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Accelerated Evolution of Nervous System Genes in the Origin of *Homo sapiens*

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Summary

Human evolution is characterized by a dramatic increase in brain size and complexity. To probe its genetic basis, we examined the evolution of genes involved in diverse aspects of nervous system biology. We found that these genes display significantly higher rates of protein evolution in primates than in rodents. Importantly, this trend is most pronounced for the subset of genes implicated in nervous system development. Moreover, within primates, the acceleration of protein evolution is most prominent in the lineage leading from ancestral primates to humans. Thus, the remarkable phenotypic evolution of the human nervous system has a salient molecular correlate, i.e., accelerated evolution of the underlying genes, particularly those linked to nervous system development. In addition to uncovering broad evolutionary trends, our study also identified many candidate genes—most of which are implicated in regulating brain size and behavior—that might have played important roles in the evolution of the human brain.

Introduction

Greatly expanded and highly complex brains are among the most defining attributes distinguishing primates, especially humans, from other mammals (Brodmann, 1912; Jerison, 1973; Finlay and Darlington, 1995). As a result of increased brain size and complexity, behavioral repertoires became much richer in primates, culminating in highly sophisticated cultural behaviors in humans such as language, tool use, and social learning (Spuhler, 1959; Matsuzawa, 2001).

In past decades, researchers have devoted significant efforts toward understanding the evolutionary processes that gave rise to the distinct features of the human brain. Traditionally, such efforts have focused on the anatomical and physiological differences between the human brain and that of the other taxa, as well as the behavioral manifestations of these differences (Jerison, 1973; Byrne and Whiten, 1988; Aiello and Dean, 1990; Matsuzawa, 2001). More recently, the genetic basis of brain evolution has emerged as a topic of considerable discussion. Of particular interest are questions regarding what genes underlie brain differences between humans and other species, and how changes in these genes led to specific alterations in brain biology. As yet, these important questions remain poorly explored. In this study, we probe these questions by comparative genomics studies utilizing both primates and nonprimate species.

It has long been noted that brains of various extant and extinct primates display remarkable variation in size, organization, and behavioral output (Noback and Montagna, 1970; Armstrong and Falk, 1982; Byrne and Whiten, 1988; Matsuzawa, 2001). This is particularly true for the evolutionary lineage leading from ancestral primates to humans, in which the increase in brain size and complexity was remarkably rapid and persistent throughout the lineage (Jerison, 1973; Walker et al., 1983). In contrast, for most nonprimate mammalian orders, the extent of intra-ordinal brain differences is much more limited (Brodmann, 1912; Pagel and Harvey, 1989). For example, the encephalization quotient, a rough measure of brain size scaled allometrically to body size, can differ by more than an order of magnitude between humans and nonhuman primates, but varies much less between species of any nonprimate order (Williams, 2002). Thus, the phenotype of the nervous system has apparently undergone far greater evolutionary changes in primates than most other mammals.

Extrapolating from these observations, we hypothesized that the intensified phenotypic evolution of the brain seen in primates might have a molecular correlate—that is, genes involved in nervous system biology might display more dynamic molecular evolutionary changes in primates relative to nonprimate mammals. We further surmised that within primates, the lineage leading from ancestral primates to humans might exhibit more dramatic evolutionary changes than other primate lineages, on the basis that the increase in brain size and complexity is most profound in the lineage leading to humans.

In this study, we compared the evolutionary rates of an extensive set of nervous system-related genes between primates and rodents. To obtain evolutionary rates in primates, we compared sequences between human and the Old World monkey, macaque. We note that even though much discussion of human evolution has focused on human-chimpanzee comparisons, the strong sequence similarities between these two species results in high stochastic uncertainty in the estimation of evolutionary rates. This is likely to reduce the statistical power in detecting interesting evolutionary signatures. Human-macaque comparisons, in contrast, offer much more accurate rate estimation because of the considerably greater sequence divergence. For the nonprimate mammalian order, we used rodents, with rat and mouse as the species chosen for comparison. The evolutionary time separating human and macaque (20–25 million

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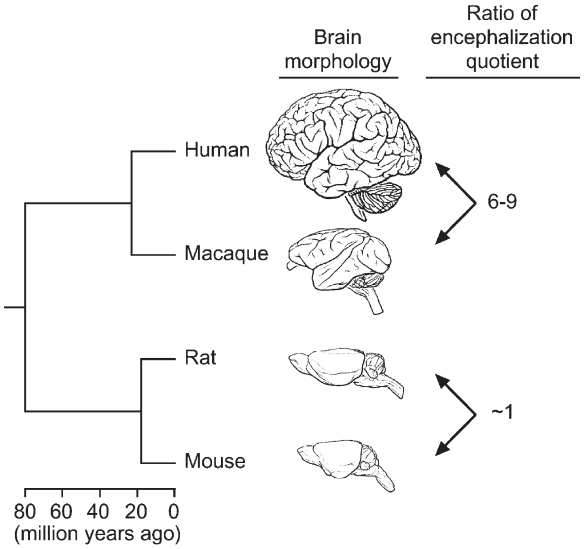

Figure 1. Phylogenetic Relationship of the Four Taxa Used in the Study

Ratios of encephalization quotient (brain size allometrically scaled to body size) between taxa are indicated following published data (Williams, 2002). Brains of different taxa are not drawn to scale of absolute size. Estimated evolutionary time separating these four taxa is depicted.

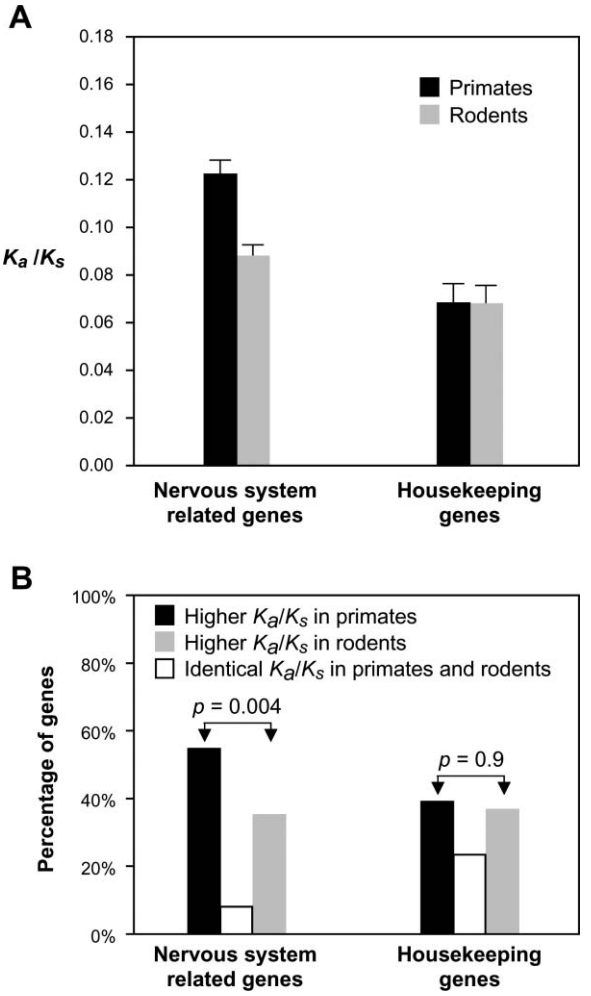

Figure 2. Evolution of Nervous System Genes and Housekeeping Genes in Primates and Rodents

(A) Evolutionary rates in primates and rodents.

(B) Percentage of genes that evolved with higher K_a/K_s in one or the other mammalian order.

The p values indicate the statistical significance of primate-rodent disparities.

years) is grossly comparable to that separating rat and mouse (16–23 million years) (Kumar and Hedges, 1998; Springer et al., 2003). However, point mutation rates are lower in primates than in rodents (Gibbs et al., 2004), which results in the synonymous sequence divergence between human and macaque being about half that between rat and mouse. Despite the fact that human-macaque sequence divergence is less, the size and complexity of the brain differ profoundly between these two primates while remaining grossly comparable between the two rodents (Figure 1). Comparisons of these four taxa should, therefore, allow us to interpret any molecular evolutionary differences of nervous system genes between primates and rodents within the meaningful context of contrasting evolutionary outcomes in brain phenotypes between these two mammalian orders.

By comparing nervous system genes across the four aforementioned taxa, we demonstrate that the average rate of protein evolution as scaled to neutral divergence is indeed considerably faster in primates than in rodents and that this trend is most pronounced for the subset of genes implicated in nervous system development. We further show that within primates, such evolutionary acceleration is much greater in the lineage leading from ancestral primates to humans relative to lineages leading to nonhuman species. Thus, the dramatic evolution of nervous system phenotype in primates, particularly humans, is indeed correlated with salient molecular evolutionary footprints in the underlying genes.

Results

Evolution of Nervous System Genes

We used multiple criteria to compile a list of genes as broadly representative of nervous system biology as possible. First, we performed extensive literature searches to obtain a set of genes demonstrated to play important roles in the nervous system. Second, we used databases of expressed sequence tags (ESTs) and SAGE tags (Velculescu et al., 1999) to identify a group of genes expressed exclusively or predominantly in the brain. Lastly, we included a set of genes implicated in various diseases of the nervous system, such as brain malformations, mental retardation, and neurodegeneration. Many of the genes appear to function exclusively in the nervous system whereas others may also play roles in additional tissues. In either case, the prominent involvement of these genes in the nervous system makes them good candidates for our study. By sequencing and bioinformatics, we obtained orthologous sequences for 214 such genes in all of the four taxa chosen for this study (Supplemental Table S1 at http://www.cell.com/cgi/content/full/119/7/1027/DC1/). We note that these genes are scattered randomly across the genome. Be-

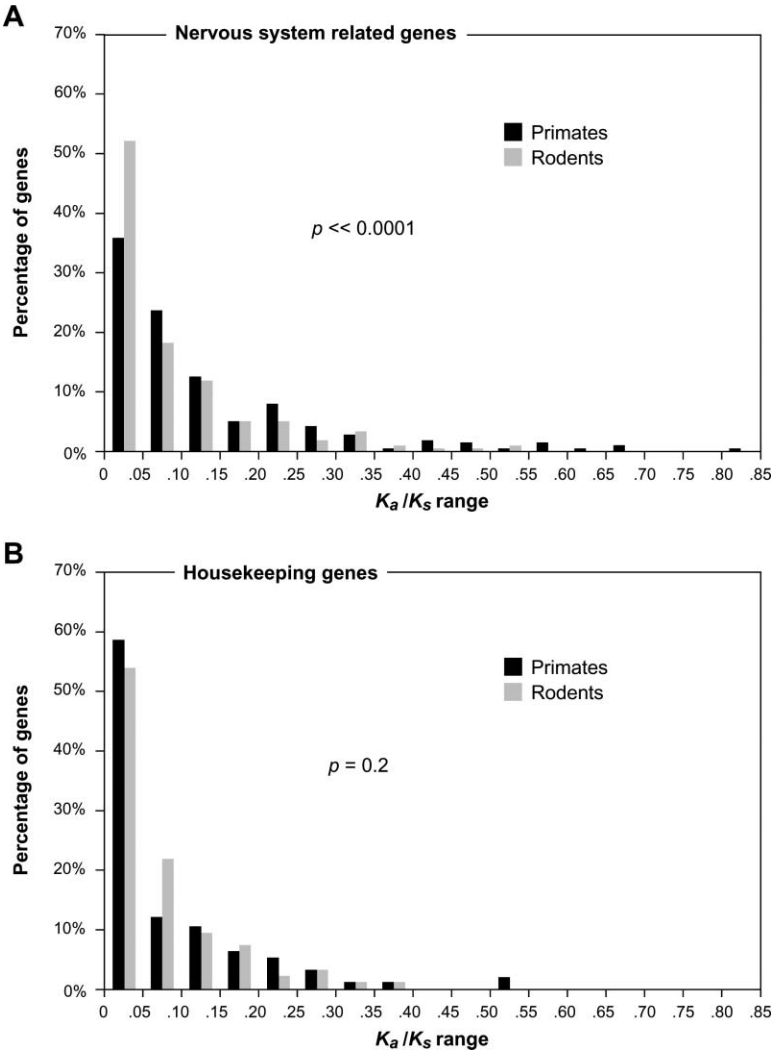

Figure 3. The K_a/K_s Distributions of Nervous System Genes and Housekeeping Genes in Primates and Rodents

(A) Nervous system-related genes.
(B) Housekeeping genes.
The p values indicate the statistical significance of primate-rodent disparities.

cause the acquisition of these genes was done without prior knowledge of their evolutionary properties, the findings discussed below are not due to selective sampling of genes with desirable evolutionary parameters.

The pace of protein evolution as scaled to neutral divergence is commonly approximated by the ratio between nonsynonymous (K_a) and synonymous (K_s) substitution rates (Li, 1997). To infer K_a/K_s ratios of genes in primates, we compared human and macaque orthologs. For rodent K_a/K_s, rat and mouse sequences were compared. The average K_s of these genes is 0.065 $\pm$ 0.028 (mean $\pm$ SD) for the primate comparison and 0.158 $\pm$ 0.063 for the rodents, in close agreement with previous reports (Yi et al., 2002; Gibbs et al., 2004). Notably, the average K_a/K_s of these genes is substantially higher (by 37%) in primates than in rodents (Figure 2A), and the disparity is statistically highly significant ($p << 0.0001$ by Fisher's exact test). As discussed below, additional statistical tests further corroborated the significance of this disparity. This result indicates that the average rate of protein evolution for these genes after scaling to neutral divergence is faster in primates than in rodents by a significant margin.

We next counted the number of genes that showed higher K_a/K_s in primates than rodents, or vice versa. We found that, not surprisingly, there were substantially more genes with higher K_a/K_s in primates than the other way around (118 versus 77; Figure 2B). Such a departure from parity is statistically significant ($p = 0.004$ by the binomial test). This observation argues that the higher average K_a/K_s in primates is contributed to by a large fraction of these nervous system genes beyond just a few outliers.

Finally, we compared the K_a/K_s distributions between primates and rodents. We found that primates have far fewer genes in the very low K_a/K_s range (i.e., $K_a/K_s \leq 0.05$) as compared to rodents, and more genes in the high K_a/K_s range (Figure 3A). Statistical tests confirmed that the primate distribution differed significantly from the rodent distribution ($p << 0.0001$ by the Wilcoxon signed-rank test).

Evolution of Housekeeping Genes

The significantly higher average K_a/K_s of nervous system genes in primates is suggestive of adaptive evolution. However, this observation in itself is by no means a definitive proof of adaptive evolution because it could also arise from relaxed functional constraint. The classi-

cal (and most stringent) test of adaptive evolution requires K_a/K_s greater than 1. Yet, none of the genes sampled here have K_a/K_s greater than 1. In fact, the observation of overall low K_a/K_s is consistent with previous reports that nervous system genes tend to experience strong evolutionary constraint (Duret and Mouchiroud, 2000). Such constraint, which curbs K_a/K_s to levels substantially lower than 1, would mask the effect of adaptive evolution. We therefore sought additional evidence of adaptive evolution by examining the evolution of a set of housekeeping genes. Given that housekeeping genes perform basic cellular functions that are likely conserved across different species, they should have evolved predominantly under constraint (and experiencing little positive selection). If housekeeping genes also show higher K_a/K_s in primates, then it would cast doubt on the interpretation that the elevated K_a/K_s of nervous system genes in primates is the consequence of positive selection. We compiled a list of housekeeping genes that satisfied two stringent criteria. First, they must be involved in the most basic cellular functions such as metabolism and protein synthesis. Second, they must exhibit ubiquitous expression based on EST and SAGE databases (Velculescu et al., 1999). By sequencing and bioinformatics, we obtained orthologs for 95 such genes across the four taxa, which are scattered randomly across the genome (Supplemental Table S2 at http://www.cell.com/cgi/content/full/119/7/1027/DC1/). The average K_s of these genes is 0.061 $\pm$ 0.032 (mean $\pm$ SD) for the primate comparison and 0.171 $\pm$ 0.067 for the rodents, which closely parallels the nervous system genes. But unlike the nervous system genes, the average K_a/K_s of the housekeeping genes in primates is very similar to—and statistically indistinguishable from—that in rodents (Figure 2A). Additionally, the fraction of genes with higher K_a/K_s in primates is comparable to that with higher K_a/K_s in rodents (37 versus 35; Figure 2B). Finally, the K_a/K_s distributions of these genes are not statistically distinct between primates and rodents (Figure 3B). This finding indicates comparable levels of selective constraint on housekeeping genes between primates and rodents. It therefore argues that the considerably higher average K_a/K_s of nervous system genes in primates is not a part of a nonspecific, genome-wide phenomenon.

Classification of Nervous System Genes

The above results still leave open two possible interpretations. One is stronger positive selection on nervous system genes in primates than rodents. The other is weaker functional constraint on these genes in primates. We argue that the possibility of weaker constraint seems unlikely, on the basis that the primate nervous system is far more complex (and therefore likely demanding greater precision in gene function) relative to the rodent nervous system. This consideration notwithstanding, we searched for additional evidence that might differentiate between positive selection and relaxation of constraint. To this end, we focused on two categories of genes that are particularly relevant to the understanding of nervous system evolution. One comprises genes whose functions are strongly biased toward nervous system development. The other consists of genes biased toward the routine physiological operations and maintenance of the nervous system.

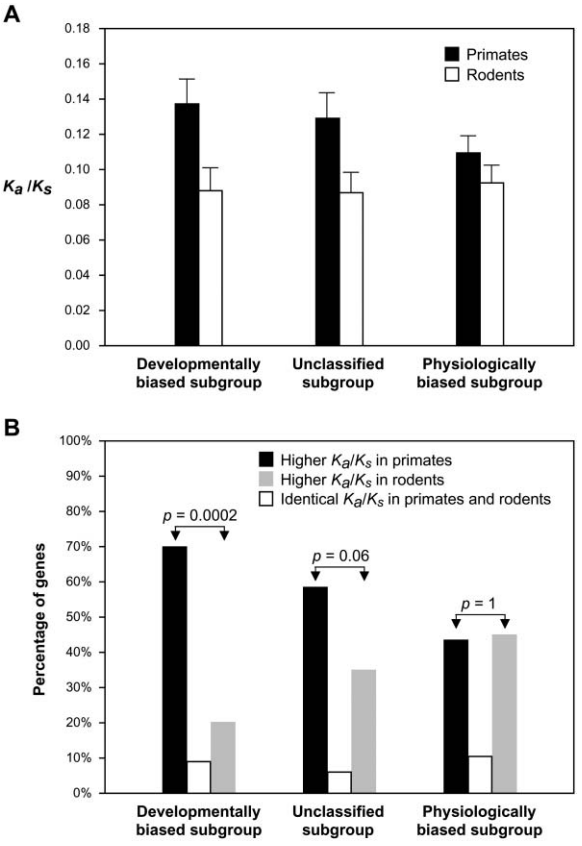

Figure 4. Evolution of Different Functional Subgroups of Nervous System Genes

(A) Evolutionary rates in primates and rodents.

(B) Percentage of genes that evolved with higher K_a/K_s in one or the other mammalian order.

The p values indicate the statistical significance of primate-rodent disparities.

The evolution of the primate brain is characterized by extensive structural modifications, which are necessarily achieved through changes in the molecular programs that underlie brain development. If the higher K_a/K_s of nervous system genes in primates is indeed the consequence of positive selection, then such selection is likely to have impinged more intensely on the developmentally biased genes. The result would be even greater primate-rodent K_a/K_s disparity (in the direction of higher primate K_a/K_s) for the developmental genes, and perhaps less K_a/K_s disparity for the physiological genes. To test this hypothesis, we classified our nervous system genes into subgroups whose functions are biased toward either nervous system development or physiology. We took several cautionary measures to minimize the inherent uncertainty in the functional classification of genes. First, we imposed stringent definitions on both subgroups. Genes were included in the developmentally biased subgroup only if a preponderance of evidence, particularly in vivo gain- or loss-of-function studies, had demonstrated unequivocal roles of these genes in nervous system development. On the other hand, genes were placed in the physiologically biased category only if a combination of biochemical, pharmacological, and

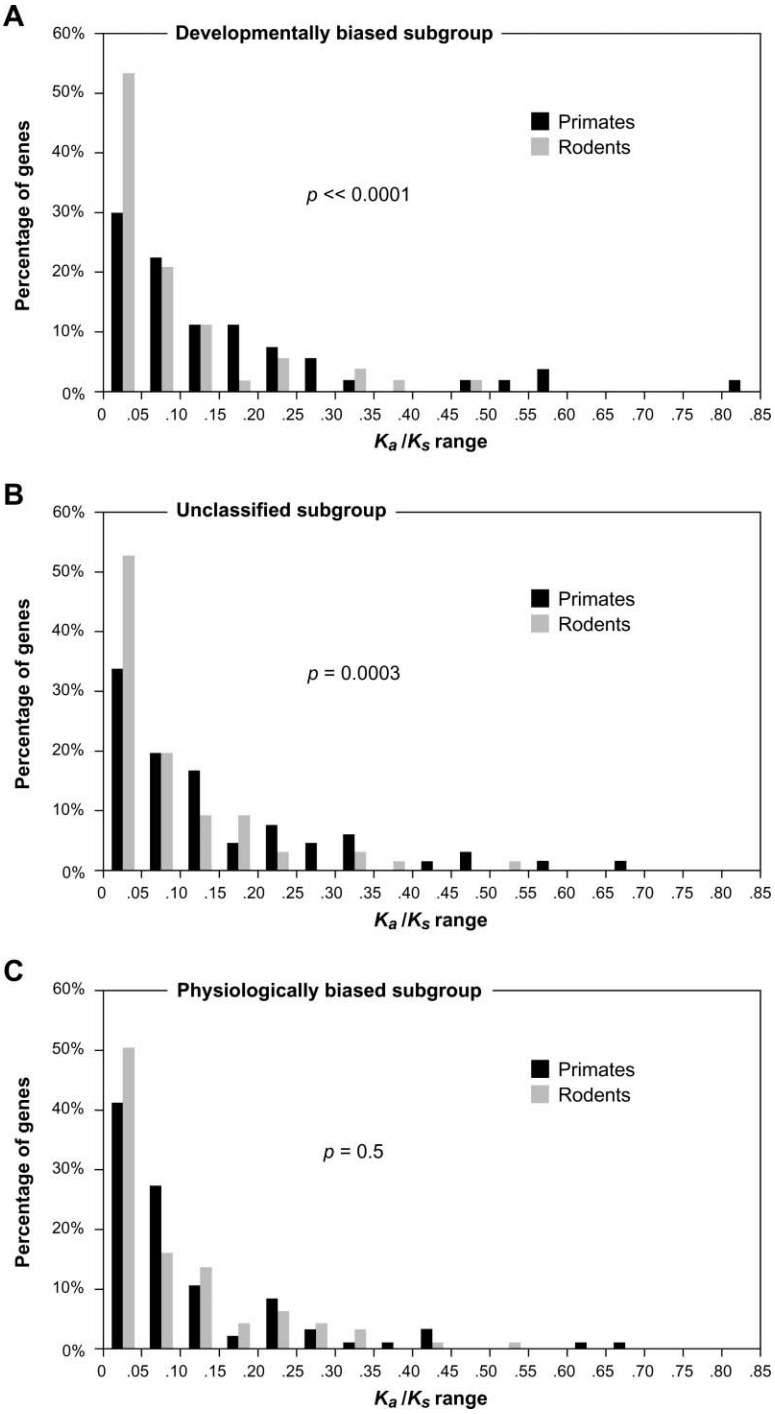

Figure 5. The K_a/K_s Distributions of Three Subgroups of Nervous System Genes in Primates and Rodents

(A) Developmentally biased subgroup.
(B) Unclassified subgroup.
(C) Physiologically biased subgroup.
The p values indicate the statistical significance of primate-rodent disparities.

genetic evidence had shown that their predominant functions lie in the routine operation and maintenance of the nervous system. Second, we created an "unclassified" subgroup to encompass all the genes that could not be clearly assigned to the first two categories, either because of insufficient functional data or because they appear to be prominently involved in both neural development and physiology. Third, classification of genes was performed blind to the evolutionary properties of these genes.

The nervous system genes were partitioned into these three subgroups without any overlap between categories. The developmentally biased subgroup contained 53 genes that included patterning signals of the developing nervous system, downstream components of such signals, transcription factors that specify neuronal phenotypes, and regulators of neural precursor proliferation, apoptosis, differentiation, migration, and morphogenesis. The physiologically biased subgroup had 95 genes, comprised predominantly of neurotransmitters, their synthesis enzymes and receptors, neurohormones, voltage-gated ion channels, synaptic vesicle compo-

nents, factors involved in synaptic vesicle release, metabolic enzymes specific to neurons or glia, and structural components of the nervous system. The unclassified subgroup contained the remaining 66 genes. Notably, the developmentally biased subgroup showed even greater K_a/K_s disparity between primates and rodents than did the entire set of nervous system genes. The average K_a/K_s of this subgroup is significantly higher (by 53%) in primates than in rodents ($p = 0.002$ by Fisher's exact test; Figure 4A). In addition, the great majority of developmental genes exhibited higher K_a/K_s in primates whereas only a small fraction displayed higher K_a/K_s in rodents (37 versus 11), which is a significant departure from parity ($p = 0.0002$ by the binomial test; Figure 4B). In contrast to the developmental genes, the physiologically biased subgroup exhibited much less primate-rodent K_a/K_s disparity (Figure 4A). Furthermore, the number of genes in this subgroup with higher K_a/K_s in primates is comparable to that with higher K_a/K_s in rodents (42 versus 43; Figure 4B). Indeed, the reason that the average K_a/K_s of the physiological subgroup is slightly higher in primates can be attributed to a subset of outliers with markedly higher K_a/K_s in primates than in rodents (these outliers are discussed later).

Interestingly, the unclassified subgroup shows evolutionary parameters that are intermediate between the developmental and the physiological subgroups. This is true when considering K_a/K_s values (Figure 4A) or the number of genes with higher K_a/K_s in either primates or rodents (39 versus 23; Figure 4B). We next compared K_a/K_s distributions between primates and rodents for each subgroup. For the developmental subgroup, primates showed a marked deficiency of genes in the lowest K_a/K_s range (i.e., $K_a/K_s \leq 0.05$) as compared to rodents, but a relative excess of genes in the higher K_a/K_s range (Figure 5A). In particular, the very top K_a/K_s ranges ($K_a/K_s > 0.5$) contain only primate, and no rodent genes. This notable primate-rodent disparity is statistically highly significant ($p << 0.0001$ by the Wilcoxon signed-rank test). In contrast, K_a/K_s distributions of the physiological genes are much more similar between primates and rodents and are not statistically distinct (Figure 5C). For the unclassified subgroup, the K_a/K_s distributions again exhibit an intermediate level of primate-rodent disparity (Figure 5B).

The higher K_a/K_s of nervous system genes in primates means that there is an overabundance of amino acid substitutions (after scaling to neutral divergence) in primates as compared to rodents. A rough estimate suggests an excess of 1–2 amino acid substitutions per nervous system gene in primates than would have occurred if the average K_a/K_s in primates was similar to (rather than significantly higher than) the average rodent K_a/K_s. The excess becomes 3–4 substitutions per gene in primates when considering only the developmental subgroup.

Genes with Marked Evolutionary Rate Disparities between Primates and Rodents

To identify candidate genes whose molecular evolutionary changes might bear particular relevance to brain evolution, we searched for genes with the most marked K_a/K_s disparities between primates and rodents. Using a p value of 0.05 as a cutoff, we obtained a set of 24 outlying genes with significantly higher K_a/K_s in primates than in rodents (hereon referred to as "primate-fast outliers") (Table 1A).

As expected, the developmental subgroup has the highest proportion of outliers (9 out of 53, or 17%). The physiological subgroup contains 9 outliers among 95 genes (9%), while the unclassified subgroup has 6 outliers among 66 genes (9%). Interestingly, a preponderance of these outliers appeared to be involved in controlling brain size or behavior. Mouse knockout of *CASP3* exhibits severe overgrowth of the brain; *LHX1* knockout shows absence of brain and other anterior structures; and *NRCAM* knockout leads to reduced cerebellum size. Perhaps even more interesting are the observations that mutations in human *ASPM*, *MCPH1*, *PAFAH1B1*, and *SHH* all result in severe reductions in brain size (microcephaly). Hence, 7 of the outliers are implicated in controlling brain size. Mouse knockout of *DVL1* displays defective social behavior; *PEG3* knockout shows impaired maternal behavior; *ADCYAP1* knockout exhibits altered anxiety state; knockouts of *GDI1*, *GRIN2A*, or *CSPG3* show deficits in learning or neural correlates of learning; knockouts of *CHRM5*, *DRD2*, or *OPRM1* exhibit defects in acquiring reward-mediated behavior; and mutation in *AANAT* alters circadian rhythm. Thus, 10 of the outliers are involved in regulating behavior.

It is remarkable that 17 out of the 24 primate-fast outliers are linked to the regulation of either brain size or behavior. This trend suggests that genes controlling brain size or behavior are preferential targets of positive selection during primate evolution. The functional specificity of these outliers adds additional credence to the notion that the higher K_a/K_s of nervous system genes in primates is likely the consequence of adaptive evolution.

For the developmental and unclassified subgroups, removal of the primate-fast outliers only moderately reduced the overall primate-rodent K_a/K_s disparities (data not shown). This suggests that for these two subgroups, the higher average K_a/K_s in primates is contributed to by many genes, and not just the primate-fast outliers. For the physiological subgroup, however, removal of the outlying genes actually led to higher average K_a/K_s in rodents than in primates (by nearly 10%). This hints at the possibility that, overall, physiological genes might actually be slightly more conserved in primates, except for a small subset of genes that underwent adaptive evolution (and hence exhibiting much higher K_a/K_s in primates).

Using the same statistical cutoff, we also obtained 3 rodent-fast outliers, considerably fewer than the primate-fast outliers (Table 1B). Such a dramatic disparity is consistent with the tendency of nervous system genes to have higher K_a/K_s in primates than in rodents. Among the 95 housekeeping genes, only two showed significant K_a/K_s disparities between primates and rodents, and both had higher K_a/K_s in rodents (Supplemental Table S2 online). This reinforces the notion that housekeeping genes evolved under levels of selective constraint that tended to remain steady across different mammalian lineages.

Comparison between Human Lineage and Macaque Lineage

Increases in brain size and complexity are evident in the evolution of many primate lineages (Jerison, 1973).

However, this increase is far more dramatic in the lineage leading to humans than in other primate lineages (Williams, 2002). If the higher average K_a/K_s of nervous system genes in primates (based on human-macaque comparison) is indeed the product of adaptive evolution, then one might expect this accelerated evolution to be more dramatic in the lineage leading from human-macaque ancestors to humans than the lineage leading to macaques. To address this possibility, we followed a phylogeny-based methodology as previously described (Messier and Stewart, 1997). Specifically, we chose squirrel monkey (*Saimiri boliviensis*), a New World monkey, as an outgroup to partition human-macaque sequence divergence into the two respective branches. (Squirrel monkey can serve as a highly reliable outgroup because it is closely related to the catarrhine clade containing human and macaque; rat and mouse are too distantly related to primates to be reliable outgroups.)

We first focused on the primate-fast outliers of the nervous system genes because they have the greatest likelihood of bearing relevance to primate brain evolution. Using squirrel monkey sequences as an outgroup, we found that they have much higher average K_a/K_s in the human lineage than the macaque lineage (Figure 6A) and that the difference is statistically significant ($p = 0.004$ by Fisher's exact test). Additionally, at the level of individual genes, the great majority (20 out of 24) evolved faster in the human lineage, which is a significant departure from parity ($p = 0.002$ by the binomial test).

As a control, we also examined a set of 25 nervous system genes with comparable evolutionary rates between primates and rodents and found that these genes do not show any statistically significant K_a/K_s disparities between the human and the macaque lineages (Figure 6A).

Thus, nervous system genes with higher K_a/K_s values in primates than in rodents also have a strong tendency to have higher K_a/K_s in the human branch than in the macaque branch. That the K_a/K_s of these genes is markedly and specifically elevated along the human branch—in which the increase in brain size and complexity is most dramatic—further argues that adaptive evolution rather than relaxed functional constraint is likely responsible.

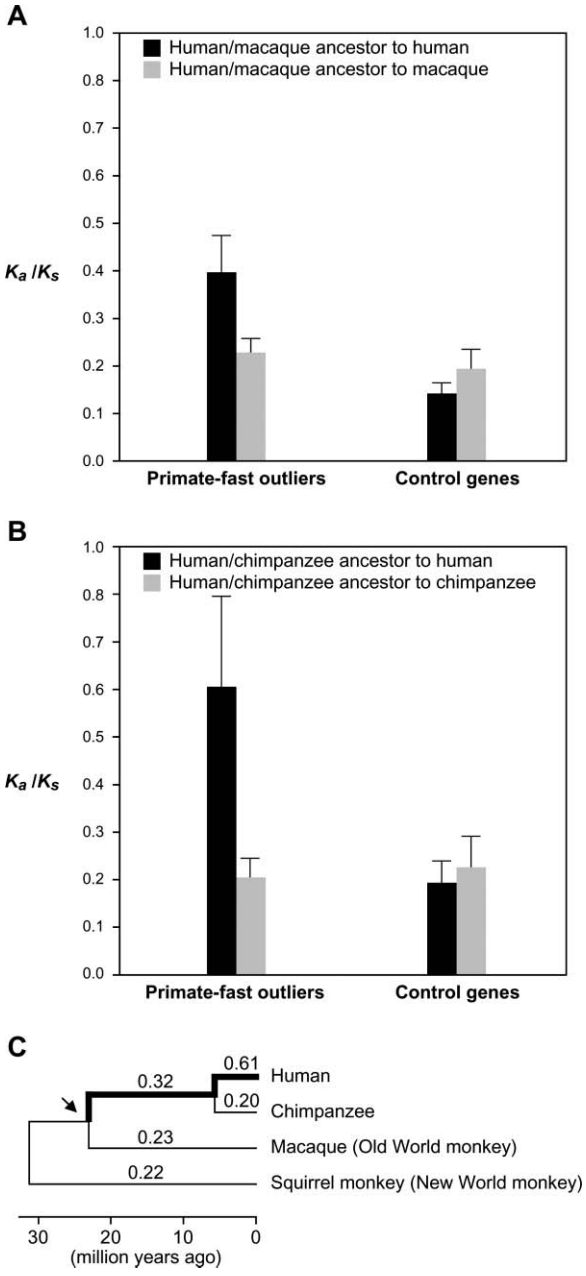

Figure 6. Evolutionary Rates of the Primate-Fast Outliers and the Control Group of Nervous System Genes in Different Primate Lineages

(A) Comparison between the lineage from human-macaque ancestor to human and the lineage to macaque.

(B) Comparison between the lineage from human-chimpanzee ancestor to human and the lineage to chimpanzee.

(C) Phylogenetic tree depicting K_a/K_s values along the primate lineage leading to humans (bolded lines) and in nonhuman primate lineages (plain lines). Note that the K_a/K_s value shown next to the squirrel monkey branch applies to the entire lineage from the catarrhine ancestor node (indicated by arrow) to squirrel monkey.

Comparison between Human Lineage and Chimpanzee Lineage

Another important question is whether nervous system genes show different K_a/K_s between the human lineage and the chimpanzee lineage after the divergence of these two lineages. To address this question, we obtained chimpanzee sequences for both the primate-fast outliers and the control group. We then used macaque as an outgroup to partition human-chimpanzee divergence into separate human and chimpanzee branches. For the primate-fast outliers, the K_a/K_s of the human branch is considerably higher than the chimpanzee branch (Figure 6B). For the control genes, the two lineages show comparable and statistically indistinguishable K_a/K_s values (Figure 6B).

An important caveat in the above analysis is ascertainment bias. The primate-fast outliers were expected to show higher K_a/K_s in the human terminal branch (i.e., from human-chimpanzee ancestors to humans) than in the chimpanzee terminal branch, due to the fact that these genes were ascertained on the basis of elevated K_a/K_s in the human-to-macaque lineage (which subsumes the human terminal branch). We therefore performed computer simulations to evaluate the extent to

Table 1. Nervous System Genes Showing Significantly Faster Evolution in Either Primates or Rodents

A. Genes Showing Faster Evolution in Primates

Gene Class	Gene Symbol	Gene Name	Function in Nervous System	Nervous System Phenotype of Mutants	Primate			Rodent			References
					Ka	*Ks*	*Ka*/*Ks*	*Ka*	*Ks*	*Ka*/*Ks*	
Developmental	*ASPM*	Abnormal spindle-like microcephaly associated	A spindle-associated protein implicated in determining cerebral cortical size, presumably by regulating neural progenitor division and differentiation	Human homozygous mutations cause primary microcephaly, which is characterized by severely reduced brain size without other overt neuropathologies or dysmorphic features.	0.020	0.041	0.488	0.083	0.238	0.349	Bond et al., 2002
	CASP3	Caspase 3	A protease involved in the activation of the apoptosis pathway during neural precursor proliferation	Mouse homozygous mutants show marked brain ventricular zone expansion, exencephaly, and ectopic neuronal structures.	0.022	0.040	0.550	0.035	0.322	0.109	Kuida et al., 1996
	DVL1	Dishevelled 1	A PDZ-domain-containing protein involved in the Wnt signaling pathway	Dominant-negative mutation of *Dishevelled* in frog causes failure of neural axis formation. Mouse homozygous mutants show defects in social behavior, such as huddling, whisker trimming, and nest building, and in sensorimotor gating.	0.009	0.137	0.066	0.002	0.100	0.020	Sokol, 1996; Lijam et al., 1997
	LHX1	LIM homeo box 1	A transcription factor essential in organizing the anterior structures during development	Mouse homozygous mutants lack brain and other anterior head structures, but show normal development in the remaining body axis.	0.006	0.075	0.080	0.002	0.141	0.014	Shawlot et al., 1995
	MCPH1	Microcephalin	Implicated in the control of brain size, presumably by affecting the proliferation of neural progenitors	Human homozygous mutation leads to primary microcephaly.	0.040	0.048	0.833	0.070	0.146	0.479	Jackson et al., 2002
	NRCAM	Neuronal cell adhesion molecule	A cell adhesion molecule involved in developmental signaling of the nervous system	Mouse homozygous mutants show failure of cerebellar granule cells to extend neurites in vitro and reduced cerebellum size in vivo.	0.019	0.085	0.224	0.009	0.177	0.051	Sakurai et al., 2001
	NTRK3	Neurotrophic tyrosine kinase receptor, type 3	A tyrosine kinase receptor for neurotrophin 3	Mouse homozygous mutants fail to develop proprioceptive sensory neurons.	0.003	0.068	0.044	0.000	0.134	0.000	Klein et al., 1994
	PAFAH1B1	Platelet-activating factor acetylhydrolase, 1B, alpha subunit	An acetylhydrolase implicated in microtubule function during neuronal migration	Human heterozygous mutations cause severe defects in neuronal migration, leading to significantly reduced brain size (microcephaly) and lack of brain folding (lissencephaly). Mouse heterozygous mutants show impaired neuronal migration during development.	0.005	0.048	0.104	0.000	0.057	0.000	Reiner et al., 1993; Cahana et al., 2001
	SHH	Sonic hedgehog	A signaling molecule involved in specifying ventral structures of the central nervous system, and in driving the expansion of the developing brain	Human heterozygous mutations cause severely reduced brain size (microcephaly) and fusion of the two cerebral hemispheres (holoprosencephaly). Mouse homozygous mutants lack ventral structures of the central nervous system, display severe underdevelopment of the brain and holoprosencephaly.	0.029	0.091	0.319	0.021	0.163	0.129	Belloni et al., 1996; Roessler et al., 1996; Chiang et al., 1996
Physiological	*AANAT*	Arylalkylamine N-acetyltransferase	An enzyme that converts serotonin to N-acetylserotonin, the penultimate step in melatonin synthesis	Mouse homozygous mutants (found naturally in many inbred lines) have altered activity levels and circadian behavior.	0.032	0.079	0.405	0.023	0.266	0.086	Roseboom et al., 1998
	ADCYAP1	Adenylylcyclase-activating peptide1	An adenylylcyclase-stimulating hormone secreted from hypothalamus	Mouse homozygous mutants show remarkable behavioral changes including hyperactivity, explosive jumping, increased exploratory behavior, and less anxiety.	0.074	0.113	0.655	0.034	0.191	0.178	Hashimoto et al., 2001
	CHRM5	Acetylcholine receptor, muscarinic, 5	A member of the muscarinic subtype of acetylcholine receptors	Mouse homozygous mutants show defective reward/withdrawal response to morphine, and failure in acetylcholine-mediated dilation of cerebral blood vessels.	0.021	0.034	0.618	0.018	0.118	0.153	Yamada et al., 2001

Table 1. Continued.

Gene Class	Gene Symbol	Gene Name	Function in Nervous System	Nervous System Phenotype of Mutants	Primate			Rodent			References
					Ka	*Ks*	*Ka/Ks*	*Ka*	*Ks*	*Ka/Ks*	
	CHRNA2	Cholinergic receptor, neuronal nicotinic, a2	A member of the nicotinic subtype of acetylcholine-gated ion channels	Not available.	0.036	0.124	0.290	0.016	0.339	0.047	Elliott et al., 1996
	CHRNA5	Cholinergic receptor, neuronal nicotinic, a5	A member of the nicotinic subtype of acetylcholine-gated ion channels	Not available.	0.015	0.062	0.242	0.011	0.289	0.038	Boulter et al., 1990
	DRD2	Dopamine receptor D2	A member of the dopamine receptor family	Mouse homozygous mutants show suppression of morphine-mediated reward behavior and slow movement resembling Parkinson disease.	0.005	0.042	0.119	0.000	0.115	0.000	Maldonado et al., 1997; Baik et al., 1995
	GRIK4	Glutamate receptor, ionotropic kainate, 4	A member of the kainate subtype of glutamate-gated ion channels	Not available.	0.003	0.030	0.100	0.002	0.123	0.016	Szpirer et al., 1994
	GRIN2A	Glutamate receptor, ionotropic NMDA, 2A	A member of the NMDA subtype of glutamate-gated ion channels	Mouse homozygous mutants show deficits in spatial learning and synaptic plasticity.	0.008	0.063	0.127	0.007	0.164	0.043	Sakimura et al., 1995
	OPRM1	Oxytocin receptor	A G-protein-coupled receptor for opioid ligands	Mouse homozygous mutants show defect in morphine-mediated analgesia and reward response.	0.012	0.049	0.245	0.026	0.235	0.111	Matthes et al., 1996
Unclassified	*CSPG3*	Chondroitin sulfate proteoglycan 3	A chondroitin sulfate proteoglycan implicated in neuronal adhesion and migration	Mouse homozygous mutants are overtly normal, with mild deficits in synaptic plasticity.	0.029	0.065	0.446	0.059	0.188	0.314	Zhou et al., 2001
	DPPX	Dipeptidyl peptidase IV related	A dipeptidyl-peptidase-like protein expressed predominantly in the brain	Not available	0.008	0.076	0.105	0.006	0.181	0.033	Wada et al., 1992
	GDI1	GDP dissociation inhibitor 1	A protein that inhibits RAB-mediated GDP-GTP exchange by preventing dissociation of GDP from RAB	Human mutations cause several forms of X-linked nonspecific mental retardation. Mouse homozygous mutants show impaired short-term memory and social behavior.	0.002	0.057	0.035	0.000	0.142	0.000	D'Adamo et al., 1998, 2002
	LYNX1	Lynx1	A neuronal membrane molecule highly expressed in the brain and linked to the modulation of neuronal nicotinic acetylcholine receptors	Not available.	0.030	0.086	0.349	0.000	0.221	0.000	Miwa et al., 1999
	PEG3	Paternally expressed gene 3	A maternally imprinted zinc finger protein implicated in the TNF signaling pathway	Female mutant mice show impaired nurturing behavior and reduced milk ejection due to reduced hypothalamic oxytocin neurons.	0.024	0.077	0.312	0.032	0.170	0.188	Li et al., 1999
	TTR	Transthyretin	A thyroid hormone carrier highly expressed in choroid plexus and constituting a major protein component of cerebrospinal fluid	Mouse homozygous mutants have reduced thyroid hormone levels but are overtly normal.	0.035	0.060	0.583	0.041	0.273	0.150	Episkopou et al., 1993
B. Genes Showing Faster Evolution in Rodents											
Developmental	*ASCL1*	Achaete-scute complex like 1	A transcription factor involved in the development of olfactory, autonomic, and enteric neurons	Mouse homozygous mutants die at birth and lack olfactory and autonomic neurons.	0.000	0.111	0.000	0.024	0.189	0.127	Guillemot et al., 1993
	NEUROD2	Neurogenic differentiation 2	A transcription factor involved in inducing neural precursor cells to undergo neuronal differentiation	Mouse homozygous mutants die a few weeks after birth and show reduced cerebellar granular cell layer.	0.001	0.049	0.020	0.047	0.136	0.346	Olsen et al., 2001
Physiological	*PPT1*	Palmitoyl-protein thioesterase 1	An enzyme that removes palmitate groups from lipid-modified proteins	Mouse homozygous mutations develop motor defects such as spasticity and die by 10 months of age. Human homozygous mutations cause neuronal ceroid lipofuscinosis.	0.000	0.054	0.000	0.025	0.253	0.253	Vesa et al., 1995; Gupta et al., 2001

which this ascertainment bias would result in elevated K_a/K_s in the human terminal branch. They showed that for the primate-fast outliers, ascertainment bias would indeed lead to an average K_a/K_s of the human terminal branch being higher than that of the chimpanzee branch. However, the actual K_a/K_s disparity between the human and the chimpanzee terminal branches is greater than that expected from ascertainment bias alone ($p = 0.04$; see Experimental Procedures). This suggests that ascertainment bias is unlikely to fully account for—though it clearly contributes to—the observed disparity in K_a/K_s between the human and the chimpanzee terminal branches.

With sequences of the primate-fast outliers available in four primate taxa (human, chimpanzee, macaque, and squirrel monkey), we constructed a phylogenetic tree and calculated K_a/K_s for each segment of the tree (Figure 6C). Clearly, the segments that lie along the lineage leading to humans (bolded in Figure 6C) have notably higher K_a/K_s than segments that branch away from this lineage.

The above data reinforce the notion that K_a/K_s values of nervous system genes in primates are especially elevated in the lineage leading from ancestral primates to humans, and that this trend has likely continued through recent human evolution.

Discussion

In this study, we examined the molecular evolution of an extensive set of nervous system-related genes in primates. We demonstrated that their average rate of protein evolution as scaled to neutral divergence (i.e., the K_a/K_s ratio) is significantly higher in primates than in rodents. One possible interpretation is adaptive evolution of these genes in primates, but it could also be due to relaxed functional constraint. We note, however, that brain size and complexity are much greater in primates than in rodents, which likely places stiffer demands on the functional precision of genes. It is therefore difficult to envision the relaxation of functional constraint as a major force in the evolution of the primate nervous system. This argument notwithstanding, we sought additional evidence that might bolster the case of adaptive evolution.

First, we examined a large set of housekeeping genes and noted that there is no significant primate-rodent disparity in the K_a/K_s of these genes. This argues that the primate-rodent K_a/K_s disparity seen in nervous system genes is not a nonspecific, genome-wide phenomenon.

Second, we classified our nervous system genes into functional categories. We found that the subgroup of nervous system genes with developmentally biased functions displayed much greater primate-rodent K_a/K_s disparity than the entire set of genes. In contrast, the K_a/K_s of genes that function predominantly in the routine physiological operations and maintenance of the nervous system showed much less primate-rodent disparity. The latter observation argues against reduced functional constraint on the primate nervous system per se, and together, these results are more consistent with the notion of adaptive evolution.

Third, we found that the average K_a/K_s of primate-fast outliers (i.e., those nervous system genes exhibiting significantly higher K_a/K_s in primates than in rodents) is considerably higher in the lineage leading from human-macaque ancestors to humans than the lineage leading to macaques. Furthermore, these same genes were also found to have evolved with much higher K_a/K_s in the human terminal branch than the chimpanzee branch after human-chimpanzee divergence. This disparity was not seen in a control set of nervous system genes that evolved at comparable rates between primates and rodents.

Fourth, mutations in many nervous system genes, including those with significantly higher K_a/K_s in primates, have been shown to cause severe nervous system defects in humans (Table 1A). This obviously does not support the notion of functional relaxation in these genes during human evolution.

Fifth, there is no evidence of recent duplications involving any of the genes studied (data not shown), which rules out the possibility of increased genetic redundancy for these genes in primates.

Finally, concurrent with the present study, more detailed evolutionary analyses were performed on two genes included in this study, *ASPM* and *MCPH1*, which have since been published by us and other groups (Zhang, 2003; Evans et al., 2004b; Kouprina et al., 2004; Evans et al., 2004a; Wang and Su, 2004). These detailed analyses, motivated by the observation that these two genes are involved critically and specifically in regulating brain size during development (Bond et al., 2002; Jackson et al., 2002), indeed revealed multiple lines of evidence in support of their adaptive evolution in primates and particularly in the primate lineage leading to humans. These include (1) significantly higher K_a/K_s in primates than in nonprimate mammals in addition to rodents, (2) much higher K_a/K_s in the primate lineage leading to humans than in the other primate lineages, (3) a preponderance of evolutionary signatures supporting the presence of positive selection in the lineage leading to humans, such as $K_a/K_s > 1$ for portions of this lineage and highly significant departure from the neutral expectation of the McDonald-Kreitman test (McDonald and Kreitman, 1991), and (4) evidence that strong positive selection tends to be focused within specific domains of these genes. Other genes not included in this study, such as *FOXP2*, *AHI1*, and *GLUD2*, have also revealed a possible link between alterations in protein sequences and phenotypic evolution of the human brain (Enard et al., 2002b; Ferland et al., 2004; Burki and Kaessmann, 2004).

Collectively, the above results argue against the possibility of relaxed functional constraint on the primate nervous system. Instead, they are more consistent with the interpretation that higher K_a/K_s of nervous system genes in primates—especially along the lineage leading to humans—is a reflection of adaptive evolution.

Indeed, as first recognized by Charles Darwin, adaptive evolution must have played a key role in driving the acquisition of greater cognitive powers in humans (Darwin, 1871). It is therefore reasonable to suppose that positive selection on genes involved in nervous system biology should have operated more intensely during the descent of humans than in species showing less dramatic cognitive evolution. However, researchers

have not been able to make a priori predictions regarding how intensified selection on the nervous system might have molded the molecular evolution of the primate genome. For example, it has remained a matter of speculation as to whether brain evolution involved a small number of key mutations in a few genes or a very large number of mutations in many genes (Carroll, 2003). It was also not known whether evolutionarily important mutations have occurred predominantly in regulatory sequences or coding regions (King and Wilson, 1975; McConkey et al., 2000; McConkey, 2002; Olson and Varki, 2003; Carroll, 2003), though preliminary data suggest that gene expression patterns of the human brain might have evolved rapidly (Enard et al., 2002a; Caceres et al., 2003; Uddin et al., 2004). Whereas our study does not address all these important questions, it does argue that the evolution of the brain in primates and particularly humans is likely contributed to by a large number of mutations in the coding regions of many underlying genes, especially genes with developmentally biased functions.

Might genes involved in tissues other than the nervous system also display accelerated evolution in primates? We argue that this is a distinct possibility given the precedent found in nervous system genes. In particular, accelerated evolution of genes might be found in tissue systems that are especially relevant to the adaptation of primates, such as the immune system, the digestive system, the reproductive system, the integumentary system, and the skeletal system.

Recent discussions surrounding the genetic origin of humans have placed a great emphasis on human-chimpanzee comparative genomics. Undoubtedly, this approach has revealed—and will continue to reveal—genetic differences that might underlie the biological distinctions between these two sister species (Chou et al., 1998, 2002; Enard et al., 2002b; Clark et al., 2003; Stedman et al., 2004). Because of the exceedingly high degree of sequence identity between human and chimpanzee genomes, however, comparative studies often lack statistical power, and in many cases would overlook genetic differences that bear biological relevance. The issue of weak statistical power in human-chimpanzee sequence comparisons has been noted before (Shi et al., 2003) and is supported by our simulation studies showing that the average stochastic variance in K_s as a fraction of the true underlying mutation rate is about twice in human-chimpanzee comparison as it is in human-macaque comparison (our unpublished data). Relative to human-chimpanzee comparisons, our approach offers two important advantages. First, the use of a more distant primate species for comparison with humans provides the much needed statistical power for determining the evolutionary significance of sequence changes. Second, the use of nonprimate mammals as "controls" allows for the identification of primate-specific evolutionary signatures. We therefore propose that our methodology is a valuable complement to human-chimpanzee comparisons in probing the genetic basis of human origins.

In summary, our study revealed the following broad themes that characterize the molecular evolution of the nervous system in primates and particularly in humans. First, genes underlying nervous system biology exhibit higher average rate of protein evolution as scaled to neutral divergence in primates than in rodents. Second, such a trend is contributed to by a large number of genes. Third, this trend is most prominent for genes implicated in the development of the nervous system. Fourth, within primates, the evolution of these genes is especially accelerated in the lineage leading to humans. Based on these themes, we argue that accelerated protein evolution in a large cohort of nervous system genes, which is particularly pronounced for genes involved in nervous system development, represents a salient genetic correlate to the profound changes in brain size and complexity during primate evolution, especially along the lineage leading to *Homo sapiens*. Besides revealing broad evolutionary themes, our study also identified a set of genes whose molecular evolution might have contributed to the phenotypic evolution of the brain in primates. In-depth analyses of these genes might yield further insights into how changes in specific genes contribute to the emergence of primate- or human-specific traits.

Experimental Procedures

Sequence Acquisition

Standard RT-PCR protocols were employed to amplify coding sequences from the Old World monkey, crab-eating macaque (*Macaca fascicularis*), followed by sequencing of PCR product. Amplicons were designed to be 500–700 bp in length with a minimum of 50–75 bp of overlap between adjacent amplicons. Nervous system genes were amplified from cDNA combined from all major regions of the brain. Housekeeping genes were amplified from cDNA combined from the heart, lung, liver, kidney, and the pooled brain sample. Squirrel monkey (*Saimiri boliviensis*) sequences were obtained in a similar manner from brain tissue. For chimpanzee (*Pan troglodytes*), amplification was performed on genomic DNA. PCR primers to amplify nonhuman primate genes were designed based on orthologous human cDNA sequences. If a particular set of primers failed, new primers would be designed until successful primers were obtained. In rare cases of single-nucleotide polymorphisms, the derived allele was ignored because it did not represent fixed difference between species. Additional sequences, including human, chimpanzee, macaque, squirrel monkey, rat, and mouse, were obtained from public databases.

Inference of Ancestral Sequences

The human-macaque and the human-chimpanzee ancestral sequences were inferred using the PAMP program available in the PAML v.3.13 software package as previously described (Yang et al., 1995). Orthologous sequences from human, macaque, and squirrel monkey were used to infer the human-macaque ancestral sequences. Similarly, orthologous sequences from human, chimpanzee, and macaque were used to infer the human-chimpanzee ancestral sequences. In rare cases where there was ambiguity in inferring the ancestral nucleotide (i.e., the three taxa each had a different nucleotide at a given position), the corresponding codon was disregarded from the analysis. To obtain K_a/K_s of a terminal phylogenetic branch, inferred sequences at the ancestral node of the branch were compared with sequences at the terminal node. To obtain K_a/K_s of an internal branch, inferred sequences at one ancestral node were compared with inferred sequences at the other ancestral node.

Sequence Analysis and Tests of Statistical Significance

Orthologous coding sequences were aligned in frame using the Pileup and Framealign programs from the Wisconsin Package v10.2 (Accelrys Inc., San Diego, California). The Diverge program from the same package was employed to calculate evolutionary parameters by the Li method (Li, 1993), including the total numbers of nonsynonymous (A) and synonymous (S) substitutions corrected for multiple hits and transition/transversion bias, and K_a and K_s. The average

K_a/K_s for a group of genes was calculated as the ratio of average K_a and average K_s. The error bar of average K_a/K_s was generated by bootstrap simulation. To evaluate the statistical significance that the evolutionary rates of a group of genes differ between two lineages, a 2×2 contingency table was built, with the four entries being the total *A* and *S* values in either of the two lineages. Two-tailed Fisher's exact test was then applied to the table to obtain statistical significance that evolutionary rates differed between the two lineages. One-tailed Fisher's exact test was used to test the significance by which an individual gene had significantly higher K_a/K_s in one lineage versus the other. Given that this test utilizes the total numbers of nonsynonymous and synonymous changes, it is possible that a gene might have substantially higher K_a/K_s in one lineage than in the other, and yet the difference does not reach statistical significance because the total numbers of nonsynonymous and synonymous substitutions are low (as in short genes). Conversely, it is also possible that the K_a/K_s of a gene is only moderately higher in one lineage than in the other, and yet the difference is statistically significant because of the large number of substitutions involved (as in long genes). To evaluate the significance of the inequality in the number of genes with higher K_a/K_s in one lineage versus the number of genes with higher K_a/K_s in the other lineage, the two-tailed binomial test was used. To assess the significance that two sets of K_a/K_s values had distinct distributions, we used the nonparametric Wilcoxon signed-rank test, which evaluated the likelihood of the null hypothesis that two sets of paired data were drawn from the same underlying distribution (Hollander and Wolfe, 1999). We also used the nonparametric Kolmogorov-Smirnov test for the same purpose (Hollander and Wolfe, 1999), which in all cases confirmed the results of the Wilcoxon test.

Computer Simulations

Simulations were performed to assess the extent to which the ascertainment of the primate-fast outliers would elevate the K_a/K_s of these genes in the human terminal branch (i.e., from human-chimpanzee ancestors to humans) relative to the chimpanzee terminal branch. We considered a phylogenetic tree as depicted in Supplemental Figure S1 at http//www.cell.com/cgi/content/full/119/7/1027/DC1/. Four lineages in this tree were germane to the analysis: human-chimpanzee ancestor to human, human-chimpanzee ancestor to chimpanzee, human-chimpanzee ancestor to macaque, and rat to mouse. The levels of neutral divergence in these four lineages were set at a ratio of 6:6:62:174. This ratio was set according to published genome-typical K_s rates, which are 0.012 between human and chimpanzee (Chen et al., 2001), 0.068 between human and macaque (Yi et al., 2002), and 0.174 between rat and mouse (Gibbs et al., 2004). For each outlier gene, we performed simulations under the null assumption that the substitution rate (either nonsynonymous or synonymous) after scaling to neutral divergence is constant across all four lineages. By this assumption, any enrichment or deficit of substitutions in a given lineage (including situations that would produce significantly higher human-macaque K_a/K_s than rat-mouse K_a/K_s) is the result of stochastic fluctuation. As the first step of the simulation, the total numbers of nonsynonymous (*A*) and synonymous (*S*) substitutions of the gene observed for both the human-to-macaque and the rat-to-mouse lineages were summed. The resulting numbers were then scaled up by 6/242 to correct for the addition of the chimpanzee terminal branch in the phylogeny. These corrected *A* and *S* numbers were apportioned onto the four lineages based on the 6:6:62:174 ratio to obtain the number of substitutions on each lineage as expected under the null assumption of equal evolutionary rates across lineages. For an individual lineage, simulation was performed to generate the number of substitutions that followed the Poisson distribution and with a mean being the expected number of substitutions. The subset of repetitions for which the human-macaque *A* and *S* numbers match that observed for the gene was selected for further analysis. This procedure was performed for all the primate-fast outliers, which produced one aforementioned subset of simulated data per gene. One data point per subset was then randomly selected to create a simulated outlier data set. By generating 100,000 such simulated outlier data sets, we were able to obtain the probability by which a simulated outlier data set produced *A/S* ratio disparity between the human and the chimpanzee terminal branches that was as great as or greater than the observed disparity.

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