Supporting Information

Miller et al. 10.1073/pnas.1117943109

SI Materials and Methods

Sample. ***Humans.*** Human brain specimens from the Yakovlev–Haleem collection were fixed in 10% (vol/vol) formalin, prepared in whole-brain serial celloidin sections, cut at a uniform thickness of 35 μm, and stained using the Loyez technique for myelinated fibers (1). Tissue from the National Institute of Child Health and Human Development Brain and Tissue Bank was fixed in 10% buffered formalin, cut at a uniform thickness of 40 μm, and stained using a modified Gallyas technique for myelinated fibers (2). Tissue from the laboratory of Nenad Šestan at Yale University (New Haven, CT) was snap-frozen in isopentane and stored at −80 °C (detailed in ref. 3).

Chimpanzees. The chimpanzee brain specimens were collected postmortem from various research institutions and fixed by immersion in 10% buffered formalin for variable lengths of time, transferred to a PBS solution containing 0.1% sodium azide, and stored at 4 °C. Blocks of ∼3 cm were sectioned perpendicular to the pial surface containing a single gyrus from the regions of interest in the left hemisphere (right hemisphere was used in a single case; Table S1). Tissue blocks were cryoprotected by immersion in buffered sucrose solutions up to 30%, embedded in tissue medium, frozen in a slurry of dry ice and isopentane, and sectioned at 40 μm. Every 10th section (400 μm apart) was stained for Nissl substance with a solution of 0.5% cresyl violet to visualize cytoarchitecture. An adjacent 1-in-10 series of sections was stained using a modified Gallyas silver impregnation method to reveal myelinated axons (2) (Table S1).

Human brain specimens ($n = 24$) in the Yakovlev–Haleem slide collection were prepared using the Loyez method, as were control chimpanzee specimens ($n = 5$). The Loyez protocol follows incubation in hematoxylin and alcohol with a developer consisting of potassium ferricyanide, sodium borate, and water, with a rinsing step with ammonium hydroxide, to visualize myelinated fibers (1). Chimpanzee brain sections ($n = 20$) were prepared by using a modified Gallyas silver impregnation protocol, as were control human sections ($n = 9$). The Gallyas protocol follows incubation in pyridine and acetic anhydride with a developer consisting of formalin, ammoniacal silver nitrate, and paraformaldehyde, a silver granule cleansing step with low concentrations of acetic acid, and a bleaching step using potassium ferricyanide, to visualize myelinated fibers. An adjacent 1-in-10 series of sections in all samples were stained for Nissl.

Stereology. Myelinated fiber length density (MFLD) in both samples was quantified by the same observer (D.J.M.) using a computerized stereology system consisting of a Zeiss Axioplan 2 (for chimpanzees) or Nikon E1000M (for humans) microscope and StereoInvestigator software (MBF Bioscience). By using adjacent Nissl-stained sections, we confirmed the cytoarchitecture of regions of interest and located the white matter–gray matter interface. Beginning at a random starting point, three myelin-stained sections equidistantly spaced within 1,200 μm (chimpanzees) or 1,050 μm (humans) were selected for analysis. MFLD was evaluated using the SpaceBalls probe, a 6-μm sampling hemisphere for lineal features combined with a fractionator sampling scheme (4). Fibers were marked where they intersected the outline of the hemispheric probe. Sampling hemispheres were placed in a systematic random fashion every 700 × 700 μm to cover the region of interest with ∼30 frames per section, and mean mounted section thickness was measured at every 10 sampling locations. The analysis was performed under Koehler illumination at 60× (humans), 63× (chimpanzees), or 100× (control samples). To obtain MFLD, the total fiber length was divided by the planimetric measurement of the reference volume that was sampled, as calculated by the StereoInvestigator software.

Western Blot Analysis. Protein expression analysis of tissue sections adjacent to those used for stereology was performed in chimpanzees by Western blot assay. Frozen tissue samples from human were used for Western blotting. Tissue (50–100 mg) was homogenized in radioimmunoprecipitation assay buffer (pH 7.6) containing 2% SDS and protease inhibitors, and the contents were incubated at 100 °C for 20 min followed by incubation at 60 °C for 2 h. The tissue lysates were then centrifuged at 15,000 × *g* for 20 min at 4 °C. Protein concentrations were measured with the DC protein assay (Bio-Rad) after detergent solubilization. Protein samples were diluted 1:1 in Laemmli sample buffer and boiled for 10 min. Samples were separated on NuPAGE Novex 4% to 12% Bis-Tris gel (Invitrogen). The proteins were electrotransferred to a PVDF membrane in Tris-glycine-methanol buffer. The membrane was blocked for 1 h at room temperature in a blocking solution mixture with 5% nonfat dry milk in 0.05% Tween 20 and Tris-buffered saline solution (pH 8.0). The membrane was washed with Tris-buffered saline solution with Tween 20 on a shaker at room temperature three times for 10 min and incubated overnight at 4 °C with the following antibodies: rabbit anti–myelin-associated glycoprotein (MAG; 1:300; LifeSpan BioSciences), mouse anti–2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP; 1:300; Abcam), anti–β-actin polyclonal antibody (1:1,000; Santa Cruz Biotechnology), and anti-GAPDH polyclonal antibody (1:500; Imgenex) in 1% nonfat dry milk in Tris-buffered saline solution with Tween 20. After repeated washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or donkey anti-rabbit IgG (1:3,000; Santa Cruz Biotechnology) overnight at 4 °C. After washing, immunoreactivity was visualized by using a chemiluminescent substrate (ECL; Amersham Biosciences; Figs. S2 and S3*C*). Densitometry analyses were performed to quantify signals generated by Western blotting with Scion Image software. In chimpanzees, the immunodetected bands were then normalized to total protein in each sample. To account for blot-to-blot variation in exposure and film development, three concentrations of a blotting standard were loaded onto each gel. The standard comprised a mixture of protein samples from the four cerebral cortex regions of each individual chimpanzee used in the study. The intensity of the bands for each unknown sample was normalized to this standard. In human samples, densitometric measurements were normalized to an anti–β-actin loading control.

1. Yakovlev PI, Lecours A (1967) The myelogenetic cycles of regional maturation of the brain. *Regional Development of the Brain in Early Life*, ed Minkowski A (Blackwell Science, Oxford), pp 3–70.
2. Pistorio AL, Hendry SH, Wang X (2006) A modified technique for high-resolution staining of myelin. *J Neurosci Methods* 153:135–146.
3. Kang HJ, et al. (2011) Spatio-temporal transcriptome of the human brain. *Nature* 478: 483–489.
4. Mouton PR, Gokhale AM, Ward NL, West MJ (2002) Stereological length estimation using spherical probes. *J Microsc* 206:54–64.

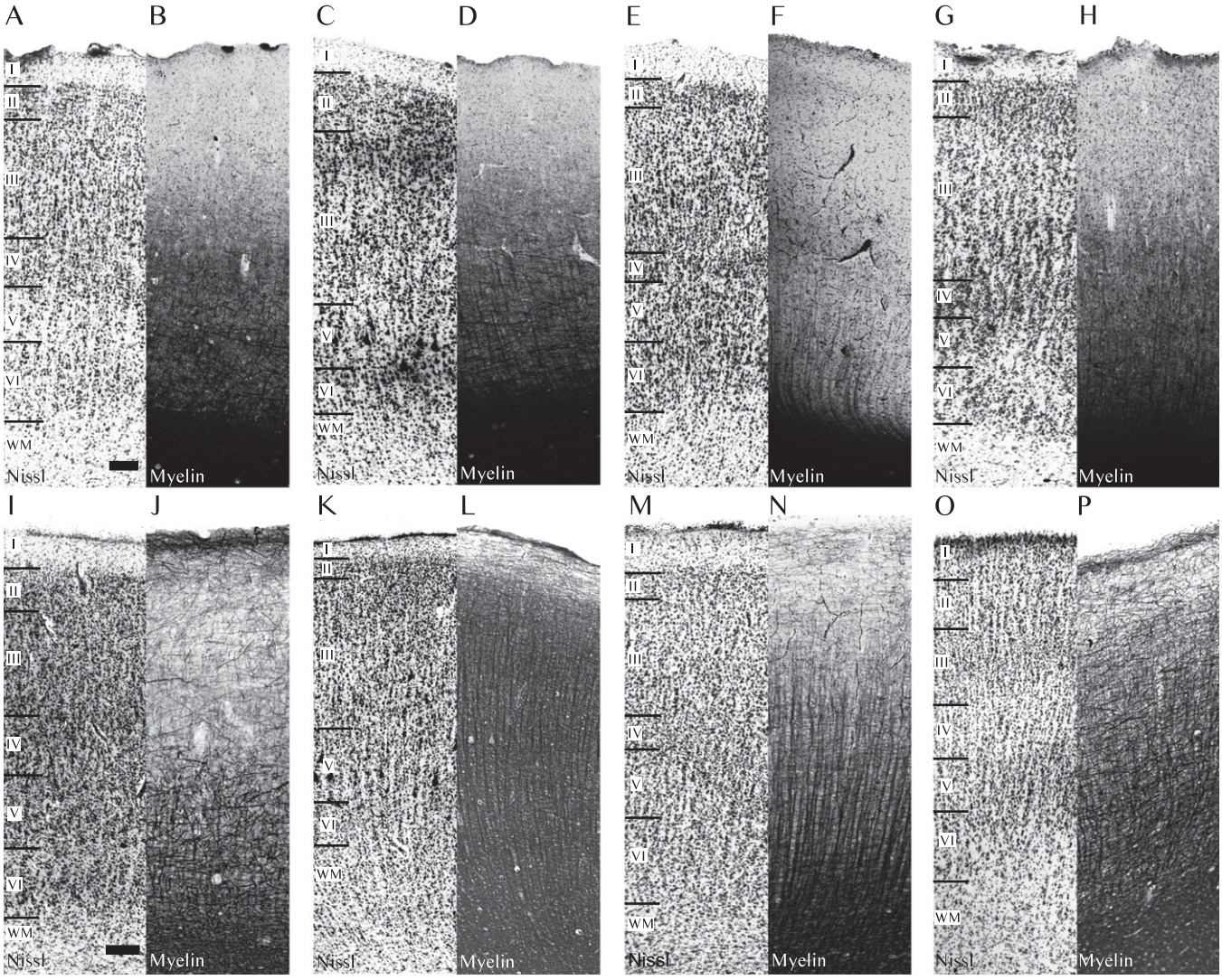

Fig. S1. Neocortical regions of interest in adult humans and chimpanzees. Representative sections from each region of interest (somatosensory, *A, B, I,* and *J*; motor, *C, D, K*, and *L*; frontopolar, *E, F, M,* and *N*; and visual, *G, H, O, P*) stained for cytoarchitecture (Nissl) and myelinated axons (myelin) in adjacent sections in adult humans (*A–F*) and chimpanzees (*G–L*). Nissl-stained sections are subdivided into layers I to VI. White matter (WM) is demarcated at the bottom of the cortex. (Scale bar: 200 μm.)

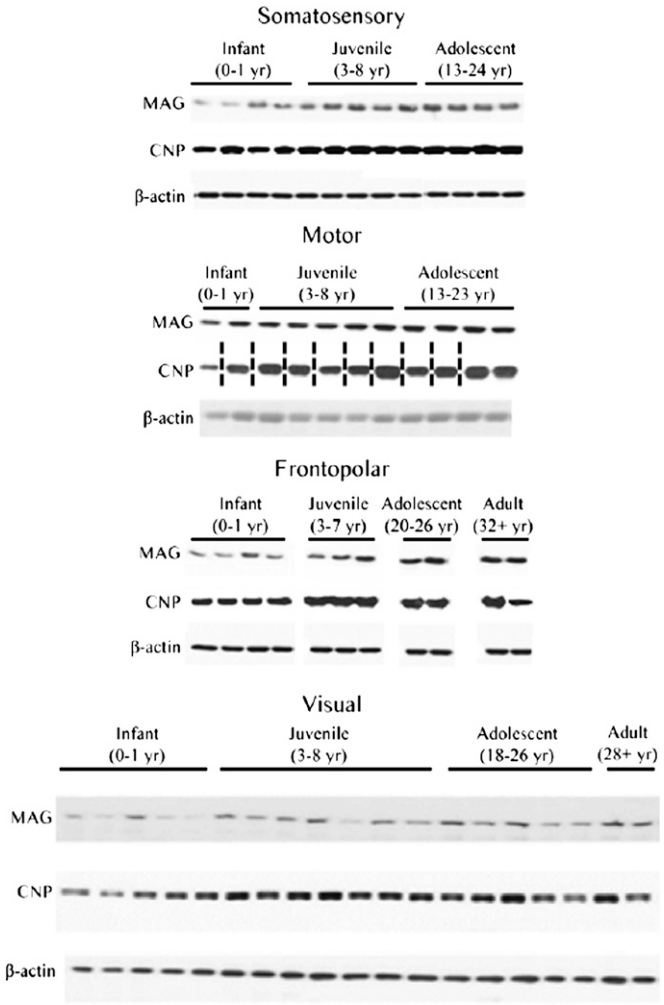

Fig. S2. Immunoblots depicting MAG and CNP protein expression in humans. Representative Western blots of MAG and CNP extracted from frozen human neocortex, arranged by age in years (0–1 y, infant; 3–8 y, juvenile; 13–26 y, adolescent/young adult; and ≥28 y, adult) and region of interest (somatosensory, areas 3a, 3b, 1, and 2; motor, area 4; frontopolar, area 10; and visual, areas 17/V1 and 18/V2). Vertical bars on the blot specify where only one band per sample was used for the representative figure. In the original blot, samples were run in duplicate. Adjustments of brightness were applied to whole images of gels immunoblotted with anti-CNP and anti–β-actin antibodies to facilitate densitometry analysis.

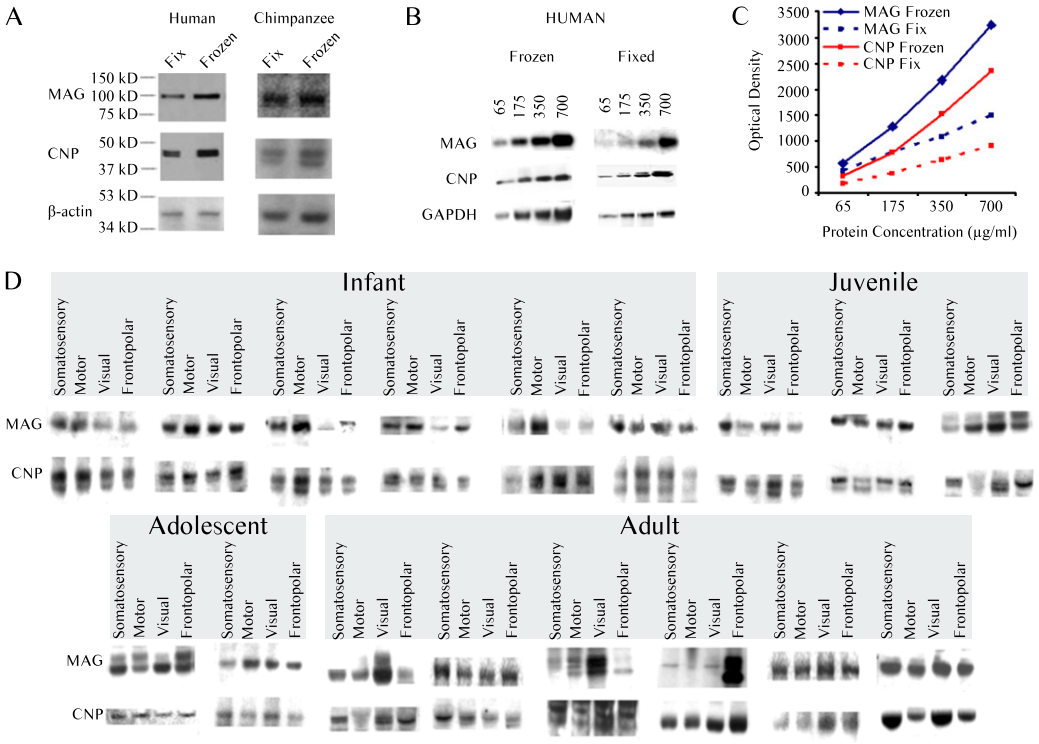

Fig. S3. MAG and CNP protein expression profile in chimpanzee neocortical regions: Western blot analysis validation and representative immunoblots. (*A*) SDS/PAGE pattern of MAG and CNP extracted from formalin-fixed tissue is comparable to that extracted from fresh frozen tissue, as compared between and within species. (*B*) Representative immunoblot of MAG and CNP protein expression analysis in frozen and formalin-fixed tissue producing a linear relationship between protein concentrations. (*C*) Plot of optical density standard curve in Western blot assays used to determine that tissue samples fell within the linear portion of the densitometric curve. (*D*) Representative Western blots of MAG and CNP, arranged by age (0–2 y, infant; 5–6 y, juvenile; 9–11 y, adolescent; and ≥17 y, adult) in years and region of interest (somatosensory, area 3b/3a/1/2; motor, area 4; frontopolar, area 10; and visual, areas 17/V1 and 18/V2).

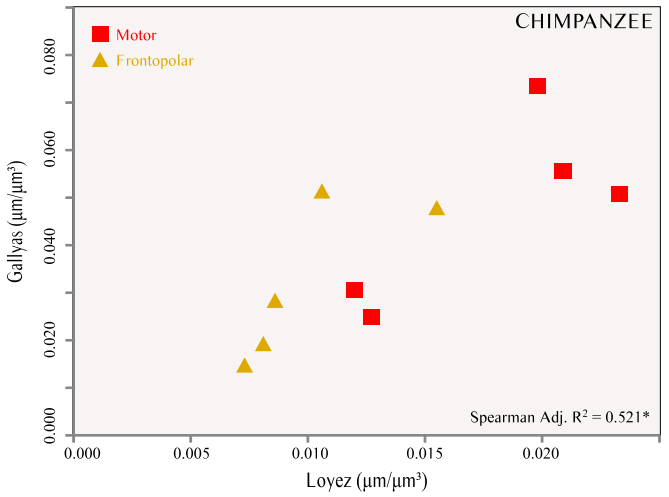

Fig. S4. Interprotocol correlation in chimpanzees. Correlation between the same chimpanzee specimens stained using the Gallyas (*y*-axis) (1) and Loyez (*x*-axis) (2) protocols (n = 5 per region of interest; units are $\mu m/\mu m^3$). Squares represent motor cortex area (area 4) and triangles represent frontopolar cortex (area 10). Nonparametric Spearman adjusted R^2 is reported (*$P < 0.05$).

1. Pistorio AL, Hendry SH, Wang X (2006) A modified technique for high-resolution staining of myelin. *J Neurosci Methods* 153:135–146.
2. Yakovlev PI, Lecours A (1967) The myelogenetic cycles of regional maturation of the brain. *Regional Development of the Brain in Early Life*, ed Minkowski A (Blackwell Science, Oxford), pp 3–70.

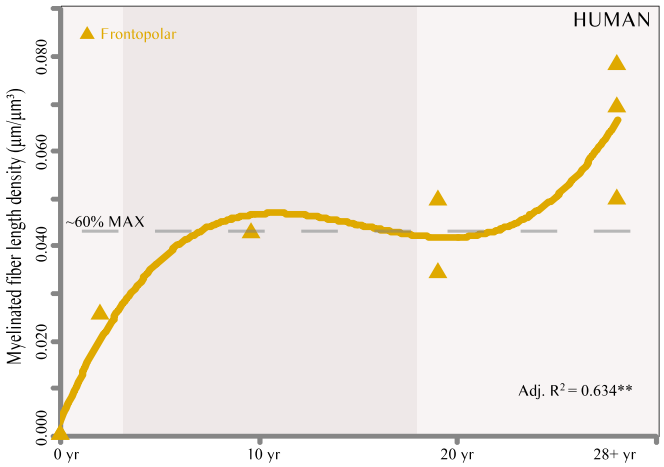

Fig. S5. Developmental trajectory of MFLD in human controls. Best-fit curves for data in human control sections stained by using the Gallyas protocol for myelin by age in years (0–1 y, infant; 3–8 y, juvenile; 13–26 y, adolescent/young adult; ≥28 y, adult). The shaded vertical area represents time between weaning and full sexual maturation. Triangles represent frontopolar cortex (area 10). Dashed line represents 60% of maximum adult MFLD in the sample (**$P < 0.01$; $n = 9$).

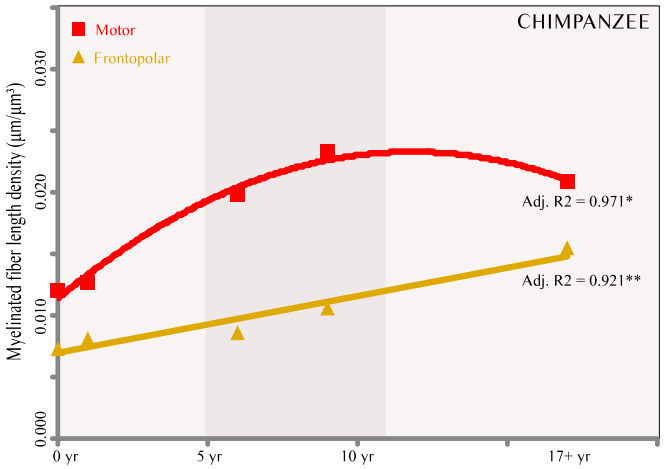

Fig. S6. Developmental trajectory of MFLD in chimpanzee controls. Best-fit curves for data in chimpanzee control sections stained using the Loyez protocol for myelin (1) (*SI Materials and Methods*) by age in years. The shaded vertical area represents time between weaning and full sexual maturation. Squares represent motor cortex (area 4) and triangles represent frontopolar cortex (area 10). (*$P < 0.05$ and **$P < 0.01$; $n = 5$ per region of interest).

1. Yakovlev PI, Lecours A (1967) The myelogenetic cycles of regional maturation of the brain. *Regional Development of the Brain in Early Life*, ed Minkowski A (Blackwell Science, Oxford), pp 3–70.

Table S1. Specimens used in this study

Species	Age	Sex	Hemisphere	Stain
Human	Stillborn	M	Left	Loyez
Human	Stillborn	F	Left	Loyez
Human	Stillborn	F	Left	Loyez
Human	Stillborn	F	Left	Loyez
Human	Stillborn	F	Left	Loyez
Human	Stillborn	F	Left	Gallyas
Human	Stillborn	M	Left	Gallyas
Human	6 wk	F	Left	Loyez
Human	4 mo	M	Left	Western blot
Human	7 mo	M	Left	Loyez
Human	9 mo	M	Left	Loyez
Human	10 mo	M	Left	Western blot
Human	1 y	F	Left	Western blot
Human	2.8 y	Unknown	Left	Gallyas
Human	3 y	M	Left	Western blot
Human	3 y	F	Left	Western blot
Human	3.5 y	M	Left	Loyez
Human	4 y	F	Left	Loyez
Human	4 y	F	Left	Loyez
Human	5 y	M	Left	Loyez
Human	6 y	M	Left	Loyez
Human	6.5 y	M	Left	Loyez
Human	8 y	M	Left	Western blot
Human	9.5 y	F	Left	Loyez
Human	9.6 y	F	Left	Gallyas
Human	11 y	F	Left	Loyez
Human	13 y	F	Left	Western blot
Human	16 y	F	Left	Loyez
Human	18 y	M	Left	Western blot
Human	19 y	M	Left	Gallyas
Human	19 y	M	Left	Gallyas
Human	20 y	M	Left	Loyez
Human	22 y	M	Left	Western blot
Human	23 y	F	Left	Loyez
Human	23 y	M	Left	Western blot
Human	25 y	Unknown	Left	Western blot
Human	26 y	Unknown	Left	Western blot
Human	28 y	M	Left	Loyez
Human	28.5 y	M	Left	Gallyas
Human	32 y	F	Left	Loyez
Human	32 y	Unknown	Left	Western blot
Human	41 y	F	Left	Gallyas
Human	44 y	M	Left	Gallyas
Human	44 y	M	Left	Loyez
Human	47 y	F	Left	Loyez
Human	47 y	Unknown	Left	Western blot
Human	50 y	M	Left	Loyez
Chimpanzee	Stillborn	F	Left	Gallyas
Chimpanzee	Stillborn	F	Left	Gallyas/Western blot
Chimpanzee	Stillborn	M	Left	Gallyas
Chimpanzee	Stillborn	M	Left	Gallyas
Chimpanzee	1 wk	M	Left	Gallyas/Western blot
Chimpanzee	2 wk	M	Left	Gallyas/Western blot
Chimpanzee	1 y	Unknown	Left	Western blot
Chimpanzee	1 y	M	Left	Gallyas/Loyez
Chimpanzee	1.5 y	Unknown	Left	Gallyas/Western blot
Chimpanzee	2 y	F	Left	Gallyas/Western blot
Chimpanzee	5 y	M	Left	Gallyas/Western blot
Chimpanzee	5.3 y	M	Right	Gallyas/Western blot
Chimpanzee	6 y	M	Left	Gallyas/Loyez/Western
Chimpanzee	9 y	M	Left	Gallyas/Loyez/Western
Chimpanzee	11 y	M	Left	Gallyas/Loyez/Western
Chimpanzee	17 y	M	Left	Gallyas/Western blot

Table S1. Cont.

Species	Age	Sex	Hemisphere	Stain
Chimpanzee	19 y	M	Left	Gallyas/Western blot
Chimpanzee	19 y	F	Left	Gallyas/Western blot
Chimpanzee	27 y	M	Left	Gallyas/Western blot
Chimpanzee	35 y	F	Left	Gallyas/Western blot
Chimpanzee	41 y	F	Left	Gallyas/Loyez/Western

Table S2. Percent of maximal, mature adult MFLD during different developmental stages (mean ± SEM)

	Human			Chimpanzee		
Category	Infant (0–1 y)	Juvenile (3–9 y)	Adolescent/ young adult (11–23 y)	Infant (0-2 y)	Juvenile (5–7 y)	Adolescent/ young adult (9–11 y)
No. of individuals	8	7	4	9	3	2
Somatosensory	2.4 ± 2.1	45.1 ± 4.3	65.7 ± 7.2	14.6 ± 6.6	58.4 ± 14.2	97.3 ± 23.8
Motor	3.7 ± 2.2	61.3 ± 2.3	71.4 ± 13.8	18.2 ± 7.8	82.3 ± 25.5	106.3 ± 18.1
Frontopolar	0.1 ± 0.1	28.3 ± 1.9	61.5 ± 7.8	16.2 ± 7.9	57.1 ± 17.1	93.4 ± 49.0
Visual	0.3 ± 0.2	33.0 ± 1.6	61.4 ± 5.6	17.9 ± 8.8	74.7 ± 23.1	86.9 ± 25.2
Control frontopolar	1.5*	52.5*	64.2*	49.7*	55.5†	68.4†
Control motor	—	—	—	59.1*	94.7†	111.4†

*n = 2.
†n = 1.

Table S3. Best-fit regression functions for protein expression data

Function	Function	Adjusted R^2	P value
Humans			
CNP			
Somatosensory	Linear	0.149	0.029*
Motor	Linear	−0.007	0.387
Frontopolar	Quadratic	0.425	0.046*
Visual	Cubic	0.273	0.051*
MAG			
Somatosensory	Linear	0.174	0.02*
Motor	Linear	−0.021	0.559
Frontopolar	Quadratic	0.530	0.038*
Visual	Linear	0.169	0.006†
Chimpanzees			
CNP			
Somatosensory	Linear	0.155	0.043*
Motor	Quadratic	0.106	0.074
Frontopolar	Linear	0.113	0.110
Visual	Quadratic	0.053	0.118
MAG			
Somatosensory	Quadratic	0.158	0.129
Motor	Quadratic	0.159	0.052*
Frontopolar	Linear	0.541	0.001*
Visual	Linear	0.173	0.061

Values calculated by using hierarchical multiple regression analysis with adults as endpoint (*Materials and Methods*). Parametric Spearman adjusted R^2 values are reported. Significant at *$P < 0.05$ or †$P < 0.01$.