Effects of chronic renal failure on brain drug transporters in rats

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Brain drug transporters in renal failure

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Number of text pages: 18
Number of tables: 2
Number of figures: 6
Number of references: 54
Number of words in Abstract: 229 words
Number of words in Introduction: 727 words
Number of words in Discussion: 1146 words

transporting polypeptide, PBS: Phosphate Buffered Saline, PCR: polymerase chain reaction, P-gp: P-glycoprotein, PMSF: Phenylmethylsulfonylfluoride, PTH: parathyroid hormone, qPCR: quantitative PCR
ABSTRACT

Studies demonstrated that chronic renal failure (CRF) affects the expression and activity of intestinal, hepatic and renal drug transporters. Such drug transporters are expressed in brain cells and at the blood-brain-barrier (BBB) where they limit the entry and distribution of drugs in the brain. Perturbations in brain drug transporters equilibrium by CRF could lead to central drug toxicity. This study evaluates how CRF affects BBB drug transporters using a 5/6 nephrectomised rat model. Protein and mRNA expression of influx transporters (organic anion transporting polypeptide [Oatp], organic anion transporter [Oat]), and efflux transporters (p-glycoprotein [P-gp], multidrug resistance related protein [Mrp], breast cancer resistance protein [Bcrp]) was measured in CRF and control rat brain. Intra-cerebral accumulation of radio-labelled benzylpenicillin, digoxin, doxorubicin and verapamil was used to evaluate BBB drug permeability. Protein expression of the transporters was evaluated in rat brain endothelial cells (RBEC) and astrocytes incubated with control and CRF rat serum. We demonstrated significant decreases (30-50%) in protein and mRNA levels of Bcrp, Mrp2-4, Oat3, Oatp2-3 and P-gp in CRF rat brain biopsies as well as in astrocytes and RBEC incubated with CRF serum. These decreases did not correlate with in vivo changes since BBB permeability of benzylpenicillin was decreased by 30% in CRF rats while digoxin, doxorubicin and verapamil permeabilities were unchanged. It thus appears that even with decreased drug transporters, BBB integrity and function is conserved in CRF.
INTRODUCTION

In recent years, many studies have demonstrated that chronic renal failure (CRF) significantly affects the expression and activity of intestinal, hepatic and renal drug transporters via uremic mediators, thus, altering the pharmacokinetics of many drugs (Huang et al., 2000; Laouari et al., 2001; Sun et al., 2004; Sun et al., 2006; Naud et al., 2007; Naud et al., 2008; Naud et al., 2011). Such drug transporters are also expressed in brain cells and at the blood-brain-barrier (BBB) where they limit the entry and distribution of drugs in the brain (see Figure 1) (Loscher and Potschka, 2005c; Loscher and Potschka, 2005b; Loscher and Potschka, 2005a; Ohtsuki and Terasaki, 2007). Two major drug transporter families have been identified: ATP-binding cassette (ABC) transporters and solute carriers (Slc). The most important ABC transporters at the BBB are multidrug resistance related proteins (Mrp), breast cancer resistance protein (Bcrp) and P-glycoprotein (P-gp). Bcrp, Mrp2-4-5 and P-gp are expressed on the luminal side and they mediate the efflux of molecules from the brain capillary cell into the blood (Kusuhara and Sugiyama, 2005; Loscher and Potschka, 2005c; Deeken and Loscher, 2007; Oswald et al., 2007; Eyal et al., 2009). Mrp4 was also found on the abluminal side of bovine brain capillaries where it is thought to mediate the efflux of molecules from the capillary cell into the brain, but this was not reported in rat and human brain (Kusuhara and Sugiyama, 2005; Loscher and Potschka, 2005c; Deeken and Loscher, 2007; Oswald et al., 2007). As for solute carriers, the most important found at the BBB are the organic anion transporter 3 (Oat3) and the organic anion transporting proteins 2 and 3 (Oatp2-3). Oat3 is located on both
membranes and mediates drug efflux from the brain into the blood (Kikuchi et al., 2003; Mori et al., 2003; Mori et al., 2004; Loscher and Potschka, 2005c; Deeken and Loscher, 2007; Oswald et al., 2007). Furthermore, it was recently hypothesized that Oat3 is also implicated in the influx of drugs from the blood to the brain (Ose et al., 2009). Oatp2 is expressed at the luminal and abluminal membranes, while Oatp3 was found only at the luminal membrane of brain capillary cells, and they both mediate bi-directional transport of drugs (Loscher and Potschka, 2005c; Deeken and Loscher, 2007; Ohtsuki and Terasaki, 2007; Ose et al., 2010). Perturbations in brain drug transporter equilibrium caused by CRF could lead to central toxicity of drugs.

To this day, there are no studies reporting the effects of CRF on brain drug transporters. However, indirect evidence suggests that CRF could be implicated in modulating their expression. Indeed, neurotoxicity of cephalosporin and penicillin derivates was reported in patients with altered renal function despite normal plasma concentrations (Chatellier et al., 2002; Ferrara et al., 2003; Alpay et al., 2004). Drug transporters expressed at the BBB normally prevent the entry of these drugs into the brain (Kikuchi et al., 2003). It was demonstrated that beta-lactam antibiotics and cephalosporins are substrates of Oat3 and Mrps in the kidney and the brain (Kikuchi et al., 2003; Ueo et al., 2005; Ci et al., 2007; Nozaki et al., 2007). Interestingly, Oat3 is also known to extract at least three uremic toxins from the brain: p-aminohippuric acid (PAH), indoxyl sulfate (IS) and 3-carboxyl-4-methyl-5-propyl-2-furonapropionate (CMPF) (Ohtsuki et al., 2002; Kikuchi et al., 2003;
Tahara et al., 2005; Deguchi et al., 2006). Finally, it was demonstrated that central and systemic inflammation, which are present in CRF (Oettinger et al., 1994; Sunder-Plassmann et al., 1994; Ziesche et al., 1994; Akahoshi et al., 1995; Higuchi et al., 1997), can downregulate the expression of P-gp at the BBB and in the liver, leading to drug accumulation in the brain and plasma (Zhao et al., 2002; Goralski et al., 2003; Petrovic et al., 2007).

The objective of this study was to investigate the effects of CRF on the expression and activity of the major drug transporters expressed at the BBB and in the brain. To address this, we have measured 1) the protein and mRNA expressions of Oat3, Oatp2-3, Mrp2-4-5 and P-gp in brain tissue biopsies from CRF and control rats, 2) the protein expression of the same transporters in rat astrocytes and rat brain endothelial cells (RBEC) incubated with sera from control and CRF rats, and 3) the in vivo intra-cerebral accumulation of $^{14}$C-benzylpenicillin, $^3$H-digoxin, $^{14}$C-doxorubicin and $^3$H-verapamil four substrates of brain drug transporters.
MATERIALS AND METHODS

Experimental model

Male Sprague-Dawley rats (Charles River, Portage, MI), weighing 176 to 225 g were housed in the Research Center animal care facility and maintained on Harlan Teklad rodent diet (Harlan Teklad Global Diets, Montreal, Canada) and water ad libitum. Animals were allowed an acclimatization period of at least 7 days before the first nephrectomy was performed. All the experiments were conducted according to the Canadian Council on Animal Care guidelines for care and use of laboratory animals and under the supervision of our local animal care committee.

Experimental protocol

Studies were performed in two groups of animals: control pair-fed and CRF. Drug transporter protein expression and mRNA levels were measured in at least 6 rats per group. Drug transporter activity was measured in vivo through the cerebral accumulation of $^{14}$C-benzylpenicillin, $^3$H-digoxin, $^{14}$C-doxorubicin and $^3$H-verapamil in at least 3 rats per group, per experiment.

Chronic renal failure was induced by two-stage 5/6 nephrectomy as previously described (Leblond et al., 2001). Every animal had free access to water, but in order to limit the effects of CRF-induced malnutrition, control pair-fed rats were fed the same amount of chow that CRF rats ate on the previous day. We have previously demonstrated that the caloric reduction in control animals induced by pair-feeding did not influence the expression of drug transporters (unpublished
data). At day 41 post-nephrectomy, the rats were housed in metabolic cages and urine was collected for 24 hours to determine the clearance of creatinine. Rats were sacrificed by decapitation 42 days after nephrectomy for organ and blood collection, or underwent a radio-labeled drug accumulation study. Following sacrifice, the brain was immediately excised, rinsed in ice-cold saline then flash-frozen in liquid nitrogen. Samples were stored at -80°C until membrane preparation or mRNA extraction. Blood was collected for the measurement of serum creatinine and urea and for bioassays with cultured rat brain endothelial cells (RBEC) and astrocytes.

**Preparation of crude brain membranes**

Frozen rat brain biopsies (500 mg) were homogenized in 3 mL of homogenizing buffer (250 mM sucrose, 10 mM HEPES and 10 mM Tris-HCl, pH 7.4) containing 0.1 mM PMSF using a Potter-Elvehjem tissue grinder (Wheaton Science Products, Millville, NJ). Differential centrifugation was used to obtain a crude membrane fraction (Chow et al., 2010). Briefly, the brain homogenates were centrifuged at 9,000 g for 10 min at 4°C. The supernatant was then spun at 33,000 g for 60 min at 4°C and the resulting pellet was resuspended in phosphate buffered saline containing 0.1 mM PMSF. Samples were then sonicated on ice for 10 seconds to ensure homogeneity. Protein concentration was determined using the method of Lowry et al. (Lowry et al., 1951), using bovine serum albumin as a reference protein. Aliquots were stored at -80°C up to Western blot analysis.
Western blot analysis

Major brain drug transporters were assessed by Western blot analysis following a previously described protocol (Leblond et al., 2002; Naud et al., 2007; Naud et al., 2008; Naud et al., 2011). Protein expression in control animals was arbitrarily defined as 100%.

P-gp and Mrp2 were detected using monoclonal antibodies from ID Labs (London, Ontario, Canada): C219 and M2 III-6 respectively. Oat3, Oatp2 and Oatp3 were detected using antibodies from Alpha Diagnostic International (San Antonio, TX). Anti-Bcrp was from Abbiotec (San Diego, CA), Anti-Mrp4 was from Novus Biological (Littleton, CO) and anti-Mrp5 from Abnova (Walnut, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as a loading control, was detected using rabbit anti-GAPDH from Abcam (Cambridge, MA).

mRNA analysis

Total RNA was extracted from frozen tissue using Trizol reagent (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer’s protocol. RNA concentration was determined by measuring absorbance at a wavelength of 260nm. One µg of total RNA was used to prepare cDNA by reverse transcription using SuperScript® VILO™ cDNA Synthesis Kit from Invitrogen. The mRNA encoding for P-gp (mdr1a), Mrp2 and Oatp2 was measured by quantitative Real-Time PCR (qPCR) as previously described (Naud et al., 2007; Naud et al., 2008; Naud et al., 2011). Other genes were measured
using appropriate TaqMan gene expression assays from Applied Biosystems (Carlsbad, CA). Table 1 shows the TaqMan gene expression assays used for the quantification of mRNA for each transporter. PCR products were analyzed using the ΔΔCT method (Livak and Schmittgen, 2001) using GAPDH as standard gene.

**In vivo accumulation of radio-labeled drugs in brain**

The intra-cerebral accumulation of radio-labeled drugs was used to determine the activity of the blood-brain-barrier drug transporters. Four different drugs were tested: 14C-benzylpenicillin, an antibiotic and a substrate for Oat3 and Mrps (Kikuchi et al., 2003; Choi et al., 2009), 3H-digoxin a cardiac glycoside and a substrate for Oatps and P-gp (Goralski et al., 2003), 14C-doxorubicin, an anthracycline antibiotic used in cancer chemotherapy and substrate of P-gp (Chan et al., 2004), and 3H-verapamil, a calcium channel blocker and substrate of P-gp (Chan et al., 2004). Digoxin (0.5 mg/kg) was administered via intra-peritoneal injection, benzylpenicillin (5 mg/kg CTL, 1 mg/kg CRF) via intra-muscular injection, and doxorubicin (5 mg/kg) (Hsieh et al., 2008) and verapamil (0.1 mg/kg) (Hendrikse et al., 1998) were administered in the tail-vein. All were injected with an approximate 1:1500 hot/cold ratio so the mg amount of radioactive compound wouldn’t factor into the final dosage calculation. To evaluate cerebral accumulation, similar plasma concentrations of drugs need to be attained and therefore CRF rats received a lower dose of benzylpenicillin because of its reduced clearance in CRF. Rats were anesthetized using isoflurane 30 minutes (doxorubicin), 60 minutes (verapamil) or 2 hours...
(benzylpenicillin and digoxin) post-injection and a cardiac puncture was performed to recover the maximum volume of blood in heparinised tubes to obtain plasma. Brains were harvested, rinsed in cold phosphate buffered saline, weighted, minced and placed in 2 mL of Solvable solution (PerkinElmer, Waltham, MA) at 56°C overnight. Dissolved brain volumes were noted and radioactivity was counted on a Wallac 1409 beta-counter (Perkin Elmer) in 1 mL of dissolved brain tissue and 2 mL of plasma after addition of 4 mL ScintiSafe Plus Scintillant (Fisher, Ottawa, Ontario, Canada). The results were calculated as a ratio between the radioactivity count (CPM) for 1 g of brain tissue and the radioactivity count for 1 mL of plasma. The mean for the CTL groups was arbitrarily defined as 100%.

**Effects of uremic serum on brain drug transporters**

In order to determine whether uremic serum could affect the expression and function of blood-brain-barrier drug transporters, we incubated rat brain endothelial cells (RBEC) and astrocytes with serum from nephrectomised rat and evaluated the drug transporters’ protein expression.

**Isolation and culture of rat astrocytes**

Rat astrocytes were isolated and cultured as previously described by Perriere *et al.* (Booher and Sensenbrenner, 1972; Perriere et al., 2005). When cells reached confluency, the standard culture medium was changed to culture medium containing 10% serum from either control or nephrectomised rats (1
After 48 h of culture, the culture medium was recovered under sterile conditions to be used for RBEC conditioning and cells were trypsinized, pelleted and homogenized in phosphate buffered saline containing 0.1 mM PMSF. Protein concentrations were determined using the Micro BCA Protein Assay Kit from Thermo Fisher Scientific (Rockford, IL). 100 ug was loaded for Western Blot analysis of each studied transporter.

**Isolation and culture of rat brain endothelial cells (RBEC)**

Rat brain endothelial cells (RBEC) were isolated and puromycin-purified for 72 h as previously published (Perriere et al., 2005). Culture medium was then changed, for a 24 h period, to endothelial cell basal medium (ECBM) containing 20% bovine plasma-derived serum (BPDS), 2 ng/mL basic fibroblast growth factor (bFGF), 500 ng/mL hydrocortisone (HC) and 1% Penstrep (a mixture of 20 000 U/mg penicillin and streptomycin)(Perriere et al., 2005). Four days after seeding, the culture medium was again changed to a 50/50 mix of pre-described ECBM culture medium and culture medium recovered from astrocyte cultures containing 10% foetal bovine serum to simulate co-culture with astrocytes. Finally, the culture medium was changed 48h later to a new 50/50 mix of pre-described ECBM containing 20% serum from CTL or nephrectomised rats and astrocyte pre-conditioned medium with sera from CTL of CRF rats. 8-(4-chlorophenylthio)-cAMP (250 µmol/L) and the cAMP phosphodiesterase-4-specific inhibitor RO 20 1724 (17.5 µmol/L) were added to the final mix. After 48 h of culture, cells were trypsinized, pelleted and homogenized in phosphate buffer.
buffered saline containing 0.1 mM PMSF. Protein concentrations were
determined using the Micro BCA Protein Assay Kit from Thermo Fisher Scientific
(Rockford, IL). 100 ug were loaded for Western Blot analysis of each studied
transporter.

Other assays.
Blood and urine chemistries were determined with an Architect C1600 clinical
analyzer (Abbott, Saint-Laurent, QC, Canada).

Statistical analysis.
Results are expressed as mean ± SD. Differences between groups were
assessed using an unpaired Student’s t test or an ANOVA test. Significant
ANOVA was followed by a Scheffe post hoc comparison of groups. The threshold
of significance was p<0.05.
RESULTS

Biochemical parameters and body weight in control and CRF rats (Table 2)

Table 2 presents the biochemical parameters and body weights of both groups of rats. Compared to control animals, CRF rats had higher levels of serum creatinine and urea and lower creatinine clearance (reduced by 80%, p<0.001). There was no difference in body weight between control and CRF rats.

Protein expression of brain drug transporters in CRF rats (Figure 2).

Figure 2 presents the measured protein expression of various drug transporters in crude brain membranes of control and CRF rats. The expression level of Bcrp, Mrp2, Mrp4, Oat3, Oatp2, Oatp3 and P-gp were significantly decreased from 20 to 50% in CRF rat crude brain membranes as shown by Western blot. The only measured protein that remained stable was Mrp5. We found significant correlations between protein expression and the rat clearance of creatinine for Bcrp (R=0.426, p<0.05), Mrp2 (R=0.798, p<0.001), Mrp4 (R=0.594, p<0.005), Oat3 (R=0.557, p<0.05), Oatp2 (R=0.626, p<0.01), Oatp3 (R=0.566, p<0.005) and P-gp (R=0.587, p<0.005).

mRNA expression of brain drug transporters in CRF rats (Figure 3).

Figure 3 presents results from the analysis of mRNA coding for brain drug transporters. There was significant decreases in the expression of Bcrp (35%), Mrp2 (40%), Mrp4 (30%), Oat3 (40%), Oatp2 (35%), Oatp3 (70%) and P-gp (35%) mRNA in CRF rats brain compared to controls. Again, only Mrp5 mRNA
remained stable between the groups.

In vivo accumulation of radiolabelled drugs in the brain of rats with surgically-induced chronic renal failure (Figure 4).

Two hours post-administration, digoxin plasma radioactivity was similar in control and CRF rats, while the plasma radioactivity of benzylpenicillin was slightly elevated by 17% in CRF rats compared to control animals. There was 33% less $^{14}$C-benzylpenicillin in the brain of CRF rats. On the other hand, no significant differences in $^{3}$H-digoxin, $^{14}$C-doxorubicin or $^{3}$H-verapamil brain accumulation were observed between CTL and CRF rats. These data were obtained via a ratio of radioactivity for 1 g of brain tissue/1 mL of plasma.

Protein expression of drug transporters in rat brain endothelial cells (RBEC) incubated with serum from CRF rats (Figure 5).

Protein expression of drug transporters was measured in rat brain endothelial cells incubated for 48 h with serum from control or CRF animals. There was a significant decrease in the expression of Bcrp (40%), Mrp2 (25%), Mrp4 (45%), Oat3 (35%), Oatp2 (35%), Oatp3 (25%) and P-gp (30%) protein when incubated with CRF serum compared to controls. On the other hand, the expression of Mrp5 was unaltered. These results were similar to those obtained in vivo in CRF rats.

Protein expression of drug transporters in rat astrocytes incubated with
serum from CRF rats (Figure 6).

Protein expression of drug transporters was measured in rat astrocytes incubated for 48 h with serum from control or CRF animals. Significant decreases in the expression of Bcrp (25%), Mrp2 (25%), Mrp4 (30%), Oat3 (60%), Oatp2 (40%), Oatp3 (50%) and P-gp (30%) protein were observed when incubated with CRF serum compared to controls. The expression of Mrp5 remained unaltered. These results were similar to those obtained in vivo in CRF rats.
DISCUSSION

This study demonstrated a significant (20-50%) decrease in protein and mRNA expression of Bcrp, Mrp2 and 4, Oat3, Oatp2 and 3, and P-gp in crude rat brain membranes from CRF rats compared to CTL rats, while Mrp5 was unchanged. Similar reductions were observed in RBEC and astrocytes incubated with serum from nephrectomised rat serum, compared to control. Influx and efflux drug transporters expression at the BBB and in the brain thus seem equally affected by CRF. As for BBB permeability, we observed a 30% decrease in the BBB permeability to benzylpenicillin in nephrectomised rats and no change in the BBB permeability to digoxin, doxorubicin and verapamil as measured by their brain/plasma radioactivity ratio.

We found significant correlations between the clearance of creatinine and the expression of all downregulated transporters at the protein level indicating that the changes in transporters are related to the severity of the renal failure. Also, we have demonstrated that the modifications in drug transporter expression occurred in response to one or many factors present in the sera from uremic rats in at least two brain cell types, as demonstrated by the changes in protein expression of transporters in astrocytes and RBEC, which are similar to those observed in CRF rats’ brain biopsies.

To our knowledge, no group has studied the impact of renal failure on the expression of brain drug transporters. However, many studies have reported a
change in drug transporters in various organs in CRF (Huang et al., 2000; Laouari et al., 2001; Sun et al., 2004; Sun et al., 2006; Naud et al., 2007; Naud et al., 2008; Naud et al., 2011). In some of these studies, uremic circulating factors seemed involved in the expression and activity changes. While no specific factor has been identified, many potential factors include uremic toxins such as CMPF, IS or PAH and proinflammatory cytokines which are known to be elevated in CRF (Oettinger et al., 1994; Sunder-Plassmann et al., 1994; Ziesche et al., 1994; Akahoshi et al., 1995; Higuchi et al., 1997). Indeed, uremic toxins have been shown to inhibit drug uptake by Oat1, Oat3 and Oatp2 in the rat liver, kidney and brain (Deguchi et al., 2004; Sun et al., 2004; Deguchi et al., 2006; Sun et al., 2006). Also, proinflammatory cytokines such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor α (TNFα) can modulate drug transporters’ expression and activity in various organs including at the BBB (Petrovic et al., 2007; Morgan et al., 2008). For example, in vitro studies have demonstrated that IL-1β and IL-6 can decrease the expression of P-gp and Bcrp in brain capillaries endothelial cells and that IL-6 can decrease P-gp expression in cultured rat astrocytes (Ronaldson and Bendayan, 2006; von Wedel-Parlow et al., 2009; Poller et al., 2010). Proinflammatory cytokines could thus play a role in the BBB drug transporters’ expression downregulation observed in CRF.

Despite significant decreases of brain transporter expression in CRF rats, we did not observe in vivo brain accumulation of 3 different substrates, namely digoxin, doxorubicin and verapamil. Such discrepancy has also been observed in
inflammation studies. Indeed, previously published data demonstrated that systemic inflammation, as seen in CRF, moderately downregulated (-24%) the protein expression of P-gp at the BBB 6h after the induction of inflammation by endotoxin (Zhao et al., 2002). In that study, P-gp expression returned to normal 24h post-inflammation (Zhao et al., 2002). They observed increased plasma and brain concentrations of doxorubicin in mice 6h after the induction of inflammation compared to control. However, endotoxin-induced systemic inflammation did not significantly change the brain/plasma concentration ratio of doxorubicin (Zhao et al., 2002). The authors thus conclude that endotoxin-induced systemic inflammation has no effect on BBB integrity and doxorubicin transport across the blood–brain barrier in mice (Zhao et al., 2002). Also, another study demonstrated that central nervous system inflammation produces a 50% decrease in P-gp mRNA expression and an almost 100% decrease in Oatp2 mRNA in the rat brain 6h post-induction (Goralski et al., 2003). This group used $^{3}$H-digoxin as a probe for P-gp function in vivo and observed, 6 and 24h post inflammation induction, an increase in brain and plasma $^{3}$H-radioactivity (Goralski et al., 2003). However, the brain/plasma radioactivity ratio was unchanged (Goralski et al., 2003). Based on these results, and since CRF is a state of chronic inflammation, it could explain why, like Zhao and Goralski, we observe decreased protein and mRNA expression of P-gp and Oatp2 in the brain of nephrectomised rats but no intracerebral accumulation of digoxin, doxorubicin or verapamil.

We also observed a 30% decrease in the BBB permeability to
benzylpenicillin in nephrectomised rats compared to controls as measured by the total brain/plasma radioactivity ratio. *In vitro*, studies showed that benzylpenicillin is a substrate of Mrp2 and Oat3, but no blood to brain transporter has been identified (Kikuchi et al., 2003; Choi et al., 2009). As mentioned earlier, Oat3 is involved in the transport of uremic toxins from the brain (Ohtsuki et al., 2002; Kikuchi et al., 2003; Tahara et al., 2005; Deguchi et al., 2006). In fact, this transporter is typically thought to play a role in the efflux of drugs from the brain. However, a recent study hypothesized that it is also involved in the uptake of Ro 64-0802, a pharmacologically active form of oseltamivir, from the blood to the brain (Ose et al., 2009). It could, thus, be responsible for the blood to brain uptake of substrates like benzylpenicillin or uremic toxins as well as for their brain to blood transport. This hypothesis is supported by a study performed in the 1960s that demonstrated that probenecid, a well known Oat3 inhibitor, limits the entry of benzylpenicillin into rat brain as shown by a decrease in brain/plasma concentration ratio of benzylpenicillin when co-administered with probenecid (Fishman, 1966). The 30% decrease in the brain/blood radioactivity ratio of benzylpenicillin observed in the brain of nephrectomised rats indicates a more efficient efflux of the drug compared to its uptake. However, Mrp2 (efflux) and Oat3 (influx and efflux) were equally reduced by 40% in the brain of nephrectomised rats compared to controls. If the hypothesis of Oat3 involvement in the blood to brain influx of benzylpenicillin and uremic toxins is correct, the greater functional reduction in benzylpenicillin brain uptake could be attributed to competition with accumulating uremic toxins such as CMPF, IS and PAH for
blood to brain transport by Oat3 preventing benzylpenicillin entry into the brain. However, it is also possible that Mrp2 plays a more important functional role in the BBB permeability of benzylpenicillin causing a greater efflux of the drug compared to its influx.

In conclusion, even though we reported significant reductions in the expression of influx and efflux brain drug transporters, it appears that the BBB integrity and permeability to drugs is preserved in renal failure as shown by the unchanged intra-cerebral accumulation of digoxin, doxorubicin and verapamil and by the reduced accumulation of benzylpenicillin.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Naud, Laurin, Pichette

Conducted experiments: Naud, Michaud, Beauchemin, Leblond, Laurin

Performed data analysis: Naud, Leblond, Pichette

Wrote or contributed to the writing of the manuscript: Naud, Leblond, Pichette
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This work was financed by La Fondation Hôpital Maisonneuve-Rosemont (La néphrologie et son Impact). V Pichette is supported by a career award from FRSQ.

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Figure Legends

Figure 1: Blood-brain-barrier drug transporters. Bcrp: Breast cancer resistance protein, Mrp: multidrug resistance related protein, Oat: organic anion transporter, Oatp: organic anion transporting polypeptide, P-gp: p-glycoprotein. Mrp4 was found on the abluminal side of bovine brain capillaries where it is thought to mediate the efflux of molecules from the capillary cell into the brain, but this was not reported in rat and human brain hence the dotted line (Kusuhara and Sugiyama, 2005; Loscher and Potschka, 2005c; Deeken and Loscher, 2007; Oswald et al., 2007). Oatps are widely known to be bidirectional transporters hence the arrows indicating both directions (Loscher and Potschka, 2005c; Deeken and Loscher, 2007; Ohtsuki and Terasaki, 2007; Ose et al., 2010). It was recently hypothesized that Oat3 mediates import and export from the brain (Ose et al., 2009). Figure based on information from (Kikuchi et al., 2003; Mori et al., 2003; Kusuhara and Sugiyama, 2005; Loscher and Potschka, 2005c; Deeken and Loscher, 2007; Ohtsuki and Terasaki, 2007; Oswald et al., 2007; Eyal et al., 2009; Ose et al., 2009).

Figure 2: Protein expression of various brain drug transporters in CRF rats. A. Protein bands are expressed in densitometry units. The densitometry units measured for transporters in control (CTL) (white bars) and chronic renal failure (CRF) (black bars) rats were standardized by dividing them by the value obtained for GAPDH. The standardized densitometry units of control rats were arbitrarily defined as 100%. Graphic shows the mean expression in rats.
expressed as percent of controls ± SD of 12 rats in each group. **p<0.01, ***p<0.001 compared to control rats. B. Representative western blots for each transporter. Each blot presents bands for two CRF (left) and two control (right) rats.

**Figure 3: mRNA expression of various brain drug transporters in CRF rats.**

mRNA encoding brain drug transporters in control (CTL) (white bars) and chronic renal failure (CRF) (black bars) rats were measured by quantitative real time PCR. mRNA levels are expressed in relative quantities using the ΔΔCT method (Livak and Schmittgen, 2001) with gapdh as a reference gene. The mean relative quantity of control rats was arbitrarily defined as 100% (dotted line). Graphic shows the mean expression in rats expressed as percent of controls ± SD of 12 rats in each group. *p<0.05, **p<0.01 compared to control rats.

**Figure 4: In vivo accumulation of radio-labeled drugs in the brain of rats with surgically-induced chronic renal failure.** Brain/plasma radioactivity (counts per minute) ratio for 1 g brain tissue/1 mL plasma in control (CTL) (white bars) and chronic renal failure (CRF) (black bars) rats. CRF rats received a fifth of the CTL rats’ dose of benzylpenicillin, but had a 17% (p<0.05) higher plasma radioactivity of benzylpenicillin. However, with equal dosing between the groups, digoxin, doxorubicin and verapamil plasma radioactivity was similar in the CTL and CRF rats, respectively. The mean ratio for CTL animals was arbitrarily
defined as 100%. Data are expressed as mean ± SD of at least 4 rats in each group. ***p<0.001 compared to CTL rats.

**Figure 5: Protein expression of various drug transporters in rat brain endothelial (RBEC) cells incubated with the serum of CRF rats.** Protein bands are expressed in densitometry units. The densitometry units measured for transporters in control (CTL) (white bars) and chronic renal failure (CRF) (black bars) rats were standardized by dividing them by the value obtained for GAPDH. The standardized densitometry units of control rats were arbitrarily defined as 100% (dotted line). Graphic shows the mean expression in rats expressed as percent of controls ± SD of 10 different rat sera in each group. *p<0.05, **p<0.01, ***p<0.001 compared to control rats.

**Figure 6: Protein expression of various drug transporters in astrocytes incubated with the serum of CRF rats.** Protein bands are expressed in densitometry units. The densitometry units measured for transporters in control (CTL) (white bars) and chronic renal failure (CRF) (black bars) rats were standardized by dividing them by the value obtained for GAPDH. The standardized densitometry units of control rats were arbitrarily defined as 100% (dotted line). Graphic shows the mean expression in rats expressed as percent of controls ± SD of 10 different rat sera in each group. **p<0.01, ***p<0.001 compared to control rats.
Tables and figures

Table 1: TaqMan gene expression assays used for Real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>TaqMan Gene Expression Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcrp</td>
<td>abcg2</td>
</tr>
<tr>
<td></td>
<td>Rn00710585_m1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>gapdh</td>
</tr>
<tr>
<td></td>
<td>Rn99999916_s1</td>
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<tr>
<td>Mrp4</td>
<td>abcc4</td>
</tr>
<tr>
<td></td>
<td>Rn01465702_m1</td>
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<tr>
<td>Mrp5</td>
<td>abcc5</td>
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<tr>
<td></td>
<td>Rn00588341_m1</td>
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<tr>
<td>Oat3</td>
<td>slc22a8</td>
</tr>
<tr>
<td></td>
<td>Rn00580082_m1</td>
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<tr>
<td>Oatp3</td>
<td>slco1a5</td>
</tr>
<tr>
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<td>Rn00578150_m1</td>
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</table>
Table 2. Characteristics of control and CRF rats

<table>
<thead>
<tr>
<th></th>
<th>Control (n=32)</th>
<th>CRF (n=32)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight (grams)</strong></td>
<td>352.8 ± 29.5</td>
<td>333.0 ± 54.5</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Serum creatinine (μmol/L)</strong></td>
<td>55.4 ± 10.5</td>
<td>205.0 ± 90.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Creatinine clearance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μL/100 g of body weight/min)</td>
<td>309.1 ± 82.4</td>
<td>70.1 ± 35.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Serum urea (mmol/L)</strong></td>
<td>4.5 ± 1.0</td>
<td>38.4 ± 17.9</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are the mean ± SD. Measurements were made at the time of sacrifice.

Twenty-four hour urinary collection was begun the day before. Blood urea and creatinine, and urine creatinine were determined with an Architect C1600 clinical analyzer (Abbott, Saint-Laurent, QC, Canada).
Figure 1
Figure 2B

<table>
<thead>
<tr>
<th></th>
<th>CRF</th>
<th>CTL</th>
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</thead>
<tbody>
<tr>
<td>Gapdh</td>
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<td></td>
</tr>
<tr>
<td>Bcrp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mrp2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mrp4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mrp5</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>CRF</th>
<th>CTL</th>
</tr>
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<tbody>
<tr>
<td>Oat3</td>
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<td></td>
</tr>
<tr>
<td>Oatp2</td>
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<tr>
<td>Oatp3</td>
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<td></td>
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<tr>
<td>P-gp</td>
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<td></td>
</tr>
</tbody>
</table>
Figure 3

The bar chart shows the mRNA expression levels of various transporters as a percentage of control. The following transporters are displayed:

- Bcrp: 65.1%
- Mrp2: 60.3%
- Mrp4: 68.8%
- Mrp5: 700%
- Oat3: 62.7%
- Oatp2: 64.9%
- Oatp3: 31.6%
- P-gp: 66.1%

The chart includes error bars indicating the variability of the data.
Figure 4