NIH Blueprint NHP Atlas

TECHNICAL WHITE PAPER:
MICROARRAY PROFILING OF 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 in brain regions associated with high-level cognitive, mnemonic and emotional functions as well as neurodevelopmental disorders linked to disruption of these capacities.

The primary data component of the NHP atlas is genome-wide transcriptional profiling aimed at the identification of transcriptional programs differentially regulated in specific brain regions at different stages of brain maturation. This profiling was performed in rhesus macaque in two stages, focused on pre- and postnatal development respectively, that together provide a full developmental timecourse from early brain formation through adulthood. The methodological approach aimed to isolate and analyze all histologically discrete developmental and mature partitions (*e.g.*, layers, nuclei) of the neocortex (primary visual cortex and medial prefrontal cortex), hippocampus, striatum and amygdala. Laser microdissection was used to isolate these zones from tissue sections, and RNA isolated from these samples was amplified and used for DNA microarray analysis using Affymetrix GeneChip Rhesus Macaque Genome Arrays.

For transcriptome analysis of prenatal development, laser microdissected samples were profiled in four independent, timed-pregnancy-derived biological replicate specimens (2 males, 2 females) at each of six prenatal developmental stages (E40, E50, E70, E80, E90, and E120). These timepoints were selected to coincide with peak periods of neurogenesis for the different layers of primary visual cortex based on birthdating experiments. Based on Nissl and marker staining, discrete proliferative and postmitotic developmental compartments were independently profiled in each developing brain region.

For transcriptome analysis of postnatal development, laser microdissected samples were profiled in three independent biological replicate specimens at each of four postnatal developmental stages representing the neonate (0 months), infant (3 months), juvenile (12 months) and post-pubertal adult (48 months). Based on Nissl staining, discrete nuclei and layers of each structure were independently profiled at each timepoint. A comparable transcriptional analysis of the same set of developmental stages and anatomical structures was also generated from freshly macrodissected primary visual cortex, medial prefrontal cortex, hippocampus, amygdala, and ventral striatum.

This document describes details about the generation of these microarray data, starting with the specimen preparation through microarray data generation and data processing. All data are freely accessible for downloading.

LASER MICRODISSECTION SAMPLE ISOLATION AND MICROARRAY DATA GENERATION

Frozen postmortem tissue samples from prenatal rhesus macaque (*Macaca mulatta*) were provided by the Time-Mated Breeding Program at the California National Research Primate Center (CNPRC; http://www.cnprc.ucdavis.edu). For the purpose of RNA analysis, prenatal brain material was acquired following fetectomy using Standard Operating Procedures (SOPs) at the CNPRC. Extensive health, family lineage and dominance information was maintained on all animals in the Time-Mated Breeding

Program. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at UC Davis.

Frozen postmortem tissue samples from postnatal male rhesus macaque were provided by CNPRC. For the purpose of generating postnatal histological and *in situ* hybridization (ISH) data, as well as gene expression 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 were maintained on all animals in the outdoor enclosures. All procedures were approved by the IACUC at UC Davis.

For a subset of E40 specimens, following specimen collection, the calvarium was frozen intact in an isopentane/dry ice slurry maintained at -40°C to -45°C, gradual freezing at a steady rate. For the majority of E40 specimens, the specimens were embedded in OCT (optimal cutting temperature compound) during the freezing process. In brief, chilled OCT was placed around the calvarium. A disposable embedding chamber was filled with approximately 5 mm^3 chilled OCT. The specimen was carefully oriented and centered in the OCT, posterior surface down in the OCT. Then, the specimen was aligned along the medial/lateral axis using the bilateral ocular fiduciaries as a frame of reference. Next, the specimen was aligned in the coronal plane. After alignment along all axes, OCT was added to encase the specimen in its entirety. The top of the specimen was covered with approximately 3 mm of OCT. The chamber containing the specimen was directly placed onto a level bed of dry ice. The specimen and OCT were allowed to freeze completely. After demarcation of the orientation of the brain in the OCT block, the frozen tissue block was stored at -80°C.

For the E50 specimens, following removal of the brain from the skull, the whole brain was frozen intact in an isopentane/dry ice slurry maintained at -40°C to -45°C, gradual freezing at a steady rate. For a subset of E50 specimens, the specimens were embedded in OCT during the freezing process. In brief, chilled OCT was placed around the intact brain. Freestanding aluminum bars were assembled onto a Teflon coated plate and sized to the appropriate specifications for the E50 brain. The internal chamber was filled with approximately 5 mm^3 chilled OCT. The specimen was carefully oriented and centered in the OCT dorsal surface down in the OCT. Then, the specimen was aligned along the medial/lateral axis using the longitudinal fissure as the frame of reference. Next, the specimen was aligned in the coronal plane. After alignment along all axes, OCT was added to encase the specimen in its entirety. The top of the specimen was covered with approximately 3 mm of OCT. The Teflon plate containing the specimen was directly placed onto the level bed of dry ice. The specimen and OCT were allowed to freeze completely. After demarcation of the orientation of the brain in the OCT block, the aluminum bars were removed and the frozen tissue block was stored at -80°C.

For the E70, E80, E90, and E120 specimens, the hemispheres were bisected along the midline and individually frozen by placing the medial aspect of each hemisphere down onto an aluminum-Teflon coated plate that was slowly lowered into an isopentane/dry ice slurry maintained at -40°C to -45°C. Only approximately a third of the tissue was submerged in the slurry to allow the tissue to gradually freeze and to keep freezing artifacts to a minimum. Frozen hemispheres were stored at -80°C.

Depending on the prenatal timepoint, different approaches were taken for generating coronal slabs. When possible, the number of slabs per specimen was kept to a minimum. The E40, E50, and E70 specimens were not slabbed. For E80, the first slab contained up through the temporal pole and the second slab contained the occipital pole. For E90, the first slab contained the frontal lobe anterior of the temporal pole and the second slab contained temporal pole posterior through the occipital lobe. For E120, three coronal slabs were made. The first slab consisted of the frontal lobe anterior of the temporal pole. The second slab consisted of the temporal pole posterior to the cerebellum including all of the mid-brain. The third slab included primarily the occipital lobe.

For postnatal brains, after dissection brains were sectioned into coronal slabs approximately 1 to 1.5 cm in thickness and the left hemisphere was prepared for sectioning onto microscope slides for ISH. Structures for microarray analysis were isolated from the right hemisphere slabs, and these samples were then frozen at -80°C until processed further.

Prenatal macaque laser microdissection and RNA isolation

Tissue from male and female specimens (per timepoint n = 4 animals, 2 males and 2 females) was selected for further thin sectioning and laser microdissection (LMD). Frozen tissue was 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 for all prenatal timepoints. In addition, for the E40, E50, E70, E80, and E90 timepoints, a 1:10 *GAP43* and 1:10 *ENC1 in situ* hybridization (ISH) series was generated for neuroanatomical reference. For E120, a 1:10 acetylcholinesterase 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 desiccated in a vacuum for 1 hour, then frozen at -80°C until microdissection. Laser microdissection was performed on a Leica LMD6000 (Leica Microsystems, Inc., Bannockburn, IL), using the Nissl stain and *GAP43* and *ENC1* ISH or acetylcholinesterase histological staining as a guide to identify target brain regions. **Table 1** and **Table 2** provide a list of structures collected for gene expression analysis from E50, E70 and E120. In most cases, samples from 4 replicates per age were collected from the subdivisions listed.

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.

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 prenatal E50, E70, and E120 passed samples was 7.1. Samples were failed when the Bioanalyzer traces showed degraded 18S and 28S bands, with RINs typically lower than 4.5 failing. In most cases, 2 ng of total RNA was used as the input amount for the labeling reaction.

Table 1. Neocortical and Hippocampal Prenatal Macaque Laser Microdissection Samples.

Structures	Acronym	E50	E70	E120
marginal zone of rostral cingulate cortex	rCGmz	x	x	x
outer cortical plate of rostral cingulate cortex	rCGcpo		x	
layer II of rostral cingulate cortex	rCG2			x
layer III of rostral cingulate cortex	rCG3			x
inner cortical plate (infragranular layer) of rostral cingulate cortex	rCGcpi	x		
layer V of rostral cingulate cortex	rCG5		x	x
layer VI of rostral cingulate cortex	rCG6		x	x
subplate zone of rostral cingulate cortex	rCGsp	x	x	x
intermediate zone of rostral cingulate cortex	rCGiz	x	x	x
outer fiber (plexiform) zone of rostral cingulate cortex	rCGofz	x	x	
inner fiber (plexiform) zone of rostral cingulate cortex	rCGifz		x	
subventricular zone of rostral cingulate cortex	rCGsz	x		x
outer subventricular zone of rostral cingulate cortex	rCGszo		x	
inner subventricular zone of rostral cingulate cortex	rCGszi		x	
ventricular zone of rostral cingulate cortex	rCGvz			x
outer ventricular zone of rostral cingulate cortex	rCGvzo	x	x	
inner ventricular zone of rostral cingulate cortex	rCGvzi	x	x	
molecular layer of dentate gyrus (cortex)	DGmo			x
granular layer anlage of dentate gyrus (cortex)	DGgra	x		
granular layer of dentate gyrus (cortex)	DGgr		x	x
subgranular zone of dentate gyrus (cortex)	DGsg		x	x
polyform layer of dentate gyrus (cortex)	DGpf			x
dentate migratory stream	DMS	x		
marginal zone of CA1	CA1mz	x		
stratum lacunosum-moleculare of CA1	CA1lm		x	x
stratum radiatum of CA1	CA1ra		x	x
hippocampal plate of CA1	CA1cp	x		
stratum pyramidale of CA1	CA1py		x	x
hippocampal subplate of CA1	CA1sp	x	x	
stratum oriens of CA1	CA1or			x
hippocampal intermediate zone of CA1	CA1iz		x	
hippocampal subventricular zone of CA1	CA1szi	x	x	
hippocampal ventricular zone of CA1	CA1vz	x	x	x
stratum pyramidale of CA2	CA2py		x	x
stratum pyramidale of CA3	CA3py		x	x
pyramidal layer of subiculum	Spy		x	x
cortical hem	Hem	x	x	
marginal zone of V1	V1mz	x	x	x
layer II of V1	V1-2			x
layer III of V1	V1-3			x
layer IVA of V1	V1-4A			x
layer IVB of V1	V1-4B			x
layer IVCa of V1	V14Ca			x
layer IVCb of V1	V14Cb			x
inner cortical plate (infragranular layer) of V1	V1cpi	x		
layer V of V1	V1-5		x	x
layer VI of V1	V1-6		x	x
subplate zone of V1	V1sp	x	x	x
intermediate cell dense zone of V1	V1icd		x	
outer fiber (plexiform) zone of V1	V1ofz		x	
transitory migratory zone of V1	V1tmz		x	
intermediate zone of V1	V1iz	x	x	x
subventricular zone of V1	V1sz	x		x
outer subventricular zone of V1	V1szo		x	
inner subventricular zone of V1	V1szi		x	
ventricular zone of V1	V1vz			x
outer ventricular zone of V1	V1vzo	x	x	
inner ventricular zone of V1	V1vzi	x	x	

Table 2. Amygdaloid and Striatal Prenatal Macaque Laser Microdissection Samples.

Structures	Acronym	E50	E70	E120
amygdaloid complex	Amy	x		
medial division of central nucleus	CEm		x	x
lateral division of central nucleus	CEl		x	x
periamygdaloid cortex (cortical amygdaloid nucleus)	PAC			x
medial nucleus	Me		x	x
lateral nucleus	L		x	x
basal nucleus (basolateral nucleus)	B		x	x
accessory basal nucleus (basomedial nucleus)	AB		x	x
paralaminar nucleus	PL			x
amygdaloid intramedullary gray	IMG		x	x
lateral ganglionic eminence-cortex border	LGEcx	x	x	
lateral ganglionic eminence	LGE	x	x	x
medial ganglionic eminence	MGE	x	x	x
caudal ganglionic eminence	CGE	x	x	x
caudate nucleus	Ca	x	x	x
putamen	Pu	x	x	x
nucleus accumbens	NAC	x	x	x
external segment of globus pallidus	GPe	x	x	x
internal segment of globus pallidus	GPi	x	x	x
internal capsule	ic	x	x	x
dorsal lateral geniculate nucleus	DLG	x	x	
dorsal lateral geniculate nucleus, magnocellular layers	DLGmc			x
dorsal lateral geniculate nucleus, parvocellular layers	DLGpc			x
dorsal lateral geniculate nucleus,koniocellular layers	DLGkc			x

Postnatal macaque laser microdissection and RNA isolation

Tissue from male monkeys (n = 3 animals) was selected for further thin sectioning and laser microdissection (LMD). Frozen structures were cryosectioned at 14 µm onto 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 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 3** provides a detailed list of the postnatal 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 3. Postnatal Macaque Laser Microdissection Samples.

Structure	Total Structures Captured	Subdivisions
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.

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.

Histological stains

Nissl
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.

Acetylcholinesterase
A modified acetylcholinesterase protocol was used to assist in neuroanatomical delineations in E120. Unlike acetylcholinesterase staining in fixed tissue, staining in fresh frozen tissue does not elucidate cholinergic fibers commonly seen in the cortex, but instead provides demarcation of various subcortical nuclei. Acetylcholinesterase staining was performed using a direct coloring thiocholine method combined with a methyl green nuclear counterstain to improve tissue visibility (Karnovsky and Roots, 1964). Glass slides with fresh frozen tissue sections were removed from 4°C, allowed to equilibrate to room temperature, fixed in 10% neutral buffered formalin (NBF) and washed briefly in ultra-pure water. Sections were then incubated for 25 minutes in a solution of acetylthiocholine iodide, sodium citrate, cupric sulfate, and potassium ferricyanide in a

0.1M sodium acetate buffer (pH 6.0), washed in 0.1M Tris-HCl buffer (pH 7.2), and incubated with 0.5% diaminobenzidine (DAB) in 0.1M Tris-HCl with 0.03% hydrogen peroxide for 8 minutes. Slides were incubated in 0.2% methyl green, briefly dipped in 100% ethanol, cleared with Formula 83 and coverslipped with DPX. After drying, slides were cleaned prior to digital imaging.

In situ hybridization (ISH)
High-throughput colorimetric ISH methods are essentially described in detail elsewhere (see Lein *et al*., 2007) and in the In Situ Hybridization white paper located under the Documentation tab. In brief, fresh frozen tissue sections (from either E40, E50, E70, E80, or E90) on slides were fixed in 4% PFA in PBS, acetylated, and dehydrated through graded alcohols. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Proteinase K digestion at 0.0135 unit/ml was done. Digoxigenin (DIG)-labeled riboprobes (either *GAP43* or *ENC1*) were hybridized at 63.5°C for 5.5 hr, followed by stringency washes and a series of enzymatic reaction steps for detection and amplification of DIG signal. Sections were washed with EDTA, fixed in 4% PFA, and washed with acid alcohol (70% ethanol, adjusted to pH = 2.1 with 12 N HCl) to reduce background signal. Slides were coverslipped with Hydromatrix, subjected to quality control analysis and cleaned prior to digital imaging.

Digital imaging and processing of histologically stained sections
Digital imaging was done using the ScanScope XT (Aperio Technologies Inc., Vista, CA). Final resolution of images was 1 µm/pixel. All images were databased and preprocessed, then subjected to quality control analysis to ensure optimal focus and that no process artifacts were present in the images. Images that passed this initial QC were further assessed to ensure that the staining data were as expected. Once all QC criteria were met, images became available for annotation of anatomic structures.

mRNA profiling
Prenatal samples passing RNA quality control (QC) were amplified and profiled. Briefly, samples were amplified and labeled using a custom 2 cycle Ultra Low Input procedure, using components of MessageAmp II aRNA Amplification kit (AM1751) for the first amplification cycle (using oligo dT), and components of MessageAmp II Biotin Enhanced Single Round aRNA Amplification kit (AM1791) for the second amplification cycle (using both random hexamers and oligo dT). For prenatal samples, 2ng of total RNA was added to the initial reaction mix together with 250ng of pBR322 (Invitrogen). Following the first cycle of IVT, the plasmid carrier was removed with a DNaseI (Qiagen) treatment. The first cycle IVT products were purified using the Qiagen MinElute Kit (Qiagen). Input into the second cycle was normalized to 400ng. 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. QC assessment failed a small number of microarray samples. Sample amplification, labeling, and microarray processing were performed by Covance in Seattle, WA.

Postnatal 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. For postnatal samples, 5 ng of total RNA was added to the initial reaction mix together with 250ng 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 150ng for postnatal samples. Hybridization was to catalog GeneChip Rhesus Macaque Genome Arrays. 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
For postnatal samples, 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, $\log_2$ 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. The purpose of normalization was to minimize the effects of these non-biological biases while keeping biological variance intact so that within and across brain comparisons primarily reveal differences and similarities that are biologically relevant. As a data-driven QC 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 microarray was similar to the other microarrays in the batch. The same QC process was applied over multiple batches to identify outliers.

POSTNATAL MACAQUE MACRODISSECTION SAMPLE ISOLATION AND MICROARRAY DATA GENERATION

Frozen postmortem tissue samples from male rhesus macaque (*Macaca mulatta*) were provided by the 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, ½-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 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 of interest 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.

Tissue structures were homogenized in TRIZOL. The aqueous phase was removed and further processed for RNA isolation, using a modified version of Ambion's bead-based MagMAX-96 Total RNA Isolation kit, done on the MagMAX Express instrument. RNA samples were examined using a Bioanalyzer to assess RNA quality and concentration. Microarray data generation was performed by Covance (Seattle, WA) using 50 ng total RNA starting material and Affymetrix GeneChip Rhesus Macaque Genome Arrays.

Sixty microarrays were generated in total, comprising five neuroanatomical structures across four developmental timepoints in triplicate. The structures and ages are listed in **Table 4**.

Table 4. Postnatal Macaque Macrodissection Experimental Design.

Neuroanatomical Structure	Ages	Replicates/Age
Medial prefrontal cortex	0, 3, 12, 48 months	3
Primary visual cortex	0, 3, 12, 48 months	3
Amygdala	0, 3, 12, 48 months	3
Hippocampus	0, 3, 12, 48 months	3
Ventral striatum	0, 3, 12, 48 months	3

For processing the microarray data, Affymetrix microarray data was read in using the BioConductor "affy" package. Probe level expression was summarized using the RMA (Robust Multi-array Average) normalization method (Bolstad *et al*.) that consisted of background subtraction, $\log_2$ transformation, and quantile normalization. No further normalization was applied since all macrodissected samples were hybridized in a single batch.

ACCESSING MICROARRAY DATA

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

for analysis. Prenatal microarray data can be downloaded as raw Affymetrix .CEL files along with sample metadata.

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