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Gene expression profiling of neurochemically-defined regions of the human brain by *in situ* hybridization-guided laser capture microdissection

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Abstract

Laser capture microdissection (LCM) permits isolation of specific cell types and cell groups based upon morphology, anatomical landmarks and histochemical properties. This powerful technique can be used for region-specific dissection if the target structure is clearly delineated. However, it is difficult to visualize anatomical boundaries in an unstained specimen, while histological staining can complicate the microdissection process and compromise downstream processing and analysis. We now introduce a novel method in which *in situ* hybridization (ISH) signal is used to guide LCM on adjacent unstained sections to collect tissue from neurochemically-defined regions of the human postmortem brain to minimize sample manipulation prior to analysis. This approach was validated in nuclei that provide monoaminergic inputs to the forebrain, and likely contribute to the pathophysiology of mood disorders. This method was used successfully to carry out gene expression profiling and quantitative real-time PCR (qPCR) confirmation from the dissected material. When compared to traditional micropunch dissections, our ISH-guided LCM method provided enhanced signal intensity for mRNAs of specific monoaminergic marker genes as measured by genome-wide gene expression microarrays. Enriched expression of specific monoaminergic genes (as determined by microarrays and qPCR) was detected within appropriate anatomical locations validating the accuracy of microdissection. Together these results support the conclusion that ISH-guided LCM permits acquisition of enriched nucleus-specific RNA that can be successfully used for downstream gene expression investigations. Future studies will utilize this approach for gene expression profiling of neurochemically-defined regions of postmortem brains collected from mood disorder patients.

Keywords

human; serotonin; norepinephrine; postmortem; microarray

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Introduction

Microarray-based gene expression profiling studies have been successfully conducted in the human brain (Mirnics et al., 2001; Baranzini, 2004). Such studies require extracted RNA from dissected brain tissue, and two dissection approaches have been used: 1) gross dissection of anatomically defined brain regions, and 2) laser capture microdissection (LCM) of specific cell populations. While gross dissection assumes homogeneity within anatomically defined brain regions, transcriptional expression may vary greatly within a certain brain structure, as it consists of various cell types, neurons of different neurochemical phenotypes, as well as those with different projection targets and afferent inputs. LCM has the advantage of allowing precise acquisition of discrete populations of cells from histological brain samples, such as an individual nucleus (Bonaventure et al., 2002). To define areas to be used for LCM, a variety of histochemical stains have been used (Torres-Munoz et al., 2004; Greene et al., 2005). However, such staining procedures increase complexity of the tissue processing protocol and can negatively impact the outcome of downstream gene expression studies (Kerman et al., 2006).

To circumvent this problem we have developed a simple method that minimizes processing of tissue sections used for LCM. It utilizes *in situ* hybridization (ISH) and histological staining on adjacent sections for visualization of: 1) major anatomical landmarks, and 2) neurochemically-defined cell populations that define regions of interest. We validated this approach in the postmortem human brain in areas that contain serotonergic (dorsal [DR] and median [MR] raphe) and noradrenergic (locus coeruleus [LC]) neurons. These areas were chosen because they: 1) are relatively small and circumscribed and are well-suited for LCM; 2) are adjacent to each other and allow us to directly evaluate the anatomical resolution of our dissections; and 3) likely play a prominent role in the pathophysiology of neuropsychiatric disorders. Our data demonstrate that this method can be effectively applied for anatomically accurate microdissections, and then combined with downstream microarray and quantitative real-time PCR (qPCR) gene expression measures.

Methods

Subjects

Acquisition of postmortem human brain samples, tissue processing, and procedures for microarray experiments have been previously described (Evans et al., 2003; Evans et al., 2004; Li et al., 2004; Tomita et al., 2004; Choudary et al., 2005). Ten subjects without known psychiatric diagnoses were chosen for ISH-guided LCM (Table 1).

Tissue processing and ISH-guided LCM

Brainstem blocks were cryostat-sectioned (-20°C) in the coronal plane at $10\text{ }\mu\text{m}$ and stored at -80°C . Pairs of adjacent sections $500\text{ }\mu\text{m}$ apart were then processed for radioactive ISHs to detect serotonin (SERT; NM_001045.2; pos.705-1789) and norepinephrine (NET; NM_001043; pos.1-1974) transporter mRNAs as previously published (Neal et al., 2001; Lopez-Figueroa et al., 2004). SERT and NET ISH signals were used to define the boundaries of DR, MR, and LC (Fig. 1). Specificity of labeling was confirmed by absence of signal utilizing sense riboprobes (data not shown).

Following 72-hour exposure to radiosensitive film, the same tissue sections were stained with luxol fast blue combined with cresyl violet. Slide sets from all subjects were then aligned to match along the anterior-posterior axis using anatomical landmarks from the histochemically stained slides and ISH signals.

LCM was performed on unstained sections adjacent to those processed for ISH and histochemical staining. For raphe studies we identified a common region 1.5 mm in length at the mid-caudal level of the SERT signal in all subjects (~ +25 to +27 mm from obex (Paxinos and Huang, 1995)). We collected a total of 9 slides (1 section/slide) from 3 equally-spaced levels (levels 500 μ m apart, 3 adjacent slides/level). For LC studies, we collected a total of 4 slides, 500 μ m apart from within a 2 mm common region of the mid-rostral portion of the nucleus (~ +25 to +27 mm from obex; (Paxinos and Huang, 1995)). For each subject a total of 9 DR nuclei, 9 MR nuclei and 8 LC nuclei (bilateral collection) were collected. Slides were thawed and dehydrated prior to LCM as previously described (Kerman et al., 2006).

LCM was performed with an AutoPix instrument (Molecular Devices, Sunnyvale, CA); laser settings ranged from 50-75mW (power), 1,500-3,500 ms (duration) and 200-250 mV (intensity). Position of anatomical landmarks (e.g. the fourth ventricle and medial longitudinal fasciculus) from histochemically-stained images, and SERT or NET ISH signals were visually projected onto sections used for LCM. Regions in unstained sections that corresponded to boundaries of DR, MR, and LC were then microdissected under a 4x objective using CapSure macrocaps (Molecular Devices) (Fig.1). Each cap contained 3 MR, 3 DR, or 4 LC microdissected areas.

Micropunches

In order to compare data generated by ISH-guided LCM with an established microdissection method using the micropunch technique, brainstem blocks from 2 additional subjects (both 79 years old, male, and Caucasian; 5.5 and 15 hrs. post-mortem intervals [PMI]) were collected and yielded two samples from LC and one each from DR and MR. Brainstem blocks of these subjects were coronally sectioned on a cryostat (at -20°C) to a thickness of 250 μ m and then stored at -80°C until further processing. Immediately prior to the dissections, sections were warmed up to -25°C for 30 min and then placed on a TCP-2 thermoelectric cold plate (Thermoelectrics Unlimited, Wilmington, DE). After quick visual inspection, regions of interest (LC, DR and MR) were dissected using a micropuncher tool (3 mm diameter). Dissected tissue from 4 sections were combined for each of the three nuclei and stored on dry ice. RNA was extracted using RNAqueous isolation kit (Applied Biosystems, Foster City, CA), quantified with Quant-iT™ PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, and then processed for hybridization to gene expression microarrays as described below. Obtained data were compared to all samples from their respective regions collected using ISH-guided LCM.

RNA isolation and amplification

In LCM samples RNA extraction and isolation were performed using the PicoPure RNA Isolation kit (Molecular Devices) according to manufacturer's instructions including DNase treatment. For each nucleus, RNA extracts from the same subjects (3 caps for raphe nuclei and 2 for LC) were combined before purification. RNA quality was evaluated on a 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA), and different regions of resultant RNA electropherograms were quantified according to the method of Schoor et al. (Schoor et al., 2003)

RNA samples were then subjected to two rounds of amplification (RiboAmp OA RNA kit, Molecular Devices) and subsequent biotin labeling (Perkin Elmer, Waltham, MA). After the first round of amplification a portion of amplified double-stranded cDNA was saved for qPCR (see Methods below). Following two amplification rounds, 15 μ g of biotinylated aRNA from each nucleus was then hybridized to HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) per manufacturer's instructions. Biotinylated RNA obtained from micropunched tissue was hybridized to HG-U133A arrays (Affymetrix).

Microarray data analysis

Affymetrix CEL files were analyzed using Robust Multi-Chip Average (RMA) and Affymetrix Microarray Suite 5 (MAS5) calls algorithm. Affymetrix chip description files were replaced by custom probe set mapping files (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/genomic_curated_CDF.asp) that independently reassigned all Affymetrix probe sets to an updated UniGene cluster (Build No. 199). RMA output files containing log₂-transformed intensity values for gene transcripts were statistically analyzed using Prism 3.0 software (GraphPad, San Diego, CA). Genes were considered for further analysis only if detected in ≥ 50% of all subjects according to MAS5CALLS algorithm.

Based on low microarray percent present call rates (> 2*SD away from the mean) the following subjects were excluded: subject 3706 (DR analysis), subject 3433 (LC analysis), subject 3416 (DR and MR analysis).

For gene expression comparison between LCM-dissected and micropunched nuclei, only probe sets for genes present on both array types (HG-U133 Plus 2.0 and HG-U133A) were considered. Due to the small sample size, genes were considered for analysis if detected by MAS5CALLS algorithm.

Additionally, we mined our LCM microarray data for the expression of nonmonoaminergic genes uniquely expressed in LC, DR, or MR. Criteria for uniquely enriched genes in one or two of the three nuclei were: detection in at least 66% of subjects according to MAS5CALLS and a log₂ microarray intensity of 5.5. Genes with a MAS5CALLS rate between 66 and 33% were not considered for nucleus-enrichment analysis.

Quantitative Real-Time PCR (qPCR)

QPCR employing SYBR Green chemistry was used to confirm microarray results. Amplification reactions and fluorescence quantification were performed in real time using a Bio-Rad iCycler (BioRad, Hercules, CA) in combination with a SYBR-488 detection protocol using a touchdown PCR approach (Kerman et al., 2006). Amplification reactions were carried out in 96 well PCR plates (Bio-Rad). Each well contained 5 µl of amplified double-stranded cDNA (aDNA; 50 pg/µl) that was set aside following the first round RNA amplification. Prior to amplification concentration of aDNA was quantified for each sample using Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Each qPCR well also contained 5 µl of forward and reverse strand primers (final concentration: 500 nM) and 10 µl of iQ SYBR Green Supermix (Bio-Rad).

All samples were run in triplicate and an average cycle threshold (Ct) was calculated for each sample. Replicates that were ≥ 1 Ct away from the mean Ct were excluded; the mean Ct included only the remaining duplicates. Subjects that produced only one Ct value were excluded from further analysis. Since input amount of aDNA was equivalent across all samples, raw Ct values were inversely proportional to the levels of gene expression. We chose this approach rather than normalization to housekeeping genes because of the potential for differential expression of such reference transcripts (Dheda et al., 2005; Wong and Medrano, 2005). A similar approach in which standardized DNA input amounts for qPCR were used has recently been validated (Libus and Storchova, 2006).

Relative fold changes were calculated according to the following formula: $2^{-(C_{ta} - C_{tb})}$ in which C_{ta} is cycle threshold in a region of interest and C_{tb} is cycle threshold in a reference region. Following amplification, specificity of each reaction was confirmed by the presence of a single peak on the melting curve, plotted as the negative derivative of fluorescence during incremental

increases in well temperature. No template controls, in which aDNA was replaced with distilled H₂O, did not yield amplification products.

Primer Design

Genomic DNA and mRNA sequences were downloaded from NCBI LocusLink at <http://www.ncbi.nlm.nih.gov/LocusLink/> for: DDC, COMT, MAOA, MAOB, SERT, TPH2, and VMAT2. PCR primer pairs were designed to anneal within 500 bp of the 3' end and to generate a single amplicon between 75 and 150 bp in size using Primer3 software (Rozen and Skaletsky, 2000). For each amplicon predicted secondary structure was minimized using DNA Mfold (Zuker, 2003) (<http://www.bioinfo.rpi.edu/applications/mfold/>). Primer sequences are listed in Table 2. Their performance was validated via serial dilutions and amplification efficiency testing using human genomic DNA or aDNA from human brainstem sections. DNA concentration was quantified using PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Statistical Analysis

Differences in gene expression were quantified as fold changes calculated in relation to either the mean intensity (for microarray data) or mean Ct (for qPCR data) of a reference group. In most cases values obtained from LC samples were used for reference; for MAOA and TPH2 gene expression in LC was not examined and so DR values were used for reference. Values > 1 represent upregulation in expression, while those < 1 represent downregulation relative either to LC or DR.

GraphPad Prism 3.0 software was used for Analysis of variance (ANOVA) comparison of microarray intensity values and fold changes calculated based on microarray and qPCR data. Significant ($p < 0.05$) main effects were followed up with Bonferroni's multiple comparison post-hoc tests; significance level was set at $p < 0.05$.

Linear regression analyses across nuclei between microarray intensities and qPCR Cts of common monoaminergic genes were performed using GraphPad Prism 3.0 software.

Results

LCM Compared to Micropunches

Our initial gene expression studies focused on determining the utility of using LCM as compared to the traditional micropunch method. Analysis of microarray data revealed considerably higher present call rates and signal intensities for all three nuclei in the LCM-obtained samples versus the micropunch samples (Table 3). Furthermore, average gene expression intensities were 2.4- (LC), 3.7- (DR), and 2.2- (MR) fold higher in LCM-dissected material as compared to micropunches.

Analysis of specific monoaminergic mRNAs revealed substantial enrichment in expression in the LCM-obtained material (Table 4). In this analysis signal intensity values were normalized to the average intensity of three housekeeping genes: ACTB, GAPD, PPIA, which are routinely used as reference transcripts in gene expression studies (Eisenberg and Levanon, 2003; Kerman et al., 2006). All of the genes exhibited a large increase in their signal intensities in the LC, ranging from about 3- to almost 40- fold greater in the LCM material (Table 4). In the raphe nuclei, the two receptor mRNAs that were examined (ADRA2A and HTR2A) were only detected in the LCM tissue, while signal intensities of the other transcripts were 3- to over 50- fold higher in the tissue obtained from LCM dissection than from micropunches (Table 4). Taken together these data indicate increased sensitivity of the LCM method as compared to the micropunch collected samples. They also suggest greater dynamic range using this

methodology that may facilitate detection of smaller differences in expression of rare transcripts. Having demonstrated its improved performance as compared to the micropunch method, we applied ISH-guided LCM to examine gene expression differences among specific brainstem nuclei with defined neurochemical properties and known gene expression differences.

RNA Quality

Initial assessment of RNA quality was performed using the Agilent BioAnalyzer instrument, and visual inspection of the BioAnalyzer electropherograms revealed clear 28S and 18S peaks (Fig. 2). To determine whether extracted RNA was of acceptable quality for downstream gene expression analyses, we quantified different regions of each electropherogram according to the method of Schoor, et al. (Schoor et al., 2003). These authors compared results of microarray- and qPCR- based gene expression studies in samples with various levels of RNA degradation and demonstrated that valid results can be obtained if electropherograms from input RNA contain: 1) less than 65% of their signal in the pre-18S peak of the trace, and 2) more than 4% of the signal in the 28S peak region (Schoor et al., 2003). Such analysis of our samples revealed that the pre-18S peak region contained: $44.3 \pm 2.4\%$ (DR), $45.3 \pm 2.6\%$ (MR), and $47.4 \pm 4.0\%$ (LC) of total signal, while the 28S peak region contained: $10.0 \pm 1.7\%$ (DR), $9.4 \pm 0.9\%$ (MR), and $10.8 \pm 0.9\%$ (LC) of total signal. For technical reasons we were not able to quantify these parameters in 3 of the samples. However, downstream gene expression measures (e.g. microarray intensity values, microarray present call rates and qPCR Cts) were not different in these samples. Taken together these data indicate that RNA extracted from our samples was suitable for valid microarray- and qPCR- based gene expression studies.

ISH-Guided LCM

We then set out to determine the anatomic precision of ISH-guided LCM by examining expression of genes known to be differentially enriched within the sampled brain regions. This analysis revealed differential expression of such mRNAs across the three nuclei, with tyrosine hydroxylase (TH), dopamine β -hydroxylase (DBH), and NET mRNA detected only in LC (Fig. 3). Conversely, expression of tryptophan hydroxylase 2 (TPH2), SERT, and monoamine oxidase B (MAO-B) mRNA was greatly enriched within DR and MR. Expression of aromatic amino acid decarboxylase (DDC), catechol-o-methyl transferase (COMT), monoamine oxidase A (MAO-A), and vesicular monoamine transporter 2 (VMAT2) mRNA was detected in all three regions. However, expression of phenylethanolamine N-methyltransferase (PNMT) mRNA, an adrenergic marker, was not detected in any of the three nuclei.

QPCR analyses confirmed microarray findings as regional differences in gene expression were consistently replicated by both methods (Fig. 4). Interestingly, changes in expression as detected by microarrays and qPCR were in agreement in terms of direction of change (i.e. upregulation vs. downregulation across nuclei), but were consistently greater in magnitude as detected by qPCR (note the differences in the scale of y-axes in Fig. 4). In addition, we found significant negative correlations between microarray intensity values and qPCR Cts (Fig. 5). As expected increased microarray intensity values (indicating increased gene expression) correlated with smaller Ct values (Fig. 5), further validating our gene expression findings.

To determine whether ISH guided-LCM method may also be used to identify novel non-monoaminergic genes that are expressed in the three regions included in our study we mined our data for such mRNAs. Results of this analysis are presented in Table 5. A number of genes that are uniquely expressed in these nuclei were identified. Consistent with our findings, previous studies in rodents have also documented enrichment of RGS3 and RGS4 expression in the LC (Gold et al., 1997) and that of VIP in the raphe nuclei (Hill et al., 1994; Hill et al., 2003).

Discussion

The present study introduces a method for using ISH to guide LCM of neurochemically-defined regions of the postmortem human brain for subsequent gene expression profiling by microarrays and confirmation by qPCR. We demonstrate that ISH-guided LCM leads to increased detection of specific mRNAs and to increased sensitivity in subsequent microarray studies when compared to samples harvested with the micropunch technique from the same nuclei. ISH-guided LCM was anatomically precise as evidenced by the patterns of expression of genes known to be specifically expressed in the different brain regions examined in our study. Regional differences in the expression of monoaminergic transcripts detected by microarrays were confirmed by subsequent qPCR validation. In addition, we present preliminary data suggesting that this approach may be used to identify novel transcripts uniquely expressed in the regions of interest.

Micropunch dissection is a fast method to obtain macroscopically identified tissue that may be used for gene expression profiling. It is relatively simple and can be used to microdissect multiple specimens in a short period of time. In contrast, LCM is more labor-intensive, requires additional reagents, tissue processing, and added expenses. We, therefore, set out to determine whether an LCM approach may be advantageous. Our data indicate a substantial improvement in the overall sensitivity and the dynamic range of microarray-based expression profiling of LCM-collected samples as compared to those collected with micropunches. Despite limited micropunch replicates that were used in this initial study, the results are compelling. Compared to micropunches the LCM approach yields: 2.2-3.7- fold increase in overall gene expression, nearly 50% increase in the number of detected transcripts, and as much as a 50-fold increase in enrichment of mRNAs known to be selectively expressed in the regions included in this study. We also observed that two of three housekeeping genes had higher microarray signal intensities in micropunched tissue compared to LCM-obtained material (data not shown). This finding suggests that overabundance of few non-specific genes in micropunched tissue masks the signal for specific, monoaminergic genes. In addition, RNA integrity of micropunch and LCM tissue was assessed as 3' to 5' signal intensities ratio of one internal control gene -- GAPDH. This analysis indicated comparable high RNA quality of micropunch and LCM samples for all three nuclei (Supplemental Material, Table 1). Taken together our results indicate that the limited anatomical resolution by micropunch dissections leads to decreased detection of specific transcripts, whereas ISH-guided LCM enhances sample homogeneity as reflected in increased sensitivity and enrichment of specific transcripts.

RNA quality is an important factor for subsequent gene expression profiling methods, as severely degraded RNA can negatively impact outcomes of gene expression studies (Tomita et al., 2004; Copois et al., 2007). Evaluation of postmortem RNA quality is not trivial, as measures, such as 18S/28S ratio can be misleading since they quantify indicators of ribosomal RNA to infer the quality of mRNA species, which are usually much smaller in size and may be differentially susceptible to degradation (Schoor et al., 2003; Copois et al., 2007; Weis et al., 2007). Studies utilizing postmortem tissues are faced with increased challenges mainly due to the delay between death and brain collection that can negatively impact RNA integrity and make it nearly impossible to obtain non-degraded RNA. Therefore, an important consideration is whether such studies are compatible with gene expression studies, and whether partially degraded input RNA can be used for valid gene expression analyses. To address this issue Schoor and colleagues investigated effects of RNA degradation on gene expression as measured by qPCR and microarrays. As a measure of RNA quality, they quantified total signal within the different regions of BioAnalyzer electropherograms and concluded that RNA samples are compatible with gene expression analyses if: 1) the region between marker and 18S peaks is less than 65% of the overall signal area, and 2) the 28S peak constitutes more than 4% of the overall signal (Schoor et al., 2003). Using such cut-off criteria they demonstrated

similar gene expression profiles between intact and partially degraded RNA samples (Schoor et al., 2003). Electropherograms from postmortem samples in our study show only a moderate degree of degradation and fulfill the criteria for acceptable RNA quality (~45% in pre-18S region and ~10% in 28S region) as defined by Schoor and colleagues.

One limitation of LCM is that increasingly complex tissue processing protocols that are often required for tissue visualization can compromise downstream sample processing and subsequent analyses (Kerman et al., 2006). To circumvent this problem, while still maintaining the necessary anatomical precision of microdissection, we used: 1) images of ISH autoradiograms from adjacent sections, and 2) images of adjacent sections stained with luxol fast blue and cresyl violet to guide LCM. We were able to successfully implement this strategy in an anatomically precise way as evidenced by our gene expression data. Clear differences in the expression of genes that are specifically associated with serotonergic and/or noradrenergic transmission were detected. For example, consistent with previous reports we detected expression of TH and NET in the noradrenergic LC but not in the serotonergic DR and MR (Eymin et al., 1995). On the other hand, TPH2 (Patel et al., 2004) and SERT (Austin et al., 1994) mRNAs are primarily expressed in the raphe nuclei, which was also confirmed in our study. Furthermore, PNMT, the synthetic enzyme for epinephrine, is not expressed in serotonergic or noradrenergic neurons (Carton et al., 1989) and, as expected, its mRNA was not detected in any of the nuclei that we examined.

In addition to these unique mRNAs, serotonergic and noradrenergic transmitter systems also share several enzymes, including MAO-A and MAO-B (Jahng et al., 1997). However, their relative content differs between the two systems. Compared to MAO-B, MAO-A mRNA is more abundant in the human LC, while the converse is true within DR and MR (Saura et al., 1996; Ordway et al., 1999). These findings were also confirmed by our microarray (Fig. 3) and qPCR (Fig. 4) analyses.

Taken together these data validate ISH-guided LCM method and demonstrate the feasibility of combining it with gene expression profiling and qPCR validation. Its increased anatomical resolution leads to more precise sampling, which in turn enriches expression of specific mRNAs and improves sensitivity and dynamic range of microarray-based gene expression profiling. We also present preliminary evidence that this method can be applied to identification of uniquely enriched transcripts within the three monoaminergic regions included in this study (Table 5). Though these data are preliminary and will require additional confirmation, they are consistent with previous reports that documented similar expression patterns for VIP (Hill et al., 1994; Hill et al., 2003), RGS3 (Gold et al., 1997), and RGS4 (Gold et al., 1997) in the rodent brain.

As noradrenergic and serotonergic neurotransmission is implicated in the etiology of major depression (Maas et al., 1987; Leonard, 2000) and bipolar disorder (Swann et al., 1999), use of this ISH-guided LCM approach has the potential to uncover novel gene expression alterations in these illnesses in key monoaminergic brain regions. Having validated ISH-guided LCM on brain regions with clear anatomical borders and known neurochemical differences, we also plan to apply this method to gene expression studies of human postmortem brain areas that are less defined by anatomical landmarks but more by their neurochemical content (such as neuropeptides).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Brain sections containing noradrenergic and serotonergic brainstem nuclei. A — coronal brainstem section stained with luxol fast blue combined with cresyl violet. Dashed square area in panel A is shown at higher magnification in B. It illustrates the region containing locus coeruleus, as identified by the dark-colored melanin-containing cells. Adjacent sections to the one shown in A were processed for norepinephrine transporter (C) and serotonin transporter (D) *in situ* hybridizations. Panels E and F display sections that were adjacent to those in C and D, respectively, from which locus coeruleus (bilaterally; E) and dorsal and median raphe (F) have been removed by laser capture microdissection. In F note that the microdissected dorsal aspect contained the dorsal raphe nucleus, whereas the removed ventral area represented the

median raphe nucleus. All scale bars - 1 mm. Abbreviations: 4V -fourth ventricle, ctg - central tegmental tract, DR — dorsal raphe nucleus, LC - locus coeruleus, mlf - medial longitudinal fasciculus, MR — median raphe nucleus, PnO -pontine reticular nucleus, oral part, scp - superior cerebellar peduncle.

Figure 2.

RNA electropherograms from LCM-dissected samples. Representative electropherograms from LC, DR, and MR are shown (numbers refer to subject identifiers). Locations of marker, 18S, and 28S peaks are shown with arrows. Such traces were used to calculate area under the curve for different portions of the electropherogram as part of RNA quality assessment (see text for details).

Figure 3.

Expression of specific noradrenergic and serotonergic mRNAs. Comparisons of gene expression intensities were sorted into four classes: synthetic enzymes (A), transporters (B), and degradative enzymes (C). * - $p < 0.05$ vs. LC, # - $p < 0.05$ vs. MR (one-way ANOVA with Bonferroni's multiple comparison post-hoc tests). Abbreviations: COMT — catechol-O-methyl transferase, DBH — dopamine β -hydroxylase, DDC — aromatic amino acid decarboxylase, MAO-A — monoamine oxidase A, MAO-B — monoamine oxidase B, NET — norepinephrine transporter, n. d. — not detected, PNMT — phenylethanolamine-N-methyl transferase, SERT — serotonin transporter, TH — tyrosine hydroxylase, TPH2 — tryptophan hydroxylase 2, VMAT2 — vesicular monoamine transporter 2.

Figure 4.

Comparison of differences in gene expression as detected by microarrays and qPCR. For each gene fold changes were calculated relative to mean expression value (intensity measure for microarrays, and Ct for qPCR) in LC. In the case of MAOA and TPH2, qPCR was not performed in LC. Data for DR and MR are presented for these transcripts; differences in gene expression were calculated relative to DR. Note the difference in the scale of y-axes between microarrays and qPCR plots, which is due to the larger magnitude of changes detected by qPCR. * - $p < 0.05$ as compared to LC; # - $p < 0.05$ vs. MR.

Figure 5.

Correlations between qPCR and microarray results. Relationship between qPCR cycle thresholds and log₂ array intensities is depicted for 5 monoamine genes that are expressed in all three nuclei. Linear regression analysis was performed and deviation from zero was statistically evaluated. Goodness of linear regression fit (R^2) were 0.49, 0.16, 0.29, 0.79, 0.89 for DCC, COMT, MAOA, MAOB, and SERT, respectively. Correlations for all 5 genes were statistically significant ($p < 0.05$).

Table 1
List of subject characteristics. Averages $\pm$ S.E.M.: age (49.5 $\pm$ 5.2); PMI (21.0 $\pm$ 1.5); pH (6.95 $\pm$ 0.04). Previous investigations have demonstrated that low tissue pH can profoundly alter patterns of gene expression (Li et al., 2004; Tomita et al., 2004; Atz et al., 2007). Therefore, in the present study we utilized only brain samples with a pH $\geq$ 6.81. Abbreviations: F - female, M - male, PMI - post-mortem interval, med. cond. - medical condition.

subject	race	age	sex	PMI (hours)	brain pH	cause of death
2169	Caucasian	18	M	22	6.97	accident
2292	Caucasian	55	M	15	6.89	sudden med. cond.
2805	Caucasian	45	M	21	6.86	sudden med. cond.
3228	Pacific Islander	39	M	18.15	6.81	sudden med. cond.
3433	Caucasian	39	M	30	7.02	accident
3516	Caucasian	41	M	22.5	7.01	sudden med. cond.
3519	African American	65	M	13.5	6.88	sudden med. cond.
3520	Caucasian	74	F	18.5	7.21	sudden med. cond.
3588	Caucasian	56	M	24.5	6.98	sudden med. cond.
3706	Caucasian	63	M	24.5	6.88	sudden med. cond.

Table 2

Primers used for qPCR. Gene names, symbols, and NCBI accession numbers are shown for the seven transcripts that were examined. Abbreviations: bp — base pairs, Tm — melting temperature.

Gene	Symbol	Accession number	Primers (5',3')	Tm (°C)	Product size (bp)
Dopa decarboxylase	DDC	NM_000790	AGAGAGGGAGTAGGAGTGAAAGC AGTTCCATGAAGGCAGGATG	58.25 60.07	108
Catechol-O-methyltransferase	COMT	NM_000754	GTGCTCCTGCTGACCTTCTG AACAAATCCAGTGTTCAGTTTC	58.77 59.35	76
Monoamine oxidase A	MAOA	NM_000240	ACACGAAATTGGAGCTGAGG CAGGGCCAAAGATCTATCAGC	60.25 59.80	124
Monoamine oxidase B	MAOB	NM_000898	TCCCCAATTATCCTCCTTCAC AGACACACCCGCACAAAACAG	47.62 50.00	143
Solute carrier family 18 (vesicular monoamine), member 2	SLC18A2 (VMAT2)	NM_003054	CCAGTGACACAACTCATCCAG GTTGGCAATCGACCAATAACC	59.16 60.20	109
Tryptophan hydroxylase 2	TPH2	NM_173353	TCAATTACCCGTCCTTCTC AGGTCCTGCACCATTTTC	58.98 59.97	98
Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	SLC6A4 (SERT)	NM_001045	TTCTCCCTCCAAAGTGAATG GGGGAATCCATGGAAATAAG	60.04 58.17	120

Microarray results for ISH-guided LCM samples and those obtained using the micropunch approach. Comparisons were made for samples obtained from LC, DR, and MR. Present call rates, unadjusted log₂ signal intensities, and the resulting average differences in expression (expressed as fold changes) between ISH-guided LCM and micropunch-dissected samples are displayed for all three nuclei. Note substantial increases in the percentage of mRNAs that are detected and an overall increase in signal intensity in ISH-guided LCM samples from all 3 regions.

Table 3

	Locus Coeruleus		Dorsal Raphe		Median Raphe	
	LCM	micropunch	LCM	micropunch	LCM	micropunch
% present call rate	61.9	40.4	64.5	30.9	59	33.6
log ₂ signal intensity	5.90	4.61	6.52	4.62	5.77	4.16
fold change difference	2.44		3.72		2.22	

Expression comparison of common monoaminergic marker genes between LCM and the micropunch method. Gene expression was normalized to the average expression of three housekeeping genes: β -actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPD), and peptidylprolyl isomerase A (PPIA); results are expressed as percentage of average signal intensity of the housekeeping genes. In addition, the ratio of normalized expression between tissue obtained from LCM and the micropunch method was calculated for each gene and nucleus. Note substantial enrichment in the expression of these genes in samples obtained with LCM. Abbreviations: n.d. - not detected; ADRA2 - alpha-2A adrenergic receptor; COMT - catechol-O-methyltransferase; DDC - dopa decarboxylase, MAOA - monoamine oxidase A; MAOB - monoamine oxidase B; HTR2A - serotonin receptor 2A.

Table 4

RefSeq Number	Gene symbol	LOCUS COERULEUS			DORSAL RAPHE			MEDIAN RAPHE		
		LCM (%)	punch (%)	ratio LCM/punch	LCM (%)	punch (%)	ratio LCM/punch	LCM (%)	punch (%)	ratio LCM/punch
NM_000681	ADRA2A	19.5	1.8	10.6	3.9	n.d.	-	7.2	n.d.	-
NM_000754	COMT	8.8	2.2	4.1	9.4	2.7	3.5	10.4	2.7	3.8
NM_000790	DDC	8.7	2.6	3.4	19.7	5.9	3.3	24.0	5.1	4.7
NM_000240	MAOA	53.4	2.5	21.4	17.3	1.7	10.4	20.4	1.8	11.2
NM_000898	MAOB	235.2	10.7	21.9	750.8	27.9	26.9	1101.8	21.9	50.4
NM_000621	HTR2A	21.5	0.5	39.1	30.3	n.d.	-	14.6	n.d.	-

Non-monoamine genes with selectively enriched mRNA expression in monoaminergic nuclei. List of genes as detected by microarrays that exhibit enriched mRNA expression in LC, DR, or MR as indicated by “x”.

Table 5

RefSeq_ID	Symbol	Gene Name	LC	DR	MR
NM_006950	SYN1	synapsin I		x	x
NM_003381	VIP	vasoactive intestinal peptide		x	x
NM_003888	ALDH1A2	aldehyde dehydrogenase 1 family, member A2		x	
NM_000830	GRIK1	glutamate receptor, ionotropic, kainate 1		x	
NM_004982	KCNJ8	potassium inwardly-rectifying channel, subfamily J, member 8		x	
NM_004417	DUSP1	Dual specificity phosphatase 1		x	
NM_004823	KCNK6	Potassium channel, subfamily K, member 6		x	
NM_001841	CNR2	Cannabinoid receptor 2	x	x	
NM_006651	CPLX1	Complexin 1	x	x	
NM_000756	CRH	Corticotropin releasing hormone	x	x	
NM_006143	GPR19	G protein-coupled receptor 19	x	x	
NM_000891	KCNJ2	Potassium inwardly-rectifying channel, subfamily J, member 2	x	x	
NM_004978	KCNC4	Potassium voltage-gated channel, Shaw-related subfamily, member 4	x	x	
NM_016524	SYT17	Synaptotagmin XVII	x	x	
NM_000743	CHRNA3	Cholinergic receptor, nicotinic, alpha polypeptide 3	x		
NM_000813	GABRB2	Gamma-aminobutyric acid A receptor, beta 2	x		
NM_000208	INSR	Insulin receptor	x		
NM_021106	RGS3	Regulator of G-protein signalling 3	x		
NM_005613	RGS4	Regulator of G-protein signalling 4	x		