Ecological trait differences are associated with gene expression in the primary visual cortex of primates

Trisha Marie Zintel
University of Massachusetts Amherst https://orcid.org/0000-0001-7356-0867
John J. Ely
MAEBIOS
Mary Ann Raghanti
Kent State University
William D. Hopkins
University of Texas MD Anderson Cancer Center Michale E Keeling Center for Comparative Medicine and Research
Patrick R. Hof
Icahn School of Medicine at Mount Sinai Friedman Brain Institute
Chet C. Sherwood
The George Washington University
Jason M. Kamilar
University of Massachusetts Amherst
Amy L. Bauernfeind
Washington University in Saint Louis School of Medicine
Courtney C. Babbitt (cbabbitt@bio.umass.edu)

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Ecological trait differences are associated with gene expression in the primary visual cortex of primates

Trisha M. Zintel [1,2], John J. Ely[3], Mary Ann Raghanti[4], William D. Hopkins[5], Patrick R. Hof[6,7], Chet C. Sherwood[8], Jason M. Kamilar[9,10], Amy L. Bauernfeind[11,12], and *Courtney C. Babbitt[1,2]

[1]Department of Biology, University of Massachusetts Amherst, Amherst, MA;
[2]Molecular and Cellular Biology Graduate Program, University of Massachusetts Amherst, Amherst, MA;
[3]MAEBIOS, Alamogordo, NM;
[4]Department of Anthropology, Kent State University, Kent, OH;
[5]Keeling Center for Comparative Medicine and Research, The University of Texas, MD Anderson Cancer Center, Bastrop, TX;
[6]Nash Family Department of Neuroscience and Friedman Brain Institute, Icahn School of Medicine at Mount Sinai, New York, NY;
[7]New York Consortium in Evolutionary Primatology,
[8]Department of Anthropology and Center for the Advanced Study of Human Paleobiology, The George Washington University, Washington, DC;
[9]Department of Anthropology, University of Massachusetts Amherst, Amherst, MA;
[10]Organismic and Evolutionary Biology Graduate Program, University of Massachusetts Amherst, Amherst, MA;
[11]Department of Neuroscience, Washington University School of Medicine in St. Louis, St. Louis, MO;
[12]Department of Anthropology, Washington University in St. Louis, St. Louis, MO

*Corresponding author:
Courtney C. Babbitt, Ph.D.
Dept. of Biology
611 North Pleasant St.
Amherst, MA, 01003, USA
413-545-5574
cbabbitt@bio.umass.edu

ABSTRACT

Background: Primate species differ drastically from most other mammals in how they visually perceive their environments, which is important for foraging, predator avoidance, and detection of social cues. Although it is well established that primates display diversity in color vision and various ecological specializations, it is not understood how visual system characteristics and ecological adaptations may be associated with gene expression levels within the primary visual cortex (V1).

Results: We performed RNA-Seq on V1 tissue samples from 28 individuals, representing 13 species of anthropoid primates, including hominoids, cercopithecoids, and platyrrhines. We explored trait-dependent differential expression (DE) by contrasting species with different visual system phenotypes and ecological traits. Between 4-25% of genes were determined to be differentially expressed in primates that varied in type of color vision (trichromatic or polymorphic di/trichromatic), habitat use (arboreal or terrestrial), group size (large or small), and primary diet (frugivorous, folivorous, or omnivorous). DE analyses revealed that humans and chimpanzees showed the most marked differences between any two species, despite the fact that they are only separated by 6-8 million years of independent evolution. Pathway enrichment analyses of DE genes demonstrated that changes in cellular metabolic pathways (e.g. glycolysis) contribute to altered gene expression in primate V1 more than neuron-specific processes (e.g. synaptic signaling). The exception to this trend is between human and chimpanzee, which exhibited DE for a number of processes related to cholinergic and GABAergic synaptic signaling.

Conclusions: Our data significantly expand the number of primate species for which V1 expression data exists. These results show a combination of species-specific and trait-dependent differences in the evolution of gene expression in primate V1. We also show that human-specific changes in brain gene expression extend to the primary visual cortex in a manner similar to that reported of other brain regions.

KEYWORDS

Brain, evolution, metabolic, phenotype, genomics, visual cortex, gene expression

BACKGROUND

In most mammals, vision is critical for foraging, predator avoidance, and mate recognition [1-5]. However, primates are distinguished from other mammals by unique visual traits, including high visual acuity and variation in color perception [2, 5-9]. Primates with forward-facing eyes have high orbital convergence and correspondingly enhanced binocular vision resulting in greater depth perception (stereoscopic vision) and increased visual acuity [10]. Primates also possess a uniquely specialized fovea, a localization of cone receptors in the retina, that allows for greater resolution [11, 12]. Among primates, visual acuity is highest in diurnal haplorrhine species [13-17]. Trichromatic color vision is a hallmark of hominoid and cercopithecoid species, but color vision varies significantly across platyrrhine species as well as within some species that have polymorphic sex-linked di/trichromacy [18].

Higher visual acuity and a shift to trichromatic color vision are hypothesized to confer a number of benefits across primate species. High visual acuity has been suggested to be adaptively favorable in relation to diet (i.e. detecting insects) as well as for arboreal species navigating tree limbs [10]. At the level of opsin gene conservation across primates and tree shrews, typical light conditions that require enhanced visual acuity are the same as those in which color vision is favored, representing a putative adaptive link between enhanced acuity and trichromacy [8]. The evolution of a third opsin gene increases the range of detectable wavelengths (especially in the range of red hues) and has been hypothesized to be influenced by foraging and social pressures [19, 20]. The foraging hypothesis stresses the importance of detecting red ripe fruits and young leaves against a green foliage background [1-3, 21, 22]. The social signal hypothesis states that wavelength sensitivity for red hues is important for accurate interpretation of skin color changes indicative of receptivity, sociality, or health [19, 23, 24]. Evidence in a lemur species with mixed populations of trichromats and dichromats showed that there is a group level benefit when at

least a single trichromatic individual is present, likely linked to the ability to locate ripe fruit during the dry season, thus positively influencing fitness [22].

The primary visual cortex (V1) is the first cortical region in the visual pathway and where visual inputs converge from both eyes. Inputs from both eyes that impart binocular vision are critical for detecting edge orientation, thus playing an important role in depth perception [9, 25]. Increased orbital convergence allowing for binocular vision correlates with an increase in size in brain regions associated with visual perception [26]. Neuronal density within V1 of primates is more than twice that of other mammals, reflecting the importance of vision for primates [9, 10]. There is also known variation in V1 neuronal cytoarchitecture across hominoid species, including molecular differences in cytochrome oxidase staining in layer 4A [27, 28] and interneuron density [29, 30] as measured by immunohistochemistry. Taken together, these findings may indicate an adaptive advantage of visual processes that could differentially influence V1 function in primates. However, we still have little knowledge about the genetic mechanisms driving V1 variation across species and how this correlates with differences in visual acuity and perception.

Given the high degree of homology between humans and chimpanzees at the nucleotide and protein levels, King and Wilson [31] hypothesized that the extensive phenotypic differences must be due to changes in gene expression rather than coding-sequence evolution. Investigations of differential expression (DE) across primates have indeed demonstrated that *cis*-regulatory changes are critical for large-scale differences in phenotype [32-36]. Gene expression tends to vary across different bodily tissues, while different brain regions have region-specific profiles indicative of localized regulation [37-42]. Enrichments for neuron-specific processes (e.g. synaptic signaling) and metabolism (e.g. carbohydrate and lipid metabolism) have been reported in human brain gene expression compared to chimpanzee and rhesus macaque [33-35] and may be indicative of region-specific differences in neural function.

Few investigations of interspecific gene expression have focused on differences in the primate visual cortex [38, 43-45]. Khaitovich et al. [38] investigated several brain regions, including V1, with the

primary focus on determining the extent of region-specific differences in expression between three primate species (human, chimpanzee, and macaque). These authors found that regions of the cerebral cortex, including V1, differ significantly from other brain regions, such as the cerebellum, and that the degree of intraspecific variation in expression between these brain regions was significantly greater in human than chimpanzee. Furthermore, they reported that in the cerebral cortex of both species, V1 was an outlier among other regions investigated (dorsolateral prefrontal cortex, anterior cingulate cortex, and Broca's area) [38]. Another study found coexpression networks specific to V1 in both humans and chimpanzees and found the greatest degree of interspecific conservation in V1 compared to any other region investigated [45]. The authors attributed these findings to the sensory nature of V1's function for which humans and chimpanzees presumably differ very little [45]. A comparison of transcription between human, macaque, and mouse V1 using microarrays found that primate (human and macaque) V1 expression was conserved in comparison to mouse, suggesting that the conserved genomic profiles of V1 extends beyond primates [43]. These studies represent important initial investigations of V1 gene expression, however, they investigated few primate species (humans, chimpanzees, rhesus macaques) and emphasized other brain regions over V1.

To understand primate visual system adaptations better at the gene expression level, we used RNA-Seq to quantify gene expression in V1 of 13 primate species across three major clades (hominoids, cercopithecoids, and platyrrhines), significantly increasing the number of primates for which V1 gene expression data exists. This broad phylogenetic sampling of phenotypically diverse primates allowed us to explore how differences in gene expression were associated with variation in traits that contribute to a species' visual perception, including differences in species-typical color vision (trichromats vs polymorphic di/trichromats), and ecological variables such as habitat use (arboreal vs terrestrial), average group size (large vs small), and typical diet (folivore vs frugivore vs omnivore). We also evaluated the influence of sex on gene expression in V1. We found significantly differentially expressed genes in V1 when comparing global gene expression based upon extremes in species-typical traits for color vision, habitat use, group size and diet, but not sex. Pathway enrichment analyses of genes exhibiting differential

expression (DE) showed that expression changes correlated with variation in visually-relevant traits as well as expression differences between species are largely driven by altered metabolic signaling. Human and chimpanzee V1 expression notably differs from other species, and though they do not differ in the visual ecological traits investigated here, they are divergent from one another in important neurological signaling processes.

RESULTS

Humans and chimpanzees exhibit the most variation in V1 gene expression.

RNA-Seq was conducted on V1 tissue collected from 28 individuals from 13 primate species, including hominoids, cercopithecoids, and platyrrhines (Figure 1, SI Table 1; additional details in Methods). Despite the fact that gene expression does not evolve in the same manner as nucleotide sequence [46], due to tissue- and cell-type-specificity within an organism, V1 gene expression profiles overall grouped by species and phylogenetic clade (Figure 2). Interestingly, there was greater variation in V1 transcriptomes within hominoids than cercopithecoids or platyrrhines, predominantly due to distances observed among samples representing human and chimpanzee (Figure 2A). Thus, the expression profiles of other hominoids (orangutan, gorilla, and siamang) were more similar to that of cercopithecoids and platyrrhines than to human and chimpanzee (Figure 2A). Hierarchical clustering of whole transcriptomes revealed similar results, with most samples per species clustering generally according to phylogeny (Figure 2B). The exceptions were for the single representative samples of gorilla, saki monkey, squirrel monkey, and orangutan. Additionally, the two pig-tailed macaque samples clustered away from the other cercopithecoid species. These multivariate analyses indicate conservation in the overall expression profiles of primates with the exception of human and chimpanzee. There were no overt technical factors influencing the out grouping of human, chimpanzee, or pig-tailed macaque samples (e.g. individual sex or age, sample hemisphere of origin, RNA or cDNA library quality, read number, alignment percentages, SI Table 1). Observed human and chimpanzee divergence from other species was consistent with other

studies [32, 35, 41, 42] and highlighted that the increased distinctiveness of brain gene expression with proximity to the human lineage extends to V1.

Next, we performed pairwise differential expression (DE) analyses between species to determine the proportion of genes that were statistically significantly differentially expressed (SI Table 2). There was a positive correlation between number of genes exhibiting DE and phylogenetic distance (Pearson's r = 0.6, p = 0.0001), demonstrating that the number of genes exhibiting DE increases with phylogenetic distance (SI Table 2). However, humans exhibit noticeably greater numbers of genes displaying DE when compared to chimpanzee (n= 3,648) and siamang (n= 3,846; SI Table 2), despite being relatively closely related. Notably, chimpanzee and siamang do not exhibit as high a degree of difference from one another (n= 2,771; SI Table 2). This suggested that there is significant difference in V1 expression with phylogenetic proximity to humans, and not more generally in the hominoid clade. These results demonstrated that there is a significant degree of DE in V1 across primate species, despite its sensory function.

Metabolic and neuronal signaling are enriched in V1 DE.

To determine what biological processes are enriched in genes displaying DE in V1, we performed pathway enrichment analyses for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms of pairwise DE comparisons (Table 2; SI Table 3). To obtain an overall view of global processes enriched in primate V1 DE, we grouped specific pathways into broad parent KEGG categories for "signaling", "immune", "disease", "neuron-specific", "metabolic", and "other", generally following the established hierarchy already present in the KEGG ontology (SI Table 3; for more details on thresholding and grouping of pathways into parent categories, see Methods). Overall, there was a wide variety of KEGG pathways enriched in these DE comparisons related to inter- and extracellular signaling processes associated with growth and development, as well as more specific pathways related to cellular metabolism (e.g. glycolysis) and neuron-specific signaling (e.g. synaptic signaling; SI Table 3). To assess whether metabolic and neural processes routinely differ in the gene expression of primate V1, as well as

what interspecies DE comparisons deviated from that trend, we determined what proportion of all the pathways enriched were metabolic or neuron-specific signaling pathways. On average, metabolic pathways accounted for 23.8% of enrichments while neural pathways accounted for only 7.6% of enrichments (SI Table 3). Similarly, enrichments for metabolism were present in all interspecies DE comparisons (34/34 pairwise species comparisons), while enrichments for neuron-specific processes were present in only 23/34 or 68% of comparisons (SI Table 3). Interestingly, there was a reduction in the proportion of metabolic enrichments observed in the human-chimpanzee DE comparison coinciding with an increase in the number of neuron-specific enrichments (Table 1; SI Table 3). This suggests that altered expression in neural signaling deviates humans and chimpanzees more than any other two species.

Table 1. Metabolic pathways are most significantly enriched in interspecies DE comparisons with the exception of human-chimpanzee.

Interspecies-DE Comparison	KEGG Term	KEGG Parent Category	Genes	Enrichment P-Value	P-Value Rank
Human - Chimpanzee	Cholinergic synapse	**Neuronal (SS)**	40	0.00001	1
Human - Chimpanzee	GABAergic synapse	**Neuronal (SS)**	31	0.00011	4
Human - Siamang	Neuroactive ligand receptor interaction	**Neuronal (GEN)**	75	0.00086	3
Human - Siamang	Metabolic pathways	Metabolic (GEN)	272	0.00730	9
Human - Baboon	Metabolic pathways	Metabolic (GEN)	364	0.00001	1
Human - Baboon	Fatty acid metabolism	Metabolic (LIP)	20	0.00558	7
Human - Rhesus macaque	Steroid biosynthesis	Metabolic (LIP)	11	0.00118	5
Human - Rhesus macaque	Purine metabolism	Metabolic (NT)	55	0.00206	7
Human - Rhesus macaque	Nicotinate and nicotinamide metabolism	Metabolic (C&V)	13	0.00465	10
Human - Marmoset	Metabolic pathways	Metabolic (GEN)	349	0.00046	3
Human - Marmoset	Nicotinate and nicotinamide metabolism	Metabolic (C&V)	15	0.00122	6
Human - Spider monkey	Metabolic pathways	Metabolic (GEN)	326	0.00024	1
Human - Spider monkey	Steroid biosynthesis	Metabolic (LIP)	11	0.00142	3
Human - Spider monkey	Purine metabolism	Metabolic (NT)	54	0.00549	7

A table of the top-10 most significantly enriched KEGG pathways in interspecies DE comparisons between human and other species, abbreviated to show only metabolic (green) and neuron-specific (purple) pathways. '# Genes' refers to the number of genes exhibiting DE in that pathway. P-value rank refers to the rank of the enrichment within the top 10 for each comparison. Table is sorted by interspecies DE comparison. Abbreviated from SI Table 3. Neuronal pathways are bolded. Abbreviations: SS – synaptic signaling; GEN – general; LIP – lipid; NT – nucleotide; C&V – cofactor & vitamin.

Synaptic signaling pathways included in the neuron-specific parent group of enriched pathways included glutamatergic, cholinergic, serotonergic, and GABAergic synaptic signaling as well as "synaptic vesicle cycling" and "long-term potentiation" (SI Table 3). Other neuron-specific KEGG pathways that were enriched included axon guidance, neurotrophin signaling, and oxytocin signaling (SI Table 3). All eight DE comparisons of chimpanzee to other species had higher proportions of neuron-specific pathway enrichments of any interspecies DE comparison (SI Table 3). The DE comparison between human and chimpanzee had the most neuron-specific enrichments of any interspecies DE comparison (n =12; SI Table 3), including cholinergic and GABAergic synapse among the top ten most significantly enriched pathways ($p < 0.0001$; Table 1). In contrast, there were no neuron-specific pathways enriched in the top ten KEGG pathways for any other human interspecies DE comparisons (except neuroactive ligand receptor interaction in the DE comparison between human and siamang; Table 1). These results highlight that differences in neuron-specific processes in the V1 are not common across all primates but are more prominent with proximity to human, and most notably between human and chimpanzee.

The V1 region is highly specialized in cytoarchitecture and structure [9], and previous studies have determined that many of the V1-specific differences in gene expression between species are for genes involved in determining this structure [43]. Therefore, in addition to KEGG pathway enrichments, we conducted pathway enrichment analyses for Gene Ontology (GO) cellular components (CC) for pairwise DE comparisons to determine if there were differences in primate V1 related to specific neuron cellular parts. In many interspecific comparisons, including, but not limited to human vs. chimpanzee, there were several GO CC enrichments related to plasma membrane-associated complexes of known importance for intercellular signaling in the brain, including ATP-coupled ion channels and various neurotransmitter plasma membrane receptors and intermembrane transporters "clathrin-sculpted glutamate transport vesicle membrane", "ionotropic glutamate receptor complex", and "ciliary neurotrophic factor receptor complex" (SI Table 4). There were also enrichments for GO CC terms for specialized cell projections that characterize neural cells, including components of dendrites (e.g. dendritic branch point) and types of axons ("C fiber"), as well as for synapse parts and neurofilaments (SI

Table 4). Interestingly, DE comparisons between human and chimpanzee and other species are the only interspecies DE comparisons enriched for dendrite cellular parts. Similarly, only chimpanzee DE comparisons are enriched for axon cellular parts (SI Table 4). Enrichments for GO cellular components corroborate KEGG pathway enrichments and further show that neural signaling complexes critical for neurological signaling and structure, such as plasma membrane associated channels, dendrites, and axons, differ among primate V1.

To determine how metabolic gene expression differed from global gene expression in primate V1, we subset our expression matrix to include only genes involved in metabolic KEGG pathways (n = 1039, Figure 3). The heatmap generated by clustering species based on distance between expression values indicated that expression profiles of metabolic genes result in clustering of species according to clades. (Figure 3). That is, all platyrrhines, hominoids, and cercopithecoids grouped together with no overt pattern in the metabolic KEGG pathways to which the genes belong (bottom color-coded bar, Figure 3). This demonstrated that, while there were significant differences in metabolic gene expression in primate V1, species-specific metabolic patterns still contained substantial phylogenetic signal. To elucidate what metabolic processes distinguished each clade, we identified which pathways demonstrated DE between species of different clades, but not between species belonging to the same clade. Carbohydrate metabolism appears to be the most distinguishing set of metabolic pathways between clades. Hominoids differ from platyrrhines in citrate (TCA) cycle gene expression and differ from both cercopithecoids and platyrrhines for glyoxylate and decarboxylate metabolism (SI Table 3). In contrast, platyrrhines differ from both cercopithecoids and hominoids for amino sugar metabolism and glycolysis/gluconeogenesis gene expression (SI Table 3). One noticeable exception of a carbohydrate metabolic pathway that differed consistently between species' comparisons of all three cross-clade combinations but never between two species of the same clade was fructose and mannose metabolism, which displayed DE between 13 interspecies comparisons, including six comparisons of cercopithecoids to platyrrhines, five comparisons of hominoids to cercopithecoids, and two comparisons of hominoids to platyrrhines (SI Table 3). Taken together, these results show that first, metabolic changes in primate V1 are common across primate

species in a manner consistent with phylogeny and second, that the metabolic gene expression differences involved are primarily related to carbohydrate metabolism. In summary, variation in primate V1 gene expression is not limited to neural signaling processes but seems to be driven to a large extent by altered metabolic signaling, although neural signaling appears to contribute more to differences in expression with proximity to humans.

Visual cortex DE is correlated with differences in color vision, group size, diet, and habitat use. We leveraged the wealth of phenotypic and behavioral data available for the sampled primate species to help understand correlations between V1 gene expression and variation in species-typical color vision, habitat use, diet, group size, and individual differences in sex (summarized in Figure 1; details in Methods and SI Table 1) [4, 10, 47-50]. These phenotype-DE comparisons were conducted by grouping all samples according to the species-typical state of their color visual system (trichromats or polymorphic di/trichromats), habitat use (primarily arboreal or terrestrial), generalized diet (primarily frugivorous, folivorous, or omnivorous), and group size (primarily living in small or large groups) and determining genes exhibiting DE between the phenotype-extremes. We reasoned that this would be a conservative estimate of differential expression, because grouping such disparate species likely introduces more variability ("noise") than comparing two discrete species and results in fewer genes exhibiting DE in these phenotype comparisons. Of these trait-based DE comparisons, we detected DE based upon differences in color visual system, primary habitat use, group living size, and primary diet (frugivore-folivore, folivore-omnivore, frugivore-omnivore) but not sex in the samples used for this study (Table 2, SI Table 2). The highest proportion of trait-based DE was for differences in color vision (25.3%) and the lowest was for the diet-based

Table 2. Proportions of DE genes in primate species V1 vary depending on the compared phenotypes.

Phenotype-DE Comparison	#	%
Color vision	3173	25.25
Habitat-use	2501	19.91
Group-size	1704	13.56
Folivore-frugivore	534	4.25
Folivore-omnivore	664	5.28
Frugivore-omnivore	991	7.89
Sex	35	0.28

Pairwise list of phenotype-based DE comparisons. '#' refers to the number of genes exhibiting DE in that pathway; % is the percentage of all expressed genes. Abbreviated from SI Table 2.

296 comparisons (4.3-7.9%; Table 2, SI Table 2). While many of the parent categories of pathways were
297 enriched in all phenotype-DE comparisons, there were unique enrichments for specific pathways
298 depending on the phenotype compared (Table 3). It is important to note that, for some traits investigated
299 here, there is a clear influence of phylogeny on phenotypic differences based purely on the species for
300 which we have samples (further discussed in Methods). Because we cannot parse out how much this
301 influences the phenotype-DE comparisons, we are conservative in our interpretations of these analyses.
302 Color vision has long been hypothesized to be adaptive in primates with numerous
303 complementary and competing hypotheses about the pressures influencing its evolution (reviewed in [18].
304 When comparing expression between species that differ in color vision, 25.3% (n = 3173) of genes

Table 3. KEGG pathways enriched in only one phenotype-DE comparison.

Phenotype-DE Comparison	KEGG Term	KEGG Parent Category	Genes	Enrichment P-Value
Metabolic Pathways				
Color vision	Biosynthesis of amino acids	AA	21	0.004
Diet: frugivore -omnivore	Glycine serine and threonine metabolism	AA	5	0.046
Group size	Histidine metabolism	AA	6	0.013
Color vision	Glycolysis Gluconeogenesis	CAR	17	0.030
Diet: frugivore - folivore	Retinol metabolism	C&V	5	0.030
Color vision	Sphingolipid metabolism	LIP	13	0.028
Diet: folivore - omnivore	Steroid hormone biosynthesis	LIP	6	0.012
Group size	Fatty acid degradation	LIP	8	0.031
Habitat Use	alpha Linolenic acid metabolism	LIP	7	0.030
Habitat Use	Ether lipid metabolism	LIP	11	0.020
Habitat Use	Glycerophospholipid metabolism	LIP	20	0.013
Color vision	Pyrimidine metabolism	NT	26	0.012
Diet: folivore - omnivore	Purine metabolism	NT	12	0.015
Neuron-Specific Pathways				
Diet: frugivore -omnivore	Serotonergic synapse	SS	12	0.010
Diet: frugivore -omnivore	Synaptic vesicle cycle	SS	8	0.012
Group size	GABAergic synapse	SS	14	0.016

"Uniquely enriched" KEGG terms are shown for each phenotype-DE comparison investigated. KEGG parent categories do overlap, with the exception of "Cofactors & Vitamins". 'Genes' refers to the number of genes exhibiting DE in that pathway. Abbreviated from SI Table 3. Abbreviations: AA – amino acid; CAR – carbohydrate; LIP – lipid; NT – nucleotide; C&V – cofactor & vitamin; SS – synaptic signaling

displaying DE (Table 2; SI Table 2). For our samples, the investigated hominoid and cercopithecoid species were monomorphic trichromats in all individuals, while the platyrrhine species were polymorphic with trichromatic homozygous females, dichromatic males or dichromatic heterozygous females (Figure 1; SI Table 1). Despite color vision being sexually dimorphic in some of the platyrrhine species, very few genes were significantly differentially expressed in V1 between sexes (0.28% DE, n = 35; Table 2; SI Table 2). This may be due to our inclusion of all sampled individuals when comparing expression differences between sex, not just the species that were sexually dimorphic for trichromacy. We did not investigate intersex differential expression of only the polymorphic di/trichromatic species because we had only nine individuals and sex was not evenly distributed across these four species (SI Table 1). There were no neuronal-specific KEGG signaling pathways enriched in genes displaying DE between trichromatic species and polymorphic di/trichromatic species but there were a number of metabolic pathways enriched, including amino acid, nucleotide, glycolysis/gluconeogenesis, and sphingolipid metabolic pathways (SI Table 3). Notably, color vision was the only phenotype-DE comparison enriched for the encompassing, large KEGG pathway of "metabolic pathways" and "carbon metabolism" and included 226 genes (SI Table 3). This was 8-fold more genes than any other KEGG pathway enrichment, suggesting extensive metabolic differences in the V1 of primates differing in color vision. However, given the clear phylogenetic influence on these samples (Figure 1, SI Table 1), we cannot resolve whether these differences are truly due to divergent color visual perception or rather how hominoids and cercopithecoids differ from platyrrhines in V1.

We investigated DE between primarily arboreal and terrestrial species due to the role of V1 in processing signals derived from navigating and perceiving the environment [9]. The primarily arboreal species included all four platyrrhine species and two hominoid species (orangutan and siamang) while the primarily terrestrial species included all four cercopithecoid species and three hominoid species (human, chimpanzee, and gorilla; Figure 1, SI Table 1). There were 2,501 genes with DE in V1 associated with differences in primate habitat use (either arboreal or terrestrial; 19.9%, Table 2; SI Table 2). Neural KEGG pathways enriched in species differing in habit use included neuroactive ligand receptor

interaction, long-term depression, and retrograde endocannabinoid signaling (SI Table 3). This phenotype DE comparison was enriched primarily for lipid metabolic pathways (SI Table 3). Lipids are important in the brain for long-term energy storage, membrane structure, extracellular signaling, and enhanced propagation of neural signaling (e.g. myelination) [51]. Habitat use DE enrichments for lipid metabolism may suggest that these processes differ significantly in V1 as it responds to visual stimuli from divergent interactions with habitat, such as enhanced V1 processing of depth perception for arboreal versus terrestrial species.

We also explored possible expression differences in V1 that correlate with differences in sociality, using group size as a proxy variable. For the included species, the "small group" included three platyrrhines species (spider monkey, marmoset, and saki monkey), and three hominoid species (siamang, gorilla, and orangutan) that typically have fewer than 20 individuals per group on average and the "large group" included all four cercopithecoid species, one platyrrhine species (squirrel monkey) and two hominoid species (human and chimpanzee) that typically have greater than 20 individuals per group (Figure 1, SI Table 1). We found that 13.6% (n = 1704) genes exhibited DE between species that differed in group size (Table 2; SI Table 2). Categorical enrichment determined genes displaying DE between species differing in group size had more neural processes enriched than any other DE comparison based upon trait except for diet (see below), including neuroactive ligand receptor interaction, long-term depression, retrograde endocannabinoid signaling, and GABAergic synapse (SI Table 3). Of the genes exhibiting DE between large and small group living species, metabolic KEGG enrichments included amino acid sugar and nucleotide sugar metabolism and fatty acid degradation (SI Table 3). Because both long-term depression and GABAergic synaptic signaling function in inhibitory signaling [52], enrichments for both of these processes may suggest a significant difference in inhibitory signaling in the V1 among primates differing in group size, which may be due in part to differential visual responses necessary for navigating socially complex environments.

To compare V1 expression between species that differ by diet, we categorized our species as primarily frugivorous, primarily folivorous, or omnivorous [50]. The primarily frugivorous species

included two hominoid species (chimpanzee and orangutan) and three platyrrhine species (spider monkey, marmoset, and saki monkey) while we had two primarily folivorous hominoid species (gorilla and siamang; Figure 1, SI Table 1). The omnivorous species included all four cercopithecoid species, a single platyrrhine species (squirrel monkey) and a single hominoid species (human; Figure 1, SI Table 1). We calculated DE among the three possible comparisons: folivore-frugivore (4.3% DE, n = 534), folivore – omnivore (5.3%, n = 664), and frugivore – omnivore (7.9%, n = 991; Table 2; SI Table 3). Folivorous species differed from omnivorous species in in tryptophan, purine, and steroid metabolic KEGG pathways (SI Table 3). Folivorous species differed from frugivorous species in carbohydrate and protein digestion and retinol and tryptophan metabolism (SI Table 3). Frugivorous primates displayed much higher numbers of genes exhibiting DE in V1 than frugivore-folivore and folivore-omnivore comparisons, though this may be due to a lack of folivorous species in the available samples (only siamang and gorilla were folivorous; Figure 1; SI Table 2). Frugivorous-omnivorous DE was enriched for serotonergic synaptic signaling and was the only diet comparison enriched for any neuron-specific KEGG pathways (SI Table 3). Frugivores differed from omnivores metabolically for amino acid metabolism (SI Table 3). Given that serotonin levels have an established link to diet [53] and tryptophan is a known precursor for serotonin [54], the finding that both tryptophan and serotonergic signaling are only enriched in DE between species differing in diet may suggest that diet has an impact on V1 serotonin signaling by way of altered tryptophan metabolism.

All trait DE comparisons were enriched for at least one pathway in the metabolic parent categories but not for neural signaling (SI Table 3). Our results suggested that neural processes drive differences in V1 gene expression in species that differ in group size, habitat use, and diet while metabolic differences are more responsible for V1 DE between species differing in color vision (SI Table 3). Our approach correlating DE with phenotype suggested an association between V1 expression and distinctive differences in visually relevant traits.

DISCUSSION

Although some studies of V1 gene expression compared it both to other regions of the brain, as well as across species [38, 43-45], no other investigations of brain gene expression included the diversity and number of primate species studied here. As such, this represents one of the first in-depth investigations of how V1 gene expression differs across a variety of primate species. We found that while global gene expression in primate V1 clusters largely by phylogenetic relatedness, the greatest degree of variation in expression is within hominoids, largely due to divergence of humans and chimpanzees from other one another as well as other hominoid species. Carbohydrate metabolic processes seem to be driving expression differences across the primate tree while neural processes are more conserved in V1. A deviation from this trend is the expression differences between human and chimpanzee. These species are outliers from all other species and display a relatively large amount of intraspecific variation in global gene expression profiles as well as in specific pathways related to synaptic signaling and neuronal cell projections important for maintaining the complex neurological signaling networks key to brain function. In addition to global and interspecific differences in expression, we were able to link V1 DE to differences in visually-relevant phenotypes.

The enrichment for neuron-specific processes in V1 DE, primarily between human and chimpanzee, is consistent with previous findings of enrichments for similar processes, such as synaptic signaling, in differential gene expression that have been reported in various studies of human, chimpanzee, and rhesus macaque brains [33-35]. Also, metabolic processes similar to those enriched here have been found to differentiate primate brain gene expression in previous studies of a smaller array of primate species [37, 38, 43, 55], including that humans specifically differ largely in metabolic processes related to aerobic glycolysis [34, 35]. Taken together, our results across a broad number of primate species show that these trends for metabolic and neural signaling differences in the brain extend to V1.

Given that V1 is a primary sensory cortex and in view of subtle interspecies differences in visual perception between closely related primates, little divergence might be expected over shorter evolutionary times. Consistent with this, we found a significant correlation for greater numbers of genes exhibiting DE

between more distantly related species. Our finding that interspecies DE tends to increase with evolutionary distance has been observed previously in a study of gene expression across ten species (including six hominoids) and six tissue types [39]. Furthermore, we observed a trend for genes involved in metabolic signaling to be differentially expressed consistently across interspecies DE comparisons, regardless of phylogenetic distance, while processes specific to neural signaling were far less common. Because the most metabolically demanding feature of the brain is synaptic transmission, metabolic differences are not mutually exclusive with neural signaling processes, but this is an interesting trend nonetheless. Our findings suggest that altered gene expression of neuron-specific pathways in V1 did not consistently contribute to functional differences between closely-related species. In contrast, altered metabolic processes do contribute (e.g. oxidative phosphorylation is enriched in genes exhibiting DE between all cercopithecoid species; SI Table 2). The sole exception to this generalization is that neuron-specific processes were among the most significantly enriched pathways in genes exhibiting DE between human and chimpanzee.

Within primates, selective differences in the genome can be linked to diet and metabolism, suggesting selection has optimized different metabolic processes in lineage-dependent ways [33-35, 56-59]. The human brain is more energetically costly than that of other primates, utilizing ~ 20% of the body's metabolic resources, compared to non-human primate brains that use less than 10% [60, 61]. Importantly, allometry alone does not explain the increase in human brain appropriation of glucose metabolism at this proportion [62-64]. Our results are consistent with previous findings that humans differ in brain gene expression from chimpanzees for neuron-specific processes related to synaptic transmission and metabolic processes involved in aerobic glycolysis [34, 35, 37, 38, 43, 55]. These data demonstrate that, like other brain regions [32, 35, 41, 42], human lineage-specific neurological changes are present in the visual cortex.

Understanding V1 gene expression differences based upon phenotypic variation related to vision could elucidate the neurological implications of differences in vision and the selective pressures hypothesized to be linked to these traits.. Although there are important caveats to our analysis of

435 phenotype-based DE, primarily the inability to account for phylogenetic influences (see Methods for more
436 details), this still represents a significant effort to link proximate gene expression differences in the brain
437 with evolved variation in ecological traits. DE among species differing in color vision was enriched for a
438 number of metabolic processes but not neural signaling processes, perhaps suggesting that any expression
439 differences in V1 influenced by differences in color visual perception are driven more by metabolic
440 differences than by synaptic signaling. However, this phenotype-DE comparison has strong phylogenetic
441 inertia and we were unable to determine if this difference represented divergent color perception or a
442 phylogenetic difference between platyrrhines and cercopithecoids and hominoids.
443 Furthermore, we chose to investigate the link between group size differences and V1 gene
444 expression due to the hypothesized influences of social behavior on primate brain evolution and the
445 possible link to required differences in visual perception among group living primates [19]. While V1 is
446 not explicitly involved in behavioral processes, there were enrichments for pathways known to be
447 involved in behavior. Primates exhibit extensive variation in social traits, and a number of genes (and
448 associated pathways) have been hypothesized to be linked to these behaviors, such as those involved in
449 social bonding or empathy [arginine vasopressin receptor 1A (*AVPR1A*), oxytocin receptor (*OXTR*), and
450 dopamine receptor (*DRD4*)] [65]. Oxytocin signaling changes in response to social interactions is well
451 documented [66, 67] and variations in coding sequence for oxytocin receptor and their associated
452 influence on social behavior have been observed in rhesus macaques [68]. While group size was not
453 enriched for "oxytocin signaling pathway" or "dopaminergic synapse", the genes *DRD4* and *OXTR* were
454 both differentially expressed in the DE comparison of species differing in group size, alternatively
455 included in the enriched category "neuroactive ligand receptor interaction" (SI Table 3). Our results
456 showed that genes putatively important in primate social evolution display significant DE in V1 in
457 primates differing in group size.
458 Future comparisons of multiple brain regions may determine if the observed trends of altered
459 gene expression between phenotypically distinct primates and between species are V1-specific or a brain
460 region-independent observation. However, the phenotypes investigated here (color vision variation,

arboreality and terrestrially, group size and sociality, and diet) do not have implications solely for primate vision evolution and thus are not expected only to impact on visual cortex gene expression evolution. Instead, they have likely played important roles in other brain regions and perhaps the brain overall. Additionally, the hypothesized importance of the evolutionary trajectories of these phenotypes are not mutually exclusive of one another. That is, the evolution of trichromatic color vision has likely been influenced by an interplay of diet, arboreality, social signals and other important primate traits. Because our methods could parse out the influences of each of these traits on V1 gene expression evolution, we remained conservative in our interpretations of the association between phenotype evolution and gene expression evolution. Furthermore, given the highly organized and specific cytoarchitecture of the V1 [9], and previously determined influence of structural genes on V1-specifc gene expression in comparison to other regions [43], it is possible that V1-region specific changes in gene expression are linked to maintaining or fine-tuning this cytoarchitecture. However, studies of V1 function are largely limited to tissue-level functional, mechanistic, and cytoarchitectural investigations [69-71] and primarily only in rhesus macaques, resulting in a very limited understanding of the gene expression changes that accompany altered systems-level function. This, in addition to the lack of other brain regions for comparison in our current study across such disparate primate species and the previous findings that V1 gene expression is similar to other brain regions except cerebellum in a limited number of primate species (rhesus macaque, chimpanzee, human) [38], we limit our conclusions about V1 region-specific gene expression and its link to variation in visually relevant phenotypes.

CONCLUSIONS

We investigated the interaction between genotype and phenotype by examining the correlation between gene expression and phenotypic and behavioral traits, including habitat use, color visual system, group size, and diet in a broad sampling of primate species, including many understudied species (e.g. siamang, squirrel monkey, and spider monkey). We determined that neural and metabolic processes

known previously to differ between species in other brain regions also demonstrate interspecies and trait-based differences in V1. We showed that human and chimpanzee are outliers for V1 gene expression, differing significantly more in neuron-specific processes related to synaptic signaling than other species do. Although these species appear to be the most divergent, they do not exhibit any major differences in the visually-relevant phenotypes investigated here for which we were able to determine significant expression differences. Future studies that include other primate taxa could further investigate the link between differences in primate vision evolution and visual cortex expression differences.

Because primates exhibit many unique visual system traits compared to other mammals, understanding the genetic basis for primate visual systems in the V1 region would provide valuable insights into the evolutionary trajectory of those traits. Our data indicate that there is also a correlated difference in gene expression in the initial processing center of visual signals in the brain. We also show that humans differ in brain gene expression in the V1 in a manner similar to other regions. Further investigation of overlap between DE and signals of selection can provide information about which expression changes are adaptive.

METHODS

Samples and Library Preparation. Tissue samples were collected from 28 individuals representing 13 primate species (n = 1-3 per species; Figure 1). Frozen brain samples from captive adult primates free of known neurological disorders were obtained from various research institutions and zoos as a part of the National Chimpanzee Brain Resource (NCBR) (see SI Table 1 for sample-specific details). All individuals had been cared for according to Federal and Institutional Animal Care and Use guidelines and died of natural causes. All tissue was collected and stored at -80°C with postmortem intervals of less than 8 hours. Further details about the samples can be found in SI Table 1. Due to the opportunistic nature of sampling such a phylogenetically broad group of primates, there is unavoidable variation in age and sex of some individuals sampled (SI Table 1).

Each sample was dissected from the cortex of the medial aspect of the occipital pole, surrounding the calcarine sulcus. The thin, striate cortex of V1 was visually identified in each sample. All dissections included the grey matter of V1, extending a small extent into the underlying white matter. Tissue samples were homogenized using a TissueLyser (Qiagen) prior to total RNA extraction using an RNeasy Plus Mini Kit (Qiagen), including a DNase step to remove residual DNA. Total RNA was analyzed for quality using the Agilent Bioanalyzer system (Agilent RNA 6000 Nano kit). RNA integrity varied among our samples due sampling from deceased primates, but there was no bias for species. Using the NEBNext Poly(A) Magnetic mRNA Isolation Kit (NEB), mRNA was isolated from intact total RNA, and cDNA libraries were made from each sample using the NEBNext RNA Library Prep Kit for Illumina (NEB). Library quality was assessed using the Agilent DNA 1000 kit. Pooled samples were sequenced using the Illumina NextSeq 500 platform to produce 75 base pair reads, yielding a minimum of 20 million reads per sample.

Read Mapping and Quantification. Quality-filtered reads were aligned to available primate genomes with Bowtie2 using default parameters for gapped alignments [72]. Current species-specific ENSEMBL genomes were used (*Homo sapiens,* GRCh38.p10; *Pan troglodytes,* CHIMP2.1.4; *Gorilla gorilla,* gorGor3.1; *Pongo abellii,* PPYG2; *Papio anubis,* PapAnu2.0*; Nomascus leucogenys,* Nleu1.0; *Macaca mulatta,* Mmul_8.0.1; *Callithrix jacchus,* C_jacchus3.2.1) [73, 74]. For species for which there is no publicly available reference genome (*Pongo pygmaeus, Symphalangus syndactylus*, *Macaca nemestrina*, *Erythrocebus patas*, *Saimiri sciureus, Pithecia pithecia,* and *Ateles fusciceps*), reads were mapped to the closest related primate for which there was a genome available. Specifically, *M. nemestrina* and *E. patas* reads were mapped to *M. mulatta, S. syndactylus* to *N. leucogenys*, *A. fusciceps*, *S. sciureus*, and *P. pithecia* to *C. jacchus*, and *P. pygmaeus* to *P. abellii*. Mapping percentages were ≥ 80% using default "—local" Bowtie2 parameters. The "—very-sensitive-local" parameter was used to increase accuracy and alignment percentage of the samples with the lowest mapping percentages (< than 85%; 2 *A. fusciceps,* 1 *S. syndactylus*, and 1 *S. sciureus* samples), and all increased to ≥ 83.5% (see SI Table 1). HT-Seq [75] was used to quantify counts per gene for each sample, using ENSEMBL gene transfer files (GTFs)

corresponding to the same genome build used for alignment [76]. For each species, homologous genes were matched to the ENSEMBL human reference set of genes using biomaRt [77]. These were subsequently filtered to the 12,564 genes from all species that have high orthology confidence, all of which also had high homolog percent identity to the human query genes as well as gene order conservation scores, as previously determined by ENSEMBL [76]. This resulted in removal of genes from all transcriptomes considered if they did not meet orthology confidence in all species. Finally, we used the R package edgeR [78] to filter out low-expressed genes (counts per million (CPM) > 1 in 1/28 samples), resulting in 12,330 expressed orthologs in our dataset.

Clustering Analyses. We used clustering analyses to determine the variation in our samples. We produced a principal coordinates analysis (PCoA) using the expression profiles of all of the protein coding genes for each of our samples (n = 1-3 per species). To further visualize patterns within our data, we produced phenograms by performing hierarchical clustering of the PCoA distances. The PCoA and dendrograms show that our samples largely cluster by species and clade.

Differential Expression Analysis. Differential expression (DE) analyses were performed for multiple pairwise comparisons using a generalized linear model (GLM) in the R package edgeR (SI Table 2) [78]. Normalization of data in EdgeR for DE analyses ensured that DE is not dependent on original tissue size. Gene expression was considered significantly different at a false discovery rate (FDR) of less than 5%. DE between species only included species for which we had more than one sample, including human, chimpanzee, siamang, olive baboon, rhesus macaque, pig-tailed macaque, patas monkey, spider monkey, and marmoset (SI Table 2). To determine if there was a relationship between number of genes exhibiting DE between species, we used a Pearson correlation between numbers of genes exhibiting DE and divergence times reported by 10k Trees of the last common branching point of the tree between the two species compared (SI Table 2; [79]. The multi-factor GLM allowed us to analyze all data at once to detect DE between species as well as between groups of samples for which the species are known to have distinct differences in visually relevant phenotypes, data for which were collected from the literature [4, 10, 47-50]. These species-typical data were used to label each of our samples for color visual system

(trichromats or polymorphic tri/dichromats), average group size (less than 20 individuals per group or greater than 20 individuals per group), primary diet (frugivorous, folivorous, or omnivorous), and primary habitat (arboreal or terrestrial — mapped onto a phylogeny in Figure 1; listed in SI Table 1). For each of these phenotype contrasts, all samples of the same species-typical phenotype were grouped together and compared to the group of samples with the opposing species-typical phenotype (e.g. all samples from primarily frugivorous species were grouped together and compared to all samples from primarily omnivorous species for the phenotype-DE comparison frugivorous versus omnivorous diet). DE was also performed on each sample by sex (male or female; SI Table 1). Given the comparative, discovery-based nature of our analyses, we did not correct p-values of DE genes for the multiple interspecies or phenotype-DE comparisons, though many would have remained significant.

Our goal was to investigate differences in V1 gene expression between species, but also to attempt to link V1 expression differences with differences in broad, species-level phenotypes. It is important to note that there a number of caveats to this approach relevant to interpretation of results. We used species-typical categories for each of the phenotypes we were interested in correlating with V1 gene expression because we do not have the relevant phenotypic information on each of the individuals from which our brain samples were obtained. Importantly, our samples are from captive living animals that did not necessarily experience the wild, ecological condition typical for their species. Thus, our study design allows us to interpret minimal innate differences in V1 gene expression that may be related to past evolutionary influences from visual ecological pressures, but not from dynamic experience-dependent differences, which would probably yield even stronger signals of ecological correlations. For these reasons, we labeled our samples for phenotype using broad, categories (e.g. primarily arboreal species vs primarily terrestrial species rather than more discrete measurements) and we determined DE by grouping samples by species-typical phenotype and comparing between phenotypically distinct groups. This leads to another important caveat for our phenotype-DE comparisons, which was that our analyses did not allow us to account for phylogeny. We "mapped" these traits onto a phylogeny in Figure 1 to provide transparency about some of the clear influences of phylogeny on our divergent phenotype groups. For

example, the investigated hominoid and cercopithecoid species are monomorphic for trichromatic color vision in all individuals regardless of genotype or sex, while the platyrrhine species are polymorphic with trichromatic homozygous females, dichromatic-males or dichromatic-heterozygous females (Figure 1; SI Table 1). With these caveats in mind, we were conservative in our interpretation of these analyses.

Categorical Enrichment Analysis of Differentially Expressed Genes. Genes identified as DE for each interspecies and phenotype comparison were subsequently used for pathway enrichment with Kyoto Encyclopedia of Genes and Genomes (KEGG) [80] to determine differences in signaling and as well as Gene Ontology (GO) cellular component (CC) categorical enrichment analyses [81, 82] to further determine the cellular locality of differences. Enrichments were obtained using EnrichR with *crisp* data sets and filtered by those that have an enrichment p-value less than .05 [83, 84]. To determine the most biologically relevant enrichments, KEGG pathway enrichments were further filtered to include only those with five or more genes per enrichment and GO CC enrichments were further filtered to only include those with ten or more genes per enrichment. Due to the discovery-based nature of our research question and the filtering of categories based upon gene number, we did not restrict our analyses to only categories with significant adjusted p-values for multiple comparisons, of which there were fewer. Similarly, GO CC enrichment terms were also grouped into parent categories related to cellular structures (e.g., "neural cell projection") is a larger category containing GO CC terms such as "axon" and "dendritic branch point" which are encompassed in the established GO CC hierarchy of "cell projections" (SI Table 4). The parent category of KEGG "neuron-specific processes" includes enrichments for pathways that are unique to the brain and the majority of these were for synaptic processes (specific neurotransmitters, synaptic activity, and synaptic vesicle function; SI Table 3). Metabolic pathways include those related to the metabolic breakdown or synthesis of all major macromolecules, including that of carbohydrates (e.g. "glycolysis/gluconeogenesis"), lipids (e.g., "fatty acid metabolism"), amino acids (e.g. "glycine, serine, and threonine metabolism"), and nucleotides (e.g., "purine metabolism" ; SI Table 3). We report all significant categorical enrichments (SI Table 3, 4), but in our results, largely focus on enrichments for pathways specifically involved in neuronal and metabolic signaling, as previous work has found that these

processes exhibit DE and signatures of positive selection in *cis*-regulatory regions when comparing human, chimpanzee, and rhesus macaque brains [33-35] and the importance of metabolism in the brain provides cellular energy and critical synthesis and breakdown of macromolecules [85-89].

LIST OF ABBREVIATIONS

DE - differential expression

V1 - primary visual cortex

GO - Gene Ontology

BP - biological processes (BP)

CC - cellular components

KEGG - Kyoto Encyclopedia of Genes and Genomes

GLM – generalized linear model

PCoA - principal coordinates analysis

CPM – counts per million

DECLARATIONS

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All read data in the form of FASTQ files have been submitted to the National Center for Biotechnology Information's (NCBI) Short Read Archive (SRA) with accession code PRJNA526359.

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Authors' contributions: JJE, MAR, WDH, PRH, CCS, CCB, and ALB contributed to sample acquisition. TMZ and CCB generated the gene expression datasets. TMZ, JMK, CCB, and ALB contributed to analyses and writing of the manuscript. All authors provided intellectual content to and have read and approved this manuscript for publication.

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FIGURE AND TABLE LEGENDS

Figure 1 | Phenotypic traits of species for which V1 gene expression was investigated. Phenotypic traits for color vision, habitat use, and group size are mapped on the phylogeny and diet is depicted to the right

of species names. Species without a diet indicator were coded as omnivorous. The tree was generated using 10k Trees Version 3 [79] and Mesquite [90].

Figure 2 | Humans and chimpanzees are the most divergent in V1 gene expression. A) principal coordinate analyses (PCoA) of V1 transcriptomes color-coded by species. Shapes of points indicate clade: triangles for hominoids, squares for cercopithecoids, and circles for platyrrhines. B) Hierarchical clustering of V1 transcriptomes of each sample with bootstrap values and the individual sample number in brackets.

Figure 3 | Expression profiles of metabolic genes in primate V1 cluster by clade. Clustering of expression profiles of 1,039 metabolic genes in primate V1. Highly correlated genes (columns) cluster together and samples (rows) cluster based on Euclidean distance between expression values. Only species for which there were greater than one sample per species were used. Averages of expression per gene were calculated across replicates per species. The bottom bar represents membership in the color-coded KEGG metabolic pathways for each gene in the heatmap.

SUPPLEMENTAL TABLE LIST:

Table Name	Content
Supplemental Table 1	Sample-specific information.
Supplemental Table 2	Full table of numbers of genes exhibiting DE for all pairwise DE comparisons.
Supplemental Table 3	Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichments for each pairwise DE comparison. All pathways enriched are significant ($p<.05$) and include 5 or greater genes. Specific KEGG term, ID number and parent group are listed.
Supplemental Table 4	Gene ontology (GO) cellular component (CC) enrichments for each pairwise DE comparison. All CC's are significant ($p<.05$) and contain 10 or greater genes. Specific CC terms, IDs, and parent group are listed.

Figures

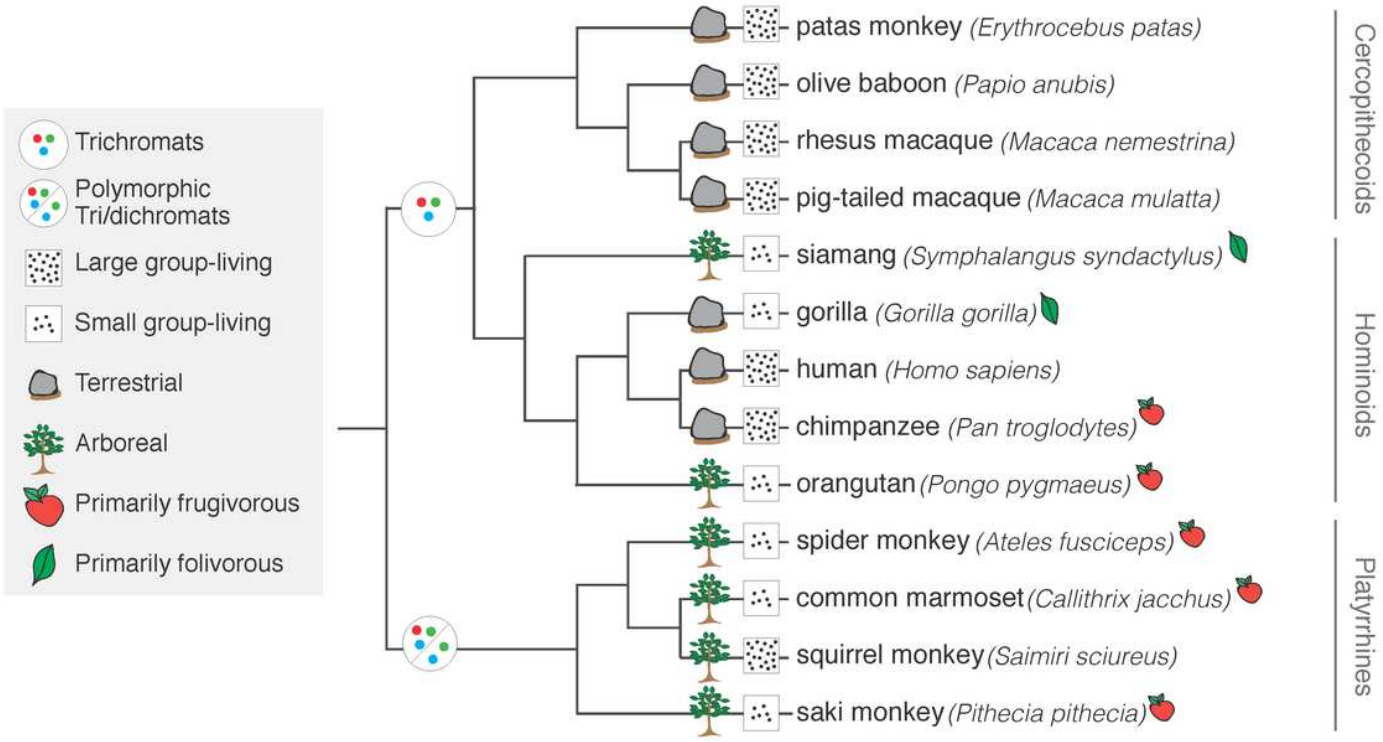

Figure 1

Phenotypic traits of species for which V1 gene expression was investigated. Phenotypic traits for color vision, habitat use, and group size are mapped on the phylogeny and diet is depicted to the right of species names. Species without a diet indicator were coded 887 as omnivorous. The tree was generated using 10k Trees Version 3 [79] and Mesquite [90].

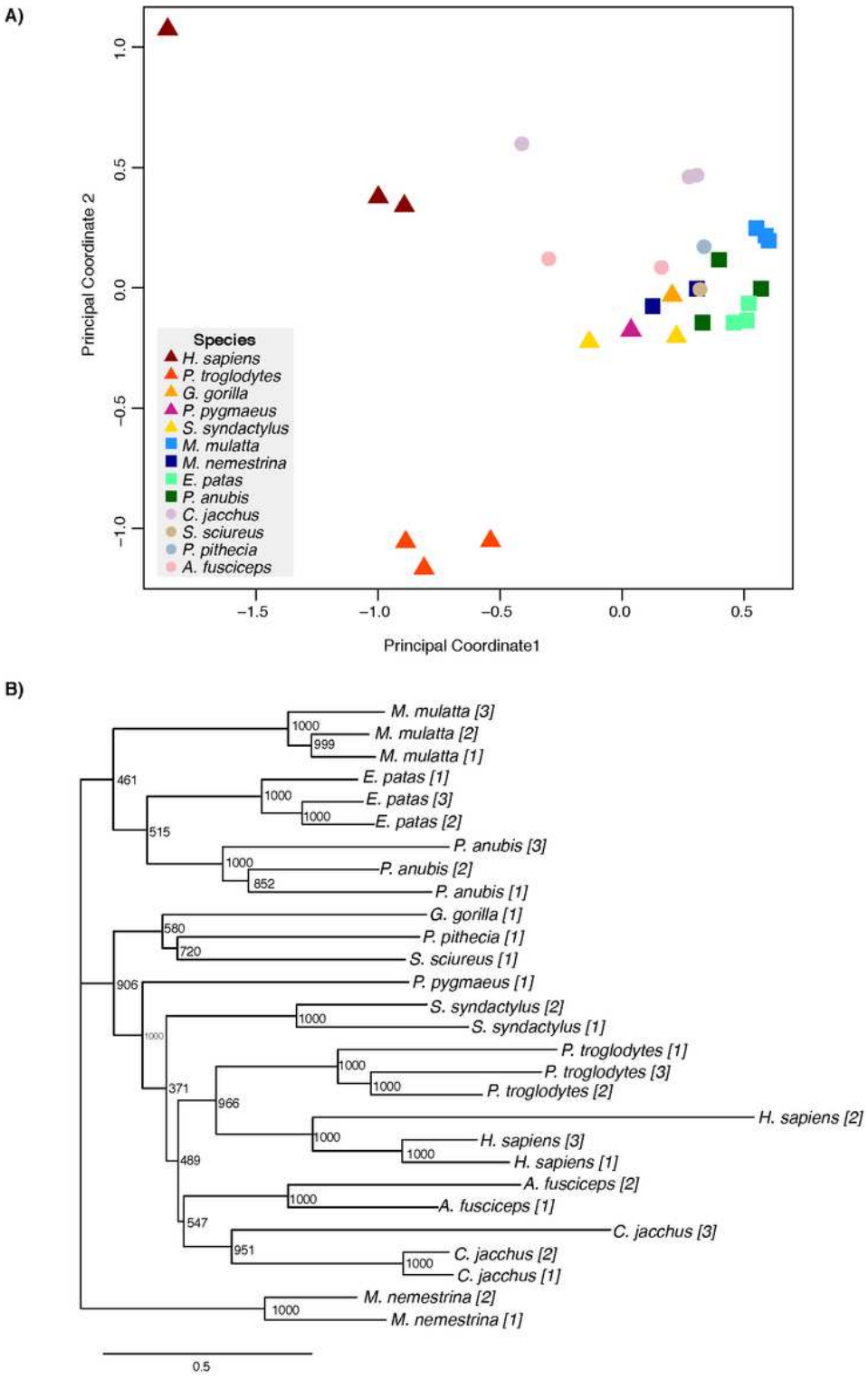

Figure 2

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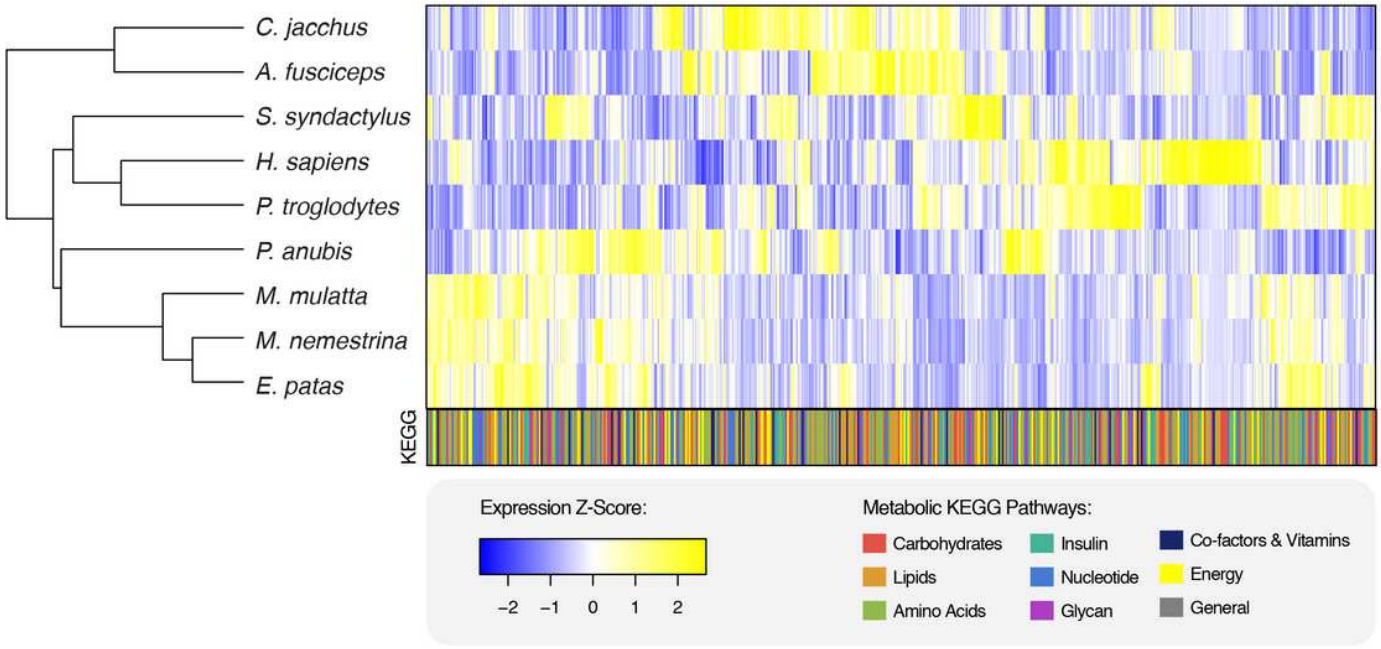

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Supplementary Files

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