Characterisation of Substance P and Transient Receptor Potential Melastatin Channel Messenger RNA and Protein Expression in Acute and Chronic Neurological Disorders

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Appendix A

Gene Expression in FFPE Tissue

A.1 Pilot Studies

A.1.1 Background

The tissue archives of pathology laboratories represent a vast resource of disease-specific biological material: they house an abundant supply of FFPE tissue for which response to treatment and clinical outcome is already known. Recent advances in molecular biology have made it possible to reliably isolate and amplify mRNA from FFPE tissue, which presents a tremendous opportunity to retrospectively quantify transcription levels of specific genes, and then correlate the molecular findings with clinical and histological observations (Abrahamsen et al., 2003; Van Deerlin et al., 2002; Lewis et al., 2001; Lehmann and Kreipe, 2001).

Several groups have reported the detection and quantification of mRNA in FFPE tissue (Mangham et al., 2006; Choi et al., 2004; Shabaana et al., 2001; Finke et al., 1993; Stanta and Schneider, 1991), including CNS tissue (Kunz et al., 2006; Usarek et al., 2005; Qin et al., 2003; Cummings et al., 2001; Kingsbury et al., 1995). However, due to the fact that tissue has been fixed in formaldehyde, nucleic acids in FFPE tissue are likely to be highly fragmented (Lehmann and Kreipe, 2001). Of particular relevance to brain tissue, other factors, such as fixation time, tissue pH and post-mortem interval can influence the quality of RNA extracted from FFPE tissue (Van Deerlin et al., 2002). However, a study by Imbeaud et al. (2005) found that samples of similar, even poor, RNA integrity were comparable in real-time RT-PCR studies.

Given that our laboratory has access to FFPE human brain tissue, with next-of-kin consent for research, dating back several decades and representing a wide variety of both acute and chronic neurological pathologies (plus many age-matched normal controls), this presented a unique opportunity for us to conduct a large mRNA quantification study for our genes of interest.

A.1.2 Experimental Procedures

In the present study, several different methods were tested in order to find the most reliable way of isolating RNA from FFPE tissue. Factors considered were reproducibility, RNA yield, RNA quality and experimental time. Three commercial kits were trialled: Ambion RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues, Invitrogen Pure-Link FFPE RNA Isolation Kit, and Qiagen
RNA Extraction

RNA was extracted from FFPE tissue using the commercial kits listed above, according to each manufacturer’s directions. In order to effectively compare each commercial kit, the same FFPE tissue blocks were used across all preliminary experiments. Two rat brain TBI FFPE blocks and two rat brain sham FFPE blocks were selected, and 8 x 10 μM slices of each block were used for each kit. In brief, each commercial kit followed the same basic RNA extraction protocol: remove paraffin (usually with xylene), lyse with proteinase K, heat, add proprietary buffers, bind RNA to a spin column, wash and elute. Following extraction, RNA was treated with Turbo DNA-Free (Ambion) to ensure no contaminating gDNA was present. Next, the RNA extracted using the three different kits was subjected to microcapillary electrophoresis using the Agilent Bioanalyzer in order to determine RNA concentration and integrity.

RNA Results

RNA concentrations from the four samples using the three different commercial kits are shown in Table A.1. In general, yields of RNA suitable for downstream applications were obtained with the Ambion and Qiagen kits, but the Invitrogen kit produced low yields (all ≤ 7 ng/μL). At this point, the Invitrogen samples were abandoned because of their extremely low RNA concentrations. From experience, RNA samples with very low concentrations (less than about 50 ng/μL), that are also highly degraded, are difficult to reliably amplify using PCR (N. Cook, R. Vink, C. Van Den Heuvel, unpublished results). Bioanalyzer analysis showed that all RNA samples were of a similar integrity (RIN range 2.1 - 2.5; mean 2.3), see Table A.2. Given that the lowest possible RIN value is 1.0 (representing totally degraded RNA), the samples in the current experiment could be considered to be highly degraded. However, according to report by Imbeaud et al. (2005), these samples should be comparable when analysed by real-time RT-PCR because they all have similar RNA integrities.

Table A.1: Concentrations of RNA Extracted from Rat Brain FFPE Tissue

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ambion (ng/μL)</th>
<th>Qiagen (ng/μL)</th>
<th>Invitrogen (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>103</td>
<td>127</td>
<td>7</td>
</tr>
<tr>
<td>Sample 2</td>
<td>92</td>
<td>37</td>
<td>3</td>
</tr>
<tr>
<td>Sample 3</td>
<td>170</td>
<td>209</td>
<td>7</td>
</tr>
<tr>
<td>Sample 4</td>
<td>44</td>
<td>17</td>
<td>2</td>
</tr>
</tbody>
</table>

Table A.2: RINs of RNA Extracted from Rat Brain FFPE Tissue

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ambion</th>
<th>Qiagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>2.4</td>
<td>2.2</td>
</tr>
<tr>
<td>Sample 2</td>
<td>2.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Sample 3</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Sample 4</td>
<td>2.5</td>
<td>2.1</td>
</tr>
</tbody>
</table>

To provide an understanding of the differences in quality and yield of RNA extracted from FFPE tissue compared to fresh frozen tissue, an electropherogram from the Agilent Bioanalyzer has been included, comparing both FFPE tissue RNA and frozen tissue RNA (see Figure A.1). The red trace shows high quality RNA extracted from frozen tissue, with minimal degradation (RIN 8.6), while the blue trace shows highly degraded RNA extracted from FFPE tissue (RIN 2.3). Fluorescence units (FU) on the y-axis and the area under the traces are indicative of RNA yield;
this electropherogram clearly shows that a much larger yield of RNA was obtained from the frozen tissue sample. The two red peaks in the frozen tissue RNA trace are representative of ribosomal RNA, which are not visible at all in the FFPE tissue RNA trace. The x-axis, showing time in seconds, signifies the size of the extracted RNA fragments: as with gel-based electrophoresis, smaller RNA fragments migrate through the capillaries earlier than large RNA fragments, and therefore, smaller fragments are measured earlier. As time progresses, the size of the detected RNA molecules increases. Figure A.1 shows that the FFPE sample comprises low quantity and quality RNA, in contrast with the frozen tissue RNA sample.

Figure A.1: Overlaid electropherograms from the Agilent 2100 Bioanalyzer. Red trace shows high quality RNA extracted from frozen tissue, with minimal degradation (RIN 8.6). Blue trace (indicated by arrow) shows highly degraded RNA extracted from FFPE tissue (RIN 2.3).

A.1.3 Real-time RT-PCR

Two-Step Assay Initially, two-step real-time PCR standard curve assays were set up as described in the Materials and Methods Chapter (Sections 3.2.6 and 3.2.7). A standard curve was prepared with pooled FFPE tissue cDNA, using β2MG primers. A 10 ng sample of frozen tissue cDNA served as a positive control. Amplification curves from one PCR assay can be seen in Figure A.2. The low R² value of this assay (0.33) indicates that the standards were not easily fitted onto a line of best fit, and that the results obtained are not likely to be reliable. Figure A.2 clearly shows that the positive control was detected many cycles before the FFPE cDNA samples. Furthermore, there is no clear standard curve; all concentrations of FFPE tissue cDNA are clustered together instead of being separated as in Figure 2.3a (in Chapter 2). After several attempts at producing a standard curve of acceptable PCR efficiency and R² values with no success, the two-step PCR assay was discontinued.

Figure A.2: Amplification curves from two-step real-time RT-PCR using FFPE tissue RNA. PCR efficiency was 45% in this assay, with an R² value of 0.33.

One-Step Assay A report by Talantov et al. (2006) describes the successful quantification of mRNA levels from FFPE tissue using one-step real-time PCR, where the RT and real-time PCR steps are performed sequentially in the one tube. Therefore, a one-step real-time PCR assay was attempted in the present study, using the Invitrogen SuperScript III Platinum SYBR Green One-Step qRT-PCR kit, according to the manufacturer’s directions. The starting amounts of
Figure A.3: Amplification and standard curves from one-step real-time RT-PCR using FFPE tissue RNA.

Pooled FFPE tissue RNA used to prepare the standard curve were: 400 ng, 200 ng, 100 ng and 50 ng. β2MG was the gene of interest in the assay. Amplification curves and standard curve are shown in Figures A.3a and A.3b, respectively. The results of the one-step real-time PCR were vastly improved over the two-step assay: the serial dilutions of the standard curve are clearly discernable on both curves. Despite this, the PCR efficiency (70 %) and R² value (0.37) were too low to be classed as optimised, and reliable PCR results would not be obtained under these conditions. Although the one-step real-time PCR assay was repeated several more times, PCR efficiency and R² values were not improved.

Conclusions

Gene expression analysis using FFPE tissue has great potential to increase our understanding of human disease, particularly diseases of the CNS, where fresh tissue is not always readily available. However, there are inherent problems with the RNA extracted from fixed tissue: the fixation process causes RNA degradation and cross-linking of RNA with proteins. Several groups have reported optimised methods of extracting nucleic acids from FFPE tissue (Körbler et al., 2003; Coombs et al., 1999; Masuda et al., 1999; Krafft et al., 1997). While it may be possible to generate reliable gene expression data from fixed tissue using real-time RT-PCR (Bediaga et al., 2008; Koch et al., 2006), the conditions in our laboratory did not generate FFPE tissue that could be used reliably for RNA-based experiments. Factors such as fixation time, type of fixative used, tissue processing methods, tissue pH and age of FFPE blocks could account for this. Therefore, after testing several different methods, the FFPE tissue gene expression analysis study was discontinued, and fresh frozen tissue used instead for all RNA-based experiments. It may have been possible to characterise the FFPE tissue RNA using semi-quantitative PCR, however, we chose to analyse frozen tissue using real-time RT-PCR technology because real-time RT-PCR technology is much more sensitive and reliable than semi-quantitative gel-based PCR (see Chapter 2). Unfortunately, this restricted the human component of the study, because only a limited amount of fresh frozen human brain tissue is available for research purposes.
Appendix B

Clinical Information

B.1 Details of Post Mortem Human Brain Tissue

B.1.1 TBI Tissue

Clinical TBI cases were grouped into survival times of < 5 hours or 5 - 24 hours based on detailed neuropathological information. All cases had donor or next-of-kin approval for research and this study was approved by the University of Adelaide Human Ethics committee.

Gene Expression Analysis Fresh frozen human TBI tissue, plus age- and sex-matched control tissue (temporoparietal cortex tissue; coronal section, 1 cm from mamillary bodies) was obtained from the Victorian Brain Bank Network. Case details have been summarised in Table B.1.

Immunohistochemistry FFPE TBI tissue (left and right parietal cortex and hippocampus), plus age- and sex-matched control tissue was obtained from the IMVS Tissue Pathology Laboratory. Each case was previously assessed by neuropathologists at the IMVS. Case details have been summarised in Table B.2. Note that cases with diffuse axonal injury (as indicated by APP+ immunostaining) were chosen wherever possible. However, since DAI develops over time (Van Den Heuvel et al., 1999; Abou-Hamden et al., 1997), some cases with a very acute survival time did not have APP+ immunostaining.

B.1.2 PD Tissue

Gene Expression Analysis Fresh frozen human PD tissue was received from the South Australia Brain Bank. Fresh frozen age- and sex-matched control tissue was supplied by the Victorian Brain Bank Network. Five brain areas were obtained from each case: SN, GP, caudate, putamen, and MTG. Case details have been summarised in Tables B.3.
Table B.1: Details of Human TBI & Control Fresh Frozen Brain Tissue Cases

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age</th>
<th>Gender</th>
<th>Type of Incident</th>
<th>Survival Time</th>
<th>PMI (h)</th>
<th>Pathology(^\d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBI 1</td>
<td>63</td>
<td>M</td>
<td>Fall</td>
<td>&lt; 5 hours</td>
<td>70</td>
<td>L, C, IVH</td>
</tr>
<tr>
<td>TBI 2</td>
<td>51</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>60</td>
<td>TBI</td>
</tr>
<tr>
<td>TBI 3</td>
<td>27</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>84</td>
<td>TBI</td>
</tr>
<tr>
<td>TBI 4</td>
<td>49</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>107</td>
<td>TBI</td>
</tr>
<tr>
<td>TBI 5</td>
<td>45</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>43</td>
<td>SAH, SDH, C, PH</td>
</tr>
<tr>
<td>TBI 6</td>
<td>21</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>100</td>
<td>SAH, SDH</td>
</tr>
<tr>
<td>TBI 7</td>
<td>41</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>114</td>
<td>TBI</td>
</tr>
<tr>
<td>TBI 8</td>
<td>57</td>
<td>F</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>97</td>
<td>SAH, C, PH</td>
</tr>
<tr>
<td>TBI 9</td>
<td>49</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>103</td>
<td>SAH</td>
</tr>
<tr>
<td>TBI 10</td>
<td>34</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>66</td>
<td>TBI</td>
</tr>
<tr>
<td>TBI 11</td>
<td>41</td>
<td>F</td>
<td>Trauma</td>
<td>&lt; 5 hours</td>
<td>95</td>
<td>TBI</td>
</tr>
<tr>
<td>TBI 12</td>
<td>57</td>
<td>F</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>87</td>
<td>SDH, SAH</td>
</tr>
<tr>
<td>TBI 13</td>
<td>56</td>
<td>M</td>
<td>MVA</td>
<td>5 - 24 hours</td>
<td>65</td>
<td>TBI</td>
</tr>
<tr>
<td>TBI 14</td>
<td>16</td>
<td>M</td>
<td>MVA</td>
<td>5 - 24 hours</td>
<td>85</td>
<td>SAH, SDH, C</td>
</tr>
<tr>
<td>TBI 15</td>
<td>78</td>
<td>M</td>
<td>Fall</td>
<td>5 - 24 hours</td>
<td>47</td>
<td>SAH, C</td>
</tr>
<tr>
<td>TBI 16</td>
<td>75</td>
<td>M</td>
<td>Fall</td>
<td>5 - 24 hours</td>
<td>89</td>
<td>S, H</td>
</tr>
<tr>
<td>TBI 17</td>
<td>46</td>
<td>M</td>
<td>Fall</td>
<td>5 - 24 hours</td>
<td>136</td>
<td>TBI, HI</td>
</tr>
<tr>
<td>TBI 18</td>
<td>18</td>
<td>M</td>
<td>MVA</td>
<td>5 - 24 hours</td>
<td>79</td>
<td>TBI</td>
</tr>
<tr>
<td>TBI 19</td>
<td>64</td>
<td>M</td>
<td>Fall</td>
<td>5 - 24 hours</td>
<td>61</td>
<td>SAH, SDH, C</td>
</tr>
<tr>
<td>TBI 20</td>
<td>61</td>
<td>M</td>
<td>Fall</td>
<td>5 - 24 hours</td>
<td>40</td>
<td>SAH, SDH, C</td>
</tr>
<tr>
<td>Control 1</td>
<td>59</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>43</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 2</td>
<td>52</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>52</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 3</td>
<td>48</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>50</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 4</td>
<td>78</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>46</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 5</td>
<td>64</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>24</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 6</td>
<td>51</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>64</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 7</td>
<td>60</td>
<td>F</td>
<td>N/A</td>
<td>N/A</td>
<td>48</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 8</td>
<td>59</td>
<td>F</td>
<td>N/A</td>
<td>N/A</td>
<td>30</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 9</td>
<td>67</td>
<td>F</td>
<td>N/A</td>
<td>N/A</td>
<td>24</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 10</td>
<td>63</td>
<td>F</td>
<td>N/A</td>
<td>N/A</td>
<td>30</td>
<td>Normal Brain</td>
</tr>
</tbody>
</table>

\(^\d\)Detailed neuropathological information was not available for some cases (designated 'TBI'). MVA, motor vehicle accident; L, lacerations; C, contusions; IVH, intraventricular haemorrhage; SAH, subarachnoid haemorrhage; SDH, subdural haematoma; PH, petechial haemorrhage; S, cerebral swelling; H, brain herniation; HI, hypoxic/ischaemic brain injury.
Table B.2: Details of Human TBI & Control FFPE Brain Tissue Cases

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age</th>
<th>Gender</th>
<th>Type of Incident</th>
<th>Survival Time</th>
<th>Pathology‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBI 1</td>
<td>46</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>SAH, L, PH, IVH</td>
</tr>
<tr>
<td>TBI 2</td>
<td>20</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>SAH, C, PH, IVH</td>
</tr>
<tr>
<td>TBI 3</td>
<td>20</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>SAH, C, PH</td>
</tr>
<tr>
<td>TBI 4</td>
<td>67</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>SAH, L, C, IVH, APP+</td>
</tr>
<tr>
<td>TBI 5</td>
<td>34</td>
<td>M</td>
<td>Head Trauma</td>
<td>&lt; 5 hours</td>
<td>SAH, C, APP+</td>
</tr>
<tr>
<td>TBI 6</td>
<td>33</td>
<td>M</td>
<td>MVA</td>
<td>&lt; 5 hours</td>
<td>SAH, L, C, IVH, APP+, HI</td>
</tr>
<tr>
<td>TBI 7</td>
<td>69</td>
<td>M</td>
<td>Fall</td>
<td>5 - 24 hours</td>
<td>SAH, L, C, SDH, S, ICP, APP+</td>
</tr>
<tr>
<td>TBI 8</td>
<td>20</td>
<td>F</td>
<td>Head Trauma</td>
<td>5 - 24 hours</td>
<td>SAH, C, PH, ICP, APP+</td>
</tr>
<tr>
<td>TBI 9</td>
<td>18</td>
<td>F</td>
<td>Head Trauma</td>
<td>5 - 24 hours</td>
<td>SAH, L, C, IVH, APP+, HI</td>
</tr>
<tr>
<td>Control 1</td>
<td>88</td>
<td>F</td>
<td>N/A</td>
<td>N/A</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 2</td>
<td>27</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 3</td>
<td>39</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 4</td>
<td>28</td>
<td>F</td>
<td>N/A</td>
<td>N/A</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 5</td>
<td>22</td>
<td>F</td>
<td>N/A</td>
<td>N/A</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 6</td>
<td>20</td>
<td>M</td>
<td>N/A</td>
<td>N/A</td>
<td>Normal Brain</td>
</tr>
</tbody>
</table>

‡MVA, motor vehicle accident; SAH, subarachnoid haemorrhage; L, lacerations; PH, petechial haemorrhage; IVH, intraventricular haemorrhage; C, contusions; APP+, amyloid precursor protein positive immunostaining; HI, hypoxic/ischaemic brain injury; SDH, subdural haematoma; S, cerebral swelling; ICP, raised intracranial pressure.

Table B.3: Details of Human Fresh Frozen Brain Tissue Cases for PD Study

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age</th>
<th>Gender</th>
<th>PMI (h)</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD 1</td>
<td>85</td>
<td>F</td>
<td>12</td>
<td>Idiopathic PD</td>
</tr>
<tr>
<td>PD 2</td>
<td>73</td>
<td>F</td>
<td>18</td>
<td>Idiopathic PD</td>
</tr>
<tr>
<td>PD 3</td>
<td>88</td>
<td>F</td>
<td>14</td>
<td>Idiopathic PD</td>
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<tr>
<td>PD 4</td>
<td>76</td>
<td>F</td>
<td>19</td>
<td>Idiopathic PD</td>
</tr>
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<td>PD 5</td>
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<td>PD 6</td>
<td>74</td>
<td>M</td>
<td>3</td>
<td>Idiopathic PD</td>
</tr>
<tr>
<td>Control 1</td>
<td>75</td>
<td>F</td>
<td>N/A</td>
<td>Normal Brain, scant plaques</td>
</tr>
<tr>
<td>Control 2</td>
<td>75</td>
<td>F</td>
<td>N/A</td>
<td>Normal Brain, occasional tangles</td>
</tr>
<tr>
<td>Control 3</td>
<td>73</td>
<td>M</td>
<td>N/A</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 4</td>
<td>85</td>
<td>F</td>
<td>N/A</td>
<td>Normal Brain, scant plaques</td>
</tr>
<tr>
<td>Control 5</td>
<td>81</td>
<td>M</td>
<td>N/A</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 6</td>
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<td>F</td>
<td>N/A</td>
<td>Normal Brain</td>
</tr>
<tr>
<td>Control 7</td>
<td>72</td>
<td>M</td>
<td>30</td>
<td>Normal Brain</td>
</tr>
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</table>
Appendix C

Rat TBI Micrographs

In Chapter 6, TRPM channel immunohistochemistry was carried out in rat and human TBI tissue. Considering that the TBI component of the present thesis was associated with many experimental variables (i.e. experimental and clinical TBI, cortex and hippocampus, different survival time points and four proteins of interest), a large number of digital images of stained tissue sections were generated. We believed that there were too many images to include in Chapter 6. Colour deconvolution analysis was used to semiquantify antigen content of each image, and TRPM channel expression results were presented graphically in Chapter 6.

Representative TRPM channel immunohistochemistry micrographs for rat TBI and sham sections have been included in the current appendix, while human TBI and control sections have been included in Appendix D. H & E staining of rat TBI and sham animals was carried out as described in the Materials and Methods Chapter (Section 3.2.4) and representative micrographs are also included here.

Therefore, the following images, representing a time course of TBI plus sham animals, are contained in the present appendix:

1. Figures C.1, C.2 and C.3: H & E staining of cerebral cortex and hippocampus (CA1 region and dentate gyrus).
2. Figures C.4 and C.5: TRPM2 staining of cerebral cortex and hippocampus (dentate gyrus).
3. Figures C.6 and C.7: TRPM3 staining of cerebral cortex and hippocampus (dentate gyrus).
4. Figures C.8 and C.9: TRPM7 staining of cerebral cortex and hippocampus (dentate gyrus).
5. Figures C.10 and C.11: TRPM6 staining of cerebral cortex and hippocampus (dentate gyrus).
Figure C.1: H & E staining of the cerebral cortex of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. Sham surgery and 1 hour TBI survival resulted in minimal dark cell change within cortical neurons, however, dark cell change, pyknosis and vacuolisation are clearly evident in the other TBI survival times. Scale bar = 25 μm.
Figure C.2: H & E staining of the hippocampus (CA1 region) of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. Dark cell change is clearly evident within the TBI neurons but is not seen in the sham section. Scale bar = 25 μm.
Figure C.3: H & E staining of the hippocampus (dentate gyrus) of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. Sham surgery resulted in minimal dark cell change with preservation of cell density and architecture. Following TBI, dark cell change, loss of cell density and vacuolisation are present. Scale bar = 25 μm.
Figure C.4: TRPM2 staining of the cerebral cortex of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. Colour deconvolution analysis revealed that TRPM2 protein was significantly increased in the 2 day, 3 day and 7 day TBI groups compared to shams, with a trend to increase in the 5 day group. TRPM2 immunoreactivity is clearly increased within injured neurons at these time points (signified by arrows). Scale bar = 25 μm.
Figure C.5: TRPM2 staining of the hippocampus (dentate gyrus) of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. The results from colour deconvolution analysis showed significant elevations in TRPM2 protein at all time points between 2 days and 7 days following TBI. TRPM2 immunoreactivity is increased within injured neurons at these TBI time points compared to shams (designated by arrows). Scale bar = 25 μm.
Figure C.6: TRPM3 staining of the cerebral cortex of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. Colour deconvolution analysis showed a significant increase in TRPM3 protein at the 3 day, 5 day and 7 day TBI time points compared to shams. Increased TRPM3 immunoreactivity can be seen within injured neurons at these time points (signified by arrows). Scale bar = 25 μm.
Figure C.7: TRPM3 staining of the hippocampus (dentate gyrus) of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. TRPM3 protein was significantly reduced in the 1 hour, 3 hour and 5 hour TBI groups compared to shams, as revealed by colour deconvolution analysis. Reduced TRPM3 immunoreactivity can be seen within neurons and in the parenchyma at these time points. Scale bar = 25 μm.
Figure C.8: TRPM7 staining of the cerebral cortex of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. Colour deconvolution analysis showed significant elevations in TRPM7 protein at 5 hours and all time points between 2 days and 5 days following TBI, compared to shams. Arrows point to increased TRPM7 immunoreactivity within injured neurons at these TBI time points. Scale bar = 25 μm.
Figure C.9: TRPM7 staining of the hippocampus (dentate gyrus) of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. The results from colour deconvolution analysis showed significant reductions in TRPM7 protein in the 5 hours and 1 day TBI groups compared to shams. A decrease in TRPM7 immunoreactivity within injured neurons can be observed at these TBI time points. Scale bar = 25 μm.
Figure C.10: TRPM6 staining of the cerebral cortex of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. Colour deconvolution analysis revealed a significant increase in TRPM6 protein at 5 days post-TBI compared to shams, and trends to increase at 1 hour, 1 day and 3 days. An increase in TRPM6 immunoreactivity within injured neurons can be observed at these TBI time points (shown by arrows). Scale bar = 25 μm.
Figure C.11: TRPM6 staining of the hippocampus (dentate gyrus) of sham and a time course of TBI animals. (a) Sham, (b) TBI 1 hour, (c) TBI 3 hours, (d) TBI 5 hours, (e) TBI 1 day, (f) TBI 2 days, (g) TBI 3 days, (h) TBI 5 days, (i) TBI 7 days. Colour deconvolution analysis showed significant increases in TRPM6 protein at 5 hours, 1 day and 7 days following TBI compared to shams. Elevations in TRPM6 immunoreactivity within injured neurons can be seen at these time points (signified by arrows). Scale bar = 25 μm.
Appendix D

Human TBI Micrographs

In Chapter 6 of the present thesis, we carried out TRPM channel immunohistochemistry in human TBI and control tissue. Colour deconvolution analysis was used to semi-quantify antigen content of each section, and results were presented graphically in Chapter 6. However, since the TBI component of the present thesis was associated with many experimental variables, leading to a large number of digital images of stained tissue sections, we have included representative TRPM channel immunohistochemistry micrographs for human TBI and control cases in the present appendix.

H & E staining of all TBI and control cases was carried out as described in the Materials and Methods Chapter (Section 3.2.4). Representative H & E micrographs of the parietal cortex and hippocampus (CA1 region and dentate gyrus) have also been included in the current appendix. Microscopic brain reports prepared by IMVS neuropathologists describe contusions, haemorrhage, red cell change and terminal ischaemic damage in the TBI cases in these brain regions.

Therefore, the following images, representing human TBI and control cases, are contained in the present appendix:

1. Figures D.1 and D.2: H & E staining of parietal cortex and hippocampus (CA1 region and dentate gyrus).
2. Figure D.3: TRPM2 staining of parietal cortex and hippocampus (dentate gyrus).
3. Figure D.4: TRPM3 staining of parietal cortex and hippocampus (dentate gyrus).
4. Figure D.5: TRPM7 staining of parietal cortex and hippocampus (dentate gyrus).
Figure D.1: H & E staining of the parietal cortex of (a) control case, (b) TBI < 5 hour survivor and (c) TBI 5 - 24 hour survivor. Scale bar = 25 μm.

Figure D.2: H & E staining of the hippocampus (CA1 region) of (a) control case, (b) TBI < 5 hour survivor and (c) TBI 5 - 24 hour survivor. H & E staining of the hippocampus (dentate gyrus, DG) of (d) control case, (e) TBI < 5 hour survivor and (f) TBI 5 - 24 hour survivor. Scale bar = 50 μm for CA1 images and 25 μm for DG images.
Figure D.3: TRPM2 immunoreactivity following clinical TBI, in the parietal cortex of (a) control, (b) TBI < 5 hour survivor, (c) TBI 5 - 24 hour survivor; and the hippocampus (dentate gyrus) of (d) control, (e) TBI < 5 hour survivor, (f) TBI 5 - 24 hour survivor. There were no changes in TRPM2 immunoreactivity in the parietal cortex between TBI cases and controls, as revealed by colour deconvolution analysis. However, a significant decrease in TRPM2 protein was observed in the hippocampus of < 5 hour TBI cases. This reduction in immunoreactivity can be observed in micrograph (e). Scale bar = 25 μm.

Figure D.4: TRPM3 immunoreactivity following clinical TBI, in the parietal cortex of (a) control, (b) TBI < 5 hour survivor, (c) TBI 5 - 24 hour survivor; and the hippocampus (dentate gyrus) of (d) control, (e) TBI < 5 hour survivor, (f) TBI 5 - 24 hour survivor. Colour deconvolution analysis revealed no changes in TRPM3 immunoreactivity in the parietal cortex between TBI cases and controls. However, a significant decrease in TRPM3 protein was observed in the hippocampus of < 5 hour TBI cases, with a trend to decrease in the 5 - 24 hour TBI group. A reduction in TRPM3 immunoreactivity can be clearly seen within neurons of micrographs (e) and (f). Scale bar = 25 μm.
Figure D.5: TRPM7 immunoreactivity following clinical TBI, in the parietal cortex of (a) control, (b) TBI < 5 hour survivor, (c) TBI 5 - 24 hour survivor; and the hippocampus (dentate gyrus) of (d) control, (e) TBI < 5 hour survivor, (f) TBI 5 - 24 hour survivor. The results of colour deconvolution analysis showed a significant increase in TRPM7 protein in the parietal cortex of < 5 hour TBI cases compared to controls, and no changes in the hippocampus. This increase in TRPM7 immunoreactivity can be observed within injured neurons in micrograph (b), designated by arrow. Scale bar = 25 μm.
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