SUPPLEMENTAL MATERIAL

Supplemental Methods

Modeling early life Na-exposure effects on adult-onset vascular disease. Tg25-rats are inbred Dahl salt-sensitive (Dahl S) rats transgenic for human cholesteryl ester transfer protein (CETP)[1]. All transgenic rats used for study are heterozygous, Tg25. They are maintained on Harlan-2018 defined regular rat diet (Harlan Inc, IA) which contains 0.23% NaCl and are not stroke-prone on this diet. We used 8-10 week old, pre-hypertensive/normotensive, and non-stroke-prone nontransgenic females mated with non-stroke prone, heterozygous Tg25-males. Dams were placed on Purina Laboratory Rodent 5001 regular rat chow (Purina Mills LabDiet, MO) containing 0.4%Na, from hereon referred to as 0.4%Na-exposure, at the desired experimental time point of onset. Tg25-male and Tg25-female littermates from 10-12 litters were used for study groups per Na-exposure onset. As reference, lifespan of Tg25-rats maintained on 0.23%Na throughout life, C_F, were used as described[1].

For early-life 0.4%Na-exposure spanning fetal life (X_F), dams were placed on 0.4%NaCl-Purina 5001 regular-rat chow (0.4%Na-exposure) 1 week prior to mating and maintained on this diet through gestation and lactation, and thereafter throughout life. Later onsets of 0.4%Na-exposure were done at representative developmental categories: at weaning (X_W) or 3 weeks of age for both Tg25-males and females, and young adult at 8 weeks of age (X_A) for Tg25-females. Other nutrients in 0.4%Na-Purina 5001 'constant-nutrition' rat chow and 0.23%Na-Harlan-2018 defined-regular rat chow are similarly within normal levels for rodents. All animal procedures were approved by the IACUC at Boston University School of Medicine.

Monitoring of Stroke Phenotype. Appearance of neurological deficits defined stroke onset, which then prompted euthanasia through deep anesthesia followed by collection of blood and vital tissues for analysis. Rats which died overnight and not observed pre-death were eliminated from the study. These rats were not subjected to any experimental manipulations in order to eliminate post-mortem confounders. At onset of stroke, brains were removed, examined for

visible hemorrhages, and immersion fixed in fresh phosphate buffered saline-buffered 4% paraformaldehyde. Non-stroke deaths defined by the absence of neurological deficits were also noted.

Physiological and Biochemical Analyses. These were done as described[21]. Different rat study groups were set-up for physiological and biochemical analyses, followed by isolation of brain microvessels at set time points. Tail vein bleeds were done at 12-weeks of age under half-dose anesthesia. Fresh plasma was used for analysis of total plasma cholesterol, triglycerides and high density lipoprotein levels done in duplicates as described[1]. Since these were not different between different test and control groups, and with previously described values, further subfractionation by ultracentrifugation was not done. Tail cuff blood pressures were obtained (Coda-2 System, Kent Scientific) under half-dose anesthesia in order to eliminate stress. Values were obtained within a set range of heart rate: 350-450 beats per minute, in order to eliminate confounding variations, and within a set time from anesthesia administration. Since differences were larger than 10mmHg, intra-aortic telemetric blood pressure measurements were not necessary.

Interleukin-18 plasma levels were measured by ELISA using platelet poor plasma samples collected prior to euthanasia at the onset of stroke and frozen and stored until completion of the study groups. Plasma from control age-and sex-matched non-stroking rats were also isolated. Interleukin-18 levels were measured using rat-specific IL-18 ELISA (Biosource International Inc, CA) according to manufacturer's specifications. ELISA testing was done in triplicate using the same-batch of 96-well plates, and run concurrently using the same reagents, controls and standards.

FACS analysis of CD11b+ activated leukocytes was done using rat-reactive anti-CD11b antibody (BD Biosciences, CA) applied to 50 microliters of whole blood anticoagulated with EDTA. Anti-cd11b antibody was added within 15 seconds of blood drawing into EDTA containing tubes, incubated on ice in the dark for 1 hour. Lysis of red blood cells, wash, and fixation were then done following manufacturer's specifications (BD Biosciences, CA). FACS

analysis was done gating for CD11b+ fluorescence and side scatter, thus distinguishing activated CD11b+ neutrophils (greater side scatter due to granularity) from CD11b+ monocytes.

Histopathology and Immunohistochemical Analysis. After inspection of all brain surfaces for hemorrhages, all brains were immersion fixed in fresh PBS-buffered 4% paraformaldehyde and processed for paraffin embedding and 6-micron serial sectioning. Twenty to fifty serial sections were obtained from each region (4 for cerebrum, 2 for cerebellum). For younger age rats, serial sagittal sections were obtained from the whole brain. H&E and Masson-trichrome stained sections were analyzed and digital photomicrographs (Zeiss Axioskop 2) obtained for documentation and analysis. Immunohistochemical analysis was done as described[1] using rat-reactive antibodies on serial sections of the brain at the different time points (GFAP, C-19 #sc-6170, myeloperoxidase C-16 #sc-16128, Sta. Cruz Biotech, Inc., CA). DAB was used as the chromogen using the Goat ImmunoCruz Staining system (#sc-2053, Sta. Cruz Biotech, Inc., CA).

Ex-vivo 11.7T Magnetic Resonance Imaging (MRI). MR-Imaging of rat brains exhibiting neurological deficits (n = 4/group: Tg25-males and females) was performed using an 11.7T Avance 500 wide bore spectrometer (Bruker, Billerica, MA), fitted with a gradient amplifier for imaging (maximum gradient strength 906.6 mT/m) and 20-mm birdcage coil tuned to 500.13-MHz for proton. After fixation in PBS-buffered 4% paraformaldehyde, brains were suspended and imaged in Fomblin. For detection of micro-hemorrhages, a gradient recalled echo sequence was used. Typical parameters were TR=500 ms, TE=5 ms, slice thickness 0.5 mm, pixel resolution 32 microns. T2-weighted sequences were used to assess ischemia; TR=2000, TE=25 ms, pixel resolution 78 microns. Data were processed with Paravision^{tm} software provided by the vendor.

Ultrasound micro-imaging of rat carotid artery disease. Vevo770 ultrasound micro-imaging (VisualSonics, Inc., Toronto, Canada) using a RMV708 scanhead (50 micron resolution)

mounted onto a rail-clamp system was performed on stroke-prone rats at the onset of neurological deficits (n = 4/group: X_FTg25-males and females). Rats were anesthetized and maintained on 0.8% isoflurane-medical air. We evaluated right and left common carotid arteries, their bifurcation and branches, internal and external carotid arteries. Partial occlusions were confirmed by Doppler flow disturbances. Cardiac function was assessed to eliminate cardiac cause of death in stroking rats.

Isolation of Brain Microvessels (bmv). Brain microvessels from rat cerebrum and cerebellum were isolated as described[2]. Isolated brain microvessels were visualized and photographed to confirm bmv isolation. BMVs were quantified by weight, and normalized to weight of corresponding cerebrum or cerebellum.

Statistical Analysis. Descriptive statistics, normality testing and statistical analyses were done using GraphPad PRISM-4 analysis software (GraphPad Software, Inc. CA). All data passed normality testing. To compare study groups, one-way analysis of variance (ANOVA) was performed followed by Tukey's test for all-pairwise comparison or Bonferroni's multiple comparison test for selected pairs when applicable. Differences in lifespan/stroke onset were also subjected to survival analysis using survival curve analysis by log rank testing. Two-way ANOVA was performed to test for interaction between sex and early-life 0.4%Na-exposure. Paired t-tests were performed to evaluate mean X_F-SBP and increment rise in SBP comparing Tg25-males and females at 2-, 3-, and 4-months of age. All statistical tests were evaluated at the $P < 0.05$ level of significance.

Summary Sequence of Analyses of different rat study groups.

Cohort – A. Step-1. Study of stroke occurrence and onset induced by early life 0.4%Na- exposure				
	Tg25 F	Tg25 M	Tg25 F FACS	Tg25 F IL-18
a. X_F	14	19	7 control age-matched pre-stroke: 7	7 control age-matched pre-stroke: 7
b. X_W	13	19		
c. X_A	19	nd		
d. C_F	14	10		
Step-2. Brain histology and immunohistochemistry time point studies of X_FTg25-males and females				
	X_FTg25 F	X_FTg25 M	C_FTg25 F	C_FTg25 M
a. at stroke onset [from step-1a]	4	4	4 [stroke(-): age-matched to X_F]	4 [stroke(-): age-matched to X_F]
b. 2m of age	3	3		
c. 3m of age	3	3		
Cohort – B. Step-3. Time point analysis of systolic blood pressure (SBP), total plasma cholesterol (TC), total plasma triglyceride (TG) and isolation of brain microvessels (bmvs)				
	SBP, TC, TG, bmvs		SBP	
	X_FTg25 F	X_FTg25 M	C_FTg25 F	C_FTg25 M
2m	8	10	8	10
3m	6	9	6	12
4-4.5m	4	8	9	6
	X_ATg25 F			
4-4.5m	5			
Cohort – C. Step-4. Ex vivo 11.7T-MRI of fixed stroke+ X_F rat brains				
	Tg25 M	Tg25 F		
at stroke onset	4	4		
Cohort – D. Step-5. Ultrasound micro-imaging of left and right carotid arteries, and cardiac function of X_F rats at stroke onset				
	Tg25 M	Tg25 F		
a. at stroke onset	4	4		
Note: MRI and more recently, ultrasound micro-imaging are recent technology cores, which were not available during Cohorts A and B studies. nd, not done; M, males; F, females; X_F, fetal 0.4%Na-exposure; X_W, 0.4%Na-exposure from weaning; X_A, 0.4%Na-exposure from 8 weeks of age; C_F, fetal 0.23%Na-exposure.				

Supplemental Tables

Supplemental Table 1: Comparative analysis of early-life 0.4%Na-exposure effects on Tg25-males and females.

	X_F: exposure from fetal stage		X_W: exposure from weaning	
Genotype Sex (n)	Tg25 F (14)	Tg25 M (19)	Tg25 F (13)	Tg25 M (19)
Stroke observed	+	+	+	70%
mean duration of 0.4%Na-exposure (days)	99	128	95	156
Lifespan (days) (mean ± sem)	99 ± 4	128 ± 4	116 ± 4	177 ± 7
lower 95% CI	90	119	107	161
upper 95% CI	108	138	126	192
One-way ANOVA	$P < 0.0001$			
	Bonferroni's Multiple Comparison test for sex-differences: X_FM *vs* X_FF: $P < 0.001$ X_WM *vs* X_WF: $P < 0.001$			
Two-way ANOVA [early-life 0.4%Na-exposure x sex] effects on stroke onset:				
early-life 0.4%Na-exp	20.9% of variance		$P < 0.0001$	
sex-effects	38.9% of variance		$P < 0.0001$	
interaction	4.6% of variance		$P < 0.008$	
All data sets passed normality test; ANOVA, analysis of variance; F, female; M, male; Tg25, transgenic rat; X_F, fetal 0.4%Na-exposure; X_W, 0.4%Na-exposure from weaning; n = number of rats.				

Supplemental Table 2.

Cardiovascular disease risk factors comparing X_F[0.4%Na] to control C_F[0.23%Na] Tg25-male and female rats at 2m-, 3m- and 4m-of age.

Age	2m		3m		4m	
sex (n)	F (8)	M (10)	F (8)	M (9)	F (4)	M (8)
X_F SBP	191.0 ± 21.2	173.7 ± 22.1	203.4 ± 24.3	214.6 ± 17.6	212.2 ± 18.2	204.9 ± 10.6
X_F TC	63.6 ± 15	242.5 ± 77	58.4 ± 20	280.6 ± 4	31.1 ± 19	264.7 ± 58
X_F TG	344.4 ± 94	750.8 ± 388	402.5 ± 172	821.6 ± 67	304.8 ± 93	2165.0 ± 570
sex (n)	F (8)	M (10)	F (6)	M (12)	F (9)	M (6)
C_F SBP	100.0 ± 5.2	110.0 ± 7.1	152.3 ± 18.8	186.4 ± 12.6	163.6 ± 18.9	207.8 ± 19.3
Δ SBP	91.0	63.7	50.7	28.2	48.6	-2.9

C_F, fetal 0.23%Na-exposure; X_F, fetal 0.4%Na-exposure; F, Tg25-female; M, Tg25-male; m, months; SBP, tail cuff systolic blood pressure (mmHg); Δ SBP, [X_FSBP – C_FSBP] mmHg; TC, 24-hour fasting total plasma cholesterol (mg/dl; TC mg/dl ⁄ 38.67 = mmol/L); TG, 24-hour fasting total plasma triglyceride (mg/dl; TG mg/dl ⁄ 88.57 = mmol/L). Mean ± standard deviation; n = number of rats per group.

Supplemental Table 3. Time-course of Histological Changes in Rat Brains

X_F Developmental Programming	neuronal pyknosis + eosinophilia	micro-hges	pmn+	macro-hges	Neurological deficits
At stroke onset (n = 4)					
Tg25 F	+1 to +2	+1 to +2	+	+	+
Tg25 M	+1 to +2	+1 to +2	+	+	+
At 3months of age (n = 3)					
Tg25 F	+ ½ to +2	-	-	-	-
Tg25 M	+ ½ to +1	-	-	-	-
At 2months of age (n = 3)					
Tg25 F	+ ½ to 2	+ ½	-	-	-
Tg25 M	+ ½	-	-	-	-

X_F, fetal 0.4%Na-exposure; hge, hemorrhages; pmn+, neutrophil adhesion and/or transmigration present; macro-hges, hemorrhagic-necrotic lesions and/or visible hemorrhages on gross inspection; neurological deficits, observed paresis, paralysis and/or seizure; Tg25, transgenic; F, female; M, male. Grading of microscopic findings in 8 coronal serial sections spanning 4 representative step-segments of the cerebrum; grade = cumulative area occupied by pyknotic/eosinophilic neurons and microhemorrhages: 0, absent; + ½ (< one 1000x oil-immersion high power field, hpf); +1 (1-2 1000x hpf); +2 (3-4 1000x fhp); +3 (>4 1000x hpf).

Supplemental References

1. Herrera VLM, Tsikoudakis A, Didishvili T, Ponce LRB, Bagamasbad P, Gantz D, Herscovitz H, Van Tol A, Ruiz-Opazo N. Analysis of gender-specific atherosclerosis susceptibility in transgenic[hCETP]25^{DS} rat model. *Atherosclerosis*. 2004;177:9-18.

2. Song L, Pachter JS. Culture of murine brain microvascular endothelial cells that maintain expression and cytoskeletal association of tight junction-associated proteins. *In Vitro Cell Dev Biol Anim*. 2003;39:313-320.