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Title

Developmental changes in hepatic OCT1 protein expression from neonates to children

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Running title page

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Abstract

Organic cation transporter 1 (OCT1) plays an important role in the disposition of clinically-important drugs, and the capacity of OCT1 activity is presumed to be proportional to the protein expression level in organ tissues. Presently, knowledge of OCT1 protein expression in children is very limited, especially among neonates and small infants. Here, we report on the characterization of OCT1 protein expression in neonatal, infant and pediatric liver samples performed by Immunoblot analysis. OCT1 protein expression was detected in liver samples from neonates as early as postnatal day 1 – 2. This youngest group showed significantly lower OCT1 expression normalized by GAPDH (0.03 ±0.02 arbitrary unit (AU), mean ± SD, N=7) compared to samples aged 3 - 4 weeks (0.08 ±0.03 AU, N=5, $^{**}P< 0.01$), 3 - 6 months (0.23 ± 0.15 AU, N=7, $^{**}P< 0.01$), 11 months - 1 year (0.42 ± 0.32 AU, N=6, $^{**}P< 0.01$), and 8 - 12 years (1.00 ± 0.44 AU, N=7, $^{**}P< 0.01$). These data demonstrate an age-dependent increase in OCT1 expression from birth up to 8-12 years of age, and the findings of this study contribute to the understanding of OCT1 functional capacity and their effect of the disposition of OCT1 substrates in neonates and small infants.

Introduction

Organic cation transporter 1 (OCT1, alternative gene name *SLC22A1*) is known to contribute to the disposition of clinically-important drugs such as morphine (Tzvetkov et al., 2013), oxaliplatin (Zhang et al., 2006), and metformin (Umehara et al., 2007). Cellular expression of OCT1 is localized to sinusoidal membranes of hepatocytes (Koepsell et al., 2007) and likely, also to basolateral membranes of kidney proximal tubule cells (Kimura et al., 2002; Mulgaonkar et al., 2013).

Recently, we demonstrated that OCT1 plays a key role in morphine clearance among pediatric patients above 6 years old (Fukuda et al., 2013). Meanwhile, there are multiple pharmacokinetics studies of morphine in neonates and infants; however, the mechanistic understanding of what is driving maturation and variability in these populations has remained to be addressed despite a high frequency of use in these developing patients for whom organ size and function typically change rapidly. Contemporary studies relating to OCT1 expression generally report on levels of OCT1 mRNA transcript and do not describe protein abundance, a deficit that has been mentioned with some emphasis by the Pediatric Transporter Working Group (Brouwer et al., 2015). Given the linkage between OCT1 protein expression, hepatic function, and the capacity for morphine clearance, this knowledge gap prompted us to investigate the developmental changes of OCT1 protein expression in neonates and infants. Here we report on the developmental trajectory of hepatic OCT1 protein expression in pediatric subjects, with emphasis given toward neonates and small infants. Importantly, the results of the present study, were of sufficient resolution to distinguish differences in OCT1 protein expression in neonatal subjects compared to those at an older age.

Materials and Methods

Human Liver Samples

Liver tissue samples from pediatric patients including neonates (total N = 36 subjects) were provided by the Better Outcomes for Children Biorepository, administered by the CAP-accredited Cincinnati Biobank. Use of provided tissue and all experimental procedures were approved by the Biobank Tissue Use Committee. All samples provided were previously characterized as either healthy tissue or relatively healthy tissue adjacent to diseased or abnormal tissue by institutional pathologists, and were de-identified prior to their release. All tissue samples were maintained at -80^{o} C both prior to and following their release from the biorepository up until the time of their processing for assay. All tissue handling including weighing of tissue and subdividing of larger tissue samples were performed frozen over dry ice to prevent thawing prior to analysis.

Donor information

The age of individual donors at the time of tissue collection was between 1 day postnatal up to 12 years old (N= 32). Of this total, N=7 samples were collected from donors aged between days 1 and 2 postnatal, N=5 samples came from donors aged between 3 – 4 weeks, N= 7 aged between 3 – 6 months, N= 6 between 11 months – 1 year, and N=7 samples were from donors aged between 8 and 12 years. These age ranges were used to bin expression data in the following analysis. The percentages of male and female donors were 53% and 47%, respectively. Racial percentages in donors were as follows: 47% White; 6% Black; 22% other; and 25% unknown.

Crude Membrane Isolation

Membrane fractions were prepared from frozen pediatric liver tissue by methods previously reported, with some modification (Ogihara et al., 1996). Tissue sample portions weighing between 20 - 50 mg were maintained at 0^o C while being rapidly minced with a clean razor blade to particles of $\leq$ 1 mm^3 in size. Minced tissue was immediately dispersed within a 15-fold volume (µL/mg) of pre-chilled (0^o C), filter-sterile homogenization buffer containing 250 mM sucrose, 6 mM HEPES (pH 7.4), 0.4 mM Sodium Deoxycholate (Pierce, Rockford IL), and freshly-added 1× Protease Inhibitor Cocktail (Sigma, St. Louis, MO) and 1 mM PMSF (Amresco, Solon, OH). Disruption of minced liver tissue was performed by homogenization on ice using a Polytron model: PT 1300D homogenizer (KINEMATICA, Luzern, Switzerland) equipped with the PT-DA 03/2EC-E05 dispersing aggregate set to 18,000 RPM for 30 seconds, followed by 2 rounds of freeze/thawing over dry ice. Homogenates were centrifuged at 3000 RCF and 4^o C for 15 minutes to sediment cellular debris, and the resulting supernatant was retained and combined with an additional 50 µL wash of the debris pellet using fresh homogenization buffer. Membranes were precipitated from the combined supernatant and wash supernatants by centrifugation at 100,000 RCF for 1 hour at 4^oC in an Optima MAX-XP Ultracentrifuge (Beckman Coulter, Brea, CA). After careful removal of supernatant, the membrane pellets were suspended in 8 volumes (µL/mg) of a sample buffer containing 50 mM Mannitol, 20 mM HEPES (pH 7.5), and 0.5% (M/V) of sodium lauroyl sarkosinate (Sarkosyl). Membranes were re-suspended and homogenized by 10 passes each through the bore of a 25 G followed a 27 G hypodermic needle (BD Biosciences, San Jose, CA). The re-suspended membrane fractions were assayed for protein concentration, and stored in aliquots at -80^o C until time of assay. Regarding the membrane recovery, there was no significant difference between

the 5 age groups studied (1-2 days, 3-4 weeks, 3-6 months, 11 months -1 year, 8-12 years of age). This finding was consistent with the previous report by Prasad et al (Prasad et al., 2016) using pediatric (n=69) and adult (n=41) livers.

Immunoblot Analysis and Quantitation of OCT1 Protein in Human Liver Samples

Protein concentrations of liver membrane fractions were determined in triplicate using the Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) following the manufacturer's protocol for 96-well plate format. Immunoblot analysis for OCT1 (SLC22A1) and control protein Glyceraldehyde-6-Phosphate Dehydrogenase (GAPDH) abundance in isolated membrane fractions were performed essentially as described previously (Hahn et al., 2014) with the following modifications: to preserve integrity of OCT1 protein, denaturation of membranes in Laemmli sample buffer (BioRad, Hercules, CA) was performed for 30 minutes at 37^{o} C, and proteins resolved by PAGE electrophoresis were transferred to PVDF membranes for downstream blotting. Immunoblot analysis was then performed using antibodies specific for OCT1 (Genetex, Irvine, CA, SLC22A1 [2C5] mAb - item GTX80400, lot 821505071) and GAPDH (Genetex). The peptide sequence recognized by this OCT1 antibody showed 82% homology (including peptide tag as a part of recombinant antigen) for human OCT1 using the BLAST search.

OCT1 genotyping

OCT1 genotyping was conducted according to the method reported by Fukuda et al. (Fukuda et al., 2013). Briefly, DNA was prepared from liver samples using the AllPrep DNA/RNA/Protein Mini kit (Qiagen, Hilden, Germany). A TaqMan probes were used for genotyping of single nucleotide polymorphisms (SNPs) in the genes of OCT1, including Arg61Cys (rs12208357),

Gly401Ser (rs34130495), Gly465Arg (rs34059508) and the deletion of Met420 (rs72552763). In addition, OCT1 intron SNP (rs622342) was also determined. The genotyping was performed using commercially available TaqMan assays (Applied Biosystems, CA, USA).

Statistical Analysis

Background-subtracted densitometry data were collected from 16-bit linear tagged image files of individual blot exposures using the FIJI image analysis platform (Schindelin et al., 2012) and initial data were composed in MS Excel. Expression levels for all donor samples were calculated and expressed as an arbitrary unit (AU) based on GAPDH- and membrane protein amount-normalized OCT1 signal density and expressed as a multiple of the 8-12 year group (N= 7) mean value. Statistical differences in OCT1 expression were evaluated based on the nonparametric comparison of each pair by means of the Wilcoxon method using JMP® statistical software (Version 10, SAS Institute Inc., Cary, NC).

Results and Discussion

OCT1 protein expression was detectable in hepatocyte membranes of donors from all ages included in the study, between postnatal day 1 up to 12 years of age (Figure 1). OCT1 protein expression increased with donor age across this developmental time frame (Figure 2). The observed change in OCT1 expression was non-linear in relation to donor age, supporting the concept of rapid expression changes during development. The rate of OCT1 expression increase was reduced over time. A rapid increase of OCT1 protein was observed between samples aged 1-2 days and 3-4 weeks, although expression levels were lower for both groups compared to the older children. OCT1 expression normalized by GAPDH in the youngest samples at 1 - 2 days (mean ± SD, 0.03 ± 0.02 AU, N=7) was significantly lower than in all other age groups studied, being inclusive of samples aged 3 - 4 weeks (0.08 ± 0.03 AU, N= 5, $^{**}P < 0.01$), 3 - 6 months (0.23 ± 0.15, N= 7, $^{**}P < 0.01$), 11 months - 1 year (0.42 ± 0.32 AU, N= 6, $^{**}P < 0.01$), and 8 - 12 years (1.00 ± 0.44 AU, N= 7, $^{**}P < 0.01$). The 3 - 4 weeks group also possessed significantly lower OCT1 protein compared to the 11 months - 1 year ($^{**}P < 0.01$) and 8 - 12 years ($^{**}P < 0.01$) groups. The 3 – 6 months and the 11 months - 1 year groups were significantly lower OCT1 protein expression compared to the 8 – 12 years ($^{**}P<0.01$ and $^{*}P<0.05$, respectively). OCT1 expression normalized by membrane protein amount increased in a similar age-dependent manner.

According to the OCT1 genotype, pediatric donors were classified into three assumed phenotype groups: wild type (having two *1 genotypes, n=19), heterozygotes (having one *1 genotype, n=10) and homozygotes (N=3) (Figure 3). Nies et al (Nies et al., 2009) reported that OCT1-Arg61Cys variant (rs12208357) strongly correlated with decreased OCT1 protein expression ($P<0.0001$). In this study, we have four donors having this variant as heterozygotes, as shown in

Figure 3. Although OCT1 expression level showed a decreasing trend according to the presence of the variant in the 8-12 age group, the sample size was too small to determine the genetic effect on the OCT1 protein expression.

The results of the present study are consistent with a previous report by Prasad et al (Prasad et al., 2016), that found significantly lower OCT1 protein levels in hepatic membranes of neonates (age 0-28 days) and infants (age 29 days to 1 year), as compared to older children, adolescents, and adults. A limitation of the referenced work however, is its inability to discern age-dependent differences in OCT1 expression among donors less than 1 year old owing to a low number (N = 4) of samples available. Similarly, a limitation of the present study was the lack of available adult tissue from which to compare OCT1 expression levels. Notably however, Klaassen and Lauren reported previously that there is no significant difference in liver OCT1 mRNA expression levels between children aged 7 and older, and adults (Klaassen and Aleksunes, 2010). In addition, Nies et al. reported a significant correlation between OCT1 protein and mRNA transcript levels in normal adult liver (Nies et al., 2009). It is therefore postulated that OCT1 protein expression may reach mature levels after 7 years of age. In order to capture the accurate ontogeny profile of hepatic OCT1 protein in humans, further accumulation of OCT1 protein expression data across the developmental age range is needed, in addition to considering the relationships of gender, race, and OCT1 genotypes.

In summary, the key findings of the current study include the observation that OCT1 protein is expressed in neonatal liver as early as day 1 postnatal, that hepatic OCT1 protein expression increased rapidly from birth through early infancy and up to 8 years of age. This observation suggests that the contribution of OCT1 transporter function to drug disposition may likewise increase rapidly in the neonatal period in conjunction with OCT1 protein expression.

This knowledge will be utilized as an age-dependent system physiological parameter for physiologically-based pharmacokinetics modeling to simulate the pharmacokinetic profiles of OCT1 substrates in neonates.

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Authorship Contributions:

Participated in research design: Hahn, Emoto, Vinks, and Fukuda

Conducted experiments: Hahn

Contributed new reagents or analytical tools: Hahn

Performed data analysis: Hahn, Emoto, and Fukuda

Wrote or contributed to the writing of the manuscript: Hahn, Emoto, Vinks, and Fukuda

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Figure legends:

Figure 1. Example immunoblot of organic cation transporter 1 (OCT1).

Isolated crude membrane fractions (20 µg protein per lane) prepared from liver tissue from human donors aged between 1 day postnatal and 12 years were resolved by SDS-PAGE and analyzed for expression of OCT1 by immunoblot as described under "materials and methods". Target protein molecular mass were estimated from protein mass standard (protein ladder) included in lanes flanking liver membrane fractions. Upper: OCT1 appeared mainly around 70 kilodaltons (kDa) in all samples analyzed in this study. An additional band at approximately 50 kDa was shown in some, but not all samples. These band signals at 70 kDa and 50 kDa represent the glycosylated and un-glycosylated forms of OCT1 respectively as discussed by Nies et al (Nies et al., 2009). Lower: Immunoblot signal for internal standard protein GAPDH appeared as a distinct band of approximately 42 kDa and did not vary by tissue donor age.

Figure 2. Comparative analysis of hepatic membrane OCT1 protein levels by donor age.

Relative levels of OCT1 protein were quantitated by immunoblot of isolated membrane fractions from liver tissues collected from donors between postnatal day 1 and 12 years of age. Data are binned by donor age into 5 discrete age groups as described under "Materials and Methods". OCT1 levels were calculated from membrane GAPDH (A, C) - and membrane protein (B, D)-normalized OCT1 signal density from a minimum of 3 immunoblots per sample. Final OCT1 values were normalized to the mean protein level for subjects aged 8-12 years and expressed in Arbitrary Units (AU).

(A, B) Relative OCT1 protein levels for individual samples at each age group. Each symbol represents individual data and bars represent the group mean value ± SD.

(C, D) Statistical analysis of differential OCT1 protein expression by age group. Statistical differences in OCT1 expression were evaluated based on the nonparametric comparison of each pair by means of the Wilcoxon method using JMP® statistical software (Version 10, SAS Institute Inc., Cary, NC). Significant differences are reported as *P* value; where none are reported, associated groups were not significantly different in OCT1 protein concentration.

Figure 3. Relative levels of hepatic membrane OCT1 protein levels by assumed phenotype groups.

Pediatric donors were classified into three assumed phenotype groups: wild type (having two *1 genotypes, n=19, A), heterozygotes (having one *1 genotype, n=10, B) and homozygotes (N=3, C). Relative OCT1 protein levels, which were calculated from membrane GAPDH-normalized OCT1 signal density, for individual samples at each age group. Each symbol represents individual data. * shows individual donor having the OCT1-Arg61Cys variant (rs12208357).

Figure 1

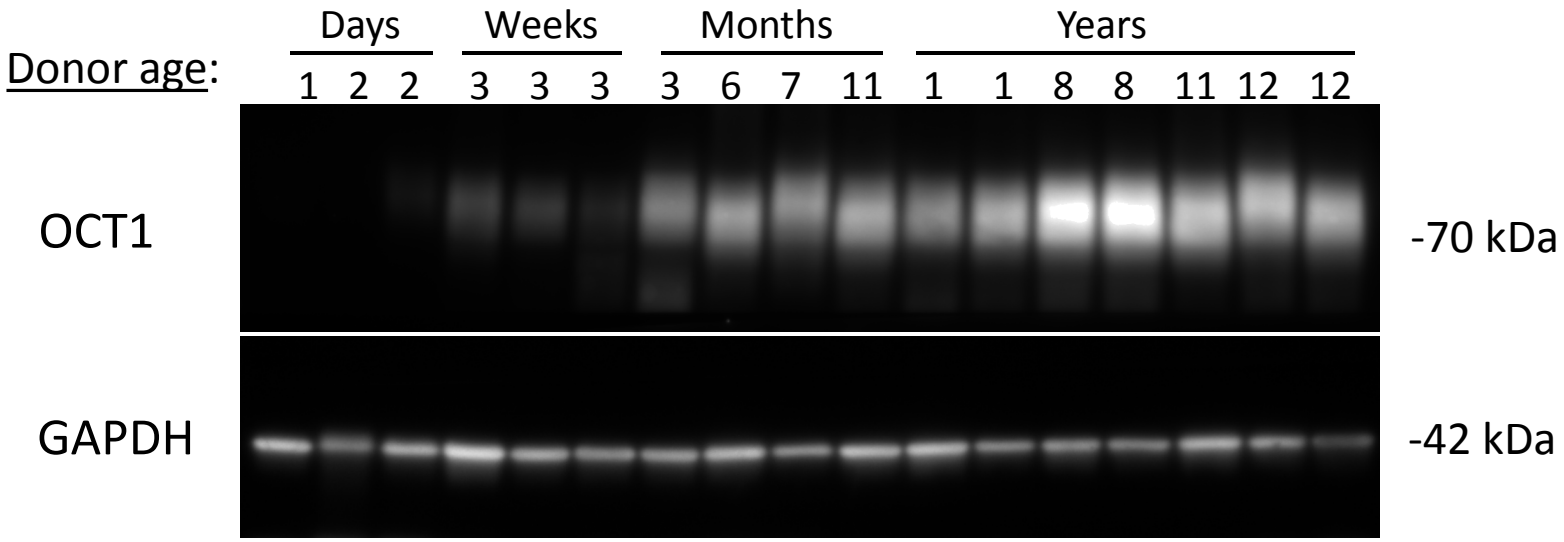
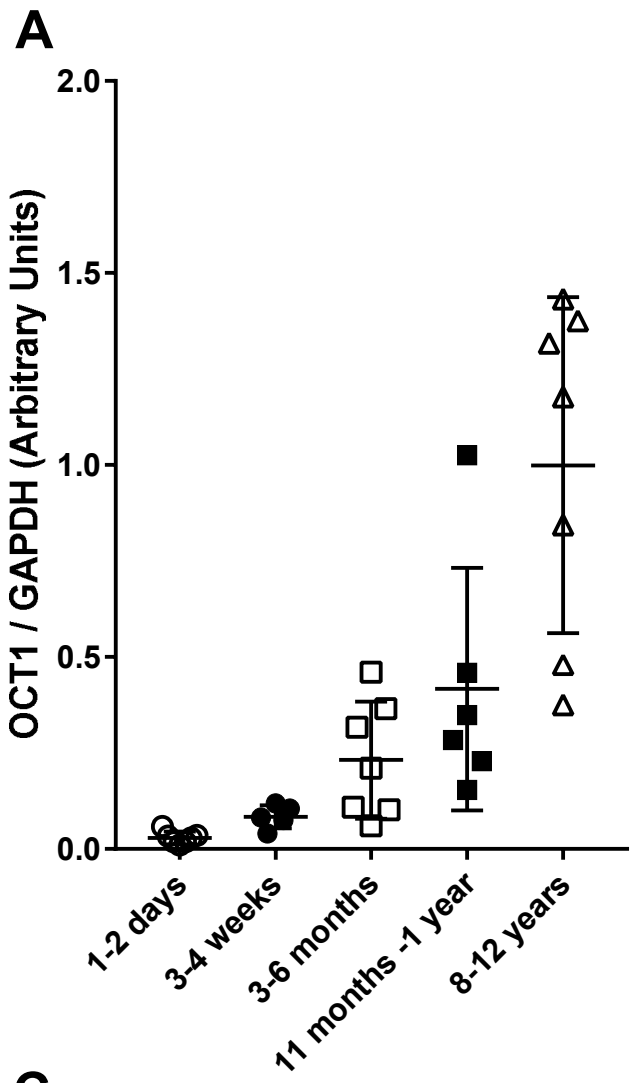
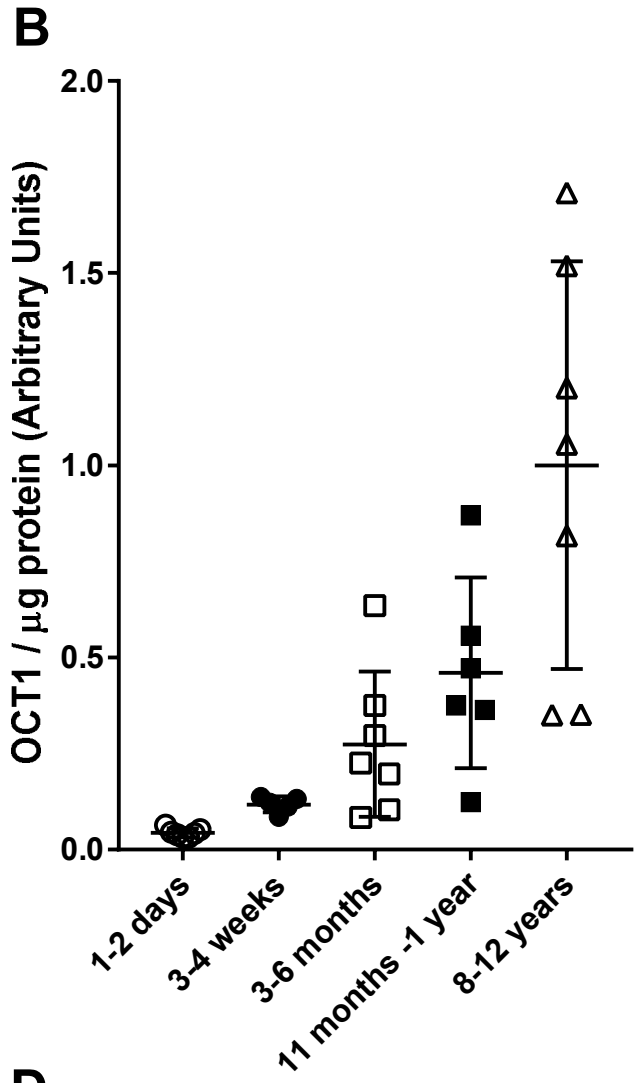

Days
Weeks
Months
Years
Donor age:
1 2 2 3 3 3 3 6 7 11 1 1 8 8 11 12 12
OCT1
-70 kDa
GAPDH
-42 kDa

Figure 2





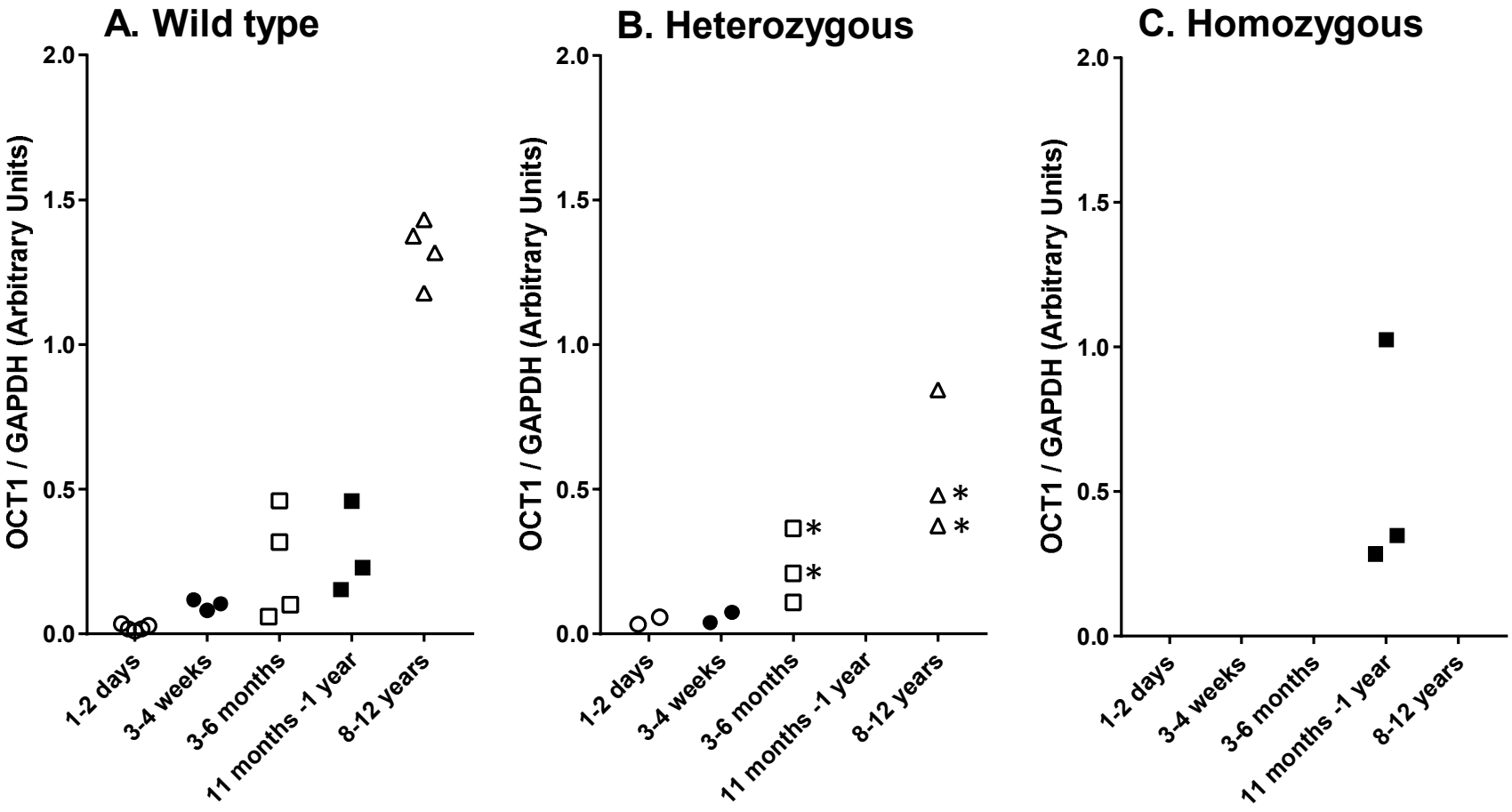
C

	1-2 days	3-4 weeks	3-6 months	11-24 months
1-2 days				
3-4 weeks	**P < 0.01*			
3-6 months	**P < 0.01*			
11-24 months	**P < 0.01*	**P < 0.01*		
8-12 years	**P < 0.01*	**P < 0.01*	**P < 0.01*	**P < 0.05*

D

	1-2 days	3-4 weeks	3-6 months
1-2 days			
3-4 weeks	**P < 0.01*		
3-6 months	**P < 0.01*		
11-24 months	**P < 0.01*	**P < 0.05*	
8-12 years	**P < 0.01*	**P < 0.01*	**P < 0.05*

Figure 3


A. Wild type
B. Heterozygous
C. Homozygous
OCT1 / GAPDH (Arbitrary Units)
2.0
1.5
1.0
0.5
0.0
1-2 days
3-4 weeks
3-6 months
11 months -1 year
8-12 years
*