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Effect of Insulin and an Erythropoietin-Derived Peptide (ARA290) on Established Neuritic Dystrophy and Neuronopathy in Akita (*Ins2^{Akita}*) Diabetic Mouse Sympathetic Ganglia

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Abstract

The Akita mouse is a robust model of diabetic autonomic neuropathy which develops severe diabetes following beta cell death, which occurs reproducibly at 3–4 weeks of age, and maintains the diabetic state without therapy for as long as 11 additional months. Neuritic dystrophy and neuronopathy involving prevertebral sympathetic superior mesenteric and celiac ganglia begin to develop within the first two months of onset of diabetes and are progressive with time. We have examined the effect of insulin implants resulting in normoglycemia and injections of ARA290, a small erythropoietin peptide which has no effect on glycemic parameters, on the reversal of established neuritic dystrophy and neuronopathy. We have found that 4 weeks of insulin therapy beginning at 2 months of diabetes resulted in normalization of blood glucose, body weight and HbA1c. Insulin therapy successfully reversed established neuritic dystrophy and neuronopathy to control levels. Numbers of sympathetic neurons were not significantly changed in either 3 month diabetic or insulin treated Akita mice. Treatment with ARA290 for 7 weeks beginning at 4 months of diabetes did not result in altered metabolic severity of diabetes as measured by blood glucose, body weight or HbA1c levels. ARA290 treatment was able to decrease neuritic dystrophy by 55–74% compared to untreated diabetics or in comparison to a separate group of diabetic animals representing the 4 month treatment onset point. Surprisingly, there was no effect of ARA290 on ganglionic neuron number or ongoing neuronopathy (pale/degenerating neurons) in diabetic Akita mice during this time period. The development of neuroprotective EPO-like peptides may provide a possible future therapy for this debilitating complication of diabetes; however, it appears that discrete elements may be differentially targeted by the diabetic state and may require selective therapy.

Keywords

diabetes; Akita mouse; neuritic dystrophy; neuronopathy; erythropoietin; insulin; sympathetic ganglia

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INTRODUCTION

Autonomic neuropathy is an increasingly recognized problem in human diabetes which may result in cardiovascular, genitourinary, sudomotor and alimentary symptoms (Rundles, 1945) or remain undetected as subclinical disease. Studies of prevertebral sympathetic ganglia in autopsied diabetic human subjects demonstrate its pathologic hallmark, neuroaxonal dystrophy, which consists of terminal axonal swellings containing a distinctive admixture of subcellular elements (Duchen et al., 1980; Schmidt et al., 1993), superimposed on a mild, poorly characterized decrease in neuronal density (Schmidt et al., 1993). These defects may disconnect or misconnect ganglionic neurons and, particularly for prevertebral ganglia serving the viscera, contribute to the loss of integrated autonomic reflexes which depend on intraganglionic connections. Rodent models of diabetic sympathetic autonomic neuropathy show significant correspondence with human pathology, developing dystrophic axons in prevertebral ganglia in the presence of relative, but not absolute, preservation of sympathetic neurons (Schmidt, 2001; Schmidt et al., 2003, 2008).

We have shown that non-obese diabetic (NOD) and streptozotocin-treated NOD/severe combined immune deficient (STZ-Rx NOD/SCID) mice develop dramatic axonal as well as dendritic pathology (i.e., “neuritic dystrophy”) within a few weeks of onset of diabetes (Schmidt et al., 2003, 2008). However, once diabetic, neither NOD nor STZ-Rx NOD/SCID mice survive long enough to test the ability of therapeutic agents to correct established neuropathy, clinically a more relevant paradigm than prevention of neuropathy. We have recently begun to investigate the Akita (*Ins2^{Akita}*) mouse model to address this deficiency (Schmidt et al., 2009). Akita mice are characterized by a spontaneous dominant mutation in the *insulin 2* gene on a C57BL/6 mouse background which results in a tyrosine for cysteine substitution, disrupting a disulfide bridge required for proper insulin folding. This defect initiates an unfolded protein response and culminates in pancreatic β -cell apoptosis in the absence of obesity, insulinitis or insulin resistance (Yoshioka et al., 1997; Mathews et al., 2002; Ron, 2002; Izumi et al., 2003). Heterozygous Akita mice are reproducibly and severely hyperglycemic (males more severely than females) and hypoinsulinemic beginning at 3-4 weeks of age but remain viable in the absence of exogenous insulin treatment for as long as 11 months, far longer than 5-8 weeks of severe hyperglycemia which can be maintained in NOD and STZ-treated NOD/SCID models over which time animals become increasingly debilitated and fragile. Within 2 months of onset of diabetes, male Akita-diabetic mice show marked neuritic dystrophy in prevertebral sympathetic ganglia identical in appearance and anatomic distribution to that which develops in other mouse models. In addition, neurons in Akita mouse prevertebral sympathetic ganglia show an unusual perikaryal alteration characterized by the accumulation of membranous aggregates and minute mitochondria and loss of rough endoplasmic reticulum culminating eventually in neuronal degeneration.

Initially discovered as a mediator of erythropoiesis, for some time erythropoietin (EPO) has been recognized to have neuroprotective effects on a variety of animal models of CNS and PNS neurodegeneration. Neuroprotection by EPO (Hoke, 2006) has been described in acrylamide and cisplatin toxic neuropathies (Bianchi et al., 2007; Keswani et al., 2004a; Melli et al., 2006), HIV sensory neuropathy (Keswani et al., 2004b) and, particularly pertinent to our studies, experimental diabetic somatic neuropathy (Bianchi et al., 2004; Tam et al., 2006). Using our STZ-treated NOD/SCID mouse model, we recently showed (Schmidt et al., 2008) that EPO and carbamylated erythropoietin (CEPO) prevented the development of experimental diabetic autonomic neuropathy which is thought to reflect a poorly understood role of these agents in neuroprotection independent of a hematopoietic effect. In the experiments described here we have demonstrated the effect of insulin implants on the reversal of established neuritic dystrophy and neuronal degeneration

following a course of 4 weeks of therapy. Additionally, we show that the use of a non-erythropoietic EPO-derived peptide ARA290 (Brines et al., 2008) results in a salutary effect on the frequency of neuritic dystrophy but did not prevent neuronal degeneration and loss in Akita prevertebral ganglia suggesting that these pathologic features may have a different pathogenesis.

MATERIALS AND METHODS

Animals

The C57BL/6J-Ins2Akita animals used were obtained from the Jackson Laboratory. All animals were housed and cared for in accordance with the guidelines of the Washington University Committee for the Humane Care of Laboratory Animals and with National Institutes of Health guidelines on laboratory animal welfare and EC Directive 86/609/EEC for animal experiments. All mice were allowed standard rat chow and water ad libitum and maintained on a 12/12 hour light/dark cycle.

Treatments

Insulin Treatment—Male Akita mice became diabetic at 3-4 weeks of age and were maintained untreated for two additional months at which time they were separated into two groups. The first group received insulin containing subcutaneous implants (Linbit mouse implant, LinShin, Canada) at a dose recommended based on body weight, [2 implants for 20 gram animals with an additional implant for each 5 grams of body weight (total range 2-5 implants)]. The second diabetic group received blank insulin-free implants as did C57BL6J control mice. Animals were followed by weekly measurement of body weight and blood glucose measurements for 4 weeks at which time animals were euthanized by decapitation following ketamine/xylazine treatment. HbA1c measurements were made at the time of euthanasia.

EPO-peptide (ARA290) Treatment—Once male Akita mice spontaneously developed diabetes they were allowed to survive for 15-16 weeks without therapy to an age of 18-19 weeks. At that time diabetic and control animals were separated into two groups: 1) ARA290-treated mice which received daily injections of ARA290 (MW 1258, 36.1 microgram/kg body weight /day, subcutaneously, 5 days/week, Arai Pharmaceuticals); and, 2) saline treated mice. Animals were followed by measurement of blood glucose and body weight for 7 additional weeks at which time they were euthanized. HbA1c measurements were made at the time of euthanasia.

Tissue Preparation

Male Akita and age-matched C57BL/6J control mice were anesthetized with ketamine/xylazine, the abdominal cavity opened, overlying organs displaced and immersed in cold modified Karnovsky's fixative containing 3% glutaraldehyde and 1% paraformaldehyde in sodium cacodylate buffer pH 7.4. Fixation was continued for several days at 4°C in the same fixative. The celiac (CG) and superior mesenteric (SMG) ganglia were dissected, cleaned of extraneous tissue and rinsed in sodium cacodylate buffer. The CG were postfixed in phosphate cacodylate-buffered 2% OsO₄ for 1 hour, dehydrated in graded ethanols with a final dehydration in propylene oxide and embedded in EMBED-812 (Electron Microscopy Sciences, Hatfield, PA). One micron thick plastic sections were examined by light microscopy after staining with toluidine blue. Ultrathin sections (~90 nanometers thick) of individual ganglia were cut onto formvar coated slot grids, which permits visualization of entire ganglionic cross sections. Sections were post stained with uranyl acetate and Venable's lead citrate and viewed with a JEOL model 1200EX electron microscope (JEOL,

Tokyo, Japan). Digital images were acquired using the AMT Advantage HR camera (Advanced Microscopy Techniques, Danvers, MA).

Morphometric Studies

Quantitation of Dystrophic Neurites—Dystrophic elements are typically intimately related to individual neuronal perikarya and, therefore, we have routinely expressed their frequency as the ratio of numbers of lesions to numbers of nucleated neuronal cell bodies. This method, used in our previous studies (Schmidt et al., 2003; Schmidt et al., 2008), substantively reduced the variance in assessments of intraganglionic lesion frequency. In addition, its simplicity permits the quantitative ultrastructural examination of relatively large numbers of ganglia and identification of robust changes which are they infrequent enough to complicate typical non-biased counting. In the presence of neuron loss this method may overestimate the frequency of neuritic dystrophy, although loss of principal sympathetic neurons, thought to be the source of many intraganglionic dystrophic neurites, may conversely result in parallel changes in dystrophic neurites and neuron number and an unchanged ratio. In our current animal studies an entire cross section of the SMG or CG was scanned at 20,000X magnification and the number of dystrophic neurites and synapses was determined by an investigator blinded to the identity of individual animals. Dystrophic neurites consist of swollen axons, synapses, dendritic spines or dendrites containing a variety of organelles including: 1) axonal tubulovesicular aggregates; 2) pallid ribosome poor cytoplasm in dendrites; 3) axons with admixed normal and degenerating subcellular organelles and multivesicular bodies; 4) axonal neurofilaments; and, 5) nearly pure aggregates of minute dendritic mitochondria. The number of nucleated neurons (range: 50-100 neurons examined in each ganglionic cross section) was determined simultaneously. The frequency of ganglionic neuritic dystrophy was expressed as the ratio of number of dystrophic neurites to the number of nucleated neurons in the same cross section.

SMG Neuronal Counts—The SMG were processed through graded alcohols and embedded in paraffin. Sections were taken sequentially at 5 micron thickness to include the entire ganglion. Paraffin sections were stained with hematoxylin and eosin. Neuronal counts were made using the non-biased method of Silos-Santiago et al. (2005) from every 3rd section as described in our previous publication (Schmidt et al., 2009). The numbers of neurons were determined by counting only cells with an obvious nucleus containing one or more nucleoli using a 60X objective on an Olympus BX51 photomicroscope and corrected for split nuclei estimated by multiplying the raw data by the correction factor, 0.96 which is the same in controls and Akita mice.

Determination of Numbers of Pale Degenerating Neuronal Cell Bodies—Numbers of neuronal cell bodies with pale cytoplasm in the presence or absence of nuclear abnormality (as shown in the Results section, Fig 3) were determined in blinded fashion by light microscopy using a 60X objective of random sections of hematoxylin and eosin (H&E) stained SMG previously used to count total neuron number. The frequency of neuronopathy is expressed as the number of pale neurons as a percentage of the total number of nucleoli containing nucleated cell bodies (typically 400-800 neurons) evaluated for each mouse.

Statistical Analysis

Statistical analysis was performed using a two tailed T-test as well as a two way ANOVA and Bonferroni post-test.

RESULTS

Metabolic Parameters

Insulin Experiment—Initial blood glucose and body weight values were comparable in the groups of Akita mice at the time of initiation of treatment. Animals in the insulin treatment group showed increased body weight and diminished blood glucose values during treatment and at sacrifice compared with untreated diabetics (Table 1) a result also confirmed by determination of HbA1c, a measure of the ambient glucose values over the course of the experiment. At the end of the experiment body weights, blood glucose and HbA1c values of insulin treated Akita animals were significantly different from saline treated diabetics. Insulin treated diabetic mice did not differ from the non-diabetic control group in blood glucose and HbA1c values but were somewhat lighter than controls.

ARA290 Experiment—Initial blood glucose and body weights were comparable in both groups of Akita mice and controls at the time they were separated into ARA290 and saline treatment groups. ARA290-treated diabetics and saline-treated diabetic animals did not differ significantly in body weight or blood glucose during the course of the experiment and body weight, blood glucose and HbA1c values at the end of the experiment (Table 2). Similarly, ARA290 treated and saline treated control groups did not differ in body weights, blood glucose concentration or levels of HbA1c; as a result, we have combined the ARA290 and saline treated controls into one control group.

Neuropathology of Prevertebral Sympathetic Ganglia in Diabetic Akita Mice

Neuritic Dystrophy—Ultrastructural examination of prevertebral celiac sympathetic ganglia (CG) of diabetic Akita and age-matched C57BL6 controls showed principal sympathetic neurons surrounded by neuropil composed of an admixture of axons and dendrites. Akita mice diabetic for a total of 3-6 months (i.e., the duration of diabetes at the conclusion of the insulin and ARA290 experiments, respectively) demonstrated numerous swollen dystrophic elements (Fig 1) which were typically located immediately adjacent to neuronal cell bodies, often within their satellite cell sheaths and distorting their perikaryal contours. As in our previously published mouse studies (Schmidt et al., 2003, 2008, 2009) dystrophic neurites exhibited a variety of ultrastructural patterns based on differences in their content of subcellular organelles. A common pattern consisted of neurites, mostly axons, containing normal and degenerating organelles (Fig 1A-C), an appearance dominated by the accumulation of autophagosomes which contained a variety of subcellular organelles including dense core neurotransmitter containing vesicles (arrow, Fig 1C). Dystrophic neurites often exhibited a disoriented microtubular cytoskeleton (arrowhead, Fig 1C). The most typical dystrophic appearance consisted of collections of large numbers of mitochondria (Fig 1D, E). The majority of mitochondria-filled dystrophic neurites were dendrites, as identified by synapses ending upon them (arrow, Fig 1E), the presence of significant numbers of ribosomes or lipopigment. Accumulated mitochondria in dystrophic neurites were small and dense with poorly demarcated cristae (Fig 1F). Occasional neurites (1-2% of the total), typically axons, contained aggregates of neurofilaments. Occasional dystrophic swellings contained large numbers of coarse to delicate tubulovesicular elements, some with clefts, or were represented by lucent neurites with pale cytoplasm and small mitochondria.

The ultrastructural appearance of dystrophic neurites in the CG of saline and ARA290 treated Akita and control mice were identical in appearance, differing only in frequency.

Quantitative Analysis of the Frequency of Neuritic Dystrophy

Since dystrophic neurites are present in diabetic and, although quite rarely, in control mice, we have used an ultrastructural quantitative method to compare them and permit the characterization of the effect of insulin and ARA290. The numbers of dystrophic neurites were counted as described in the Materials and Methods section and expressed as a ratio (numbers of dystrophic elements/numbers of nucleated neurons).

Insulin Experiment—Dystrophic neurites were markedly increased in frequency in saline-treated diabetic Akita mice (Fig 2) after 3 months of diabetes in comparison to non-diabetic controls which had received blank implants. Insulin treatment significantly diminished neuritic dystrophy to approximately 26% that of untreated diabetic, a value not significantly different from non-diabetic controls (Fig 2). Compared to Akita mice diabetic for two months, i.e., the starting point of insulin treatment, there is statistically significant 55% improvement in the frequency of neuritic dystrophy in 4 weeks of treatment (Fig 2).

ARA290 Experiment—The frequency of neuritic dystrophy in saline treated diabetics was increased in comparison to a combined group of saline treated and ARA290 treated controls (Fig 2) which did not differ. ARA290 treated Akita diabetic mice showed 38% of the frequency of neuritic dystrophy of untreated diabetics. Compared to Akita mice diabetic for 4 months, i.e., the starting point of ARA290 treatment, there was a statistically significant 57% improvement in the frequency of established neuritic dystrophy in 7 weeks of treatment (Fig 2).

Neuronopathy: Principal sympathetic neurons of control and most diabetic neurons had prominent spherical nuclei with delicate chromatin and nucleoli, and Nissl substance which extended from the nucleus to the plasmalemma. Light microscopic and ultrastructural examination of neuronal perikarya of Akita mice showed a population of neurons with a continuum of degenerative changes. Scattered neurons showed cytoplasmic pallor (Fig 3A-C) in light microscopic H&E stained SMG culminating in frank degeneration (Fig 3C). One micron thick toluidine blue stained plastic embedded sections showed scattered neurons with marked pallor (arrows, Fig 3D, E) which often contained dark cytoplasmic bodies (arrow, Fig 3E) and a change in nuclear shape and nucleoplasm. The ultrastructural equivalent of the pale neurons seen by light microscopy consists of loss of Nissl substance and replacement of normal mitochondria with minute mitochondria (Fig 3F). Later changes in neurons involved the accumulation of densely stained membranous cytoplasmic bodies (arrows, Fig 3G, H) or, rarely, frankly degenerating cell bodies with degenerating nuclei (arrowheads, Fig 3G, H).

Comparison of mitochondrial ultrastructure between those of normal appearing perikarya of Akita mouse CG (Fig 4A,C) and those in pale, degenerating neurons (Fig 4B,D) showed that the mitochondria of degenerating neurons were significantly smaller and denser than in normal appearing perikarya and were accompanied by dramatically decreased rough endoplasmic reticulum. Cristae appeared fewer in number in diabetic perikaryal mitochondria, however, the preservation of the tissue did not permit further, more sophisticated structural analysis.

Quantitation of pale neurons (with or without darkly stained inclusions) showed they represented 1-2% of total numbers of neurons at 3 and 6 months (i.e., duration of diabetes at time of death) of saline treated diabetes (Tables 3 and 4). Treatment with insulin implants normalized the frequency of pale neurons to control levels (Table 3). At the starting 2 month diabetic interval pale neurons involved approximately 2% of the total (Table 3), as measured from available plastic sections, (a measurement which tends to be more sensitive to detection of pallor too subtle to be appreciated in H&E stained sections) which demonstrated

normalization of the frequency of established lesions with 4 weeks of therapy. In contrast, treatment with ARA290 did not have a statistically significant effect on the frequency of pale neurons (Table 4) compared to saline treated Akita diabetic ganglia.

In order to determine the fate of pathologically altered neurons we determined the number of neurons in the SMG of Akita mice diabetic for 3 or 6 months and age-matched controls. The plexiform nature of the SMG and CG, however, complicates the reproducibility of dissection and increases the variability of neuronal counts. Saline treated Akita mice did not show neuron loss in the insulin treatment experiment (Table 3), likely reflecting its shorter duration. Approximately half of sympathetic neurons were lost in both saline- and ARA290-treated diabetic Akita mouse in comparison to controls. ARA290 treated mice showed comparable neuron loss to saline-treated diabetic mice (Table 4). At the starting point (i.e., mice diabetic for 4 months) pale neurons involved approximately 7% of the total (Table 4), again measured from plastic sections.

DISCUSSION

The use of the Akita mouse model offers significant practical advantages over previously examined NOD, STZ Rx-NOD/SCID and STZ-induced diabetic C57BL6 mouse models as illustrated by the current experiments. Akita mice rapidly develop neuritic dystrophy comparable ultrastructurally and in anatomic distribution to these previously characterized in other mouse models (Schmidt et al., 2003) far in excess of very low baseline levels in age-matched non-diabetic C57BL6/J controls (Schmidt et al., 2009). They do not require the use of an exogenous toxin for induction of diabetes, develop diabetes along a reproducible time course, are hardy and robust maintaining severe hyperglycemia for extended intervals in the absence of excessive mortality, and are not obese or insulin resistant. The explanation for the differences in the survival of Akita mice compared to NOD and STZ-induced NOD/SCID mouse models is not known. These characteristics make them ideal for the analysis of the effect of novel therapeutic agents used on established neuritic dystrophy, i.e., using a more clinically relevant reversibility paradigm.

Neuritic Dystrophy

We have shown that 4 weeks of insulin therapy is able to normalize established neuritic dystrophy in Akita diabetic prevertebral ganglia which demonstrates the capability for reversal of even striking alterations of neurites, in this case with short intervals of normoglycemia. Previous experiments in the STZ-diabetic rat have also provided evidence for the reversal of established dystrophic lesions in ileal mesenteric nerves and prevertebral ganglia with pancreatic islet transplantation, chronic insulin therapy resulting in near normoglycemia and chronic IGF-I therapy but not following small doses of insulin not producing a significant effect on the severity of hyperglycemia (Schmidt et al., 1983, 1999). ARA290 resulted in a significant 57-62% decrease in neuritic dystrophy in our current reversal experiments in comparison to the starting and ending frequency of neuritic dystrophy, respectively. In comparison, EPO and CEPO largely prevented neuritic dystrophy when given in a preventative paradigm; however, these experiments involve different mouse strains, nature of the agent administered and time course. Although ARA290 has been reported to have the same neurotrophic effects as EPO and CEPO in recent experiments (Patel et al., 2008), it may not be as capable in this use, necessitating future studies of a dose response/time course.

Mitochondrial Pathology—Changes in the number and size of mitochondria in the neuronal cell body and dendrites are reproducible findings in prevertebral sympathetic ganglia of mouse models of diabetic autonomic neuropathy (Chung et al., 2003; Schmidt et

al., 2003, 2008). Studies have shown a role for a dynamic network of mitochondrial fission/fusion in the determination of mitochondrial number, size and function which contribute to a variety of neurodegenerative processes (Baloh, 2008; Chan, 2006; Frank, 2006). Mutations in the fusion inducing protein mitofusin-2 in cultured dorsal root ganglia (DRG) results in the accumulation of fragmented mitochondria in abnormal clusters in both neuronal cell bodies and proximal axons, possibly reflecting defects in axonal transport (Baloh et al., 2007; Baloh, 2008). Collections of axonal mitochondria have also been reported in the DRG of STZ-diabetic rats in vivo and in DRG cultured in high glucose media, thought to result from upregulation of the fission-related protein Drp1 (Leininger et al., 2006). Increased energy demand may also induce fusion and division of mitochondria in perikaryal and dendritic sites; small mitochondria may translocate better in response to sites of increased energy demand and help in the local regulation of energy balance (Karbowski and Youle, 2003). Decreased ATP can stimulate fission or fragmentation (Knott et al., 2008) as can increased levels of nitric oxide, i.e., nitrosative stress, which may secondarily result in ATP decline, further synthesis of ROS, the overexpression of Drp1 and neuronal injury (Barsoum et al., 2006; Yuan et al., 2007).

Although it has been proposed that hyperglycemia results in mitochondrial hyperpolarization and elevated production of ROS (Nishikawa et al., 2000) in cultured endothelium, others have found that the DRG mitochondrial inner membrane potential is depolarized in adult STZ-diabetic rats. Such defects may be associated with impaired Ca^{2+} buffering and have a role in shaping Ca^{2+} transients in diabetic neurons (Hall et al, 1995; Huang et al., 2002, see Fernyhough et al., 2010 for review). Interestingly, functional differences in susceptibility to Ca^{2+} overload have also been described between synaptic and nonsynaptic mitochondria (Brown et al., 2006). In addition, mitochondrial respiration involving the full electron transport chain is significantly decreased in STZ-diabetic rat DRG (Fernyhough et al., 2010).

Neuronopathy

Normalization of the metabolic severity of diabetes by insulin in our current experiments results in reversal of the degenerative pallor in the cell bodies in comparison to untreated diabetics. Neither insulin- nor saline-treated Akita mice show evidence of neuronal cell loss in mice diabetic for 3 months, although saline-treated Akita mice show substantial loss of neurons by 6 months of untreated diabetes. In comparison to the substantial effect of ARA290 on neuritic dystrophy in Akita diabetic mice, the agent failed to prevent the development of pale degenerating neurons and did not prevent the loss of neurons by the end of 7 weeks of treatment.

Our previous studies using a variety of genetic and STZ-induced diabetic mouse models show only rare degenerating neurons and in STZ-induced rat model degenerating neurons are even rarer, with non-biased counting techniques failing to show neuron loss in prevertebral SMG or paravertebral superior cervical ganglia (Schmidt, 2001). In comparison, Akita mice show clear evidence of a continuum of neuronal cell body pathology beginning as patchy loss of rough endoplasmic reticulum, through the accumulation of distinctive membranous aggregates and culminating in neuronal degeneration. We have noted that this pattern of neuron loss is not apoptotic with its characteristic nuclear fragmentation; rather, it has some features of autophagy based degeneration and is reminiscent of guanethidine-induced sympathetic degeneration in neonatal rats (Schmidt et al., 1990) which has an autoimmune basis, although degenerating neurons in the Akita model lack a compelling inflammatory infiltrate. Although degenerating sympathetic neurons are not as prominent in other diabetic mouse models as in Akita mice, we have encountered scattered neurons containing similar membranous aggregates as well as rare (<1%) pale neurons in STZ-induced diabetic C57BL6 mice

(Obrosova et al, 2010). Perikaryal structural changes are striking in the SMG and CG of the Akita mouse and result in the loss of approximately 30-50% of SMG and CG neurons over 6-8 months (Schmidt et al, 2009). Degeneration of sympathetic neuronal cell bodies has been inconspicuous in our human studies although we reported a small (~14%) decrease in neuronal density (Schmidt et al., 1993) in the prevertebral sympathetic ganglia of diabetic patients. The reported loss of 10-20% of DRG neurons in diabetic mice is in contrast to neuronal preservation in STZ-diabetic rat DRG (Zochodne et al., 2008) and may reflect increased neuronal vulnerability in murine diabetes.

Neuropathology - Functional Significance

Autonomic nervous system function is largely unstudied in the Akita mouse model. Although an investigation of gastroparesis demonstrated more rapid stomach emptying in the Akita mouse, no changes in gastric myenteric neurons, density of intramuscular peripherin immunoreactive axons or numbers of interstitial cells of Cajal was reported (Ouyang et al., 2007). Studies of cardiac function in Akita mice have characterized apparent parasympathetic dysfunction which actually reflects hypoinsulinemia-induced decrease in atrial expression of downstream mediators resulting in decreased carbamylcholine stimulation of potassium current (Park et al, 2009). Recent studies of somatic neuropathy in Akita mouse have shown alterations in SNCV and MNCV within a week of onset of hyperglycemia (de Preux Charles et al, 2010; Choeiri et al., 2005)

Diabetic mice, rats and Chinese hamsters develop symptomatic gastrointestinal autonomic dysfunction variously characterized as gastroparesis, diabetic diarrhea, intestinal transmucosal ion flux defects interfering with fluid transport, and increased small intestinal transit time (Diani et al., 1979; Chang et al, 1985; Chang et al, 1986; Cellek et al, 2003; Ordog et al, 2000; Malagelada, 2004). In many cases these functional changes are accompanied by sympathetic neuritic dystrophy (Schmidt et al., 1983, 1989, 2003; Schmidt and Plurad, 1986). Electrophysiologic studies (Chang et al, 1985; Chang et al, 1986) have established that noradrenergic innervation of the small bowel and mucosal ion flux is abnormal in STZ-diabetic rat ileum, that it reflects loss of functional noradrenergic innervation, shows a defect in response to tyramine-induced release of norepinephrine, and is accompanied by denervation hypersensitivity-induced adrenoceptor changes. These physiologic defects parallel the distribution and time course of sympathetic neuritic dystrophy in Chinese hamster and rat prevertebral sympathetic ganglia and streptozotocin (STZ)-diabetic rat ileal mesenteric nerves (Yagihashi and Sima, 1985; Schmidt and Plurad, 1986; Schmidt et al., 1989). The loss of the ileal brake reflex in STZ-diabetic rat, by which lipid rich ileal contents reflexively influence gastric motility (Malagelada, 2004), may also involve abnormal reflex integration in prevertebral sympathetic ganglia. Nonetheless, diabetic gut dysfunction is multifactorial and also includes selective damage to nitrergic (Cellek et al., 2003) and intrinsic peptidergic axons (Ballmann et al, 1985; Belai & Burnstock, 1987) and loss of interstitial cells of Cajal in the stomach (Ordog et al., 2000).

Erythropoietin

The results of the current studies demonstrate a clear salutary effect but not normalization of neuritic dystrophy by ARA290, an 11 amino acid peptide composed of adjacent amino acids forming the aqueous face of EPO helix B. ARA290 treatment is also neuroprotective in models of sciatic nerve injury, ischemic stroke and renal ischemia-perfusion (Hand and Brines, 2010; Brines et al., 2008, Patel et al., 2008) and is currently in clinical development. ARA 290, even at very high dosages, has no effect on hematopoiesis or any toxicity in rabbits, rats, or mice (Brines et al., 2008). Similar studies in diabetic rat somatic nerves have shown that EPO produces a salutary effect on altered mechanical and thermal nociception, biochemistry and electrophysiology (Bianchi et al., 2004; Roesler et al., 2004). EPO

neuroprotection from excitotoxic, apoptotic and oxidative stress results from the stimulation of a heteroreceptor complex comprising both the EPO receptor and a common β -receptor subunit, also known as CD131, which is present on neurons and Schwann cells and is ultimately mediated by the PI3K/Akt cascade (Brines et al., 2000, 2004; reviewed by Brines and Cerami, 2005; Siren et al., 2001; Lipton, 2004). We have previously established the presence of EPO receptors on cultured rat prevertebral sympathetic neurons (Schmidt et al., 2008) and have unpublished evidence for EPO stimulation of phosphoAkt/total Akt ratio of sympathetic neurons in culture. EPO is thought to be synergistic with IGF-I in the activation of the PI3K/Akt pathway, whose function is diminished in somatic and vagus nerves of rats with STZ-diabetes (Cai and Helke, 2003; Huang et al., 2005). We have shown that IGF-I reverses dystrophic pathology in the ileal mesenteric nerves and prevertebral ganglia of STZ-diabetic rat (Schmidt et al., 1999) in the absence of an effect on the severity of the diabetic metabolic state.

A number of studies have shown EPO deficiency in diabetic patients with anemia which may occur early in diabetic nephropathy (Bosman et al., 2001, 2002). Serum EPO levels were decreased in type 1 diabetic patients with postural hypotension in comparison to age- and duration-matched type 1 diabetics free of complications as well as non-anemic, non-diabetic controls and patients with iron deficiency anemia (Winkler et al., 1999). A blunted EPO response to severe anemia has also been described as a result of autonomic neuropathy in studies of patients with both types 1 and 2 diabetes (Ricerca et al., 1999; Cotroneo et al., 2000; Spallone et al., 2004; Saito et al., 2007).

Although experience with EPO is extensive with relative safety (Sowade et al., 1998), there are reported side effects of EPO administration including accelerated hypertension, risk of thrombosis (Drueke et al., 2006; Rao and Stamler, 2002; Singh et al., 2006; Spivak, 2001) and possible promotion of tumor growth (Crawford, 2007; Henke et al., 2006; Steensma and Loprinzi, 2005), prompting the FDA to issue a warning for the use of erythropoiesis stimulating agents in oncology patients. Although these issues are currently unresolved, it may prove that agents such as ARA290 which have neuroprotective but not erythropoietic effects may provide the neuroprotective effects of EPO without triggering adverse effects such as pathological thrombosis or promoting growth of tumors.

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Abbreviations

CEPO	carbamylated erythropoietin
CG	celiac ganglion
EPO	erythropoietin
NOD	non-obese diabetic
STZ	streptozotocin
SMG	superior mesenteric ganglion
STZ-Rx NOD/SCID	streptozotocin-treated NOD/severe combined immune deficient

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Highlights (revised)

- Akita mice develop severe chronic type 1 diabetes for as long as 11 months
- Neuritic dystrophy and neuronopathy involve prevertebral sympathetic ganglia
- Insulin therapy reverses diabetes, normalizing neuritic dystrophy and neuronopathy
- Erythropoietin mimetic ARA290 reverses neuritic dystrophy but not neuronopathy

Figure 1. Ultrastructural appearance of neuritic dystrophy in the SMG and CG of treated and untreated Akita mice

A) Terminal axon (arrow) containing numerous autophagosomes which enclose normal and degenerating organelles. [5.5 month saline treated diabetic CG]

B,C) Swollen neurite containing numerous dense core vesicles, seen better at higher magnification in C. Some autophagosomes contain large numbers of synaptic vesicles (arrow, C) and are surrounded by disorganized cytoskeleton (arrowhead, C). [3 month saline treated diabetic CG]

D-F) Dilated neurites containing large numbers of small mitochondria, some of which represent postsynaptic dendrites contacted by presynaptic nerve terminals (E, arrow). Accumulated mitochondria are shown at higher magnification in F. [D: 3 month insulin treated diabetic CG; E,F: 5.5 month saline treated diabetic]

Magnification bars A-E = 1 micron; F = 500nm.

Figure 2. Effect of Insulin and ARA290 on the Frequency of Established Neuritic Dystrophy in Akita Diabetic and Control Celiac Ganglia

Values represent the means $\pm$ SEM

Statistical Comparison: * = $p < 0.002$; # = $p < 0.01$ vs. insulin-treated Akita

** = $p < 0.001$ vs. ARA290-treated Akita

Figure 3. Light Microscopic and Ultrastructural Appearance of the SMG and CG of Treated and Untreated Diabetic Akita Mice

A-C) Progressive changes in neurons of the diabetic ARA290 treated and untreated Akita SMG begin with pallor (arrows, A, B) and result in neuronal degeneration (arrow, C). [A: ARA290 treated diabetic Akita mouse; B, C: saline treated diabetic Akita mouse]

D, E) Pale neurons (arrows) show pallor caused by loss of Nissl substance with later perikaryal accumulation of membranous whorls (arrow, E) [one micron plastic toluidine blue sections of ARA290 treated Akita mouse CG]

(Magnification A-E, bar = 30 microns)

F) A typical pale neuron shows loss of Nissl substance and increased numbers of minute mitochondria [Electron micrograph, ARA290 treated Akita mouse SMG]

G, H) Degenerating neurons may contain large membranous aggregates (arrows, G,H) and disintegrating nuclei (arrowheads, G,H) [Electron micrographs, saline treated 3 month diabetic Akita mouse CG]

Magnification: F, G (bar = 4 microns); H (bar = 1 micron)

Figure 4. Ultrastructural appearance of perikaryal and dendritic mitochondriopathy in the CG of Akita mice

A,C) Typical normal neuronal perikaryon of celiac ganglion of diabetic Akita mouse demonstrates a spherical nucleus, large amounts of rough endoplasmic reticulum and normal appearing mitochondria, seen at higher magnification in C. [Electron micrographs, 5.5 month saline treated diabetic CG]

B,D) Pale degenerating neuron contains a slightly misshapen nucleus, scattered small, dark mitochondria in pale cytoplasm with depleted rough endoplasmic reticulum, seen at higher magnification in D [Electron micrographs, 5.5 month saline treated diabetic CG] (Magnification A,C, bar = 4 microns; B,D, bar = 500nm)

Table 1

Effect of Insulin Treatment on Akita Mouse Metabolic Parameters

Experimental Group	HbA1c (%)	Body Weight (g)	Blood Glucose (mg%)
Saline Treated Akita (n=7)	7.3 ± 0.4 ^a	23.6 ± 0.7 ^a	559 ± 17 ^a
Insulin Treated Akita (n=5)	5.0 ± 0.4 ^c	26.0 ± 0.4 ^{b,c}	149 ± 8 ^c
Saline Treated Controls (n=7)	4.6 ± 0.2	30.1 ± 1.2	131 ± 5
Akita – Diabetic 2 months (n=5) (starting point of treatment)	---	27.0 ± 1.5	558 ± 19

Values represent the means ± SEM of n mice.

Statistical Comparison:

^a p≤0.001

^b p≤0.05 vs. control

^c p≤0.01 vs. saline treated Akita

Table 2

Effect of ARA290 Treatment on Akita and Control Mouse Metabolic Parameters

Experimental Group	HbA1c (%)	Body Weight (g)	Blood Glucose (mg%)
Saline Treated Akita (n=8)	9.3 ± 0.6 ^a	21.5 ± 0.7 ^a	597 ± 3 ^a
ARA290 Treated Akita (n=8)	9.6 ± 0.5 ^a	22.0 ± 0.6 ^a	595 ± 5 ^a
Combined Saline + ARA290 Treated Controls (n=18)	4.0 ± 0.1	31.3 ± 0.6	203 ± 13
Akita 4 month diabetic (starting point of treatment) (n=4)	---	24.7 ± 1.1	560 ± 19

Values represent the means ± SEM of n mice.

Statistical Comparison:

^a p≤0.001 vs. control

Table 3

Effect of Insulin Treatment on Reversal of Neuronal Pathology in Akita Mouse Prevertebral Sympathetic Ganglia

Experimental Group	Neuron Number	Degenerating Neuron Frequency
	(neurons/ganglion)	(%)
Saline Treated Akita (n=7)	8015 ± 775	0.96 ± 0.26 ^a
Insulin Treated Akita (n=5)	6954 ± 836	0.03 ± 0.03 ^b
Saline Treated Controls (n=7)	5903 ± 667	0 ± 0
Akita (2 Month Diabetic = starting point of treatment) (n=5)	---	2.2 ± 0.4 (plastic sections)

Values represent the means ± SEM of n mice.

Statistical Comparison:

^a p≤0.01 vs. control

^b p≤0.01 vs. saline-treated Akita

Table 4

Effect of ARA290 Treatment on Reversal of Neuronal Pathology in Akita Mouse Prevertebral Sympathetic Ganglia

Experimental Group	Neuron Number	Degenerating Neuron Frequency
	(neurons/ganglion)	(%)
Saline Treated Akita (n=6-7)	4435 ± 692 ^b	2.1 ± 0.4 ^a
ARA290 Treated Akita (n=8)	4373 ± 724 ^a	1.5 ± 0.1 ^a
Combined Saline & ARA290 Treated Controls (n=8-10)	8442 ± 729	0.04 ± 0.01
Akita - 4 month Diabetic (starting point for Rx) (n=4)	---	7.0 ± 1.3 (plastic section data)

Values represent the means ± SEM of n mice.

Statistical Comparison:

^a
p≤0.001

^b
p<0.01 vs. control