Gorman, 2009).
Hippocampal and P19-derived neurons overexpressing senataxin
showed increased levels of phosphorylated Akt and GSK3β at
the growth cone, while in senataxin-depleted neurons the amount of phosphorylated proteins at the growth cone decreased.
Significantly, the effects of senataxin overexpression were pre-
vented when FGF8 signalling was inhibited, while exogenous
FGF8 (FGF8b) reversed the effects of senataxin silencing in hippo-
campal neurons, demonstrating the functional connection be-
 tween senataxin and FGF8. This connection is particularly
relevant in view of the role played by FGF8 in neurons and
places senataxin at the crossroads of key developmental events.
FGF8 is highly expressed in vivo, in the early phases of neuronal
differentiation and it is essential for the development of vertebrate
forebrain (Garel et al., 2003), midbrain and cerebellum (Liu and
Joyner, 2001; Nakamura et al., 2005; Mason, 2007). FGF8 is also
extensively expressed in late embryonic stages and in adult brain
in most central and peripheral neurons (Tanaka et al., 2001). Thus,
in addition to a prominent role in the early organization of the
CNS, a direct biological activity on neurons has also been
reported (Gill and Tsai, 2006). The connection between FGF8
and senataxin is intriguing from the perspective of the different
senataxin-related neurodegenerative diseases. Indeed, although
partial and preliminary, the brain expression data available for
senataxin (Chen et al., 2006) correlate with the FGF8 expression
pattern and, significantly, with the brain areas involved in the
SETX-related AOA2 and ALS4 diseases. The time-window of
FGF8 expression also correlates with the age at onset of symptoms
of the two diseases, between the first and second decade and
both with slow progression.

Senataxin silencing in neurons grossly mimics the loss of func-
tion condition of recessive AOA2 mutations, at least of those
associated with protein loss. Indeed, in view of the heterogeneity

Figure 9 Continued
GSK3B, phospho-MAP1B and actin antibodies (right-hand side). Experiments were performed in three setxwt clones independently with
comparable results. Shown is a representative blot. (F) Effect of senataxin on neurite outgrowth during neuronal differentiation. Setxwt
and control stable clones were induced to differentiate with retinoic acid and after 4 days aggregates were dissociated and plated onto
poly-l-lysine culture plates. On Day 8, cells were photographed and RNA was extract and used to analyse MAP1B and GAP43 expression
levels by real-time polymerase chain reaction. Undifferentiated P19 cells stably transfected with the vector alone were used as endogenous
controls (arbitrarily set at 1). Results shown are the mean ± SEM of three independent experiments each one performed in duplicate, using
three independent clones for each vector (***P < 0.01). Scale bar = 100 μm. UNT = untreated cells.
among the effects of similar AOA2 mutations (Suraweera et al., 2007; Airoldi et al., 2010), generalization of the loss of function effect to all AOA2 mutations lacks experimental demonstration and could still be premature. However, even with these limitations, the loss of senataxin activities due to protein loss in both the nucleus and cytoplasm here postulated may represent the preliminary molecular bases of the neurodegenerative effects in wide areas of central and peripheral nervous systems associated with the AOA2 disease. In contrast, senataxin haploinsufficiency (if definitely confirmed by testing other protein functions) associated with a correct protein localization, as we observed with the ALS4-dominant mutations, may reasonably have a milder and more restricted effect primarily on motoneurons not directly related to neurite growth.

The relevant role of senataxin in these processes is also strengthened by the finding that even in the absence of neuronal commitment, senataxin overexpression was sufficient to trigger FGF8 expression, activate its signalling, induce neurite formation and even to synergize with a differentiating stimulus (retinoic acid). This finding mirrors previous evidence of effects on neurite elongation by overexpression of a constitutively active Akt in undifferentiated PC12 cells (Namikawa et al., 2000), further reinforcing the relationship between Akt and senataxin in neuritogenesis.

Figure 10  Role of senataxin in fibroblast growth factor 8 (FGF8), bone morphogenetic protein-4 (BMP4) and retinoic acid (RA) signalling pathways. The cartoon integrates the data present in the literature with our data indicating that senataxin affects cell death and neurite formation by modulating FGF8 expression levels and its downstream signalling. The cartoon thus represents a possible integration among FGF8, BMP4 and retinoic acid signalling and the role played by senataxin in it. Blue, red and black lines refer to signalling events controlled by FGF8, retinoic acid and BMP4, respectively, in a positive (arrows) or negative (↓) way. The main outcomes on key biological events in neurons are indicated at the bottom. Retinoic acid induces neuronal patterning and differentiation and axon outgrowth by binding to the nuclear retinoic acid receptors (RARs) and activating a cascade of basic helix-loop-helix neurogenic transcription factors (Maden, 2007; Soprano et al., 2007). Moreover, retinoic acid induces apoptosis through Bcl-2 downregulation, activation of caspase 3 and 9, cytochrome c release from the mitochondria and DNA fragmentation (Nizuma et al., 2006). BMP4 inhibits neuronal differentiation and induces cell death through the phosphorylation and activation of Smad1 that activates the transcription of several genes, such as Msx2, Hes1/5 and Ids, promoting apoptosis and inhibition of basic helix-loop-helix neurogenic transcription factors (Ying et al., 2003; Gambino et al., 2006). Retinoic acid enhances BMP signalling by increasing the expression levels of BMP2, BMP4 (Kendall et al., 2005) and BMP4 receptors (Fujita et al., 1999). FGF8 promotes neuronal differentiation and survival by activating an retinoic acid-independent cascade involving phosphoinositide 3-kinase (PI3K)-Akt and Ras-mitogen-activated protein kinase pathways, leading to enhanced basic helix-loop-helix neurogenic factors transcription and inhibition of retinoic acid and BMP4-induced apoptosis (Pera et al., 2003; Mason, 2007). Indeed, Akt activation determines the phosphorylation and inactivation of caspase 9, Bad (Datta et al., 1997; Cardone et al., 1998) and glycogen synthase kinase 3β (GSK3β). The inactivation of GSK3β promotes neuronal survival through the activation of HSF-1, Myc, β-catenin, CREB and eIF2B (Grimes and Jope, 2001; Beurel and Jope, 2006). Finally, FGF8 phosphorylate Smad1 in an extracellular signal-regulated kinase-1/2 mitogen-activated protein kinases dependent way, preventing its translocation to the nucleus and thus leading to inhibition of the BMP pathway (Pera et al., 2003).
Overall, these data allow us to propose a model for senataxin activity in neuronal differentiation and cell death, having FGF8, retinoic acid and bone morphogenetic protein-4 as key players, which is schematically represented and described in Fig. 10. This model integrates the bulk of data previously produced on these factors and the related signalling pathways in different in vivo and in vitro systems, and places senataxin as a new player in the model. We show how senataxin acts on the FGF8 expression levels and thereby influences its related downstream differentiation signalling with cytoprotective effects. These effects are lost in cases of loss-of-function mutations in SETX. Based on the predicted role of senataxin in RNA transcription, splicing and/or RNA translation processes, the postulated modulatory effect of senataxin may be exerted either directly on FGF8 transcription and/or splicing or indirectly on other factor(s) regulating FGF8 expression. Likewise, a regulatory role of senataxin on FGF8 RNA translation cannot be excluded.

The scheme proposed in Fig. 10 describes only the experimentally proven interactions of senataxin and its related signalling systems. The involvement of all additional factors and multiple signalling systems known to regulate neuronal fate in vivo, e.g., morphogens such as Wnt (Baker et al., 1999; Wilson et al., 2001) and Shh (Vaillant and Monard, 2009) or proteins with neutralizing activity (noggin, chordin and follistatin) (Stern et al., 2005), although not indicated, does not affect the general architectural interplay proposed in this model per se (Melton et al., 2004).

In conclusion, the elucidation of the signalling events controlled by senataxin and their interactions with pathways relevant in neuronal differentiation provides a novel framework for the FGF8-related neuronal differentiation that can be further enriched depending on the additional players identified and on the system examined. Each single step of the proposed pathway needs to be investigated for a possible role in the pathogenesis and/or progression of all SETX-related diseases.

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