Age-Dependent Activity of the Uptake Transporters Ntcp and Oatp1b2 in Male Rat Hepatocytes: From Birth Till Adulthood

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ABSTRACT

Recognition of the role of hepatic drug transporters in elimination of xenobiotics continues to grow. Hepatic uptake transporters, such as hepatic isoforms of the organic anion-transporting polypeptide (Oatp) family as well as the bile acid transporter Na+-taurocholate cotransporting polypeptide (Ntcp) have been studied extensively both at the mRNA and protein expression levels in adults. However, in pediatric/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 Oatp. 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. In contrast, 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 onward. As a consequence, developmental changes in the kidney and liver cause differences in xenobiotic response between children and adults and even between different pediatric 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 (P450) isoenzymes, of which the ontogeny has been extensively investigated. In fetal liver, the total P450 content accounts for 30–60% of adult levels; however, these isoenzymes develop in a particular developmental pattern. For instance, the activity of CYP3A is higher before birth, whereas postnatally it declines to adult levels within 2 years. In contrast, CYP3A4, the main hepatic P450 isoenzyme involved in drug metabolism, reaches adult levels by the first year of life (Akorn and McNamara, 2002; Allegaert et al., 2008; de Wildt, 2011).

Several studies have revealed that drug transporters also undergo ontogenetic 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 Na+-taurocholate cotransporting polypeptide (Ntcp), 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. (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 organic anion-transporting polypeptide (Oatp) 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 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 pediatric donors. Nonetheless, reliable preclinical animal data regarding ontogeny of drug

ABBREVIATIONS: ANOVA, analysis of variance; LC-MS, liquid chromatography–mass spectrometry; NaFluo, sodium fluorescein; Ntcp, Na+-taurocholate cotransporting peptide; OATP/Oatp, organic anion-transporting polypeptide; P450, cytochrome P450; TCA, taurocholic acid.
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 prediction in juvenile animals and/or pediatric 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 generate more accurate clearance predictions compared with 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. [3H]Taurouric acid (TCA) (specific activity, 4.6 Ci/mmol) and scintillation cocktail were obtained from PerkinElmer Life Sciences (Boston, MA). Sodium fluorescein (NaFluo) was purchased from Union Chimique Belge (Leuven, Belgium). 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, and 1% silicon oil, sodium pyruvate, choline chloride, choline bicarbonate, glibenclamide, pyruvic acid, rifamycin SV, and taurocholic sodium salt were purchased from Sigma-Aldrich (Schnelldorf, Germany). Mineral oil was purchased from MP Biochemical (Illkirch, France). Triton X-100, collagenase (type IV), silicon oil, sodium pyruvate, choline chloride, choline bicharbonate, glibenclamide, pyruvic acid, rifamycin SV, and taurocholic sodium salt were sourced from Sigma-Aldrich (Schnelldorf, Germany). Mineral oil was purchased from Acros Organics (Geel, Belgium).

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, and 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.

Hepatocyte Isolation. Rats aged 2–7 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 intraperitoneally. For pups (P7–P10), a mixture of 40–50 mg ketamine and 5 mg xylazine per kg body weight was used, whereas for pups younger than 7 days, 100–300 μl mixture of 10 mg ketamine and 0.8 mg xylazine per kg body weight was given intraperitoneally. Rats were sacrificed at different days ranging from 2 to 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 (500 × g for 3 minutes at 4°C, and the pellet was resuspended in William’s E medium containing 5% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomyacin. Hepatocytes were counted using a hemocytometer, and cell viability was determined using trypan blue. Freshly isolated rat hepatocytes were subsequently resuspended in Krebs Henseleit Buffer (KHB): 118 mM NaCl, 5.17 mM KCl, 1.2 mM CaCl₂, 2H₂O, 1.2 mM MgCl₂·6H₂O, 23.8 mM NaHCO₃, 12.5 mM HEPES, 5 mM glucose, and 5 mM Na-pyruvate (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 (4 million/ml) were preincubated for 15 minutes at 37°C. For experiments in which nonsaturable uptake rates were determined, 350 μl inhibitor solution was added during preincubation time. Subsequently, 175 μl substrate solution was added to initiate the incubation at 37°C, which lasted 90 seconds for NaFluo and 60 seconds for TCA. Triplicate 200 μl aliquots of the suspension were immediately transferred to 1.5-ml ice-cold Eppendorf tubes, containing 700 μl oil layer (a mixture of silicone oil and mineral oil 82:18) above 300 μl 8% NaCl solution (2 N NaOH for [3H]TCA). The tubes were immediately centrifuged twice for 2 minutes at 14,000 g using a table-top centrifuge and afterward frozen on dry ice. The pellets obtained were solubilized in 300 μl 0.5% Triton X-100 (in phosphate-buffered saline), and the fluorescence was measured using fluorescence spectroscopy (excitation, 494 nm; emission, 520 nm) in a Tecan Infinite M200 plate reader (Männedorf, Austria) for determination of NaFluo concentration. For [3H]TCA samples, the obtained pellets were placed in a scintillation vial containing 2 ml scintillation cocktail, and radioactivity was quantified using liquid scintillation spectrometry (Wallac 1410, Turku, Finland). During the course of this study, a liquid chromatography–mass spectrometry (LC-MS) method was developed to analyze TCA uptake in suspended hepatocytes without need for radiolabeled compound. Quantification of TCA in hepatocyte pellets from incubations performed after this LC-MS method implementation was extracted with chilled methanol/water containing 1 μM deuterated cholic acid. The extracted samples were centrifuged for 20 minutes at 14,000 rpm, and supernatants were analyzed with a Thermo Fisher Scientific LC-MS/MS system (Thermo Fisher, 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). A Kinetix XB-C18 column (2.6 μm, 50 × 2.10 mm) with a KrudKatcher ultra HPLC in-line filter (Phenomenex, Utrecht, The Netherlands) was used. The injection volume was 25 μl. The chromatographic conditions were 400 μl/min flow rate for 4 minutes, 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 minute to 0.3 minute; subsequently, the ratio of A:B changed to 95:5 in 2.1 minutes, which was maintained for the following 0.9 minute. Then the column was re-equilibrated for the last 0.7 minute 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 deuterated cholic acid. Linearity was obtained over a TCA concentration range of 0.016–1 μM. Analytical methods were validated with nominal quality control 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 14.3–18.5%. The interday inaccuracy varied from −0.9

<table>
<thead>
<tr>
<th>Table 1: Experimental conditions used to isolate hepatocytes from rats at various ages using a two-stage collagenase perfusion</th>
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<tbody>
<tr>
<td>Rat Age</td>
</tr>
<tr>
<td>Anesthesia</td>
</tr>
<tr>
<td>Cannula size (G)</td>
</tr>
<tr>
<td>Ca²⁺ free buffer (+ collagenase): flow rate, ml/min</td>
</tr>
<tr>
<td>First-stage perfusion duration (Ca²⁺ free buffer), min</td>
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<tr>
<td>Collagenase in perfusate, mg/ml</td>
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<tr>
<td>Second-stage perfusion duration (plus collagenase), min</td>
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</tbody>
</table>
uptake kinetics was described with equation 1:

\[ V = \frac{V_{\text{max}} \cdot C}{K_m + C} \]  

(1)

with \( V_{\text{max}} \) and \( K_m \) representing the kinetic parameters for saturable uptake (Michaelis-Menten). Net cellular uptake clearances were calculated by \( V_{\text{max}}/K_m \). 

Net cellular uptake clearances were calculated by subtracting uptake in the presence of inhibitors from total uptake at 37°C. The Spearman rank correlation coefficient equaled 0.8 with age. This is consistent with previously reported data (Deschenes 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.** Analysis of variance (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 of <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 fivefold, 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%) (Fig. 1A) and to avoid interbatch variability. As shown in Fig. 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 nonlinear fashion, corresponding to saturable uptake according to the Michaelis-Menten equation for all ages (Fig. 2; Supplemental Fig. 1, A–K). The corresponding kinetic parameters are listed in Table 2. The average \( K_m \) values across different ages, except at week 3, remained constant, whereas marked differences in \( V_{\text{max}} \) values were noted (one-way ANOVA, \( P = 0.2 \) and 0.0003 for \( K_m \) and \( V_{\text{max}} \), respectively). The net TCA clearance, as shown in Fig. 3 and Table 2, increases rapidly during the weaning period and reaches 63% by week 4 postnataally. The Spearman rank correlation coefficient equaled 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 rifampycin SV, glibenclamide, and ritonavir at concentrations of 200, 20, and 40 \( \mu \)M, 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 (Fig. 4; Supplemental Fig. 2, A–P). As observed for Ntcp activity, the average \( K_m \) values for NaFluo uptake remained constant with age and \( V_{\text{max}} \) values gradually increased after weaning (one-way ANOVA, \( P = 0.9 \) and 0.0003 for \( K_m \) and \( V_{\text{max}} \), respectively). As shown in Fig. 5, the net NaFluo remains remarkably low in young animals, that is, 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 Oatp were compared with mRNA and protein expression profiles of corresponding transporter isoforms in rat hepatocytes. As shown in Fig. 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 (Fig. 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 0.86 were found for Oatp1a1, Oatp1a4, and 1b2, respectively (Fig. 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 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 (Figs. 2 and 4; Supplemental Figs. 1 and 2). Based on net clearance, different ontogeny profiles were observed for Ntcp and Oatp (Figs. 3 and 5). The activity of Ntcp-mediating TCA clearance developed during weaning, whereas Oatp-mediating NaFluo clearance showed a biphasic profile and was found to develop after weaning. Furthermore, transporter activities showed a similar profile of maturation as observed for mRNA and protein expression (Fig. 6), 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

**TABLE 2**

Parameter values for taurocholic acid uptake kinetics in suspended rat hepatocytes at different ages

Values are the mean (±S.D.) of three different batches of hepatocyte preparation (except two batches at <1 week and 1 week). Statistical significance is indicated by *P < 0.05, **P < 0.01, and ***P < 0.001 analysis of variance (and Dunnett’s post hoc), compared with values at 8 weeks old (adult rat) as control condition.

<table>
<thead>
<tr>
<th>Parameter</th>
<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>( K_m ) (mM)</td>
<td></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>( V_{max} ) (pmol/(min × million cells))</td>
<td>1024.5 ± 319.0***</td>
<td>2032.5 ± 828.0*</td>
<td>1038.2 ± 431.0***</td>
<td>1213.7 ± 272.5***</td>
<td>3494.0 ± 514.3</td>
<td>3887.0 ± 639.3</td>
<td></td>
</tr>
<tr>
<td>CL (( V_{max}/K_m )) (µl/(min × million cells))</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>
<td></td>
</tr>
<tr>
<td>( K_d ) (µl/(min × million cells))</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>
<td></td>
</tr>
</tbody>
</table>
included in the measurement. In addition, plasma membrane protein may not be fully operative, that is, not glycoslated (Hardikar et al., 1995), because enzymes mediating protein glycosylation are undergoing ontogenic regulation (Oda-Tamai et al., 1989, 1991).

The age-dependent uptake clearance of TCA (Fig. 3) is in agreement with previous data regarding TCA clearance in humans and rodents (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, whereas 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, as follows: first, in the presence of extracellular sodium (normal buffer); in the second setup, in the absence of extracellular sodium (buffer where sodium was 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 physiologic 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 bile salt export pump). The sodium-dependent bile acid transporter (Asbt) and the heterodimeric organic solute transporter αβ (Ost αβ), 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, sodium-dependent bile acid transporter mRNA increases up to 400-fold between postnatal days

Fig. 3. Age dependency of clearance values representing net uptake of TCA in suspended rat hepatocytes. Net uptake clearances were calculated from $V_{\text{max}}/K_m$ ratios describing the saturable component of TCA uptake. Representative profiles are shown in Fig. 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 (± S.D.) of clearance values measured in individual batches ($n = 3$) of rat hepatocytes. Vertical dotted line represents the cutoff value at the age of 26 days to indicate the end of weaning period.

Fig. 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 (A: 2-, B: 21-, C: 29- and D: 55-days-old rats). Points represent mean (± S.D., $n = 3$) values measured for net uptake. Lines represent best fit to experimental data according to the equations described in Materials and Methods.
analysis of variance (and Dunnett’s post hoc) competition, compared with values at 8 weeks old (adult rat) as control condition.

TABLE 3
Parameter values for sodium fluorescein 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><strong>Km (μM)</strong></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><strong>Vmax [pmol/(min × million cells)]</strong></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><strong>CL (Vmax/Km) [μl/(min × million cells)]</strong></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><strong>Kd [μl/(min × million cells)]</strong></td>
<td>0.3 ± 0.2***</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>
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</table>

Fig. 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 Fig. 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 (±S.D.) of clearance values measured in individual batches (n = 3) of rat hepatocytes. Vertical dotted line represents the cutoff value at the age of 26 days to indicate the end of weaning period.

Compared with Ntcp, activity of Oatp revealed a considerably slower pattern of development. Uptake clearance of NaFluo was significantly lower during the weaning period and increased gradually toward 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 (r² = 0.86). In contrast, poor correlation was seen with Oatp1a1 (r² = 0.62) and Oatp1a4 (r² = 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 with the other isoforms (Csaky et al., 2011). This conclusion is supported by our previously published work on the

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characterization of NaFluo as an Oatp/OATP probe substrate, showing that NaFluo uptake was mediated by OATP1B1/3 in transfected Chinese hamster ovary 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 Oatp family plays 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 Oatp, 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 Xenopus Laevis oocytes (Meier-Abt et al., 2004; Herraez et al., 2009). Petzinger et al. (1979) 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 (Ziegler et al., 1979). Consistently, uptake of unconjugated bile acid such as cholate is mediated by Oatp/OATP, especially Oatp1b2 (Meier et al., 1997; Csanky et al., 2011).

For both transporters investigated in the present study (Ntcp/Oatp1b2), the affinity (as reflected by the \( K_m \); Tables 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 physiologic 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 most likely involved. For instance, it has been reported that activation of peroxisome proliferator-activated receptor \( \alpha \) by natural fatty acids leads to downregulation of NTCP and OATP (Li and Chiang, 2009). Because rat milk is richer in fat content compared with adult rat food (Sniff R/M-H standard food for rats; ssniff Spezialdiäten Gmbh, Soest, Germany), 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 pediatric populations, leading to safer and more effective drug therapies for children.

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Authorship Contributions
Participated in research design: Fattah, Annaert.
Conducted experiments: Fattah.
Contributed new reagents or analytic tools: Fattah, Annaert.
Performed data analysis: Fattah, Annaert.
Wrote or contributed to the writing of the manuscript: Fattah, Augustijns, Annaert.
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