Aging causes collateral rarefaction and increased severity of ischemic injury in multiple tissues

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Supplemental Materials

Expanded Materials and Methods

Animals. Male, 3-, 16-, 24- and 31-months-old C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, Maine) or the National Institute of Aging. Experiments were conducted according to IACUC approvals and NIH guidelines.

Hindlimb ischemia. Femoral artery ligation (FAL) was performed as described.[1,2] Mice were anesthetized with 1.25% isoflurane/O_2 and the hindlimbs depilated. Rectal temperature was maintained at 37.0±0.5ºC. The right femoral artery was exposed through a 2mm incision without retraction and with minimal tissue disturbance. A 7-0 ligature was placed distal to the origin of the lateral caudal femoral and superficial epigastric arteries (the latter was also ligated) and proximal to the genu artery. The femoral artery was transected between the sutures and separated 1-2 mm. The wound was irrigated with saline, closed and cefazolin (50mg/kg, im), furazolidone (topical) and pentazocine (10mg/kg, im) were administered.

***Laser Doppler perfusion imaging*.** As detailed,[1,2] under 1.125% isoflurane/O_2 anesthesia and 37±0.5ºC, perfusion imaging (Moor Instruments, Wilmington, DE) of the plantar foot (index of the overall leg perfusion) and adductor thigh region (index of perfusion of superficial collaterals in the thigh) were performed before, immediately after, and 3 and 7 days after FAL on the ligated and non-ligated leg. Regions of interest were drawn to anatomical landmarks.[1,2] To account for variation in ambient light and temperature and arterial pressure, perfusion was expressed as a ratio of ligated to non-ligated leg.

Muscle function and ischemia. At 3 and 7 after FAL, animals were evaluated for right hindlimb appearance score (index of ischemia): 0, normal; 1–5, cyanosis or loss of nail(s), where the score is dependent on the number of nails affected; 6–10, partial or complete atrophy of digit(s), where the score reflects number of digits affected; 11, partial atrophy of forefoot].[1,2] Hindlimb use scores (index of muscle function) were obtained: 0, normal; 1, no toe flexion; 2, no plantar flexion; 3, dragging foot.[1,2]

Hindlimb histology and morphometry. Seven days after surgery, the center-most regions of the adductor and gastrocnemius muscles of the ligated and non-ligated legs were excized after heparinization and pressure-perfused exsanguination (100 mmHg), maximal dilation and paraformaldehyde fixation.[1,2] Paraffin sections (5 microns thick) of the adductor were stained with modified cyano-Massons elastin stain to obtain lumen diameter at the ~midpoint of the anterior and posterior gracilis muscle collaterals. Adjacent sections were immuno-stained for smooth muscle α-actin to obtain the number of vessels (primarily collaterals) crossing the midpoint of the adductor musculature.[5] Adjacent sections were also immuno-stained for $CD3^+$ and $CD11b^+$ cells in the peri-collateral region[1], and for phospho-eNOS[4] and phospho-VASP to quantify levels within gracilis collaterals. Skeletal muscle capillary density, fiber size and number were obtained in the gastrocnemius muscle from sections stained with lectin.[1,5] Morphometry of digital microscopic images was used to obtain the above parameters.

Postmortem cerebral artery micro-angiography. As described before,[1-3] 7 days after FAL, the abdominal aorta was cannulated retrograde and the circulation was heparinized, perfusion-cleared at 100 mmHg with phosphate-buffered saline (PBS, pH 7.4) containing adenosine (10 mg/ml) and papaverine (4 mg/ml), followed by fixation with 4% paraformaldehyde (PFA). The dorsal calvarium and dura mater were removed to expose the pial circulation. A second catheter was placed retrograde into the thoracic aorta, and a polyurethane solution with a viscosity sufficient to minimize capillary transit (1:1 resin-to-methylethyl ketone, PU4ii, Vasqtec, Zurich, Switzerland) was infused with the aid of a stereomicroscope to insure filling of all

collaterals. 4% PFA was applied topically, and the polyurethane was allowed to cure. After post-fixation overnight in 4% PFA, the pial circulation was imaged and digitized (Leica MZ16FA, Leica Microsystems, Bannockburn, IL). As described previously,[1-3] all collaterals connecting the middle and anterior cerebral artery (MCA, ACA) trees of both hemispheres were analyzed (ImageJ, NIH) for number, lumen diameter (D) at the midpoint, length and tortuosity (vector length/axial length (l)), and collateral circuit resistance ($l/n \cdot D^4$) was calculated.[3] Number and diameter of the distal-most Type I or Type II arterioles (not connecting or connecting to a collateral, respectively) of the MCA were also quantified. Total cortex area and territories of the MCA, ACA and posterior cerebral artery (PCA) were determined from dorsal images.[3] The number of penetrating arterioles branching from pial collaterals into the cortex was determined.[3]

Mouse middle cerebral artery occlusion and infarct volume. The right middle cerebral artery trunk was exposed at its position midway between the zygomatic arch and the pinna of the ear and permanently occluded by micro-cautery.[3] 3 days later, the mouse received an overdose of ketamine (100 mg/kg ip) and xylazine (15 mg/kg ip). The brain was removed, sliced into 1 mm coronal sections, and incubated in a PBS solution containing 2% 2,3,5 triphenyltetrazolium chloride (TTC) at 37°C for 20 min. Sections were washed in PBS, fixed in 10% formalin and imaged (MZ16FA, Leica). Infarct volume was calculated as the sum of the cortical volume devoid of TTC in each section, and expressed as a percent of total right cortex volume. Collateral remodeling was measured by determining diameter of the ACA-to-MCA collaterals on the ligated and non-ligated cerebral hemispheres.[3]

Immunohistochemistry and circulating blood differential analysis. Tissue preparation and histological procedures have been detailed previously.[1,5] Vessel density was determined by smooth muscle actin (SMA) staining in the adductor (semimembranosus) muscle. The staining was performed using M.O.M Kit (detecting mouse primary antibodies in mouse tissue - Vector). Briefly, the slides were incubated with 3% of H_2O_2 for 5 minutes and washed 2 times with PBS. Sections were incubated with blocking serum for one hour and with monoclonal mouse anti-human smooth muscle actin (1:200 - Dako) for 30 minutes. Slides were washed in PBS and incubated for 10 minutes with secondary anti-mouse IgG M.O.M reagent. DAB vector peroxidase substrate kit was applied as the detection system. SMA-positive vessels in the mid-zone of the semimembranosus muscle were counted as an index of collateral number.[5] CBC analysis was performed by the Pathology Core of the University of North Carolina.

Western blot. 3-months-old and 24-months-old mice were sacrificed one day after FAL and the mesenteric artery was collected. The mesenteric artery was homogenized in lysis buffer (50mM HEPES,150mM NaCl,10% glycerol,1.5mM MgCl2,1mM EDTA,1%NP40). Total protein was measured by BCA kit (Pierce). 80ug protein was resolved using a 4-20% Tris-Glycine gel and was blotted on nitrocellulose membrane. The membrane was blocked with 5% milk and then incubated with primary antibody against nitrotyrosine (1:500, Cell Biolabs – Nitrotyrosine Immunoblot Kit) and actin (1:500, Sigma) at 4C overnight and with corresponding secondary antibody for 1 hour at room temperature. The signal was detected using an enhanced chemiluminescence substrate (Pierce).

***Statistics*.** Data (means ± SEM) were subjected to ANOVA followed by Dunn-Bonferroni corrected *t*-tests, or Student's *t*-tests, as indicated in the figures.

Supplemental References

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2. Chalothorn D, Faber JE. Strain-dependent variation in native collateral function in mouse hindlimb. *Physiol Genomics*. 2010;42:469-79.
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Supplemental Table I. Granulocytes lower 7 days after MCA occlusion. Aging increases platelets (PLT).

		WBC 10^3/ul	LYMF 10^3/ul	GRAN 10^3/ul	MONO 10^3/ul	LYMF %	GRAN %	MONO %	HCT %
3 months	**Mean**	5.86	4.75	↓ 0.48#	0.64	80.70	↓ 8.86#	10.44	35.63
	SE	0.43	0.38	0.09	0.09	2.87	1.48	1.43	2.15
24 months	**Mean**	5.99	4.84	↓ 0.41#	0.76	75.69	10.31	14.00	32.90
	SE	1.07	0.94	0.06	0.11	5.98	3.36	2.64	1.02

		MCV fl	RBC 10^6/ul	HGB g/dl	MCH pg	MCHC g/dl	RDW %	MPV fl	PLT 10^3/ul
3 months	**Mean**	40.68	8.75	13.88	15.93	39.19	18.96	6.51	563.6
	SE	0.32	0.50	0.74	0.28	0.82	0.37	0.08	70.3
24 months	**Mean**	39.50	8.32	13.41	16.14	40.90	18.83	6.56	↑ 1089.3*
	SE	0.40	0.22	0.29	0.21	0.58	0.38	0.06	70.6

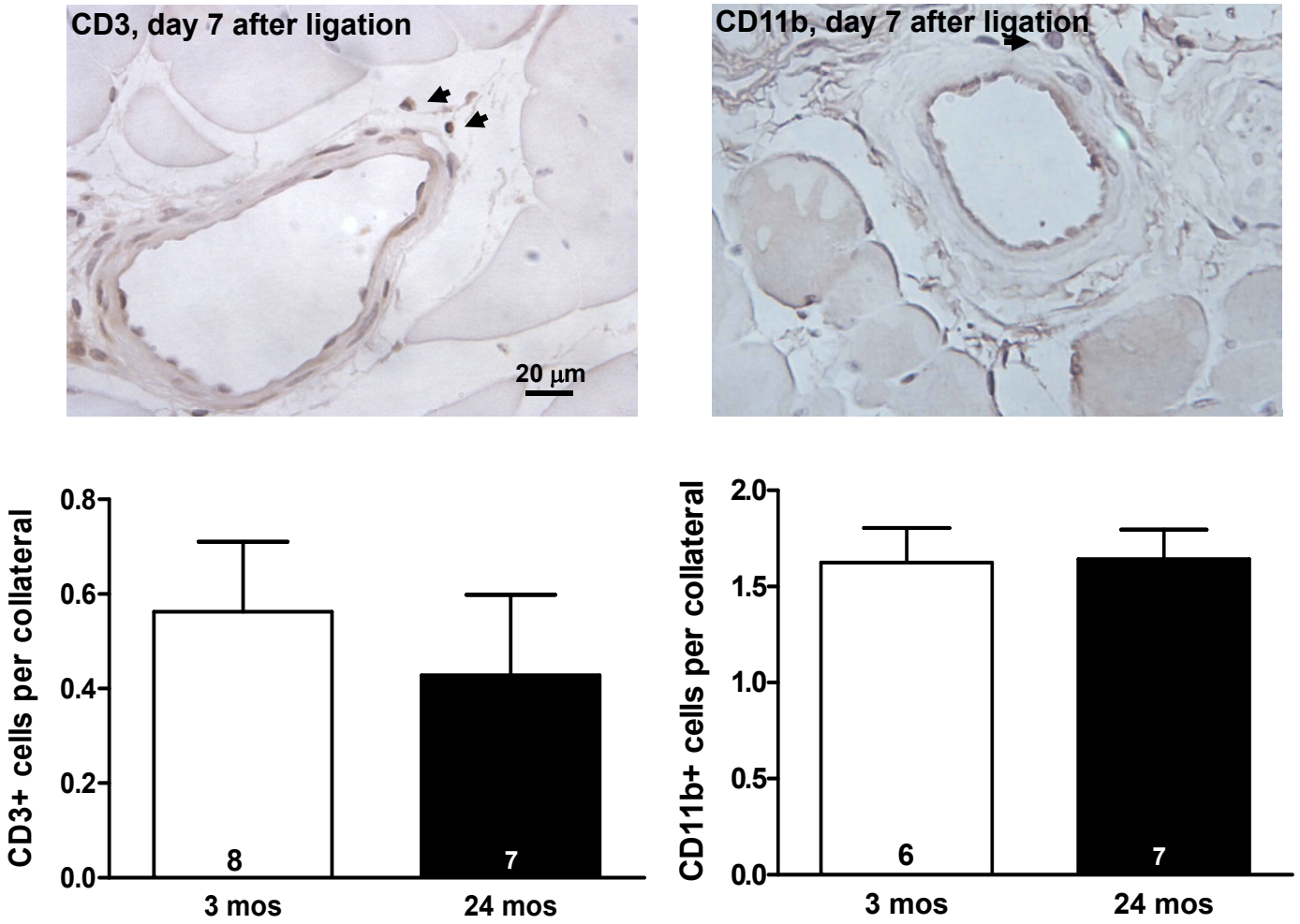
3-months-old, n=8; 24-months-old, n=8. SE, standard error of mean; MCV, mean corpuscular volume; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume. Blood samples were taken 3 days after permanent MCA occlusion. *, $p < 0.05$ vs 3-months-old group. #, $p < 0.05$ vs no-MCA occlusion control mice (Table 2). Granulocyte decrease may reflect accumulation in infarcted cerebral tissue, since same decrease observed in 3- and 24-months-old groups (dotted box = trend) and no decrease seen in 6-months-old control group shown in Supplemental Table 2. These data show that there are no differences in circulating leukocytes in 24-months-old mice, consistent with lack of a difference in T-cell and macrophage accumulation around remodeling collaterals (Suppl Fig I), to account for the observed decrease in collateral remodeling. Thrombocytosis, a marker of inflammation consistent with decreased eNOS activity and increased nitrotyrosine (Fig 6) , supports reduced NO and increased oxidative stress known to characterize the aged vasculature.

Supplemental Table II. CBC blood analysis in 5-6 month old control mice that did not receive MCA occlusion.

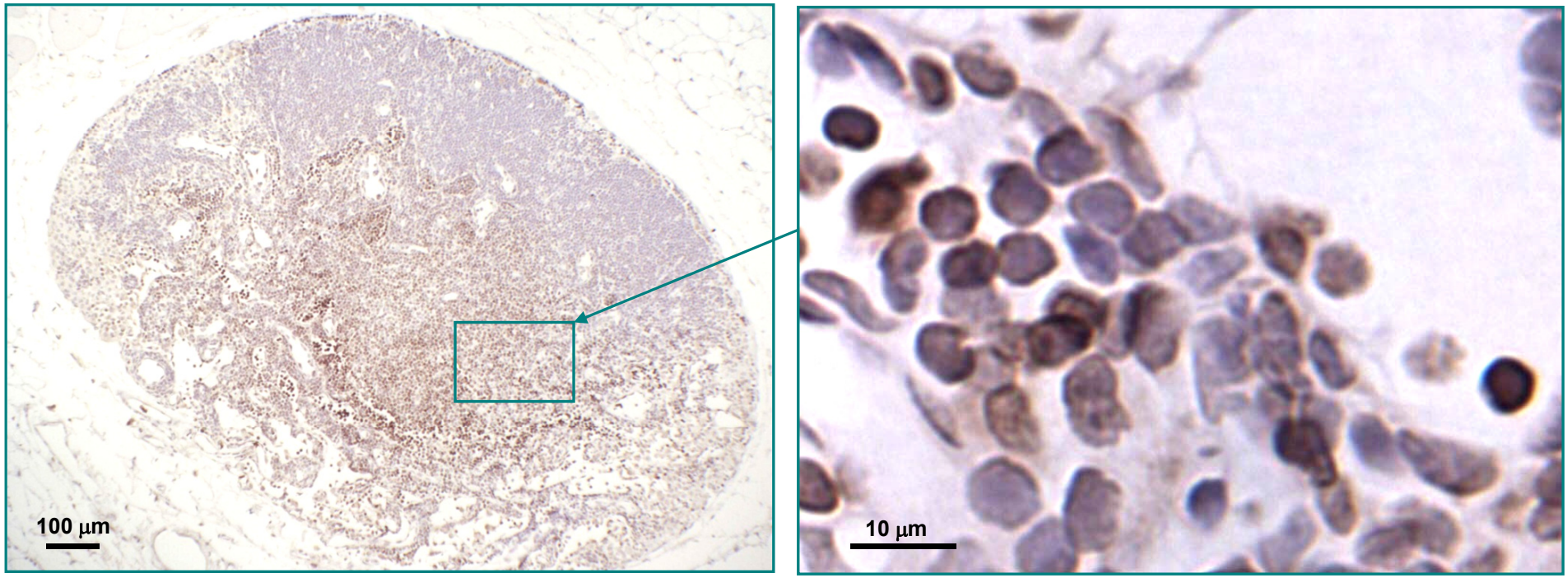
		WBC 10^3/ul	LYMF 10^3/ul	GRAN 10^3/ul	MONO 10^3/ul	LYMF %	GRAN %	MONO %	HCT %
Non-surg Ctrl	Mean	6.80	5.11	0.97	0.80	71.76	17.16	12.31	38.54
	SE	1.11	0.99	0.16	0.10	3.76	2.39	0.87	0.77

		MCV fl	RBC 10^6/ul	HGB g/dl	MCH pg	MCHC g/dl	RDW %	MPV fl	PLT 10^3/ul
Non-surg Ctrl	Mean	41.55	9.28	14.36	15.47	37.28	19.60	6.01	472.1
	SE	0.64	0.15	0.23	0.17	0.24	0.44	0.10	23.9

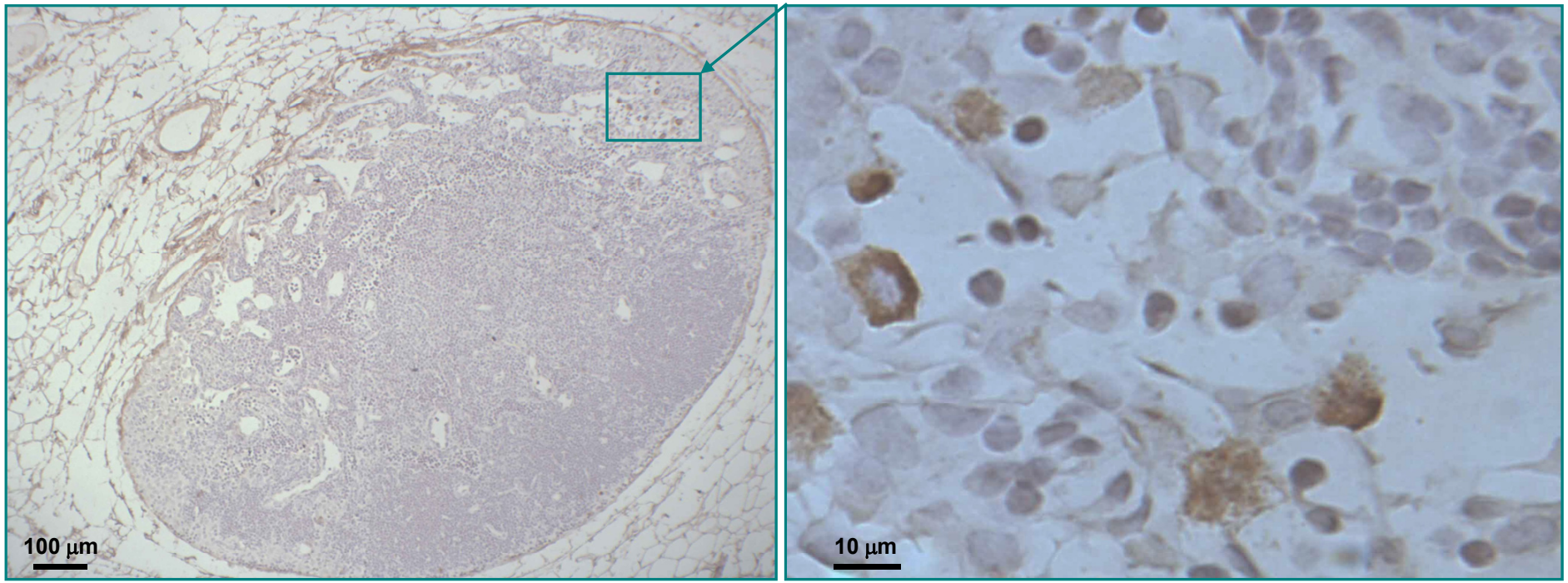
C57BL/6J, n=10. SE, standard error of the mean; MCV, mean corpuscular volume; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume.



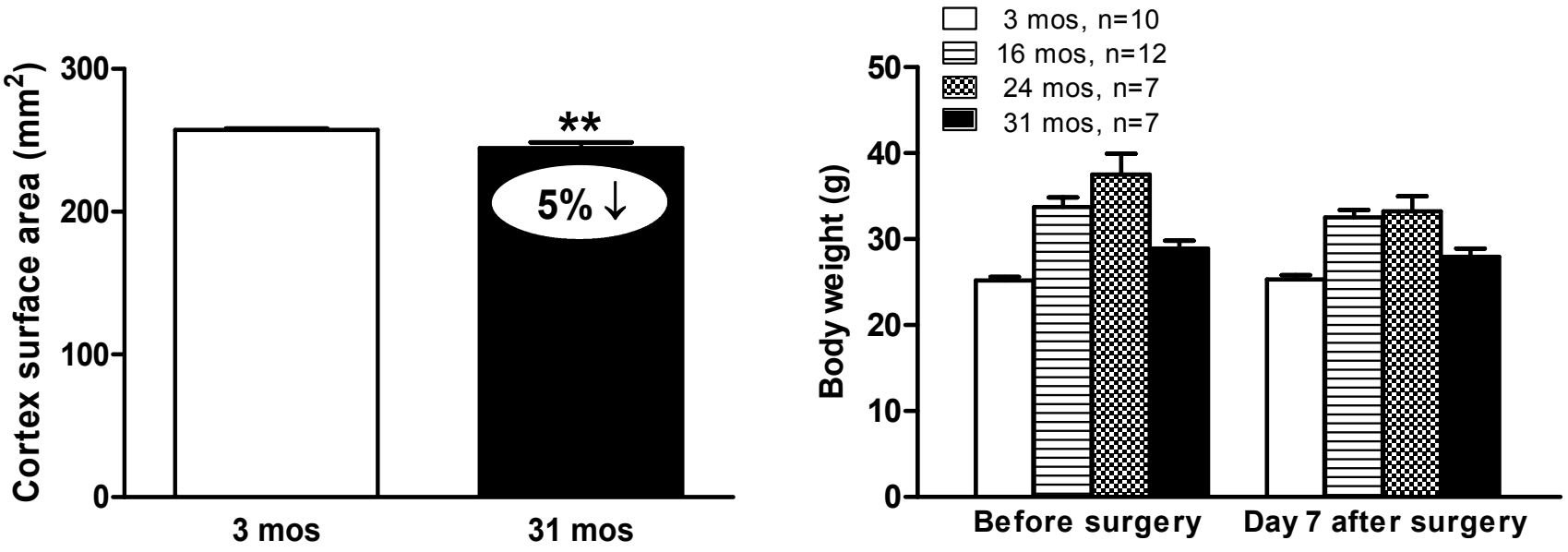
Supplemental figure I. Aging does not impair peri-collateral leukocyte accumulation. No change in T cell (CD3+) or macrophage (CD11b) recruitment to remodeling collaterals, detected by immunohistochemistry of gracilis collaterals at day-7 after ligation. Arrow indicates positives cells. Magnification same for both sections.



Supplemental figure II. Positive control for CD3 immunohistochemistry (pan-T cell marker), showing $CD3^+$ T cells in lymph node.



Supplemental figure III. Positive control for CD11b immunohistochemistry, showing CD11b^{+} macrophages in lymph node.



Supplemental figure IV. 5% decrease in cortex surface area is consistent with 5% decrease in MCA tree territory (Figure 5), indicating a much smaller decrease in brain size with advanced aging than the 22% decline in collateral number and 30% decrease in diameter (Figure 3). That is, the latter decreases cannot be ascribed to reduced brain size with aging. Body weight data show no significant loss of body weight after FAL surgery, indicating good recovery from surgery and the mild stress caused by Doppler and use/appearance score determinations on day-3 and day-7. 31-months-old group shows expected decline in baseline body weight (before FAL) with advanced age.