Final report:

Epithelial exosomes: Key modulators of neural function in human trigeminal nerves?

Background

Epithelial cells of the oral cavity play particularly important roles in protecting the host from a wide variety of ingested insults including chemicals, pollutants and pathogens. It is recognised that the epithelium itself responds to injury or infection during the progression of disease and that epithelial tissues are well innervated. However, although interactions between epithelial and neuronal cells have been shown to be important in tissue differentiation, regulation of local inflammation and repair of damaged tissue, the molecular mechanisms responsible have yet to be fully elucidated.

Emerging evidence points to a burgeoning role for extracellular vesicles (exosomes), to be an important route for non-contact cell-cell communication between cells of different lineages. In this project we studied the role of epithelial exosomes on the regulation of neuronal ion channels to gain a unique understanding of human epithelial-neuronal communication *in vitro*, fulfilling a knowledge gap in our current understanding of trigeminal pain mechanisms.

Pain emanating from the trigeminal nerves is said to be the most excruciating pain suffered by humans and is often associated with awakening from sleep. Recent research on the expression of specialised ion channels on the trigeminal nerve has highlighted the need to undertake more extensive studies on ion channel expression/functionality with the aim of elucidating their role in pain sensations. However, a major obstacle in the study of the trigeminal nerve in humans is that peripheral biopsy tissues contain only the nerve endings (the cell bodies are housed in the trigeminal ganglion).

In order to facilitate biologically relevant studies in human cells we have utilised a ground breaking approach using redundant human dental pulp tissue from which to derive nerve cells containing cell bodies (peripheral neuronal equivalents; PNEs) *in vitro*. We have used this PNE model to study the expression of the transient receptor potential (TRP) family of ion channels (previous work funded by the Pain Relief Foundation); a family of evolutionarily conserved ligand-gated ion channels that contribute to the detection of a range of physical and chemical stimuli. An improved understanding of the modulation of neuronal TRP expression and functionality in the epithelial inflammatory milieu, will help to guide regulation of these channels as promising targets for future pain therapy.

We hypothesised that epithelial exosomes modulate TRP channel expression and functionality in human trigeminal nerve fibres to mediate hyperalgesic responses associated with chronic and neuropathic oro-facial pain conditions.

Preparation of epithelial exosomes

To date there have been no previously published studies on the influence of epithelial exosomes on neural function. Indeed the preparation of exosomes from various tissues and cell culture media is a rapidly increasing area of research interest in which new methods are constantly being developed and challenged.

Three different exosome preparation kits were tested for the extraction of exosomes from the conditioned medium of exosomes.

Invitrogen Total Exosome Isolation Reagent (from Cell Culture Media) [4478359]

miRCURY Exosome Cell/Urine/CSF Kit [76743]

ExoQuick-TC PLUS

In addition an ultracentrifugation protocol was used for exosome preparation.

Our results showed that the ultracentrifugation method was superior to the kit-based methods for the preparation of epithelial exosomes. The exosomes were characterised by their expression of the exosomal marker CD63 and their size distribution as outlined below.

Optimisation of anti-CD63 antibody concentration and blocking method for oral epithelial exosome detection

In order to reliably detect epithelial exosomes by Western blotting the antibody concentration and blocking conditions were optimised (Figure 1).

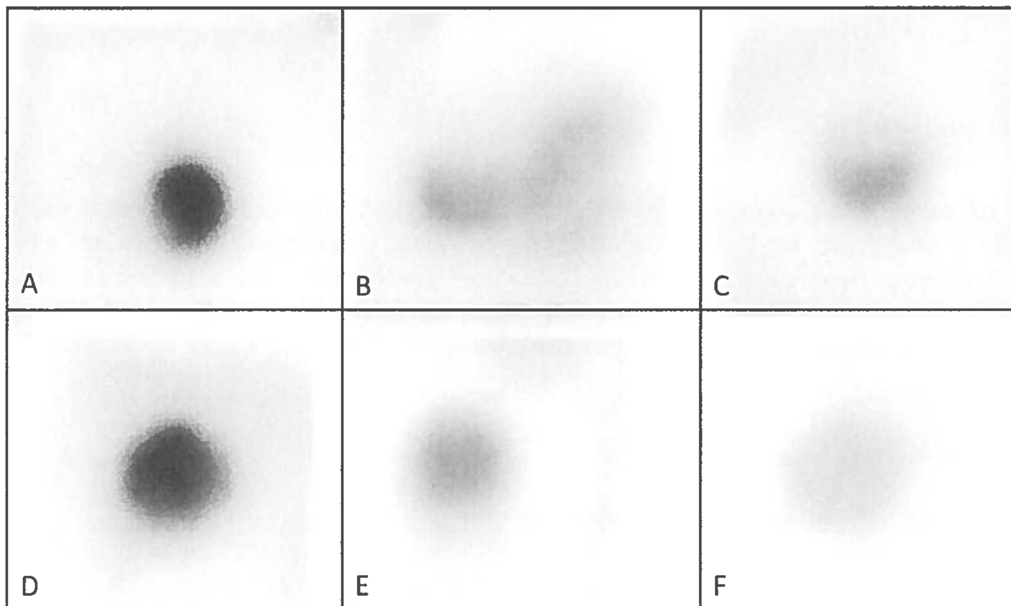

Figure 1: Dot blot to optimise the ideal concentration of CD63 antibody and the ideal blocking method for exosome detection by Western blotting. (A-C) Blocked in 5% bovine serum albumin, (D-F) blocked in 5% non-fat milk. Anti-CD63 at (A&D) 1 in 250 dilution, (B&E) at 1 in 500 dilution and (C&F) at 1 in 1000 dilution.

Western blotting confirmed exosome production by oral epithelial cells and its modulation by bacterial and viral mimics

Following the optimisation of blotting conditions, exosomes were prepared from epithelial cells using the ultracentrifugation technique and the exosome preparation was then subject to Western blotting with anti-CD63 antibody (Figure 2). The results show that exosome production was enhanced following stimulation with Toll-like receptor (TLR) agonists such as the viral mimetic Poly I:C and the bacterial TLR agonists lipopolysaccharide (LPS) and lipoteichoic acid (LTA).

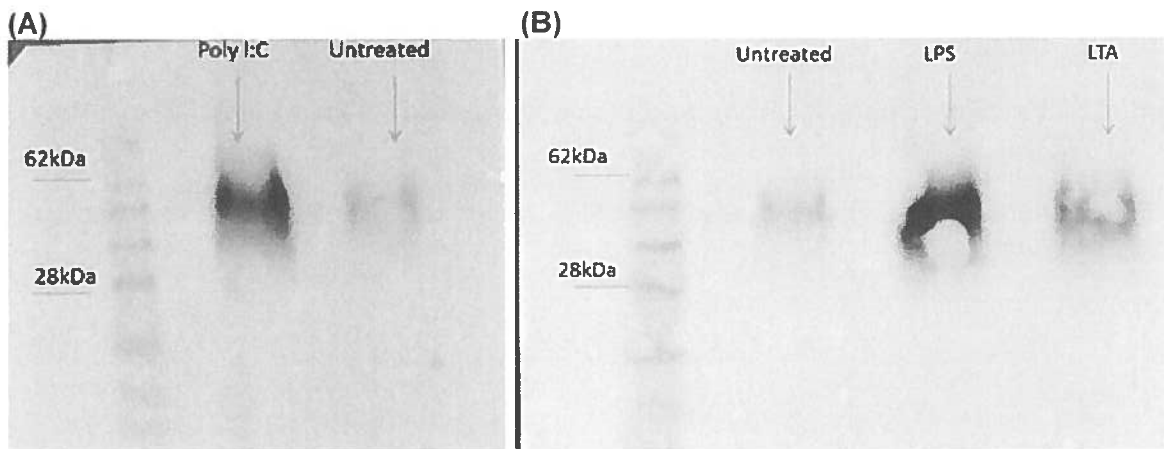

Figure 2: Western blotting of exosomes exposed to TLR agonitst (A) Exosomes prepared from untreated epithelial cells and those from epithelial cells that had been previously stimulated with the viral mimetic Poly I:C. (B) Exosomes prepared from untreated epithelial cells and those from epithelial cells that had been previously stimulated with LPS (Gram negative bacteria) or LTA (Gram positive bacteria). Exposure to viral and bacterial mimics causes enhanced exosome detection by Western blotting.

Transmission electron microscopy of oral epithelial exosomes

Size characterisation of epithelial exosomes by transmission electron microscopy (TEM) is one of the required techniques in order to truly confirm that the vesicles can be defined as exosomes. By definition exosomes should be in the size range of approximately 100 nm. TEM showed that extracellular vesicles prepared from oral epithelial cells were indeed of the correct size to be defined as exosomes.

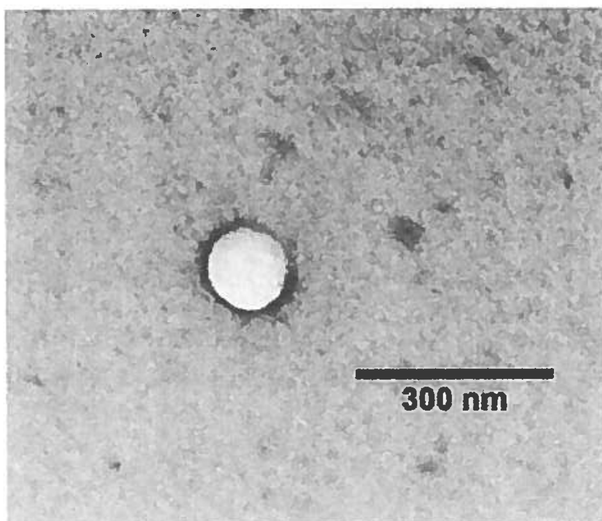

Figure 2. TEM image of an exosome isolated from oral epithelial cells. The TEM image shows that the exosome morphology is round and that the exosomes are around 100nm in size.

Functional effects of exosomes on neuronal TRP channels

In order to investigate the main hypothesis of the grant it was necessary to expose PNEs to exosomes and then monitor their responses to the TRPA1 agonist cinnamaldehyde. Our results show that indeed exosomes prepared form oral epithelial cells have the ability to enhance TRP channel neuronal responsiveness (Figure 4). This is the first data to date showing changes in neuronal function as a result of exposure to exosomes and represents a novel mechanism that could guide the modulation of these channels as promising targets for future pain therapy.

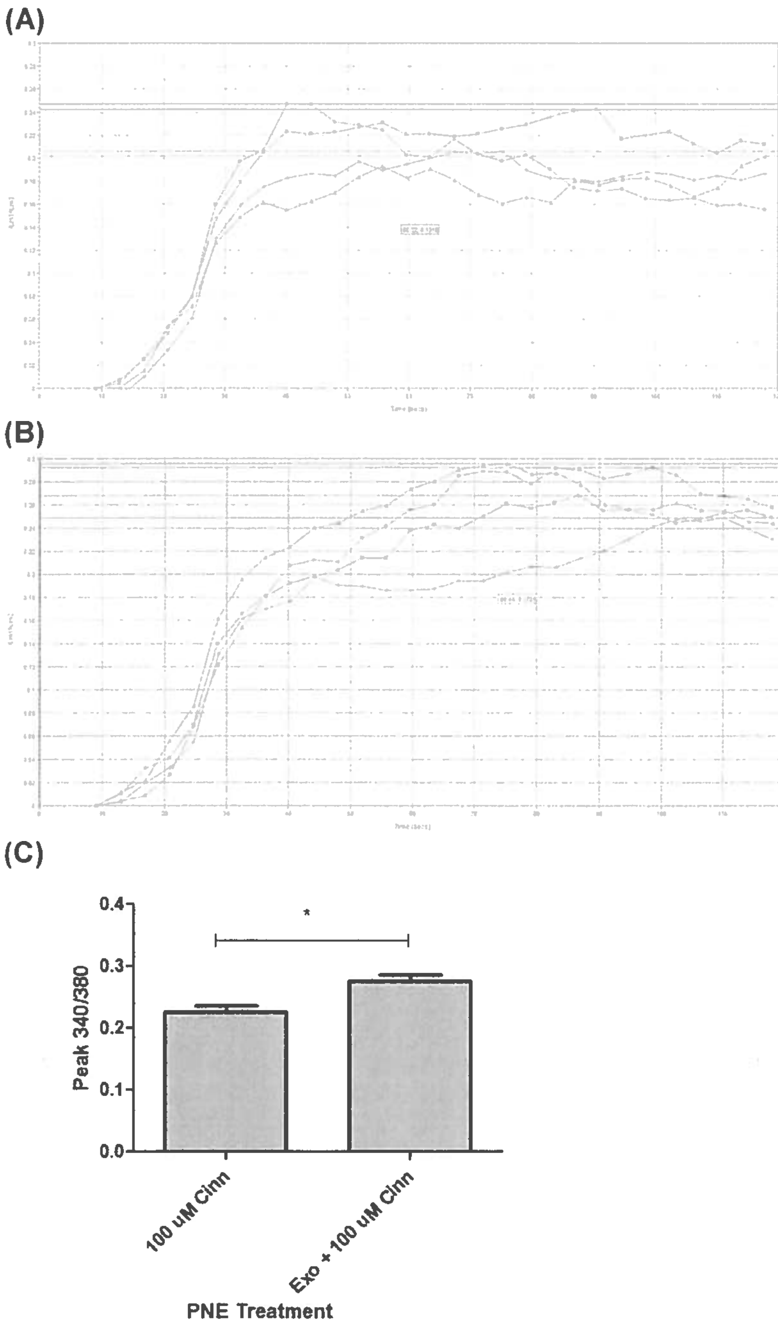

Figure 4. Flexstation calcium imaging for TRPA1 response to (A) 100 uM cinnemaldehyde alone and (B) following exosome pre-treatment for 30 mins followed by 100 uM cinnemaldehyde. Responses were enhanced following exosome pre-treatment (C) Bar chart summary of responses showing statistical significance between untreated and exosome-treated responses in PNEs.

Additional validation experiments (Figure 5) were undertaken to show the maximum and minimum responses in PNEs to ionomycin and buffer respectively. It was particularly important to show that the buffer alone did not show a calcium response. Several of the exosome preparations obtained from kits used a salting out procedure which interfered with the Flexstation calcium imaging and thus had to be avoided. The results in Figures 4 and 5 were from exosomes prepared by ultracentrifugation, which we found to be the method of choice for isolation of epithelial exosomes.

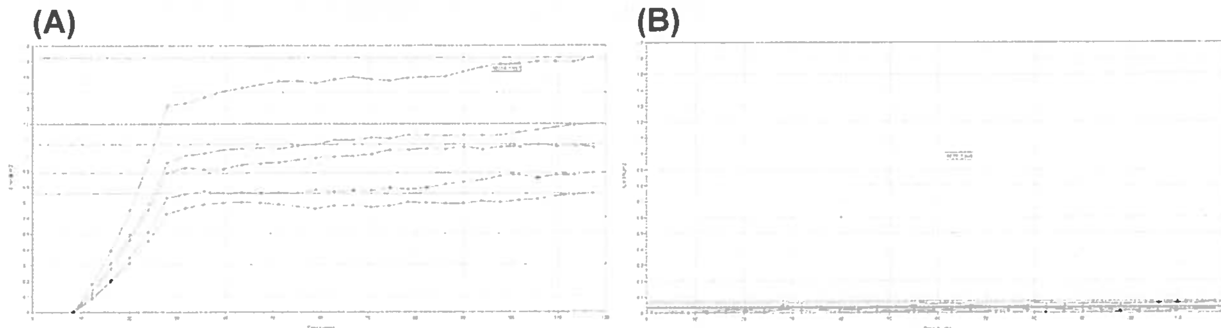

Figure 5. Flexstation calcium imaging controls (A) Response to the ionophore, ionomycin (maximum response) (B) Response to buffer alone (minimum response).

A potential role for exosomal miRNAs in neuronal TRP channel expression and function

Exosomal miRNA preparation form isolated exosomes is carried out using techniques that are in routine use in our laboratory. We have yet to analyse the miRNA signature of epithelial exosomes and undertake the locked nucleic acid (LNA) technology experiments. We aim to continue this work based on the exciting results obtained in Figure 4.

Summary:

We have produced novel data to support our hypothesis that epithelial exosomes modulate TRP channel expression and functionality in a PNE model of human trigeminal nerve fibres. To date this project has resulted in entirely new data on the modulation of TRP channel function by epithelial exosomes and we aim pursue this work further and publish it in due course.

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