# SUPPLEMENT S2: RT-QPCR METHODS

## Table S2.1 – Experimental design

| Definition of experimental and control groups | Experimental: Madison (MSN) mouse strain. Control: outbred hsd:ICR (ICR) mouse strain. |
| Number in each group | 8 MSN, 8 ICR. All mice were male. |
| Assay carried out by core or investigator’s laboratory | Carried out in investigator’s laboratory. |
| Authors’ contributions to qPCR section | C. Michael Saul: all molecular work, half of dissection work, writing. Griffin M. Gessay: half of dissection work. Stephen C. Gammie: bred mice, provided funding and lab space, writing. |

## Table S2.2 – Tissue Samples

| Description | Fresh frozen whole hippocampus. |
| Volume or Mass of sample | See table S2.10. |
| Dissection Type | Gross dissection of hippocampal tissue from brain. |
| Processing Procedure | Animals were euthanized by cervical dislocation under isoflurane anesthetic, decapitated, and their hippocampi were immediately dissected from their brains. |
| If frozen, how quickly? | Samples frozen immediately on dry ice upon dissection. |
| Sample storage conditions | Stored at -80°C for no more than 12 weeks prior to RNA extraction. |

## Table S2.3 – Nucleic Acid Extraction

| Procedure and/or instrumentation | Mortar and pestle disruption, guanidinium thiocyanate-phenol-chloroform extraction, and spin column cleanup and purification. |
| Name of kit and details of any modifications | Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Kit (catalog number 732-6830) used according to the manufacturer’s specifications. |
| Sources of additional reagents used | Chloroform (Acros Organics, catalog number AC42355-0250) Ethanol (Fisher Scientific, catalog number BP2818-500) |
| Details of DNase treatment | On-column treatment with DNase I according to manufacturer's specifications. |
| Contamination assessment of input RNA | NanoDrop curves used to assess presence of presence of protein, salt, and organic contaminants. All curves indicated clean samples. |
| Nucleic acid quantification | See table S2.10. |
| Instrument and method of nucleic acid quantification | NanoDrop spectrophotometer, absorbance at 260nm. |
| Purity (A$_{260}$/A$_{280}$) | See table S2.10. |
| Yield | See table S2.10. |
| RNA integrity instrument | Agilent RNA 6000 Nano Chips with Agilent BioAnalyzer 2100. |
| RIN | See table S2.10. |
| Inhibition testing | C$_q$ dilution, 1:8 using Ywhaz. As expected, diluted samples ran ~3 cycles behind undiluted samples. |
### Table S2.4 – Reverse Transcription

<table>
<thead>
<tr>
<th><strong>Complete reaction conditions</strong></th>
<th>500µM dNTP mix, 20mM Tris-HCl (pH 8.4), 50mM KCl, 5mM MgCl₂, 2.5mM dT 20mers, 10mM DTT, 2U/µL RNaseOUT, 10U/µL SuperScript III RT.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amount of RNA and reaction volume</strong></td>
<td>25µL reactions, 2µg total RNA used in each reaction. 1.25µL RNase H added after reaction termination.</td>
</tr>
<tr>
<td><strong>Priming oligonucleotide and concentration</strong></td>
<td>oligo-dT 20mers, final reaction concentration of 2.5mM.</td>
</tr>
<tr>
<td><strong>Temperature and time</strong></td>
<td>Prior to cDNA synthesis, RNA, primers, and dNTPs were denatured together at 65°C for 5 min. cDNA synthesis took place at 50°C for 50 min followed by an 85°C reaction termination step for 5 min. After reaction termination, the RNase reaction ran at 37°C for 20 min.</td>
</tr>
<tr>
<td><strong>Manufacturer of reagents and catalog number</strong></td>
<td>Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR (catalog number 18080-051)</td>
</tr>
<tr>
<td><strong>C_q with and without RT</strong></td>
<td>See table S2.10 for no RT C_q.s. While some gDNA contamination is present, its effect on experiments are negligible and stochastic according to hypothesis testing on no RT controls. This contamination is mostly a source of random error.</td>
</tr>
<tr>
<td><strong>Storage conditions of cDNA</strong></td>
<td>Stored at -80°C for no longer than 6 months.</td>
</tr>
</tbody>
</table>

### Table S2.5 – qPCR Target Information

<table>
<thead>
<tr>
<th><strong>Gene symbol</strong></th>
<th>See table S2.11.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Accession number</strong></td>
<td>See table S2.11.</td>
</tr>
<tr>
<td><strong>Location of amplicon</strong></td>
<td>See table S2.11.</td>
</tr>
<tr>
<td><strong>Amplicon length</strong></td>
<td>See table S2.11.</td>
</tr>
<tr>
<td><strong>In silico specificity</strong></td>
<td>All primers screened for specificity using NCBI Primer-BLAST.</td>
</tr>
<tr>
<td><strong>Homologs amplified</strong></td>
<td>No primers amplified pseudogenes or retropseudogenes.</td>
</tr>
<tr>
<td><strong>Sequence alignment</strong></td>
<td>Aligned in NCBI Primer-BLAST.</td>
</tr>
<tr>
<td><strong>Location of each primer by exon or intron</strong></td>
<td>See table S2.11.</td>
</tr>
<tr>
<td><strong>Targeted splice variants</strong></td>
<td>Each primer set targets all splice variants for every transcript of interest as they are documented in the NCBI RefSeq RNA database.</td>
</tr>
</tbody>
</table>

### Table S2.6 – qPCR oligonucleotides

<table>
<thead>
<tr>
<th><strong>Primer sequences</strong></th>
<th>See table S2.11.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Probe sequences</strong></td>
<td>Not applicable; dsDNA binding dye chemistry used.</td>
</tr>
<tr>
<td><strong>Location and identity of any modifications</strong></td>
<td>No modifications.</td>
</tr>
<tr>
<td><strong>Manufacturer of oligonucleotides</strong></td>
<td>UW-Madison Biotechnology Center DNA Synthesis Facility.</td>
</tr>
<tr>
<td><strong>Purification method</strong></td>
<td>Standard desalting and lyophilization.</td>
</tr>
</tbody>
</table>
### Table S2.7 – qPCR protocol

<table>
<thead>
<tr>
<th><strong>Complete reaction conditions</strong></th>
<th>2X Bio-Rad SsoFast EvaGreen Super Mix without ROX (catalog number 172-5204) used with no modifications.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reaction volume and amount of cDNA/DNA</strong></td>
<td>20µL reactions; 2µL 1:5 diluted cDNA used in each reaction.</td>
</tr>
<tr>
<td><strong>Primer Concentration</strong></td>
<td>500nM forward and 500nM reverse primer for all primer sets.</td>
</tr>
<tr>
<td><strong>Mg(^{2+}) concentration</strong></td>
<td>3.0mM MgCl(_2).</td>
</tr>
<tr>
<td><strong>dNTP concentration</strong></td>
<td>200µM each of dATP, dTTP, dCTP, and dGTP. 800µM dNTP total.</td>
</tr>
<tr>
<td><strong>Polymerase identity</strong></td>
<td>Bio-Rad SsoFast Taq Fusion Polymerase.</td>
</tr>
<tr>
<td><strong>Polymerase concentration</strong></td>
<td>Proprietary concentration.</td>
</tr>
<tr>
<td><strong>Buffer identity and manufacturer</strong></td>
<td>Bio-Rad qPCR buffer provided with SsoFast EvaGreen Supermix.</td>
</tr>
<tr>
<td><strong>Exact buffer chemistry</strong></td>
<td>Proprietary composition.</td>
</tr>
<tr>
<td><strong>PCR additives used</strong></td>
<td>No additives used.</td>
</tr>
<tr>
<td><strong>Manufacturer of plates and catalog number</strong></td>
<td>Applied Biosystems MicroAmp Fast 96-Well Reaction Plates (catalog number 4346907).</td>
</tr>
<tr>
<td><strong>Complete thermal cycling parameters</strong></td>
<td>Incubation stage: 30s at 95°C. Cycling stage: 40 cycles, 3 steps: 5s at 95°C, 20s at annealing temperature (see table S2.11 for the specific annealing temperature used with each primer set), and 20s at 72°C.</td>
</tr>
<tr>
<td><strong>Reaction setup</strong></td>
<td>Manual using Eppendorf single channel adjustable volume pipettes.</td>
</tr>
<tr>
<td><strong>qPCR instrument</strong></td>
<td>Applied Biosystems StepOnePlus.</td>
</tr>
</tbody>
</table>

### Table S2.8 – qPCR validation

<table>
<thead>
<tr>
<th><strong>Evidence of optimization</strong></th>
<th>Prior to analysis, we ran each primer set at several annealing temperatures. We used the annealing temperature with the earliest C(_q) and the highest efficiency.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Specificity</strong></td>
<td>Stringent <em>in silico</em> testing of primers prior to qPCR using Primer-BLAST, dissociation curve test of specificity <em>in vitro</em>.</td>
</tr>
<tr>
<td><strong>C(_q) of NTC</strong></td>
<td>C(_q) &gt; 40 for all NTCs for all genes.</td>
</tr>
<tr>
<td><strong>Calibration curves with slope and y-intercept (m, b)</strong></td>
<td>See table S2.12.</td>
</tr>
<tr>
<td><strong>Efficiency calculated from slope</strong></td>
<td>See table S2.12.</td>
</tr>
<tr>
<td><strong>r(^2) of calibration curve</strong></td>
<td>See table S2.12.</td>
</tr>
<tr>
<td><strong>Linear dynamic range (LDR)</strong></td>
<td>See table S2.12.</td>
</tr>
<tr>
<td><strong>C(_q) variation at Limit of Detection</strong></td>
<td>LOD measurements not necessary for relative quantification.</td>
</tr>
<tr>
<td><strong>Evidence for LOD</strong></td>
<td>LOD measurements not necessary for relative quantification.</td>
</tr>
<tr>
<td><strong>If multiplex, efficiency and LOD for each assay</strong></td>
<td>Not applicable; dsDNA binding dye chemistry used.</td>
</tr>
</tbody>
</table>
Table S2.9 – Data analysis

<table>
<thead>
<tr>
<th>qPCR analysis program</th>
<th>Relative Expression Software Tool (REST)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method of Cq determination</td>
<td>Used ABI StepOnePlus software to determine ABI’s Cq value.</td>
</tr>
<tr>
<td>Outlier identification and disposition</td>
<td>Our experiments contain no outliers.</td>
</tr>
<tr>
<td>Results from NTC</td>
<td>All NTCs have no amplification.</td>
</tr>
<tr>
<td>Justification of number and choice of reference genes</td>
<td>The combination of Sdha and Ywhaz was found to be the most stable combination of reference genes by Gubern et al. (2009).</td>
</tr>
<tr>
<td>Description of normalization</td>
<td>Data normalized for baseline fluorescence.</td>
</tr>
<tr>
<td>Number and stage (reverse transcription or qPCR) of technical replicates</td>
<td>3 qPCR technical replicates.</td>
</tr>
<tr>
<td>Statistical methods for results significance</td>
<td>Randomization test for significance.</td>
</tr>
<tr>
<td>Software (source, version) of stats</td>
<td>StepOnePlus 2.1; REST 2009.</td>
</tr>
</tbody>
</table>

Table S2.10 – Sample quality control

<table>
<thead>
<tr>
<th>ID</th>
<th>Strain</th>
<th>Tissue Mass</th>
<th>RNA Concentration</th>
<th>RNA Yield</th>
<th>Cq (RT/no RT)†</th>
<th>A260 : A280</th>
<th>RIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MSN</td>
<td>35.8 mg</td>
<td>222.34 ng/µL</td>
<td>35.574 µg</td>
<td>18.20/36.78</td>
<td>2.11</td>
<td>8.2</td>
</tr>
<tr>
<td>2</td>
<td>MSN</td>
<td>36.7 mg</td>
<td>248.83 ng/µL</td>
<td>39.813 µg</td>
<td>17.84/40.00</td>
<td>2.12</td>
<td>8.3</td>
</tr>
<tr>
<td>3</td>
<td>MSN</td>
<td>35.7 mg</td>
<td>271.41 ng/µL</td>
<td>43.426 µg</td>
<td>18.01/31.40</td>
<td>1.99</td>
<td>8.3</td>
</tr>
<tr>
<td>4</td>
<td>MSN</td>
<td>35.8 mg</td>
<td>235.50 ng/µL</td>
<td>37.680 µg</td>
<td>17.75/38.96</td>
<td>2.12</td>
<td>8.2</td>
</tr>
<tr>
<td>5</td>
<td>MSN</td>
<td>60.0 mg</td>
<td>454.53 ng/µL</td>
<td>72.725 µg</td>
<td>18.09/36.62</td>
<td>2.08</td>
<td>8.4</td>
</tr>
<tr>
<td>6</td>
<td>MSN</td>
<td>48.2 mg</td>
<td>331.09 ng/µL</td>
<td>52.974 µg</td>
<td>17.88/35.23</td>
<td>2.11</td>
<td>8.2</td>
</tr>
<tr>
<td>7</td>
<td>MSN</td>
<td>41.0 mg</td>
<td>290.64 ng/µL</td>
<td>46.502 µg</td>
<td>17.83/38.10</td>
<td>2.11</td>
<td>8.5</td>
</tr>
<tr>
<td>8</td>
<td>MSN</td>
<td>46.0 mg</td>
<td>263.10 ng/µL</td>
<td>42.096 µg</td>
<td>17.78/38.97</td>
<td>2.11</td>
<td>8.3</td>
</tr>
<tr>
<td>9</td>
<td>ICR</td>
<td>50.7 mg</td>
<td>347.06 ng/µL</td>
<td>55.530 µg</td>
<td>18.03/34.77</td>
<td>2.09</td>
<td>8.5</td>
</tr>
<tr>
<td>10</td>
<td>ICR</td>
<td>32.2 mg</td>
<td>215.83 ng/µL</td>
<td>34.533 µg</td>
<td>17.66/36.45</td>
<td>2.12</td>
<td>8.2</td>
</tr>
<tr>
<td>11</td>
<td>ICR</td>
<td>44.2 mg</td>
<td>261.85 ng/µL</td>
<td>41.896 µg</td>
<td>17.77/33.17</td>
<td>2.13</td>
<td>8.3</td>
</tr>
<tr>
<td>12</td>
<td>ICR</td>
<td>38.2 mg</td>
<td>247.66 ng/µL</td>
<td>39.626 µg</td>
<td>17.83/39.20</td>
<td>2.12</td>
<td>8.3</td>
</tr>
<tr>
<td>13</td>
<td>ICR</td>
<td>42.0 mg</td>
<td>278.96 ng/µL</td>
<td>44.634 µg</td>
<td>17.97/37.30</td>
<td>2.11</td>
<td>8.9</td>
</tr>
<tr>
<td>14</td>
<td>ICR</td>
<td>37.2 mg</td>
<td>274.48 ng/µL</td>
<td>43.917 µg</td>
<td>17.98/38.91</td>
<td>2.12</td>
<td>8.7</td>
</tr>
<tr>
<td>15</td>
<td>ICR</td>
<td>30.7 mg</td>
<td>236.85 ng/µL</td>
<td>37.896 µg</td>
<td>17.83/36.06</td>
<td>2.12</td>
<td>9.2</td>
</tr>
<tr>
<td>16</td>
<td>ICR</td>
<td>43.3 mg</td>
<td>288.02 ng/µL</td>
<td>46.083 µg</td>
<td>18.02/35.53</td>
<td>2.12</td>
<td>9.4</td>
</tr>
</tbody>
</table>

†RT versus no RT data were collected using the reference gene Ywhaz. Cq = 40 indicates no amplification detected.

*No amplification was detected in at least 1 of the replicates in these no RT controls.
### Table S2.11 – Primers

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>RefSeq Accession</th>
<th>Primer Sequence (5’-3’)</th>
<th>Product Length</th>
<th>Amplicon Location</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ywhaz</td>
<td>NM_01174</td>
<td>F: TCCTATTCCCTCTGGGACAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ATGGAAAGCTACATTACGGTT</td>
<td>92 bp</td>
<td>Exon 5; 2432-2523</td>
<td>58°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3’ UTR)</td>
<td></td>
</tr>
<tr>
<td>Sdha</td>
<td>NM_02328</td>
<td>F: CCGCTCCTACTGATAAGGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGCCAACTCAATCCCTTAC</td>
<td>179 bp</td>
<td>Exon 12; 2015-2193</td>
<td>58°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(ORF, 3’ UTR)</td>
<td></td>
</tr>
<tr>
<td>P2x7</td>
<td>NM_011027</td>
<td>F: CGAATTATGGCACCGTCAAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TCTCCGTACCTCTGCTATG</td>
<td>150 bp</td>
<td>Exons 1, 2; 234-383</td>
<td>57°C</td>
</tr>
<tr>
<td>Epor</td>
<td>NM_010149</td>
<td>F: GTCCGATTCTGGCATCTCA</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>R: GGACAAGGCTGTTCATAG</td>
<td>107 bp</td>
<td>Exon 8; 1519-1625</td>
<td>58°C</td>
</tr>
<tr>
<td>Fhit</td>
<td>NM_010210</td>
<td>F: CAAACGATTTCCCAAGGTTAA</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>R: GGGTACAATAAAGAGTGGTAG</td>
<td>89 bp</td>
<td>Exon 7; 697-763</td>
<td>58°C</td>
</tr>
<tr>
<td></td>
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<td>(3’ UTR)</td>
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</tr>
<tr>
<td>Cmklr1</td>
<td>NM_00815</td>
<td>F: ATCTTACACCACCATGCCACG</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTATACACACTGAAAGAGAC</td>
<td>95 bp</td>
<td>Exon 3; 2003-2097</td>
<td>58°C</td>
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<td></td>
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<td>(3’ UTR)</td>
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<tr>
<td>Npsr1</td>
<td>NM_175678</td>
<td>F: GTAGAGGGAACTAAAGGAATT</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>R: TAGACAAAGACCTGAGGAGAT</td>
<td>106 bp</td>
<td>Exon 10; 2866-2971</td>
<td>57°C</td>
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<td></td>
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<td>(3’ UTR)</td>
<td></td>
</tr>
<tr>
<td>Tac1</td>
<td>NM_009311</td>
<td>F: ACGCAGTATCTTTATCGCTCC</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>R: AGAATCACAAGGGCGTTAC</td>
<td>167 bp</td>
<td>Exon 7; 502-668</td>
<td>58°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3’ UTR)</td>
<td></td>
</tr>
<tr>
<td>Cat</td>
<td>NM_009804</td>
<td>F: TTCCCACTTGAGATAGTGGTGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTGAAAGCAACCAACACGG</td>
<td>119 bp</td>
<td>Exon 13; 2358-2476</td>
<td>56°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3’ UTR)</td>
<td></td>
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</tbody>
</table>

### Table S2.12 – qPCR quality control

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>PCR Efficiency</th>
<th>Linear Dynamic Range</th>
<th>r&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Slope</th>
<th>y Intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ywhaz</td>
<td>98.375%</td>
<td>C&lt;sub&gt;q&lt;/sub&gt;: 17.60-27.71</td>
<td>0.998</td>
<td>-3.362</td>
<td>25.020</td>
</tr>
<tr>
<td>Sdha</td>
<td>97.271%</td>
<td>C&lt;sub&gt;q&lt;/sub&gt;: 17.97-28.19</td>
<td>0.998</td>
<td>-3.389</td>
<td>25.472</td>
</tr>
<tr>
<td>P2x7</td>
<td>96.354%</td>
<td>C&lt;sub&gt;q&lt;/sub&gt;: 27.06-33.29</td>
<td>0.989</td>
<td>-3.412</td>
<td>34.583</td>
</tr>
<tr>
<td>Epor</td>
<td>99.573%</td>
<td>C&lt;sub&gt;q&lt;/sub&gt;: 25.42-31.47</td>
<td>0.998</td>
<td>-3.332</td>
<td>32.743</td>
</tr>
<tr>
<td>Fhit</td>
<td>104.902%</td>
<td>C&lt;sub&gt;q&lt;/sub&gt;: 26.25-33.86</td>
<td>0.990</td>
<td>-3.210</td>
<td>33.346</td>
</tr>
<tr>
<td>Cmklr1</td>
<td>98.375%</td>
<td>C&lt;sub&gt;q&lt;/sub&gt;: 27.92-35.92</td>
<td>0.994</td>
<td>-3.362</td>
<td>35.382</td>
</tr>
<tr>
<td>Npsr1</td>
<td>99.305%</td>
<td>C&lt;sub&gt;q&lt;/sub&gt;: 30.68-34.69</td>
<td>0.989</td>
<td>-3.339</td>
<td>38.136</td>
</tr>
<tr>
<td>Tac1</td>
<td>97.414%</td>
<td>C&lt;sub&gt;q&lt;/sub&gt;: 23.69-29.79</td>
<td>0.997</td>
<td>-3.385</td>
<td>31.181</td>
</tr>
<tr>
<td>Cat</td>
<td>97.228%</td>
<td>C&lt;sub&gt;q&lt;/sub&gt;: 28.24-34.31</td>
<td>0.987</td>
<td>-3.390</td>
<td>35.793</td>
</tr>
</tbody>
</table>