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POSTMORTEM INTERVAL EFFECT ON RNA AND GENE EXPRESSION IN HUMAN BRAIN TISSUE

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Abstract

Banked tissue is essential to the study of neurological disease but using postmortem tissue introduces a number of possible confounds. Foremost amongst these are factors relating to variation in postmortem interval (PMI). Currently there are conflicting reports on how PMI affects overall RNA integrity, and very few reports of how gene expression is affected by PMI. We analyzed total RNA extracted from frozen cerebellar cortex from 79 deceased human subjects enrolled in the Banner Sun Health Research Institute Brain and Body Donation Program. The PMI, which ranged from 1.5 to 45 hours, correlated with overall RNA quality measures including RNA Integrity Number (RIN) ($r = -0.34$, $p = 0.002$) and RNA quantitative yield ($r = -0.25$, $p = 0.02$). Additionally, we determined the expression of 89 genes using a PCR-based gene expression array (RT² ProfilerTM PCR Array: Human Alzheimer's Disease; SABiosciencesTM, Frederick, MD). A greater proportion of genes had decreased rather than increased expression with increasing PMI (65/89 vs 20/89; $p < 0.0001$). Of these, transcripts from the genes ADAM9, LPL, PRKCG, and SERPINA3 had significantly decreased expression with increasing PMI ($p < 0.01$). No individual gene transcripts had significantly increased expression with increasing PMI. In conclusion, it is apparent that RNA degrades progressively with increasing PMI and that measurement of gene expression in brain tissue with longer PMI may give artificially low values. For tissue derived from autopsy, a short PMI optimizes its utility for molecular research.

Keywords

RIN; RNA; postmortem; brain; gene expression; Alzheimer's disease; neurological disease; research; methods

INTRODUCTION

Molecular biologists rely on intact RNA obtained from diseased and control human tissue to determine differences in gene expression associated with disease. Obtaining intact RNA is particularly difficult for research into neurological disease as tissue must often be obtained postmortem, making it impossible to ensure that RNA is unaltered and introducing numerous possible confounds. A variety of antemortem factors may influence the quality of harvested tissue including fever, hypoxia-ischemia and acidosis, while critical postmortem variables include postmortem interval (PMI), brain or cerebrospinal fluid pH, ambient temperature in the postmortem period, harvesting procedures, storage temperature and accidental or systematic thawing and refreezing.

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Previous research has drawn conflicting conclusions about the postmortem stability of RNA in brain tissue. Many report that global measures of total RNA quality are relatively stable over a wide range of postmortem intervals (Morrison and Griffin 1981; Perrett et al. 1988; Leonard et al. 1993; Cummings et al. 2001; Yasojima et al. 2001; Preece and Cairns 2003; Li et al. 2004; Tomita et al. 2004; Ervin et al. 2007; Durrenberger et al. 2010) while some others report a definite loss with increasing time (Beach et al 2008; Broniscer et al 2010).

Studies of subtypes of RNA have also had varying conclusions, with some indicating generalized stability (Sajdel-Sulkowska et al. 1988; Gilmore et al. 1993; Kingsbury et al. 1995; Johnston et al. 1997; Mathern et al. 1997; Schramm et al. 1999; Miller et al. 2004; Ervin et al. 2007; Popova et al 2008) while others have reported significant loss of individual mRNA transcripts with increasing PMI (Johnson et al. 1986; Lukiw et al. 1990; Burke et al. 1991; Ragsdale and Miledi 1991; Ross et al. 1992; Eastwood et al. 1994; Pardue et al. 1994; Harrison et al. 1995; Castensson et al. 2000; Bauer et al. 2003; Barrachina et al. 2006; Durrenberger et al. 2010). These studies have generally investigated small numbers of mRNA species and therefore general conclusions are not possible.

The conflicting evidence as to whether RNA appreciably degrades postmortem indicates that further investigations are needed. In the current study, we isolated and analyzed RNA from a large set of brain samples from the Banner Sun Health Research Institute Brain and Body Donation Program in order to determine if there is a relationship between PMI and measures of global RNA integrity. Subsequently, we utilized a PCR-based gene expression array to determine how PMI affects the expression of a large set of individual genes.

MATERIALS AND METHODS

Tissue Samples

Cerebellar cortex samples were obtained from the Banner Sun Health Research Institute Brain and Body Donation Program (Beach et al. 2008). Institutional Review Board-approved consent was obtained from all brain donors or their legal representatives. At the time of brain removal, the cerebellar peduncles were severed, separating the cerebellum from the brainstem. Each hemispheric cerebellum was then sliced in the parasagittal plane into 4-5 segments, each about 0.75 cm thick. These segments were then quickly frozen by placing on a sheet of dry ice, following which they were stored in vacuum-sealed plastic bags in freezers held at -70 to -80 degrees C, for periods of time ranging from 1 to 17 years. All samples used were chipped from frozen lateral cerebellar cortex slices while holding the sample on a sheet of dry ice. The samples were placed in chilled polyethylene microcentrifuge tubes and weighed (25-30 mg per sample).

Subject Variables

The time of death was obtained from the subject's caregiver and the time of brain removal was also noted. The elapsed time between death and brain removal was defined as PMI.

Subject demographic information including gender and age was self-reported upon program enrollment. Information about neurological disease was obtained from standardized neurological examinations and examination of private medical records, in combination with neuropathological examination after death. A full description of these procedures is given in an earlier publication (Beach et al 2008).

Study Subject Selection

Samples were taken from 79 deceased subjects chosen by searching the database for cases that would represent a wide range of PMI and a wide range of freezer storage times.

Selection of cases was performed blind to other subject characteristics including age, gender, diagnosis and results of earlier research studies (Table 1). These 79 subjects were used to assess RNA Integrity Index (RIN) and RNA quantitative yield. The subjects were categorized by clinicopathological diagnosis (Table 2) as Alzheimer's disease (AD), Parkinson's disease (PD), cerebrovascular disease (VASC), non-Alzheimer's neurodegenerative disease (NONAD) and normal control subjects (non-demented and without a major neurological diagnosis during life). The diagnosis of AD was made if the subject was demented and met National Institute on Aging - Reagan Institute criteria of "intermediate" or "high" (1997). The diagnosis of PD was made according to Gelb et al (1999). Subjects were assigned to the category of VASC if they met diagnostic criteria (Roman et al, 1993) for vascular dementia (11 subjects), had large acute or subacute cerebral infarcts (4 subjects) or acute intracranial hemorrhage (2 subjects). The diagnoses of NONAD conditions were made according to a published algorithm (Dickson 2005); cases included within this category included 6 with progressive supranuclear palsy, 2 with Pick's disease, 2 with dementia lacking distinctive histology and one with amyotrophic lateral sclerosis. Three subjects had diagnoses that did not fall into any of the above categories and hence were excluded from the comparison in Table 2. The diagnoses of these 3 subjects were glioblastoma multiforme, leukemic brain infiltrates and multiple sclerosis. Seventeen subjects classified here as VASC or NONAD also met diagnostic criteria for AD; for the between-group analyses presented here these were excluded from the AD group.

Of the 79 subjects used to assess RNA integrity and yield, only those with a diagnosis of AD were used to examine gene expression changes, in order to minimize gene expression changes due to disease state. Of the 43 AD cases in the overall series, we excluded, prior to conducting the assays, all cases with RIN less than 7.0 in order to avoid cases with unusual RNA degradation. This resulted in the exclusion of three cases. One additional case was excluded on the basis of a low RNA concentration. This left 39 cases for the study of gene expression (Table 1).

RNA Isolation and Analysis

The RNeasy Mini Kit (Qiagen, Valencia, CA) was used to extract total RNA from the tissue samples following the manufacturer's instructions. Homogenization of the tissue samples was achieved by sonication of the frozen sample in a denaturing guanidine-thiocyanate-containing buffer. The samples were first centrifuged through genomic DNA elimination columns. Then ethanol was added and the mixture was centrifuged through a silica membrane. The RNA was eluted from the membrane using 100 μ l of water. RNA measures including RIN and RNA concentration were determined using the RNA 6000 Nano Labchip Kit (Agilent Biotechnologies, Santa Clara, CA), the Agilent 2100 Bioanalyzer platform and the manufacturer's software. RNA yield was calculated multiplying the RNA concentration (ng/ μ l) by the total sample volume (100 μ l) and then dividing by the sample weight (g).

Gene Expression Analysis

First strand cDNA synthesis was achieved using the RT² First Strand Kit (SABiosciencesTM, Frederick, MD) following the manufacture's instructions and including a genomic DNA elimination step and external RNA controls. For each sample, 168 ng of total RNA was reverse transcribed. Real-Time PCR was performed using the RT² ProfilerTM PCR Array: Human Alzheimer's Disease (SABiosciencesTM, Frederick, MD) according to the manufacture's instructions on the Mx3000P real-time PCR platform with software version 3.00 (Stratagene, La Jolla, CA). For each sample, the PCR experimental cocktail was prepared by mixing 102 μ l of diluted First Strand cDNA Synthesis Reaction, 1248 μ l of H₂O, and 1350 μ l of RT² SYBR[®] Green/ROXTM qPCR Master Mix (SABiosciencesTM, Frederick, MD). Each 96-well RT² ProfilerTM PCR Array: Human

Alzheimer's Disease contains 84 wells with a real-time PCR assay for different genes related to Alzheimer's disease, 4 wells with assays for different housekeeping genes, a genomic DNA control, 3 replicate Reverse Transcription controls, and 3 replicate Positive PCR controls. Each well received 25 μ l of PCR experimental cocktail. A two-step cycling program was used consisting of an initial activating step of 10 minutes at 95°C and a second step of 40 cycles of denaturation (95°C, 15 sec) and annealing (60°C, 1 min). Finally, each plate underwent a melting curve program (95°C, 1 min; 55°C, 30 sec; 95°C, 30 sec). The baseline for each plate was automatically determined by the software program. A threshold value of 1607 was used across all arrays. C_t values were calculated as the cycle when each well first crossed the set fluorescence threshold. Gene expression level was defined as the inverse of the C_t value. C_t values greater than 35, or those not detected, were set to 35.

Statistical Analysis

The analyses of the relationship between PMI and global RNA measures, as well as the relationship between PMI and quantitative expression of individual gene transcripts, were done using Pearson correlations. T-tests were performed when comparing means from two groups while one-way analysis of variance was used when comparing means of several groups. The overall proportion of individual genes under- or over-expressed was compared using the one sample goodness-of-fit test. Proportional differences between groups were examined using Fisher's Exact Test. The significance level for all tests was set at $p < 0.05$ except for the correlations involving individual gene expression levels, for which the significance level was set at $p < 0.01$. Statistical testing was performed using GB-Stat (Dynamic Microsystems, Silver Springs, MD) and Microsoft Office Excel 2003 (Microsoft, Redmond, WA). Figures were created using Prism 5.01 for Windows (GraphPad, La Jolla, CA). Gene expression group differences were tested using the SABiosciences™ web-based PCR data analysis program (SABiosciences™, Frederick, MD).

RESULTS

Both measures of overall RNA quality were significantly related to PMI. Values for RIN negatively correlated with PMI ($r = -0.34$, $p = 0.002$) (Fig. 1a). RNA yield also negatively correlated with PMI ($r = -0.25$, $p = 0.02$) (Fig. 1b). Neither RIN nor RNA yield correlated with subject age, gender or diagnostic group (Table 2). Prolonged freezer storage did not reduce either RIN or RNA yield.

Twenty genes were over-expressed and 65 were under-expressed with increasing PMI (Table 3). This difference in proportionate under- and over-expression was significant ($p < 0.0001$). Four gene transcripts were not detected. Transcripts from the genes ADAM9, LPL, PRKCG, and SERPINA3 had significantly decreased expression with increasing PMI ($p < 0.01$). There were no transcripts with significantly increased expression with increasing PMI.

DISCUSSION

The results presented here, along with previously-published data, indicate that autopsies with shorter PMI result in a larger fraction of molecular-quality usable human tissue. Using RNA gel electrophoresis as a qualitative measure of RNA quality, we previously reported (Beach et al 2008) that with a median PMI of 3.1 hours, 82% of samples from our brain bank were suitable for RNA studies. Programs with average PMIs of 16 hours or more have reported that only one-third to one-half of cases have RNA suitable for molecular research methods (Vanderburg et al. 2005; Vonsattel et al. 1995). In a just-published study, Broniscer et al (2010) analyzed normal human brain tissue from 33 autopsied subjects with a median PMI of 7.7 hours and found, using a cutoff RIN value of 7.5 for "minimally-degraded"

RNA, that 58% of all samples were minimally degraded. Comparing these results to those of the present study, where the median PMI was 3.5 hours, 87% of samples met this criterion ($p < 0.001$ for the comparison between the two studies). When Broniscer et al divided their samples into those with PMI of less than or greater than 5 hours, 70% of samples from the short-PMI autopsies had a RIN greater than or equal to 7.5 compared to only 21% of the samples from the longer-PMI autopsies ($p < 0.01$). We also found a definite difference in RIN between autopsies with PMI greater or less than 5 hours as 96% of samples from our shorter-PMI autopsies had RIN greater than or equal to 7.5 vs only 74% of those from the longer-PMI autopsies ($p < 0.05$).

There have been several previous reports by groups that found only weak or no correlations between PMI and global RNA quality measures (Johnson et al. 1986; Perrett et al. 1988; Cummings et al. 2001; Stan et al. 2006; Grunblatt et al. 2009). Possible reasons for these conflicting studies include varying sample sizes and statistical power, different methods and inconsistency of RNA isolation and differences in between-subject variability. Inter-individual differences in postmortem human tissue studies are typically large and often prevent the attainment of statistical significance. Much of the between-subject variability may be due to highly variable agonal conditions (Burke et al. 1991; Harrison et al. 1995; Kingsbury et al. 1995; Johnston et al. 1997; Li et al. 2004; Tomita et al. 2004; Durrenberger et al. 2010). We have found that the methods used for isolating RNA are critical. We formerly extracted RNA using Trizol and were obtaining RIN values much lower than we now obtain using the Quiagen RNeasy Mini Kit. Additionally, consistency of technique is essential to avoid introducing excess variability in isolation efficiency. Tissue variability in ribonuclease activity may also be a factor, for studies comparing different tissue types.

As we have examined only one brain region, it is possible that these findings may not apply to all brain regions. Two groups, however, have found no significant RNA quality differences between different regions of the same brains (Preece and Cairns, 2003; Li et al, 2004). We did not find that there were differences in measures of overall RNA integrity in relation to either age or gender. One group (Preece and Cairns 2003) has reported a “significant but inconsistent” relationship between mRNA levels and age at death and also reported that lower mRNA values were obtained for females than for males. Another group found no clear relationship amongst these factors (Tomita et al 2004).

The results of the present study also indicate that increasing PMI has widely varying effects on individual gene transcripts but with an overall general tendency to decreased apparent transcription levels with increasing PMI. Testing commonly studied AD gene and housekeeping gene expression, we found that a significantly greater proportion of genes show decreased expression, rather than increased expression, with increasing PMI. Of the 89 genes studied, the expression levels of 4 were significantly decreased with increasing PMI: ADAM metallopeptidase domain 9 (ADAM9), lipoprotein lipase (LPL), protein kinase C, gamma (PRKCG), and serpin peptidase inhibitor, clade A, member 3 (SERPIN3). These results are similar to those of multiple other groups that have also found considerable transcript-to-transcript variability in the relationship between gene expression and PMI (Johnson et al. 1986; Lukiw et al. 1990; Burke et al. 1991; Ragsdale and Miledi 1991; Ross et al. 1992; Eastwood et al. 1994; Pardue et al. 1994; Harrison et al. 1995; Castensson et al. 2000; Bauer et al. 2003; Barrachina et al. 2006; Durrenberger et al. 2010). Some of this variability is undoubtedly due to the great range in transcript physiological half-lives *in vivo*, ranging from 15 minutes to greater than 50 hours (Buesa et al. 2004) but there is clearly a definite influence of PMI on some transcripts.

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Figure 1. Correlation between PMI and (a) RIN and (b) RNA yield measured as the weight of RNA isolated (μg) by tissue sample weight (g). Dashed lines show 95% confidence intervals.

Table 1

Study subject and sample data for the global RNA and gene expression studies. Shown for all cells except those relating to n and gender are means, range and standard deviation.

<i>n</i>	Global RNA Analysis	RT-PCR Gene Expression Analysis
	79	39
Age	61 – 96; 81.2 ± 7.6	72 – 95; 83.4 ± 6.3
Gender	M = 54; F = 25	M = 26; F = 13
PMI, hours	1.5 – 45; 6.6 ± 6.5	1.5 – 45; 5.8 ± 7.4
RIN	0 – 9.8; 8.5 ± 1.6	7.4 – 9.7; 8.9 ± 0.6
RNA Yield, µg/g tissue	26 – 1531; 541 ± 310	36.3 – 1020; 469 ± 267

Table 2

Diagnostic categorization of subjects used for the global RNA studies. Shown for all cells except those relating to n and gender are means, range and standard deviation. One-way analysis of variance found no significant differences between groups in terms of RIN or RNA yield. AD = Alzheimer’s disease; PD = Parkinson’s disease; VASC = cerebrovascular disease; NONAD = Non-Alzheimer’s disease neurodegenerative condition; CONTROL = elderly subject without major neurological diagnoses. Seventeen subjects classified here as PD, VASC or NONAD also met diagnostic criteria for AD; for the depicted analyses these were not included with the AD group. See text of Methods section for explanation of diagnostic categorization.

	AD	PD	VASC	NONAD	CONTROL
<i>n</i>	26	11	15	11	12
Age	72 - 93; 81.8 ± 6.6	63-89; 76.9 ± 7.5	70 - 95; 84.0 ± 7.2	61 - 85; 76.8 ± 7.4	70 - 96; 85.0 ± 7.4
Gender	M = 17; F = 9	M = 9; F = 2	M = 12; F = 3	M = 7; F = 4	M = 6; F = 6
PMI, hours	1.8 - 15.0; 5.2 ± 4.0	1.8 -18.5; 9.3 ± 5.2	1.5 -45; 6.4 ± 11.0	2.8 - 25; 9.0 ± 6.9	2.0 - 15.5; 6.7 ± 5.3
RIN	6.9 -9.7; 8.9 ± 0.7	7.4 - 9.4; 8.6 ± 0.7	7.4 - 9.7; 8.8 ± 0.8	5.4 - 9.8; 8.4 ± 1.4	0 - 9.5; 8.0 ± 2.6
RNA Yield, µg/g tissue	43 - 1003; 465 ± 261	69.3 - 775; 439 ± 238	69 - 1000; 551 ± 307	36 - 931; 531 ± 251	73 - 1412; 657 ± 221

Table 3

RT-PCR Array Results. Quantitative gene expression for each transcript is correlated with PMI, the results are shown as a correlation coefficient (R). Blank cells are for transcripts with insufficient RNA to measure. For translation of gene name abbreviations, see the US National Library of Medicine website EntrezGene (www.ncbi.nlm.nih.gov/gene).

Gene	Gene Expression (C_t^{-1}) vs PMI	
	R	P-value
A2M	0.023	0.889
ABCA1	-0.135	0.414
ACHE	-0.036	0.828
ADAM9	-0.423	0.007*
APBA1	-0.036	0.828
APBA3	-0.128	0.439
APBB1	0.044	0.790
APBB2	-0.224	0.170
APH1A	0.033	0.843
APLP1	-0.042	0.801
APLP2	-0.235	0.150
APOA1	0.103	0.535
APOE	-0.176	0.285
APP	-0.346	0.031
APPBP1	-0.177	0.282
BACE1	-0.140	0.397
BACE2	-0.190	0.246
BCHE	-0.343	0.032
BDNF	-0.080	0.628
CASP3	-0.256	0.116
CASP4	-0.054	0.744
CDC2	0.130	0.429
CDK5	-0.090	0.584
CDKL1	0.013	0.940
CHAT	0.250	0.125
CLU	0.042	0.798
CTSB	-0.200	0.223
CTSC	-0.205	0.210
CTSD	-0.292	0.071
CTSG	0.207	0.205
CTSL1	-	-
EP3000	-0.229	0.162
ERN1	0.034	0.838
GAP43	-0.255	0.117
GNAO1	-0.322	0.046

Gene	Gene Expression (C_t^{-1}) vs PMI	
	<i>R</i>	<i>P</i> -value
GNAZ	−0.244	0.134
GNB1	−0.367	0.022
GNB2	−0.181	0.270
GNB4	−0.216	0.187
GNB5	−0.284	0.079
GNG10	−0.285	0.079
GNG11	-	-
GNG3	−0.218	0.182
GNG4	−0.177	0.282
GNG5	−0.289	0.074
GNG7	−0.192	0.243
GNG8	0.158	0.337
GNGT1	0.242	0.138
GNGT2	0.175	0.288
GSK3A	−0.096	0.561
GSK3B	−0.258	0.114
HSD17B10	−0.332	0.039
IDE	−0.160	0.330
IL1A	0.210	0.199
INS	-	-
INSR	−0.332	0.039
LPL	−0.446	0.004 *
LRP1	−0.091	0.583
LRP6	−0.254	0.118
LRP8	−0.124	0.452
MAP2	−0.394	0.013
MAPT	−0.396	0.013
MPO	0.217	0.185
NCSTN	−0.188	0.252
PKP4	−0.225	0.169
PLAT	0.097	0.557
PLAU	0.201	0.220
PLG	0.298	0.066
PRKCA	−0.400	0.012
PRKCB1	−0.079	0.635
PRKCD	−0.385	0.016
PRKCE	−0.170	0.302
PRKCG	−0.419	0.008 *
PRKCI	−0.093	0.573
PRKCQ	-	-

Gene	Gene Expression (C_t^{-1}) vs PMI	
	<i>R</i>	<i>P</i> -value
PRKCZ	−0.191	0.243
PSEN1	−0.364	0.023
PSEN2	0.085	0.607
SERPINA3	−0.468	0.003 *
SNCA	−0.282	0.082
SNCB	0.042	0.800
UBQLN1	−0.318	0.049
UQCRC1	−0.298	0.065
UQCRC2	−0.232	0.154
B2M	−0.324	0.044
HPRT1	−0.253	0.121
RPL13A	−0.309	0.056
GAPDH	−0.406	0.010
ACTB	−0.405	0.011

*
 $p < 0.01$