Age-dependent activity of the uptake transporters Ntcp and Oatp1b2 in male rat hepatocytes: from birth till adulthood

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Running title:

Age-dependent activity of hepatic uptake transporters

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List of nonstandard abbreviations:

FBS, fetal bovine serum;

GLB, glibenclamide;

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

NaFluo, sodium fluorescein;

NTCP/Ntcp, Na⁺-Taurocholate Co-transporting Peptide (Human/Rat);

OATP/Oatp, Organic Anion Transporting Polypeptide (Human/Rat);

PBS, phosphate buffered saline;

RIF SV, rifamycin SV;

RTV, ritonavir;

SLC, Solute-Linked Carrier;

TCA, taurocholic acid.
Abstract

Recognition of the role of hepatic drug transporters in elimination of xenobiotics continues to grow. Hepatic uptake transporters, such as for instance hepatic isoforms of the Oatp (Organic anion transporting polypeptide) family as well as the bile acid transporter Ntcp (Na\(^+\)-taurocholate co-transporting polypeptide) have been studied extensively both at the mRNA and protein expression levels in adults. However, in paediatric/juvenile populations there continues to be a knowledge gap about the functional activity of these transporters. Therefore, the aim of this study was to examine the functional maturation of Ntcp and Oatp isoforms as major hepatic transporters. Hepatocytes were freshly-isolated from rats aged between birth and 8 weeks. Transporter activities were assessed by measuring the initial uptake rates of known substrates: taurocholate (TCA) for Ntcp and sodium fluorescein (NaFluo) for Oatps. Relative to adult values, uptake clearance of TCA in hepatocytes from rats aged 0, 1, 2, 3 and 4 weeks reached 19, 43, 22, 46 and 63%, respectively. On the other hand, Oatp-mediated NaFluo uptake showed a considerably slower developmental pattern: uptake clearance of NaFluo in hepatocytes from rats aged 0, 1, 2, 3, 4 and 6 weeks were 24, 20, 19, 8, 19 and 64%, respectively. Maturation of NaFluo uptake activity correlated with the previously reported ontogeny of Oatp1b2 mRNA expression, confirming the role of Oatp1b2 for NaFluo uptake in rat liver. The outcome of this project will help in understanding and predicting age-dependent drug exposure in juvenile animals and will eventually support safe and more effective drug therapies for children.
Introduction

It is known that various organs, including those involved in xenobiotic clearance pathways, remain in the developing phase from birth onwards. As a consequence, developmental changes in the kidney and liver cause differences in xenobiotic response between children and adults and even between different paediatric populations. Over the past several years, the complementary roles of drug transporters and drug metabolizing enzymes to constitute elimination pathways have been recognized. Drug transporters are trans-membrane proteins capable of controlling molecular influx and efflux of xenobiotics, thus determining the access of these xenobiotics to metabolizing enzymes and excretory mechanisms. Information regarding the ontogenic profiles of drug transporters is presently scarce and mostly still limited to the gene and protein expression level. An increase in the activity of phase I and II metabolic enzymes occurs within the first year of human life (Anderson, 2010). The most important group of enzymes mediating phase I reactions are cytochrome P450 (CYP) isoenzymes of which the ontogeny has been extensively investigated. In fetal liver the total CYP content accounts for 30-60% of adult levels, however, these isoenzymes develop in a particular developmental pattern. For instance, the activity of CYP3A7 is higher before birth, while postnatally it declines to adult levels within two years. On the other hand, CYP3A4, the main hepatic CYP isoenzyme involved in drug metabolism, reaches adult levels by the first year of life (Alcorn and McNamara, 2002; Allegaert et al., 2008; de Wildt, 2011).

Several studies have revealed that drug transporters also undergo ontogenic regulation, but most studies to date have focused on gene and protein expression and to a lesser extent on determination of age-dependent transporter activities. For instance, the mRNA and protein expression levels of Ntcp (Na⁺-taurocholate co-transporting polypeptide), the prime hepatic bile
acid uptake transporter, are detectable at gestational age and increase to adult levels within a few hours after birth (Hardikar et al., 1995; Gao et al., 2004). However, the increase in activity of the same transporter when measured using taurocholate as a probe substrate, followed a more gradual profile with age (Suchy and Balistreri, 1982). Consistently, Gao et al (Gao et al., 2004) reported that the expression profiles do not closely correlate with the activity profiles, which can be attributed to the role of post-translational processes. For other uptake transporters such as those belonging to the Oatp (Organic anion transporting polypeptide) family, the expression level in rat liver is detectable a few days after birth and it increases to adult level within a few weeks. Macias and co-workers (Macias et al., 2011) found that the ontogeny-associated changes in mRNA expression of the main Oatp isoforms in rat liver follow different patterns; most of these Oatp isoforms appeared to remain at a low level during early postnatal days and increase progressively toward adult level (week 8).

To our knowledge, very few studies are available illustrating age-dependent activity of uptake transporters. Moreover, the majority of research in this area was performed using animal tissue, although limited experiments have been performed on human tissue (Smits et al., 2013). This is due to the fact that the required in vitro models for studying transporter activity such as primary hepatocytes are not available for neonates and to a very limited extent from older paediatric donors. Nonetheless, reliable preclinical animal data regarding ontogeny of drug transporters have their particular value, mainly for supporting toxicity profiling during drug development (i.e. in juvenile toxicity studies). For instance, age-dependent transporter activity data may prove instrumental in improving physiology-based pharmacokinetic (PBPK) prediction in juvenile animals and/or paediatric populations.
To this end, the aim of the present study was to generate high-resolution ontogenic profiles of the hepatic uptake transporters Ntcp and Oatp1b2. As isolated hepatocytes in suspension have been shown to maintain at least some transporter activity, they constitute one of the most biorelevant in vitro tools for determination of hepatic drug uptake clearance, and are thus frequently used for this purpose (Maeda and Sugiyama, 2010; De Bruyn et al., 2011b). Furthermore, rapidly spinning aliquots of drug-hepatocyte incubates through an oil layer generates more accurate clearance predictions compared to the ‘media loss’ assay (Soars et al., 2009). For this reason, this oil-spin method was applied in the present study on hepatocytes isolated from rat livers at various ages.
Materials and Methods

Materials

\(^{[3]}H\)Taurocholic acid (TCA) (specific activity, 4.6 Ci/mmol) and scintillation cocktail were obtained from PerkinElmer Life Sciences Inc. (Boston, MA). Sodium fluorescein (NaFluo) was purchased from UCB. William’s E Medium, L-glutamine, penicillin-streptomycin mixture (contains 10,000 IU potassium penicillin and 10,000 μg streptomycin sulfate per ml in 0.85% saline), Fetal Bovine Serum (FBS), and Phosphate Buffered Saline 1x (PBS) were purchased from Lonza Verviers SPRL (Verviers, Belgium). HEPES (4-(2-hydroxy)-1-piperazine-ethanesulfonic acid) was purchased from MP Biochemical (Illkirch, France). Triton X-100, collagenase (Type IV), silicon oil, sodium pyruvate, choline chloride, choline bicarbonate, glibenclamide, pyruvic acid, rifamycin SV and taurocholic sodium salt were sourced from Sigma-Aldrich (Schnelldorf, Germany). Mineral oil was purchased from Acros organics.

Animals

Male Wistar rats were used for hepatocyte isolation from whole liver. Male pups (≤ 26 days old, weaning period as indicated with dotted line in the Figures) were kept with their mothers until the day of sacrifice. The rats were housed according to the relevant Belgian and European laws, guidelines and policies for animal experiments, housing and care in the Central Animal Facilities of the University. Approval for this project was granted by the Institutional Ethical Committee for Animal Experimentation.

Hepatocytes isolation

Rats aged > 7 days were anesthetized with an appropriate dose based on their body weight (Table 1). The anesthesia consisted of a mixture of ketamine and xylazine at a dose of 120 mg ketamine and 24 mg xylazine per kg body weight, given intra-peritoneally. For pups (P7–P10), a mixture
of 40–50 mg ketamine and 5 mg xylazine per kg body weight was used, while for pups younger than 7 days, 100 – 300 μl of a mixture of 10mg ketamine and 0.8 mg xylazine in 1000 μl water was given intra-peritoneally. Rats were sacrificed at different days ranging from 2-56 days old. Hepatocytes were isolated using a two-step collagenase perfusion, as described previously (Annaert et al., 2001) with slight modification for the isolation of hepatocytes from juvenile rats (Table 1). After isolation, cells were centrifuged (50 g) for 3 min at 4°C and the pellet was re-suspended in William’s E medium containing 5 % FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin. Hepatocytes were counted using a hemocytometer and cell viability was determined using Trypan blue. Freshly-isolated rat hepatocytes were subsequently re-suspended in Krebs Henseleit Buffer: NaCl 118 mM, KCl 5.17 mM, CaCl₂·2H₂O 1.2 mM, MgCl₂·6H₂O 1.2 mM, NaHCO₃ 23.8 mM, HEPES 12.5 mM, glucose 5 mM and Na-pyruvate 5 mM; pH 7.4 and were kept on ice until initiation of the experiment. For sodium depletion experiments, NaCl, NaHCO₃ and Na-pyruvate in KHB were replaced by choline Cl, choline HCO₃ and pyruvic acid, respectively.

**Uptake experiments**

Prior to starting substrate uptake experiments 175 μl cell suspensions (4million/ml) were pre-incubated for 15 min at 37°C. For experiments where non-saturable uptake rates were determined, 350 μl inhibitor solution was added during pre-incubation time. Subsequently, 175 μl substrate solution was added to initiate the incubation at 37°C which lasted 90 sec for NaFluo and 60 sec for TCA. Triplicate 200 μl aliquots of the suspension were immediately transferred to 1.5 ml ice-cold Eppendorf tubes, containing 700 μl of an oil layer (a mixture of silicone oil and mineral oil 82:18) above 300 μl of 8% NaCl solution (2N NaOH for [³H]TCA). The tubes were immediately centrifuged twice for 2 min at 14,000 g using a table top centrifuge and afterwards frozen on dry
ice. The pellets obtained were solubilised in 300 μl 0.5% Triton X (in PBS) and the fluorescence was measured using fluorescence spectroscopy (ex 494 nm; em 520 nm) in a Tecan Infinite M200 plate reader (Austria) for determination of NaFluo concentration. For [3H]TCA samples, the obtained pellets were placed in a scintillation vial containing 2 ml of scintillation cocktail and radioactivity was quantified using liquid scintillation spectrometry (Wallac 1410, Finland). During the course of this study, an LC-MS method was developed to analyse TCA uptake in suspended hepatocytes without need for radiolabelled compound. Quantification of TCA in hepatocyte pellets from incubations performed after this LC-MS method implementation, were extracted with chilled methanol/water containing 1 μM deuterated cholic acid (d₄-CA). The extracted samples were centrifuged for 20 min at 14,000 rpm and supernatants were analyzed with a Thermo Fisher Scientific LC-MS/MS system (Thermo Fischer, Breda, The Netherlands) as described below. Uptake clearances were normalized for the cell density during the incubation and expressed as μl (ml)/(min × million cells).

**Determination of TCA by LC-MS**

The Thermo Fisher Scientific system consisted of a TSQ Quantum Access™ mass spectrometer coupled with Accela™ U-HPLC system (Thermo Fischer, Breda, The Netherlands). A Kinetex XB-C₁₈ column (2.6 μm, 50 x 2.10 mm) with a KrudKatcher ultra HPLC in-line filter (Phenomenex®, The Netherlands) was used. The injection volume was 25 μl. The chromatographic conditions were: 400 μl/min flow rate for 4 min, with the mobile phase consisting of (A) methanol and (B) 5 mM ammonium formate buffer (pH adjusted to 3.5 with formic acid). The gradient A:B (v/v) was programmed as follows: 5% A was running at 0 min to 0.3 min, subsequently the ratio of A:B changed to 95:5 in 2.1 min, which was maintained for the following 0.9 min. Then the column was re-equilibrated for the last 0.7 min with the initial ratio
of mobile phase. Analysis was done in the negative electrospray ionization mode. TCA concentrations in samples were determined by calculating the area ratio of analyte to the internal standard d^4-CA. Linearity was obtained over a TCA concentration range of 0.016-1 μM. Analytical methods were validated with nominal QC sample concentrations of 0.02, 0.1 and 0.5 μM. The intraday inaccuracy varied from 4.6 to -5.2%. The repeatability expressed by % CV was 14.3 to 18.5%. The interday inaccuracy varied from -0.9 to + 0.37% and the interday repeatability (% CV) for analysis at different days was 6.0 to 9.6%.

Data analysis

Net uptake values were obtained by subtracting uptake in hepatocytes in the presence of inhibitors (or under conditions of Na^+-depletion for TCA uptake) from total uptake at 37°C in rat hepatocytes. Net cellular uptake kinetics was described with equation 1:

$$V = \frac{V_{\text{max}} \times C}{K_m + C}$$

with $V_{\text{max}}$ and $K_m$ representing the kinetic parameters for saturable uptake (Michaelis-Menten). Net cellular uptake clearances were calculated by $V_{\text{max}}/K_m$. $K_d$ values (representing the non-saturable uptake process) were calculated from the slope of the linear part of the uptake rate-substrate concentration profile as observed in the presence of inhibitors or Na^+-depletion. The Solver tool in Microsoft Excel 2007 and GraphPad Prism v. 5.00 for Windows software, were used for non-linear regression analysis. Experimental $1/SD^2$ values were used for weighing. Expression profiles were obtained from previously published data (Hardikar et al., 1995; Gao et al., 2004; Macias et al., 2011). The transporter expression data were obtained from the graphs with Plot Digitizer (v. 2.1, Sourceforge.net) and linear interpolation was applied to obtain expression values reflecting the exact ages of the rats used in the present study.
Statistics

ANOVA and Dunnett's post-hoc were used to evaluate statistical differences between parameter estimates describing transport kinetics at various age groups. Spearman rank correlation analysis was performed in GraphPad Prism v. 5.00 for Windows to measure the strength of association between two ranked variables, *in casu* uptake clearance and age. A *p* value < 0.05 was used as criterion for statistical significance.
Results

Isolation of hepatocytes from rats at different ages

The application of isolated hepatocytes in suspension in the present work required additional methodological optimization to allow hepatocyte preparation from young animals. As presented in Table 1 several parameters of the two-step collagenase perfusion commonly used to isolate hepatocytes from adult rats were modified to successfully isolate hepatocytes from younger rats. The amounts of both ketamine and xylazine were reduced by two- and five-fold, respectively. Additional changes dealt with isolation parameters such as buffer flow rate, cannula size, etc. By combining proper perfusion conditions and applying them to specific rat ages, the consistent quality of hepatocytes across all ages was achieved. Finding a proper isolation condition was crucial for obtaining hepatocyte suspensions with adequate viability (> 80%) (Figure 1A) and to avoid inter-batch variability. As shown in Figure 1B the yields of hepatocytes per gram of liver decreased with increasing liver weight with age. This is consistent with previously reported data (Deschenes et al., 1980; Barter et al., 2007).

Ontogeny of Ntcp activity in rat hepatocytes

Maturation of Ntcp activity was determined with TCA as substrate (Meier et al., 1997). To exclude contribution by other transport pathways, net concentration dependent uptake rates of TCA were calculated by subtracting uptake in the presence of choline buffer from total uptake in sodium containing buffer at 37°C. Net initial uptake rates were concentration-dependent in a non-linear fashion, corresponding to saturable uptake according to the Michaelis-Menten equation for all ages (Figure 2A-D, supplemental data Figure 1A-K). The corresponding kinetic parameters are listed in Table 2. The average Km values across different ages, except at week 3, remained constant, whereas marked differences in Vmax values were noted (1-way ANOVA p = 0.2 and...
0.0003 for Km and Vmax, respectively). The net TCA clearance as shown in Figure 3 and Table 2, increases rapidly during the weaning period and reaches 63% by week 4 postnatally. The Spearman rank correlation coefficient equalled 0.8 ($p = 0.0002$) indicating that the variables age and TCA clearance are correlated.

**Ontogeny of activity of Oatp isoforms in rat hepatocytes**

To profile maturation of the transport activity of hepatic Oatp isoforms, NaFluo was used as a probe substrate (De Bruyn et al., 2011a). NaFluo net uptake clearances were determined in the presence of a cocktail of known Oatp inhibitors (Fattinger et al., 2000; Shitara et al., 2002; Ye et al., 2008). The cocktail of inhibitors consisted of rifamycin SV, glibenclamide and ritonavir at concentrations of 200, 20 and 40 μM, respectively. In the presence of this cocktail, the NaFluo uptake in adult rat hepatocytes was inhibited by more than 50% (data not shown). NaFluo net uptake clearances were calculated by subtracting NaFluo uptake measured in the presence of inhibitors from total uptake at 37°C (Figure 4A-D, supplemental data Figure 2A-P). As observed for Ntcp activity, the average Km values for NaFluo uptake remain constant with age and Vmax values gradually increased after weaning (1-way ANOVA: $p = 0.9$ and 0.0003 for Km and Vmax, respectively), (Table 3). As shown in Figure 5 the net NaFluo remains remarkably low in young animals, i.e. below 30% of adult level, with noticeable increase after the weaning period (Spearman rank correlation coefficient = 0.98).

**Comparison between functional maturation and expression for Ntcp and hepatic Oatp isoforms**

The obtained age-dependent activity profiles for Ntcp and Oatps were compared with mRNA and protein expression profiles of corresponding transporter isoforms in rat hepatocytes. As shown in Figure 6A, Ntcp expression levels were at adult level immediately at birth, whereas activities
lagged behind to increase at later age. Oatp activities as well as expression of hepatic Oatp isoforms were lower during early postnatal life and increased as a function of age after weaning (Figure 6B). Net uptake clearance values for NaFluo were plotted against mRNA expression of the three main Oatp isoforms in rodent liver: $r^2$ values of 0.62, 0.43 and 0.85 were found for Oatp1a1, Oatp1a4 and 1b2, respectively (Figure 7).
Discussion

We have presently determined the functional ontogeny of the hepatic uptake transporters Ntcp and Oatp1b2 by determining uptake clearance of probe substrates in suspended rat hepatocytes. Hepatic uptake of TCA and NaFluo was measured for different concentrations at all ages in order to have accurate estimates for intrinsic uptake clearance from kinetic parameters (Kusuhara and Sugiyama, 2010). We have observed saturable substrate (TCA and NaFluo) accumulation across different ages ranging from 2 to 57 days old (Figure 2 and 4, supplemental Figure 1 and 2). Based on net clearance, different ontogeny-profiles were observed for Ntcp and Oatps (Figure 3 and 5). The activity of Ntcp mediating TCA clearance developed during weaning, while Oatps mediating NaFluo clearance showed a biphasic profile and found to develop after weaning. Furthermore, transporter activities showed a similar profile of maturation as observed for mRNA and protein expression (Figure 6 A/B), but were shifted to older age. Immaturity in protein localization and/or intrinsic functionality may explain these differences. Protein expression is usually measured by either Western blot or immunohistochemistry, which are often not sufficiently specific to measure plasma membrane protein, thus intracellular protein is also included in the measurement. In addition, plasma membrane protein may not be fully operative i.e. not glycoslated (Hardikar et al., 1995), because enzymes mediating protein glycosylation are undergoing ontogenic regulation (Oda-Tamai et al., 1989; Oda-Tamai et al., 1991).

The age-dependent uptake clearance of TCA (Figure 3) is in agreement with previous data regarding TCA clearance in human and rodent (Suchy and Balistreri, 1982; Suchy et al., 1985; Olinga et al., 1998). In these studies, the ontogeny of TCA uptake clearance was determined in sodium-driving conditions while it has been noted that TCA uptake is also mediated, albeit to a lesser extent, by other transporters (Van Dyke et al., 1982; Meier et al., 1997). Therefore, we
determined TCA uptake in two conditions: first in the presence of extracellular sodium (normal buffer) and in the second setup in the absence of extracellular sodium (buffer where sodium replaced by choline, causing indirect inhibition). Interestingly, TCA uptake clearance in sodium-free buffer also showed age dependence (Table 2). This observation might be due to the contribution of other pathways and/or transporters such as Oatp1a1 (Kullak-Ublick et al., 1994; Kanai et al., 1996; Meier et al., 1997), or the possibility that expression/function of the Na+/K+ pump changes with age (Suchy et al., 1986).

The physiological implications of the ontogeny of Ntcp as observed in the present study are expected to play a role in age-dependent bile acid disposition in early life. Bile acids, after being biosynthesized in the liver are excreted into the bile by canalicular transporters (mainly BSEP/Bsep, bile salt export pump). The sodium-dependent bile acid transporter (ASBT/Asbt) and the heterodimeric organic solute transporter (OSTα/β, Ostα/β), expressed on the apical and basolateral enterocytic membrane, reabsorb most of these excreted bile acids (90-95%) prior to hepatic reuptake by NTCP/Ntcp. At the level of the intestine, ASBT mRNA increases up to 400-fold between postnatal day 7 and 28, which is in agreement with increasing taurocholate absorption at the weaning period (Little and Lester, 1980; Barnard et al., 1985; Ferdinandusse et al., 2005). The mechanism underlying this stage-specific regulation requires further investigation. Our data indicate reduced Ntcp immediately after birth, and are thus consistent with the observation of the physiological cholestasis in neonates (Shneider, 2001; Venigalla and Gourley, 2004).

Furthermore, as recent data obtained in our laboratory have indicated that NTCP/Ntcp transports the antifungal drug micafungin in human/rat (Yanni et al., 2010), the present Ntcp ontogeny data also expected to have pharmaco-therapeutical implications. Micafungin, which is also used in
neonates, is shown to be cleared three times more rapidly in neonates compared to the adults. Our group previously identified the 8-fold higher free fraction of micafungin in neonatal serum as a major reason to explain the higher neonate micafungin clearance (Yanni et al., 2011). Based on the results obtained in the present study (Table 2), the reduced Ntcp activity in the first week of neonatal life is expected to translate into reduced Ntcp-mediated micafungin uptake immediately after birth. Therefore, assuming a similar developmental pattern for NTCP/Ntcp, the 3-fold increase in micafungin clearance in neonates is likely the net result of 8-fold increase in micafungin free fraction in serum attenuated by decreased hepatic uptake of micafungin in newborns. Further experiments regarding NTCP function in neonatal liver are required to confirm this hypothesis.

Compared to Ntcp, activity of Oatps revealed a considerably slower pattern of development. Uptake clearance of NaFluo was significantly lower during the weaning period and increased gradually towards the adult level after 4 weeks of postnatal age. In addition, NaFluo uptake clearance with age showed a good correlation with mRNA expression (Macias et al., 2011) of the Oatp1b2 isoform ($r^2=0.86$). In contrast, poor correlation was seen with Oatp1a1 ($r^2=0.62$) and Oatp1a4 ($r^2=0.43$). This strongly suggests that NaFluo clearance in rat liver is mostly mediated by Oatp1b2 which is more closely related to human hepatic OATP1B1/1B3 as compared to the other isoforms (Csanaky et al., 2011). This conclusion is supported by our previously published work on the characterization of NaFluo as an Oatp/OATP probe substrate, showing that NaFluo uptake was mediated by OATP1B1/3 in transfected CHO cells but not by OATP2B1 (De Bruyn et al., 2011a). Our present finding thus revealed a somewhat unexpected approach to verify the role of single transporter isoform in transport of a given (drug) substrate.
Most drug transporters work synergistically with drug metabolizing enzymes in eliminating drugs and it is known that metabolizing enzymes are not fully developed at birth and undergo different ontogenic regulation (de Zwart et al., 2008). Both elimination mechanisms can ultimately protect the liver from toxic xenobiotics. The Oatps play an important role in the hepatic uptake of a number of endogenous compounds and clinically important drugs. Development of elimination capacity which is handled by Oatps might alter the incidence of cholestasis and severity of drug toxicity in the liver of newborns and neonates. For example phalloidin uptake is shown to be mediated by Oatp1b2 and OATP1B1/3 expressed in X. Laevis oocytes (Meier-Abt et al., 2004; Herraez et al., 2009). Petzinger and co-workers stated that the hepatocytes from newborn rats were less sensitive to phalloidin than adult hepatocytes and this might be due to lower uptake of phalloidin into the immature hepatocytes. In a parallel experiment carried out by the same group, they showed that the sensitivity of the hepatocytes to phalloidin toxicity coincided with increasing bile acid (cholate and glycocholate) uptake by rat hepatocytes (Petzinger et al., 1979; Ziegler et al., 1979). Consistently, uptake of unconjugated bile acid such as cholate is mediated by Oatp/OATP specially Oatp1b2 (Meier et al., 1997; Csanaky et al., 2011).

For both transporters investigated in the present study (Ntcp/Oatp1b2), the affinity (as reflected by the Km, Table 2 and 3) of the respective probe substrates, remained constant across age. This suggests that these transporters are representing major uptake pathways for these probes irrespective of rat age. In contrast, a significant increase in the maximum velocity was observed. This phenomenon was seen in previous work which mentioned that the number of drug transport proteins (“transport sites”) may increase with age (Suchy and Balistreri, 1982). As mentioned earlier in the discussion, the developmental changes in protein glycosylation are expected to have an effect on the activity of the drug transporters. Recent observations have revealed the role of
protein glycosylation in terms of transporters trafficking to the plasma membrane (Tanaka et al., 2004; Yao et al., 2012).

Many physiological and environmental factors are expected to contribute to the expression and function of Ntcp and Oatp1b2 during development. The underlying mechanisms and pathways have not been fully elucidated, but the effects of hormones and fat intake on the expression and function of nuclear transcription factors (and consequently the drug transporters) are likely involved. For instance, it has been reported that activation of peroxisome proliferator-activated receptor α (PPARα) by natural fatty acids leads to down-regulation of NTCP and OATP (Li and Chiang, 2009). Since rat milk is richer in fat content compared to adult rat food (Ssniff R/M-H standard food for rats, GmbH), the fat composition in the milk possibly limits transporter expression, hence activity, in early life.

In conclusion, our data indicate that the activities of Ntcp and Oatp1b2 develop according to a transporter-specific pattern. The outcome of this project will help in accurate prediction of exposure of juvenile animals to drug candidates that are substrates for these transporters. Ultimately, this work will contribute to a better understanding of drug disposition in paediatric populations, leading to safer and more effective drug therapies for children.
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Authorship contribution

Participated in research design: Fattah and Annaert.

Conducted experiments: Fattah.

Contributed new reagents and analytic tools: Fattah and Annaert.

Performed data analysis: Fattah and Annaert.

Wrote or contributed to the writing of the manuscript: Fattah, Augustijns and Annaert.
References


Footnotes

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Figure legends

**Figure 1:** Characterization of hepatocytes isolated from rats at different ages.  
**A:** viability of hepatocytes as a function of age. Closed circles represent percent of viable cells (measured by the Trypan blue method) obtained during isolations for the purpose of optimization of the isolation procedure, while open circles represent viabilities (%) of hepatocyte suspensions used for determination of transporter activities in the present study.  
**B:** yields of viable hepatocytes used in the present study (triangles). Closed and open points identify data obtained during optimization of isolation procedure and data for batches used in uptake experiments, respectively. Vertical dotted line represents the cut-off value at the age of 26 days to indicate the end of weaning period.

**Figure 2:** Concentration-dependent uptake of TCA in freshly-isolated rat hepatocytes in suspension. Saturable (‘net’) TCA uptake was obtained by subtracting uptake observed in choline buffer (under condition of Na⁺-depletion) from total uptake at 37°C. The data shown were obtained in representative batches of suspended rat hepatocytes (day 2, 21, 29 and 55 days old rats). Points represent mean (± SD, n = 3) values measured for net uptake. Lines represent best fit to experimental data according to the equations described in the *Materials & Methods* section.

**Figure 3:** Age-dependency of clearance values representing ‘net’ uptake of TCA in suspended rat hepatocytes. Net uptake clearances were calculated from Vmax/Km ratios describing the saturable component of TCA uptake. Representative profiles are shown in Figure 2. The solid line represents linear regression. Statistical evaluation was performed by Spearman’s rank correlation analysis yielding r = 0.8. Each point represents the mean (± SD) of clearance values measured in individual batches (n = 3) of rat hepatocytes. Vertical dotted line represents the cut-off value at the age of 26 days to indicate the end of weaning period.
Figure 4: Concentration-dependent uptake of NaFluo in freshly-isolated rat hepatocytes in suspension. Saturable (‘net’) NaFluo uptake was obtained by subtracting uptake observed in the presence of cocktails of inhibitors (ritonavir + glibenclamide + rifamycin SV) from total uptake at 37°C. The data shown were obtained in representative batches of suspended rat hepatocytes (day 2, 21, 29 and 55 days old rats). Points represent mean (± SD, n = 3) values measured for net uptake. Lines represent best fit to experimental data according to the equations described in the Materials & Methods section.

Figure 5: Age-dependency of clearance values representing ‘net’ uptake of NaFluo in suspended rat hepatocytes. Net uptake clearances were calculated from Vmax/Km ratios describing the saturable component of NaFluo uptake. Representative profiles are shown in Figure 4. The solid line represents linear regression. Statistical analysis was performed by Spearman rank correlation analysis on segmented data sets (segment 1: birth till end of weaning; segment 2: end of weaning till adulthood). Each point represents the mean (± SD) of clearance values measured in individual batches (n = 3) of rat hepatocytes. Vertical dotted line represents the cut-off value at the age of 26 days to indicate the end of weaning period.

Figure 6: Comparison of expression levels (literature data) of Ntcp and hepatic Oatp isoforms with their activity levels (present study) in rat hepatocytes at different ages. A: comparison between Ntcp mRNA (black line) and protein expression (dark gray line, protein expression determined by immunohistochemistry; light gray line, protein expression determined by Western blot) (Hardikar et al., 1995; Gao et al., 2004) and Ntcp activity (dashed black line, TCA net uptake clearance, present work). B: comparison between Oatp1a1 (black line), 1a4 (dark gray line) and 1b2 (light gray line) mRNA expression (Macias et al., 2011) and Oatp activity (dashed
black line, NaFluo net uptake clearance, present work). Vertical dotted line represents the cut-off value at the age of 26 days to indicate the end of weaning period.

**Figure 7:** Correlation between NaFluo net uptake clearance and mRNA (Macias et al., 2011) expression of Oatp1a1 (A), 1a4 (B) and 1b2 (C), respectively.
Tables:

Table 1: Experimental conditions used to isolate hepatocytes from rats at various ages using a two-stage collagenase perfusion

<table>
<thead>
<tr>
<th>Rat age</th>
<th>&lt; 1 week</th>
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<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
<th>&gt; 5 weeks</th>
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<td>100 – 300 μl of a</td>
<td>40-50 mg/kg ketamine + 5 mg/kg xylazine; ip</td>
<td>120 mg/kg ketamine + 24 mg/kg xylazine; ip</td>
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<tr>
<td>mixture of 10 mg</td>
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<tr>
<td>ketamine and 0.8 mg</td>
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<tr>
<td>xylazine, qs. to 1ml</td>
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<tr>
<td>with sterile water</td>
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<tr>
<td><strong>Cannula size (G)</strong></td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24/16</td>
<td>16</td>
</tr>
<tr>
<td><strong>Ca++ free buffer</strong></td>
<td></td>
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<tr>
<td>(+ collagenase): flow</td>
<td>4-8</td>
<td>8</td>
<td>10-15</td>
<td>15</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>rate, ml/min</td>
<td></td>
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<tr>
<td><strong>First-stage perfusion</strong></td>
<td></td>
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<tr>
<td>duration (Ca++ free</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td>8</td>
<td>10</td>
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<tr>
<td>buffer), min</td>
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<tr>
<td><strong>Collagenase in</strong></td>
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<tr>
<td>perfusate, mg/ml</td>
<td>0.8-1</td>
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<tr>
<td><strong>Second-stage perfusion</strong></td>
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<tr>
<td>duration (plus</td>
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<td></td>
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<tr>
<td>collagenase). min</td>
<td>4-8</td>
<td></td>
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</tr>
</tbody>
</table>
Table 2: Parameter values for TCA uptake kinetics in suspended rat hepatocytes at different ages.

<table>
<thead>
<tr>
<th>Rat age</th>
<th>&lt; 1 week</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Km (μM)</strong></td>
<td>25.6 ± 1.4</td>
<td>22.5 ± 12.8</td>
<td>21.1 ± 5.4</td>
<td>12.0 ± 0.8</td>
<td>28.0 ± 10.6</td>
<td>19.3 ± 5.7</td>
</tr>
<tr>
<td><strong>Vmax [pmol/(min*million cells)]</strong></td>
<td>1024.5 ± 319.0***</td>
<td>2032.5 ± 828.0*</td>
<td>1038.2 ± 431.0***</td>
<td>1231.7 ± 272.5***</td>
<td>3494.0 ± 514.3</td>
<td>3887.0 ± 639.3</td>
</tr>
<tr>
<td><strong>CL (Vmax/Km) [μl/(min*million cells)]</strong></td>
<td>42.0 ± 15.4*</td>
<td>95.4 ± 7.7</td>
<td>49.3 ± 16.1*</td>
<td>101.9 ± 16.6</td>
<td>138.8 ± 56.4</td>
<td>220.9 ± 103.1</td>
</tr>
<tr>
<td><strong>Kd [μl/(min*million cells)]</strong></td>
<td>0.2 ± 0.2**</td>
<td>0.7 ± 0.9**</td>
<td>2.7 ± 1.4**</td>
<td>3.2 ± 2.8**</td>
<td>9.1 ± 4.6</td>
<td>12.8 ± 2.5</td>
</tr>
</tbody>
</table>

- Values are the mean (± SD) of three different batches of hepatocytes preparation. (except #: two hepatocyte preparations).
- Statistical significance is indicated by *p < 0.05, **p < 0.01 and ***p < 0.001 Anova (and Dunnett's post-hoc), compared to values at 8 weeks old (adult rat) as control condition.
Table 3: Parameter values for NaFluo uptake kinetics in suspended rat hepatocytes at different ages.

<table>
<thead>
<tr>
<th>Rat age</th>
<th>&lt; 1 week</th>
<th>1 week</th>
<th>2 weeks</th>
<th>3 weeks</th>
<th>4 weeks</th>
<th>6 weeks#</th>
<th>8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km (μM)</td>
<td>55.9 ± 39.6</td>
<td>42.4 ± 17.3</td>
<td>56.8 ± 12.2</td>
<td>46.6 ± 1.5</td>
<td>44.6 ± 24.9</td>
<td>48.5 ± 8.4</td>
<td>38.2 ± 13.6</td>
</tr>
<tr>
<td>Vmax [pmol/(min*million cells)]</td>
<td>67.0 ± 18.2**</td>
<td>48.1 ± 6.8***</td>
<td>59.4 ± 40.1***</td>
<td>25.2 ± 11.2***</td>
<td>51.2 ± 24.1***</td>
<td>196.2 ± 7.8</td>
<td>246.5 ± 101.1</td>
</tr>
<tr>
<td>CL (Vmax/Km) [μl/(min*million cells)]</td>
<td>1.5 ± 0.7***</td>
<td>1.3 ± 0.5***</td>
<td>1.2 ± 1.0***</td>
<td>0.5 ± 0.2***</td>
<td>1.2 ± 0.2***</td>
<td>4.1 ± 0.9**</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>Kd [μl/(min*million cells)]</td>
<td>0.3 ± 0.2***</td>
<td>0.3 ± 0.1***</td>
<td>0.3 ± 0.1***</td>
<td>0.3 ± 0.1***</td>
<td>1.0 ± 0.5*</td>
<td>1.4 ± 0.1</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

- Values are the mean (± SD) of three different batches of hepatocyte preparation. (except #: two hepatocyte preparations).
- Statistical significance is indicated by *p < 0.05, **p < 0.01 and ***p < 0.001 Anova (and Dunnett's post-hoc), compared to values at 8 weeks old (adult rat) as control condition.
Figure 2

(A) 2 days old

(B) 21 days old

(C) 29 days old

(D) 55 days old

TCA uptake [pmol/(min*million cells)]

Concentration [μM]
Figure 3

Spearman $r = 0.8$
Figure 5

Spearman $r = 0.98$

NaFluo uptake CL [µl/(min*million cells)]

Age [Days]
Figure 6

(A) Relative level [% of adult] of Ntcp mRNA, Ntcp IHC, Ntcp WB, and TCA uptake CL over age [Days].

(B) Relative level [% of adult] of Oatp1a1 mRNA, Oatp1a4 mRNA, Oatp1b2 mRNA, and NaFluo uptake CL over age [Days].
Figure 7

A

Oatp1a1

NaFluo uptake CL [% of adult]

r² = 0.6199

Oatp1a1 expression [% of adult]

B

Oatp1a4

NaFluo uptake CL [% of adult]

r² = 0.4297

Oatp1a4 expression [% of adult]

C

Oatp1b2

NaFluo uptake CL [% of adult]

r² = 0.8594

Oatp1b2 expression [% of adult]