Early-Life Sodium Exposure Unmasks Susceptibility to Stroke in Hyperlipidemic, Hypertensive Heterozygous Tg25 Rats Transgenic for Human Cholesteryl Ester Transfer Protein

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Background—Early-life risk factor exposure increases aortic atherosclerosis and blood pressure in humans and animal models; however, limited insight has been gained as to end-organ complications.

Methods and Results—We investigated the effects of early-life Na exposure (0.23% versus 0.4% NaCl regular rat chow) on vascular disease outcomes using the inbred, transgenic [hCETP]25 Dahl salt-sensitive hypertensive rat model of male-predominant coronary atherosclerosis, Tg25. Rather than the expected increase in coronary heart disease, fetal 0.4% Na exposure (≤2 g of Na per 2-kcal/d diet) induced adult-onset stroke in both sexes (ANOVA P<0.0001), with earlier stroke onset in Tg25 females. Analysis of later onset of 0.4% Na exposure resulted in decreased stroke risk and later stroke onset despite longer 0.4% Na exposure durations, which indicates increasing risk with earlier onset of 0.4% Na exposure. Histological analysis of stroke-positive rat brains revealed cerebral cortical hemorrhagic infarctions, microhemorrhages, neuronal ischemia, and microvascular injury. Ex vivo MRI of stroke-positive rat brains detected cerebral hemorrhages, microhemorrhages, and ischemia with middle cerebral artery distribution and cerebellar noninvolvement. Ultrasound microimaging detected carotid artery disease. Prestroke analysis detected neuronal ischemia and decreased mass of isolated cerebral but not cerebellar microvessels.

Conclusions—Early-life Na exposure exacerbated hypertension and unmasked stroke susceptibility, with greater female vulnerability in hypertensive, hyperlipidemic Tg25 rats. The reproducible modeling in stroke-prone Tg25 rats of carotid artery disease, cerebral hemorrhagic infarctions, neuronal ischemia, microhemorrhages, and microvascular alterations suggests a pathogenic spectrum with causal interrelationships. This “mixed-stroke” spectrum could represent paradigms of ischemic-hemorrhagic transformation and/or a microangiopathic basis for the association of ischemic lesions, microhemorrhages, and strokes in humans. Together, the data reveal early-life Na exposure to be a significant modifier of hypertension and stroke disease course and hence a potentially modifiable prevention target that deserves systematic study. (Circulation. 2009;119:1501-1509.)

Key Words: stroke ■ sodium chloride, dietary ■ experimental animal models ■ hypertension ■ risk factors

Coronary heart disease and stroke remain in the top 3 causes of death in the United States despite clinical advances addressing their major risk factors, hypertension and hyperlipidemia. This persistence mandates the study of pathogenic paradigms not addressed in current intervention and prevention approaches.

One such pathogenic paradigm is the impact of early-life risk factor exposure on increasing susceptibility to adult-onset cardiovascular diseases. Maternal hypercholesterolemia increases childhood (from age 1 to 13 years) aortic atherosclerosis, even in the presence of normcholesterolemia. Likewise, maternal hyperglycemia increases risk for type 2 diabetes mellitus in humans. Notably, the effects of maternal hypercholesterolemia on atherosclerosis have been corroborated in controlled animal model studies in rabbits, apolipoprotein E knockout mice, and low-density lipoprotein–receptor knockout mice, whereas maternal environmental tobacco smoke exposure increased adult atherogenesis in apolipoprotein E knockout mice.
However, we note that these studies did not analyze the impact on end-organ disease. Analysis of the impact of early-life Na exposure lags behind, however, despite the significance of hypertension as a risk factor for coronary heart disease and especially stroke.\textsuperscript{11,12} Although intrauterine growth retardation and low birth weights have been found to be associated with hypertension in humans,\textsuperscript{13,14} epidemiology studies in humans studying the impact of common in utero exposures have not reported any maternal diet component that affects long-term blood pressure in offspring.\textsuperscript{15} Notably, however, given that salt sensitivity and salt resistance are both present in patient populations, the epidemiological detection of early-life Na exposure effects will be difficult unless patients are stratified according to genetic predisposition to salt sensitivity. Before successful genetic stratification of hypertension, animal model studies of human essential (polygenic) hypertension are necessary for the investigation of the role of early-life Na exposure effects on hypertension and its target-organ complications.

Studies in spontaneously hypertensive rats (SHRs) found that prenatal exposure to a 5% Na diet compared with a 0.1% Na diet exacerbated hypertension at 4 months of age\textsuperscript{16}; however, a similar study using a 3% Na diet found that hypertension was unchanged in 5- and 6-month-old SHRs, which survived until 14 to 15 months of age, but that prenatal exposure to a low 0.1% Na diet lowered blood pressure significantly.\textsuperscript{17} We note that all SHRs were maintained on 0.8% Na diets from weaning,\textsuperscript{16,17} with no reports of stroke occurrence. Not surprisingly, minimal effects were observed in Sprague-Dawley rats, an outbred normotensive strain, in a test of prenatal exposure to a 3% Na\textsuperscript{18} and 8% Na\textsuperscript{19} diet. However, these studies did not investigate the role of early-life Na exposure on adult-onset, hypertensive end-organ diseases, which must be addressed given the importance of hypertension in exacerabating coronary heart disease\textsuperscript{20} and increasing the risk for stroke.\textsuperscript{11,12}

The potential significance of the study of early-life risk factor exposure on “adult-onset” disease course is of high impact, because early-life exposure that alters the disease course is a priori a major confounder for genetic studies if not taken into account. More importantly, the elucidation of early-life modifiers of adult-onset disease pathogenesis, given identical genetic predisposition, carries major health significance owing to its potential efficacy, cost-effectiveness, and accessibility as a target mandate for prevention of adult-onset disease. Animal modeling studies are therefore needed that recreate the likely human clinical scenario: normotensive, normolipidemic mothers eating a balanced diet that follows current dietary sodium recommendations (2 g of Na per 2-kcal/d diet) but whose offspring carry disease-susceptibility genotypes.

Here, we tested the hypothesis that in prehypertensive, normolipidemic dams, differential sodium intake affects the disease course of adult-onset hypertension and vascular end-organ disease in genetically predisposed offspring. Using the Tg[hCETP25-Dahl-S] transgenic rat model of polygenic hypertension, hypercholesterolemia, and hypertriglyceridemia with coronary atherosclerosis predominant in males (Tg25),\textsuperscript{21} we investigated whether early-life Na exposure could exacerbate coronary atherosclerosis through acceleration of salt-sensitive hypertension, just as differential adult-onset Na exposure alters the course of coronary atherosclerosis.\textsuperscript{22} Surprisingly, early-life 0.4% Na exposure unmasked susceptibility to stroke in both Tg25 male and female rats, with greater vulnerability in females despite equivalent blood pressure levels and less hyperlipidemia in Tg25 females than in males.

**Methods**

**Modeling Early-Life Na Exposure Effects on Adult-Onset Vascular Disease**

We used heterozygous Tg25, inbred Dahl salt-sensitive rats transgenic for human cholesteryl ester transfer protein (CETP)\textsuperscript{21} exposed to regular rat chow containing 0.4% Na (0.4% Na exposure) at the desired experimental time point of onset: Fetal (Xf), at weaning (Xw), and at 8 weeks of age for early adulthood (Xa). Tg25 rats maintained on 0.23% Na regular rat chow throughout life (C0) served as the reference group.\textsuperscript{21} All animal procedures were approved by the institutional animal care and use committee at Boston University School of Medicine. Details and the study group analysis sequence are provided in the online-only Data Supplement.

**Monitoring of Stroke Phenotype**

The appearance of neurological deficits such as seizures, paralysis, or paresis defined stroke onset. Details are provided in the online-only Data Supplement.

**Physiological and Biochemical Analyses**

Physiological and biochemical analyses were performed as described previously,\textsuperscript{21} with more details specified in the online-only Data Supplement.

**Histopathology and Immunohistochemical Analyses**

Histopathology and immunohistochemical analyses were done as described previously,\textsuperscript{21} with more details given in the online-only Data Supplement.

**Ex Vivo 11.7-Tesla MRI**

A gradient recalled echo sequence was used to assess hemorrhages, and a T2-weighted sequence was used to assess ischemia with an 11.7-Tesla Avance 500 wide-bore spectrometer (Bruker, Billerica, Mass). Details are provided in the online-only Data Supplement.

**Ultrasound Microimaging of Rat Carotid Artery Disease**

Fifty-micrometer-resolution ultrasound images were obtained with a Vevo770 imaging system (VisualSonics, Inc, Toronto, Canada). Details are given in the online-only Data Supplement.

**Isolation of Brain Microvessels**

Brain microvessels from rat cerebrum and cerebellum were isolated as described previously.\textsuperscript{21} Details are given in the online-only Data Supplement.

**Statistical Analysis**

All statistical analyses were done with Prism-4 (GraphPad Software Inc, La Jolla, Calif). Details are given in the online-only Data Supplement.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Plasma triglyceride levels, Tukey's multiple comparison test was
CETP.21,26 To investigate whether the onset of 0.4% Na diet
ogenic rat model, which is a polygenic hypertensive, hyper-
eliminate genetic variability, we used the inbred Tg25 trans-
polygenic nature of human salt-sensitive hypertension and to
exacerbate adult-onset cardiovascular disease in genetically
surviving adult-onset stroke, marked by neurological deficits such
as seizures, paresis, or paralysis, than sex-matched CF control
rats (Figures 1A and 1B). Time-course analysis of early-life
stroke risk and a later onset of stroke than XF Tg25 males,
conception (CF). The differential impact on phenotype out-
come of later onset of 0.4% Na exposure despite equivalent or
longer 0.4% Na exposure duration would be informative as to
the impact of early-life 0.4% Na exposure.

Because Tg25 males have worse hypertension, hyperlipid-
emia, and coronary atherosclerosis than age-matched Tg25
females, which exhibit minimal if any coronary atheroscle-
rosis,21 we expected that early-life Na exposure would in-
crease hypertension and exacerbate coronary artery disease in
Tg25 males owing to hypertension-atherosclerosis interac-
tions, as observed in the high-expresser Tg[hCETP]53 rat
line.22 Surprisingly, Xf Tg25 male and female rats exhibited
more adult-onset stroke, marked by neurological deficits such as
seizures, paresis, or paralysis, than sex-matched CF control
rats (Figures 1A and 1B). Time-course analysis of early-life
0.4% Na exposure revealed that Xf Tg25 males had less
stroke risk and a later onset of stroke than Xf Tg25 males,
despite a longer duration of 0.4% Na exposure in Xf Tg25
females (Figure 1B). In females, time-course analysis of the
onset of 0.4% Na exposure showed that Xf and Xw Tg25
females exhibited similar stroke risk and stroke onset, but Xf
Tg25 females exhibited decreased stroke risk and later stroke
onset (P<0.001), despite an almost 2-fold increase in 0.4% Na
exposure duration in the Xf Tg25 female rats (Figure 1A). Together,
these observations indicate early-life vulnerability to 0.4% Na exposure, with females showing a longer window

Results
Early-Life Na Exposure Unmasks Susceptibility to Stroke
We investigated whether early-life sodium exposure would
exacerbate adult-onset cardiovascular disease in genetically
salt-sensitive, hypertensive rats. We limited Na exposure to
fall within the range of standard salt levels contained in
regular rat chow (0.23% to 0.4% NaCl) to simulate human
dietary salt intake. We note that 0.4% NaCl content is a
time-tested, accepted level in regular rat chow on which
time-course analysis of early-life Na exposure revealed that Xf Tg25
females exhibited decreased stroke risk and later stroke
onset (P<0.001), despite an almost 2-fold increase in 0.4% Na
exposure duration in the Xf Tg25 female rats (Figure 1A). Together,
these observations indicate early-life vulnerability to 0.4% Na exposure, with females showing a longer window

Figure 1. Early-life 0.4% Na exposure effects on stroke onset and hypertension in Tg25 female and male rats. A, Lifespan (●) and Na
exposure duration (●) in Tg25 females by study group: Xf, Xw, Xs, and control Cs. Strokes were observed (+) in Xf and Xw rats, much
later and in even fewer (30%) Xs rats, and even later and in even fewer (7%) Cs rats.21 Mean±SEM; ANOVA P<0.0001; **Tukey's multiple
comparison P<0.001 vs Xf; #not significant. B, Lifespan (●) and Na exposure duration (●) in Tg25 males by study group: Xf, Xw, and
control Cs. Strokes were observed (+) in Xf males and in 70% of Xw males with later onsets, but not in Cs rats.21 Mean±SEM; ANOVA
P<0.0001; **Tukey's multiple comparison P<0.001 vs Xf. C, SBP level (in mm Hg; ●) in Xf Tg25 females (F+) and males (M+) at 2, 3,
and 4 months of age (paired t test P<0.03); mean±SD. Corresponding increment rise in mean SBP (●) in Xf rats compared with Cs rats
(Xf–Cs) at 2, 3, and 4 months (paired t test P<0.03). By 2-way ANOVA, early-life Na exposure contributed to 60% of SBP variance in
females (P<0.0001) and 15% of SBP variance in males (P<0.0001). D, Plasma levels (mean±SD) of total plasma cholesterol (●) and
total plasma triglyceride (●) of Xf rats at 2, 3, and 4 months of age (paired t test P<0.001 for each respective time point). For total
plasma triglyceride levels, Tukey's multiple comparison test was P<0.05 at 2 months, P<0.01 at 3 months, and P<0.001 at 4 months.
(Data specifics are provided in the online-only Data Supplement.)
Sex-Specific Effects of Early-Life Na Exposure on Hypertension

To gain further insight into determinants of the observed greater vulnerability of females to early-life Na exposure–induced adult-onset stroke, we investigated levels of hypertension and combined hyperlipidemia in age-matched Xf and Cf Tg25 male and female rats. Systolic blood pressure (SBP) analysis revealed equivalent levels between Xf Tg25 male and female rats at 2, 3, and 4 months of age (Figure 1C; online-only Data Supplement Table II). Notably, however, analysis of the increment rise in SBP at 2-, 3-, and 4-month time points revealed significant differences between males and females (Figure 1C), which suggests that early-life Na exposure exacerbated hypertension more in Xf Tg25 female rats, letting them “catch up” to the typically higher SBP levels in males. This observation is supported by 2-way ANOVA of the early-life Na exposure×sex effects on SBP, with early-life Na exposure contributing to 60% of SBP variance in females compared with 15% in males (Figure 1C). Analysis of total plasma cholesterol and triglyceride levels of [Xf]Tg25 males and females detected significant differences between sexes at 2, 3, and 4 months of age, but females consistently had lower levels of both total plasma cholesterol and triglyceride (Figure 1D; online-only Data Supplement Table II). We also noted that levels observed were lower than reported previously.21

Stroke Phenotype Characterization

To identify the stroke phenotype unmasked by early-life Na exposure, multifaceted studies were performed. Visual examination of the brains at the onset of stroke, marked by seizures, paresis, and/or paralysis, revealed hemorrhages in different regions of the cerebral cortex, which were confirmed on histological analysis (Figures 2A through 2F). Histological hematoxylin-and-eosin–stained (data not shown) and Masson trichrome–stained serial section analysis revealed acute hemorrhages (Figures 2B and 2C) associated with neutrophil adhesion and transmigration (Figures 3A through 3C), as well as concurrent subacute hemorrhages characterized by hemorrhagic-necrotic areas with inflammatory cell infiltrates (Figures 2E and 2F). Both acute and subacute hemorrhages were associated with microhemorrhages that were found primarily in the cerebral cortex (Figures 2A through 2F), as well as in the hippocampus, basal ganglia, and white matter. Areas surrounding cerebral microhemorrhages showed evidence of ischemia with neuronal pyknosis, eosinophilia, and microvacuolation in the surrounding tissue (Figures 3D and 3E) in contrast to nonaffected areas (Figure 3F). Microvessels that exhibited microhemorrhages also exhibited endothelial denudation (Figure 3A) and discontinuous glial fibrillary acidic protein–positive astrocytic endfeet (Figures 4A through 4C), concordant with postischemic loss of microvascular integrity.27

Interestingly, neutrophil transmigration without red blood cell extravasation and perivascular edema (Figures 3A and 3B) was observed, in accordance with previous observations of neutrophil recruitment before loss of microvascular integrity.28 Transmigrated neutrophils immunostained positively for myeloperoxidase (Figure 3B), concordant with the concept that postischemic neutrophil transmigration might promote further vascular injury and lead to additional or larger hemorrhage (Figure 3C) via myeloperoxidase-H2O2-halide system–induced toxicity.29 Areas of subacute hemorrhagic infarction also exhibited positive myeloperoxidase immunostaining (Figure 4D) in the neuropils (Figure 4E), as well as in monocytes and macrophages (Figure 4F), which suggests continued myeloperoxidase-mediated tissue injury after the 48-hour postischemic period. Compared with age-matched

Figure 2. Representative stroke-positive rat brain with acute (A through C), and acute plus subacute (D through F) intracerebral hemorrhages. A, Gross inspection of multiple hemorrhagic lesions in right frontal lobe. B, Microscopic analysis of multiple intracerebral hemorrhages (Masson trichrome stain). C, Cortical microhemorrhages and hemorrhagic infarction (☆), transmigrated neutrophils (arrows; viewed at higher magnification in Figure 3A; Masson trichrome stain). D, Gross inspection of hemorrhage in the left temporal lobe. E, Microscopic analysis of hemorrhagic-necrotic lesion and inflammatory response (Masson trichrome stain). F, High-power magnification of Figure 2E showing area of hemorrhagic-necrotic lesion and inflammatory response. Scale bar = 5 mm in A and D; 400 μm in B and E; and 25 μm in C and F.
prestroke and non–stroke-prone C57 Tg25 female rats, we detected increased numbers of circulating CD11b-positive activated neutrophils (P<0.0001; Figures 5A through 5C) and increased plasma interleukin-18 levels (P<0.0001; Figure 5D) at the onset of neurological deficits in stroke-positive Tg25 female rats, which suggests a systemic proinflammatory process in postischemic hemorrhagic transformation.

Histological Time-Course Analysis of Stroke-Prone Rat Brains
Comparative microscopic examination of rat study groups detected acute macrohemorrhages, subacute hemorrhagic-necrotic lesions, and neutrophil recruitment only at the onset of stroke-associated neurological deficits (online-only Data Supplement Table III). Microhemorrhages were detected mainly at stroke onset and occasionally in 2- and 3-month-old Tg25 females, but not in Tg25 males of similar ages (online-only Data Supplement Table III), consistent with the observations that stroke occurs later in males (Figures 1A and 1B). Features of acute ischemia, such as neuronal pyknosis and eosinophilia, were detected at prestroke time points, which suggests that ischemia likely precedes microhemorrhages (online-only Data Supplement Table III).

MRI Analysis of Stroke-Positive Brains
To further characterize the stroke phenotype, we applied 32-μm/pixel–resolution MRI at 11.7 Tesla to fixed rat brains. Using a gradient recalled echo sequence that detects heme products, MRI detected multiple cortical intraparenchymal microhemorrhages and larger hemorrhages in the subcortical white matter (Figure 6A). Gradient recalled echo MRI also detected microhemorrhages in the hippocampus and thalamus (data not shown). Analysis of T2-weighted images detected a relatively extensive region of abnormal high signal (Figure 6B). Quantification of intensity of T2-weighted imaging, highlighting pixels with intensity >70% of maximal signal, better delineated the high-signal area, consistent with ischemia with a middle cerebral artery distribution (Figure 6C), thus revealing that microhemorrhages occurred within ischemic tissue. Together with histological observations, the MRI findings of multiple mixed cortical and white matter hemorrhages and microhemorrhages within the middle cerebral artery ischemic distribution demonstrate the stroke phenotype in the stroke-prone Tg25 (Tg25sp) model to be that of ischemic-hemorrhagic stroke.
Ultrasonographic Detection of Carotid Artery Atherosclerosis

To investigate the cause of the ischemia following a middle artery distribution detected on ex vivo MRI, we used a 50-μm-resolution ultrasound system to investigate putative carotid artery disease at the onset of stroke in another set of XF rats. Ultrasonography detected carotid artery disease in the internal carotid artery (Figure 7) or at the carotid bifurcation and ruled out concomitant heart failure (data not shown).

Figure 6. Representative ex vivo high-resolution MRI of representative stroke-positive brain detected cerebral hemorrhages and microhemorrhages within ischemic area. A, Axial image of gradient recalled echo sequence detected hemorrhage in the subcortical white matter and multiple hemorrhages in the cerebral cortex. B, T2-weighted image detected a relatively extensive region of abnormal high signal. C and D, Two representative slices showing quantification of intensity of T2-weighted image, highlighting pixels with intensity >70% of maximal signal, depicts area of ischemia that follows the arterial distribution of the middle cerebral artery. E, Parametric map showing T2 relaxation within the tissue with a Carr-Purcell-Meiboom-Gill (CPMG) sequence. The core area of the lesion is depicted with prolonged T2 relaxation times (white-red) and is less extensive than the area indicated on the T2-weighted images (C and D).

Figure 7. Representative high-resolution ultrasound microimaging detected carotid artery disease at stroke onset in stroke-prone rats. A, Ultrasound image of control age-matched non-stroke-prone (C57) female showing no lesions or occlusion in common carotid artery (CCA), external carotid artery (ECA), or internal carotid artery (ICA). B, Ultrasound image of Xc stroke-prone Tg25 female at the onset of stroke depicting an occlusive carotid artery lesion (circle) in the ICA.

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These observations support the MRI findings of ischemia with middle cerebral artery distribution and suggest that stroke pathogenesis in this model likely involves the synergistic interactions of embolic and low-flow hemodynamic factors known to be contributory to stroke pathogenesis in large-artery atherosclerotic disease.50

Prestroke Brain Changes in Microvascular Mass
To investigate the hypothesis that prestroke changes exist in brain microvessels that increase ischemic damage and risk for hemorrhagic transformation, we investigated putative prestroke changes in brain microvessels. At the onset of stroke, histological serial-section analysis of cortical areas with ischemic neurons demonstrated a decrease in the number of brain microvessels (0 to 1 per 1000× high-power field; Figures 3D and 3E), in contrast to nonischemic cortical areas on the same section, which exhibited 2 to 3 brain microvessels per 1000× high-power field (Figure 3F). To assess putative prestroke changes in brain microvessel density, we isolated brain microvessels (Figures 5E and 5F) and compared different study groups and prestroke time points. The study of brain microvessel mass gives a total representative picture of brain microvessel status in specific brain regions.

In prestroke rats at 4 to 4.5 months of age, cerebral cortical microvessel density, measured as cortical brain microvessel mass/cortex weight, was significantly different among age-matched Xr females, Xr females, and Xr males (Figure 5G). Interestingly, analysis of total cerebral cortical microvessel density at earlier time points (2 and 3 months of age) did not detect differences in Xr study groups (Figure 5H). In accordance with the absence of cerebellar lesions, cerebellar brain microvessel number was not altered among study groups (data not shown).

Discussion
Early-Life 0.4% Na Exposure Unmasks Stroke Susceptibility With Greater Female Vulnerability
Early-life 0.4% Na exposure unmasks susceptibility to adult-onset stroke in Tg25 rats in contrast to later-life 0.4% Na exposure, despite longer durations of 0.4% Na exposure in the latter situation. Without early-life 0.4% Na exposure, Tg25 females and males did not exhibit adult-onset stroke.21 We note, however, that genetic susceptibility to hypertension plays a critical role in early-life 0.4% Na exposure–induced stroke susceptibility, because lifetime 0.4% Na Purina 5001 rat chow consumption does not induce strokes in other rat strains, including the SHR strain. These observations highlight the importance of gene-environment interactions and the impact of early-life exposure on disease course in genetically predisposed individuals. The data also demonstrate the role of sex-specific factors, because given identical early-life Na exposure and genetic backgrounds, similar levels of blood pressure, and less hyperlipidemia, Tg25 females exhibited earlier onsets of stroke and a longer window of vulnerability to early-life Na exposure–induced risk for adult-onset stroke than Tg25 males.

Modeled Paradigms in the Stroke-Prone Tg25 Rat Model
The detection of hemorrhagic infarctions, carotid artery disease, and artery-specific ischemic distribution, encompassing multiple microhemorrhages and intraparenchymal hemorrhages, as well as microvascular pathology, together simulates human ischemic-embolic strokes with hemorrhagic transformation, the clinical association of microhemorrhages and ischemic stroke,31 and the coexistence of microangiopathy, microhemorrhages, and acute spontaneous intraparenchymal hemorrhage in humans.32 The reproducible modeling of these features in the inbred Tg25sp rat model provides compelling evidence that these events are interrelated pathogenically and supports the hypothesis that carotid artery disease embolic and hemodynamic events act synergistically to increase stroke risk.30

More importantly, the modeling of spontaneous carotid artery disease and ischemic stroke with hemorrhagic transformation in Xr 0.4% Na-exposed Tg25 rats or Tg25sp rats, which affected both females and males, provides a much needed new animal model of spontaneous, ischemic stroke with hemorrhagic transformation. This stroke model provides the biological context of 2 clinically relevant stroke risk factors, polygenic hypertension and hyperlipidemia, that are missing in commonly used stroke models induced by the acute injection of blood or collagenase33,34 or by acute arterial ligation–induced or microsphere-induced ischemia.35,36 As such, the Tg25sp model provides a compelling model system to analyze the prestroke changes that lead to ischemic strokes, postischemic hemorrhagic transformation, and progression of microhemorrhages to intraparenchymal hemorrhages.

Additionally, the Tg25sp stroke model differs from the prototype stroke-prone SHRsp rat stroke model, because the latter exhibits hypertensive arterial fibrinoid necrosis, greater male susceptibility,37,38 and distinct vascular, neuronal, and parenchymal histopathological changes.39,40 Given that women were reported to have more strokes than men in 2004, with 91 274 deaths due to strokes in females compared with 58 800 deaths in males,1 the fact that the Tg25sp rat model mirrors this trend validates Tg25sp rats as a model system to study sex-specific stroke mechanisms and test corresponding sex-specific stroke intervention approaches.

Implication of a Brain Microvascular Paradigm in Stroke Pathogenesis
The prestroke decrease in microvascular mass, microvascular paucity associated with neuronal ischemia, loss of endothelial integrity, and astrocyte endfoot contiguity collectively implicate a microvascular paradigm that could contribute to continuing cycles of microvascular dysfunction and ischemia after the initial ischemic stroke event. Coupled with interleukin-18–mediated activation of neutrophils, neutrophil transmigration, and myeloperoxidase release, microvascular paucity and dysfunction could lead to increased susceptibility for ischemic hemorrhagic transformation. Together, these observations support the hypothesis that a hemorrhage-prone angiopathy underlies multiple cerebral microhemorrhages,41 which on progression could underlie the observed coexistence of microhemorrhages with acute intraparenchymal hem-
orrages in humans and the predictive association of microhemorrhages with new or fatal strokes in patients with ischemic strokes. These multiple concordances between the Tg25sp model and clinical observations support a microvascular paradigm in stroke pathogenesis.

Study Limitations
Observations are currently limited to rat modeling wherein hypertension is not treated at SBP >160 mm Hg, and hyperlipidemia is due to CETP transgenic expression in a susceptible genetic background. Despite severe hypertension, the Tg25sp rat model of stroke does not exhibit cerebellar hemorrhage, which is a feature of hypertensive hemorrhagic strokes in humans. Although the data revealed a microvascular paradigm in stroke pathogenesis, investigation of the impact of early-life Na exposure on the kidney and carotid artery vascular wall will be necessary. Additionally, future studies will be required to elucidate the mechanisms underlying the observed sex-specific differences. We qualify that the observation of strokes rather than coronary heart disease does not imply the nonimportance of Na exposure in coronary heart disease, because strokes occurred much earlier than the expected onset of pathogenesis of coronary heart disease in this model.

Clinical Implications and Conclusions
The present findings demonstrate that early-life Na exposure within levels that comply with current recommended daily sodium intake is a key modifier of hypertension and its target-organ complications in genetically predisposed individuals and reveal greater susceptibility in females. When unaccounted for, changes in disease course induced by differential early-life Na exposure will confound genetic analyses of adult-onset hypertension and hypertensive end-organ complications. Such confounders most likely underlie the shortfall in identifying hypertension or stroke susceptibility genes despite large-cohort studies. The modeling of cerebral microvascular abnormalities, microhemorrhages, hemorrhagic infarction, and intraparenchymal hemorrhages as a spectrum suggests pathogenic interrelationships, which, given the new Tg25sp rat stroke model, can now be investigated systematically as to both mechanism(s) and new diagnostic and therapeutic approaches. The use of clinically pertinent diagnostic modalities (MRI and ultrasound carotid artery imaging, fluorescent-activated cell sorter analysis, and circulating biomarkers) and the correlation of these findings with molecular and histopathological observations in Tg25sp rats could facilitate clinical translation of model-derived mechanistic insight. Most importantly, given that stroke risk persists despite successful antihypertensive therapies, the data support the mandate for further study of early-life Na exposure as a potential high-impact prevention target to decrease the incidence of adult-onset hypertension and stroke.

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Disclosures
None.

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CLINICAL PERSPECTIVE

Despite major inroads that have been made into the treatment of hypertension and stroke, hypertension remains the leading risk factor for stroke, and stroke remains the third-leading cause of mortality. Validated animal models of stroke are needed for the identification of stroke mechanisms and hence the elucidation of much needed mechanism-based intervention and prevention strategies. A recently characterized rat stroke model, Tg25sp, provides several advantages. As a model of spontaneous stroke that recreates 2 key risk factors, hypertension and hyperlipidemia, it provides an experimental system to investigate new targets for prevention and intervention strategies, as well as an animal model to test new drugs and diagnostic modalities with systematic histological corroboration. These critical tasks are impossible to perform in human studies. As a complex stroke model that spans a spectrum of carotid artery disease, ischemic lesions, microhemorrhages, intraparenchymal hemorrhages, and microvascular alterations, stroke-prone Tg25 rats prove, through reproducible modeling, that the association of these same pathologies in humans is causally interrelated rather than just coincident, thus giving experimental support for systematic study to determine prognosis for future stroke events. As a model that exhibits strokes in females, Tg25sp rats provide a key experimental tool for the investigation of sex-specific mechanisms and intervention and prevention strategies. Most importantly, the demonstration of the impact of early-life Na exposure on adult-onset hypertension and stroke highlights the need to study Na intake during gestation as a key prevention strategy. Current new studies fall short of the most vulnerable period for adult-onset stroke, which is in utero development.
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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\(^2\). 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 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™ 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.

<table>
<thead>
<tr>
<th>Cohort – A.</th>
<th>Step-1. Study of stroke occurrence and onset induced by early life 0.4%Na- exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td></td>
<td>Tg25 F</td>
</tr>
<tr>
<td>a. X_\text{F}</td>
<td>14</td>
</tr>
<tr>
<td>b. X_\text{W}</td>
<td>13</td>
</tr>
<tr>
<td>c. X_\text{A}</td>
<td>19</td>
</tr>
<tr>
<td>d. X_\text{F}</td>
<td>14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cohort – B.</th>
<th>Step-2. Brain histology and immunohistochemistry time point studies of X_\text{F} Tg25-males and females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>X_\text{F} Tg25 F</td>
</tr>
<tr>
<td>a. at stroke onset [from step-1a]</td>
<td>4</td>
</tr>
<tr>
<td>b. 2m of age</td>
<td>3</td>
</tr>
<tr>
<td>c. 3m of age</td>
<td>3</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Cohort – C.</th>
<th>Step-3. Time point analysis of systolic blood pressure (SBP), total plasma cholesterol (TC), total plasma triglyceride (TG) and isolation of brain microvessels (bmvs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td></td>
<td>SBP, TC, TG, bmvs</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X_\text{F} Tg25 F</td>
</tr>
<tr>
<td>2m</td>
<td>8</td>
</tr>
<tr>
<td>3m</td>
<td>6</td>
</tr>
<tr>
<td>4-4.5m</td>
<td>4</td>
</tr>
<tr>
<td>X_\text{A} Tg25 F</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cohort – D.</th>
<th>Step-4. Ex vivo 11.7T-MRI of fixed stroke+ X_\text{F} rat brains</th>
</tr>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Tg25 M</td>
</tr>
<tr>
<td>at stroke onset</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cohort – D.</th>
<th>Step-5. Ultrasound micro-imaging of left and right carotid arteries, and cardiac function of X_\text{F} rats at stroke onset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tg25 M</td>
</tr>
<tr>
<td>a. at stroke onset</td>
<td>4</td>
</tr>
</tbody>
</table>

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_\text{F}, fetal 0.4%Na-exposure; X_\text{W}, 0.4%Na-exposure from weaning; X_\text{A}, 0.4%Na-exposure from 8 weeks of age; C_\text{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.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex (n)</th>
<th>X_F: exposure from fetal stage</th>
<th>X_W: exposure from weaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg25</td>
<td>F (14)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>M (19)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>99 ± 4</td>
<td>128 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>116 ± 4</td>
<td>177 ± 7</td>
</tr>
<tr>
<td></td>
<td>lower 95% CI</td>
<td>90</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>upper 95% CI</td>
<td>108</td>
<td>138</td>
</tr>
</tbody>
</table>

One-way ANOVA

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:

<table>
<thead>
<tr>
<th>Factor</th>
<th>Percentage of Variance</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>early-life 0.4%Na-exp</td>
<td>20.9%</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>sex-effects</td>
<td>38.9%</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>interaction</td>
<td>4.6%</td>
<td>P &lt; 0.008</td>
</tr>
</tbody>
</table>

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\textsubscript{F}[0.4\%Na] to control C\textsubscript{F}[0.23\%Na] Tg25-male and female rats at 2m-, 3m- and 4m-of age.

<table>
<thead>
<tr>
<th>Age</th>
<th>2m</th>
<th>3m</th>
<th>4m</th>
</tr>
</thead>
<tbody>
<tr>
<td>sex (n)</td>
<td>F (8)</td>
<td>M (10)</td>
<td>F (8)</td>
</tr>
<tr>
<td>X\textsubscript{F} SBP</td>
<td>191.0 ± 21.2</td>
<td>173.7 ± 22.1</td>
<td>203.4 ± 24.3</td>
</tr>
<tr>
<td>X\textsubscript{F} TC</td>
<td>63.6 ± 15</td>
<td>242.5 ± 77</td>
<td>58.4 ± 20</td>
</tr>
<tr>
<td>X\textsubscript{F} TG</td>
<td>344.4 ± 94</td>
<td>750.8 ± 388</td>
<td>402.5 ± 172</td>
</tr>
</tbody>
</table>

| sex (n) | F (8) | M (10) | F (6) | M (12) | F (9) | M (6) |
| C\textsubscript{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\textsubscript{F}, fetal 0.23\%Na-exposure; X\textsubscript{F}, fetal 0.4\%Na-exposure; F, Tg25-female; M, Tg25-male; m, months; SBP, tail cuff systolic blood pressure (mmHg); Δ SBP, [X\textsubscript{F}SBP – C\textsubscript{F}SBP] 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

<table>
<thead>
<tr>
<th>XF Developmental Programming</th>
<th>neuronal pyknosis + eosinophilia</th>
<th>micro-hges</th>
<th>pmn+</th>
<th>macro-hges</th>
<th>Neurological deficits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At stroke onset (n = 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tg25 F</td>
<td>+1 to +2</td>
<td>+1 to +2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tg25 M</td>
<td>+1 to +2</td>
<td>+1 to +2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>At 3months of age (n = 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tg25 F</td>
<td>+ ½ to +2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tg25 M</td>
<td>+ ½ to +1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>At 2months of age (n = 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tg25 F</td>
<td>+ ½ to 2</td>
<td>+ ½</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tg25 M</td>
<td>+ ½</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

XF, 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 hpf); +3 (>4 1000x hpf).
Supplemental References
