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# A Ketogenic Diet Reduces Metabolic Syndrome-Induced Allodynia and Promotes Peripheral Nerve Growth in Mice

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## Abstract

Current experiments investigated whether a ketogenic diet impacts neuropathy associated with obesity and prediabetes. Mice challenged with a ketogenic diet were compared to mice fed a high-fat diet or a high-fat diet plus exercise. Additionally, an intervention switching to a ketogenic diet following 8 weeks of high-fat diet was performed to compare how a control diet, exercise, or a ketogenic diet affects metabolic syndrome-induced neural complications. When challenged with a ketogenic diet, mice had reduced bodyweight and fat mass compared to high-fat-fed mice, and were similar to exercised, high-fat-fed mice. High-fat-fed, exercised and ketogenic-fed mice had mildly elevated blood glucose; conversely, ketogenic diet-fed mice were unique in having reduced serum insulin levels. Ketogenic diet-fed mice never developed mechanical allodynia contrary to mice fed a high-fat diet. Ketogenic diet fed mice also had increased epidermal axon density compared all other groups. When a ketogenic diet was used as an intervention, a ketogenic diet was unable to reverse high-fat fed-induced metabolic changes but was able to significantly reverse a high-fat diet-induced mechanical allodynia. As an intervention, a ketogenic diet also increased epidermal axon density. *In vitro* studies revealed increased neurite outgrowth in sensory neurons from mice fed a ketogenic diet and in neurons from normal diet-fed mice given ketone bodies in the culture medium. These results suggest a ketogenic diet can prevent certain complications of prediabetes and provides significant benefits to peripheral axons and sensory dysfunction.

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## Introduction

The growing epidemic of obesity and diabetes has led to a dramatic increase in various pain syndromes and a personal as well as economic burden. A common complication associated with diabetes and metabolic syndrome is a loss of small fibers in the skin and increased pain (Callaghan and Feldman, 2013; Smith and Singleton, 2013). With the development of metabolic syndrome, some have proposed that there is a loss of axonal regenerative capacity leading to a loss of small fibers that can occur with diabetes (Singleton et al., 2015). Prediabetes can be modeled in rodents using a high-fat diets and consumption of high-fats and carbohydrates lead to metabolic alterations and changes in sensory function similar to changes human patients, including obesity, elevated blood glucose, insulin resistance, and mechanical allodynia (Groover et al., 2013; Guilford et al., 2011; Hoke, 2012; Obrosova et al., 2007). Physical activity can improve many of these symptomatic changes; including reversing mechanical allodynia induced by a high-fat diet (Groover et al., 2013). However, the mechanism(s) by which exercise leads to metabolic and sensory nerve benefits is poorly understood.

Previous research has demonstrated that exercise and a high-fat diet create distinctive metabolic phenotypes both systemically and in the peripheral nervous system (Cooper et al., 2016). Both exercise and a ketogenic diet intervention are attractive approaches as both can increase fat oxidation (Horowitz and Klein, 2000; Paoli, 2014). Additionally, both exercise and ketogenic diet can stimulate anti-inflammatory signaling cascades and reduce chronic inflammation that occurs in response to a high-fat diet (Ruskin et al., 2009).

High-fat, low carbohydrate ‘ketogenic’ diets are a rapidly emerging intervention for a wide array of clinical diseases (Klein et al., 2014; Masino and Ruskin, 2013; Wheless, 2008). Historically, a ketogenic diet was popularized in the treatment of epilepsy after it was noted that patients who fasted observed reduced seizure occurrence (Wheless, 2008). Relevant to peripheral nerve function, only a limited number of investigations have examined how a ketogenic diet impacts mechanical and thermal sensation (Galdino et al., 2014; Ruskin et al., 2009; Ruskin et al., 2013; Ziegler et al., 2005). Caloric restriction increases ketone metabolism and can be a pro-growth signaling agent in the hippocampus and dentate gyrus (Lee et al., 2002a; Lee et al., 2002b). However, the mechanism and function of increased neuronal growth is still under investigation.

Here, we examined several parameters of peripheral nervous system function from mice fed a ketogenic diet. We have previously reported that mice fed a high-fat and carbohydrate-rich diet develop negative symptoms related to peripheral nerve function similar to early peripheral neuropathy in prediabetes (Cooper et al., 2016; Groover et al., 2013; Guilford et al., 2011). In the current study, we provide evidence that mice fed a ketogenic diet fail to develop mechanical allodynia similar to mice fed a high-fat diet. Additionally, after 8 weeks of a high-fat diet, a ketogenic diet can reverse established mechanical allodynia. Lastly, we

investigated the potential of a ketogenic diet to promote peripheral nerve outgrowth and noted that a ketogenic diet or ketone body supplementation increases axon growth of primary sensory neurons. Together, these results suggest that a ketogenic diet may be an attractive intervention for metabolic and diabetic peripheral neuropathy.

## Materials & Methods

### Diet and Mice

Seven-week-old male C57/BL6 #027 mice were purchased from Charles River (Wilmington, Mass) and maintained on a 12:12h light/dark cycle in the research support facility at the University of Kansas Medical Center. All mice were given ad libitum access to food and water and were fed either a standard chow diet (8604; Envigo, Madison Wisconsin; 14% kcals from fat, 32% protein, and 54% carbohydrate), a high-fat diet (07011; Envigo; 54% kcals from vegetable shortening (hydrogenated) and corn oil fat, 21% protein and 24% carbohydrate), or a ketogenic diet (96355; Envigo; 90.5% kcals from vegetable shortening (hydrogenated) and corn oil fat, 9.2% protein, and 0.3% carbohydrate). Standard diet energy was calculated as previously described (Groover et al., 2013).

Two separate experiments to examine the effects of dietary and exercise interventions on peripheral nerves were conducted. The first experiment included mice, referred to as the “challenge cohort”, included control-fed sedentary (CF-Sed); high-fat sedentary (HF-Sed); high-fat exercise (HF-Ex); ketogenic (Keto) groups of mice. After baseline behavioral testing was complete, mice were separated and the groups were then challenged with different diets and/or access to running wheels.

The second experiment included groups of mice referred to as “intervention cohort” in which all mice were fed a high-fat diet for eight weeks (notated as HF-CF-Sed, HF-HF-Sed, HF-HF-Ex, HF-Keto). After eight weeks and baseline behavioral testing, mice were then separated and the groups were given different diets or interventions. Control-fed, high-fat-fed and ketogenic-fed mice were all pair housed for the entirety of the study. Exercise mice were singly housed to allow for analysis of running amounts for each mouse and given access to a voluntary running wheel and were allowed to run for the remainder of the study. All mice were 8 weeks of age at the start of each experiment. A timeline of diet and exercise challenge and intervention cohorts are displayed in Figure 1. All studies were in accordance with NIH guidelines and conformed to protocols approved by the institutional Animal Care Committee.

### Blood Measurements

Mice underwent assessments for weight and blood glucose (glucose diagnostic reagents; Sigma, St. Louis, MO) biweekly after a 3 hour fast (Groover et al., 2013). Additionally, at the time of sacrifice following a 3 hour fast, blood was drawn from the chest cavity and allowed to clot for 30 min on ice, spun at 3,000g for 30 min at 4 °C and serum drawn off and frozen at −80 °C until insulin was analyzed by ELISA (Alpco; Salem, NH). Blood ketones (β-Ketone blood test strips; Precision Xtra; Abbott Laboratories; Chicago, IL) were measured at baseline, week 1, 4, 8, and at sacrifice following a 3 hour fast.

After 4 weeks, an intraperitoneal glucose tolerance test (IPGTT) was performed after a 6 hour fast. Animals were given 1g glucose/kg body weight. Blood glucose levels were measured via tail clip immediately before glucose injection, and 15, 30, 60, and 120 minutes thereafter.

### Body Composition

Body composition to assess fat mass was measured using the EchoMRI-100 (EchoMRI, Houston, TX). The first cohort of mice's body composition was determined immediately before sacrifice. Mice in the obesity intervention groups had body composition determined biweekly from baseline testing until sacrifice.

### Sensory Behavior Testing

Sensory behavior assessments were carried out at baseline and biweekly time points for all mice. Dietary fed mice were examined for both mechanical and thermal sensitivity, while mice in the obesity intervention groups were only examined for mechanical sensitivity. Mechanical sensitivity was assessed using Von Frey monofilaments as previously described (Groover et al., 2013). Thermal thresholds were assessed by placing mice in individual clear plastic cages on a Hargreaves's apparatus and a 4.0 V radiant heat source was applied three times to the hind paw as previously described (Groover et al., 2013).

### Intraepidermal Nerve Fiber (IENF) Density

Footpads were collected and processed from all mice using protocols previously described for IENF density (Groover et al., 2013).

### Neurite Outgrowth

Lumbar DRGs 4-6 neurons were harvested and dissociated to a single cell suspension as previously described (Malin et al., 2007). Mice were fed the control, high-fat, or ketogenic diet described above for four weeks prior to DRG dissection. Upon plating, all mice were given Nutrient Hams F-12 media with 10mM glucose for 4 days (Gibco). Additional culture experiments utilized chow-fed mice in which DRG neurons were plated and were given F12 media custom supplemented with varying levels of glucose (0mM, 5mM, 10mM) and/or the ketone (R)-(-)-3–hydroxybutyric acid (5mM or 10mM) (Sigma). Ketone levels were selected to mirror glucose levels to reduce energy availability differences effecting neurite outgrowth and to mirror physiological levels seen in human patients on a nutritional ketogenic diet (Koppel and Swerdlow, 2017). Following the 4 days in culture, neurons were fixed with 4% paraformaldehyde for 10 minutes. Immunohistochemistry was performed with SMI-312 (Covance, Emeryville, CA), a pan-axonal marker, to visualize neurites and counterstained with nuclear marker, Hoechst 33342 (Invitrogen). Coverslips were mounted on slides and imaged. Neurite outgrowth area was quantified using Image J. A stereological grid was superimposed on images of the cultures, and the number of neurites crossing exactly through intersections of the grid was counted, as was the number of neuronal cell bodies producing neurites. Three regions of interest were imaged per coverslip, and three coverslips per group were analyzed for each animal and the neurite area per neuron was calculated according to the following equation (Blacklock et al., 2005):

$$\frac{\left(\frac{neurite\ intersections}{total\ grid\ intersections}\right) \times total\ grid\ area}{neurons\ extending\ neurites = neurite\ area(\mu m^2) per\ neuron}$$

### Statistical Analysis

All data is presented as mean ± SEM. Data was analyzed for normality utilizing D'Agostino & Pearson normality test or Kolmogorov-Smirnov test where appropriate. Data was analyzed using a one-way ANOVA, two-factor ANOVA, or repeated measures ANOVA with post hoc comparisons analyzed using Fisher's test of least square difference where appropriate. Statistical significance was defined as $p < 0.05$ and all statistics were run using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA).

## Results

### Measures of Obesity and Prediabetes

**Challenge Cohort—**High-fat-sedentary mice weighed significantly more than all other groups from week 2 ($p<0.01$ for all groups) until the completion of the study. High-fat-exercise and ketogenic mice also gained significantly more weight than control-fed sedentary mice across the final 6 weeks of the study ($p<0.01$ for both groups), though both groups remained below high-fat-sedentary mice weights (main effect of group and time: $p<0.0001$) (Fig. 2a). High-fat-exercise and ketogenic mice consumed the greatest amount of energy throughout the study (Fig. 2b). High-fat-sedentary mice displayed a significant increase in fat mass as a percent of their body weight compared to all other groups (ANOVA $p<0.0001$; high-fat-sedentary vs. control-fed sedentary $p<0.0001$; high-fat-sedentary vs. high-fat-exercise $p<0.0001$; high-fat-sedentary vs. ketogenic $p=0.0028$). Additionally, high-fat-exercise and ketogenic mice have increased fat mass as compared to control-fed sedentary mice (high-fat-exercise vs. control-fed sedentary $p<0.0001$; ketogenic vs. control-fed sedentary $p<0.0001$), though less than high-fat-sedentary. Though there was no difference in body weight, ketogenic mice had greater fat mass than high-fat-exercised mice ($p=0.0420$) (Fig. 2c). Blood ketones were consistently elevated in ketogenic diet-fed mice compared to all other groups, which peaked at 2 weeks on the ketogenic diet (Main effect of group and time: $p<0.0001$; week 2 $p<0.0001$ for all groups) (Fig. 2d).

High-fat-sedentary, high-fat-exercise, and ketogenic-diet fed mice had mildly elevated blood glucose levels compared to control-fed sedentary mice beginning after 2 weeks of dietary challenge for high-fat-sedentary and ketogenic mice ($p=0.05$ and $p=0.03$) and 4 weeks for high-fat-exercise mice ($p=0.001$) (main effect of group and time: $p<0.0001$) (Fig. 3a). High-fat-sedentary mice displayed significantly increased blood glucose levels during IPGTT at 60 ($p=0.001$) and 120 minutes ($p<0.0001$) compared to control-fed mice (main effect of diet and group: $p<0.0001$). Ketogenic-fed mice glucose levels were significantly reduced after 120 minutes compared to high-fat-sedentary mice ($p=0.04$) (Fig 3b). Fasting insulin was significantly elevated in high-fat-fed mice (main effect: $p<0.0001$; high-fat-sedentary vs. control-fed sedentary $p<0.0001$; high-fat-sedentary vs. ketogenic $p<0.0001$; high-fat-exercise vs. control-fed sedentary $p<0.0001$; high-fat-exercise vs. ketogenic $p<0.0001$), but

was not altered in ketogenic diet-fed mice compared to control-fed sedentary mice (Fig. 3c). HOMA-IR, a measure of beta cell function and insulin resistance, was significantly increased in high-fat-fed mice but was unaltered in ketogenic-diet fed mice (main effect: $p<0.0001$; high-fat-sedentary vs. control-fed sedentary $p<0.001$; high-fat-sedentary vs. ketogenic $p<0.0001$; high-fat-exercise vs. control-fed sedentary $p<0.0001$; high-fat-exercise vs. ketogenic $p<0.0001$) (Fig 3d).

**Intervention Cohort—**Following 8 weeks of being fed a high-fat diet to induce prediabetes symptoms and mechanical allodynia, mice were divided into 4 unique groups to receive a dietary and/or exercise intervention. High-fat-sedentary and ketogenic-diet fed mice weighed significantly more than control-diet fed mice from 2 weeks post intervention (high-fat-fed $p=0.004$; ketogenic-fed $p=0.04$) until the completion of the study. High-fat-exercise mice also gained significantly more weight than mice switched to a control diet beginning at 6 weeks post intervention ($p=0.009$) (main effects of group and time: $p<0.0001$) (Fig. 4a). Control-fed sedentary mice were the only group that had a decrease in body weight from baseline measures at the start of the interventions. Ketogenic diet-fed mice consumed the greatest amount of energy, mirroring their increased body weight compared to other groups (Fig. 4b). Increases in fat mass mirrored bodyweight increases, as high-fat-sedentary and ketogenic-diet fed mice displayed significant elevations in fat mass as a percent of their body weight beginning at 2 weeks post intervention ($p<0.001$ for all groups) (main effect of time: $p=0.0025$; main effect of group $p<0.0001$). High-fat-exercised mice maintained their fat mass below high-fat-sedentary and ketogenic mice, yet above control-fed sedentary mice beginning at 4 weeks post intervention ($p<0.001$ for all groups) (Fig. 4c). Mice switched to a control-fed sedentary diet had significantly less fat mass compared to all other groups.

Only mice switched to a control-fed sedentary diet displayed a decrease in blood glucose levels beginning after 2 weeks after the dietary change (main effect of time and group: $p<0.0001$; 2 weeks $p<0.01$ for all groups) (Fig. 4d). Fasting insulin levels at sacrifice were significantly reduced in control-fed sedentary mice compared to high-fat-sedentary (ANOVA $p=0.04$; control-fed sedentary vs. high-fat-sedentary $p<0.05$) and high-fat-exercised (control-fed sedentary vs. high-fat-exercise $p<0.01$) mice. Mice switched to a ketogenic diet had insulin levels between control-fed sedentary and high-fat-sedentary mice, although not significantly different (Fig. 4e). Only mice switched to a control-fed sedentary diet showed a decrease in HOMA-IR as compared to high-fat-sedentary (ANOVA $p=0.05$; high-fat-sedentary $p=0.05$) and high-fat-exercise ($p<0.01$) mice (Fig. 4f).

## Sensory Thresholds and Mechanical Allodynia

**Challenge Cohort—**Both high-fat-fed sedentary and high-fat-fed exercised mice developed mechanical allodynia, though only high-fat-fed sedentary mice were statistically different (main effect of group: $p<0.001$; high-fat-sedentary vs. control-fed sedentary $p=0.0268$; high-fat-exercise vs. control-fed sedentary $p=0.0565$; high-fat-sedentary vs. ketogenic $p=0.0482$; high-fat-exercise vs. ketogenic $p=0.094$). However, mechanical thresholds in high-fat-exercised mice to return towards baseline threshold levels after 8 weeks, whereas high-fat-fed sedentary mice maintained mechanical allodynia (high-fat-

sedentary vs. control-fed sedentary p =0.0421; high-fat-sedentary vs. ketogenic p=0.0161). At 12 weeks, high-fat-sedentary mice displayed mechanical allodynia, while high-fat-exercise returned to baseline mechanical thresholds (high-fat-sedentary vs. ketogenic p=0.0136; high-fat-sedentary vs. control-fed sedentary p=0.0702; high-fat-sedentary vs. high-fat-exercise p=0.0658) (Fig. 5a). Throughout the course of the study, no groups displayed any significant changes in thermal sensitivity (Fig. 5b).

**Intervention Cohort—**At the point of introducing the dietary and exercise interventions, all mice had developed mechanical allodynia as a result of the previous 8 weeks consuming a high fat diet. However, mice switched to a ketogenic diet displayed a significant elevation in mechanical thresholds within 4 weeks to levels near standard baseline value for hind paw sensation (main effect of time: p=0.008 main effect of group: p<0.0001; ketogenic vs. high-fat-sedentary p=0.0033; ketogenic vs. control-fed sedentary p=0.0119; ketogenic vs. high-fat-exercise p=0.0426) (Fig. 5c). No other groups displayed changes in their mechanical sensitivity following an interventional change in their diet.

### Epidermal Axon Changes

**Challenge Cohort—**IENF densities were altered by both diet and exercise, although in opposing fashions. IENF density was decreased by exercise (ANOVA p<0.0001; control-fed sedentary vs. high-fat-exercise p=0.0002; high-fat-sedentary vs. high-fat-exercise p=0.0017; ketogenic vs. high-fat-exercise p<0.0001) and increased with a ketogenic diet (control-fed sedentary vs. ketogenic p=0.0496; high-fat-sedentary vs. ketogenic p=0.0225; high-fat-exercise vs. ketogenic p<0.0001) (Fig. 6c). A subset of epidermal axons that express the NGF receptor, TrkA were also analyzed and the expression of this neurotrophin receptor was unaltered by diet or exercise.

**Intervention Cohort—**Mice placed on a ketogenic diet following a high fat diet displayed an increase in IENF density compared to all groups (ANOVA p<0.0001; ketogenic vs. control-fed sedentary p=0.0158; ketogenic vs. high-fat-sedentary p=0.0033; ketogenic vs. high-fat-exercise p<0.0001). High-fat-exercise mice displayed decreased IENF density relative to control-fed sedentary mice (p=0.0004) (Fig. 6d). Analysis of TrkA+ fibers displayed no changes in fiber density by diet or exercise intervention.

**Neurite Outgrowth:** Following four weeks of consuming either a control-, high-fat-, or ketogenic-diet, lumbar DRG neurons were harvested from mice and grown in 10mM glucose F12 media. Neurons from mice fed a ketogenic diet displayed enhanced neurite outgrowth compared to mice fed a control (p=0.0197) or high-fat (p=0.001) diet (ANOVA p=0.003) (Fig. 7c). Supporting this data, neurons harvested from control diet-fed mice displayed enhanced neurite outgrowth when grown in media containing 5mM glucose and 5mM ketones (p=0.001) and 10mM ketone and 0mM glucose (p=0.0343) compared to 10mM glucose F12 media (ANOVA p=0.011) (Fig. 7d).

## Discussion

The growing epidemic of obesity and metabolic syndrome is coupled with an increase in distal symmetric sensorimotor polyneuropathy (DSPN), one of the most common forms of

peripheral neuropathy. This has driven an emergence of research to combat these changes in sensation driven by these disorders (Cooper et al., 2016; Groover et al., 2013; Guilford et al., 2011; Obrosova et al., 2007). Currently, there are few approaches that have been successful in preventing or reversing axonal loss associated with diabetes-associated neuropathy (Calcutt et al., 2017; Christianson et al., 2007; Li et al., 2012; Ma et al., 2014; Turkiew et al., 2017). Our results reaffirm that a high-fat diet leads to numerous metabolic changes representative of prediabetes, including mechanical allodynia. Importantly, the consumption of a ketogenic diet reversed pre-existing mechanical allodynia related to the long-term consumption of a high-fat diet independent of changes in other metabolic parameters. The work presented here also shows that a ketogenic diet can increase cutaneous innervation, even in mice with obesity and metabolic syndrome. Importantly, these results suggest for the first time that a ketogenic diet may improve nociception and axonal degeneration associated with obesity and prediabetes that may be translatable to clinical interventions to address a number of painful conditions.

### Ketogenic Diet and Metabolic Changes

Previous studies have demonstrated that a high-fat diet can serve as a valuable experimental model of obesity, increased fat mass, and insulin resistance (Cooper et al., 2016; Groover et al., 2013; Guilford et al., 2011). Here, mice challenged with a high-fat diet predictably developed increased body weight and fat deposition, mildly increased blood glucose and increased insulin levels. While consuming the high fat diet, exercise was able to reduce increases in body weight and fat deposition by a high-fat diet despite no changes in elevated blood glucose and insulin levels. Thus, exercise was able to blunt several but not all metabolic changes associated with a high-fat diet consistent with previous studies (Cooper et al., 2016). Similar to exercise, a ketogenic diet also resulted in comparatively reduced body weight and fat deposition but was unable to lower blood glucose. One important distinction is that the ketogenic diet did not lead to hyperinsulinemia similar to a high-fat diet or exercised mice on a high-fat diet, consistent with previous studies (Paoli et al., 2013).

Another important aspect of this study analyzed whether a ketogenic diet could be used as an intervention to improve/reverse poor metabolic control and pre-existing nociceptive changes induced by a high-fat diet. As noted above, a high-fat diet leads to symptoms resembling prediabetes and only an interventional change to a control diet was able to return mice to normal levels of metabolic biomarkers. Surprisingly, mice fed a ketogenic diet continued to increase their body weight, fat deposition, blood glucose and insulin similar to sedentary mice continued on a high-fat diet. Contrary to our expectations, exercise initiated eight weeks as an intervention was unable to correct metabolic maladies. As an intervention, high-fat-exercised mice saw no reduction in insulin levels; however previous work has shown that exercise in high-fat-fed mice can improve glucose tolerance (Groover et al., 2013). Though exercise as an intervention blunted increases in some metabolic measures, its overall effect exhibited maintenance of the starting metabolic status without improvements to normal levels. Mice switched back to a control-diet saw improvements in all metabolic parameters, suggesting the control diet may be the most potent intervention for improving metabolic indicators. Together, these results are somewhat surprising as neither mice given

exercise or switched to a ketogenic diet led to significant improvements in metabolic parameters.

## Sensory Changes Accompanying a High-fat or Ketogenic Diet

The impact of a ketogenic diet on sensation and neuropathy has received little attention. Previous research reported changes in thermal sensitivity in both juvenile and adult rats when fed a ketogenic diet (Ruskin et al., 2009; Ruskin et al., 2013). In our study, mechanosensation was quite sensitive to dietary and metabolic changes. Similar to previous studies, mice challenged with a high-fat diet developed long-lasting mechanical allodynia (Cooper et al., 2016; Groover et al., 2013). Importantly, exercise was able to reverse mechanical allodynia in mice challenged with a high fat diet similar to previous studies (Cooper et al., 2016; Groover et al., 2013). An important point from this study is that mice challenged with a ketogenic-diet never develop mechanical allodynia. This suggests that, despite the elevated fat content in both the ketogenic and high-fat diets, these diets affect mechanical sensation very differently. In concordance, an intervention with a ketogenic diet after consuming a high-fat diet led to remarkable improvements in mechanosensation. Interestingly, mice switched to a control diet as an intervention had improvements in metabolic parameters, but not in mechanical thresholds. We postulate that the failure of a control diet as an intervention to improve mechanosensation could be due to metabolic memory of peripheral pathways. If true, it is plausible to suggest that a ketogenic diet may be able to overcome or reset metabolic memory in sensory components that convey mechanosensation, and possibly reduced allodynia or pain associated with prediabetes.

## A Ketogenic Diet Promotes Nerve Growth

A common complication associated with diabetes and metabolic syndrome is a loss of epidermal axons and pain (Callaghan and Feldman, 2013; Smith and Singleton, 2013). A common hypothesis is that in prediabetes and diabetes, regenerative capacity is lost in axons, leading to a loss of small fibers and small fiber neuropathy (Singleton et al., 2015). The present results may offer an attractive new intervention to prevent epidermal axon loss and perhaps promote increased nerve growth. Our analysis of IENF density in mice challenged with different diets revealed that a ketogenic diet leads to an approximate 10% increase in IENF density compared to mice fed a high-fat diet. This was also true in mice switched to a ketogenic diet as an intervention. I*n vitro* assessments of neurite outgrowth revealed that DRG neurite growth was enhanced in mice fed a ketogenic diet for 4 weeks. Finally, additional *in vitro* experiments in mice fed a control diet revealed that DRG neurons supplemented with either ketones by themselves, or ketones and glucose displayed increased neurite outgrowth compared to DRG neurons in media containing only glucose. We hypothesize that a ketogenic diet and ketone bodies themselves may alter neuronal metabolism that promotes axon integrity and/or axon growth. Future studies are needed to determine if a ketogenic diet has similar actions in humans and the intracellular mechanisms that may stimulate axon growth.

## Conclusion

Our current research suggests there are key alterations in the peripheral nervous system that are sensitive to metabolic insults, and that these alterations often do not mirror whole body

metabolic changes. In addition, we have shown that consumption of a ketogenic diet positively impacted mechanosensation, reduced elevations in insulin and promoted axon growth. Intriguingly, consumption of a ketogenic diet increased IENF density compared to a high-fat or control diet. Assessment of axonal growth revealed that ketone bodies increased neurite outgrowth, with the greatest growth occurring with a combination of glucose and ketone bodies as fuel sources. We propose that the peripheral nervous system responsible for normal sensation has a separate micro- metabolic environment that is not reflective of systemic metabolic changes and can respond positively to interventions and can override potential metabolic memory complications associated with sensation and neuropathy. These results suggest that features of neuropathy may be improved by consuming a ketogenic diet, and this may have implications for increasing lost epidermal fibers associated with neuropathy.

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## Highlights

- A ketogenic diet can reverse high-fat-induced mechanical allodynia
- Peripheral nerves are sensitive to metabolic change that may not mirror systemic metabolism
- A ketogenic diet and ketones stimulates sensory axon growth in vitro and in vivo

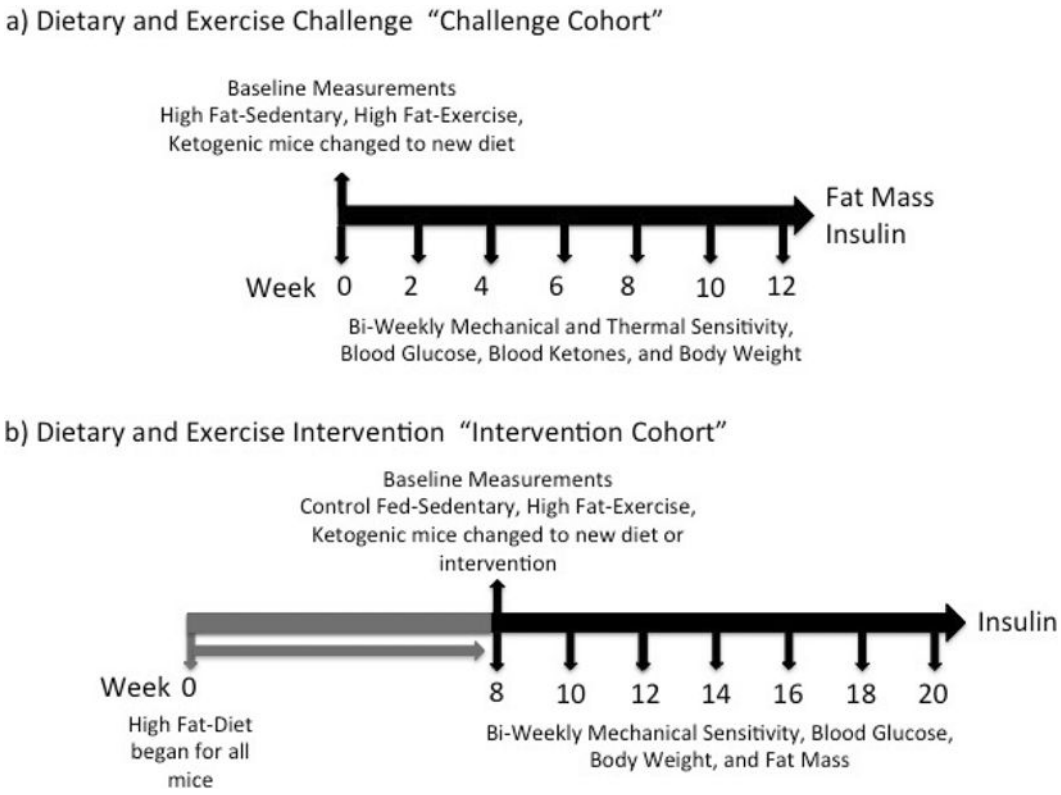


**Figure 1. Experimental Timeline of Cohorts**

a) Challenge cohort: All mice remained on a control diet until baseline testing was completed, then mice were changed to experimental diets and exercise for 12 weeks. b) Intervention cohort: all mice remained on a high fat diet for 8 weeks, at which point baseline testing was completed, mice were divided into different intervention groups and provided different diets and exercise for 12 additional weeks.

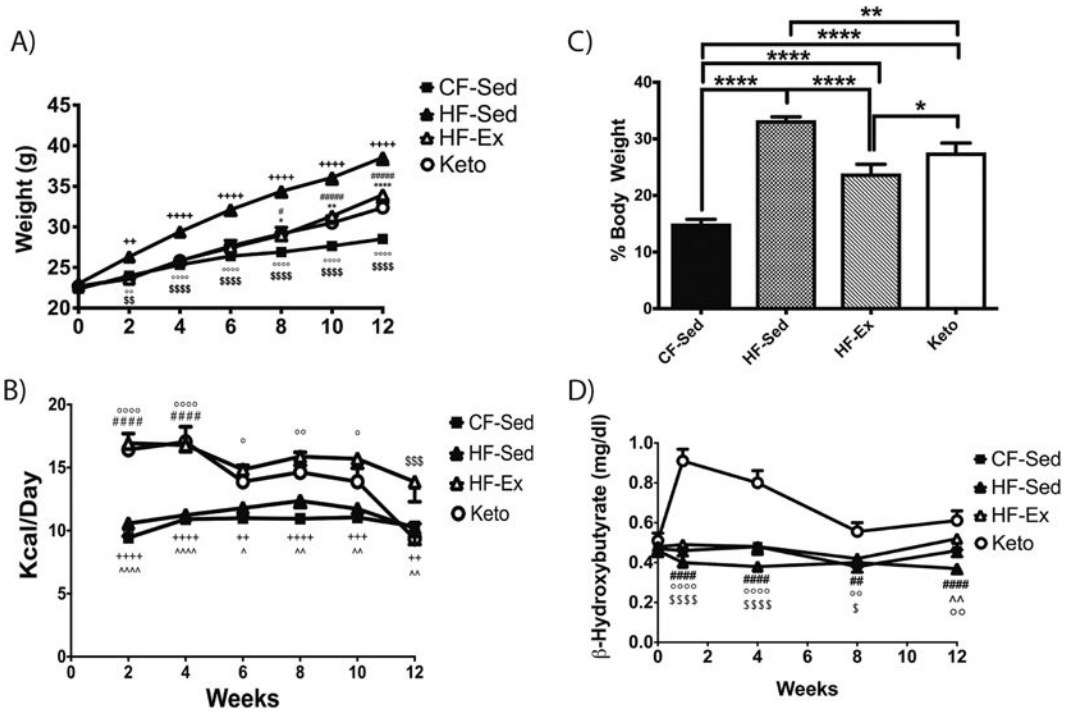


**Figure 2. A Ketogenic Diet Leads to a Different Metabolic Profile than a High-fat Diet**

(a) A high-fat diet causes weight gain relative to all groups, whereas exercised or ketogenic diet-fed mice displayed significant weight gain relative to standard diet controls (n=18 for all groups). (b) High-fat exercised and ketogenic-diet fed mice displayed increased energy intake relative to CF-Sed or HF-Sed mice (n=18 for all groups) (c) Body composition analysis performed by EchoMRI of fat mass following 12 weeks of diet and exercise revealed that a high-fat diet causes increased fat mass in mice relative to all groups, while exercised or a ketogenic diet-fed mice display fat mass levels more similar to CF-Sed control mice. Ketogenic diet-fed mice displayed increased fat mass compared to HF-Ex mice (n=18 for all groups) (d) Ketogenic diet-fed mice had increased serum ketone levels after 1 week, with a decrease over time. However, ketone levels remained slightly elevated compared to all other groups All data presented as mean ± SEM *:CF-Sed vs. HF-Sed #: Keto vs. HF-Sed ^: HF-Sed vs. HF-Ex +: HF-Ex vs. CF-Sed °: Keto vs. CF-Sed $: Keto vs. HF-Ex * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

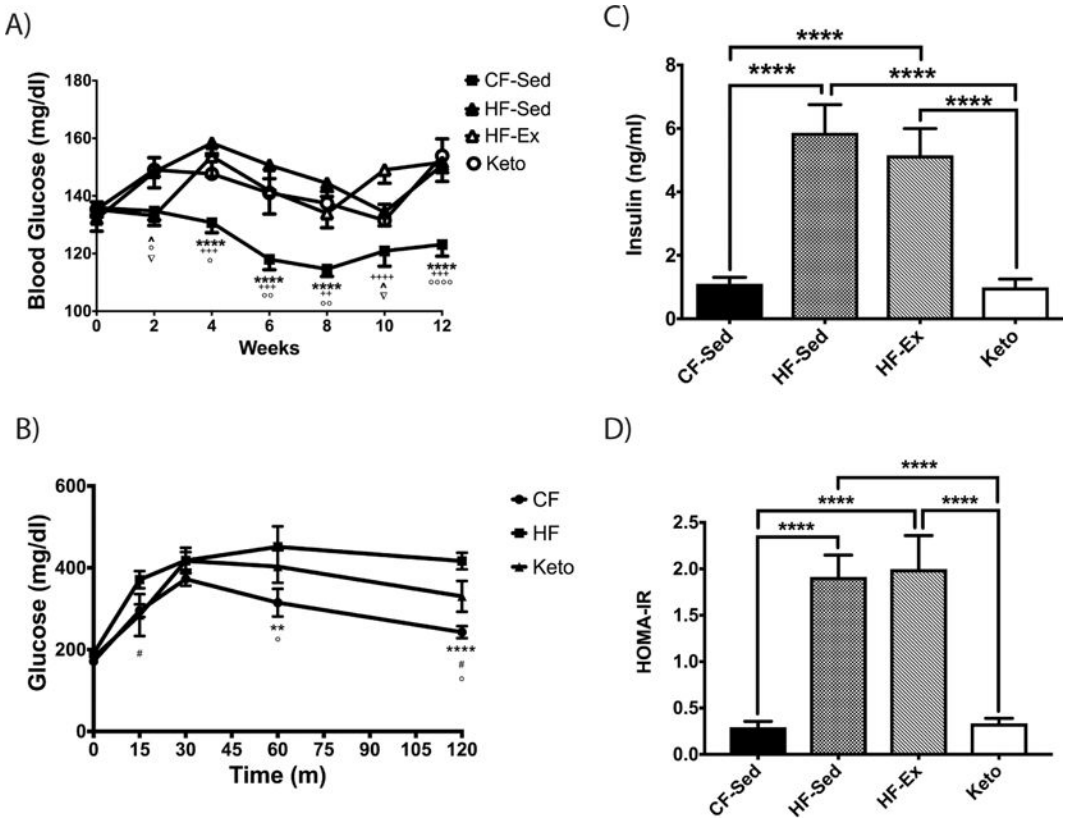


**Figure 3. A Ketogenic Diet Does Not Alter Glucose Tolerance and Fasting Insulin Similar to A High-fat Diet**

(a) Compared to control-diet fed mice, moderate increases in blood glucose levels were evident in mice fed a high-fat diet, a high-fat diet plus exercise and mice fed a ketogenic diet. Frank hyperglycemia never developed in any group (n=18 for all groups). (b) Following 12 weeks of dietary challenge, high-fat diet fed sedentary mice had a reduced IPGTT relative to control-diet fed mice, while ketogenic-diet fed mice displayed no significant changes in IPGTT compared to control diet-fed mice (n=5 for all groups). (c) After 12 weeks, a high-fat diet increased serum insulin levels compared to both ketogenic-diet fed and control-diet fed mice. (d) A high-fat diet, even with exercise increased HOMA-IR values compared to both control- and ketogenic-diet fed mice following 12 weeks of diet or exercise. All data presented as mean ± SEM *:CF-Sed vs. HF-Sed #: Keto vs. HF-Sed ^: HF-Sed vs. HF-Ex +: HF-Ex vs. CF-Sed °: Keto vs. CF-Sed $: Keto vs. HF-Ex * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

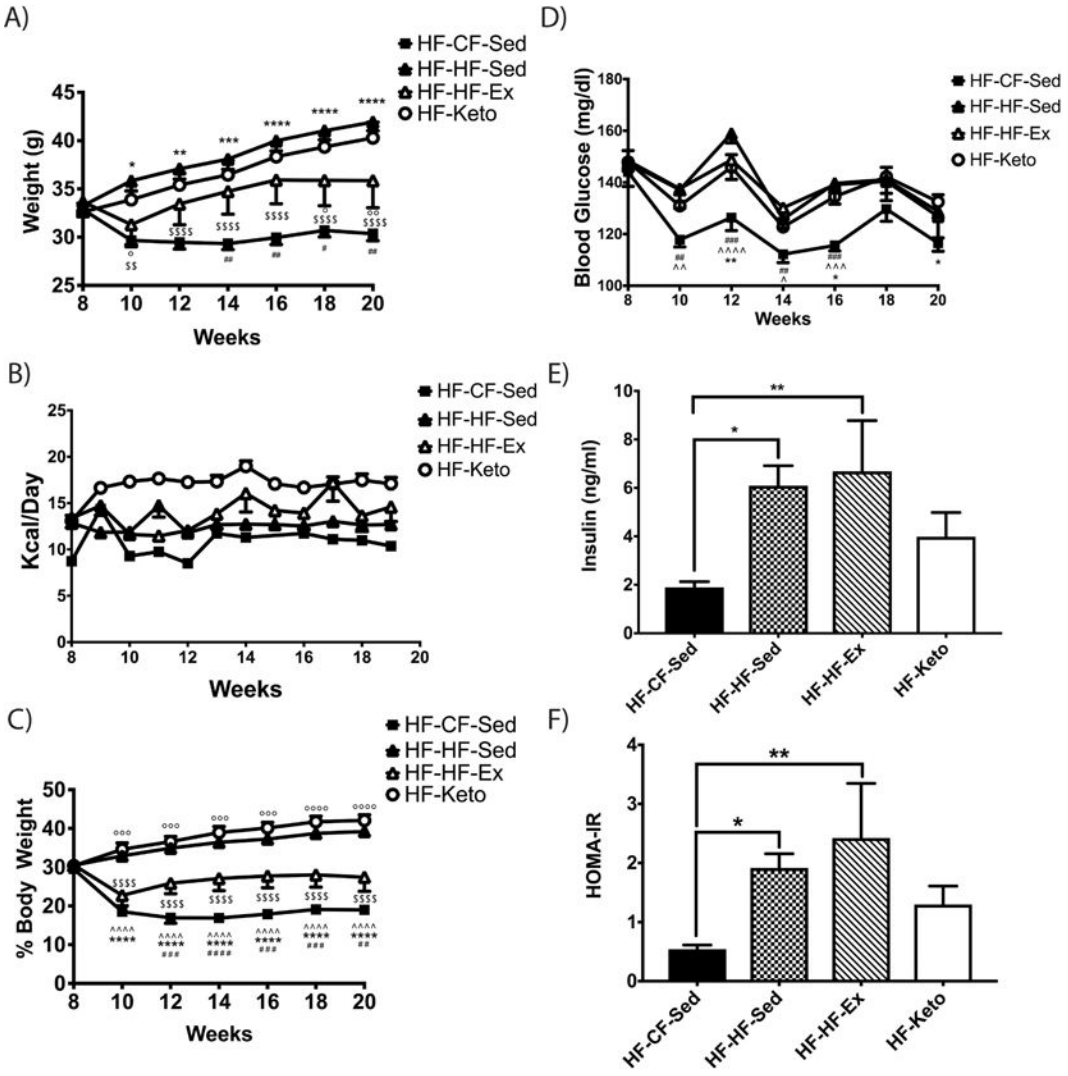


**Figure 4. A Ketogenic Diet Obesity Intervention Does Not Reverse Obesity Measures but Decreases Insulin**

(a) Following 8 weeks of a high-fat diet, mice remaining on a high-fat diet or those switched to a ketogenic diet continued to gain weight for the remainder of the study. Mice given access to a running wheel maintained their weight, while those switched to a control diet lost weight (b) Following 8 weeks of a high-fat diet, mice switched to a ketogenic diet still consumed the most energy per day (c) Mice given access to a voluntary running wheel or a control diet had decreased fat mass (n=10 for all groups) d) Following 8 weeks of a high-fat diet, only a control-fed sedentary intervention reduced blood glucose levels (n=10 for all groups) (e) Insulin levels were normalized by switching mice back to a control diet, whereas maintenance on a high fat diet (exercised or not) kept insulin levels elevated. Mice switched to a ketogenic diet fell in between levels in control-diet and high fat diet-fed mice. (f) Following 8 weeks of a high-fat diet, only an intervention using control-fed diet was able to normalize HOMA-IR. Ketogenic-diet fed mice again fell between mice fed a control-diet or a high fat diet. All data presented as mean ± SEM #: HF-Sed vs. Keto ^: HF-Sed vs. HF-Ex °: CF-Sed vs. Keto ∇: HF-Ex vs. Keto * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

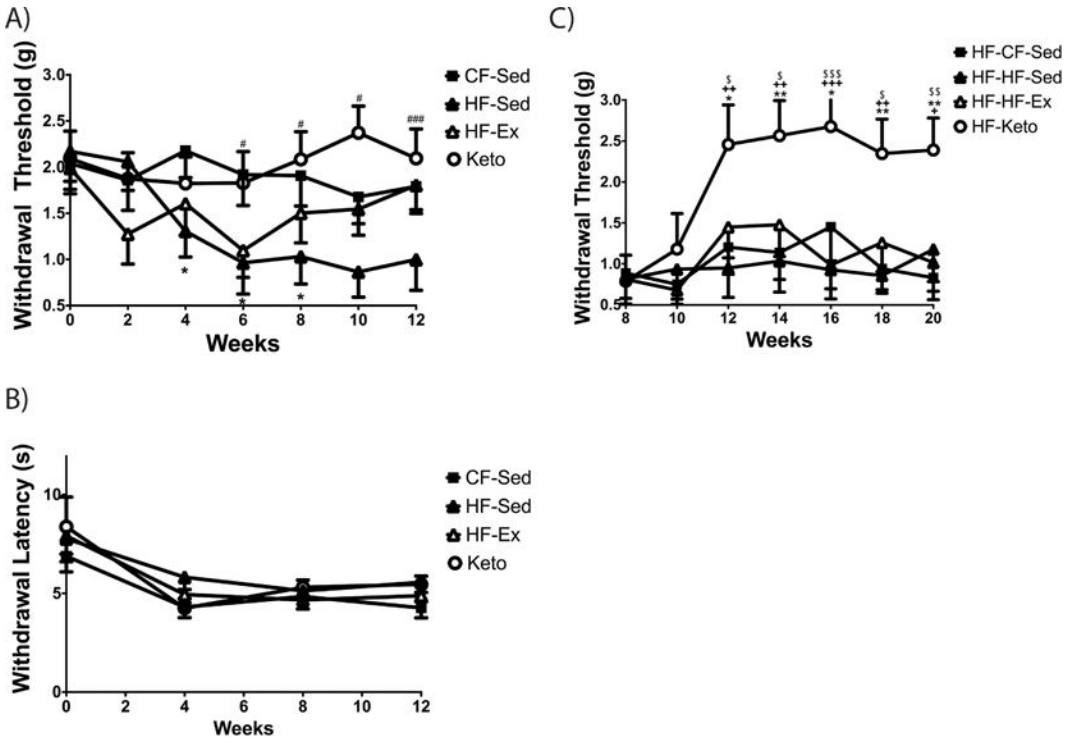


**Figure 5. A Ketogenic Diet Prevents and Reverses Diet-Induced Mechanical Allodynia**

(a) Mechanical paw withdrawal thresholds reveal that mechanical allodynia develops in mice challenged with a high-diet compared to mice fed a control diet. (b) Thermal sensitivity was unaffected by diet or exercise in any group. (c) As an intervention, mice fed a ketogenic diet has significant reversal of mechanical allodynia. All data presented as mean ± SEM *: CF-Sed vs. HF-Sed #: Keto vs. HF-Sed * $: Keto vs. HF-Ex * $p<0.05$; ** $p<0.01$; *** $p<0.001$

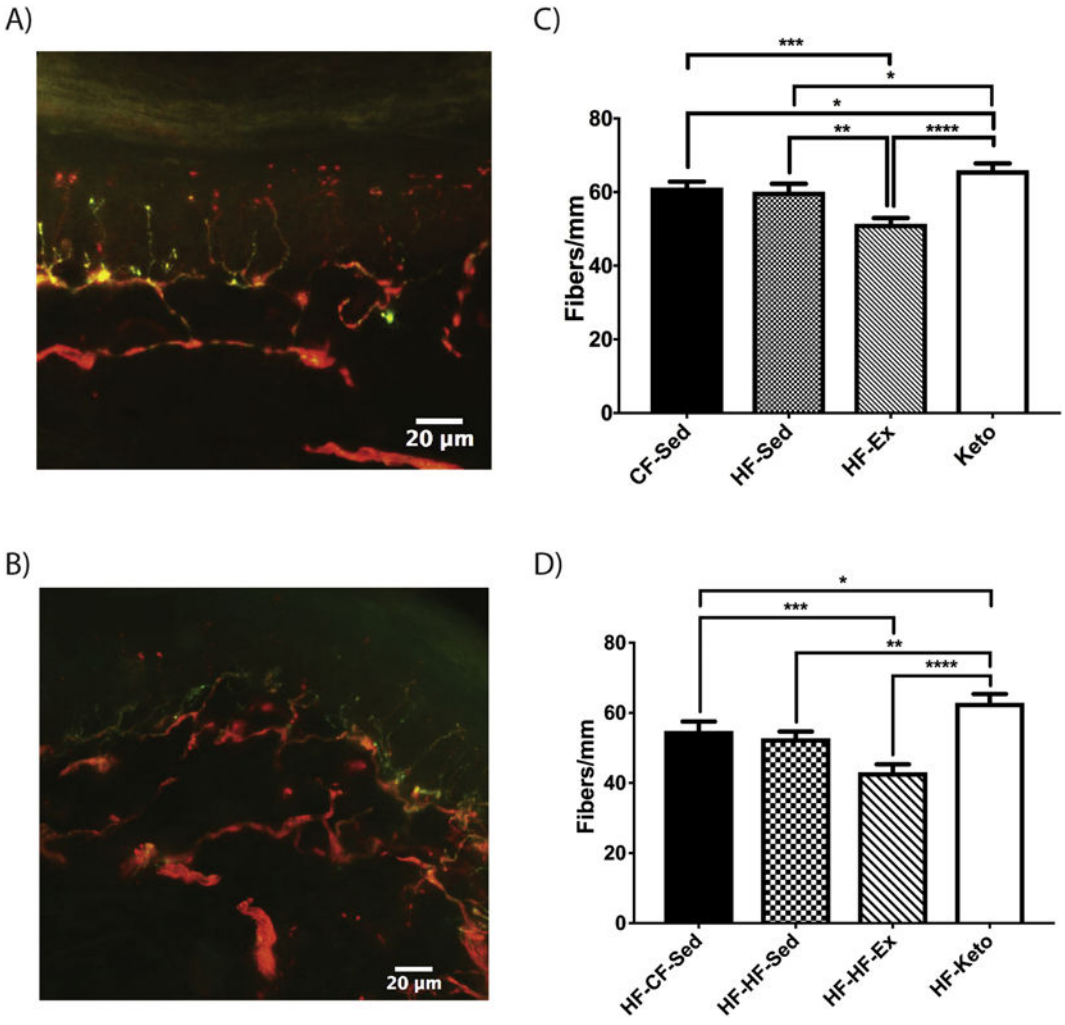


**Figure 6. IENFD Is Increased By A Ketogenic Diet**

(a) A ketogenic-fed mouse footpad stained with the panaxonal antibody protein gene product 9.5 (PGP) (red) and NGF receptor TrkA (green) following 12 weeks of diet in the challenge cohort (b) ketogenic-fed mouse PGP fibers 12 weeks following the intervention from a high-fat diet in the intervention cohort (c) In the challenge cohort, ketogenic-fed mice have increased epidermal nerve fiber density compared to all other groups. High-fat-fed exercised mice displayed decreased nerve fiber density compared to all groups (d) in the intervention cohort, ketogenic-fed mice also displayed increased epidermal nerve fiber density compared to all other groups. High-fat-exercised mice displayed decreased nerve fiber density compared to control-fed sedentary mice. All data presented as mean ± SEM * $p<0.05$; ** $p<0.01$; *** $p<0.001$, **** $p<0.0001$

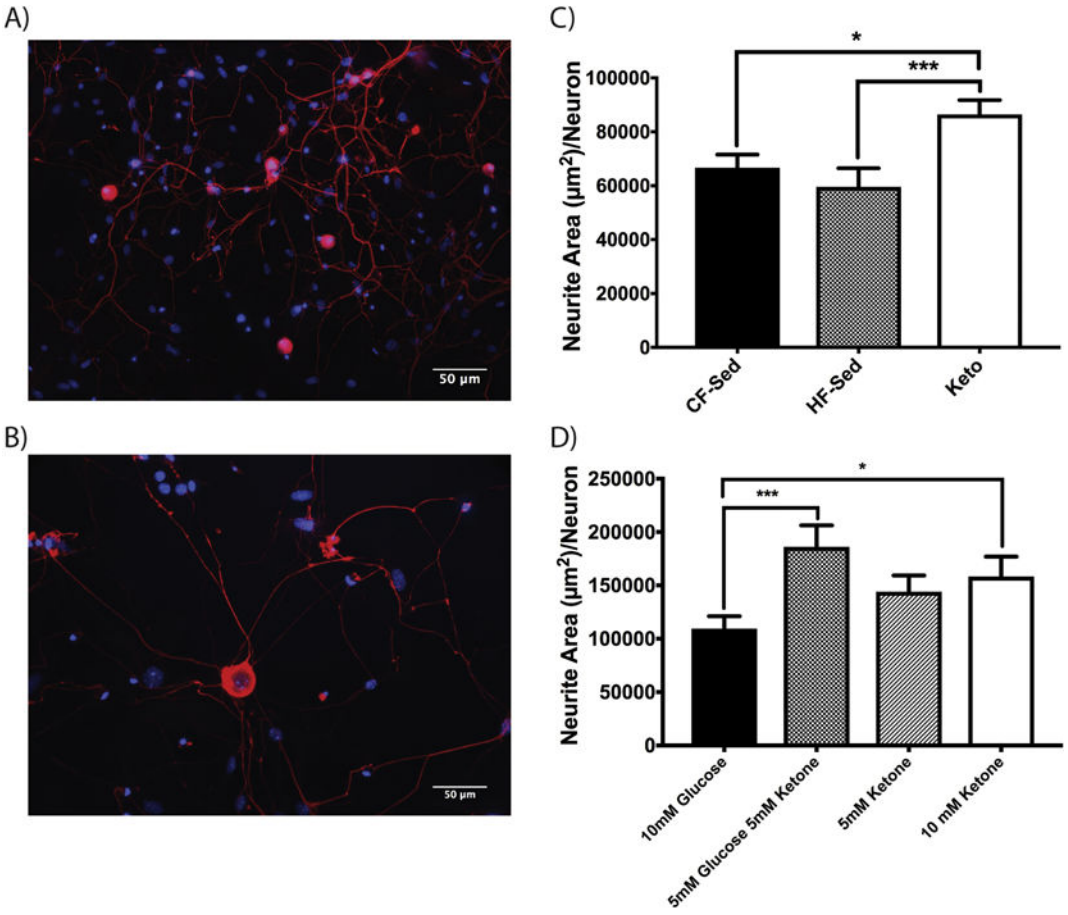


**Figure 7. Neurite Outgrowth Is Increased By Ketone Supplementation**

(a) Neurite outgrowth of primary DRG from ketogenic-fed mice following 4 days of culture in 10mM glucose Nutrient Hams F12 media (b) Neurite outgrowth of primary DRG from control-fed mice cultured in Nutrient Hams F12 media with 5mM glucose and 5mM ketone (c) Mice fed a ketogenic diet one month prior to culture display improved neurite outgrowth as compared to mice fed a control or high-fat diet when all placed in 10mM glucose F12 media (d) DRG cultured in F12 media supplemented with 5mM ketones and 5mM glucose or 10mM ketones display improved neurite outgrowth as compared to traditional 10mM glucose media. All data presented as mean ± SEM * $p<0.05$; *** $p<0.001$