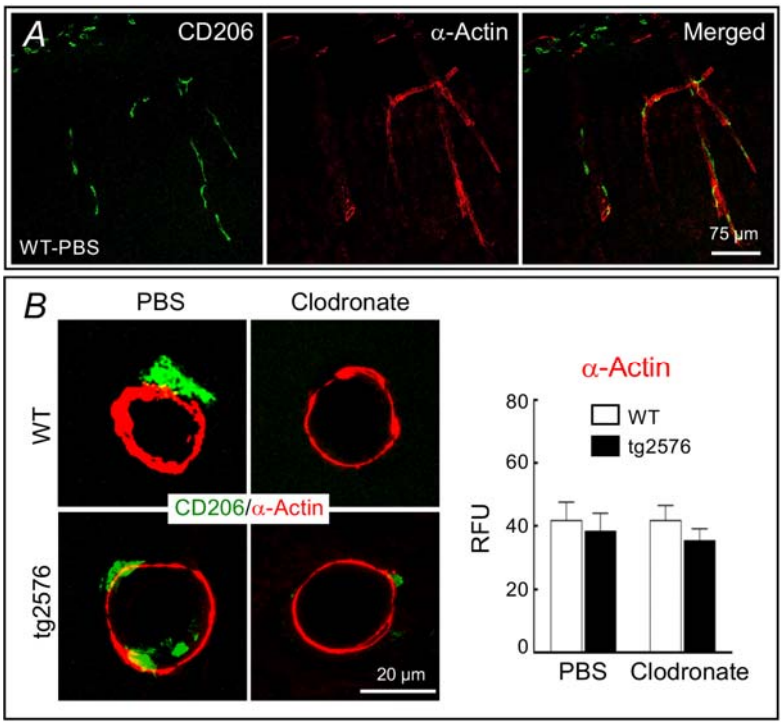
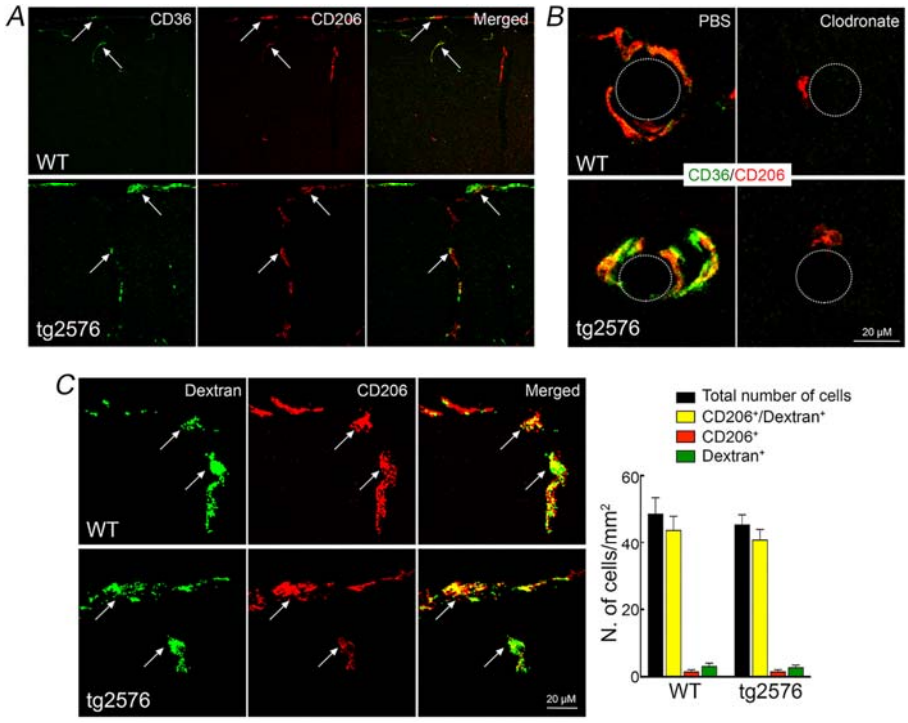
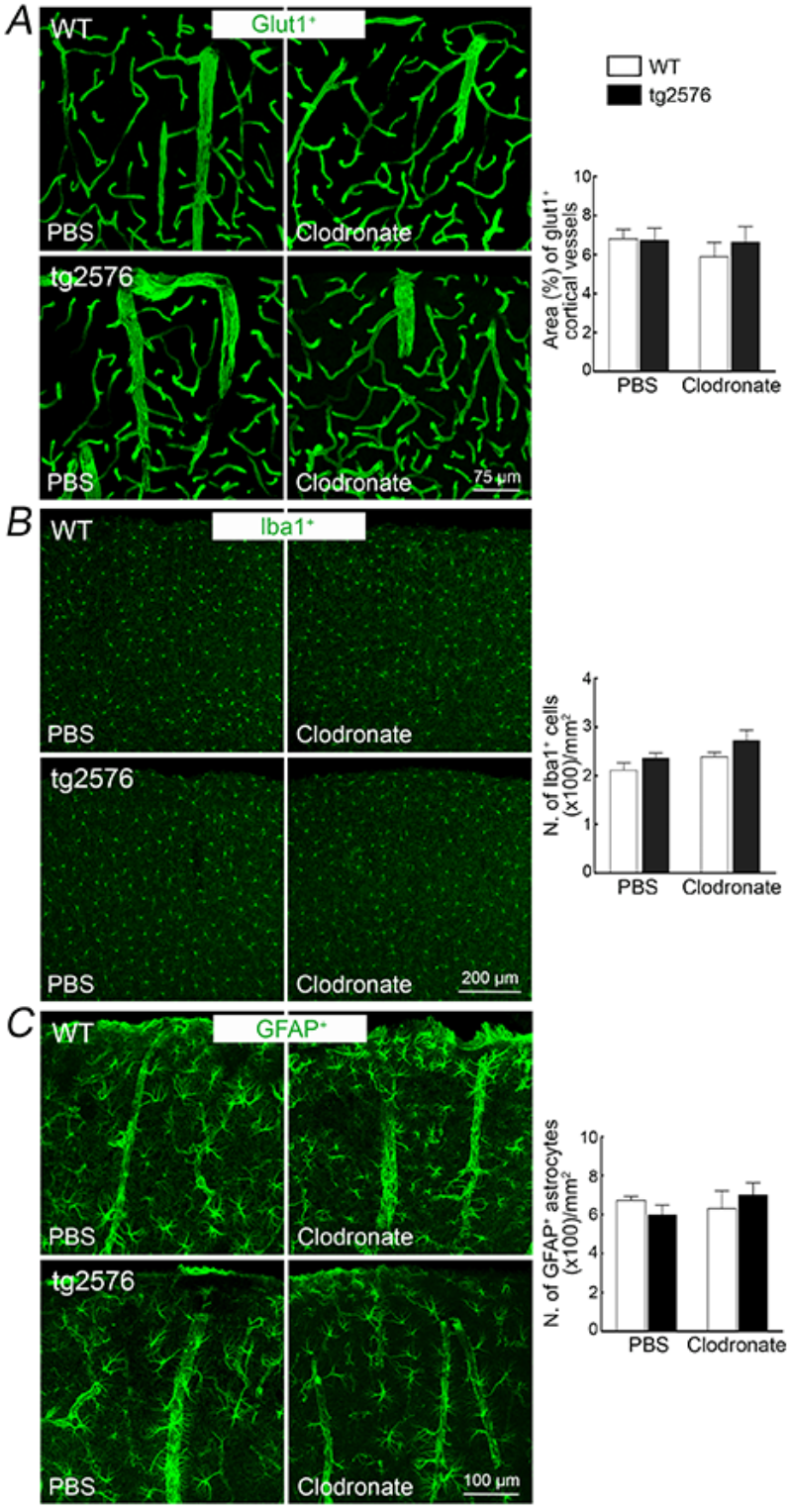
SUPPLEMENTAL MATERIAL



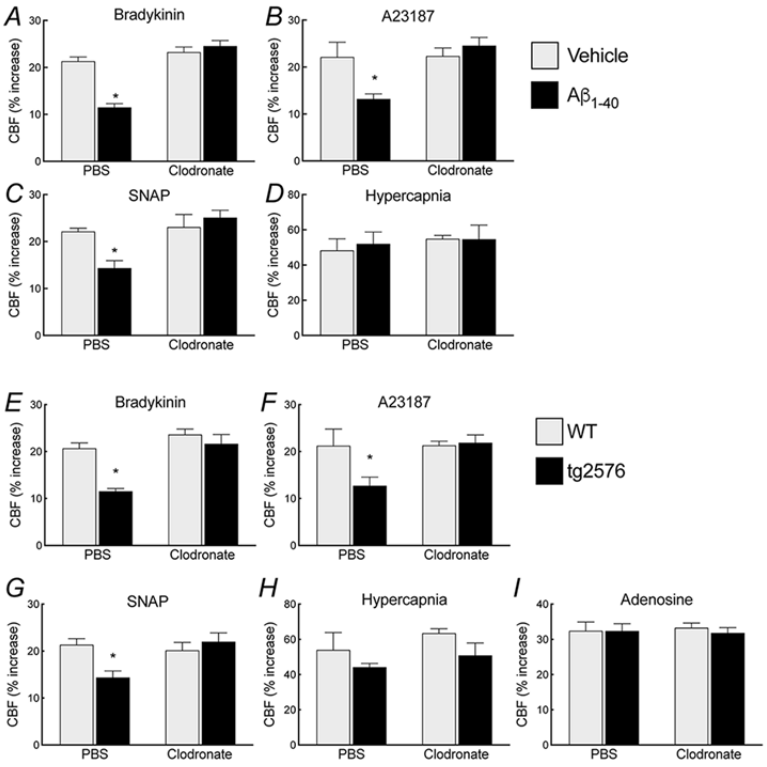
Online Figure I: PVM are associated with resistance arterioles: CD206+ PVM are juxtaposed along penetrating arterioles identified by the smooth muscle cell marker α-actin (**A**), both in WT and in Tg2576 mice (**B**). Clodronate depletes PVM but does not affect the smooth muscle cell marker α-actin (**B**). α-actin immunoreactivity, is not affected by icv injection of clodronate in WT and tg2576 mice (**B**). RFU, relative fluorescence units $p>0.05$, student's t-test; n=5/group.



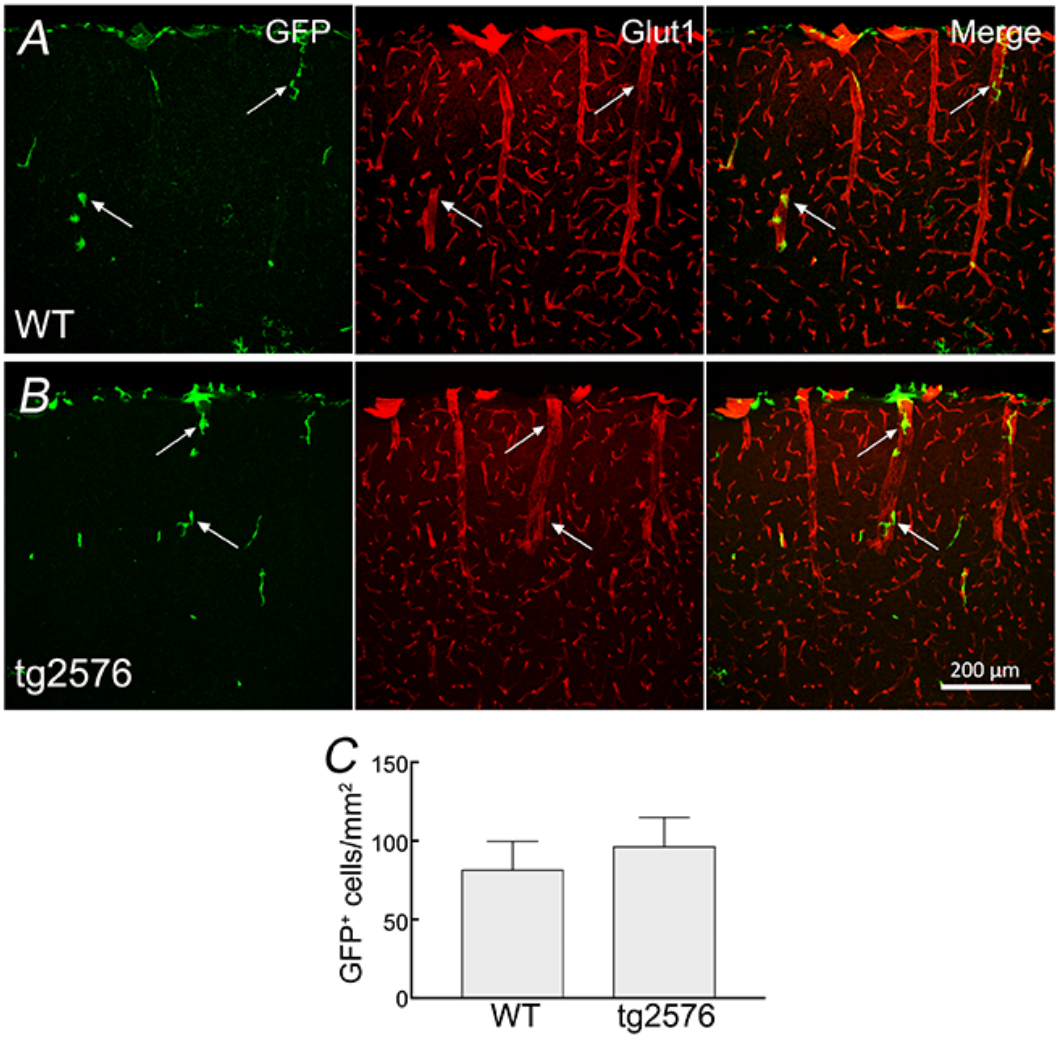
Online Figure II: CD36 expression in CD206^{+} PVM and co-localization between CD206 and dextran in WT and Tg2576 mice. CD206-labelled PVM also expressed CD36, both in WT and Tg2576 mice (**A,B**). Merged images illustrating that clodronate depletes CD36/CD206 double-positive cells both in WT and Tg2576 mice (**B**). More than 90% of dextran-positive cells also express CD206 (**C**). * $p<0.05$, analysis of variance and Tukey's test; n=5/group.



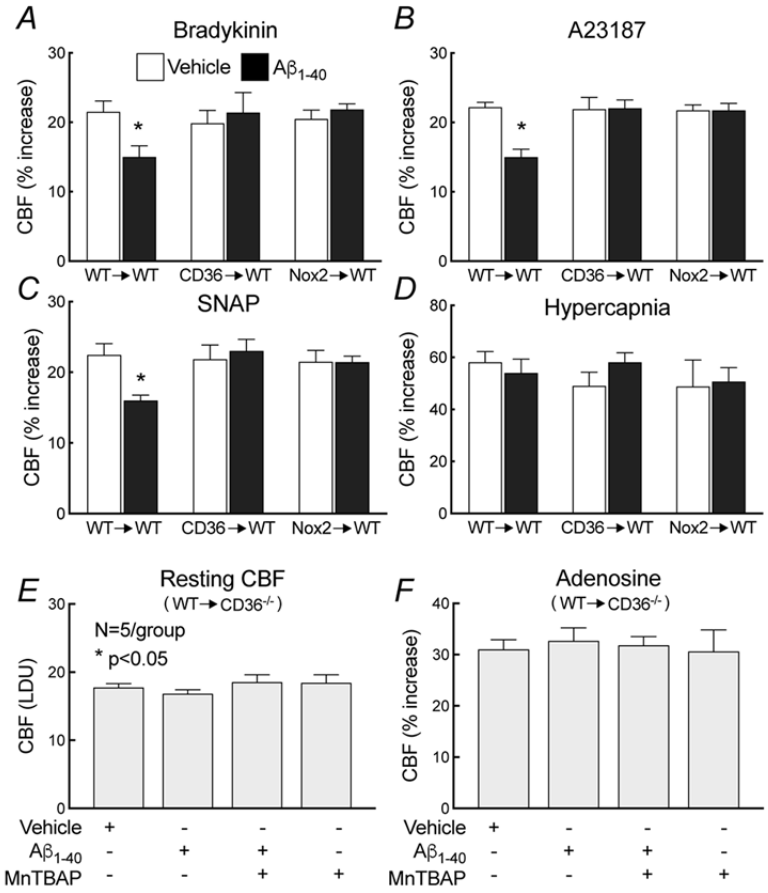
Online Figure III: Clodronate does not affect cerebral microvessels, Iba1+ macroglia/macrophage, and astrocytes. Microvessels identified by the endothelial marker Glut1^{+}(**A**), microglia/macrophages (Iba1^{+})(**B**), and astrocytes (GFAP^{+})(**C**) in somatosensory cortex are not affected by clodronate-liposomes, both WT and tg2576 mice. $p>0.05$, analysis of variance and Tukey's test; n=5/group.



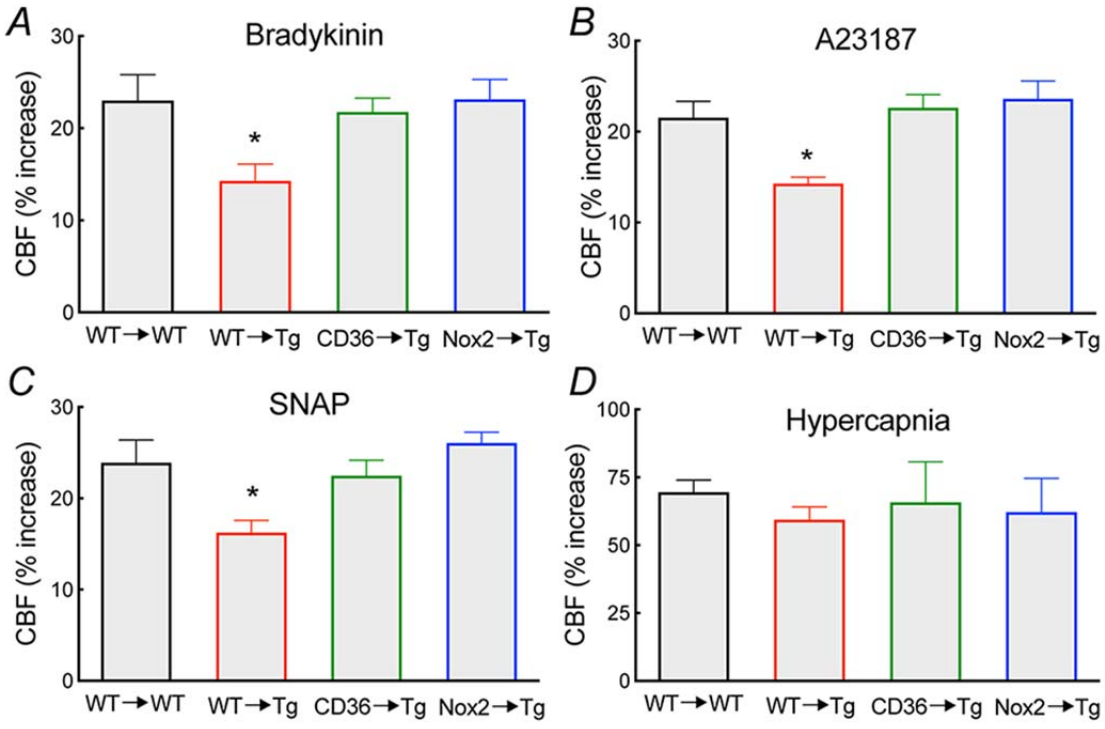
Online Figure IV: Effect of clodronate on cerebrovascular responses in WT mice with $A\beta_{1\text{-}40}$ superfusion, and in Tg2576 mice. The attenuation of cerebrovascular response to topical application of bradykinin (**A**), the calcium ionophore A23187 (**B**), or the NO donor SNAP (**C**) induced by neocortical superfusion of $A\beta_{1\text{-}40}$ is not observed in WT mice treated with clodronate. The CBF response to hypercapnia (pCO_2 50-60 mmHg) is not affected by $A\beta_{1\text{-}40}$ or clodronate treatment **(D)**. Clodronate also rescues CBF responses in Tg2576 mice (**E-G**). The CBF response to hypercapnia or adenosine is not attenuated in Tg2576 mice and is not altered by clodronate (**H,I**) . *$p<0.05$ from vehicle or WT, ANOVA and Tukey's test; n=5-6/group.



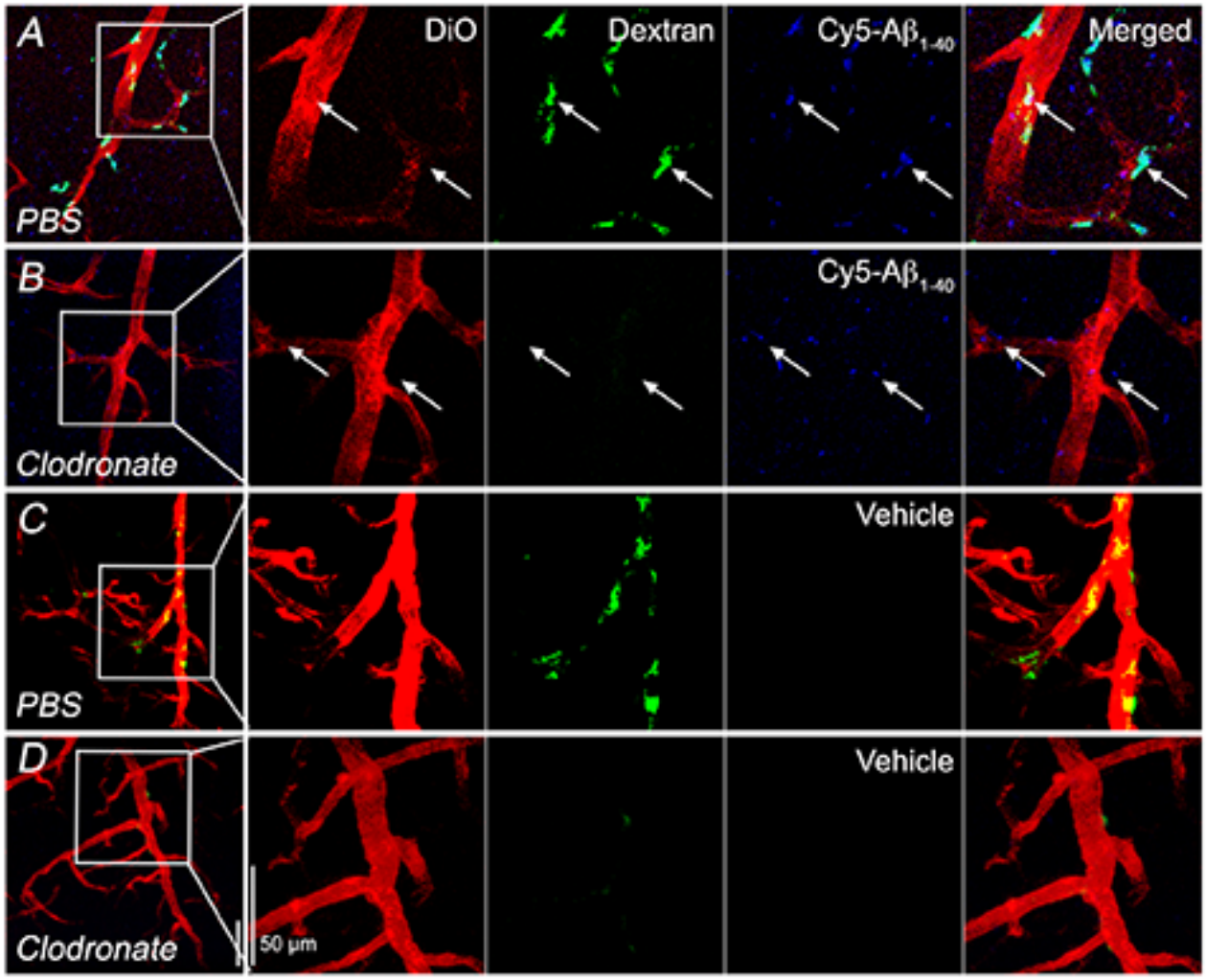
Online Figure V: Number of perivascular distribution of GFP+ cells after GFP+ BM transplant in WT and Tg2576 mice: WT and Tg2576 mice were transplanted with GFP+ BM and the repopulation of the perivascular space of PVM was determined and quantified 2 months later. The pattern of perivascular localization (**A,B**) and number (**C**) of GFP+ PVM is comparable in both groups. p>0.05, student's test; n=5-6/group.



Online Figure VI: Deletion of CD36 or Nox2 in PVM ameliorates the neurovascular dysfunction induced by $A\beta_{1\text{-}40}$ superfusion in WT mice. $A\beta_{1\text{-}40}$ superfusion attenuates CBF responses to bradykinin, A23187 and SNAP (**A-C**). However, these cerebrovascular effects of $A\beta_{1\text{-}40}$ are not observed in CD36→Tg and Nox2→Tg mice (**A-C**). The CBF response to hypercapnia is not affected in all the chimeras (**D**). Resting CBF and response to adenosine are not affected by Aβ superfusion or MnTBAP treatment in all groups; *p<0.05 from vehicle ANOVA and Tukey's test; n=5-6/group.



Online Figure VII: Deletion of CD36 or Nox2 in PVM counteracts the neurovascular dysfunction in Tg2576 mice. Transplant of CD36-/- or Nox2-/- BM into Tg2576 mice rescues the attenuation in the CBF response to bradykinin (**A**), A23187 (**B**), or SNAP (**C**). The CBF response to hypercapnia is not affected in the chimeras. *p<0.05 from WT➔WT, CD36➔Tg, or Nox2➔Tg; ANOVA and Tukey's test; n=5-6/group.



Online Figure VIII: Circulating $A\beta_{1\text{-}40}$ enters the perivascular space and reaches PVM . Vessels are visualized with DiO (red) and PVM are identified by the presence of phagocytized dextran (green). In WT mice treated with PBS, Cy5-labelled $A\beta_{1\text{-}40}$ infused into the carotid artery ($icA\beta_{1\text{-}40}$) is observed in the perivascular space in close association with PVM (**A**). In mice treated with clodronate, the signal from Cy5-labelled $A\beta_{1\text{-}40}$ is still present in the brain, but is no longer associated with dextran-positive PVM, which are depleted (**B**). As a control, when vehicle is injected into the carotid artery of mice treated with PBS-liposomes (**C**) or clodronate (**D**) the Cy5 signal is not observed.

MATERIALS AND METHODS

Mice

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine. Experiments were performed in 3-4 month-old transgenic mice overexpressing the Swedish mutation of the amyloid precursor protein (APP) (Tg2576)[1] or age-matched WT littermates, referred to as WT mice. In BM chimera experiments $CD36^{-/-}$ and $Nox2^{-/-}$ mice were used as BM donors. In some studies, GFP+ mice (JAX Stock #006567) were used as BM donors. All mice were males and derived from in house colonies[2-5].

Intracerebroventricular injection of clodronate or dextran

Liposomes containing clodronate or phosphate buffered saline (PBS) were administered intracerebroventricularly (icv) as previously described[6, 7]. Isoflurane-anesthetized mice were placed in a stereotaxic frame. Ten µl of clodronate-liposomes (7 mg/ml) or PBS-liposomes (vehicle) were injected into the cerebral ventricles with a glass micropipette through a burr hole drilled on the right parietal bone. Mice were used in the experiments 5-7 days later, when PVM depletion is well developed and stable[6, 7]. In some experiments, PVM were identified by their ability to phagocytize dextran[6, 8]. For dextran injections, 10 µl of Alexa Fluor® 680 dextran (10,000 MW, anionic, fixable, ThermoFisher Scientific, D34680; 2.5 mg/ml) in saline or saline alone were injected and PVM labeling was examined 24 hrs later.

Labeling cortical blood vessels with DiO

Cortical blood vessels were labeled with the lipophilic dye DiO [DiOC18(3) (3,3'-Dioctadecyloxacarbocyanine Perchlorate)], as described[9]. Briefly, mice were anesthetized (5% isoflurane) and transcardially perfused with PBS (2 ml) followed by DiO (1:50, V-22886, Molecular Probes; 5ml/mouse) and then by 4% paraformaldehyde (PFA). Brains were harvested and post-fixed in 4% PFA overnight, and then cut (thickness 150 µm) using a vibratome and examined under the confocal microscope (Leica SP5).

Immunohistochemistry

Mice were anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and perfused transcardially with PBS followed by 4% PFA in PBS. Brains were removed, stored overnight, and sectioned in a vibratome (section thickness: 40 µm). Free-floating brain sections were permeabilized with 0.5% Triton X-100 and non-specific binding was blocked with 1% of normal donkey serum. Sections were randomly selected and incubated with the primary antibodies CD206 (clone MR5D3, rat polyclonal, 1:200, Serotec), Glut-1 (rabbit polyclonal, 1:200, Calbiochem), Iba-1 (rabbit polyclonal, 1:500, Wako Chemicals), or GFAP (mouse monoclonal, 1:1000, Sigma) overnight at 4°C. After washing, brain sections were incubated with a Cy5- or a FITC-conjugated secondary antibody (1:200; Jackson ImmunoResearch Laboratories), mounted on slides and imaged with a confocal microscope (Leica SP5). The specificity of the immunofluorescence was verified by omission of the primary and/or secondary antibody or blocking of the antigen. All quantifications were performed by investigators blinded to the treatment on randomly selected fields within the somatosensory cortex.

Identification and quantification of PVM in somatosensory cortex

PVM were identified by well established criteria, including expression of CD206, ability to phagocytize dextran and perivascular location[6, 7, 10] (fig 1). The association with cortical blood vessels was confirmed by co-labeling with the endothelial marker Glut-1 (rabbit polyclonal, 1:200, Calbiochem), the smooth muscle marker α-actin or DiO[9]. For $CD206^{+}$ PVM, randomly

selected fields (20x objective; 4 confocal images/mouse; n=5 mice/group) within the somatosensory cortex were analyzed. For dextran^{+} PVM, a representative coronal section from each mouse was reconstructed from tiled images taken with the confocal microscope, and the whole somatosensory cortex (n=3/group) was analyzed. ImageJ (NIH) was used for all image analyses.

Cerebrovascular ROS measurement

ROS production was assessed *in vivo* by hydroethidine (HE) microfluorography[2-4, 6, 11]. To assess ROS production in PVM, mice were first injected icv with clodronate- or PBS-liposome and 5-7 days later with dextran (see above). The day after dextran injection, in WT mice HE (2 μM in Ringer; Invitrogen) with or without Aβ (5 μM) was superfused over the somatosensory cortex. In tg2576 mice, HE (10 mg/kg) was injected into the jugular vein. BM-transplanted mice were injected with icv dextran one day before ROS measurement. Sixty minutes after HE administration, mice were injected with DiO to label cerebral blood vessels as described above. Coronal brain sections were then cut through the cortex underlying the cranial window, and ROS dependent fluorescence associated with blood vessels or PVM was quantified as described previously[2-4, 6, 11].

Bone marrow transplant

Procedures for BM transplant have been previously described[6, 12] and are only summarized. Whole-body irradiation was performed in 7 weeks-old mice (Nordion Gammacell 40 Exactor). Eighteen hours later, mice were transplanted with BM cells ($2x10^6$, i.v.) isolated from the donor CD36$^{-/-}$, Nox2$^{-/-}$, and WT controls. Mice were housed in cages with sulfamethoxazole (0.12%; w/v) and trimethoprim (0.024%) added to drinking water for the first 2 weeks. Reconstitution of BM cells was verified 5 weeks after irradiation by testing the percentage of positive CD36 or Nox2 genomic DNA in isolated blood leukocytes[12]. Reference primers sequences were as follows: m_ICAM1_prom.3, 5′-GGACTCACCTGCTGGTCTCT-3′ and m_ICAM1_prom.4, 5′-GAACGAGGGCTTCGGTATTT-3′; target primers sequences were as follows: CD36_1, 5'- -3' and CD36_2, 5'- -3', m_Cybb_gt_1, 5'-CTGCTCACCAGCCTCTCTCTA-3' and m_Cybb_gt_2, 5'-CTGGAACCCCTGAGAAAGGAG-3' (Invitrogen). qRT-PCR was conducted with 20 ng of DNA, in duplicate 15 μl reactions using the Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Scientific). Chimerism was >95% for both CD36$^{-/-}$ and Nox2$^{-/-}$ BM chimeras. A PCR cycling protocol consisting of 15 s at 95°C and 1 min at 60°C for 45 cycles was used for quantification CD36 or NOX2 relative expression levels were calculated by 2 (−ΔΔ CT) method For studies of PVM number and distribution after BM transplant in Tg2576 mice, the BM of transgenic mice expressing GFP was transplanted into irradiated Tg2576 mice or littermates.

General Surgical Procedures for CBF measurement

As described in detail elsewhere[2, 4, 11], mice were anesthetized with isoflurane (induction, 5%; surgery, 2%) and maintained with urethane (750 mg/kg; i.p.) and α-chloralose (50 mg/kg; i.p.). A femoral artery was cannulated for recording of arterial pressure and collection of blood samples for blood gas analysis. The trachea was intubated and mice were artificially ventilated with a mixture of N_2 and O_2. Arterial blood pressure (80-90 mmHg), blood gases (pO_2, 120-140 mmHg; pCO_2, 30–40 mmHg; pH, 7.3-7.4), and rectal temperature (37°C) were monitored and controlled. Throughout the experiment the level of anesthesia was monitored by testing motor responses to tail pinch. The somatosensory cortex was exposed through a small craniotomy (2x2 mm). The dura was removed, and the exposed cortex was continuously bathed with a modified Ringer's solution (36-37°C; pH: 7.3-7.4)(see ref.[13] for composition). CBF was continuously monitored at the site of superfusion with a laser-Doppler probe (Vasamedic, St. Paul, MN) positioned stereotaxically on the neocortical surface and connected to a computerized data acquisition system. CBF values were expressed as percent increase relative

to the resting level. Resting CBF is reported as arbitrary laser-Doppler perfusion units (LDU). Zero values for CBF were obtained after the heart was stopped by an overdose of isoflurane at the end of the experiment.

Experimental protocol for CBF experiments

CBF recordings were started after arterial pressure and blood gases were in a steady state. CBF responses to whisker stimulation were recorded while gently stroking the whiskers with a cotton-tipped applicator for 60 sec. All pharmacological agents were dissolved in a modified Ringer's solution. To test endothelium-dependent responses, the endothelial nitric oxide (eNOS)-dependent vasodilator acetylcholine (10 μM, Sigma), the Ca^{2+} ionophore A23187 (3 μM; Sigma) or bradykinin (50 μM; Sigma) was topically superfused for 3-5 min and the evoked CBF increases recorded. To test smooth muscle reactivity, CBF response to adenosine (400 μM, Sigma) or the NO donor S-Nitroso-N-acetyl-DL-penicillamine (SNAP; 50 μM, Sigma) were examined[2, 4, 11]. The increase in CBF produced by hypercapnia was tested by introducing 5% CO_2 in the ventilator to increase arterial pCO_2 up to 50-60 mmHg. Once a stable increase in CBF was obtained for 3-5 min, pCO_2 was returned to normocapnia.

Measurement of Aβ

Aβ was measured using an ELISA-based assay, as described previously[2, 3, 5]. Briefly, the left hemispheres from the mice used for CBF studies were sonicated in 1% SDS with protease inhibitors and centrifuged. The supernatant contained SDS-soluble Aβ peptides. The pellet was sonicated in 70% formic acid and centrifuged as above. The formic acid extract was neutralized by a 1:20 dilution into 1 M Tris phosphate buffer (pH 8.0). $Aβ_{1-40}$ and $Aβ_{1-42}$ concentrations (pM) were determined in supernatant (SDS-soluble) and in the formic acid extract of the pellet (SDS-insoluble) using the BAN-50/BA-27 and BAN-50/BC005 sandwich ELISA assay.

Statistics

Sample size was determined by power analysis based on previous published works published by our lab on CBF regulation. Animals were randomly assigned to treatment or control group and analysis was performed in a blinded fashion. Group difference was analyzed using Student's *t* test (paired or unpaired) or analysis of variance (ANOVA) with Tukey's test, as appropriate. Data are expressed as mean±SEM and statistical difference was considered significant at $p<0.05$.

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