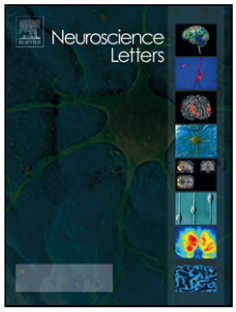

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Pinprick hypo- and hyperalgesia in diabetic rats: Can diet content affect experimental outcome?

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Highlights

- **Pinprick pain threshold was studied in a rat model of diabetes**
- **Diabetic animals developed either pinprick hypoalgesia or pinprick hyperalgesia**
- **The pain phenotype switch coincided with a change in diet content at a supplier animal facility**
- **Diet consumed from childhood may modify progression of disease into adulthood.**

Abstract

Existing literature concerning the effect of experimentally-induced diabetes on pain thresholds in rodent models remains controversial. In this work, we describe a phenotypical switch from streptozotocin-induced pinprick hypoalgesia to hyperalgesia observed in the same laboratory, in the same strain of rats, obtained from the same vendor, and measured by the same technique carried out by the investigators. This switch was observed around January 2015, at the time when there was a change in the diet of rats at the Radley North Carolina Charles River facility . These data support the contention that diet may significantly modify disease progression, including progression of signs of diabetic neuropathy.

Abbreviations: PDPN, painful diabetic polyneuropathy; PST, pinprick sensitivity threshold; STZ, streptozotocin

Key words: Streptozotocin-induced diabetes; diabetic neuropathy; evoked pricking pain; hyperalgesia; hypoalgesia.

Introduction

Painful diabetic polyneuropathy (PDPN) is a frequent complication of diabetes mellitus. Both evoked and spontaneous pain symptoms and their response to specific pain medications vary greatly among subjects with PDPN. Thus, an appropriate stratification of patients and a thorough understanding of underlying pathogenic mechanisms are needed [1;2]. A change in sensitivity to pinprick stimuli is one such elusive symptom/sign of PDPN. In humans, superficial pinprick-evoked pain sensations are primarily mediated by thinly myelinated Aδ-nociceptors, and serve a protective function by triggering a withdrawal reaction from potentially harmful stimuli [3;4]. In the majority of the patients with DPN and PDPN, pinprick sensitivity is either suppressed (pinprick hypoalgesia) or unchanged [5-7] . However, there is a substantial fraction of PDPN patients (up to 19%) that develop mechanical and punctate hyperalgesia (exaggerated pain on normally moderate nociceptive stimulus) [2;8]. The question as to why some PDPN subjects develop pinprick hyperalgesia, while others do not, remains elusive.

Examination of existing literature concerning streptozotocin (STZ)-induced diabetes in rodents also does not add much clarity to this question. In most of these animal studies, superficial sensitivity to punctate mechanical stimuli was assayed using the von Frey filament technique and measurements of paw withdrawal threshold or frequency. With rare exception [9;10], the vast majority of these experiments reported development of tactile hyperalgesia in diabetic animals (see references in [11]. The von Frey filament paw withdrawal test is prone to experimental bias, and results from this test are difficult to interpret as pure responses to pricking stimuli (as opposed to withdrawal responses to innocuous touching or tickling) [12]. However, our laboratory has previously provided evidence of mechanical hyperalgesia in STZ-treated rats

examined with modified, sharp tip von Frey filaments producing pinprick-like responses [13]. Observation of different results from different laboratories are most commonly attributed to specifics in testing procedures, duration and severity of diabetes induced, animal strain or sub-strain used, or other unquantified laboratory-specific confounding factors (for a more complete discussion of this issue, see [11]. In this work, we report data suggesting that animal diet may also play a critical role in phenotype expression (pinprick threshold changes) in a STZ model of diabetes in rats.

Materials and Methods

This work is based on observations made during our studies of the effect of analgesic compounds on nociceptive pain thresholds conducted in rats during the period from 2013 to 2016 in our laboratory.

Animals

All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arkansas for Medical Sciences and performed in compliance with the guidelines of the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (8-10 week-old; 250-350g; Charles River laboratories, NC) were housed three per cage with *ad libitum* access to food (22/5 Rodent Diet; Teklad Diets, Madison, WI) and water in rooms provided by the Institutional Division of Laboratory Animal Resources. Rats were randomly assigned to control or diabetic groups (averaging 4-6 rats per group in each experiment). Diabetes was induced in animals fasted overnight by injection of STZ (Sigma Aldrich, St. Louis, MO). STZ was dissolved in 10mM

citrate buffer (pH 4.0) immediately before injection and administered intraperitoneally (i.p., 1 ml/kg) at a dose of 65 mg/kg. Control animals received citrate buffer injection only (1 ml/kg).

All STZ-treated animals developed diabetes, which was confirmed by measuring blood glucose concentrations obtained by tail-prick 6 to 7 days after STZ injection (colorimetric Contour Next blood glucose monitoring system; Bayer HealthCare, Mishawaka, IN, USA). Diabetes was defined according to guidelines established by Standards of Medical Care in Diabetes, as the state of casual/random blood plasma glucose concentration equal or exceeding 11.1 mM (*e.g.*, measured at any time of day without regard to time since last food intake) [14]. As in humans, this criterion is also routinely used in rats as an alternative to fasting glucose or 2 hr oral glucose tolerance test measurements to reliably distinguish between normal, prediabetic and overt diabetic states [10;11].

Behavioral testing

All experiments were conducted during 2 to 3 weeks following STZ-induction of diabetes and at equivalent times in respective control animals. Behavioral tests were carried out between 10 AM and 12 PM. Animals were allowed to acclimate to the room environment for one hr prior to the beginning of the tests. At least one training session prior to behavioral testing was conducted to familiarize rats with the test procedures.

Pinprick sensitivity thresholds (PST) were measured as described previously [15] using a sewing needle (150 µm at the tip with an angle of 30 degrees) attached to a hand-held force transducer (Fort 1000, World Precision Instruments, Inc., USA). Force transducer-generated signals (pinprick force waveforms) were recorded using a differential amplifier ISO-80 (World Precision Instruments, Sarasota, FL) digitized at 40 kHz and analyzed using a micro 1401 analog-to-digital

converter and signal-4 software (Cambridge Electronic Design, Cambridge, UK). Rats were placed in an elevated testing cage with a wire mesh bottom and allowed to acclimate for 15 min. During the test session, each trial consisted of six pinprick stimuli that were administered at 2 to 6 sec intervals to the central area on the ventral aspect of each hind paw. During the trial, care was taken to avoid applying the probe to a previously tested surface of the paw. The threshold force was defined as peak force that produced paw withdrawal, or a 50 g cut-off force value for trials in which animals failed to demonstrate a withdrawal response. Threshold readings (total of 12/rat/trial) were filtered for outliers using the mean ± SD rule, averaged and expressed as mass units (g) before further analysis.

Statistical analysis

Statistical analysis was conducted using Prizm 5 (GraphPad Software, Inc. La Jolla, CA) and Origin 9.0 (OriginLab, Northampton, MA) software. Non-parametric analyses were used when data in any comparison group failed a Shapiro-Wilk normality test. In all other cases, parametric statistical tests were used, as appropriate. Effects were considered as statistically significant at $p < 0.05$. Data in figures are expressed as mean ± SEM (standard error of the mean).

Results

Figure 1A shows PST values measured in control and diabetic rats 8 days following STZ-injection. In control animals, the mean PST value was 10.2 ± 0.4 g (n = 38). However, in diabetic animals, PST threshold changes could be divided into two distinct groups. In the first group of diabetic rats (DB1 group) that were purchased and studied before 2015, significant pin-prick hypoalgesia (mean PST = 36 ± 2.0 g; DB1; n = 58) was observed. In marked contrast, in studies

that began in January, 2015, diabetic animals (DB2 group) that were purchased from the same vendor, and studied under the same protocol as the DB1 rats, demonstrated stable pin-prick hyperalgesia (DB2, mean PST = 5.6 ± 0.3 g; n = 18). PST values were normally distributed around respective means in both normal and DB2 groups, but not in the DB1 group. Between-all groups, PST differences were statistically significant (by the Kruskal-Wallis test followed by Dunn's multiple comparison test: $p < 0.01$ for DB1 vs normal or vs DB2 groups and $p < 0.05$ for normal vs DB2 rats).

In **Figure 1B**, PST was measured in subsets of normal, DB1 and DB2 rats (n=13, 10 and 6 rats, respectively), studied repeatedly during the 2nd to 3rd week following STZ or vehicle injections. In all three groups of animals, PST values were stable over time, showing no effects of repetitive testing (RM Friedman test, followed by Dunn's multiple comparison test; $p > 0.05$). These data also demonstrate that once either pinprick hypoalgesia (DB1) or pinprick hyperalgesia (DB2) has developed in diabetic rats, it is maintained at the same level for at least 1 week.

Comparative characteristics of hypoalgesic (DB1) and hyperalgesic (DB2) diabetic rats are listed in **Table 1**. Baseline PST measures, weight, and blood glucose levels measured at the end of the first week after STZ injection, were not different in rats that developed pinprick hyperalgesia or pinprick hypoalgesia. However, the ratio of blood glucose to body weight (Diabetes Severity Index; **Table 1**) was significantly lower ($p < 0.05$ by Mann Whitney test) in hyperalgesic animals when compared to hypoalgesic animals. These observations suggest that the severity of diabetes may also influence the pain phenotype in this particular test.

Discussion

Pain symptoms and results of quantitative sensory testing vary greatly among subjects with PDPN [2;7;8]. Similarly, there is a great variation in existing literature with regard to changes in pain thresholds observed in experimentally induced diabetic animals (see [11]. The reasons for this variability remain obscure. In the present study, we describe a phenomenon of phenotypic switch in pain from hypoalgesia to hyperalgesia encountered while working with STZ-induced diabetic rats in our laboratory during the period 2013 - 2016. In these studies, diabetic rats studied before January, 2015 developed pinprick hypoalgesia, while those studied after January, 2015 developed pinprick hypoalgesia (Fig. 1). Remarkably, this phenotypic switch occurred despite uniformity in animal housing and experimental conditions, including diabetes induction and pain testing protocols/procedures. Furthermore, multiple experiments confirming this phenotypic switch were conducted during the 3-year period by several independent observers, thus significantly reducing or eliminating any potential bias.

Compared to hyperalgesic animals, rats that developed pinprick hypoalgesia appeared to have a more severe state of disease (**Table 1**). The observed phenotypic switch remained, without any possible explanation, until consultations with the vendor were initiated. During these discussions, it was revealed by the vendor that the experimental observations reported in this study occurred at the time when an adjustment in the rodent 5L79 diet took place by the vendor in January of 2015 at the Radley North Carolina Charles River facility.

Most diet adjustments reported by the vendor were minor (within 30% change in a particular constituent content; Table 2) and did not affect caloric content, or composition of proteins, fat or carbohydrates (1% and 8% increase and 2% decrease in the new formulation, respectively, compared to the old formulation). Several other diet changes, however, such as a decrease in chromium content and an increase in selenium and pyridoxine concentration in the newly

formulated diet, appears to be of potential significance. For example, pyridoxine overdose is a known cause of sensory neuropathy [16], while both chronic chromium insufficiency [17], or excess selenium [18] levels have been suggested as factors promoting the severity of the diabetes. With regard to other important diet modifications, increases in thiamine and cobalamin concentrations might be expected to contribute to an amelioration of the diabetic condition [19;20]. However, during our review of the literature, no studies directly addressing either negative or positive correlations between carotene or vitamin B5 intake and either diabetes or neuropathy were found. Finally, it is well documented that consumption of a high-fat diet is a risk factor for development of diabetes and also predeposes experimental animals to development of exaggerated sensitivity to mechanical and thermal stimuli in various chronic and acute pathological conditions [21-24]. In this respect, it should be noted that in the new diet reported here, monosaturated fatty acids were increased 36%, the greatest difference when compared to changes in other fat constituents (**Table 2**). However, unlike that previously shown for polysaturated fatty acids, there are no data reported to date that implicate involvement of monosaturated fatty acids in modulating pain responses [24]. It should also be reiterated that all animals used in this study were fed the same diet (22/5 Rodent Teklad Diet) beginning one week prior to induction of diabetes and maintained on this diet thereafter. Thus, if changes in disease phenotype observed here were indeed associated with alterations in diet produced by the North Carolina Charles River facility, this importantly suggests that diet consumed during development may lead to long-term, perhaps irreversible, disease-modifying effects in adulthood.

As previously discussed by others in great detail [25-27], there are many factors that may influence behavioral testing outcomes. Due to carefully controlled experiments in the present study, the observed phenotype switch cannot be attributed to differences in protocol design,

experimenter bias (including effect of experimenter's gender; [27]), experimental season, testing room temperature and humidity, testing time of the day or housing conditions. Although genetic drift occurring in the Charles River SD rat colony cannot be excluded, and would require extensive experimental validation, the most likely explanation for the phenotypic switch in pain modulation reported here is an alteration in animal diet content, as described.

Conclusions

We describe here a phenotypical switch from streptozotocin-induced pinprick hypoalgesia to hyperalgesia, observed in the same laboratory, in the same strain of rats obtained from the same supplier, and measured by the same technique and by the same investigators. This switch was observed at a time when a modification of animal diet initiated by the Radley North Carolina Charles River facility was carried out (January of 2015). Thus, these data support the hypothesis that diet consumed during development may critically modify the disease progression in adulthood, specifically the progression of diabetic neuropathy in a diabetic rat model. Although the work presented suggests that alteration in animal diet content contributes to the observed phenotypic switch in pain modulation, it should be noted that genetic drift occurring in the Charles River SD rat colony cannot be excluded as an additional contributing mechanism.

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Disclosures and Contribution

Declarations of interest: none. JSKY - conducted the study, analyzed the data, and drafted the manuscript; ND - participated in experiments and helped analyze the data; AWW - participated in experiments and helped analyze the data; PLP - helped with the data analysis and the manuscript writing and editing; PAC - contributed equally with MD to the study design, the data analysis, discussion and interpretation, and the writing of the manuscript; MD - is the principal investigator in this study who contributed to the study design, the data analysis, discussion and interpretation, and the drafting and preparation of the manuscript. All authors had read and approved the final version of the article.

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Figure Legends

Figure 1: Pinprick thresholds of diabetic (2^{nd}-3^{rd} week post-STZ administration) and age-matched normal control rats

A. Mean PST values measured in control and diabetic rats 8 days after STZ or vehicle injection. Data are presented as Whisker plot boxes (percentile values are labeled next to Whisker plot shown in panel A (left); median and mean values are represented by horizontal bars and dark squares within the box, respectively).

B. Mean PST values measured in the subsets of normal, DB1 and DB2 rats (filled circles, opened triangles and opened circles, respectively) studied repeatedly during 2nd – 3rd week of experimentation.

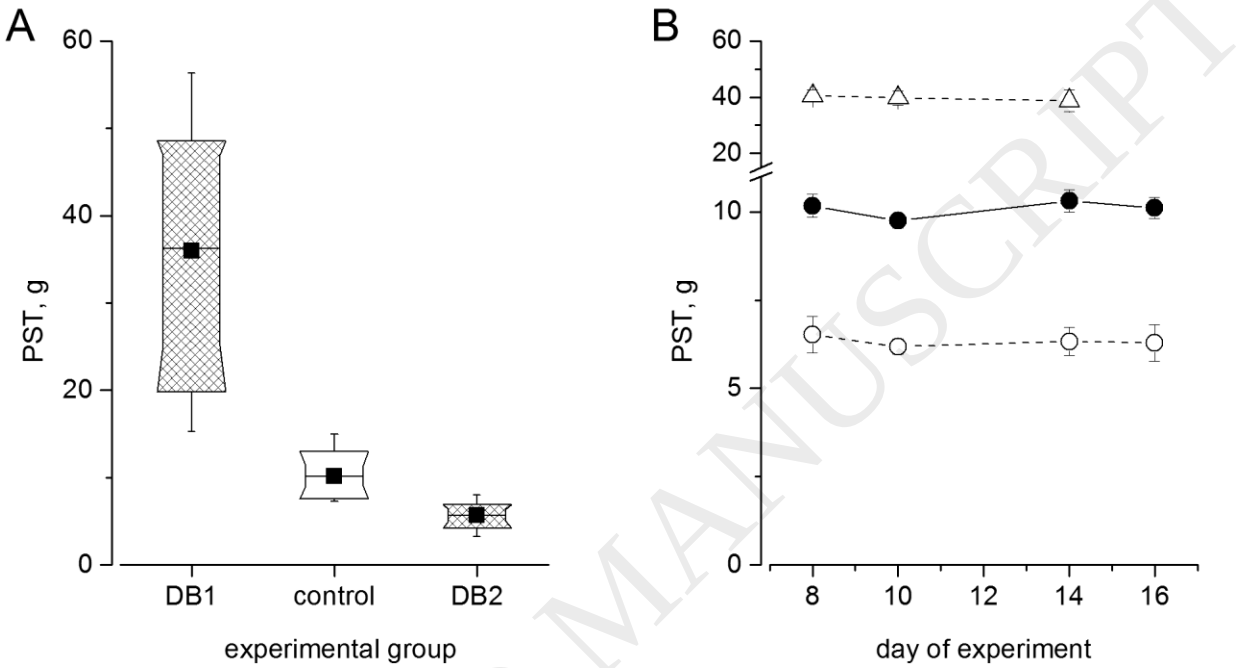

Table 1: Characteristics of DB1 and DB2 rat cohorts

Parameter	DB1 (n = 34)	DB2 (n = 18)	Two-tailed Mann Whitney test, p (U) values
PST 2nd week of DB, g	36.1 ± 2.3	5.6 ± 0.3	<0.001 (0)
PST before STZ, g	10.4 ± 0.8	10.6 ± 0.2	0.431 (249)
Random glucose (RG), mM	27.8 ± 1.3	32.4 ± 0.7	0.082 (215)
Body weight (BW), g	256 ± 7	254 ± 10	0.825 (294)
Diabetes Severity Index (BW/RG), g/mM	10.1 ± 0.6	8.1 ± 0.6	0.022 (186)

Table 2: Radley North Carolina Charles River facility, 5L79 rat and mouse diet changes*

Diet component	Before 01/21/2015	After 01/21/2015	Percent decrease/increase
Amino acids			
Methionine, %	0.48	0.38	-20.8%
Aspartic acid, %	1.67	2.03	+21.6%
Lipid and carbohydrate metabolism-related			
Linoleic acid, %	1.9	1.6	-16.8%
Monounsaturated Fatty acids, %	1.4	1.9	+35.7%
Minerals			
Chromium, ppm	1.4	0.01	-99.3%
Selenium, ppm	0.27	0.46	+70.4%
Vitamins			
Carotene, ppm	2.8	0.9	-67.9%
Pantothenic acid, B5, ppm	24	36	+50.0%
Thiamine, B1, ppm	92	500	+443.5%
Pyridoxine, B6, ppm	12	72	+500.0%
Cobalamin, B12, mcg/kg	19	130	+584.2%

**Only major category of the diet constituents subjected to the greatest changes (decrease, first line or increase, second line in concentration) are shown in the table. Also, four vitamins whose concentrations were increased in the new diet formulation by more than 50% are listed. For all remaining and unlisted dietary components, concentration percent change differences between old and new diet formulations were within ±30% limits.*