Progress/Final Report Template

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Title of Project: Do metallothioneins promote and direct regenerative growth of epidermal nerve fibres in diabetic peripheral neuropathy?

Summary: *(approximately 1,000 words)*

The objective of this project was to develop a rat model of diabetic neuropathy, and to then administer metallothionein (MTII) or its synthetic analogue, emtinB, to ask whether these agents could restore either anatomical or functional innervation to epidermal nervous system. Thus, a major part of this work was in development and evaluation of a clinically relevant model of neuropathy. Diabetes was established by supplementing rats with a high fat diet, and administering dual injections of STZ to specifically ablate insulin producing β pancreatic cells. We confirmed that diabetic neuropathy with characteristic mechanical allodynia becomes established in these rats, and are in the process of establishing the progression of neuropathy over the timecourse of disease.

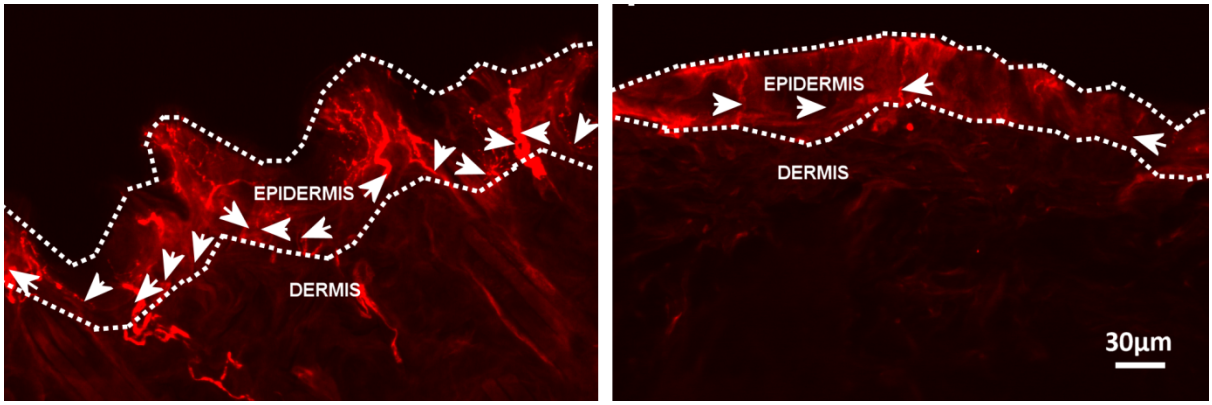

Figure 1. Representative punch biopsies of hindlimb skin taken above the gastrocnemius in rats prior to treatment.
*Hindlimb biopsy from **(A)** control rat, and **(B)** diabetic rat (6 weeks after confirmation of diabetes). Hindlimb skin is highly elastic and retracts after biopsy increasing the apparent ENF density. Confocal microscope images show epidermal nerve fibres (denoted by arrows) stained by anti-βIII-tubulin (red). Qualitative analysis suggests that diabetic rats showed a decrease in skin ENF density indicating established neuropathy (quantitative analysis not yet complete). Thinning of the epidermis is also noted in diabetic skin.*

The course of diabetes was monitored with weekly blood glucose tests. Nerve function was characterised by testing nociception of the hindlimb plantar surface with von Frey monofilaments which allowed for the development of neuropathy to be detected. Examination of punch biopsies (Fig 1) from the hindlimbs demonstrated that the model of neuropathy clearly exhibited the typical loss of epidermal nerve fibres and thinning of the epidermis. Metallothionein (0.3mg/kg, 3 times weekly) and emtinB (1mg/kg, 3 times weekly) was therefore commenced on day 1 of week 10, and continued for 4 weeks.

Loss of epidermal nerve fibres is associated with the development of mechanical allodynia, but is not indicative of the severity of disease. As such, von Frey monofilament testing of mechanical sensitivity was performed to evaluate the development and extent of mechanical allodynia. Typical nociceptive-like withdrawal of the hindlimb occurs when a pressure of 26 grams is applied with von Frey monofilaments (Fig 2). A drop in pressure required to elicit a withdrawal response indicates an increase in mechanical sensitivity and thus development in mechanical allodynia. Characteristic development of mechanical allodynia was observed in diabetic rats, visualised by deviation of mechanical threshold in response to von Frey monofilaments compared to the control group by week 6 (Fig 2). An unforeseen difficulty in functional evaluation arose from the finding that control rats developed tolerance to the repeated stimuli from von Frey monofilaments around 6 weeks, and because a similar effect was observed in the diabetic groups (albeit delayed), it was difficult to ascertain the onset of mechanical allodynia, which would have ordinarily marked the onset of the treatment intervention. Mechanical tolerance occurs when a stimulus is repeated, such that a reduction of the reaction or response occurs over a period of time (ie. a greater mass of monofilament was required to elicit a paw withdrawal response over time). In control rats this phenomenon was observed around 6 weeks. In diabetic rats, tolerance was observed to progress much more gradually, and may have been offset by the development of mechanical allodynia (Fig 3). Due to the development of tolerance to von Frey monofilaments over time, data from rats commencing treatment after week 14 (as a result of delayed onset of diabetes) was omitted from these graphs. Furthermore, the remaining numbers in this dataset were too small to subject to statistical analysis appropriately. Thus, we conclude that the use of Von Frey testing is not suited to longitudinal studies in rats. The tolerance effect made interpretation of the mechanical threshold difficult and likely masked any therapeutic effects one may have observed in response to MTII or EmtinB. Future experiments should not use von Frey testing at such regular intervals, and treatments should be administered earlier to overcome the likelihood of observing tolerance. As a result of the issues surrounding the development of tolerance, and delayed onset of diabetes in some animals, only a few animals from each cohort who had treatment commenced at precisely the same time could be directly compared (fig 3). However, these numbers were too small to perform statistical analyses.

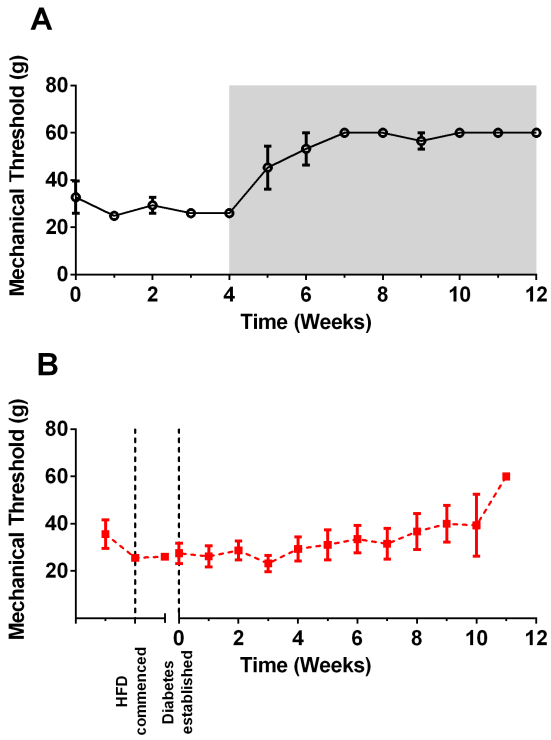

Figure 2. Characterisation of functional changes in sensation in diabetic neuropathy in non-diabetic and STZ/HFD diabetic rats
Average mass of von Frey monofilament required to elicit a paw withdrawal response in rat hindpaws prior to treatment interventions (saline, EmtinB or MTII). ***(A)*** *Control non-diabetic rats began to develop tolerance to the repeated stimuli after 4 weeks of testing. Grey shaded area indicates tolerance development.* ***(B)*** *Pooled diabetic rat data responses to von Frey fibres from all diabetic groups prior to introduction treatment. Data normalised for varied onset of diabetes. The development of tolerance in diabetic rats develops gradually.*

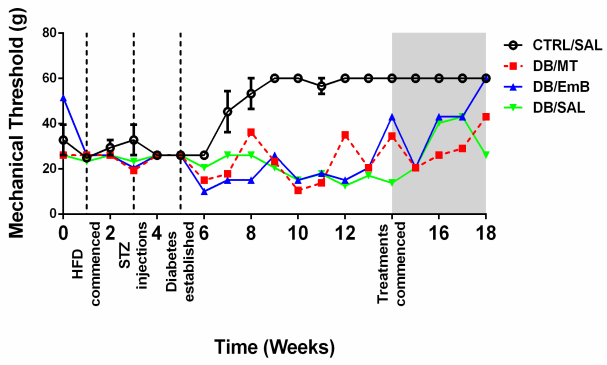

Figure 5.3 Mechanical thresholds before and after treatment with MTII, *EmtinB and saline*
Average mass of von Frey monofilament required to elicit a paw withdrawal response in control rat hindpaws in control rats and diabetic rats. Grey shaded area indicates treatment period. Divergence in mechanical sensitivities between control and diabetic groups begins from week 6. (MT, metallothionein; EmB, emtin B; SAL, saline; CTRL, non-diabetic rats; DB, diabetic rats).

Table 5.1 Average blood glucose prior to, and at end of, 4 week treatment period.

	CTRL/SAL	DB/SAL	DB/MTII	DB/EmB
Average blood glucose at week 14, prior to treatment with MT, saline or EmtinB (mmol/L)	8.4	>21*	>27*	>26*
Average blood glucose at week 18, at end of	7.8	>26*	>33*	>29*

treatment with MT, saline or EmtinB (mmol/L)				

**Glucose measurement device has a threshold of 33.3, and as such SEM cannot be calculated.* (MT, metallothionein; EmB, emtin B; SAL, saline; CTRL, non-diabetic rats; DB, diabetic rats)

We are currently examining histological outcomes of the various cohorts of animals.

Hypothesis vs Findings

Given the observation of mechanical tolerance, we cannot yet report on the functional aspect of the therapeutic intervention in diabetic neuropathy. Histological evaluation is not complete but we expect to have this finished in the near future. It is worth noting that in a parallel project to this, neuropathy was induced in rats by acute capsaicin administration. In that model, metallothionein (MTII) treatment clearly increased anatomical and functional nerve fibre regeneration into the epidermis, providing the first *in vivo* evidence to support our hypothesis that this class of agents has the potential to improve outcomes in neuropathy *per se*. In the current project, we have made considerable progress in developing and assessing the rat model of diabetic neuropathy and we are now in a position to perform larger scale studies to test our primary hypothesis. We are pleased to announce that *based on our outcomes in this pilot study funded by the Brain Foundation*, we successfully obtained an NH&MRC grant in this years' October funding announcement for commencement in 2015 (Project code 1089252, $421,398, over 3 years).

Unanswered Questions

In light of the observed pre-diabetic neuropathy symptoms, an earlier therapeutic intervention in an animal model of diabetic neuropathy is warranted.

What these research outcomes mean

We have made considerable progress in the development and evaluation of a rat model of diabetic neuropathy and we see clear anatomical and functional differences in epidermal nerve fibres between diabetic and untreated animals. We are now in a more realistic position to examine the effect of metallothionein and emtinB on the regeneration of epidermal nerve fibres in this model.

Please submit this report as a PDF using the following naming convention:

West Adrian – Regenerative Nerve Growth in Diabetic Neuropathy.PDF

For example: Smith Jane – The anatomy of the Brain.PDF