A Potential Role for Glucose Transporters in the Evolution of Human Brain Size

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Abstract

Differences in cognitive abilities and the relatively large brain are among the most striking differences between humans and their closest primate relatives. The energy trade-off hypothesis predicts that a major shift in energy allocation among tissues occurred during human origins in order to support the remarkable expansion of a metabolically expensive brain. However, the molecular basis of this adaptive scenario is unknown. Two glucose transporters (*SLC2A1* and *SLC2A4*) are promising candidates and present intriguing mutations in humans, resulting, respectively, in microcephaly and disruptions in whole-body glucose homeostasis. We compared *SLC2A1* and *SLC2A4* expression between humans, chimpanzees and macaques, and found compensatory and biologically significant expression changes on the human lineage within cerebral cortex and skeletal muscle, consistent with mediating an energy trade-off. We also show that these two genes are likely to have undergone adaptation and participated in the development and maintenance of a larger brain in the human lineage by modulating brain and skeletal muscle energy allocation. We found that these two genes show human-specific signatures of positive selection on known regulatory elements within their 5'-untranslated

region, suggesting an adaptation of their regulation during human origins. This study represents the first case where adaptive, functional and genetic lines of evidence implicate specific genes in the evolution of human brain size.

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Introduction

The brain is a metabolically expensive tissue [Kety, 1957; Holliday, 1986; Peters et al., 2004] whose energetic requirements scale positively with size [Aiello and Wheeler, 1995]. The approximately 2-fold increase in brain size during hominin evolution thus imposed a substantially increased metabolic demand [Aiello and Wheeler, 1995]: the brain metabolism of modern humans accounts for 20–25% of the resting metabolic rate in adults and 60% in infants while it is only 7–8% in other primate species [Leonard et al., 2007]. In addition, it has been suggested that the human brain is not only more energetically expensive due to its size, but also presents an increased metabolic demand because of its elevated neuronal activity [Caceres et al., 2003; Uddin et al., 2004, 2008; Oldham et al., 2006].

In principle, a proportionally larger and more energetically expensive brain could be supported through an increase in the basal metabolic rate, but no evidence of such a relationship has been found in humans and other

primates [McNab and Eisenberg, 1989; Isler and van Schaik, 2006]. Beginning with the original expensive-tissue hypothesis by Aiello and Wheeler [1995], it has been proposed instead that metabolic support for the expansion of brain size along the hominin lineage was accomplished by redirecting energy from other metabolically 'expensive' tissues such as the gut [Aiello and Wheeler, 1995] and skeletal muscle [Leonard et al., 2003].

These energy trade-off scenarios [Isler and van Schaik, 2006] required major physiological and dietary changes during human origins (online suppl. fig. 1; for all suppl. material see www.karger.com/doi/10.1159/000329852). Several studies investigating energy trade-offs have focused on changes in the relative proportion of tissue mass, with increases in the brain counterbalanced by decreases in other high-demand tissues [Aiello and Wheeler, 1995; Leonard et al., 2003]. In particular, the energy trade-off scenario suggested by Leonard et al. [2003] invokes a shift in energy allocation between skeletal muscle and brain. In this scenario, a decrease in skeletal muscle mass would allow the redirection of energy to an expanded central nervous system. However, more recent analyses indicate that, although humans seem undermuscle compared to primates in general, their relative muscle mass is not significantly different from that of other hominoids [Isler and van Schaik, 2006].

Another possibility is that the energy trade-offs were accomplished in part at the molecular level. Since glucose is the primary source of energy in the postnatally developing and adult mammalian brain [Maher et al., 1994], we reasoned that changes in the tissue-specific expression of genes that carry out the transport and metabolism of carbohydrates might shift energy allocation between the brain and other 'expensive' tissues. Among the many genes that might be implicated in this reallocation, members of the sugar transporter facilitator family (*SLC2A*) are particularly intriguing candidates due to their essential function as gatekeepers of hexose distribution and utilization in all mammals.

The function of the proteins encoded by these genes is to passively transport sugar metabolites through the membranes of most cells.

Within this family of about a dozen genes, *SLC2A1* and *SLC2A4* are particularly noteworthy because of their tissue-specific expression. *SLC2A1* encodes the primary glucose transporter in the brain while *SLC2A4* is mainly expressed in skeletal muscle and adipose tissue [Wood and Trayhurn, 2003; Uldry and Thorens, 2004; Herman and Kahn, 2006; Huang and Czech, 2007]. Other members of this gene family are expressed in different tissues

or have a minor role in hexose transport (table 1). Thus, *SLC2A1* and *SLC2A4* are well positioned to play a role in metabolic trade-off by virtue of their tissue-specific expression domains in the brain and skeletal muscle.

In this paper, we investigate and propose a possible link between *SLC2A1* and *SLC2A4* and the evolution of human brain size by showing (1) that tissue-specific gene expression changed during human origins in a manner consistent with a reallocation of energy from skeletal muscle to the brain, and (2) that these genes show human-specific signatures of positive selection, consistent with adaptation.

Materials and Methods

Sequence Data and Gene Compartment Annotations

SLC2A1, *SLC2A4* and intronic data were downloaded from the human (*Homo sapiens*; assembly hg18 of March 2006), chimpanzee (*Pan troglodytes*; assembly panTro2 of March 2006), orangutan (*Pongo pygmaeus abelii*; assembly ponAbe2 of July 2007), and macaque (*Macaca mulatta*; assembly rheMac2 of January 2006) genomes from the University of California Santa Cruz Genome Bioinformatics website (UCSC: <http://genome.ucsc.edu/>).

5'- and 3'-UTRs and exons were defined from the UCSC annotations, Ensembl annotations (<http://www.ensembl.org/>), and canonical sequences from experimental studies [Boado and Pardridge, 1999]. For each gene, we identified the most 5' transcription starting site (TSS). 5'-Flanking regions were defined as the 5-kilobase (kb) region upstream of the most 5' TSS. For neutral proxy (see Detecting Signatures of Positive Selection), we used introns from genes within a 100-kb window centered in the middle of the gene of interest. We excluded first introns because they are known to often contain regulatory elements, and 100 bp at each extremity of introns to eliminate splicing signal sites [Sorek and Ast, 2003]. We included a maximum of 2,500 bp from any one intron, drawn from the edges, since some long introns have been shown to contain regulatory elements in their center [Blanchette et al., 2006]. Intronic identity of a sequence was determined by overlapping all the known transcript isoforms and taking their intersection (segments identified as introns are always introns for all transcripts). We aligned all sequences using the computer program TBA (for 'Threaded Blockset Aligner'; http://www.bx.psu.edu/miller_lab/). We used these intronic sequences as neutral proxies to test for positive selection on noncoding regions. Chimpanzee, orangutan and macaque sequences with a quality score less than 40 were masked.

Tissue and RNA Samples

Human total RNA samples were acquired from Biochain (<http://www.biochain.com/>). Chimpanzee and macaque tissue samples were obtained from Southwest Foundation for Biomedical Research and New England Regional Primate Research Center, respectively (online suppl. table 1). Chimpanzee and macaque total RNAs were extracted from tissues according to the manufacturer's recommended protocol using different Qiagen® kits: RNeasy® Kit for liver samples, RNeasy Fibrous Tissue Kit for skel-

Table 1. Signatures of positive selection in the human lineage (p values)

Gene name	5'-flanking	5'-UTR	Exons	3'-UTR	Sugar [Uldry and Thorens, 2004; Wood and Trayhurn, 2003]	Main tissue of expression [Wood and Trayhurn, 2003]
<i>SLC2A1</i>	1.00	0.02*	1.00	0.08	Glc	brn, ery
<i>SLC2A2</i>	0.51	0.84	1.00	0.14	Glc	lvr, pcs, int, kdy
<i>SLC2A3</i>	0.97	1.00	1.00	0.48	Glc	bn
<i>SLC2A4</i>	0.93	0.01*	1.00	0.85	Glc	skm, hrt, wad, bad
<i>SLC2A5</i>	1.00	1.00	1.00	0.32	Frc	int, tst, kdy
<i>SLC2A6</i>	1.00	1.00	1.00	1.00	Glc	brn, spl, leu
<i>SLC2A7</i>	1.00	NA	1.00	NA	Frc	?
<i>SLC2A8</i>	1.00	1.00	1.00	1.00	Glc	tst, brn, oth
<i>SLC2A9</i>	0.52	0.09	1.00	0.61	Frc	lvr, kdy
<i>SLC2A10</i>	0.19	NA	1.00	1.00	Glc	lvr, pcs
<i>SLC2A11</i>	?	?	?	?	Glc, Frc	hrt, skm
<i>SLC2A12</i>	1.00	0.97	1.00	0.87	?	hrt, pro, skm, sin, wad, brn

Asterisks indicate significant p values (<0.05). 'NA' tests could not be performed because there was no available information about UTRs. '?' tests could not be done because the macaque orthologue could not be confidently identified. Glc = Glucose; Frc = fructose. Note that many of these genes are expressed more broadly at lower levels such as *SLC2A1*. Main tissue of expression: brn = brain, ery = erythrocytes, lvr = liver, pcs = pancreas, skm = skeletal muscle, hrt = heart, wad = white adipose tissue, bad = brown adipose tissue, int = intestine, tst = testes, kdy = kidney, spl = spleen, leu = leucocytes, pro = prostate, sin = small intestine, oth = other tissues, ? = unknown. See online supplementary tables 7 and 8 for more details.

etal muscle samples and RNeasy Lipid Tissue Kit for cerebral cortex samples. We synthesized first-strand cDNA from total RNA aliquots using a High Capacity cDNA Reverse Transcription Archive Kit (P/N 4368813) from Applied Biosystems® according to the manufacturer's protocol.

Primer Design

For primer design, we chose an exonic region conserved across humans, chimpanzees and macaques that is present in all the known transcript isoforms. We ensured that this region is unique in the genome by blasting the nucleotide sequence to the human genome (http://www.ensembl.org/Homo_sapiens/bblastview). We designed primers using Primer3 [Rozen and Skaletsky, 2000] with the following parameters: 100- to 150-bp product long, primer Tm min 58°C, Opt 59°C, max 61°C; primer size min 17 bp, opt 20 bp; max 23 bp. Finally, we blasted these primer sequences to the human and chimpanzee genomes to ensure that they are unique. Standard curves were performed for each set of primers using cDNA from an IMR32 cell line, a neuroblastoma that expresses both genes. The points on the standard curve were designed as a dilution series of 8 different concentrations with consecutive points varying by a factor of 2. Primer efficiencies were estimated as follows:

$$Eff = \frac{10^{-1/slope}}{2} \times 100$$

where *slope* is the regression line slope. Primer sets were kept for $0.95 \leq Eff \leq 1.0$ and $R^2 \geq 0.99$: 0.956 (*SLC2A1*); 0.965 (*SLC2A4*); 0.992 (*HMBS*); 0.965 (*EIF2B2*). The following primers were used:

5'-GTCGGAGTCAGAGTCGCAGT-3' (*SLC2A1* forward); 5'-TG-AACGATTTTCATGGTTGC-3' (*SLC2A1* reverse); 5'-CTTCGA-GACAGCAGGGGTAC-3' (*SLC2A4* forward); 5'-ACAGTCATCA-GGATGGCACA-3' (*SLC2A4* reverse); 5'-GGCAATGCGGCTG-CAA-3' (*HMBS* forward); 5'-GGGTACCCACGCGAATCAC-3' (*HMBS* reverse); 5'-TCAAGATTATCCGGGAGGAG-3' (*EIF2B2* forward); 5'-ATGGAAGCTGAAATCCTCGT-3' (*EIF2B2* reverse).

Quantitative Real-Time Polymerase Chain Reaction

We conducted quantitative real-time polymerase chain reaction (q-RT-PCR) measurements on an ABI PRISM 7000 (Applied Biosystems) with each reaction containing 15 µl 2× ABGene Absolute q-PCR SYBR® Green Mix, 0.75 µl for each primer (10 µM), 1 µl of cDNA template, and PCR-quality water to reach a total volume of 30 µl. The q-RT-PCR program started with a hot start at 95°C for 15 min, followed by 40 cycles of a 15-second melt at 95°C, and a 30-second annealing/elongation at 60°C. After 40 cycles were completed, a dissociation curve was created from 50 to 90°C. We ran the reactions in technical triplicates.

Within plates, expression was normalized with a set of two normalizer genes that have been chosen because of their performance in a geNorm™ analysis on brain, muscle and liver tissues for humans and chimpanzees (*EIF2B2* and *HMBS*) [Fedrigo et al., 2010]. To convert raw results into relative expression, we used a modified delta-delta Ct method [Pfaffl, 2001; Vandesompele et al., 2002; Hellemans et al., 2007]. This method propagates standard deviation at every step (standard deviation caused by pipet-

ting and instrument errors). When the experiment was repeated several times (i.e. the same technical triplicates on a different plate), we selected the experiment with the least amount of technical artifacts (i.e. smallest propagated standard deviation). Since apparent variation in gene expression between plates can be caused by instrument and reagent variation, we also measured the expression of all the genes on cDNA from IMR32 cells on each plate for inter-run calibration [Hellemans et al., 2007]. Tukey's HSD test was used to determine significant differences between species for each tissue.

Detecting Signatures of Positive Selection

Using a likelihood ratio test based on a modified and improved branch-site model [Zhang et al., 2005] that allows for relaxed constraints, we estimated and compared the substitution rate, using the Hasegawa-Kishino-Yano HKY85 model [Hasegawa et al., 1985], within noncoding sequences with the rate of selected introns (ζ) for the quartet macaque-orangutan-chimpanzee-human [Wong and Nielsen, 2004; Haygood et al., 2007]. We fitted and contrasted two models: a null model, which assumes three site classes with negative selection ($\zeta < 1$) on every branch, neutral evolution ($\zeta = 1$) on every branch, and a class that allows relaxed constraint on the branch of interest (online suppl. table 2); an alternative model, which assumes four site classes with negative selection ($\zeta < 1$) on all branches, neutral evolution ($\zeta = 1$) on every branch, and two classes with positive selection on the branch of interest ($\zeta > 1$) while on the other branches sites are neutrally evolving or constrained (online suppl. table 3). We compared these two models using a likelihood ratio and χ^2 test with 1 degree of freedom. We kept the best of 10 fittings to avoid local optima. We also analyzed exonic/coding sequences using the same quartet of species as for the noncoding sequence analyses and an improved branch-site likelihood method for coding sequences [Zhang et al., 2005]. The main difference is that we compared the substitution rates of nonsynonymous and synonymous sites (dn/ds or ω). We contrasted two models similar to the ones described above: a null model that allows relaxed constraint and an alternative model with positive selection ($\omega > 1$) on the branch of interest (online suppl. tables 2, 3). For both noncoding and coding sequences, we focused on detecting positive selection on the human branch but we also scanned for signatures of positive selection on the chimpanzee branch in order to contrast the signal for genes that have a significant p value on the human branch.

An important statistical concern arises regarding stochasticity of the evolutionary process, especially for the noncoding sequence analyses where introns are used as neutral proxies. Indeed, intronic sequences are thought to generally evolve neutrally and thus homogeneously, but some introns contain regulatory elements comprising a subset of nucleotide sites that are more slowly evolving than neutral sites. The presence of these slower sites in the neutral proxy dataset can inflate ζ and increase the false-discovery rate. In order to eliminate this potential source of error, we constructed 100 bootstrap replicates from the intronic data, performed the test for selection on 5'-flanking regions and -UTRs with each of the bootstrap replicates and considered the median p value as an indicator of signature of positive selection.

For the regions under positive selection in the human lineage ($p < 0.05$), we identified sites that are responsible for this signal, using a Bayes Empirical Bayes approach [Yang et al., 2005] to identify sites under positive selection with a posterior probability

$\Pr(\zeta > 1) > 0.75$. To insure that the sites driving the signal of positive selection are fixed in human populations, we compared them to the human polymorphism database (dnSNP build 130) [Sherry et al., 2001]. We implemented the tests for selection and the Bayes Empirical Bayes approaches in HyPhy [Pond et al., 2005] (<http://www.hyphy.org>). HyPhy scripts are available at: <http://www.biology.duke.edu/wraylab/Resources.html>.

We also did a survey of the other glucose/fructose transporters of this family. We downloaded, annotated and processed the data and tested for positive selection on the human branch as described previously.

Testing for Factors That Can Increase the Substitution Rate

There are numerous factors that can inflate the false-positive rate for detecting signatures of positive selection. The sequence data collection and test for selection procedures we have implemented attempt to account for many of them (orthology, sequence quality, intron substitution rate and relaxation of constraint). An additional source of bias can come from a differential proportion of methylated CpG sites between the regions of interest and the neutral proxy (introns). Indeed, bases present in CpG dinucleotides are hypermutable when methylated [Krawczak et al., 1998]. Thus, if the regions of interest contain more methylated CpG dinucleotides than the introns, the estimate of ζ will be inflated. However, not all CpG sites are methylated and therefore hypermutable. In order to verify if there is such a mutational bias in regions for which we detected signatures of positive selection, we partitioned sites of these regions into CpG susceptible sites and CpG nonsusceptible sites and test whether or not their substitution rate is significantly different. We defined CpG susceptible sites as any sites for any of the four species that are preceded by a C or followed by a G. For testing for a potential different evolutionary rate between these two types of sites, we performed a likelihood ratio test, contrasting a null model in which the transversion/transition ratio κ is constrained to be the same in the two partitions, and an alternative model in which this ratio is allowed to differ between the two partitions: the constrained null hypothesis $H_0: \kappa_{CpG} = \kappa_{non-CpG}$ and the full alternative model $H_A: \kappa_{CpG} \neq \kappa_{non-CpG}$. We used the HKY85 [Hasegawa et al., 1985] model to estimate all parameters. We computed the likelihood statistic $2(\log L_A - \log L_0)$ and a p value (χ^2 test with one degree of freedom). We also performed a parametric bootstrap to verify the χ^2 test.

Results

Differential Expression

As a first step in understanding the role of *SLC2A1* and *SLC2A4* may have played in the evolution of human brain size, we measured the expression of these two genes in three tissues (cerebral cortex, skeletal muscle and liver) for three species and four biological replicates each: human, chimpanzee and rhesus macaque. We found that the mean expression of *SLC2A1* and *SLC2A4* is strongly enriched in cerebral cortex and skeletal muscle, respectively, in all three species (fig. 1). This result is consistent

Fig. 1. Interspecific differences in gene expression are consistent with the predictions of the energy trade-off hypothesis. Expression of *SLC2A1* is higher in human than chimpanzee and macaque cerebral cortex; expression of *SLC2A4* is similarly higher in chimpanzee than in human and macaque skeletal muscle. Relative gene expression is plotted for human (Hsap), chimpanzee

(Ptro), and macaque (Mmul) tissues: cerebral cortex, liver and skeletal muscle. Error bars represent standard deviation. Asterisks indicate significant expression differences according to Tukey's HSD test: * $p < 0.05$; ** $p < 0.005$. See online supplementary table 4 for detailed results.

with previous studies in humans [Wood and Trayhurn, 2003; Uldry and Thorens, 2004; Huang and Czech, 2007], and suggests that the tissue-specific expression of both genes has been maintained at least since the divergence of humans and chimpanzees, and perhaps much longer [Wilson-O'Brien et al., 2010].

Despite maintaining tissue specificity, both genes show clear differences in the relative levels of expression between species. *SLC2A1* expression in cerebral cortex is about 3.2-fold higher in human than chimpanzee repli-

cates (adjusted p value = 0.0057). Conversely, *SLC2A4* expression in skeletal muscle is about 1.6-fold higher in chimpanzee than in human replicates (adjusted p value = 0.0245). In contrast, there are no significant differences in the expression of *SLC2A1* in skeletal muscle and *SLC2A4* in cerebral cortex between species, and neither shows evidence of differential expression between species in liver tissue. These results suggest that the proportionally higher levels of *SLC2A1* in human cerebral cortex and of *SLC2A4* in chimpanzee muscle are not the result

of generally elevated expression levels for the two genes, but instead represent tissue- and species-specific changes in gene regulation.

Macaques were used as an outgroup for polarizing expression differences between humans and chimpanzees in an attempt to sketch an evolutionary scenario. However, caution is indicated in using the macaque data for this purpose due to the large phylogenetic distance separating Old World monkeys from great apes. Macaque replicates show the lowest expression level in all tissues and for both genes although these differences are not always significant. These results are consistent with an increase in *SLC2A1* expression in the cerebral cortex on the human branch. For *SLC2A4*, it is more complicated as two equally parsimonious scenarios can explain the expression pattern: an increase in *SLC2A4* expression in skeletal muscle during great ape origins, followed by a decrease in expression on the human branch or an increase on the chimpanzee branch (see Discussion).

It is possible that collecting tissue samples from an organ as structurally complex as the brain could introduce a systematic bias if we mistakenly drew samples for one species from one brain region and samples for another species from a different brain region with a distinct gene expression profile. This could produce an observation of differential *SLC2A1* expression whose true biological basis lies in intraorgan regional differences rather than species-specific differences in gene expression. To test for this possibility, we measured *SLC2A1* expression of one macaque for four distinct areas of the brain (prefrontal cortex, occipital lobe, parietal lobe and temporal lobes). We observed less variation among cortical regions from the same individual than in the same brain region between individuals of the same species and between species (online suppl. fig. 2). This result suggests that a combination of heterogeneous tissue sampling of the cerebral cortex and variable spatial expression of the gene is not likely the cause of the observed interspecies differences in tissue-specific expression.

Signatures of Positive Selection

Next, we tested for signatures of branch-specific positive selection within the protein-coding regions of *SLC2A1* and *SLC2A4* as well as noncoding sequences around both genes, including the 5-kb region immediately upstream of the transcription start site and both 5'- and 3'-UTRs. Because the ability to discriminate branch-specific differences in substitution rates increases with more taxa, we based these tests for selection on sequences from four primate species: human, chimpanzee, orang-

utan and rhesus macaque. We tested for signatures of positive selection using the modified branch-site method. We applied a similar method for coding and noncoding sequences. Basically, we compared the substitution rate of the sequence of interest with the substitution rate of a neutrally evolving sequence. This rate ratio was used as an index of nonneutral sequence change. For coding sequences, nonsynonymous/synonymous ratio was used (ω or dn/ds ratio), and for noncoding sequences, the substitution rate ratio of the sequence of interest and neighboring introns in a 100-kb window was used (ζ ratio). A likelihood ratio test was constructed by comparing an alternate model that allows the rate ratio to be greater than 1 (positive selection) on the branch of interest with a null model that does not allow it on any branch [Wong and Nielsen, 2004; Haygood et al., 2007] (see Materials and Methods).

As we previously noted in a study based on sequences from three species [Haygood et al., 2007], neither *SLC2A1* nor *SLC2A4* shows evidence of human or chimpanzee branch-specific positive selection within the 5'-flanking region, where many *cis*-regulatory elements are known to reside [Crawford et al., 2006]. Also in agreement with previous studies by others [Clark et al., 2003; Nielsen et al., 2005; Kosiol et al., 2008] (online suppl. table 5), we found no evidence of positive selection within protein-coding exons of either gene along the human and chimpanzee lineages. In contrast to these earlier studies, we found that the 5'-UTRs of both *SLC2A1* and *SLC2A4* show an elevated substitution rate and significant evidence for signatures of positive selection, specifically along the human lineage but not the chimpanzee branch (fig. 2). In order to further investigate this observation, we performed a sliding-window analysis. We detected a human-specific elevated substitution rate concentrated in the 5'-UTR (online suppl. fig. 3). Within these regions, we used a Bayes Empirical Bayes method [Yang et al., 2005] to detect noncoding sites with high posterior probability of being under positive selection [$\Pr(\zeta > 1) > 0.75$] in the human lineage. We also checked whether these sites are conserved in human populations using polymorphism data from the dbSNP database [Sherry et al., 2001]. We found that all the sites identified by our test for positive selection are fixed in human populations for both genes (online suppl. table 6), suggesting that they occurred prior to the origin of modern humans.

Mutational biases can influence tests for selection, and susceptible CpG sites in particular can be a concern in noncoding sequences near transcriptional start sites because these regions often show an elevated GC base com-

Fig. 2. Patterns of sequence substitution indicate positive selection on the 5'-UTR of two glucose transporters. Both *SLC2A1* and *SLC2A4* show evidence of positive selection in their 5'-UTR on the human branch, but not on the chimpanzee branch. Tests on other gene compartments of *SLC2A1* and *SLC2A4* revealed no evidence of positive selection. As summarized in table 1, there were no larger signatures of positive selection in the human lineage on any gene compartment for any of the other 10 glucose/fructose transporters of the *SLC2A* gene family. Human tissue = Dark grey and chimpanzee tissue = light grey. Asterisks indicate significant p values (<0.05).

position. In order to test whether hypermutability of CpG sites could bias the detection of signatures of positive selection within *SLC2A1* and *SLC2A4* 5'-UTRs, we compared the substitution rate of susceptible CpG sites and nonsusceptible CpG sites by contrasting two hypotheses: a null hypothesis for which the substitution rate is the same for both types of sites, and an alternate hypothesis for which the substitution rate is different. The likelihood ratio test of these two models does not reject the null hypothesis (p value = 0.54), strongly suggesting there is no evidence of a mutational bias caused by CpG sites in *SLC2A1* and *SLC2A4* 5'-UTRs. Therefore, the signal of positive selection we have detected cannot be explained by an elevated mutation rate in 5'-UTR CpG sites.

If the signatures of positive selection on the 5'-UTRs of *SLC2A1* and *SLC2A4* are associated with an adaptive change in the allocation of energy among tissues, we would expect other members of this gene family to show no evidence of positive selection. We therefore tested for signatures of positive selection on coding and noncoding regions among all 12 glucose/fructose transporter genes of the *SLC2A* family. Strikingly, the 5'-UTRs of *SLC2A1* and *SLC2A4* are the only genic regions that show evi-

dence of human-specific positive selection across the entire family (table 1). This result demonstrates that these two genes do not simply belong to a family with generally rapid sequence substitution.

Discussion

The Observed Differences in Expression Have Phenotypic Consequences

Both *SLC2A1* and *SLC2A4* show tissue-specific differences in expression between humans and chimpanzees that are consistent with the expectations of the energy trade-off hypothesis. There is no a priori way to know whether the magnitude of these differences, an approximately 1.6- to 3.2-fold change, is biologically significant. However, genetic evidence indicates that *SLC2A1* and *SLC2A4* are strongly dose dependent in humans, and clearly demonstrate that expression differences of this magnitude have important phenotypic consequences. Partial to complete loss-of-function mutations in *SLC2A1* lead to Glut-1 deficiency syndrome in humans (OMIM 606777). This syndrome is characterized by a low glucose

level (hypoglycorrhacia) and a low to normal lactate level in the cerebrospinal fluid – a product of decreased glucose availability [Wang et al., 2006]. Mild hypomorphic alleles of *SLC2A1* result in cognitive impairments and infancy-onset seizures while severe alleles (thought to be null or nearly complete loss of function) result in acquired microcephaly despite otherwise normal brain development and do not seem to affect other organs [De Vivo et al., 1991; Wang et al., 2000; Brockmann et al., 2001; Klepper and Leidencker, 2007]. These clinical phenotypes are the consequence of insufficient glucose transport across the blood-brain barrier [Wang et al., 2000; Brockmann et al., 2001; Klepper and Leidencker, 2007]. A wild-type/null heterozygous genotype leads to brain size reduction, most likely because neurons literally starve during postnatal brain growth. This is caused by decreased glucose availability for astrocytes, resulting in reduced production of lactate, which is the primary energy source for neurons [Wang et al., 2006]. The homozygous null condition has never been observed in humans, presumably because it results in embryonic lethality. The phenotypes of transgenic mice confirm these observations: heterozygous mice develop symptoms similar to the human Glut-1 deficiency syndrome, while homozygous knockouts die during late embryonic development [Wang et al., 2006]. Importantly, this haploinsufficiency means that the approximately 3.2-fold difference in expression between the human and chimpanzee cerebral cortex is larger than that of the human hemizygote, implying that a difference in expression of this extent is physiologically and developmentally critical. As with *SLC2A1*, the genetic data demonstrate that the difference in *SLC2A4* expression between humans and chimpanzees in skeletal muscle has potential phenotypic consequences. Indeed, *SLC2A4* heterozygous knockout mice present symptoms that include increased serum glucose, hyperinsulinemia and increased blood pressure, and demonstrate the importance of the regulation of this gene for whole-body glucose and lipid metabolism [Charron and Katz, 1998; Charron et al., 2005], and specifically that downregulation of the major glucose transporter in skeletal muscle increases the amount of glucose available to other organs.

Differential expression between humans and chimpanzees could be explained by an increase or a decrease in expression in one or the other species. Measuring expression in the rhesus macaque allowed us to tentatively polarize these differences. Cortical expression of *SLC2A1* in the macaque is slightly lower but not significantly different from that in the chimpanzee, suggesting

increased expression on the human branch. For skeletal muscle, *SLC2A4* expression in the macaque is significantly lower than in both humans and chimpanzees. These data are consistent with a human-specific decrease in expression following an ape-specific increase in expression. It is important to note that macaques and humans diverged about 25 million years ago [Dennis, 2005] and that this makes direct comparisons with apes problematic since additional expression changes could have evolved and obscure the true polarity of change. Despite this limitation, the use of macaques as an out-group for interpreting differences in gene expression is generally the only practical choice because obtaining tissues from multiple individuals of other apes is rarely possible.

The Evolution of Muscle and Brain Energetics

In principle, a trade-off in energy allocation among tissues could be accomplished in several ways. It has previously been suggested that humans have less muscle mass relative to body mass compared with primates in general [Leonard et al., 2003]. This observation could explain the shift in energy allocation from muscle to brain tissue on the human lineage by reducing the ratio of skeletal muscle to overall body mass. However, more recent analyses indicate that humans are not undermuscle compared with other apes [Isler and van Schaik, 2006]. A change in the cost of muscle recruitment during locomotion would also explain this energy trade-off, by allowing more energy to be redirected to the brain without a decrease in muscle mass. Based on a study in birds, Isler and van Schaik [2006] suggested that a change in locomotor costs may have played a role in human brain evolution. Indeed, it has been reported that the energetic costs of locomotion are higher in chimpanzees than in humans [Sockol et al., 2007]: human walking is about 75% less costly than quadrupedal and bipedal walking in chimpanzees, mostly due to anatomical and gait differences. In line with the energy trade-off hypothesis, it has been suggested that locomotor energetics could have played an important role in the evolution of bipedalism [Sockol et al., 2007].

In a similar line of thought, given a certain amount of glucose in the bloodstream, an increase in the amount of *SLC2A1* or *SLC2A4* protein per gram of tissue will result in more glucose being captured by that tissue and less by other tissues. An increase in *SLC2A1* expression in brain and a decrease in *SLC2A4* in skeletal muscle would work synergistically to shift the relative allocation of glucose between two of the most energetically demanding tissues

in the human body, such that more energy is available to the brain.

Interestingly, imaging studies suggest a higher metabolic rate for brain tissue in conscious state in humans than in macaques [Bohnen et al., 1999; Bentourkia et al., 2000; Cross et al., 2000; Noda et al., 2002], implying that the higher metabolic cost of the human brain is a function not only of its size but also of its neuronal activity [Caceres et al., 2003; Uddin et al., 2004, 2008; Oldham et al., 2006].

Signature of Positive Selection on Regulatory Regions

The trade-off in energy allocation that allowed for a larger brain in humans was likely adaptive since it may have allowed for the evolution of important cognitive functions. Thus, mutations contributing to an increase in brain size during human evolution may have been under positive selection. Consistent with this expectation, we found that the 5'-UTRs of both *SLC2A1* and *SLC2A4* show evidence of positive selection on the human branch (fig. 2). 5'-UTRs often contain regulatory elements that influence transcription as well as elements involved in posttranscriptional regulation and message stability [Mignone and Pesole, 2007; Xue et al., 2008]. The signatures of positive selection we have detected in 5'-UTRs suggest human-specific adaptations of *SLC2A1* and *SLC2A4* regulation. Importantly, transfection assays in cultured human endothelial brain cells have previously demonstrated the importance of the 5'-UTR in *SLC2A1* expression [Boado and Pardridge, 1999], and a region overlapping the *SLC2A4* 5'-UTR is necessary for transcriptional regulation in cultured adipocytes [Armoni et al., 2003]. Thus, the regions showing evidence of positive selection contain regulatory elements that are important for the expression of both *SLC2A1* and *SLC2A4*.

Because several factors can give rise to spurious signatures of positive selection on nucleotide sequences, it is important to consider whether they contribute to the accumulation of mutations within the 5'-UTRs of *SLC2A1* and *SLC2A4*: (1) poor-quality sequences in one taxon could resemble an accelerated substitution rate. This is a particular concern with the chimpanzee and macaque genomes, where coverage and reading quality are more uneven, so we masked bases with a quality score less than 40 to avoid this problem; (2) gene duplication and incorrect orthology assignments are another source of misleading signatures. We addressed this issue by using whole-genome pairwise alignments from UCSC, mapping regions of interest (liftover chain files) and checked for mapping errors, chromosomal and gene synteny (i.e.

identical flanking genes) as previously described [Haygood et al., 2007]; (3) conserved functional sites in the neutral proxy can inflate the ζ ratio. We used introns as neutral proxies, because they are the fastest evolving sequences of the genome [Keightley et al., 2005]. In order to further reduce the chance of including functional sequences, we excluded first introns, eliminated potential splicing signal sites and included a maximum of 2,500 bp from any one intron [since some long introns have been shown to contain regulatory elements in their center; Blanchette et al., 2006]; (4) relaxation of constraint is also characterized by an increase in substitution rate, and can be misinterpreted as positive selection. Our null model accounts for this scenario as described in Materials and Methods; (5) methylated CpG sites are hypermutable and their presence can therefore inflate the detection of positive selection. We showed that susceptible CpG sites do not evolve faster than nonsusceptible CpG sites in the 5'-UTRs of both *SLC2A1* and *SLC2A4*, which addresses this concern.

Thus, reasonable alternative explanations do not account for elevated rates of nucleotide substitution within the 5'-UTRs of *SLC2A1* and *SLC2A4* during human origins, suggesting that they are most likely due to positive selection. Consistent with this interpretation, the signatures of positive selection overlap known *cis*-regulatory elements of both genes [Boado and Pardridge, 1999; Armoni et al., 2003], the expression of both genes has changed on the same branch showing the elevated rates of sequence substitution, and signatures of positive selection are restricted to just these 2 members of the 12-gene *SLC2A* family.

The Genetic Basis for Human Brain Size Evolution

The fossil record clearly shows a steady expansion of human brain size that extended over a period of several million years [Schoenemann, 2006]. This suggests a genetic model involving multiple mutations of relatively small effect that were fixed across a protracted interval of time, rather than a few mutations of large effect. The causal mutations almost certainly involved two kinds of genes: those that altered brain development [Vallender et al., 2008] and those that provided metabolic support [Aiello and Wheeler, 1995; Leonard et al., 2003; Isler and van Schaik, 2006]. A few genes have been implicated in the developmental component of increased brain size based on evidence of positive selection [Evans et al., 2005; Mekel-Bobrov et al., 2005] although the functional and trait consequences of these mutations are not known. Identifying the relevant mutations affecting brain devel-

opment is challenging since many critical events happen in utero and during the immediate postnatal period. However, the metabolic consequences of an expanded brain extend into adult life, making this component of the genetic basis more accessible for functional analysis. To the best of our knowledge, the present study is the first to implicate specific genes as mediating the metabolic trade-off that would be necessary to support the expansion of the human brain.

A major caveat of our expression analysis is that genetic changes might not be the only contributors to the observed differences in gene expression. Indeed, it has been shown that environmental factors, such as diet and training conditions, often have an effect on gene expression [Gibson, 2008; Idaghdour et al., 2008; Somel et al., 2008; Hodgins-Davis and Townsend, 2009]. Controlling for environmental conditions is feasible with model organisms such as mice [Somel et al., 2008], but it is not possible with chimpanzees and humans. This is especially a potential issue for skeletal muscle samples, where age, sex and fiber type distribution are factors that interact to influence SLC2A4 protein density [Gaster et al., 2000a, b; Gaster, 2007]. However, while it does not rule out the effect of diet or training conditions, the fact that our test for positive selection on regulatory regions is consistent with the expression pattern is supportive of our evolutionary interpretations.

Conclusion

Three lines of evidence suggest that regulatory mutations in *SLC2A1* and *SLC2A4* contributed to the evolution of a larger brain during human origins by mediating a shift in energy allocation from skeletal muscle to brain: (1) cross-species comparisons of gene expression reveal tissue-specific changes in expression levels that are consistent with the energy trade-off hypothesis; (2) genetic evidence from humans and the mouse indicates that expression differences of this magnitude have an effect on normal brain development and maintenance (*SLC2A1*), and on muscle function and blood glucose levels (*SLC2A4*), and (3) sequence comparisons of known regulatory regions for both genes are consistent with human-specific adaptations of gene regulation. These results raise some interesting questions for subsequent study, including whether expression levels during development differ between species, which cells in the brain are most directly affected by the change in *SLC2A1* expression, precisely which mutations account for the expression

change, and whether there are expression differences among skeletal muscles between species that may be related to both energy trade-offs and the energetic cost of locomotion. Moreover, it seems unlikely that *SLC2A1* and *SLC2A4* were the only genes involved in metabolism that contributed to the energy trade-off. Many other genes that mediate carbohydrate and lipid metabolism also show evidence of positive selection on their putative regulatory regions during human origins [Haygood et al., 2007]. In addition, as hypothesized by Aiello and Wheeler [1995], a gut size reduction may have contributed to human brain evolution. In consequence, we should also expect genes implicated in digestive system energy transport, physiology and development to be entangled in a complex network of energy trade-offs between the brain and several expensive tissues. Exploring the history of mutations in these genes during human origins, and particularly their functional, trait and fitness consequences, will shed light on the evolution of one of the most distinctive of all human traits.

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