The Connexin40 A96S Mutation Causes Renin-Dependent Hypertension

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ABSTRACT
Deletion of the gap-junction–forming protein connexin40 leads to renin-dependent hypertension in mice, but whether observed human variants in connexin40, such as A96S, promote hypertension is unknown. Here, we generated mice with the A96S variant in the mouse connexin40 gene. Although mice homozygous for the A96S mutations had normal expression patterns of connexin40 in the kidney, they were hypertensive, had sixfold higher plasma renin concentrations, and had 40% higher levels of renin mRNA than controls. Renin-expressing cells were aberrantly located outside the media layer of afferent arterioles, and increased renal perfusion pressure did not inhibit renin secretion from kidneys isolated from homozygous A96S mice. Treatment with a low-salt diet in combination with an ACE inhibitor increased renin mRNA levels, plasma renin concentrations, and the number of aberrantly localized renin-producing cells. Taken together, these findings suggest that the A96S mutation in connexin40 leads to renin-dependent hypertension in mice. Modulation of renin secretion by BP critically depends on functional connexin40; with the A96S mutation, the aberrant extravascular localization of renin-secreting cells in the kidney likely impairs the pressure-mediated inhibition of renin secretion.


Recent evidence suggests that the gap-junction–forming protein connexin40 (Cx40) is important for the regulation of renin release from the kidney.1–3 Renin-secreting juxtaglomerular cells express high levels of Cx40 together with Cx37 and Cx43 and form multiple gap junctions between each other and with neighboring mesangial and endothelial cells.4 Deletion of Cx40 coding DNA in renin-secreting cells probably interrupts the negative feedback control of renal perfusion pressure on renin secretion, leading to dysregulation of renin release.1,2 Inappropriate high levels of circulating renin likely contribute to the development of severe hypertension in mice lacking Cx40.1–3 In humans, it has been reported that polymorphisms in the promoter region of the Cx40 gene may be associated with an increased risk for hypertension.5 We have recently found that renin-secreting cells in the human kidney express high levels of Cx40, leading to the hypothesis that Cx40 may be similarly relevant for the regulation of renin secretion and BP in humans as in mice.6 In patients, mutations in the Cx40 gene that lead to reduced electrical coupling of gap junctions formed by Cx40 have been described. One out of these is an alanine to serine exchange located at position 96, which lies within the highly conserved pore-forming region of the protein.7 This germ-line mutation was detected in a patient with extended periods of atrial fibrillation, most likely because Cx40 also functions in electric signal
propagation in the heart. Interestingly, at the time of assessment, at age 61 years, the patient was being treated for hypertension with no sign of diabetes or hyperlipidemia (M. Gollob, personal communication). Under the assumption that the regulation of renin secretion from human and mouse kidneys may follow similar general lines, we wanted to determine the consequences of the A96S mutation in the Cx40 gene on renin and BP regulation in mice. Thus, we have generated and analyzed mice bearing the A96S mutation in the Cx40 gene. Furthermore, we were interested in gaining more insight into the mechanisms responsible for Cx40-dependent BP regulation in mice harboring the Cx40A96S loss-of-function mutation. For this purpose we evaluated the possibility that the changes in the function of renin-producing cells induced by deletion of Cx40 may not necessarily be due to defective gap-junctional coupling, but may instead be caused by another deletion-associated defect such as the loss of scaffold function of Cx40. Such a scaffold function of Cx40 protein has recently been demonstrated for endothelial cells in which Cx40 directly interacts with membrane-bound nitric oxide synthase. Overall, the new Cx40A96S transgenic mice offer the possibility to investigate Cx40-associated mechanisms in renin production and secretion in more detail than Cx40 null mice regarding template functions of the Cx40 protein.

RESULTS

Generation of Cx40A96S Mice

For targeted mutation of the mouse Cx40 gene we used the nonconditional Cx40A96S vector shown in Figure 1A. The vector construct contains an eGFP reporter gene whose expression is linked to the expression of the Cx40A96S gene via an internal ribosomal entry site (IRES) cassette. Because of the viability of Cx40-deficient mice, we used a nonconditional approach that leads to a ubiquitous expression of the mutated protein in the transgenic mice. The vector was transfected into HM1 embryonic stem (ES) cells for homologous recombination with the mouse Cx40 locus. After selection of vector-containing ES cell clones via the neomycin resistance gene of the Cx40A96S vector, positively recombined ES cells were verified by PCR and Southern blot analyses (data not shown). Blasto-

**Figure 1.** Generation of nonconditional Cx40A96S mice. (A) Homologous recombination of the nonconditional Cx40A96S vector with the genomic locus of the Cx40 gene leads to the recombinant allele mCx40A96SNeo. Besides the 5’ and 3’ homologous regions, the vector consists of the mutated Cx40A96S sequence followed by an IRES-eGFP construct and a neomycin resistance gene flanked by frt sites. Flp-mediated deletion of the neomycin resistance gene results in the genotype mCx40A96S. (B) Southern blot analysis with an internal probe on SacI digested liver DNA from different genotypes verified the homologous recombination in Cx40A96S mice. Detection of a 5.5-kb fragment indicates the Cx40 wild-type allele, a 7-kb fragment the Cx40A96S allele, and a 8.9-kb fragment the Cx40A96SNeo allele. (C) PCR genotyping of Cx40A96S mice results in a 290-bp fragment for the Cx40 wild-type allele, a 350-bp fragment for the Cx40A96S allele, and a 680-bp fragment for the Cx40A96SNeo allele.
cyst injections of three correctly recombined ES cell clones yielded germ-line transmission chimeras. Breeding of these animals with C57BL/6 mice resulted in heterozygous Cx40 +/A96SNeo offspring, which were used for the generation of a Cx40 A96S/A96S mutated mouse line with ≈87.5% C57BL/6 genetic background. Deletion of the neomycin resistance gene was achieved by mating Cx40 +/A96SNeo animals with Flp-recombinase–expressing mice, yielding the genotype Cx40 +/A96S (Figure 1A, lower part). The different genotypes were tested by Southern blot analyses (Figure 1B) and PCR (Figure 1C).

**Analyses of Cx40A96S Mice**

The mice of the three genotypes (wt/wt, wt/A96S, and A96s/A96S [wt, wild type]) were not different in body weights (28.3 ± 3.2, 29.6 ± 2.3, and 27.8 ± 2.9 g, respectively) nor in kidney weights (196 ± 24, 198 ± 15, and 183 ± 18 mg, respectively). Moreover, there was no difference in fertility and survival rate between the three genotypes.

Because mutation of the Cx40 protein may alter its cellular processing and trafficking, we therefore first analyzed the expression pattern of Cx40 in the kidneys of homozygous Cx40A96S mice. We found punctate immunoreactivity of Cx40 in renin-expressing cells and in glomerular cells but a more line-shaped immunoreactivity in the interior of arterioles (Figure 2). The same staining pattern was seen in wild-type kidneys in which also renin-expressing cells, mesangial cells, and preglomerular endothelial cells stained positive for Cx40 (Figure 3). We conclude from these findings that the correct membrane insertion of mutated Cx40 protein is not disturbed. As a first indicator for major changes in the cardiovascular system, we measured systolic BP in the wild-type, heterozygous, and homozygous Cx40A96S mice. Homozygous Cx40A96S mice had an increased BP relative to wild-type mice, while heterozygous Cx40A96S mice had a normal BP (Figure 4). The elevated BP in Cx40A96S mice could be markedly lowered by treatment with a low salt diet (0.02% w/w NaCl) in combination with the ACE inhibitor enalapril (Figure 4). To determine if the increased BP could be related to an activated renin-angiotensin system in Cx40A96S mice, we determined plasma renin concentrations, as a measure for renin secretion, in the three genotypes. Basal plasma renin concentrations of homozygous Cx40A96S mice were increased about sixfold over wild-type controls (Figure 5, upper part). PRC values of heterozygous Cx40A96S mice tended to be higher, without reaching the level of significance (P > 0.05) (Figure 5, upper part). The high PRC values in homozygous Cx40A96S mice were not paralleled by accordingly elevated renin gene transcript levels in the kidney. mRNA abundance of basal kidney renin was only moderately increased by about 40% in homozygous Cx40A96S mice (P < 0.05) (Figure 6, upper part). Heterozygous Cx40A96S mice exhibited normal kidney renin mRNA levels. Enhanced renin secretion in combination with high BP, as seen in homozygous Cx40A96S mice, suggests that the renal pressure control of renin secretion is altered in these animals. We therefore determined the pressure dependency of renin secre-

![Figure 2.](image-url)
The number of renin-expressing cells markedly increased outside the vessel walls in the juxtaglomerular interstitium and occasionally in the glomerular stalk (Figure 8D). In wild-type animals renin-expressing cells recruited in a typical fashion along the media layer of afferent arterioles in a retrograde fashion (Figure 8C). The localization of renin-expressing cells in heterozygous Cx40A96S mice was very similar to wild-type mice both under basal conditions and under challenge by low salt in combination with ACE inhibition (not shown).

The number of renin-expressing cells in response to challenge was paralleled by changes of kidney renin mRNA abundance, which increased to similar levels in all three genotypes (Figure 6, lower part). Plasma renin concentrations reflecting renin secretion rates strongly increased to similar final values in response to challenge in all three genotypes (Figure 5, lower part).

Analyses of Cx40A96S Transfected HeLa Cells

To study the coupling properties of homotypic mouse Cx40A96S gap-junction channels, we generated stable HeLa cell transfectants expressing mouse Cx40A96S and we performed microinjection analyses with neurobiotin. In comparison to cells expressing the Cx40 wild-type protein,9 (Figure 9A) cells expressing the mutated Cx40A96S protein (Figure 9B) displayed clearly reduced neurobiotin transfer between neighboring cells as indicated by smaller coupling areas. Immunostaining of Cx40A96S protein verified the correct localization of the mutated protein at contact membranes of neighboring cells (Figure 9C) as described previously for HeLa cells expressing the mouse Cx40 wild-type protein.10 Thus, the A96S mutation does not impair the transport and insertion of the Cx40 protein.
into the plasma membrane. Statistical evaluation of the dye transfer experiments revealed an approximately 96% loss of cell-cell coupling in Cx40A96S cells compared with Cx40 wild-type cells (Figure 9D).

DISCUSSION

Our data show that the human A96S mutation of the connexin40 gene leads to hypertension and to abnormal renin regulation in mice. We found in transfected HeLa cells that expression of mouse Cx40A96S protein caused a similar decrease of gap-junctional coupling (approximately 96%) as expression of the human Cx40A96S protein in transfected N2A cells. Furthermore, the mouse Cx40A96S protein was located in contact membranes of transfected HeLa cells, suggesting that the mutated protein is normally transported intracellulary.

The overall renin-related phenotype of Cx40A96S mice is between that reported for mice lacking the Cx40 protein globally and that reported for renin cell–specific deletion of Cx40. The apparent difference between the reports on global and renin cell–specific deletion of Cx40 comprises ambient plasma renin activities and degree of basal renin expression, both of which are lower in mice with renin cell–specific deletion of Cx40 than in mice with global Cx40 deletion, while the stimulability of the renin system is greater in mice with renin cell specific deletion of Cx40.

We meanwhile realized that the renin phenotype of young mice with global Cx40 deletion is similar to that of renin cell–specific deletion of Cx40 and aggravates with increasing age/persistence of hypertension. In any case, all animal models, namely, global Cx40 deletion, renin cell–specific deletion of Cx40 and Cx40A96S missense mutation, share in common hypertension, absolute or relative renin hypersecretion, absence of pressure control of renin secretion, and aberrantly localized renin-producing cells. In contrast to the Cx40 deleted mice, however, mice bearing the Cx40A96S mutation expressed the mutated Cx40 protein in the kidneys in a cellular distribution pattern that was not different from that of wild-type mice. In particular, renin-producing cells, endothelial cells, and mesangial cells incorporated the mutated Cx40 protein in the plasma membrane.

The combination of the findings of our present and of our previous studies now strongly suggest that the gap-junctional functions rather than possible scaffold functions of Cx40 are relevant for proper regulation of renin expression and secretion. Consequently, we can conclude for the first time that the main cause for the hypertension of Cx40 defective mice is most likely a loss of Cx40 gap-junctional coupling properties. We furthermore infer from our results that intact gap-junctional coupling of renin-secreting cells via Cx40 gap junctions is essential for the final localization of renin-secreting cells and for the control of renin secretion by BP via the renal perfusion pressure. In this regard not only gap-junctional channels containing exclusively Cx40 protein subunits could play an important role. It has been described that Cx40 forms heteromeric channels with Cx37 and Cx43, which are also expressed in renin-expressing cells, although it is not clear if Cx40/Cx43 heteromeric channels play a functional role. In the absence of any wild-type Cx40 protein the mutated Cx40A96S protein...
could also affect the coupling properties of all heteromeric channels. Consequently, any gap-junctional coupling among renin-expressing cells themselves and the neighboring cells could be impaired, which may contribute to the renal phenotype described here. This suggestion is in accordance with the results of Gollob et al., who described a transdominant negative effect of the missense mutation on the coupling properties in cultured cells coexpressing both the mutated Cx40A96S and the wild-type Cx43 protein. The nature of intercellular signals passing through Cx40-containing gap junctions in renin-secreting cells to determine their localization and their pressure sensitivity needs to be investigated in future experiments.

Apart from this central question this study also opens further aspects that need to be considered. We have previously reported that the yet unknown mechanisms of renin expression and secretion as well as the correct localization of renin-expressing cells. Therefore, the number of aberrant renin-expressing cells in Cx40A96S kidneys could be increased by a treatment that induces the well-known retrograde recruitment of renin-expressing cells in wild-type kidneys. We infer from this finding that the yet unknown signals that induce renin expression in resident renin precursor cells are in principle also active in aberrantly located precursor cells.

In summary, the analysis of Cx40A96S mice provided new insights into the mechanisms underlying renin-dependent hypertension. Functional coupling through Cx40 channels is required for regulatory mechanisms of renin expression and secretion as well as the correct localization of renin-expressing cells.

**CONCISE METHODS**

**Construction of the Nonconditional Cx40A96S Vector**

The Cx40A96S mutation identified in a patient was generated by PCR mutagenesis and cloned into the vector pH85 in pBluescript (8.5-kb HindIII fragment from the phage clone 2A-40 in pBluescript) which contained the coding sequence of mCx40 together with the corresponding 5’ and 3’ homologous regions. In this step the Cx40 wild-type gene was exchanged by the Cx40A96S sequence using Nhel/Acc digestions. To limit the size of the targeting vector and to enable a PCR screening of recombined ES cell clones, the 3’ homologous region of the vector pH85 in pBluescript was shortened to 1.5 kb by partial digestion with AccI and XhoI. Furthermore, for more efficient homologous recombination, the 5’ homologous region of pH85 in pBluescript was elongated with a 1.2-kb PCR fragment amplified from genomic DNA using the primers (Cx40_5’HR_for: 5’-ggg atc acg tgt gct tgt cc3’ and Cx40_5’HR_rev: 5’-ctt gca tgt tgt tat gcc cac tg-3’) by AccI/HindIII digestion. Downstream of the Cx40A96S coding region we inserted the IRES-eGFP construct from the vector IRES-eGFP-Zeo17 by EcoRI/EcoRV and AccI digestions, respectively. In the final step we cloned the neomycin resistance gene driven by a PGK (phosphoglycerate kinase) promoter and flanked by frt (Fip recognition target) sites from the vector pBluescript: frt-Neo-frt by AflII digestions, respectively. For more efficient homologous recombination, the 5’ homologous region of pH85 in pBluescript was elongated with a 1.2-kb PCR fragment amplified from genomic DNA using the primers (Cx40_5’HR_for: 5’-ggg atc acg tgt gct tgt cc3’ and Cx40_5’HR_rev: 5’-ctt gca tgt tgt tat gcc cac tg-3’) by AccI/HindIII digestion. Downstream of the Cx40A96S coding region we inserted the IRES-eGFP construct from the vector IRES-eGFP-Zeo17 by EcoRI/EcoRV and AccI digestions, respectively. In the final step we cloned the neomycin resistance gene driven by a PGK (phosphoglycerate kinase) promoter and flanked by frt (Fip recognition target) sites from the vector pBluescript: frt-Neo-frt into the vector described above by Sall and AflIII digestions, respectively. The final nonconditional Cx40A96S vector was analyzed by...
Figure 9. Coupling of Cx40A96S gap junctions is impaired. Coupling analysis of homotypic Cx40A96S gap-junction channels in stably expressing HeLa cells. Injection of neurobiotin (brown) into single cells (marked with black asterisks) leads to intercel-lular dye transfer between neighboring cells. Cx40WT-expressing cells (A) show clearly higher transfer properties than Cx40A96S cells (B); bar 20 μm. Immunoreactivity of Cx40A96S (green) in stably transfected HeLa cells (C) indicates the localization of the mutated protein in contact membranes of neighboring cells (marked with arrow); bar 20 μm. Statistical analysis of the coupling experiments reveals a 96% loss of coupling among Cx40A96S HeLa cells compared with Cx40WT-expressing HeLa cells (D). Data are means ± SEM of ≥20 injections in each cell line.

Screening of ES Cell Clones

For transfection of HM1 ES cells via electroporation (0.8 kV, 3 μF), 300 μg of the nonconditional Cx40A96S vector were linearized by NolI digestion. Screening of positively transfected ES cells was carried out with 350 μg/μl G418-neomycin (Invitrogen, Karlsruhe, Germany). Resulting ES cell clones were tested by two different PCRs. PCR1 amplifies the 3′ homologous region (Cx40_3′HR_for: 5′-cga tac gtc gga ggg aag cca tcc-3′ and Cx40_3′HR_rev: 5′-ccc cca tca aat ctg cca ccc-3′) and PCR 2 the Cx40A96S gene (Cx40A96S_5′ of NheI: 5′-ggg gca ggc tca tct tgt gaa gct c-3′ and Between_IREs_and_Cx40: 5′-gat gca tgc ctc acg cgt-3′). ES cell clones which were positive in both PCRs were further characterized by Southern blot analyses for recombination at the 5′ homologous region (external probe) as well as for single integration of the vector construct (internal probe). DNA extracted from PCR positive clones was digested with NsiI (external probe) or PstI and SpeI (internal probe). After electrophoresis in an agarose gel the digested DNA was transferred on Hybond-N+ membranes (Amersham Biosciences, Buck, U.K.) and fixed to the membrane via cross-linking by exposure to UV light. The external and internal probes were radioactively labeled with α-32PdCTP (Amersham Biosciences). Hybridization with the membranes was performed in Quick-Hyb solution (Stratagene, La Jolla, CA) at 6°C for 2 hours. The external probe was generated by PCR on genomic DNA with primers amplifying a 985-bp fragment of the 5′ homologous region of the Cx40 gene (ext._probe_for: 5′-ctt tgc cct gtc ctg cat g-3′ and ext._probe_rev: 5′-gac aca ttc ttc cag ggt tgg g-3′). The internal probe consisted of a 872-bp AflII-IpsII-fragment from the coding region of Cx40.

Generation of Cx40A96S Mice

Three positively recombined ES cell clones were injected into C57BL/6 blastocysts. Resulting high-extent fur colored chimeras were bred with C57BL/6 mice and agouti colored offspring were genotyped by PCR analyses of isolated tail DNA. Heterozygous Cx40 +/A96SNeo mice were backcrossed several times to increase the C57BL/6 genetic background to at least 87.5%. Additionally, Cx40 +/A96S mice were mated to Flp-recombinase–expressing mice to delete the neomycin resistance gene via the ftr/Flp system. To verify correct homologous recombina-tion in Cx40A96S transgenic mice by Southern blot analyses, DNA was extracted from livers of mice with different genotypes and digested with SacI. Analyses with the internal probe were performed as described for screening of ES cell clones. For PCR genotyping a combination of three different primers (Between_cgFP_and_Frt_for: 5′-ggt act atg ccc atc ctc ttg-3′; Between_Neo_and_Frt_for: 5′-ggg tga tag agg cag acc tac-3′; and 3′_HR_rev: 5′-gca tga agg ctg aga cat c-3′) resulted in a 290-bp fragment for the Cx40 wild-type allele, a 350-bp fragment for the Cx40A96S allele and a 680-bp fragment for the Cx40A96SNeo allele.

Treatment of Mice

All experimental animals were performed according to the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and were approved by the local ethics committee. Mice were kept under standard housing conditions with a 12-hour/12-hour dark/light cycle, and with food (0.4% w/w NaCl, Ssniff, Soest, Germany) and water ad libitum. Salt depletion was induced by feeding a low-salt diet (0.02% w/w NaCl, Ssniff) in combination with the ACE-inhibitor enalapril (10 mg/kg added to the drinking water) for 7 days. This treatment regimen was expected to strongly enhance renin gene expression and renin secretion. Blood samples (75 μl) for the determination of the plasma renin concentration were taken from the tail vein into hematocrit tubes containing 1 μl of 125 mmol/L EDTA to prevent clotting. Thereafter, the mice were deeply

restriction mapping and partial sequencing (AGOWA, Berlin, Germany). The functionality of the ftr sites was tested by transformation of the targeting vector into Flp-recombinase expressing Escherichia coli bacteria.19
anesthetized with sevoflurane and killed by cervical dislocation. The kidneys were removed quickly. One was frozen and stored in liquid nitrogen for further determination of different mRNAs and the contralateral kidney was cryoconserved for the preparation of cryosections. In addition, the kidneys from two animals of each group were perfusion-fixed with 4% paraformaldehyde for the preparation of paraffin sections.

Measurement of Arterial Pressure
Measurements of the systolic arterial pressure were performed noninvasively by tail cuff manometry (TSE, Germany). Before the first BP determination the animals were habituated to the experimental procedure by placing them into the holding device on 5 successive days. BP was measured daily over 1 week under each treatment regimen.

Determination of Renin mRNA by Real-Time PCR
Total RNA was isolated from the frozen kidneys as described by Chomczynski and Sacchi. The CDNA was synthesized by MMLV reverse transcriptase (Superscript, Invitrogen). For quantification of renin mRNA expression real time RT-PCR was performed using a Light Cycler Instrument (Roche Diagnostics Corp) and the Quanti-Tect SYBR Green PCR kit (Qiagen) and GAPDH as a control. The primers were chosen to span over two exon-intron borders, to avoid amplification of genomic DNA. To verify the accuracy of the amplification, a melting curve analysis was performed after amplification and PCR products were analyzed on an ethidium bromide–stained agarose gel. For amplification of mouse renin and β-actin cDNAs, the following primers were used: renin: 5'-atg aag ggg tgt ctt gtt ggg-3' (sense), 5'-atg cgg gga ggg tgg gca cct-3' (antisense); β-actin: 5'-cgg gat ccc cgc cct agg cac cag ggt g-3' (sense), 5'-gga att agg ctt ggg tgt tga agg tct caa a-3' (antisense).

Determination of Plasma Renin Concentration
For determination of plasma renin concentration the blood samples taken from the tail vein were centrifuged and the plasma was incubated for 1.5 hours at 37°C with plasma from bilaterally nephrectomized male rats as renin substrate. The generated AngI [ng/ml per min] was determined by RIA (Byk & Diasorin Diagnostics, Germany) as described previously. Renin secretion rates were calculated as the product of the renin activity and venous flow rate [ml/min*g kidney weight].

Immunohistochemical Analyses for Renin, α-Smooth Muscle Actin, and Cx40
The expression of renin and α-smooth muscle actin were localized by immunohistochemical methods. In brief, kidneys were perfusion-fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Immunolabeling was performed on 5-μm paraffin sections. After being blocked with 10% horse serum and 1% BSA in PBS, sections were incubated with either anti-renin (generated by Davids Immunotechnologie, Regensburg, Germany) or anti–smooth muscle actin (Beckman Coulter, Immunotech, Marseille, France) overnight at 4°C, followed by incubation with a fluorescence secondary antibody (Dianova, Hamburg, Germany). Antibodies used for immunostaining of Cx40 and α-smooth muscle actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Kidneys were frozen unfixed in TissueTek OCT embedding medium (Sakura Finetek, Heppenheim, Germany) and sectioned at 5 μm with a cryostat. Without further storing, sections were fixed in methanol at −20°C for 20 minutes, washed three times in PBS, and blocked in a buffer containing PBS, 1% BSA, and 10% horse serum for 30 minutes. Primary antibodies were diluted in the same blocking solution using anti-Cx40 (1:100), anti-renin (1:200), and anti–α-smooth muscle actin (1:10) in respective combinations, incubating sections at 4°C overnight. On the next day, sections were washed three times in PBS containing 1% BSA and incubated with combinations of Cy2, TRITC, or Cy5 secondary antibodies (Dianova, Hamburg, Germany) for 90 minutes at room temperature, diluted 1:400, 1:300, and 1:400, respectively. After being washed in PBS, sections were mounted with Dakocytomation Glycergel mounting medium (Dako, Glostrup, Denmark) and viewed with an Axiosvert Microscope (Zeiss, Jena, Germany).

Generation of Cx40A96S-Expressing HeLa Cells
Coupling-deficient HeLa cells were stably transfected by lipofection with a vector construct containing the Cx40A96S coding sequence under the control of a CMV (cytomegalovirus) promoter. Furthermore, the vector included a puromycin resistance gene driven by a SV40 (Simian virus 40) promoter for the selection of successfully transfected clones.

Immunofluorescence Analyses for Cx40 in HeLa Cells
Cx40A96S stably expressing HeLa cells were cultivated in Dulbecco modified Eagle medium with 10% FCS on coverslips in a 24-well plate up to a confluency of 80 to 90%. The cells were fixed in ethanol at −20°C for 10 minutes, washed two times in PBS, and blocked with 5% newborn goat serum, 5% BSA, and 0.1% Triton X-100 in PBS at 4°C overnight. Primary rabbit Cx40 antibodies (BioTrend, Cologne, Germany) were diluted 1:400 in blocking so-
lution and the cells were incubated for 1 hour at room temperature. On the next day, the cells were washed three times with PBS and incubated with Alexa Fluor 488 secondary antibodies (Invitrogen, Darmstadt, Germany) for 1 hour at room temperature, diluted 1:1000 in blocking solution. After being washed three times in PBS, the coverslips were mounted with Glycergel mounting medium (Dako, Glostrup, Denmark) and viewed with a Laser Scanning Microscope (Zeiss, Jena, Germany).

Microinjection Analyses of HeLa Cells

All HeLa cells were cultivated up to confluency and washed in PBS before microinjection to remove dead cells. During the microinjection of neurobiotin with iontophoresis, the cells were kept at 37°C. For each cell line tested, at least 20 injections were performed and analyzed. After the last injection the cells were fixed with 1% glutaraldehyde in PBS for 5 minutes at room temperature, washed three times with PBS, and permeabilized with 2% Triton X-100 in PBS at 4°C overnight. On the next day, the cells were washed twice with PBS and incubated in 0.1% horseradish peroxidase–conjugated Avidin D (Vector Laboratories, Burlingame, CA) solution for 90 minutes at room temperature. After three washing steps with PBS, the staining of neurobiotin-containing cells was performed by incubation of the cells in DAB (diaminobenzidine) staining solution (0.01% hydrogen peroxide in 0.06% DAB in TBS) for a few seconds at room temperature until brown-colored cells appeared. The staining reaction was stopped by washing three times in PBS and the cells were microscopically viewed and counted.

Statistical Analysis

Values are presented as means ± SEM. For in vivo experiments differences between groups were analyzed by ANOVA test and Bonferroni’s test for multiple comparisons. For individual BP changes induced by low salt treatment and for cell culture experiments paired t test was applied. P < 0.05 was considered statistically significant.

ACKNOWLEDGMENTS

The study was financially supported by grants of the German Research Foundation: WA 2137/2-1, SFB 699, and W:270/33-1 as well as SFB 645, project B2, to K.W. I.L. gratefully acknowledges a Ph.D. stipend of the Jürgen-Manchot-Foundation.

DISCLOSURES

None.

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