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The Effects of Nanoparticle Injections of Insulin on the Myelin Sheath to Axon Area Ratio and Neuronal Function in Diabetic Rat Sciatic Nerve

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Abstract

Diabetic neuropathy is a complication of diabetes that causes nerve degeneration. Research indicates that insulin binds to receptors on the nerves to encourage growth; therefore, a plausible method to counteract this degeneration is by delivering insulin to the degenerating nerves. Nanoparticles (InsNP) are one such method of delivery. InsNPs allow for accurate depositing of insulin at the targeted site. The effects of diabetic neuropathy were observed using the ratio of the area of myelin sheath to the area of axon(m:a) and electrophysiological measures of individual proprioceptor responses to muscle stretch from sciatic nerve of rats of various treatment groups. Data from untreated diabetic rats (3wk) and diabetic rats with InsNP injected in triceps surae muscles was reanalyzed and sham-injected control groups were added. It has been observed that diabetes decreases the myelin axon ratio. The use nanoparticle injections containing insulin can shift the myelin axon ratio towards the non-diabetic control ratios. To ensure the effects were a result of the InsNP injections, and not the injection itself, a control group was added: diabetic rats with triceps surae injections of phosphate buffered saline (PBS). The data show that m:a was reduced in the 3wk diabetic+ PBS injected rats(3.6+/-0.05 vs. 5.3+/-0.12 for 3wk diabetic, p=0.00002;8.9+/-0.07 untreated, p=0.00002; 5.7+/-0.09 for 3wk diabetic+1 wk InsNP, p=0.89, ANOVA, post hoc unequal N HSD), therefore, not explaining the recovery toward untreated values of m:a in the InsNP injected rats. In future experiments, the goal is to investigate a less invasive method of introducing the InsNP, test formulations of nanoparticles to possibly increase insulin release stability and to test the effectiveness of insulin-like growth factor (IGF).

Keywords: Nanoparticle, Injections, Insulin

1. Introduction

Diabetic neuropathy refers to a collection of clinically diverse disorders affecting the nervous system, with differing anatomic features, clinical courses, and phenotypes[5]. Neuropathy is characterized in part by a reduction in nerve conduction velocity, an increase in sensory thresholds and reduction in action potential amplitude[12]. The myelin sheath is a plasma membrane of Schwann cells that wrap around an axon in a spiral fashion. It acts as an electrical insulator maintaining nerve conduction[9]. Damage to the myelin sheath results in reduction of nerve conduction and action potential amplitude[1].

Streptozotocin (STZ)-induced hyperglycemia is typically used to study the structural and functional changes of the nervous system attributed to type I diabetes[11]. Tonra and colleagues demonstrated that STZ rats had blood glucose level of over 300 mg/dl within 2 days of STZ injection and lower body weight compared to control. These results remained true for the whole 10 weeks of their experiment. It was also reported that STZ rats showed a decrease in motor conduction velocity and sensory conduction velocity 4 weeks after the injection. It has also been reported[10] that STZ injected mice showed significant and progressive sensorimotor dysfunction 5 weeks post STZ injection. When

analyzing the innervation patterns of the diabetic mice a range of abnormalities suggesting axon degeneration were observed. The effects of short-term hyperglycemia on individual proprioceptor function have not yet been examined. Darling and colleagues [10], demonstrated that reflexes mediated by proprioceptors have been altered, and may be due, at least in part to dysfunction of proprioceptor activity.

Currently, there are few effective treatments for the neurodegeneration and dysfunction found in diabetic patients. Nanoparticles are a potential therapeutic treatment for diseases like diabetic peripheral neuropathy. This is because nanoparticles encapsulate a drug and deliver the drug to a specific target site in a prolonged, time-released fashion depending on the formulation of the encapsulating layer. The effectiveness of a nanoparticle depends on its size, the coating or membrane of the particle, and the environment in which the particles are used. It has been shown that modifying a nanoparticle coating with a hydrophilic flexible polymer allows for the particle to be biodegradable but prevents it from being instantly attacked by the immune system. Previous research (Li, et. al., 2001) suggests that nanoparticles could be an effective carrier for protein and peptide drug delivery, for example when applied in treatment of spinal cord injury in rats[7].

The present study aims to use known electrophysiologically-recorded responses to mechanical perturbations to establish the effects of hyperglycemia on proprioceptor function, and the ability to apply nanoparticles containing insulin to ameliorate those effects in short term diabetes (3-6 weeks).

2. Materials and Methods

2.1 Subjects

Adult male and female Wistar and Sprague-Dawley rats were used. Rats were separated into 5 groups. 1) *Untreated:* which served as a healthy control group, these rats did not receive STZ injections or nanoparticle injections. 2) *Short term-Disease control:* rats that received an STZ injection to induce diabetes (see below). They were tested three weeks following induction of diabetes (3wk). 3) *Long term- Disease control:* rats that received an STZ injection to induce diabetes (see below). They were tested six weeks following induction of diabetes (6wk). 4) *Experimental Group:* rats that received an STZ injection to induce diabetes. Three weeks later (3wk), these rats received a treatment of nanoparticles containing insulin (InsNP; see below) via intramuscular injection (IM). They were then tested one week after the treatment date (3wk/1wk InsNP). 5) *Injection control*: rats received an STZ injection to induce diabetes. Three weeks after the STZ injection they received a treatment of nanoparticles containing phosphate buffered saline and were tested one week following NP injection (3wk/1wk pbs). This group served as a control to ensure that any effects recorded were due to insulin and not the injection or the PLGA coating.

2.1.1 diabetes induction

Rats were anesthetized by inhalation of 3% Isoflurane. Diabetes was induced using streptozotocin via intraperitoneal injection at 50 mg/kg in citrate buffer (pH 4.0). Streptozotocin kills the beta islet cells in the pancreas, thus stopping insulin secretion. Following a 48 hour period, rat blood glucose level was checked and urinalysis was conducted to confirm diabetes (a blood glucose level >300 mg/dL.

2.1.2 nanoparticle fabrication and injection

Nanoparticles were fabricated following the protocol developed by Li, et. al., (2001, see also Kim et. al., 2008). The particles consisted of an organic layer of polylactide-co-glycolide (PLGA) in a 75:25 ratio mixed with an aqueous layer of human recombinant insulin (Sigma-Aldrich) dissolved in phosphate buffered saline (pH 7.0). For injection control, PLGA nanoparticles contained only phosphate buffered saline. The two solutions were emulsified, freeze dried and stored at -20 degrees C until injected. This formulation allowed for the contents to be released over the course of 14 days. Ten injections of 0.01mL for a total volume of 0.1 mL (4 mg/ml InsNP/phophate buffered saline, pH 7.0) were made intramuscularly with a fine bore sterile needle (26G) in the left triceps surae of the rats.

2.1.3 electrophysiology

Afferent firing behavior was recorded using intra-axonal penetration of individual triceps surae muscle spindle, or group Ia sensory afferents. These afferents respond to muscle stretch along with the velocity with which the muscle is stretched. Characteristic afferent firing patterns in response to isometric muscle twitch contraction and ramp-hold-release stretches were recorded and analyzed, and also used to identify the type of afferent recorded[3,4].

2.1.4 statistical analysis

Data were analyzed using STATSOFT's *Statistica* software applying ANOVA with post hoc Honest Significant Difference ($p < 0.05$).

3. Results

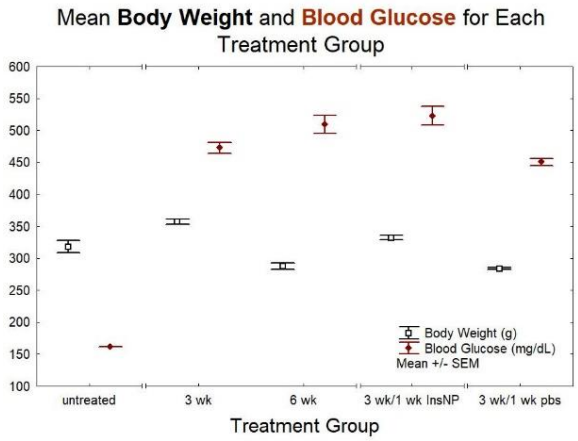

Figure 1**.** The mean (+/- S.E) of the body weight and blood glucose levels are shown in this graph. Blood Glucose was measured in (mg/dL) and body weight was measured in (g). Measurements were taken 2 days after STZ injection. (ANOVA with post hoc HSD $p < 0.05$)

Body weight and blood glucose levels were measured in order to verify hyperglycemia. A significant increase in blood glucose levels was observed within 24 hours of STZ injection. There was an increase in mean body weight within the 3wk diabetic control group (356.9 ± 5.8 g) in comparison to the untreated control (318.2 ± 6.1g) . The 6wk diabetic control group (287.7 ± 5.8 g) showed a decrease in mean body weight in comparison to the untreated control (318.2 ± 6.1 g). Neither of these differences were significant. Injection with insulin encapsulated nanoparticles had little effect on body weight. The experimental group (3wk/1wk InsNP) did not show a significant difference in body weight (332.1 ± 6.2 g)

All groups were significantly different than the untreated control group (162.0 ± 33.3 md/dL) in terms of blood glucose level. Both short-term (3wk) and long-term (6wk) diabetic control groups were not significantly different from each other (427.8 ± 11.8 mg/dL and 509.7 ± 11.8 mg/dL respectively). The experimental group (3wk/1wk InsNP) was both significantly different (522.9 ± 12.6 mg/dL) from the short-term diabetic control (3wk) (427.8 ± 11.8 mg/dL) and the injection control (3wk/1wk PBS) (450.6 ± 18.8 mg/dL).

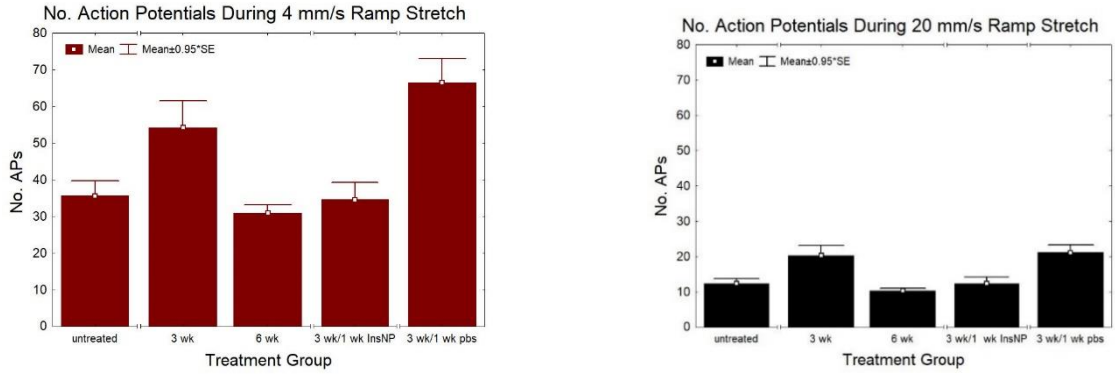

Figure 2. Recording of afferents firing within the triceps surae in response to isometric muscle twitch contraction. Stretches were done at two different speeds (4 mm/s and 20 mm/s). (Mean +/- SEM; ANOVA with Unequal N HSD post hoc analysis; significantly different (*) $p < 0.05$.) (**) signifies significantly different from disease control (3wk). (*) signifies significantly different from Untreated control.

The number of action potentials that occurred during slow muscle and fast muscle stretch were recorded intra-axonally. Similar differences were seen between each group in both slow and fast muscle stretches. In comparison to the untreated control group (35.6 ± 5.9 at 4 mm/s and 12.4 ± 2.0 at 20 mm/s) , the (3wk) diabetic control group (54.1 ± 5.3 at 4 mm/s and 20.3 ± 2.0 at 20 mm/s) had a significant increase in the number of action potentials that occurred in both respective movements. The experimental group (3wk/1wk InsNP) group showed almost no difference in the number of action potential recorded (34.6 ± 5.4 at 4 mm/s and 12.4 ± 2.0 at 20 mm/s) in comparison to the untreated group (35.6 ± 5.9 at 4 mm/s and 12.4 ± 2.0 at 20 mm/s). However, the experimental group (3wk/1wk InsNP) had a significantly lower number of action potentials in comparison to the injection control group (3wk/1wk PBS) during slow stretch (4 mm/s). During fast stretch (20 mm/s) the experimental group (3wk/1wk InsNP) was had a significantly higher number of action potentials in comparison to the long-term diabetic control group (6wk) (30.8 ± 5.7 at 4 mm/s and 10.3 ± 2.0 at 4 mm/s). The injection control group (3wk/1wk PBS) had the highest number of action potentials (66.5 ± 6.4 at 4 mm/s and 21.1 ± 2.4 at 20 mm/s) occur and was significantly different than all other groups during slow muscle stretch.

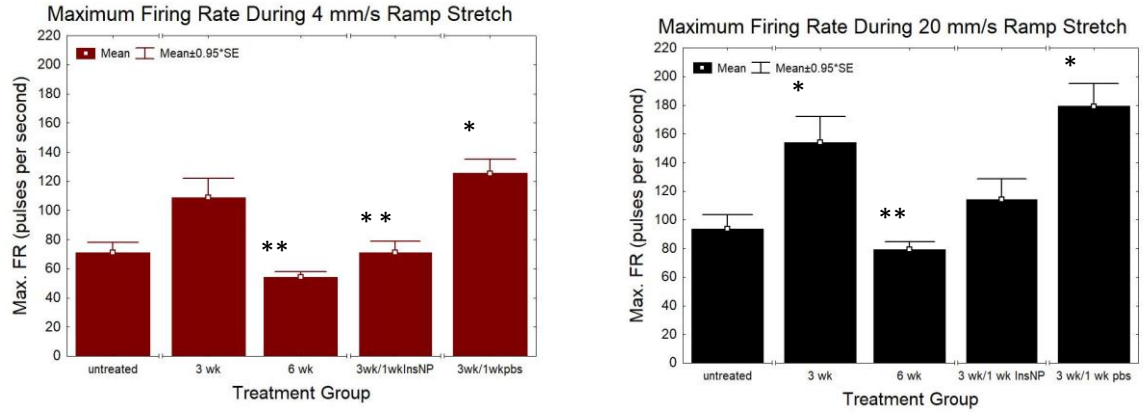

Figure 3. Recording of afferents firing within the triceps surae in response to isometric muscle twitch contraction. Stretches were done at two different speeds (4 mm/s and 20 mm/s). Measurements represent the number of pulses per second. (Mean +/- SEM; ANOVA with Unequal N HSD post hoc analysis; significantly different (*) $p < 0.05$. (**) signifies significantly different from disease control (3wk). (*) signifies significantly different from Untreated control.

The maximum instantaneous firing rate represents the maximum number of action potentials that occur during a 1 second hold using a ramp-hold-release stretch at two different speeds. The differences between each group were very similar at in both slow and fast muscle stretch. The results showed similarity to the Figure 2. Number of action potentials. In comparison to the Untreated control group (71.2 ± 9.8 at 4 mm/s and 93.5 ± 13.8 at 20 mm/s), the (3wk) short-term diabetic group showed a significant increase (109.0 ± 8.9 at 4 mm/s and 154.1 ± 13.5 at 20 mm/s) in the max number of action potentials. The long-term diabetic control (6wk) group (54.1 ± 10.0 at 4 mm/s and 79.4 ± 14.2 at 20 mm/s) was significantly different than the short-term diabetic control (3wk) and the injection control group (3wk/1wk PBS). The experimental group (3wk/1wk InsNP) group performed (71.0 ± 9.0 at 4 mm/s and 114.04 ± 13.8 at 20 mm/s) very similar to the untreated control group showing only a significant difference to the short-term diabetic control group during slow stretch (4 mm/s). The injection control group (3wk/1wk PBS) had the highest maximum firing rate (125.5 ± 10.8 at 4 mm/s and 179.2 ± 17.0 at 20 mm/s) and was significantly different than the untreated control and experimental group (3wk/1wk InsNP) group.

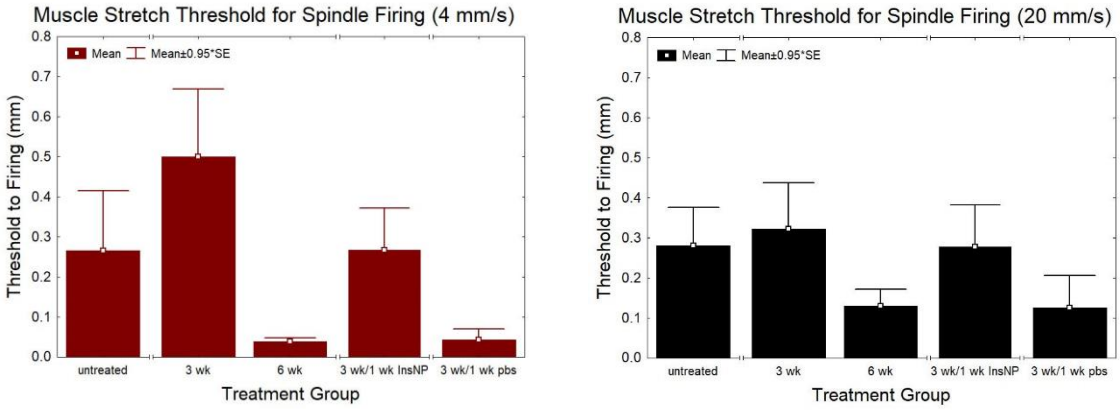

Figure 4. Represents the length in which the triceps surae was stretched until the first action potential occurred. Stretches were done at two different speeds (4 mm/s and 20 mm/s) measurements were taken in millimeters (mm). (Mean +/- SEM; ANOVA with Unequal N HSD post hoc analysis; significantly different (*) $p < 0.05$.)

The threshold length to fire an action potential is a measurement that signifies the distance in which the triceps surae is stretched until an action potential occurs. This test provided comparable information about the sensitivity of the muscle. When comparing the mean values for the control groups, the (3wk) short-term diabetic control group had a higher (.50 ± .12 mm at 4 mm/s and .32 ± .10 mm at 20 mm/s) threshold than the untreated control (.27 ± .13 mm at 4 mm/s and .28 ±.10 mm at 20 mm/s). The experimental group (3wk/1wk InsNP) group had a threshold length (.27 ± .12 mm at 4 mm/s and .28 ± .10 mm at 20 mm/) that was not significantly different from any other groups. The group with the lowest threshold and least variation was the injection control group (3wk/1wk PBS0 (.04 ±.14 mm at 4 mm/s and .13 ± .11 mm at 20 mm/s) group it was also not significantly different when compared to the other groups.

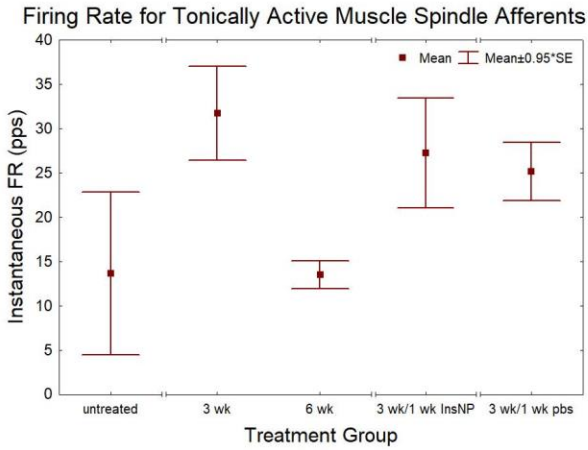

Figure 5**.** The background firing rate of individual triceps surae afferents in DRt L4 or L5. (Mean +/- SEM; ANOVA with Unequal N HSD post hoc analysis; significantly different (*) $p < 0.001$.)

Many Ia afferents afferents fire tonically under normal conditions. Due to an elevation of blood glucose in a short time frame (3wk) afferents had a higher firing rate in comparison to the untreated control. The 3wk/1wk InsNP and 3wk/1wk PBS groups both had higher firing rates than the untreated control group and similar firing rates to 3 wk afferents. Six-week (6wk) hyperglycemic rats had lower tonic firing rates, but closer to that of the untreated afferents. The tonic firing of these afferents would obscure determination of the threshold length sensitivity found in figure 4.

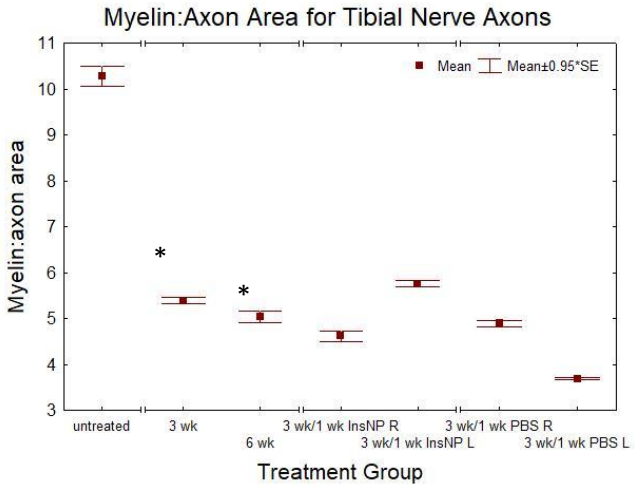

Figure 6. Myelin Axon area for tibial nerve axons. Ratio is determined by dividing the area of the axon by the area of the myelin sheath. (Mean +/- SEM; ANOVA with Unequal N HSD post hoc analysis; significantly different (*) $p < 0.001$.) (*) signifies significantly different from Untreated control.

Nanoparticle injections were done in the left triceps surae of the rats. The right leg muscles serve as an internal "untreated" control, as they were not injected with either the insulin nor PBS encapsulated nanoparticles. When comparing the left (treated) vs. right (untreated) afferent structure of the 3wk/1 wk InsNP myelin: axon ratio there was a significant increase($5.76 \pm .05$ for left leg and $4.61 \pm .13$ for right leg), moving closer to the untreated rat ratio. The left 3wk/1 wk InsNP afferents were also greater than all other groups' ratios, indicating that insulin did have a role in preserving or restoring myelin: axon ratio. The injection control group (3wk/1wk PBS) showed that the treated side (left side) has a significantly lower myelin axon ratio ($3.7 \pm .03$) in compression to the control side ($4.9 \pm .06$) (right side).

4. Discussion

The rat model of STZ injection does mimic the symptoms and characteristics of type 1 diabetes. Urinalysis and blood glucose data confirms hyperglycemia within 48 hours of injection. This model can also be used to help determine the effect of type I diabetes on afferent function and structure of proprioceptors. Afferent function can be readily identified by recording from individual axons and determining firing patterns that are characteristic following ramp-hold-release muscle stretch and nerve stimulation[3,4], the resultant function of which are under studied in literature in the face of hyperglycemia.

4.1 Short-Term Vs. Long-Term Diabetes

Short-term diabetes (3wk) increased both the number of action potentials and the maximum firing rate of afferents in response to ramp stretch. There was a contradictory increase in the threshold of muscle stretch to fire an action potential. This could be due to the increase in tonic instantaneous firing rate.

4.2 Effect Of Stz Injection On Electrophysiological And Structural Function

Long-term diabetes (6wk) decreased both the number of action potentials and the maximum firing rate of afferents in response to ramp stretch. An increase in the sensitivity to stretch was also seen in the stretch threshold stretch. This could also be due to the increase in tonic instantaneous firing rate.
To determine if structural changes occurred the myelin: axon ratio was measured on a cross-section of tibial nerve carrying the afferents that were tested electro-physiologically, among other afferents. Both short-term and long-term diabetic rats (3wk and 6wk) showed reduced ratios, indicating some effect on not only physiological function but structure at an early timepoint in disease.

4.3 Measuring The Effectiveness Of Insulin Nanoparticle Treatment

Following confirmation of changes in early diabetes, application of insulin containing nanoparticles were employed at the 3wk timepoint of diabetes and allowed to degrade over one week using a formulation that could deliver drug for up to two weeks (75:25 PLGA).

Comparing electrophysiological and structural measurements, as with the 3wk and 6wk afferents, the insulin nanoparticle-treated afferents had structure and function return toward untreated control values. This was also observed when measuring afferents on the untreated side in the treated animals.

Insulin nanoparticle injection (3wk/1wk InsNP) returned the number of action potentials and the maximum firing rate back to normal control values. The muscle stretch threshold of the experimental group (3wk/1wk InsNP) was also similar to the control group, leading to the conclusion that insulin nanoparticle injection can prevent functional changes after short-term diabetic neuropathy symptoms.

When comparing the treated and untreated myelin axon ratio of the experimental group (3wk/1wk InsNP) it was observed that the particles were able to increase the myelin axon ratio. However, the value of this ratio is not close to the ratio of the untreated rats. This means that the particles are not able to fully restore structural changes.

5. Future Directions

The injection control group (3wk/1wk PBS) performed the worst in both electrophysiological and structural test. This group had the highest number of action potentials and maximum firing rate in response to ramp stretch. It also had the lowest threshold for spindle firing which may represent hypersensitivity. In terms of myelin:axon ratio, this group had the lowest ratio. In fact the treated (left) side of the injection control group had a lower ratio than the untreated side. The treated side only received nanoparticles that contained PBS, therefore this does not explain for the decrease in structure and function. It is plausible that the injection itself could detrimental to the nerve of the rat.

Considering long term diabetes of both types demonstrate peripheral neuropathy, even when well controlled, therapeutic regimens for treating the peripheral neuropathy directly are being explored. Research done on Alzet minipump use show delivery of insulin and IGFs can help reduce neuropathic changes[6]. Here, we explored use of

time-release capsules of nanometer size to deliver drugs to the area of peripheral nerve shown to be affected first, the neuron terminals. Direct placement of nanoparticles in the muscle was effective in reducing changes in electrophysiology and structure following a short duration of application.

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