Adaptive Evolution of Four Microcephaly Genes and the Evolution of Brain Size in Anthropoid Primates

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Abstract

The anatomical basis and adaptive function of the expansion in primate brain size have long been studied; however, we are only beginning to understand the genetic basis of these evolutionary changes. Genes linked to human primary microcephaly have received much attention as they have accelerated evolutionary rates along lineages leading to humans. However, these studies focus narrowly on apes, and the link between microcephaly gene evolution and brain evolution is disputed. We analyzed the molecular evolution of four genes associated with microcephaly (ASPM, CDK5RAP2, CENPJ, MCPH1) across 21 species representing all major clades of anthropoid primates. Contrary to prevailing assumptions, positive selection was not limited to or intensified along the lineage leading to humans. In fact we show that all four loci were subject to positive selection across the anthropoid primate phylogeny. We developed clearly defined hypotheses to explicitly test if selection on these loci was associated with the evolution of brain size. We found positive relationships between both CDK5RAP2 and ASPM and neonatal brain mass and somewhat weaker relationships between these genes and adult brain size. In contrast, there is no evidence linking CENPJ and MCPH1 to brain size evolution. The stronger association of ASPM and CDK5RAP2 evolution with neonatal brain size than with adult brain size is consistent with these loci having a direct effect on prenatal neuronal proliferation. These results suggest that primate brain size may have at least a partially conserved genetic basis. Our results contradict a previous study that linked adaptive evolution of ASPM to changes in relative cortex size; however, our analysis indicates that this conclusion is not robust. Our finding that the coding regions of two widely expressed loci has experienced pervasive positive selection in relation to a complex, quantitative developmental phenotype provides a notable counterexample to the commonly asserted hypothesis that cis-regulatory regions play a dominant role in phenotypic evolution.

Key words: ASPM, MCPH1, CDK5RAP2, CENPJ, brain, neurogenesis, primates.

Introduction

The expansion of the brain, and in particular the neocortex, is a major hallmark of primate evolution (Jerison 1973; Martin 1990). After correcting for allometric scaling with body mass, primates have larger brains than most other mammals (Martin 1990; Barton 2006b) and both absolute and relative brain size have increased along multiple, independent primate lineages (Montgomery et al. 2010). The adaptive significance and anatomical basis of the diversity of primate brains has long been studied using comparative methods (for review, see Falk and Gibson 2001; Finlay et al. 2001; Barton 2006a), but the investigation of the genetic basis of primate brain expansion has only begun relatively recently and is currently a topic of intense interest.

The convergent evolution of increased brain size in different lineages provides an opportunity to study whether the independent evolution of complex traits involves convergence at the molecular level (Arendt and Reznick 2007) and may provide insights into lineage-specific evolution, for example, on the human lineage. Both scans of brain-expressed genes in published primate genomes (Dorus, Vallender, et al. 2004; Shi et al. 2006; Yu et al. 2006; Wang et al. 2007) and studies of candidate genes (e.g., Enard et al. 2002; Burki and Kaessmann 2004; Wang et al. 2005) have mostly focused on identifying changes along the lineage leading to humans and have largely ignored convergent increase in brain size in multiple primate lineages.

One group of genes of particular interest in relation to the evolution of gross brain size is the microcephaly genes. Autosomal recessive primary microcephaly is a congenital disorder characterized by reduced growth of the cerebral cortex in the absence of environmental, metabolic, or cytogenetic etiologies (Bond and Woods 2006; Cox et al. 2006). In humans, it is inherited as a recessive Mendelian trait involving at least eight loci, of which five have now been identified at the molecular level: ASPM, MCPH1, CDK5RAP2, CENPJ (Jackson et al. 1998; Bond et al. 2002, 2005; Thornton and Woods 2009) and the more recently identified STIL (Kumar et al. 2009).

The five genes are expressed in the fetal brain during neurogenesis (Bond et al. 2002, 2005; Jackson et al. 2002; Kouprina et al. 2005; Kumar et al. 2009). ASPM, CDK5RAP2,
and \textit{CENPJ} all have roles in centrosome or microtubule formation (Bond and Woods, 2006; Cox et al. 2006; Fish et al. 2006; Buchman et al. 2010) and can affect neurogenic mitosis by influencing the spindle pole and astral microtubule network (Fish et al. 2006; Fong et al. 2008; Cormier et al. 2009; Buchman et al. 2010). \textit{MCPH1} functions in the DNA damage response pathway and apoptosis (Rickmyre et al. 2007; Wood et al. 2007, 2008) and may also affect the timing of cell cycle progression (Bunk et al. 2007). Both apoptosis and cell cycle length are known to have significant roles in brain development (Roth and D’Sa 2001; Calegari and Huttner 2003). The function of \textit{STIL} is less well studied, but it localizes to the centrosome and has some functional similarities to \textit{ASPM} (Kumar et al. 2009; Thornton and Woods 2009).

The main hypotheses for how the number of neurons could increase during brain expansion (Rakic 1988, 1995; Caviness et al. 1995; Kriegstein et al. 2006) rely on switches between symmetric and asymmetric cell divisions, via changes in spindle pole orientation, at a particular stage of neurogenesis. The functions of microcephaly genes are therefore consistent with the developmental mechanisms proposed to have facilitated brain expansion (Götz and Huttner 2005; Cox et al. 2006; Kriegstein et al. 2006). Notably, the phenotypes exhibited by individuals with microcephaly show that these loci affect cortical surface area, not thickness, consistent with a role in regulating the size of the neural progenitor pool (Desir et al. 2008). Interestingly, recent studies in humans identified single nucleotide polymorphisms in \textit{ASPM}, \textit{CDK5RAP2}, and \textit{MCPH1} associated with total brain size or cortical surface area (Wang et al. 2008; Rimol et al. 2010) but not cortical thickness, an observation that again is consistent with a role in controlling the size of the neural progenitor pool (Montgomery and Mundy 2010; Rimol et al. 2010).

Previous studies of the molecular evolution of the first four microcephaly loci to be identified supported the hypothesis that they have been subject to positive selection (Zhang 2003; Evans, Anderson, Vallender, Choi, and Lahn 2004; Evans, Anderson, Vallender, Gilbert, et al. 2004; Kouprina et al. 2004; Wang and Su 2004; Evans et al. 2006) but provided no direct evidence that the loci were involved in brain evolution as brain size was not incorporated into their analyses and did not include a diverse phylogenetic sample of species. A recent study analyzed \textit{ASPM} evolution in relation to brain size in primates and concluded that branches with high relative telencephalon volume (reported as cerebral cortex) were associated with positive selection on \textit{ASPM} (Ali and Meier 2008).

For a comprehensive understanding of the role of genes in primate brain evolution, broad comparisons across the primate phylogeny incorporating relevant phenotypes are needed (Carroll 2003; Goodman et al. 2005; Barton 2006a; Pollen and Hoffmann 2008; Vallender 2008). An important issue is which aspects of brain phenotype are most salient. Measures of brain size corrected for body size (i.e., relative brain size) are frequently used in studies investigating brain evolution as these take into account the strong correlation between brain and body mass (Barton 2006b). However, given the implied functions of the four microcephaly genes in regulating the proliferation and survival of neurons, absolute brain mass may be a more relevant phenotypic measure as in primates it increases linearly with the total number of neurons (Herculano-Houzel et al. 2007). In agreement with quantitative genetic analysis of brain and body size allometry (Lande 1979), it has recently been shown that primate brain and body size differ in their evolutionary trajectories (Montgomery et al. 2010) suggesting that these two traits must be developmentally and genetically decoupled to some extent despite their closely correlated evolution. Crucially, because primate neocortical neurogenesis is largely restricted to prenatal development (Rakic 1988, 2002; Bhardwaj et al. 2006) and microcephaly is primarily a disorder of fetal brain growth (Cox et al. 2006), microcephaly gene evolution should be more closely related to neonatal brain size than to adult brain size. Postnatal brain growth is largely driven by gliogenesis (Low and Cheng 2006), axon growth (Sauvageot and Stiles 2002), and myelination (Sowell et al. 2001) rather than by production of new neurons. There are only two known sites in the primate brain, which are small and noncortical, in which substantial postnatal neurogenesis occurs (Jabes et al. 2010). Indeed, apoptosis eliminates large numbers of neurons (Buss et al. 2006). Variation in these and other nonneurogenic processes will reduce the relationship between brain size and neuron number as development progresses, weakening any association with the molecular evolution of genes under selection in relation to prenatal neurogenesis. Indeed, patterns of postnatal brain growth vary considerably across primates (Leigh 2006). Finally, if there is an association with adult brain size, given the specific effect of microcephaly on the development of the cerebral cortex and their functions in cortical neurogenesis (Cox et al. 2006; Thornton and Woods 2009), we might also predict a stronger association with adult neocortex size than adult whole brain size. Unfortunately, there are insufficient comparative data available on volumes of neonatal brain regions to test this hypothesis.

Alternative hypotheses to explain the high evolutionary rates of microcephaly genes also exist. The four loci are widely expressed throughout the body and \textit{ASPM}, \textit{CDK5RAP2} and \textit{CENPJ} are particularly highly expressed in the testis (Bond et al. 2005; Kouprina et al. 2005), where many genes have been shown to be under sexual selection in primates (Dorus, Evans, et al. 2004; Clark and Swanson 2005; Ramm et al. 2008). However, the precise function of these genes in testes development and function is still unknown. For \textit{ASPM}, a possible ciliary function led to the suggestion of a role in sperm flagellar movement that may affect sperm locomotion and hence be targeted by sexual selection (Ponting 2006). If the microcephaly genes do have important roles in the testes or sperm, their high rates of evolution may be associated with levels of sexual selection and be unrelated to changes in brain size. Hence, explicit tests are required before the molecular evolution of microcephaly loci can be linked to brain evolution.
Here, we investigate the molecular evolution of *ASPM*, *CDK5RAP2*, *CENPJ*, and *MCPH1* in relation to brain size in anthropoid primates. First, we test whether these loci are under positive selection across anthropoids and whether or not different anthropoid clades have experienced different selective regimes. Second, we explore the association between the rate of molecular evolution of microcephaly genes and measures of brain size, predicting a positive and stronger association between these genes and absolute brain mass than measures of relative brain size, we calculated relative brain mass by performing a phylogenetically controlled regression analysis between log(brain mass) and log(body mass), and for neocortex, log(neocortex volume) was regressed against log(rest of brain volume) separately (following Barton 1998). For testis, log(testis mass) was regressed against log(body mass). These analyses were performed using a phylogenetically controlled regression using phylogenetic generalized least squares (PGLS) models in BayesTraits (Pagel et al. 2004) with maximum likelihood and 1,000 runs for each analysis. Residual values from the regression line were calculated for each taxon, and these were used as values of relative brain size and relative testis mass in all subsequent analyses. All phenotypic data are provided in table S1 (Supplementary Material online), and additional phenotypic analyses are presented in the supplementary information (Supplementary Material online). With PGLS, the phylogeny is converted into a variance-covariance matrix, where the diagonal of the matrix gives information on the path length from root-to-tips (the “variance”) and the off-diagonal values of the matrix provide information on the shared evolutionary history of any pair of species, that is the time from the root to the last common ancestry (the “covariance”) (Pagel 1997, 1999; Freckleton et al. 2002). With PGLS, the variance-covariance matrix is included into the error term of the regression model, and the resulting estimated regression parameters (i.e., slopes and intercepts) are “phylogenetically controlled” (Pagel 1997, 1999; Freckleton et al. 2002).

**Materials and Methods**

**Phenotypic Data**

Data for body, brain mass, and volumes of specific brain regions were obtained from previously published data (Bauchot and Stephan 1969; Stephan et al. 1981; Zilles and Rehkemper 1988), leading to a data set of 37 primate genera including 14 catarrhines, 12 platyrhines, one tarsier and 10 strepsirhines (supplementary table S1, Supplementary Material online). Data on neonatal brain size (22 taxa) were obtained from Capellini et al. (submitted). Data on testis size, a commonly used phenotypic correlate for sperm competition and sexual selection (Harcourt et al. 1995; Ramm and Stockley 2010), to test the hypothesis that these loci may have been under sexual selection. We find that whereas all four loci have been targets of selection throughout primate evolution, *ASPM* and *CDK5RAP2* but not *MCPH1* and *CENPJ* show positive associations with absolute neonatal brain but not with any measure of relative brain size or relative testis size, suggesting a role in the evolution of total neuronal number.

**Phylogeny**

We used a genus level composite phylogeny of primates using published trees. The topology is taken from Goodman et al. (2005) for haplorhine primates and Horvath et al. (2008) for strepsirhines. Proportional branch lengths were obtained from recent studies of primate divergence dates (Purvis 1995; Page and Goodman 2001; Poux and Douzery 2004; Opazo et al. 2006) scaled to agree with dates of divergence for the deeper primate nodes estimated by Steiper and Young (2006). The tree obtained therefore has branch length information in time and is ultrametric (supplementary fig. S1, Supplementary Material online).

**Laboratory Methods**

Genomic DNA samples had previously been extracted from tissue samples using Qiagen kits. Sequence data from previous studies and primate genomes were collected from the online databases, GenBank and Ensembl. From these sequences, primers were designed using Primer3Plus (Untergasser et al. 2007). We sequenced exons that had previously been shown to have accelerated rates of evolution or contained a large proportion of the coding sequence. For *ASPM*, we sequenced exons 3 and 18, totaling 6,235 bp (60% of the coding region). For *MCPH1*, three exons were sequenced: 8, 11, and 13, totaling 1,556 bp (62% of the coding region). Exons 2 and 7 were sequenced for *CENPJ*, totaling 1,556 bp (52% of the coding region). We sequenced 7 of 38 exons of *CDK5RAP2*: exons 12, 20, 21, 24, 25, 32, and 33 (total 2,120 bp; 37% of the coding sequence). Polymerase chain reactions and sequencing on both strands were performed using standard protocols (for further details and primers, see supplementary information and table S2, Supplementary Material online).

Sequences were edited in SEQMAN v. 5.05 (DNASTAR Inc.) and aligned and checked in ClustalW in MEGA 4.0 (Tamura et al. 2007). Exons of each locus were concatenated and subsequently analyzed together; alignments are available on request. Sequences were obtained for 5 apes, 5 Old World monkeys, and 10 New World monkeys, representing all major clades of anthropoid primates, shown in figure 1. Where phenotypic data were not available for the species sequenced, we used closely related congeneric species for which data were available. Newly sequenced data have been submitted to GenBank (for accession numbers, see table S3, Supplementary Material online). We used the Strepsirhines *Microcebus murinus* and...
**Otolemur garnettii**: Ensembl IDs are shown in table S3 (Supplementary Material online).

**Molecular Evolution**
A common measure used to infer selection pressures acting on coding regions of genes is the ratio of rates of nonsynonymous to synonymous fixed base changes. Estimation of dN/dS ratios ($\omega$) was carried out using a codon-based maximum likelihood method (PAML version 4; Yang 2007). Several analyses were performed to test the hypothesis that the four loci have experienced positive selection across primates, in particular, in relation to brain size evolution. Nested models are compared using the likelihood ratio test statistic ($-2(\text{loglikelihood}_1 - \text{loglikelihood}_2)$) to critical values of the chi-square distribution and degrees of freedom as the difference in the number of parameters estimated by each model.

**Site and Branch Models**
To detect positive selection across primates, we implemented the site models. These allow the $\omega$ to vary among sites but not across lineages (Nielsen and Yang 1998; Yang et al. 2000). Model M1a (nearly neutral) allows sites to fall into two categories with $\omega < 1$ (purifying selection) and $\omega = 1$ (neutral evolution), whereas model M2a (positive selection) allows sites to fall into three categories with $\omega < 1$, $\omega = 1$, and $\omega > 1$ (positive selection) (Yang et al. 2005).

In addition, we used the branch models to test whether the dN/dS of lineages leading to humans was significantly higher than nonhuman lineages and whether dN/dS
significantly differed between Apes, Old World Monkeys, and New World Monkeys.

**Root-to-Tip dN/dS and Gene–Phenotype Associations**

The branch models were used to estimate the average dN/dS ratio from the ancestral anthropoid to each terminal species tip. These values were then set as species data and used in a PGLS regression with measures of brain size in BayesTraits, as explained above (Pagel 1999; Pagel et al. 2004; Organ et al. 2007). Previous analyses that have tested for correlations between phenotypes and dN/dS ratios using similar methods have typically used the dN/dS of the terminal branch (e.g., Dorus, Evans, et al. 2004; Nadeau et al. 2007). However, although a species’ phenotype reflects the whole phenotypic evolution, the dN/dS of the terminals branch does not reflect the whole genotypic evolution. The root-to-tip dN/dS is more inclusive of the evolutionary history of a locus and is a property of the species tip rather than the terminal branch and is therefore more suitable for regressions against phenotypic data from extant species. In addition, by analyzing the rate of evolution since the last common ancestor of the species in our data set, all branches are the same length and therefore not subject to temporal effects on dN/dS (Wolf et al. 2009). One assumption of regression analysis is that the residuals of the model are normally distributed. As the residuals of the regression using dN/dS ratios were not normally distributed, we used log-transformed dN/dS to improve normality. Residuals of regression analysis with log-transformed dN/dS did not violate assumptions of normality and constant variance.

First, we examine the relationship between microcephaly gene molecular evolution and the evolution of absolute and relative neonatal brain size. As we specifically hypothesize a positive association between brain size and the selection pressure on these loci, the significance of the regression coefficient was determined using a one-tailed t-test. A significant negative association would suggest an increase in “purifying” selection has acted on a locus as brain mass increased and, although interesting, would suggest that the locus does not contribute to the genetic basis of “change” in that phenotype and could not explain why the locus has evolved adaptively. Hence, we meet both recently suggested requirements for justifying the use of one-tailed tests; we explain why we hypothesize an association in a particular direction, and why the opposite pattern can be treated the same as a nonsignificant trend in the expected direction (Ruxton and Neuhäuser 2010).

As the size of our data set for this analysis is limited by the availability of neonatal brain size data and the imperfect overlap between phenotype and gene sequence data sets, it is highly likely that the small sample size will result in low power to reject the null hypothesis. To minimize the chances of Type I errors, we restrict our analyses to a small number of critical tests, and we determine the specificity of relationships for microcephaly genes by testing for associations with other genes having no known role in neurogenesis. In particular, we test for associations with alternative phenotypes and test for associations between the evolution of genes with no known role in neurogenesis with brain size. In addition, a Jackknife approach was taken to test the robustness of the associations found and to identify any outliers that have a dominant effect on the slope of the regression.

We subsequently explored the relationship with volume of adult whole brain and neocortex. This analysis was performed to test our hypothesis that genes involved in neurogenesis should be more strongly associated with neonatal than adult brain mass and that if there is an association with adult brain size it may be stronger for neocortex size than whole brain size. Comparisons of nonnested models were performed using Akaike Information Criterion (AIC: calculated as \(2 \times \text{number of parameters} + (2 \times \log(\text{likelihood}))\)) to identify the best supported model, where a lower value indicates a better fitting model, and a difference between models greater than two suggests a substantial difference (Burnham and Anderson 2002).

In addition to the standard dN/dS ratios, we used multiple regressions to investigate the association between phenotype and dN while controlling for dS. Here, we predict a negative association between brain size and dS given known relationships between dS and life-history traits, such as body size (Nikolaev et al. 2007). Conversely, a locus that is a target of selection in relation to brain size may show a positive association with dN. Both approaches examine variation in dN and dS relative to one another, but they make different assumptions about the nature of the underlying relationship. For example, a significant dN/dS–phenotype relationship suggests an association between phenotypic evolution and selection acting on a locus and may be obtained when both change together in a tightly correlated fashion but with one changing at a faster rate than the other (so that the ratio correlates with the absolute value of the changes), whereas in this case, a multiple regression would show no significant correlation. Hence, differences in the results obtained may be informative about the nature of the gene-phenotype correlation.

In most cases, the phenotypic data is based on a small number of individuals, and the degree of interspecific variation is unknown. However, where interspecific variation greatly exceeds intraspecific variation, as is expected to be the case for brain size, results of comparative analyses are not biased by intraspecific variation (see Nunn and Barton 2001). It is also likely that error introduced by sampling small numbers of individuals will lead to an underestimate of correlation coefficients between two traits (Nunn and Barton 2001; Ives et al. 2007).

Finally, we used branch-site models to test for associations between positive selection and brain evolution, but this method did not produce informative results (see supplementary information, Supplementary Material online).

**Results**

Our full data set comprises sequence data from 21 species for each gene including representative species from all
Table 1. Site Models Detecting Positively Selected Sites for Anthropoid Primates.

<table>
<thead>
<tr>
<th>Gene</th>
<th>N</th>
<th>lnL (null/M1a)</th>
<th>lnL (positive selection/M2a)</th>
<th>LRT Statistic</th>
<th>P Value</th>
<th>Proportion of Sites, ω &gt; 1</th>
<th>ω</th>
<th>Positively Selected Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPM</td>
<td>21</td>
<td>-20522.7</td>
<td>-20487.0</td>
<td>71.4</td>
<td>&lt;0.001</td>
<td>0.0228</td>
<td>5.395</td>
<td>105, 107, 1437, 1558, 2016, 2185</td>
</tr>
<tr>
<td>CDK5RAP2</td>
<td>21</td>
<td>-7858.2</td>
<td>-7818.7</td>
<td>79.0</td>
<td>&lt;0.001</td>
<td>0.0650</td>
<td>4.418</td>
<td>853, 881, 929, 930, 948, 964, 977, 1581, 1683, 1687, 1688, 1690</td>
</tr>
<tr>
<td>CENPJ</td>
<td>21</td>
<td>-7695.8</td>
<td>-7684.1</td>
<td>23.4</td>
<td>&lt;0.001</td>
<td>0.0225</td>
<td>3.897</td>
<td>527, 813</td>
</tr>
<tr>
<td>MCPH1</td>
<td>21</td>
<td>-6375.3</td>
<td>-6369.0</td>
<td>12.6</td>
<td>0.002</td>
<td>0.0558</td>
<td>2.574</td>
<td></td>
</tr>
</tbody>
</table>

* Positively selected sites identified using Bayes empirical Bayes are shown in the right-hand column, numbered according to the full human coding sequence. Only sites with P > 95% are presented; sites in bold have P > 99%. LRT, likelihood ratio test.

major anthropoid clades and 11–17 newly sequenced species for each locus. The coverage of full coding sequence in the data set comprises 60% for ASPM (from 2 exons), 37% for CDK5RAP2 (from 7 exons), 52% for CENPJ (from 2 exons), and 62% for MCPH1 (from 3 exons) (see Materials and Methods and supplementary table S3, Supplementary Material online). Previous data sets for CDK5RAP2 and CENPJ have included only four species (Evans et al. 2006). With the exception of recent ASPM study by Ali and Meier (2008), which was published after the completion of our data set, analyses of this gene have considered 3 (Zhang 2003), 7 (Evans, Anderson, Vallender, Gilbert, et al. 2004), and 8 species (Kouprina et al. 2004), whereas studies of MCPH1 have included 7 (Evans, Anderson, Vallender, Choi and Lahn 2004) and 13 species (Wang and Su 2004). These studies were particularly lacking in a diverse range of New World Monkeys. Although we have sequenced only partial coding sequences, we have focused on regions of the gene which contain functionally important domains or which have previously been shown to have accelerated rates of evolution. Our data set therefore allows us to examine how widespread selection on these regions has been across anthropoids and to explore the relationship between this selection and phenotypic evolution.

Pervasive Adaptive Evolution in Microcephaly Genes

We first examined whether a signal of positive selection is present in four microcephaly loci by performing site model tests using a codon-based maximum likelihood method (Yang 2007; table 1). All four genes showed evidence for positive selection across anthropoids, with estimated omega of 2.57–5.39 at 2.28–6.50% of sites across the loci. The most significant results are for ASPM and CDK5RAP2 that have 2.3% and 6.5% of sites having an omega of 5.39 and 4.42, respectively. In CDK5RAP2, a notable feature is the clustering of sites identified as being under positive selection using Bayes empirical Bayes (P > 95%) (positions 929–977 and 1683–1690), these mostly fall within an SMC domain (Evans et al. 2006), these domains are thought to play a role in the chromosome segregation, regulation, and repair (Hirano 2006), but the functional significance of these particular sites is unknown.

Previous authors have proposed that ASPM and MCPH1 have evolved at a higher rate along lineages leading from the last common ancestor of apes to modern humans than on other lineages (Evans, Anderson, Vallender, Choi, and Lahn 2004; Evans, Anderson, Vallender, Gilbert, et al. 2004). We tested this explicitly using a branch model and found that the evolutionary rate of change in lineages leading to humans does not significantly differ from that in other lineages (table 2a), which is congruent with the fact that the proportional change in brain mass along this lineage is also not exceptional compared with the rest of primate brain size evolution (Montgomery et al. 2010). In addition, we tested whether the evolutionary rate of the four loci differed between apes, Old World Monkeys, and New World Monkeys and found no significant differences (table 2b). Finally, we performed site models to test for positive selection on each of the three clades separately. Although the results are influenced by differences in the number of sequences and the time depth for each clade, we found no instance where positive selection was found in Apes and not in Old or New World Monkeys (supplementary table S4, Supplementary Material online).

Associations between Gene Evolution and Brain Size

To explicitly test the link between the molecular evolution of microcephaly genes and brain size, we performed several tests. We first performed a phylogenetically controlled regression analysis in BayesTraits (Pagel 1999; Pagel et al. 2004) between the root-to-tip dN/dS, estimated using the branch models, and neonatal brain mass.

We found a significant association between the molecular evolution of CDK5RAP2 and absolute neonatal brain mass (t = 1.95, P = 0.039, R² = 0.255) but no significant association between ASPM, CENPJ, or MCPH1 and this trait (table 3). For ASPM, however, Callithrix represented a strong outlier (fig. 2, supplementary table S5, Supplementary Material online), and when this species was removed, the association between the dN/dS of ASPM and neonatal brain mass became significant (t = 2.42, P = 0.018, R² = 0.369). Callithrix, and the other Callitrichids, show high rates of evolution of ASPM (supplementary table S5, Supplementary Material online) but have the smallest brain masses among the anthropoid primates (Stephan et al. 1981); however, this is due to a secondary reduction in brain mass in this taxon (Ford 1980; Montgomery et al. 2010). The significance of the regression between ASPM and neonatal brain mass was not affected by the removal of any other species. From here on, unless otherwise stated, regressions for ASPM were performed without the
Callitrichids. Removing Callithrix does not reveal a significant association with neonatal brain mass for either CENPJ ($t_{10} = 0.81, P = 0.218, R^2 = 0.061$) or MCPH1 ($t_{10} = -0.55, P = 1.000, R^2 = 0.029$). The high root-to-tip MCPH1 dN/dS ratio of Pan (1.39) appeared to be heavily influenced by a small number of synonymous substitutions on the terminal Pan branch (1 synonymous change, compared with 6 synonymous changes on the Homo branch), and we note previous studies have found a much lower dN/dS ratio on the terminal Pan lineage using the full coding sequence (Evans, Anderson, Vallender, Gilbert, et al. 2004; Wang and Su 2004). We therefore repeated the regressions excluding the Pan data point but still found no significant result (data not shown). No locus showed any association with relative neonatal brain size (Table 3).

We next explored whether the association with brain mass can be observed for adult phenotypes by performing regressions of root-to-tip dN/dS with absolute and relative neocortex and whole brain size. We found no significant association with any measure of absolute brain size for any locus (supplementary table S6, Supplementary Material online). To confirm this result, we performed the same regressions but using only the species used in the neonatal regressions. Using this reduced data set, we subsequently found a significant association between both absolute neocortex and whole brain mass with ASPM (whole brain: $t_{10} = 2.732, P = 0.010, R^2 = 0.427$; neocortex: $t_{10} = 2.980, P = 0.007, R^2 = 0.470$) and CDK5RAP2 (whole brain: $t_{11} = 2.131, P = 0.028, R^2 = 0.292$; neocortex: $t_{11} = 2.00, P = 0.035, R^2 = 0.267$) but not MCPH1 or CENPJ (not shown). To identify the cause of this discrepancy, we performed a reverse jackknife, repeating the regressions with the full data set of adult phenotypes and removing each species, one at a time (supplementary table S7, Supplementary Material online). We found that for both ASPM and CDK5RAP2, the removal of Papio results in a significant association with absolute neocortex and whole brain mass (ASPM: whole brain: $t_{15} = 2.063, P = 0.028, R^2 = 0.221$; neocortex: $t_{14} = 2.128, P = 0.0257, R^2 = 0.244$; CDK5RAP2: whole brain: $t_{18} = 2.418, P = 0.013, R^2 = 0.245$, neocortex: $t_{17} = 2.238, P = 0.019, R^2 = 0.228$), whereas removing any other species does not have this affect (supplementary table S5, Supplementary Material online), suggesting Papio is an outlier to a general trend.

We subsequently added the Callitrichids back into the regressions with ASPM and find that the addition of any one Callitrichid or all three together substantially reduces or negates the association (addition of Saguinus alone: $t_{16} = 0.612, P = 0.274$, Leontotheicus alone: $t_{16} = 1.842, P = 0.042$, Callithrix alone: $t_{16} = 0.612, P = 0.265$, all three: $t_{18} = 0.302, P = 0.2383$). In contrast, after removal of all three Callitrichids, the association with CDK5RAP2 is still significant ($t_{15} = 0.612, P = 0.042$) and removal of any single species (removal of Saguinus: $t_{17} = 2.010, P = 0.031$, Leontotheicus: $t_{17} = 2.359, P = 0.015$, Callithrix: $t_{17} = 2.010, P = 0.031$) or any pair of Callitrichids (retention

### Table 2. Branch and Site Model Tests on Separate Anthropoid Clades.

<table>
<thead>
<tr>
<th>Gene</th>
<th>dN/dS lines leading to Homo</th>
<th>dN/dS “nonhuman” lines</th>
<th>lnL(M2)</th>
<th>lnL(M0)</th>
<th>Likelihood Ratio Statistic</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPM</td>
<td>0.489</td>
<td>0.466</td>
<td>-21351.430</td>
<td>-21351.451</td>
<td>0.042</td>
<td>0.838</td>
</tr>
<tr>
<td>CDK5RAP2</td>
<td>0.729</td>
<td>0.676</td>
<td>-8718.412</td>
<td>-8718.440</td>
<td>0.054</td>
<td>0.816</td>
</tr>
<tr>
<td>CENPJ</td>
<td>0.623</td>
<td>0.503</td>
<td>-8664.800</td>
<td>-8664.986</td>
<td>0.373</td>
<td>0.542</td>
</tr>
<tr>
<td>MCPH1</td>
<td>0.793</td>
<td>0.579</td>
<td>-7301.153</td>
<td>-7301.505</td>
<td>0.704</td>
<td>0.401</td>
</tr>
</tbody>
</table>

#### Model 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>dN/dS ApeS</th>
<th>dN/dS OWM</th>
<th>dN/dS NWM</th>
<th>lnL(M2)</th>
<th>lnL(M0)</th>
<th>Likelihood Ratio Statistic</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPM</td>
<td>0.473</td>
<td>0.415</td>
<td>0.479</td>
<td>-21350.890</td>
<td>-21351.451</td>
<td>1.122</td>
<td>0.571</td>
</tr>
<tr>
<td>CDK5RAP2</td>
<td>0.781</td>
<td>0.627</td>
<td>0.687</td>
<td>-8717.931</td>
<td>-8718.440</td>
<td>1.018</td>
<td>0.601</td>
</tr>
<tr>
<td>CENPJ</td>
<td>0.582</td>
<td>0.549</td>
<td>0.522</td>
<td>-8663.445</td>
<td>-8664.986</td>
<td>3.081</td>
<td>0.114</td>
</tr>
<tr>
<td>MCPH1</td>
<td>0.606</td>
<td>0.724</td>
<td>0.538</td>
<td>-7300.305</td>
<td>-7301.505</td>
<td>2.401</td>
<td>0.030</td>
</tr>
</tbody>
</table>

### Table 3. Phylogenetically Controlled Regression Analysis between Root-to-Tip dN/dS and Brain Size in Anthropoid Primates.

<table>
<thead>
<tr>
<th>Gene</th>
<th>n</th>
<th>r-Statistic</th>
<th>P Value</th>
<th>R^2</th>
<th>t-Statistic</th>
<th>P Value</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPM</td>
<td>13</td>
<td>0.040</td>
<td>0.484</td>
<td>0.000</td>
<td>0.797</td>
<td>0.221</td>
<td>0.055</td>
</tr>
<tr>
<td>ASPM (without Callitrichids)</td>
<td>12</td>
<td>2.420</td>
<td>0.018</td>
<td>0.369</td>
<td>0.967</td>
<td>0.178</td>
<td>0.086</td>
</tr>
<tr>
<td>CDK5RAP2</td>
<td>13</td>
<td>1.955</td>
<td>0.039</td>
<td>0.255</td>
<td>-0.500</td>
<td>1.000</td>
<td>0.022</td>
</tr>
<tr>
<td>CENPJ</td>
<td>13</td>
<td>0.631</td>
<td>0.270</td>
<td>0.035</td>
<td>-1.204</td>
<td>1.000</td>
<td>0.116</td>
</tr>
<tr>
<td>MCPH1</td>
<td>13</td>
<td>-0.697</td>
<td>1.000</td>
<td>0.042</td>
<td>-1.126</td>
<td>1.000</td>
<td>0.103</td>
</tr>
</tbody>
</table>

NOTE.—OWM, Old World Monkeys; NWM, New World Monkeys.
of *Saguinus*: $t_{16} = 1.886$, $P = 0.039$, *Leontopithecus*: $t_{16} = 1.988$, $P = 0.032$, *Callithrix*: $t_{16} = 2.460$, $P = 0.012$) does not significantly affect the association. This confirms Callitrichids are outliers to the general trend for *ASPM* but not for *CDK5RAP2*. It is notable that *Leontopithecus* has a much smaller effect on the *ASPM* result than *Saguinus* and *Callithrix* suggesting a complex pattern of selection on this locus, more data will be required to tease out the pattern within Callitrichids.

In addition, we repeated the analysis using relative measures of adult brain and neocortex sizes. For *ASPM*, there was a significant association with relative adult brain size on both the full data set ($t_{19} = 1.77$, $P = 0.046$, $R^2 = 0.142$; without Callitrichids: $t_{15} = 2.241$, $P = 0.020$, $R^2 = 0.239$) and the reduced data set used for regressions with neonatal data ($t_{10} = 2.59$, $P = 0.013$, $R^2 = 0.402$). However, in both cases, the result was dependent on the human data point and when this was removed the association is no longer significant (adult: $t_{18} = 0.574$, $P = 0.287$, $R^2 = 0.018$; without Callitrichids: $t_{15} = 0.923$, $P = 0.185$, $R^2 = 0.054$; neonate: $t_{18} = 0.880$, $P = 0.200$, $R^2 = 0.079$). No other significant relationship was detected (supplementary table S6, Supplementary Material online).

We next used AIC to determine the best supported model for the associations between *ASPM* and *CDK5RAP2* and neonatal and adult brain mass using only the species for which data are available for both phenotypes. For both loci, the regression with neonatal brain mass has a substantially lower AIC than whole brain mass (*ASPM*: neonate AIC = 12.19, adult AIC = 14.67) suggesting a closer association with neonatal brain mass. Comparing adult neocortex and whole adult brain size, we found no substantial difference (*ASPM*: neocortex AIC = 7.43, whole

Fig. 2 Phylogenetically controlled regressions between root-to-tip dN/dS and absolute neonatal brain mass: for (a) *ASPM*, (b) *CDK5RAP2*, (c) *CENPJ*, and (d) *MCPH1*. Data points are raw species values, the phylogenetically controlled regression line was estimated in BayesTraits and superimposed on top of species data. For *ASPM*, two lines are displayed, the dashed line shows the regression when the outlier, *Callithrix* (labeled C) is included and the solid line shows the regression when it is excluded.
AIC = 7.40; CDK5RAP2: neocortex AIC = 16.29, whole AIC = 14.67). Hence, both the results are for ASPM and CDK5RAP2 are highly consistent.

To further explore the relationship between brain evolution and the molecular evolution of the microcephaly genes, we performed multiple regressions with neonatal brain size and root-to-tip dN and dS (log transformed) as independent variables. Significant negative partial regression coefficients were found, as predicted, for the three cytoskeletal genes and dS (one-tailed ASPM: $t_9 = -2.958, P = 0.007$; CDK5RAP2: $t_9 = -1.919, P = 0.042$; CENPJ: $t_9 = -1.859, P = 0.046$) but interestingly not for MCPH1 ($t_9 = -1.294, P = 1.000$). Neither CENPJ nor MCPH1, which show no association between brain size and dN/dS, show a significant association with dN (CENPJ: $t_9 = 0.049, P = 0.481$; MCPH1: $t_9 = -2.322, P = 1.000$). For ASPM, we do see an association between dN and neonatal brain size (ASPM: $t_9 = 2.032, P = 0.035$) but we do not find a significant association for CDK5RAP2 ($t_9 = 1.127, P = 0.142$). This suggests the association between ASPM and dN/dS may be driven predominantly by an accelerated dN, whereas the association for CDK5RAP2 may have a more complex basis.

As a final analysis, we used the branch-site test to detect positive selection on branches along which brain size is estimated to have expanded greatly (see Ali and Meier 2008). However, as discussed in the supplementary information (Supplementary Material online), we found this method was not informative as it is possible to get a positive result by selecting branches at random. This is likely to be due to positive selection acting across the phylogeny, as demonstrated by our site model tests, and strongly questions the strength of the methodology used and results obtained by Ali and Meier (2008), namely that episodes of adaptive evolution of ASPM are specifically associated with expansion of the relative size of the telencephalon.

### Controls for Specificity of Gene–Phenotype Associations

To exclude the possibility that the gene–phenotype correlations reported above are coincidental, we investigated eight loci (supplementary table S8, Supplementary Material online), with no known function in neurogenesis, for which data were already available for a reasonably large number of species ($n = 10–20$) across the anthropoid phylogeny. This control set includes both genes that have previously been shown to have experienced positive selection across anthropoids and genes which appear to be under purifying selection (supplementary table S9a, Supplementary Material online). We tested for an association between the root-to-tip dN/dS of these loci and absolute adult and neonatal brain mass in the same way as described above. No locus was found to have a significant association with either phenotype (supplementary table S9b, Supplementary Material online), and neither did the removal of any one species result in a significant change in the regression slope and an positive association with brain size (data not shown). This suggests that the significant results presented above are unlikely to be Type I errors.

### Discussion

#### Molecular Evolution of Microcephaly Genes and Brain Evolution

Studying the molecular basis of convergent phenotypes has enhanced our understanding of the evolutionary genetics of adaptation and the constraints that act on phenotypic evolution (Arendt and Reznick 2007). Here, we show that independent increases in brain mass across anthropoids may share a common genetic basis. By sampling a substantial number of phylogenetically diverse species, we have demonstrated that positive selection acted on four microcephaly loci across the anthropoid phylogeny and was not, as previously reported, restricted to lineages leading to humans. To our knowledge, our study is the first to implement robust codon-based models to test for positive selection acting on these loci across anthropoids.
(A previous study reporting such a finding [Wang and Su 2004] used Model 3 in PAML which is not a robust test for positive selection [Anisimova et al. 2002].) This is a striking result as pervasive positive selection is considered rare. However, as brain size has increased multiple times independently and is likely to have been under strong selection in all major groups of anthropoids (Montgomery et al. 2010), such widespread positive selection on genes involved in the evolution of brain size should perhaps be expected.

We explored whether this selection is relevant to gross brain size evolution using phylogenetically controlled regressions which show that the average dN/dS across ASPM and CDKSRAP2 is significantly related to absolute neonatal brain mass and that in both cases a relatively large proportion of the variance is explained (R² = 0.369 and 0.255, respectively). Furthermore, two key predictions of a gene’s involvement in prenatal neurogenesis were verified for ASPM and CDKSRAP2: 1) an association with absolute brain mass as this correlates closely with total neuron number (Herculano-Houzel et al. 2007) and 2) a stronger association with neonatal than adult brain mass as cortical neurogenesis is largely restricted to prenatal development (Rakic 1988, 2002; Bhardwaj et al. 2006).

These results are not explained by a general association with body mass nor can they be attributed to random or genome-wide effects because no associations were found with two other microcephaly genes (CENPJ and MCPH1) and eight control genes. Thus, although the results are only marginally significant, our control tests and the highly consistent pattern observed in the significant results strongly suggest that the associations found are unlikely to be Type 1 errors. The results using multiple regressions suggest that while positive selection on ASPM has brought about an increase in dN/dS mainly through an acceleration in dN relative to dS, the pattern for CDKSRAP2 may be more complex. This suggests that caution should be exercised in interpreting dN/dS ratios, and we recommend the use of supplementary analyses such as multiple regression to disambiguate correlations involving this measure. Together with the demonstration of positive selection in the site analyses, these results imply that adaptive evolution on ASPM and CDKSRAP2 has been involved in independent changes in brain size along multiple lineages during primate evolution through a role in prenatal neurogenesis.

Although we detect a general positive association for ASPM and CDKSRAP2, there are notable outliers, which may suggest a more complex relationship between the evolution of these loci and brain size. For both loci, Papio has a much lower dN/dS than would be predicted given our results and the size of its the brain in this species. Assuming the association between ASPM, CDKSRAP2 and brain evolution have a genuine, functional basis, the Papio discrepancy may indicate brain expansion can occur independently of the evolution of these loci. However, as Papio is not represented in our neonatal data set, we cannot say whether it is an outlier due to pre- or postnatal developmental processes. The high rate of evolution of ASPM during Callitrichid evolution is also striking. A plausible hypothesis is that this acceleration is related to phyletic dwarism and the reduction of brain size in this clade (Ford 1980; Montgomery et al. 2010). The lower rate of evolution of CDKSRAP2 in the Callitrichids raises the interesting possibility that different selection pressures have acted on these loci during episodes of brain size reduction. Further work is required to address these issues.

Despite demonstrating CENPJ and MCPH1 have experienced pervasive positive selection during anthropoid evolution, we found no significant relationship between either locus and any measure of absolute or relative brain mass. This potentially indicates an interesting dichotomy in evolutionary roles among microcephaly genes and raises the issue of whether these loci are involved in the evolution of other traits or more specific aspects of brain phenotype that were not considered here. It is important to emphasize that the phenotypes on which selection for CENPJ and MCPH1 is acting in primates has not been established, and this study provides no evidence that they are involved in the evolution of gross measures of size of the whole brain or neocortex.

Integrating Comparative Genetics and Neurobiology

Our results are consistent with models of how neuron number might evolve. A single additional round of proliferative, symmetric divisions of neuroepithelial cells in the ventricular zone would double the number of neurons in the cortex (Rakic 1988, 1995; Caviness et al. 1995). Neuroepithelial cells have apical–basal polarity, and the switch from proliferative, symmetric to neurogenic, asymmetric divisions is controlled by the orientation of the spindle pole during mitotic division (Chenn and McConnell 1995; Götz and Huttner 2005). An alternative, but not mutually exclusive, model places greater emphasis on prolonged intermediate progenitor cell division in the subventricular zone, which may also occur by changes in spindle pole orientation (Kriegstein et al. 2006). In addition, as brain size expands, neural progenitors become increasingly elongated (Smart et al. 2002; Fish et al. 2008) and selection may be acting on cytoskeletal genes in response to the need to maintain the precision of spindle orientation during mitotic division of these highly elongated cells in larger branched species (Zhang 2003; Kouprina et al. 2004; Fish et al. 2008). In this way, selection on these loci may be in response to the evolution of larger brains rather than causing the change in brain size. Although it is also possible to envisage scenarios where the change in spindle orientation itself leads to the production of additional neurons, whether the role of these loci in brain size evolution is causative or responsive is yet to be determined. Both scenarios are consistent with the results presented here.

Notably, there is strong agreement between the results of studies investigating the expression of these loci (Bond et al. 2002, 2005; Jackson et al. 2002; Kouprina et al. 2005), their function (Bond and Woods 2006; Cox et al. 2006;
Brain size.

significant roles in the evolution of neuron number and brain size.

Implications for Evolutionary Genetics of Adaptation

The inferred role of ASPM and CDK5RAP2 in the evolution of primate brain mass has implications for our understanding of the evolutionary genetics of adaptation. First, they provide evidence that a complex, polygenic quantitative phenotype evolved by convergence at the molecular level (Cresko et al. 2004; Mundy 2005; Arendt and Reznick 2007). Second, our results go against a commonly asserted hypothesis that evolution of form occurs primarily through changes in cis-regulatory sequences (King and Wilson 1975; Carroll 2005, 2008; Wray 2007).

Changes in cis-regulatory sequences are proposed to be more important for phenotypic evolution than their modular nature limits pleiotropic effects (Carroll 2005; but see Hoekstra and Coyne 2007; Lynch and Wagner 2008; Stern and Orgogozo 2008). In fact the evolution of brain mass has been singled out as an example where regulatory evolution is likely to be the predominant evolutionary mechanism: “the evolution of complex traits such as brain size . . . must have a highly polymorphic and largely regulatory basis” (Carroll 2005; emphasis ours). It is therefore interesting that the microcephaly genes are expressed throughout the body (Bond et al. 2005; Kouprina et al. 2005) but are subject to positive selection. If ASPM and CDK5RAP2 are in fact involved in brain evolution, how are pleiotropic effects avoided?

The first point to note is that the pathology of primary microcephaly itself shows that pleiotropic effects of microcephaly gene disruption can be limited to the brain (Bond and Woods 2006). Both ASPM and CDK5RAP2 are alternatively spliced (Kouprina et al. 2005; Buchman et al. 2010), which may reduce pleiotropic effects (Hughes 2006; Hoekstra and Coyne 2007; Lynch and Wagner 2008). Alternatively, the evolution of ASPM may not affect nonneural cells either due to the elongated cell morphology of neuroepithelial cells (Fish et al. 2006) or cell-dependent recruitment factors (Van der Voet et al. 2009). These explanations are not mutually exclusive and provide plausible mechanisms to reduce the pleiotropic effects of protein evolution (Hoekstra and Coyne 2007; Lynch and Wagner 2008). More broadly, research is showing a diversity of mechanisms for the possible genetic basis of aspects of primate brain evolution, including coding sequence evolution (e.g., this study, Wang et al. 2005; Vallender and Lahn 2006; Uddin et al. 2008), gene duplication (Burki and Kaessmann 2004; Marques-Bonet et al. 2009), noncoding RNA evolution (Zhang et al. 2008), and changes in regulatory sequences and gene expression (Khaitovich et al. 2005, 2006; Rockman et al. 2005; Gilad et al. 2006; Prabhakar et al. 2006; Haygood et al. 2007; Somel et al. 2009). Indeed, the mosaic nature of brain structure evolution in mammals indicates a complex genetic basis to changes in brain size (Barton and Harvey 2000).

Conclusions

We have presented evidence implicating ASPM and CDK5RAP2 in the evolution of brain size across anthropoid primates, a result which is consistent with an effect of the two loci on neurogenesis via mitotic spindle orientation. Despite showing that CENPJ and MCPH1 have been subject to positive selection, we find no evidence to link these loci to the evolution of gross brain size, and the mechanism of selection acting on these loci is therefore unresolved. This study demonstrates the importance of including phenotypic data and a phylogenetically broad range of species when attempting to associate the evolution of genes with brain size evolution (Carroll 2003; Goodman et al. 2005; Barton 2006a; Pollen and Hoffmann 2008; Vallender 2008). This point is especially pertinent to the literature on human genetic evolution where claims are often based on differences between humans and chimpanzees or a small number of nonhuman primates. The results also clearly highlight the importance of including measures of neonatal brain size in studies of primate brain evolution. Finally, our results suggest a conserved genetic basis for brain evolution in primates, providing an important example where genetic basis of a complex developmental phenotype has involved coding sequence evolution.

Supplementary Material

Supplementary information, figure S1, and tables S1–S9 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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References


