Placenta passage of the thyroid hormone analogue DITPA to male wild-type and Mct8 deficient mice

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MCT8 deficiency causes severe X-linked intellectual and neuropsychological impairment associated with abnormal thyroid function tests (TFTs) producing thyroid hormone (TH) deprivation in brain and excess in peripheral tissues. The TH analogue diiodothyropropionic acid (DITPA) corrected the TFTs abnormalities and hypermetabolism of MCT8 deficient children but did not improve the neurological phenotype. The latter result was attributed to the late initiation of treatment. Therefore, we gave DITPA to pregnant mice carrying Mct8 deficient embryos, in order to determine whether DITPA, when given prenatally, crosses the placenta, and affects the serum TFTs and cerebral cortex of embryos.

After depletion of the endogenous TH, Mct8 heterozygous pregnant dams, carrying both wild-type (*Wt*) and Mct8 deficient (*Mct8KO*) male embryos were given DITPA. Effects were compared to those treated with L-T_4. With DITPA treatment, serum DITPA concentration was not different in the two genotypes, which produced equal effect on serum TSH levels in both groups of pups. In contrast, with L-T_4 treatment, TSH did not normalize in *Mct8KO* pups while it did in the *Wt* littermates and dams despite higher concentration of serum T_4. Finally, both treatments similarly modulated the expression of the TH-dependent genes *Shh*, *Klf9* and *Aldh1a3* in brain. Thus, the ability of DITPA to cross the placenta, its thyromimetic action on the expression of TH dependent genes in brain and its better accessibility to the pituitary than L-T_4, as assessed by serum TSH, make DITPA a candidate for the prenatal treatment of MCT8 deficiency.

Mutations in the *monocarboxylate transporter 8* (*MCT8*, *SLC16A2*) gene, located on the X chromosome, produce in males a syndrome of severe psychomotor retardation and unusual thyroid function tests (TFTs) abnormalities consisting of high serum T_3, low rT_3, low T_4, and normal or slightly elevated TSH (1, 2). The earliest neurological manifestation in the first few months of life is truncal hypotonia with poor head control, progressing to spastic quadriplegia, lack of speech and poor communication skills. Based on the well-established role of thyroid hormone (TH) in brain development (3) it is assumed that in the absence of MCT8, impaired transport of TH across the blood-brain barrier and into neurons of the central nervous system (CNS) has devastating consequences for neural differentiation and proper brain function (4). At the same time, the high serum T_3 in MCT8 deficiency increases energy expenditure resulting in failure to maintain weight despite adequate calorie intake. This hypermetabolic state is generated by tissues that are not predominantly MCT8 dependent for TH transport, including liver and skeletal muscle (5). This coexistence of TH deficiency and excess in patients with MCT8 deficiency (1, 2) has challenged the treatment using simple hormonal replacement.

To circumvent this therapeutic dilemma, TH analogues that exert thyromimetic actions by entering cells indepen-

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dently of MCT8 were sought. As the TH analog diiodothyropropionic acid (DITPA) corrected the effect of TH deprivation in CNS of Mct8 deficient (*Mct8KO*) mice without causing hormonal excess in peripheral tissues (6), DITPA was given on a compassionate basis to four children with MCT8 deficiency for 26–40 months (7). This treatment (2.1–2.4 mg/kg*d), initiated at 8.5–25 months of age, normalized the TFTs and reduced the hypermetabolism and the tendency for weight loss. Although none of the children treated with DITPA developed seizures, there was no measurable improvement in the neurodevelopment deficit. This is likely due to the irreversible neurological damage incurred during the fetal and very early neonatal life (7–9) that is not improved by postnatal treatment with DITPA (7). The ability to diagnose MCT8 deficiency prenatally, raises the possibility of prenatal treatment of MCT8 deficiency with the potential of improving and even preventing the otherwise inevitable intellectual and neuropsychiatric disabilities.

To test this possibility, pregnant Mct8 heterozygous ($Mct8^{+/-}$) mice were treated with DITPA. The capacity of DITPA to cross the placenta and its effects on TFTs and expression of TH-responsive genes in the cerebral cortex of newborn pups were determined. Results indicate that DITPA does cross the placenta and reaches the brain, providing a rationale for prenatal use of DITPA in human mothers carrying a MCT8 deficient fetus.

Materials and Methods

Experimental animals

Procedures carried out on mice were approved by the University of Chicago Institutional Animal Care and Use Committee. *Mct8KO* mice were generated and housed as described previously (1). All pregnant dams were heterozygous ($Mct8^{+/-}$), bearing both male *Wt* ($Mct8^{+/y}$) and *Mct8KO* ($Mct8^{-/y}$) littermates used in the study. Experiments were performed on P0 (day of parturition). Genotypes were identified as described previously (1). Endogenous production of TH in pregnant dams and embryos was suppressed, starting at gestational day 10 (E10) with low iodine diet (Harlan Teklad Co., Madison, WI) and the addition of 0.5% perchlorate and 0.02% methimazole in the drinking water (LoI/MMI/ClO_4). Starting at E12 and until delivery, separate groups of pregnant mice, with suppressed endogenous TH production, were given sc, once daily DITPA [0.3 mg/100g of body weight (BW)*d] or L-T_4 (2 μg/100gBW*d). Based on previous studies, these doses represent replacement regimens for male *Wt* mice of the same strain with suppressed production of endogenous TH using the same method. Pups from untreated dams (baseline) and pups from dams treated with only LoI/MMI/ClO_4 served as controls. Blood samples from dams were obtained on the day of delivery, 20 and 24 hours after the last injection of DITPA and L-T_4, respectively. Pups were anesthetized, and after blood sampling from the jugular vein, were euthanized by decapitation. Tissues were collected, immediately frozen on dry ice and stored at -80C. All groups contained 8 to 12 animals.

Measurement of serum concentrations of DITPA TSH and iodothyronines and tissue content of DITPA

TSH and T_4 were measured by RIA and DITPA by tandem mass spectroscopy as reported previously (6, 10).

Extraction and measurement of tissue mRNA

Cerebral cortex was collected from male *Wt* and *Mct8KO* mice born to untreated dams (baseline), treated with LoI/MMI/ClO_4 (MMI), LoI/MMI/ClO_4 and L-T_4 (MMI+T_4), and LoI/MMI/ClO_4 and DITPA (MMI+DITPA) to study gene expression. Total RNA was extracted and mRNAs were measured by quantitative PCR (qPCR) as previously described (5). The sequences of primers used to measure sonic hedgehog (*Shh*), Kruppel-like factor (*Klf9*), aldehyde dehydrogenase type 1a3 (*Aldh1a3*) mRNAs are provided in a Supplemental Table. The housekeeping gene, RNA polymerase II (*RpII*), was used as internal control.

Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis of multiple groups performed using ANOVA. The Student's *t* test was used when there were only two groups to compare. $P \geq .05$ was considered not to be significant (NS).

Results

DITPA concentration in serum and content in tissues (Figure 1). To determine whether DITPA crossed the placenta and if it was equally delivered to embryos of both genotypes, we measured DITPA levels in serum of *Wt* and *Mct8KO* littermates at P0 as well as in serum of $Mct8^{+/-}$ dams on the day of parturition. Pups of both genotypes had similar serum DITPA concentrations (*P* NS). However, they were significantly higher than that of their dams, 9.8-fold and 6.2-fold for *Wt* mice and *Mct8KO* mice, respectively; $P < .001$ (Figure 1A). Thus, DITPA crosses the placenta and is equally delivered to littermates of the two genotypes. Moreover, DITPA shows a longer persistence in serum of newborns than their $Mct8^{+/-}$ dams.

We assessed how DITPA is distributed in central and peripheral tissues of the newborn pups. *Mct8KO* mice showed a 1.6-fold higher DITPA content in cerebral cortex compared to *Wt* animals ($P < .05$, Figure 1B), but not in liver (*P* NS, Figure 1C). These results demonstrate that DITPA enters into fetal tissues and, in particular, accumulates in cortex of *Mct8KO* mice.

Effects of treatments with L-T_4 and DITPA on TFTs (Figure 2). We investigated whether and how DITPA, given

prenatally, affects TFTs at birth. As previously described (10), at baseline and compared to *Wt* littermates, *Mct8KO* newborn mice showed relative hyperthyroidism with lower TSH ($P < .001$) (Figure 2A) and higher T_4 ($P < .01$) (Figure 2C). The suppression of endogenous TH by administration of LoI/MMI/ClO_4 increased the serum TSH levels in newborn pups of both genotypes to an equal degree (*P* NS, Figure 2A). Compared to TH-deprived animals, treatment with L-T_4 produced the expected decrease in serum TSH levels in dams and *Wt* pups ($P < .001$) bringing the values to their respective mean baseline levels (*P* NS). In contrast, L-T_4 failed to bring the TSH in *Mct8KO* pups to pretreatment level, remaining high ($P < .001$, Figure 2A) despite the higher levels of serum T_4 ($P < .001$, Figure 2C). Contrary to L-T_4, DITPA suppressed serum TSH levels to the same extent in the newborn pups of both genotypes (Figure 2A). These results indicate that DITPA given during pregnancy has a better accessibility to the hypothalamo-pituitary feedback system of *Mct8KO* embryos, and a stronger inhibitory effect on the TSH production than L-T_4. Of note, DITPA significantly decreased serum TSH levels ($P < .001$ vs MMI group) in *Mct8*$^{+/-}$ dams but not to the same extent as the L-T_4 treatment ($P < .001$ vs MMI) (Figure 2B). Moreover, the DITPA treated *Mct8*$^{+/-}$ dams showed higher serum TSH levels compared to both *Wt* and *Mct8KO* newborn mice ($P < .001$).

Effects of DITPA on cerebral cortex of newborn mice (Figure 3). To test the effect of DITPA on cerebral cortex of embryos, we measured the expression of TH-dependent genes at birth. As previously reported (10), and in contrast to adult mice, expression of the genes *Shh* and *Klf9*, positively regulated by TH, was higher in untreated (baseline) *Mct8KO* mice by 2.0-fold ($P < .01$) and 2.1-fold ($P < .001$), respectively, compared to the *Wt* animals. Similarly, the *Aldh1a3*, a gene negatively regulated by TH, was

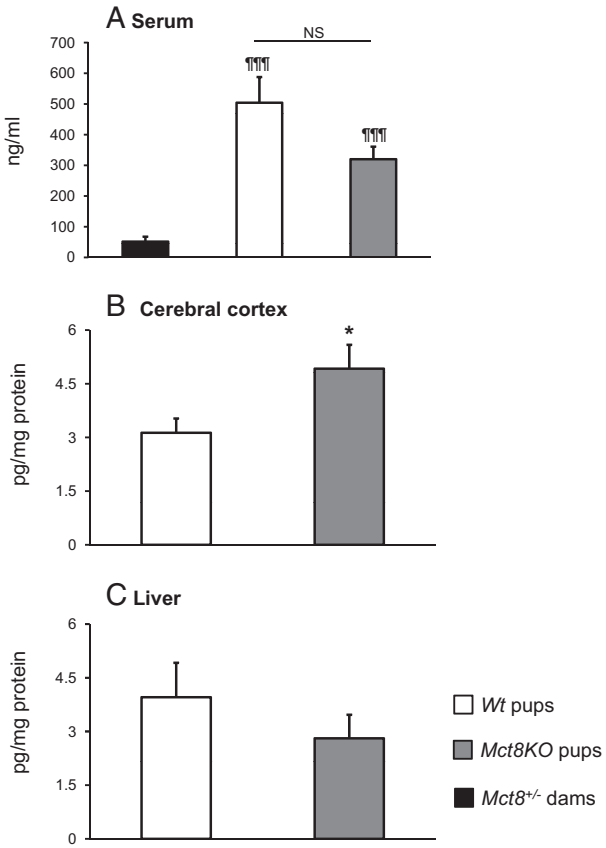

Figure 1. DITPA content in serum and tissues of *Mct8*$^{+/-}$ dams, and *Wt* and *Mct8KO* offspring at P0. Data are expressed as mean ± SE. Statistical differences between *Wt* and *Mct8KO* pups are indicated by * above the *Mct8KO* bars. In panel A, statistical differences from the values in *Mct8*$^{+/-}$ dams are indicated by ¶ above each value bar. *, $P < .05$; ¶¶¶, $P < .001$. NS not significant ($P > .05$)

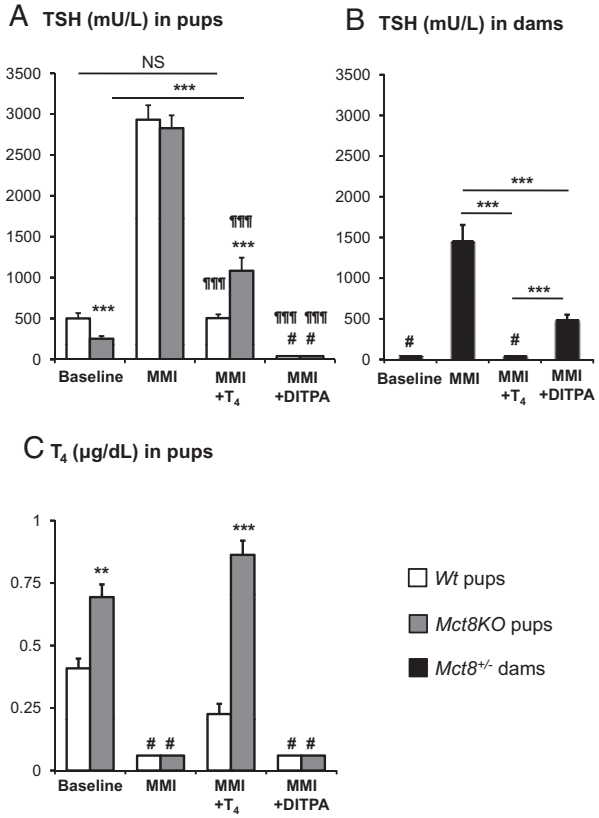

Figure 2. Comparison of the effect of L-T_4 and DITPA on serum TSH concentrations of A) *Wt* and *Mct8KO* pups at P0, and B) *Mct8*$^{+/-}$ dams after delivery, and C) on serum T_4 levels of *Wt* and *Mct8KO* pups at P0. Data are expressed as mean ± SE. Statistical differences between *Wt* and *Mct8KO* pups for each treatment group are indicated by * above the *Mct8KO* bars. In panel A, statistical differences from the values in *Mct8*$^{+/-}$ dams, in panel B, are indicated by ¶ above each value bar. The symbol # above bars indicate TSH and T_4 values suppressed below limits of the assay sensitivity. Treatment with LoI/MMI/ClO4 is indicated as MMI on the abscissa. **, $P < .01$; ***, $P < .001$; ¶¶¶, $P < .001$. #, below the limit of detection of 20 mU/L for TSH and of 0.1 µg/dl for T_4.

reduced by 23% ($P < .05$). As expected, TH deprivation significantly decreased *Shh* ($P < .01$) and *Klf9* ($P < .001$) to the same extent in *Wt* and *Mct8KO* mice and increased *Aldh1a3* ($P < .001$) mRNA content in both genotypes to the same proportion. Treatment with both L-T_4 and DITPA significantly reversed the changes induced by TH deprivation of all three genes (*Shh* $P < .01$; *Klf9* and *Aldh1a3* $P < .001$ for both L-T_4 and DITPA): in L-T_4-treated group, *Shh*, *Klf9* and *Aldh1a3* genes did not show significant difference between the *Wt* and *Mct8KO* mice, whereas DITPA treatment induced a higher *Shh* gene expression, by 1.8-fold ($P < .05$), in *Mct8KO* than in *Wt* mice. All treatments had the same effect on *Klf9* and *Aldh1a3* gene expression in both genotypes. These results indicate that DITPA, given prenatally under the current experimental conditions, enters the brain of *Mct8KO* embryos abolishing, by and large, the differences in gene expression between the genotypes.

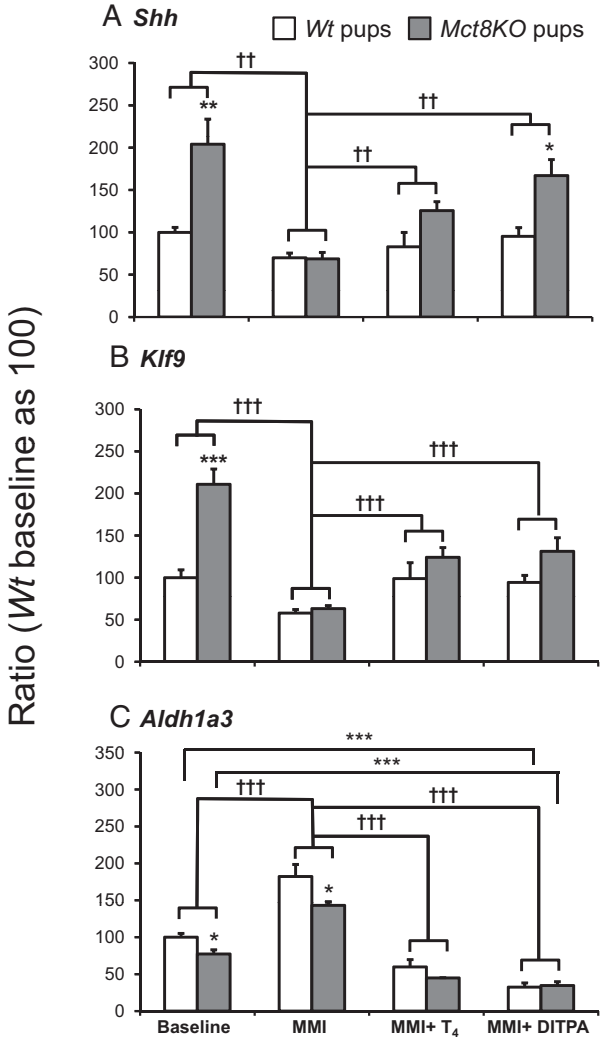

Figure 3. Comparison of the effect of treatment with LoI/MMI/ClO_4 alone and in combination with L-T_4 and DITPA on expression of A) *Shh*, B) *Klf9* and C) *Aldh1a3* genes in cerebral cortex of *Wt* and *Mct8KO* mice at P0. Data are expressed as mean ± SE. Statistical differences between *Wt* and *Mct8KO* mice for each treatment group are indicated by * above the SE bar of the *Mct8KO* groups of mice. Statistical differences among different groups are also indicated by * above the horizontal lines indicating the groups being compared. The results of 2-way ANOVA performed to determine the effect LoI/MMI/ClO_4 treatment compared to baseline and the effect of T_4 or DITPA given the hypothyroid mice regardless of genotype, is indicated by † above the horizontal lines . Treatment with LoI/MMI/ClO_4 is indicated as MMI on the abscissa. *, $P < .05$; ** and †† $P < .01$, *** and ††† $P < .001$.

Discussion

The failure of DITPA to rescue the neurological phenotype of MCT8 deficient patients was ascribed to the late initiation of treatment (7). If true, earlier treatment with DITPA could prevent the brain damage caused by the TH abnormalities during embryonic life. It was with this thought in mind that we conducted the present study to investigate the effects of prenatal exposure to DITPA, compared to that of L-T_4, on correcting the TFTs abnormalities and TH action in cortex of *Mct8KO* mice. The choice of L-T_4, rather than L-T_3, was based on the greater accessibility of L-T_4 to brain (6, 11). The major findings in this study are that DITPA crosses the placenta to reach equally the blood circulation of *Wt* and *Mct8KO* mice and that prenatal exposure to DITPA has significant effect on serum TSH levels and expression of TH dependent genes in cerebral cortex of embryos.

Analysis and interpretation of data must take into account the fact that during perinatal life *Mct8KO* mice, from embryonic day 17 to postnatal day 3, have high T_4 and manifest the effect of central and peripheral tissue TH excess (10). In contrast, adult *Mct8KO* mice manifest changes typical of central TH deprivation (1, 2, 6).

DITPA suppressed serum TSH levels to the same extent in both genotypes, confirming its thyromimetic effect. On the other hand, the physiological dose of L-T_4 was unable to reduce the TSH concentrations in *Mct8KO* mice to the same level as in *Wt* mice despite elevated serum T_4 levels, confirming the resistance of the hypothalamo-pituitary axis to L-T_4 during the perinatal period of life (10). This resistance was not observed in L-T_4-treated *Mct8*$^{+/-}$ pregnant mice that have suppressed serum TSH levels (Figure 2B). In pregnant mice, 2 μg of L-T_4 was enough to suppress the TSH whereas in *Mct8KO* adult mice 4 μg did not even normalize TSH levels (6). Similar to the serum, DITPA content in liver did not show statistical difference between the two genotypes of newborn mice, as it occurs in adult mice (6). Contrary to adult mice treated with

DITPA (6), we observed a 2.1-fold ($P < .05$) difference in DITPA levels between cortex and liver in *Mct8KO* newborn mice and a higher level of DITPA in cortex of *Mct8KO* mice compared to *Wt* littermates. Two possible explanations are offered. First, tissue-specific transporters might increase DITPA content in cerebral cortex through an increased influx and/or a decreased efflux. Second, tissue-specific TH-binding proteins could "trap" DITPA in the cortex. Regarding the latter possibility, we recently showed that, at birth, expression of μ-crystallin, an intracellular TH-binding protein (12), was not significantly different between *Wt* and *Mct8KO* mice (10).

Besides the effects on serum TSH levels, the thyromimetic action of DITPA is further confirmed by the response of TH dependent genes to the treatment. Indeed, *Shh*, *Klf9* and *Aldh1a3*, during DITPA treatment, showed similar modifications as in the case of L-T_4 treatment when compared to the TH-deprived mice: increased expression for the positive *Shh* and *Klf9* genes and decreased expression for the negative *Aldh1a3* gene. The fact that *Shh* gene in DITPA-treated *Mct8KO* newborn mice showed a significantly higher expression than in the *Wt* littermates might reflect either the higher concentration of DITPA in cerebral cortex, a greater sensitivity of some genes to DITPA, or an accumulation of DITPA in specific areas of the cortex where the *Shh* is more expressed. Taking into consideration the suppression of the serum TSH levels, the *Aldh1a3* gene expression and the increased DITPA content in cerebral cortex, an excessive dose of the analog seems available to the embryos. The DITPA dose used in this study was physiological to adult male *Wt* mice and slightly higher than that given to children with MCT8 deficiency (7). Considering the relative hyperthyroid status achieved in the cortex of newborn *Mct8KO* pups, as indicated by the high levels of Shh mRNA and the suppressed levels of serum TSH and Aldh1a3 mRNA, a lower dose of DITPA could be sufficient.

The capacity of DITPA to cross the placenta, to enter fetal tissues and to mimic TH action at pituitary and cortical levels in addition to the beneficial effects on the hypermetabolism already described in humans (7), makes this compound particularly suitable for prenatal treatment of MCT8 deficiency in humans. Indeed, another TH analog, 3,3′,5,5′-tetraiodothyroacetic acid (TETRAC), has been recently tested in Mct8 deficient mice during early postnatal life (13). TETRAC treatment did promote TH-dependent neuronal differentiation in some brain areas. However, it was ineffective in suppressing TRH in the hypothalamus and, in contrast to DITPA, did not significantly ameliorate the peripheral tissue thyrotoxicity of *Mct8KO* mice (6). On the other hand, treatment with propylthiouracil (PTU) in combination with T_4 that blocks endogenous TH production and D1-mediated conversion of T_4 to T_3 in peripheral tissues have been tested in older MCT8 deficient children (14–17). This treatment ameliorates the symptoms of thyrotoxicosis without any improvement of the neurological impairments. However, the potential side-effects associated with PTU (18–20) make this treatment less desirable for prenatal treatment of MCT8 deficiency.

The effects that prenatal treatment with DITPA could have on the MCT8 carrier pregnant mother require consideration. When treated with DITPA, *Mct8*$^{+/-}$ pregnant dams showed less decline in serum TSH levels compared to L-T_4-treated dams (Figure 2B). Furthermore, the decline of TSH in *Mct8*$^{+/-}$ pregnant dams was lesser than that of their litter (Figure 2B vs 2A) suggesting a shorter half-life of DITPA in adult mice, possibly caused by an age-dependent difference in DITPA metabolism. This is supported by the 6- to 9-fold lower serum DITPA level in pregnant dams than their offspring. This decreased effect of DITPA on *Mct8*$^{+/-}$ pregnant dams provides a predominant effect on the embryos. It allows the administration of relatively small doses to MCT8 heterozygous carriers with optimal effect on the fetus.

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