Cardiovascular and Renal Effects of High Salt Diet in GDNF+/- Mice with Low Nephron Number

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• Renal vasculature • Tubular transporter

Abstract
Aims: To test the suggested association of low nephron number and later development of renal and cardiovascular disease we investigated the effects of high sodium diet in heterozygous GDNF+/- mice. Methods: Aged wild type and GDNF+/- mice were grouped together according to high sodium (HS, 4%) or low sodium (LS, 0.03%) diet for 4 weeks. The heart, the aorta and the kidneys were processed for morphometric and stereological evaluations and TaqMan PCR. Results: On HS GDNF+/- mice showed significantly higher drinking volume and urine production than wt and mean arterial blood pressure tended to be higher. Heart weight was higher in GDNF+/- than in wt, but the difference was only significant for LS. HS significantly increased cardiac interstitial tissue in GDNF+/-, but not in wt. On LS GDNF+/- mice had significantly larger glomeruli than wt and HS led to an additional two fold increase of glomerular area compared to LS. On electron microscopy glomerular damage after HS was seen in GDNF+/-, but not in wt. Dietary salt intake modulated renal IL-10 gene expression in GDNF+/-.

Conclusion: In the setting of 30% lower nephron number HS diet favoured maladaptive changes of the kidney as well as of the cardiovascular system.

Introduction
A direct involvement of the kidney in the development of hypertension has been discussed for a long time and evidence was initially provided by experimental studies in rats [1] and sheep [2]. In 1988 Brenner and colleagues supposed an association between
nephron number and blood pressure in humans [3] and hypothesized that any reduction in nephron number is accompanied by hyperfiltration of the remaining glomeruli, followed by glomerular enlargement, glomerular and then systemic hypertension. These maladaptive changes result in progressive glomerulosclerosis thus establishing a vicious circle [3]. In line with the "Brenner hypothesis" an association of low nephron number, glomerular enlargement and development of hypertension was shown in various animal models [2, 4–6] as well as in two autopsy studies in humans [7, 8]. In the study by Keller et al. [7] in Caucasian patients with essential hypertension the number of glomeruli per kidney was significantly lower than in age, weight and gender matched controls. In parallel, mean glomerular volume was more than twice as high in hypertensive than in normotensive control patients indicating compensatory glomerular enlargement. The results of this study were confirmed in a subsequent larger autopsy study in Caucasian Americans, whereas interestingly in African Americans an association between glomerular number and blood pressure was not found [8].

A substantial body of epidemiological and experimental evidence has accumulated strongly implicating salt as having a causal role in the genesis of cardiovascular disease, in particular essential hypertension. The prevalence of salt sensitivity in the general population, however, is largely unknown. Of note, in African Americans a greater prevalence of salt sensitivity was found compared to Caucasians. The reasons for this observation are not entirely clear; however, intrinsic or hypertension-induced renal abnormalities that limit natriuretic capacity, reduced Na⁺/K⁺-ATPase pump activity, other membrane ion transport disturbances, differential exposure to psychological stressors, greater insulin resistance, and dietary factors (reduced Ca²⁺ and K⁺ intake) have all been suggested as possibly playing a role [9]. Whether reduced nephron number is also involved is currently explored in animal models of low nephron number [10]. One such model is the GDNF+/- mouse with an approximately 30% lower number and mild hypertension in later life [11, 12]. GDNF is a crucial factor for nephrogenesis; it directly induces branching of the ureteric bud. Complete deficiency of GDNF in GDNF knockout mice leads to bilateral kidney agenesis and death within the first 24 hours [11, 13]. In our own studies early changes in glomerular cells and capillaries were seen at the age of 26 weeks, but no differences in postglomerular structures, i.e. expression and distribution of tubular transporter proteins were noted [14].

Thus, in summary there is human data in specific populations supporting the Brenner hypothesis of nephron underdosing as one potential cause of essential hypertension, but neither the pathomechanisms involved nor the modulatory effects of dietary factors, i.e. high salt intake, are known. Therefore, it was the aim of the present study to investigate the effects of dietary salt modification by low and high salt diet in the GDNF+/- model with 30% lower nephron on cardiovascular and renal structure and function. Moreover, we aimed to analyse alterations in those regulatory systems that might be involved in sodium handling when salt intake is varied in the setting of low nephron number, i.e. the renin angiotensin system (RAS), the endothelin (ET) system, some inflammatory markers and selected tubular sodium transporters.

**Material and Methods**

**Animals and tissue preparation**

GDNF+/- mice were initially kindly provided by Prof. K. Krieglstein and Dr. S. Hermann, Department of Neuroanatomy, University of Göttingen, Germany. The mouse model was originally described by Pichel et al. [13]. Tail tissue was obtained at weaning for genotyping using PCR analysis as described before [14]. All animal experiments were performed in accordance with the guidelines of the American Physiological Society and were approved by the local government authorities (Regierung von Mittelfranken, AZ # 54-2531.31-3/07).

In a *first* experiment 34 GDNF+/- (15 male, 19 female) mice were used with 35 wildtype C57B6 (wt, 15 male, 20 female) serving as controls. Mice were housed under maintained conditions (22±2°C, 12-hour dark/light cycle). At the start of the experiment, the mice were randomly assigned into 2 different treatment
groups. One animal group (16 GDNF+/-, 19 wildtype) received a high salt diet (HS, 4%) and 0.9% saline as drinking water; the other group (18 GDNF+/-, 16 wildtype) received low salt (LS) diet containing 0.03% salt and normal tap water. Both regimens were given over a period of 4 weeks. Before sacrifice, all mice were equipped with a carotid artery catheter under isoflurane anesthesia and intraarterial blood pressure was measured in conscious mice 2 hours after anesthesia [15]. Thereafter, both kidneys were taken for histological analysis and assessment of mRNA expression. From the above animals which mostly were 49-53 weeks of age the group of younger animals (n=3 per group, 27-34 weeks of age) were used for determination of serum aldosterone levels using a radioimmunoassay (Radim Aldosterone MAIA; Radim Deutschland GmbH, Germany) [16]. After blood was drawn these animals were perfusion fixed with glutaraldehyde as described below.

A second group of animals (9 wt and 7 GDNF+/-, age 70 weeks) were used for detailed histological analyses on semithin sections as well as detailed analysis of the renin angiotensin system (RAS). Here, mice were also treated with the above mentioned HS (n=8) or LS (n=8) diet for 4 weeks. Afterwards, the study was terminated by perfusion fixation via the left ventricle under deep ketamine/xylazine anesthesia with glutaraldehyde. The kidneys and the heart were further analyzed using morphometry and stereology [17]. Before fixation urine samples were obtained in all animals for determination of urinary sodium, potassium, chloride, urea, creatinine and albumine (Autoanalyzer, Hitachi Systems, Munich, Germany). The heart was taken out and processed using the orientator technique [17]. Both kidneys were taken out, decapsulated, weighed and dissected in a plane perpendicular to the interpolar axis yielding slices of 1mm width. For further morphometric and stereological analyses and electron microscopy 5 small pieces of the right kidney were selected by area weighted sampling for embedding in Epon-Araldite. From the resulting resin blocks, semithin sections of 0.5µm were prepared and stained with methylene blue and basic fuchsin. The remaining kidney slices were embedded in paraffin; 2-µm sections were cut and stained with hematoxylin/eosin (HE), perjodic acid Schiff (PAS) and Sirius red (fibrous tissue stain). Thereafter, the stained kidney sections were investigated by morphometry and stereology [14]. For qualitative electronmicroscopical investigations of the kidney several semithin and ultrathin sections per animal (n=4 animals per group) were prepared and stained with methylene blue or uranyl acetate / lead citrate, respectively. Ultrathin sections were then qualitatively and quantitatively investigated using a Zeiss electron microscope EM 906 (Zeiss Co., Oberkochen, Germany) at various magnifications [14].

**Immunohistochemistry**

**Antibodies.** Formalin fixed tissues (experiment 1) were dehydrated by bathing in increasing concentrations of methanol, followed by 100% isopropanol. After embedding in paraffin, 2-µm sections were cut (Leitz SM 2000 R microtome, Leica Instruments, Nussloch, Germany), deparaffinised and rehydrated in decreasing concentrations of ethanol. For immunohistochemistry, endogenous peroxidase activity were blocked (3% H₂O₂ in TBS, 20min, room temperature), kidney sections were then layered with the primary antibody and incubated at 4°C over night. The following antibodies were used: PCNA (mouse IgG, clone PC 10, DakoCytomation Co, Hamburg, Germany; dilution 1:500), SMA (anti human smooth muscle actin, monoclonal mouse, DakoCytomation Co, Hamburg, Germany; dilution 1:200), Collagen IV (Southern Biotechnology Associates, Birmingham, AL, USA, goat antibody, dilution 1:100) and fibronectin (Gibco; rabbit antibody, 1:200), NCC (thiazide-sensitive sodium chloride cotransporter; antibody gift from Fife DJ, Portland, Oregon, USA, rabbit, dilution 1:500), Na⁺/Ca²⁺-exchanger (anti Na⁺/Ca²⁺-exchanger antibody transmembrane segment 5 and 6, monoclonal mouse; Swant, Bellinzona, Switzerland, dilution 1:200). Thereafter, incubation with the appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, USA) and HRP-conjugated avidin-biotin complexes (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, USA) was performed. Aminoethyl Carbazole (ABC) solution substrate (DakoCytomation Co, Hamburg, Germany) served as chromagen. All sections were counterstained with Mayer’s hemalaun solution (Merck, Darmstadt, Germany) and examined using light microscopy. Detection of the monoclonal mouse antibody was enhanced using a M.O.M. Kit (Vector Laboratories, Burlingame, USA) according to the manufactures instructions to eliminate background staining from endogenous mouse immunoglobulin.

**Morphological investigations of the kidneys**

All morphological investigations were performed in a blinded manner, i.e. the observer was unaware of the experimental protocol.
Indices of renal damage. To quantify mesangial matrix accumulation and sclerosis of the glomerular tuft, a score of 0 to 4 was determined on PAS and HE stained paraffin sections as described before [14]: score 0: normal glomerulus, score 1: mesangial expansion or sclerosis involving up to 25% of the glomerular tuft, score 2: glomerular sclerosis of 25 to 50%, score 3: glomerular sclerosis 50 to 75% and/or segmental extracapillary fibrosis or proliferation, score 4: global sclerosis >75%, global extracapillary fibrosis or complete collapse of the glomerular tuft. Tubulointerstitial changes, i.e. tubular atrophy, tubular dilatation, interstitial inflammation and fibrosis, and vascular damage, i.e. wall thickening and necrosis of the vessel wall, were assessed on HE stained paraffin sections at a magnification of 100x using a similar semi-quantitative scoring systems from 0-4 as described before [14]. In brief, for determination of the tubulointerstitial damage 10 fields per kidney were randomly sampled and graded as follows: grade 0 - normal tubulointerstitial structure; grade 1 - lesions involving less than 25% of the area; grade 2 - lesions affecting 25 to 50%; grade 3 - lesions involving more than 50% up to 75% and grade 4 with tubulointerstitial damage in almost the entire area. Similarly, for the vascular damage score interlobular and smaller arteries were graded according to the following scheme: grade 0 - no wall thickening; grade 1, 2, 3 – mild, moderate and severe wall thickening, respectively; grade 4 – fibrinoid necrosis of the vascular wall. Glomerular size was assessed by directly measuring glomerular area in 50 randomly selected glomeruli using a semiautomatic image analysing system (Optimas, SIS, Münster, Germany).

Analysis of glomerular capillarisation and cellularity on semithin sections. Five semithin sections per animal were analyzed in terms of glomerular capillarisation and cellularity using the point counting method and a 121 point eyepiece (Zeiss Co., Oberkochen, Germany) at a magnification of 1000x with oil immersion as previously described [16]. In brief, length density (L_v) of glomerular capillaries was determined according to the standard stereological formula L_v = 2 * Q_v with Q_v being the number of capillary transects per area of the capillary tuft. Total length of glomerular capillaries per kidney (L_v(K)) was then derived from L_v and the total glomerular volume: V_glom = V_tissue + V_cortex. Total volume of the capillary tuft (V_v) was also calculated using point counting. The number of podocytes, endothelial and mesangial cells per glomerulus was assessed at least 25 glomeruli per animal from density per volume (N_v) and volume density of the cell type (V_v) according to N_v = k / β * N_vcell * V_vglomer / V_vglomer. The mean cell volume (v_v) was calculated with v_v = V_vglomer * V_glom / N_glom [16].

Intrarenal arteries. The length density (L_v) of renal cortical arteries was determined according to the equation L_v = c * Q_v at a magnification of 200x. Briefly, on paraffine sections Q_v was determined as the number of vessels per cortical area. The area and lumen of cortical vessels were determined using planimetry and a semiautomated image analysis system (Soft Imaging Systems, Münster, Germany). In all SMA stained kidney sections, the minimal and maximal vessel diameters (D_min and D_max, respectively) were measured; c was determined as D_max / D_max. The vessel area A_vessel and lumen area A_lumen were determined with the equations A_vessel = π * (D_vessel / 2)^2 and A_lumen = π * (D_lumen / 2)^2; respectively (D_vessel: distance from one outer site of the vessel to the opposite site; D_lumen: distance from one inner site of the vessel to the opposite site; D_max: largest vessel diameter; D_min: smallest vessel diameter). The lumen of the vessels was calculated as the difference of the two.

Morphological investigations of the heart

In the 70 weeks old animals (experiment 2) uniformly random sampling of the heart was achieved by preparing a set of equidistant slices of the left ventricle and the interventricular septum with a random start. Two slices of the left ventricle were selected by area weighted sampling and processed according to the orientator method [17]. Stereological analysis was performed on 8 random samples of differently orientated sections of the left ventricular myocardium including the interventricular septum as described in details [17]. Volume density of cardiomyocytes, interstitial tissue and intramyocardial capillaries; length density and total length of intramyocardial capillaries: Eight systematically subsampled orientator sections per animal (a total of 96 test areas per animal) were investigated using a Zeiss eyepiece with 100 points for point counting resulting in a single mean value per heart. Volume density (V_v) was obtained according to the equation V_p = V_v (with V_p is point density) and length density (L_v) of intramyocardial capillaries was determined using the equation L_v = 2 * Q_v (where Q_v is area density, i.e. the number of capillary transects per area of myocardial reference tissue). Reference volume was the total myocardial tissue exclusive of non-
capillary vessels. Total length of capillaries per heart ($L_{\text{cap tot}}$) was calculated using the formula: $L_{\text{cap tot}} = L_{V} \times V$ with $V = m/\delta$ and $\delta = 1.04 \text{ g/cm}^3$. Intercapillary distance (ICD) was calculated according $ICD = \left(\frac{\sqrt{4 \times Q_A}}{\sqrt{3}}\right)/2$.

Real-time PCR of the kidney

To evaluate relative mRNA expression levels, total RNA was obtained from frozen kidney tissue with RNeasy Mini columns (Qiagen, Hilden, Germany). Primers for 18s, TGF-ß1, endothelin, endothelin receptor A, fibronectin, IL-10, MCP-1, osteopontin, renin, angiotensinogen, angiotensin receptor 1, NCC, ENaCα were designed with the primer design software Primer Express 3 (Applied Biosystems, Weiterstadt, Germany) and synthesized (MWG-BIOTECH AG, Ebersberg, Germany) as described previously [14]. In addition, primers for endothelin receptor B (fw 5’- CAG GAA GAA GAG CGG TAT GCA-3’; rev 5’- AGG ACC AGG CAG AAG ACT GTC T-3’) and angiotensin receptor 2 (fw 5’- ACA GAA TTA CCC GTG ACC AAG TC-3’; rev 5’- CAA ATG ATG AAT GCC AAC ACA A-3’) were used in this study. Primers were tested for target- specificity and amplification efficiency following standard quality protocols provided by Applied Biosystems (Weiterstadt, Germany). Reverse transcription reactions and Real-time PCR were performed using Power SYBR Green (Applied Biosystems, Weiterstadt, Germany) on a 7500 Fast Real time PCR system (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer’s instructions. Real-time PCR data were analyzed using the SDS v1.3 software (Applied Biosystems). To compare the expression levels among the groups, the relative expression of target gene mRNA levels was calculated using the comparative delta Ct (threshold cycle number) method. Normalization was conducted against the endogenous 18s rRNA levels as a housekeeper, showing similar mean 18s CT values in all investigated groups and a inner group standard deviation below 0.9 CT.

Analysis of data

All statistical analyses were performed with SPSS 13 (SPSS Inc., Chicago, USA). Data are given as means ± standard deviation (SD). After testing for homogenous distribution ANOVA was used for comparison of means followed by appropriate post-hoc test. The results were considered significant if the probability of error (p) was <0.05.

Results

1. Animal data (Tab. 1,2, Fig. 1A-C)

High salt diet increased diuresis and albuminuria in GDNF+/- with low nephron number: At the beginning and at the end of the study the body weight was comparable in all 4 groups. Relative kidney weight, however, was significantly higher in wt type animals than in GDNF+/- . On HS diet it was significantly increased in both groups. Food intake was absolutely similar in all 4 groups whereas water intake was significantly higher in both HS groups compared to LS. Hb, S-Na+, S-K+, S-Cl- and S-glucose were not significantly different in the 4 groups. As expected, in the HS groups S-aldosterone levels were significantly lower than in LS, there was no genotype specific difference, however (Tab. 1). On LS (109.4±9.33 mmHg) and HS (114.1±7.06 mmHg) diet GDNF+/- mice tended to show slightly, but not significantly higher mean arterial blood than in wt animals (105.2±7.7 mmHg and 105.7±8.23 mmHg, Fig. 1 A). On HS diet GDNF+/- showed markedly higher daily diuresis than wt mice on HS and both LS groups (Fig. 1B). On LS diet no significant differences in 24h albuminuria between GDNF+/- and wt were seen (Fig. 1C). On HS diet, however, GDNF+/- showed significantly higher albuminuria per day (Fig. 1C) as well as albumin per creatinine excretion (Tab. 2). As expected urinary Na+ and Cl- excretion was significantly higher after HS diet with significantly higher values in GDNF+/- than in wt controls. Urinary K+ excretion was significantly higher in GDNF+/- under HS diet than in HS wt and both LS groups. In contrast, urinary Ca2+ excretion was significantly lower in GDNF+/- under HS than in HS wt and both LS groups. Urine osmolarity was not different in the 4 groups (Tab. 2).
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**Table 1. Animal data – Body weight, kidney weight and serum parameters**

|                           | High salt wt (n=19) | High salt GDNF+-/- (n=16) | Low salt wt (n=16) | Low salt GDNF+-/- (n=18) | ANOVA |
|---------------------------|---------------------|---------------------------|-------------------|--------------------------|-------|
| Body weight [g]           | 31.2±1.13           | 30.1±1.19                 | 28±0.97           | 29.7±1.18                | ns    |
| Relative kidney weight [mg/g] | 14.2±0.59 *#       | 12.7±0.56 #              | 12.5±0.57 *       | 10.4±0.46                | p<0.0001 |
| Food intake [g]           | 3.52±0.37           | 3.74±0.51                 | 3.44±0.36         | 3.46±0.34                | ns    |
| Water intake [g]          | 18.5±5.67 #         | 31.16±14.64 #            | 8.09±3.54         | 6.83±2.69                | p<0.0001 |
| Haemoglobin [g/dl]        | 15.03±3.08           | 14.04±4.01               | 14.51±0.73        | 14.07±1.15               | ns    |
| S-Na [mmol/l]             | 146±5.02            | 147.2±5.62               | 144±4.88          | 145.9±1.91               | ns    |
| S-K [mmol/l]              | 3.82±0.41           | 3.8±0.48                 | 3.95±0.45         | 3.99±0.53                | ns    |
| S-CI [mmol/l]             | 115.8±2.25          | 115.5±3.12               | 116.6±3.41        | 116.1±2.36               | ns    |
| S-glucose [mg/dl]         | 117.6±42.92         | 155.7±50.41              | 211.3±87.41       | 207.5±71.43              | ns    |
| S-lactate [mg/dl]         | 81.5±38.6           | 66.4±33.18               | 66.2±16.04        | 72.2±24.01               | ns    |
| S-aldosterone [pg/ml]     | 40.99±9.00 #        | 35.98±8.00 #             | 1636±91.46        | 1513±110.1               | p<0.0001 |

* p<0.05 versus GDNF+-/-; # p<0.05 versus low salt (LS); #: n=3 animals per group

**Table 2. Animal data – Urinary parameters**

|                           | High salt wt (n=19) | High salt GDNF+-/- (n=16) | Low salt wt (n=16) | Low salt GDNF+-/- (n=18) | ANOVA |
|---------------------------|---------------------|---------------------------|-------------------|--------------------------|-------|
| Osmolarity [mosm/kg]      | 1433±688.8          | 1175±599                  | 2393±699          | 2020±588.4               | ns    |
| Urinary Na+ [µmol/mg Crea]| 4619±168.8 *#       | 6572±604.2 #             | 130.7±14.65       | 140.3±16.23              | p<0.0001 |
| Urinary K+ [µmol/mg Crea] | 1031±87.2 *         | 1516±127.8 #             | 1082±115.2        | 989.1±93.5               | p<0.005 |
| Urinary Cl- [µmol/mg Crea]| 4211±501.0 *#       | 8508±1689 #              | 2857±373.7        | 310.7±33.5               | p<0.0001 |
| Urinary Ca++ [µmol/mg Crea]| 20.28±3.81 *       | 10.01±9.36               | 15.73±5.75        | 14.21±4.81               | p<0.05 |
| Urinary Crea [mg/dl]      | 10.24±1.39 #        | 7.76±1.82                | 39.33±6.8         | 30.58±2.99               | p<0.0001 |
| Albumin/Crea ratio [mg/g] | 19.73±27.37 *       | 247.1±431.4 #            | 7.38±3.61         | 7.85±4.56                | p<0.05 |

* p<0.05 versus GDNF+-/-; # p<0.05 versus low salt (LS)

Fig. 1. Renal alterations in GDNF+-/- and wildtype mice under LS and HS diet. A: Mean arterial blood pressure. Please note that there is a tendency to higher values in GDNF+-/- animals, but the differences are not significantly different. B: Daily diuresis (ml / 24 h). Diuresis is markedly increased in GDNF+-/- on high salt diet. Due to the high standard deviation, however, this difference is not significant. C: Urinary albumin excretion (µg/24 h). Urinary albumin excretion is markedly increased in GDNF+-/- on high salt diet compared to all other groups (* p<0.05). D: Glomerular area (µm²). Glomerular size measured by mean glomerular area is significantly higher in GDNF+-/- than in wildtype animals on both low and high salt diet (** p<0.01 LS wt vs. GDNF+-/-; *** p<0.005 HS wt vs. GDNF+-/-).
2. High salt diet increased myocardial fibrous tissue in 70 weeks old GDNF+/- with low nephron number (Fig. 2, Tab. 3)

Compared to wt mice GDNF+/- mice with lower nephron number showed higher cardiac (Fig. 2A) and left ventricular weight (Tab. 3) under both LS and HS diet indicating cardiac hypertrophy; due to higher standard deviation, however, this difference was only statistically significant for LS diet (Fig. 2A). Detailed analysis of myocardial structure on semithin sections revealed significantly (p<0.05) higher interstitial myocardial fibrous tissue in GDNF+/- than in wt under HS, but not under LS diet (tab. 3, Fig. 2B-F). In contrast, no significant differences were seen in myocardial capillary supply and wall thickening of intramyocardial arterioles (tab. 3).

3. High salt diet further increased glomerular size, but induced only mild renal structural injury in GDNF+/- with low nephron number (Tab. 4, Fig. 3)

Glomerular area, however, as one parameter of glomerular size was already significantly (p<0.01) higher in GDNF+/- compared to wt controls under LS diet (Fig. 1D). After HS diet this difference was even more pronounced (p<0.001) (Fig. 3A-D). Already on light microscopy, a mild increase in glomerular cellularity and matrix deposition was seen in both GDNF+/- groups (Fig. 3B,D) compared to wt controls (Fig. 3A,C). On electron microscopy this effect was even more pronounced in GDNF+/- on HS (Fig. 3F) compared to HS diet in wildtype animals (Fig. 3E). Increased glomerular matrix deposition was also investigated by sirius red stain, confirming that matrix accumulation was significantly increased in GDNF+/-.
Fig. 2. Cardiac alterations in GDNF+/- and wildtype mice under LS and HS diet. A: Cardiac weight (g). The absolute heart weight is markedly higher in both GDNF+/- groups indicating cardiac hypertrophy with the difference being significant on low salt diet. B: Volume density of the cardiac interstitial tissue (%). High salt diet leads to significantly higher percentage of myocardial interstitial tissue compared to wildtype animals. C-F: Representative histology of the myocardium on semithin sections demonstrates slightly increased interstitial tissue (arrow) in the hearts of GDNF+ on high salt diet (F) compared to wildtype animals (E,C) and GDNF+/- on low salt diet (D).

Fig. 3. Renal histological alterations in GDNF+/- and wildtype mice under low salt (LS) and high salt (HS) diet. Representative light microscopy (A-D) and electron microscopy (E,F) GDNF+/- and wildtype controls on HS and LS diet. On hematoxylin eosin (HE) stains there is a mild increase in mesangial matrix and cellularity in GDNF+/- animals on both low (B) and high salt diet (D) compared to the respective wildtype groups (A,C). These findings are confirmed on the ultrastructural level. In addition, a mild thickening in glomerular basement membrane is seen in GDNF on high salt (F) compared to wildtype animals on high salt diet (E).
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animals with HS diet compared to all other groups (tab. 4). Of note, HS diet did not show any significant effect on glomerular cell number and volume or glomerular capillarisation in GDNF+/- animals compared to wt mice (tab. 5) No significant differences were found with respect to all renal damage scores for tubulointerstitial (TSI), vascular damage (VSI) and mesangiolysis (MSI) (data not shown). In parallel, there was no significant difference in glomerular or tubulointerstitial proliferation between the groups, as assessed by PCNA staining. Glomerular or tubulointerstitial inflammation was not present in any of the groups.

Under HS diet tubular expression of the Na\(^+\)-Ca\(^{2+}\) exchanger was significantly higher in wt, but not in GDNF+/- mice compared to both LS groups. Length density, total length and wall-to-lumen ratio of intrarenal arteries did not differ between the 4 groups.

4. Salt intake modulates renal IL-10 production in GDNF+/- with low nephron number (tab. 6)

On HS diet the renin angiotensin system (RAS) was found to be downregulated as the intrarenal mRNA expression of renin, Angiotensin II (AngII) receptor 1, AngII receptor 2

| Table 5. Morphological alterations of the kidney – results of semithin sections |
|---------------------------------|----------|----------|----------|----------|----------|
|                                | High salt wt (n=6) | High salt GDNF+/- (n=6) | Low salt wt (n=8) | Low salt GDNF+/- (n=7) | ANOVA      |
| Length density of glomerular capillaries [1/mm²] | 13485±1899 | 12409±3405 | 14058±1940 | 14000±511 | ns        |
| Volume density of glomerular capillaries [%] | 30.8±4.56 | 28.08±4.86 | 28.96±5.79 | 27.77±7.58 | ns        |
| Total volume of glomerular capillaries [10⁶µm³] | 97.4±25.11 | 119.3±47.4 | 119.6±28.06 | 100.3±26.63 | ns        |
| Number of mesangial cells per glomerulus | 218.2±56.5 | 297.5±87.02 | 238.7±58.82 | 297.7±109.9 | ns        |
| Mean volume of mesangial cells [µm³] | 53.3±34.49 | 37.2±19.08 | 43.15±23.78 | 38.41±21.61 | ns        |
| Number of endothelial cells per glomerulus | 147.5±30.06 | 208.5±65.07 | 187.6±63.22 | 192.8±71.54 | ns        |
| Mean volume of glomerular endothelial cells [µm³] | 76.23±36.40 | 57.79±18.39 | 65.29±30.83 | 58.73±22.36 | ns        |
| Number of podocytes per glomerulus | 167.1±63.31 | 196.1±59.41 | 190.5±83.71 | 233.7±100 | ns        |
| Mean volume of podocytes [µm³] | 96.81±43.16 | 69.14±14.44 | 86.95±38.02 | 55.88±21.64 | ns        |

| Table 6. Results of TaqMan PCR of kidneys |
|---------------------------------|----------|----------|----------|----------|----------|
|                                | High salt wt (n=13) | High salt GDNF+/- (n=15) | Low salt wt (n=11) | Low salt GDNF+/- (n=10) | ANOVA      |
| Fibronectin | 2.56±0.39 | 3.94±0.58 | 3.33±0.35 | 3.08±0.45 | ns        |
| TGF-B1 | 1.46±0.18 | 1.97±0.22 | 1.66±0.12 | 1.89±0.15 | ns        |
| IL-10 | 2.54±1.13 | 2.19±0.51# | 2.21±0.59* | 6.89±2.20 | <p>0.05 |
| Osteopontin | 2.00±0.30 | 3.41±0.76 | 2.43±0.27 | 2.17±0.24 | ns        |
| MCP-1 | 1.25±0.37 | 1.33±0.20 | 0.82±0.11 | 2.26±0.88 | ns        |
| Endothelin (ET) | 1.99±0.25 | 2.76±0.40 | 2.24±0.18 | 2.27±0.18 | ns        |
| ET1-receptor A | 1.36±0.17# | 2.01±0.21# | 1.93±0.16 | 1.76±0.16 | <p>0.05 |
| ET1-receptor B | 1.36±0.18 | 1.80±0.23 | 1.71±0.15 | 1.56±0.15 | ns        |
| Renin | 0.48±0.08# | 0.50±0.08# | 1.80±0.22 | 1.61±0.25 | <p>0.0001 |
| Angiotensinogen | 1.33±0.18 | 1.48±0.23 | 1.48±0.10 | 0.26±0.14 | ns        |
| AngII receptor 1 | 1.46±0.21 | 0.76±0.19 | 2.27±0.24 | 2.17±0.26 | <p>0.051 |
| AngII receptor 2 | 1.03±0.15# | 1.12±0.14* | 1.91±0.21 | 1.56±0.28 | <p>0.01 |
| NCC | 1.63±0.29 | 2.06±0.19 | 2.26±0.26 | 1.97±0.22 | ns        |
| ENaCα | 1.02±0.13 | 1.18±0.12# | 1.87±0.16 | 1.70±0.14 | <p>0.0001 |

* p<0.05 versus GDNF+/-; # p<0.05 versus low salt; + p<0.05 vs. wt low salt; NCC: thiazide-sensitive sodium chloride cotransporter; ENaCα: epithelial Na⁺ channel alpha
were lower compared to LS diet. Also AngII receptor 2 expression was significantly lower in HS compared to LS wt and in HS GDNF+/- than in LS wt. Apart from that no genotype specific differences were noted. Under HS diet, IL-10 mRNA was downregulated in GDNF+/-, but not in wt mice. In contrast, ET1-receptor A (ET-RA) gene expression was significantly (p<0.05) higher in GDNF+/- and significantly lower in wt under HS compared to LS diet. As expected, gene expression of ENaCα was significantly (p<0.05) downregulated under HS diet in both genotypes whereas mRNA expression of NCC, the thiazide sensible transporter, was not affected by HS diet. Of note, significant differences due to the genotype and salt intake were only found for IL-10 which was significantly lower in wt compared to GDNF+/- under LS whereas there were no differences under HS diet. In contrast, no differences in the renal mRNA expression of fibronectin, TGFβ1, osteopontin, MCP-1, ET, ET1 receptor B, angiotensinogen (Tab. 6) as well as iNOS, eNOS, IL-6 (data not shown) were found.

**Discussion**

An association between low birth weight and renal and cardiovascular disease in later life was found in experimental and clinical studies [18, 19]. One potential explanation was that low birth weight is associated with low nephron number [8], i.e. a condition that predisposes to the development of glomerular enlargement, progressive glomerular damage, glomerular and finally systemic hypertension and associated cardiovascular disease (3,7,8,20). The detailed pathomechanisms, however, that underlie the above mentioned vicious circle are not fully understood. One long standing potential explanation that was also proposed by Brenner and colleagues in their seminal paper was inappropriate sodium retention due to the reduced capacity of hyperfiltrating nephrons to excrete salt [3]. In order to further investigate this idea we used high (HS) and low salt (LS) diet in GDNF+/- mice with approximately 30% lower nephron number and mild hypertension in later life and wildtype (wt) controls [11, 12]. We were particularly interested in effects of HS diet on cardiovascular and renal function and structure in this animal model of low nephron number. To this extent serum and urinary parameters, blood pressure, renal structure and gene expression of specific markers were analysed in GDNF+/- and wt control animals on a C57BL6 background after 4 weeks on either HS or LS diet.

Our findings confirm and extend earlier findings of Ruta and coworkers [10] in GDNF+/- animals on a mixed 129Sv-C57/BL6 background. We found that 4 weeks of HS diet induced mildly higher blood pressure, marked diuresis and significant albuminuria in GDNF+/- with low nephron number compared to wt controls. HS diet also further increased glomerular enlargement and induced mild glomerular damage in GDNF+/- mice whereas tubulointerstitial and vascular damage scores were not altered. On the ultrastructural level increased mesangial matrix was also seen in GDNF+/- on HS compared to LS diet confirming mild glomerular damage. Of potential importance we identified IL-10 as a new salt-sensitive gene that might play an important role in extracellular volume and blood pressure homeostasis.

The above observations in an animal model of low nephron number are in line with the so-called „multi-hit theory“ of Nenov et al. [21] which postulates that a low nephron number is a predisposition for the subsequent development of renal disease, i.e. the first hit in the pathogenetic cascade. If in addition a second even subtle injury occurs (i.e. the second hit), the likelihood for a progressive kidney disease increases. Thus, any significant reduction in nephron number could facilitate the development of either kidney disease or hypertension in the setting of additional injury i.e. by obesity, diabetes mellitus or increased salt intake. Our finding of significantly higher urinary production in GDNF+/- compared to wildtype control animals under HS diet argue for a concentration defect of the kidney with lower nephron number. Of note, in addition to more marked albuminuria as a sign of increased permeability of the glomerular filter significantly higher urinary excretion of Na+, K+, Cl- and Ca2+ were seen in GDNF+/- compared to wildtype mice under HS diet.
Much to our surprise we observed cardiac and left ventricular hypertrophy in GDNF+/- with low nephron number which obviously was independent of salt loading since it was even more pronounced and significant in LS diet. This finding is in contrast to the study of Ruta et al. [10] who did not find cardiac hypertrophy after high salt diet (5%) in GDNF+/- on a mixed background. In view of the somewhat higher blood pressure values in both GDNF+/- groups it is possible, however, that cardiac hypertrophy is at least to some extent blood pressure dependent. Thus, in our view this issue of blood pressure development and salt sensitivity has to be addressed in further studies using telemetric blood pressure measurements. Of note, HS diet induced significant higher myocardial fibrous tissue in GDNF+/-, but not in wt mice confirming earlier data on a profibrotic effect of HS [22] via induction of subset of genes encoding for proteins involved in inflammation and extracellular matrix remodeling [23].

A new and interesting finding is that salt intake could modify renal IL-10 gene expression in GDNF+/-mice with lower nephron number. Since IL-10 is an anti-inflammatory cytokine produced by monocytes, macrophages and TH2 lymphocytes it is tempting to speculate that this finding could indicate a link between salt, the immune system and presumably also blood pressure as postulated recently by Machnik and coworkers [24]. Of note, despite changes in IL-10 we did not see alterations in interstitial inflammatory cell infiltrates. Of course much more data has to be collected in further studies in order to provide more evidence for this novel and intriguing finding.

Of note, AT1 gene expression in the kidney was downregulated by high-salt diet in mice of both genotypes albeit to a higher degree in GDNF+/- . We speculate that this somewhat different regulation should be understood in the context of a maladaptive response to high-salt in GDNF+/- but we do not consider it likely that differential AT1 regulation does play a causal role, considering the lack of an altered response to AT1 blockers in GDNF+/- reported by Shweta et al. [25].

Some aspects of the study deserve specific comments: When interpreting the data one has to keep in mind the nature of the animal model of low nephron number. There are definitely differences between the most widely used animal model of low nephron number, i.e. the intrauterine growth restriction (IUGR) of the rat by low protein diet of the mother [26, 27], and our genetic model in mice where nephron underdosing was induced by lack of GDNF during nephrogenesis. In our genetic model of GDNF+/- mice with low nephron number no changes in tubular sodium transporters were seen under HS or LS diet. This is in contrast to earlier studies in the IUGR model of low nephron number in which upregulation of NaK2Cl and Na+ cotransporters was reported [28]. In view of the difference in mean arterial blood pressure between GDNF+/- and wt mice (+ 5.2 mmHg in LS and + 8.4 mmHg in HS diet) and the data of Ruta et al. [10] the issue of salt dependency of blood pressure requires further studies. It is obvious that blood pressure measurements in mice are difficult to perform and to interpret. Despite we used intraarterial measurements in conscious animals it is possible that small, but significant differences could easily be missed by one spot measurement as in the present study. Further experiments using telemetric blood pressure measurements are definitely required. It is of note, however, that salt loading could aggravate renal injury independent of any blood pressure effect. In the animal model of adriamycin induced nephropathy [29], in the uninephrectomized spontaneously hypertensive rat (SHR) as well as in SHR with chronic allograft nephropathy [30] salt loading was found to aggravate kidney injury [31]. It is also known that in hypertensive patients the response to salt restriction is very variable and the pathomechanisms are speculative [32]. As shown in clinical studies obesity, pre-existent kidney disease, age, ethnicity, metabolic syndrome and diabetes predispose to salt sensitivity [31]. Unfortunately, in these patients nephron number is not known. The fact that obese patients are particularly responsive to salt restriction supports the idea of low nephron number as a predisposing factor since obesity is regarded as a situation of at least relative nephron underdosing due to a mismatch between kidney weight and size and body mass.
Conclusion

Our data in a genetic model of low nephron number provide evidence for mildly higher blood pressure, marked diuresis and albuminuria under high salt diet possibly pointing to a concentration defect and a disturbance of the glomerular filtration barrier as maladaptive renal changes. Whether under specific conditions like reduced nephron number dietary salt modification interferes with inflammatory or immune system, respectively, as suggested by the differences in IL-10 gene expression between GDNF+/- and wt animals requires further, more sophisticated studies.

Conflict of Interests

The authors declare no conflict of interests.

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