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## Potassium channels related to primary aldosteronism: expression similarities and differences between human and rat adrenals

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### Abstract

Three potassium channels have been associated with primary aldosteronism (PA) in rodents and humans: KCNK3 (TASK-1), KCNK9 (TASK-3), and KCNJ5 (Kir3.4). Mice with deficiency in *Kcnk3* and *Kcnk9* have elevated aldosterone production and blood pressure. In humans, adrenal tumors with somatic mutations in *KCNJ5* cause PA. However, there are very few reports on the expression patterns of these genes in humans versus rodents. Herein, we compared human and rat mRNA expression (by quantitative real-time polymerase chain reaction (qPCR) and protein levels (by immunohistochemistry) across three tissues (adrenal, brain, heart) and two laser-captured adrenal zones (zona glomerulosa, zona fasciculata). Our findings show that expression patterns of KCNK3, KCNK9, and KCNJ5 are inconsistent between rats and humans across both tissues and adrenal zones. Thus, species variation in the expression of PA-related potassium channels indicates an evolutionary divergence in their role in regulating adrenal aldosterone production.

### Keywords

aldosterone; primary aldosteronism; potassium (K<sup>+</sup>); zona glomerulosa; zona fasciculata

## 1. Introduction

### 1.1. Primary aldosteronism (PA)

Aldosterone controls sodium homeostasis, and its physiological production in the adrenal zona glomerulosa (ZG) is tightly controlled by the renin-angiotensin-aldosterone-system. However, in about 8% of hypertensive patients, aldosterone is autonomously produced due to aldosterone-producing adenoma (APA) or idiopathic hyperaldosteronism, both of which are major causes of primary aldosteronism (PA) (Chao et al., 2013; Young, 2007). It is important to detect and treat PA in hypertensive patients, since aldosterone excess in PA

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patients is associated with a higher likelihood of cardiovascular complications (Milliez et al., 2005).

## 1.2. KCNK3 and KCNK9 (TASK-1 and TASK-3)

Circulating potassium ( $K^+$ ) is well recognized as one of the major regulators of ZG aldosterone production. ZG cells respond to small changes in circulating  $K^+$  because they are depolarized by  $K^+$  and selectively permeable to  $K^+$ , such that they effectively act as  $K^+$  electrodes (Guagliardo et al., 2012). Therefore,  $K^+$  channels significantly influence ZG cell membrane potential. Intriguingly, it has been suggested that each species may utilize a different set of  $K^+$  channels to maintain membrane potential in ZG cells (Guagliardo et al., 2012). KCNK3, or TWIK-related acid-sensitive  $K^+$  channel (TASK)-1, and KCNK9 (TASK-3) are both members of the KCNK family of two-pore-forming domain  $K^+$  channels, which are also known as  $K^+$ -selective leak channels (Goldstein et al., 2001). Members of this channel family produce weak rectifying currents and are generally active at resting cell membrane potentials. These channels generate background (or “leak”)  $K^+$  conductances that play a key role in maintaining negative membrane potential (Bayliss et al., 2003). A recent study demonstrated that human KCNK5 (TASK-2) mRNA and protein have lower expression in APAs compared with the normal human adrenal cortex, and that H295R cells transfected with a KCNK5 dominant-negative mutants increased *CYP11B2* expression and aldosterone production (Lenzini et al., 2014). Furthermore, other studies have shown that mice lacking *Kcnk3* (Heitzmann et al., 2008), *Kcnk9* (Guagliardo et al., 2012; Penton et al., 2012), or both *Kcnk3* and *Kcnk9* (Davies et al., 2008) in the adrenal develop PA. From these findings, it has been hypothesized that KCNK3 and KCNK9 may play an important role in PA in humans; however, expression patterns of these  $K^+$  channels have not previously been characterized in the human adrenal gland to this date.

## 1.3. KCNJ5 (Kir3.4)

Potassium inwardly-rectifying channel, subfamily J, member 5 (KCNJ5 or Kir3.4) is a G protein-activated inwardly-rectifying  $K^+$  (Kir) channel, forming tetrameric complexes of two membrane-spanning domains. Like other Kir channels, KCNJ5 regulates cell membrane potential (Hibino et al., 2010). Recently, *KCNJ5* gene somatic mutations have been found in human APAs (Choi et al., 2011). KCNJ5 may have an important role in physiological and pathological aldosterone production because KCNJ5 protein is strongly expressed in the human ZG and in APA (Choi et al., 2011; Monticone et al., 2012). Furthermore, it was reported that normal (non-mutated) KCNJ5 participated in AngII-stimulated aldosterone production in the human adrenocortical cell line HAC15 (Oki et al., 2012). However, despite these studies in humans, detailed analyses of KCNJ5 expression in mouse and rat adrenal glands have not been described.

## 1.4. Summary of this study

In this study,  $K^+$  channel (KCNK3, KCNK9, KCNJ5) expression patterns were compared between two species: humans and rats. By quantitative real-time polymerase chain reaction (qPCR) and immunohistochemical analyses, we characterized mRNA and protein expression levels across three tissues (adrenal, brain, heart) and three adrenal zones [ZG,

zona fasciculata (ZF), medulla]. Intriguingly, our results demonstrated a divergence between humans and rats in the expression of PA-related K<sup>+</sup> channels.

## 2. Materials and Methods

### 2.1. Human tissue samples

For human tissue comparison, human tissue RNA samples (four adrenal, three brain, and three heart) were purchased from Clontech (Mountain View, CA), BioChain Institute (Newark, CA), System Biosciences (Mountain View, CA), and Becton, Dickinson and Company (Franklin Lakes, New Jersey). For human *KCNK9* qPCR, two additional brain RNA samples were used. RNA was then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA).

### 2.2. Human adrenal collection for laser capture microdissection (LCM) and immunohistochemistry (IHC)

Use of the human tissues for LCM and IHC was approved by the Institutional Review Board of Georgia Regents University. After obtaining informed consents from the families, normal human adrenal samples were collected from renal transplantation donors at Georgia Regents University. Extracted adrenals were immediately transferred into cold Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, 1:1; Gibco, NY). Then, the adrenal glands were cleaned of adherent tissues and one piece each of 3–4 mm-thick adrenal was frozen in embedding media (Tissue-Tek Optimum Cutting Temperature (O.C.T.) compound; Sakura Finetek U.S.A, Inc., Torrance, CA) for LCM or fixed in 10% formaldehyde, embedded in paraffin, and serially sectioned in 5 μm sections onto glass microscope slides for IHC, as previously reported (Nishimoto et al., 2012). A total of nine human adrenals were used for LCM (six male, two female): Donor Adrenal Normal (DAN) 24 (male, 30 years old), #28 (female, 43 years old), #31 (male, 29 years old), #32 (male, 17 years old), #45 (female, 37 years old), #47 (male, 46 years old), #48 (male, 39 years old), #50 (male, 43 years old), and #55 (male, 19 years old). Hypertension data, unfortunately, is not available for these deceased donors. Frozen adrenal glands in O.C.T. compound were cut in 7 μm serial sections onto Superfrost Plus Microscope Slides (Fisher Scientific, Loughborough, UK) or PEN-Membrane Slides (Leica, Germany). These sections were stained with cresyl violet (Sigma, Rochester, NY) by following the manufacturer's ribonuclease-free protocol in the HistoGene LCM Frozen Section Staining Kit (Molecular Devices, Sunnyvale, CA); the xylene wash step was excluded when staining PEN-Membrane Slides. To collect enriched populations of aldosterone-producing ZG cells, the first of the five to seven serially sectioned slides was immunostained for CYP11B2 as a reference, and five to seven layers of CYP11B2 positive cells immediately beneath the capsule were carefully laser captured from the adjacent unstained sections. ZF cells were captured from lipid-rich cells in the middle layer. Adrenal medulla cells were also captured. RNAs from ZG, ZF, and medulla cells were isolated using the PicoPure RNA isolation kit (Molecular Devices) as previously described (Nishimoto et al., 2012). Total RNA (1–10ng) from ZG, ZF, and medulla samples was submitted to the University of Michigan DNA Sequencing Core (UMDSC) MicroArray Core Facility for amplified cDNA preparation. RNA amplification and reverse transcription were performed using the Ovation Pico WTA System V2 (NuGEN Technologies, Inc., San

Carlos, CA). The cDNA was then purified using the QIAquick PCR Purification Kit (Qiagen, Germantown, Maryland) and used for qPCR analyses.

### 2.3. Rat tissue samples

All rat procedures carried out were reviewed and approved by the University of Michigan's University Committee on the Use and Care of Animals or the Georgia Regents University Institutional Animal Care and Use Committee. For tissue comparison, 5 male and 4 female Sprague-Dawley (SD) rats were acquired from the Rodent Recycling Program at the University of Michigan Medical School. These male and female rats were sacrificed by rapid decapitation at an age of average  $\pm$  S.E.M:  $10.2 \pm 1.1$  and  $10.9 \pm 1.1$  weeks, respectively. Whole adrenal tissue, segments of brain cortex, and segments of heart left ventricle were extracted, cleaned of surrounding connective tissues, and homogenized with 600 $\mu$ L of RLT Buffer Plus (QIAGEN, Germantown, Maryland) containing 1% beta-mercaptoethanol (SIGMA Life Science, St. Louis, MO) as samples of 'adrenal', 'brain', and 'heart', respectively. RNA from the homogenized tissue was isolated using the RNeasy Plus Mini Kit (QIAGEN) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit.

### 2.4. Rat adrenal collection for LCM and IHC

As previously described, five eleven-week-old male SD rats (Harlan) on a normal sodium (NS) diet were sacrificed, and their adrenal glands were extracted and frozen in O.C.T. compound for LCM or fixed in 4% paraformaldehyde and paraffin-embedded for IHC. Cells from rat ZG and ZF cells were laser captured and amplified cDNA was prepared (Nishimoto et al., 2012).

### 2.5. Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed using cDNA from human and rat tissues and amplified cDNA from laser captured ZG and ZF cells from human and rat adrenal gland (see 2.1. to 2.3.). For qPCR, either 5 ng (human/rat tissues) of cDNA or 1 ng (human/rat isolated ZG/ZF) of amplified cDNA was mixed with Fast Universal PCR Master Mix (Applied Biosystems) and primer/probe mix specific for each gene, except for human *KCNK9*. Human *KCNK9* primers were mixed with cDNA as mentioned above and SYBR Green PCR master mix (Applied Biosystems). In instances where 1 ng of the isolated adrenal zones showed no detectable signal, we repeated the reactions using 5 ng of template. Except for human *CYP11B2* and human *KCNK9*, all primer/probe mixes were purchased from Applied Biosystems: human *KCNK3*, Hs00605529\_m1; human *KCNJ5*, Hs00168476\_m1, rat *Cyp11b2*, Rn02396730\_g1; rat *Kcnk3*, Rn04223042\_m1; rat *Kcnk9*, Rn00755967\_m1; and rat *Kcnj5*, Rn01789221\_mH. For human *CYP11B2* qPCR, primer/Taqman probe mix was prepared as previously reported (Ye et al., 2007). Human *KCNK9* was amplified using specific primers designed in the 3' UTR of human *KCNK9* transcript variant 1 (NM\_001282534); forward 5'-ACC TTT CCA GCC AGA CAG AG - 3' and reverse 5' - AAG GAG GAG ATC CAT GCT TC - 3'. Peptidylprolyl isomerase A (*PPIA*, cyclophilin A) transcript was used for normalization of sample loading (Hs99999904\_m1 for human cDNA and Rn03302269\_gH for rat cDNA).

## 2.6. IHC

IHC was performed for human and rat serial tissue sections using pH 9 Target Retrieval Solution (Dako, Glostrup, Denmark), an EnVision horseradish peroxidase-labeled secondary antibody (Dako), and 3, 3'-diaminobenzidine (brown color) for visualization, as previously reported (Nishimoto et al., 2012). For positive control tissue, a rat heart 4% paraformaldehyde-fixed paraffin-embedded section (see 2.4) was used. For all tissues, negative controls were run omitting the primary antibody from the protocol. Primary antibodies, dilutions, and suppliers are listed in Table 1.

## 2.7. Statistics for qPCR

The  $2^{-Ct}$  method was used to calculate fold changes (Livak and Schmittgen, 2001). DNA levels that were not detectable by RT-PCR were assigned a Ct value of 40 for analysis, and the S.E.M. of fold change was calculated based on delta delta Ct values. Statistical differences were determined either by student's t-test across adrenal zones or by one-way ANOVA across tissues using delta Ct values in SigmaPlot 12.5 software (Systat Software, Inc), as previously reported (Nishimoto et al., 2014). P values below 0.05 were considered statistically significant.

## 3. Results

### 3.1. Tissue and zone confirmation

In order to confirm the aldosterone-producing ability in whole adrenal tissue and ZG, we first compared CYP11B2 expression levels in three tissues (adrenal, brain, and heart) and two zones (ZG and ZF). In tissue comparisons, *CYP11B2* mRNA in human adrenal, expressed as [mean  $\pm$  S.E.M.], was: 9414.5  $\pm$  6944.6-fold and 2788.6  $\pm$  2057.0-fold higher than that in brain (1.0  $\pm$  0.7,  $p < 0.001$ ) and heart (1.0  $\pm$  0.8,  $p < 0.001$ ), respectively (Figure 1A). In human zone comparisons, *CYP11B2* in ZG was 207.4  $\pm$  330.2-fold higher than that in ZF (1.0  $\pm$  1.0,  $p < 0.01$ ) (Figure 1B). Consistent with *CYP11B2* mRNA expression, IHC showed distinct CYP11B2 protein expression in the ZG, as previously reported (Nishimoto et al., 2010) (Figure 1C). As expected, the rat tissue and adrenal zone expression was similar to that in humans. *Cyp11b2* mRNA in rat adrenal was 10287.9  $\pm$  9377.0-fold and 3466.7  $\pm$  3159.8-fold higher than those in brain (1.0  $\pm$  0.9,  $p < 0.001$ ) and heart (1.0  $\pm$  1.2,  $p < 0.001$ ), respectively (Figure 1D). In rat zone comparisons, *Cyp11b2* in ZG was 125914.3  $\pm$  45735.1-fold higher than that in ZF (1.0  $\pm$  2.7,  $p < 0.001$ ) (Figure 1E). CYP11B2 protein was clearly expressed in the ZG, as previously reported (Mitani et al., 1994) (Figure 1E). In summary, CYP11B2 expression in human and rat tissues and zones was confirmed, ensuring that tissue collection and laser capturing were accurate.

### 3.2. KCNK3 (TASK-1) expression in human and rat adrenal glands

*Kcnk3* expression levels have been partially described in mouse (Davies et al., 2008), but have not been characterized in the human or rat adrenal gland. *KCNK3* mRNA in human adrenal was 17.7  $\pm$  4.0-fold and 44.3  $\pm$  10.0-fold higher than those in brain (1.0  $\pm$  0.3,  $p < 0.001$ ) and heart (1.0  $\pm$  0.1,  $p < 0.001$ ), respectively (Figure 2A). In human zone comparisons, *KCNK3* in ZG was 4.0  $\pm$  2.0-fold higher than in ZF (1.0  $\pm$  0.8-fold,  $p = 0.17$ ) (Figure 2B). Consistent with *KCNK3* mRNA expression, KCNK3 protein displayed slightly

higher expression in ZG compared to ZF (Figure 2C). In rat tissue comparisons, *Kcnk3* mRNA was also high in adrenal but higher in heart. *Kcnk3* in adrenal was  $19.6 \pm 4.3$ -fold ( $p < 0.001$ ) and in heart was  $43.0 \pm 5.1$ -fold ( $p < 0.001$ ) higher than that in brain ( $1.0 \pm 0.1$ ) (Figure 2D). In rat zone comparisons, *Kcnk3* in ZG was  $4.9 \pm 0.4$ -fold higher than that in ZF ( $1.0 \pm 0.6$ ,  $p < 0.05$ ) (Figure 2E), which differs from a mouse analysis in a previous report (Davies et al., 2008). Consistent with mRNA expression patterns, KCNK3 protein was expressed in the ZG (Figure 2F). Thus, the expression patterns of KCNK3 in human and rat were clearly determined. We found similar KCNK3 expression patterns between human and rat adrenals.

### 3.3. KCNK9 (TASK-3) expression in human and rat adrenal glands

Similar to *Kcnk3*, *Kcnk9* expression levels have been partially described in mouse (Davies et al., 2008) but have not been previously characterized in rat or human adrenal. In human tissue comparisons, *KCNK9* was highest in brain at  $83.7 \pm 78.0$ -fold and  $1.2 \pm 1.1$ -fold higher than heart ( $1.0 \pm 0.9$ ,  $p < 0.01$ ) and adrenal ( $1.0 \pm 0.3$ ,  $p = 0.826$ ), respectively, but not significantly different from the adrenal (Figure 3A). In human zone comparisons, *KCNK9* was highest in the medulla, at  $177.5 \pm 92.5$ -fold higher than ZG ( $1.0 \pm 1.4$ ,  $p < 0.01$ ), and not detectable in ZF (Figure 3B). Consistent with the mRNA expression patterns, KCNK9 protein was detected in human adrenal medulla (Figure 3C). Of note, the IHC images of the adrenal cortex and adrenal medulla in Figure 3C were taken from the same adrenal section. Thus, these tissue regions were processed by IHC under the same conditions. In contrast, for rat tissue, although the expression level was not different between adrenal and brain, *Kcnk9* mRNA in adrenal ( $1073.9 \pm 190.5$ -fold) was higher than that in heart ( $1.0 \pm 0.9$ ,  $p < 0.05$ ) (Figure 3D). Furthermore, rat zone comparisons showed that *Kcnk9* levels in ZG were  $35.6 \pm 6.9$ -fold above ZF ( $1.0 \pm 0.5$ ,  $p < 0.01$ ) (Figure 3E). Consistently, IHC displayed high KCNK9 protein expression in rat ZG (Figure 3F). In summary, KCNK9 expression patterns differed between human and rat adrenal.

### 3.4. KCNJ5 (Kir3.4) expression in human and rat adrenal glands

After the important discovery of *KCNJ5* mutations in human aldosterone-producing adenomas (Choi et al., 2011), a number of human KCNJ5 studies have been performed, including reports on KCNJ5 localization by IHC (Monticone et al., 2012). However, KCNJ5 expression in the mouse and rat adrenal gland has not previously been characterized. In human tissue, *KCNJ5* mRNA was highest in adrenal at 287.3-fold and  $10.2 \pm 1.7$ -fold higher than brain ( $1.0 \pm 0.7$ ,  $p < 0.001$ ) and heart ( $1.0 \pm 0.2$ ,  $p < 0.001$ ), respectively (Figure 4A). Human zone comparisons showed  $6.5 \pm 3.7$ -fold higher *KCNJ5* levels in ZG above ZF ( $1.0 \pm 0.5$ ,  $p < 0.05$ ) (Figure 4B). Consistent with mRNA expression patterns and previous reports, KCNJ5 protein was expressed in human ZG (Figure 4C). On the other hand, rat tissue comparisons showed *Kcnj5* mRNA highest in heart at  $59.7 \pm 9.9$ -fold and  $55.2 \pm 9.1$ -fold higher than adrenal ( $1.0 \pm 0.3$ ,  $p < 0.001$ ) and brain ( $1.0 \pm 0.1$ ,  $p < 0.001$ ), respectively (Figure 4D). Furthermore, in rat zonal comparisons, *Kcnj5* mRNA was not detectable in either ZG or ZF (Figure 4E). IHC was consistent, showing distinct KCNJ5 expression in heart tissue and minimal signal in the ZG and ZF. Faint signal is considered to be non-specific background staining (Figure 4F). Of note, the IHC in the Figure 4F was simultaneously



performed in rat adrenal and heart under the same conditions. In general, KCNJ5 expression patterns differed between human and rat across both tissues and adrenal zones.

## 4. Discussion

### 4.1. Summary and impact

Adrenal aldosterone production is tightly regulated by angiotensin II and circulating  $K^+$ . Both agonists act through intracellular calcium signaling pathways to stimulate adrenal aldosterone biosynthesis and release. Recent studies have demonstrated that gene mutations in several  $K^+$  channels alter intracellular calcium signaling, leading to excess adrenal aldosterone production. These include mouse genetic deletion of *Kcnk3* and *Kcnk9* and human germline or somatic mutations in the ion selectivity filter of *KCNJ5*. Because of the importance of these channels in rodents and humans, we undertook the current study in mRNA and protein expression patterns of KCNK3, KCNK9, and KCNJ5 in rat and human adrenal. Our findings suggest that species differences should be considered when studying the role of these channels in adrenal aldosterone production.

### 4.2. KCNK3 and KCNK9 (TASK-1 and TASK-3)

**4.2.1. Rodent**—KCNK3 and KCNK9 have recently been identified as important regulators of  $K^+$  conductance related to the maintenance of ZG cell membrane potential. Genetic deletion of *Kcnk3* and/or *Kcnk9* in mice removes an important background  $K^+$  current that leads to elevated adrenal aldosterone production and a model of PA. Furthermore, *Kcnk3* and *Kcnk9* knockout mice did not exhibit suppression of aldosterone production with dietary sodium loading. In normal mice, *Kcnk3* mRNA was localized to the ZG and ZF, while *Kcnk9* mRNA was expressed primarily in the ZG and medulla (Davies et al., 2008). In our immunohistochemical analyses of rats, KCNK3 protein was localized primarily in the ZG and outer ZF, and KCNK9 protein was localized in the ZG. We also found that *Kcnk3* mRNA was expressed at significantly higher levels in the ZG compared with the ZF, suggesting a role in aldosterone production in rats, as has previously been demonstrated in mice. Furthermore, we saw no sex difference in rat adrenal gland *Kcnk3* or *Kcnk9* expression (Supplemental Figure 1). In addition, we determined that *Kcnk9* mRNA is expressed in the ZG at significantly higher levels than those of the ZF, consistent with previous demonstrations of the key regulatory role of KCNK9 protein in  $K^+$  conductance in ZG cells. In summary, our zonation studies demonstrate that increased KCNK3 and KCNK9 expression in the ZG is consistent between mice and rats.

**4.2.2. Human**—The role of KCNK3 in human adrenal aldosterone production is not clear. Little is known regarding KCNK3 expression in the human adrenal other than in comparison to KCNK family members. These studies suggest that KCNK3 is more highly expressed compared with other KCNK members. Microarray analysis has shown that this pattern holds true in not only normal adrenals, but also in APA and H295R adrenal cells. Genetic knockdown of *KCNK3* in adrenal H295R cells resulted in upregulation of steroidogenic acute regulatory (StAR) protein and aldosterone synthase. These cells exhibited increased intracellular calcium, resulting in CaMK activation and increased CYP11B2 expression (Nogueira et al., 2010). As for other KCNK family members, lower levels of KCNK5 have

been associated with APA and increased aldosterone synthesis (Lenzini et al., 2014), suggesting that KCNK5 plays an important role in adrenal aldosterone production. Our expression studies of KCNK3 in the adrenal cortex suggest a trend towards higher expression in ZG versus ZF in human, based on higher (but not statistically significant) mRNA fold expression and apparent stronger immunohistochemical staining in ZG versus ZF. On the other hand, KCNK9 was not detected in human ZF, while positive and significant expression was observed in the medulla and in brain tissue. However, the relatively low mRNA expression of *KCNK9* transcripts in the ZG, especially compared with the medulla, suggests a possibly lesser role in adrenal aldosterone production.

**4.2.3. Species Comparison**—KCNK3 expression patterns appear similar between human and rat adrenals, while KCNK9 expression patterns are inconsistent between rodents and humans. In rats, *Kcnk3* mRNA expression levels in ZG cells are higher than those in ZF, and similarly in humans, there is a possible trend towards higher *KCNK3* mRNA expression levels in the ZG compared to the ZF. Our studies localized rat KCNK3 protein mainly to ZG and outer ZF cells, while previous studies showed mouse *Kcnk3* mRNA throughout the adrenal cortex. Based on our findings, the tissue distributions for *KCNK3* and *KCNK9* among adrenal, brain, and heart differ somewhat between rats and humans. Furthermore, absolute levels of *KCNK9* transcripts appear to be higher in the rat adrenal than in the human adrenal (Supplemental Figure 2). Within the adrenal, *Kcnk9* in both rats and mice is primarily expressed in ZG, with little expression in ZF. Humans, on the other hand, have undetectable levels of *KCNK9* in ZF and relatively low levels in ZG, suggesting an important aldosterone-regulating role of KCNK9 in rodents but possibly a lesser role in humans.

### 4.3. KCNJ5

**4.3.1. Rodent**—KCNJ5, particularly in humans, has been recently pinpointed as a key regulator of ZG cell membrane potential, and several mutations in the channel have been linked to adrenal disorders. However, its role in rodent adrenal aldosterone production has not been determined. The role of KCNJ5 in mice has been previously studied in the heart (Liang et al., 2014; Mesirca et al., 2013) and in a mouse cardiac muscle cell line (Nobles et al., 2010). In rats, KCNJ5 has also been studied in cardiac tissue (Atkinson et al., 2013; Bingen et al., 2013), and in kidney tissue of obese rats (Kang et al., 2013). However, to date, no studies have investigated the role of KCNJ5 in the adrenal cortex or in aldosterone regulation in rodents. Our study suggests that, at least in rats, *Kcnj5* mRNA and KCNJ5 protein are not expressed in the adrenal cortex. This suggests that KCNJ5 does not play a role in adrenal steroid production in rats.

**4.3.2. Human**—KCNJ5 and other K<sup>+</sup> channels maintain the negative membrane potential of human ZG cells under basal conditions. Mutations in and near the selectivity filter of KCNJ5 result in loss of selectivity, leading to sodium entry, membrane depolarization, calcium mobilization, and aldosterone synthesis. 30–60% of APAs have mutations in the selectivity filter of KCNJ5 (Gomez-Sanchez and Oki, 2014). KCNJ5 overexpression in the HAC15 adrenocortical cell line downregulated membrane voltage, intracellular calcium, and *CYP11B2* mRNA. Furthermore, activation of KCNJ5 blocked angiotensin II-stimulated



membrane voltage and suppressed aldosterone production (Oki et al., 2012). In agreement with earlier data, KCNJ5 protein was expressed in both ZG and the outer ZF, with higher *KCNJ5* mRNA expression in ZG. These findings are consistent with the previously established role of KCNJ5 in the regulation of aldosterone production, including effects of mutations.

**4.3.3. Species Comparison**—While *Kcnj5* mRNA is expressed at undetectable levels in rat adrenal ZG and ZF, KCNJ5 in humans is more strongly expressed in ZG compared with ZF, and its key role in normal and pathological aldosterone production has been described in many reports.

#### 4.4. Overall Summary

The current study supports the finding that while several KCNK family members (especially KCNK9) appear to dominate regulation of ZG K<sup>+</sup> currents in mice and rats, KCNJ5 appears to play this important role in humans. Our data reveal distinctly different expression patterns of these K<sup>+</sup> channels between humans and rats. Species variation in the expression patterns of these K<sup>+</sup> channels highlights a possible evolutionary divergence in their regulation of aldosterone production. Furthermore, this inconsistency across species suggests that rodents may not be ideal models for some aspects of human adrenal disease, including the role of KCNJ5 in normal or pathologic production of aldosterone production.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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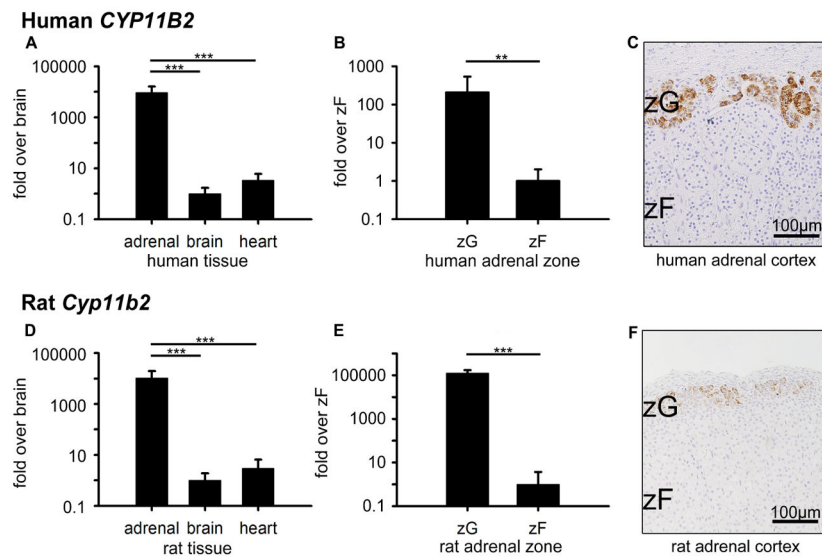
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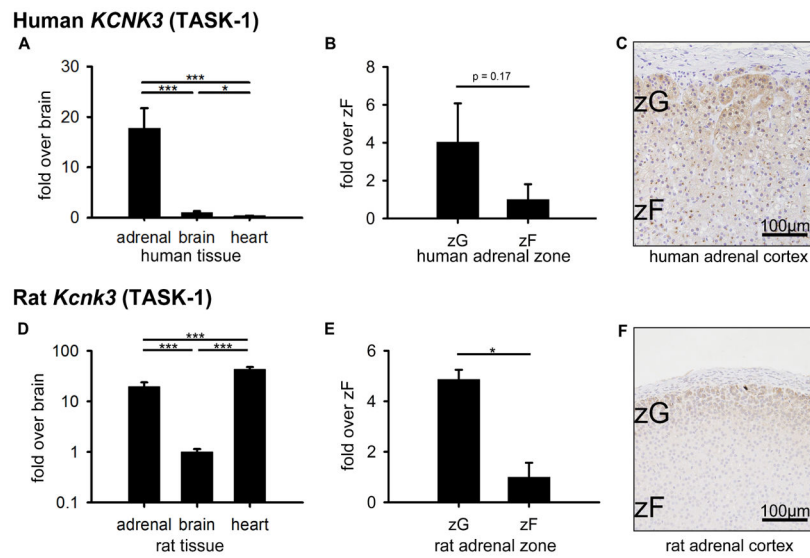
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### Highlights

- We examine three potassium channels related to primary aldosteronism in humans and rats.
- Expression patterns of primary aldosteronism-related potassium channels differ between these species.
- Species variation should be considered when studying the role of these channels.

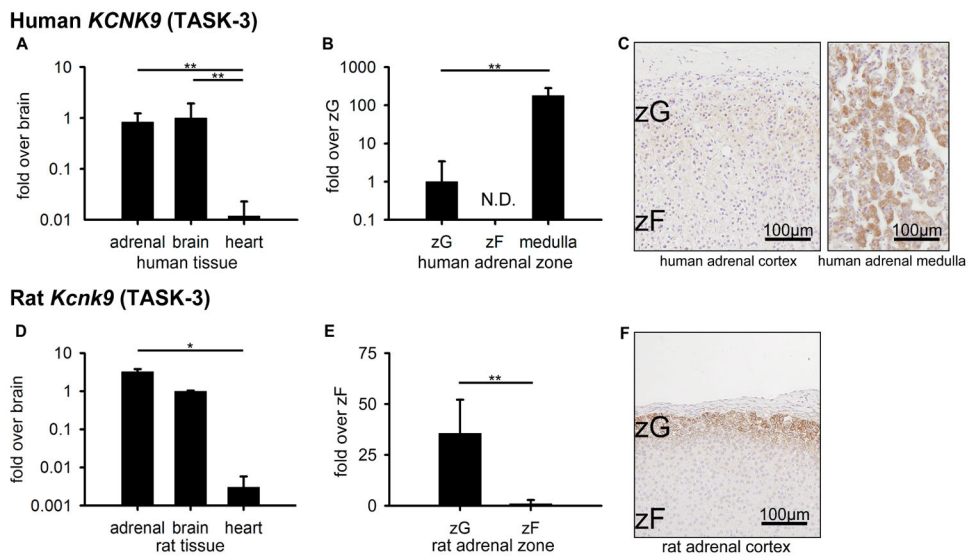


**Figure 1.** *CYP11B2* expression in human (A–C) and rat (D–F). (A, D) Quantitative RT-PCR analysis for *CYP11B2* mRNA across adrenal, brain, and heart tissues. (B, E) Quantitative RT-PCR analysis for *CYP11B2* mRNA across ZG and ZF adrenal zones. Values represent mean  $\pm$  S.E.M. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (C, F) Immunohistochemical analyses of *CYP11B2* protein localization in ZG and ZF adrenal zones, plus hematoxylin nuclear counterstain.

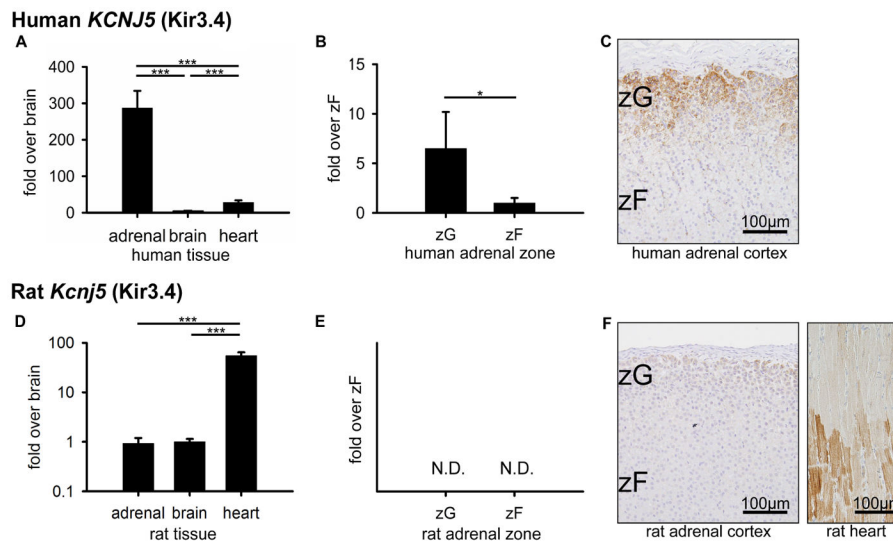


**Figure 2.** *KCNK3* (TASK-1) expression in human (A–C) and rat (D–F). (A, D) Quantitative RT-PCR analysis for *KCNK3* mRNA across adrenal, brain, and heart tissues. (B, E) Quantitative RT-PCR analysis for *KCNK3* mRNA across zG and zF adrenal zones. Values represent mean  $\pm$  S.E.M. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (C, F) Immunohistochemical analyses of *KCNK3* protein localization in zG and zF adrenal zones, plus hematoxylin nuclear counterstain.





**Figure 3.** *KCNK9* (TASK-3) expression in human (A–C) and rat (D–F). (A, D) Quantitative RT-PCR analysis for *KCNK9* mRNA across adrenal, brain, and heart tissues. (B, E) Quantitative RT-PCR analysis for *KCNK9* mRNA across ZG, ZF, and adrenal medulla. Values represent mean  $\pm$  S.E.M. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (N.D.) Not detectable by quantitative RT-PCR. (C) Immunohistochemical analyses of *KCNK9* protein localization in human ZG and ZF adrenal zones and human adrenal medulla (positive control), plus hematoxylin nuclear counterstain. Note: the immunohistochemistry was performed in human ZG, ZF, and medulla simultaneously under the same conditions. (F) Immunohistochemical analysis of *KCNK9* protein localization in rat ZG and ZF adrenal zones, plus hematoxylin nuclear counterstain.



**Figure 4.** *KCNJ5* (Kir3.4) expression in human and rat. (A, D) Quantitative RT-PCR analysis for *KCNJ5* mRNA across adrenal, brain, and heart tissues. (B, E) Quantitative RT-PCR analysis for *KCNJ5* mRNA across ZG and ZF adrenal zones. Values represent mean  $\pm$  S.E.M. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (N.D.) Not detectable by quantitative RT-PCR. (C) Immunohistochemical analyses of *KCNJ5* protein localization in human ZG and ZF adrenal zones, plus hematoxylin nuclear counterstain. (F) Immunohistochemical analysis of *KCNJ5* protein localization in rat ZG and ZF adrenal zones and rat heart tissue (positive control), plus hematoxylin nuclear counterstain. Note: the immunohistochemistry was performed in rat adrenal and rat heart simultaneously under the same conditions.

**Table 1**

Primary antibodies used in IHC, human and rat

	Target	Reference number/Citation	Source	Dilution
<b>HUMAN</b>	CYP11B2	(Gomez-Sanchez et al., 2014)	Dr. Celso Gomez-Sanchez, M.D. (Univ. of Mississippi Medical Center)	1:100
	KCNK3 (TASK-1)	NBP1-83070	Novus Biologicals	1:100
	KCNK9 (TASK-3)	37678	Signalway Antibody	1:1000
	KCNJ5 (Kir3.4)	HPA017353	SIGMA Life Science	1:100
<b>RAT</b>	CYP11B2	(MacKenzie et al., 2000)	Dr. Celso Gomez-Sanchez, M.D. (Univ. of Mississippi Medical Center)	1:200
	KCNK3 (TASK-1)	NBP1-83070	Novus Biologicals	1:1000
	KCNK9 (TASK-3)	LS-B3980	LifeSpan Biosciences	1:1000
	KCNJ5 (Kir3.4)	HPA017353	Sigma-Aldrich	1:200

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