Osmoregulatory defect in adult mice associated with deficient prenatal expression of *six2*

S Jack Somponpun, Brittany Wong, Thomas E Hynd, Benjamin Fogelgren, Scott Lozanoff

Department of Anatomy, Biochemistry, and Physiology, John A Burns School of Medicine, University of Hawaii at Manoa, Honolulu HI 96813

Running title: Impaired renal function in adult mice lacking embryonic *six2*

Address correspondence and request offprints to:

S Jack Somponpun, PhD
John A Burns School of Medicine
Department of Anatomy, Biochemistry, & Physiology
651 Ilalo Street, BSB 167
Honolulu, HI 96813
Tel: 808-692-1420 (office)
Tel: 808-692-1796 (lab)
Fax: 808-692-1951
Email: Jack Somponpun [suwit@hawaii.edu]
Abstract

Suboptimal kidney development resulting from a genetic deficit in nephron number can have lifelong consequences that may lead to cardio-renal complications when exposed to secondary insults in later life. To determine whether the inherited reduced renal reserve compromises the ability to handle osmotic stress in the adult animal, we challenged the heterozygous 3H1 Brachyrrhine (Br/+ ) mouse that displays heritable renal hypoplasia associated with reduced embryonic six2 expression, to a solution of 2% NaCl for 5d or to fluid restriction for 48h. Blood chemistry, fluid intake, and physiologic parameters including renal measurements were determined. Systemic hypertonicity by prolonged salt loading led to significant increases in plasma osmolality (pOsm) and plasma sodium (pNa+) along with polydipsia and polyuria with significant urine concentrating defect that was resistant to dDAVP-treatment in the adult Br/+ , compared to the wild-type littermates. The Br/+ mouse also developed a significant increase in blood urea nitrogen at baseline that was further elevated when given 2% NaCl. Fluid restriction for 48h further enhanced pOsm and pNa+ responses although the Br/+ was evidently able to produce small output of concentrated urine at this time. Hypothalamic cFos expression was appropriately activated in the Br/+ in response to both osmotic challenges, indicating an intact central neuroendocrine pathway that was not affected by the lack of congenital six2 expression. Collectively, our results demonstrate impaired osmoregulatory mechanisms consistent with chronic renal failure in the Br/+ and indicate that six2
haploinsufficiency has a direct consequence on postnatal fluid and electrolyte handling associated with fluid imbalance.

**Key words:** nephrogenesis, glomeruli, nephron endowment, renal hypoplasia, vasopressin
Introduction

The kidney is a vital organ that serves as a homeostatic regulator of fluids and electrolytes. This regulation is achieved primarily by the nephron, the functional subunit of the kidney. Chronic renal failure (CRF) or the progressive loss of kidney function occurs when the kidney no longer maintains a critical number of functional nephrons. CRF may arise during the course of numerous diseases, but might also occur as a result of insufficient nephron number at birth [1, 4, 21]. While kidney disease affects populations worldwide, its incidence is disproportionately high among certain population such as in African-Americans [14] and the Pacific Islanders for reasons that are not entirely clear but may be related to reduced renal reserve associated with suboptimal nephron differentiation during development.

Several morphogenetic factors interact specifically during the critical period of kidney organogenesis to determine and give rise to final nephron formation (see reviews [2, 3, 22]). In an effort to understand how the kidney develops nephrons and achieves a critical number that adequately regulates and maintains fluid balance in the organism, we are investigating the role of an embryonic transcription factor *sine oculis 2 (six2)* in the establishment of nephron number. *Six2* belongs to the homeodomain-containing transcription factors of the *Six* family that are expressed and function to regulate a variety of developmental processes principally during embryonic induction [9]. Its expression pattern in embryonic tissues includes the frontal region of the head mesenchyme, the cells
in the hindbrain, and the developing pituitary arising from Rathke’s pouch [13]. In the developing kidney, *six2* expression is restricted primarily to the mesenchymal populations in the nephrogenic zone where loss of *six2* expression is known to reduce the number of the progenitor cells [17].

Previous work in our laboratory established a colony of adult heterozygous 3H1 Brachyrrhin (Br/+) mice that display heritable renal hypoplasia (RH) associated with diminished expression of *six2* during renal development as a result of a radiation-induced mutation [5, 11]. The homozygous *Br/Br* mutants die neonatally due to the severity of their genetic defect, but the heterozygous *Br/+* mutants survive to adulthood. As a consequence, the *Br/+* mice demonstrate reduced nephron number and develop CRF and hypertensive phenotype when compared to the wild-type littermates [6]. Because RH is inherited in a haploinsufficient manner in the adult *Br/+* animal, this makes it uniquely valuable for identification of genes that are associated with this renal disease as well as for complications arising from CRF. Interestingly, while the heterozygous *Br/+* mice survive into adulthood and inherit renal insufficiency with reduced nephron number, no obvious abnormalities were reported in the *six2*-heterozygous transgenic and the *six2*-nullizygous mice are embryonic lethal [17]. This suggests that additional factors interact specifically with *six2* to contribute to reduced nephron number, but does not eliminate renal formation altogether. Thus, we have a valuable model of adult RH of embryonic origin that can be used to address a number of important biological questions, including the role of sub-
optimal kidney development in long-term blood pressure regulation, for instance. Additionally, this strain is particularly useful since it can be subjected to experimental alterations to determine the consequence of reduced nephron number without surgical intervention such as those associated with nephrectomy models, thus reducing variables inherent to surgery.

In the current study, we characterize and investigate the functional role of reduced embryonic *six2* expression on adult renal function and body fluid homeostasis. We demonstrate a significant loss of fluid homeostatic control in the adult *Br/+* when exposed to a prolonged high salt loading or fluid restriction. We also determined whether the defect in osmoregulation inherent to the *Br/+* mouse arises exclusively from renal inadequacies associated with under expression of *six2* or whether there is also a neural component that is involved. The results demonstrate that brain osmoregulatory mechanisms are not affected by the lack of *six2* expression and suggest that the impaired fluid regulating ability observed in the adult *Br/+* lies in the structural abnormality associated with reduced nephron number consequent on diminished nephrogenesis.

**Materials and Methods**

**Animals**

Adult male and female mice (20-30g, 12-16 week old) of 3H1 background and *Br/+* were allowed to acclimatize and maintained on a 12h light/dark cycle with lights on at 6:00 A.M., with ambient temperature at 20-23°C for 5 days prior
to experimentation. All female mice were examined for vaginal plugs. The date when the plugs were first identified is designated as E0.5. At the appropriate embryonic stage, the gestational female was anesthetized with isoflurane inhalant and cervical dislocation performed. The embryos were then removed. All animals were used in compliance with federal, state, and local laws and institutional regulations. The experimental protocol was approved by the University of Hawaii Institutional Animal Care and Use Committee (IACUC). Adequate measures were taken to minimize pain and discomfort in the animals throughout the study.

**Kidney dissection, mRNA collection and qRT-PCR for six2**

For E13.5 and E17.5 embryos, the uterus was removed and placed in 1x PBS. Under an Olympus SZ-CTV dissecting microscope, each embryo was dissected from the uterus and placed in 1x PBS in its own 35 mm tissue culture dish. Using microforceps, the viscera of the abdomen were carefully removed as not to damage the posterior abdominal wall. Once the nephric duct was identified, it was resected laterally to expose the kidney, gonad and adrenal gland. With the forceps, the kidney was gently loosened from the underlying and surrounding tissue. Post-natal day 2, 7 and 27 mice were euthanized and kidneys dissected from the posterior abdominal wall using micro forceps under room light.
Total mRNA was extracted from intact E13.5, E17.5 and post-natal day 2 kidneys. From the post-natal day 7 and 27 kidneys, renal cortex tissue was dissected from approximately the level of the renal pelvis using a double-bladed razor blade (1.5mm between blades). All tissue samples were placed immediately and individually in 200µL of RNA later (Sigma) and stored at 4°C for one to seven days before processing. A total of 37 mice (19 embryos, 18 post-natal) derived from reciprocal 3H1 x Balb Br/+ crosses were collected. Each litter contained +/+ , Br/+ and Br/Br embryos, as determined by genotyping [5]. mRNA from kidney tissue samples was extracted using Qiagen’s RNeasy Mini Kit according to the included protocol for animal tissues. Total RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) and the included protocol.

qRT-PCR reactions (25µL final volume) were performed in triple replicates with 1µL of cDNA, 1µL of each primer and 12.5µL of Bio-Rad’s IQ SYBR Green Supermix with the MyiQ iCycler thermocycler and single color real-time PCR detection system (Bio-Rad). Primers to amplify Six2 (Six2-f: 5’-CTC ACC ACC ACG CAA GTC AGC AAC-3’; and Six2-r: 5’-CAC CGA CTT GCC ACT GCC ATT GAG-3’) and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GAPDH-f: 5’-TGC ACC ACC ACC TGC TTA GC-3’; and GAPDH-r: 5’-GAC TGT GGT CAT GAG-3’) were used. The thermocycle profile used was an initial denaturation at 94°C for 2min, followed by 35 cycles of 94°C for 15sec (denaturation), 59°C for 30sec (annealing), 72°C for 60sec (extension)
and 79°C for 6sec (quantification of fluorescence). Product-specific amplification of Six2 and GAPDH was confirmed by melting curve analysis. The threshold cycle, C(t), was established at the linear portion of the log scale curve and the ratio of Six2 to GAPDH was calculated using the $2^{-\Delta\Delta C(t)}$ method [10].

**Experimental fluid perturbation** To characterize and elucidate possible mechanisms underlying the osmoregulatory defect in the Br/+ mouse, the following experiments were carried out.

1) **Hypertonic saline loading and fluid deprivation** To induce systemic hypertonicity, adult 3H1 wild-type and Br/+ mice were given 2% NaCl solution as a sole source of their fluid intake for 5d. Non-treated control animals continued on tap water until being sacrificed. Food was available ad libitum throughout the experiment. For fluid deprivation, mice were deprived of access to drinking water by removing the water bottles 48h before being sacrificed. Urine production, plasma samples, fluid intake, and body weight changes were monitored and recorded throughout the experiment using mouse metabolic isolator unit (MMC100, Hatteras Instruments). Urine osmolality (uOsm) and plasma osmolality (pOsm) were determined by freezing point depression (Fiske Micro-Osmometer). Plasma sodium (pNa⁺) was determined using EasyElectrolytes Na/K/Cl, Medica. Blood urea nitrogen (BUN) was determined using iSTAT® Portable Clinical Analyzer, HESKA according to the manufacturer's instruction. The kidneys from salt loaded animals were collected at termination of experiment and prepared for routine light microscopy and studied following H & E staining.
2) **Water loading and 1-deamino-8-D-arginine vasopressin (dDAVP) experiment**

Adult 3H1 wild-type and Br/+ mice were loaded with sterile irrigation water at 5% BW i.p. and concomitantly injected with either vehicle (100µl of 0.9% saline s.c.) or dDAVP (100µl of 1.0µg/kg s.c.). Spot urine samples were collected at 0, 6, and 24h following water loading for subsequent urinalyses.

**Hypothalamic cFos expression**

The expression of brain cFos was determined in the hypothalamic supraoptic nuclei (SON) and in the forebrain circumventricular organ neurons. Experiments were performed as previously described [18]. Briefly, at the end of salt loading and fluid deprivation experiments, mice were anesthetized with pentobarbital and perfused transcardially with physiological saline, followed by phosphate-buffered 4% paraformaldehyde, pH 6.7 (Sigma). After fixation, brains and kidneys were removed and cryostat sections (30µM thick) were obtained in a one-in-four series and stored in cryoprotectant solution until processing. For brain tissue, a single set of sections was used to localize Fos-immunoreactivity (-ir) in SON nuclei. Quantification of Fos expression in the forebrain nuclei surrounding the anterior third ventricle included cells of the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO) nuclei. Cryoprotectant was removed from freely floating tissue sections with multiple rinses of 0.05 M buffered saline (KPBS, pH 7.4). The tissues were subsequently incubated with a rabbit polyclonal antiserum raised against amino acids 4-17 of human c-Fos (Ab-5, Calbiochem) at a 1:35,000 dilution made up in KPBS with 0.4% Triton X-100.
for 60 min at room temperature (RT) then 72 h at 4°C. The sections were rinsed with multiple KPBS, incubated with a biotinylated donkey anti-rabbit serum (Jackson ImmunoResearch) at a 1:600 dilution in KPBS with 0.4% Triton X-100 for 1 h at RT. After several rinses, tissues were incubated in avidin-biotin complex solution for 1 h at RT. Primary antibody was localized using a conventional immunoperoxidase method with a 15-min exposure to nickel sulfate plus diaminobenzidine-HCl in sodium acetate solution in the presence of H₂O₂. This yielded a blue-black reaction product in the nuclear compartment. Sections were mounted on poly-L-lysine-treated slides, air-dried overnight, and coverslipped. Cells that expressed nuclear blue-black immunoprecipitants after DAB-nickel reaction were considered positive. cFos expression was quantified in each nucleus by counting the number of Fos-positive nuclei in one or more sections from each animal. Only intensely stained nuclei were counted to avoid interference with background staining, and the same threshold was applied to count all sections of Fos-ir. For each variable, the mean value from multiple measurements from each animal was calculated, and these values were used to calculate group means.

**Statistical analysis** Student’s t-test (or rank sum test), one-way ANOVA (or Kruskal-Wallis analysis on rank), and two-way ANOVA with repeated measures were used as appropriate to determine the statistical significance between groups. The α-value was set at $P<0.05$. Results are expressed as group means±SEM.
Results

*Haploinsufficient expression of renal six2 is non-linear and contributes to a large degree of variation in nephron number seen in the adult Br mouse*

We previously reported a haploinsufficient expression of *six2* in the E13.5 *Br* kidneys when compared to the wild-type littermates [5]. Given a large variation in the glomerular number seen in the colony of adult *Br/+* mice, we re-evaluated and further examined the expression of *six2* at an additional stage (E17.5) from the *Br* kidneys and standardized it to the level expressed in E13.5 kidneys. As shown in Fig 1, *six2* expression in the wild-type embryo decreased as development proceeds and became undetectable by postnatal D7. Similarly, the expression of *six2* in *Br/+* kidneys decreased over the course of the development and was gone by postnatal D2. While comparison of relative expression of *six2* transcripts demonstrates a haploinsufficient expression (1.0:0.5:0.0) for *Br* at E13.5 as initially reported, this relationship was not maintained in E17.5 kidneys. Instead, the expression of *six2* in the *Br* mouse declined at a more rapid pace at E17.5, compared to that in E13.5 kidneys. This demonstrates that, although haploinsufficient initially, the decrease in *six2* expression over the course of development in the *Br* kidney is not linear. Thus, we do not necessarily expect a 50% decrease in the glomerular number in the *Br/+* mouse post-natally.
Consequences of reduced embryonic six2 expression and impaired nephrogenesis – abnormal handling of fluid and electrolyte in post-natal life

To demonstrate the functional consequence of sub-optimal metanephric development associated with reduced embryonic six2 expression on body fluid regulation, we challenged adult heterozygous Br/+ to either 2% NaCl solution as a source of their fluid intake for 5d or to total fluid deprivation for 48h with ad lib food intake. While 3H1 wild-type animals were able to maintain their pOsm and pNa+ against the rising concentration of salt intake associated with 5d of 2% NaCl loading, their pOsm and pNa+ were significantly increased when fluid-deprived for 48h. In the Br/+ animal, pOsm was already elevated while pNa+ remained relatively unchanged at baseline. In contrast, a significant increase in pOsm and pNa+ was developed in the Br/+ in response to 5d of 2% NaCl and further elevated with 48h of fluid deprivation. The increases in response to fluid challenges observed in the Br/+ were significantly higher than those seen in the wild-type mice (Table 1).

Blood urea nitrogen (BUN) was determined in a separate experiment to assess the kidney function in these animals. In the Br/+, there was significantly elevated level of BUN at baseline that may contribute to the increase in basal pOsm. The increase in BUN was further enhanced when the animals were exposed to 2% NaCl loading for 72h. In contrast, no change in BUN was observed in the 3H1 wild-type animals in response to 2% NaCl intake.
Additionally, while the elevated BUN in the Br/+ slightly decreased after the animals had been replete with water for an additional 2-day post-NaCl treatment, their BUN never returned to the resting baseline value (Fig 2). Collectively, the results indicate inadequate osmoregulation and decreased renal function in the adult Br/+ mouse consequent on reduced expression of embryonic six2.

**Impaired urine concentrating ability, polyuria, and polydipsia in the adult Br/+ indicative of chronic renal failure**

We monitored and recorded fluid intake, body weight change and performed 24h urine collection over the course of 5d of high salt challenge as well as during 48h of fluid deprivation using the mouse metabolic isolator unit. In response to prolonged salt loading, the Br/+ consumed significantly higher amount of 2% NaCl over a 24h period, compared to the wild-type. In fact, Br/+ animals were already consuming higher amount of tap water at baseline, suggesting heightened dipsogenic response and partial nephrogenic DI phenotype in the Br/+ colony. The higher consumption of 2% NaCl solution may have accounted for the higher pNa⁺ seen in the Br/+ compared to the wild-type. The average amount of fluid intake remained unchanged in the 3H1 wild-type animal when challenged with 2% NaCl (Table 1).

The higher fluid intake observed in the Br/+ mouse is associated with a corresponding output in dilute urine, suggesting that the Br/+ animals had a
reduced urine concentrating capacity when challenged with systemic hypertonicity (Table 1). Further, to directly test their urine concentrating ability, animals of both genotypes were water loaded to suppress the endogenous vasopressin (AVP) production and concurrently given a supraphysiological dose of V2R agonist, dDAVP. In response to exogenous dDAVP, the wild-type animals increased their urine osmolality from 1500±146 to 2747±119 mOsm/kg H₂O (p<0.05). In contrast, Br/+ mice only partially increased their urine osmolality in response to dDAVP (1422±126 to 1686±201 mOsm/kg H₂O), consistent with partial nephrogenic DI. Urine osmolality in all animals returned to their baseline values after 24h. Thus, while wild-type animals fully increased urine concentration in response to exogenous dDAVP, the Br/+ mouse modestly responded to the treatment and was only able to partially concentrate their urine, suggesting a reduced renal concentrating ability in these animals (Fig 3). Overall, the results indicate an impaired urine concentrating ability that is resistant to dDAVP treatment in the Br/+ animals, consistent with physiologic feature prominent in CRF.

In response to fluid restriction for 48h, animals of both genotypes were able to produce a concentrated urine, but while the 3H1 wild-types were relatively resistant to systemic hyperosmolality, the Br/+ mouse were unable to protect themselves against plasma hypertonicity associated with fluid restriction (Table 1).
Finally, both 3H1 wild-type and Br/+ mice displayed a significant body weight loss when fluid deprived for 48h. Both groups of animals also lost weight in response to 5d of hypertonic saline consumption, although the degree of weight loss observed in the Br/+ animal was markedly steep and occurred more rapidly than in the wild-type controls. These changes in body weight agree well with the degree of hypertonic difference between the two groups of animals when challenged with high salt consumption (Table 1).

**Morphological defect in the adult Br/+ kidney is exaggerated with salt loading**

The adult Br/+ mouse exhibits renal hypoplasia, characterized by a smaller volume and fewer numbers of nephrons with compensatory sclerosis and hypertrophy in the remaining glomeruli. Further, treatment with hypertonic saline loading led to a progressive enlargement of the nephron in the adult Br/+ mouse. To quantitatively demonstrate changes in kidney morphology, glomerular surface area was compared between samples using standard stereological analysis (DisectorZ, www.akuaware.com). There was a significant increase in the glomerular surface area in the kidneys from Br/+ mice that were treated with high-salt consumption, compared to the wild-type animals (wild-type non-salt: 10173±819 μm², wild-type salt treatment: 11891±336 μm², Br/+ non-salt: 15523±1635 μm², and Br/+ salt treatment: 21042±2043* μm² (p<0.05). Thus, while salt loading had minimal effect on 3H1 +/- mice, the same treatment
qualitatively caused an increased size of Bowman’s capsule with vacuoles forming in mesangial cells in the Br/+ nephrons (Fig 4).

*Increased hypothalamic cFos expression to fluid challenges in the Br/+ suggests intact osmoregulatory brain center that is not affected by the lack of embryonic six2 expression*

To further establish whether the abnormal handling of fluids and osmolytes inherent to the Br/+ mouse arises exclusively from reduced renal glomerular number or whether such osmoregulatory defects are secondary to malfunction of brain regulatory systems, a quantitative immuno-analysis was undertaken to determine the pattern of neural activity in response to experimental fluid perturbation. A marked increase in pOsm/pNa⁺ in response to the prolonged hypertonic saline load resulted in a modest increase in Fos expression in SON neurons of animals of both genotypes. However, this did not achieve statistical significance. Conversely, Fos expression was significantly up-regulated following fluid deprivation for 48h in SON of both wild-types and Br/+ animals, although the degree of Fos activation was much more substantial in the Br/+ relative to non-stimulated and salt-treated mice (Fig 5a and 6). Therefore, it is evident that the brains of Br/+ animals remained functionally intact and appropriately expressed Fos in the SON nuclei, particularly in response to the combined plasma hypertonicity-volume depletion associated with fluid restriction.
We also determined the expression of Fos in the osmoreceptive neurons of the forebrain circumventricular organs, including the SFO and OVLT nuclei. Similar to the response observed in SON nuclei, immuno-analyses revealed a significant increase in the number of Fos-expressing cells in both nuclei in response to fluid deprivation and, to a lesser extent, salt loading (Fig 5b and 5c). The results suggest intact osmosensitivity of these forebrain neurons. Further, gross histological analysis of the rest of brain tissues collaborated and revealed no significant alterations in most brain structures that were examined (data not shown). Taken together, the results suggest that absence of embryonic six2 expression did not alter the central neuroendocrine pathway controlling osmoregulation and indicates that the impaired fluid regulating ability in the adult Br/+ derives exclusively from renal inadequacy associated with reduced nephron number.

**Discussion**

Successful regulation of body fluid is essential for proper function of most cells in the body and depends primarily on the normal function of the kidney in establishing extracellular fluid homeostasis. Because renal filtering capacity is determined by the number of nephrons, it is crucial that the embryonic metanephros (the mammalian kidney) develops properly to achieve an optimal number of nephrons. While generally assumed, not all kidneys possess the same number of nephrons and that the number of functional nephrons varies greatly among individuals [8, 12, 15]. Since this variability appears to be present early in
neonates [23], it suggests that final nephron number is developmentally established during the period of embryogenesis.

While progress has been made to uncover factors and mechanisms that regulate nephron differentiation and branching morphogenesis, precisely which genes give rise to the final number of nephron in the developing kidney remains unknown. As demonstrated previously in transgenic mice harbouring the mutated gene, absence of \textit{six2} upsets the delicate balance between pools of native mesenchymes and the differentiated epithelial cells, leading to irreversible loss of nephron subunits that result in RH at birth [17], indicating the critical role of \textit{six2} in nephron development. Indeed, RH associated with decreased nephron number has been linked with chronic hypertension in human newborns [1].

In the current study, we demonstrate a developmental decrease in embryonic renal \textit{six2} expression that is absent in adult tissue. This is evident in both wild-type and \textit{Br/+} animals. Further, while \textit{Br/+} kidneys contain half as much of \textit{six2} transcript at E13.5 compared to the wild-types indicating haploinsufficiency, the level of \textit{six2} was found to decrease more rapidly such that the haploinsufficient expression is no longer maintained at E17.5 stage. Since the metanephros proceeds through development by continuously expanding itself over successive rounds of repetitive branching, the rapid disappearance of \textit{six2+} progenitors in the \textit{Br/+} and, almost immediately, in the \textit{Br/Br} embryos probably accounts for the non-linear expression of \textit{six2} at a later stage as these \textit{six2+} cells continue its rapid proliferation as usual in the wild-type metanephros. In other
words, it is the relative difference in the amount of $six2^+$ cells amongst the growing mix of others during the exponential growth of the embryonic kidney that results in the non-linear expression change of $six2$ over nephrogenesis.

The rapid decline in $six2$ expression at the later stage of development possibly accounts for the more than 50% decrease in glomeruli post-natally with relatively high variability in nephron number that is evident among the adult $Br^+/+$ colony. In fact, we previously reported variable nephron numbers that ranged from 13,000-24,000 per kidney in the 3H1 wild-types, and about 700-3,200 per kidney in the $Br^+/+$ mice [6]. These observations support the contention that nephron number is variable even in the general population. That such variability is enhanced among the $Br^+/+$ colony (>4fold variation) provides observational evidence that further suggests the possible role of $six2$ in the determination of final nephron number.

In this study, we challenged the adult $Br^+/+$ mice to two specific experimental fluid perturbations and provide evidence that indicates an osmoregulatory defect that persists well into adulthood, including hypernatraemia, plasma hypertonicity, polydipsia and polyuria with significantly dilute urine output that fails to respond fully to exogenous dDAVP. The attenuated renal response indicates an impaired urine concentrating ability that, together with a significant increase in BUN at rest and under stimulated
conditions, further indicate reduced renal function reflecting suboptimal nephron number development.

Although the Br/+ clearly develops a dDAVP-resistant urine concentrating defect that typically characterizes the progression of CRF [7, 20], they were nonetheless able to concentrate their urine when fluid deprived for 48h. The residual urinary concentrating ability in response to fluid deprivation is probably due to an increased AQP2 signalling in the remaining nephrons [19]. Indeed, while the Br/+ mouse was physically able to withstand and survived sustained high salt consumption, they were much more vulnerable to fluid restriction and predictably died shortly after 48h fluid restriction if water was not returned soon (Somponpun S.J., personal observation). Overall, changes in physiologic parameters observed in the current study are consistent with renal insufficiency associated with CRF in the adult Br/+ mice.

Histo-morphological defects such as cysts and distended renal tubules were consistently observed in the Br/+ mouse. We previously demonstrate the smaller total kidney volume, smaller glomerular density/number ratio, and larger glomerular surface area confirming nephropathy in the mutant mouse [6]. Prolonged exposure to high salt loading in the current experiment exacerbates condition and causes a progressive enlargement of the nephron in the Br/+, compared to the wild-type mice.
Since *six2* demonstrates broad expression during embryogenesis, we also determined whether the diminished *six2* expression in the *Br/+* mouse might, in addition to causing renal dysmorphology, affect the central neuroendocrine pathway critical for osmoregulation. The elevated pOsm and pNa⁺ provoked by prolonged salt loading for 5d led to an appropriate, though modest, increase in Fos expression in SON nuclei in both the *Br/+* and the wild-type mice. In contrast, up-regulation of Fos was clearly established in SON neurons in response to 48h fluid restriction, indicating that these nuclei remain functionally intact and are capable of responding to signals generated by the combined hyperosmotic and hypovolemic stress associated with fluid deprivation. The SFO and OVLT nuclei in the basal forebrain also expressed Fos appropriately, suggesting that the osmosensitive mechanism residing in the forebrain circumventricular organ neurons remain able to sense the osmotic changes in response to both salt loading and fluid deprivation. Since the lack of *six2* is known to reduce glomerular number and *Br/+* mice display reduced renal nephrons compared to wild-type mice, our data suggest that the osmoregulatory defect in these animals likely resides in the kidney’s inability to reabsorb sufficient fluid to maintain a constant plasma osmolality when exposed to osmotic challenges while the hypothalamic magnocellular neurons in the SON and the basal forebrain nuclei are intact and were not developmentally altered by the lack of embryonic *six2* expression in the *Br/+* animal. This is important because it isolates the kidney as the primary target of *six2* and sets up an opportunity for us to further investigate and attempt to restore *six2* expression to the *six2*-deficient *Br/+* mouse in our
ongoing gene replacement protocol. We are currently generating a transgenic rescue mouse re-expressing *six2* in our *Br* colony to test the hypothesis that successful re-expression of *six2* leads to a restoration of nephron number, thus rescuing the defective renal function *in vivo*, thereby, confirming the role of *six2* in nephron endowment.

**Conclusion and Perspective**

Successful metanephric development is crucial to achieving appropriate postnatal renal function. Results from the current study strongly argue herein the role of *six2* as an important embryonic regulatory gene of nephron development and suggest that *six2* may constitute the genetic basis for renal nephron number. We have shown that reduced expression of *six2* during embryonic development initiates glomerular dysplastic changes that are associated with the subsequent development of multicystic kidneys with nephron deficit leading to fluid and electrolyte imbalance in the adult.

The deficit in the number of functioning nephrons marks the 3H1 *Br/+* mouse as an experimentally useful model to study the role of nephron numbers in renal diseases and establishes the adult *Br/+* colony as one of only a few existing non-surgical models of CRF. They not only allow for an investigation into genetic attributes of initial nephron differentiation in the embryo but also for physiologic defects that develop in the affected adult. Since a deficit in nephron endowment leads to increased mean arterial pressure, owing to reduced glomerular filtration rate, the current study also allows us to establish an
experimental mouse model for understanding the role of reduced nephron numbers in the genesis of cardiovascular diseases including high blood pressure. As demonstrated in a recent experimental model, difference in nephron endowment has functional consequences on the regulation of blood pressure and renal abnormality [15, 16].

Ongoing experiments are directed at identifying factors that are the targets of *six2* as well as genes associated with RH and complications arising from this renal disease. Determining such markers associated with *six2* might allow for potential clinical applications for an early detection of renal ailments or to identify subpopulation that might be more susceptible to renal damage because of the reduced nephron number.
Acknowledgement

This work was supported by HCF 08PR-43033, NIH/NCRR 5P20RR024206, NIH/NCRR 1U54RR026136-01A1, and NIH/NCRR U54RR022762 (to S.J. Somponpun), K01DK087852-01 (to B. Fogelgren) and 1R01-DK-064752 (to S. Lozanoff). We thank Mari Kuroyama for her technical support and Jin Seon Kim for her quantitative study on the kidney glomerular surface area.
References


Legends

**Figure 1** Comparison of *six2* expression among the wild-type, *Br*/*+, and *Br/*Br kidneys. Expression of *six2* decreases during development and is absent from adult tissue. While quantitative PCR analyses demonstrate a haploinsufficient expression (1.0:0.5:0.0) for the *Br* at E13.5, this relationship was not maintained at E17.5. Instead, *six2* in the *Br* kidney decreases more rapidly at E17.5 and indicates that the decrease in *six2* expression over the course of development in the *Br* kidney is not linear. This might account for the more than 50% decrease in glomeruli post-natally with relatively high variability in nephron number among the adult *Br/*+ colony.

**Figure 2** Comparison of blood urea nitrogen (BUN) levels in the 3H1 wild-type and *Br*/*+ mice. Significant increases in BUN were observed in the *Br*/*+ mice at rest and under stimulated conditions with prolong salt loading, indicating reduced renal function associated with suboptimal nephron development in the *Br*/*+ animals.

**Figure 3** Urine osmolality in response to water load suppression with or without i.p. exogenous dDAVP. The 3H1 wild-type animals fully concentrated their urine in response to exogenous dDAVP following 5% body weight water load suppression. In contrast, the *Br*/*+ mice responded modestly to the dDAVP treatment and were only able to partially concentrate their urine. The results
indicate an impaired urine concentrating ability that is resistant to dDAVP treatment in the \textit{Br}l/+ animals, consistent with partial diabetes insipidus.

\textbf{Figure 4} Kidney histology with H & E stain. There is a progressive enlargement of the nephron due to prolonged salt loading in 3H1 wild-type (A,B) and \textit{Br}l/+ (C,D) mice. Salt loading shows minimal effect on the wild-type (compare wild-type control (A) with wild-type salt-loading (B)) while the same treatment caused an expansion of Bowman’s capsule with vacuoles forming in mesengial cells in \textit{Br}l/+ nephrons (D). Arrows indicate glomerulus. Scale bar = 300\textmu M.

\textbf{Figure 5} Cell counts of Fos-immunopositive cell nuclei in SON (A), OVLT (B), and SFO (C). Fos expression was activated in response to prolonged salt loading and significantly elevated by fluid deprivation in all brain regions examined in both genotypes of mice (\textit{*P} \leq 0.05). This indicates an intact central neuroendocrine pathway in response to fluid perturbation that is not affected by the lack of congenital \textit{six}2 expression in the \textit{Br}l/+ animal.

\textbf{Figure 6} Representative photomicrographs of Fos-immunoreactive cells in SON of 3H1 wild-type (A,B,C) and \textit{Br}l/+ mice (D,E,F). While Fos was minimally expressed in water-replete animals of either genotype (A,D), it was activated in animals made hyperosmotic with high salt consumption (B,E) and became significantly increased with fluid deprivation (C,F). Scale bar: 100\textmu m. OC, optic chiasm, SON, supraoptic nucleus.
Table 1. Comparison of physiologic parameters among 3H1 wild-type and heterozygous Brachyrrhine (Br/+) mice exposed to either 5d of 2% NaCl ingestion (S) or 48h of fluid deprivation (DH) relative to non-treated controls (NS)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild-type</th>
<th></th>
<th></th>
<th>Br/+</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
<td>S</td>
<td>DH</td>
<td>NS</td>
<td>S</td>
</tr>
<tr>
<td>Posm, mOsmol/kgH₂O</td>
<td>325.4±3.27</td>
<td>332.5±2.22</td>
<td>343.5±2.84</td>
<td>344.8±2.47</td>
<td>384.2±9.65*</td>
</tr>
<tr>
<td>PNa, meq/l</td>
<td>131.9±1.48</td>
<td>136.9±1.97</td>
<td>140.1±0.46</td>
<td>134.2±1.24</td>
<td>145.6±2.16*</td>
</tr>
<tr>
<td>Uosm, mOsmol/kgH₂O</td>
<td>1461±105.70</td>
<td>2650±114.80*</td>
<td>3048±96.69**</td>
<td>1415±71.61</td>
<td>1260±110.70</td>
</tr>
<tr>
<td>Initial B.W., grams</td>
<td>33.96±2.29*</td>
<td>33.85±1.50**</td>
<td>41.73±1.43^</td>
<td>25.19±1.22</td>
<td>22.79±0.78</td>
</tr>
<tr>
<td>Final B.W., grams</td>
<td>33.45±2.08</td>
<td>31.52±1.32</td>
<td>38.16±1.28</td>
<td>25.53±1.13^</td>
<td>19.14±0.82**</td>
</tr>
<tr>
<td>Total ΔB.W., grams</td>
<td>-0.51</td>
<td>-2.33</td>
<td>-3.57</td>
<td>+0.34</td>
<td>-3.65</td>
</tr>
<tr>
<td>%B.W. loss</td>
<td>1.50</td>
<td>6.90</td>
<td>8.55</td>
<td>NA</td>
<td>16.02</td>
</tr>
<tr>
<td>Fluid intake, grams</td>
<td>0.93±0.03</td>
<td>1.01±0.05</td>
<td>1.58±0.09*</td>
<td>2.15±0.31**</td>
<td></td>
</tr>
</tbody>
</table>

Fluid intake, body weight change and urine collection were performed using the mouse metabolic isolator unit. Values are mean±SEM. P<sub>osm</sub>, P<sub>Na</sub> and U<sub>osm</sub> are plasma osmolality, plasma sodium and urine osmolality, respectively. P<sub>osm</sub> and P<sub>Na</sub> were determined from blood drawn at termination of experiment. B.W. is body weight. Fluid intake was calculated per 10g body weight average over 24h.

P<sub>osm</sub>  *  P<0.05 Br/+ (S) vs. Wild-type (NS,S,DH)
  **  P<0.01 Br/+ (DH) vs. Wild-type (NS,S,DH) and Br/+ (NS)

P<sub>Na</sub>  *  P<0.01 Br/+ (S) vs. Wild-type (NS) and Br/+ (NS)
  **  P<0.05 Br/+ (DH) vs. Wild-type (NS,S,DH) and Br/+ (NS)

U<sub>osm</sub>  *  P<0.001 Wild-type (S) vs. Wild-type (NS) and Br/+ (NS,S)
  **  P<0.001 Br/+ (DH) vs. Wild-type (NS) and Br/+ (NS,S)
  ^  P<0.001 Wild-type (DH) vs. Wild-type (NS) and Br/+ (NS,S)
Initial B.W.  
* \( P < 0.05 \) Wild-type (NS) vs. Br/+ (NS,S,DH)  
** \( P < 0.05 \) Wild-type (S) vs. Br/+ (NS,S,DH)  
^ \( P < 0.05 \) Wild-type (DH) vs. Wild-type (S) and Br/+ (NS,S,DH)

Final B.W.  
* \( P < 0.05 \) Br/+ (NS) vs. Wild-type (NS,DH)  
** \( P < 0.001 \) Br/+ (S) vs. Wild-type (NS,S,DH)  
^ \( P < 0.001 \) Br/+ (DH) vs. Wild-type (NS,S,DH)

Fluid intake  
* \( P < 0.05 \) Br/+ (NS) vs. Wild-type (NS)  
** \( P < 0.001 \) Br/+ (S) vs. Wild-type (NS,S)
Fig 5a SON

* +/+ DH > +/+ NS & Br/+ NS, p < 0.05
** Br/+ DH > +/+ NS, S, DH & Br/+ NS, S; p < 0.05

Fig 5b OVLT

* +/+ DH > +/+ NS, S & Br/+ NS, S, p < 0.01
** Br/+ DH > +/+ NS, p < 0.05

Fig 5c SFO

* Br/+ DH > +/+ NS, S, DH & Br/+ NS, p < 0.01
Table 1. Comparison of physiologic parameters among 3H1 wild-type and heterozygous Brachyrrhine (Br/+ ) mice exposed to either 5d of 2% NaCl ingestion (S) or 48h of fluid deprivation (DH) relative to non-treated controls (NS)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild-type</th>
<th>Br/+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
<td>S</td>
</tr>
<tr>
<td>Posm, mOsmol/kgH$_2$O</td>
<td>325.4±3.27</td>
<td>332.5±2.22</td>
</tr>
<tr>
<td>PNa, meq/l</td>
<td>131.9±1.48</td>
<td>136.9±1.97</td>
</tr>
<tr>
<td>Uosm, mOsmol/kgH$_2$O</td>
<td>1461±105.70</td>
<td>2650±114.80*</td>
</tr>
<tr>
<td>Initial B.W., grams</td>
<td>33.96±2.29*</td>
<td>33.85±1.50**</td>
</tr>
<tr>
<td>Final B.W., grams</td>
<td>33.45±2.08</td>
<td>31.52±1.32</td>
</tr>
<tr>
<td>Total ΔB.W., grams</td>
<td>-0.51</td>
<td>-2.33</td>
</tr>
<tr>
<td>%B.W. loss</td>
<td>1.50</td>
<td>6.90</td>
</tr>
<tr>
<td>Fluid intake, grams</td>
<td>0.93±0.03</td>
<td>1.01±0.05</td>
</tr>
</tbody>
</table>

Fluid intake, body weight change and urine collection were performed using the mouse metabolic isolator unit. Values are mean±SEM. P$_{\text{osm}}$, P$_{\text{Na}}$ and U$_{\text{osm}}$ are plasma osmolality, plasma sodium and urine osmolality, respectively. P$_{\text{osm}}$ and P$_{\text{Na}}$ were determined from blood drawn at termination of experiment. B.W. is body weight. Fluid intake was calculated per 10g body weight average over 24h.

P$_{\text{osm}}$ * P<0.05 Br/+ (S) vs. Wild-type (NS,S,DH)  
** P<0.01 Br/+ (DH) vs. Wild-type (NS,S,DH) and Br/+ (NS)

P$_{\text{Na}}$ * P<0.01 Br/+ (S) vs. Wild-type (NS) and Br/+ (NS)  
** P<0.05 Br/+ (DH) vs. Wild-type (NS,S,DH) and Br/+ (NS)

U$_{\text{osm}}$ * P<0.001 Wild-type (S) vs. Wild-type (NS) and Br/+ (NS,S)  
** P<0.001 Wild-type (DH) vs. Wild-type (NS) and Br/+ (NS,S)
^ P<0.001 Br/+ (DH) vs. Wild-type (NS) and Br/+ (NS,S)
Initial B.W.  *  $P<0.05$ Wild-type (NS) vs. Br/+ (NS,S,DH)
    **  $P<0.05$ Wild-type (S) vs. Br/+ (NS,S,DH)
    ^  $P<0.05$ Wild-type (DH) vs. Wild-type (S) and Br/+ (NS,S,DH)

Final B.W.   *  $P<0.05$ Br/+ (NS) vs. Wild-type (NS,DH)
    **  $P<0.001$ Br/+ (S) vs. Wild-type (NS,S,DH)
    ^  $P<0.001$ Br/+ (DH) vs. Wild-type (NS,S,DH)

Fluid intake  *  $P<0.05$ Br/+ (NS) vs. Wild-type (NS)
    **  $P<0.001$ Br/+ (S) vs. Wild-type (NS,S)