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Metabolic changes in human brain evolution

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Abstract

Because the human brain is considerably larger than those of other primates, it is not surprising that its energy requirements would far exceed that of any of the species within the order. Recently, the development of stem cell technologies and single-cell transcriptomics provides novel ways to address the question of what specific genomic changes underlie the human brain's unique phenotype. In this review, we consider what is currently known about human brain metabolism using a variety of methods from brain imaging and stereology to transcriptomics. Next, we examine novel opportunities that stem cell technologies and single-cell transcriptomics provide to further our knowledge of human brain energetics. These new experimental approaches provide the ability to elucidate the functional effects of changes in genetic sequence and expression levels that potentially had a profound impact on the evolution of the human brain.

KEYWORDS

chimpanzee, energy, gene expression, genomics, glucose metabolism, neuron, primate

1 | WHAT IS KNOWN ABOUT HUMAN BRAIN METABOLISM AND WHY IS IT IMPORTANT TO CONSIDER IN EVOLUTION?

The most notable feature of the human brain is its large size. The human brain is about 3.5 times larger than that of our closest living ancestors, the chimpanzees (~1,350 g compared to ~380 g).[1] Although it makes up only 2% of total body mass, the brain of an adult modern human uses about 15–20% of the body's total resting metabolism.[2] The proportion of energy needed by the human brain far exceeds the energetic requirements of other adult primates that allocate between 2 and 10% of their body's resting energy to their brains during adulthood.[3] From an evolutionary perspective, the importance of the modern human brain requiring so much energy cannot be understated and has inspired numerous anthropological theories attempting to address how additional energy is made available to the human brain. For example, researchers have noted that a greater amount of energy could be made available by increasing the input of energy either by improving diet quality or cooking food.[4–9] In addition, a tradeoff in energy between the brain and other metabolically expensive organs, including musculature or the digestive tract, may have freed additional energy that could be allocated to the brain.[6,10]

Even though the energetic needs of the adult human brain likely had a profound impact on the evolution of modern human anatomy and diet, it is even more astounding to consider the metabolic needs of the human brain before maturity is reached. To understand how the energetic cost of the human brain changes over development, one needs to consider the rate of brain growth and how the metabolic demand of the organ changes per unit mass (Figure 1). The brain of a human newborn is only about 25% of the volume of an adult's brain, but brain growth occurs rapidly during the first 2 years of life[1,12] (Figure 1a). Human brain growth slows after the first couple of years, and by the age of about 7 years, the volume of the human brain is about 90% that of an adult.[11]

Humans—like all other mammals—are born with roughly the same number of neurons that they possess in adulthood (Box). Although postnatal neurogenesis occurs in mammalian brains, it is observed within only a few regions of the brain and is not thought to appreciably increase the total number of neurons.[13–15] While the total number of neurons in the brain stays fairly constant, the postnatal growth of the brain is due to enlargement and increasing complexity of those

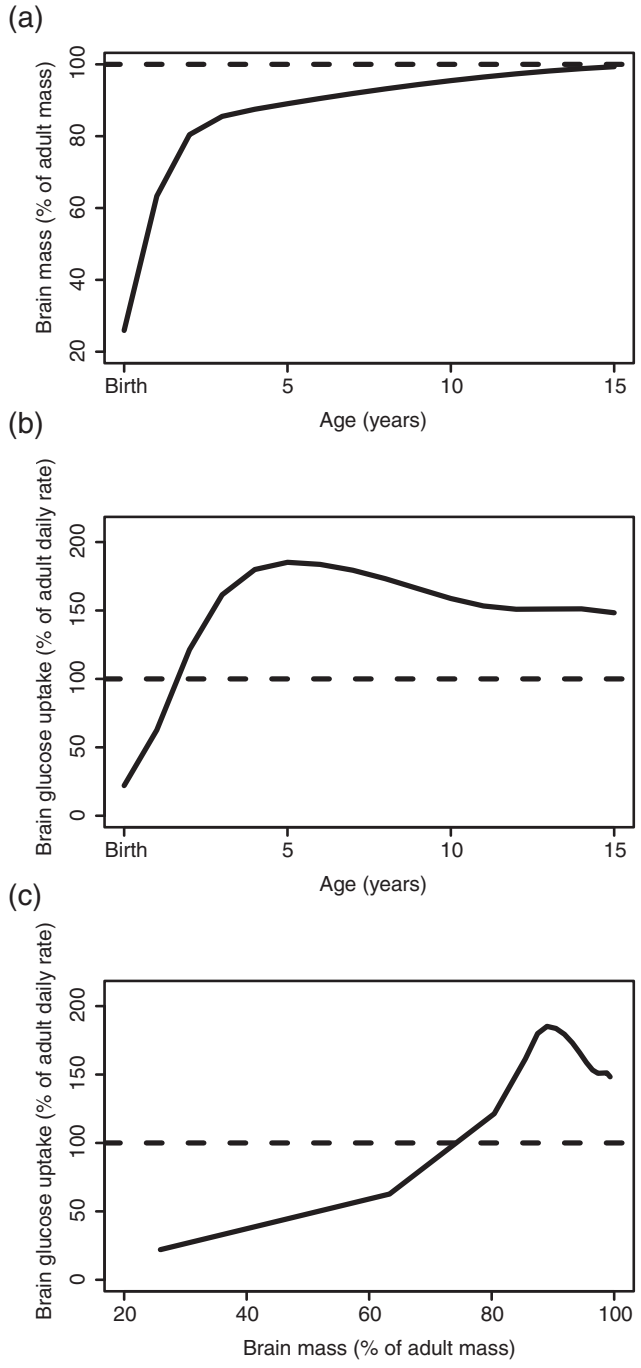

FIGURE 1 Graphs of brain mass and metabolism plotted over time. (a) Brain mass, as a percentage of adult mass, increases over development and reaches within 90% of the adult value by ages 6 and 7. (b) Glucose uptake, as a percentage of adult daily rate, dynamically changes over the course of development, reaching its peak at age 5. (c) Percent adult glucose uptake per percent adult mass highlights the high metabolic cost of the brain when it is between 80 and 90% of its adult mass. In (a–c), the dashed line indicates the adult level. All data are sourced from Ref. [11] and is plotted as the mean of the male and female values that were reported

neurons. In all regions of the brain, synapses proliferate, axons elongate, subcortical axons myelinate, and dendrites branch, creating more complex neurons.[16] However, the duration of neuronal maturation, defined as the time it takes each region to display adult levels of synapses and other structural elements, is heterogenous across different regions of the cerebral cortex.[17–20] Human primary sensory and motor cortices mature by 3–4 years of age, but regions of the prefrontal cortex and other association cortices are later to develop, with some not reaching maturity until the second decade of life.[21–23] Chimpanzees are similar to humans in that neurons within their primary sensory and motor cortices reach maturity at an earlier age than other parts of the cerebral cortex, and neurons within association cortices display a more protracted neurodevelopment with some regions not reaching maturity until about 12 years of age.[24–27] In contrast, the neurons across all regions of macaque monkeys' cerebral cortex mature shortly after birth, without protracted development of association cortices.[28–30] Compared to chimpanzees and macaques, the prolonged postnatal maturation of neurons within human association cortices is thought to be related to increased cortical plasticity and behavioral flexibility of the human species.[31]

Positron emission tomography (PET) is a technique in which metabolic processes can be quantified within the body by measuring uptake of a radioactive tracer. The tracer that is commonly used in studies investigating brain metabolism is glucose, the sugar typically used as fuel for energy production by all tissues of the body. Remarkably, PET studies tracking glucose in the brain find that the metabolic requirements of the brain through development mimic the trajectory of neuronal maturation.[32–34] In humans, the amount of energy used by the human brain, as defined by its total glucose uptake, increases from birth and peaks around the age of 5, when it requires 66% of the body's resting metabolic energy[11](Figure 1b). Interestingly, the developing human brain requires almost twice as much energy per gram of tissue before adult brain size has been reached (Figure 1c). Although PET studies are not performed on immature chimpanzees to allow the developmental brain glucose uptake to be assessed, the duration of neuronal maturation would predict that the peak metabolic uptake of chimpanzees would occur a couple of years earlier than humans.[35,36] PET studies have been performed in macaque monkeys and indicate that the species has its highest rate of brain glucose metabolism at birth.[34] Figure 2 illustrates the overall trajectory of glucose uptake in human and macaque brain over development as compared to what is predicted from chimpanzee neuronal maturation.

It is a common assumption that the brain becomes more energetically costly with increased activity. Surprisingly, local energy uptake of the adult brain changes by only about 5% in response to brain activity,[37] suggesting that vast majority of the metabolic expense of the brain is not caused by increased activity.[38,39] If so, what is so energetically costly about the developing brain? In addition to being used as a source of fuel, glucose can also be processed through various anaerobic pathways to create biomolecules that are essential for the growth and maturation of neurons.[35,36,40,41] PET studies of immature humans and macaque monkeys have shown that their brains metabolize glucose anaerobically at very high rates and that maximum glucose uptake is temporally coincident with the periods of the

BOX 1 Glossary of key terms used in this review

Adaptation: Here, defined at the DNA sequence level, as an excess of changes between species in one part of the genome, as compared to other, putatively neutral, regions of those genomes.

Aerobic metabolism (or oxidative phosphorylation): The process of producing energy (ATP) from oxygen and glucose. In sequence, the steps of aerobic metabolism are glycolysis, citric acid (Kreb's) cycle, and the electron transport chain. Aerobic metabolism produces ~ 36 molecules of ATP for every molecule of glucose.

Anaerobic metabolism: The process of producing energy without oxygen through the glycolytic pathway. Anaerobic metabolism produces ~ 2 molecules of ATP for every molecule of glucose. Another product of glycolysis, pyruvate, can be used in biosynthetic pathways.

Astrocyte: These are the most numerous cell type within the central nervous system. They perform tasks from axon guidance during development, support synapses metabolically, control the blood brain barrier, and regulate blood flow.

ATP: Adenosine-5′triphosphate, the body's primary source of energy.

Enhancers: A short (50–1,500 bp) region of DNA that can be bound by proteins to increase transcription of a gene.

Functional genomics: Genomic studies that investigate the relationship between genotype and phenotype on a genome-wide scale. Studies examine the functional output of the DNA sequence encoded in the genome through a range of processes such as transcription, translation and epigenetic regulation.

Human accelerated regions: Small segments of the human genome that are conserved throughout vertebrate evolution but are strikingly different in humans.

Glucose: The sugar that is most commonly metabolized to produce energy.

iPSCs: Induced pluripotent stem cells. Derived from somatic cells by either reprogramming or dedifferentiating cells by the introduction of specific pluripotency genes, altering the original somatic cells to a state similar to that of embryonic stem cells.

Lactate dehydrogenase: An enzyme that plays and important role in cellular respiration, the process by which glucose is converted into usable energy.

Long noncoding RNAs: RNA transcripts with lengths exceeding 200 nucleotides that are not translated into protein.

Neuron: Neurons are a specialized cell within the nervous system that transmit information to other nerve cells, muscle, or gland cells.

Organoid: An organoid is a three-dimensional multicellular in vitro tissue construct that mimics its corresponding in vivo organ, which then can be used to investigate aspects of that organ function in the tissue culture dish.

Positron emission tomography: A technique in nuclear medicine functional imaging that is used to observe metabolic processes in the body.

Promoter: A region of DNA that leads to initiation of transcription of a particular gene.

RNA-Seq: RNA sequencing. RNA-Seq is an experimental protocol that uses next-generation sequencing technologies to sequence RNA molecules within a biological sample; this allows the researcher to both determine the primary sequence of the RNA and relative abundance of each RNA type in the sample.

scRNA-Seq: Single-cell RNA sequencing provides the expression profiles of individual cells using RNA-Seq.

TH^+ interneurons: Tyrosine hydroxylase-expressing interneurons. TH^+ interneurons are a neuronal subtype expressed in the neocortex and striatum. This neuronal subtype expresses an enzyme, tyrosine hydroxylase, critical for the production of dopamine and norepinephrine, neurotransmitters involved in higher cognitive processes, such as working memory and attention.

highest synaptogenesis and myelination in these species[34,35] (Figure 1). These results suggest that the high "metabolic cost" of the brain during development may be the result of the brain repurposing glucose into other organic molecules as a substrate for its own growth. Because human neurodevelopment is prolonged compared to nonhuman primates, humans require elevated levels of glucose for a longer duration.[35,42,43]

The metabolic requirements of the human brain, both in adulthood and during development, are substantial. Several lines of evidence suggest that humans may benefit from molecules that have evolved during the primate lineage to potentially allow species to make and use energy more efficiently. For example, many studies in anthropoid primates have reported adaptive evolution in coding genes that are important to aerobic energy production.[44–48] Although the functional consequences of these changes are unclear, these proteins are located within the electron transport chain of mitochondria and their sequences are generally conserved in mammals, suggesting these alterations may alter the efficiency of energy production.[46] Another molecule that has changed during primate evolution in a way that may benefit energetic efficiency is LDH. LDH is an enzyme that catalyzes

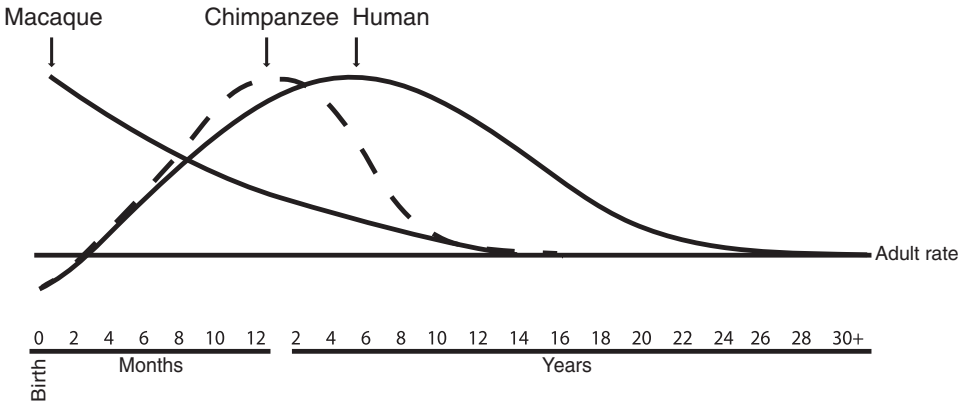

FIGURE 2 Time course of whole brain glucose uptake in the human and macaque brain (solid lines) over development based on positron emission tomography imaging data.[33] Whole brain glucose uptake for the chimpanzee brain over development (dashed line) is projected based on the timing of synaptogenesis and myelination.[36] A *y*-axis is not indicated because the precise metabolic rates of chimpanzee brain over development is not known

the conversion of pyruvate to lactate, or the reverse reaction, depending upon the isoform that is present. Both pyruvate and lactate are used in metabolic pathways, but the aerobic pathway that uses pyruvate produces 15 times the amount of energy than the anaerobic pathway that uses lactate. Within forebrain regions of large-brained haplorhine primates, including tarsiers, monkeys, apes, and humans, the isoform of LDH that supports aerobic metabolism (LDH-B) is expressed in greater proportion to the isoform that supports anaerobic metabolism (LDH-A).[49,50] The ratio of LDH-B to LDH-A is particularly elevated in humans and chimpanzees suggesting that expression of the LDH isozyme favoring aerobic metabolism may contribute to energetic efficiency that allows the energy needs of the brain to be met.[50]

As reviewed in the preceding paragraphs, the current state of knowledge regarding how adult human brain metabolism differs from that of other primate species is derived from whole brain PET scans, stereological studies comparing neuronal and glial densities, and research on the expression of specific molecules with known functions in energetic pathways. When considering how human brain metabolism differs from nonhuman primates over the course of neurodevelopment, most data are inferred from the timing of synaptic proliferation and pruning and myelination of axons from tissue samples of deceased individuals. Regardless of whether one is evaluating energy use in adulthood or development, the questions of inquiry are limited by the accessibility of data. While recent, shared ancestry makes chimpanzees the most valuable point of comparison with humans, practical considerations limit the amount and quality of data that can be acquired from this species. For example, performing PET imaging on any nonhuman primate requires the animal to be under anesthesia[51] restricting how comparable the data are to awake humans during resting state. Moreover, studies that aim to sample from brain tissue samples of subadult chimpanzees for stereological or molecular research is, at best, extremely limited in possible data points due to tissue availability.

With the rise of affordable and efficient methods of exploring patterns of gene expression came attractive opportunities for researchers of primate comparative neurobiology. The ability to gather expression levels of hundreds to tens of thousands of transcripts at once is an efficient use of rare tissue samples. Before we review how these technologies have advanced our knowledge of human brain metabolism, we will summarize what is known broadly about transcriptomic differences between humans and chimpanzees.

2 | HOW HAVE GENE EXPRESSION STUDIES ADVANCED OUR UNDERSTANDING OF BRAIN ENERGETICS AND EVOLUTION?

Upon observing the striking degree of similarity in amino acid sequence between human and chimpanzee proteins, King and Wilson[52] theorized that the profound phenotypic differences between humans and chimpanzees could not be attributed to changes in genetic sequence, but were more likely the result of changes in gene regulation. Indeed, King and Wilson's hypothesis has borne out in the results of comparative whole transcriptome sequencing studies of humans and chimpanzees where the gene sequences are very similar, but we observe distinct differences in magnitude, location, and timing of gene expression. One method to assay changes in gene expression, RNA sequencing (RNA-Seq), can address questions in highly complex tissues, since thousands of genes can be quantified at once and the covariation of genes can be assessed within a single sample. The human brain is known to have a vast diversity of neurons that vary in structure and function, resulting from astounding epigenomic and transcriptomic variation.[53–55] RNA-Seq allows us to assess this incredible diversity in gene expression, and additionally compare how human gene expression profiles differ from non-human primates.

To date, most studies have focused on a trio of human, chimpanzee, and rhesus macaque to assess how gene expression varies within and between various different organs and tissues,[56–59] including the brain.[56,60–64] These exemplar species have also been critical in expanding studies of gene expression to include other levels of regulation, such as alternative splicing,[65] methylation,[66,67] histone modifications,[68,69] metabolites,[70,71] and noncoding RNA (ncRNA),[60] or miRNAs[72,73] and structural variation.[74] These studies have found that genes involved in metabolism, gene regulation, and cell–cell communication have changed expression levels significantly in humans compared to chimpanzees, and that the other levels of regulation add small, but measurable, differences distinct from the changes in gene expression.

Although the typical objective of comparative primate studies of the brain is to find unique changes of the genome that may account for novel human phenotypes, the limited sampling (i.e., the triad of humans, chimpanzees, and macaques) may distort rates of evolution through the great apes, giving us an incomplete picture of how gene expression has shaped modern primates. Two possible approaches to address this issue are (a) to increase sampling within the apes to include other species that have at least one representative high-quality publicly available genome and to expand our knowledge of intraspecific variation from these species,[74,75] and (b) increased sampling across primates more generally, leveraging species that have both high-quality genomes and human disease-related data resource. Currently, there are several primate species that could be used to increase primate diversity in RNA-Seq studies, including the marmoset (*Callithrix jacchus*), African green monkey (*Chlorocebus aethiops*) and olive baboon (*Papio anubis*).[76] Genome assemblies for multiple lemur species (e.g., *Microcebus murinus*) will allow even broader taxon sampling through the full time-scale of primate evolution.

Generally, including a greater diversity of primate taxa to comparative genomic investigations will be as important to understanding the evolution of gene expression in the brain as it has been for contextualizing phenotypic and behavioral primate traits.[76,77] Diverse taxon sampling across primates will enable examination of both microevolutionary processes seen in intraspecific variation to macroevolutionary processes affecting interspecific differences.[76]

3 | HOW HAVE GENOMIC STUDIES ADVANCED OUR UNDERSTANDING OF COMPARATIVE BRAIN ENERGETICS?

Although RNA-Seq is able to quantify thousands of genes, focused analyses on specific classes of genes provide insight into which neuronal functions may be enriched in certain samples. For genes that support glucose metabolism, there is a consistent pattern of up-regulation in the human brain compared with that of nonhuman primates, including enrichment for categories such as oxidative phosphorylation, electron transport, and other nuclear encoded genes that function in the mitochondria.[78–80] These results are insightful from the perspective of human brain evolution in that the upregulation of genes in the adult human neocortex suggests that, per unit mass, the human brain is more metabolically expensive than the brains of other adult primates.[81] Such a finding indicates that the human brain would violate Kleiber's law, a well-established physiological principle that states that organs become more metabolic efficient as mass increases (metabolic rate scales to the ¾ power of mass).[82] Because Kleiber's law would predict expression of metabolic genes would be less dense in human brain compared to other primates, gene expression studies suggest that the energetic expense of the brain exceeds the upper limit of metabolism predicted by Kleiber.

In humans, sequence changes found within the promoter regions of genes supporting glucose metabolism outnumber those of chimpanzees.[83] Specific promoter regions that contain evidence of positive selection in humans are associated with the genes *HK1*, *GCK*, *GPI*, and *PFKFB3*, all of which are involved in glycolysis and the citric acid (Kreb's) cycle, two of the three steps of aerobic energy production.[83] These results reveal positive selection acting on the putative regulatory regions of genes in categories supporting carbohydrate metabolism, glycolysis, and other sugar metabolism in humans compared with other primates. Furthermore, there is a neotenous pattern of genes supporting neuronal development in humans compared to nonhuman primates.[63,73,84] Surprisingly, however, the genes that show neotenous expression patterns do not include genes that support energy metabolism. Although it is puzzling that energetic genes would not be dynamically regulated throughout development, these genes may indeed be regulated on a cell-specific scale.

4 | FINDING FUNCTIONAL HUMAN ADAPTIVE CHANGES AT A GENOME-WIDE SCALE

Despite King and Wilson's early insight into the biology underlying differences between humans and chimpanzees, it is still challenging to test their prediction. Ideally, we would do this by experimentally linking changes in gene expression to both (a) phenotypic changes in cell culture or in animal models and (b) specific changes in genome sequence, especially with brain phenotypes. In the broader literature, there are some high-profile examples where very recent (<10,000 years) metabolic adaptations at the DNA level found in specific human populations have then been experimentally tested (e.g., *LCT*,[85] *EPAS1*,[86] and *FADS1/2*[87,88]). Likewise, examples combining evidence of selection at the DNA level and experimental data at deeper timescales, such as during the divergence of the human lineage from chimpanzee, have tended to focus on differences in genes or transcripts such as transcription factors involved in the early stages of brain development and patterning (e.g., neuronal PAS domain protein 3 [*NPAS3*][89] and *HAR1A*,[90] reviewed in Ref. 91).

Researchers need to combine diverse types of evidence to support links between genome to phenotype change in the brain, which we can illustrate by one of these examples, the changes in the gene *NPAS3*. For *NPAS3*, there is evidence that (a) the rate of sequence evolution is accelerated in humans in multiple introns of this gene, as compared to other species[89]; (b) the gene is critical for normal neurodevelopment, based on data from mouse brain ontogeny[92,93]; (c) the gene has functional associations to diseases, such as schizophrenia, as explored by the medical literature[94]; (d) experiments of zebrafish and mouse model systems of the human-accelerated regions show quantifiable changes when introduced experimentally in in vivo brain phenotypes.[89] By linking all of these strands of evidence, we can begin to make the functional links between changes at the genome level to appreciable differences in phenotype of the human brain.

For our purposes here, it is important to note that none of these genes listed as examples are directly implicated in brain metabolism; instead they deal with other metabolic adaptations linked to new environmental opportunities or challenges or brain development. There are still challenges between identifying changes in the genome

or transcriptome and linking those to functions in an organ as complex as the brain. The final sections of this review will highlight new technologies that may make this experimental link from genotype to phenotype more accessible to researchers.

5 | HOW DO WE FIND MORE ADAPTIVE CHANGES IN THE HUMAN GENOME RELATED TO BRAIN EVOLUTION?

By looking for heritable changes in gene sequence that may drive different levels of gene expression across species, we can begin to appreciate how data derived from genomics contributes to the evolution of the modern human phenotype (Figure 3a–d). Several studies have found that regions of the human genome are under positive selection,[83] and a greater proportion of these regions under positive selection are located in regulatory regions compared to protein-coding regions[95](Figure 3a). However, the challenge is locating putatively functional regulatory sequences since they are not as conserved as genes, can move to new locations over evolutionary time, and are scattered throughout the genome. Multiple approaches have been used when investigating adaptive changes in noncoding regions of the human genome as compared to other primate species. One study, discussed above, looked for accelerated changes in rates of sequence evolution in promoter regions (proximal 5,000 base pairs) of nearby protein-coding regions,[83] but many others have taken comparative genomic approaches to identify positive selection as rapid change in the genome in humans relative to other species (human accelerated regions [HARs], reviewed in Ref. 96; Figure 3b). HARs are short DNA sequences that have acquired significantly more DNA substitutions than expected in the human lineage as compared to other species.[97] A few HARs have been studied in more detail,[90,98] and a subset seems to be developmental enhancers,[99] possibly regulating novel human phenotypes. More than 3,000 noncoding HARs have been identified to date,[100] providing a wealth of regulatory regions with putative human-specific activity whose affects need to be explored experimentally. In another complementary approach, ortholgous functional regulatory elements (such as gene promoters or enhancers) in primates have been examined to find which of these elements that have been gained or lost in humans as compared to nonhuman primates[101,102](-(Figure 3c). We can then also explore how those regulatory elements might be coordinated within functional networks,[69] with an output of changed gene expression levels (Figure 3d). Regardless of the precise methodological approach, these studies have found specific regions of the human genome that have undergone positive selection at different times in primate evolution.

Although these adaptive changes in sequence evolution may affect phenotypic changes anywhere in the body, a number of studies have considered the evolution of gene regulatory regions that affect the brain specifically. It is surprising given the degree of behavioral divergence between humans and nonhuman primates that the brain shows more constraint in gene expression compared to other organs.[59,103] Yet, data from gene expression studies and scans of genome sequence have revealed that a number of neural phenotypes may have undergone positive selection during human evolution.[83,97,104–106] Many genes that are differentially expressed between human and chimpanzees have also been shown to display cell-specific expression, which is typically not robust enough to be assessed in interspecific RNA-Seq studies performed on whole tissue samples.[107,108] Understanding how selective changes to the genome are correlated with changes in phenotype is a broad and exciting challenge.[91] The energetic differences between brain and primate species seem to be a promising starting point to better understand these connections and how evolution has shaped them in human evolution.

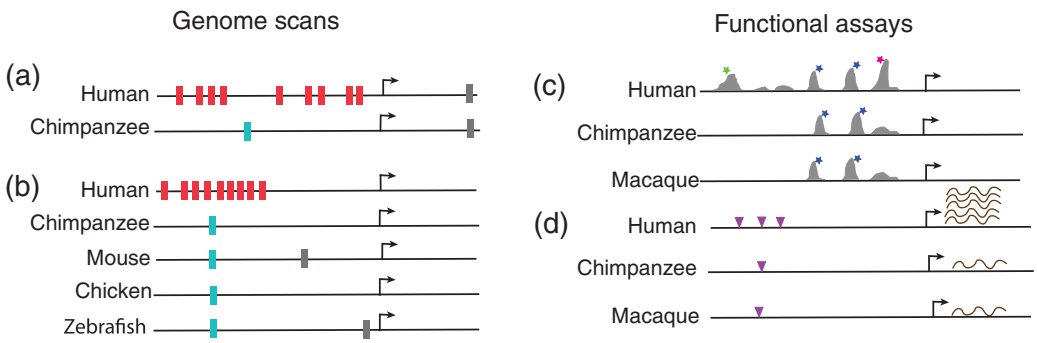

FIGURE 3 Schematics of tests for global changes in the genome that is specific to humans using comparative genomics. Black line is DNA, and the black arrow marks the start of a gene. (a,b) *Genome scans*: DNA sequence-based tests for changes on the human branch. (a) Scans for selection in noncoding regions in multiple species looking for an excess of species-specific change (red and cyan boxes), as compared to local neutral changes (gray boxes). (b) Human accelerated regions, smaller sequences with an excess of changes as compared to deeper evolutionary comparisons (here vertebrates), where the sequences show strong conservation. (c,d) *Functional assays*: Using functional genomics (e.g., CHiP-Seq, RNA sequencing [RNA-Seq]) to find regions of the human genome that are working differently to other species with in a tissue or time point. (c) Pileups of short read sequencing data displayed as gray peaks. The higher the peaks, the more active the genomic element. Here, between human, chimpanzee, and macaque we can find (1) peaks unique to humans (green star), (2) shared peaks (blue stars), and (3) differentially expressed peaks, here higher in humans (magenta star). (d) Transcription factor binding difference (purple arrows) can lead to increased gene transcription into mRNA (brown ribbons), which can be measured by RNA-Seq [Color figure can be viewed at wileyonlinelibrary.com]

6 | EMERGING OPPORTUNITIES TO UNDERSTAND CONSEQUENCES OF MOLECULAR CHANGES IN HUMAN BRAIN EVOLUTION

Transcriptomic studies of the human brain have revealed vast complexity at the level of different brain regions,[109] different populations of cell types,[110] and individual neurons.[111] One challenge in understanding how changes in gene expression have influenced human brain evolution had been the difficulty of interpreting results in light of the regional, laminar, and cellular diversity in the brain. RNA-Seq analyses are typically performed on brain tissue samples that pool all of the mRNA from all cells present, including neurons, glia, and cells comprising blood vessels and blood. Because of this heterogeneity, interpreting specific functional results from these samples can be challenging, if not impossible. Now, however, for researchers interested in the evolution of humans and nonhuman primates, there are new possibilities for testing how changes in gene expression might lead to important phenotypic changes at the level of the cell, tissue, or organism by creating or isolating single cell types from cell lines or tissues of interest. When brain tissues of human and nonhuman primates are produced by stem cells and/or isolated into individual cells, researchers may discover a fine-grained understanding of the functional differences in human and nonhuman primate cells and how differences in gene expression are correlated with these different functions. In this section, we discuss new opportunities in brain cell type studies using (a) induced pluripotent stem cell (iPSC)-derived cells and (b) single-cell RNA-Seq (scRNA-Seq).

Current stem cell technologies include the ability to produce primate iPSCs derived from primate fibroblasts and other somatic cells. Comparisons of gene expression between iPSCs of different species have found them to be a useful tool for studying interspecific differences, including those between closely related primates,[112–114] without confounding effects of cell type of origin on gene expression.[115] These cells can be made into different germ layer primordia,[116] or differentiated into terminal cells types, such as heart muscle,[112,117] endoderm,[118] or neural cells.[74] An example of the workflow and the resulting cells from these transformations is shown in Figure 4, where iPSCs have been transformed into neural progenitor cells (Figure 4b,c), and those cells have then additionally been induced toward a fate of a neuron or astrocyte (Figure 4d,e) (T. Zintel and C.C. Babbitt, unpublished data).

scRNA-Seq pairs well with stem cell methodologies by assessing genomic expression of single cells (either neurons or glia) of brain tissue and thus simplifying an inherently complex biological system.[111,119] scRNA-Seq presents an opportunity to distinguish gene expression profiles of specific cell types in the brain,[110,111] which presents an advantage of being able to correlate these profiles to the other sources of data, including morphology, immunohistochemistry, in situ hybridization, and electrophysiology.[120] An example of a recent study is presented in the following paragraph.

Sousa et al.,[107] leveraged this approach to explore species-specific distribution of neurotransmitter systems. By performing RNA-Seq and in situ hybridization on samples of neocortex and striatum, the authors found that expression patterns of specific genes involved in the biosynthesis and signaling of the neurotransmitter, dopamine, were differentially expressed among humans, chimpanzees, and macaques. The authors theorized that differences they found in their transcriptomic results could be due to the quantity and distribution of specific neurons, tyrosine hydroxylase postive (TH^+) interneurons, known to have variable distributions in primates.[121] To explore this possibility, the authors performed scRNA-Seq on iPSC-derived TH^+ interneurons and found they had comparable profiles. They were then able to show that the migratory patterns of immature TH^+

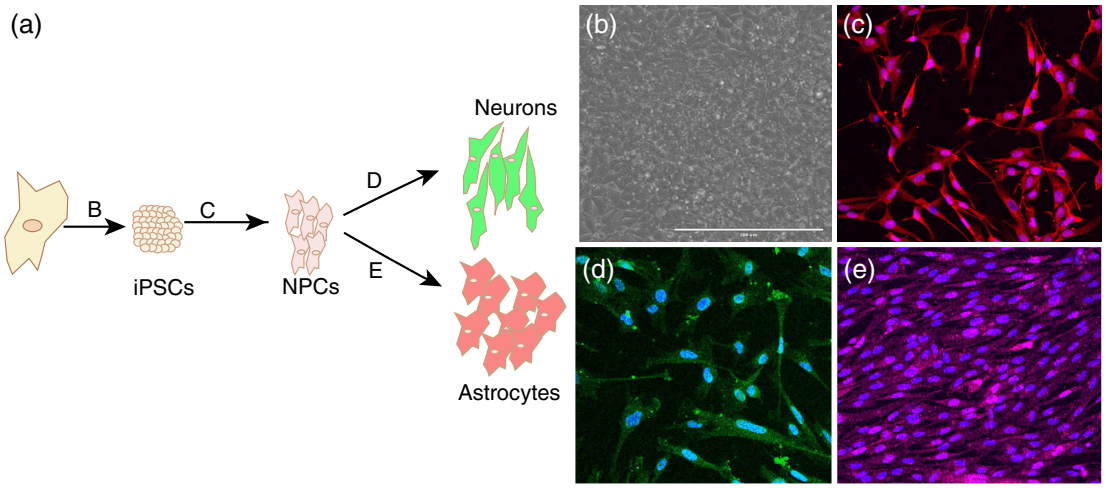

FIGURE 4 Differentiation and maturation of a human iPSC line into cell types. (a) Schematic of differentiation protocol for induced pluripotent stem cell (iPSC)-derived neurons and astrocytes. Letters in the left panel correspond with image labels on the right. (b) Human iPSCs prior to differentiation, (c) iPSC-derived neural progenitor cells (NPCs) stained for NPC-marker PAX6 (red), (d) iPSC-derived mature neurons with neuron-marker MAP2 (green), (e) iPSC-derived mature astrocytes with astrocyte-specific marker S100ß (pink). All insets show DNA in the nucleus counterstained blue (DAPI), with the scale bar shown at 200 μm. Unpublished data from T. Zintel and C.C. Babbitt (University of Massachusetts Amherst) [Color figure can be viewed at wileyonlinelibrary.com]

interneurons differed in humans compared to chimpanzees, potentially accounting for the higher density of these cells within human neocortex and striatum in adulthood. The authors propose that the distribution of neurons associated with the dopaminergic system could contribute to distinct aspects of human cognition, including working memory, reasoning, and overall intelligence.

Other initial investigations comparing human and nonhuman primate iPSCs have combined iPSC and scRNA-Seq to focus on developmental processes and their signatures in specific cell types using scRNA-Seq.[74,119,122] Kronenberg and colleagues[74] found hundreds of differentially expressed genes specific to excitatory neurons and radial glial cells in humans compared to chimpanzees, using available scRNA-Seq data. Furthermore, the same study found that downregulated genes were likely to occur near structural variants in the genome, whereas upregulated genes were associated with gene duplications.[74]

Some of the current challenges in using either iPSCs or scRNA-Seq are in the matching of developmental stage of neurons between samples and in understanding how representative cells derived from iPSCs are matched with cells to those within the complex tissues of the actual organs. For example, the differentiation protocols using iPSCs yield developmental stages that are more immature than their in vivo counterparts.[123] For scRNA-Seq, the statistical quantification of data sets is still improving so that data sets are not skewed by transcripts that are expressed at low levels or biased in other ways (sparse data sets).[124] In addition, the magnitude of intraindividual and intraspecific biological variation of each cell type will be important to understand and quantify before interpreting how the neuronal transcriptomes of disparate species differ. Yet, there are many exciting possibilities in comparative functional studies of human and other primate iPSC-derived neural cells, such as species-specific differences in gene expression, experimental manipulation of those cells to identify species-specific responses (e.g., treatments with glucose, growing cells in hypoxic conditions), and comparing species-specific responses to those found in modern human neurological diseases from patient-derived iPSCs.[123]

As with the synthesis of all of the layers of metabolic data from the cell to organ level that were discussed at the beginning of this review, there are challenges in combining multiple complex layers of genomic data, from DNA sequence to functional assays, and how they combine to effect organismal phenotype. Even understanding the biological importance of multiple genomic ("multiomic") data sets from the same samples (e.g., gene expression and chromatin modification) across tens of thousands of genes, and other genomic regions, from a small population of single cells is a real challenge both biologically[125] and computationally.[126] The goal of multiomics approaches is to correlate higher-order biological impacts of these changes across the genome. For example, single-cell multiomics technologies can reveal heterogeneity between cells at multiple molecular layers within a population of cells and reveal how this variation impacts the different "omics" data sets.[124] The analysis of data sets generated by multiomic techniques have the potential to enable a deeper understanding of the biological processes and mechanisms driving differences between cells, how they are linked with brain function, and how these levels of genome function and interactions can change over evolutionary time.

7 | OTHER FUNCTIONAL TESTS ON THE HORIZON: LINKING GENOTYPE TO ORGANISMAL PHENOTYPES

Other functional tests on the horizon will allow researchers to understand the simplified systems of single cells and iPSC-derived cell types in higher-dimensional and realistic experimental environment. In combination with these other genomic techniques, it is now also possible to assess functional changes in the genome through assaying changes in transcription factor binding (e.g., ahromatin immunoprecipitation [ChIP] with sequencing [ChIP-Seq] or assay for transposase-accessible chromatin using sequencing [ATAC-Seq])[116] or using massively parallel enhancer screens[127,128] to understand the differential functions of thousands of noncoding regulatory elements at once, and how the control of gene expression has changed in primate and human evolution. Clustered regularly interspaced short palindromic repeats (CRISPR) and related gene-editing technologies test what sequence changes perturb or constrain gene expression within specific cell types in the lab, permitting the experimental testing of both biomedical and comparative questions.[129,130]

One of the most exciting technologies might be in the creation of organoids. Organoids can be derived from iPSCs that differentiate to form an organ-like tissue of multiple cell types that self-organize to form a structure similar to the organ.[131] We now have the ability to create, combine, and test the differentiated iPSCs from multiple species in these organoid cell culture systems.[119,122] These organoid protocols can closely recapitulate the cellular organization and gene expression events observed in developing tissue,[132] though not necessarily adult systems. They have the potential to both illuminate critical, but subtle, differences between species. Recently, comparative genomics between humans and chimpanzee cerebral organoids have found nuanced species-specific differences in gene expression,[119] as well as at the cellular level.[122] For example, changes in the expression of genes underlying cellular division and orientation can lead to subtle, yet, possibly important, changes in brain structures.[122] These initial results suggest that organoid systems will be critical for understanding the subtle changes that had phenotypic consequences in human brain evolution. Comparative organoid genomics can also highlight functional differences of unknown and dynamically expressed transcripts. A comparative brain organoid-based study found functional long ncRNAs (lncRNAs) that are differentially expressed in human cortical differentiation, as compared to chimpanzee, orangutan, and rhesus macaque.[133] They showed that some lncRNAs are expressed in different cell types over different windows of developmental time, suggesting important and different roles in human and nonhuman primate brain development. These techniques can also be applied combinatorially to ask how enhancer function changes (using CHiP-Seq or enhancer screens) in a brain (or brain organoid) over developmental time,[134] and then how that has evolved to differ between species.

8 | FUTURE PROSPECTS

We are in an exciting time for comparative primate genomics as changes at the level of the genome and cell are brought together to attempt to understand the basis for uniquely human phenotypes. We can now examine the functional impacts of changes throughout the genome and how those are translated to changes in specific cell types. Linking genotypic change to differences in phenotype is especially critical for heterogenous and complex structures like many of those in the brain. Understanding how these genomic changes have affected overall metabolic production or efficiency in primate and human evolution has also been challenging due to the interplay of different cell types in fueling neuronal function. The new experimental approaches described here may represent powerful steps in allowing us to elucidate the functional impacts of these changes that potentially had a profound impact on the evolution of the human brain.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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