NIH Blueprint NHP Atlas

TECHNICAL WHITE PAPER:
LASER CAPTURE MICRODISSECTION AND MICROARRAY PROFILING OF POSTNATAL BRAIN DEVELOPMENT IN RHESUS MACAQUE

OVERVIEW

Non-human primates provide experimentally tractable model systems that allow carefully controlled analysis of important developmental processes relevant for human health and disease. The goal of the NIH Blueprint Non-Human Primate (NHP) Atlas is to provide a detailed analysis of gene expression in the normal developing primate brain from birth through adulthood in brain regions associated with high-level cognitive, mnemonic and emotional functions as well as neurodevelopmental disorders linked to disruption of these capacities.

A major component of the NHP atlas is genome-wide transcriptional profiling aimed at the identification of transcriptional programs differentially active at different stages of brain maturation in neonates, infants, juveniles and adults. The second phase of this profiling consists of microarray profiling of finely dissected tissue samples from subdivisions of the medial prefrontal cortex, primary visual cortex, hippocampus, amygdala and ventral striatum. Each subdivision is profiled in three independent biological replicate specimens at each of four postnatal developmental stages (0, 3, 12 and 48 months) using Affymetrix GeneChip Rhesus Macaque Genome Arrays.

This document describes details about the generation of these microarray data, which are freely accessible for download.

SAMPLE ISOLATION AND MICROARRAY DATA GENERATION

Frozen postmortem tissue samples from male rhesus macaque (*Macaca mulatta*) were provided by the California National Primate Research Center (CNPRC; http://www.cnprc.ucdavis.edu). For the purpose of generating histological and *in situ* hybridization (ISH) data, as well as RNA analysis by microarray, brain regions were systematically collected from well-characterized rhesus monkeys born and raised at the CNPRC in outdoor, half-acre enclosures that provide a naturalistic setting and normal social environment. Extensive health, family lineage and dominance information is maintained on all animals in the outdoor enclosures. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at UC Davis.

After dissection, brains were sectioned into coronal slabs approximately 1 to 1.5 cm in thickness. The left hemisphere was prepared for sectioning onto microscope slides for ISH. Structures for microarray analysis were scalpel dissected from the right hemisphere slabs, and these samples were then frozen at -80°C until further processed. Structures were isolated with the greatest precision possible based on gross anatomical structure, with minimal white matter inclusion for cortical structures.

Laser microdissection and RNA isolation

Tissue from male monkeys (n = 3 animals) was selected for further thin sectioning and laser capture microdissection (LCM). Frozen structures were cryosectioned at 14 µm onto polyethylene naphthalate (PEN) slides (Leica Microsystems, Inc., Bannockburn, IL) and a 1:10 Nissl series was generated for neuroanatomical reference. After drying for 30 minutes at room temperature, PEN slides were frozen at -80°C. Slides were lightly Nissl stained with cresyl violet to allow cytoarchitectural visualization. Slides were fixed in ice-cold 70% ethanol for 30 seconds, washed 15 seconds in nuclease-free water, stained in 0.7% cresyl violet in 0.05% NaOAc, pH 3.4 for 2 minutes, nuclease-free water for 15 seconds, followed by 15

seconds each in 50%, 75%, and 95% ethanol, followed by 20 seconds in 100% ethanol, and then a final 100% ethanol wash for 25 seconds. Slides were air-dried for 2 minutes, and dessicated in a vacuum for 1 hour, then frozen at -80°C until needed for microdissection. Laser microdissection was performed on a Leica LMD6000 (Leica Microsystems, Inc., Bannockburn, IL), using the Nissl stain as a guide to identify target brain regions. Table 1 provides a detailed list of the substructures collected for gene expression analysis. Samples from 3 replicates per age (0, 3, 12, and 48 months) were collected from the subdivisions listed.

Table 1. Samples collected for gene expression analysis.

Structure	Total Structures Captured	Divisions
Medial Prefrontal Cortex	18	Anterior cingulate gyrus (Area 24, 32): layer 2, 3, 4, 5, 6 Rectal gyrus (Area 14): layer 2, 3, 4, 5, 6 Orbital gyrus (Area 13): layer 2, 3, 4, 5, 6 Dorsolateral prefrontal cortex (Area 9 and 46): layer 2, 3, 4, 5, 6
Ventral Striatum	8	Nucleus accumbens Islands of Calleja Olfactory tubercle Caudate nucleus Putamen Globus pallidus; internal segment Globus pallidus; external segment Internal capsule
Amygdala	10	Central amygdaloid nucleus Anterior amygdaloid area Basolateral amygdaloid nucleus Basomedial amygdaloid nucleus Lateral amygdaloid nucleus Paralaminar amygdaloid nucleus Medial amygdaloid nucleus Anygdalopiriform transition area Amygdalohippocampal area Ventral anterior cortical nucleus of the amygdala
Hippocampus	10	CA1: pyramidal layer, stratum radiatum, stratum oriens CA2: pyramidal layer CA3: pyramidal layer CA4 Dentate gyrus: granule cell layer, subgranular zone, polymorph layer Subiculum
Primary Visual Cortex	14	V1: layer 1, 2, 3, 4A, 4B, 4Ca, 4Cb, 5, 6, white matter V2: layer 2, 3, 4, 5, 6

Microdissected tissue was collected directly into RLT buffer from the RNeasy Micro kit (Qiagen Inc., Valencia, CA) with ß-mercaptoethanol. Samples were volume-adjusted with water to 75 µl, vortexed, centrifuged, and frozen at -80°C.

For the Nissl neuroanatomical reference slides, slides were stored at 37°C for 1-5 days prior to staining. Sections were defatted with xylene or the xylene substitute Formula 83, and hydrated through a graded series containing 100%, 95%, 70%, and 50% ethanol. After incubation in water, the sections were stained with 0.213% thionin, then differentiated and dehydrated in water and a graded series containing 50%, 70%, 95%

and 100% ethanol. Finally, slides were incubated in xylene or Formula 83, and coverslipped with the mounting agent DPX. After drying, slides were cleaned prior to digital imaging.

RNA was isolated for each structure following the manufacturer's directions for the RNeasy Micro kit. RNA samples were eluted in 14 µl and 1 µl was run on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA) using the Pico assay. Due to low sample volume and incompatibility of the eluant with the Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), samples were quantitated using the Bioanalyzer concentration output. This was done by running a 1ng/µl RNA standard on the same Pico chip and then dividing the sample concentration output by the output of the standard concentration. The average RNA Integrity Number (RIN) of all hippocampus, amygdala, ventral striatum, medial prefrontal cortex and visual cortex passed samples was 6.1. Samples were failed when the Bioanalyzer traces showed degraded 18S and 28S bands, with RINs typically lower than 5 failing. In most cases, 5 ng of total RNA was used as the input amount for the labeling reaction.

mRNA profiling

Samples passing RNA quality control (QC) were amplified and profiled as described in Winrow *et al*. (2009) with a few modifications. Briefly, samples were amplified and labeled using a custom 2 cycle version, using 2 kits of the GeneChip HT One-Cycle cDNA Synthesis Kit from Affymetrix. Five ng of total RNA was added to the initial reaction mix together with 250 ng of pBR322 (Invitrogen). *In vitro* transcription (IVT) for the first cycle was performed using a 5X MEGAscript T7 Kit (Ambion). Following the first round of IVT, the plasmid carrier was removed with a DNaseI (Qiagen) treatment. The first round IVT products were purified using the Qiagen MinElute Kit (Qiagen). Input into the second round was normalized to 150 ng. Hybridization was to catalog GeneChip Rhesus Macaque Genome Arrays from Affymetrix containing 52,803 probe sets/sequences. For detailed information about this macaque microarray, see the Affymetrix web site (http://www.affymetrix.com/browse/products.jsp?productId=131496&navMode=34000&navAction=jump&aId=productsNav#1_3). Labeling and scanning were completed following the manufacturer's recommendations and profiles were normalized using robust multi-array (RMA). Sample amplification, labeling, and microarray processing were performed by Covance in Seattle, WA.

MICROARRAY PROCESSING AND NORMALIZATION

BioConductor "affy" package was used to read in the Affymetrix microarray data and to summarize the probe level data into RMA expression measure (Bolstad et al.) through background normalization, log2 transformation, and quantile normalization. ComBat (http://statistics.byu.edu/johnson/ComBat/) (Johnson *et al*.) was applied in order to reduce the systematic bias across multiple batches. As a data-driven Quality Control process, for each batch, we applied clustering/MDS (multi-dimensional scaling) to detect any outlier in the batch by checking whether samples from the same structure/age were grouped together. IAC (inter-array-connectivity) was also calculated to numerically measure how one array was similar to the other arrays in the batch. The same QC process was applied over multiple batches to identify outliers.

ACCESSING MICROARRAY DATA

Microarray data can be downloaded either as raw Affymetrix .CEL files per each LCM tissue sample or as an archive file containing normalized expression values as well as probe and sample metadata necessary for analysis.

REFERENCES

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Microarray probe generation, hybridization and scanning were performed by Covance (Seattle, WA).