Supplementary Information

Carotid Artery Disease in Post-Stroke Survivors and Effects of Enriched Environment on Stroke Pathology in a Mouse Model of Carotid Artery Stenosis

Yoshiki Hase, Tuomo M. Polvikoski, Masafumi Ihara, Mai Hase, Rayyan Zafar, William Stevenson, Louise M Allan, Abdel Ennaceur, Karen Horsburgh, Xavier Gallart-Palau, Siu Kwan Sze, Raj N Kalaria

Assessment of cerebral small vessel disease (SVD) and cerebral amyloid angiopathy (CAA)

Vascular pathology scores were derived from the presence of vascular lesions in four brain areas, including the frontal lobe at the level of the olfactory bulbs, temporal lobe at level of the anterior hippocampus, and basal ganglia at level of mamillary body. Lesions including arteriolosclerosis, cerebral amyloid angiopathy, perivascular haemosiderin leakage, perivascular space dilatation in the deep and juxtacortical white matter, myelin loss, and cortical micro (>0.5 cm) and large (<0.5 cm) infarcts were recorded with increasing severity resulting in greater scores [1]. Cerebral amyloid angiopathy (CAA) was assessed by immunohistochemistry using anti-amyloid beta (Aβ) antibody (4G8, 1:1000, Signet Laboratories, MA, USA). The scoring method was based on our previous consensus criteria [2].

Animals and Surgical Procedures

Adult male C57BL/6J mice (~25g) were purchased from the Charles River, UK. The mice were housed in groups on a 12 hrs day and 12 hrs night cycle (6am–6pm, day; 6pm–6am, night) and were given access to food and water ad libitum. A total of 74 mice were randomly selected for either bilateral common carotid artery stenosis (BCAS, n=41) or sham (n=33) surgery. BCAS surgery was performed as described previously [3, 4]. Animal experiments and data analyses were performed under investigator blinded conditions by 2 or more observers. The surgical and animal housing procedures were pre-approved by the Home Office, UK based upon ASPA: The Animals (Scientific Procedures) Act 1986, UK and performed in accordance with the guidelines stipulated by the ethical committee of Newcastle University and also adhering to ARRIVE guidelines.
**Enriched Environment (EE) and animal groups**

One week after surgery, the BCAS and sham mice were randomly assigned to six subgroups, three different levels of EE per main group for 12 weeks: standard housing no EE, limited exposure to EE and full-time exposure to EE. Standard housing denotes normal housing conditions, which incorporated a paper house and shredded tissue. EE cages had extra toys in addition to the standard housing e.g. running wheels, hanging chains, igloos, and a paper tunnel. Limited exposure to EE was performed as described previously [5]. Briefly, for the first four weeks, mice were transferred to the EE cages for 3 hours daily in the morning from 9am to noon. From 5th week to 12th week after BCA surgery, mice experienced EE for 3 hours, 3 days a week. Full-time EE group was exposed to EE every day for 24 hours over the entire 12 weeks [4].

**Cognitive Behaviour and Working Memory Testing**

Cognitive function (mainly working memory) was assessed in each mouse from 13 weeks post BCAS surgery during a period of 20 days, using a novel 3-dimensional (3D) 9-arm radial maze (3D-RAM) essentially as described previously [4]. The number of arm entries, number of arm repeats in each session were recorded. The probability of each pair of arm repeats in 9 arm entries were then calculated (Figure 4). The final arm repeat score shown in this study represent the average of the scores in 10 consecutive sessions from day 11 to day 20, as in this period, mice were acclimatised to the maze and the score reflect cognitive functions in each animal accurately. We also performed correlation analysis between arm repeat score and infarct volume in the frontal lobe and frontal cortex in each BCAS mice.

**Quantitative Proteomic Analysis by TMT6-based LC-MS/MS**

Brain tissues (~40 mg/group) including the white matter from BCAS and sham animals were retained for molecular analysis [6]. Tissues with washed metallic beads were suspended in 200 μL of buffer (0.5% (w/v) sodium deoxycholate (SDC) in 100 mM ammonium acetate (AA) at pH 6.0 supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) in Eppendorf safe-lock tubes (Hamburg, Germany) for homogenization in a bullet blender homogenizer (Next Advance, NY, USA) at high intensity for 5 min at 4 °C. Protein extracts
were then purified by acetone precipitation for 30 min at -20 °C. Purified protein pellets were further resuspended in 100 mM AA buffer for subsequent proteomic sample preparation. Reduction, alkylation and tryptic digestion of proteins was performed [7]. After digestion, the tryptic peptides were dried using an Eppendorf vacuum concentrator. Dried tryptic peptides were then labelled with TMT6 tags per manufacturer’s protocol (Thermo Scientific, IL, USA). TMT tags were distributed as follows: 126 Sham Standard Control, 127 Sham Environmental Enrichment (EE), 128 Sham 3H (EE), 129 BCAS EE, 130 BCAS Standard and 131 BCAS 3h EE. The individual labelled peptide samples were mixed together, dried, desalted and vacuum dried for HPLC fractionation on a XBridge C18 column (4.6 × 200 mm, 5 μm, 300 Å) (Waters, MA, USA) at a flow rate of 1.0 ml/min using HPLC as previously described [8]. The LC-MS/MS analysis was performed using Dionex Ultimate 3000 RSLC nanoLC system coupled to a Q-Exactive apparatus (Thermo Fisher, MA, USA) as described previously [8]. Two μg of labelled peptides in each fraction were injected into an acclaim peptide trap column via the autosampler of the Dionex RSLC nanoLC system for the LC-MS/MS analysis.

Database search and data analysis for Proteomic Analysis by TMT6-based LC-MS/MS

Raw files were directly imported into the PD with deisotope and deconvolution in MS/MS spectra. The processed MS/MS spectra were queried against a Uniprot mouse database (downloaded on 18th February 2016 with 117522 total sequences and 54026430 total residues) using both SequestHT and Mascot search engines. The parameters set were enzyme: trypsin; maximum miss cleavage: 2; precursor mass tolerance: 10 ppm; fragment mass tolerance: 0.02Da; fixed modification: carbamidomethylation at Cys and TMT6 at peptide N-terminal and Lys; dynamic modification: Oxidation at Met, deamidation at Asp and Gln; The peptide spectral matches (PSMs) were further processed by “percolator” algorithm where target FDR (strict) was set as 0.01, target FDR (relaxed) was set as 0.05. The relative protein quantitation based on the TMT reporter ions was performed by the default method for TMT 6plex labelling in the consensus workflow of PD2.2. The results were exported to a text file that were then imported to Microsoft Excel for further analysis. Regulation cut-offs to identify differential protein expression between BCAS and sham groups were established based on the calculated percentage coefficient of variation (% CV) of protein ratios as previously indicated [9]. A total 95 % of the proteins identified in our study displayed a ratio % CV <50 %, therefore only proteins with % CV >50 were considered.
as differentially regulated. Furthermore, only those differentially regulated proteins, which showed a False Discovery Rate (FDR) adjusted p-value (q-value) <0.001 were considered.

**Statistical Analysis**

In the box plots, the boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Error bars above and below the box indicate the 90th and 10th percentiles respectively.

**Supplementary References:**


12. Pan Y, Thapa D, Baldissera L, Jr., Argunhan F, Aubdool AA, Brain SD. Relevance of TRPA1 and TRPM8 channels as vascular sensors of cold in the cutaneous microvasculature. Pflugers Arch 2018; 470: 779-786

### Supplementary Table: Effects of EE on Brain Proteins in Sham and BCAS Animals

<table>
<thead>
<tr>
<th>Protein Description / Gene symbol</th>
<th># peptides[^1]</th>
<th>MW [kDa]</th>
<th>Sham-3hrs</th>
<th>Sham-Full</th>
<th>BCAS-Std</th>
<th>BCAS-3hrs</th>
<th>BCAS-Full</th>
<th>Brain infarcts[^4]</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td>Thioredoxin domain-containing protein 5 / TXNDC5</td>
<td>13</td>
<td>46.4</td>
<td>0.86</td>
<td>0.4</td>
<td>1.15</td>
<td>1.26</td>
<td>0.59*</td>
<td>Contributes to endothelial dysfunction</td>
<td>[10]</td>
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<tr>
<td>Peroxisomal targeting signal 1 receptor / PEX5</td>
<td>11</td>
<td>66.8</td>
<td>0.9</td>
<td>0.15</td>
<td>1.03</td>
<td>1.11</td>
<td>0.5*</td>
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<td>COMM domain-containing protein / COMMD9</td>
<td>8</td>
<td>21.8</td>
<td>0.06</td>
<td>0.02</td>
<td>1.13</td>
<td>1.21</td>
<td>0.14*</td>
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<td>Synapse-associated protein 1 / SYAP1</td>
<td>7</td>
<td>41.3</td>
<td>1.06</td>
<td>0.21</td>
<td>1.42</td>
<td>1.57</td>
<td>0.48*</td>
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<td>OTU domain-containing protein 6B / OTUD6B</td>
<td>5</td>
<td>37.2</td>
<td>0.01</td>
<td>0.18</td>
<td>1.17</td>
<td>1.27</td>
<td>-0.32</td>
<td>Decrease in deubiquitination causes neuroprotection in hypoxia</td>
<td>[11]</td>
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<tr>
<td>Transmembrane protein 132A / TMEM132A</td>
<td>7</td>
<td>110.2</td>
<td>0.03</td>
<td>0.08</td>
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<td>1.12</td>
<td>-0.19</td>
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<td>Mesoderm development candidate 1 / MESD1</td>
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<td>37.8</td>
<td>-0.35</td>
<td>-0.25</td>
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<td>1.17</td>
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<tr>
<td>Snapin / SNAPIN</td>
<td>4</td>
<td>15</td>
<td>0.13</td>
<td>0.14</td>
<td>1.06</td>
<td>1.06</td>
<td>-0.01</td>
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<td>TRPM8 channel-associated factor 1 / TCAF1</td>
<td>5</td>
<td>102.4</td>
<td>-0.05</td>
<td>0.22</td>
<td>1.02</td>
<td>1.3</td>
<td>-0.01</td>
<td>Cold/pH sensor that regulates vasoconstriction [12]</td>
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<tr>
<td>Serpin B8 / SERPINB8</td>
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<td>42.1</td>
<td>0.18</td>
<td>0.11</td>
<td>1.73</td>
<td>1.97</td>
<td>-0.33</td>
<td>Levels increase in neuronal tissues under hypoxia conditions [13]</td>
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</tr>
</tbody>
</table>

1. Peptides exclusively identified for the protein. 2. Abundance log2 ratio in all cases compared to sham. A restrictive cut-off range based on 50% CV was established to indicate significant regulation. All the proteins included in this list shown q-values (FDR adjusted p-values) lower than 0.001. 4. Potential role(s) of differentially regulated proteins in the apparition/progression of brain infarcts based on the reference(s) provided. * indicates significant regulation in sham-EE and BCAS-EE compared to Sham and BCAS respectively.