Molecular and cellular reorganization of neural circuits in the human lineage

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To better understand the molecular and cellular differences in brain organization between human and nonhuman primates, we performed transcriptome sequencing of 16 regions of adult human, chimpanzee, and macaque brains. Integration with human single-cell transcriptomic data revealed global, regional, and cell-type-specific species expression differences in genes representing distinct functional categories. We validated and further characterized the human specificity of genes enriched in distinct cell types through histological and functional analyses, including rare subpiallal-derived interneurons expressing dopamine biosynthesis genes enriched in the human striatum and absent in the nonhuman African neocortex. Our integrated analysis of the generated data revealed diverse molecular and cellular features of the phylogenetic reorganization of the human brain across multiple levels, with relevance for brain function and disease.

Although the human brain is about three times as large as those of our closest living relatives, the nonhuman African great apes (chimpanzee, bonobo, and gorilla), increased size and neural cell counts alone fail to explain its characteristic functionalities (1–5). The brain has also undergone microstructural, connectional, and molecular changes in the human lineage (1–5), changes likely mediated by divergent spatiotemporal gene expression (6–17). Here, we profiled the miRNA and small noncoding RNA transcriptomes of 16 adult brain regions involved in higher-order cognition and behavior of human (Homo sapiens); chimpanzee (Pan troglodytes), our closest extant relative; and rhesus macaque (M) (Macaca mulatta), a commonly studied nonhuman primate. We integrated these profiles with single-cell transcriptomic data from the human brain (18, 19), histological data on January 8, 2018 http://science.sciencemag.org/ Downloaded from
respectively, in the human brain, with many displaying region-specific patterns (Fig. 1B and Fig. S12). Independently validated examples include **PKD2L1** (up-regulated in neocortical areas except primary motor cortex), a gene encoding an ion channel (21); **MET** (up-regulated in prefrontal cortex), a gene implicated in autism spectrum disorder (22); **ZP2** (up-regulated in cerebellum), a gene encoding a protein mediating sperm-egg recognition (23); and several miRNAs (Fig. 1C and Figs. S11 and S12).

**Species differences in gene coexpression patterns**

To extract additional biologically relevant information, we applied weighted gene coexpression network analysis (WGCNA) to generate modules of genes with similar variation across regions and/or species. We identified 229 mRNA modules, many of which exhibited regional and/or species-specific expression patterns (Figs. 2, A and B, and Table S5). For example, genes in module 92 (M92) and M32 are respectively up-regulated and down-regulated in human neocortex, and M130 genes are up-regulated in human striatum, hippocampus, and amygdala (Fig. S13). M130 includes tyrosine hydroxylase (**TH**) and **DOPA** (3,4-dihydroxyphenylalanine) decarboxylase (**DDC**), both involved in dopamine biosynthesis (Fig. S13F). Human-specific modules were enriched for genes associated with categories and pathways such as “thrombospondin N-terminal-like domains” and “alternative splicing” (Table S5).

We also clustered all miRNAs based on their individual correlations to the average expression profile of each mRNA module (Fig. S4A and Table S6). Because the expression of each miRNA might correlate with multiple mRNA modules, module pairings were refined using a transcriptome-wide high-throughput sequencing approach combined with cross-linking immunoprecipitation (HTS-CLIP) map of miRNA binding sites in the human brain (24) (Fig. S14B and Table S7). We identified 37 stable miRNA modules, with several pairs of miRNA/mRNA modules exhibiting opposing regional and/or species-specific enrichment for potential miRNA-mRNA target predictions (Fig. S14C to E).

**Cell-type specificity of differentially expressed genes**

To investigate differential gene expression patterns at the cellular level, we integrated our data sets with single-cell RNA sequencing (RNA-seq) data generated from the human neocortex (18, 19) and validated findings via immunohistochemistry or in situ hybridization. We found that many of the genes displaying species- and/or region-specific patterns also exhibited cell-type-specific expression. For example, **PKD2L1** is enriched in excitatory projection neurons (Fig. 3, A and C), **TH** is expressed in a subset of somatostatin (**SST**)–expressing inhibitory interneurons in human and macaque neocortical deep layers and white matter (Fig. 3, B and D), and **ZP2** is up-regulated in the granule cells of the human cerebellum (Fig. S11, C and D). Additionally, we found cell-type-specific enrichment among WGCNA modules, including human-specific M81 and M162, which were composed of genes enriched in a subset of neocortical excitatory projection neurons (Fig. 2B, right panels, and Table S5).

**Species differences in neurotransmitter receptor gene expression**

The species- and region-specific expression patterns of several genes associated with neurotransmission prompted us to investigate whether there were broad interspecies differences in the coexpression networks and genomic sequences of genes encoding receptors underlying excitatory
inhibitory, or modulatory signaling (fig. S15, A to D). Gene coexpression networks of the cholinergic and serotonergic systems differed among the three species (figs. S15, E and F). Although the dopaminergic system did not have enough genes for reliable network construction, we found that DRD1, DRD2, and DRD3, genes encoding dopamine receptors, exhibited human-specific down-regulation in striatum (fig. S10). By contrast, excitatory glutamatergic and inhibitory γ-aminobutyric acid (GABA)-ergic systems’ genes exhibited conserved networks among species, and their coding sequences were more conserved than the coding sequences of genes with similar expression levels (figs. S15 to S17 and table S8).

Fig. 2. Conserved and species-specific gene coexpression modules. (A) Number of WGCNA modules (numbers on gray background; see table S5) clustered by differential expression across brain regions, species, and interspecies differences across regions (interaction). Analysis of variance of eigengene Bonferroni-adjusted $P < 0.01$, solid line; $\geq 0.01$, dashed line. (B) (Left) Enrichment of gene expression for modules (columns) in several cell types (rows) based on human single-cell transcriptome data (18, 19), sorted by unsupervised hierarchical clustering to show similarities among modules. (Right) Species-specific modules showing human (red), chimpanzee (blue), or macaque (green) up-regulation (normal font) or down-regulation (italics) relative to the other two species exhibit distinct patterns of cell-type–associated gene expression.

Fig. 3. Cellular specificity of neocortical human and chimpanzee-specific differential expression. (A and B) Radar plots depicting neocortical neuron cell-type enrichments of (A) human- or (B) chimpanzee-specific differences of genes associated with (i) neuropsychiatric disorders; (ii) neurotransmitter biosynthesis, degradation, and transport proteins; and (iii) encoding ion channels (table S10). Only genes expressed in the respective cell type are plotted. The distance of each gene from the center represents differential expression between human and the average of chimpanzee and macaque (red) or between chimpanzee and the other two species (blue). The direction of triangles denotes up- or down-regulation; filled triangles represent cell-type–specific expression (Pearson correlations > 0.5). (C) In situ hybridization shows that PKD2L1 is expressed in pyramid-shaped cell bodies of excitatory projection neurons of human, but not chimpanzee or macaque, neocortex. (D) TH-immunopositive interneurons (filled arrowheads) are present in neocortex of human and macaque, but not chimpanzee, where only TH+ midbrain dopaminergic axons (open arrowheads) are present. Scale bar, 30 μm.
Species differences in dopamine biosynthesis gene expression
We next investigated dopamine biosynthesis and signaling genes. TH and DDC displayed humanspecific (H > C = M) up-regulation in the striatum (Fig. 4A). TH also displayed chimpanzee-specific down-regulation (C < H = M) in the neocortex (Fig. 4A). An extended analysis of RNA-seq data (25) independently validated the down-regulation of TH mRNA in chimpanzee neocortex compared with human, as well as the down-regulation of TH expression in the neocortex of bonobo and gorilla, but not orangutan (Fig. S1A).

Analyses of cis-regulatory elements active near the TH gene in the adult human, chimpanze, and macaque brain (26) revealed no differences that would explain observed TH expression patterns. We hypothesized that the species-specific TH expression patterns might be explained by changes in the number and distribution of TH-expressing interneurons, which have been previously identified in telencephalic regions and shown to vary in chromosome across species (27–29), including depletion in the prefrontal cortex of nonhuman great apes (28). Therefore, we quantified TH-immunopositive (TH+) interneurons (Fig. S19, A to C) on an independent set of 45 adult brains from nine primate species (table S9). Consistent with our transcriptome data, humans have a higher fraction (Tukey’s honest significance test; all P < 0.05) of TH+ interneurons in both the dorsal caudate nucleus and putamen (striatum) when compared with all other analyzed nonhuman primates (Fig. 4C and fig. S20A). Furthermore, we found neocortical TH+ interneurons in all analyzed areas of human, all monkey species, and orangutan (Fig. 4C and fig. S20, B and D), but only TH+ fibers in all analyzed neocortical areas of chimpanzee, bonobo, and gorilla (Fig. 4C and fig. S20B). We found no differences in the number of TH+ interneurons in human, chimpanze, gorilla, and macaque olfactory bulbs (Fig. S19D).

Molecular profiling of human TH+ interneurons
To further explore the phenotype of adult human neocortical TH+ interneurons, we performed immunohistochemistry and in situ hybridization. TH+ interneurons expressed GAD1, the GABA synthesis enzyme (Fig. 4B), but were lacking canonical markers of neocortical interneuron subtypes such as SST, PV, NPY, NOS1, CALB1, and VIP (Fig. S21), as well as ETV7, which is required for differentiation of dopaminergic neurons in multiple species (30), or its homolog, ETV5 (figs. S21, H and I). Most TH+/GAD1+ interneurons co-expressed DDC (62.54 ± 1.0%) (Fig. 4B), the enzyme that converts L-DOPA to dopamine, but not dopamine-β-hydroxylase (DBH) (fig. S18), the enzyme that converts dopamine to noradrenaline, indicating that a subset of TH+ interneurons are able to produce dopamine but not noradrenaline.

Developmental origin of human TH+ interneurons
To gain insight into the development of TH+ interneurons, we analyzed the regional expression of TH across human brain development using the BrainSpan RNA-seq data set (www.brainspan.org). The highest TH expression is observed in striatum and increases steadily from early fetal development (period 2, as defined in (31)) to young adulthood (period 13) (Fig. 5A). The up-regulation of TH expression is observed in neocortex, hippocampus, and amygdala and increases perinatally (periods 7 [late fetal development] and 8 [early infancy]) and remains stable in neocortex. In addition, TH expression increases from early childhood (period 10) to young adulthood in the amygdala and hippocampus (Fig. 5A).

Using immunohistochemistry, we detected TH+ axons in striatum as early as late midfetal development (fig. S22), and occasional bipolar TH+ interneurons were first observed in the external capsule and neocortical white matter in the newborn human (Fig. 5B). The neonatal chimpanzee brain displayed the same pattern of unmyelinated TH+ fibers in the external capsule (Fig. 5B), but no TH+ interneurons were detected in the neocortex.
Fig. 5. Human telencephalic TH⁺ interneurons are of subpallial origin and start to express TH protein perinatally. (A) TH expression in human neocortex (NCX), HIP, AMY, and STR throughout development. The shaded area corresponds to a confidence interval of 50%. (B) Immunohistochemistry reveals TH⁺ axons in external capsule (arrowheads), STR, and NCX of newborn (38 pcw) human and chimpanzee brains. Bipolar TH⁺ interneurons (filled arrowhead) are present in parallel with myelin basic protein (MBP)⁻/TH⁺ (arrows) and TH⁺/MBP⁺ (open arrowheads) fibers in the external capsule. No TH⁺ cells were detected in chimpanzee external capsule. Scale bar, 1 cm. (C) Schematic of dissection of ganglionic eminences [lateral (LGE), medial (MGE), and caudal (CGE)] and neocortical regions within the ventral forebrain (subpallium), known to generate interneurons (22–34), and neocortical proliferative zones, which may also generate interneurons in humans (39). We found TH⁺ interneurons coexpressing canonical markers of distinct progenitor lineages within ganglionic eminences (NKX2-1, NR2F2, or SP8) (Fig. 5D). BrdU (bromodeoxyuridine) birth-dating confirmed that TH⁺ interneurons are derived independently in the human lineage (homoplasy) or, in a less-likely scenario, may have occurred in the common ancestor of African apes before being reabsorbed in the human lineage. (D) TH⁺ cells from ganglionic eminences also express NKX2-1, NR2F2, or SP8, and are BrdU⁺, DDC⁺, and GAD1⁺. TH⁺ interneurons in the neocortical culture are SP8⁺, but BrdU⁻ (bottom right). Scale bar, 20 μm. (E) Percentage of TH⁺/BrdU⁺ cells in culture from MGE, LGE, CGE, and NCX. Error bars, SEM. Pairwise t tests were performed and corrected for multiple testing using Bonferroni correction. *P < 0.05; **P < 0.01.

To identify the birthplace of TH⁺ interneurons, we prepared primary cell cultures from 17 to 18 postconceptional week (pcw)–old human brains (Fig. 5C) of lateral, medial, and caudal ganglionic eminences (LGE, MGE, and CGE) of the ventral forebrain (subpallium), known to generate interneurons (22–34), and neocortical proliferative zones, which may also generate interneurons in humans (39). We found TH⁺ interneurons coexpressing canonical markers of distinct progenitor lineages within ganglionic eminences (NKX2-1, NR2F2, or SP8) (Fig. 5D). BrdU (bromodeoxyuridine) birth-dating confirmed that TH⁺ interneurons are generated by ganglionic eminence, but not neocortical, progenitors (Fig. 5, D and E), indicating that TH⁺ interneurons are derived from diverse subpallial lineages and are developmentally heterogeneous. Similar to adult neocortical TH⁺ interneurons, subpallial-derived TH⁺ interneurons also coexpressed GAD1 and DDC (Fig. 5D). Neocortical TH⁺ interneurons were mainly SP8⁺ (77.78 ± 12.11%), with a smaller NR2F2⁺ (22.22 ± 8.78%) subpopulation and were all BrdU⁺, indicating that they began to migrate into neocortex before 17 pcw but express TH protein later in development (Fig. 5, D and E).

In vitro characterization of human TH⁺ interneurons

To further characterize TH⁺ interneuron development and properties, we asked whether TH⁺ interneurons could be generated from human iPSCs using a differentiation protocol for cortical excitatory projection neurons and inhibitory interneurons (fig. S23) (see the supplementary materials). Immunofluorescence confirmed the presence of TH⁺ cells coexpressing GAD1 and SP8, but not SST, PVALB, NR2F2, or NKX2-1, confirming that iPSC-derived TH⁺ interneurons display a similar molecular profile to TH⁺ interneurons from the adult neocortex and neocortical primary culture (fig. S24). Complementary analysis of a single-cell RNA-seq data set from human embryonic stem cell–derived cortical interneurons (35) revealed that many TH-expressing cells coexpress GABAergic marker genes GAD1/2 at all time points, as well as SST, ETV1, and ETV5, transiently at early time points (fig. S25).

We characterized human iPSC-derived TH⁺ interneurons by assessing their ability to produce and transport dopamine using immunofluorescence, a monoamine uptake assay, and high-performance liquid chromatography. We found that 73.14 ± 10.02% of 80 days in vitro (DIV) TH⁺ interneurons that had taken up a monoamine-imitating fluorophore were DDC⁺ (Fig. 6, A and B) and consequently could produce and transport dopamine in vitro. Commensurate with these observations, we detected dopamine in conditioned culture media from iPSC-derived and LGE primary neural cultures, both of which contained TH⁺/DDC⁺ interneurons, but not in control culture media (Fig. 6C).

Discussion

Our analysis of transcriptomic data revealed global, regional, and cell-type–specific species expression differences in protein-coding and noncoding genes. Genes with human-specific differential expression patterns include those encoding transcription factors, ion channels, and neurotransmitter biosynthesis enzymes and receptors. Changes in the regional and cellular expression patterns of these genes could affect function of neural circuits by altering transcription of other genes, intrinsic electrophysiological properties, or synaptic transmission.

Neuromodulatory systems show broad expression differences between species. One example includes a rare and molecularly heterogeneous subpopulation of interneurons expressing dopamine biosynthesis genes TH and DDC, which are enriched in the human striatum and neocortex as compared with nonhuman African apes. These cells originate in the subpallial ganglionic eminences and likely migrate into the striatum and neocortex during late prenatal and early postnatal development. We also observed an increase in TH expression during postnatal development and young adulthood, suggesting that TH expression and/or the migration of TH⁺ interneurons may be dynamically regulated and protracted.

The absence of TH⁺ interneurons from the cortex of nonhuman African apes [see also (28)], and their decreased density in the striatum of nonhuman primates, may result from several mechanisms. First, these cells could have been lost due to genetic disruptions affecting interneuron migration, differentiation, or survival (32–34). These disruptions may have occurred in the common ancestor of African apes before being reabsorbed in the human lineage (homoplasy) or, in a less-likely scenario, may have occurred independently in the Gorilla and Pan lineages. A second possibility is that these interneurons are present in the nonhuman African ape cortex but do not express TH, do so only transiently, or die before our ability to detect them. Commensurate with this possibility, the molecular profile of mouse cortical SST-positive interneurons is malleable (36), and sensory stimuli can cause a switch from the production of TH and dopamine to SST in rat hypothalamic interneurons (37).
Finally, TH⁺ interneurons of nonhuman African apes may have lost their ability to deviate to the cortex from the rostral migratory stream. Indeed, some human TH⁺ interneurons migrating via the rostral migratory stream to the olfactory bulb divert to the prefrontal cortex (38), and our observation of SP⁺/TH⁺ coexpression is consistent with a rostral migratory stream origin. However, other routes of migration are possible, as suggested by our observation of TH⁺ interneurons in the external capsule of newborn human brain.

Neuromodulatory transmitters, in particular dopamine, are involved in distinctly human aspects of cognition and behavior, such as working memory, reasoning, reflective exploratory behavior, and overall intelligence. By analyzing brain regions involved in these processes, we show that evolutionary modifications in gene expression and the distribution of neurons associated with neuromodulatory systems may underlie cognitive and behavioral differences between species. Cortical TH⁺ interneurons are depleted in patients affected by Parkinson’s disease (39) or dementia with Lewy bodies (40), and these alterations may contribute to cognitive impairments.

As these results demonstrate, the resource we present here may aid future studies on the evolution and neuroscience of primates.

REFERENCES AND NOTES

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SUPPLEMENTARY MATERIALS
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Fig. 6. Human telencephalic TH⁺ interneurons synthesize and transport dopamine in vitro.

Human iPSC-derived neurons were incubated with a fluorophore-labeled synthetic monoamine. (A) TH⁺ (red) and DDC⁺ (blue) immunolabeled interneurons (arrowheads) that transported monoamine-imitating fluorophore (green) in vitro. Scale bar, 10 μm. (B) Percentage of neurons that took up the fluorophore and were positive for both the uptake assay and TH. This population is composed of DDC⁺ (blue) or DDC⁻ (red) interneurons. (C) Concentration of dopamine detected by high-performance liquid chromatography in the unused control (Ctrl) cell culture medium and the conditioned media from LGE and iPSC-derived cultures. Error bars, SEM. Dunnett’s test, ***P < 0.001.
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The makings of the primate brain
Although nonhuman primate brains are similar to our own, the disparity between their and our cognitive abilities tells us that surface similarity is not the whole story. Sousa et al. overlaid transcriptome and histological analyses to see what makes human brains different from those of nonhuman primates. Various differentially expressed genes, such as those encoding transcription factors, could alter transcriptional programs. Others were associated with neuromodulatory systems. Furthermore, the dopaminergic interneurons found in the human neocortex were absent from the neocortex of nonhuman African apes. Such differences in neuronal transcriptional programs may underlie a variety of neurodevelopmental disorders.

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