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Genetics of cortico-cerebellar expansion in anthropoid primates: a comparative approach

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Abstract

What adaptive changes in brain structure and function underpin the evolution of increased cognitive performance in humans and our close relatives? Identifying the genetic basis of brain evolution has become a major tool in answering this question. Numerous cases of positive selection, altered gene expression or gene duplication have been identified that may contribute to the evolution of the neocortex, which is widely assumed to play a predominant role in cognitive evolution. However, the neocortex co-evolves with other, functionally inter-dependent, regions of the brain, most notably the cerebellum. The cerebellum is linked to a range of cognitive tasks and expanded rapidly during hominoid evolution, independently of neocortex size. Here we demonstrate that, across primates, genes with known roles in cerebellum development are just as likely to be targeted by selection as genes linked to cortical development. In fact, cerebellum genes are more likely to have evolved adaptively during hominoid evolution, consistent with phenotypic data suggesting an accelerated rate of cerebellar expansion in apes. Finally, we present evidence that selection targeted genes with specific effects on either the neocortex or cerebellum, not both. This suggests cortico-cerebellar co-evolution is maintained by selection acting on independent developmental programs.

Introduction

The proximate basis of primate brain expansion and increased cognitive performance lies in changes in gene function and regulation. Identifying the genetic basis of phenotypic change can provide insights into how developmental mechanisms evolve, how they are constrained, and how changes at a cellular level contribute to broad scale anatomical evolution (1). This potential for dissecting the biological basis of brain and behavioural evolution motivates many genomic comparisons across primates (2). These have identified numerous genes associated with brain development with high rates of evolution (3–9), divergent expression profiles (10–13) or duplicated sequence (14–17), either across primates or during recent human evolution. In several of these cases the genetic changes have been demonstrated to have functional effects on neuronal proliferation or maturation (7,8,15,18,19). These results highlight potential cellular adaptations driving changes in brain size, and provide a powerful means of investigate human-specific adaptations.

The majority of these examples investigate genes linked to neocortical evolution, reflecting the widely held assumption that the neocortex has a predominant role in 'higher' cognition (20). However, the neocortex co-evolves with other brain components with which it is functionally connected, suggesting a complete understanding of primate brain expansion will not be found by focusing solely on neocortex development (21–23). Of particular importance is the relationship between the neocortex and cerebellum. Across primates cortico-cerebellar co-evolution pervades biological levels, occurring at a coarse volumetric scale, at the level of individual nuclei, and at the cellular level (21,22,24).

In humans, the cerebellum is increasingly recognised to be important for both motor and 'higher cognitive' function, including the capacity to plan and execute complex behavioural sequences (20,23,25). Similarly, across primates, cerebellar expansion is linked to extractive foraging, independently of neocortex volume (23). The importance of the cerebellum in primate brain evolution is further bolstered by comparative analyses that demonstrate a rapid, non-allometric expansion of the cerebellum relative to neocortex size in hominoids that is indicative an adaptive change in the cortico-cerebellar functional relationship (26–29). Hominin evolution is also characterised by reciprocal expansion of the neocotex and cerebellum, with recent modern humans being distinguished from our early modern humans by an increase in cerebellum volume relative to the neocortex (30).

The importance of coordinated cortico-cerebellum expansion suggests that we must look beyond neocortical evolution to obtain a full picture of the genetic architecture of primate brain evolution. The likely action of positive selection on genes involved in

cerebellum development is further suggested by the accelerated rate of evolution of *AHI1*, a gene associated with developmental disorders of the cerebellum, during human evolution (31). In addition, genetic approaches may be useful in addressing long-standing debates about the constraints governing co-evolving brain networks. For example, do the neocortex and cerebellum co-evolve through genetically independent developmental changes maintained by selection (*sensu* Barton and Harvey (21))? Or are they the result of pleiotropic genetic effects shared across anatomical boundaries (*sensu* Finlay and Darlington (32))? If the latter is true, do these pleiotropic effects reflect an evolutionary constraint, or have they evolved to maintain the functional relationship between components during brain expansion? These questions will be central to providing a full understanding of brain evolution, and also address questions of long standing interest in evolutionary biology on the trade-offs between adaptation and constraint in the genetic basis of composite traits.

Here we ask three questions that provide an initial assessment of the role of genes controlling the development of the neocortex and cerebellum in anthropoid brain evolution. First, we ask whether genes with known roles in the development of the cerebral cortex, which is predominantly composed of the neocortex, are more likely to be targets of positive selection than those affecting cerebellum development. Second, whether patterns of molecular evolution mirror non-allometric changes in component size. Finally, whether these genes evolve in a manner suggesting a specific evolutionary association with either brain component to explore whether cortico-cerebellar co-evolution is maintained by selection acting on a common or independent set of genes.

Methods

Data collection

We obtained a list of human genes with known roles in 'cerebral cortex development' or 'cerebellum development' from two ontology databases; Amigo (33) (GO:0021987 and GO:0021549) and EBI's QuickGO (34) (GO:0021987 and GO:0021549). These GO terms have the best-matched definitions among those relevant for each component. In anthropoids, the neocortex comprises the vast majority of the cerebral cortex (~90%; Stephan et al. 1981). This GO search resulted in 198 Amigo and 300 QuickGO genes associated with cerebral cortex development, and 144 Amigo and 222 QuickGO genes associated with cerebellum development, which are not mutually exclusive, that were then combined to form the starting gene set. This starting human gene set was then used to obtain 1:1 orthologs from 11 anthropoid genomes (Figure 1A) using a reciprocal best hit BLASTn (36) approach between

each species and the human coding sequence with an e-value cut-off of $1e^{-10}$ and a minimum percentage identify of 30. 11-way 1:1 ortholog sets were aligned using codon aware PRANK v 140615 (37), converted to phylip format in preparation for PAML analyses (38) and filtered using a conservative alignment filtering program SWAMP v 1.0 (39) to remove regions of poorly aligned or error-rich sequences on a branch-specific basis. SWAMP was run twice, first using a threshold of 5 and a window size of 15, and then a second run with a threshold of 2 with a window size of 3, with a minimum sequence length of 300 bases and *interscan* masking for both runs. The final, strict and conservatively filtered 11-species dataset consisted of three non-overlapping groups: 53 genes with known roles cerebral cortex development, 47 genes with known roles in cerebellum development, and 10 genes with known roles in both (Table S1).

Selection analyses

Estimation of *dN/dS* ratios (ω), a common measure of the strength of selection acting on a protein coding gene, was carried out using a codon-based maximum likelihood method (PAML v.4) (38). Nested models were compared using the likelihood ratio test statistic (−2[loglikelihood1 – loglikelihood2]) to critical values of the χ^2 distribution and degrees of freedom as the difference in the number of parameters estimated in each model. We compared the frequency of positive selection acting on genes associated with cerebral cortex and cerebellum development in two ways. First, we used the site model tests of positive selection ('Model 8/8a') to identify genes evolving under positive selection across anthropoids. The site models allow ω to vary across sites, but not across branches. Second, we used the branch-site models ('new model A') to identify genes under increased positive selection in hominoids (Figure 1A). The branch-site models allow ω to vary across both sites and branch categories defined a priori. We repeated this test for accelerated rates of evolution in hominoids using a branch model test, where ω is fixed across sites but varies between branch categories. For each analysis, the percentage of each category of gene that experienced selection were compared using a Z-test and more conservative Fisher's Exact Test.

Gene-phenotype co-evolution

We sought to test the link between the molecular evolution of our gene-set and the evolution of neocortex and cerebellum size using a phylogenetic, comparative approach (40). Branch models in PAML were used to calculate the root-to-tip *dN/dS* for each species. These were

then regressed against the phenotypic trait of interest using a Phylogenetic Generalised Least Squares (PGLS) regression implemented in BayesTraits (41), that corrects for the non-independence of inter-specific data. Each gene was regressed against neocortex volume and cerebellum volume as predictor variables simultaneously. This permits the identification of genes with either a specific co-evolutionary association with either neocortex or cerebellum size, or genes that co-vary with both traits independently. We also repeated the analyses including rest-of-brain volume as an additional predictor variable, which lead to similar results. In all cases root-to-tip *dN/dS* and brain component volumes were $\log_{10}$-transformed.

Results

What proportion of genes with known roles in cerebral cortex or cerebellum development are targeted by positive selection?

Across anthropoids, the average rate of evolution of genes with known roles in the development of the cerebellum does not differ from those that function in the development of the cerebral cortex, which is predominantly comprised of neocortex (Mann-Whitney $U_{(98)}$ = 1167.5, Z = 0.539, p = 0.590) (Table S2). Genes linked to the development of both brain components do not differ from those specifically linked to cerebral cortex ($U_{(61)}$ = 296.5, Z = 0.592, p = 0.554) or cerebellum development ($U_{(55)}$ = 241.5, Z = 0.136, p = 0.892). Site-model tests for positive selection acting at a subset of codons also identify a similar proportion of genes associated with cerebral cortex or cerebellum development with evidence of positive selection across anthropoids. 3/47 cerebellum genes (6.4%) are significant at a nominal α of 0.05, compared to 5/53 cerebral cortex genes (9.4%). These proportions are not significantly different (Fisher's Exact Test, p = 0.716, Z-test: z = 0.5613. p = 0.575) (Table, 1; Figure 1B; Table S3). The same conclusion is reached after correcting for multiple testing using the false discovery rate (FDR) (42), after which the site-model test is significant for two genes linked to cerebellum development (*RPGRIP1L*, *PCNT*) and one gene linked to cerebral cortex development (*TACC2*). None of the 10 genes with annotated function in the development of both brain components show evidence of positive selection. These results suggest genes affecting cerebellum development are just as likely to be targeted by positive selection as those affecting cerebral cortex development.

Do rates of molecular evolution reflect rates of brain component expansion?

A significantly greater proportion of cerebellum genes (14/47, 29.8%) than cerebral cortex genes (6/53, 11.3%) experienced an accelerated rate of evolution in hominoids, taking a

nominal α of 0.05 (Fisher's Exact Test, p = 0.026, Z-test: z = 2.304, p = 0.021) (Table 1; Figure 1C; Table S4). This result is also reflected in the branch-site test where the proportion of cerebellum genes (6/47, 12.8%) with evidence of episodic positive selection in hominoids again exceeds the proportion of cerebral cortex genes (2/53, 3.8%). In this case the trend does not reach significance (Fisher's Exact Test, p = 0.143, Z-test: z = 1.654, p = 0.099) (Table S5). However, one assumption of the branch-site test is the absence of positive selection in the background (non-hominoid) branches (43). After excluding genes with evidence of positive selection across anthropoids under the site-model test (Table S3), the trend observed in the branch-site test becomes significant at $p < 0.05$ (Fisher's Exact Test, p = 0.049, Z-test: z = 2.136, p = 0.032) (Table 1; Figure 1D). These contrasting proportions suggest the strength of positive selection acting on genes controlling cerebellum development increased during hominoid evolution, a clade in which the rate of cerebellar expansion significantly accelerated (29), without a corresponding acceleration in cerebral cortex expansion (21,44).

Is selection associated with interspecific variation in the size of specific brain components?

We identify 11/47 cerebellum genes (23.4%) that co-evolve with cerebellum volume, after controlling for neocortex volume, of which 4 are significant at $p < 0.001$ and 2 (*RPGRIP1L*, *ATRN*) remain significant after FDR-correction (Table 1; Figure 2; Table S6). We re-nalaysed the top four genes associated with cerebellum volume, separating *dN* and *dS* whilst controlling variation in neocortex volume, to test if the association is driven by variation in *dN*. For 3/4 genes we find a significant partial regression with *dN* (*ATRN* $t_5 = 3.789$, p = 0.006; *EZH2* $t_5 = 3.990$, p = 0.005*; KNDC1* $t_5 = 3.586$, p = 0.008), the remaining locus showed a non-significant trend (*RGRIP1L* $t_5 = 1.582$, p = 0.087). Only one cerebellum gene (2.1%) shows an association with neocortex volume, which is a significantly lower proportion (Fisher's Exact Test, p = 0.004, Z-test: z = 3.091, p = 0.002). 1/53 cerebral cortex genes (*DICER1*) shows evidence of co-evolution with neocortex volume (1.9%), whilst 6 (11.3%) show evidence of an association with cerebellum size (Table S6). This is not a significant difference in proportion (Fisher's Exact Test, p = 0.113, Z-test: z = 1.955, p = 0.050), only one of these associations is significant at $p < 0.001$ and none survive FDR-correction. One of the ten genes annotated as functioning in both neocortex and cerebellum development (*GART*) shows an association with cerebellum, but not neocortex, volume (Table S6). Similar results were obtained when rest-of-brain was included in the regression model. No gene shows an association with variation in both neocortex and cerebellum

238 volume, regardless of the gene category or regression model. These results suggest selection
239 acts on genes associated with phenotypic variation specific to each brain component.
240
241 **Discussion**
242 Primate brain expansion reflects increases in the volume and neuron number of co-evolving
243 structures (21). This pattern of distributed adaptation must be reflected in the molecular
244 evolution of genes controlling brain development. Our results provide two contributions to
245 understanding the genetic basis of brain evolution. First, we provide evidence that genes
246 associated with cerebral cortex development are no more likely to have evolved adaptively
247 across anthropoids than cerebellum genes. Indeed, significantly more cerebellum genes
248 experienced an increased rate of evolution in hominoids, consistent with the non-allometric
249 expansion of the cerebellum, but not the neocortex, in apes (29). Second, we provide
250 evidence that the selection regimes shaping these genes are linked to the evolution of specific
251 brain components.
252 Our results raise the possibility that a significant proportion of the genetic changes
253 that underpin adaptive evolution of primate brain size, structure and cognition will affect
254 aspects of non-cortical development. This conclusion is consistent with evidence of adaptive
255 expansion in cerebellum volume during hominoid evolution (29), and is further supported by
256 a phylogenetic analysis of lineage-specific shifts in gene expression across mammals that
257 found an over-abundance of hominoid-specific expression shifts in genes expressed in the
258 cerebellum (10). These results suggest future comparative studies of primate gene expression
259 should not be limited to samples derived from cortical tissue, and functional tests of
260 candidate genes targeted by positive selection should consider the phenotypic relevance of
261 changes in gene function in non-cortical structures.
262 Of course, our results are wholly dependent on the quality of gene ontology
263 annotation. Our incomplete knowledge of gene function means that it is inevitable that the
264 genes included in this study reflect a minority of those that influence cerebral cortex and
265 cerebellum development. However, gene ontology datasets are routinely used in post-hoc
266 tests for functional enrichment where the same caveats apply, here we have simply used the
267 available data to facilitate tests of specific hypotheses. We know of no reason why either
268 gene set may be biased in a way that would produce contrasting patterns of results, but cannot
269 formally rule out this possibility. Finally, in this study we adopted a conservative approach,
270 analysing only genes with strict 11-way 1:1 orthologues in published anthropoid genomes

and removing short and/or poorly aligned sequence. This further reduces the gene set, but produces more reliable estimates of selection regimes (39).

Our analyses highlight several genes with patterns of molecular evolution that link them to inter-specific differences in cerebellum volume. These include two genes, *RGRIP1L* and *ATRN,* with a particularly strong signal of co-evolution between the strength of selection acting on their coding sequence and cerebellum volume, independently of variation in neocortex or rest-of-brain volume, in our gene-phenotype association tests (Figure 2). *RGRIP1L* is one of a small number of loci linked to Joubert Syndrome, a rare genetic disorder associated with severe hypoplasia of the mid-hindbrain and cerebellum (45,46). The cellular role of *RGRIP1L* appears to be in the correct function of the cilia and basal bodies (47,48) which are necessary for expansion of the cerebellar neural progenitor pool (49). Disruption of *ATRN*, an E3 ubiquitin ligase, in *Mus* causes vacuolization and degeneration of the cerebellum (50,51). Another ubiquitin ligase, *UBE3A*, has been implicated in brain and cognitive development (52) and interacts with *ASPM*, a key regulator of brain size (53). A third gene, *EZH2*, also shows evidence of a phenotypic association with cerebellum volume but is narrowly non-significant after FDR correction. *EZH2*, functions to regulate neuronal migration of precerebellar neurons during the development of the cortico-cerebellar connectivity (54).

We also find multiple genes with significantly accelerated rates of evolution in hominoids, coincident with an accelerated rate of cerebellar expansion (29). Several of these genes also show an association with cerebellum volume in our gene-phenotype association tests at a nominal α threshold of 0.05. These include *AGTPBP1*, disruption of which causes cerebellar Purkinje cell degeneration (55), *PCNT,* which causes primordial dwarfism with microcephaly (56), *TH*, a gene linked to two disorders which affect motor control, Segawa Syndrome and Parkinson's (57,58), and *MYO16* and *GART* which have putatively been linked to Autistic Spectrum Disorders and Down Syndrome respectively (59,60).

Finally, we identify patterns of molecular evolution that may implicate a separate group of genes in the evolution of neocortex size. Only one locus shows a phenotypic association with neocortex volume; *DICER1*, which regulates neurogenesis in the developing cerebral cortex in a time dependent manner (61,62). Notably, the two genes with the strongest evidence for episodic positive selection in hominoids belong to the same gene family. *TACC1* and *TACC2* are both associated with regulation of nuclear migration and are required for normal patterns of self-renewal in neural progenitors. TACCs interact with a centrosomal protein, CEP120, to regulate nuclear migration and the self-renewal of cortical neural

progenitor cells by controlling microtubule growth (63). A similar function is thought to mediate the influence of microcephaly genes on brain development (18,64).

Beyond functional affects of individual genes, our approach has the potential to tackle fundamental questions about how composite or modular tissues, such as the brain, evolve. For example, two models of brain evolution dominate debates surrounding the adaptive significance of variation in brain structure. One model proposes a conserved developmental program drives a 'concerted' pattern of brain evolution, with selection shaping the overall size of the system but not individual components(32,65). An alternative model instead argues that different brain regions evolve independently of overall brain size to meet species-specific behavioural needs, resulting in a 'mosaic' pattern of brain evolution, but may co-evolve due to functional interdependence (21,66). These two models implicitly make contrasting predictions about the genetic architecture of brain structure. The concerted model predicts variation in the size of different brain regions will be determined by genetic correlations between those structures, i.e. variation at a common set of genes. The mosaic model instead predicts that the development of different brain regions must be at least partially distinct in order to facilitate independent evolution.

In recent years these predictions have been tested using quantitative genetics within a range of vertebrates, either using wild pedigrees (67–69), inbred strains (70) or divergent populations/domestic breeds (71,72). In support of the mosaic brain hypothesis, these have found little evidence for widespread genetic co-variation between major brain components. Similarly, large genome-wide association studies within humans that have identified independent genetic bases associated with brain regions (73,74). Quantitative genetics assesses the phenotypic associations of standing genetic variation within populations and their relevance to macroevolution depends on the relative frequency at which selection acts on *de novo* mutations that may cause different patterns of genetic correlation. Our results therefore complement these intra-specific studies, providing the first inter-specific test designed to identify genes associated with neocortex and/or cerebellum evolution. These two structures show a consistent pattern of co-evolution across primates (21,22,24), reflecting their functional inter-dependence (23,25). Our analyses did not identify any gene that co-varies with both structures, but does identify multiple genes with a specific association with either the cerebellum or neocortex. This suggests the coevolution of these structures is unlikely to be solely due to genetic integration or pleiotropy. If more broadly true, this conclusion bolsters the interpretation of cortico-cerebellar coevolution as indicating adaptive co-evolution, maintained by selection acting on distinct developmental pathways.

Comparative functional analysis of the genes highlighted by our analyses will be necessary to confirm and extend these conclusions. These will also be needed to address key questions beyond functional effects. For example, when disrupted, several of the genes highlighted by our analyses affect the development of multiple organs. For example, both *AGTPBP1* and *PAFAH1B* have known roles in spermatogenesis (75,76), whilst disruption of *PCNT* can affect global somatic growth (56). If these genes do have a specific evolutionary role in cerebellar development, how are these pleiotropic effects avoided? Similar questions have been raised over previous candidate genes (3,18), further emphasising the importance of coupling comparative and functional data.

In summary, we have presented an analyses aimed at providing an initial assessment of the strength of selection targeting genes with development roles in distinct brain regions. Although our understanding of gene ontology is incomplete, we illustrate how this information can be used to test hypotheses in a phylogenetic comparative setting, in addition to post-hoc enrichment analyses. We highlight that there is currently no evidence that selection is limited or biased towards genes affecting cerebral cortex development, and encourage evolutionary geneticists to adopt a cohesive view of brain evolution that encompasses the recognised importance or non-allometric expansion of non-cortical regions, and to tackle the central question of co-evolution and relative genetic independence of brain components. Finally, we further illustrate the potential of hypothesis driven comparative genetics in dissecting the genetic basis of phenotypic evolution. The ever-increasing numbers of sequenced genomes will permit increasingly powerful analyses of the targets of selection and their phenotypic relevance.

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585 **Figure legends:**

586

587 **Figure 1 A)** Phylogeny of the 11 anthropoids included in the study: hominoid lineages are
588 shown in orange. **B-D)** Pie-charts showing the proportion of genes significant (darker colour)
589 under each test for cerebral cortex (red) and cerebellum genes (green). The p-value from a
590 Fisher's Exact test comparing the groups is shown below.

591

592 **Figure 2 Phenotypic associations for two genes A) *RGRIP1L* and B) *ATRN*.** The $\log_{10}$-
593 transformed root-to-tip (rtt-) *dN/dS* is plotted against residual cerebellum volume, calculated
594 from a PGLS regression of cerebellum against neocortex volume. The black line shows the
595 result of a phylogenetically-controlled regression between rtt-*dN/dS* and residual cerebellum
596 volume. This figure is for illustrative purposes, in the main analyses the two brain
597 components were included as variables in a multiple regression.

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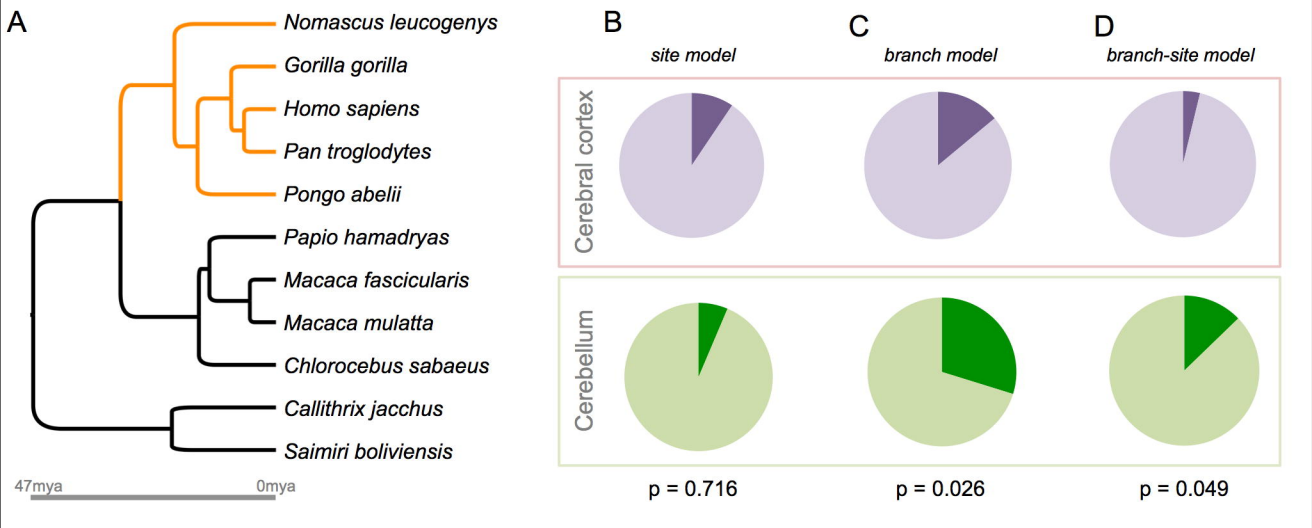
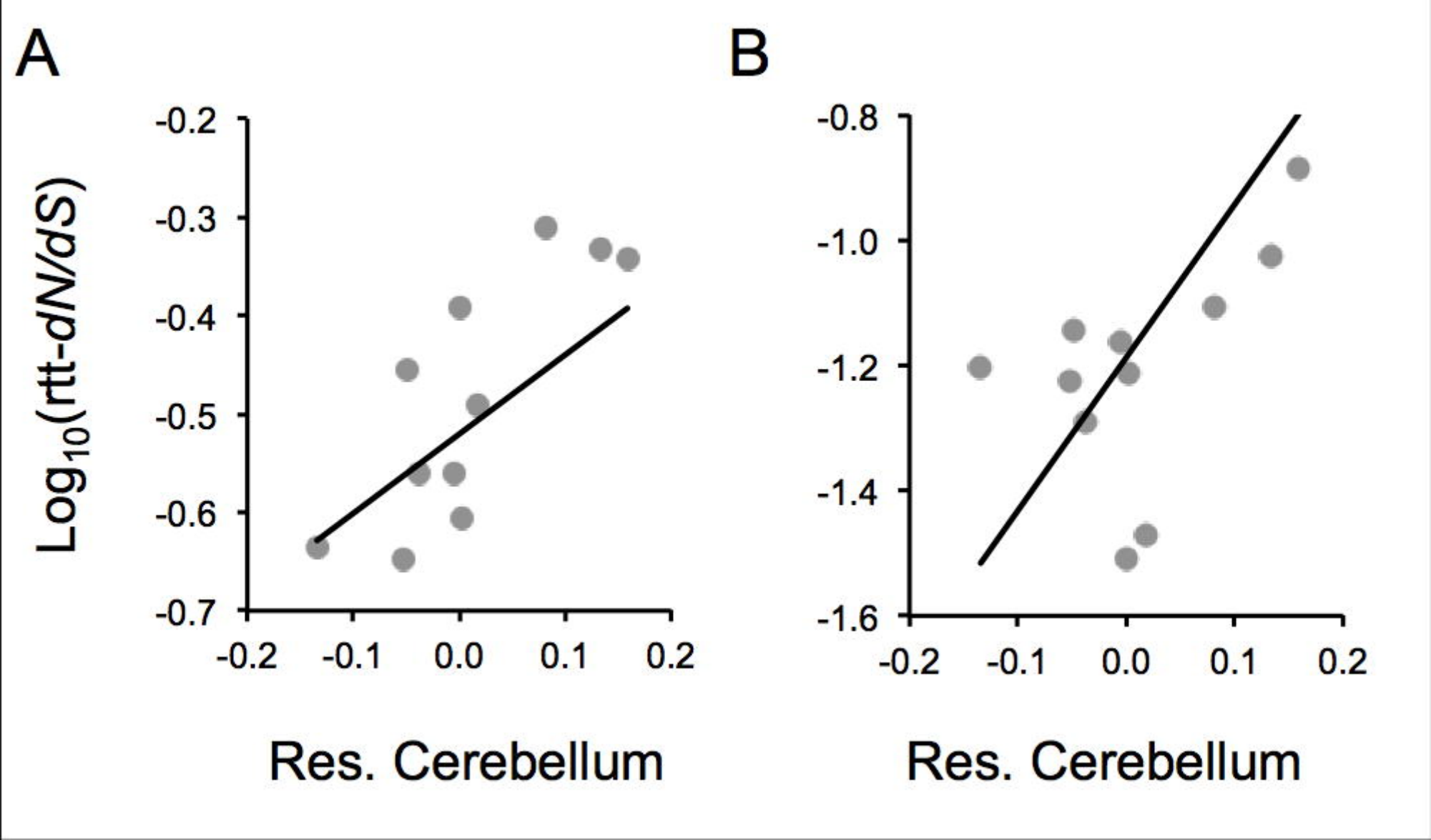
Tables

Table 1: summary of results of *dN/dS* analyses and tests of positive selection

Test	Inference	Significant results (proportion)		Difference in proportion	
		Cerebral cortex (n = 47)	Cerebellum (n = 53)	Z-test	Fisher's Exact Test
Site model test	Pervasive positive selection	5 (9.4%)	3 (6.4%)	z = 0.561, p = 0.575	p = 0.716
Branch model test	Accelerated evolution in apes	6 (11.3%)	14 (29.8%)	z = 2.304, p = 0.021	p = 0.026
Branch-site model test (all)	Episodic positive selection in apes	2 (3.8%)	6 (12.8%)	z = 1.654, p = 0.099	p = 0.143
Branch-site model test (restricted[1])	Episodic positive selection in apes	0 (0.0%)	4 (10.0%)	z = 2.136, p = 0.049	p = 0.032

[1] excludes genes with significant site-model results


A
Nomascus leucogenys
Gorilla gorilla
Homo sapiens
Pan troglodytes
Pongo abelii
Papio hamadryas
Macaca fascicularis
Macaca mulatta
Chlorocebus sabaeus
Callithrix jacchus
Saimiri boliviensis
47mya
0mya
B
site model
C
branch model
D
branch-site model
Cerebral cortex
Cerebellum
p = 0.716
p = 0.026
p = 0.049


A
B
Log10(rtt-dN/dS)
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-1.0
-1.2
-1.4
-1.6
-0.2
-0.1
0.0
0.1
0.2
Res. Cerebellum
Res. Cerebellum