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# Rescue of the mineralocorticoid receptor knock-out mouse

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**Abstract** The mineralocorticoid receptor knock-out mouse (MR–/–), resembling inborn pseudohypoaldosteronism, dies 8–12 days after birth in circulatory failure with all the signs of terminal volume contraction. The present study aimed to examine the functional defects in the kidney and colon in detail and to attempt to rescue these mice. In neonatal (nn) MR–/– the amiloride-sensitive short-circuit current in the colon was reduced to approximately one-third compared to controls (MR+/+ and MR+/–). In isolated in vitro perfused collecting ducts the amiloride-induced hyperpolarization of the basolateral membrane ($V_{bl}$) of nn MR–/– was similar to that of controls, but urinary $Na^+$ excretion was markedly increased to 4.3 µmol/day·g (BW). Based on this measured urinary $Na^+$ loss we tried to rescue nn MR–/– mice by injecting NaCl twice daily (3.85 µmol/g BW), corresponding to 22 µl of isotonic saline/g BW subcutaneously. This regimen was continued until the animals had reached a body mass of 8.5 g. Thereafter, in addition to normal chow and tap water, NaCl drinking water (333 mmol/l) and pellets soaked in 333 mmol/l NaCl were offered. Unlike the untreated nn MR–/– most of these mice survived. The adult animals were examined between days 27 and 41, some were used for breeding. When compared to age-matched controls the growth of MR–/– was delayed until day 20. Then their growth curve increased in slope and reached that of controls. MR–/– retained their $Na^+$-losing defect. Amiloride's effect on urinary $Na^+$ excretion was not significant in MR–/– mice and the effect on $V_{bl}$ in isolated cortical collecting ducts was attenuated. The renin-producing cells were hypertrophic and hyperplastic. Plasma renin and aldosterone concentrations were significantly elevated in MR–/– mice. These data indicate that MR–/– can be rescued by timely and matched NaCl substitutions. This enables the animals to develop through a critical phase of life, after which they adapt their oral salt and water intake to match the elevated excretion rate; however, the renal salt-losing defect persists.

**Key words** Aldosterone · Amiloride · Epithelial $Na^+$ channel · Glucocorticoids · NaCl metabolism · Pseudohypoaldosteronism

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## Introduction

There are two main causes of inborn pseudohypoaldosteronism type 1 in man: a defect in the epithelial and amiloride-sensitive $Na^+$ channel (ENaC); and a defect of the mineralocorticoid receptor [8,14]. Recently a mineralocorticoid receptor knock-out mouse (MR–/–) has been generated and some of the functional characteristics of these mice have been described [3]. They fail to thrive early after birth and die between days 8 and12 with all the signs of massive salt and volume loss: they lose weight; have fixed skin folds; their haematocrit is excessively high; as are plasma renin, angiotensin II, and aldosterone levels. Their glomerular filtration rate (GFR) is not altered initially, but their fractional $Na^+$ excretion is enhanced. Surprisingly, the messenger RNAs for the ENaC subunits α, β, and γ were not altered in the kidney or in the colon [3]. Amiloride-inhibited $Na^+$ absorption was reduced in colon and kidney, but was not abolished. These data suggest that (1) MR controls $Na^+$ absorption distal to ENaC mRNA and (2) that even in these animals some amiloride-sensitive ENaC function is preserved.

With the present study we wanted to examine the defect in colonic and kidney function in the neonatal (nn) MR–/– mouse in more detail. In addition, based on the

previous findings that MR–/– represents a partial $Na^+$ absorptive defect, we calculated the deficits and substituted NaCl and water by two daily injections of an isotonic NaCl solution. This regimen rescued the MR–/– mice, and their $Na^+$ absorptive capacity could then be examined close to adulthood. The present data indicate that MR–/– mice, once rescued by an adequate parenteral NaCl and water substitution through the critical neonatal phase, can compensate by adapting their eating and drinking habits to the greater urinary $Na^+$ loss. They do, however, keep their renal (and colonic) $Na^+$ transport defect.

## Materials and methods

### The MR–/– mice and the rescue protocol

MR–/– mice were bred from heterozygous pairs of animals (MR+/–) [3]. The functional studies of the colon and kidney were performed at days 4–8 after birth. The experiments on the rescued adult MR–/–, MR+/– and MR+/+ mice were done on days 27–41 after birth.

For the rescue protocol all mice (MR–/–, MR+/+, MR+/–) were injected subcutaneously with an isotonic NaCl solution twice daily from day 5 until they had reached a body mass of 8.5 g. This mass was reached in MR+/+ between days 14 and 19, between days 14 and 23 in MR+/–, and between days 24 and 30 in MR–/–. The amount of NaCl injected was calculated from the GFR of neonatal mice [approximately 1 µl/(min·g BW)] and the fractional $Na^+$ excretion of approximately 2.7% in MR–/– [3]. This amounts to a renal $Na^+$ loss of 4.3 µmol/day·g (BW). To be on the safe side and also to compensate for other minor $Na^+$ losses (e.g. via the gut) we injected twice per day 3.85 µmol/g BW, each injection corresponding to 22 µl of isotonic saline solution. After the animals had reached a body mass of 8.5 g injections were stopped and thereafter the mice (all groups) had the choice between normal tap water and 333 mmol/l saline and normal chow as well as chow soaked in 333 mmol/l saline. We did not measure $Na^+$ intake but we can assume that it was probably 1–1.5 times the amount secreted (see below).

The animals were examined between days 27 and 41 with respect to their plasma $Na^+$, $K^+$, $Ca^{2+}$, creatinine, renin and aldosterone concentrations, their fractional urinary excretion of $Na^+$ ($FE_{Na}$), $K^+$ ($FE_K$) and GFR. In addition the kidneys were examined by light and electron microscopy on 1-µm-thick plastic-embedded and ethylene-blue-stained sections. Colonic function was examined using a specifically designed [16] miniature Ussing chamber.

### Renal clearance studies

For the clearance measurements the animals were anaesthetized by thiopental-Na (Trapanal, 5 g/l in physiological saline solution, given subcutaneously at 50 mg/kg BW) and ketamin-HCl (Ketanest, 10 g/l in water, injected intraperitoneally at 100 mg/kg BW). The animals were placed on a heated table. All surgical manoeuvres were done whilst observing the preparation through a lens (2–10×). Urine samples were taken by puncturing the urinary bladder through a small abdominal incision. Blood samples were taken either from a tail vein or as a terminal venous blood sample (usually 50–100 µl) by puncturing the right atrium. The samples were collected into a heparinized capillary or syringe. Blood was centrifuged immediately. Kidneys and colon as well as trachea were removed and preserved in ice-cold Ringers solution for examination within 2 h. The animals were killed by bleeding under anaesthesia.

Plasma $Na^+$ and $K^+$ concentrations as well as those in urine samples were examined by flame photometry using $Li^+$ as an internal standard. The procedures had to be scaled down to the available plasma volumes (<10 µl per sample). Plasma and urine creatinine were determined by the Jaffé reaction (Boehringer, Ingelheim, Germany). The procedure was scaled down to 30 µl plasma. Plasma renin and aldosterone were determined as described previously [3].

### In vitro perfused proximal tubules and collecting ducts

The kidneys were cut into 0.5-mm-thick slices and dissected manually. Straight proximal tubules (neonatal) and cortical (adult), medullary and papillary (neonatal) collecting ducts were identified by their morphological criteria and topology [17]. They were transferred into a bath chamber mounted into the stage of an inverted microscope (Zeiss Axiovert, Zeiss, Jena, Germany). Tubules were cannulated and perfused in vitro as described previously [9, 10]. One end of the tubule was freely accessible and immobilized by a suction pipette. The luminal perfusate could be exchanged by a fluid exchange system. The tubules could be approached by patch pipettes or microelectrodes from the basal cell pole and, at the open end, also from their luminal pole.

Transepithelial voltage ($V_{te}$) was measured with reference to the bath [10] and transepithelial resistance ($R_{te}$) was estimated by pulsed current injections through one of the barrels of the double-barrelled perfusion pipette. This current injection caused a voltage deflection ($\Delta V_{te}$). The other measured parameters were: inner and outer tubule radius, the length of the perfused segment and the resistivity of the used perfusion solution. Using the appropriate cable equation $R_{te}$ could be calculated [10]. From $R_{te}$ and $V_{te}$ the equivalent short-circuit current ($I_{sc}$) can be calculated as: $I_{sc}=V_{te}/R_{te}$. The bath (1 ml) was perfused at a rate of 20 ml/min.

### Patch-clamp investigations in isolated perfused renal tubules

Patch pipettes were manufactured as customary in this laboratory [4]. They had tip diameters between 0.5–1 µm and input conductances of 5–8 MΩ when filled with a solution containing (in mmol/l): $K^+$ 120, $Cl^-$ 32, gluconate 91, ATP 1, $Na^+$ 10.8, $HPO_4^{2-}$ 4.8, $H_2PO_4^-$ 1.2, EGTA 1, $Ca^{2+}$ 1.3, $Mg^{2+}$ 1. This solution was titrated with KOH to a pH of 7.2. Nystatin (100 mg/l) was added to this solution to obtain slow-whole-cell recordings [13]. As usual for intact tubule preparations, the probability of obtaining tight seals was extremely low. The reference electrode was of the flowing type and consisted of an Ag/AgCl pellet in 1 mol/l KCl. The KCl flow rate was approximately 0.1 µl/min. The patch-clamp amplifier (Fröbe/Busche this institute) was set to zero-current-clamp mode. Hence, the membrane voltage was recorded.

### Impalement of collecting ducts and tracheal mucosal cells

Because of the low yield of successful patch-clamp recordings we have also used impalement techniques, as customarily done in our laboratory [10]. The microelectrodes for impalement were pulled on a vertical Narishige puller (Narishige, London, UK). The microelectrodes had an input resistance of 80–120 MΩ when filled with 1 mol/l KCl. They were advanced to the mucosa by a micromanipulator (Leitz, Göttingen, Germany, or Luigs and Neumann, Ratingen, Germany) and impalement was aided by a piezo stepper (Physik Instrumente, Waldbronn, Germany). Basolateral membrane voltage ($V_{bl}$) was monitored by a high-impedance electrometer (LMPR, List, Darmstadt, Germany) or by the patch-clamp amplifier (this institute) in zero-current mode and recorded on a pen recorder. The microelectrode resistance was monitored continuously by pulsed current injection. Impalements were only accepted if: (1) the tip voltages were <5 mV; (2) the voltage deflection upon impalement was abrupt; (3) the recorded $V_{bl}$ was stable for at least 3 min and $V_{bl}$ returned to the baseline within ±2 mV upon withdrawal of the microelectrode; and (4) voltages of >–30 mV were not considered further.

For impalements of tracheal cells the trachea was cut open and placed into a chamber mucosal side up. It was pressed by a nylon mesh to the bottom of the chamber. The bath chamber was mounted into the stage of an inverted microscope (Zeiss IM 35, Zeiss, Jena, Germany). The bath solution was identical to that used for the perfusion experiments of renal tubules. The bath exchange rate was 0.3 Hz. The cells were impaled from the mucosal side and luminal membrane voltage ($V_l$) was recorded. The criteria for data evaluation were as stated in the last paragraph.

### Ussing chamber measurements from the colon

To allow for the measurement of extremely small samples of colonic mucosa, a recently developed micro Ussing chamber [16] was used. This chamber has an aperture of 0.5 mm. It consists of a Perspex tissue slide with an aperture and a fixation spring to press the tissue against the slide. The slide is equipped with two rubber gasket rings and can be inserted into the corresponding slot of the actual chamber. Two Ag/AgCl pellets connected to agar bridges were used to monitor the transepithelial voltage ($V_{te}$) via a differential amplifier (Fröbe, this institute). Two Ag/AgCl electrodes were used to inject current pulses (≅500 nA) produced by a pulse generator (Fröbe, this institute) connected to a resistor. The solutions could be exchanged on both sides of the tissue with a time constant of approximately 0.5 min.

All in vitro experiments were performed at 37°C by thermostating the perfusion solutions and chambers.

### Solutions

The normal bath, Ussing chamber, and luminal perfusion solution contained (in mmol/l): NaCl 140, $K_2HPO_4$ 1.6, $KH_2PO_4$ 0.4, $CaCl_2$ 1.3, $MgCl_2$ 1, D-glucose 5. For proximal tubules this solution was used with and without 5 mmol/l D-glucose, alanine or glycine. The preservation solution for kidney tubules was (all in mmol/l): NaCl 115, $NaHCO_3$ 25, $K_2HPO_4$ 1.6, $KH_2PO_4$ 0.4, $CaCl_2$ 1.3, $MgCl_2$ 1, acetate 5. This solution was gassed with 5% $CO_2$/95% $O_2$. All substances were of the highest grade of purity available (Sigma, Aldrich, Steinheim, Germany). 293B, a blocker of $K_V$LQT1 channels, was synthesized as described previously [15].

### Statistics

The data are presented as mean values ±SEM (number of observations or animals). Paired as well as unpaired Student's *t*-test were used as appropriate. The significance level was set to $p<0.05$.

## Results

### The $V_{bl}$ of isolated perfused collecting tubules of neonatal mice.

Previous data [3] as well as the present data (see below) suggest that renal tubular $Na^+$ absorption is reduced in neonatal (nn) MR–/– mice, leading to an increased $FE_{Na}$. In addition, amiloride's effect on $FE_{Na}$ was attenuated in nn MR–/– animals in our previous study [3]. This would suggest that $Na^+$ absorption in collecting ducts is reduced. To examine this more directly we have perfused isolated fragments of medullary and papillary collecting ducts of nn mice in vitro, measured $V_{bl}$ (patch-clamp and impalement data) and examined the effect of luminal perfusion of 5 µmol/l amiloride, a concentration at which epithelial $Na^+$ channels (ENaC) should be inhibited almost completely [7]. Figure 1A summarizes the data for nn MR+/+, MR+/–, and MR–/–. In nn MR+/+ $V_{bl}$ was –59±15.4 mV and amiloride hyperpolarized to –63.3±14.4 mV ($n$=3). In nn MR+/– $V_{bl}$ was –49.5± 2.9 mV before and –52.3±2.6 mV after amiloride ($n$=36). Upon removal of amiloride $V_{bl}$ returned to control values. In nn MR–/– the data were indistinguishable: $V_{bl}$ was –52.5±2.6 mV under control conditions and amiloride hyperpolarized significantly to –58.1±2.4 mV ($n$=54). This finding was surprising inasmuch as a reduced amiloride-inhibited $Na^+$ absorption was expected to occur in the collecting duct on the basis of previous clearance data [3].

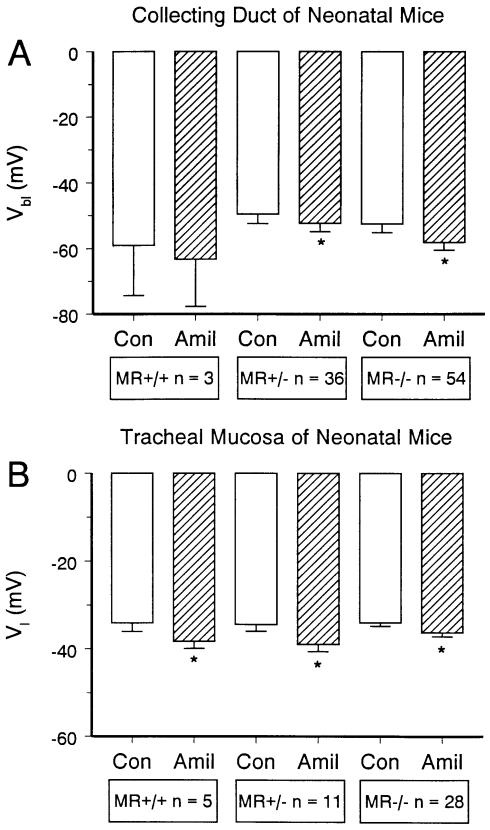


**Fig. 1. A** The effect of amiloride (*Amil*, 10 µmol/l, lumen, *hatched bars*) on basolateral membrane voltage ($V_{bl}$) of in vitro perfused collecting ducts of neonatal MR+/+, MR+/– and MR–/– mice. Mean values ± SEM (number of observations). *Statistical significance. Note that amiloride has a comparable effect in collecting ducts from control and MR–/– mice. **B** The effect of amiloride (10 µmol/l, lumen, *hatched bars*) on luminal membrane voltage ($V_l$) of tracheal mucosa of neonatal MR+/+, MR+/– and MR–/– mice. Mean values ± SEM (number of observations). *Statistical significance. Note that amiloride hyperpolarizes all three types of cells comparably

### The $V_{bl}$ of isolated perfused proximal tubules in neonatal mice

One might argue that this defect in nn MR–/– could be localized anywhere in the nephron where amiloride exerts an effect. Along these lines we have shown recently

for rat kidney that amiloride inhibits $Na^+$ absorption also in the proximal tubule via its effect on ENaC channels [20]. Therefore, we examined whether amiloride has any effect in the proximal straight tubules of nn MR+/+, MR+/– mice and whether this putative effect is attenuated in nn MR–/– mice. Whilst the addition of 5 mmol/l D-glucose, alanine or glycine to the lumen depolarized all tubules by 6.9±0.7 mV ($n$=32), 4.2±0.7 mV ($n$=16), and 4.3±0.7 mV ($n$=10) respectively, amiloride (5 µmol/l) had no effect in any of the three groups of nn mice: –40.8±1.6 versus –40.9±1.6 mV ($n$=32). This indicates that the $Na^+$-losing defect in nn MR–/– is not localized in the proximal tubule.

## The $V_l$ of tracheal mucosal cells of neonatal mice

The regulation of ENaC channels in the mucosa of the trachea is not quite clear at this stage [1]. It was thought interesting to examine whether the regulation of ENaC is defective in nn MR–/– animals, although there is evidence to suggest that ENaC is regulated by glucocorticoids rather than mineralocorticoids in this tissue [18,19]. To clarify this issue we performed impalement studies on this tissue from nn control and MR–/– mice and examined the effect of amiloride. The data are shown in Fig. 1B. The mean value of $V_l$ was –34.0±1.95 mV in nn MR+/+ cells. Amiloride (5 µmol/l) induced a significant hyperpolarization to –38.3±2.5 mV ($n$=5). This effect was entirely reversible. The data for nn MR+/– and MR–/– tracheal cells were indistinguishable. Under control conditions $V_l$ was –34.3±1.6 mV ($n$=11) and –33.9±0.8 mV ($n$=28), respectively. Amiloride induced a significant hyperpolarization to –38.9±1.6 mV and –36.2±0.9 mV, respectively. These data suggest that ENaC function in tracheal mucosal cells is normal in nn MR–/– mice.

## The $I_{sc}$ of colonic mucosa from neonatal mice

Previously we have shown that amiloride's effect on $V_{te}$ in the distal colon of the intact animal is attenuated in nn MR–/– mice [3]. We wanted to study more closely colonic function in the isolated colonic mucosa mounted in a micro Ussing chamber. First we examined whether the stimulation of secretion by cAMP [forskolin 10 µmol/l + isobutylmethylxanthine (IBMX) 0.1 mmol/l] and by carbachol (CCH, 0.1 mmol/l) as well as the inhibition of secretion by the chromanol 293B [15] (a blocker of basolateral $K_VLQT_1$ channels) is influenced in nn MR–/–. These data are summarized in Fig. 2A–D. The control tissue (MR+/+, MR±) and that of MR–/– showed very similar responses. Forskolin (10 µmol/l) and IBMX (0.1 mmol/l) increased lumen-negative $I_{sc}$ significantly in all three series. The cAMP-activated lumen-negative $I_{sc}$ was inhibited almost completely by the chromanol 293 B (blood side 10 µmol/l) (Fig. 2D). The inhibition by 293B was concentration dependent with half-maximal inhibition occurring at approximately 2 µmol/l (Fig. 2D).

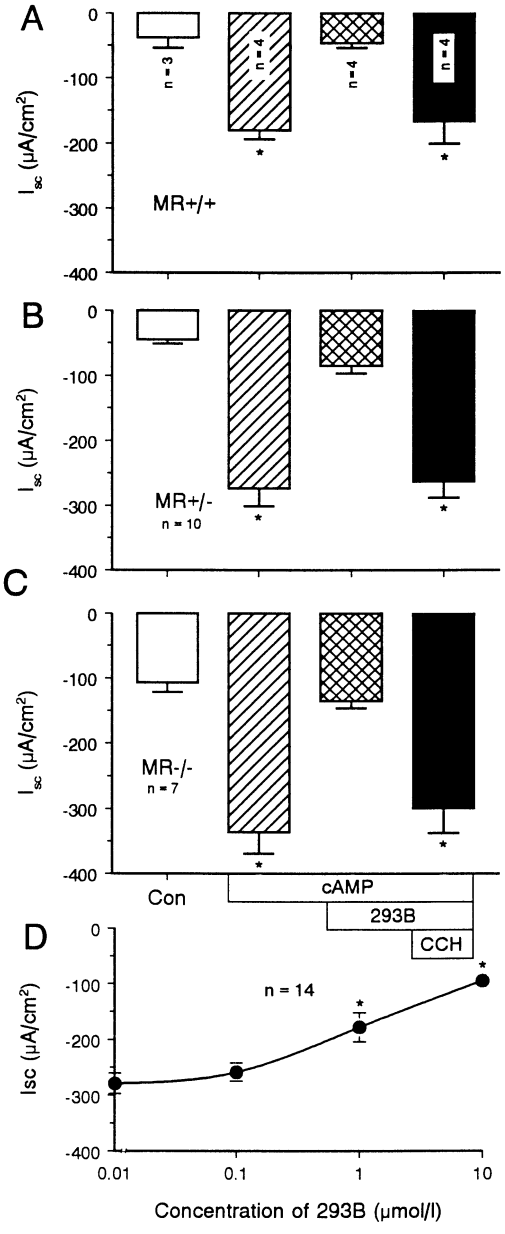


**Fig. 2A–D** The effect of forskolin/isobutylmethylxanthine (IBMX) (10 µmol/l, 0.1 mmol/l, bath, abbreviated here as *cAMP*); the chromanol 293B (10 µmol/l, bath, a blocker of $K_VLQT_1$ channels) and carbachol (*CCH*, 0.1 mmol/l) on equivalent short circuit current ($I_{sc}$) of neonatal mouse colonic mucosa (all in the presence of 10 µmol/l amiloride, mucosal side). **A–C** Data for MR+/+, MR+/– and MR–/– mice. Mean values ± SEM (number of observations). *Statistical significance. Note that CCH stimulates $I_{sc}$ even in the presence of 293B. **D** Concentration/response curve for the chromanol 293B on cAMP-mediated $I_{sc}$. The $I_{sc}$ is plotted as a function of bath 293B concentration. Note that $Cl^-$ secretion is qualitatively similar to that of adult rats and rabbits [5] and that it is unaltered in MR–/– mice

In the presence of 10 µmol/l forskolin, 0.1 mmol/l IBMX and 293B, CCH (0.1 mmol/l, blood side) still had its stimulatory effect on $Cl^-$ secretion. Thus, these data indicate that the colonic function of these neonatal mice, with respect to $Cl^-$ secretion, is qualitatively similar to that of the adult mouse, rat or rabbit [5,12].

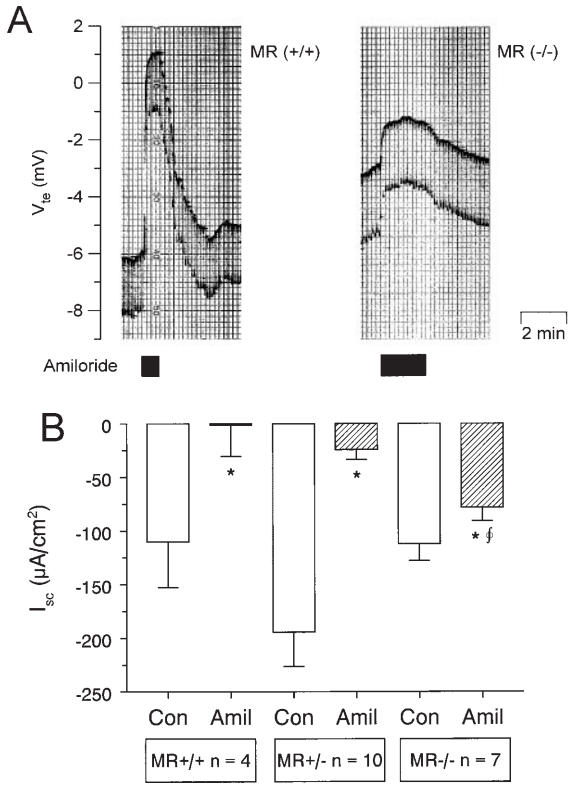


**Fig. 3A,B** The effect of amiloride (5 µmol/l, lumen) on the $I_{sc}$ of neonatal mouse colonic mucosa of MR+/+, MR+/– and MR–/– mice. **A** A typical Ussing chamber experiment for colonic mucosae of MR+/+ and MR–/– mice. $Cl^-$ secretion is directly proportional to $I_{sc}$ and transepithelial potential ($V_{te}$). At the time indicated 10 µmol/l amiloride was added to the mucosal perfusate. An inhibitory effect is seen in both tissues. The effect is much stronger in the mucosa of the MR+/+ mouse. **B** Mean values of $I_{sc}$ ±SEM (number of observations) under control conditions (*Con*) and in the presence of amiloride (*Amil*, mucosal side, 10 µmol/l). *Statistical significance within group. §Unpaired significant difference between MR–/– and control mice (MR+/+, MR+/–). Note that amiloride has a significant inhibitory effect in all three groups but that the effect is reduced significantly to approximately 30% in MR–/– mice. The transepithelial resistance was between 35 and 40 Ω cm² under control conditions

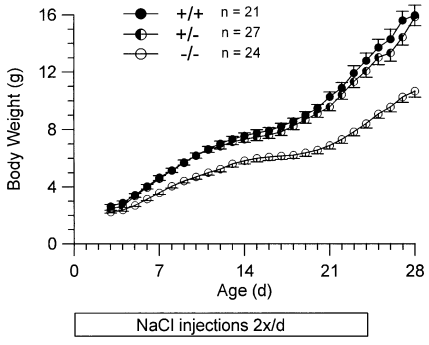


**Fig. 4** Growth curves in MR+/+ (*filled circles*), MR+/– (*half filled circles*), and MR–/– mice (*open circles*). Body weight (*BW*) is plotted as a function of postnatal age. Mean values ±SEM, number of animals. The mice have been injected with physiological saline according to the protocol described in Materials and methods. Note that unlike in our previous study [3] the MR–/– are now rescued. They grow more slowly initially but start to catch up with respect to their growth rate after day 20

Next we examined amiloride's effect on $I_{sc}$ in the colonic mucosa of all three types of neonatal mice (MR+/+, MR+/– and MR–/–). The data are summarized in Fig. 3. A typical record for an nn MR+/+ and an nn MR–/– colonic mucosa is depicted in Fig. 3A. Amiloride (10 µmol/l, mucosal side) had an inhibitory effect on $V_{te}$ and hence $I_{sc}$. However, the effect was less marked in the MR–/– tissue. In the pooled (Fig. 3B) studies of nn MR+/+ mice the $I_{sc}$ was –110±43 µA/cm² under control conditions and amiloride (10 µmol/l) reduced this lumen-negative current significantly to –1.4±29.1 µA/cm² (*n*=4). This effect was entirely reversible (data not shown). In the studies of nn MR+/– mice the results were indistinguishable: $I_{sc}$ was –194±32 µA/cm² under control conditions and amiloride (10 µmol/l) reduced this lumen-negative current significantly to –24.3±9.2 µA/cm² (*n*=10). In nn MR–/– mice $I_{sc}$ was –111±15.6 µA/cm² under control conditions. Most importantly, the amiloride inhibition, albeit statistically significant, was less and $I_{sc}$ was reduced only to –78.3±12.3 µA/cm² (*n*=7). Thus, the amiloride-sensitive $I_{sc}$ was only approximately 30% of that seen in control animals, which fits well with what we have shown recently for the transepithelial voltage in distal colon in vivo and the fractional renal excretion in neonatal MR–/– [3].

## Rescue of MR–/– mice by NaCl application

A first rescue series was started with 13 MR–/– mice. The injection and feeding protocol is described above. Injections were necessary from day 5 until ≅21 days. Two of these 13 mice died for unknown reasons within the first week of treatment. All other animals survived, compared to a survival of 0% after day 12 in untreated neonatal MR–/– mice [3]. Of the 11 survivors 7 mice were studied at days 24–34 after birth and sacrificed after the experiment. Of the remaining four mice one died at day 56 and another one at day 90. The remaining two mice have been used for breeding. In an additional series we have bred and rescued another 14 MR–/– mice. Thus, the success of this protocol was ≅90%.

Figure 4 displays the growth curves for MR+/+, MR+/– and MR–/– mice. The two control curves (MR+/+ and MR+/–) are very similar. They are triphasic: a rapid growth phase between days 0 and 10 was followed by a slower one (days 11–19). Thereafter, i.e. after weaning, growth sped up and levelled off after days 50–60 (not shown). The rescued MR–/– mice started with a slightly lower birth weight. Their growth was slowed more markedly during days 12–20. Thereafter, their growth rate almost matched that of the control group. The two sets of growth curves are parallel after day 30 (not shown). The weight deficit of the MR–/– group was approximately 4–5 g. Apart from the fact that MR–/– mice drank certain amounts of saline instead of tap water and

**Table 1** Basic observations of control MR+/+, MR+/– and MR–/– mice (day 27–41). Mean values ±SEM (number of observations). (*BW* Body weight, *Crea* creatinine, *Aldo* aldosterone)

| Parameter | MR+/+ | MR+/– | MR–/– |
|---|---|---|---|
| Age (days) | 33±0.6 (11) | 32±0.8 (13) | 31±1.8 (8) |
| BW (g) | 18±0.5 (11) | 17±0.8 (13) | 13±1.1 (8)* |
| $[Crea]_P$ (µmol/l)§ | 66±2 (10) | 67±4 (13) | 85±5 (8)* |
| $[Na^+]_P$ (mmol/l) | 149±3 (9) | 143±0.7 (6) | 145±2.3 (6) |
| $[K^+]_P$ (mmol/l) | 5.65±0.24 (8) | 5.38±0.20 (6) | 7.21±0.27 (6)* |
| $[Ca^{2+}]_P$ (mmol/l)§ | 2.3±0.15 (10) | 2.7±0.09 (13) | 3.0±0.10 (8) |
| Haematocrit | 0.49 ( 0.01 (4) | 0.49 ( 0.01 (4) | 0.49 ( 0.01 (12) |
| pH§ | 7.20±0.03 (8) | 7.21±0.02 (10) | 7.21±0.03 (5) |
| $pO_2$ (mmHg)§ | 75±9 (7) | 76±7 (9) | 99±29 (3) |
| $pCO_2$ (mmHg)§ | 55±7 (8) | 53±3 (10) | 51±4 (4) |
| Aldo (mg/l)§ | 1.63±0.70 (4) | 2.98±0.45 (3) | 17.1±1.94 (6)* |
| Renin (µg AI/ml·h)§ | 5.20±2.28 (4) | 6.17±1.79 (3) | 83.8±32.5 (6)* |
| GFR (µl/min·g BW) | 2.5±0.4 (11) | 2.5±0.5 (13) | 2.2±0.4 (6) |

§Blood taken from a terminal venous sample. *Statistical significance of differences (unpaired *t*-test, versus MR+/+)

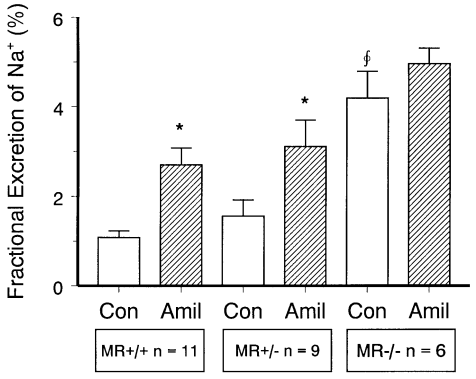


**Fig. 5** Fractional $Na^+$ excretion in adult MR+/+, MR+/–, and MR–/– mice in the absence and presence of amiloride (5 mg/kg BW, subcutaneously). Mean values ±SEM (number of mice). *Statistical significance within group. §Statistical difference between MR–/– and MR+/+, MR+/– mice. Note that amiloride has a significant inhibitory effect only in MR+/+ and MR+/– mice. In MR–/– mice fractional $Na^+$ excretion is significantly higher than in the control mice (*§*). Amiloride has no detectable effect in this series

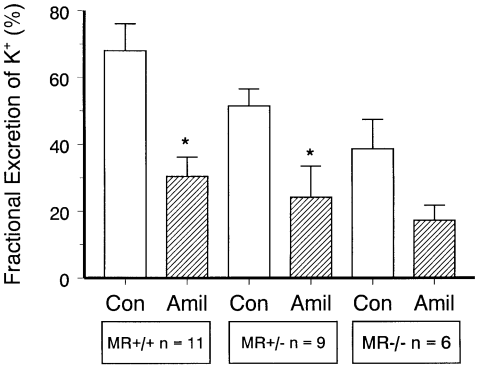


**Fig. 6** Fractional $K^+$ excretion in adult MR+/+, MR+/–, and MR–/– mice in the absence and presence of amiloride (5 mg/kg BW, subcutaneously). Mean values ±SEM (number of mice). *Statistical significance. Note that amiloride has a significant inhibitory effect in MR+/+ and MR+/– mice. In MR–/– mice fractional $K^+$ excretion is lower than in the control mice. Amiloride has no significant effect in this series

preferred to eat some of the pellets soaked in 333 mmol/l saline, we did not recognize any striking difference in behaviour between these mice and the control groups. Also their mating behaviour was not different from that of adult MR+/– and MR+/+ mice.

## The renal clearances of adult rescued MR–/– and control mice

The general parameters, measurements of electrolytes in plasma, of pH and blood gasses, and the measurements of plasma renin activity and plasma aldosterone are depicted in Table 1. Several points deserve comments. The mean age at the day of the experiment was similar (27–41 days) in all three groups. Therefore, the mean mass of the MR–/– group was lower than that of the MR+/+ and MR+/– groups. The plasma creatinine level was slightly and significantly higher in the MR–/– group. This may indicate that MR–/– mice have higher catabolic rates. GFR, measured here as creatinine clearance, was very similar in all three groups, 2.2–2.5 µl/min·g (BW), indicating that glomerular filtration is not compromised in the MR–/– group.

Unlike the plasma $K^+$ concentration in normal neonatal mice [2,3], that of adult mice was almost normal (M+/+ and M+/–). Significantly higher values were measured in MR–/– mice. The plasma $Na^+$ concentration was normal in all three groups. The hyperkalemia of MR–/– mice is easily explained by their persisting ENaC channel defect (see below). There was, however, no overt sign of volume contraction in these animals and the haematocrit was normal. Blood pH, $pO_2$ and $pCO_2$ were in the expected range for a central venous blood sample, especially when considering that some degree of respiratory acidosis was expected in these anaesthetized animals. These parameters were indistinguishable in all three groups. Plasma renin activity and plasma aldosterone were significantly elevated in MR–/– mice. This may indicate that these mice have a $Na^+$ deficit due to their persisting defect (see below), but it is also possible that the high aldosterone and renin concentrations are due to a reduced mean blood pressure (not measured in this study) or due to the hyperkalemia.

$FE_{Na}$ in the presence and absence of amiloride is depicted in Fig. 5 for all three groups. Under control condi-

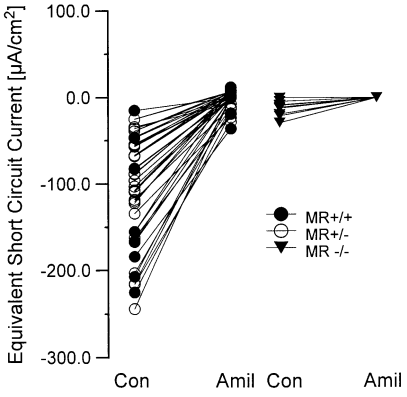


**Fig. 7** Measurements of $I_{sc}$ in isolated in vitro perfused cortical collecting ducts of adult MR–/– mice and the respective controls (MR+/+ and MR+/–) under control conditions (*Con*) and in the presence of amiloride (*Amil*, 5 µmol/l, lumen). Each individual experiment is shown. Note that amiloride has a significant inhibitory effect in all three series, but that the effect in MR–/– mice is significantly smaller than in MR+/+ and MR+/– mice

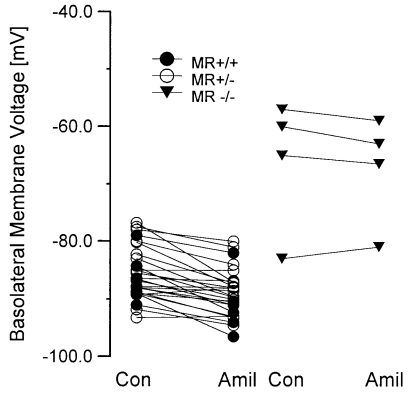


**Fig. 8** Measurements of basolateral membrane voltage in isolated in vitro perfused cortical collecting ducts of adult MR–/– mice and the respective controls (MR+/+ and MR+/–) under control conditions (*Con*) and in the presence of amiloride (*Amil*, 5 µmol/l, lumen). Each individual experiment is shown. Note that amiloride has a significant hyperpolarizing effect in MR+/+ and MR+/– mice but not in MR–/– mice

tions, $FE_{Na}$ was 1.08±0.15% ($n$=11) in MR+/+ mice and slightly higher in the heterozygous mice (MR+/–): 1.56±0.36% ($n$=9). These fairly high values are probably due to the fact that these animals also spontaneously enhanced their $Na^+$ intake because they had access to NaCl drinking water and NaCl-soaked chow. However, $FE_{Na}$ of the MR–/– mice was significantly higher, 4.19±0.60% ($n$=6), reflecting their $Na^+$ absorption defect. The effect of amiloride (5 mg/kg BW, i.p, 1–2 h prior to the measurements) was significant only in both control series (MR+/+, MR+/–), where it increased $FE_{Na}$ to 2.70±0.38% ($n$=11) and to 3.11±0.59% ($n$=9), respectively. Amiloride had no effect on $FE_{Na}$ in MR–/– mice: 4.96±0.35% ($n$=6).

Figure 6 depicts the fractional excretion for $K^+$ ($FE_K$). In MR+/+ and MR+/– baseline fractional kaliuresis was very similar: 68±8.1% ($n$=11) and 51±5.1% ($n$=9), respectively. In MR–/– mice $FE_K$ was lower: 39±8.7% ($n$=6, significantly different from the MR+/+ group), reflecting their transport defect in the principal cells of the collecting duct. Amiloride reduced $FE_K$ significantly in both control groups (MR+/+ and MR+/–) to 30±5.8% ($n$=11) and 24±9.3% ($n$=9), respectively. Amiloride had no significant effect in the MR–/– animals, even though it reduced $FE_K$ in five out of six experiments and the mean $FE_K$ after amiloride was only 17±4.5% ($n$=6). These data indicate that MR–/– mice show a strong natriuretic effect and less kaliuresis. Amiloride produces little additional natriuresis. It may still have a small effect on $K^+$ excretion, but this does not reach statistical significance.

## Experiments on isolated perfused cortical collecting tubules of adult MR–/– mice in comparison to control mice

The results on $I_{sc}$ ($I_{sc}=V_{te}/R_{te}$) are summarized in Fig. 7. $I_{sc}$ under control conditions was significantly larger in MR+/+ and MR+/– mice when compared to MR–/– mice: –134±22 µA/cm² ($n$=11, MR+/+) and –100±10 µA/cm² ($n$=34, MR+/–) as compared to only –10±2.9 µA/cm² ($n$=11) in MR–/– mice. Amiloride's effect on $I_{sc}$ was significant in all three groups, although the inhibitory effect was very small in MR–/– mice $\Delta I_{sc}$ was –10±2.9 µA/cm² and was significantly larger in MR+/+ and MR+/– mice, i.e. –125±20 µA/cm² ($n$=11) and –99±9.3 µA/cm² ($n$=34), respectively.

We also measured $V_{bl}$ by impalement in some of these tubules. The results are depicted in Fig. 8. The baseline $V_{bl}$ was already close to $E_K$ in the two control series: MR+/+ –87±1.3 mV ($n$=8) and MR+/– –84±1.7 mV ($n$=21), respectively. It was less hyperpolarized in MR–/– mice: –66±5.8 mV ($n$=4). Amiloride induced a significant hyperpolarization only in the control series: $V_{bl}$ after amiloride was –91±1.5 mV ($n$=8) in MR+/+ mice and –87±1.3 mV ($n$=21) in MR+/– mice. There was no effect in the four measurements in MR–/– mice: $\Delta V_{bl}$ 1.1±1.1 mV.

The data obtained in the studies of in vitro perfused cortical collecting ducts of adult mice therefore indicate that amiloride has a small effect in MR–/– mice, but that this effect is very limited and only picked up in a statistically significant way by the $I_{sc}$ measurements.

## Structural studies of the kidneys of adult MR–/– mice in comparison to control mice

The structural investigation has been done by light and electron microscopy on 1-µm-thick plastic-embedded methylene-blue-stained sections. Thereby the granules of juxtaglomerular renin-synthesizing cells were stained in dark blue (black in Fig. 9, data are only shown for MR–/– kidneys). The general finding was a hypertrophy and hyperplasia of renin-producing cells. Every nephron was involved. The hyperplasia of renin-producing cells began at the end of portions of interlobular arteries and

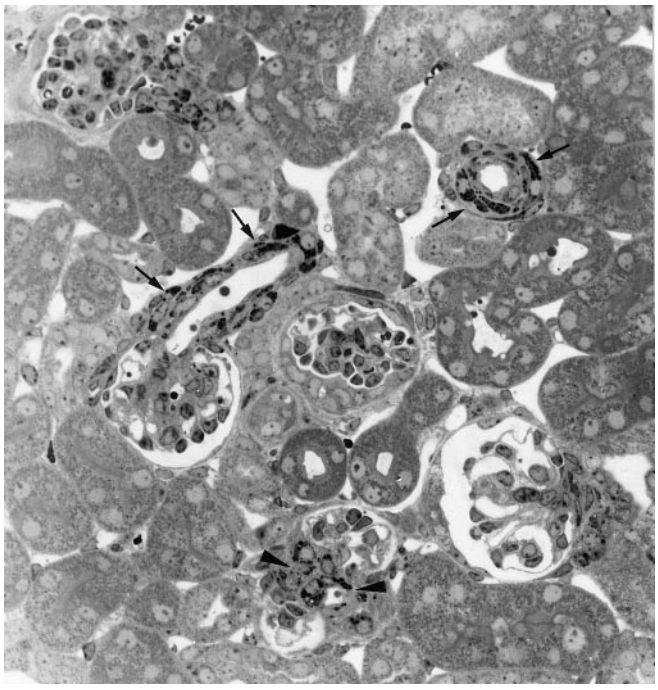

**Fig. 9** Structure of adult MR–/– mouse kidney. Micrograph of kidney slice of a MR–/– mouse. Magnification: full width of graph corresponds to 250 µm. Note the hyperplasia and hypertrophy of renin producing cells in afferent arterioles (*arrows*) but also in mesangial cells (*arrow heads*)

involved the entire afferent arteriole as well as many of the extraglomerular mesangial cells. Even mesangial cells (proper) within the glomerular stalk contained such granules. By electron microscopy the hypertrophy and hyperplasia of smooth muscle cells along the afferent arterioles was clearly seen. Afferent arterioles were enclosed by up to four layers of smooth muscle cells. In addition to the dense accumulation of renin granules, the hypertrophy of the protein-synthesizing machinery, i.e. the rough endoplasmic reticulum, was prominent. A similar cell hypertrophy was encountered in extraglomerular as well as in mesangial cells proper. Many of those cells exhibited a dense accumulation of renin granules. Otherwise the microvasculature of the kidney and the glomeruli were normal in appearance.

## Discussion

### The neonatal MR–/– mouse

The present study was undertaken to examine in more detail the three epithelia that absorb $Na^+$ via ENaC. Our previous data indicated that $FE_{Na}$ is enhanced in MR–/– mice and that $V_{te}$ in the intact colon was reduced. Furthermore, and to our surprise, amiloride still exerted a limited effect in kidney and colon, which amounted to approximately one-third of that seen in the control animals [3]. We now wanted to examine the mechanisms of $Na^+$ absorption in more detail and intended to include measurements in adult MR–/– mice.

From the perspective that $FE_{Na}$ is enhanced in neonatal MR–/– mice, the findings from isolated in vitro perfused collecting ducts reported here came as a complete surprise: the amiloride-induced hyperpolarization was comparable in MR–/– and MR+/+ as well as MR+/– mice. This might be due to one or more of the following reasons: (1) the $Na^+$ absorptive current may in fact be attenuated in these collecting ducts, but the voltage change induced by amiloride may be unaltered if total whole-cell conductance were diminished in these MR–/– cells; (2) the $Na^+$ absorptive defect may be localized in a different nephron segment; (3) the methods used here may not be sufficiently sensitive to pick up the quantitative differences between MR+/+, MR+/– and MR–/– tubule segments. At first glance the last two explanations may appear unlikely, because the collecting duct is one of the key targets of aldosterone in the kidney. However, we were unable to examine the cortical collecting duct in the present study and our data were obtained from medullary and papillary collecting duct, which were the only collecting duct segments that we could dissect from these neonatal kidneys. To examine this aspect more closely we also checked whether amiloride-sensitive $Na^+$ absorption occurs in the proximal straight tubule, where we have previously found amiloride-inhibited $Na^+$ absorption in the rat [20]. There was no indication for such a mechanism in the present study. Thus, we are left with two alternative interpretations: the $Na^+$ absorptive defect resides in the cortical collecting duct and we were unable to see it, or this defect is further upstream between the straight proximal tubule and the collecting duct. The present data from the adult mice suggest that the former explanation is valid (see below).

The present data obtained from tracheal mucosal cells indicate that there is no detectable difference between neonatal control and MR–/– mice. This fits with the recently developed concept that the expression of ENaC in respiratory tract is under glucocorticoid control [18,19]. Glucocorticoid concentrations have not been measured here, but it is highly likely that they are elevated with the circulatory stress present in neonatal MR–/– mice.

Our previous studies of the colon of intact neonatal MR–/– and MR+/+ or MR+/– mice have indicated that the in situ voltage response to luminally applied amiloride is attenuated in MR–/– mice [3]. In the present Ussing chamber experiments this could be studied more closely.

First we examined whether the secretory pathway, which also influences ENaC function [6,11], is affected in MR–/– mice. This was not the case. The present data from the three groups of neonatal mice indicate that the mechanisms of cAMP- and acetylcholine-mediated $Cl^-$ secretion are already mature at this stage. Furthermore, these data confirm our previous observation in rabbit and rat [5] that CCH exerts a strong secretory effect even in the presence of 293B when $Cl^-$ secretion has been previously stimulated by cAMP. This indicates that CCH activates a $K^+$ channel other than $K_VLQT_1$ [12].

With respect to amiloride-inhibited $Na^+$ absorption in the colon the present data indicate that it is largely attenuated in MR–/– mice. However, still some 30% of the amiloride-sensitive $Na^+$ current of control mice is observed in MR–/–. This corresponds closely to our previous findings on urinary $FE_{Na}$ in these animals [3]. In this study the amiloride-inhibited $Na^+$ absorption of MR–/– also amounted to approximately 30%. These data indicate that even in neonatal MR–/– mice there is residual ENaC function. The fact that additional glucocorticoid injections produced a partial rescue of MR–/– in previous studies [3] might suggest that this residual ENaC function is glucocorticoid mediated. Along the same lines it has been shown here and even more directly in previous studies that glucocorticoids are the main regulatory principle for ENaC in respiratory epithelial cells [18,19].

## Rescue of MR–/– mice by NaCl injections

The present and previous studies [3] permit estimation of the daily $Na^+$ deficit in MR–/– mice. We substituted 1.8 times the calculated amount with two daily subcutaneous injections of physiological saline. This simple rescue worked surprisingly well, and ≅90% of the mice lived until the functional tests were carried out between days 27 and 41.

The rescued MR–/– mice showed a definite lag in growth initially, followed by almost normal growth rates after day 20, although the final adult weights were significantly lower than those of the control groups. Compared to another rescue protocol, namely the injection of glucocorticoids [3], the present approach was much more successful. Obviously glucocorticoids, albeit able to sustain some ENaC function, cannot cure the renal defect completely. The success of the present rescue suggests that the major pathophysiological determinant of MR–/– mice survival is in fact compensation of the urinary, and to a lesser extent that of extrarenal, $Na^+$ losses. When the MR–/– mice reach a certain weight and start to feed from other sources but milk they successfully correct their defect.

## The $Na^+$ balance of adult MR–/– mice

The present data from the adult MR–/– mouse clearly indicate that the defect in renal $Na^+$ absorption persists. $FE_{Na}$ was between 4% and 5% which is greater than the maximum usually achieved by amiloride administration (see Fig. 5). This corresponds to an absolute amount of $Na^+$ excretion of 0.31 mmol/day, i.e. approximately 2.1 ml isotonic $Na^+$ solution per day or, as the urine $Na^+$ concentration in these animals was <200 mmol/l, approximately 1.5 ml hypertonic $Na^+$ solution. Hence, these animals require more than 10–15% of their body weight of hypertonic $Na^+$ drinking solution per day. This calculation is conservative in the sense that it completely ignores extrarenal $Na^+$ losses.

With this defect the animals apparently and constantly stay in $Na^+$ deficit. This may explain why renin and aldosterone are upregulated. The high renin and aldosterone levels may also be caused by hyperkalemia and a fall in systemic blood pressure (not recorded here). The chronic $Na^+$ deficit is obviously also one plausible cause of the morphological changes that comprise a strong hyperplasia and hypertrophy of renin-producing cells.

The hyperkalemia seen in neonatal MR–/– mice [3] persisted in adult mice. This is probably caused by decreased principal cell function in the collecting duct. To the extent that $FE_{Na}$ was increased one might expect a reduced $K^+$ secretion, a reduced $FE_K$ and hence hyperkalemia. The hyperkalemia may contribute to the marked increase in plasma aldosterone levels.

In the adult, unlike in the neonatal MR–/– mouse, we had no difficulty in localizing the defect in $Na^+$ absorption to the cortical collecting duct. Also the inhibitory effect of amiloride was clearly attenuated, albeit not completely absent, at this nephron site. Hence, the data obtained by clearance measurements and in the in vitro perfusion studies of the cortical collecting duct are in good agreement.

## Why, unlike the neonates, do adult MR–/– mice cope with their defect?

One reason is probably that the NaCl supply during milk feeding is simply not sufficient to cope with the renal losses in conjunction with the net needs required for growth. In addition, with the maturation of the kidney less of the filtered NaCl load is handled by the collecting duct than during the neonatal period. This is easily apparent from the data obtained from control mice of both age groups. In the neonatal period $FE_{Na}$ in the presence of amiloride goes up to 6–8%, whilst it is only 3–4% in adult animals. Therefore, from the kidney's point of view, the $Na^+$ absorptive defect in the collecting duct is pathophysiologically much more relevant in the immature (neonatal) when compared to the adult kidney.

Finally, the MR defect obviously does not lead to a complete loss of ENaC function. In the neonatal and also in the adult MR–/– mouse some amiloride-sensitive ENaC function was demonstrable. The underlying mechanism is not clear at this stage.

In conclusion, MR–/– mice show a partial ENaC defect in colon and kidney. This ENaC defect causes severe renal $Na^+$ losses and is life limiting in these mice 8–12 days after birth. A balanced substitution of NaCl can rescue these animals and help them to live into adolescence and adulthood. Then, these animals compensate for their persisting $Na^+$ losses by adequate dietary intake. They apparently never fully compensate and live in a constant state of $Na^+$ deficit. This pathology resembles closely what is known of pseudohypoaldosteronism and MR receptor defect in Man.

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