Neuritin mediates NGF-induced axonal regeneration and is deficient in experimental diabetic neuropathy

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Running title: Neuritin mediates axonal regeneration

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Abstract

Objective. Axonal regeneration is defective in both experimental and clinical diabetic neuropathy contributing to loss of axonal extremities and neuronal dysfunction. The mechanisms behind this failure are not fully understood, however a deficit in neurotrophic support and signaling has been implicated.

Research Design and Methods. We investigated the expression of neuritin (also known as candidate plasticity gene 15, cpg15) in the sensory nervous system of control rats and rats with streptozotocin-induced diabetes using microarray, PCR, Western blotting and immunocytochemical analysis. The functional role of neuritin in sensory neurons in vitro was assessed using silencing RNA.

Results. Neuritin was expressed by a population of small diameter neurons in the dorsal root ganglia (DRG) and was anterogradely and retrogradely transported along the sciatic nerve in vivo. Nerve Growth Factor (NGF) treatment induced an increase in the transcription and translation of neuritin in sensory neurons in vitro. This increase was both time and dose dependent and occurred via MAP kinase or PI3K activation. Inhibition of neuritin using silencing RNA abolished NGF-mediated neurite outgrowth, demonstrating the crucial role played by neuritin in mediating regeneration. Neuritin levels were reduced in both the DRG and sciatic nerve of rats with 12 weeks of streptozotocin-induced diabetes, and these deficits were reversed in vivo by treatment with NGF.

Conclusions. Manipulation of neuritin levels in diabetes may therefore provide a potential target for therapeutic intervention in the management of neuropathy.
Axonal regeneration is defective in both experimental (1;2) and clinical (3;4) diabetic neuropathy. This may be instrumental in the pathogenesis of diabetic neuropathy, but its mechanism is unclear and multifactorial – a combination of oxidative and biochemical stress, neurotrophin deficits, impaired synthesis and transport of cytoskeletal components and formation of intracellular and extracellular AGEs (5-7).

Neurotrophins play an important role in promoting neuronal survival, differentiation, function and repair. It is well established that they regulate axonal growth in sensory neurons both regenerative growth in response to injury and collateral sprouting of uninjured nerve terminals (8-11). In diabetes, it is thought that a reduction in neurotrophin production and support contributes, in part, to the failure in axonal regeneration and pathogenesis of diabetic neuropathy (12). Levels of NGF are reduced in peripheral target tissue in experimental (13) and clinical diabetes (14) and retrograde axonal transport is also impaired (12;15;16).

NGF stimulates neurite outgrowth in sensory neurons in vitro via activation of high-affinity tyrosine kinase (TrkA) or low-affinity p75 receptor (17;18) and intracellular activation of both the Raf-ERK and PI3K pathways is involved (19-21). However, the downstream mechanisms by which NGF exerts its neuritogenic effects are still not fully understood.

Neuritin was first identified and characterized in a screen for genes regulated in the rat hippocampal dentate gyrus by kainate-induced seizures (22;23). Neuritin is also upregulated in hippocampal and cortical neurons in vivo and in vitro by the neurotrophin BDNF (22) and induces neurite outgrowth and arborization in cultures of embryonic rat hippocampal and cortical neurons (22), Xenopus motoneurons (24) and PC12 cells (25). When overexpressed in Xenopus optical tectal neurons, neuritin elicits dendritic and axonal extension (26) and synaptic maturation (27). Taken together, this evidence of dynamic regulation of neuritin coupled with neurotrophic effects, suggests that neuritin may play a role in neurotrophin-dependent axonal regeneration in the sensory nervous system.

In this study we characterize the expression and function of neuritin in the rat sensory nervous system of control rats and rats with streptozotocin (STZ)-induced diabetes. We demonstrate that neuritin is expressed by sensory neurons, down-regulated in STZ-diabetes and is upregulated by NGF in vitro and in vivo. Furthermore, we demonstrate that upregulation of neuritin is essential for NGF-mediated neurite outgrowth.

Methods and Materials

Animals

All experiments were conducted in accordance with UK Home Office Regulations and with the Animals (Scientific Procedures) Act 1986. Male Wistar rats (250-300g on arrival, Charles River UK) were used in all studies. Diabetes was induced by a single intraperitoneal injection of STZ (Sigma, St. Louis, MO), freshly dissolved in normal saline, at a dose of 55 mg/kg, administered the morning after an overnight fast. Three days later, blood glucose was monitored (BM Acutest strips; Roche Diagnostics, Mannheim, Germany) and any STZ-treated rats with blood glucose < 15 mmol/l were rejected from the study. Animals were then group housed with full access to food and water for 12 weeks.
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**Animal Study 1 – effects of diabetes on gene expression by microarray profiling**

Gene expression in the DRG was compared between two groups of rats, one control group and one after 12 weeks diabetes. DRG were dissected out from spinal segments L4 and L5 and the four ganglia pooled from each rat. Ganglia from groups of 3 rats were further pooled to give enough RNA for purification, derivatization and hybridization. Pooled ganglia were extracted and the RNA processed and hybridized to Affymetrix U34 rat microarray chips exactly as described elsewhere (28).

**Animal Study 2 – effects of NGF treatment on neuritin expression in diabetic rats**

Rats were randomly assigned to 4 groups (10 rats per group), designated as follows: control untreated, untreated diabetic, diabetic given 0.1 mg/kg NGF and diabetic given 0.5 mg/kg NGF. NGF treatment began 8 weeks after induction of diabetes and was maintained for 4 weeks until the rats were killed. Purified human recombinant NGF (Genentech, San Francisco, CA, USA) was given 3 times per week by subcutaneous injection at the back of the neck. At the end of 12 weeks rats were killed and L4/5 DRG and sciatic nerve removed and processed for quantitative PCR or Western blotting.

**Animal Study 3 – Axonal transport of neuritin and effect of NGF**

In order to study axonal transport control, diabetic rats (as treated in Study 2) were anaesthetized with isoflurane (2% in oxygen), and, under sterile conditions, the left sciatic nerve was exposed at mid-thigh level. The nerve was ligated using prolene sutures (Ethicon, Johnson & Johnson, Brussels, Belgium), the wound was closed and animals recovered under observation (29). Twenty-four hours later, animals were deeply anaesthetized with sodium pentobarbitol (Sagatal, 60 mg/kg, i.p.) and perfused transcardially with 0.9% heparinised saline (0.9% NaCl, 50 units/ml heparin) followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB). Sciatic nerves were removed, post-fixed for 2 hours at 4°C then cryoprotected in 30% sucrose (in 0.1 M PB overnight at 4°C). Nerves were frozen in OCT embedding medium (VWR, UK) on liquid nitrogen and stored at -80°C until processing. Alternatively, animals were humanely killed and 5mm segments were cut from the constricted sciatic nerve - two segments were taken proximal and two distal to the ligature, and anatomically matching segments were cut from the contralateral intact nerve. Samples were frozen on liquid nitrogen prior to analysis.

**Sensory neuron culture**

Adult male Wistar rats (250g, Charles River, UK) were killed by concussion followed by decapitation. Dissociated sensory neurons were prepared as previously described (30). Neurons were suspended in modified Bottenstein and Sato’s medium (BS; containing 0.1 mg/ml transferrin, 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite, and 1 µg/ml BSA, 0.01 mM cytosine arabinoside and 10 pM insulin in Ham’s F12). Neurons were seeded onto either Lab-Tek chamber slides (Nunc, VWR) or 35mm petri-dishes coated with 2 µg/ml laminin (Sigma, L2020). Neurons were plated for one hour prior to treatment with NGF (Sigma; 0.1, 1 or 10 ng/ml in BS) or BS alone as a control for 24 hours in 5% CO2 at 37°C. For studies using functional inhibitors of MEK 1/2 (U0126; 10 µM) and its control U0124 (10 µM; Calbiochem) and phosphatidylinositol 3-kinase
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(LY294002; 10 µM; Calbiochem), neurons were treated with each inhibitor 1 h following plating, then treated with NGF (10ng/ml; Sigma) for 24 hours at 37 °C.

Neuritin siRNA transfection of adult rat sensory neurons
siRNA was introduced into cells using the AMAXA nucleofection system (AMAXA Inc, Maryland, USA). Following dissociation, sensory neurons were suspended in 100 µl rat neuron nucleofector solution containing either a scrambled sequence of siRNA with no homology to any known proteins (50 nM; Ambion) or 50 nM of a combination of three neuritin siRNAs:

5’ GUGCGAUGCGACUUUAAGtt 3’
5’ GGGCUUUUCAGACUGUUUGtt 3’
5’ GGCAGCUUAUUCGAACUCUtt 3’

Neurons were transfected using program 0-03 of the AMAXA nucleofection system (transfection efficiency was determined to be 70% using pmaxGFP positive control plasmid (Amaxa)), resuspended in BS, treated with NGF and incubated for 48 hours at 37 °C.

Quantitative RT-PCR of neuritin and calcitonin gene-related peptide levels
Total RNA was extracted from sensory neurons, measured and quantities normalised before being reverse transcribed to cDNA using Moloney-murine leukaemia virus reverse transcriptase (M-MLV RT). qPCR analysis of neuritin and CGRP levels were carried out on samples in triplicates using the SYBR Green Master mix kit (Molecular probes, USA) and the ABI Prism 7000 sequence detection system (Applied Biosystems Inc., Foster City, CA). The primers were designed using Primer Express™ 1.0 software (Applied Biosystems Inc; Foster City, CA) and primer sequences available on request. Quantitative PCR data are expressed as cycle thresholds rather than normalising expression to a housekeeping gene, to prevent errors introduced by choosing an erroneous housekeeping gene whose expression may itself be altered by the experimental conditions (diabetes or neurotrophin treatment).

Western blotting
Samples were homogenized in ice-cold lysis buffer (Tris-HCl pH 7.4, NP-40 (1%), sodium orthovanadate (2 mM), sodium fluoride (10 mM), EGTA (2 mM), sodium pyrophosphate (10 mM), 1mM PMSF and protease inhibitor cocktail (Sigma, UK). Samples (30 µg protein/lane) were separated by SDS-PAGE using 16.5% acrylamide gels. Proteins were transferred to nitrocellulose membranes by semi-dry electrophoresis. Non-specific binding was blocked by incubation in Tris-buffered saline containing 0.05% Tween-20, 5% casein (Sigma, UK) and 5% BSA (Fraction V, Sigma, UK), for one hour at room temperature and then incubated overnight at 4°C with anti-neuritin (1:500; R&D systems). Blots were washed and then incubated in HRP-conjugated anti-goat IgG (Cell Signaling; 1:5000) for 1 hour at room temperature. Protein bands were visualized using an Enhanced Chemiluminescence kit and quantified using densitometric analysis (Scion image software version alpha 4.0.3.2, USA).

Immunocytochemistry
Sensory neurons were fixed with ice-cold 4% paraformaldehyde for 30 min, and then washed three times with PBS. Fixed cells were incubated with anti-β (III) tubulin (Sigma, 1:1K), a pan-neuronal marker to label neuronal cell
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bodies and neurites, or anti-neuritin (1:100, R&D systems) overnight at 4°C. Cells were washed and incubated with FITC-conjugated donkey anti-mouse or donkey anti-goat (1:200, Jackson ImmunoResearch) respectively for 1 hour at room temperature. Longitudinal sections (8 µm) of sciatic nerve or DRG were incubated for one hour at room temperature with 5% donkey serum, 0.01% Triton X-100 in PBS, then in anti-neuritin (1:100, R&D systems) and anti-TrkA (1:1000; Chemicon) for 24-48 hours at 4°C. Sections were washed with PBS, then incubated for 1 hour in TRITC- or biotin-conjugated donkey anti-goat and Cy3-conjugated donkey anti-rabbit (1:400; Jackson ImmunoResearch), followed by 1 hour in FITC-conjugated extravidin (1:400; Vector labs). Sections were mounted in Vectorshield containing DAPI (Vector Labs). Immunofluorescence was viewed using a Leica fluorescence microscope and images acquired using a Hamamatsu camera with Wasabi software.

**Neurite outgrowth analysis**

For each experimental condition, images of 30 neurons were acquired from randomly selected fields of view. The mean number of neurite-bearing cells (defined as those with neurites longer than 1.5 times cell body diameter) was calculated from these images. The length of the longest neurite from each cell was calculated using SigmaScan software (SPSS, UK), as was a measure of total neurite outgrowth. A series of concentric circles was overlaid onto an image of each neuron, and the number of times neurites crossed each circle was calculated. This gave us both a measure of total neurite outgrowth (total number of crosspoints) and neurite branching structure (crosspoints related to distance from the cell body). All these measurements were repeated in at least 3 independent cultures.

Data are expressed in all graphs as mean ± s.e.m. Statistical analysis was conducted using Prizm software using ANOVA followed by Dunnett’s post-hoc test.

**Results**

**Neuritin is expressed by sensory neurons**

We first established that neuritin was expressed in adult control rat DRG, using conventional PCR and Western blotting techniques (data not shown). Using immunocytochemical analysis we examined the distribution of neuritin immunoreactivity (IR) in adult rat lumbar (L4/5) DRG. Neuritin-IR was clearly localized within the cytoplasm of a population of sensory neurons in the DRG (Fig 1A). Small diameter sensory neurons expressed higher cytoplasmic levels of neuritin-IR (Fig 1A, arrows) than large diameter neurons (Fig 1A, asterisks). Neuritin-IR colocalised with TrkA, the high-affinity receptor for nerve growth factor (NGF; Fig 1B). Immunofluorescence was abolished by omission of the primary antibody, indicating the neuritin-IR to be primary-antibody specific (data not shown). Neuritin-IR was also maintained in cultures of dissociated adult rat sensory neurons treated with control media (in the absence of neurotrophic support, Fig 1C) or with Nerve Growth Factor (Fig 1D). Neuritin-IR was detected in neuronal cell bodies and in puncta throughout the neurites (Fig 1D, E).

**Neuritin is anterogradely and retrogradely transported in rat sciatic nerve in vivo**

We used immunohistochemistry to characterize axonal transport of neuritin in the sciatic nerve of control adult rats. Ligation of the sciatic nerve caused an increase in neuritin levels proximal and distal to the ligation site (Fig 2A). Immunohistochemical analysis showed that neuritin-IR increased in axons
proximal and distal to the ligature site (Fig 2A, B, arrows) whilst contralateral, non-ligated sciatic nerve showed no neuritin (Fig 2C). Accumulations were also quantified by western blotting (see below). Neuritin is therefore transported bidirectionally along axons in the sciatic nerve.

**Neuritin is upregulated by Nerve Growth Factor**

Since neuritin was expressed in predominantly small diameter sensory neurons, we investigated the effect of NGF on neuritin expression. Quantitative real time-PCR was used to assess neuritin mRNA levels in sensory neurons treated for 24 hours with NGF (0.1, 1, and 10 ng/ml). NGF caused a dose-dependent increase in neuritin mRNA (Fig 3A) and protein (Fig 3B). Quantification of neuritin protein levels using densitometric analysis showed that these NGF-mediated increases in neuritin were significant compared to control (Fig 3C).

The observation that neuritin is expressed in adult rat sensory neurons, particularly small diameter neurons, and that its expression is increased in response to NGF in vitro, indicates a potential role for neuritin in mediating downstream actions of NGF.

**Neuritin is upregulated via activation of MAP kinase and/or PI3K pathway**

To investigate the intracellular signaling pathways responsible for the NGF-mediated upregulation of neuritin, we pretreated dissociated sensory neurons with either a MEK1/2 inhibitor (U0126), a negative control for U0126 (U0124) or a PI3K inhibitor (LY294002) prior to stimulation with NGF (Fig 4). It can clearly be seen that from the photomicrographs that inhibition of either MEK1/2 or PI3K abolishes NGF-mediated neurite outgrowth (Fig 4, C, D). Inhibition of TrkA with k252A (10mM) also reduced neuritin upregulation (data not shown). U0124 had no effect on neurite outgrowth (data not shown). The levels of neuritin in neurons treated with these inhibitors was determined using Western blotting (Fig 4 E), and quantified using densitometric analysis (Fig 4F). The NGF-mediated upregulation of neuritin was significantly reduced to below control values by U1026 (whilst the negative control, U1024 had no effect). The PI3K inhibitor, also prevented the NGF-mediated upregulation of neuritin, but did not reduce the basal level of neuritin in sensory neurons (Fig 4 E, F).

These results indicate the NGF-mediated neurite outgrowth can be prevented using inhibitors of both MEK1/2 and PI3K. These inhibitors prevent the NGF mediated upregulation of neuritin, a factor we have shown to be crucial in mediating NGF induced neurite outgrowth.

**Neuritin expression is reduced in diabetes**

Since diabetes is associated with a decrease in NGF levels we hypothesized that neuritin levels may also be compromised in diabetes. We compared neuritin gene expression levels in DRG of control rats with those of rats with 12 weeks STZ-diabetes (9 rats, 3 chips per condition) and found a significant reduction in mRNA from 2756 to 1548 (Affymetrix units; p<0.05; Affymetrix accession number U88958_at). This decrease in diabetes was also seen at the protein level using Western blot analysis (Fig 5). This deficit in neuritin was reversed by treatment with NGF (p<0.02 for the higher NGF dose, Fig 5).

Diabetic rats lost weight and were hyperglycemic (blood glucose: controls: 6.0 ± 0.6 mmol/l vs diabetic untreated 33.0 ± 3.2 mmol/l). Treatment with NGF did not affect the raised blood glucose in diabetic rats, though the higher NGF dose exacerbated body weight loss (data not shown). NGF was
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Neuritin mediates NGF-mediated neurite outgrowth

In order to determine the functional role of neuritin, we utilised a gene silencing approach. Sensory neurons were transfected with siRNA using electroporation with AMAXA Nucleofector™ (31). Adult rat sensory neurons were transfected with either a cocktail of three neuritin siRNAs or a scrambled sequence siRNA with no homology to any known genes or untransfected to act as control. Neurons were treated for 48 hours with NGF (10ng/ml) or control media alone.

Transfection with a scrambled sequence siRNA did not affect the NGF-induced upregulation of neuritin mRNA (Fig 7A) or protein (Fig 7B, C). However, transfection with neuritin siRNA caused a significant reduction in both neuritin message (Fig 7A) and protein (Fig 7B, C), to below control neuronal values, indicating the specificity of the siRNA.

To assess the contribution of neuritin to the regenerative response, we utilized an in vitro culture system of dissociated adult sensory neurons treated with NGF to promote neurite outgrowth (30;32;33). Following 48 hours in culture neurons began to extend neurites in the absence of exogenous neurotrophins (Fig 8A). Neurons typically extended only a few neurites and these were unbranched and relatively short (316 ± 29 µm; Fig 8E).

Exogenous NGF produced robust neurite outgrowth, and this outgrowth was not affected by transfection of neurons with scrambled siRNA (Fig 8B, D-F). A greater number of neurons extended neurites in the presence of NGF compared to control neurons (NGF: 76% vs control: 59% neurons with neurites). Furthermore, neurites were significantly longer (533 ± 36 µm, p<0.05; Fig 8B, D) and more complex (Fig 8B, E, F). In contrast, transfection of neurons with neuritin siRNA reduced the number of neurite-bearing cells to 48% (in the presence of NGF). The length of the longest neurite extended in response to NGF was significantly reduced (281 ± 22 µm), and neurite density, as measured by total crosspoints, was significantly inhibited compared to neurons transfected with the scrambled siRNA (Fig 8 E, F). Silencing neuritin, therefore, reduced neurite outgrowth to a level equivalent to that observed in the absence of exogenous NGF.

Since this inhibition of neurite outgrowth by neuritin siRNA could have reflected simple interference with NGF signaling, we examined the expression of another NGF-responsive gene, which is not part of the neurite outgrowth response to the neurotrophin. Calcitonin gene-related peptide (CGRP) is upregulated by NGF both in vitro and in vivo and responds to the neurotrophin in assumed not to attenuate the severity of the diabetic state.

As illustrated in Figure 2 neuritin is axonally transported in control sciatic nerve. We compared neuritin levels in sections proximal and distal to a ligature (and equivalent section from contralateral unconstituted nerve) in control and diabetic rats using Western blotting and densitometric analysis (Fig 6). In control rats the proximal accumulation (P1+P2) was 65.9 ± 3.5 (arbitrary units, mean ± SEM) and distal accumulation (D1+D2) was 34.1 ± 3.5 (Fig 6A). In untreated diabetic rats the proximal accumulation (13.2 ± 2.5) was significantly reduced (p<0.001), though the distal accumulation (23.3±7.3) was not (Fig 6B). NGF treatment of diabetic rats (0.5 mg/kg) increased proximal accumulation (38.5±4.2), such that it was significantly higher (p<0.003) than that seen in untreated diabetic rats, but still significantly lower (p<0.003) than that seen in controls. Distal accumulation was also increased numerically by NGF (41.7±11.0), such that it did not differ significantly from the equivalent values for the other two groups (Fig 6C).
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intact (i.e. not regenerating) primary afferent neurons (34). Neither the scrambled siRNA nor the neuritin siRNA had any effect on NGF-mediated upregulation of CGRP mRNA (Fig 7C), confirming that the knockdown of neuritin by siRNA was specific, other NGF-mediated signaling events were unaffected by the silencing of neuritin.

Together these data indicate that neuritin is upregulated by NGF, and plays a functional role in mediating neurite outgrowth of sensory neurons.

Discussion

This investigation shows reduced expression and axonal transport of neuritin in sensory neurons of STZ-induced diabetic rats. These deficits were prevented by treatment with NGF. The in vitro studies show that neuritin is instrumental in the translation of NGF signals to promote neurite outgrowth in adult rat sensory neurons. Inhibition of neuritin using silencing RNA abolished NGF-mediated neuritogenesis.

A deficit in NGF expression by sensory neuron targets is well established in experimental diabetic neuropathy (13;15;35). Furthermore, replacement therapy with exogenous NGF in diabetic rats normalizes key molecular and functional aspects of the neuropathy (36;37). Hence, it is likely that the neuritin deficit reported here in diabetic rats is secondary to a deficit in endogenous NGF. It remains to be determined whether other stimuli such as hypo-insulinaemia may also contribute to the deficit. The precise mechanism of action of NGF on neuritin expression has not been fully elucidated, but appears to be dependent on MAPK and/or PI3-K activation.

Neurotrophins have previously been shown to regulate neuritin gene expression in a number of other cell types. Brain derived neurotrophic factor and Neurotrophin-3 caused an upregulation of neuritin in hippocampal and cortical neurons (22). It has also been demonstrated that hypoxia induces neuritin mRNA transcription in human microendothelial cells (HMEC-1) (38). Neuritin was identified in a microarray screen of NGF-regulated genes in rat pheochromocytoma cells (39) and has recently been found to promote neuritogenesis in this cell line (25). In this study we demonstrate that NGF upregulates neuritin in adult rat sensory neurons which mediates neurite outgrowth.

Sequencing of the promoter region of the mouse neuritin gene has found at least nine binding sites for transcription factors, including three sequences similar to cyclic AMP response element binding protein (CREB), three sequences similar to AP-1 binding sites, a TPA-responsive element (TRE), and two sequences similar to the early growth response (EGR) family binding site (40). These transcription factors have been implicated in NGF mediated neurite outgrowth (41;42).

Stimulation of TrkA via NGF leads to activation of several intracellular signaling pathways including Ras-Raf-MEK-ERK and P13K-AKT (43). Inhibition of both MEK and PI3K inhibited neurite outgrowth in this study, and also prevented NGF-mediated upregulation of neuritin. The MEK1/2 inhibitor, U0126, reduced neuritin levels to below control levels – possibly indicating inhibition of an endogenously produced factor in the culture system. The PI3K inhibitor reduced NGF-upregulation of neuritin levels, but not below control levels. This may indicate two pathways by which neuritin is upregulated – an NGF-mediated ERK/AKT pathway, and an unidentified factor produced in culture (possibly by neurons themselves or contaminating Schwann cells or fibroblasts) maintaining a ‘constitutive’ expression of neuritin via ERK activation.
The mechanism of the neuritogenic action of neuritin remains unknown. Neuritin is a GPI-linked protein, without transmembrane or cytoplasmic domains. There are examples of such GPI-linked proteins acting as heterophilic or homophilic adhesion molecules, e.g. glypican-1 a heparin sulphate proteoglycan, acts as a co-receptor for slit, FGF and laminin (44). Neuritin, however, lacks the immunoglobulin G domain common to GPI-linked adhesion molecules, but does show some structural similarity to ephrins, a family of GPI-linked ligands which act as guidance molecules activating their receptor by cell-cell contact (45).

There is conflicting evidence regarding the functional role of GPI-cleaved, soluble neuritin. Infection of Xenopus tectal cells with full length neuritin promoted dendritic arborisation, in contrast to infection with truncated neuritin lacking the GPI consensus signal (26). However, transfection of rat hippocampal neurons with truncated neuritin did promote neuritogenesis (22). Expression of full length neuritin in Xenopus motor neurons increased the rate of axonal outgrowth by promoting axonal branching and reducing axonal retraction from presynaptic sites, indicating that neuritin modulates both axonal structure and function (24). Neuritin, in either truncated, secreted, or full-length version, was found to alter the morphology of NIH 3T3 cells producing neurite-like outgrowths and arborization (46). We found that neuritin is localised in discrete puncta within neurites particularly at branch points and growth cones. Since, neuritin promotes extension and branching of neurites, it will be of interest to determine whether this occurs through interactions of neuritin with the extracellular matrix at points of focal adhesions. It is interesting to note that a recent study of small fiber innervation of the cornea in diabetic patients detected reduced branching as a significant change associated with the earliest stage of neuropathy (47). This may derive from changes in neuritin expression in peripheral fibres.

Axonal regeneration is defective in both experimental (1;48;49) and clinical (3;50) diabetic neuropathy. Furthermore, when measurements are focused on fibers of appropriate phenotype, NGF supplementation is shown to reverse the regeneration deficit in experimental diabetes (51). However potential therapeutic effects of NGF treatment in diabetic neuropathy were negated due to the deleterious painful side-effects observed in phase II clinical trials (52). It is likely that NGF induced expression of neuritin in diabetes forms an important part of the normalization of nerve function by NGF, it will now be important to determine whether manipulation of neuritin levels in sensory neurons following injury or neuropathy may provide a potential target for therapeutic intervention in the management of peripheral nerve trauma or neuropathy.
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Reference List

10. Lindsay, RM: Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. J Neurosci 8:3337-3342, 1988
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Figure legends

Fig 1. Neuritin is expressed by adult rat sensory neurons. Representative micrographs show that neuritin-immunoreactivity (IR) is present in sensory neurons in L4/5 DRG. A number of small-diameter neurons show higher levels of neuritin-IR (arrows, A) than large diameter neurons (asterisks, A). Neuritin-IR colocalises with TrkA-IR (B). Dissociated sensory neurons express high levels of cytoplasmic neuritin-IR in culture (C). Neurons extend neurites in response to stimulation with Nerve Growth factor (10 ng/ml, 24 hours) and neurites express punctate neuritin-IR (D, E). Scale bars = 50 µm (A-D) 10 µm (E).

Fig 2. Neuritin is anterogradely and retrogradely transported in rat sciatic nerve. Adult rats received a unilateral sciatic nerve ligature at mid-thigh level for 24 hours. Longitudinal sections through paraformaldehyde-fixed sciatic nerve at the constriction site were immunostained for neuritin (A, B). Neuritin-IR can clearly be seen to accumulate within axons (arrows A) at the ligature site, proximal (A, B) and distal (A) to the ligature – indicating bidirectional axonal transport of neuritin. There was little neuritin-IR observed in contralateral nerve (C). Scale bars = 1mm (A), 0.5mm (B, C).

Fig 3. Nerve Growth Factor causes a dose-dependent upregulation of neuritin in sensory neurons. Dissociated sensory neurons were plated for 24 hours with either NGF (0.1, 1, 10 ng/ml) or control media. Neuronal lysates were analyzed for neuritin mRNA using qualitative rt-PCR (A) or protein using Western blotting (B) with densitometric analysis (C). Total ERK levels were determined for loading controls (B). NGF caused a significant dose-dependent increase in neuritin mRNA levels at all concentrations compared to control levels in the absence of NGF (o: represent data from 3 individual experiments; - represents mean value; A; *p<0.001). NGF also caused a significant increase in neuritin protein levels (B, C; *p<0.05, *** p<0.001, n=3 independent experiments) compared to control levels. Statistical analysis was conducted using ANOVA with TUKEY posthoc test.

Fig 4. NGF upregulates neuritin via MAP kinase or PI3K activation. Dissociated sensory neurons were treated with a MEK specific inhibitor (U0126; 10µM, Calbiochem) the negative control for MEK inhibitor (U0124; 10µM, Calbiochem) or a PI3K inhibitor (LY294002; 10µM, Calbiochem) for one hour then NGF (10ng/ml) was added for 24 hours. Neurons were either visualized by immunostaining for beta III tubulin (representative micrographs, A-D) or lysed and neuritin levels assessed by Western blotting (E, F). Both U0126 (C) and LY294002 (D) inhibited NGF-mediated neurite outgrowth (B). U0124 did not have any effect on NGF-mediated neurite-outgrowth (data not shown). Neuritin levels were significantly reduced in cells treated with NGF and U0126 and NGF and LY294002, compared to NGF alone (E, F, *p<0.01). This indicates that neuritin-mediated neurite outgrowth in response to NGF is mediated via the MEK1/2 and/or PI3K pathways.

Fig 5. Diabetes-induced decrease in neuritin in the DRG is reversed by treatment with NGF in vivo. Dorsal root ganglia from control rats, rats with 12 weeks streptozotocin-induced diabetes (untreated or treated for last 4 weeks with NGF (0.1 or 0.5mg/kg s.c. 3 times per week)), were lysed and analyzed for neuritin expression using Western blot analysis (A, B). There was a decrease in neuritin levels in rats
Neuritin mediates axonal regeneration with diabetes, this decrease was reversed or prevented by treatment with NGF 0.5mg/kg. Protein levels are derived from scanned Western blots and are expressed relative to the control mean value (B, * indicates p<0.01 by comparison with the untreated diabetic group using one-way ANOVA with post hoc TUKEY’S tests).

Fig 6. NGF treatment restores the deficit in axonally transported neuritin in the sciatic nerve. Ligated and intact sciatic nerves from control or STZ-diabetic rats were cut into 5mm segments proximal and distal to the ligature and samples processed for Western blotting to assess neuritin levels (see schematic diagram, D). Neuritin accumulated both proximal and distal to the ligature in control rats (A). In untreated diabetic rats the proximal accumulation was significantly reduced though the distal accumulation was not (B). NGF treatment (0.5 mg/kg)of diabetic rats increased proximal and distal accumulation of neuritin (C).

Fig 7. Transfection of sensory neurons with silencing RNA (siRNA) against neuritin prevents NGF-upregulation of neuritin. Neurons were transfected with either a siRNA against neuritin, or a scrambled sequence siRNA as a control and treated with NGF (10ng/ml) for 48 hours, or untreated to act as control. Quantitative PCR analysis of neuritin gene expression showed that transfection with the scrambled control sequence did not prevent the NGF-mediated increase in neuritin mRNA (A, *p<0.001 compared to control). As expected, transfection with neuritin siRNA prevented the NGF-mediated increase in neuritin mRNA (# p<0.001 compared to scrambled siRNA plus NGF) and significantly reduced neuritin mRNA levels compared to control levels (A * p<0.01). Neuritin siRNA also prevented the NGF-mediated upregulation of neuritin protein (B, representative Western blot against neuritin, using samples obtained from 3 independent experiments and densitometric analysis (# p<0.001, compared to scrambled siRNA plus NGF, *p<0.001 compared to control). Total ERK levels were determined for loading controls (B). Note, transfection of neurons with neuritin siRNA does not affect NGF-induced transcription of calcitonin gene-related peptide (C), indicating the specificity of the siRNA approach. Statistical analysis was performed using ANOVA followed by TUKEY post hoc test.

Fig 8. NGF-mediated neurite outgrowth is prevented by neuritin siRNA. Dissociated adult rat sensory neurons were transfected with either a siRNA against neuritin (C) or a control scrambled sequence siRNA (B), and incubated in the presence (B, C) or absence (A) of NGF (10 ng/ml; 48 hours). Neurons were fixed in 2% paraformaldehyde, immunostained for (III) tubulin, a pan-neuronal marker which labels all cell bodies and neurite outgrowth. Neurite outgrowth was assessed and quantified (D - F). Control untransfected neurons extend highly branched neurites when stimulated with NGF, transfection with the scrambled sequence siRNA did not prevent this NGF-induced neurite outgrowth (B, D - F). In contrast, neurons transfected with neuritin siRNA did not exhibit the same pattern of neurite outgrowth in response to NGF (C, D - F), with neurons extending significantly shorter neurites than neurons transfected with scrambled siRNA (C, D). Total neurite density was quantified by a series of concentric circles overlaid onto an image of a neuron, the number of times neurites crossed each circle was calculated. Data is expressed as mean total number of crosspoints at all circles ± s.e.m from at least 3 independent experiments (E). Scale bars = 50 µm.
Fig 1

A. Neurtitin-IR
B. TrkA-IR
C. in vitro control
D. in vitro + NGF
E.
Fig 2
Neuritin mediates axonal regeneration

Fig 3
Fig 4

Neuritin mediates axonal regeneration
Fig 6
Neuritin mediates axonal regeneration

Fig 7
Fig 8

Neuritin mediates axonal regeneration