

# Interim report on project titled: In-vitro assessment of the efficacy of the nootropic nefiracetam to enhance plasticity after experimental demyelination

## 1: Progress to date

Our original experimental plan was to assess the use of nefiracetam to reverse the secondary effects of demyelination which may underlie cognitive deficits seen in multiple sclerosis. To do this, we used an in-vitro model of a complex neuronal network (specifically organotypic slice culture of the hippocampus from postnatal day 9 rats). We used exposure to lysophosphatidylcholine (LPC) as a demyelinating agent in our system, as this has been previously used to induce demyelination in organotypic cultures of brain tissue, and this is independent of inflammatory responses to cause demyelination, which themselves may change the underlying neuronal network function..

Figure 1 shows the results of our initial experiments. Exposure to 125 µg/ml LPC in the medium for 18 hours produced a marked decrease in myelin basic protein immunofluorescence 48 hours after termination of the LPC exposure. At the same time, the LPC exposure caused decreased expression of PSA-NCAM, a neural cell adhesion molecule which is critically involved in the plasticity processes underlying learning and memory, and acetylated alpha tubulin, the stable form of tubulin which is present at higher density in stable, intact axons. The loss of expression in both these plasticity related proteins was reversed when the cultures were treated with 10 µM nefiracetam commencing immediately after LPC exposure. Surprisingly, and more importantly, nefiracetam exposure also completely reversed the loss of myelin basic protein, suggesting nefiracetam can reverse the demyelination itself.

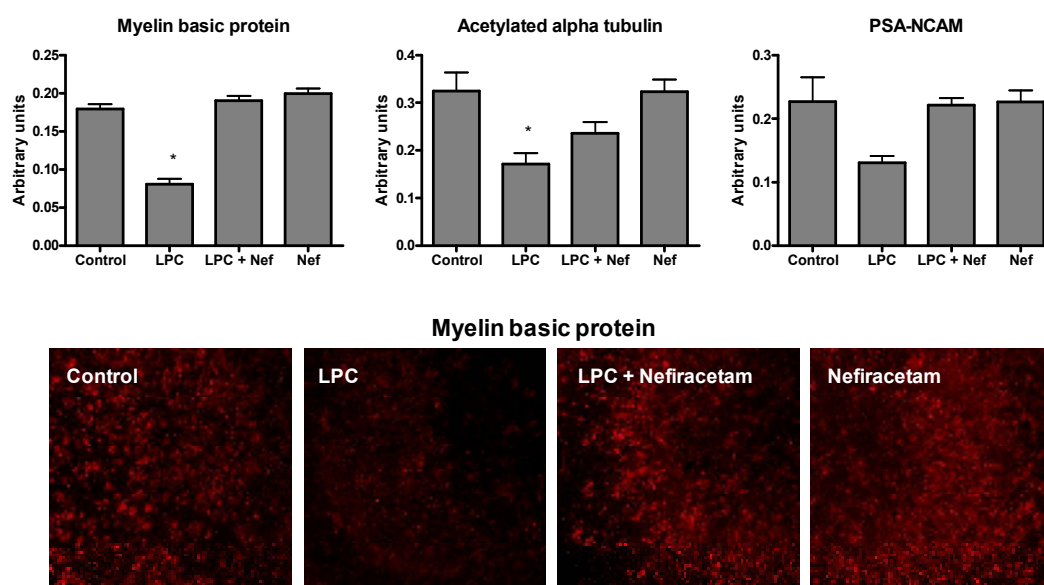


Figure 1: Nefiracetam normalises expression of myelin basic protein, acetylated alpha tubulin and polysialylated neural cell adhesion molecule (PSA-NCAM)

The effect of nefiracetam on myelin itself was completely unanticipated; there has been no suggestion in published literature or our own previous work which suggested nefiracetam might cause remyelination. As a result of these initial findings we decided to change emphasis of the project to focus on detailed characterization of our demyelination model, and the effect of nefiracetam on remyelination.

### **Characterization of the LPC demyelination model in organotypic hippocampal cultures.**

We carried out further analysis of the effect of LPC on myelin in our cultures in order to validate the model. Specifically, we examined the dose response relationships (at doses of 62.5  $\mu\text{g/ml}$ , 125  $\mu\text{g/ml}$ , 250  $\mu\text{g/ml}$ , 500  $\mu\text{g/ml}$ , or 1mg/ml LPC) between LPC exposure and loss of myelin basic protein immunofluorescence, keeping a constant exposure time of 18 hours. Myelin basic protein was evaluated by measuring the percentage area of each confocal fluorescent image which was covered in myelin basic protein, and the staining density (indicating protein density) in the myelinated areas. In general, we found that LPC caused a loss of myelin basic protein positive tissue (area) more than causing a decrease in protein in the tissue where myelin basic protein was present, indicating selective damage to oligodendrocytes.

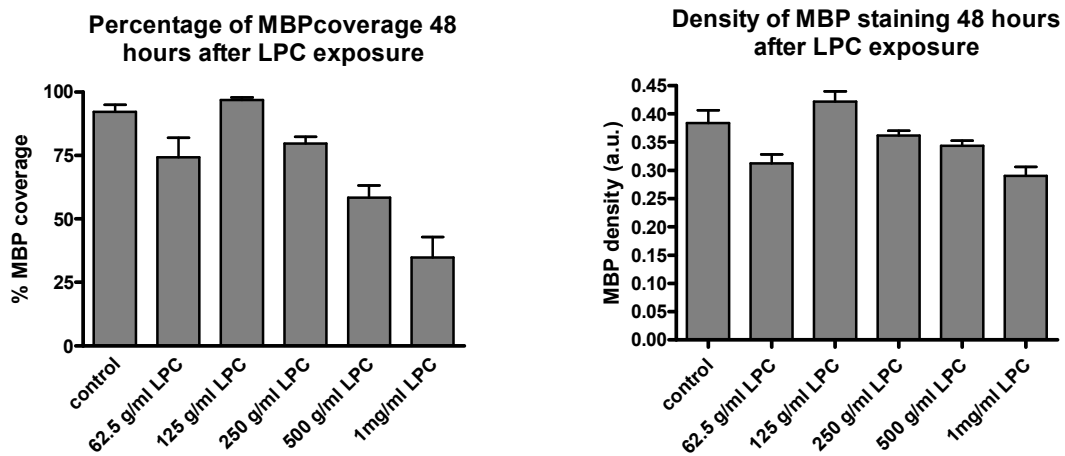


Figure 2. Dose response relationships between myelin basic protein staining and LPC dose 48 hours after termination of LPC exposure

We also examined the time course of demyelination following LPC exposure. We found that LPC produced demyelination which was evident immediately after exposure. This persisted over several days, recovering to baseline levels between 48 and 72 hours after exposure. The time course of demyelination and remyelination at a dose of 250  $\mu\text{g/ml}$  LPC is shown in figure 3.

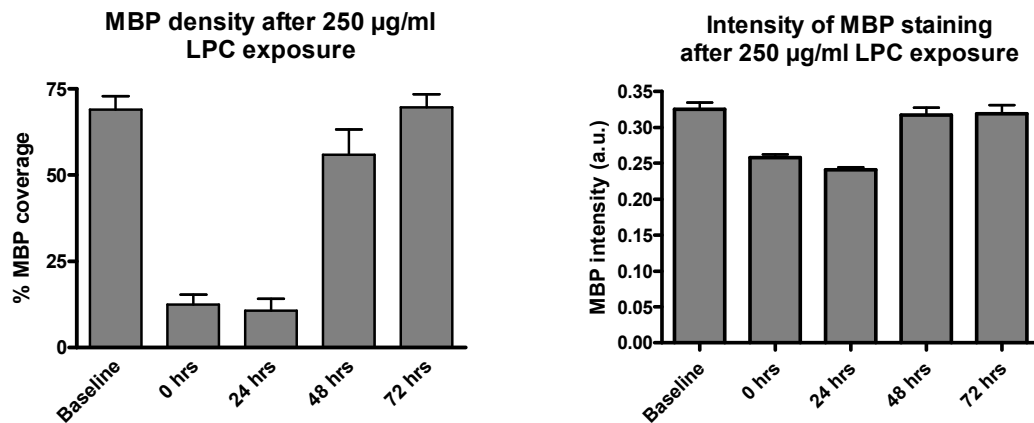


Figure 3: Exposure to 250 µg/ml LPC for 18 hours produces a major decrease in myelin which recovers between 48 and 72 hours after exposure

Additionally, we validated the selectivity of LPC in damaging oligodendrocytes by assessing the general toxicity of LPC in SHY cells and mixed primary hippocampal cultures. We found no evidence of general toxicity of LPC to SHY cells after 18 hours of 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, or 1mg/ml exposure to LPC. We only found evidence of toxicity to neuronal cells after 18 hour 1mg/ml LPC exposure, but not at the lower doses, including the doses we use in evaluating the effect of nefiracetam.

### **Evaluation of nefiracetam efficacy in reversing LPC induced demyelination.**

After establishing the time course of the LPC induced demyelination / remyelination cycle, we examined specifically how this was affected by nefiracetam treatment. Nefiracetam was again applied as soon as the LPC exposure ceased; this precludes the possibility that the nefiracetam effect is linked to an LPC specific mechanism. We found significant recovery of myelin as early as 24 hours after the end of the LPC exposure. An example of the effect of nefiracetam on LPC induced demyelination is shown in figure 4.

## Nefiracetam accelerates MBP recovery after 250 µg/ml LPC exposure

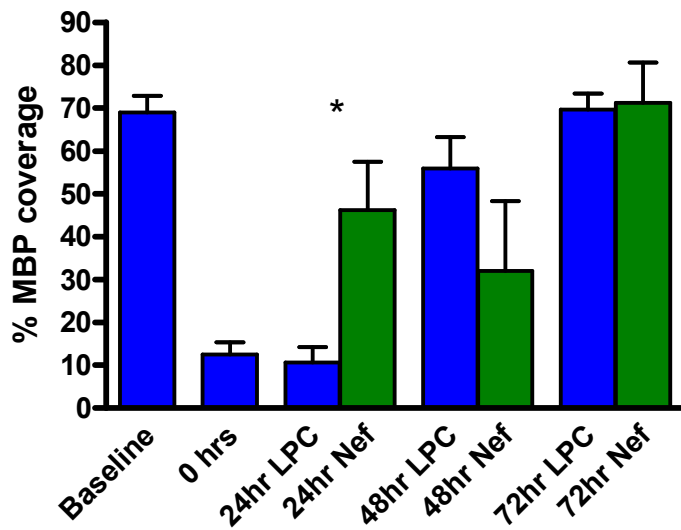


Figure 4. Nefiracetam causes significant remyelination 24 hours after LPC exposure

These findings are particularly promising considering nefiracetam is already licensed for use in humans for other purposes; it is a drug which is well tolerated with a very good safety profile, and that the effect we see is independent of immune modulation. These findings suggest we may have identified nefiracetam as the first therapeutic agent which can induce remyelination (and possibly prevent demyelination) in multiple sclerosis without suppressing the immune system.

## 2: Expenditure to date

Expenditure on materials is outlined in the table below:

Confocal microscopy costs	€1,039.36
Organotypic culture inserts	€570.40
Fluo-4	€312.60
Lysophosphatidylcholine	€86.30
Primary Antibodies	€790.68
Secondary fluorescent antibodies	€226.31
Animals	€493.76
Molecular analysis reagents	€459.00
<b>TOTAL</b>	<b>€3,978.41</b>

Additionally, a total of €60,000 (€5,000/month) has been allocated for salary costs over the duration of the project. This total amount includes gross salary, employers PRSI and pension contribution.

## 3. Abstracts submitted.

On the 22<sup>nd</sup> October 2009, a provisional patent application was filed in the US for the use of nefiracetam to treat demyelinating diseases, based on our findings described above. This allows us a period of 1 year to add additional supporting data to the patent claim before the evaluation of the patent, which will begin in October 2010.

Due to the requirement for non-disclosure because of the patent, no abstracts have been submitted relating to this study. Papers will be submitted after the patent evaluation.

## 4. Work plan to completion date.

A number of additional experimental plans are currently underway, to be completed by the end of January 2010. These experiments will give us a more complete description of the efficacy of nefiracetam reversal of demyelination in-vitro.

### 1. Evaluating the effect of nefiracetam pre-treatment on LPC demyelination.

Thus far, we have shown nefiracetam is effective in causing recovery of myelin when applied after the damage has occurred. We are currently evaluating whether nefiracetam treatment commencing before the LPC exposure is effective in preventing the damage from occurring. This is done using the same experimental approach as we have used in the experiments described above.

**2. Assessment of functionality of nefiracetam supported remyelination.** We are currently assessing whether the recovery of myelin basic protein after nefiracetam

treatment represents functional remyelination. To do this, we are carrying out LPC demyelination and nefiracetam exposure as described above, and immunofluorescently staining for both myelin basic protein and neurofilament H, a marker of axons. This will allow us to differentiate between functional myelin (associated with axons) and myelin expressed ectopically and not associated with axonal fibres.

**3. Assessment of neuronal network function after LPC exposure with and without nefiracetam.** We are also carrying out experiments to assess the degree of functional alteration of the neuronal network in the organotypic hippocampal cultures after LPC exposure, and the effect nefiracetam has on this. Here, we load neurons in organotypic cultures with the calcium dye fluo-4, and can assess network function by the degree of spontaneous activity (measured by spontaneous calcium fluctuations) occurring in the culture network.