Author's response to reviews

Title: Differential expression of the capsaicin receptor TRPV1 and related novel receptors TRPV3, TRPV4 and TRPM8 in normal human tissues and changes in traumatic and diabetic neuropathy

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Author's response to reviews: see over
Dear Editor,

Re – Manuscript 1985653446121310 - Differential expression of the capsaicin receptor TRPV1 and related novel receptors TRPV3, TRPV4, and TRPM8 in normal human tissues and changes in traumatic and diabetic neuropathy.

Many thanks for the most helpful reviews of our manuscript. We now submit a revised version in accord with all the comments and suggestions from the reviewers. We also include a point-by-point response below, with altered text provided in italic/bold/highlight.
Point-by-point response to comments from reviewers

Reviewer 1

1. ‘Findings of distribution in peripheral nervous system to be compared with literature’

Response: The discussion has been updated to include a comparison of results with other publications and references updated accordingly:

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The wide distribution of TRPV4 receptor in both small and large neurons matches observations in mice [47], and the specific localisation of TRPM8 in small/medium neurons is consistent with a previous report in rodents [46].

In rodents, TRPM8 and the cold activated receptor TRPA1 are also detected in sub-populations of small neurons. In mouse DRG, TRPM8 mRNA does not co-express with many of the classical markers of nociception including TRPV1[48]. However, TRPA1 is found in nociceptive sensory neurons in DRG and colocalises with TRPV1, CGRP and SP but not with TRPM8 in rat [9] [47]. Co-expression of TRPA1 with TRPV1 could explain the paradoxical heat sensation which may be experienced on exposure to a very cold stimulus.
Reviewer 2

1. ‘The presentation of the manuscript can be improved by inclusion of a summary of results in Table format.’

Response: A new table (Table 2) summarising the results has been included and the text updated as follows:

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**Results**

*A summary of results obtained in the tissues examined are presented in tabular form (Table 2).*

**Table 2**

<table>
<thead>
<tr>
<th>Nerve injury</th>
<th>TRPV1</th>
<th>TRPV3</th>
<th>TRPV4</th>
<th>TRPM8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypersensitive skin</strong></td>
<td>Increased (brachial plexus)</td>
<td>Increased (brachial plexus)</td>
<td>Positive</td>
<td>Positive. Reduced</td>
</tr>
<tr>
<td><strong>DRG injury</strong></td>
<td>Increased</td>
<td>Not detected</td>
<td>Fibres</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Decreased (see Smith et al 2002)</td>
<td>Decreased (see Smith et al 2002)</td>
<td>Unchanged</td>
<td>Increased?</td>
</tr>
<tr>
<td><strong>Diabetic skin</strong></td>
<td>Decreased nerve fibres</td>
<td>Nerve fibres not detected</td>
<td>Nerve fibres - very few</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Negative keratinocytes</td>
<td>Trend for decreased keratinocytes</td>
<td></td>
<td>Unchanged</td>
</tr>
<tr>
<td><strong>Neuropathic nerve</strong></td>
<td>Positive fibres</td>
<td>None detected</td>
<td>Not examined</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>Spinal cord</strong></td>
<td>Decreased</td>
<td>Not examined</td>
<td>Positive</td>
<td>Unchanged</td>
</tr>
<tr>
<td></td>
<td>Positive nerve fibres in dorsal horn</td>
<td>Positive motor neurones</td>
<td>Positive (weak) motor neurones</td>
<td>Weak fibres in dorsal horn and roots</td>
</tr>
<tr>
<td><strong>Dorsal roots</strong></td>
<td>Positive fibres</td>
<td>Not detected</td>
<td>Positive fibres</td>
<td>Positive fibres</td>
</tr>
<tr>
<td><strong>Ventral roots</strong></td>
<td>Not detected</td>
<td>Positive fibres</td>
<td>Positive fibres</td>
<td>Positive fibres</td>
</tr>
</tbody>
</table>
2. ‘Negative controls for antibodies should be presented in first two figures and authors to comment on ‘not nuclei-excluded’ staining in Figure 1.’

Response: Peptide blocking was described in the methods and has been updated in more detail as follows:

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Specificity controls included pre-incubation of primary antibodies with homologous antigen at $10^{-1}$ to $10^{-6}$ mg per ml of diluted antibodies prior to immunostaining. Specificity of antibodies to TRPV1 and TRPV3 has been described in a previous publication [4].

In addition, a representative photomicrograph for the inhibition of TRPM8 has been included (see new Figure 2D) and the legend to figure updated accordingly.

3. ‘In Figure 1, there are lots of stainings that are not nuclei excluded’

Response: The text has been updated to include a comment on ‘not nuclei-excluded’ staining as follows:

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Small dot-like structures in the tissue surrounding the sensory neurons, apparent with antibodies to TRPV4 and TRPM8 (Figures 1 and 2), probably represents immunoreactivity in nerve fibres cut transverse to the plane of the sections.

4. ‘Title is vague’

Response: The title has been revised to be more specific as follows:

Differential expression of the capsaicin receptor TRPV1 and related novel receptors TRPV3, TRPV4, and TRPM8 in normal human tissues and changes in traumatic and diabetic neuropathy.

5. ‘More quantitation is required’

Response: Results have been updated to include median values and ranges for all quantification and percentage of motor neurones included for TRPV3 as follows:
Immunoreactivities for both TRPV3 and TRPV1 (% area immunoreactive nerve) were significantly increased after injury [median (range) TRPV3: control 5.25 (0.9 – 6.9), injured 15.0 (5.8 – 20.1) p< 0.001; TRPV1; control 17.6 (12.1 – 20.6), injured 24.1 (18.6 – 32.0) p< 0.001; Fig 4 A, C] and although image analysis of the nerve marker peripherin showed no change, % ratios of TRPV3 or TRPV1 to peripherin were significantly increased also [median (range) % TRPV3:PPN, control 17.6 (2.9 – 19.4), injured 50.1 (25.0 – 67.9), p< 0.001; % TRPV1:PPN, control 55.7 (31.9 – 82.3), injured 89.8 (63.4 – 100.0), p< 0.001; Fig 4B, D].

Four out of 9 samples showed strong TRPV3-immunoreactivity in motor neurones and fibres in the ventral horn of the spinal cord (Fig 6C). In these positive samples, counts of TRPV3 motor neurones were 33% of total.

6. ‘Provide a micrograph for peripherin as a control’

Response: Figure 8 has been updated to include a photo of peripherin in control skin (see new Figure 8) and text and legend to figure adjusted accordingly as follows:

Few TRPV4-immunopositive nerve fibres were detected in skin samples. Positive but patchy immunostaining of TRPV4 basal keratinocytes was observed (Fig 8E). Sub-epidermal fibres were detected using antibodies to peripherin (PPN; Fig 8F).
TRPV3-immunoreactive basal keratinocytes in control (A) and diabetic (B) skin.

TRPV1-immunoreactive dermal and epidermal (arrows in C) fibers in control (C) and diabetic (D) skin. TRPV4 - immunoreactivity in keratinocytes (E) and nerve marker (peripherin PPN) in control skin (F). Scale bar = 50µm A, B, D, E; 25µm C, F.

7. ‘More labels can be added for some figures’

Response: Figures have been updated with individual labels to improve clarity (see new Figures 2, 5, 6, 9 and 10.)