Inventory of Supplemental Items

Supplemental Figures and Legends (5)

Suppl. Fig. 1. Results of control experiment to support CNS-selectivity of liposome-mediated macrophage ablation. Complementary to Fig. 1.

Suppl. Fig. 2. Evidence for perivascular cell involvement in the immune regulation of cerebrovascular expression of mPGES-1, a terminal enzyme in prostaglandin E_2 synthesis. Complementary to Fig. 2.

Supp. Fig. 3. View of individual confocal channels of images shown in Fig. 2 to show detail of prostaglandin E_2 localization in identified vascular cell types.

Suppl. Fig. 4. Complement to Fig. 2, showing that medullary neurons responsive to immune challenges display catechoamine phenotypes (adrenergic or noradrenergic).

Suppl. Fig. 5. Effects of perivascular cell ablation on changes in body temperature and activity of IL-1 or LPS administered during the subjective day. These data complement those shown in Fig. 5, and were specifically requested by Reviewer #1.

Supplemental Experimental Procedures and References (3 pages)

Provides details of standard or non-critical aspects of methodology.

Supplemental Data

Dual Roles for Brain Macrophages in Immune Challenge Effects on Central Stress Circuitry and Acute Phase Responses

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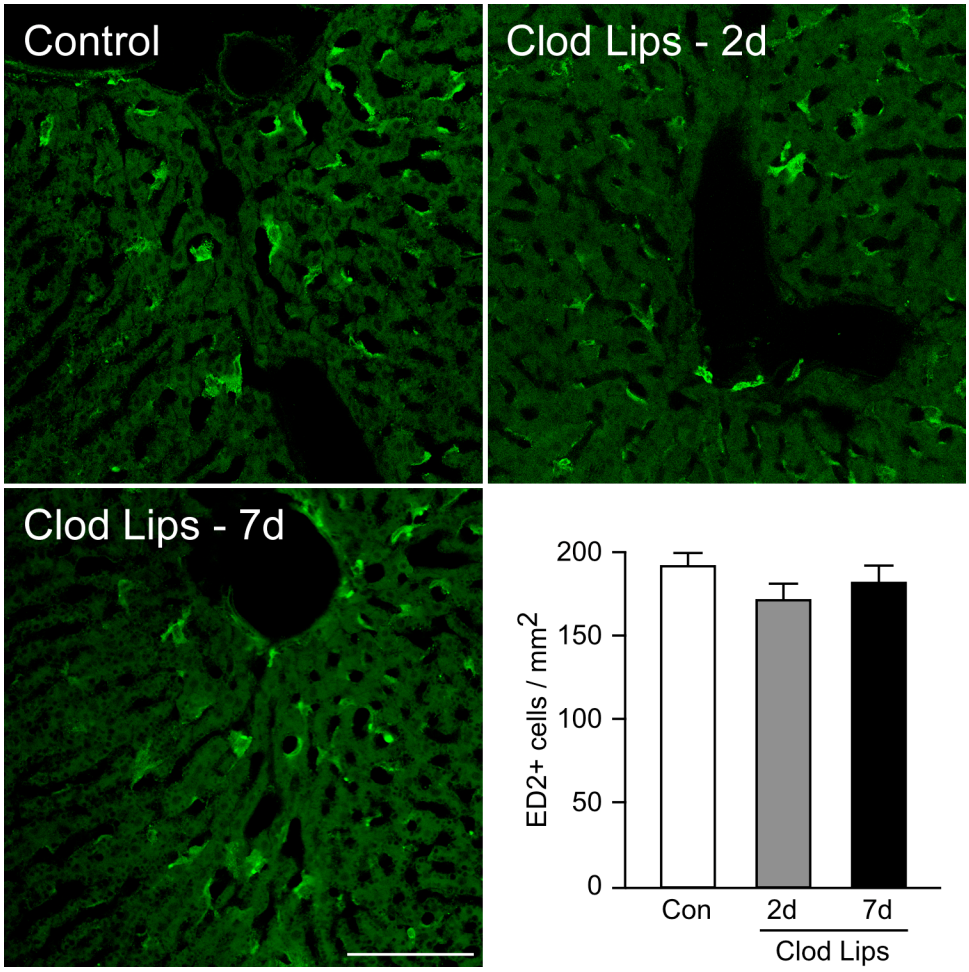
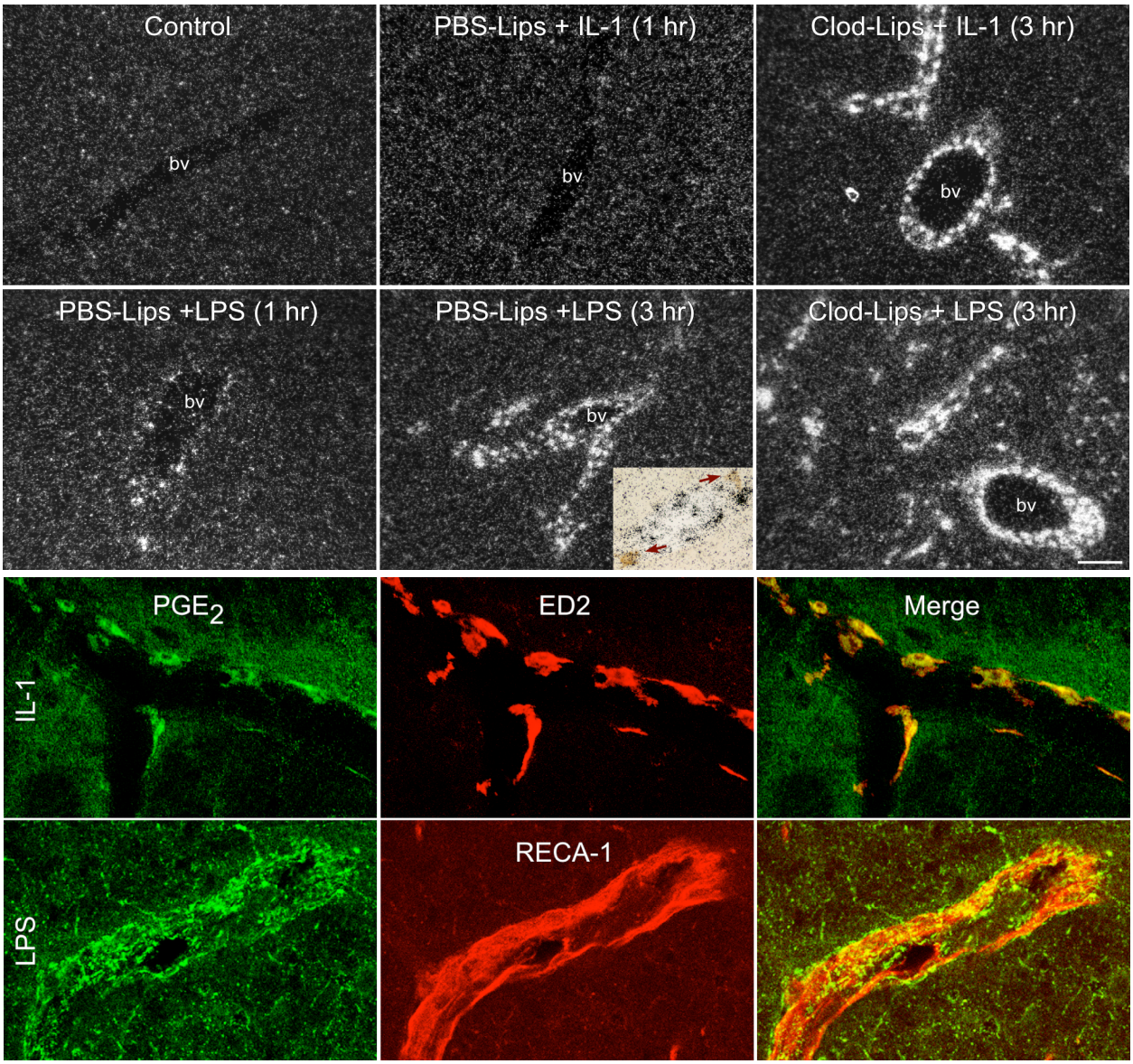
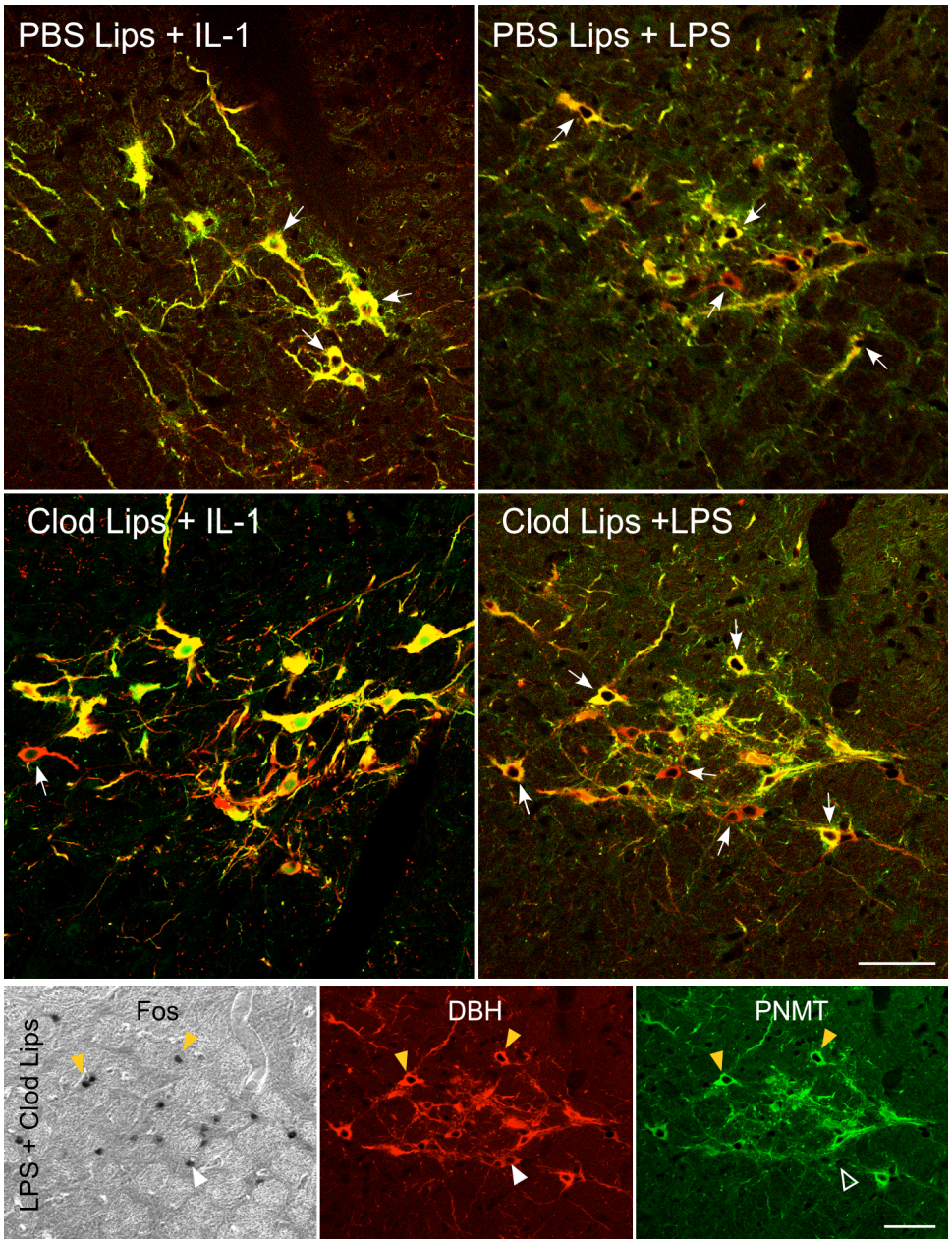
Supplemental Figures



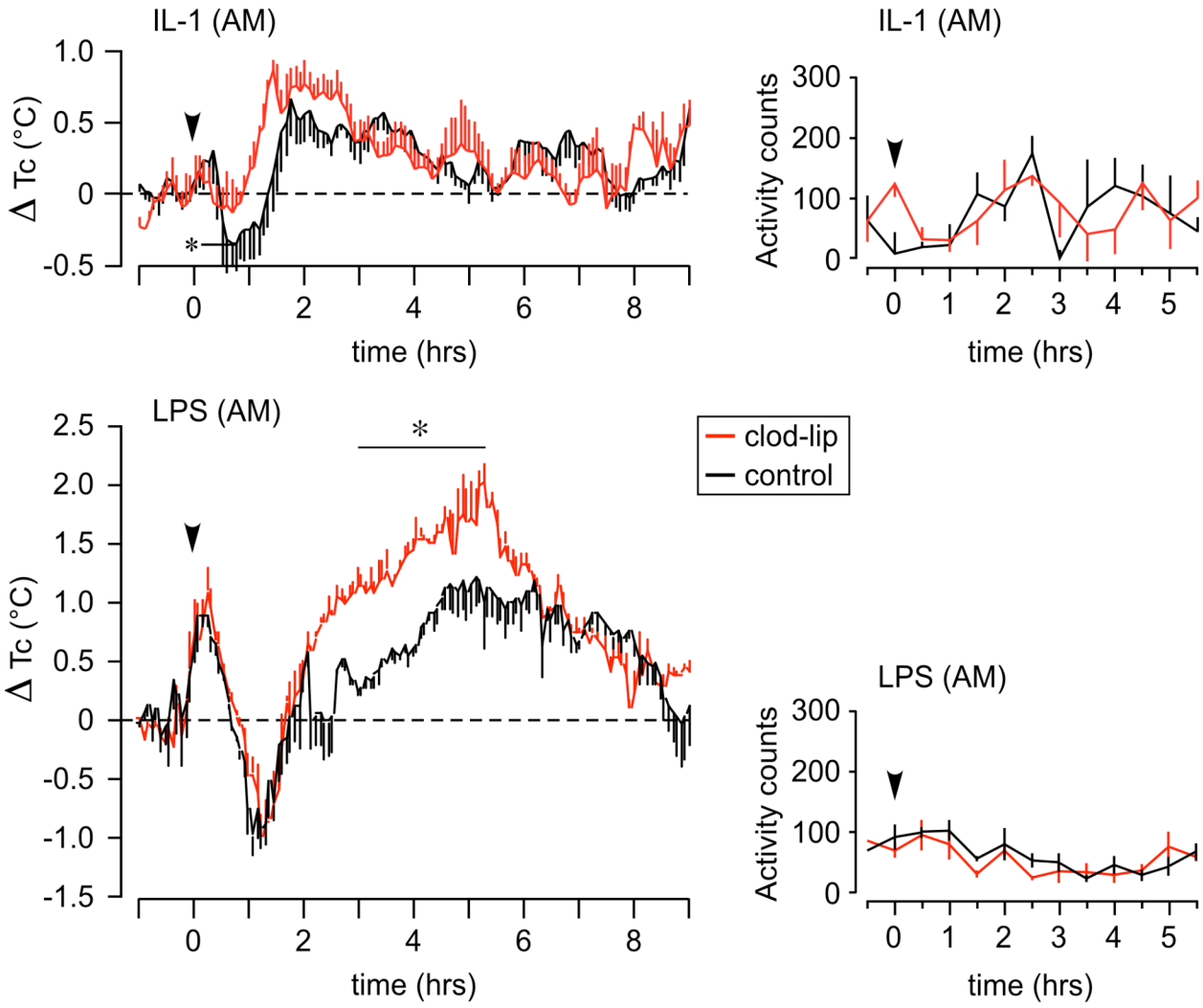
Supplemental Figure 1, related to Figure 1. Effects of central liposome treatment on hepatic macrophages. Immunofluorescence images of ED2-stained Kupffer cells in the liver of an untreated control rat, and ones injected icv with Clod-Lips 2 or 7 d earlier. At neither time point does the density of labeled cells differ significantly from control values (histogram at lower right; n=3 per group), supporting the selective targeting of brain macrophages. Scale bar = 100 µm.



Supplemental Figure 2, related to Figure 2. Further evidence for differential regulation of PGE_2 production in cerebrovascular cell types. *Top*: Vascular mPGES-1 mRNA expression as a function of treatment condition. Vascular mPGES-1 transcripts are not detectable at 1 hr (not shown) and only very scarcely at 3 hr after IL-1 challenge in control liposome pretreated rats. By contrast, mPGES-1 expression is upregulated mildly at 1 hr, and more substantially at 3 hr, after LPS. This includes induction in ED2-labeled PVCs (arrows in inset). In brain macrophage-depleted animals, both challenges elicit much more robust mPGES-1 mRNA responses. Scale bars: 100 µm. *Bottom*: Immune challenge-induced PGE_2-ir in vascular cell types. Expanded confocal images from the middle panels of Fig. 2, bottom, to show individual channels and merged images of sections from rats treated with IL-1 (top row) or LPS (bottom) that were co-stained for markers of perivascular (ED2) or endothelial cells (RECA-1; red), PGE_2-ir (green), and merged channels (right). Doubly stained elements appear as yellow. IL-1-induced PGE_2 production localizes quite discretely to PVCs, while that seen following LPS includes punctate labeling that localizes in part to endothelial cells.



Supplemental Figure 3, related to Figure 3. Aminergic phenotype of medullary immune-responsive neurons. *Top four panels*: Merged confocal images of sections through the A1/C1 region of the ventrolateral medulla from rats in various treatment groups labeled concurrently for challenge-induced Fos (black nuclear immunoperoxidase labeling) and the catecholaminergic markers DBH (red fluorescence) and/or PNMT (green fluorescence). Examples of challenge-responsive adrenergic (yellow cytoplasm with dark nucleus) and noradrenergic (red with dark nucleus) are highlighted with arrows. IL-1- and LPS-induced Fos expression localizes predominantly to catecholaminergic neurons, and is differentially affected in these cell types (reduced and enhanced, respectively) by Clod-Lip pretreatment. *Bottom*: Individual channels from the lower right (Clod Lips + LPS) image, showing examples of individual neurons labeled for all three markers (i.e., LPS-responsive adrenergic neurons; yellow arrowheads) or for Fos and DBH only (i.e., LPS-responsive noradrenergic neuron; white arrowheads). Scale bar: 100 µm.



Supplemental Figure 4, related to Figure 5. Liposome effects on fever and lethargy induced by daytime administration of IL-1 or LPS. Organized like Fig. 5, except that immune challenges were administered during the lights-on portion of the illumination cycle. *Left*: Mean ± SEM change in core body temperature (Tc; data points in 3 min bins) of rats pretreated with control (black) or Clod Lips (red), and challenged (arrowhead) with iv injection of IL-1 (top) or LPS (bottom) 1 hr before lights out. Brain macrophage ablation eliminated an early hypothermic response to IL-1, but did not significantly affect the magnitude or duration of subsequent fever. By contrast, LPS-induced fever was enhanced at 3-5.5 hrs post-injection. *Right*: Locomotor activity data (averaged into 30 min bins) from the same animals. In both intact and brain macrophage-depleted rats, neither immune challenge affected the low basal levels of activity exhibited during the morning hours. n = 9 per group. *, differs significantly from control liposome group, $p<0.05$.

Supplemental Experimental Procedures

Antisera and controls. Fos protein was detected using a rabbit polyclonal antiserum raised against a synthetic peptide corresponding to 4-17 residues of human Fos protein (Oncogene Sciences; cat. no. Ab-5,). Staining in normal and immune-challenged rats was eliminated by preadsorbing the antiserum overnight with 30 μM synthetic immunogen. COX-2 was detected using a polyclonal antiserum raised in goat against a synthetic peptide corresponding to residues 584-604 of the carboxy terminus of the rat COX-2 precursor (Santa Cruz; cat. no. sc-1747). Labeling with this serum conformed to sites of basal and stimulated COX-2 mRNA and was abolished following preadsorption with the immunizing peptide at 40 μM. PVCs were identified using a monoclonal antibody raised against the ED2 macrophage differentiation antigen, produced by immunization with rat splenocytes (Serotec; cat. no. MCA342R). The ED2 antigen has been found to conform to a member of the group B scavenger-receptor cysteine-rich family, CD163. This antiserum stained only cells of macrophage morphology associated with blood vessels in brain and peripheral tissues. Iba 1, used here as marker for parenchymal microglia, was localized using a rabbit polyclonal antiserum generated against the synthetic peptide, PTGPPAKKAISELP (Wako; cat. no. 019-19741). This antiserum recognizes a single band of appropriate Mr (~17 KDa) in Western blots, and immunolocalization in normal brain includes cells typical microglial morphology, as well as PVCs and meningeal macrophages (Imai and Kohsaka, 2002). Dopamine-β-hydroxylase (DBH) was localized using a monoclonal antibody raised against purified bovine DBH (Chemicon; cat. no. MAB308, clone 4F10.2). Phenylethanolamine-N-methyltransferase (PNMT) was detected using a rabbit polyclonal antiserum against purified bovine adrenal PNMT (Howe et al., 1980). Specificity of DBH and PNMT was assessed by comparison of labeling patterns with the consensus distributions (Hokfelt et al., 1984), and with material from the same animals prepared for hybridization histochemical localization of the cognate mRNAs.

Cell counts were generated by sampling regularly spaced series of sections through cell groups of interest, and correcting for double counting error (Abercrombie, 1946).

Hybridization histochemistry. *In situ* hybridization was performed using ^{35}S-labeled sense (control) and antisense cRNA probes labeled to similar specific activities. These included probes generated from a full-length (1.2 kb) CRF cDNA (Dr. K. Mayo, Northwestern University), and a rat mPGES-1 cDNA fragment encompassing nt 4-571 (Dr. A. Ericsson-Dahlstrand, AstraZeneca R&D Södertälje, Sweden). Identity of the cloned cDNAs was confirmed by DNA

sequencing. Sections were mounted onto poly-L-lysine-coated slides and dried under vacuum overnight. They were postfixed with 10% paraformaldehyde for 30 minutes at room temperature, digested with 10 µg/ml proteinase K for 15 minutes at 37°C, and acetylated for 10 minutes. Probes were labeled to specific activities of 1–3 x 10^9 dpm/µg and applied to the slides at concentrations of ~10^7 cpm/ml, overnight at 56°C in a solution containing 50% formamide, 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, 0.05% tRNA, 10 mM dithiothreitol, 1x Denhardt's solution, and 10% dextran sulfate, after which they were treated with 20 µg/ml of ribonuclease A for 30 minutes at 37°C and washed in 15 mM NaCl/1.5 mM sodium citrate with 50% formamide at 70°C. Slides were then dehydrated and exposed to X-ray films (Kodak Biomax MR, Eastman Kodak) for 18 hours. They were coated with Kodak NTB-2 liquid emulsion and exposed at 4°C for 3–4 weeks, as determined by the strength of signal on film. Slides were developed with Kodak D-19 and fixed with Kodak rapid fixer. Quantification of relative levels of CRF mRNA was carried out by densitometry, as previously described (Ericsson et al., 1994).

Electron Microscopy. Rats that received icv of control or Clod-Lips as described above were perfused 7 d later with saline followed by either 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0; n= 3/group) or 2% paraformaldehyde and 2.75% acrolein in 0.1 M phosphate buffer (n=3). 50 µm thick vibratome sections were taken from comparable regions of hypothalamus and medulla, and series of paraformaldehyde/acrolein-fixed sections were prepared for preembedding immunolocalization of the ED2 antigen as described (Schiltz and Sawchenko, 2002). Sections were fixed in 1% osmium tetroxide with 1.5% potassium ferricyanide, dehydrated with ethanol and propylene oxide and infiltrated with Spurr's resin. The sections were embedded, thin-sectioned, and counterstained with uranyl acetate and lead citrate. The material was examined in a JEOL 100 CX II transmission electron microscope.

Intravenous HRP injections. Groups of rats pretreated with icv injection of clodronate or control liposomes (n=3) were fitted with jugular catheters. 2 d later, they received iv injections of 100 µl of an HRP solution (Sigma Type VI at 120 mg/ml in saline). One hr after injection, they were perfused and complete series of sections were prepared for histochemical localization of HRP activity, using diaminobenzidine as the chromogen and a nickel-enhanced glucose oxidase method.

PGE_2 assays. Tissue PGE_2 levels were measured by enzyme immunoassay (EIA) using reagents in kit form (Prostaglandin E_2 EIA Kit-Monoclonal; Cayman Chemical Company). Rats were rapidly anesthetized and briefly perfused transcardially with saline to minimize confounding effects of circulating PGE_2 sources. Hypothalamus and medulla were dissected, snap-frozen and stored at -80° C until analysis. Samples were sonicated in 300 *µ*l homogenization buffer (0.1M phosphate buffer, pH7.4, 1mM EDTA and 10uM indomethacin) and purified by incubation in ethanol at 4x sample volume for 5 min at 4° C and then centrifugation at 3,000 x *g* for 10 min. They were acidified with glacial acetic acid to pH 3.5 and PGE_2 was extracted using SPE (C-18) columns rinsed with methanol and water. After application of samples, columns were washed with water and hexane and PGE_2 was eluted with ethyl acetate. Samples were then evaporated to dryness under nitrogen and resuspended in EIA buffer. Levels of PGE_2 were measured in a 96-well plate and read at 405 nm. The sensitivity of the assay was 15 pg/ml; intra- and interassay coefficients of variation were 6.6% and 15.5%, respectively, at 62.5 pg/ml.

Supplemental References

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