

## Microneurography in Diabetic Mice

### Final Report

The goal of this Pilot/Feasibility study was to adapt microneurography to studies of diabetic peripheral neuropathy in the mouse and measure spontaneous action potential discharge in nociceptive C-fibers of hyperglycemic NOD/Ltj and C57BL/6-*Ins2*<sup>AKita/+</sup>/J mice and normoglycemic (control, age- and sex-matched) NOD/Ltj and C57BL/6-*Ins2*<sup>+/+</sup>/J mice.

Microneurography is an electrophysiologic extracellular recording technique which was invented in Sweden in the 1970's and in subsequent decades was employed with great success in studies of sensory coding by primary afferent fibers in healthy human volunteers.<sup>13</sup> Microneurography has also been applied for studies in patients with peripheral neuropathic pain, including patients with diabetic neuropathy<sup>16, 18, 19</sup>. Recently, the microneurography technique was adapted for use in rats.<sup>12</sup>

We proposed that this minimally invasive technique offered a unique opportunity to bridge human and rodent studies of the peripheral neural mechanisms which are responsible for pain in diabetic neuropathy. Our goal was to refine the microneurography technique further to point where it could be used in mice. We reasoned that comparison of the patterns of C-fiber discharge measured with microneurography in diabetic humans who experience spontaneous burning pain to C-fiber activity measured in different strains of diabetic mice would permit the selection of the most representative mouse model.

We proposed to develop the microneurography technique for mice because widely-used conventional electrophysiological tests (nerve conduction studies) are incapable of providing information on the presence/absence of action potential discharge in nociceptive primary afferent C-fibers. Unlike microneurography, the conventional tests measure only the amplitude and latency of the compound action potential produced by the fastest conducting (A $\beta$ ) fibers (which supply low-threshold mechanoreceptors), not the much smaller and slower conducting C fibers (which supply nociceptors). Diabetes-related changes in the conduction velocity (and compound action potential amplitude) of A $\beta$  fibers, while indicative of nerve disease, do not explain the ongoing burning pain, cold and heat allodynia / hyperalgesia found in many patients with diabetic neuropathy.<sup>11, 17, 18</sup> To explain the burning pain, allodynia and hyperalgesia which plague patients with diabetic neuropathy,<sup>11, 17, 18</sup> one needs to measure action potential discharge in nociceptive C fibers.

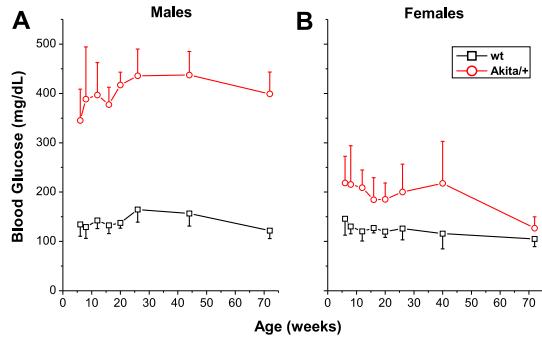
The microneurography technique is particularly suited for recording from C fibers. Microneurographic studies in patients with painful diabetic neuropathy<sup>18, 19</sup> reported the presence of abnormal spontaneous action potential discharge in C-fiber nociceptors. In patients with heat allodynia/hyperalgesia the spontaneous discharge increased in a skin temperature-dependent manner. In addition, a heat-dependent switch from a single- to a double-spike pattern of action potential discharge was observed suggesting that the heat hyperalgesia is due to an increase in the frequency of firing in C-fiber nociceptors.<sup>18</sup> While cold allodynia (burning pain evoked by gentle cooling of the skin) is among the positive neurological signs found in patients with diabetes and animals with experimental diabetes,<sup>1, 7, 9, 14, 20</sup> few people are aware of this paradoxical feature of diabetic neuropathy and the neural mechanisms remain to be elucidated. Our recent microneurographic study in humans suggest that a novel physiological subtype of C fiber may be a major player in cold allodynia.<sup>3</sup>

The proposed AMCC Pilot and Feasibility Study was to adapt the microneurography technique for recordings from C fiber afferents in mice and determine if spontaneous action potential discharge measured by microneurography in C-fiber nociceptors could be used as a surrogate measure for burning pain in mice.

## RESULTS

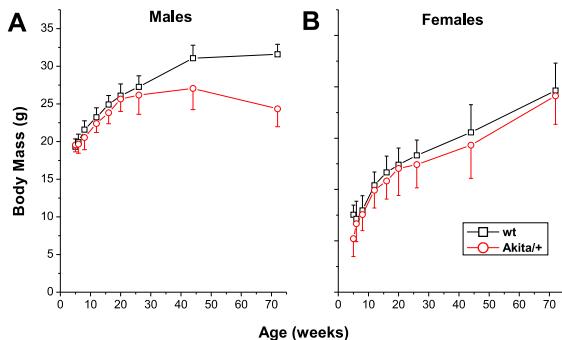
**R.1. Husbandry.** We purchased C57BL/6-*Ins2*<sup>Akita</sup>/J mouse breeders from the Jackson Laboratory and established a colony at the Oregon Health and Science University, Department of Comparative Medicine, animal facility. The colony was propagated by mating C57BL/6-*Ins2*<sup>Akita/+</sup>/J males (heterozygous for the C96Y mutation in insulin2 gene) to wild-type (C57BL/6-*Ins2*<sup>+/+</sup>/J) females.

In preliminary experiments we confirmed literature reports that C57BL/6-*Ins2*<sup>Akita/+</sup>/J mice develop hyperglycemia at an early age and that hyperglycemia is more pronounced in C57BL/6-*Ins2*<sup>Akita/+</sup>/J males than females (Fig. 1).



**Fig. 1.** Glucose levels in the blood of C57BL/6-*Ins2*<sup>Akita/+</sup>/J and wild-type mice. Blood samples were withdrawn from the saphenous vein and analyzed with a Freestyle Freedom Blood Glucose Monitoring System (Abbott Diabetes Care). Longitudinal mean  $\pm$  s.d. measures, N=7-8 animals / genotype and sex, red  $\circ$  symbols: C57BL/6-*Ins2*<sup>Akita/+</sup>/J mice; black  $\square$  symbols: wild-type (C57BL/6-*Ins2*<sup>+/+</sup>/J) mice.

We found that the diabetic male C57BL/6-*Ins2*<sup>Akita/+</sup>/J mice gain body mass (weight) more slowly than their non-diabetic (wild-type) male littermates and begin to lose body mass at approximately the age of 1 yr (Fig. 2A). Female C57BL/6-*Ins2*<sup>Akita/+</sup>/J mice gain (and retain) body mass at a rate very similar to their wild-type female littermates (Fig. 2B).



**Fig. 2.** Body mass of C57BL/6-*Ins2*<sup>Akita/+</sup>/J and wild-type (C57BL/6-*Ins2*<sup>+/+</sup>/J) mice as a function of age and sex. Longitudinal mean  $\pm$  s.d. measures, N= 8 males / genotype and 7 females / genotype. Data after wk 40 is based on fewer animals (6 males / genotype and 3 females / genotype, respectively). Red  $\circ$  symbols: C57BL/6-*Ins2*<sup>Akita/+</sup>/J mice; black  $\square$  symbols: wild-type (C57BL/6-*Ins2*<sup>+/+</sup>/J) mice.

All these observations are in agreement with previous husbandry reports for the *Ins2*<sup>Akita/+</sup> mouse.<sup>15</sup>

Unfortunately, because the review process for 2009 AMDCC Pilot/Feasibility proposals was delayed by the review of ARRA proposals, the funding for our Pilot/Feasibility proposal was not approved in time to allow us to maintain and utilize the first colony of Akita mice. We had to start anew.

The second colony of Akita mice, established with a new set of C57BL/6-*Ins2*<sup>Akita</sup>/J mouse breeders from the Jackson Laboratory, showed blood glucose and body mass dependence on genotype and sex that was remarkably similar to the results obtained for the first colony (not illustrated).

## R.2. Behavioral testing.

We performed longitudinal experiments involving behavioral testing of 6 litters in order to determine if diabetic *Ins2*<sup>Akita/+</sup> mice develop signs of sensory neuropathy. Non-diabetic (*Ins2*<sup>+/+</sup>) siblings of the respective sex served as controls. Measurements of sensitivity of the hind paws to mechanical, radiant heat, and evaporative cooling stimuli were taken at 4-wk intervals, starting at the age of 4-6 wk all the way up to the age of 26 wk.

*Mechanical sensitivity* was determined by measuring the the hind paw withdrawal threshold for stimulation of the plantar surface with nylon monofilaments (von Frey hairs). The up and down procedure for assessment of mechanical allodynia was used to determine the filament strength that was needed to evoke a response in 50% of the trials.<sup>5</sup> This strength is indicated in Fig. 3 as the “50% threshold.” We found no indication for the presence of mechanical allodynia in the diabetic (*Ins2*<sup>Akita/+</sup>) mice. On the contrary, at the age of 26 wk the male *Ins2*<sup>Akita/+</sup> and most of the female *Ins2*<sup>Akita/+</sup> siblings showed increased 50% thresholds (reduced sensitivity) to punctate mechanical stimulation of the hind paw (Fig. 3). The finding of reduced punctate mechanosensitivity is in line with a previous report on diabetic Akita mice.<sup>21</sup>

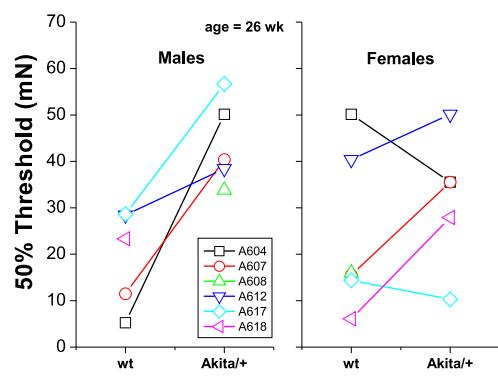


Fig. 3. Hind paw sensitivity to punctate mechanical stimuli is reduced in diabetic (Akita  $+/+$ ) mice. Males and females are compared separately (left and right panels). Different colors signify different litters. Symbols represent the average value of the 50% threshold for all the males (or females) of the same genotype in that litter. Note that diabetic males in all litters (and diabetic females in most litters) had higher 50% response thresholds compared with their wild-type littermates.

*Heat sensitivity* was assessed by measuring the latency of paw withdrawal from a radiant stimulus<sup>8, 10</sup> applied with a commercially available instrument (ITCC, Model 33T). Measurements at the ages of 4, 8, 12, 16, 20, and 26 weeks provided no evidence for heat hyper- or hypoalgesia in either the male or the female diabetic (*Ins2*<sup>Akita/+</sup>) siblings (Fig. 4). The lack of any change in sensitivity to noxious heat stimuli is in agreement with the literature.<sup>21</sup>

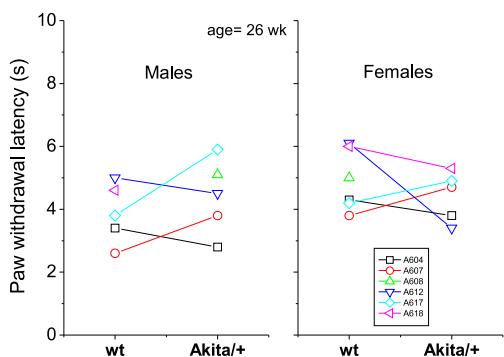
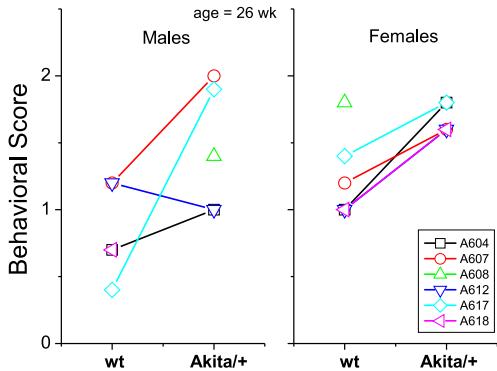


Fig. 4. Hind paw sensitivity to radiant heat stimuli. The same animals / litters as in Fig. 3 (indicated by the same color coding). Note lack of consistent difference in withdrawal latencies between wild-type and diabetic siblings.

*Cold allodynia* was measured using a behavioral assay that employed gentle cooling stimulus which was produced by the evaporation of a 50  $\mu$ l drop of acetone placed on the plantar surface of the hind paw.<sup>6</sup> Each mouse was exposed to the cooling stimulus 5 times (with a minimum of 15 min between stimulus repetitions) and its nocifensive behavior scored on an integer scale ranging from 0 to 2 (0 = a rapid transient lifting, licking, or shaking of the hindpaw, which subsides immediately; 1 = lifting, licking, and/or shaking of the hind paw, which continues beyond the initial application, but subsides within 5 s; 2 = protracted, repeated lifting, licking, and/or shaking of the hindpaw). The 5 individual scores obtained for a given mouse during a particular test session were averaged to obtain the mean score for that animal and session.



**Fig. 5. Hind paw sensitivity to gentle cooling.** Same animals as in Figures 3 and 4 (indicated by the same color coding). Note the prevalence of higher behavioral scores for Akita/+ males and females (compared with their wild-type littermates) signaling the presence of cold allodynia in diabetic mice.

Fig. 5 demonstrates the novel finding that by the time the diabetic *Ins2*<sup>Akita/+</sup> mice are 26 wk old they show behavioral signs of cold allodynia. This finding is of considerable interest because diabetes in humans causes burning pain and cold allodynia in humans is perceived as pain of burning quality.

**R.3. Microelectrode recording of sensory nerve fiber activity.** We began to explore the feasibility of performing microneurography in mice. Mice were anesthetized with isoflurane, placed on a custom-made stage inside a Faraday cage and the sciatic nerve exposed at the level of the sciatic notch. All surgery was done under observation through a dissection microscope. The animal was grounded using a silver/silver chloride pellet electrode. Nerve activity was recorded as the voltage difference between the tip of a low impedance microelectrode (indifferent lead), inserted in connective tissue, and the tip of a tungsten microelectrode (active lead) inserted into the nerve under observation through a dissection microscope and slowly advanced with the help of a remotely controlled micromanipulator. Both leads were connected to the preamplifier (10x) headstage of a differential amplifier (EX-1, Dagan Corp.). The nerve signal was post-amplified (2,000x for a total gain of 20,000x) and filtered by the differential amplifier (typical bandpass 0.3-2 kHz). Power line-related noise in the recording was eliminated in real time by subtracting noise replica using HumBug (Quest Scientific). The conditioned signal was lead to a computer interface (PCI-6036E Multifunction board, National Instruments or Digidata 1340A, Axon Instruments) and digitized by a personal computer running Qtrac, a program written by Prof. H. Bostock (© Institute of Neurology, University College, London).<sup>2,4</sup>

In preliminary experiments, we were able to record (Fig. 6) individual (all-or-none) action potentials fired by fibers in the 3 conduction velocity groups (A $\beta$ , A $\delta$ , and C), but long-term stability of the recording was not adequate for running the fiber subtype identification protocol.

In subsequent electrophysiological experiments, we spent a great deal of effort until we finally found a satisfactory way to mechanically stabilize the anesthetized mouse and its leg for a longer term nerve recording.

Next we sought to optimize the signal-to-noise (S/N) ratio of the microneurography recording. We attempted recordings using commercially available tungsten microelectrodes (Frederick Haer Co.) We

examined 3 different values of nominal tip impedance ( $\sim 100$ ,  $\sim 300$ , and  $\sim 1000$  k $\Omega$ ), but neither delivered S/N ratio sufficient for recording of spontaneous action potential discharge in single C fibers of the mouse (the main goal of this Pilot/Feasibility study).

Because of the technical difficulties in the mouse, we decided to establish in our laboratory microneurography recordings in the rat. Using the same commercially available microelectrodes, we were able to record from both Type 1A (polymodal nociceptor) and Type 1B (mechano-insensitive nociceptor) C fiber afferents in the rat (Fig. 7, next page).

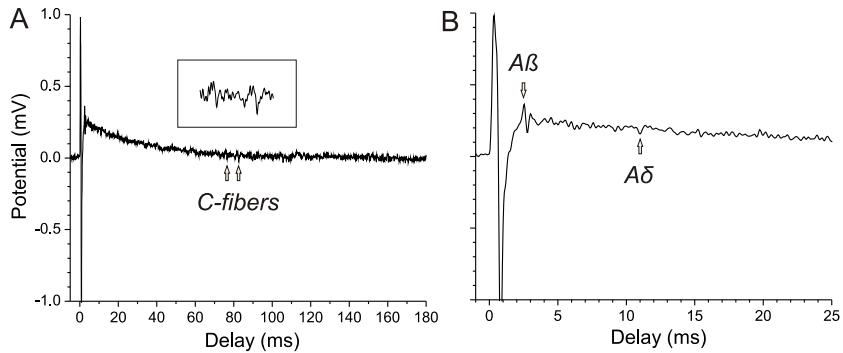


Fig. 6. Nerve microneurography in the mouse. **A:** Two C-fiber action potentials (peak-to-peak amplitude 40  $\mu$ V) near the 80 ms delay ( $\uparrow\uparrow$ ) are shown enlarged in the inlay; **B:** A $\beta$ - and A $\delta$ -fiber action potentials from the trace in panel A shown on an expanded time scale. Cutaneous electrical stimuli had the form of constant current pulses, 300  $\mu$ A amplitude, 0.5 ms duration and produced the stimulus artifact near time zero. Conduction distance to the recording site = 36 mm.

**Conclusion:** We have established in our laboratory rat microneurography, which could be used to study the peripheral neural substrate of diabetic sensory neuropathy in the rat. Our laboratory is currently the only one in the New World capable of performing microneurography in the rat. Additional time and effort, which will be needed to test intermediate values of microelectrode impedance (and possibly also varying the shape of the microelectrode tip using customized microelectrodes), will be required in order to obtain adequate signal resolution for microneurography in the mouse.

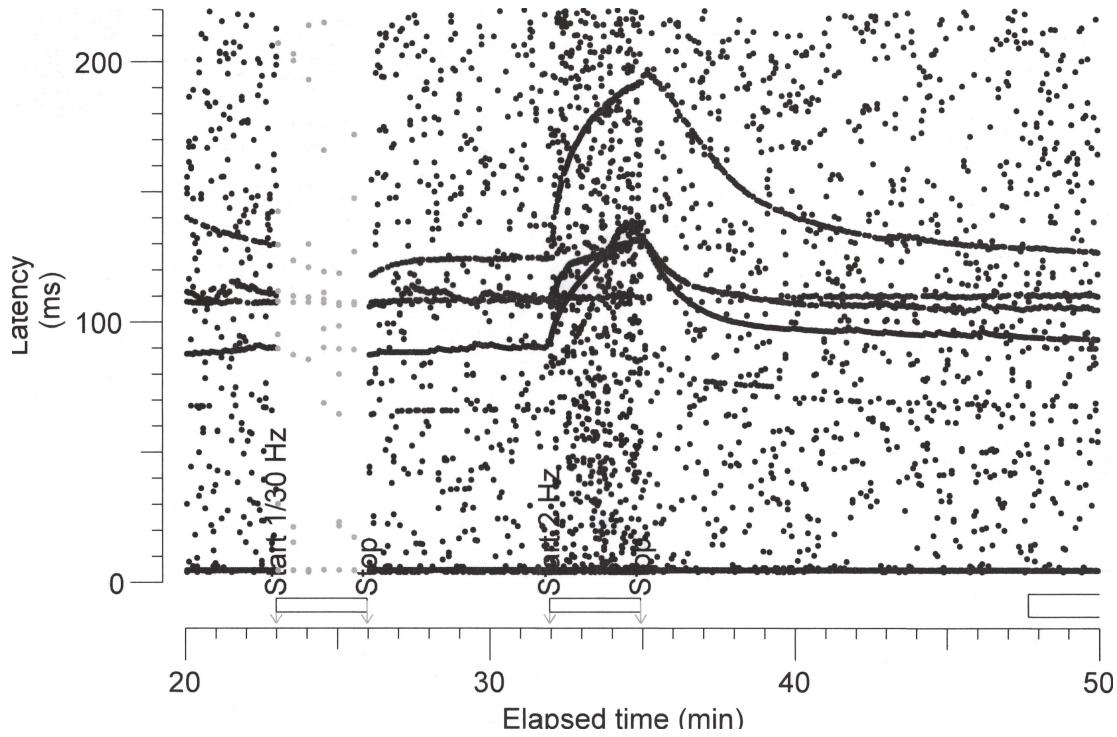


Fig. 7. Raster display of latencies for all peaks above an arbitrary level detected in a microneurography recording from the sciatic nerve of the rat. The recording at this site contained at least 4 identifiable C fibers (note 4 series of dots forming near horizontal lines at different conduction latencies, relative to the horizontal line formed by the stimulus artifact near 0 latency). For stimulation at 0.25 Hz (standard) throughout the experiment, the fastest conducting C fiber had an action potential conduction latency of ~90 ms and the slowest had a latency close to 125 ms. Due to activity-dependent slowing of conduction, the latencies for all the C fibers increased during the 3-min period of stimulation at 2 Hz (which occurred between minutes 32 and 35 of elapsed time in the experiment). Note that the slowest C fiber showed gradual slowing also (while the other units did not) when the 0.25 Hz stimulation resumed following a 3-min period of stimulation at the lower frequency of 1/30 Hz (the “pause”, which occurred between minutes 23 and 26 in the experiment). The slowing following the “pause” in stimulation is characteristic of Type 1B (mechano-insensitive) C-fiber nociceptors, while the lack of such slowing is characteristic of Type 1 A (mechanosensitive, polymodal) C-fiber nociceptors (cf. George et al., *J. Physiol.* 578: 213-32, 2007). One of the Type 1A nociceptors discharged action potentials spontaneously, as indicated by the rapid fluctuation in conduction latency (caused by activity-dependent slowing). The different behavior of the units is readily resolved from each other and distinguished from background noise.

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