**ONLINE METHODS**

1. **Introduction**

In this section we provide further information regarding the study design, materials and methods. The materials and methods section provide detailed description of the collection, dissection methods, and quality control assessments of human, chimpanzee, and macaque postmortem tissue used in this study. We provide technical descriptions of data generation and analyses using different platforms.

1. **Procurement and Sampling of Postmortem Human, Chimpanzee, and Macaque Brains** 
   1. **Tissue procurement**

Human (*Homo sapiens*) brain specimens were collected postmortem from 6 adult donors [21 years old (Y) female, 23 Y male, 30 Y female, 36 Y male, 37 Y male, 40 Y female]. The postmortem interval (PMI) was defined as hours between time of death and time when tissue samples were frozen. Tissue was collected after obtaining parental or next of kin consent and with approval by the institutional review boards at the Yale School of Medicine. Tissue was handled in accordance with ethical guidelines and regulations for the research use of human brain tissue set forth by the NIH (<http://bioethics.od.nih.gov/humantissue.html>) and the WMA Declaration of Helsinki (<http://www.wma.net/en/30publications/10policies/b3/index.html>). Appropriate informed consent was obtained and all available non-identifying information was recorded for each specimen.

Chimpanzee (*Pan troglodytes*) brain samples were collected postmortem from 5 adult specimens (23 Y female, 31 Y female, 31 Y male, 27 Y male, 23 Y male). Brains specimens were obtained from the Alamogordo Primate Facility (Holloman Air Force Base, New Mexico, USA). All chimpanzees suffered sudden death with no prolonged agonal state and for reasons other than their participation in this study, and without any relation to the tissue used.

Rhesus macaque (*Macaca mulatta*) brain samples were collected postmortem from 5 adult specimens (11 Y female, 11 Y female, 11 Y female, 7 Y male, 7 Y male). All experiments using non-human primates were carried out in accordance with a protocol approved by Yale University’s Committee on Animal Research and NIH guidelines. The present study also complies with the definition of research exempt from the NIH Chimpanzee Research Use Panel (NOT-OD-14-024) regarding the use of chimpanzees or chimpanzee biomaterials. For detailed information on sample processing and quality control see Supplemental Information.

* 1. **Neuropathological evaluation of human and non-human primate (NHP) brains**

All clinical histories, tissue specimens, and histological sections were evaluated to assess for hypoxia, cerebrovascular incidents, tumors, microbial infections, neurodegeneration, demyelination, and metabolic disease. Tissue samples from the brain specimens analyzed in the study were fixed in 4% paraformaldehyde and processed for histological examination using Nissl stain and immunohistochemistry. No obvious signs of neuropathological alterations were observed in any of the human, chimpanzee, or macaque specimens. Human specimens were collected and processed as part of the BrainSpan project ([www.brainspan.org](http://www.brainspan.org)).

* 1. **Selection criteria for brain specimens**

To better ensure consistency between samples and decrease potential variation due to ante- and postmortem conditions, specific selection criteria were arbitrarily established. The aim was to collect tissue specimens from clinically unremarkable donors. The following selection criteria were strictly adhered to when deciding whether to exclude or include each human brain specimen:

* Only brains free of obvious malformations or lesions were collected. Disqualifying characteristics included prominent intraparenchymal hemorrhage and ischemia, infection, periventricular leukomalacia, abnormal meninges, dysplasia, hypoplasia, alterations in the pial or ventricular surface, and extensive white matter lesions.
* Samples were excluded if microscopic analysis revealed extensive neuronal loss, neuronal swelling, glioneuronal heterotopias, or dysmorphic neurons and neurites.
* Samples that tested positive for Hepatitis B, Hepatitis C, or HIV were excluded.
* Specimens were excluded if excessive drug or alcohol abuse was reported. All specimens with any known neurological or psychiatric disorders, or any prolonged agonal conditions (coma, prolonged pyrexia, hypoxia, seizures, prolonged dehydration, hypoglycemia, and multiple organ failure) were not included in this study. Other excluding factors included ingestion of neurotoxic substances at the time of death, suicide, severe head injury, significant hemorrhages, widespread vascular abnormalities, ischemia, tumors, stroke, congenital neural abnormalities, and signs of neurodegeneration (spongiosis, amyloid plaques, neurofibrillary tangles, Lewy bodies, and amyloid angiopathy).

We collected tissue specimens from chimpanzees and macaque that were not used for brain invasive procedures and had no signs of malformations or lesions. Chimpanzees were tested negative for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus. Macaques were tested negative for herpes B virus and tuberculosis.

* 1. **Tissue dissection**

Different dissection procedures were used for each specimen depending on whether the samples were frozen (human and chimpanzee specimens) or fresh (macaque specimens). Human brain regions were sampled as described in Kang et al.21. Chimpanzee and macaque brain regions were sampled using anatomical landmarks described in the DeLucchi51 and Saleem and Logothetis52 reference atlases, respectively.

* + 1. **Regional sampling from fresh brain specimens**

Macaque brains were chilled on ice for 15–30 minutes prior to sectioning. Brains were placed ventral side up onto a chilled aluminum plate (1 cm-thick) on ice. The brainstem and cerebellum were removed from the cerebrum by making a transverse cut at the junction between the diencephalon and midbrain. Next, they were sectioned to obtain 1 cm thick serial, coronal sections. The tissue slabs were snap frozen in isopentane (J.T. Baker)/dry ice at -30 to -40ºC and stored at -80ºC.

* + 1. **Regional sampling from frozen brain specimens**

Human and chimpanzee brain specimens and tissue slabs were microscopically inspected and the desired region was demarcated, then dissected using a dental drill (AnyXing, 300D) and a Lindemann Bone Cutter H162A.11.016 or diamond disk saw (Dental Burs USA; r = 11 mm) on a 1 cm-thick aluminum plate over dry ice. Dissected tissue samples were stored at -80ºC prior to further processing.

* 1. **Histological verification of tissue sampling**

To verify that the brain region or NCX area of interest is properly and consistently sampled, we occasionally also collected small tissue blocks, adjacent to the tissue sample dissected for the RNA extraction. These tissue blocks were then fixed in 4% paraformaldehyde for 48 h, sectioned on a vibratome or cryostat, and Nissl-stained to verify the identities of dissected adjacent tissue (data not shown).

* 1. **Tissue pulverization**

To ensure proper representation of the region of interest, frozen tissue samples were pulverized in liquid nitrogen using a ceramic mortar and pestle (Fisher Scientific, cat# 12-961C and 12-961-5C). Pulverized samples were transferred to chilled wide-mouth cryogenic vials (Nalgene, cat# 03-337-7B) and stored at -80ºC until used for RNA extraction.

1. **RNA Isolation, Library Preparation, Sequencing, and Quality Assessment**
   1. **RNA extraction**

A bead mill homogenizer (Bullet Blender, Next Advance) was used to lyse the pulverized tissue. Each pulverized tissue sample was transferred to a chilled safe-lock microcentrifuge tube (Eppendorf). A mass of chilled stainless steel beads (Next Advance, cat# SSB14B) equal to the mass of the tissue was added to the tube. Two volumes of lysis buffer were added to the tissue and beads. Samples were mixed in a Bullet Blender for 1 min at a speed of 6. Samples were visually inspected to confirm desired homogenization and then incubated at 37°C for 5 min. The lysis buffer was added up to 0.6 ml, and samples were mixed in the Bullet Blender for 1 min. Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen) for mRNA-sequencing and using mirVana kit (Ambion) for small RNA-sequencing. Total RNAs from two macaques (RMB 218 and RMB 219) were also extracted using mirVana kit. Each sample was subjected to a DNase treatment (TURBO DNase, Ambion) as per manufacturer’s instructions.

Optical density values of extracted RNA were measured using NanoDrop (Thermo Scientific) to confirm an A260:A280 ratio above 1.9. RIN was determined for each sample using Bioanalyzer RNA 6000 Nano Kit (Agilent), depending upon the total amount of RNA.

* 1. **Library preparation for mRNA-sequencing**

cDNA libraries were prepared using the mRNA-Seq Sample Kit (Illumina) as per the manufacturer’s instructions with some modifications. Briefly, polyA RNA was purified from 1 to 5 µg of total RNA using (dT) beads. Quaint-IT RiboGreen RNA Assay Kit (Invitrogen) was used to quantitate purified mRNA with the NanoDrop 3300. Following mRNA quantitation, 2.5 µl spike-in master mixes, containing five different types of RNA molecules at varying amount (2.5 × 10-7 to 2.5 × 10-14 mol), were added per 100 ng of mRNA53. The spike-in RNAs were synthesized by External RNA Control Consortium (ERCC) consortium by in vitro transcription of de novo DNA sequences or of DNA derived from the *B. subtilis* or the deep-sea vent microbe *M. jannaschii* genomes and were a generous gift of Dr. Mark Salit at The National Institute of Standards and Technology (NIST). These were used both to track the brain regions and to normalize expression levels across experiments. Each sample was tagged by adding a pair of spike-in RNAs unique to the region from which the sample was taken. Also, an additional three common spike-ins were added for controlling sequencing error rates, which is not influenced by SNP existence (Supplementary Table 9). The mixture of mRNA and spike-in RNAs were subjected to fragmentation, reverse transcription, end repair, 3’-end adenylation, and adapter ligation to generate libraries of short cDNA molecules. The libraries were size selected at 200-250 bp by gel excision, followed by PCR amplification and column purification. For RMB 218 and 219, we used TruSeq RNA Sample Prep Kit to generate cDNA libraries as per the manufacturer’s instructions with the similar modification of adding spike-in RNAs before the mRNA fragmentation. The TruSeq Kit allows addition of 12 different types of index, thus the multiplexing of samples when they are sequenced. The final product was assessed for its size distribution and concentration using Bioanalyzer DNA 1000 Kit.

**3.3** **Library preparation for small RNA-sequencing**

The TruSeq Small RNA Sample Kit (Illumina) was used to prepare cDNA libraries per manufacturer instructions. Briefly, 1 µg of total RNA was ligated with 3’- and then 5’-adapters, followed by reverse transcription and PCR amplification. The PCR utilizes 48 different types of primer that will add 48 different index sequences to the adapters. Each library was assessed for the presence of desired micro RNA population by Bioanalyzer High Sensitivity DNA Kit. We pooled 21 samples (16 samples from one brain and 5 technical replicates) with distinct indexes, which allow subsequent retrieval of each sample from multiplexed sequencing runs. Each pooled library was size selected by gel excision. The final product was assessed for its size distribution and concentration using Bioanalyzer DNA 1000 Kit.

**3.4 Sequencing**

We used Illumina Genome Analyzer IIx (GAIIx) for mRNA-sequencing by loading one sample per lane. For RMB 218 and 219, we used HiSeq 2000 by loading 4 samples per lane. For small RNA-sequencing, we used Illumina HiSeq 2000 by loading 21 samples per lane. For GAIIx sequencing, the library was diluted to 10 nM in EB buffer and then denatured using the Illumina protocol. The denatured libraries were diluted to 12 pM, followed by cluster generation on a single-end Genome Analyzer IIx (GAIIx) flow cell (v4) using an Illumina cBOT, according to the manufacturer's instructions. The Illumina GAIIx flow cell was run for 75 cycles using a single-read recipe (v4 sequencing kits) according to the manufacturer's instructions.

For HiSeq 2000 sequencing, the library was diluted to 10 nM in EB buffer and then denatured using the Illumina protocol. The denatured libraries were diluted to 15 pM, followed by cluster generation on a single-end HiSeq flow cell (v1.5) using an Illumina cBOT, according to the manufacturer's instructions. The HiSeq flow cell was run for 75 cycles for RMB218 and 219 mRNA-sequencing, and for 50 cycles for small RNA-sequencing using a single-read recipe (v2 sequencing kit) according to the manufacturer's instructions.

1. **mRNA Sequencing Data Analysis**
   1. **mRNA sequencing alignment, annotation, and expression quantification** 
      1. **Reference genomes and read alignment**

The exporter files generated from Illumina's CASAVA pipeline were converted to fastq file. Reads were then filtered by quality score and mapped to the human (*Homo sapiens*, Feb. 2009 hg19), chimpanzee (*Pan troglodytes*, Oct. 2010 panTro3), and rhesus macaque genomes (*Macaca mulatta*, Jan. 2006 rheMac2), as appropriate, using TopHat54 without providing junction annotation.

* + 1. **Generation of inter-species annotation**

To calculate the gene expression, we first created a composite gene model, using RSEQtools55, based on human Gencode v1056. In the composite model, the overlapped exons in the same gene were merged to one to avoid double counting.

To find the non-human primate orthologous sequences for human mature mRNAs, we applied XSAnno pipeline we previously developed and independently tested57 to generate annotation of human-chimpanzee and human-macaque orthologs, respectively. The orthologs shared by human, chimpanzee, and macaque were obtained by intersecting the lists of human-chimpanzee and human-macaque orthologs. The XSAnno pipeline incorporated whole genome alignment and local alignment with multiple filters. The LiftOver58 parameter “-minMatch” was set to 0.98 for human and chimpanzee and 0.913 for human and macaque, based on bootstrapping. The inter-species PID and PL were both set to 0.95 for human and chimpanzee. The inter-species PID and PL were both set to 0.9, for human and macaque. The 1:1:1 human-chimpanzee-macaque annotation based on Gencode v10 is available for download at http://hbatlas.org/xsanno/.

* + 1. **Expression quantification**

After the reads were mapped to the reference genomes, the expression level of genes and exons were quantified by count number and RPKM (reads per kilobase of exon model per million mapped reads)59 using RSEQtools. Only genes with count number greater than 1 and RPKM greater than 1 in at least 3 samples were included in further analysis.

* 1. **Quality control assessment and detection of outliers**

Several quality control measures were implemented throughout sample preparation and transcriptome data generation steps as described in Kang et al.21. Samples that failed to pass the quality control measures were reprocessed or removed from the downstream analyses.

* + 1. **Number of uniquely mapped reads**

The number and the percentage of uniquely mapped reads were calculated for each sample. Samples with less than 5 million uniquely mapped reads were excluded or re-sequenced.

* + 1. **Uniformity of reads’ coverage along the annotated genes**

All genes were evenly divided into 100 segments from 5’- to 3’-end. We calculated the number of nucleotides mapped to each segment. Only genes with more than 1000 nucleotides mapped were used. The percentage of nucleotides (PN) mapped to each segment was calculated as the number of nucleotides in each segment divided by the total number of nucleotides mapped to the whole gene. The median of segmental PN for all genes was calculated to represent segmental PN of a specific sample. The quartiles of sample segmental PN were then plotted, with solid and dash lines representing median, upper, and lower quartiles, respectively.

* + 1. **Hierarchical clustering and batch effect correction**

All statistical analyses were performed using R. Hierarchical clustering was performed using function *hclust*, with 1-correlation as distance. Hierarchical clustering of log2-RPKM clearly separated samples by species, as well as RNA extraction protocols. To normalize the expression values, we corrected batch effects using R package *ComBat*60. We then built a hierarchical clustering with the normalized data, which resulted in a clustering by species and regions (Supplementary Fig. 1e).

* + 1. **Principal component analysis**

We performed a PCA for all samples included in the study, and for samples belonging to each species separately, using the function *prcomp*. The first three components were plotted using the R package *rgl*61(Fig. 1).

* + 1. **Effects of RIN on mRNA expression**

To investigate the effects of main factors and confounders on the data, we plotted the number of genes with log2(rawRPKM+1) > 1 and log2(rawRPKM+1) > 10 against RIN. No significant correlation was found between the number of expressed genes (log2(rawRPKM+1) > 1) and RIN (Pearson’s correlation = 0.3) and weak correlation was observed between very highly expressed genes (log2(rawRPKM+1) > 10) and RIN (Pearson’s correlation = 0.6).

* 1. **Identification of differentially expressed (DEX) genes**

DEX genes were computed with the R package *DESeq*62. In this package, the count data were modeled with a negative binomial distribution to address the problem of overdispersion.

* + 1. **Identification of inter-species DEX genes**

Inter-species DEX genes were computed for each brain region. Five generalized linear model of the negative binomial family with log link were fitted for each gene. The fitted models were listed as follows:

* Model A: count ~ species + batch (3 levels of *species*: human, chimpanzee, macaque)
* Model H: count ~ species + batch (2 levels of *species*: human, chimpanzee/macaque)
* Model C: count ~ species + batch (2 levels of *species*: chimpanzee, human/macaque)
* Model M: count ~ species + batch (2 levels of *species*: macaque, human/chimpanzee)
* Model 0: count ~ batch

*P* values were calculated by a likelihood ratio test comparing models and inter-species DEX genes were called at a false discovery rate (FDR)63 of 0.01.

Eleven inter-species gene expression patterns were investigated per region. The genes were called at false discovery rate 0.01 for significant differential expression and 0.01 for non-significance. The models used were listed in Supplementary Table 2.

* + 1. **Identification of intra-species DEX genes**

*P* values of likelihood ratio test were computed by comparing the alternative model (count ~ region + batch) with the null model (count ~ batch), for each species. Intra-species DEX genes were called at a FDR of 0.01.

* + 1. **Hierarchical clustering of brain regions**

Hierarchical cluster analysis was performed by *hclust* function in R, using a union set of human, chimpanzee and rhesus macaque intra-species DEX genes. We used 1-correlation as the distance measure. To assess the cluster stability, we computed p values using *pvclust* package64.

* 1. **Weighted gene co-expression network analysis (WGCNA)**

Signed co-expression networks were built using the *WGCNA* package65 in R. To reduce noise, only inter- and intra-species DEX genes were included in the analysis. For all genes included, a pairwise correlation matrix was computed, and an adjacency matrix was calculated by raising the correlation matrix to a power. The power was set to 15 according to a scale-free topology criterion66. For each pair of genes, a robust measure of network interconnectedness (topological overlap measure) was calculated based on the adjacency matrix. The topological overlap based dissimilarity was then used as input for average linkage hierarchical clustering. Modules were generated by hybrid dynamic tree cutting. To obtain rare expression patterns, we set the minimum module size to 5 genes, deepSplit to 4, and the minimum height for merging modules to 0.15.

Each module was summarized by an eigengene, which is the first principal component of the scaled module expression. Thus, the module eigengene explained the maximum amount of variation of the module expression levels. The module membership (also known as module eigengene based connectivity kME) is defined as the correlation between gene expression values and the module eigengene. To obtain modules with clear clustering of expression patterns, we iteratively reassigned genes to the module with largest kME and genes with maximum kME < 0.5 were assigned to the M0.

* 1. **Comparative gene network analysis of neurotransmission systems**

We compared networks of neurotransmitter receptors across species. Networks were constructed based on pairwise correlation of receptors subunit genes from the same neurotransmission system. The preservation of the networks was evaluated with the statistics computed by *modulePreservation* function in the *WGCNA* R package67.

1. **MicroRNA Sequencing Data Analysis**
   1. **Re-annotation of chimpanzee and macaque miRNAs**

The most recent (at the time of writing this manuscript) release of miRbase v18 contains 1,523 annotated precursor-miRNA (pre-miRNA, a.k.a. hairpin) and 1921 mature miRNA sequences for human/hg19.  Unfortunately, the corresponding miRbase annotation for chimpanzee and macaque sequences is far less comprehensive, containing 600 chimpanzee and 479 macaque pre-miRNAs and 525 chimpanzee and 488 macaque mature miRNAs.  The reason for this disparity, despite the high genomic sequence identity of these very closely related species is that the chimpanzee and macaque references have not been updated since the last annotation efforts, for chimpanzee in 2009 based on the liftover of 678 human miRNAs68, and for macaque in 2008 based on the liftover of 533 human miRNAs69.

Such a disparity has been overcome in notable recent publications70 by taking human miRNAs expressed above some arbitrary threshold and lifting over these sequences to the non-human primate genomes.  Such an approach is certainly valid, but is biased, as it does not allow for analysis of miRNAs that may be present in all genomes, but not expressed in human samples.

Our re-annotation effort, described here, does not restrict the analysis in such a way.  We took all 1,523 human pre-miRNA coordinates from miRBase v18 corresponding to hg19, extended each by 100nt up- and down-stream and performed a reciprocal liftover to the chimpanzee (panTro3) and macaque (rheMac2) genomes (Supplementary Fig. 2c).  It is important to note that it is possible that this approach, in a small number of cases, is still capable of overlooking miRNAs that have evolved independently in either the chimpanzee or macaque genomes and/or novel human miRNAs that have not been included in the miRbase release information.  The liftover from human resulted in the identification of 1,469 chimpanzee and 1,386 macaque extended-hairpin-containing regions.  To allow for mutations within the hairpin and/or mature chimpanzee/macaque sequences compared to human, we performed a global-local alignment of the human hairpin sequence to the extended chimpanzee/macaque region identified by the liftover.  The results of this alignment were then taken as the chimpanzee and macaque pre-miRNA sequences.

* 1. **Small-RNA-seq read processing and mapping**

Small-RNA-seq (smRNA-seq) reads were subjected to quality-assessment using the *FastQC* software (v.0.10.1; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) both prior to and following 3'-adapter clipping.  Adapter clipping was performed using the *FastX* software (v.0.0.13; <http://hannonlab.cshl.edu/fastx_toolkit/>) using the *fastx\_clipper* tool requiring identification of at least 7 adapter bases at the 3’-end of a read before clipping and clipped reads shorter than 15nt were discarded.  The distribution of clipped read-lengths, for all samples in each species, (Supplementry Fig. 1d) clearly shows enrichment for reads in the 20-22nt size range, corresponding perfectly to the range of annotated mature-miRNAs.  Separately for each sample identical clipped-reads were counted and collapsed to a single entry using the *fastx\_collapse* tool and reads containing N's were removed.

Reads were mapped directly to the corresponding species' pre-miRNA sequences (for chimpanzee and macaque these were derived from the liftover of human sequences described above) using the Bowtie aligner71 invoked via the command-line implementation of the miRanalyzer tool72, allowing for only a single mismatched base in the alignment.  The output from this read-alignment, for each sample, is a collection of files containing the alignment outcomes (pre-miRNA ID) for each read and the read-stack covering each pre-miRNA sequence (Supplementary Fig. 2c).  The use of miRanalyzer was mostly to coordinate and streamline the process of parsing these alignments and to integrate pre-miRNA secondary-structure prediction, via *RNA-fold* provided in the Vienna software suite73, which we use to define the mature sequences.

* 1. **Identification of mature miRNA sequences**

Mature miRNA sequences were defined directly from the pre-miRNA alignments obtained using the method described above.  This was deemed the most compatible quantification strategy to the liftover method by which the pre-miRNAs were identified for the non-human samples, as it does not require a well-matched human mature miRNA to be able to quantify chimpanzee/macaque mature miRNA abundance.  An example of the read-stack in one of the human samples for miR-124 is provided in Supplementary Fig. 2c; there is a clear distribution of reads that map uniquely to the 3'-end of this sequence that may arise due to technical or biological factors.  Therefore, in defining the start and end position of the mature sequences we took the modal position of all overlapping mapped reads.  For the example case of miR-124-3p, the start position is clearly defined differently to the start position in the human miRBase reference by around 10,000 reads that start with an A at the second position of the miRBase-annotated 3'-mature sequence.

The IDs of all mature miRNA were formatted to reflect the mature sequence position with respect to the pre-miRNA sequence.  Reads seeming to suggest a mature sequence that overlapped with the hairpin loop of the pre-miRNA were excluded from further analysis as these are not supported by the canonical theory of miRNA biogenesis and processing by DROSHA/DICER.

* 1. **Expression quantification, filtering, and normalization**

Reads uniquely mapped to a single position in a single hairpin sequence were added to the total read-count for that mature miRNA.  Reads mapped to multiple ‘candidate’ mature miRNAs were processed to try and identify the most likely assignment based on reads uniquely mapped to each candidate.

The distributions of read-lengths were used to identify outlying samples. Almost all annotated mature miRNAs are between 20-22nt in length therefore samples with an abnormally small fraction of reads of this same length (compared to the other sequenced samples) were flagged for removal. In total, three human and two macaque samples were removed based on the fact that less than 25% of the sequenced reads fell in the 20-22nt size window. For reference, the average fraction of reads in this window over all samples is 38 ± 8% in human, 56 ± 0% in chimpanzee, and 53 ± 0% in macaque.

To compensate for remaining sample-level differences in read-yield, read-counts for each sample, for each miRNA, were adjusted based on the total number of sequenced reads using the ‘estimateSizeFactors’ function in the DESeq package62 in the R/bioconductor environment74.  Following this normalization, technical replicate samples were collapsed to a single replicate per brain/region by taking the mean average.  To reduce the complexity of the dataset miRNAs with very low cumulative read-counts (<50) over all samples were removed. Normalised expressions were log2-transformed and overall sample behavior was assessed by hierarchical clustering of both the absolute expressions and pairwise sample correlations (data not shown).  Sample clustering is dominated by inter-species variance however clustering of subcortical tissues within each species is apparent.

Interestingly, when clustering all expressed miRNAs, human and chimpanzee samples cluster closest leaving macaque as the outgroup; however filtering 65% of the least expressed miRNAs results in human becoming the outgroup — and filtering even more stringently to retain only the 30% highest-expressing miRNAs, the cerebellum of all three species forms the outgroup (data not shown).  This suggests that the CBC is more similar between the three species than any of the other brain regions.

Principal component (PC) analysis of the filtered and normalized expression data revealed that PC1 (78.5% of total variance) correlated strongly with brain region, PC2 (5.2% of total variance) correlated strongly with species, PC3 (3.1% of total variance) correlated strongly with chimpanzee vs. human/macaque, PC4 (1.1% of total variance) correlated slightly with differences between individuals, and PC5 (0.4% of total variance) correlated strongly with the difference between cerebellum, the visual cortex, and all other tissues (data not shown). This was implemented using the R package *prcomp* using the standardising ‘scale=T’ option to scale variables to unit variance.

* 1. **Inter- and intra-species DEX analysis**

Tests for inter-species and inter-tissue differential expression were performed using the DESeq package62.  A total of four models were fit for expressions E:

species: E ~ 1+species

tissue: E ~ 1+tissue

species+tissue: E ~ 1+species+tissue

interaction: E ~ 1+species+tissue+species\*tissue

Significance was computed using a negative-binomial model for each effect by exploiting the following combinations of these models as the null and the alternative hypotheses:

P(species): species+tissue  -  tissue

P(tissue): species+tissue  -  species

P(interaction): interaction - species+tissue

Significance values were adjusted to compensate for multiple testing using the Benjamini-Hochberg correction.  Distributions of pre- and post-adjusted significance values for each of the three tests suggest little difference in the choice of significance threshold for calling differentially expressed miRNAs with approximately 12 false-positive miRNAs DEX between species at pAdj < 1% or ~63 false positives at pAdj < 5%. Based on this minimal difference, the threshold for detecting significantly differentially expressed miRNAs between species, tissues, and their interaction was set to the more conservative <1%.

Inter-species differential expression is presented in Fig. 2f.  Only miRNAs significantly differentially expressed at the inter-species level are plotted.  For those miRNAs that exhibit a high magnitude (>2-fold change) of species-specific expression behavior are highlighted by color: red for human-specific (H>C=M, H<C=M), blue for chimpanzee-specific (C>H=M, C<H=M), and green for macaque-specific (M>H=C, M<H=C) up- or downregulation.  Various pairwise combinations are colored based on the relevant combinations of individual species-specific colors (red, blue, and green).

Post-hoc tests were performed, separately for each species between all pairs of brain regions, to determine miRNAs significantly changed between individual regions.  This was again performed using DESeq and these miRNAs intersected with the tissue-specific results of the GLM tests described above to produce lists of miRNAs significantly differentially expressed between pairs of tissues. Inter-tissue differences are presented, separately for each of the three species (red: human, blue: chimpanzee, green: macaque), based on the expression correlation of the set of miRNAs that achieved statistically significant DEX between all possible pairs of tissues (Fig. 3).  Tissues are ordered so as to match the clustering of tissues based on the mRNAs to facilitate direct comparison.

* 1. **miRNA clustering using the mRNA module eigengenes**

In order to cluster the miRNA expressions in a way that best enables integration and interpretation with the mRNA network analysis, the eigengene expression of the mRNA modules was used to correlate (Pearson) the miRNA expressions (Supplementary Fig. 6). In this way any negative influence due to the bulk differences in between the miRNA and mRNA expression distributions (which is extremely pronounced and would hinder integration of miRNA and mRNA expressions prior to clustering) is minimized. The dendrogram of miRNA distances to the mRNA eigengenes was used to define the 37 miRNA clusters presented in Fig. 4. This number of clusters resulted from maximizing the gap statistic (using the ‘clusGap()’ function in the ‘cluster’ R package) computed using bootstrapped (1000 iterations) kmeans and PAM clustering over a range of cluster numbers (1-50 clusters). The global maximum gap statistic (within this range of 1-50 clusters) was 37 (data not shown).

The average miRNA cluster profiles provided in Fig. 4d were computed from the mean average expression of each tissue in each species. These average values were further autoscaled using the scale() function in R to remove bias due to highly abundant miRNAs in each cluster.

* 1. **miRNA Target Prediction Using AGO2 HiTS-CLIP Clusters in Boudreau et al.**

In order to limit the extreme potential for false-positives in transcriptome-wide computational prediction of miRNA-mRNA target pairs we performed target prediction (using the miRanda tool) only on the regions reported as AGO2 clusters by the HiTS-CLIP analysis performed by Boudreau et al.40. These clusters are loci that should correspond to sites at which the AGO2 protein is actively bound to an mRNA transcript and, as such, present a greatly reduced search space when computationally identifying miRNAs with complementary seed sequences.

The genomic coordinates of these 3,712 clusters (defined by Boudreau et al.40 in terms of the human genome) were lifted-over to the chimp (3,583) and macaque (3,434) genomes as described in earlier sections of the Online Methods. Species-specific cluster sequences were then used as input, along with the species-specific mature miRNA sequences, to miRanda for computational screening of complementary seed sequences to produce three lists (one for each species) of miRNA/HiTS-CLIP-cluster pairs.

A series of filters were then applied to these miRNA/cluster pairs:

1. intergenic and intronic clusters were removed
2. clusters not contained within the 3’UTR were removed
3. clusters for which the miRNA target site was found to be more than +/- 10% of the cluster sequence length away from the middle of the cluster sequence were removed

Starting with a total of 11,303 human miRNA/cluster pairs these filters passed 13.4% (1,517) of the pairs that were considered in downstream analyses as ‘high-confidence’ miRNA/mRNA target pairs. Corresponding fractions of high-confidence chimpanzee and macaque targets were 13.2% and 12.7%, respectively.

Next, we compared the enrichment of these miRNA/mRNA targets in all pairs of miRNA/mRNA modules. Enrichment significance values were computed by chi-square tests an all pairs of modules and corrected for multiple testing via the Bonferroni method. The ratios used in the chi-square calculation were the total number of miRNA-mRNA pairs in the entire dataset vs. the number of possible miRNA-mRNA pairs in the module and the total number of miRNA-mRNA pairs defined by HiTS-CLIP vs. the number of HiTS-CLIP pairs in the module. Module pairs with a Bonferroni-corrected pValue < 0.05 were considered as significantly enriched for HiTS-CLIP defined target pairs (Fig. 4).

Finally, we used the combination of module pairs that exhibited negative correlation of their eigengenes and significant enrichment for HiTS-CLIP clusters to define our set of miRNA and mRNA modules with putative regulatory interaction.

* 1. **Intronic miRNA Identification and Analysis**

Coordinates were extracted from annotated features from GENCODE v10 and the chimpanzee/macaque annotations used throughout the mRNA analyses described in this supplement.  Particularly long miRNAs and features shorter than 200nt were removed to prevent mapping miRNAs to themselves.  Precursor miRNA coordinates obtained from miRBase v18 and from the liftover to the chimpanzee and macaque genomes described above were intersected with the host RNA coordinates. Expression of the miRNA across regions was, separately for each species, correlated with the expression of the host gene. Results of this analysis are presented in Supplementary Table 5 (see table legend for details).

1. **Comparative Gene Network Analysis of Neurotransmission Systems**

We compared networks of neurotransmitter receptors across species. Networks were constructed based on pairwise correlation of receptors subunit genes from the same neurotransmission system. The preservation of the networks was evaluated with the statistics computed by *modulePreservation* function in the *WGCNA* R package.

1. **Functional Enrichment Analysis**

Functional enrichment was assessed using DAVID Bioinformatics Resources (<http://david.abcc.ncifcrf.gov/>). Since the gene annotation analysis is inherently noisy, we used the Bonferroni-adjusted P-value for detection of significant categories. This adjustment is more stringent than the Benjamini-Hochberg, which was used for all of the other analyses in the study.

1. **Determination of the Genetic Relationships Among Samples**

We used the dorsolateral prefrontal cortex (DFC) samples from each individual (except for HSB 136, for which the orbital prefrontal cortex (OFC) was used, as the DFC sample was filtered out in the process of quality control) to study the relationships between species, under the assumption that one sample should represent the true phylogeny of the species.

* 1. **Mapping**

All DFC samples were mapped against the human reference genome by GEM (<http://sourceforge.net/apps/mediawiki/gemlibrary/index.php?title=The_GEM_library>), allowing mismatches based on different divergence of the species. Human samples were mapped at edit distance 1. Unmapped reads were trimmed at 50 bp and mapped at edit distance 1. Chimpanzees were mapped at edit distance 2. Unmapped reads were then mapped at edit distance 3. If still unmapped, reads were then trimmed at 50 bp and mapped at edit distance 2. Macaque samples were mapped at edit distance 2; unmapped reads were then mapped at the edit distance 4, and 6, sequentially. If still unmapped, reads were trimmed at 50 bp and mapped at edit distance 2, 4 and 6, sequentially.

* 1. **Identification of single nucleotide variants**

Regions with more than 20X coverage in all the samples were selected for the SNP calling. This threshold was selected because only one sample is used for this analysis. In total, 3,373 Kbps were used. SNP calling was performed using standard SAMtools methods75 and SNPs with more than two variants were filtered out.

* 1. **Construction of the phylogenetic tree**

PLINK76 was used to calculate a distance matrix between the samples. The distance is defined as the average number of positions in which the two individuals have a different base over the total number of variable sites. MEGA software77 was used to build a neighbor-joining tree from the distance matrix.

1. **Validation of Gene Expression**
   1. **Droplet digital PCR for gene expression quantitation**

We employed droplet digital PCR (ddPCR) to reliably quantitate gene expression. An aliquot of the total RNA that was previously extracted from each brain sample was used for secondary validation through ddPCR analysis. One µg of total RNA was used for cDNA synthesis using SuperScript III First-strand synthesis Supermix (Invitrogen) and subsequently diluted with nuclease-free water. Custom gene-specific primers and probe for each gene of interest were designed using NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and PrimerQuest tool (IDT). ddPCR was carried out using the Bio-Rad QX100 system. After each PCR reaction mixture consisting of ddPCR master mix and custom primers/probe set was partitioned into 15,000–20,000 droplets, parallel PCR amplification was carried out. Endpoint PCR signals were quantified and Poisson statistics was applied to yield target copy number quantification of the sample. Two-color PCR reaction was utilized for the normalization of gene expression by the housekeeping gene *TBP*. Sequences of primers used in validation are as follows:

***TH***: Forward: CCTGACCTGGACTTGGAC

Probe (5’FAM/ZEN/3’IBFQ): ACACCTGGTCCGAGAAGCCC

Reverse: AAGGCGATCTCAGCAATCAG

***DDC***: Forward: CAATCTCTTAGAAGTCGGTCCTATC

Probe-human (5’FAM/ZEN/3’IBFQ): ACGTGCAGCCATATGTCTTCCTTGT

Probe-chimpanzee (5’FAM/ZEN/3’IBFQ): TGCAACAAGGAAGACATGTGGCTGCA

Probe-macaque (5’FAM/ZEN/3’IBFQ): TGCAACAAGGAAGACATGTGGCTGCA

Reverse: ACTCCATTCAGAAGGTGCC

***COMT***: Forward: AGAAAGGCAAGATCGTGGAC

Probe (5’FAM/ZEN/3’IBFQ): CTACTGTGGCTACTCAGCTGTGCG

Reverse-human: GGGTTGATCTCGATGGTGATG

Reverse-chimpanzee: GGGTTGATCTCGATGGTGATC

Reverse-macaque: GGGTTGATCTCGATGGTGAGC

***MAOA***: Forward: ACAAAGTGGAGCGGCTAC

Probe (5’FAM/ZEN/3’IBFQ): CTGCTCGTTCTCCAGCCTCAACT

Reverse: GACCATTTAAGACCTCCCTAGC

***ZP2***: Forward: TGACCCCAACATCAAGCTG

Probe (5’FAM/ZEN/3’IBFQ): TCTTAGATGACTGCTGGGCGACG

Reverse: TGGGGGAAAGAGTCTGGATC

***TWIST1***: Forward: ATGTCCGCGTCCCACTAGC

Probe (5’FAM/ZEN/3’IBFQ): AGGGCCGGAGACCTAGATGTCATT

Reverse: TGTCCATTTTCTCCTTCTCTGGA

***TBP***: Forward: CCACTCACAGACTCTCACAAC

Probe (5’HEX/ZEN/3’IBFQ): CCATCACTCCTGCCACGCCA

Reverse: GTACAATCCCAGAACTCTCCG

* 1. **Immunoblotting**

We homogenized STR brain tissue by sonication on ice in PBS + protease inhibitor cocktail (Roche) followed by centrifugation to separate membranous material from soluble cytosolic proteins. After measuring protein concentration on the soluble fraction, 20 µg of proteins were electrophoresed through 4-12% NuPage gels (LifeTechnologies) and subsequently transferred to nitrocellulose (Bio-Rad) membranes for immunoblotting. Blots were blocked using 5% fat-free milk in Tris-buffered saline + 0.05% Tween-20 for 1h, and then exposed to corresponding primary antibodies targeting TH (ABCAM ab112, 1:200), DDC (ABCAM ab3905, 1:1000), or GAPDH (EnCor Biotechnology MCA-1D4, 1:2000) overnight at 4oC. After extensive washings, blotted protein was exposed to appropriate horseradish peroxidase–conjugated secondary antibodies and visualized with chemi-luminescence systems (Chemiglow). Images shown are representative of two independent experiments that showed similar results.

**9.3. Immunohistochemistry**

For validation studies, adult human, chimpanzee, and macaque brain tissue samples were fixed in 4% paraformaldehyde for 2 days at 4°C. Tissue sections were incubated in 1% hydrogen peroxide/PBS to quench endogenous peroxidase activity. Sections were washed in PBS (3 x 15 min) and incubated in blocking solution containing 5% (v/v) normal donkey serum (Jackson ImmunoResearch Laboratories), 1% (w/v) bovine serum albumin, and 0.4% (v/v) Triton X-100 in PBS for 1 h at room temperature. Primary antibodies – TWIST1 (ABCAM ab49254) and ZP2 (Sigma Aldrich HPA011296) – were diluted (TWIST1 1:100, ZP2 1:100) in blocking solution and incubated with tissues sections overnight at 4°C. Sections were washed with PBS (3 x 15 min) prior to being incubated with the appropriate biotinylated secondary antibodies (Jackson ImmunoResearch Labs) for 1.5 h at room temperature. All secondary antibodies were raised in donkey and diluted at 1:250 in blocking solution. Sections were subsequently washed in PBS and incubated with avidin-biotin-peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories) for 1 h at room temperature. Finally, sections were washed in PBS (3 x 15 min) and signals were developed using a DAB peroxidase substrate kit according to the manufacturer’s protocol (Vector Laboratories). Following washes in PBS, sections were mounted on Superfrost Plus charged slides, dried, dehydrated, and cover slipped with Permount (Fisher Scientific). Sections were digitized using AxioTome (Zeiss). Digitized images were assembled in Zeiss Zen, Adobe Photoshop, and Adobe Illustrator. Images shown are representative of two independent experiments that showed similar results.

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