SUPPLEMENTAL MATERIAL

Endoplasmic Reticulum Stress in the Brain Subfornical Organ Mediates Angiotensin-Dependent Hypertension

*Colin N. Young[1], *Xian Cao[2], Mallikarjuna R. Guruju[2], Joseph P. Pierce[3], Donald A. Morgan[4], Gang Wang[3], Costantino Iadecola[3], Allyn L. Mark[2,4] & Robin L. Davisson[1,2]

[1]Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY

[2]Cell and Developmental Biology, Weill Cornell Medical College, New York, NY

[3]Neurology and Neuroscience, Weill Cornell Medical College, New York, NY

[4]Internal Medicine, University of Iowa, Iowa City, IA

*Authors contributed equally

The authors have declared that no conflict of interest exists

Corresponding author:
Robin L. Davisson, Ph.D.
T9-014C Veterinary Research Tower
Cornell University
Ithaca, NY 14853-6401
E-mail: robin.davison@cornell.edu
Phone: 607-253-3598
Fax: 607-253-3378

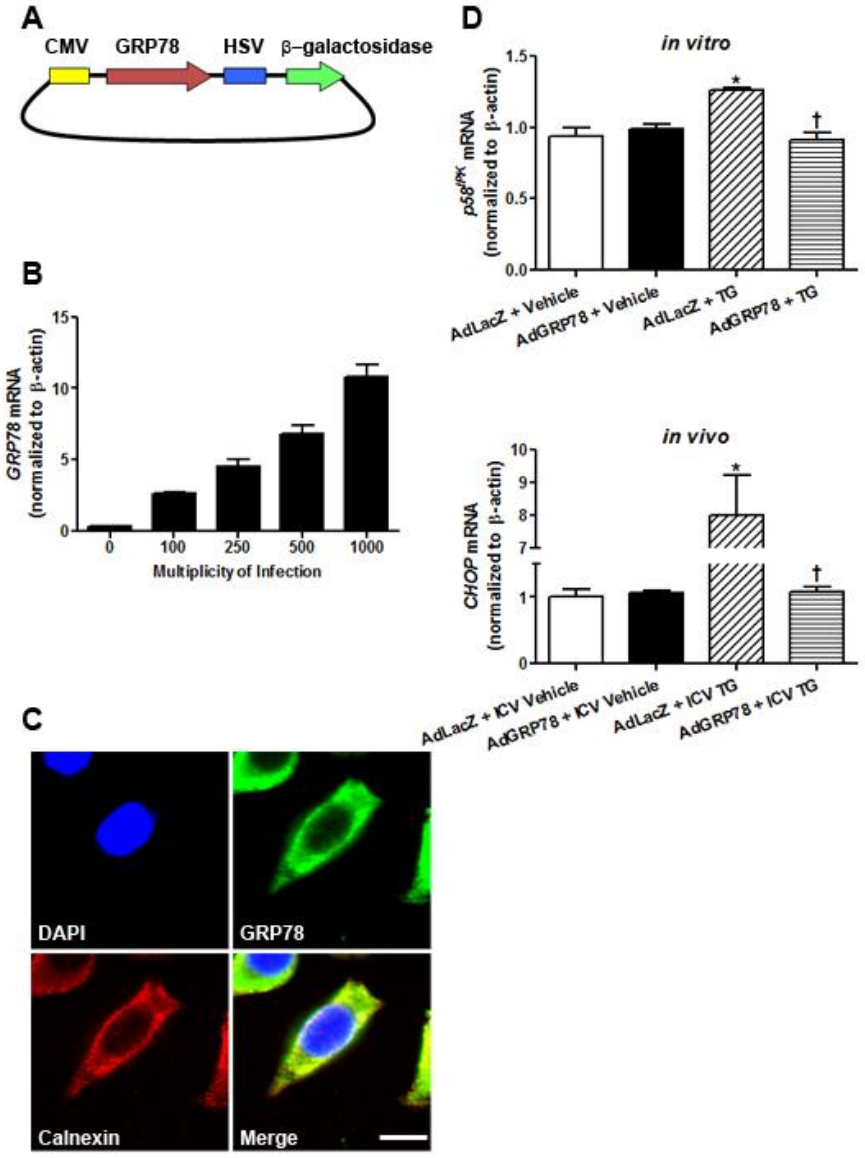

Figure S1. Ad-mediated GRP78 transgene expression in the SFO in vitro and in vivo. (A) Schematic of AdGRP78 vector containing the full-length murine GRP78 gene driven off CMV and β-galactosidase driven off RSV. (B) Real-time PCR revealed concentration-dependent (0-1000 multiplicity of infection) effects of AdGRP78 on *GRP78* transcript levels in Neuro2A cells (n=3 per concentration). (C) Representative immunohistochemistry in Neuro2A cells transfected with AdGRP78 demonstrating robust overexpression and subcellular localization of the

transgene to the ER as indicated by double-labeling with calnexin (n=3 replicates). (D) qPCR demonstrate that AdGRP78 prevents thapsigargin (TG)-induced ER stress biomarker induction in vitro (top, Neuro2A cells, n=3) and in vivo (bottom, SFO homogenates, n=3-4) compared to AdLacZ. *$p<0.05$ vs. vehicle-treated groups; †$p<0.05$ vs. AdLacZ + TG.

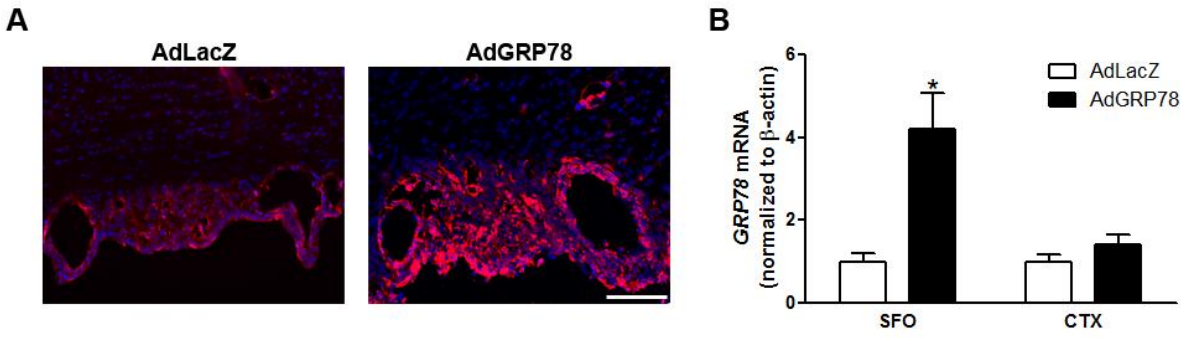

Figure S2. Ad-mediated GRP78 overexpression in the SFO. **(**A) Representative immunohistochemical images of the SFO in mice with SFO-targeted injections of either AdLacZ or AdGRP78 using an antibody against GRP78 combined with nuclear staining (DAPI) (n=4-5). Scale bar = 20μm. (B) Mice with SFO-targeted AdGRP78 demonstrate robust overexpression of the *GRP78* transcript in the SFO compared to AdLacZ-treated animals, but not in the cortex (CTX). n=3-5; *p<0.05 vs. AdLacZ.

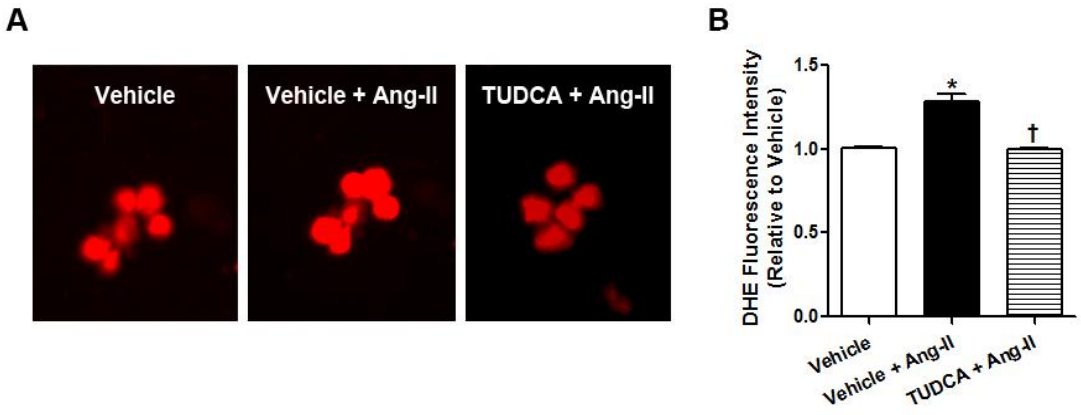

Figure S3. Ang-II-induced oxidative stress in SFO cells is blocked by the chemical ER stress inhibitor TUDCA. (A) Representative images of dihydroethidium (DHE) fluorescence in freshly isolated SFO cells treated with vehicle, vehicle + Ang-II or TUDCA + Ang-II. (B) Summary DHE data from isolated SFO cells. n=10/group; *$p<0.05$ vs. Vehicle. †$p<0.05$ vs. Vehicle + Ang-II.

DETAILED METHODS

Animals. All procedures were approved by the Animal Care and Use Committee at Cornell University and the University of Iowa. Studies were conducted in adult (8-12 wks old) male C57Bl/6 mice obtained from in-house colonies. Mice were fed standard chow and water *ad libitum*. Care of the mice met or exceeded the standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, USDA regulations, and the AVMA Panel on Euthanasia.

Pharmacological agents. The ER stress inhibitor thapsigargin (TG; EMD Chemicals,) was dissolved in dimethylsulfoxide (DMSO) and diluted with saline to the desired concentration. Tauroursodeoxycholic acid (TUDCA; EMD Chemicals) was diluted in artificial cerebrospinal fluid (aCSF) to the final concentration. The doses of TG and TUDCA used in the current study have been confirmed extensively to induce or inhibit ER stress, respectively, in vivo (1-3). Human angiotensin-II (Ang-II; Sigma-Aldrich) was diluted in 0.1% bovine serum albumin (BSA) and the α-1 adrenergic receptor agonist phenylephrine (Sigma-Aldrich) was diluted in saline to the desired concentration. The appropriate vehicle control was used for all studies utilizing TG (DMSO), TUDCA (aCSF), Ang-II (BSA) and phenylephrine (saline).

Arterial pressure and sympathetic nerve recording studies. For anesthetized recordings of arterial pressure (AP) and renal sympathetic nerve activity (RSNA) mice were anesthetized (ketamine, 91 mg/kg + xylazine, 9.1 mg/kg) i.p. Each mouse was intubated (PE-50) to allow for spontaneous respiration of oxygen-enriched air and the left carotid artery was cannulated for continuous measurements of AP. The right jugular vein was cannulated to maintain the level of

anesthesia throughout the protocol with α-chloralose (initial dose: 25 mg/kg; supplemental dose of 6mg/kg/hr). Multifiber recordings of RSNA were performed as previously described (4). Briefly, the nerves to the left kidney were identified under a dissecting microscope and then mounted on custom-made 36-gauge platinum-iridium recording electrodes (Cooner Wire Co.) and the nerve was fixed to the electrode with silicone gel (Kwik-Sil, World Precision Instruments Inc.). Following surgical procedures, the animals were allowed to stabilize prior to obtaining AP and RSNA measurements. Body temperature was maintained at 37°C throughout surgical and recording procedures. RSNA data were acquired and analyzed as previously described (4). The nerve electrodes were attached to a high-impedance probe (HIP-511, Grass Instruments Co.), amplified (10^5) with a preamplifier (Grass P5 AC), and filtered (100-1000Hz). The amplified, filtered neural signal and AP were routed through an analogue-digital converter (Powerlab model 8S, AD Instruments Inc.) to a computer for continuous recording and data analysis. Background noise was determined from the residual RSNA signal following euthanasia.

For conscious recordings of AP mice were anesthetized (ketamine, 150 mg/kg + xylazine, 15 mg/kg, ip) and instrumented with radiotelemetry probes (TA11PA-C10, Data Sciences International) as previously described (5). Briefly, the radiotelemetric catheter was implanted in the thoracic aorta via the left common carotid artery, and the body of the probe was placed in a subcutaneous pocket on the right flank. The wound was closed and sutured, and body temperature was maintained at 37°C throughout surgical procedures and during recovery using a heating pad. For certain experiments, radiotelemetric probes were implanted in the same surgical session as adenoviral gene transfer and/or lateral cerebroventricular cannulation (see below). Mice remained undisturbed in their home cages for a minimum of 7 days to achieve full

recovery of normal circadian rhythm and cardiovascular parameters before obtaining recordings of AP. For certain experiments TG was administered through the brain cannula at a dose of 1 µg/day in 1 µl. In separate protocols, following baseline AP recordings, mice were implanted with 14 day osmotic minipumps (ALZET, Durect Corporation) loaded with Ang-II (600ng/kg/min), phenylephrine (30 µg/kg/day) or appropriate vehicle controls as described (6-9). For studies using brain infusion of TUDCA, administration of this drug (5 µg/day in 1 µl) began on the day of osmotic minipump implantation and continued throughout the 2 wk infusion period.

Lateral cerebroventricle cannulation and targeting of the SFO with adenoviral vectors. Mice were instrumented with an indwelling lateral cerebroventricle (ICV) cannula (PlasticsOne Inc.) for brain injection of pharmacological agents as previously described (9). The animals were anesthetized (ketamine, 150 mg/kg + xylazine, 15 mg/kg, ip), placed in a stereotaxic device and the dorsal surface of the skull was visualized under a dissecting microscope. Stereotaxic coordinates for the placement of the ICV cannulae were 0.3 mm rostral, 1.0 mm lateral to Bregma and 3.2 mm ventral from the dorsal surface of the skull. Mice were given a minimum of 7 days to the recover prior to ICV administration of pharmacological agents and/or other experimental measurements.

Recombinant adenoviral vectors encoding 78 kDa glucose-regulated protein (AdGRP78, 6×10^7 plaque-forming units/ml) and titer-matched AdLacZ were obtained from the Iowa Gene Transfer Vector Core (IGTVC, see below). Targeting of the adenoviral vectors to the SFO was performed as described extensively by our laboratory (6, 9-14). Briefly, mice were anesthetized and placed in a stereotaxic device as above. The adenovirus was targeted to the SFO using

pulled glass pipettes and a custom pressure injection system. The glass pipette tip was lowered 3.2 mm ventral from the dorsal surface of the skull at 0.3 mm rostral and 1.0 mm lateral to Bregma. Mice were given a minimum of 7 days for surgical recovery. Using this method, we have shown highly localized and robust transgene expression in the SFO by 3 days post-injection that remains stable at high levels for at least 6 weeks (13).

Adenoviral-mediated overexpression of GRP78. A recombinant adenoviral vector encoding the full-length cDNA of mus musculus GRP78 (Genbank ID: NM_022310) was engineered and then generated by the IGTVC. The adenovirus was based on the human Ad serotype 5 in which the E1a and E1b replication genes have been deleted (15). GRP78 was under the control of the CMV promoter and in the same construct the reporter gene β-galactosidase was driven off the RSV promoter (Fig S1A). To evaluate AdGRP78 potency and efficacy, several experiments were performed. First, Neuro2A cells were infected with serial dilutions of AdGRP78 (0-1000 multiplicity of infection). 48 hours after infection cells were collected and qPCR analysis was performed as described below using a primer set for GRP78 (Fig S1B). Second, for in vitro validation of the subcellular localization of AdGRP78, Neuro2A cells were infected with the construct (500 MOI) and were then examined using immunocytochemistry as described below (Fig S1C). To test the efficacy of the virus in vitro, Neuro2A cells were transfected with AdGRP78 or titer-matched AdLacZ. 24 hours later cells were treated with TG (300nM) or control DMSO and were then collected for qPCR analysis of the ER stress biomarker $p58^{IPK}$ 24 hours later (Fig S1D). For in vivo validation of the efficacy of the virus, AdGRP78 or AdLacZ was targeted to the SFO as described above and mice were instrumented with an ICV cannula.

Following 7 days of surgical recovery mice were treated ICV with TG (1 µg) or control DMSO and brains were then harvested for qPCR analysis of the ER stress biomarker *CHOP* (Fig S1D).

Quantitative real-time PCR (qPCR). Mice were euthanized by decapitation and brains were removed and immediately placed on dry ice. Micropunches of the SFO, OVLT and CTX were isolated. Tissue from two mice was pooled per biological sample in all such studies. Total RNA was isolated by Trizol® (Invitrogen) extraction and reverse transcribed using random hexamer primers. Template samples of 25 ng were subjected in triplicate to qPCR (ABI 7500FAST system) using Power SYBR Green (Applied Biosystems). All primers were derived from *mus musculus* (National Center for Biotechnology Information GenBank). Primer sequences used were:

p58^{IPK}	F: 5'-GTGGCATCCAGATAATTTCCAG-3'
	R: 5'-GAGTTCCAACTTCTGTGGAAGG-3'
CHOP	F: 5'-ATATCTCATCCCCAGGAAACG-3'
	R: 5'-TCTTCCTTGCTCTTCCTCCTC-3'.
GRP78	F: 5'- TTCAGCTGTCACTCGGAGAAT-3' R: 5'- ATATCTCATCCCCAGGAAACG -3'.
β-actin	F: 5'-CATCCTCTTCCTCCCTGGAGAAGA-3'
	R: 5'-ACAGGATTCCATACCCAAGAAGGAAGG-3'

β-actin was used as a calibrator gene in all experiments. For each target gene set, the average expressed isoform in each category was expressed relative to the calibrator and the relative fold-change compared to the calibrator was calculated using the comparative ΔΔCt method (16).

Western Blot and Phos-tag gels. Micropunches of the SFO were isolated (SFO from three mice were pooled per biological sample). Protein was isolated in mammalian tissue lysis buffer

(Sigma-Aldrich) with protease inhibitor (Sigma-Aldrich). Western analyses for GRP78 was performed using standard procedures (7) and expressed relative to β-actin. 15 µg of protein was loaded per sample lane and GRP78 antibodies (Abcam) were used at 1:10000 dilution. Phos-tag gels for phosphorylated PERK (p-PERK) analysis was performed in accordance with previous studies with slight modifications (17). Briefly, 15 to 20 µg of protein were loaded onto a modified SDS-PAGE gel containing 3.5 µM Phos-tag (Wako Pure Chemical Industries, Ltd.) with the following running conditions: 15 mA for 15 min followed by 5 mA for 9.5 h. Before transferring, gels were soaked in ice-cold transfer buffer with 1mM EDTA for 15 min. The rest of the protocol was identical to traditional Western Blot. The PERK antibody (Cell Signaling Technology) was applied at 1:500 in 2% BSA/TBS-tween. p-PERK was detected at 170kDa versus un-phosphorylated PERK at 140kDa. PERK blots were re-probed with HSP90 (Santa Cruz Biotechnology Inc.) as a position/loading control. Data were expressed as a percentage of p-PERK relative to total PERK (p-PERK + u-PERK).

Immunohisto/cytochemistry. Immunocytochemistry of cultured Neuro2A cells was performed 24 hours after transfection with AdGRP78 by fixing cells at 80% confluence with 4% paraformaldehyde (PFA) in 1X PBS for 20 minutes at RT. The cells were then washed twice with 1X PBS followed by sequential incubation with primary antibodies for GRP78 (1:200, Abcam) and the ER protein calnexin (1:100, Santacruz) followed by Alexa-Fluor-conjugated antibodies (Invitrogen) in 0.1 Triton X-100 and 1XPBS buffer. Cells were counterstained with DAPI and mounted with Prolong Gold Antifade. Images were obtained with a Zeiss Axio Imager.

Immunohistochemistry was used for *in situ* detection of AdGRP78 in mice that had undergone SFO-targeted AdGRP78 or AdLacZ (see above). Briefly, mice were perfused transcardially with 37°C saline followed by ice-cold 4% PFA. Brains were removed and stored in 30% sucrose overnight. Cryosections (20μm) were obtained and mounted directly onto glass slides. AdGRP78 and AdLacZ samples were processed on the same slide. Sections were incubated with a rabbit anti-mouse GRP78 antibody (1:100, Abcam) followed by donkey anti-rabbit-conjugated secondary antibody (1:100, Invitrogen). Images were obtained using a Zeiss Axio Imager. GRP78 overexpression was confined to the SFO as shown previously.

Ultrastructural analysis of rough ER (RER) in SFO neurons. Male C57Bl/6 mice receiving systemic Ang-II (n=3) or vehicle (n=3) infusions for 14 days were anesthetized and perfused with acrolein (3.8%) and paraformaldehyde (2%). The brains were postfixed and vibratome-sectioned as described previously (18). Sections from each group were postfixed in 2% osmium, dehydrated, and flat-embedded in Embed 812 (Electron Microscopy Sciences Inc.)., and portions containing the SFO were thin sectioned to 70 nm. Micrographs of neuronal somal profiles containing RER in randomly selected SFO neuropil fields were then collected (6 micrographs per animal, containing 6-11 RER profiles each). Each RER profile was characterized in terms of a circularity index (defined as $4\pi \times \text{Area}/ \text{Perimeter}^2$), whereby a value of 1 indicates a perfectly round profile and values approaching 0 indicate increasingly elongated profiles. In this regard, distended RER profiles will display values closer to 1 whereas normally arranged RER profiles will exhibit a value closer to 0. In addition, the ribosomal density (ribosomes / μm) was calculated as the number of distinct, clearly identifiable ribosomes contacting a given RER

profile, divided by the length of the perimeter of that profile. For all calculations, ImageJ was used to determine profile area and perimeter.

Reactive oxygen species (ROS) detection. ROS production was assessed in dissociated SFO cells and in SFO sections using dyhidroethidium (DHE) as an indicator (6). For in vitro ROS detection in SFO cells, mice were sacrificed using CO_2, and the brains were removed and quickly transferred to a chamber containing ice-cold sucrose artificial cerebrospinal fluid (s-aCSF) composed of (in mM): 26 $NaHCO_3$, 1 NaH_2PO_4, 3 KCl, 5 $MgSO_4$, 0.5 $CaCl_2$, 10 glucose, and 248 sucrose, oxygenated with 95% O_2 and 5% CO_2, pH 7.35. Coronal slices (300μm) were then obtained using a Vibratome (Leica) and stored in a chamber filled with oxygenated lactic acid (I)-aCSF composed of (in mM): 124 NaCl, 26 $NaHCO_3$, 5 KCl, 1 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 4.5 lactic acid, pH 7.35. The SFO was dissected and transferred to an oxygenated (I)-aCSF buffer containing 0.02% pronase and 0.02% thermolysin, and then incubated at 35^0C for 1.5 hrs. SFO cells were dissociated with mild mechanical stirring and were then transferred to a glass-bottom Petri dish and perfused with oxygenated (I)-aCSF and incubated with 2μM DHE (Molecular Probes) for 30 minutes in the dark. Following 100-150 ms exposure to mercury light, fluorescence intensity was measured after addition of vehicle, vehicle + Ang-II (100nM) or TUDCA (500ug/ml) + Ang-II using a Nikon Diaphot 300 inverted microscope.

ROS production in SFO tissue was assessed by DHE microfluorography as described (6, 9). Brains from mice that had undergone SFO-targeted AdGRP78 or control AdLacZ injections were removed on day 14 of Ang-II infusion and flash frozen. Coronal sections (30μM) were taken onto chilled microscope slides. Sections were then thawed at room temperature, rehydrated with phosphate-buffered saline (PBS), and incubated for 5 minutes in the dark with DHE (1μM)

followed by a 2 minute wash with PBS. DHE fluorescence was visualized by confocal microscopy (Zeiss LSM 510). Detector and laser settings were kept constant across all samples, and control and experimental samples were always processed in parallel. Fluorescence intensity was quantified using ImageJ software and normalized to fluorescence levels observed in untreated samples as described (6, 9).

Data analysis. Data are expressed as mean ± SEM. Two-tailed unpaired t-test was used for comparisons between two groups and multiple comparisons were evaluated using an appropriate ANOVA. Post-hoc comparisons were performed using a Tukey's test when appropriate. The alpha level was set at $p<0.05$.

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