Persistent changes within the intrinsic kidney-associated NPY system and tubular function by litter size reduction

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Abstract

Background. Intraternal growth restriction (IUGR) is associated with an increased risk of renal diseases in adulthood. However, while low-birth-weight-infants often experience accelerated postnatal growth, the impact of postnatal environmental factors such as nutrition and early postnatal stressors on renal development and function remains unclear. In this context, Neuropeptide Y (NPY) may act as a critical factor. NPY is a sympathetic neuropeptide involved in blood pressure regulation and tubular function. Yet, little is known about the expression and function of endogenous NPY in the kidney and the functional relevance for the transmission of persistent postnatal-induced effects.

Methods. (1) IUGR was induced in Wistar rats by isocaloric protein restriction in pregnant dams. (2) Litter size was reduced to 6 (LSR6) or 10 (LSR10) male neonates. To differentiate the effect of postnatal nutrition and stressors, we additionally included home-cage-control animals without any postnatal manipulation. Animals were sacrificed at Day 70.
**Introduction**

Epidemiological studies have shown an association between low-birth-weight (LBW) and an increased risk of cardiovascular and renal diseases [1–6]. Several animal models have replicated the consequences of intrauterine growth restriction (IUGR). IUGR was induced either by protein restriction during gestation [7] or by uterine artery ligation [8]. Barker et al. [9, 10] coined the concept of ‘perinatal programming’ postulating that early perinatal events predispose for diseases in later life. However, epidemiological data have revealed that LBW itself is not the only factor that predisposes for diseases in later life. The mechanisms by which the pre- and postnatal environment programmes renal function are still unclear [11–14].

Newborns with LBW undergo early postnatal over-nutrition. Enhanced early nutrition of LBW infants may be adverse for the postnatal development of the kidney and hypertension [15]. The sympathetic nerve system (SNS) among other systems plays a pivotal role in the development of renal hypertrophy and tubular function [16, 17]. Data from Vásárhelyi [18] show an association between LBW and natriuresis in later life. Lenaerts et al. [16, 19, 20] and McDonough [16] indicate a role of the SNS in the aetiology of increased renal sodium excretion. The role of the early postnatal stressors, such as litter size reduction (LSR), in the development of hypertension and imbalance of renal homeostasis has not been addressed so far. Neuropeptide Y (NPY) is a 36 aminoacid conenotransmitter, which affects renal function in several direct and indirect ways. It is released from renal sympathetic nerves [21–23] and acts directly via NPY-receptors, especially Y1 and Y2 on renal tubules leading to alterations of intrarenal tubular transport processes and renin release [24]. It has been shown that NPY regulates natriuresis, diuresis [25–27] and kaliuresis via the NPY-receptor Y1 [26, 28, 29]. NPY-receptor Y1 is a G-Protein-coupled receptor, transducing extracellular stimuli into intracellular signals. One intracellular signalling pathway which plays a pivotal role is the mitogen-activated protein kinase (MAPK) 42/44 [30] and an activation of Na⁺-K⁺-ATPase [28, 31, 32]. G-protein receptors activate the phosphorylation of MAPK 42/44. Activated MAPK 42/44 pathway has been reported to regulate Na⁺/Pi cotransporter in the renal tubuli and to control the activity of the Na⁺-K⁺-ATPase. Initial evidence for a role of endogenous NPY in the regulation of renovascular function has come from studies on reserpenized pigs [32]. In addition, several studies demonstrate that NPY inhibits renin release and lowers plasma renin activity [26, 33–35] via Y1-receptor [26, 35, 36]. Renin, as part of the Renin-Angiotensin-Aldosterone-System (RAAS) plays a pivotal role in the regulation of natriuresis and kaliuresis.

Studies in rats show that neonatal perturbations can exert a significant behavioural and biological effect, which translates into various adaptational changes within the adult animal [37–39]. Most studies have focused on early stressors such as maternal deprivation, neonatal handling and infection [40–42]. They agree that such early life events alter among others, NPY release and signal transduction [43–46]. Therefore, the aim of this study was to investigate the effects of altered prenatal and early postnatal management on renal function in later life and to elucidate the possible associated alterations of endoge nous NPY-system.

**Materials and methods**

**Animal procedures**

All procedures performed on animals were done in accordance with guidelines of the American Physiological Society and were approved by the local government authorities (AZ # 621-2531.31-11/02 and AZ # 621-2531.31-14/05; Regierung von Mittelfranken). Virgin female Wistar rats were obtained from our own colony and were housed in a room maintained at 22 ± 2°C, exposed to a 12-h dark/light cycle. The animals were allowed unlimited access to standard chow (#1320; Altromin, Lage, Germany) and tap water. Two simultaneous sets of experiments, completely independent from each other were performed. The first included pre- and postnatal nutritional interventions, the second was restricted to postnatal interventions only.

(1) Intrauterine growth restriction (IUGR) was induced in Wistar rats by isocaloric protein restriction in pregnant dams as previously described [7]. They were fed either a normal protein (Co) diet containing 17.0% protein (casein) or a low-protein (IUGR) isocaloric diet containing 8.0% protein (casein) throughout pregnancy. Rats delivered spontaneously at Day 23 of pregnancy. On the first day of life, the litters were reduced to six male pups per dam. During lactation, dams were fed standard chow. The offspring were nursed by their own mothers until weaned at Day 23 to standard chow. Male animals were sacrificed at Day 70 defining four groups: IUGR 6 (six animals, three dams), Non IUGR 6 (six animals, three dams), IUGR 10 (six animals, three dams) and non-IUGR 10 (six animals, three dams).

(2) In the model of postnatal LSR, the litters were either reduced to 10 (LSR10) or 6 (LSR6) pups on the first day of life. Each group (LSR6 and LSR10) was formed of males from three to five different litters. During lactation, rat mothers were fed standard chow. The weaning was at Day 23 after birth. We included home-cage-control (HCC, mean litter size of 16) animals without any postnatal manipulation during lactation, rat mothers were fed standard chow. After weaning, animals were equally fed ad libitum with standard chow. HCC, LSR10 and LSR6 animals were sacrificed at Day 70 of life defining three groups: HCC (five animals, three dams), LSR10 (ten animals, four dams), LSR6 (eight animals, three dams).
Physiological data of animals after IUGR and/or LSR

Body weight (gram) and body length (centimeter) was obtained at different time points: birth, Day 23 and Day 70 by weighing each animal. Body length was obtained by measuring from nose to anus and tail length beginning at tail insertion at birth.

Metabolic studies

Metabolic studies including standardized analyses of renal function were performed at the age of 70 days. The following protocol was carried out at each time point: 24 h before sacrifice, the animals were housed individually in a metabolic cage for 24 h allowing exact quantification of food and water intake as well as urine excretion. Next, a retro-orbital blood sample of 2 mL was collected at the end of the 24-h period. Haemoglobin, potassium and sodium concentration in plasma were analysed. Fractional excretion of sodium (FeNa) and fractional excretion of potassium (FeK) was calculated:

\[
FeNa (\%) = \frac{(\text{urine-sodium} \times \text{plasma creatinine})}{(\text{plasma-sodium} \times \text{urine creatinine})}
\]

\[
FeK (\%) = \frac{(\text{urine-potassium} \times \text{plasma creatinine})}{(\text{plasma-potassium} \times \text{urine creatinine})}
\]

Tissue preparation

At Day 70, the experiment was terminated as previously described [7]. Then, the kidneys were excised and a portion was immediately placed frozen in liquid nitrogen. For histology, another portion of renal tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 20

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**Table 1.** Designed primer pairs and TaqMan probes used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>TaqMan probe (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY</td>
<td>forward 5’-AGCAGAGGACATGGCCAGATA-3’</td>
<td>reverse 5’-TGAATCATGGTCTCAGG-3’</td>
<td>probe 5’-(FAM)-CAATCTCATCACCAGAGATATGGCAAGA-(TAMRA)-3’</td>
</tr>
<tr>
<td>Y1</td>
<td>forward 5’-CACCTCAACCGGCACAT-3’</td>
<td>reverse 5’-TGGAATTGTTGACCTGGAAT-3’</td>
<td>probe 5’-(FAM)-TCACTCGGCTGACACCAAGG-(TAMRA)-3’</td>
</tr>
<tr>
<td>Y2</td>
<td>forward 5’-GTTGAGTTGCCGTTGATC-3’</td>
<td>reverse 5’-ACCACCTGCACCTCAACCA-3’</td>
<td>probe 5’-(FAM)-AGCATTAGACACATCCAAAGA-(TAMRA)-3’</td>
</tr>
<tr>
<td>Renin</td>
<td>forward 5’-ACAGGGCAACTTTCACTACGT-3’</td>
<td>reverse 5’-GAGAGCTTCAGGCTGATC-3’</td>
<td>probe 5’-(FAM)-AGCATTAGACACATCCAAAGA-(TAMRA)-3’</td>
</tr>
<tr>
<td>MCR</td>
<td>forward 5’-CCAAGGTACCTTCACGAGTATTTATAC-3’</td>
<td>reverse 5’-AACGAGTGCATAGACATCCAA-3’</td>
<td>probe 5’-(FAM)-AGCATTAGACACATCCAAAGA-(TAMRA)-3’</td>
</tr>
<tr>
<td>ATR1a</td>
<td>forward 5’-TGTAAAGGCCACGCCTAT-3’</td>
<td>reverse 5’-TGAGTCTCGGAAATTTGAGCTCCC-(TAMRA)-3’</td>
<td>probe 5’-(FAM)-AGCATTAGACACATCCAAAGA-(TAMRA)-3’</td>
</tr>
<tr>
<td>ATR1b</td>
<td>forward 5’-TTCTGCTGACAGTGCTTCA-3’</td>
<td>reverse 5’-GATGATGTCAGGCTTTGG-3’</td>
<td>probe 5’-(FAM)-AGCATTAGACACATCCAAAGA-(TAMRA)-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>forward 5’-ACGGGAAACCCATACCAATC-3’</td>
<td>reverse 5’-AGCAGATACCCATGTGCA-3’</td>
<td>probe 5’-(FAM)-AGCATTAGACACATCCAAAGA-(TAMRA)-3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>forward 5’-TCAAGCTGCTGACAGTCCAG-3’</td>
<td>reverse 5’-GCCCAAGGATTTCACATCCC-3’</td>
<td>probe 5’-(FAM)-AGCATTAGACACATCCAAAGA-(TAMRA)-3’</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Physiological data of animals with LSR to either 10 or 6. (A) Body weight (g) and (B) body length (cm) per week from birth until Week 3. Animals after LSR to 6 (LSR6, inverted triangles) have in the first 3 weeks after birth an increased body weight gain compared to animals with LSR to 10 (LSR10, triangles). At Weeks 2 and 3, the group with LSR6 has a significant increased body weight per week compared to the control group with LSR10 (*P < 0.05, ***P < 0.001; two-way ANOVA test and Bonferroni correction).
Fig. 2. Continues
NPY and tubular function after LSR in rats

mM Tris–Cl, 137 mM NaCl, pH 7.6). Electrolytes in serum and urine samples were analysed with the automatic analyser Integra 800 (Roche Diagnostics, Mannheim, Germany).

RNA extraction and real-time polymerase chain reaction
RNA extraction and quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR) analysis was performed as described previously [7] for NPY, NPY-receptor Y1 (Y1), Y2, mineral corticoid receptor (MCR), renin, angiotensin receptor (ATR) 1a and ATR1b. The results were confirmed by normalization to ‘housekeeping genes’ β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer pairs and TaqMan probes used are listed in Table 1. Unless otherwise indicated, the oligonucleotides for each gene investigated were designed as previously described [7].

Protein isolation and immunoblotting
Protein extraction from rat kidneys, gel electrophoresis and immunoblotting were performed as described previously [47]. Blots were probed with: rabbit anti-phospho-p42/44 MAPK (Thr202/Tyr204) (catalogue number 4370, 1:1000; Cell Signalling Technology, New England Biolabs, Frankfurt, Germany), p42/44 MAPK (catalogue number 9102, 1:1000; Cell Signalling, New England Biolabs), mouse anti-5E8 (DPPIV) (catalogue number HM302I, 1:625; Cell Sciences, Canton, MA), while goat anti-β-actin (designated F-19, catalogue number SC-1616, 1:1000; Santa Cruz Biotechnology, San Francisco, CA) served as loading control. Horseradish peroxidase-conjugated anti-rabbit (catalogue number 7074, 1:1500; Cell Signalling Technology, Minneapolis, MA) and anti-goat (catalogue number 305-035-045, 1:1500; Jackson ImmunoResearch Laboratories, Suffolk, UK) was used with a dilution 1:1500. Densiometric analysis of the bands was performed using Advanced Image Data Analyzer-Software (Version 4.15; Fuji Photo Film Co., Omiyama, Japan) and normalized to total amount of β-actin per lane. Pixel densities were corrected for background staining in the same film.

Immunohistohemistry
Immunohistohemistry was performed as previously described [7, 47]. The expression was assessed with rabbit anti-NPY (catalogue number NA1233, 1:100; Biotrend, Cologne, Germany), goat anti-NPY-receptor 1 (catalogue number Cs21990, 1:400; Santa Cruz Biotechnology, San Francisco), sheep anti-11-hydroxysteroid dehydrogenase Type 2 (11β-HSD2) (catalogue number AB1296, 1:200; Chemicon) and mouse anti-smooth muscle actin (catalogue number M0851, 1:200; Dako). Immune complexes were visualized with fluorescent secondary antibodies: Cy3 goat anti-rabbit (catalogue number 111-166-003; DCS, Hamburg, Germany), Alexa 488 donkey anti-goat (catalogue number A11055, 1:400; Jackson Immunoresearch Laboratories), Alexa 488 donkey anti-goat (catalogue number A11055, 1:400; Invitrogen, Karlsruhe, Germany) and Cy2 donkey anti-mouse (catalogue number 715-226-150, 1:400; Jackson Immunoresearch Laboratories). Cell nuclei were labelled with 4′,6-diamidino-2-phenylindole (DAPI) (catalogue number D1306, 1:1000; Invitrogen).

For the immunohistochemistry analysis of DPPIV, the sections were treated as previously explained. After quenching of endogenous peroxidase activity with 1.5% (vol/vol) H2O2 (35%), 20% methanol and 0.1 M PBS for 20 min at room temperature, sections were incubated with blocking buffer (catalogue number AL120R500; DCS, Hamburg, Germany) for 45 min at room temperature. The expression was assessed with mouse anti-5E8 (DPPIV) (catalogue number HM302I, 1:1000; Cell Sciences, Canton, MA). Immune complexes were visualized with an avidin/biotin-DAB (3,3′-diaminobenzidine) detection system (Vector Lab, Burlingame, CA, USA).

Tissue-specific DPPIV-like enzymatic activity
Synthesis of the histochemical substrate H-Gly-L-Pro-1-hydroxy-4-naphthylamide hydrochloride as well as histochemistry were conducted as previously described [48]. As a further control for specificity and in addition to the use of DPPIV-deficient animals, this histochemical activity assay was also performed using a DPPIV-specific inhibitor, which was added to the incubation solution in a final concentration of 2 μM. Light microscopy investigations were carried out on a Nikon Eclipse 80i microscope (Nikon GmbH, Duesseldorf, Germany) and representative pictures were taken with a MicroFire digital microscope camera (Optronics, Goleta, CA).

Analysis of data
The results of RT–PCR were calculated based on the ΔΔCt method as described in detail previously [7] and expressed as fold induction of mRNA expression compared to the corresponding control animal groups. Mean mRNA expression in control animals was defined as 1.0-fold induction. For quantitative immunoblot analysis, a densitometry was performed normalized to β-actin of the same animal probe.

Two tailed Mann–Whitney test, one-way analysis of variance (ANOVA) test, two-way ANOVA test, Bonferroni correction test and Dunnett’s test were used to test significance at the given time points. A P-value <0.05 was considered significant. The procedures were carried out using the GraphPad Prism software (Version 4.0; GraphPad Software, San Diego, CA). Values are displayed as means ± standard deviations of the mean.

Results
Localization of NPY, NPY-receptor Y1 and DPPIV in the kidney

NPY and Y1-receptor localization in the kidney. NPY and Y1-receptor were detected in both the renal cortex and renal medulla. NPY is localized pericellular and in the cytoplasm (Figure 2A and B). Both NPY (Figure 2A and B) and Y1-receptor (Figure 3A) were colocalized with 11β-HSD, a marker of distal tubuli and collecting ducts of the renal cortex and renal medulla. Y1-receptor was observed in the cytoplasm and in the membrane of the cells of the distal and collecting renal duct (Figure 3A).

NPY and Y1-receptor are colocalized in the kidney.

NPY and Y1-receptor were detectable in both the cells of the distal and the collecting ducts of the kidney. A coimmuno-staining demonstrated a colocalization of NPY and Y1 in the tubuli of the renal cortex (Figure 4A) and in the renal medulla (Figure 4B).

NPY and Y1-receptor are colocalized in the macula densa.

NPY was determined in the tubuli of the renal cortex and medulla by immunofluorescence. Furthermore, it was demonstrated that NPY is localized adjacent to the macula densa, characterized by a positive staining for actin and loco tipico in the renal morphology (Figure 5A). NPY and Y1-receptor are colocalized adjacent to the glomeruli in loco tipico of the macula densa (Figure 5B).

Fig. 2. Continued
Fig. 2. Localization of NPY in the kidney and the expression pattern after LSR and IUGR. (A and B) NPY expression is assessed with immunofluorescence (green), nuclear staining with DAPI (blue). The localization of NPY in the kidney was shown with colocalization with 11β-HSD (red) as a marker for the distal (A) and the collecting tubuli (B) of the kidney. Colocalization of NPY and 11β-HSD in the distal and collecting tubuli is yellow (merge). (C and D) Two independent animal models: one model of IUGR combined with LSR (C) and the other, postnatal LSR only (D). The mRNA expression of NPY is not altered after IUGR (IUGR6 versus non-IUGR6, IUGR10 versus non-IUGR10) but significantly increased after LSR to 6 (non-IUGR-6 versus non-IUGR10, IUGR6 versus IUGR10; LSR6 versus LSR10 and HCC) (C and D). (*P < 0.05, ***P < 0.001; one-way ANOVA test and Bonferroni correction).
Localization and activity of DPPIV in the kidney. DPPIV was detectable in the cortex (Figure 6A), in renal ducts (Figure 6A) and in glomeruli (Figure 6A). However, there was almost no staining for DPPIV in the medulla (Figure 6A). The activity of DPPIV was detected in the cortex and proximal and distal tubuli (Figure 6B).

IUGR and the NPY-system

IUGR after low protein diet. Low protein (LP) diet of the pregnant dams (IUGR) led to a reduction of birth weight, body length and tail length at birth in their offspring in comparison to litters of normal protein-fed dams (non-IUGR). The offspring of the IUGR mothers reached body weight of the offspring of non-IUGR mothers until Day 42. At Day 70, body weight was comparable in all groups (non-IUGR10, non-IUGR6, IUGR10 and IUGR6) (Table 2).

mRNA expression of endogenous NPY in the kidney after IUGR. No alteration of the mRNA expression of NPY was detected after IUGR without LSR (IUGR10) at Day 70 (Figure 2C). Whereas both the group after IUGR with LSR to 6 (IUGR6) and the group without IUGR but with LSR to 6 (non-IUGR6) showed an up to 10-fold induction of the mRNA expression of NPY compared with the analogical groups with LSR to 10 (IUGR10; non-IUGR10) at Day 70 after birth (Figure 2C).

The impact of postnatal LSR on the NPY-system and tubular function

Physiological data after LSR: diuresis, haemoglobin, plasma sodium and plasma sodium. Diuresis was not altered after LSR. It was comparable in LSR6, LSR10 and HCC. Neither alterations in the haemoglobin
concentration nor hyponatraemia or hypokalaemia were detectable after LSR (Table 3).

Excreted fraction of the filtered sodium (FeNa) and potassium (FeK) after LSR. Metabolic studies at Day 70 demonstrated that both FeNa and FeK (Figure 8C and D) are significantly increased after LSR6 (postnatal LSR, no IUGR). Additionally, absolute excretion of potassium (Figure 8B) but not absolute excretion of sodium (Figure 8A) was increased after LSR, while diuresis was only slightly affected (Table 3). Natriuresis and kaliuresis was linked to postnatal LSR only.

Postnatal growth after LSR. No differences in body weight or body length were observed at Day 1 after birth. Reduction of the litter size to 6 (LSR6) led to a significant increased weight in weeks 2 and 3 after birth (Figure 1A), whereas body length was not different from the control group (LSR10) (Figure 1B).

mRNA expression of NPY and NPY-receptor 1 and 2 (Y1, Y2) after LSR. A LSR to 6 after birth led to an up to 10-fold induction of the mRNA expression of NPY (Figure 2D) and a decrease of the Y1-and Y2-mRNA at Day 70 of life (Figure 3B) compared to the control group (HCC) and, respectively, LSR10.

mRNA expression of renin, MCR, ATR1a, ATR1b after LSR. Renin expression is significantly up-regulated (Figure 5C), whereas MCR and ATR1a expression (Figure 5C) did not change after LSR (LSR6, LSR10) compared to the control group (HCC). ATR1b expression was decreased after LSR to 6 compared to LSR10 and HCC (Figure 5C).

Localization and activity of DPPIV in the kidney and expression after LSR. Localization and activity of DPPIV was predominantly detectable in the renal cortex and almost not in the medulla. Protein analysis in our study demonstrated a decrease of DPPIV expression after LSR to 6 (LSR6) compared to the control group with LSR to 10 (LSR10) or without LSR (HCC) (Figure 6C).

MAPK 42/44 and its phosphorylation are dysregulated after LSR. LSR6 led to decrease of phosphorylation of MAPK 42/44 compared to the control group (LSR10, HCC). Furthermore, an increase of total MAPK 42/44 was detected after LSR to 6 (LSR6) (Figure 7).

Discussion

This is the first study demonstrating early postnatally acquired alterations of the kidney physiology. LSR immediately after birth leads to an altered expression pattern of endogenous NPY and NPY-receptor 1 (Y1), which is associated with an impaired tubular function resulting in increased natriuresis and kaliuresis at Day 70 after birth. In contrast, IUGR itself does not alter the mRNA expression of NPY.

IUGR is associated with an increased sodium excretion in the renal ducts after IUGR in piglets [49]. Furthermore, LBW leads to increased sympathetic nerve traffic [50]. That may be the link between low birth weight and altered tubular function. It is well known that the postnatal environment influences kidney development and nephron number. Bearing in mind the differences in the animal models, Wlodek et al. [51, 52] showed that both IUGR and LSR impair renal function in later life. Consistently, Boubred et al. [53] showed that early postnatal overfeeding induced...
Fig. 5. Colocalization of NPY and Y1-receptor in the macula densa and dysregulation of renin after LSR. (A) Localization of NPY is assessed with immunofluorescence. NPY (green) is localized adjacent to the macula densa (red; actin-positive staining and loco tipico). Merge shows the adjacent localization of NPY to the macula densa. Where high-magnification images (lower row) are derived from the low magnification image (upper row), the magnified area is demarcated with a solid-lined box. (B) NPY (red) and Y1-receptor (green) localization is assessed with immunofluorescence. There is co-staining adjacent to the glomerulus in loco tipico of the macula densa (yellow, arrow). (C) The mRNA expression of renin but not of MCR and ATR1a is increased after LSR to 6 (LSR6) compared to control group without LSR (HCC). Additionally the expression of ATR1b is decreased after LSR6 compared to the control. (*P < 0.05; one-way ANOVA test and Bonferroni correction).
by LSR in rat enhances postnatal nephrogenesis but the blood pressure, and the glomerulosclerosis score are still elevated. Additionally, Boubred et al. [54] demonstrated that reduced nephron number alone due to IUGR may not be sufficient to induce physiological alterations, and early postnatal overfeeding acts as a ‘second hit’. Our data shows that LSR and not IUGR itself leads to increased postnatal weight gain. Furthermore, we could demonstrate that LSR but not IUGR is associated with an up-regulation of the mRNA expression of NPY. Hence, there seems to be an association between increased postnatal weight gain due to LSR and the dysregulation of the NPY-system. Therefore, we focused this study on the effect of LSR on the NPY-system and renal function.

Fig. 6. Protein expression of DPPIV after LSR and its activity and the localization in the kidney. DPPIV is a peptidase known to cleave peptide hormones such as NPY. Therefore, we assessed immunohistochemistry to characterize the localization (A) and the activity (B) within the kidney. It is expressed and active ubiquitously in the renal cortex (A,B), in the tubuli (A,B) and in the glomeruli (A,B). Almost no staining was detectable in the renal medulla (A,B). Analysis of the protein expression was assessed with western blotting. Densitometric analysis showed a significant down-regulation of DPPIV in the animals with LSR to 6 (LSR6) compared with the control group without LSR (HCC) (D). (\*P < 0.05; one-way ANOVA test and Bonferroni correction).

Table 2. Physiological data of IUGR animals and controls with and without LSRa

<table>
<thead>
<tr>
<th></th>
<th>Non-IUGR 10</th>
<th>Non-IUGR 6</th>
<th>IUGR 10</th>
<th>IUGR 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (g)</td>
<td>6.2 ± 0.43  (n = 39)</td>
<td>5.405 ± 0.403 (n = 22)</td>
<td></td>
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</tr>
<tr>
<td>Body length at birth (cm)</td>
<td>5.0 ± 0.17 (n = 39)</td>
<td>4.88 ± 0.1 (n = 39)</td>
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</tr>
<tr>
<td>Tail length at birth (cm)</td>
<td>1.63 ± 0.95 (n = 39)</td>
<td>1.46 ± 0.1 (n = 39)</td>
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<td></td>
</tr>
<tr>
<td>Body weight at weaning Day 23 (g)</td>
<td>51.8 ± 5.8 (n = 9)</td>
<td>69.00 ± 6.1 (n = 24)***</td>
<td>50.95 ± 6.7 (n = 6)</td>
<td>60.97 ± 3.15 (n = 12)*</td>
</tr>
<tr>
<td>Body weight at Day 70 (g)</td>
<td>351.9 ± 21.28 (n = 9)</td>
<td>373.6 ± 18.07 (n = 12)</td>
<td>379.9 ± 16.52 (n = 6)</td>
<td>354.7 ± 24.41 (n = 11)</td>
</tr>
</tbody>
</table>

*Values are given as means ± standard deviations. ***P < 0.001, respectively, \*P < 0.05 versus non-IUGR. ***P < 0.001: non-IUGR10 versus non-IUGR6, \*P < 0.05: non-IUGR6 versus IUGR6.
NPY-expression is not altered yet. Maybe the up-regulation of NPY after LSR to 6 is a compensatory mechanism to the decreased expression of the Y1-receptor.

The results demonstrated in this study show that postnatal LSR to 6 alters tubular function. A significant increase of both natriuresis and kaliuresis was observed at Day 70 after birth. Additionally, a slight increment of diuresis was measured. One underlying mechanism of renal tubular function is the RAAS. Therefore, we analysed renin, the mineralcortcoid receptor, and the angiotensin (AT) receptors. No changes in the group with LSR compared to the control group without LSR were detectable with regard to mRNA expression of the mineralcorticoid receptor and AT-receptor 1a in the kidney, whereas renin mRNA expression is increased and AT-receptor 1b is decreased at Day 70 after birth. Renin release is regulated by multiple mechanisms. Several studies describe a direct effect of NPY on the renin release [33, 35, 36]. Consistent with the localization of NPY next to the macula densa—shown in our study—it can directly act through a paracrine effect on the macula densa and influence the expression of renin. According to Aubert et al. [34] NYP inhibits renin release in the kidney. Hence, the RAAS activation would be suppressed and less aldosterone is secreted. Consequently, that leads to increased natriuresis and decreased kaliuresis. The expression of one of the corresponding receptors of RAAS (AT-receptor 1b) is decreased in the LSR6-group. That can be reactive to the increased expression of renin. These findings may have an influence on renal blood flow and blood pressure. Further studies will have to address blood pressure after LSR. In our study, the expression of endogenous NPY is increased and the Y1-receptor is decreased. Consistent with the decreased expression of the Y1-receptor, we observe less phosphorylation of MAPK42/44 due to a reduced activation through G-protein coupled receptors. The activity of the NPY-system through Y1-receptors seems to be decreased. That leads to a diminished activity of Na\(^+\)-K\(^+\)-ATPase and of Na\(^+\)/Pi cotransporter in the renal tubuli. In addition, these results may explain why renin expression is not suppressed but increased. Activation of the RAAS is the consequence. That may contribute to the increase of kaliuresis.

To demonstrate that NPY is expressed endogenously in the renal tubular cells and not just as previously described in the end of the sympathetic nerves, we analysed the localization of NPY and Y1-receptor with a renal tubular marker. Both NPY and Y1-receptor are localized in the distal tubuli and collecting ducts of the renal system. We conclude that endogenous renal NPY is expressed not just in the end of sympathetic nerves but also and predominantly in the renal tubular cells itself and may have paracrine effects in the kidney.

<table>
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<th>Table 3. Physiological data of animals with and without LSR</th>
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<tr>
<td>HCC</td>
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<td>Diuresis (mL/24 h)</td>
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<td>Haemoglobin (g/dL)</td>
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<td>Na(^+)-concentration plasma (mmol/L)</td>
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<td>K(^+)-concentration plasma (mmol/L)</td>
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Fig. 7. MAPK 42/44 and its phosphorylation after LSR. Analysis of the phosphorylation of MAPK 42/44 and total MAPK 42/44 after LSR to 6 (LSR6) was assessed by western blotting. Densitometric analysis demonstrated a decreased phosphorylation of MAPK 42/44, whereas the expression of total MAPK 42/44 after LSR to 6 (LSR6) was increased. (*P < 0.05, **P < 0.01; one-way ANOVA test and Bonferroni correction).
NPY is inactivated by several peptidases, one key regulator is DPPIV. It is a peptidase with the capability of hydrolyzing postproline bonds and it cleaves NPY. Some previous studies have described an expression of DPPIV in the kidney, but none have addressed the expression pattern within the kidney. This study demonstrates that it is predominantly localized and active immunhistochemically in the renal cortex, especially in renal ducts and in the glomeruli, and also slightly in the renal medulla. Furthermore, DPPIV is significantly decreased after LSR6 on protein level. Consequently, we assume that NPY is less cleaved in the kidney of LSR6 animals due to the insufficient level of its regulating peptidase. Hence, the NPY levels are increased.

Additional extrarenal factors may influence renal function and body weight, i.e. SNS or metabolic syndrome. That may have an impact on the expression of the NPY-system and its signalling. Further investigations are necessary to elucidate these issues.

To sum up, LSR and increased postnatal weight gain lead to increased natriuresis and kaliuresis in this animal model. A potential underlying mechanism may be seen in the intrinsic kidney-associated NPY-system. On the one hand, NPY influences kaliuresis indirectly through renin via the Y1-receptor. In addition, NPY also has a direct RAAS-independent effect on kaliuresis and natriuresis via Y1+/Y2-receptor. Both receptors are G-Protein-coupled receptors acting via MAPK42/44. Due to reduced expression of Y1-receptor, its intracellular signalling through MAPK 42/44 is significantly decreased. The NPY-system regulates both kaliuresis and natriuresis and may be a potential mechanism in the tubular dysfunction after LSR.

In conclusion, we provide direct experimental evidence in rats illustrating that tubular function is influenced by the postnatal environment. Furthermore, this study also shows a potential role of the endogenous NPY-system in the regulation of tubular function as well as a mediating system transmitting early in life acquired physiological changes. Given that the NPY-system is also amenable to pharmacological and genetic manipulation in the kidney, it provides a potential target in this context.

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Conflict of interest statement. All authors declare that there is no conflict of interest concerning this study.

References

Fig. 8. Natriuresis and kaliuresis after LSR. Analysis of sodium and potassium excretion in the urine for 24 h revealed an alteration of the renal homoeostasis after LSR. An increase is shown in absolute kaliuresis (B) but no alteration of absolute excretion of sodium (A) after LSR to 6 (LSR6) compared to the control group without any LSR (HCC). Both excreted fraction of the filtered sodium (FeNa) (C) and excreted fraction of the filtered potassium (FeK) (D) were increased significantly after LSR6 compared to the control group. (*P < 0.05, **P < 0.01; one-way ANOVA test and Bonferroni correction).


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