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THE HUMAN BRAIN EVOLVING:

Paleoneurological Studies in Honor of Ralph L. Holloway

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Stone Age Institute Press · www.stoneageinstitute.org
1392 W. Dittemore Road · Gosport, IN 47433

FRONT COVER CAPTIONS

Center: Portrait of Ralph L. Holloway.
Upper left: A modern human brain.
Upper right: Ralph measuring landmarks on an endocast ca. 1976.
Lower right: Homo habilis cranium KNM-ER-1813 from Koobi Fora, Kenya (photo by Holloway).
Lower left: Ralph with an endocast of the Flores "hobbit" cranium.

Published by the Stone Age Institute.
ISBN-10: 0-9792276-3-1
ISBN-13: 978-0-9792276-3-9

CHAPTER 8

Study of Human Brain Evolution at the Genetic Level

Eric J. Vallender and Bruce T. Lahn

Abstract

As a species, *Homo sapiens* is characterized by its uniquely large and complex brain. Comparative anatomists and paleoanthropologists have done much to elucidate the phenotypic changes of the human brain over evolutionary time. Here we review the emerging understanding of the genetic basis that underlies these phenotypic changes.

Introduction

People across virtually all disciplines – philosophers, sociologists, artists, preachers and scientists alike – have grappled with the question of what it means to be human. And while today mankind may be no closer to answering the metaphysical aspects of the question, the biological underpinnings of what it means to be human are gradually coming to light.

Perhaps no single feature is as salient or of greater importance in the evolution of *Homo sapiens* as the emergence of the modern human brain. The increase in brain size correlates with advances in cognitive capabilities and an increasingly complex behavioral repertoire including complex tool use, symbolic thought and language, and artistic expression. At the heart of it, it is this increased cognitive complexity that has allowed humans to develop society, culture, and indeed the ability to ask ourselves the philosophical question of what it means to be human.

From the birth of evolutionary theories, the relationship between humans and other primate species was apparent. And while differences among the primates are legion, it is the differences in brain size and complexity that are perhaps most difficult to comprehend. The human brain is roughly eight times the relative volume of New World monkeys and approximately three times that of chimpanzees (Falk, 1986). Further, the expansion of the human brain has not been proportional, rather certain regions, including the cerebral cortex, have seen size and complexity increases even relative to other human brain regions. In particular, the prefrontal cortex, which may play an important and unique role in social behavior, has seen significant enlargement (Semendeferi et al., 2002). Understanding the distinctiveness of humans means in part understanding these differences and the mechanisms that caused them to emerge.

As approaches to understanding the human condition have varied, so too have approaches to understanding the evolutionary development of the human brain. Primatologists have studied similarities and differences in the behaviors of human and non-human primates. Comparative anatomists have noted congruities and inconsistencies across the brain. Paleoanthropologists have identified a long history of hominids leading from humanity's last common ancestor with chimpanzees through to modern man with several dead-ends thrown in for good measure. The last century also saw the development of a new science which promises to add to the growing understanding of human origins, evolutionary genetics.

While the concept of evolutionary genetics has been around since the early 1900s, it was only in the last several decades that its use in understanding human species origins, and in particular the origins of the human brain, has blossomed (Vallender et al., 2008). This modern growth has been fueled by the ability of researchers to cheaply and quickly decipher sequence genetic infor-

mation, from single genes to complete genomes. Using comparative genomics, it is possible to identify differences at the most fundamental, genetic, level between species and to probe the most basic mechanisms of evolutionary change and adaptation. Geneticists now have access to the actual and complete genomes of numerous species, human and non-human, primate and non-primate, mammal and non-mammal, and increasingly to the genomes, or at least select genotypes, of specific individuals within species. This influx in data has necessitated the development of associated tools, both for access and visualization of the information as well as techniques and methodologies for making sense of the immense quantities of data.

Coincident with the development of modern tools of genetic analysis has been the explosive growth of the neurosciences. Long studied, the complexity of the brain has refused to reveal its secrets easily. With the development of imaging, electrophysiology, genetic manipulation and whole hosts of other techniques, neuroscientists are gradually making headway into understanding the brain. In doing so, there has been increasing understanding of how specific genes contribute to specific aspects of brain development and function.

By coupling the functional information gained through the study of basic neurobiology with molecular evolution data gathered by comparative geneticists, slowly a new understanding of human origins is emerging where the random evolutionary mutations have lead to functional consequences, neurological and otherwise, that would eventually lead to the species-specific changes that characterize the emergence of the modern human brain. The subsequent sections offer a snapshot of these studies as they stand in the early part of the 21st century; an incomplete understanding to be sure, but the beginnings of the scientific, if not metaphysical, basis of what it means to be human.

Methodologies

Evolutionary change can occur by any number of mutational processes. Some of these changes are on a genetically large scale; chromosomes may come apart or fuse, as is the case for human chromosome 2 (Jauch et al., 1992), or there may be major inversions such as are seen on the Y chromosome (Lahn et al., 2001). There can be changes on a more moderate level, including the duplication or deletion of genes and genomic regions known as copy number variants. Most commonly, however, are the smallest mutations wherein only a handful of bases are added or deleted or the most common point mutations wherein only the identity of a base changes. Each of these mutations can have functional effects and has at some point in the history of human, primate, or mammalian evolution. However, they need not necessarily have functional effects. The translocation of a gene from one chromosome to another may result in a decoupling from a regulatory element or the positioning in a new and functionally relevant milieu or it may be nothing more than a change of scenery. Point mutations in particular are likely to be functionally silent (also referred to as "selectively neutral"). In order to differentiate between those mutations that are likely to be relevant to evolution and adaptation and those that are not, numerous methodologies have been developed.

Methodologies vary in many facets: what kinds of mutations they hope to identify, the timing of those mutations, and the functional nature of those mutations (Vallender, 2008; Zhai et al., 2009). Techniques devised for one type of mutation or selective event may not be relevant for others. Because of their ubiquity and a more complete understanding of their origins and downstream effects, many tests are designed to focus on point mutations. Within these tests two broad categories can be discerned, those that focus on inter-specific comparisons and those that focus on intra-specific comparisons. Inter-specific methodologies compare the fixed genetic differences between species while intra-specific methodologies utilize polymorphism data within a species to detect selective events.

Polymorphism-based approaches can come in many flavors (see for example Zeng et al., 2007). They may utilize the allele frequency spectrum (a measure of the frequency of SNPs in a population) (Fay and Wu, 2000; Fu and Li, 1993; Tajima, 1989), haplotype diversity and structure (Depaulis and Veuille, 1998; Fu, 1996; Hudson et al., 1994), linkage disequilibrium (Kelly, 1997; Sabeti et al., 2002; Slatkin and Bertorelle, 2001; Toomajian et al., 2003), population substructure (Lewontin and Krakauer, 1973), or any combination of these. In addition, the development of new tests aimed at detecting specific types of selective events in specific situations is ongoing. There are significant strengths and weaknesses to these tests even as they apply specifically to the changes associated with the emergence of the human brain. The major strength is that power is generally very strong and often specific functional mutations can be identified. Further, the tests are context independent and work equally well on coding regions and non-coding regions. The major weakness is that these tests half relative short half-lives, meaning that the selective event must have occurred fairly recently. These tests are very successful in identifying genetic changes that accompanied modern *Homo sapiens* dispersal into new environments and encounters with novel disease or the genetic and biological changes that occurred coincident with the emergence of civilization, but are less useful for identifying the genetic mutations that led to the emergence of modern humans (Vallender, 2008).

The reason for the inappropriateness of these tests in understanding the development of the human brain is fairly straightforward. These tests rely on differences within populations to identify selection, while by definition the genetic changes required for making a modern human brain are shared by all members of the species. Certainly there is some lag time wherein a signature of

the selective event will still be present though the mutation itself has fixed, but these situations are often difficult to differentiate from demographic events. Also, in the case of the human brain, the change seems to have occurred even beyond what this lag time could hope to include.

Anatomically modern humans are believed to have emerged 100,000 to 200,000 years ago and paleoanthropologists tell us that the size of the human brain was largely fixed at that time. Indeed, even 500,000 years ago *Homo heidelbergensis*, one of *Homo sapiens* direct ancestors, appears to have a cranial capacity similar to those seen in extant humans (Neill, 2007). The most recent of the major growth spurts toward the human brain appears to have occurred during the transition from the Australopithecines to early *Homo* roughly two million years ago. The genetic changes associated with this anatomical step have thus been fixed for somewhere around 100,000 generations and their polymorphic signatures eroded.

With polymorphism-based tests unable to identify the genetic changes responsible for the development of the human brain, we turn to inter-specific divergence-based tests. Immediately divergence-based suffer a failing relative to their polymorphism-based brethren. Divergence-based tests require multiple functional categories of mutation. This commonly takes the form of functional versus neutral. Our current inability to *a priori* predict functional sites in non-coding regions of the genome has restricted the use of these tests to protein-coding regions where rates of change at amino acid changing sites (d_N or K_A) can be compared to those at synonymous or non-amino acid changing sites (d_S or K_S). This ratios (d_N/d_S, K_A/K_S, or ω) is an immediate and direct measure of selection (Miyata and Yasunaga, 1980). Equal to one indicates neutral evolution such as would be predicted in a pseudogene. Less than one is indicative of negative selection or functional constraint. Greater than one is evidence of positive selection, presumably (though not necessarily for reasons enumerated below) the primary source of adaptation in the human brain.

Difficulties abound even with these tests, however. Firstly, tests are usually conducted on genes as a whole and even when positive selection occurs at one position in a gene it is often balanced by negative selection at other locations. Indeed, for nearly all genes (the MHC is a specific counter-example) negative selection to maintain overall protein structure and function generates baseline ω values around 0.2. Selection needs to be exceptionally strong to have a significant detectable effect. Another issue is the fact that the time periods that can be studied are limited. Studies necessitate two extant species (or species that one can recover DNA from which today means the same thing) and lineages for studies cannot be shortened without adding a new species. In short, a selective event must be strong enough to overwhelm both negative selection at other positions in the gene as well as extended time periods that occurred without positive selection. To say this is a tall order would not be overstating it.

There remains another difficulty unique to human-specific traits, a short lineage problem. Looking for fixed differences between humans and chimpanzees is certainly possible and has been done several times in the past (2005; Clark et al., 2003). The difficulty with short lineages, however, is that stochastic variation in the mutational process can have too great an effect to be overcome. In essence stochastic variation in the rate of synonymous mutation can result in values that are low, resulting in false positives, or values that are too high, resulting in false negatives. The low signal to noise ratio in synonymous mutations can also make achieving statistical significance all but impossible.

Here we offer only a cursory discussion of these methodologies; more through undertakings can be found elsewhere (Vallender, 2008; Zhai et al., 2009). We present this to illustrate two points. The first is how our available methodologies have affected what has been found. Current methodologies are biased towards the identification of functional mutations in protein coding regions. King and Wilson famously hypothesized early on that many of the differences observed between humans and chimpanzees would be the result of non-coding, regulatory, changes (King and Wilson, 1975). This hypothesis has almost become dogma in the field and yet most studies still focus on protein-coding mutations. Methodological limitations are the explanation why. Secondly, it is important when reading the literature on the field to understand discrepancies in findings. It is all too easy to oversimplify the question, looking for recent human positive selection, when in fact the subjects of the study are actually a great deal more nuanced. As a result, while different studies of "recent human positive selection" may indeed produce different results, it is important to ensure that they were in fact designed to answer the same question.

Protein sequence change

For reasons described above, a large number of studies hoping to elucidate the changes leading to the human brain have focused on selection on proteins. For the most part, studies of this kind (and indeed most studies presented here) couple two findings to produce the hypothesis of functional relevance in the species-specific development of the human brain. The first is a brain related function for the gene and the second is evidence of positive selection. Taken together, these offer circumstantial evidence for selection acting on the brain phenotype of the gene. This is not necessarily the case, however, and it should be noted that functional evidence for a neurological effect of a specific mutation remains few and far between. Nevertheless, while the results produced by these studies still represent hypotheses, they are well-founded. The evidence for selection is not in doubt, nor is the evidence for neurological relevance.

Many studies have taken for their starting point genes that have been implicated in neurological diseases. This is particularly true of those diseases arising from developmental changes. One particularly well studied category is the genes that have been associated with microcephaly and, in particular, primary microcephaly. Microcephaly is a developmental affliction which is characterized by a severe reduction in brain size without any major abnormalities in brain structure or architecture (Dobyns, 2002; Mochida and Walsh, 2001; Woods et al., 2005). Primary microcephaly lacks any additional abnormalities as well. The microcephalic phenotype has been considered to be atavistic because in many ways it appears to recapitulate earlier hominid features. This similarity has led to significant exploration of the genes responsible for the disease as potential contributors to the evolutionary changes that lead to the modern human brain. Primary microcephaly is a genetically heterogeneous condition that has been mapped to six loci in the human genome with specific genes and mutations identified for four of these loci: *microcephalin* (*MCPH1*), *CDK5RAP2* (*MCPH3*), *ASPM* (*MCPH5*), and *CENPJ* (*MCPH6*) (Bond et al., 2002; Bond et al., 2005; Jackson et al., 2002).

ASPM and *microcephalin* were the first two genes to be mapped to primary microcephaly loci and several groups exploring each found evidence for positive selection. While *microcephalin* is characterized by a bout of positive selection in the lineage leading from the last common catarrhine ancestor to the great apes (Evans et al., 2004a; Wang and Su, 2004), *ASPM* bears signatures of positive selection along the entire lineage leading from early primates to extant humans (Evans et al., 2004b; Kouprina et al., 2004; Zhang, 2003). Both *ASPM* and *microcephalin*, as well as *CDK5RAP2* and *CENPJ*, show elevated ω values in primates relative to rodents, while *CDK5RAP2* additionally shows particularly high rates in the human and chimpanzee terminal lineages (Evans et al., 2006).

The function of these genes has only begun to emerge. *Microcephalin* appears to be involved in DNA damage control and condensation during mitosis (Evans et al., 2006; Trimborn et al., 2004; Wood et al., 2007; Xu et al., 2004). *ASPM*, *CDK5RAP2*, and *CENPJ* are also seemingly involved in mitotic spindle formation (Bond et al., 2005; Fish et al., 2006; Kouprina et al., 2005). Indeed, all four primary microcephaly-associated genes to date appear to be involved in cell cycle control and likely manifest developmental effects on the brain through the regulation of neural precursor cell proliferation. This is perhaps particularly relevant because of a widely held belief that the changes observed in the human brain may have resulted from increases in neural precursor division during neurogenesis (Kornack and Rakic, 1998).

Interestingly another gene involved in neural cell proliferation, *ADCYAP*1, has also been identified as harboring the signature of positive selection (Wang et al., 2005). This gene has one of the highest ratios of nonsynonymous to synonymous substitutions observed between humans and chimpanzees thus far. Although ADCYAP dysfunction results in many pathologies throughout the body, its role regulating the transition from proliferative to differentiated states offers the possibility of a role for this gene's evolution in the emergence of the human brain (Dicicco-Bloom et al., 1998; Suh et al., 2001). As before, however, it is important to note that the two lines of evidence, for selection and for neural function, remain to be formally conjoined.

While primary microcephaly may be an atavistic trait, other congenital brain malformations are not. One of these abnormalities, called holoprosencephaly, can be caused by mutations in the *sonic hedgehog* (*SHH*) gene. *SHH* encodes a highly studied signaling molecule that plays a role in the development and patterning of many tissues including the skeletal and nervous systems. The gene encodes a signaling molecule as well as an auto-catalytic region. While the signaling molecule is extraordinarily conserved, the auto-catalytic domain shows a significantly increased rate of protein sequence change in primates compared to other animals (Dorus et al., 2006). In particular, the lineage leading to humans shows a rapid rate of evolution and a statistically non-random accumulation of serines and threonines, residues implicated in post-translational modifications. These findings raise again suggestions of ties to human-specific biology.

Joubert syndrome is another example of a neurological disorder where a causative dysfunctional gene has been shown to have an interesting evolutionary history. A syndrome with complex symptomologies, including cerebellar hypoplasia, on causative mutation in *AHI1* involves a protein involved in axon guidance from the brain to the spinal cord (Ferland et al., 2004). Like several of the other genes presented here, *AHI1* has been demonstrated to show accelerated rates of protein sequence change in humans since the last common ancestor with chimpanzees (Ferland et al., 2004).

Using behavioral variation as a substrate for identification of candidate genes has also proven fruitful. The X-linked *MAOA* gene encodes a protein that is responsible for the catabolism of many monoaminergic neurotransmitters including dopamine, serotonin, and norepinephrine. Variation in the gene has been associated with numerous behavioral consequences and neuropsychiatric disorders (Brunner et al., 1993; Cases et al., 1995; Kim et al., 1997; Shih et al., 1999; Sims et al., 1989). Intriguingly, in addition to variation currently segregating in humans, nonsynonymous mutations in the gene may have created a functional change in the enzyme as well (Andres et al., 2004). Of all behavioral changes, however, perhaps none is more obvious than the acquisition of language. It is unsurprising, therefore, that this significant step in the evolution of humans should also be a major emphasis for those seeking genetic correlates. While several of the microcephaly genes, *ASPM* and *microcephalin*, have been suggested to harbor roles in language (Dediu and Ladd, 2007), two

others have demonstrated more prominent roles and offer intriguing possibilities for the evolution of speech. *SRPX2* has been associated with speech processing (Roll et al., 2006) and shows an accelerated rate of protein evolution along the human lineage (though as a result perhaps of short lineage effects it falls short of statistical significance) (Royer et al., 2007). The most interesting of genes in this category, however, was also one of the first to make headlines in the search for humanness genes, *FOXP2*.

FOXP2 is a gene that has been associated with developmental verbal dyspraxia, a disorder that is characterized by difficulties in the production of language and thought to be associated with defects in the brain translating intended speech into the complex muscle movements required (Lai et al., 2001). Since this early finding in humans, *FOXP2* has also been implicated in aural communication in mice and bird song (Haesler et al., 2007; Haesler et al., 2004; Shu et al., 2005; Teramitsu et al., 2004; Teramitsu and White, 2006). While *FOXP2* is nearly perfectly conserved in amino acid sequence across mammalian species, it has undergone two nonsynonymous mutations in the human lineage since the divergence from chimpanzees (Enard et al., 2002b). Because of the extraordinary conservation across mammals, these mutations contribute to statistically significant change in humans. The mere suggestion that these mutations may have played a role in the emergence of spoken language and all that accompanied it was enough to invigorate researchers and energize the field to its current flowering.

While the studies above have focused on specific candidate genes, there has also been genomic research taking a more broad approach (2005; Bustamante et al., 2005; Clark et al., 2003; Dorus et al., 2004; Nielsen et al., 2005). One early study focused on approximately 200 genes chosen for their neurological roles or association with neurological disease. Collectively, these genes showed an increase in their rate of protein change in primates as compared to rodents, an increase not seen in a companion set of more ubiquitously expressed genes (Dorus et al., 2004). Supporting a neurodevelopmental hypothesis of human adaptation, genes with roles during brain and nervous system development showed this acceleration more pronouncedly than genes involved in neurophysiological processes. While this finding was corroborated by a later study (Khaitovich et al., 2005), other studies failed to replicate the finding (Shi et al., 2006; Wang et al., 2007). While much has been made of the differences in results, it is important to note that rather than necessarily represent conflicting findings this may instead be a result of different methodologies answering different questions. Indeed there may be differences in the evolution of neurodevelopmental and neurophysiological genes that may be reflected in the findings even if not explicitly tested for. Similarly, studies may vary in the lineage and/or time scale that they test. Studies aimed at detecting positive selection that has occurred in the last hundred thousand years are unlikely to reveal the genes that contributed to human-specific characters prior to that point. While still not providing definitive answers, these studies nevertheless can offer insight and may be useful in revealing macro trends if carefully considered.

Gene gain and loss

Evolutionary changes in protein sequence are thought to tweak effects, somehow changing the existing functions of the protein. More drastic changes are possible, however. Losses of gene function can occur through point mutations that are difficult to detect, but usually genes without function undergo fairly rapid pseudogenization making their identification straightforward. While it can be counterintuitive to imagine the loss of a gene as adaptive, and indeed not all loses of genes are strictly beneficial, this can be the case. At the same time, while the addition of new genes can be more easily reconciled with adaptation, this occurrence is mechanistically more difficult than either point mutations or gene loss, making gene gain a fairly rare event. Despite these considerations, however, evidence has been found suggesting roles for each of these processes in the emergence of the human brain.

The most pronounced gene loss in humans, and indeed all primates, is in the olfactory system (Gilad et al., 2003a; Gilad et al., 2005a; Gilad et al., 2003b; Glusman et al., 2001; Young et al., 2002; Young and Trask, 2002). While rodents are estimated to have over twelve hundred functional olfactory receptor genes, the human genome appears to harbor between a third and a quarter that amount (Young et al., 2002; Young and Trask, 2002). Rampant pseudogenization has occurred in the olfactory gene family not only in humans, but across all primate species, though there are examples of specific gene losses in humans since the last common ancestor with chimpanzees. These losses should not be surprising given the shift to a primarily visual sensory focus in primates. While it is unclear if this shift merely rendered the ultra-complex olfactory system of ancestral mammals unnecessary or if there was an active benefit to the loss of these genes, suggestive evidence exists that positive selective pressures did, at least in part, shape the current human olfactory subgenome (Gilad et al., 2003a; Gilad et al., 2005a).

A more explicit and tantalizing example of gene loss playing a major role in human brain evolution comes from the gene encoding a myosin heavy chain protein, *MYH16*. This particular heavy chain is expressed uniquely in the muscles of the head including the masticatory apparatus. In humans this gene has undergone a pseudogenization event that has been attributed to positive selection (Stedman et al., 2004). Arguments in favor of this interpretation point to the loss of the sagittal crest in humans and expansions in cranial capacity coinciding with changes in diet and masticatory needs (Neill, 2007). This hypothesis has been challenged, however, because

of a failure to reconcile the age of the mutation with the paleoanthropological data (Perry et al., 2005). While still unclear, it remains plausible that the loss of *MYH16* is related in some way to human evolution.

While gene loss occurs through genetically simple mechanisms, gene gain is more complex. Very rarely do genes emerge out of whole cloth, rather they are the result of duplication events (Ohno, 1970). With multiple copies of a gene an evolutionary relaxation of constraint occurs and, while usually resulting in a simple pseudogenization event, the duplicated gene may undergo neofunctionalization, wherein a new and unique function is imparted on the protein, or subfunctionalization, where multiple functions of the original protein are partitioned between its offspring (Force et al., 1999; Hughes, 1994; Lynch and Force, 2000; Lynch et al., 2001). Events such as these are not particularly common, but recent advances in genomic technologies have made their identification possible.

While not specific to humans, the emergence of trichromatic color vision in primates offers a striking example of duplication followed by neofunctionalization (Li et al., 1999). Most platyrrhines, and presumably ancestral primates, have dichromatic vision engendered by "blue" and "green" opsins. In the ancestor of catarrhines the X-linked "green" opsin was duplicated and neofunctionalization led to the emergence of a "red" opsin gene. This event led to the shift to trichromatic vision and has been argued to coincide with an increase in the importance of visual perception.

The *morpheus* gene family has undergone large scale duplication followed by positive selection and presumably neofunctionalization, in humans and great apes (Johnson et al., 2001). The functional changes in some members of this family are so strong as to obscure homology though studies of synteny confirm their origins. To this point, however, the function of the *morpheus* genes remain unknown. The function of the family of genes harboring the DUF1220 domain is likewise unknown, though their expression is limited to the brain and neurons (Popesco et al., 2006). Like the *morpheus* gene family, these genes have greatly expanded in number in primate species with evolutionary proximity to humans (Popesco et al., 2006). Although the functions of both of these gene families remain unclear, their dramatic and startling appearance in human lineages warrants further examination.

The most transparent example relating to changes in brain function was the duplication and subfunctionalization of the glutamate dehydrogenase genes (Burki and Kaessmann, 2004). Present in ancestral primates, *GLUD1* is responsible for the catabolism of glutamate, the chief excitatory neurotransmitter, and is broadly expressed through the body. Sometime during the lineage separating great apes from old world monkeys a retrotransposition event occurred creating a second glutamate dehydrogenase gene, *GLUD2* (Burki and Kaessmann, 2004). Unlike is parent gene, the expression of *GLUD2* is restricted to nerve tissues and the testis (Shashidharan et al., 1994). This subfunctionalization appears to have been followed by a period of positive selection optimizing enzymatic activity for its new milieu. While the phenotypic relevance of these changes remains shrouded, the evolutionary origins of this brain-specific great ape gene reveal an adaptive role.

Gene expression changes

The genetic differences between humans and chimpanzees pale in comparison with the phenotypic differences; the mutations that gave rise to these differences must have had hugely significant effects. This belief led to the hypothesis that the evolutionary action separating the species was due more to changes in gene expression and regulation rather than protein function (King and Wilson, 1975). More recently, the pleiotropic effects of mutations in brain genes have been invoked to support the same hypothesis (Carroll, 2005). While not excluding protein changes from the evolutionary process of human speciation from chimpanzees, it is clear that regulatory changes have played a significant and likely prominent role.

The primary difficulty with studying regulatory changes lies in our relative lack of understanding, borne out of the extreme lability of the cis-regulatory process and more generally a lack of *a priori* predictive power. Not only does this result in an inability to use traditional evolutionary tests of selection for fixed differences, but it often precludes even identifying potential changes. Rarely are researchers afforded the understanding of gene regulation necessary to make predictions of relevant evolutionary change. Indeed, when this is possible it is driven by an extraordinary interest in the gene itself for reasons almost never related to evolution. But while the genetics of regulatory differences are often difficult to tease apart, the readout of these effects, particularly in terms of mRNA expression is much more straightforward. Because of this an interesting dynamic has developed. While evolutionary studies of protein change focus on evidence for selection and often fall short of function, those analogous studies of regulatory change often demonstrate more clearly functional differences while struggling to prove evidence of selection.

Several disparate studies have approached this question by comparing gene expression in the brains of humans and other non-human primates (Khaitovich et al., 2006; Preuss et al., 2004). While some studies focus on specific regions of the brain, others are more broadly based (Caceres et al., 2003; Enard et al., 2002a; Khaitovich et al., 2004; Marvanova et al., 2003; Uddin et al., 2004). And though these differences in study design result in particular differences of result and may suffer from differing problems, two similar results continue to be found. Overall brain gene expression levels in humans are generally upregulated compared to chimpanzees and yet these expression patterns in the brain are more simi-

lar between the two species than gene expression profiles from other tissues. As with protein change, it seems that on the whole the brain is particularly conserved and yet specific changes necessarily have significant effects.

One difficulty with these studies comes not from the theoretical premises on which they rely, but rather on the difficulty in ensuring apples-to-apples comparisons; it can be very difficult to ensure homology between the samples being tested. This problem can take many forms. Firstly, pathological state of the samples must be determined. Because of ethical considerations and procedural difficulties, many samples used are from diseased animals or significantly aged individuals. Similarly, circumstances of death may result in confounding effects, for instance as related to circadian rhythms, seasonal differences, or menstrual cycles. More broadly, the environmental conditions in which the individual lived may profoundly affect gene expression and it goes without saying that humans and non-human primates in the best of situations live in very different environments (Myers et al., 2007). A second and related complication can be found in developmental timing. While comparing adult to adult seems straightforward, many of the most interesting and likely most important differences may be found in early development, possibly prenatal. Ensuring developmentally homologous time points is particularly difficult in non-human (and human) primates where the ages of the fetus for study cannot be controlled as in rodents. This, coupled with the general difficulties of generating cross-species timelines for development, especially when changes in this developmental timeline are precisely the variable under study, makes comparisons of developmental gene expression particularly daunting. In addition to developmental homology, anatomical homology must be considered. This is particularly relevant as regards the increasingly more refined anatomical substructures under study. As with differences in the developmental timeline between species, the issues surrounding complications that arise through changes in functional roles of specific brain regions must be addressed.

A separate, but equally important, issue that must be resolved is in the detection of mRNA levels themselves. While human array-based methodologies are largely well established and single gene studies using methods such as quantitative PCR can be developed across species, non-human primate array-based methodologies are less developed. Many large-scale studies of non-human primate gene expression rely upon xenohybridization, the hybridization of non-human primate mRNA to human probes. The relative effects of this cross-species hybridization can vary from platform to platform, gene to gene, and species to species, in all greatly complicating in unpredictable ways these studies (Gilad et al., 2005b). Luckily, these problems have a simple solution, the development of species-specific arrays, but one which still represents additional expenditures in time and money that may be difficult to overcome.

While studies which focus on the end phenotype, changes in mRNA expression, have flourished, there have also been a smaller number of studies that have proceeded from genotype to phenotype that have showed some success. The most notable among these is the evolution of an upstream cis-regulatory element in *PDYN*, a precursor of several endogenous opioidergic neuropeptides that have been implicated in many neural processes. This regulatory element shows an exceptionally rapid rate of evolutionary change in the human lineage since its divergence from chimpanzees, consistent with the effects of natural selection (Rockman et al., 2005). Further, in a cell culture system, the human regulatory element was demonstrated to significantly upregulate expression of a reporter gene compared to the orthologous chimpanzee sequence (Rockman et al., 2005). It remains to be seen whether the methodologies that were applied in the *PDYN* study will be successfully generalized, though it would appear unlikely as a perfect storm of prior knowledge, evolutionary timing, and functional assayability was necessary for its success.

It should be mentioned, however, that despite the difficulties involved, there are ongoing genomic efforts to identify regions of rapid evolution. Several genome-wide analyses have been preformed to identify regulatory regions that have undergone rapid change during human evolution (Bush and Lahn, 2008; Haygood et al., 2007; Pollard et al., 2006a; Prabhakar et al., 2006). While these studies have provided an excellent starting point and almost certainly will herald the beginning of a new focus for evolutionary genomics, at present their power for detecting positive selection, as opposed to relaxation of constraint or simply non-functional neutral evolution, is unclear. Similarly, like protein-coding changes, studies remain to be done showing the functional effects of regulatory changes. This is particularly important because, while changes in amino acid are relatively easy to visualize as having a functional effect, changes in conserved non-coding regions without clearly identified functions are not.

Before proceeding it is important to note one area of convergence between the studies of protein-coding change and regulatory evolution. Up until this point our discussion of the evolution of gene expression has focused on changes in the cis-regulatory elements themselves. Indeed, there are many reasons to believe that these changes should be most commonplace, not the least of which is their relative specificity in accomplishing a specific functional task without too many untoward side effects. And while it seems reasonable to believe that this will in fact be the substrate for major evolutionary change in gene expression, several genome-wide studies of protein change have identified a significant overrepresentation of transcription factors among genes likely to have undergone positive selection (Bustamante et al., 2005; Gibbs et al., 2007). Issues of pleiotropy raised more broadly against protein sequence evolution seem to be innumerably more relevant for transcription

factors, however.

Evolution of gene expression will certainly prove to play an important role not only in the emergence of the human brain and other human-specific characters, but in adaptation broadly. While protein-coding changes remain a low-hanging fruit and an important in and of themselves, the efforts into understanding and identifying signatures of selection on gene expression and in cis-regulatory regions will only increase.

Other substrates for change

Changes in protein sequence have long been studied for their affect on phenotypic change during evolution. And while evolutionary studies of gene expression are relatively nascent, theories of their importance are fairly well-established. However, as our understanding of genetics develops so to do potential targets for natural selection and substrates for human-specific evolutionary changes. Among these, several are worthy of brief discussion: alternative splicing, epigenetics, post-translational modifications, and non-coding RNAs.

While whole-gene gain and loss has been considered here and has long been a topic of study in molecular evolutionary literature, the emergence and loss of alternative splice variants has received less attention. With total numbers of genes in mammalian genomes much lower than initially anticipated, the role of alternative splicing has taken on a renewed importance. The emergence of new alternative splice forms may offer a loophole for the lessening of pleiotropic effects. Unfortunately relatively little is known about the evolution of alternative splice forms though research is underway (Jin et al., 2008). Part of this has been the shift in focus to genomic DNA from mRNA. As comparable cDNA libraries from different species emerge it is likely that this research will develop rapidly. Of particular note in this regard are early studies comparing human and mouse cDNAs (Takeda et al., 2008). While still evolutionarily distant for identification of human specific changes, it is important to note that the human-mouse comparison was also the beginning point for many other studies of evolutionary change in humans and mammals.

Similar to single nucleotide point mutations in cis-regulatory regions, changes in epigenetic patterns may affect gene expression differences. In fact, it may be through these mechanisms that cis-regulatory evolution occurs (at least in part). Epigenetic gene silencing in particular is important during in utero development (Keverne and Curley, 2008), a period that has changed dramatically during human evolution and during which many of the brain developmental differences between humans and non-human primates are generated. As our understanding of epigenetics emerges, it seems likely that changes in epigenetic mechanisms will be discovered that have played an adaptive role in the human brain.

As epigenetic evolution may play a role in regulatory evolution more broadly, so too may post-translational modification evolution play a role in protein sequence evolution. The functional effects of protein sequence change are typically thought to be mediated through changes in protein structure, enzymatic activity, or ligand binding. Indeed, the science of understand why protein changes result in the functional affects they do is a major endeavor in it own right. Changes in protein sequence may also result in changes in post-translational modifications. Differences in dimerization can certainly be functional, as can differences in small molecule changes. Differences in sialic acid biology resulting in glycosylation differences were among the first changes to be noted between humans and chimpanzees (Chou et al., 1998; Muchmore et al., 1998). Also noted above is a significant evolution in humans of the autocatalytic region of SHH towards serines and threonines, common substrates for post-translational modifications (Dorus et al., 2006). While far from proven, the role of post-translational modifications must be considered when looking for the mechanisms underlying human-specific traits.

One last area that has only recently emerged yet shows great promise in developing importance is in non-coding RNA genes. These RNAs are relative newcomers to the scene and yet their importance has been immediately recognized. As a means of regulating gene expression they seem likely to play a role in the processes considered here. Evolutionary changes in these genes suffer from the same pros and cons as cis-regulatory changes, and methodologies designed for one often apply to the other. Indeed, the first putatively positively selected non-coding RNA was discovered in the course of a genome-wide study of non-coding DNA. *HAR1* is an RNA gene of unknown function, yet it is expressed in the neurons of the developing neocortex (Pollard et al., 2006b). Although only 118 base pairs in length, there are 18 changes between the human and chimpanzee orthologs, roughly ten times the neutral rate (Pollard et al., 2006b). This difference is even more striking when viewed in light of the chicken-chimpanzee comparison, only two changes (Pollard et al., 2006b). It seems inconceivable that changes of this magnitude do not have some effect, and yet what that effect is remains elusive. Just as we await functional verification of protein changes, so too do we now wait functional verification of non-coding RNA changes.

Conclusion

Understanding the evolution of the human brain will not be easy. The function of the brain is so complex and such a scientifically daunting task by itself, and yet we hope to overlay on top of this another layer of complexity, evolutionary change. It is certainly not a trivial task. Yet we continue to strive to achieve this seemingly insurmountable goal because in doing so we strive to better understand ourselves. We approach the question from many angles, multiple scientific disciplines, using

diverse methodologies and techniques. Evolutionary genetics is but one of many of these.

The progress that has been made in identifying and understanding the genetic differences between humans and our closes primate relatives over the last decade has been astounding. The substrates of evolutionary change have expanded, from proteins to regulatory regions and beyond. Techniques have improved and the scale upon which these questions are considered has broadened. Yet much remains to be done.

Some of those questions that are still outstanding are resource-driven: more species, more individuals, more spatial and temporal time points, greater throughput. The question that dominates all others, however, is functional relevance. How do we demonstrate the functional relevance of the putatively human-important changes? In vitro protein functional assays may be useful for some changes, for some proteins. Cell culture based assays may give additional insight though caution must be taken in interpretation. Transgenic animals, particularly rodents, are likely to provide some clues but again contextual differences may be relevant.

There may be no simple answer to demonstrating unequivocally functional importance, but as a field this must be the goal to which we aspire. A systems biology approach to evolutionary change is difficult to envision, yet it has begun already as we consider the implications of pleiotropy on our hypotheses and theories. Dobzhansky famously said, "Nothing in biology makes sense except in the light of evolution." As the field of molecular evolution matures, we must not forget the biology underlying it.

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