Engineered zinc finger protein mediated VEGF-A activation restores deficient VEGF-A in sensory neurons in experimental diabetes

Running title: VEGF-A and experimental diabetic neuropathy

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Objective: To evaluate retrograde axonal transport of VEGF-A protein to sensory neurons following intramuscular administration of an engineered zinc finger protein activator of endogenous VEGF-A (VZ+434) in an experimental model of diabetes, and to characterize the VEGF-A target neurons.

Research Design and Methods: We compared the expression of VEGF-A in lumbar (L)4/5 dorsal root ganglia (DRG) of control rats and VZ+434 treated and untreated streptozotocin (STZ)-induced diabetic rats. In addition, axonal transport of VEGF-A, activation of signal transduction pathways in the DRG, and mechanical sensitivity was assessed.

Results: VEGF-A-immunoreactivity (-IR) was detected in small-medium diameter neurons in DRG of control rats. Fewer VEGF-A-IR neurons were observed in DRG from STZ-diabetic rats, this decrease was confirmed and quantified by Western blotting. VZ+434 administration resulted in a significant increase in VEGF-A protein expression in ipsilateral DRG, 24 hours following injection. VEGF-A was axonally transported to the DRG via the sciatic nerve. VZ+434 administration resulted in significant activation of AKT in the ipsilateral DRG by 48 hours which was sustained for 1 week post-injection. VZ+434 protected against mechanical allodynia 8 weeks post-STZ.

Conclusions: Intramuscular administration of VZ+434 increases VEGF-A protein levels in L4/5 DRG correcting the deficit observed following induction of diabetes and protects against mechanical allodynia. Elevated VEGF-A levels result from retrograde axonal transport and are associated with altered signal transduction, via the phosphatidylinositol 3'-kinase pathway. These data support a neuroprotective role for VEGF-A in the therapeutic actions of VZ+434 and suggest a mechanism by which VEGF-A exerts this activity.
The vascular endothelial growth factor (VEGF) family consists of seven secreted glycoproteins, named VEGF-A to -F and placental growth factor (PGF). The prototypic VEGF family member, VEGF-A, was first demonstrated to enhance vascular permeability and promote the proliferation, migration, and survival of endothelial cells leading to increased angiogenesis and tissue perfusion (1). In addition to its angiogenic properties, both neurotrophic and neuroprotective functions for VEGF-A have been described in both sensory (2-6) and motor neurons (7).

The VEGFs bind to, and signal through, specific receptor tyrosine kinases: VEGFR-1 (Flt-1); VEGFR-2 (KDR; Flk-1); the neuropilin receptors (NP-1 and NP-2) (8; 9) as well as via interaction with heparin sulphate proteoglycans (HSPG) (10). VEGF-A can activate both the mitogen activating kinases (MAPK) and the phosphatidylinositol 3'-kinase (PI3-K) signal transduction pathways (11), and both of these pathways have been implicated as mediators of the phenotypic abnormalities observed in sensory neurons during the development of diabetic neuropathy (12-15).

VEGF-A mRNA is alternatively spliced to express three major protein isoforms, VEGF-A121, VEGF-A165 and VEGF-A189. Both experimental and clinical studies have indicated improvements in the signs and symptoms of sensory diabetic neuropathy after intramuscular injection of a plasmid DNA encoding a single isoform of VEGF-A, VEGF_{165}-A (16-18). However, several preclinical studies suggest that the combination of all three VEGF-A isoforms may provide more potent biological activity (19-21). Given the importance of all VEGF-A isoforms, we have chosen to investigate an alternative strategy for the therapeutic application of this growth factor, namely, the activation of the endogenous VEGF-A gene through the action of an engineered zinc finger protein-transcription factor (ZFP-TF). Transfection of plasmid DNA encoding the VEGF-A—activating ZFP-TF, VZ+434, results in the expression of all three major isoforms of VEGF-A in their correct proportions (22). Moreover, it has also been demonstrated that intramuscular (i.m.) administration of VZ+434 drives therapeutic angiogenesis (23) and provides significant, and dose-related, protection of both motor and sensory nerve conduction velocity (NCV) deficits in streptozotocin (STZ)-diabetic rats (24). ZFP-TF driven VEGF-A activation thus has both neuroprotective and angiogenic effects which could contribute directly (or indirectly) to improvements in NCV.

To begin to resolve the mechanism(s) by which VEGF-A affects nerve function, we have investigated retrograde axonal transport of VEGF-A protein following i.m. administration of VZ+434 in the rat STZ-induced model of diabetic neuropathy. We show that VEGF-A is normally expressed in small-medium diameter sensory neurons of the DRG, and is down-regulated in diabetes. However, VEGF-A protein levels can be normalized by i.m injection of VZ+434. Plasmid DNA encoding VZ+434 is found in the muscle but not in the DRG, while VEGF-A protein is shown to undergo axonal transport to the DRG, 24 hours following VZ+434. In addition, we demonstrate sustained activation of AKT in the DRG, demonstrating a functional consequence to the increased VEGF-A levels in this tissue, which may underlie the neuroprotective effect of VZ+434-mediated VEGF-A activation in STZ-induced diabetic rats.

**RESEARCH DESIGN AND METHODS**

**Induction of diabetes:** All studies and procedures were licensed under the UK Animals (Scientific Procedures) Act 1986.
Diabetes was induced in adult male Wistar rats (250 – 300g, Charles River, UK) via intraperitoneal injection of freshly dissolved streptozotocin (STZ; 55mg/kg in sterile saline), administered following an overnight fast. Diabetes was verified 3 days post-STZ injection using a strip-operated reflectance photometer (OptimumPlus; MediSense, UK) to measure the animal’s tail vein blood glucose levels. Rats with blood glucose less than 15mmol/l were excluded from the study. Age- and weight-matched rats were used as non-diabetic controls. The VEGF-A activating zinc finger protein (ZFP) transcription factor VZ+434 has previously been described (22). Plasmids were formulated at a concentration of 2mg/ml in 5% poloxamer 188 (BASF, Washington, USA), 150 mmol/l NaCl and 2mmol/l Tris (pH 8.0; Sangamo BioSciences Inc, Richmond, USA).

**Study design:** Three weeks following induction of diabetes, control and STZ-diabetic rats were anaesthetized with isofluorane (2% in oxygen) and were either injected with a total of 250µg VZ+434 at 2 sites in their left gastrocnemius/soleus muscle, or left untreated.

Following injection of VZ+434, additional groups of treated and untreated diabetic rats also had their left sciatic nerve exposed and ligated with polypropylene monofilament non-absorbable sutures (Prolene, Ethicon) at mid thigh level, proximal to the site of injection. The wound was closed and animals recovered under observation. Rats were killed at selected timepoints following injection with VZ+434 (1, 2, 7 and 14 days), or for the sciatic nerve ligation studies at 1 day post ligation.

Ipsilateral and contralateral muscle, sciatic nerves and L4/5 DRG were removed. Tissue designated for Western blot analysis was snap frozen in liquid nitrogen and stored at -40ºC until processing. Sciatic nerve and DRG samples destined for immunohistochemical studies were post-fixed in 4% paraformaldehyde overnight at 4ºC, then cryoprotected in 30% sucrose in 0.1M phosphate buffer (PB) for 24 hours at 4 ºC. Tissues were then frozen in OCT embedding medium (VWR, UK) and stored at -40ºC until processing.

For sensory testing experiments, STZ-rats were either untreated or treated with 250µg VZ+434 at 4, 6 and 8 weeks post-STZ, in 2 sites in their left gastrocnemius/soleus muscle (n = 6-10 per group). Behavioural responses of STZ-diabetic rats and age-matched control rats to tactile stimulation were measured at baseline (pre-STZ), and 4, 8 and 12 weeks post-STZ.

**Western Blotting:** Western blotting was conducted as previously described (25). Blots were incubated overnight at 4ºC with primary antibodies: anti-VEGF-A (1:500, Santa Cruz), anti-total ERK (1:2000, Cell Signaling Technology), anti-phospho AKT and anti-total AKT (1:2000, Cell Signaling Technology) and anti-βIII-tubulin (1:1000, Sigma). Blots were washed, incubated in horseradish peroxidase (HRP)-linked anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technology; 1:2000) for 1 hour at room temperature. Protein bands were visualized using LumiGLO™and Peroxidase Reagent (Cell Signaling Technology, UK). The films were scanned, and the total pixel intensity for each band calculated using SigmaScan Pro5 software (SPSS, UK). Data were analysed using either One-Way Analysis of Variance (ANOVA) followed by Dunnett’s Post Hoc test, students paired t-test, or students one-sample t-test as appropriate. Data are expressed as mean + standard deviation.

**Immunohistochemistry:** 12µm longitudinal sections of sciatic nerve or L4/5 DRG were thaw-mounted onto Superfrost Plus Slides (VWR, UK). Sections were washed with PBS, then incubated in blocking
buffer (10% normal donkey serum, 0.2% Triton-X100 in PBS) for 1 hour at room temperature. Slides were incubated overnight at either 4°C or room temperature with primary antibodies (in blocking buffer): anti-VEGF-A (1:500, Santa Cruz), anti-CGRP (1:500, Sigma), anti-NF200 (1:400, Chemicon), anti-S100β (1:500, Sigma) or anti-pAkt (1:100, Promega). Slides were washed then incubated with either Cy3-conjugated donkey anti-rabbit and/or FITC-conjugated donkey anti-mouse IgG as appropriate (1:200, Jackson ImmunoResearch) for 1 hour at room temperature. In addition some slides were co-labelled with FITC-conjugated isoelectin B4 from *Griffonia Simplicifolia* (20µg/ml, Vector Labs, UK). Sections were mounted in Vectorshield containing DAPI (Vector Labs). Immunofluorescence was visualized using a Leica MPS60 DMR fluorescent microscope. Images were acquired using Hamamatsu digital camera and WASABI software and processed using Adobe Photoshop CS.

**Image Analysis:** The threshold for high VEGF-A-immunoreactivity (-IR) was determined by calculating the mean staining intensity of 4 neurons per animal, which were deemed to be positively stained (Sigma Scan Pro, SPSS). This mean intensity was then taken as the threshold value in order to determine the percentage of VEGF-A-IR neuronal profiles. All neuronal profiles were traced and mean VEGF-A-IR and feret diameter of each profile was automatically calculated (Sigma Scan Pro, SPSS) and data was used for cell size distribution analysis. Cell profiles from 4 randomly selected sections (each 100 µm apart) of ipsilateral and contralateral L4/5 DRG (processed on the same slide) from 4 animals in each treatment group were measured. The method of recursive translation was applied to these results to correct for overestimation of both large and small diameter cell profiles (26).

**Taqman and qRT-PCR:** VZ+434 plasmid DNA levels were measured in duplicate reactions containing 100 ng of DNA and TaqMan Universal PCR Mix (Applied Biosystems, Foster City, CA). Reactions were analyzed using an ABI 7300 Real Time PCR System (Applied Biosystems). Plasmid copy numbers (PCNs) were determined from a standard curve prepared from a ten-fold serial dilution of VZ+434. The background PCN level was determined from the mean of VZ+434 levels in tissues from the right (contralateral) side plus two standard deviations.

**Testing of Mechanical Thresholds:** One week prior to, and at 4, 8 and 12 weeks following induction of diabetes, the behavioural response to mechanical stimulation of the hindpaw was assessed using a dynamic plantar aesthesiometer (Ugo Basile, Italy). Paw withdrawal responses were measured in both right and left hindpaws in all animals, and are reported as the mean of three measurements per paw, taken at least 10 minutes apart. Testing was repeated over two consecutive days, with results recorded from the second day of testing. The assessor was blinded to the VZ+434-treated and untreated STZ groups.

**RESULTS**

**VEGF-A is downregulated in the DRG of STZ-diabetic rats compared to control rats:** Using Western blot and immunocytochemical analysis we compared the expression of VEGF-A in L4/5 DRG of control rats and rats three-weeks following induction of STZ-diabetes (see Table 1 for details of blood glucose levels and body weight). VEGF-A levels were significantly reduced in L4/5 DRG from rats 3 weeks post-STZ (control rats: 16.6 ± 3.2 arbitrary units versus diabetic rats: 3.9 ± 1.6 arbitrary units, p<0.0001, Fig. 1A, B)

VEGF-A-IR was clearly detected within the cytoplasm of a population of small-medium
diameter sensory neurons in the DRG of control rats (Fig. 1C, arrows) and also in motor neurons in the ventral horn of the spinal cord (data not shown). Fewer VEGF-A-IR neurons were observed in DRG from STZ-diabetic rats (Fig 1D, E. control rats: 6.5 ± 2.7 % VEGF-A-IR neurons versus diabetic rat: 1.9 ± 2.2% VEGF-A-IR neurons; p<0.01). Immunofluorescence was abolished by omission of the primary antibody, indicating the VEGF-A-IR to be primary-antibody specific (data not shown).

**Intramuscular administration of VZ+434 increases VEGF-A in the ipsilateral DRG of STZ-diabetic rats:** A single intramuscular dose of the VEGF-A-activating zinc finger protein transcription factor VZ+434, resulted in a rapid and significant increase in VEGF-A protein expression in ipsilateral L4/5 DRG (Fig. 2). This increase is evident as early as 24 hours post-injection (left: 5.2 ± 1.2 arbitrary units versus right: 1.0 ± 0.2 arbitrary units, p<0.05), VEGF-A levels were normalized by 1 week post-injection (Figure 2 A, B). VEGF-A-IR was detected in contralateral (Fig 2 C) and ipsilateral (Fig 2 D) L4/5 DRG within a population of small-medium diameter sensory neurons (Fig 2 C, D; arrows). Intramuscular observed (Fig 4), suggesting that plasmid treatment with VZ+434 generated a significant uptake is restricted to the site of injection, increase in VEGF-A in small diameter neurons resulting in upregulation of VEGF-A in the in the ipsilateral compared to contralateral DRG. muscle, consistent with previous observations VEGF-A-IR was significantly elevated in (27). Importantly, the results demonstrate neurons with diameters between 18.75-22.5μm that axonal transport of VZ+434 plasmid is compared to contralateral DRG (Fig 2 E, F, not responsible for the increased VEGF-A levels in the DRG.

**VEGF-A undergoes axonal transport in sciatic nerve in vivo:** Axonal transport of VEGF-A protein in the sciatic nerve of diabetic rats was assessed using immunocytochemistry and Western blotting techniques (Fig 5). Ligation of the sciatic nerve of diabetic rats injected distally with VZ+434 caused an increase in VEGF-A-IR both proximal and, notably, distal to the ligature site (Fig 5A). VEGF-A-IR was clearly observed in axonal profiles, a region is highlighted and shown as inset (Fig 5A, B,
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Ligation of the sciatic nerve of untreated diabetic rats, showed very little axonal accumulation of VEGF-A-IR (Fig 5E), restricted to the regions of nerve immediately adjacent to the ligature, a marked contrast to the wide distribution of VEGF-A-IR in the VZ+434-injected rats, particularly throughout the distal nerve (Fig 5A, arrows). VEGF-A-IR was absent from the contralateral nerve (Fig 5D).

Ligation of the sciatic nerve of control rats (non-diabetic) either injected with VZ+434 (Fig 5G) or left untreated (Fig 5F), revealed an accumulation of VEGF-A-IR proximal and distal to the ligature site. These observations were confirmed by western blot analysis of VEGF-A protein levels in nerve segments taken proximal and distal to the ligature (schematic diagram illustrated in Fig 5H). Protein levels were quantified by densitometric analysis. Increased VEGF-A was particularly observed in all distal segments and segments immediately proximal to ligature site in diabetic rats injected with VZ+434 (Fig 5I). In contrast, sciatic nerve samples prepared from control rats injected with VZ+434 showed a more even distribution of VEGF-A throughout the whole nerve (Fig 5J), with the greatest accumulation observed in the proximal segment adjacent to the ligature (Figure 5J). Together these data demonstrate that VEGF-A undergoes axonal transport to the DRG and that i.m. administration of VZ+434 is sufficient to overcome the deficit in VEGF-A levels observed in this tissue in this STZ-model of diabetes.

VEGF-A accumulation in the DRG is associated with activation of the PI3-K pathway: To analyze the functional consequences of elevated VEGF-A levels in the DRG we investigated intracellular signaling pathways potentially responsible for the VEGF-A mediated neuroprotective effects in diabetic neuropathy. Specifically, we investigated activation of p38 MAP Kinase, ERK-1/2 and PI3-K pathways using phospho-specific antibodies. Neither the levels nor the phosphorylation status of the MAP kinases p38 and ERK1/2 were significantly altered in the DRG of VZ+434-injected diabetic rats at any time point studied (e.g. 24 hours post-injection: p38: ipsilateral 1.2 ± 0.51 arbitrary units versus contralateral 1.0 ± 0.73 arbitrary units, p>0.05, n=4.; ERK1/2: ipsilateral 1.56 ± 0.25 arbitrary units versus contralateral 1.0 ± 0.34 arbitrary units, p>0.05, n=4).

In contrast, an increase in AKT phosphorylation was observed in the ipsilateral L4/5 DRG 48 hours post VZ+434 injection (left: 5.4 ± 2.6 arbitrary units versus right: 1.0 ± 0.4 arbitrary units, p<0.05, n=4), and activation was maintained for 1 week (left: 2.0 ± 0.6 arbitrary units versus right: 1.0 ± 0.6 arbitrary units, p<0.01, n=4; Fig 6A, B). This increase in pAKT in the ipsilateral compared to contralateral DRG was confirmed using immunocytochemical analysis (Fig 6 C-F). Ipsilateral and contralateral DRG were co-labelled with VZ+434 administration restored VEGF-A to control levels (Supp Fig 1A, B). However, this increase was markedly reduced by ligation of the sciatic nerve in 3 of 5 animals tested (mean change across all 5 animals was not significant) (Supp Fig 1A, B). Taken together with the absence of plasmid DNA encoding VZ+434 in the DRG, these data suggest that the observed normalization of VEGF-A levels in the DRG following VZ+434 treatment occurs via retrograde axonal transport of VEGF-A protein in the sciatic nerve.

Ligation of the sciatic nerve reduced the VZ+434 mediated increase in VEGF-A in the DRG: Diabetes was associated with a significant decrease in basal VEGF-A protein levels in the DRG (Figs. 1B and Supp Fig 1A, B in the online appendix available at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org), and
antibodies against VEGF-A (Fig 6 C, E) and pAKT (Fig 6 D, F). A 3-fold increase in neurons expressing pAKT-IR was observed in the ipsilateral DRG compared to the contralateral DRG (Fig 6 D, F arrows; left: 10% pAKT-IR neurons vs right 3% pAKT-IR neurons, p<0.05). Furthermore, of the pAKT-IR neurons 61% co-expressed VEGF-IR and of the VEGF-IR neurons 85% co-expressed pAKT-IR. As pAkt-IR is observed in a greater number of neurons than those possessing VEGF-IR this may suggest a paracrine action of VZ+434-generated and transported VEGF-A within the DRG.

Intramuscular administration of VZ+434 protects against mechanical allodynia: To determine whether the VZ+434-mediated increase of VEGF-A in small-medium diameter neurons has protective effects in experimental diabetic neuropathy, we investigated the functional efficacy of VZ+434 on the development of mechanical hyper- and/or hyposensitivity in STZ-diabetic rats (Fig 7). Eight weeks following the induction of diabetes, hindpaw thresholds to mechanical stimulation were significantly reduced compared to pre-STZ thresholds (Fig 7A) and age-matched control rats (Fig 7B). Similarly, paw withdrawal response thresholds from the contralateral hindlimb of STZ-rats treated with VZ+434 were reduced compared to both pre-STZ values (Fig 7A) and age-matched control rats (Fig 7C). However, VZ+434 treatments provided significant protection against mechanical allodynia in the left hindpaw 8 weeks-post-STZ (Fig 7A, D). This suggests that the VZ+434-mediated increase of VEGF-A in the DRG protects against the mechanical hypersensitivity observed in STZ-diabetes.

DISCUSSION

This study demonstrates that VEGF-A protein levels in the DRG are reduced in STZ-induced diabetic rats at early stages of the disease (3 weeks post STZ). This deficit was corrected by intramuscular administration of VZ+434, a plasmid that encodes a zinc finger protein-transcription factor capable of simulating endogenous production of VEGF-A. There was no evidence of VZ+434 plasmid DNA being transported to, or transcribed within the DRG, rather VEGF-A protein was axonally transported to sensory neurons in ipsilateral L4/5 DRG 24 hours post-injection, and was associated with altered signal transduction, via the PI3-K pathway. Treatment with VZ+434 provided unilateral protection against mechanical allodynia 8 weeks post-STZ.

VEGF-A was localized to small-medium diameter sensory neurons in the DRG of control rats, confirming previous studies (2; 5), and was down-regulated 3 weeks post-induction of diabetes. The time course of VEGF-A expression in the DRG following induction of diabetes has not been systematically characterized to date, and evidence suggests a dynamic regulation over the course of the disease. VEGF-A-IR has been reported to be upregulated in the DRG 12 weeks post-STZ in mice (28), however we found no change in VEGF-A protein levels 12 weeks-post STZ in rat (data not shown).

Receptors for VEGF-A have previously been characterized in sensory neurons (8; 9; 29). Neurons appear to lack VEGFR-1, which is largely expressed by endothelial cells (30; 31). Conflicting evidence exists about the neuronal expression of VEGFR-2 in sensory neurons. Sondell et al (2; 5; 6) describe a post-natal decrease in the percentage of VEGFR-2-IR neurons in the DRG, with less than 10% of sensory neurons expressing VEGFR-2 in adult mouse DRG. These VEGFR-2-positive neurons were of the large diameter RT97-positive population and the small diameter CGRP-IR population; no IB4 positive neurons expressed VEGFR-2 (6). In contrast other groups did not detect VEGFR-2 in sensory neurons (31). Neuropilin-1 is highly expressed in sensory
neurons (6; 31; 32) however the neuropilin receptors lack defined signaling motifs and appear to act as a co-receptor for VEGFR-2.

A decrease in VEGF-A in sensory neurons may be a functional defect associated with the pathogenesis of diabetic neuropathy. Indeed, clinical studies by Quatrinni et al., identified a significant reduction in VEGF-A levels, endothelial cell dysfunction and a loss in intraepidermal nerve fibres, in skin biopsy samples taken from patients with increasing severity of diabetic neuropathy (33). Diabetic neuropathy is a heterogeneous disease with a widely varying pathology including a neurotrophin deficit. A deficit in nerve growth factor (NGF) expression by sensory neuron targets and retrograde transport to the DRG is well established in experimental diabetic neuropathy (34-36). Indeed, the reduced availability of NGF in experimental diabetic neuropathy provided the rationale of NGF administration as a potential treatment for the disease, and normalizes key molecular and functional aspects of the neuropathy (37; 38). It has not been established whether glial cell derived neurotrophic factor (GDNF) production is similarly impaired in diabetes, however therapy with exogenous GDNF in experimental diabetes normalized cutaneous innervation in STZ-diabetic mice (39; 40). VEGF-A has demonstrated neurotrophic functions in both central and peripheral neurons (4-6), and a number of studies have demonstrated a neuroprotective role for VEGF-A in experimental diabetic neuropathy following administration of VEGF-A by gene transfer (16; 17; 24). Due to the paradox surrounding VEGF-A in relation to both macro- and microvascular complications in diabetes, targeted local delivery of VEGF-A is crucial for the treatment of diabetic neuropathy.

We have previously demonstrated that unilateral intramuscular injection of VZ+434 prevents the sensory and motor NCV deficits in the ipsilateral sciatic nerve of STZ-diabetic rats (24), and suggested a direct neuroprotective role of VEGF-A in experimental diabetic neuropathy. In this current study we extend these observations to demonstrate a further neuroprotective effect of VZ+434, namely protection against mechanical allodynia. Following intramuscular VZ+434 there is a correction in retrograde axonal transport of VEGF-A in the sciatic nerve to the DRG. As with the NCV change, this effect was restricted to the neurons ipsilateral to the injection. We also observed accumulation of VEGF-A proximal to the ligature, in accordance with other studies showing VEGF axonal transport to be bi-directional (6; 16; 41).

The mechanism of uptake of VEGF-A by sensory afferent terminals present in the gastrocnemius muscle is unclear. NGF release from cultured smooth muscle cells is elevated in the presence of contractile stimuli (42). It is feasible that, in our study, VEGF-A release from the injected skeletal muscle occurs in this way, aided by the everyday movement of the rats. We postulate that VEGF-A uptake occurs via binding to VEGFR-2, it is well established that upon ligand binding VEGFR-2 is internalized with caveolin-1 and dynamin-2 (47; 48) and undergoes intracellular trafficking (43-45) and nuclear localization, via microtubules and the endocytic pathway.

VZ+434-induced VEGF-A activates the cytoplasmic serine/threonine kinase AKT in L4/5 DRG ipsilateral to the VZ+434 injection. The downstream targets of VEGF-A/AKT activation are currently under investigation. The PI3K pathway is activated by many growth factors including IGFs, insulin and NT-3 in sensory neurons (49-52), all of which are reduced in diabetic neuropathy (13; 53; 54). Furthermore, VEGF-A increases pAKT in primary cultures of dissociated rat sensory neurons (data not shown). A study using the PI3-K inhibitor, LY294002, on mice DRG explants implicated
this pathway in sensory neuron survival, as LY294002 caused a dose-dependent cell death (55). PI3-K promotes neuronal cell survival by modulation of Bcl-2 and thus mitochondria function, and regulates energy metabolism in sensory neurons. In addition, activation of the PI3K-Akt pathway increases axonal caliber and terminal branching of sensory neurons in vitro (56) and thus plays an important role in axon morphological differentiation.

Since phosphorylation of AKT occurs in a greater number of neurons than those possessing VEGF-A-IR following VZ+434 administration, this may support the notion of both an autocrine and paracrine action of transported VEGF-A within the DRG. This could also explain how expression of VEGF-A, by small-medium diameter neurons in the DRG, may ameliorate the NCV deficits measured in large-diameter myelinated axons in diabetic neuropathy (24) - as increased pAKT-IR is observed in all populations of sensory neurons, however further experiments would be necessary to confirm this. Alternatively, NCV deficits may be ameliorated by another, as yet unidentified, mechanism such as restoration of nerve blood flow.

Our current work suggests that VEGF-A is able to modulate the PI3-K signaling cascade in the L4/5 DRG, which may underlie the neuroprotective action of VZ+434 in the management of diabetic neuropathy.

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Table 1

| Group number | Experimental details | Blood Glucose mmol/l | Body Weight (g) |
|--------------|----------------------|---------------------|-----------------|
|              |                      |                     | Start     | End       |
| **Experiment 1** |                      |                     |           |           |
| 1            | Diabetic + VZ+434    | 26.0 ± 1.7          | 324 ± 6   | 334 ± 48  |
|              | 24 hr                |                     |           |           |
| 2            | Diabetic + VZ+434    | 25.9 ± 1.6          | 280 ± 16  | 229 ± 18  |
|              | 48 hr                |                     |           |           |
| 3            | Diabetic + VZ+434    | 20.8 ± 4.2          | 317 ± 10  | 335 ± 48  |
|              | 1 wk                 |                     |           |           |
| 4            | Diabetic + VZ+434    | 23.3 ± 4.3          | 326 ± 16  | 376 ± 78  |
|              | 2 wk                 |                     |           |           |
| 5            | Control              | 7.9 ± 0.4           | 332 ± 18  | 491 ± 5   |
| **Experiment 2** |                      |                     |           |           |
| 1            | Diabetic + VZ+434    | 19.1 ± 2            | 274 ± 14  | 257 ± 20  |
|              | 24 hr                |                     |           |           |
| 2            | Diabetic + VZ+434 +  | 21.1 ± 2.1          | 287 ± 24  | 271 ± 25  |
|              | sciatic ligature 24 hr|                     |           |           |
| 3            | Diabetic + ligature 24 hr | 18.5 ± 2.3          | 278 ± 15  | 247 ± 13  |
| 4            | Diabetic untreated   | 22.6 ± 5.7          | 280 ± 16  | 270 ± 28  |
| 5            | Control untreated    | 8 ± 1.4             | 272 ± 16  | 352 ± 26  |

**Blood glucose and body weight measurements.** Two studies were performed in STZ-induced diabetic rats: Experiment 1) time course study and Experiment 2) axonal transport study. Final blood glucose levels and start and end body weights for all groups are shown in A and B. Data are expressed as means ± SD (n=4 per group).
Figure Legends

**Fig 1. VEGF-A is downregulated in the DRG of STZ-diabetic rats.** Western blot analysis of VEGF-A levels in pooled L4/5 DRG obtained from control (C) rats or diabetic rats 3 weeks post-STZ (D), show a dramatic decrease in VEGF-A in diabetes (A). Densitometric analysis of VEGF-A protein levels (normalized to tERK to correct for protein loading) shows a significant decrease post-STZ (**p<0.0001, t-test, n=5 per group, B). Representative micrographs show that VEGF-A-IR is present in sensory neurons in L4/5 DRG. A population of small-medium diameter neurons in DRG from control rat DRG show VEGF-A-IR (arrows, C, E). Fewer VEGF-A-IR neurons were observed in DRG obtained from STZ-diabetic rats (D, E). Data for individual animals (n=4 per group, **p<0.01, t-test) are shown as circles and the mean value is shown as a horizontal bar (E). Scale bar = 50 µm.

**Fig 2. Intramuscular administration of VZ+434 increases VEGF-A in the ipsilateral DRG of STZ-diabetic rats.** Diabetic rats (3 weeks post-STZ) received two intramuscular injections of the VEGF-A–activating zinc finger protein-transcription factor VZ+434 (250 µg total) into the gastrocnemius muscle and were killed at the indicated timepoints post-injection (n=4 per timepoint). VEGF-A levels in right (uninjected, contralateral limb) and left (injected, ipsilateral limb) were assessed using Western blotting (A) and quantified using densitometry (normalized to tAKT to correct for protein loading; B). Intramuscular VZ+434 caused a rapid and significant increase in VEGF-A protein expression in ipsilateral L4/5 DRG compared to contralateral by 24 hours post-injection (A, B, *p<0.05, **p<0.01 paired t-test). Increased VEGF-A-IR (C, D, arrows) was clearly detected within a population of small-medium diameter sensory neurons in the ipsilateral DRG (left, D) compared to the contralateral DRG (right, C). Cell size distribution analysis shows a significant increase in VEGF-A-IR neurons in small diameter neurons in the ipsilateral (F, *p<0.05, ANOVA) compared to contralateral DRG (E). Scale bar = 50 µm.

**Fig 3. Phenotypic characterization of VEGF-A-IR neurons in L4/5 DRG of diabetic rats post VZ+434** Representative micrographs show that VEGF-A-IR is present in sensory neurons in L4/5 DRG. Sections have been dual labeled with: (A, C, E) anti-VEGF-A and anti-CGRP (B); IB4 (D); and anti-NF200 (F). A large proportion of neurons that express VEGF-A-IR (A, C: arrows) coexpress the phenotypic markers CGRP (B, arrows) and IB4 (D, arrows). Note, very few VEGF-A-IR neurons colocalize with NF200 (asterisk, E, F) Scale bar = 50 µm.

**Fig 4. Expression of VZ+434 DNA plasmid copy number in ipsilateral and contralateral tissues.** 24 hours following unilateral intramuscular injection of VZ+434 (250 µg), tissues were harvested and DNA extracted. The background plasmid copy number (PCN) of VZ+434 was set at 2700 (derived from mean plus two standard deviations from contralateral tissue samples). Data from individual animals are shown as circles with the mean as a horizontal bar (n=8 for muscle and n=4 for DRG). Ipsilateral muscle contained significantly more copies of VZ+434 per 100ng DNA than contralateral muscle, whereas there was no significant left-right tissue difference in VZ+434 in DRG (*p<0.05, Mann-Whitney U test).

**Fig 5. VEGF-A undergoes axonal transport in the sciatic nerve in vivo.** Adult rats received a unilateral sciatic nerve ligature at mid-thigh level, proximal to the site of VZ+434 injection (250 µg, i.m.) Longitudinal sections of sciatic nerve, 24 hours post-ligation, were
immunostained for VEGF-A (red) and S100 to label Schwann cells (green; A-G). VEGF-A-IR can clearly be seen to accumulate within axons (arrows A) at the ligature site, proximal and distal to the ligature site, indicating bidirectional axonal transport. A region highlighted by a rectangle in (A) is shown at increased magnification (B). VEGF-A-IR was restricted to axons and not Schwann cells (B, C). Little VEGF-A-IR was observed in unligated contralateral nerve (D) or untreated ligated nerve (E). In contrast, VEGF-IR accumulated both proximal and distal to ligatures in both untreated (F) or treated (G) control rats. Scale bars = 1mm. Ligated sciatic nerves from VZ+434-injected STZ-diabetic rats (I) or control rats (J) were cut into 5mm segments proximal and distal to the ligature and samples processed for Western blotting to assess VEGF-A levels (see schematic diagram, H). VEGF-A accumulated both proximal and particularly distal to the ligature in VZ+434-injected diabetic rats (I). Samples prepared from control rats injected with VZ+434 showed the greatest accumulation of VEGF in proximal samples (J). Data for individual animals are shown as circles, the mean value is shown as a horizontal bar (I, J).

**Fig 6** Akt is phosphorylated in ipsilateral L4/5 DRG following VZ+434 injection. Diabetic rats (3 weeks post-STZ) treated with VZ+434 were killed at the indicated timepoints post-injection (24 hours, 48 hours, 1 week, 2 weeks; n=4 per timepoint). Phosphorylated (p-Akt) and total (t-Akt) Akt levels in right (uninjected, contralateral limb) and left (injected, ipsilateral limb) DRG were assessed using Western blotting (A) and quantified using densitometry (B). Intramuscular administration of VZ+434 caused a rapid and significant ipsilateral increase in pAkt levels in L4/5 DRG (A, B) by 48 hours post-injection (*p<0.05) and remained significantly elevated for 1 week (**p<0.001). Immunocytochemical analysis with antibodies against VEGF-A and pAkt showed co-labeling of VEGF-A-IR (C) and pAKT (D) neurons in the DRG (arrows), however, pAkt-IR neurons that did not co-localise with VEGF-IR neurons were also observed (asterisk). Reduced pAkt-IR was observed in contralateral DRG (E). Scale bar = 50 μm.

**Fig.7 VZ+434 protects against mechanical hyperalgesia.** Sensory testing was conducted prior to treatment (baseline measures) and at 4, 8 and 12 weeks post-STZ. VZ+434-treated rats received unilateral i.m injections at 4, 6 and 8 weeks post-STZ. At the 4 and 8 week timepoints, mechanical thresholds were assessed 3 days following VZ+434. At 8 weeks post STZ, a significant decrease in the paw withdrawal force (PWF) was observed in STZ-rats and in the contralateral hindpaw of VZ+434-treated STZ rats compared to their baseline measures (A, mean ± S.D, *p<0.05, n=7-9, Two way ANOVA, Bonferroni’s Multiple Comparison Test). At 8 weeks, STZ rats (B) and the contralateral hindpaw of VZ+434-treated rats (C) compared to non-diabetic controls (B,C). VZ+434 treatment (D) provided significant protection against this allodynia in the ipsilateral limb (B-D, data shown for individual animals *p<0.05; **p<0.01, n=7-9, One way ANOVA, Dunnett’s Post Hoc test).
Figure 1

A. 

B. 

C. 

D. 

E.
Figure 2

A. 

24h  48h  1 week

R  L  R  L  R  L  R  L

VEGF  IAKT  VEGF  IAKT  VEGF  IAKT

B. 

VEGF levels normalised to IAKT (arbitrary units)

24h  48h  1 week

R  L  R  L  R  L

C.

Right

D.

Left

E.

Frequency

profile diameter (µm)

F.

Frequency

profile diameter (µm)

H neurons  EGF-lr neurons
Figure 3

A. VEGF-IR
B. CGRP-IR
C. VEGF-IR
D. IB4-IR
E. VEGF-IR
F. NF200-IR

Figure 4

VEGF-A and experimental diabetic neuropathy
VEGF-A and experimental diabetic neuropathy

Figure 5

A. Diabetic ipsilateral: treated & ligated

B. Diabetic contralateral

C. Diabetic, ligated

D. Control ipsilateral: ligated

E. Control ipsilateral: treated & ligated

F. Ligature

G. Control ipsilateral: treated and ligated

H. VEGF

I. β III-tubulin

J. Control ipsilateral: treated and ligated

% total VEGF protein

Scolatic nerve segment

% total VEGF protein

Scolatic nerve segment
Figure 6

A. [Image of Western blot analysis of VEGF-A and experimental diabetic neuropathy]

B. [Bar graph showing pAKT levels normalized to AKT (relative units)]

C. Left: VEGFIR

D. Left: pAKTIR

E. Right: pAKTIR

Figure 7

A. [Graph showing force generation in different groups over time]

B. [Scatter plot showing force generation at different weeks post-STZ]

C. [Scatter plot showing force generation at different weeks post-STZ]

D. [Scatter plot showing force generation at different weeks post-STZ]