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HuR Function and Translational State Analysis Following Global Brain Ischemia and Reperfusion

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Abstract

Prolonged translation arrest in post-ischemic hippocampal CA1 pyramidal neurons precludes translation of induced stress genes and directly correlates with cell death. We evaluated regulation of mRNAs containing adenine and uridine rich elements (ARE) by assessing HuR protein and *hsp70* mRNA nuclear translocation, HuR polysome binding, and translation state analysis of CA1 and CA3 at 8 hr reperfusion after 10 min global cerebral ischemia. There was no difference between CA1 and CA3 at 8 hr of reperfusion in nuclear or cytoplasmic HuR protein or *hsp70* mRNA, or HuR polysome association, suggesting neither mechanism contributed to post-ischemic outcome. Translation state analysis revealed that 28% and 58% of unique mRNAs significantly different between 8hR and NIC, in CA3 and CA1, respectively, were not polysome-bound. There was significantly greater diversity of polysome-bound mRNAs in reperfused CA3 compared to CA1, and in both regions ARE-containing mRNAs accounted for 4% – 5% of the total. These data indicate that post-transcriptional ARE-containing mRNA regulation occurs in reperfused neurons and contributes to post-ischemic outcome. Understanding the differential responses of vulnerable and resistant neurons to ischemia will contribute to the development of effective neuroprotective therapies.

Keywords

CA1; CA3; global cerebral ischemia; HSP70; HuR; microarray; stress responses; translation state analysis

INTRODUCTION

A prolonged translation arrest (TA) correlates precisely with hippocampal CA1 pyramidal neuron death after global brain ischemia [1, 2]. Over two decades ago it was established that, in spite of copious transcription, TA in CA1 prevents translation of ischemia-induced stress genes such as *hsp70* [3, 4], *c-fos* [5], or *c-jun* [5]. We observed *hsp70* transcription to be greater in CA1 compared to the ischemia-resistant CA3 region [6]. How such high levels of transcript accumulation can occur in the absence of translation still remains a mystery, the

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COMPLIANCE WITH ETHICS REQUIREMENTS

Jeffrey J. Szymanski declares that he has no conflict of interest.

Haihui Huang declares that she has no conflict of interest.

Jill T. Jamison declares that she has no conflict of interest.

Donald J. DeGracia declares that he has no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

solution to which is expected to shed significant light on the nature of CA1 selective vulnerability [2, 7, 8].

Two mechanisms of prolonged TA have been demonstrated in post-ischemic CA1: sequestration of ribosomes in protein aggregates [9, 10] and the sequestration of mRNA away from ribosomal subunits in the form of mRNA granules [11, 12] (see Lewis et al. this issue). Colocalization studies [11, 12] support the conclusion that mRNA granules are a form of HuR granule [13, 14]. When HuR colocalized with mRNA granules during reperfusion, *hsp70* translation occurred [11]. Because HuR is a well-studied mRNA regulatory protein, we posited that differential mRNA regulation may play a role in the prolonged TA of reperfused CA1 [7].

HuR is a member of the ELAV protein family which includes HuR/HuA, HuB, HuC and HuD [15]. HuR is ubiquitously expressed in mammals [16] while other Hu family members are most highly expressed in neurons [15]. The ELAV proteins are members of a superfamily of mRNA regulatory proteins that bind to the adenine and uridine rich element (ARE), a 3' cis-acting sequence in target mRNAs [17]. ARE sequences are estimated to occur in 5% - 8% of all human transcripts [18]. This relatively high abundance suggests AREs are an important mechanism of mRNA regulation.

HuR's function was first described in the context of preventing the degradation of ARE-containing mRNAs (ARE-mRNAs) [19, 20]. It has since been discovered that HuR is a multifunctional protein. In addition to stabilizing ARE-mRNAs, HuR can mediate nuclear export of ARE-mRNAs during heat shock [21], facilitate translation of ARE-mRNAs [22, 23], and participate in stress granules [24, 25] and HuR granules [11]. HuR's function in the latter is understood in terms of an "RNA regulon" model where combinations of mRNA regulatory proteins bind mRNAs sharing common cis-acting regulatory sequences to mediate and regulate all aspects of mRNA metabolism [7, 26].

Here we investigated, in reperfused CA1 and CA3, two possible roles of HuR. First, we asked if HuR was involved with nuclear export of *hsp70*, the most prominently expressed ARE-containing mRNA in the post-ischemic brain. We observed no difference in nuclear or cytoplasmic levels of HuR protein or *hsp70* mRNA between CA1 and CA3 at 8h of reperfusion, suggesting that HuR-mediated nuclear transport is not a major regulatory mechanism during reperfusion. Second, we investigated the role of HuR in facilitating translation of ARE-mRNA in reperfused brain. We did not observe a difference in HuR distribution on polysome profiles between controls and reperfused animals. However, we performed translational state analysis in which mRNAs are extracted from polysomes and identified by microarray. Translational state analysis of CA1 and CA3 at 8 hours reperfusion showed enrichment of ARE-mRNAs, and additionally showed major differences in polysome-associated mRNAs between CA1 and CA3. These data indicate that post-transcriptional regulation contributes to outcome differences in post-ischemic neurons but is not confined exclusively to ARE-mRNAs.

MATERIALS AND METHODS

Materials

HuR (sc-5261) antiserum was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-lamin B1 (16048), rabbit anti-PABP (21060), and rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (25778) were purchased from Abcam (Cambridge, MA). Mouse anti-S6 (2317S) and rabbit anti-L7a (2403) were from Cell Signaling (Danvers, MA). Cycloheximide (CHX) (c1988) was purchased from Sigma (St. Louis, MO). All other chemicals were reagent grade.

Global brain ischemia by bilateral carotid artery occlusion with hypotension

All animal experimental protocols were approved by the Wayne State University Animal Investigation Committee and were conducted following the *Guide for the Care and Use of Laboratory Animals* (National Research Council, revised 2011). All efforts were made to reduce animal suffering and minimize the total number of animals used. Normothermic global forebrain ischemia of 10 min duration was induced in male Long Evans rats using the bilateral carotid artery (two-vessel) occlusion and hypovolemic hypotension (2VO/HT) model of Smith et al. [27], as previously described [6, 11, 12, 28]. Exclusion criteria and survival rates were as previously reported [11]. Experimental groups were: sham-operated, non-ischemic controls (NIC) and 10 min ischemia and 8 hr reperfusion (8hR). Numbers of animals and brain regions used are described below in the context of each experiment.

Microdissection of CA1, CA3 and forebrain

For all dissection procedures and all procedures involving mRNA, RNase contamination was minimized by taking the following steps. Consumables were RNase-free and containers and surfaces were treated with RNaseZap (Invitrogen, Carlsbad, CA) and rinsed x5 with sterile water. Procedures were performed under a laminar flow hood whenever possible, and all solutions were prepared with DEPC-treated water.

Hippocampal CA1 and CA3 were microdissected as previously described [28] with the above modifications to minimize RNase contamination. Experimental and control rats were anesthetized in 5% halothane and euthanized by decapitation. Brains were carefully dissected within < 1 min by removing the skull with a rongeur. Whole brains were quickly rinsed in sterile distilled water and then snap frozen in dry ice and ethanol for 15 seconds. Brains were placed in a brain blocker, allowed to thaw for ~ 5 sec, and then a 1 mm slice taken at -2.30 mm to -3.30 mm posterior to Bregma (27), containing the dorsal hippocampus. The slice was placed on a dissecting stage that had been decontaminated with RNaseZap, and CA1 and CA3 were dissected under a dissecting microscope, while maintaining the tissue in a semi-frozen state by application of dry ice to the dissecting stage. The CA3 region was separated by a vertical cut slightly medial to the curve of the CA3 followed by a second vertical cut at the lateral edge of the dorsal hippocampus. Surrounding cortex was removed to isolate CA3. CA1 was separated from the dentate gyrus (DG) by a roughly horizontal cut superior to the upper blade of the DG. The corpus callosum was removed and CA1 isolated. Dissections were placed in pre-weighed, sterile, RNase free Eppendorf tubes and immediately frozen in dry ice and ethanol. The tubes containing microdissections were weighed. Frozen tissue was removed with sterile forceps and then hand-homogenized on ice (in buffer listed below) and further processed as described below. For a single rat, pooled, bilateral dissections of CA1 or CA3 gave 10–12 mg wet weight per each region.

For studies using forebrain as input, the brain was treated identically as described above. Brain was placed in a brain blocker and cut coronally at 1 mm anterior to Bregma, and the rostral tissue was taken, and consisted of bilateral cerebral cortex frontal lobe and striatum, but lacked hippocampus.

Nuclear and post-mitochondrial supernatant fractionation

Microdissected tissue samples were homogenized at 1:15 (w/v) in a homogenization buffer of 320 mM sucrose, 1 mM MgCl₂, 1 mM NaH₂PO₄, pH 6.6, 1 mM DTT, 80 units/mL RNase inhibitor (Invitrogen), 0.5% protease inhibitor cocktail (Sigma). Homogenization used a Kontes glass dounce homogenizer with 150-μM clearance to preserve the nuclear membrane. Nuclei were pelleted by 850 g centrifugation for 10 minutes, 4°C. Supernatant was centrifuged 13000 g for 30 min, 4°C, to generate post-mitochondrial supernatant

(PMS). Nuclear pellet was washed three times and then resuspended in 1 mL homogenization buffer and mixed with 5 mL of nuclear purification buffer (2.39 M sucrose, 1 mM MgCl₂, 1 mM NaH₂PO₄, pH 6.6, 10% (v/v) Triton X-100, 1 mM DTT, 80 units/mL RNase inhibitor, 0.5% protease inhibitor cocktail). Resuspended nuclei were centrifuged 48,000 g for 1 hour, 4°C in a Beckman SW50.1 rotor. Pellets of intact nuclei were washed x3 in homogenization buffer and then resuspended at minimal volume in a lysis buffer of 5 mM EDTA, 50 mM Tris, pH 7.5, 2% SDS.

Western blotting of subcellular fractions

Protein concentrations of nuclear and PMS fractions were determined by Lowry assay. Samples were run on 10% SDS-PAGE, electroblot transferred to nitrocellulose, and western blotting was performed exactly as previously described [12]. For loading controls, SDS-PAGE gels were silver stained after electroblot transfer. Antibody conditions were: mouse anti-HuR, 1:200, 2 hr, room temperature; anti-lamin B1, 1:5000, overnight, 4°C; and anti-GAPDH, 1:1000, 1 hr, room temperature. All primary antibodies were incubated in Tris buffered saline containing 2% Tween 20 (TTBS). Films were scanned on a Scanmaker 9800XL flatbed scanner (Microtek) and densitometry performed in BioImage Intelligent Quantifier v.4.

RNA isolation and real time PCR

RNA was extracted from hippocampal subregions by guanidinium-acid-phenol using the TRIzol reagent (Invitrogen) exactly according to vendor instructions. Final isolated RNA had an A₂₆₀:A₂₈₀ ratio >1.7, and RNA integrity was confirmed with denaturing agarose gel electrophoresis. cDNA was generated using the Transcriptor First Strand cDNA Synthesis kit (Roche, Grenzach-Wyhlen, Germany). cDNA was transcribed using poly-T primers to ensure that only mature and not nascent transcripts were amplified from nuclear fractions. PCR reactions used SYBR Green quantitative PCR (qPCR) Master Mix (Stratagene, La Jolla, CA) on an Mx3000P real time PCR thermocycler. Primers for qPCR were *gapdh* (5'-ACAAGATGGTGAAGGTCGGTGTGA-3', 5'-TTGTCATTGAGAGCAATGCCAGCC-3'; 1.0 kb product) and *hsp70* (5'-TCTTGGTTGCCAACACCCAAATCC-3', 5'-AAAGGTCACTGCTAGCTCCGTGTT-3'; 0.5 kb product). *gapdh* was used to normalize *hsp70*. After an initial 10 min at 95°C, all samples were amplified for 40 cycles of one minute annealing at 55°C, 30 seconds elongation at 72°C, and 30 seconds dissociation at 95°C followed by a dissociation curve. Amplification data were analyzed in MxPro v.3.00 (Stratagene) using 2^{-ΔΔCT} method for relative quantification [29]. Threshold fluorescence was determined using a minimum cycle threshold (Ct) spread algorithm in MxPro. Averaged differences between hippocampal subregions were evaluated by two-tailed Student's t-test with p < 0.05.

Polysome profiles

Polysome profiles, which use sucrose gradient centrifugation to isolate intact polysomes from the post-mitochondrial supernatant, were generated based on the methods of MacManus, *et al.* [30] and DeGracia, *et al.* [31]. All procedures were performed on ice or in a 4°C walk-in cold room. Sucrose gradients were prepared by layering sucrose concentrations of 15%, 20%, 25%, 30%, 35%, 40%, and 45% (w/v) dissolved in gradient buffer (50 mM Tris pH 7.4, 25 mM NaCl, 5 mM MgCl₂, 100 μg/mL CHX, 5.2 μL/mL protease inhibitor cocktail, 1 mM DTT, 80 U/mL RNase inhibitor) and equilibrating overnight at 4°C. Samples were homogenized at 1:2 (w/v) in a 7-mL dounce homogenizer in cold lysis buffer (340 mM sucrose, 50 mM Tris pH 7.4, 25 mM NaCl, 5 mM MgCl₂, 100 μg/mL CHX, 5.2 μL/mL protease inhibitor cocktail, 1 mM DTT, 80 U/mL RNase inhibitor, 1% (v/v) Triton X-100). Homogenates were centrifuged 1000 g, 10 min, 4°C to pellet nuclei

and membranes. Supernatants were then centrifuged 13000 g, 30 min, 4°C to generate a post-mitochondrial supernatant (PMS). Seventy five A₂₅₄ units of PMS were layered onto each 11.5-mL sucrose gradient and centrifuged at 240,000 g, 3 hr, 4°C (Beckman SW41Ti rotor). After centrifugation, each gradient was fractionated on a Beckman fraction recovery system at 1 mL/min using peristaltic pump bottom displacement by 66% (w/w) sucrose. Polysome profile absorbance was continuously recorded by strip chart recorder via a UV flow cell at 254 nm, and sucrose gradient fractions were collected as fifteen 800 µL aliquots using an automated Beckman fraction collector. Additional control studies evaluated the polysome profile dependence on: (1) input NIC PMS amounts of 25, 50, or 75 A₂₅₄ units, and (2) ionic strength using buffers containing 15 mM EDTA.

Western blots of polysome profile fractions

Each 800 µL fraction was concentrated to ~75 µL in a 3 kDa Amicon ultrafiltration device (Millipore, Billerica, MA) according to manufacturer instructions. One fraction per lane was run on 10% SDS-PAGE and Western blotted as above. Primary antisera conditions were: anti-S6, 1:250 in TTBS plus 5% dry milk, overnight, 4°C; anti-PABP, 1:500 in TTBS, overnight, 4°C; anti-L7a, 1:1000 in TTBS plus 5% dry milk, overnight, 4°C; and anti-HuR as described above. HuR density per fraction was expressed as a percent of total HuR density per sucrose gradient. Corresponding fraction averages were compared between experimental groups by two tailed Student's t-test with $p < 0.05$.

Translation state analysis

Translation state analysis (TSA) is the isolation of polysomes by sucrose gradient centrifugation, extraction of polysome-bound mRNAs, and identification of extracted mRNAs by microarray [32, 33]. TSA input samples of microdissected CA1 or CA3 were prepared from $n = 15$ animals per NIC or 8hR group, giving four experimental groups: (1) CA1 NIC, (2) CA1 8hR, (3) CA3 NIC and (4) CA3 8hR. Five individual microdissections per group were randomly pooled to provide enough material to run a single polysome profile (Figure 3A), giving $n = 3$ polysome profiles per group. Pooled regions were homogenized, PMS prepared, and polysome profiles run, all as described above. Gradient fractions containing polysome peaks that concentrated 40S and 60S markers were pooled to give the polysome-bound fractions (bound, B); the low density fractions that lacked markers of polysomes were also pooled to provide the polysome-unbound fraction (unbound, U) (Online Resource 1, Supplemental Figure 4A). RNA was extracted from B and U fractions as described above to produce 24 samples for microarray analysis.

Microarrays

Ethanol-washed RNA pellets were resuspended in sterile water and shipped overnight on dry ice to Genome Explorations (Memphis, Tennessee) who performed microarray hybridization as follows:

RNA quality control

RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Lab-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) as per the manufacturer's instructions. Representative samples demonstrated the integrity of extracted RNA used as input for microarrays (Online Resource 1, Supplemental Figure 4B).

cDNA synthesis and labeling

RNA (5–25 ng per sample) was converted to cDNA, amplified, fragmented, and labeled with biotin using the Ovation Pico WTA, Ovation Exon Module, and Encore Biotin Module kits according to the manufacturer's instructions (NuGEN, San Carlos, CA).

Oligonucleotide array hybridization and analysis

Fragmented, biotin-labeled cDNA was hybridized for 17 hr at 45°C to GeneChip Rat Gene 1.0 ST Arrays (Affymetrix, Santa Clara CA). The Rat Gene 1.0 ST array is comprised of 722,254 unique 25-mer oligonucleotide features representing all 26,309 rat genes, where each gene is represented on the array by approximately 27 probes spread across the full transcript length. Arrays were washed and stained with phycoerythrin-conjugated streptavidin (Life Technologies, Carlsbad, CA) in a Fluidics Station 450 (Affymetrix) according to the manufacturer's recommended procedures. Fluorescence intensities were determined using a GCS 3000 7G high-resolution confocal laser scanner, and analyzed using programs resident in the Affymetrix GeneChip Operating Software suite, v.3.2. (GCOS; Affymetrix). GCOS quality control metrics included average expression across arrays box plots and relative level of expression box plots. Principal component analysis (PCA) was conducted using GeneMaths XT (Applied Maths, Austin TX).

Microarray probe summation, normalization, filtering and class comparisons

Background correction by the PM-CGBG method and probe set signal summarization using the PLIER algorithm was performed in Affymetrix Expression Console. Quantile normalization, filtering, and class comparisons to identify probe sets exhibiting statistically significant differential expression were performed using BRB-ArrayTools [34]. The filter criterion for class comparisons was $\log(\text{intensity variation}) > 75\text{th percentile}$, reducing the number of transcripts compared from 26,309 to 6,578. The following class comparisons were performed on the filtered microarray results: (1) NIC CA1B vs. 8hR CA1B, (2) NIC CA3B vs. 8hR CA3B, (3) NIC CA1U vs. 8hR CA1U, (4) NIC CA3U vs. 8hR CA3U. Class comparison criteria were: absolute signal fold change > 2 , and significance threshold $p < 0.01$ on univariate two-tailed t-test. Results are expressed here as: (1) tables of significant hits that included parametric p value, false discovery rate (FDR), fold-change, Entrez ID and gene name, and (2) Venn diagrams.

Significant hits database searches

Differentially expressed transcripts in class comparisons were tested for the presence of: (1) ARE sequences, (2) internal entry ribosome sites (IRES), and (3) transcription factor regulation by searching significant hit lists against publically accessible databases. ARE sequences were determined using the AU-rich element containing database (ARED) (<http://brp.kfshrc.edu.sa/ARED/>) [18]. IRES sites were determined by searching against IRESite (Charles University, Prague, Czech Republic; <http://iresite.org>). Transcription factor binding site enrichment was tested in MATInspector (Genomatix, Ann Arbor, MI) [35].

RESULTS

HuR and hsp70 subcellular localization

If HuR mediated ARE-mRNA nuclear export during I/R, then levels of HuR protein should decrease in nuclear and increase in PMS fractions. Further, if HuR-mediated nuclear export contributed to the outcome difference between CA1 and CA3, then different levels of HuR and *hsp70* in nuclear and PMS fractions should be detected between CA1 and CA3.

Nuclear fractions lacked the cytoplasmic protein GAPDH, and PMS was free of the nuclear marker Lamin B1 (Figure 1A). Consistent with published microscopy work [21, 36], HuR levels were higher in nuclear fractions in both NIC and 8hR groups ($n = 6$ animals per experimental group; Figure 1B, input vs. nucleus lanes). HuR in PMS was 50% greater in CA1 compared to CA3 ($p < 0.05$). However, HuR in CA1 PMS from NIC and 8hR showed no change across groups (Figure 1B). For both CA1 and CA3, the amount of HuR was not

different between the NIC and 8hR groups in unfractionated homogenate, PMS, or nuclear fractions (Figure 1C).

Levels of *hsp70* mRNA in PMS and unfractionated homogenates were assessed by qPCR (Figure 4D). Validation of qPCR is shown in Online Resource 1, Supplemental Figure 1. Mean *hsp70* mRNA x-fold increases ($\pm$ SEM) at 8hR over NIC was 25 ± 5 and 24 ± 6 in unfractionated homogenates of CA1 and CA3, respectively, which were not statistically different ($p = 0.87$). In PMS, *hsp70* increased 489 ± 171 and 424 ± 176 in CA1 and CA3, respectively, at 8hR compared to NIC, and were not statistically different ($p = 0.36$). The RT-PCR for *hsp70* mRNA in PMS showed large within-group variance. However, microarray determination (discussed in more detail below) of *hsp70* mRNA in PMS, expressed as x-fold 8hR/NIC, gave 73.8 ± 6.7 for CA1, and 68.6 ± 3.7 for CA3, which were not statistically different (p Student's t-test > 0.05), thereby confirming the RT-PCR results.

Together, these observations indicate that: (1) HuR did not detectably exit the nucleus by 8hR in either CA1 or CA3, and (2) there was no difference in *hsp70* levels in subcellular fractions between CA1 and CA3.

Polysome association of HuR after I/R

Previous studies indicate that HuR can facilitate translation of ARE-mRNAs [22, 23]. A shift of HuR to heavier fractions in polysome profiles would indicate increased polysome association. Therefore we assessed HuR polysome profile distribution in NIC compared to 8hR in forebrain ($n = 4$ per group) because: (1) forebrain provided ample tissue for running polysome profiles, and (2) forebrain behaves similarly to CA3 at 8hR after 10 min ischemia by translating *hsp70* mRNA into HSP70 protein [37, 38].

Validation of polysome profiles (Online Resource 1, Supplementary Figure 2) showed: (1) 40S and 60S markers in heavier fractions, (2) PABP concentrated in lighter fractions, (3) intact rRNA concentrated in fractions containing the 40S and 60S markers, and (4) intact *hsp70* and *gapdh* mRNAs could be extracted from polysome-containing fractions in 8hR and NICs, respectively, and (5) polysome profiles were proportional to the amount of gradient input material, and were lost in the presence of EDTA (Online Resource 1, Supplementary Figure 3).

Individual fractions of polysome profiles for NIC and 8hR groups were Western blotted for HuR. HuR concentrated in light and intermediate density fractions and was detected at low levels in heavy, polysome-containing fractions (Figure 2A). However, there was no difference in the relative HuR distribution across polysome profiles between the NIC and 8hR groups (Figure 2B, p values ranged from 0.11 to 0.96).

Microarray quality control

Integrity of extracted RNA is illustrated in Online Resource 1, Supplemental Figure 2, and was further confirmed by capillary electrophoresis (Online Resource 1, Supplemental Figure 4B). Quality control metrics of the 24 individual microarray chips indicated reproducible and consistent signal intensities amongst experimental group replicates. Box plots before normalization showed chip mean intensities fell in a small range, with no outliers (Online Resource 1, Supplemental Figure 5A). Hierarchical cluster analysis demonstrated similarities of probe intensities amongst individual samples of each experimental group (Online Resource 1, Supplemental Figure 5B). Principal component analysis showed: (1) replicate chips clustered more within than amongst experimental groups, and (2) replicates of cognate groups in CA1 and CA3 (e.g. NIC CA1B vs. NIC CA3B, etc.) clustered closer together than did non-cognate groups (Online Resource 1, Supplemental Figure 5C).

Quantitation of experimental group class comparisons

The full dataset for the defined class comparisons are provided in Online Resource 2 in the file “Online Resource 2 Szymanski et al Microarray Results.pdf”. Quantitative assessment of the 8hR vs. NIC class comparisons provided a global overview of the microarray results (Figure 3 and Table 1). Polysome-bound mRNAs from CA3 (CA3B) showed a total 1005 hits meeting the class comparison criteria, with 591 of these (59%) increased at 8hR compared to NICs (volcano plot Figure 3B). Polysome-bound mRNAs from CA1 (CA1B) had 247 total hits, with 186 (75%) increased at 8hR over NIC (volcano plot Figure 3C). Thus, there were ~ 4-fold more distinct polysome-bound mRNAs in CA3 compared to CA1. Only 95 genes overlapped between CA1B and CA3B, indicating a larger, more diverse pattern of polysome-bound mRNAs in 8hR CA3 (Venn diagram, Figure 3D).

Principal component analysis showed a clear demarcation between B and U groups (Online Resource 1, Supplemental Figure 5C). mRNAs not associated with polysomes in CA3 (CA3U) had 495 total significant hits, with 373 (75%) increased at 8hR compared to NICs (volcano plot Figure 3E). mRNAs not associated with polysomes in CA1 (CA1U) had 363 total hits, with 201 (55%) increased at 8hR over NIC (volcano plot Figure 3F). There was overlap of 68 transcripts in CA1U and CA3U (Venn diagram, Figure 3G), again indicating mostly distinct populations of mRNAs not associated with polysomes in CA1 vs. CA3.

Unique hits were counted after excluding unidentified (‘NA’) and repeated hits (Table 1). Summing expression of unique significant hits in the B and U fractions, a total of 829 transcripts were significantly different for CA3 and 331 for CA1, and overlap ratios were similar to the non-unique hit list. Unique transcripts that were polysome-bound were 72% in CA3 and 42% in CA1. Thus, 28% and 58% of unique transcripts significantly different between 8hR and NIC were not associated with polysomes in CA3 and CA1, respectively.

We next considered those transcripts that increased at 8hR over NIC (e.g. those genes to the left of $\log_2[\text{fold change}] = 0$ in the volcano plots), and plotted x-fold from highest to lowest (Figure 3H). Figure 3I plots, from highest to lowest, the x-fold values of the transcripts higher in NIC compared to 8hR (those genes to the right of $\log_2[\text{fold change}] = 0$ in the volcano plots). These plots show that only a few genes in each experimental group had very large x-fold changes. For 8hR > NIC, the top 8 genes, and for NIC > 8hR, the top 6 genes, were substantially higher than the remainder (insets, Figure 3H and 3I, respectively). For 8hR > NIC cases, the top 10 genes, in terms of x-fold increase accounted for 23%, 15%, 17% and 11% of the total x-fold increases in the CA1B, CA3B, CA1U and CA3U groups, respectively, but these represented only 4%, 1%, 3% and 2%, respectively, of the total number of transcripts that significantly increased.

The above calculations allow the following inferences: (1) populations of mRNA transcripts, whether polysome-bound or not, were qualitatively different between CA1 and CA3 at 8hR, (2) a substantial proportion of transcripts were not polysome-associated in either region, and (3) the populations of polysome-bound mRNAs were dominated by a relatively few transcripts, and all other transcripts were present in relatively smaller proportions.

Qualitative analysis of experimental group class comparisons

The full tables of class comparisons are provided in Supplemental Files. We do not perform an extensive gene ontology analysis here, which has been described in other studies [39]. Instead, based on the quantitative considerations above, we briefly discuss the top 10 hits in each experimental group.

Of the top 10 polysome-bound transcripts upregulated at 8hR compared to NIC (Table 2), 5 of the CA1B transcripts, and 2 of the CA3B transcripts, belonged to the heat shock protein

family. The second most abundant transcript in both regions was ATF3. The remaining transcripts were related to immune function, cell signaling, lipid metabolism or the extracellular matrix. There was almost no overlap between the top 10 B and U transcripts in either region, with the exceptions of: (1) Hspb1 common to CA1U and CA3U, (2) ATF3 in CA1B, CA3B and CA3U and (3) Mt1a in CA3B and CA3U. Growth arrest and DNA-damage 45 (GADD45) subunits and Hspb1 (HSP27) were notable for not being polysome-bound.

For transcripts that were significantly higher in NIC, we evaluated the top 5 (Table 3), again, for the quantitative reasons above. Out of the 20 total hits, 13 were unidentified sequences ('NA' and RGD1562229). The remainder coded neuronal (Olr1722, Kcnq2, Vom2r17), immunological (LOC288521, Ly49si2) or housekeeping (Gsta4, Man2a2) proteins.

Thus, qualitatively, the most abundant transcripts were related to heat shock and ATF3, and heat shock transcripts were represented more in CA1B than CA3B at 8hR. CA3 generally displayed greater diversity of the most abundant transcripts, whether or not the transcripts were polysome-bound.

ARE enrichment in polysome-bound and unbound groups

685 rat transcripts, out of ~ 28,000[40], or about 2% of the total rat transcriptome, have either been demonstrated to contain an ARE sequences, or are predicted to have one by computational extraction of genomic databases [18]. When the significant hit lists of transcripts upregulated at 8hR compared to NICs in CA3B and CA1B were searched in the ARED database, 27 (4.5%) and 9 (5.6%), respectively, were identified as ARE-containing, and 6 occurred in both regions (Table 1). For CA3U and CA1U, 19 (5.7 %) and 8 (4.1 %) transcripts, respectively, contained ARE sequences, with 5 overlapping between CA1 and CA3 (Table 1). In contrast, when transcripts greater in NIC compared to 8hR were searched against ARED organism, none were detected in CA1B, and 1 each was detected in CA3B (Stk39), CA1U (Dnaja6) and CA3U (Dnaja6). Thus, transcripts that increased at 8hR showed a 2 to 2.5-fold enrichment of ARE-mRNAs over the entire rat transcriptome. One member each of the top 10 lists of 8hR > NIC transcripts contained an ARE (Table 2, bolded rows), but no members of the NIC > 8hR top five list (Table 3) had AREs (the two bolded rows for CA3B are the synonymous probes for rat inducible HSP70 (<http://www.uniprot.org/uniprot/Q07439>) [41]).

Table 4 lists all ARE-containing transcripts, and specifies which hit list they were found on. Again the main observation was the greater diversity of ARE-containing transcripts in the CA3 fractions compared to CA1. The gene ontology of this list is characteristic of ARE-containing mRNAs, including stress response genes, and genes involved in basic cell regulatory processes such as cell signaling, transcriptional and mRNA regulation [18].

IRES enrichment in R/N-bound transcripts

When the list of significant hits was screened in IRESite, there was evidence for only one IRES-containing transcript in CA1B population: ODC1, encoding the ornithine decarboxylase 1 protein. Thus, consistent with a previous study [30], we found no evidence of preferential upregulation of IRES-containing mRNAs either bound or unbound to the polysomes.

Transcription factor binding site enrichment in R/N-bound transcripts

Transcription factor binding site enrichment was tested specifically for the 95 overlapping transcripts in the CA3B and CA1B (overlap in Figure 3C) in MATInspector [35]. The ten most-enriched transcription factors binding sites represented by the overlapping hits in CA1

and CA3 are shown in Table 5: four of these are uncharacterized, three are from the heat shock family of transcription factors, and one each of: the nuclear factor of activated T cells (NFAT), cMyc, and the generic TATA box.

DISCUSSION

ARE-containing mRNAs such as *c-fos* [5], *c-jun* [5], and *hsp70* [3,4] are copiously transcribed but not translated in selectively vulnerable CA1 during reperfusion. We previously showed HuR colocalized with mRNA granules in CA3 but not CA1 at 8hR, and CA3 but not CA1 neurons translated HSP70 [6,11]. Thus we suggested that TA in CA1 involved ARE-mRNA regulation [7]. The present study investigated potential molecular mechanisms underlying this correlation. We did not find evidence that HuR mediated nuclear export or altered polysome distribution during reperfusion. However, reperfused polysomes were enriched in ARE-mRNAs, present in 4% to 5% of transcripts increased at 8hR over NIC (Table 1) and found amongst the most abundant transcripts (Table 2). Further, the populations of polysome-bound mRNAs were mostly distinct between CA1 and CA3. These data support that ARE-mRNA regulation occurs during reperfusion, but is not the exclusive means of mRNA post-transcriptional regulation.

Possible roles of HuR in brain I/R

Three distinct roles have been identified for HuR, which, alone or in combination, could occur in reperfused brain: (1) stabilization [19, 20], (2) nuclear export [21], or (3) translation initiation [22, 23] of ARE-containing mRNAs. We *a priori* ruled out dysfunction in the stabilization of *hsp70* mRNA during reperfusion, because abundant *hsp70* transcription occurs in all post-ischemic neurons [4].

Nuclear export

Neuronal HuR is predominantly nuclear [19]. During cell stress, HuR can redistribute to cytoplasm [42] and mediate ARE-mRNA nucleocytoplasmic shuttling [36], including *hsp70* [24]. If this mechanism occurred during I/R, but was disrupted in CA1, then nuclear accumulation of *hsp70* would preclude its translation. However, we observed no differences in HuR or *hsp70* levels in NIC and 8hR subcellular fractions (Figures 1C & 1D). These data argue against a role for HuR nuclear export of *hsp70* during brain I/R.

Selective initiation

Other groups showed HuB redistributed on polysome gradients during TA [43], and HuD acted as a translation enhancer via eIF4A binding [44]. We previously showed *hsp70* mRNA co-immunoprecipitated with HuR [12]. Thus we considered that HuR might facilitate ARE-mRNA translation during brain I/R. As a marker of HuR interaction with the translational machinery, we assessed HuR polysome profile distribution. There was no difference between NIC or 8hR forebrain (Figure 2), even though 8hR forebrain copiously translated HSP70 (data not shown). However, this result does not definitively rule out a role for HuR in ARE-mRNA translation. A putative initiation complex containing HuR may not have co-purified with polysomes in our system. Future studies will need to investigate this possibility, the importance of which is underscored by our observation of the enrichment of ARE-mRNAs on polysomes at 8hR.

Comparison with other brain I/R microarray studies

The majority of gene expression profiling of brain I/R via microarrays has assessed focal ischemia [39], and none, to our knowledge, have compared CA1 to CA3. One study, with conditions close to those used here, assessed total RNA in whole ipsilateral hemisphere after

15 min unilateral transient global ischemia and 6 hr reperfusion in rat [45]. Comparing the ten most upregulated transcripts after I/R, seven were shared between that study and our microarray results (Table 6). We found that two of the seven, Hspb1 and GADD45 γ , were not associated with polysomes at 8hR. Four of their top 10 hits were ARE-mRNAs, two of which, Fos and FosB, were not detected here. Thus, the results broadly corresponded, and the differences can be accounted for by the different microarray input tissues.

Bound vs. Unbound mRNAs

It is now well-known that transcription and translation do not directly correlate [46]. Only one other study of brain I/R used TSA [30] and found only 36% of significant hits to be polysome-bound. In the present study, ~67% of CA3, and ~40% of CA1 significant hits were polysome-bound. Since it is impractical to validate all significant hits, all other previous brain I/R microarray studies implicitly assumed all transcriptional changes had functional consequences. At minimum, transcripts not associated with polysomes should not be included in standard hierarchical clustering pathway analyses. Large proportions of transcripts not being polysome-bound is consistent with the occurrence of TA after I/R, and with our previous observation that mRNAs is sequestered from ribosomal subunits in the form of mRNA granules [11].

CA1 and CA3 transcriptomes at 8hR

Pubmed search of the terms “transcription cerebral ischemia” gives over 1400 citations, the oldest from 1978 [47]. This long history of brain I/R transcription studies has led to the understanding that “waves” of gene expression occur over different time frames in the post-ischemic tissue. There are variant interpretations of the functional significance of these [48–51], but also consensus that they represent progressive phases of the tissue response to I/R injury. Three broad phases can be recognized: (1) An acute “preparatory” phase where normal gene expression is silenced by TA, concurrent with upregulation of stress-induced mRNAs [2]. (2) A “stress response” phase where de novo translated proteins, such as HSP70, combat existing I/R-induced damage. (3) A “clean up” phase where the glia and immune system eliminate damaged cells, and surviving cells, neuron or otherwise, rebuild damaged cell components. Successful execution of all three phases constitutes recovery from the injury, and represents the eventual “resetting” of the cells to their pre-injury phenotypes.

Even though we evaluated only at 8hR, our microarray results can be interpreted in this framework. The main finding was that CA3 showed greater mRNA diversity than CA1. The transcriptome overlap was unequal: it was a small fraction of CA3 but a large fraction of CA1 (Figure 3D and 3G). Five of the top 10 CA1B hits were heat shock proteins, but only 2 were in CA3B. This suggests that 8hR CA1 was in the “stress response” phase, while CA3 was transitioning into the “clean up” phase, as evidenced by the expression of metallothioneins (remodeling) and paracrine agents such as inhibin beta and transthyretin. We also note increased polysome-bound ATF3, increasingly recognized as a hub for cell stress responses [52].

ARE containing mRNAs

ARE-containing transcripts were greater, in terms of x-fold, at 8hR compared to NIC in both the B and U fractions (Table 4) and one each of the top 10 hits was an ARE-mRNA (Table 2). Thus ARE-mRNAs were enriched in the transcriptomes of both CA1 and CA3. That post-ischemic tissue displays “waves” of gene expression is consistent with the known functions of ARE-containing mRNAs. ARE-based regulation comes into play when cells undergo physiological or pathological transitions, such as differentiation [53], fertilization [54], or cell stress [17, 55, 56]. Our data indicate that quantitatively, the heat shock response

is the largest ARE response. However, we identified 47 ARE-containing transcripts upregulated in brain I/R, of which 29 were polysome-bound at 8hR (Table 4), thus indicating that regulation of ARE-mRNAs is not exclusive to the heat shock mRNAs.

Conclusion

Our data show ARE regulation occurs following brain I/R. Future studies will have to discern the role of HuR and more broadly, RNA regulons for ARE-mRNAs, in regulating “waves” of gene expression after I/R. It is important to study these processes in brain regions that survive 10 min ischemia, such as CA3 and cerebral cortex because it reveals the processes that allow these neurons to survive ischemia. By understanding the intrinsic mechanisms by which some neurons survive ischemia, we may eventually be able to exploit these to develop successful neuroprotection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

Assessment of HuR and *hsp70* at 10 min ischemia and 8 hr reperfusion (8hR). **(A)** Validation of subcellular fractions in unfractionated homogenate (input), nuclear fractions (nuclear), and post-mitochondrial supernatants (PMS). **(B)** Representative HuR Western blots from non-ischemic control (NIC) and 8hRCA1 PMS, with GAPDH shown as loading control. **(C)** Densitometry of NIC and 8hR HuR levels in CA1 and CA3 subcellular fractions (means $\pm$ standard deviations), as indicated. No statistically significant differences were detected between corresponding NIC and 8hR samples in any group. **(D)** Real time quantitative PCR results for *hsp70* mRNA (means $\pm$ standard error of the mean, SEM), with *gapdh* as normalizer. No statistically significant differences were detected.

Fig 2. HuR polysome profile distributions. **(A)** Representative HuR Western blots in NIC and 8hR samples. Polysome profile fraction numbers are indicated under respective lanes. HuR was detected as a smear of three bands at ~ 34 kDa. **(B)** Densitometry of average values for the percent of HuR in each fraction as a percent of total HuR density (means $\pm$ standard deviations). There were no differences between NIC and 8hR HuR in any fraction.

Fig 3.

Translational state analysis of CA1 and CA3 at 8hR. (A) Experimental design of translation state analysis of microdissected CA1 and CA3. 5 microdissections from $n = 15$ rats/group were randomly pooled to give one polysome profile (pCA1, etc.). Polysome bound (B) and unbound (U) fractions were then obtained by sucrose density centrifugation from pooled brain regions. (B) Volcano plot of B transcripts in CA3 between NIC and 8hR. (C) Volcano plot of polysome bound (B) transcripts in CA1 between NIC and 8hR. (D) Volcano plot of polysome unbound (U) transcripts in CA1 between NIC and 8hR. (E) Volcano plot of polysome U transcripts in CA3 between NIC and 8hR. In all volcano plots, blue points are statistically different between NIC and 8hR conditions. (F) Venn diagram of the numbers of

B transcripts increased at 8hR over NIC in CA1 and CA3. **(G)** Venn diagram of the numbers of U transcripts increased at 8hR over NIC in CA1 and CA3. **(H)** Plot of x-fold increase, from highest to lowest, for 8hR > NIC for all significant hits for CA1B, CA1U, CA3B, and CA3U groups. Inset shows the twenty highest x-fold increases, and dashed line demarcates very large x-fold increases of the top 6 hits particularly in CA1B and CA3B. **(I)** Plot of x-fold increase, from highest to lowest, for NIC > 8hR for all significant hits for CA1B, CA1U, CA3B, and CA3U groups. Inset shows the fifteen highest x-fold increases, and dashed line demarcates very large x-fold increases of the top 6 hits again in CA1B and CA3B.

Fig 4.
(A) Venn diagram of the numbers of ARE-containing mRNAs in polysome-bound CA1 and CA3. (B) Venn diagram of the numbers of ARE-containing mRNAs in polysome-unbound CA1 and CA3.

Table 1

Number of significant hits for experimental groups using class comparison criteria described in Materials and Methods.

Group	CA3	CA1	overlap
B total	1005	247	95
U total	495	363	68
sum	1500	610	163
% bound	67 %	41 %	
B total (unique hits)	600	139	63
U total (unique hits)	229	191	25
sum	829	330	88
% bound	72%	42%	
B 8hR > NIC	591	186	--
U 8hR > NIC	373	201	--
sum	964	387	--
% 8hR > NIC	64 %	63 %	--
B 8hR > NIC ARE	27 (4.6 %)	9 (4.8 %)	6
U 8hR > NIC ARE	19 (5.1 %)	8 (4.0 %)	5

B, polysome bound; U polysome unbound; ARE, adenine and uridine rich sequence. B and U total are all significant hits meeting class comparison criteria. B and U total (unique hits) eliminate unidentified ('NA') and duplicate hits from the B and U total lists.

Table 2

Top 10 8hR > NIC x-fold increased transcripts. ARE-mRNAs are boldface.

R>N	p-value	FDR	x-fold	Symbol	Name	EntrezID
CA1B	1.9E-03	2.0E-01	45.5	Hspa1a	heat shock 70kD protein 1A/B	294254
	1.1E-03	2.0E-01	43.5	Atf3	activating transcription factor 3	25389
	3.2E-04	1.7E-01	38.5	Hspb1	heat shock protein 1 (hsp27)	24471
	3.8E-03	2.1E-01	28.6	Pigs2	prostaglandin-endoperoxide synthase 2	29527
	1.8E-03	2.0E-01	19.2	Pcdh8	protocadherin 8	64865
	1.5E-03	2.0E-01	18.9	Hspa1b	heat shock 70kD protein 1B (mapped)	294254
	4.2E-03	2.1E-01	17.5	Zfand2a	zinc finger, AN1-type domain 2A	360772
	3.7E-03	2.1E-01	16.9	Hmox1	heme oxygenase (decycling) 1	24451
	7.4E-03	2.3E-01	16.9	Cdkn1a	cyclin-dependent kinase inhibitor 1A	114851
	9.6E-03	2.4E-01	16.7	Cryab	crystallin, alpha B	25420
CA3B	5.0E-07	5.5E-04	76.9	Hspb1	heat shock protein 1 (hsp27)	24471
	6.0E-07	5.6E-04	58.8	Atf3	activating transcription factor 3	25389
	1.3E-05	2.2E-03	55.6	Ttr	transthyretin	24856
	4.0E-07	5.3E-04	45.5	Hspa1a	heat shock 70kD protein 1A/B	294254
	2.0E-07	5.3E-04	28.6	Pigs2	prostaglandin-endoperoxide synthase 2	29527
	8.2E-05	5.7E-03	18.5	Cdkn1a	cyclin-dependent kinase inhibitor 1A	114851
	2.0E-06	1.1E-03	13.9	Inhba	inhibin beta-A	29200
	3.0E-07	5.3E-04	13.0	Mt1a	metallothionein 1a	24567
	1.9E-06	1.1E-03	13.0	Tmem27	transmembrane protein 27	57395
	3.0E-07	5.3E-04	12.8	Mt1a	metallothionein 1a	24567
CA1U	1.2E-04	5.8E-02	41.7	Mia	melanoma inhibitory activity	81510
	1.7E-04	5.8E-02	22.7	NA	NA	NA
	6.7E-03	1.4E-01	19.2	Gadd45a	growth arrest and DNA-damage-inducible, α	25112
	6.3E-03	1.4E-01	16.9	Dusp1	dual specificity phosphatase 1	114856
	4.0E-03	1.2E-01	16.4	Npas4	neuronal PAS domain protein 4	266734
	5.8E-04	6.2E-02	15.9	Hspb1	heat shock protein 1 (hsp27)	24471
	1.5E-04	5.8E-02	15.6	NA	NA	NA
	7.5E-04	6.8E-02	14.9	Zfand2a	zinc finger, AN1-type domain 2A	360772

R>N	p-value	FDR	x-fold	Symbol	Name	EntrezID
	7.2E-03	1.4E-01	14.3	Coq10b	coenzyme Q10 homolog B (S. cerevisiae)	301416
	3.0E-04	5.8E-02	12.8	Cdkn1a	cyclin-dependent kinase inhibitor 1A	114851
CA3U	6.8E-05	3.2E-02	58.8	Mia	melanoma inhibitory activity	81510
	4.8E-03	9.8E-02	27.8	NA	NA	NA
	2.8E-03	8.5E-02	25.6	Ier3	immediate early response 3	294235
	8.7E-03	1.2E-01	21.3	Gadd45β	growth arrest and DNA-damage-inducible, β	299626
	2.1E-04	4.7E-02	19.6	Cdr2	cerebellar degeneration-related 2	308958
	2.9E-05	2.7E-02	18.2	Hspb1	heat shock protein 1	24471
	5.3E-04	5.4E-02	17.9	NA	NA	NA
	1.7E-03	7.6E-02	16.9	Gadd45α	growth arrest and DNA-damage-inducible α	25112
	2.9E-04	4.8E-02	16.4	Atf3	activating transcription factor 3	25389
	1.1E-03	7.0E-02	15.4	Mt1a	metallothionein 1a	24567

Table 3

Top five transcripts that were higher by x-fold in NIC compared to 8hR.

N>R	p-value	FDR	x-fold	Symbol	Name	EntrezID
CA1B	4.8E-03	2.1E-01	6.52	Olr1722	olfactory receptor 1722	406016
	3.9E-03	2.1E-01	6.34	NA	NA	NA
	2.8E-03	2.0E-01	6.05	NA	NA	NA
	8.5E-03	2.4E-01	5.49	LOC288521	similar to Leukosialin precursor	288521
	5.5E-03	2.1E-01	5.25	NA	NA	NA
CA3B	1.1E-06	9.0E-04	20.03	NA	NA	NA
	1.7E-06	1.1E-03	18.29	NA	NA	NA
	5.0E-06	1.8E-03	11.39	NA	NA	NA
	2.1E-06	1.1E-03	9.25	NA	NA	NA
	2.2E-03	2.8E-02	9.24	Gsta4	glutathione S-transferase alpha 4	300850
CA1U	2.8E-03	1.0E-01	7.04	NA	NA	NA
	6.1E-03	1.4E-01	6.91	Man2a2	mannosidase 2, alpha 2	308757
	4.7E-03	1.3E-01	6	NA	NA	NA
	5.2E-03	1.3E-01	5.94	Kcnq2	potassium voltage-gated channel, KQT-like subfamily, member 2	170848
	3.9E-03	1.2E-01	5.29	Ly49si2	immunoreceptor Ly49si2	494207
CA3U	9.7E-03	1.2E-01	20.12	NA	NA	NA
	9.5E-03	1.2E-01	15.87	NA	NA	NA
	8.2E-03	1.1E-01	11.56	Vom2r17	vomerolateral 2 receptor, 17	690716
	9.6E-03	1.2E-01	9.41	RGD1562229	similar to hypothetical protein FLJ40298	498431
	5.7E-03	1.0E-01	9.39	NA	NA	NA

Table 4

ARE-containing transcripts for CA3 and CA1 polysome bound (3B, 1B, respectively) and polysome unbound (3U, 1U, respectively) marked by “x”. ARE classification group is that of [18].

Entrez ID	Gene	ARE	Group	Location	3B	1B	3U	1U
113976	Acs14	AAATATTATATAA	5	1513–1525			x	
60332	Akap14	TAAATATTATTGA	5	69–81	x			
83477	Bcl10	TATTATTTAAAAA	5	785–797			x	
58942	Caenb4	ATATATTATTATCA	5	1208–1220	x			
26989	Cadps	ATGTAITTTATAAT	5	722–734	x			
25542	Ccl3	TTTAAITTTATTTT	5	193–205			x	
361226	Cd83_p	ATTTATTTTTTTA	3	628–640	x			
364183	Cno	ATTTATATATTTA	3	273–285			x	
313409	Dnajc6_p	TGTTATTTATTTAA	5	1645–1657				
114856	Dusp1	TTATTTATTTAAC	4	103–115	x	x	x	x
311406	Dusp2	TTATATTTATATA	5	549–561	x	x	x	x
306860	Gcnl2	ATATTTATTTAAT	4	1918–1930	x			
192254	Grasp	ATTTATTTATTTATCA	2	26–42			x	x
25433	Hbegf	ATATATTTAGTTT	5	631–643	x	x		
295398	Hiat1_p	TATTATTTAAAAA	5	933–945	x			
24472;294254	Hspalal1b	ATTTATTTACTTA	3	967–979	x	x		
294235	Ier3	ATTTATGTATTTA	3	210–222			x	x
64194	Insig1	AAATATTTATTAT	5	971–983			x	
293497	Kcid13	TATTATTTATTTAT	5	466–478			x	
305792	Kcid6_p	AAATATTTATATTT	5	531–543			x	
114764	Mapre1	TAAATATTTAAAAAT	5	1144–1156			x	
171347	Mat2a	TTTTATTTAGTAA	5	730–742	x	x	x	
288616	MGC94190	ACATATTTATATAA	5	108–120	x			
306081	Pcdh20_p	TGTTATTTATATA	5	219–231			x	
305549	Peli1	AAATATTTATAAAT	5	118–130	x	x	x	
83722	Plk2	TTTTATTTATTAC	5	503–515	x			

Entrez ID	Gene	ARE	Group	Location	3B	1B	3U	1U
58936	Plk3	ACATTTATTATT	4	266–278	x			
361676	Pnpla2_p	AATTATTTATTAT	5	340–352	x			
501563	Prkx	ATTGTTTATTTA	3	543–555	x	x		
NA	Psm3	TTAGATTTTATTT	5	730–742				
309029	RGD1305014	TTTATTTATTCA	5	323–335	x			
362220	RGD1306991	TTATATTTAGAAA	5	326–338	x			
301419	RGD1311269_p	CAATATTTATATT	5	171–183	x			
84583	Rgs2	ATAAATTTATTT	5	654–666	x	x		x
363103	Slc17a5	TTTATTTATCAT	5	22–34			x	
79212	Slc6a1	AAATATTTATACA	5	1983–1995	x			
307766	Smarca5_p	TTTGATTTTATTT	5	236–248	x			
361233	Ssr1	ATACATTTATATT	5	874–886		x		
54348	Slk39	AGTTATTTATAAT	5	1323–1335				
117526	Tbp	ATTATTTATATT	5	236–248			x	
289307	Tfb2m	ATTATTTACTTT	5	394–406		x		
308134	Tmem35	TTTGATTTTATTA	5	90–102			x	
302965	Tnfrsf12a	TGATATTTATTTT	5	480–492	x	x	x	x
294331	Ube2g2_p	TTGTATTTATTAA	5	1242–1254				x
303336	Wsb1	TTTTATTTATCTT	5	815–827	x			
362990	Zcrtb1	TCATATTTTATTT	5	14-Feb			x	
79426	Zip36	TAGTATTTATATA	5	622–634	x			x

Table 5

Top ten enriched transcription factors in the overlapping set of 95 hits between CA1B and CA3B experimental groups identified by MATInspector database search.

Transcription Factor	Number of Regulated Transcripts	Score (p<)
SMTTTTGT motif (uncharacterized)	14	7 e-09
Heat Shock Factor 1	13	6 e-08
Nuclear Factor of Activated T Cells	24	8 e-08
TTCYRGAA motif (uncharacterized)	11	8 e-08
Heat Shock Factor 2	9	1 e-06
MYC	16	1 e-06
Heat Shock Factor 6	8	1 e-06
TATA Binding Protein	18	1 e-06
AACTTT motif (uncharacterized)	22	1 e-06
TTCNRGNNNTTC motif (uncharacterized)	7	1 e-06

Table 6

Top 10 hits at 6hR in ipsilateral hemisphere from [45].

x-fold	Symbol	Name
250	Hspa1b *%	heat shock 70kD 1B
16	<u>Hspb1</u> *	heat shock protein 1
13	Fos %	FBJ murine osteosarcoma viral oncogene homolog
13	Atf3 *	activating transcription factor 3
10	Egr2	Early growth response 2
9	Ccl2 *	Chemokine ligand 2
9	<u>GADD45γ</u> *	Growth arrest and DNA-damage- inducible 45 gamma
8	Tnfrsf12a * %	Tumor necrosis factor receptor superfamily, 12a
8	FosB %	FBJ murine osteosarcoma viral oncogene homolog B
8	Hmox1 *	heme oxygenase (decycling) 1

‘*’ denotes transcripts detected in present study. Underlined symbol names indicate transcript was in the polysome unbound (U) fraction in present study. ‘%’ indicates ARE-containing mRNA.