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Impaired hepatic uptake by organic anion-transporting polypeptides is associated with hyperbilirubinemia and hypercholanemia in *Atp11c* mutant mice

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Running title: Impaired hepatic uptake by Oatps in *Atp11c* mutant mice

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List of abbreviations:

BAs; bile acids, Bsep; bile salt export pump, $CL_{bile, liver}$; biliary clearance normalized by the liver concentration, $CL_{bile, plasma}$; biliary clearance normalized by circulating plasma concentration, CL_{total}; total plasma clearance, CM; canalicular membrane, $C_{ss, liver}$; liver concentrations at steady-state, $C_{ss, plasma}$; plasma concentrations at steady-state, $E_2$17βG; 17β estradiol 17β-D-glucuronide, LPMs; liver plasma membranes, Mrp2; multidrug resistance associate protein 2, NaK; Na^+, K^+-ATPase α1 subunit, Ntcp; Na^+-taurocholate co-transporting polypeptide, Oatps; organic anion transporting polypeptides, PFIC; progressive familial intrahepatic cholestasis, qPCR; quantitative PCR, SEM; standard error of the mean, TA; taurocholic acid, $V_{ss, bile}$; biliary excretion rate at steady-state,

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Abstract

Biliary excretion of organic anions such as bile acids (BAs) is the main osmotic driving force for bile formation, and its impairment induces intrahepatic cholestasis. We investigated the involvement of Atp11c in the hepatic transport of organic anions using *Atp11c* mutant mice, which exhibit hypercholanemia and hyperbilirubinemia. Pharmacokinetic analysis following a constant intravenous infusion in *Atp11c* mutant mice showed decreased hepatic sinusoidal uptake and intact biliary secretion of [^{3}H]-17β estradiol 17β-D-glucuronide ([^{3}H]-$E_2$17βG). Consistent with this result, compared with cells and membranes from control mice, isolated hepatocytes and liver plasma membranes (LPMs) from *Atp11c* mutant mice had a much lower uptake of [^{3}H]-$E_2$17βG and expression of organic anion-transporting polypeptides (Oatps), which are transporters responsible for hepatic uptake of unconjugated BAs and organic anions including bilirubin–glucuronides. Uptake of [^{3}H]-TC into hepatocytes and expression of Na^+-taurocholate cotransporting polypeptide in LPMs, which mediates hepatic uptake of conjugated BAs, was also lower in the *Atp11c* mutant mice. Bile flow rate, biliary BA concentration, and expression of hepatobiliary transporters did not differ between *Atp11c* mutant mice and control mice. These results suggest that Atp11c mediates the transport of BAs and organic anions across the sinusoidal membrane, but not the canalicular membrane, by regulating the abundance of transporters. *Atp11c* is a candidate gene for genetically undiagnosed cases of hypercholanemia and hyperbilirubinemia but not of intrahepatic cholestasis. This gene may influence the pharmacological and adverse effect of drugs because Oatps regulate their systemic exposure.

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Introduction

The biliary excretion of bile acids (BAs) and glutathione from the hepatocyte is the main osmotic driving force for bile formation. Transport of BAs across the sinusoidal membrane is mediated by uptake transporters, Na^+-taurocholate cotransporting polypeptide (Ntcp) (Hagenbuch and Meier, 1994; Hagenbuch et al., 1991) and organic anion transporting polypeptides (Oatp) (Meier and Stieger, 2002; Trauner and Boyer, 2003). After reaching the canalicular membrane (CM), monovalent taurine- and glycine-conjugated BAs (such as taurocholic acid (TC)) and sulfated BAs amidates and bile salt ethereal glucuronides (divalent) are secreted into bile by the bile salt export pump (Bsep) (Byrne et al., 2002; Gerloff et al., 1998; Hayashi et al., 2005b; Noe et al., 2002) and the multidrug resistance-associated protein 2 (Mrp2) (Akita et al., 2001; Kuipers et al., 1988; Takikawa et al., 1991), respectively, both of which are ATP-binding cassette transmembrane transporters located on the CM. Impaired transport of BAs across the CM causes intrahepatic cholestasis, a state of reduced bile flow that is an acquired or inherited syndrome, and results in hepatocellular damage because a high concentration of BAs is toxic.

One of the most severe forms of intrahepatic cholestasis is progressive familial intrahepatic cholestasis (PFIC), a heterogeneous group of inherited autosomal recessive liver diseases. This group of diseases is characterized by intrahepatic cholestasis and jaundice in the first year of life and progression to severe cholestasis with sustained intractable itching, jaundice, watery diarrhea, and failure to thrive, which eventually leads to liver failure and death before adulthood (Davit-Spraul et al., 2009; Morotti et al., 2011). PFIC is subdivided based on the serum GGT level and the causal genes (Davit-Spraul et al., 2009). *ATP8B1* and *ABCB11* have been identified as the genes responsible for PFIC associated with a normal GGT level (Bull et al., 1998; Strautnieks et al., 1998). However, about one-third of affected individuals with a normal GGT level have no mutations in either gene (Davit-Spraul et al., 2010). In PFIC patients with mutations in *ABCB11*

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encoding BSEP, biliary BAs secretion is impaired because of the compromised expression of BSEP and/or abolished transport activity of BSEP (Hayashi et al., 2005a; Wang et al., 2002). ATP8B1 is a member of the P4 subfamily of P-type adenosine triphosphatases (P4-ATPases) and translocates aminophospholipid from the outer leaflet to the inner leaflet in lipid bilayer of biological membranes (Paulusma et al., 2008; Ray et al., 2010; Takatsu et al., 2014). It has been suggested that a deficiency in ATP8B1 causes secondary suppression of BSEP-mediated BAs excretion into bile by disrupting the well-organized aminophospholipid asymmetry in the CM (Paulusma et al., 2009; Paulusma et al., 2006) or through mass action related to the decreased expression of BSEP mRNA (Chen et al., 2004).

Considering these underlying mechanisms, we have begun to search for agents that have therapeutic potency in patients with mutations in *ATP8B1* and *ABCB11*. We have published experimental and clinical evidence that 4-phenylbutyrate, a drug used to treat ornithine transcarbamylase deficiency, has an additional pharmacological effect that increases the expression of BSEP on the CM (Hayashi and Sugiyama, 2007) and improves liver function and/or pruritus in these PFIC patients (Hasegawa et al., 2014; Naoi et al., 2014). The development of therapeutic methods to treat other PFIC patients and understanding of the mechanism underlying intrahepatic cholestasis caused by inherited genetic defects require the identification of other genes that are causally associated with PFIC in the remaining 30% of patients with a normal GGT level.

Recently, two independent groups have constructed mutant mice that have inherited an N-ethyl-N-nitrosourea-induced point mutation in *Atp11c* (Siggs et al., 2011a; Yabas et al., 2011) that encodes a P4-type ATPase and functions as an aminophospholipid flippase (Segawa et al., 2014; Takatsu et al., 2014; Yabas et al., 2011). These mutant mice developed cell-intrinsic arrest of adult B lymphopoiesis and presented with anemia and hyperbilirubinemia (Siggs et al., 2011a; Yabas et al., 2014; Yabas et al., 2011). Subsequent detailed analysis of hyperbilirubinemia involving bone marrow transplantation and biochemical

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testing indicated that the hyperbilirubinemia derived from a deficiency in the function of Atp11c in nonhematopoietic cells. *Atp11c* mutant mice showed elevated total plasma BAs and bilirubin levels, but normal GGT level, compared with control mice (Siggs et al., 2011b). Considering that *Atp11c* is expressed predominantly in the liver (Siggs et al., 2011b), in the present study, we used *Atp11c* mutant mice to examine whether *ATP11C* could be the causal gene of PFIC associated with a normal GGT level. The influence of Atp11c deficiency on hepatobiliary transport was evaluated *in vivo* using a constant intravenous infusion of [^{3}H]-TC and [^{3}H]-17β estradiol 17β-D-glucuronide ([^{3}H]-$E_2$17βG) into *Atp11c* mutant mice and subsequent pharmacokinetic analysis and *in vitro* biological, histological, and transport functional experiments using livers from the mutant mice.

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Materials and Methods

Reagents and antibodies

[^{3}H]-TC (2 Ci/mmol) and [^{3}H]-$E_2$17βG (55 Ci/mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). Antibodies against Atp11c, Bsep, and Ntcp were purchased from Abcam (Cambridge, MA). Anti-Mrp2, and Na^+, K^+-ATPase α1 subunit (NaK) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antisera to Mrp3, Oatp1b2, and Oatp1a4 were raised in rabbits as described previously (Hirohashi et al., 2000; Ose et al., 2010; Sasaki et al., 2004). All other chemicals were of analytical grade.

Mice

The mouse strain with an *Atp11c*^{emptyhive} allele, which contains an X-linked N-ethyl-N-nitrosourea-induced point mutation in *Atp11c* on a pure C57BL/6J background

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(C57BL/6J-*Atp11c*^{m1Btlr}/Mmjax) (Siggs et al., 2011a; Siggs et al., 2011b), was obtained from the Mutant Mouse Regional Resource Center (www.mmrrc.org). This strain was maintained by breeding of hemizygous male mice (*Atp11c*$^{emptyhive/Y}$) with heterozygous female mice (*Atp11c*$^{emptyhive/+}$). At 9-14 weeks of age, the mice were fasted for 12 h and used for the experiments.

Genotyping of offspring was performed using genomic DNA isolated from tail biopsies. The *Atp11c*^{emptyhive} allele has a 2113C>T mutation in *Atp11c* that confers resistance to restriction by MboI to *Atp11c*. A partial sequence (359 base pairs) of *Atp11c*, which included the mutated sites in the *Atp11c*^{emptyhive} allele, was amplified using PCR involving 5′-TGAGCTACAGCACTCAGCAGC-3′ (forward) and 5′-TTGGAAAAGGCGGCAGGCATAGC-3′ (reverse) as primers. The samples were digested by MboI for 1 h at 37°C, and analyzed by electrophoresis. The products from male control (*Atp11c*$^{+/Y}$) mice and female control (*Atp11c*$^{+/+}$) mice were digested into two bands of 242 and 117 base pairs, whereas those from male hemizygous (*Atp11c*$^{emptyhive/Y}$) mice and female homozygous (*Atp11c*$^{emptyhive/emptyhive}$) mice were detectable as a single band of 359 base pairs (Figure 1A). All three bands were observed in the products from female heterozygous (*Atp11c*$^{+/emptyhive}$) mice.

All animals were maintained under standard conditions with a reversed dark–light cycle and were treated humanely. Food and water were available ad libitum. The studies reported in this manuscript were carried out in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan).

Analysis of serum, liver, and bile composition

Biochemical testing of serum was outsourced to Oriental Yeast Co. (Tokyo, Japan). Lipids and BAs were extracted from serum, liver, and bile using the Bligh-Dyer method (Bligh and Dyer, 1959) and then

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subjected to enzymatic measurement of BAs concentration (Wako Pure Chemical Industries, Tokyo, Japan).

***In vivo* infusion study in mice**

Male mice (9-14 weeks old) were fasted for 12 h and then subjected to an *in vivo* infusion study using [^{3}H]-TC and [^{3}H]-$E_2$17βG as described (Hayashi et al., 2012b; Hayashi and Sugiyama, 2007; Hirano et al., 2005). [^{3}H]-TC or [^{3}H]-$E_2$17βG in saline was infused through the jugular vein cannula at a rate of 500 and 13.3 pmol/min/kg for 60 min, respectively. Blood and bile specimens were obtained at specified times. The radioactivity associated with the plasma, bile, and liver was measured in a liquid scintillation counter (LS 600SC; Beckman, Fullerton, CA) as described previously (Hayashi et al., 2012b; Hayashi and Sugiyama, 2007).

Pharmacokinetic analysis.

The total plasma clearance (CL_{total}), biliary clearance normalized by circulating plasma concentration ($CL_{bile, plasma}$), and biliary clearance normalized by the liver concentration ($CL_{bile, liver}$) were calculated from the equations $CL_{total} = I/C_{ss, plasma}$, $CL_{bile, plasma} = V_{ss, bile}/C_{ss, plasma}$, and $CL_{bile, liver} = V_{ss, bile}/C_{ss, liver}$, respectively, where I represents the infusion rate (pmol/min/kg), $C_{ss, plasma}$ represents the steady-state plasma concentrations (nM), $V_{ss, bile}$ represents the steady-state biliary excretion rate (pmol/min/kg), and $C_{ss, liver}$ represents the average steady-state hepatic concentration (pmol/g liver) (Hayashi et al., 2012b; Hayashi and Sugiyama, 2007). The steady-state plasma concentrations ($C_{ss, plasma}$) was determined as the mean of the plasma concentration of [^{3}H]-TC or [^{3}H]-$E_2$17βG at 30, 45, and 60 min. The steady-state biliary excretion rate at steady-state ($V_{ss, bile}$) was determined as the mean of the biliary excretion rate for [^{3}H]-TC or [^{3}H]-$E_2$17βG from 30 to 45 min and from 45 to 60 min. $C_{ss, liver}$ was determined as the average hepatic concentration of [^{3}H]-TC or [^{3}H]-$E_2$17βG at

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the end of the in vivo experiment.

Uptake study using isolated hepatocytes

Hepatocytes were isolated from *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice using the collagenase perfusion method (Yamazaki et al., 1993), suspended in Krebs–Henseleit buffer, and adjusted to 2.0 × 10 ^{6} cells/ml. The hepatocyte suspensions were prewarmed at 37°C for 3 min and then subjected to the uptake assay as described previously (Hirano et al., 2004). The uptake of [^{3}H]-TC and [^{3}H]-$E_2$17βG into hepatocytes at the designed time points was evaluated by measuring the amount of intracellular [^{3}H]-TC or [^{3}H]-$E_2$17βG.

Western blot analysis

Liver plasma membranes (LPMs) was prepared from *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice, loaded to the wells of 7 or 10% SDS-PAGE gel with a 3.75 % stacking gel, and then subjected to Western blot analysis as described previously (Hayashi et al., 2012a; Hayashi et al., 2012b; Hayashi and Sugiyama, 2007; Mizuno et al., 2015). Immunoreactivity was detected with an ECL Prime Western Blotting Detection Reagent (Amersham Biosciences, Piscataway, NJ). The intensity of the band indicating each protein was quantified using Multi Gauge software (v.2.0; Fujifilm, Tokyo, Japan).

Determination of mRNA levels.

Total RNA from the liver specimens of mice was isolated using ISOGEN II (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Reverse transcription was performed with ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Tokyo, Japan). The mRNA level for each protein were measured by real-time quantitative PCR (qPCR) using a LightCycler 480 system II (Roche Diagnostics,

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Mannheim, Germany) as described previously (Hayashi et al., 2012b; Hayashi and Sugiyama, 2007; Naoi et al., 2014). The following primer sequences were used: 5′-CTGTCATCAATGTGGGCAAC-3′ (forward) and 5′-CTGTTTCCATGCTGATGGTG-3′ (reverse) for Ntcp, 5′-TAGCTTGCCTCCAGTATGCCTT-3′ (forward) and 5′-ACAGGCCAAATGCTATGTATGC-3′ (reverse) for Oatp1a1, 5′-CAAGCTTTCTCCCTGCACTCTT-3′ (forward) and 5′-TCCTTCGCAGTGAGCTTCATT-3′ (reverse) for Oatp1a4, 5′-AGCAATGATCGGACCAATCCT-3′ (forward) and 5′-AACCCAACGAGCATCCTGA-3′ (reverse) for Oatp1b2, and 5′-AGAACATCATCCCTGCATCC-3′ (forward) and 5′-CACATTGGGGGTAGGAACAC-3′ (reverse) for Gapdh. Gene expression for each reaction was normalized by the internal control (Gapdh).

Immunohistochemistry

The liver was removed from *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice, cut into small pieces, embedded in OCT compound (Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen. Ten-micrometer-thick sections were prepared from the frozen sections using a cryostat (Leica CM 1950; Leica, Solms, Germany), mounted onto cover glasses (Matsunami Glass Ind. Ltd., Osaka, Japan), air-dried, fixed in 4% paraformaldehyde/PBS for 10 min, permeabilized in 0.1% saponin/PBS for 10 min, blocked with 3% BSA/PBS for 30 min, and stained with primary antibodies for 2 h and then with the appropriate Alexa Fluor-conjugated secondary antibodies for 1 h. These staining procedures were performed at room temperature. The specimens were mounted with VECTASHIELD mounting medium (Vector Laboratories Inc., Burlingame, CA) and then visualized by confocal microscopy using a Leica TCS SP5 II laser scanning confocal microscope.

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Statistical analysis

Experiments were repeated at least three times, and the data are presented as mean ± standard error of the mean (SEM). *p*-values between two variables were calculated at the 95% confidence level using unpaired Student's *t* tests. The data were analyzed using Prism software (GraphPad Software, La Jolla, CA).

Results

Expression of Atp11c in LPMs from mice

LPMs were prepared from *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice and analyzed by Western blot analysis. The immunosignal derived from the antibody against Atp11c was observed as two bands, about 120 and 90 kDa, in LPMs from *Atp11c*$^{+/Y}$ mice, but was undetectable in those from *Atp11c*$^{emptyhive/Y}$ mice (Figure 1B). The smaller band of Atp11c could be produced by caspase-mediated cleavage (Segawa et al., 2014). As reported previously, the liver function tests showed markedly elevated concentrations of total and direct bilirubin and total BAs, and normal activity of GGT in the plasma of *Atp11c*$^{emptyhive/Y}$ mice (Siggs et al., 2011a) (Table 1). Moderate but significant increases in ALT, AST, ALP, and LAP were observed in *Atp11c*$^{emptyhive/Y}$ mice.

Hepatobiliary transport of [^{3}H]-TC and [^{3}H]-$E_2$17βG in *Atp11c*$^{emptyhive/Y}$ mice

The constant intravenous infusion study with [^{3}H]-TC, a typical BA that is taken up predominantly into hepatocytes by Ntcp and excreted into bile by Bsep, showed that the $C_{ss, plasma}$ (plasma concentrations at steady-state), $C_{ss, liver}$ (liver concentrations at steady-state), and $V_{ss, bile}$ (biliary excretion rate at steady-state) for [^{3}H]-TC did not differ significantly between *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice (Figure 2A, C and Table 2).

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Consequently, the CL_{total}, $CL_{bile, plasma}$, and $CL_{bile, liver}$ for [^{3}H]-TC, which represents its disappearance from the body, vectorial transport from the sinusoidal space to the canaliculus through hepatocytes, and hepatobiliary transport across the canalicular membrane, respectively, did not differ significantly between *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice (Table 2). The bile flow rate during the infusion assay with [^{3}H]-TC was almost the same in the *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice and was constant in both mice strains (Figure 2E).

[^{3}H]-$E_2$17βG undergoes vectorial transport through Oatps and Mrp2, which mediate the hepatic uptake of unconjugated BAs (Meier et al., 1997; van de Steeg et al., 2010) and biliary excretion of sulfated BAs amidates and BAs ethereal glucuronides (divalent) (Akita et al., 2001; Kuipers et al., 1988; Takikawa et al., 1991), respectively. To gain further insight into the hepatobiliary transport of BAs, [^{3}H]-$E_2$17βG was infused intravenously into *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice. The $C_{ss, liver}$ and $V_{ss, bile}$ of [^{3}H]-$E_2$17βG did not differ significantly between these mice (Figure 2D and Table 3), whereas the $C_{ss, plasma}$ of [^{3}H]-$E_2$17βG was 2.1-fold higher in *Atp11c*$^{emptyhive/Y}$ mice than in *Atp11c*$^{+/Y}$ mice (Figure 2B and Table 3). The bile flow rate during the infusion assay with [^{3}H]-$E_2$17βG was almost the same in the *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice and was constant in both mice (Figure 2F). Consequently, the CL_{total} and $CL_{bile, plasma}$ for [^{3}H]-$E_2$17βG were about 50 and 60% lower in *Atp11c*$^{emptyhive/Y}$ mice than in *Atp11c*$^{+/Y}$ mice (Table 3). By contrast, the $CL_{bile, liver}$ for [^{3}H]-$E_2$17βG did not differ significantly between these mice. These results suggest that *Atp11c*$^{emptyhive/Y}$ mice exhibited decreased hepatic uptake, but with normal biliary excretion of $E_2$17βG and unconjugated BAs. Consistent with these results, the bile flow rate and biliary concentrations of BAs did not differ between *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice (Table 4).

Uptake of [^{3}H]-TC and [^{3}H]-$E_2$17βG into hepatocytes from *Atp11c*$^{emptyhive/Y}$ mice

To confirm the decreased hepatic uptake of [^{3}H]-TC and [^{3}H]-$E_2$17βG in *Atp11c*$^{emptyhive/Y}$ mice,

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hepatocytes were isolated from *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice and subjected to an uptake study. Time-dependent uptake of [^{3}H]-TC and [^{3}H]-$E_2$17βG into the hepatocytes from both mice was observed, but was markedly decreased in the presence of 100 μM unlabeled compounds (Figure 3A and B). The saturable component of the hepatic uptake for [^{3}H]-TC and [^{3}H]-$E_2$17βG, which was calculated from the slope of the hepatic uptake between 0.5 and 2 min, was compromised substantially in the hepatocytes from *Atp11c*$^{emptyhive/Y}$ mice. Its value for [^{3}H]-TC and [^{3}H]-$E_2$17βG in the hepatocytes from *Atp11c*$^{emptyhive/Y}$ mice was 18% and 6% of that in cells from *Atp11c*$^{+/Y}$ mice (Figure 3C and D), suggesting decreased function of the hepatic transporters responsible for uptake of BAs and bilirubin-glucuronides in *Atp11c*$^{emptyhive/Y}$ mice.

Hepatic expression of BAs transporters in *Atp11c*$^{emptyhive/Y}$ mice

Consistent with results of the *in vitro* uptake study using the isolated hepatocytes, analysis of LPMs by Western blot analysis showed markedly decreased expression of the sinusoidal transporters responsible for BAs uptake, Oatp1b2, Oatp1a4, and Ntcp, in *Atp11c*$^{emptyhive/Y}$ mice (Figure 4A). NaK, another sinusoidal membrane protein, was unaffected in *Atp11c*$^{emptyhive/Y}$ mice, suggesting that the role of Atp11c in the maintenance of the expression of sinusoidal membrane proteins is relatively specific for Oatps and Ntcp. In contrast to the altered expression of sinusoidal transporters for BAs uptake, the abundance of Bsep and Mrp2, both of which mediate biliary excretion of BAs across the CM, did not differ between mice. Together with the finding that *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice had similar expression level of Mrp3, which mediates the basolateral efflux of BAs and bilirubin-glucuronides and is upregulated by hepatic accumulation of bile constituents, this result is consistent with the intact $CL_{bile, liver}$ for [^{3}H]-TC and [^{3}H]-$E_2$17βG and normal bile flow rate and biliary BAs concentration in *Atp11c*$^{emptyhive/Y}$ mice (Table 2–4).

mRNA expression of hepatic BAs transporters was measured using qPCR and normalized by that of

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Gapdh. The mRNA expression levels of Oatp1b2, Ntcp, and Bsep were about 20% lower in *Atp11c*$^{emptyhive/Y}$ mice than in *Atp11c*$^{+/Y}$ mice (Figure 4B). However, this difference is unlikely to explain the markedly decreased expression of Oatp1b2 and Ntcp.

The immunohistochemical analysis of liver frozen sections showed basolateral localization of Atp11c in hepatocytes (Figure 4C). The immunosignal was undetectable in specimens from *Atp11c*$^{+/Y}$ mice.

Discussion

PFIC, a heterogeneous group of inherited autosomal recessive liver diseases, is attributed to the impaired transport of BAs across the CM of hepatocytes (Chen et al., 2004; Hayashi et al., 2005a; Paulusma et al., 2009; Wang et al., 2002). The causal gene of PFIC with the normal GGT is unidentifiable in about 30% of affected individuals (Davit-Spraul et al., 2010). *Atp11c* mutant mice present with a similar phenotype to PFIC (Siggs et al., 2011a; Yabas et al., 2014; Yabas et al., 2011). To explore whether *Atp11c* is a candidate gene that can explain genetically undiagnosed PFIC associated with the normal GGT level, in the current study, we analyzed the effects of Atp11c deficiency on the vectorial transport of BAs across the hepatocytes in *Atp11c* mutant mice. Unexpectedly, *Atp11c* mutant mice showed normal vectorial transport of [^{3}H]-TC across hepatocytes, but decreased hepatic sinusoidal uptake and intact biliary secretion of [^{3}H]-$E_2$17βG (Tables 2 and 3). This result can be explained by two observations: (1) the much lower expression of Oatps, including Oatp1b2 and Oatp1a4, which are sinusoidal transporters for the hepatic uptake of BAs and organic anions, and (2) the unaffected expression of Bsep and Mrp2, which are canalicular transporters for the biliary excretion of BAs, in *Atp11c* mutant mice compared with control mice (Figure 4A). *Slco1a/1b* cluster knockout mice exhibit hypercholanemia and hyperbilirubinemia because of

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compromised hepatic uptake of BAs and reuptake of conjugated bilirubin (van de Steeg et al., 2010); therefore the reduced function of Oatps, but not of BSEP and MRP2, is probably responsible for the hypercholanemia and hyperbilirubinemia observed in *Atp11c* mutant mice. This suggests that *Atp11c* may be a causal gene responsible for genetically undiagnosed cases of hypercholanemia and hyperbilirubinemia, such as in Rotor syndrome without mutations in *SLCO1B1* and *SLCO1B3*, but not for cases of PFIC.

Oatps mediate hepatic uptake of a wide variety of structurally-unrelated compounds including clinically used drug such as HMG-CoA reductase inhibitors (statins), angiotensin II receptor blockers (sartans), and angiotensin converting enzyme (ACE) inhibitors, and thereby regulate their dispositions (Giacomini et al., 2013; International Transporter et al., 2010). Genetic polymorphisms and drug-drug interactions that significantly alter the function of OATPs affect the systemic exposure of their substrates, which causes increased pharmacological effects and adverse reactions such as statin-induced myotoxicity (Giacomini et al., 2013; Group et al., 2008; International Transporter et al., 2010). Therefore, the results of the current study imply implications of Atp11c in drug disposition and thereby in its pharmacological and adverse effects. To our knowledge, this is the first report to suggest the association of P4-ATPases with disposition, pharmacological action, and toxicity of drugs. Several coding SNPs in *Atp11c* have been reported in the dbSNP database hosted by NCBI. If these influences on function and expression of Atp11c are confirmed, Atp11c should receive more attention in the attempt to improve drug development and to overcome interindividual variations in medical therapy.

It remains unknown why the basolateral expression of Oatps and Ntcp is markedly reduced in *Atp11c* mutant mice. One possible mechanisms is that Atp11c functions to maintain the well-organized aminophospholipid asymmetry of the basolateral membrane, as is the case of ATP8B1, which translocates phosphatidylserine from the outer leaflet to the inner leaflet at the hepatocanalicular membrane and thereby

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contributes to making it a rigid, liquid-ordered membrane (Paulusma et al., 2008; Paulusma et al., 2006). Disruption of membrane fluidity because of defective Atp11c function might alter the dynamics of Oatps and Ntcp on the basolateral membrane, which may destabilize and facilitate their degradation and thereby reducing the expression of these transporters on the basolateral membrane. This hypothesis is supported by the basolateral localization of Atp11c in hepatocytes (Figure 4C) and its function at the plasma membrane of cultured cells in the translocation of aminophospholipids into the inner leaflet (Segawa et al., 2014; Takatsu et al., 2014; Yabas et al., 2011). Alternatively, Atp11c may affect the trafficking of transport vesicles containing Oatps and Ntcp to the basolateral membrane as has been shown for Atp8a1 and P4-ATPases of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Chen et al., 2010; Lee et al., 2015; Liu et al., 2008). It has been suggested that these P4-ATPases form the membrane curvature and/or recruit membrane fission protein through the unidirectional transport of phospholipids from the luminal leaflet to the cytosolic leaflet in the organelles in which they localize, which helps to generate transport vesicles. Although Atp11c localizes predominantly on the plasma membrane in HeLa cells (Takatsu et al., 2011), its minor distribution to other organelle such as the Golgi apparatus and endosome in hepatocytes may influence the budding of transport vesicles containing Oatps and Ntcp to the basolateral membrane. Future research to identify the intracellular localization of Atp11c in hepatocytes will provide further insight into the role of Atp11c in the regulation of the basolateral expression of Oatps and Ntcp.

In conclusion, we have demonstrated that Atp11c is indispensable to maintaining the appropriate expression level of Oatps and is thereby involved in the hepatic uptake of BAs and organic anions, including conjugated bilirubin, across the sinusoidal membrane. These results suggest that *Atp11c* is a candidate gene that may be responsible for genetically undiagnosed cases of hypercholanemia and hyperbilirubinemia but not of PFIC. Our results are consistent with the recent preliminary results of Naik et al., which suggested

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Oatp1b2 contributes to hyperbilirubinemia and hypercholanema in *Atp11c* mutant mice (Naik et al., 2015). Atp11c may influence disposition, pharmacological effect, and toxicity of drugs because Oatps mediate the hepatic uptake of many drugs and consequently regulate their systemic exposure. Future research with global analysis to identify membrane proteins whose expression is affected by Atp11c provides further insight into the regulatory mechanism of the sinusoidal membrane proteins by Atp11c and expands the understanding of the physiological and pharmacological importance of Atp11c.

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Acknowledgements

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Authorship contributions

Participated in research design: Hayashi

Conducted experiments: Matsuzaka

Performed data analysis: Matsuzaka, Hayashi, and Kusuhara

Wrote or contributed to the writing of the manuscript: Hayashi and Kusuhara

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This article has not been copyedited and formatted. The final version may differ from this version.

References

Akita H, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H and Sugiyama Y (2001) Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. *Biochimica et biophysica acta* **1511**(1):7-16.

Bligh EG and Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* **37**(8):911-917.

Bull LN, van Eijk MJ, Pawlikowska L, DeYoung JA, Juijn JA, Liao M, Klomp LW, Lomri N, Berger R, Scharschmidt BF, Knisely AS, Houwen RH and Freimer NB (1998) A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. *Nature genetics* **18**(3):219-224.

Byrne JA, Strautnieks SS, Mieli-Vergani G, Higgins CF, Linton KJ and Thompson RJ (2002) The human bile salt export pump: characterization of substrate specificity and identification of inhibitors. *Gastroenterology* **123**(5):1649-1658.

Chen B, Jiang Y, Zeng S, Yan J, Li X, Zhang Y, Zou W and Wang X (2010) Endocytic sorting and recycling require membrane phosphatidylserine asymmetry maintained by TAT-1/CHAT-1. *PLoS genetics* **6**(12):e1001235.

Chen F, Ananthanarayanan M, Emre S, Neimark E, Bull LN, Knisely AS, Strautnieks SS, Thompson RJ, Magid MS, Gordon R, Balasubramanian N, Suchy FJ and Shneider BL (2004) Progressive familial intrahepatic cholestasis, type 1, is associated with decreased farnesoid X receptor activity. *Gastroenterology* **126**(3):756-764.

Davit-Spraul A, Fabre M, Branchereau S, Baussan C, Gonzales E, Stieger B, Bernard O and Jacquemin E (2010) ATP8B1 and ABCB11 analysis in 62 children with normal gamma-glutamyl transferase progressive familial intrahepatic cholestasis (PFIC): phenotypic differences between PFIC1 and

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 10, 2024

Molecular Pharmacology Fast Forward. Published on September 23, 2015 as DOI: 10.1124/mol.115.100578
This article has not been copyedited and formatted. The final version may differ from this version.

PFIC2 and natural history. *Hepatology (Baltimore, Md* **51**(5):1645-1655.

Davit-Spraul A, Gonzales E, Baussan C and Jacquemin E (2009) Progressive familial intrahepatic cholestasis. *Orphanet journal of rare diseases* **4**:1.

Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF and Meier PJ (1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *The Journal of biological chemistry* **273**(16):10046-10050.

Giacomini KM, Balimane PV, Cho SK, Eadon M, Edeki T, Hillgren KM, Huang SM, Sugiyama Y, Weitz D, Wen Y, Xia CQ, Yee SW, Zimdahl H, Niemi M and International Transporter C (2013) International Transporter Consortium commentary on clinically important transporter polymorphisms. *Clinical pharmacology and therapeutics* **94**(1):23-26.

Group SC, Link E, Parish S, Armitage J, Bowman L, Heath S, Matsuda F, Gut I, Lathrop M and Collins R (2008) SLCO1B1 variants and statin-induced myopathy--a genomewide study. *The New England journal of medicine* **359**(8):789-799.

Hagenbuch B and Meier PJ (1994) Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+/bile acid cotransporter. *The Journal of clinical investigation* **93**(3):1326-1331.

Hagenbuch B, Stieger B, Foguet M, Lubbert H and Meier PJ (1991) Functional expression cloning and characterization of the hepatocyte Na+/bile acid cotransport system. *Proceedings of the National Academy of Sciences of the United States of America* **88**(23):10629-10633.

Hasegawa Y, Hayashi H, Naoi S, Kondou H, Bessho K, Igarashi K, Hanada K, Nakao K, Kimura T, Konishi A, Nagasaka H, Miyoshi Y, Ozono K and Kusuhara H (2014) Intractable itch relieved by 4-phenylbutyrate therapy in patients with progressive familial intrahepatic cholestasis type 1.

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Molecular Pharmacology Fast Forward. Published on September 23, 2015 as DOI: 10.1124/mol.115.100578
This article has not been copyedited and formatted. The final version may differ from this version.

Orphanet journal of rare diseases **9**:89.

Hayashi H, Inamura K, Aida K, Naoi S, Horikawa R, Nagasaka H, Takatani T, Fukushima T, Hattori A, Yabuki T, Horii I and Sugiyama Y (2012a) AP2 adaptor complex mediates bile salt export pump internalization and modulates its hepatocanalicular expression and transport function. *Hepatology (Baltimore, Md* **55**(6):1889-1900.

Hayashi H, Mizuno T, Horikawa R, Nagasaka H, Yabuki T, Takikawa H and Sugiyama Y (2012b) 4-Phenylbutyrate modulates ubiquitination of hepatocanalicular MRP2 and reduces serum total bilirubin concentration. *Journal of hepatology* **56**(5):1136-1144.

Hayashi H and Sugiyama Y (2007) 4-phenylbutyrate enhances the cell surface expression and the transport capacity of wild-type and mutated bile salt export pumps. *Hepatology (Baltimore, Md* **45**(6):1506-1516.

Hayashi H, Takada T, Suzuki H, Akita H and Sugiyama Y (2005a) Two common PFIC2 mutations are associated with the impaired membrane trafficking of BSEP/ABCB11. *Hepatology (Baltimore, Md* **41**(4):916-924.

Hayashi H, Takada T, Suzuki H, Onuki R, Hofmann AF and Sugiyama Y (2005b) Transport by vesicles of glycine- and taurine-conjugated bile salts and taurolithocholate 3-sulfate: a comparison of human BSEP with rat Bsep. *Biochimica et biophysica acta* **1738**(1-3):54-62.

Hirano M, Maeda K, Matsushima S, Nozaki Y, Kusuhara H and Sugiyama Y (2005) Involvement of BCRP (ABCG2) in the biliary excretion of pitavastatin. *Molecular pharmacology* **68**(3):800-807.

Hirano M, Maeda K, Shitara Y and Sugiyama Y (2004) Contribution of OATP2 (OATP1B1) and OATP8 (OATP1B3) to the hepatic uptake of pitavastatin in humans. *The Journal of pharmacology and experimental therapeutics* **311**(1):139-146.

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Molecular Pharmacology Fast Forward. Published on September 23, 2015 as DOI: 10.1124/mol.115.100578
This article has not been copyedited and formatted. The final version may differ from this version.

Hirohashi T, Suzuki H, Takikawa H and Sugiyama Y (2000) ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). *The Journal of biological chemistry* **275**(4):2905-2910.

International Transporter C, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ and Zhang L (2010) Membrane transporters in drug development. *Nature reviews Drug discovery* **9**(3):215-236.

Kuipers F, Enserink M, Havinga R, van der Steen AB, Hardonk MJ, Fevery J and Vonk RJ (1988) Separate transport systems for biliary secretion of sulfated and unsulfated bile acids in the rat. *The Journal of clinical investigation* **81**(5):1593-1599.

Lee S, Uchida Y, Wang J, Matsudaira T, Nakagawa T, Kishimoto T, Mukai K, Inaba T, Kobayashi T, Molday RS, Taguchi T and Arai H (2015) Transport through recycling endosomes requires EHD1 recruitment by a phosphatidylserine translocase. *The EMBO journal* **34**(5):669-688.

Liu K, Surendhran K, Nothwehr SF and Graham TR (2008) P4-ATPase requirement for AP-1/clathrin function in protein transport from the trans-Golgi network and early endosomes. *Molecular biology of the cell* **19**(8):3526-3535.

Meier PJ, Eckhardt U, Schroeder A, Hagenbuch B and Stieger B (1997) Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. *Hepatology (Baltimore, Md* **26**(6):1667-1677.

Meier PJ and Stieger B (2002) Bile salt transporters. *Annual review of physiology* **64**:635-661.

Mizuno T, Hayashi H and Kusuhara H (2015) Cellular Cholesterol Accumulation Facilitates Ubiquitination

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Molecular Pharmacology Fast Forward. Published on September 23, 2015 as DOI: 10.1124/mol.115.100578
This article has not been copyedited and formatted. The final version may differ from this version.

and Lysosomal Degradation of Cell Surface-Resident ABCA1. *Arteriosclerosis, thrombosis, and vascular biology* **35**(6):1347-1356.

Morotti RA, Suchy FJ and Magid MS (2011) Progressive familial intrahepatic cholestasis (PFIC) type 1, 2, and 3: a review of the liver pathology findings. *Seminars in liver disease* **31**(1):3-10.

Naik J, de Waart DR, Utsunomiya K, Duijst S, Mok KH, Oude Elferink RP, Bosma PJ and Paulusma CC (2015) ATP8B1 and ATP11C: Two Lipid Flippases Important for Hepatocyte Function. *Digestive diseases* **33**(3):314-318.

Naoi S, Hayashi H, Inoue T, Tanikawa K, Igarashi K, Nagasaka H, Kage M, Takikawa H, Sugiyama Y, Inui A, Nagai T and Kusuhara H (2014) Improved liver function and relieved pruritus after 4-phenylbutyrate therapy in a patient with progressive familial intrahepatic cholestasis type 2. *The Journal of pediatrics* **164**(5):1219-1227 e1213.

Noe J, Stieger B and Meier PJ (2002) Functional expression of the canalicular bile salt export pump of human liver. *Gastroenterology* **123**(5):1659-1666.

Ose A, Kusuhara H, Endo C, Tohyama K, Miyajima M, Kitamura S and Sugiyama Y (2010) Functional characterization of mouse organic anion transporting peptide 1a4 in the uptake and efflux of drugs across the blood-brain barrier. *Drug metabolism and disposition: the biological fate of chemicals* **38**(1):168-176.

Pang KS and Rowland M (1977) Hepatic clearance of drugs. I. Theoretical considerations of a "well-stirred" model and a "parallel tube" model. Influence of hepatic blood flow, plasma and blood cell binding, and the hepatocellular enzymatic activity on hepatic drug clearance. *Journal of pharmacokinetics and biopharmaceutics* **5**(6):625-653.

Paulusma CC, de Waart DR, Kunne C, Mok KS and Elferink RP (2009) Activity of the bile salt export pump

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Molecular Pharmacology Fast Forward. Published on September 23, 2015 as DOI: 10.1124/mol.115.100578
This article has not been copyedited and formatted. The final version may differ from this version.

(ABCB11) is critically dependent on canalicular membrane cholesterol content. *The Journal of biological chemistry* **284**(15):9947-9954.

Paulusma CC, Folmer DE, Ho-Mok KS, de Waart DR, Hilarius PM, Verhoeven AJ and Oude Elferink RP (2008) ATP8B1 requires an accessory protein for endoplasmic reticulum exit and plasma membrane lipid flippase activity. *Hepatology (Baltimore, Md* **47**(1):268-278.

Paulusma CC, Groen A, Kunne C, Ho-Mok KS, Spijkerboer AL, Rudi de Waart D, Hoek FJ, Vreeling H, Hoeben KA, van Marle J, Pawlikowska L, Bull LN, Hofmann AF, Knisely AS and Oude Elferink RP (2006) Atp8b1 deficiency in mice reduces resistance of the canalicular membrane to hydrophobic bile salts and impairs bile salt transport. *Hepatology (Baltimore, Md* **44**(1):195-204.

Ray NB, Durairaj L, Chen BB, McVerry BJ, Ryan AJ, Donahoe M, Waltenbaugh AK, O'Donnell CP, Henderson FC, Etscheidt CA, McCoy DM, Agassandian M, Hayes-Rowan EC, Coon TA, Butler PL, Gakhar L, Mathur SN, Sieren JC, Tyurina YY, Kagan VE, McLennan G and Mallampalli RK (2010) Dynamic regulation of cardiolipin by the lipid pump Atp8b1 determines the severity of lung injury in experimental pneumonia. *Nature medicine* **16**(10):1120-1127.

Sasaki M, Suzuki H, Aoki J, Ito K, Meier PJ and Sugiyama Y (2004) Prediction of in vivo biliary clearance from the in vitro transcellular transport of organic anions across a double-transfected Madin-Darby canine kidney II monolayer expressing both rat organic anion transporting polypeptide 4 and multidrug resistance associated protein 2. *Molecular pharmacology* **66**(3):450-459.

Segawa K, Kurata S, Yanagihashi Y, Brummelkamp TR, Matsuda F and Nagata S (2014) Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure. *Science* **344**(6188):1164-1168.

Siggs OM, Arnold CN, Huber C, Pirie E, Xia Y, Lin P, Nemazee D and Beutler B (2011a) The P4-type

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 10, 2024

Molecular Pharmacology Fast Forward. Published on September 23, 2015 as DOI: 10.1124/mol.115.100578
This article has not been copyedited and formatted. The final version may differ from this version.

ATPase ATP11C is essential for B lymphopoiesis in adult bone marrow. *Nature immunology* **12**(5):434-440.

Siggs OM, Schnabl B, Webb B and Beutler B (2011b) X-linked cholestasis in mouse due to mutations of the P4-ATPase ATP11C. *Proceedings of the National Academy of Sciences of the United States of America* **108**(19):7890-7895.

Strautnieks SS, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM and Thompson RJ (1998) A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nature genetics* **20**(3):233-238.

Takatsu H, Baba K, Shima T, Umino H, Kato U, Umeda M, Nakayama K and Shin HW (2011) ATP9B, a P4-ATPase (a putative aminophospholipid translocase), localizes to the trans-Golgi network in a CDC50 protein-independent manner. *The Journal of biological chemistry* **286**(44):38159-38167.

Takatsu H, Tanaka G, Segawa K, Suzuki J, Nagata S, Nakayama K and Shin HW (2014) Phospholipid flippase activities and substrate specificities of human type IV P-type ATPases localized to the plasma membrane. *The Journal of biological chemistry* **289**(48):33543-33556.

Takikawa H, Sano N, Narita T, Uchida Y, Yamanaka M, Horie T, Mikami T and Tagaya O (1991) Biliary excretion of bile acid conjugates in a hyperbilirubinemic mutant Sprague-Dawley rat. *Hepatology (Baltimore, Md* **14**(2):352-360.

Trauner M and Boyer JL (2003) Bile salt transporters: molecular characterization, function, and regulation. *Physiological reviews* **83**(2):633-671.

van de Steeg E, Wagenaar E, van der Kruijssen CM, Burggraaff JE, de Waart DR, Elferink RP, Kenworthy KE and Schinkel AH (2010) Organic anion transporting polypeptide 1a/1b-knockout mice provide

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 10, 2024

Molecular Pharmacology Fast Forward. Published on September 23, 2015 as DOI: 10.1124/mol.115.100578
This article has not been copyedited and formatted. The final version may differ from this version.

insights into hepatic handling of bilirubin, bile acids, and drugs. *The Journal of clinical investigation* **120**(8):2942-2952.

Wang L, Soroka CJ and Boyer JL (2002) The role of bile salt export pump mutations in progressive familial intrahepatic cholestasis type II. *The Journal of clinical investigation* **110**(7):965-972.

Watanabe T, Maeda K, Kondo T, Nakayama H, Horita S, Kusuhara H and Sugiyama Y (2009) Prediction of the hepatic and renal clearance of transporter substrates in rats using in vitro uptake experiments. *Drug metabolism and disposition: the biological fate of chemicals* **37**(7):1471-1479.

Yabas M, Coupland LA, Cromer D, Winterberg M, Teoh NC, D'Rozario J, Kirk K, Broer S, Parish CR and Enders A (2014) Mice deficient in the putative phospholipid flippase ATP11C exhibit altered erythrocyte shape, anemia, and reduced erythrocyte life span. *The Journal of biological chemistry* **289**(28):19531-19537.

Yabas M, Teh CE, Frankenreiter S, Lal D, Roots CM, Whittle B, Andrews DT, Zhang Y, Teoh NC, Sprent J, Tze LE, Kucharska EM, Kofler J, Farell GC, Broer S, Goodnow CC and Enders A (2011) ATP11C is critical for the internalization of phosphatidylserine and differentiation of B lymphocytes. *Nature immunology* **12**(5):441-449.

Yamazaki M, Suzuki H, Hanano M, Tokui T, Komai T and Sugiyama Y (1993) Na(+)-independent multispecific anion transporter mediates active transport of pravastatin into rat liver. *The American journal of physiology* **264**(1 Pt 1):G36-44.

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Molecular Pharmacology Fast Forward. Published on September 23, 2015 as DOI: 10.1124/mol.115.100578
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Footnote

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Figure legends

Figure 1. Genotype analysis of Atp11c knockout mice.

(A) Genotype analysis of mice that inherited the wild-type and *Atp11c*^{emptyhive} allele. A partial sequence in *Atp11c* was amplified by PCR involving genomic DNA prepared from the mice, digested by MboI, and subjected to electrophoretic analysis. (B) Atp11c expression in liver. LPMs (10 μg) was prepared from *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice and analyzed by Western blot analysis. The data shown in A and B are representative result of more than two independent experiments. *Atp11c*$^{emp/Y}$; *Atp11c*$^{emptyhive/Y}$.

Figure 2. Plasma concentration and biliary excretion rate of [^{3}H]-TC and [^{3}H]-$E_2$17βG during constant intravenous infusion into *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice.

[^{3}H]-TC (A, C, E) and [^{3}H]-$E_2$17βG (B, D, F) were infused intravenously into *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice at a rate of 500 or 13.3 pmol/min/kg for 60 min. Plasma concentrations at the designed time points (A, B) and biliary excretion rate (C, D) and bile flow rate (E, F) for the indicated time segments were measured. Each point represents the mean ± SEM of more than three mice. *; $p<0.05$, **; $p<0.01$. *Atp11c*$^{emp/Y}$; *Atp11c*$^{emptyhive/Y}$.

Figure 3. Uptake of [^{3}H]-TC and [^{3}H]-$E_2$17βG into isolated hepatocytes prepared from *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice.

The uptake of [^{3}H]TC (20 nM) (A) and [^{3}H]-$E_2$17βG (3 nM) (B) for 0.5 and 2 min into the isolated hepatocytes from the livers of *Atp11c*$^{+/Y}$ (white) and *Atp11c*$^{emptyhive/Y}$ (black) mice was measured at 37°C in the absence (circles) or presence (squares) of 100 μM unlabeled compounds. The uptake amount per minute of [^{3}H]-TC (C) and [^{3}H]-$E_2$17βG (D) in the hepatocytes was calculated from the results shown in (A, B). Representative

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results of two independent experiments are shown. Each point (A, B) and bar (C, D) represents the mean ± SEM of quadruplicate determinations. *; p<0.05, **; p<0.01. *Atp11c*$^{emp/Y}$; *Atp11c*$^{emptyhive/Y}$.

Figure 4. Hepatic expression of BAs transporters in liver.

(A) Protein expression. Upper image, LPMs (10 μg) was prepared from the liver of *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice and subjected to Western blot analysis. A representative result of three independent experiments is shown. Lower image, quantification of the amount of each transporter in LPMs. The band intensity was quantified as described in Materials and Methods. Each bar represents the mean ± SEM of three independent experiments. **, p<0.01; ***, p<0.001; a.u.; arbitrary unit. (B) mRNA expression. RNA prepared from the liver of *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice was analyzed by qPCR as described in Materials and Methods. Expression of each transporter was normalized by that of Gapdh. Each bar represents the mean ± SEM of three independent experiments. *; p<0.05; a.u.; arbitrary unit. (C) Localization of Atp11c in the liver. Frozen liver sections from *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice were subjected to immunohistochemistry as described in Materials and Methods. The lower and upper images are specimens from *Atp11c*$^{+/Y}$ and *Atp11c*$^{emptyhive/Y}$ mice, respectively. A typical image from each mouse is shown. Bar, 100 μm. *Atp11c*$^{emp/Y}$; *Atp11c*$^{emptyhive/Y}$.

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Table

Table 1. Serum biochemistry

Parameters	*Atp11c*$^{+/Y}$ (n=6)	*Atp11c*$^{emptyhive/Y}$ (n=12)	P value (significance)	
TP (g/dL)	4.8 ± 0.1	4.7 ± 0.2	7.8E-01	
ALB (g/dL)	3.1 ± 0.1	3.1 ± 0.1	9.4E-01	
AST (IU/L)	102 ± 13	177 ± 21	3.4E-02	*
ALT (IU/L)	30.8 ± 3.1	102 ± 18	1.9E-03	**
ALP (IU/L)	315 ± 15	484 ± 34	4.7E-04	***
LDH (IU/L)	709 ± 84	852 ± 68.9	2.3E-01	
GGT (IU/L)	3>	3>	N.D.	
T-CHO (mg/dL)	104 ± 2	106 ± 7	7.7E-01	
F-CHO (mg/dL)	26.2 ± 1.5	33.9 ± 3.6	6.9E-02	
E-CHO (mg/dL)	78.5 ± 3	72.9 ± 3.9	3.6E-01	
TG (mg/dL)	28.5 ± 6	24.9 ± 6.2	7.2E-01	
PL (mg/dL)	202 ± 5	184 ± 16	2.8E-01	
T-BIL (mg/dL)	0.06 ± 0.01	1.1 ± 0.2	2.5E-04	***
D-BIL (mg/dL)	0.01>	0.8 ± 0.2	N.D.	
TBA (μmol/L)	10.7 ± 6.4	79.6 ± 13.5	3.5E-04	***

TP; total protein, ALB; albumin, AST; aspartate aminotransferase, ALT; alanine aminotransferase, ALP; alkaline phosphatase, LDH; lactate dehydrogenase, GGT; gamma-glutamyl transferase, T-CHO; total cholesterol, F-CHO; free cholesterol, E-CHO; esterified cholesterol, TG; triglyceride, PL; phospholipid, T-BIL; total bilirubin, D-BIL; direct bilirubin, TBA; total bile acids
N.D.; not determined, *; P<0.05, **; P<0.01, ***; P<0.001

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Table 2. Pharmacokinetic parameters of [^{3}H]TC during constant infusion into Atp11c$^{+/Y}$ and Atp11c$^{emptyhive/Y}$

Parameters	*Atp11c* $^{+/Y}$ (n=4)	*Atp11c* $^{emptyhive/Y}$ (n=3)	P value
$C_{ss,\ plasma}$ (nM)	10.1 ± 0.9	10.5 ± 1.3	0.81
$C_{ss,\ liver}$ (pmol/g liver)	35.6 ± 4.8	33.6 ± 2.0	0.72
$V_{ss,\ bile}$ (pmol/min/kg)	428 ± 37	432 ± 46	0.95
CL_{tot} (ml/min/kg)	43.6 ± 7.2	33.7 ± 4.8	0.34
$CL_{bile,\ plasma}$ (ml/min/kg)	37.3 ± 4.6	38.6 ± 1.8	0.82
$CL_{bile,\ liver}$ (ml/min/kg)	10.2 ± 1.4	13.9 ± 1.2	0.17

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Table 3. Pharmacokinetic parameters of [^{3}H]$E_2$17βG during constant infusion into Atp11c$^{+/Y}$ and Atp11c$^{emptyhive/Y}$

Parameters	*Atp11c* $^{+/Y}$ (n=3)	*Atp11c* $^{emptyhive/Y}$ (n=3)	P value
$C_{ss,\ plasma}$ (nM)	0.488 ± 0.075	1.01 ± 0.10	0.013*
$C_{ss,\ liver}$ (pmol/g liver)	2.95 ± 0.38	3.58 ± 0.13	0.19
$V_{ss,\ bile}$ (pmol/min/kg)	14.2 ± 2.0	14.6 ± 0.6	0.85
CL_{tot} (ml/min/kg)	27.9 ± 5.1	13.7 ± 1.5	0.050*
$CL_{bile,\ plasma}$ (ml/min/kg)	23.0 ± 2.9	13.8 ± 1.1	0.040*
$CL_{bile,\ liver}$ (ml/min/kg)	3.87 ± 0.37	3.76 ± 0.25	0.82

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Table 4. Measurement of bile flow rate and biliary BA concentrations

Parameters	*Atp11c*$^{+/Y}$ (n=9)	*Atp11c*$^{emptyhive/Y}$ (n=11)	P value
Bile flow rate (μL/min/kg)	65.9 ± 11.7	61.0 ± 3.1	0.69
BA concentrations (mM)	139 ± 14	140 ± 10	0.94

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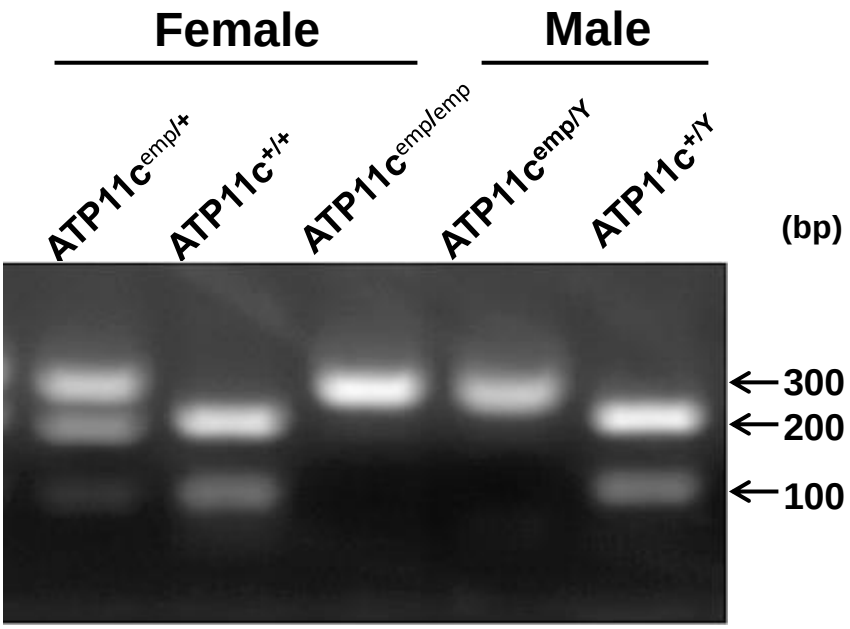
Figure 1.

A.


Female
Male
ATP11c^emp/+
ATP11c^+/+
ATP11c^emp/emp
ATP11c^emp/Y
ATP11c^+/Y
(bp)
300
200
100

B.

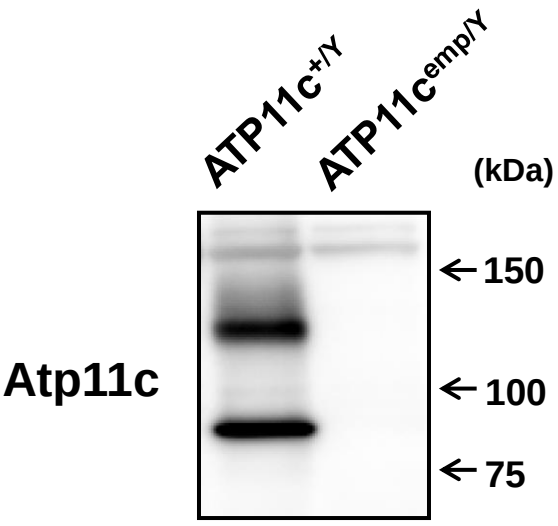

ATP11c^+/Y
ATP11c^emp/Y
(kDa)
Atp11c
150
100
75

Figure 2.

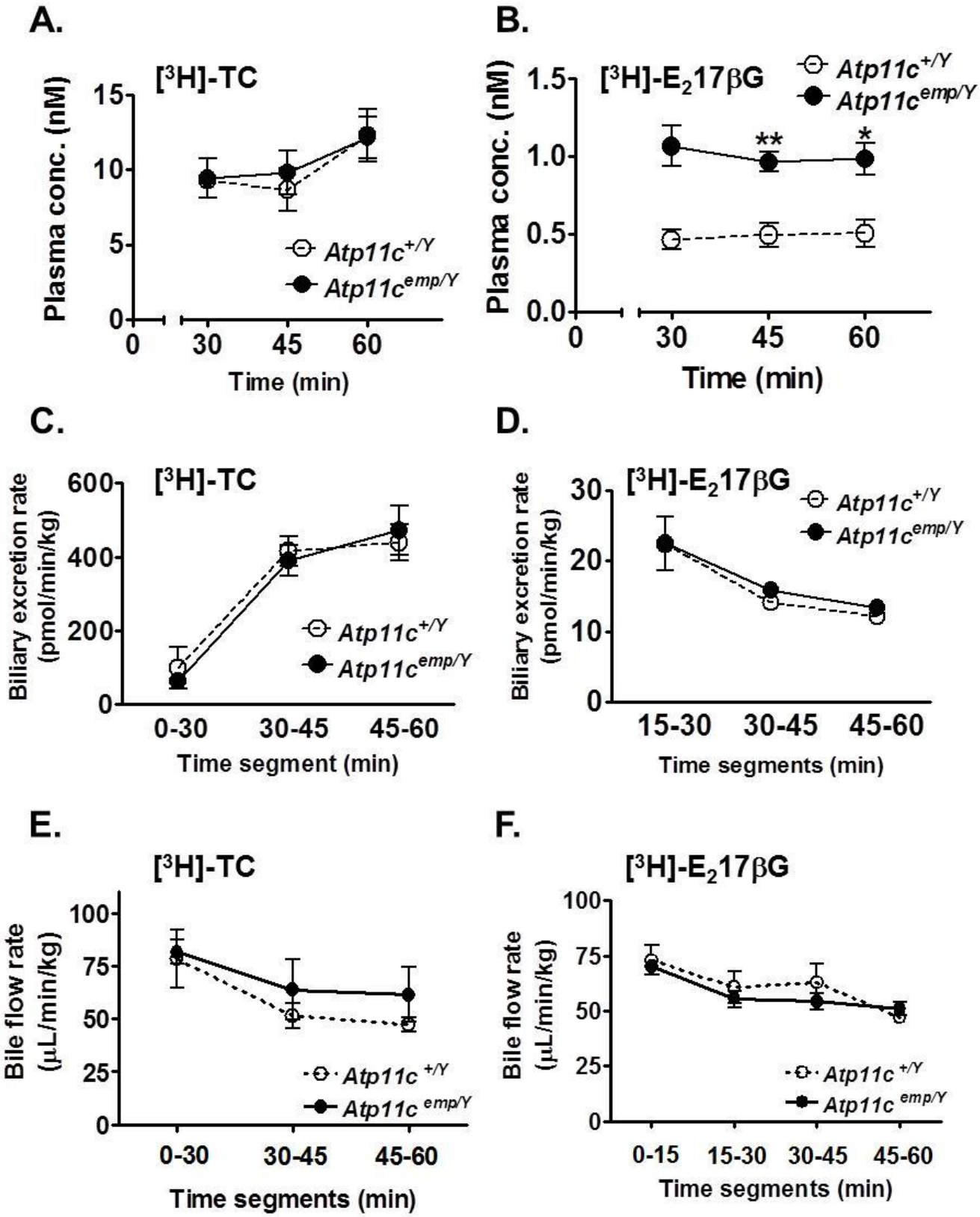

A.
[3H]-TC
Plasma conc. (nM)
15
10
5
0
0
30
45
60
Time (min)
Atp11c+/Y
Atp11cemp/Y
B.
[3H]-E2 17βG
Plasma conc. (nM)
1.5
1.0
0.5
0.0
**
*
0
30
45
60
Time (min)
Atp11c+/Y
Atp11cemp/Y
C.
[3H]-TC
Biliary excretion rate (pmol/min/kg)
600
400
200
0
0-30
30-45
45-60
Time segment (min)
Atp11c+/Y
Atp11cemp/Y
D.
[3H]-E2 17βG
Biliary excretion rate (pmol/min/kg)
30
20
10
0
15-30
30-45
45-60
Time segments (min)
Atp11c+/Y
Atp11cemp/Y
E.
[3H]-TC
Bile flow rate (μL/min/kg)
100
75
50
25
0
0-30
30-45
45-60
Time segments (min)
Atp11c +/Y
Atp11c emp/Y
F.
[3H]-E2 17βG
Bile flow rate (μL/min/kg)
100
75
50
25
0
0-15
15-30
30-45
45-60
Time segments (min)
Atp11c +/Y
Atp11c emp/Y

Figure 3.

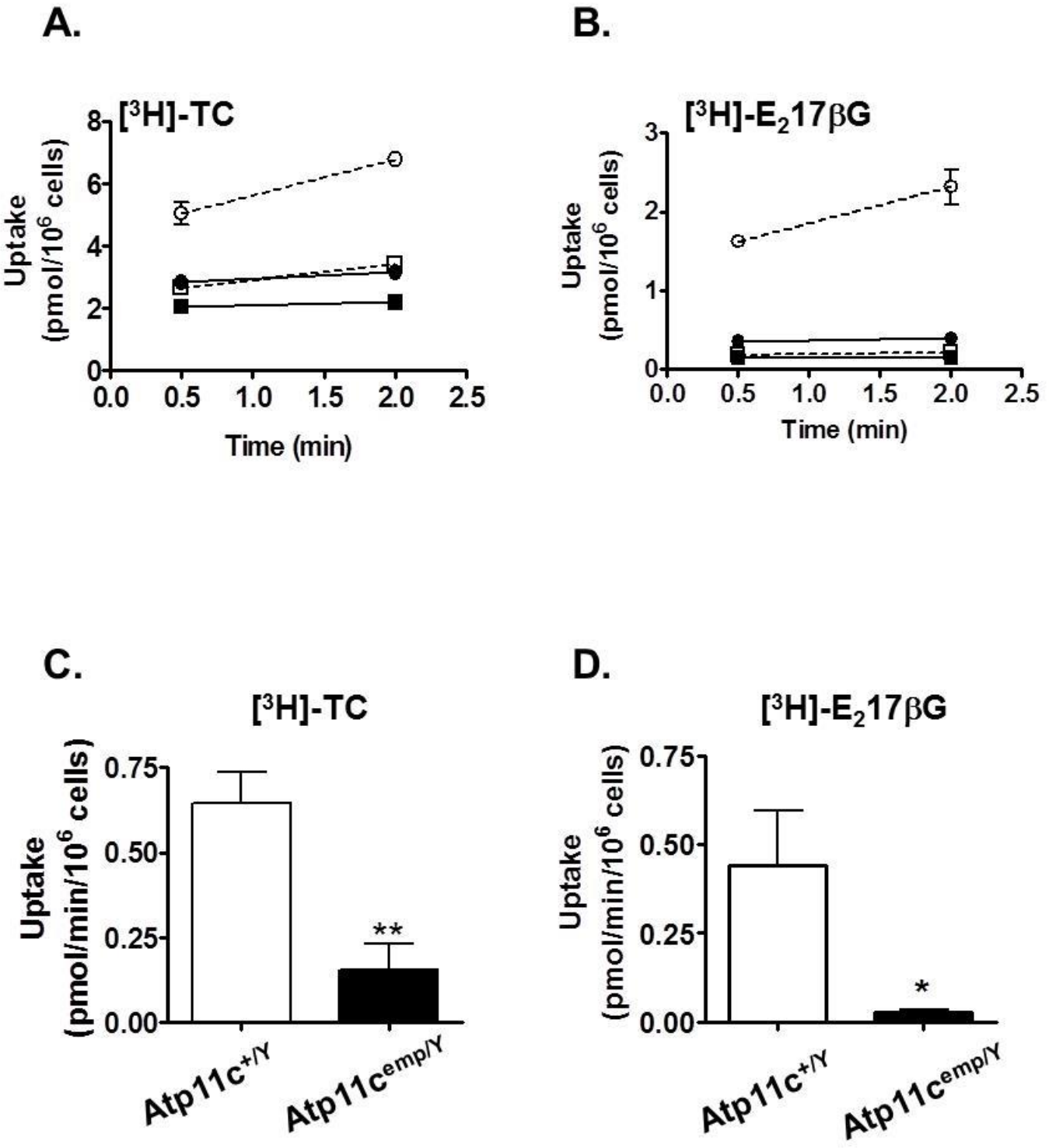
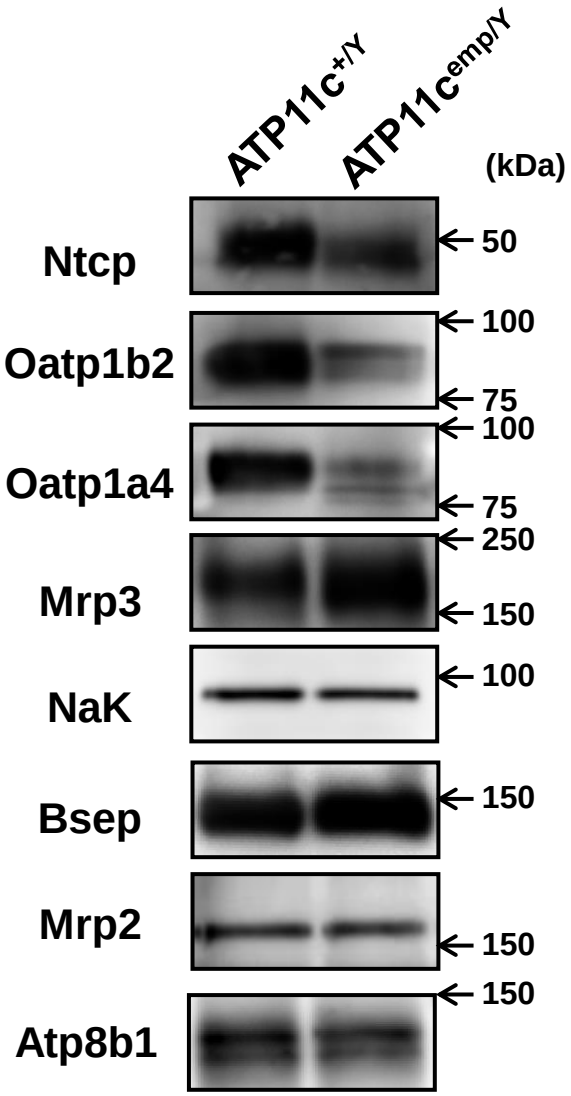

A.
[3H]-TC
Uptake (pmol/10^6 cells)
Time (min)
B.
[3H]-E2 17βG
Uptake (pmol/10^6 cells)
Time (min)
C.
[3H]-TC
Uptake (pmol/min/10^6 cells)
**
Atp11c+/Y
Atp11cemp/Y
D.
[3H]-E2 17βG
Uptake (pmol/min/10^6 cells)
*
Atp11c+/Y
Atp11cemp/Y

Figure 4.

A.


ATP11c+/Y
ATP11cemp/Y
(kDa)
Ntcp
50
Oatp1b2
100
75
Oatp1a4
100
75
Mrp3
250
150
NaK
100
Bsep
150
Mrp2
150
Atp8b1
150

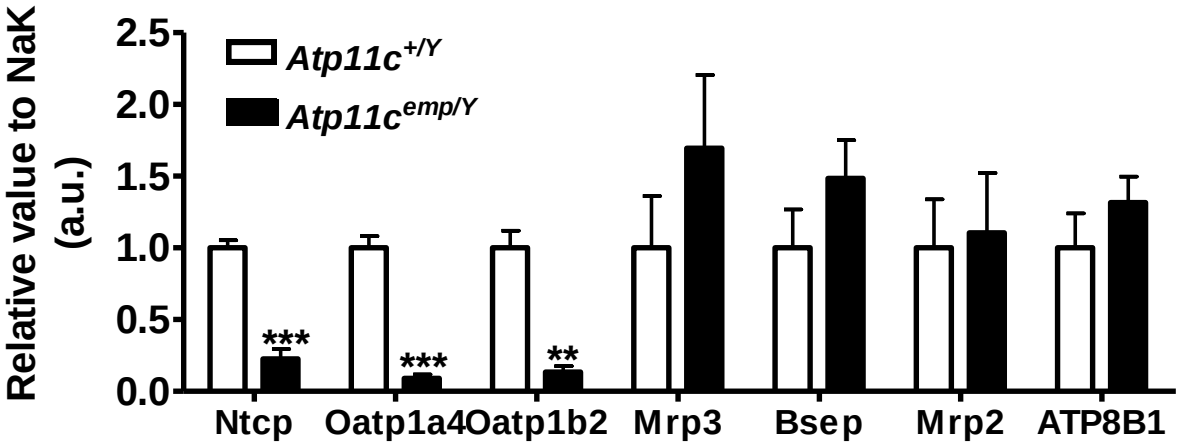
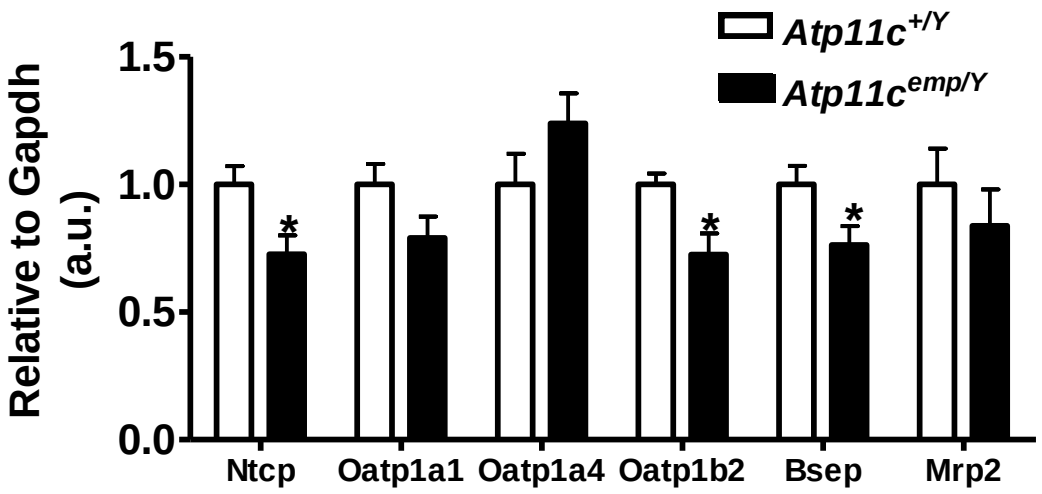
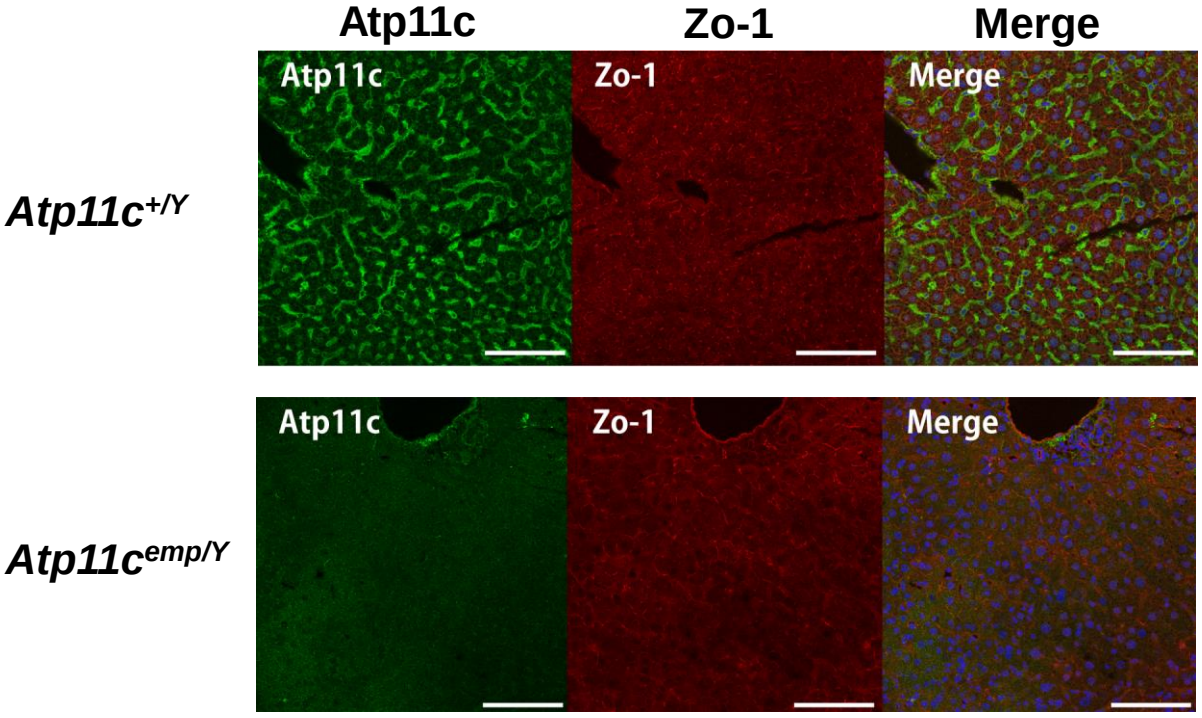

Relative value to NaK (a.u.)
2.5
2.0
1.5
1.0
0.5
0.0
Atp11c+/Y
Atp11cemp/Y
***
***
**
Ntcp
Oatp1a4
Oatp1b2
Mrp3
Bsep
Mrp2
ATP8B1

Figure 4.

B.


Atp11c+/Y
Atp11cemp/Y
Relative to Gapdh (a.u.)
1.5
1.0
0.5
0.0
*
*
*
Ntcp
Oatp1a1
Oatp1a4
Oatp1b2
Bsep
Mrp2

C.


Atp11c
Zo-1
Merge
Atp11c+/Y
Atp11cemp/Y
Atp11c
Zo-1
Merge
Atp11c
Zo-1
Merge