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Changes in Thyroid Status During Perinatal Development of MCT8-Deficient Male Mice

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Patients with the monocarboxylate transporter 8 (MCT8) deficiency syndrome presents with a severe psychomotor retardation and abnormal serum thyroid hormone (TH) levels, consisting of high T_3 and low T_4 and rT_3. Mice deficient in Mct8 replicate the thyroid phenotype of patients with the *MCT8* gene mutations. We analyzed the serum TH levels and action in the cerebral cortex and in the liver during the perinatal period of mice deficient in Mct8 to assess how the thyroid abnormalities of Mct8 deficiency develop and to study the thyroidal status of specific tissues. During perinatal life, the thyroid phenotype of Mct8-deficient mice is different from that of adult mice. They manifest hyperthyroxinemia at embryonic day 18 and postnatal day 0. This perinatal hyperthyroxinemia is accompanied by manifestations of TH excess as evidenced by a relative increase in the expression of genes positively regulated by T_3 in both the cerebral cortex and liver. An increased tissue accumulation of T_4 and T_3 and the expression of TH alternative transporters, including *Lat1*, *Lat2*, *Oatp1c1*, and *Oatp3a1* in the cortex and *Lat2* and *Oatp1b2* in the liver, suggested that Mct8 deficiency either directly interferes with tissue efflux of TH or indirectly activates other transporters to increase TH uptake. This report is the first to identify that the ontogenesis of TH abnormalities in Mct8-deficient mice manifests with TH excess in the perinatal period.

The active thyroid hormones (TH), T_3, and its precursor, T_4, are essential for the differentiation and maturation processes that constitute the normal development of mammals (1). Most TH action results from changes in gene expression mediated by the binding of T_3 to nuclear thyroid hormone receptors. Proper intracellular T_3 concentration, at different stages of life, is important in mammalian development. For this, a concerted action of TH transmembrane transporters and deiodinase activity is important for the provision of optimal intracellular T_3. Although T_3 is secreted by the thyroid gland, the main source of the circulating T_3 is through the outer ring deiodination of T_4 by type 1 deiodinase (D1), mainly expressed in the liver and kidney. Moreover, T_3 is also produced in target tissues through the outer ring deiodination of T_4 catalyzed by the type 2 deiodinase (D2). In the brain, developing cochlea, brown adipose tissue, and anterior pituitary, D2 plays an important role in providing T_3 to target cells. T_4 and T_3 are inactivated to rT_3 and T_2, respectively, by inner ring deiodination, mainly through type 3 deiodinase (D3). In fetal life, D3 is expressed in the placenta, uterus, and most fetal tissues, whereas the brain is the main site in postnatal life (2, 3).

The importance of transporters for TH action in man is best illustrated by the phenotype observed in mutations of the specific TH transporter monocarboxylate transporter 8 (*MCT8*) gene (4, 5). Patients manifest a severe neurodevelopmental defect and abnormal tissue distribution

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Abbreviations: BBB, blood-brain barrier; D1, type 1 deiodinase; D2, type 2 deiodinase; D3, type 3 deiodinase; E, embryonic day; MCT8, monocarboxylate transporter 8; Mct8KO, mice deficient in Mct8; MMI, 1-methyl-2-mercapto-imidazol; P, postnatal day; TH, thyroid hormone; Wt, wild type.

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and metabolism of TH, resulting in a thyroid phenotype characterized by low serum levels of T_4 and rT_3 and high serum levels of T_3 (6, 7).

Mice deficient in Mct8 (*Mct8KO*) faithfully replicate the TH changes observed in humans with *MCT8* gene mutations, even though they lack the severe neurological defects (8, 9). This animal model has provided a valuable tool to advance our understanding of the mechanisms that determine the thyroid phenotype in MCT8 deficiency. Brains of adult *Mct8KO* mice show low T_3 uptake, decreased T_3 content, and increased D2 and decreased D3 activities as a consequence of impaired intracellular TH transport. The resulting TH depletion in the brain produces a local hypothyroidism. The impairment of TH entry into the brain, including the hypothalamus, maintains elevated serum TSH concentrations despite high serum T_3 levels. In contrast, the liver has normal T_3 uptake due to expression of other transporters in this organ. In addition, the high D1 activity and the increased T_3 content in the liver reflect a state of local hyperthyroidism (8–10).

In this report the terms, hypothyroidism, hyperthyroidism, and euthyroidism, are used to describe the state of thyroid hormone action at the tissue level relative to the normal control: deficient, excessive, or sufficient, respectively.

Most information on the pathophysiology of the Mct8 deficiency syndrome has been derived from studies on adult and late postnatal mice. However, there are no reports on thyroid function during the perinatal period or at birth. It is unclear whether the adult thyroid phenotype of *Mct8KO* mice is a continuation of the fetal levels or whether there are unique adaptations to early life in the absence of the Mct8. Treatment of the severe neurocognitive defect in humans would require precise information on the ontogeny of thyroid function, allowing for early fetal intervention.

The role of TH in perinatal life is evident in that TH is fundamental for the development of the central nervous system assisting in brain maturation throughout gestation and in the early postnatal life (11). Consequently, during this period, either TH deficiency or excess could be detrimental for the neurological development. Indeed, as a consequence of congenital hypothyroidism, especially when associated with maternal hypothyroidism, children may have mental retardation, deafness, poor coordination and balance, abnormal fine motor movements, speech problems, spasticity with contractures, tremor, and hyperactive deep tendon reflexes (12). Similarly, fetal and neonatal hyperthyroidism have severe consequences on neurological development, causing mental and growth retardation, heart failure, accelerated bone maturation, and paroxysmal dyskinesia (13–16).

In this study, we provide an analysis of the thyroid phenotype and TH action during the immediate perinatal period of *Mct8KO* mice. We first evaluated the thyroid function tests of both *Mct8KO* and wild-type (*Wt*) mice from embryonic day (E) 17 to postnatal day (P) 7. Unexpectedly, *Mct8KO* mice showed hyperthyroxinemia at ages E18 and P0, the day prior to birth and the first day of life, respectively. Then we studied how this early hyperthyroxinemia affected the TH metabolism and action in two TH sensitive tissues: the cerebral cortex and the liver. The results indicated that the high serum T_4 levels are accompanied by an enhanced TH action in cortex at E18 and P0 and in liver at P0. Both tissues, moreover, showed changes in the expression profiles of alternative TH transporters.

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 (8). All pregnant dams were heterozygous ($Mct8^{-/+}$), bearing both male *Wt* (*Mct8*+/y) and *Mct8KO* (*Mct8*−/y) littermates. Experiments were performed on E17 and E18 and on P0, P3, P7, and P90–100 (adult) male *Wt* and *Mct8KO* mice whose genotypes were confirmed as described previously (8). Mice were anesthetized and, after obtaining a blood sample, were euthanized by decapitation or CO_2, and collected tissues were immediately frozen on dry ice and stored at −80°C. For details see Supplemental Data, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. All animal groups contained at least 8 animals.

Induction of hypothyroidism and treatment of pregnant dams with T_4

Endogenous production of TH in pregnant dams was suppressed, beginning at gestation day 10 using a low-iodine diet (Harlan Teklad Co, Madison, Wisconsin) and 0.5% sodium perchlorate ($NaClO_4$) and 0.02% 1-methyl-2-mercapto-imidazol (MMI) added to the drinking water (LoI/MMI/$NaClO_4$). To some pregnant dams, L-T_4 (2 μg per 100 g body weight) was injected sc once daily, starting at gestation day 12. Blood samples were obtained before and after delivery. Pups were handled as described above.

Tissue processing

Description is in Supplemental Data.

Measurements of iodothyronines in serum and tissues and TSH in serum

Serum total T_4, T_3, rT_3, and TSH concentrations and tissue T_4 and T_3 content were measured by RIAs as detailed in Supplemental Data. Results of T_4, T_3, rT_3 generated with the RIAs were validated by measurements using liquid chromatography

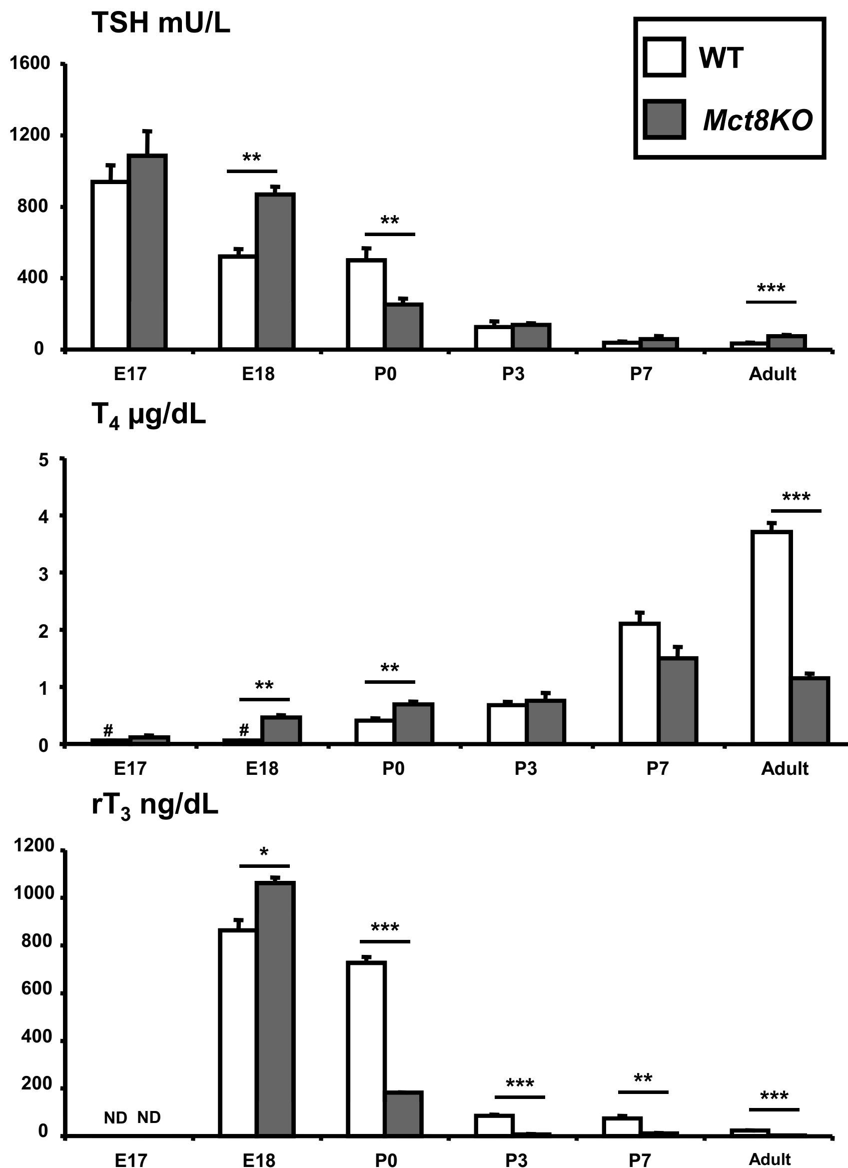

Figure 1. Serum thyroid function test of *Mct8KO* and *Wt* mice at ages E17, E18, P0, P3, P7, and in adult life. Results are expressed as mean ± SE (n = 8 for each group). *, $P < .05$; **, $P < .01$; ***, $P < .001$. For TSH in *Mct8KO* mice: E17 vs E18, $P < .05$; E17 vs P0, $P < .001$; E18 vs P0, $P < .001$. For T_4 in *Mct8KO* mice: E17 vs E18, $P < .001$; E17 vs P0, $P < .001$; E18 vs P0, $P < .001$. ND, not done because serum was not sufficient; #, undetectable;

followed by tandem mass spectrometry. Details are provided in Supplemental Data.

Measurement of specific mRNA content in tissues

Description is in Supplemental Data.

Deiodinase enzymatic activity

D1 and D2 enzymatic activities were measured as described previously (8, 17). Results, expressed in picomoles (D1) and femtomoles (D2) of radioiodide generated per hour per milligram protein, were corrected for nonezymatic deiodination in tissue-free controls.

Tissue uptake of Evans Blue

Description is in Supplemental Data.

Statistics

Statistical analysis used an unpaired 2-tailed Student's *t* test and 1-way ANOVA for multiple comparisons using GraphPad Prism software (GraphPad Inc, San Diego, California). Results are represented as mean ± SE. $P \geq .05$ was considered not to be significant (NS).

Results

Serum TH and TSH levels in *Mct8KO* vs *Wt* mice

Serum T_4, T_3, rT_3, and TSH concentrations were measured at ages E17, E18, P0 (day of birth), P3, P7, and P90–100 (referred to as adult). T_3 values were below the limit of detection of less than 10 ng/dL in embryos and through day P7 in both genotypes. In adult mice mean T_3 levels of *Mct8KO* mice were 120% higher and *Wt* mice as previously described (8). Several phases in the thyroid phenotype of the *Mct8KO* mice could be identified based on these test results (Figure 1). At E17, *Mct8KO* and *Wt* mice had comparable TSH and T_4, levels, whereas rT_3 were undetectable. At E18 and P0 *Mct8KO* mice were hyperthyroxinemic: at E18, the mean serum T_4 level was more than 760% and serum TSH 170% compared with that of *Wt* mice (100%). At P0, T_4 levels were still high at 160%, but serum TSH was reduced to 50% in comparison with the corresponding values in *Wt* mice. At P3 and P7, mean serum T_4 and TSH levels were not different between *Mct8KO* and *Wt* mice, similar to E17. Finally, adult *Mct8KO* mice showed an increased mean serum TSH level to 220% and T_4 level reduced to 30% compared with the corresponding values in *Wt* littermates. Of note, rT_3, with the only exception of E18, at which age the mean level in *Mct8KO* mice were 120%, was significantly lower, being reduced to 25%, 9%, 16%, and 14% of the corresponding values in *Wt* littermates at P0, P3, P7 and in adult mice, respectively (Figure 1).

To define the source of the fetal hyperthyroxinemia, we injected dams with L-T_4 (2 μg per 100 g body weight) after both dams and embryos were deprived of TH with LoI/

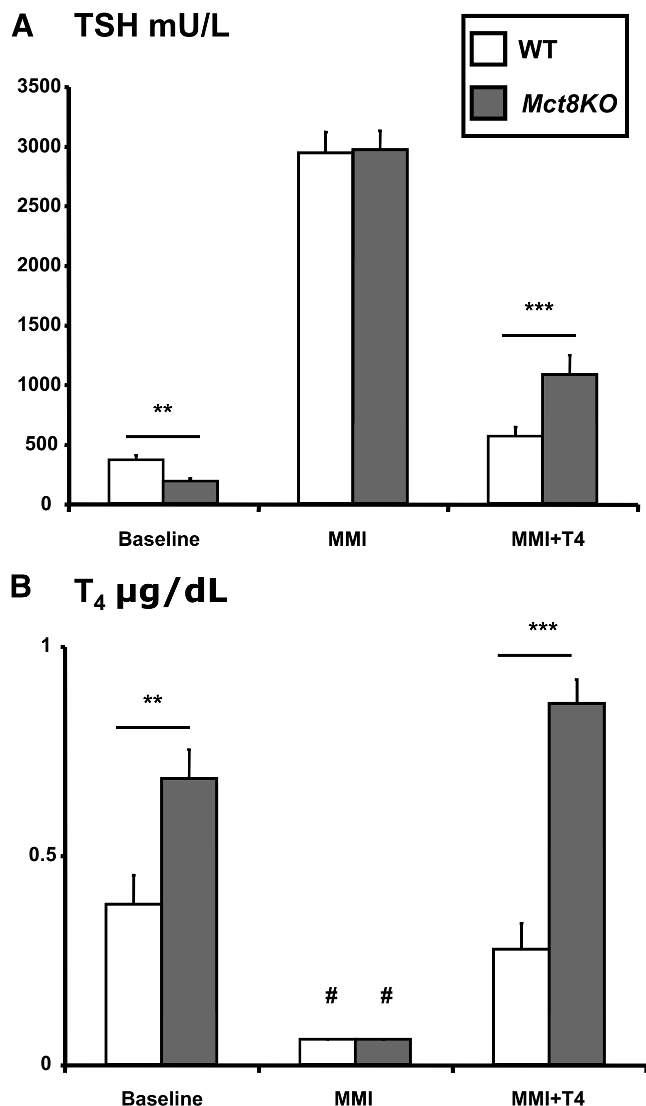

Figure 2. Serum TSH (A) and T_4 (B) levels in *Mct8KO* and *Wt* mice at P0. Values are at baseline, after LoI/MMI/NaClO4 (MMI) treatment, and after injection of 2 μg per 100 g body weight of L-T_4 in mothers treated with MMI (MMI+T_4). Results are expressed as mean ± SE (n = 8 for each group). **, $P < .01$; ***, $P < .001$. #, undetectable.

MMI/$NaClO_4$. At birth, as expected, both *Wt* and *Mct8KO* mice born to mothers treated with LoI/MMI/$NaClO_4$ but without T_4 had suppressed serum T_4 and high TSH (Figure 2). However, when these dams deprived of endogenous TH were given T_4, the mean serum T_4 level of *Mct8KO* mice was higher, 310%, than the *Wt* newborns, whereas serum TSH levels were unsuppressed, being 190% in *Mct8KO* mice compared with *Wt* littermates (Figure 2).

Role of placenta in perinatal hyperthyroxinemia

To assess the contribution (if any) of the placenta in generating the hyperthyroxinemia seen at E18 and P0 in *Mct8KO* mice, we investigated whether other TH transporters could have been responsible for increased TH transport. Gene expression of *Mct10*, *Lat1*, and *Lat2* in the placentas of *Mct8KO* and *Wt* mice obtained at E17 and E18 were not statistically different between the 2 genotypes, with the exception of *Lat1*, which was 30% higher in E17 *Mct8KO* mice than in *Wt* mice (Supplemental Figure 1B).

Effects of hyperthyroxinemia on cerebral cortex

We tested whether the unexpected hyperthyroxinemia found at E18 and P0 caused an effect on TH metabolism and action in tissues highly sensitive to TH. Ages E17, P3, and P7 were not included in the investigation because no differences in serum T_4 and TSH levels were found between the 2 genotypes. We first evaluated the thyroid status in the cortex of *Mct8KO* mice (Figure 3A). T_4 content in the rest of the brain (fornix, hippocampus, dorsal striatum, ventral striatum, thalamus, and hypothalamus) was higher in *Mct8KO* mice than in *Wt* mice by 120% and 145% at E18 and P0, respectively. At P0, in the cortex of *Mct8KO* mice, T_4 content was 160% compared with *Wt* animals, a value similar to that in the rest of the brain. In contrast, in the cortex of adult *Mct8KO* mice, T_4 content was reduced to 60% of the corresponding value of *Wt* mice (Figure 3A). Regarding T_3 content in the cortex, whereas at E18 it was 180% in *Mct8KO* mice compared with *Wt* mice, at P0 no significant difference was observed between the 2 groups of animals. The cortical T_3 content at E18 and P0 differed from that of adult *Mct8KO* mice, which was 56% compared with *Wt* mice (Figure 3B).

We then studied the D2 enzymatic activity to assess the overall thyroid status in the cortex of *Mct8KO* and *Wt* mice (Figure 3C). Enzyme activity was low and no difference was observed between the *Mct8KO* and the *Wt* mice at ages E18 and P0. In adult mice, D2 activity was increased in *Mct8KO* mice to 1311% compared with the *Wt* animals.

To investigate whether the high T_3 content at E18 had an effect on TH action in cortex, the expression of T_3-regulated genes was assessed by quantitative PCR. We analyzed the expression of 5 genes reported to be positively regulated by T_3 (18, 19): hairless (*Hr*), Sonic hedgehog (*Shh*), Kruppel-like factor 9 (*Klf9*), Semaphorin 7a (*Sema7a*), and deiodinase type 3 (*Dio3*). At E18, *Hr* mRNA expression was 140%, *Klf9* 150%, and *Dio3* 145% in *Mct8KO* mice compared with *Wt* animals, whereas *Shh* and *Sema7a* did not manifest a difference between the 2 genotypes (Figure 4). At P0, when the T_3 content has returned to values similar to those in *Wt* mice, the mRNA expression of *Hr*, *Shh*, *Klf9*, and *Sema7a* genes was still higher in the cortex of the *Mct8KO* mice, being 186%, 200%, 210%, and 180%, respectively, compared with *Wt* mice, whereas *Dio3* gene expression did not show a significant difference between the 2 genotypes (Figure 4).

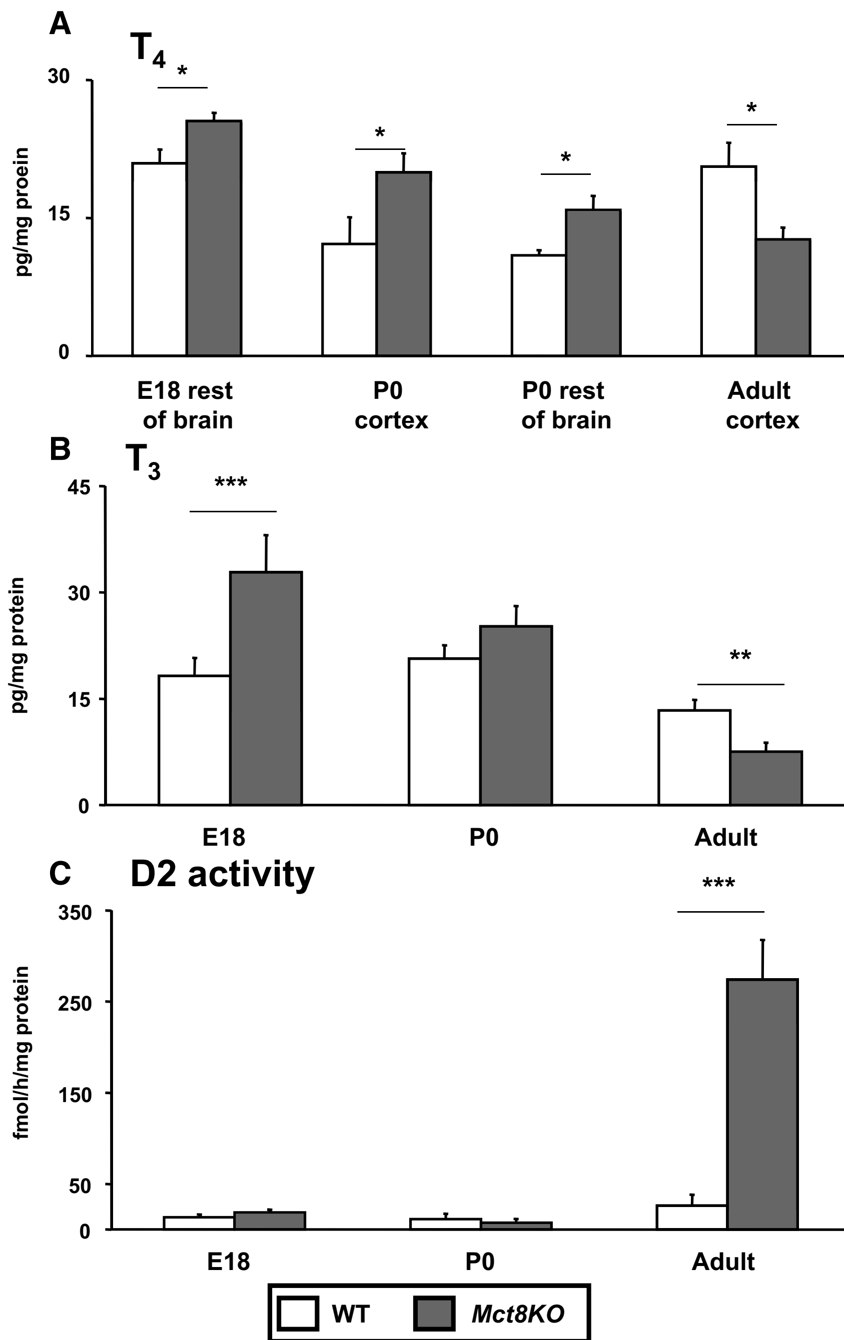

Figure 3. Thyroid status in cerebral cortex of *Mct8KO* and *Wt* mice. A, Tissue T_4 content. B, Tissue T_3 content. C, D2 activity at ages E18, P0, and adult. Results are expressed as mean ± SE (n = 8 for each group). *, $P < .05$; ***, $P < .001$.

Contrary to these findings, adult *Mct8KO* mice showed a reduction to 50%, 70%, 60%, and 53% of *Hr*, *Shh*, *Sema7a*, and *Dio3*, respectively, when compared with *Wt* mice. In adult *Mct8KO* mice, *Klf9* expression was not statistically different between the 2 groups of animals (Figure 4). Taken together, these results indicated that cortex of E18 and P0 *Mct8KO* mice manifested a hyperthyroid state in contrast to the local hypothyroidism present in the cortex of adult *Mct8KO* mice.

Additionally, in search for mechanisms that might explain the increased accumulation of TH in cortex of *Mct8KO* mice, we analyzed by quantitative PCR the mRNA expression of L-aminoacid transporter type 1 (*Lat1*) and type 2 (*Lat2*), Monocarboxylate transporter 10 (*Mct10)*, solute carrier organic anion transporter family, member 1C1(*Oatp1c1*), and member 3a1 (*Oatp3a1*), which are known TH cell transporters (20–23). In the cortex of E18 mice, only *Lat1* gene expression was significantly increased in *Mct8KO* to 139% compared with *Wt* mice. The increase of *Oatp1c1* gene expression in *Mct8KO* mice of 145% was of borderline significance (P = .055). No significant difference was observed between the 2 groups of animals for the expression of *Lat2*, *Mct10*, and *Oatp3a1*. At P0, mRNA expression of *Lat2* was 147%, *Oatp1c1* 137%, and *Oatp3a1* 150% higher in *Mct8KO* mice than in *Wt* mice. No significant difference was found between the 2 genotypes for *Lat1* and *Mct10*. In adult mice we found no statistical difference in mRNA expression for any of the transporters studied (Figure 5).

The possibility of T_3 retention by μ-crystallin, an intracellular TH-binding protein, was explored. No significant difference between the 2 genotypes was observed in the cortex of E18 and P0 mice (see Supplemental Figure 2) and was not detectable in the liver.

Finally, to investigate whether the high T_3 in the cortex of *Mct8KO* mice could have been determined by a more penetrable blood-brain barrier (BBB) than in *Wt* mice, we examined the integrity of the BBB using Evans Blue dye. At E18 none of the Evans Blue given to the mother was detected in either the cortex or liver of *Mct8KO* and *Wt* pups due to the placental barrier. At P0, Evans Blue given iv to the pups was detected in both the cortex and liver, but no statistical difference between the 2 genotypes was found. The range of values for cortex were 0.31–0.33 and 0.25–0.4 mg/g protein in *Mct8KO* and *Wt* mice, respectively, and for liver, 0.5–

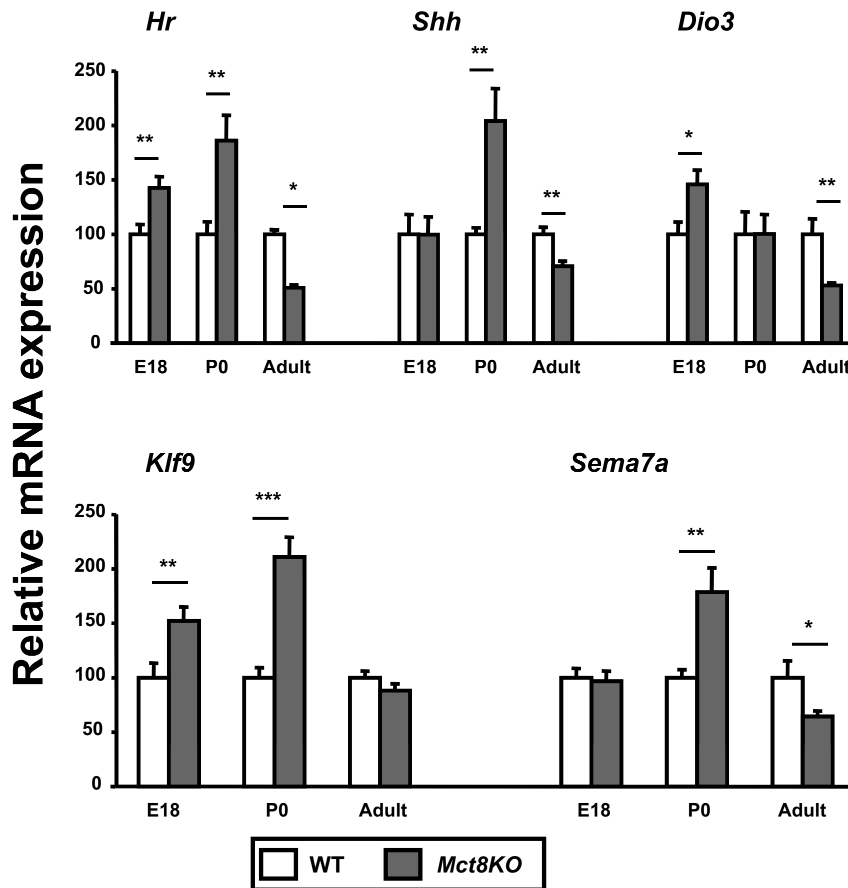

Figure 4. Effects of the hyperthyroxinemia on cerebral cortex. Gene expression of the TH-dependent genes *Hr*, *Shh*, *Dio3*, *Klf9*, and *Sema7a* in the cortex of *Mct8KO* and *Wt* mice at ages E18, P0, and in adults. Results are expressed as mean ± SE relative to the control *Wt* value set as 100 (n = 8 for each group). *, $P < .05$; **, $P < .01$; ***, $P < .001$.

0.65 and 1.0–1.2 mg/g protein in *Mct8KO* and *Wt* mice, respectively.

Effects of hyperthyroxinemia on liver

We analyzed the TH status and action in liver to investigate whether the hyperthyroid state found in cerebral cortex of E18 and P0 *Mct8KO* mice was limited to the brain. The mean liver T_4 content in E18 and P0 *Mct8KO* mice was increased by 190% and 220% compared with *Wt* littermates (Figure 6A). Hepatic T_3 content did not show significant difference between the 2 genotypes at E18 but was increased to 185% in *Mct8KO* mice at P0 (Figure 6B). The similarity in content of T_3 between the 2 genotypes at E18 was in keeping with the normal expression of the T_3-dependent genes, deiodinase type 1 (*Dio1*) and thyroid hormone-responsive Spot14 (*Spot14*) (Figure 7A). At P0, mean *Dio1* expression was 240% and *Spot14* 210% in *Mct8KO* mice compared with 100% of *Wt* mice (Figure 7A). The high content of T_3 associated with the higher expression of both *Dio1* and *Spot14* at P0 indicated that the liver was hyperthyroid in *Mct8KO* mice at birth. As expected for age, at both E18 and P0, D1 activity was minimal, but also no significant difference was seen between the 2 groups of animals (Figure 6B).

In contrast to E18 and P0 mice, the mean T_4 content of adult *Mct8KO* mice was reduced by 45% compared with *Wt* animals (Figure 6A). However, T_3 content in adult *Mct8KO* mice was 146% and D1 activity 313% compared with *Wt* animals (Figure 6, B and C).

mRNA expression of other TH transporters in the liver was measured. At E18, *Lat1* and *Mct10* gene expression was reduced in *Mct8KO* mice to 30% and 34% of the corresponding values of *Wt* mice, whereas *Lat2* and *Oatp1b2* gene expression in *Mct8KO* animals was similar to that in *Wt* littermates. At P0, *Lat2* and solute carrier organic anion transporter family, member 1b2 (*Oatp1b2*) gene expressions were higher to 280% and 630%, respectively, in *Mct8KO* mice than in *Wt* mice, whereas no significant difference in *Mct10* and *Lat1* was seen between the 2 genotypes (Figure 7B). None of the 2 groups of animals showed detectable levels of *Oatp1c1*, *Oatp3a1*, solute carrier organic anion transporter family, member 1a1 (*Oatp1a1*), and solute carrier organic anion transporter family, member 1a4 (*Oatp1a4*) transcripts in the liver.

Discussion

The aim of this study was to determine the ontogeny of the thyroid phenotype of *Mct8KO* mice starting at the perinatal period and to determine how changes of serum TH concentrations affect hormone action at the tissue level. We obtained thyroid function tests at prenatal days E17 and E18 (the last 2 days of pregnancy) and at P0 (the day of birth), P3, and P7. Furthermore, we studied the TH metabolism and action in the cerebral cortex and liver at E18 and P0 because during these days thyroid function tests showed profound and dynamic changes. Results were compared with the findings in adult mice.

The most striking and unexpected finding was the multiphasic changes of thyroid function test results in the perinatal days of life. Whereas at E17 results in *Mct8KO* mice

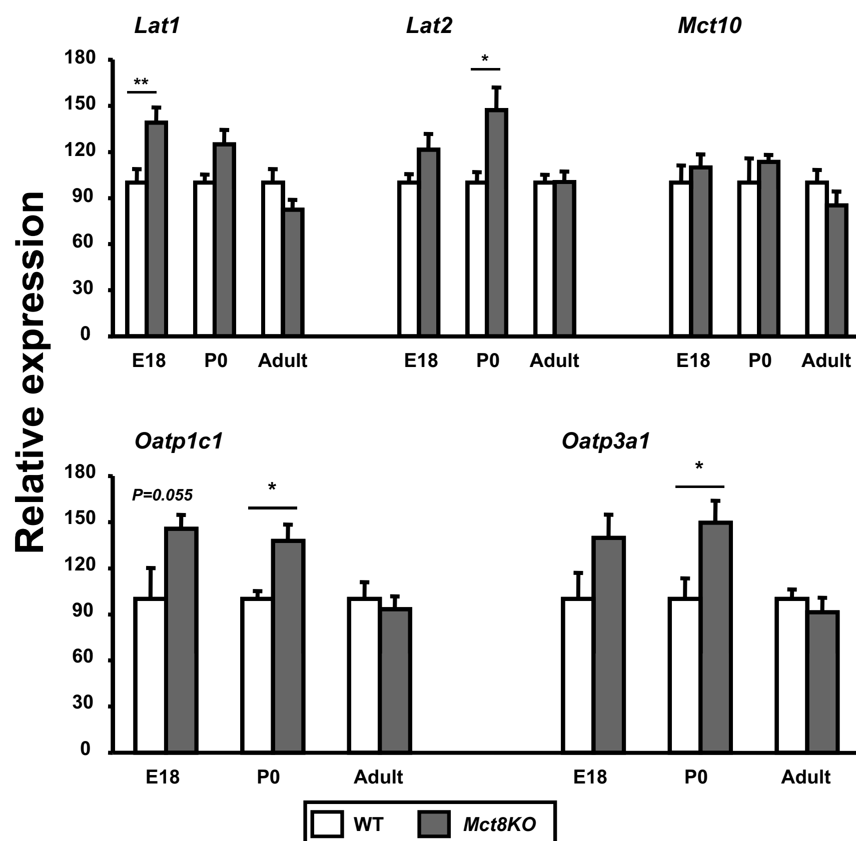

Figure 5. Gene expression of TH transporters *Lat1*, *Lat2*, *Mct10*, *Oatp1c1*, and *Oatp3a1* in the cerebral cortex of *Mct8KO* and *Wt* mice at ages E18, P0, and adults. Results are expressed as mean ± SE relative to the control *Wt* value set as 100 (n = 8 for each group). *, $P < .05$; **, $P < .01$.

were not different from those in *Wt*, a relative hyperthyroxinemic phase appeared at ages E18 and P0, followed by a return of test values to those of the *Wt* littermates at P3 and P7. This perinatal thyroid change of Mct8-deficient mice is opposite to that seen in adult mice in which serum T_4 concentration is low relative to *Wt* animals.

The first thing to consider is the origin of the relative hyperthyroxinemia of Mct8-deficient mice. TSH might not be the trigger because, if it were, it would have remained high at P0 in *Mct8KO* mice. Serum TSH levels were elevated to the same degree in *Mct8KO* and *Wt* mice at E17 and declined in both genotypes in the face of increasing T_4 (Figure 1). Consequently, the increase of T_4 is conceivably the first event to happen in the ontogenesis of the thyroid phenotype of *Mct8KO* mice. Of note, at E18 the significantly high levels of TSH in *Mct8KO* mice indicate a state of relative central hyposensitivity to circulating TH levels (Figure 1). This hyposensitivity was confirmed when physiological doses of L-T_4 administered to hypothyroid pregnant dams were unable to suppress serum TSH in newborn *Mct8KO* mice, despite higher serum T_4 levels (Figure 2), similar to those observed in untreated P0 *Mct8KO* mice (Figure 1). Therefore, the origin of the elevated T_4 has been questioned.

The possibility of an increased delivery of maternal T_4 to *Mct8KO* mice through the placenta was excluded because no important differences in alternative TH-transporter gene expression was found in placentas between the 2 genotypes (Supplemental Figure 1B). Other possibilities are decreased T_4 metabolism and/or decreased clearance. The very low hepatic D1 activity observed at this age (Figure 6C) likely prevents the consumptive effect on T_4 observed in adult mice (10). With regard to T_4 clearance, adult *Mct8KO* mice showed enhanced renal uptake and increased excretion of T_4 (24) that may not be operative in the immature perinatal period of *Mct8KO* mice. In addition, the Mct8-dependent influx and efflux of TH in specific tissues can alter the distribution of TH. In fact, the lower serum rT_3 levels in *Mct8KO* mice likely represent decreased access of T_4 to D3 expressing tissues. Although the mean serum rT_3 concentration of *Mct8KO* mice at E18 was slightly higher than that of the *Wt* mice, values were much too low relative to the corresponding high serum T_4 concentrations, the rT_3 to T_4 ratios of *Mct8KO* and *Wt* mice being 2.3 and 14.4, respectively. The possibility of accumulation of T_4 in the cortex of *Mct8KO* mice due to a difference in the BBB permeability between the 2 genotypes was also considered and the transfer of Evans Blue was studied. However, no statistical difference in the BBB permeability was found between *Mct8KO* and *Wt* mice.

The hyperthyroxinemia of the perinatal period was accompanied by a change in the TH status, suggestive of increased TH action in the cerebral cortex and liver. The cortex of E18 *Mct8KO* mice had higher T_3 and T_4 concentrations than that of the *Wt* counterpart. The excess of TH at E18 in *Mct8KO* mice was accompanied by elevated mRNA levels of *Hr*, *Dio3*, and *Klf9*. The increase in expression of genes positively regulated by T_3 was still present at P0, as indicated by the high levels of *Hr*, *Shh*, *Klf9*, and *Sema7a*, when the tissue T_3 concentration in *Mct8KO* mice was similar to that of *Wt* mice. This is expected because the effect on mRNA triggered by the high T_3 prior

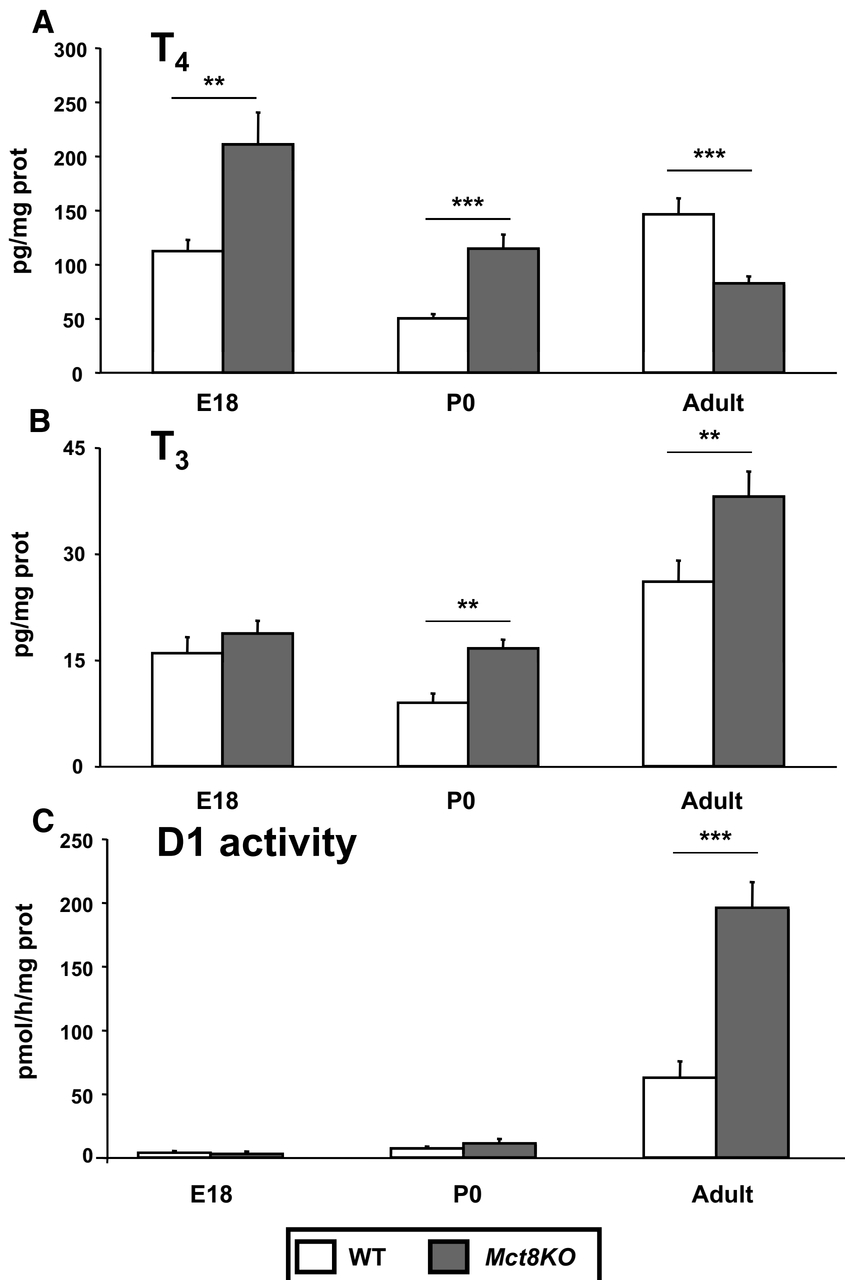

Figure 6. Thyroid status in liver of *Mct8*KO and *Wt* mice. A, Hepatic T_4 content. B, Hepatic T_3 content. C, D1 activity in liver at ages E18, P0, and adults. Results are expressed as mean ± SE (n = 8 for each group). **, $P < .01$; ***, $P < .001$.

to birth (see E18) persists on the next day, P0. Indeed, mRNA induction is downstream of T_3 stimulation, and its continued stimulation is dependent on the mRNA half-life. This is also the reason that in published experiments on gene responses to T_3, the effect persists after withdrawal of T_3 (25). The high expression of T_3-dependent genes indicated that, in the perinatal period, the cortex of *Mct8KO* mice is relatively hyperthyroid. However, the D2 activity was similarly low in both genotypes (Figure 3C), suggesting that increased generation of T_3 plays a minor role in the production of cerebral hyperthyroidism, although local increase in D2 activity that is not detected by global measurement in the entire cortex cannot be excluded.

More importantly, our data suggest a notable contribution of alternative TH transporters as a compensatory effect to the lack of Mct8. We found higher mRNA levels of *Lat1* and a trend to higher expression of *Oatp1c1* in E18 *Mct8KO* mice as well as higher mRNA levels of *Lat2*, *Oatp3a1*, and *Oatp1c1* at P0. The increased expression of alternative TH transporters in cortex of E18 and P0 *Mct8KO* mice indicates that the cortical hyperthyroidism could be the consequence of increased T_3 influx from the bloodstream rather than its local generation by D2. This hypothesis is supported by a recent study in P21 mice (26), showing that positive gene regulation by T_3 is equally and efficiently achieved by the T_3 from the circulation or by the T_3 generated from T_4 by D2. In contrast, genes regulated negatively by T_3 depend on the local generation of T_3 by D2 activity. In this respect the expression of *Aldh1a3*, a gene negatively regulated by T_3 in primary cortical neurons (19), was not affected by Mct8 deficiency at P0 (data not shown), in agreement with the source of cortical T_3 at this age (26). Moreover, the local accumulation of TH in the cortex of E18 and P0 *Mct8KO* mice could be, in part, the consequence of a reduced TH efflux in the absence of Mct8 (21, 27). Two different studies support this mechanism: 1) the fibroblasts of patients deficient in *MCT8* have impaired efflux of T_3 (28); and 2) the thyroid glands of *Mct8KO* mice have a reduced secretion of TH (29, 30). The expression of alternative TH transporters in the cerebral cortex of adult *Mct8KO* mice is not different from that in *Wt* mice. This is associated with a low content of T_3 and a reduced expression of T_3-dependent genes in the *Mct8KO* mice, supporting a role of the alternative TH transporters in perinatal life.

In line with the cortical hyperthyroidism, we found that the liver was also hyperthyroid at birth. Despite the high

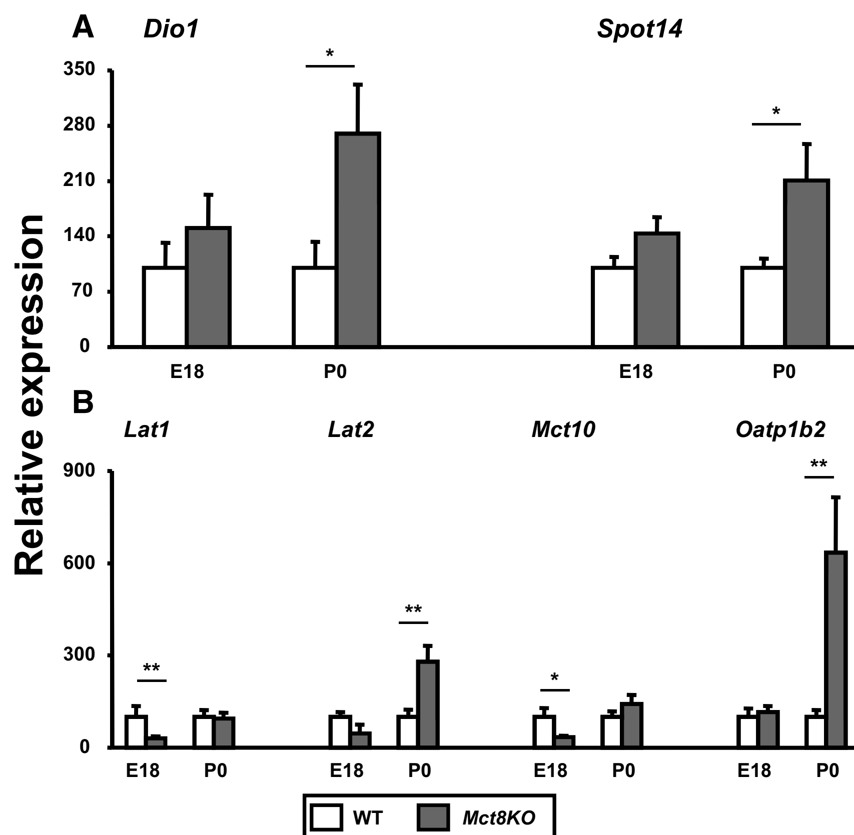

Figure 7. Effects of the hyperthyroxinemia on liver of *Mct8*KO and *Wt* mice. A, Gene expression of the TH-dependent genes *Dio1* and *Spot14*. B, Gene expression of TH transporters *Lat1*, *Lat2*, *Mct10*, and *Oatp1b2* at ages E18 and P0. Results are expressed as mean ± SE relative to the control *Wt* value set as 100 (n = 8 for each group). *, $P < .05$; **, $P < .01$.

T_4 levels, at E18 *Mct8KO* mice showed a T_3 content not different from that in *Wt* littermates. As a consequence, the expression of the T_3-dependent genes *Dio1* and *Spot14* was not different between the 2 groups of animals. In contrast, P0 *Mct8KO* mice had higher hepatic T_4 and T_3 content as well as higher *Dio1* and *Spot14* gene expression, compared with their *Wt* littermates, indicating a tissue hyperthyroid state. Because both *Mct8KO* and *Wt* newborn mice have minimal D1 activity (Figure 6C), our finding of higher hepatic T_3 content at P0 in *Mct8KO* mice may be produced by the same mechanism as suggested for the cerebral cortex, namely increased TH uptake by alternative TH transporters and impaired TH efflux. The high expression of *Lat2* and *Oatp1b2* genes in the liver of *Mct8KO* newborn mice supports the former mechanism, whereas the impaired T_3 efflux was demonstrated in the *Mct8KO* adult mice and confirmed in double-*Mct8/D1 KO* and triple-*Mct8/D1/D2KO* mice (10).

In conclusion, this study provides evidence that in perinatal life Mct8-deficient mice show an elevation of serum T_4 levels. The fact that the hyperthyroxinemia is present at E18, thus preceding birth, indicates that it is not triggered by the stress of birth. The perinatal hyperthyroxinemia is accompanied by local hyperthyroidism in the cerebral cortex and liver. This strongly contrasts with the coexistence of central hypothyroidism and peripheral hyperthyroidism in adult *Mct8KO* mice. We propose that tissue hyperthyroidism in the perinatal period is mediated by a combination of increased TH influx into tissue, through alternative transporters, and a reduced TH efflux from tissue due to the lack of Mct8. However, the generation of compound mutants in which not only Mct8 but also other TH transporters are inactivated will shed more light on the role of TH transporters during the development of *Mct8KO* mice.

These findings broaden our understanding of the pathophysiology of MCT8 deficiency, which may have implications in humans. At present, low levels of serum T_4 accompanied by normal concentrations of TSH (4, 28, 31) are the only known findings in MCT8 deficient newborns, which are often observed in prematurity and other illnesses. Moreover, the low levels of rT_3 in the early postnatal life of *Mct8KO* mice could have a useful application in the early diagnosis of MCT8 deficiency. The similarity of the neurological phenotype of MCT8 deficiency and cretins born to hypothyroid mothers suggested that an impaired transport of maternal thyroid hormone into neurons during prenatal life may lead to neurodevelopmental defects in MCT8-deficient patients (32). However, should our observations apply to humans, they add another possible event in the intrauterine life of MCT8-deficient patients. Does transient embryonal hyperthyroidism cause brain damage in these patients? Clarifying the intrauterine thyroid phenotype might help to understand the mechanisms of the neurological deficit of MCT8 deficiency and suggest new therapeutic possibilities for MCT8-deficient patients.

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1 ***Supplemental Data***

2

3 *Tissue collection processing and homogenization*

4 Newborn removed from the cage were kept at a constant temperature of 25°C using a
5 heating pad. Blood and tissues were collected between 9.30 am and 11 am, for all the time points.
6 As pregnant dams were checked (status, drinking water, food, cage bedding) last time at 7 pm the
7 day before giving birth, P0 mice were bled and sacrificed in less than 15 hour after birth.

8 Tissues from E18 and P0 mice were rapidly collected on dry ice, whereas those from
9 adult mice were collected and frozen following blood removal by perfusion with heparinated PBS
10 through a needle placed in left heart ventricle. Frozen tissues [cerebral cortex, the brain without
11 cerebral cortex, rest of brain (includes: fornix, hippocampus, dorsal striatum, ventral striatum,
12 thalamus and hypothalamus), liver and placenta], kept at -80C, were placed in the proper amount
13 of buffer (0.1 M phosphate pH 7, 1 mM EDTA and 4 mM DTT). They were homogenized in a
14 Bullet Blender (Next Advance Inc., Averill Park, NY). Each homogenate was divided into
15 aliquots used for the measurement of T_4 and T_3, mRNAs, D1 and D2 enzymatic activity and
16 protein content. Because large amount of tissue were required to measure T_4 content in brain of
17 E18 and P0 mice, most of the tissue from a single mouse was used for this measurement keeping
18 only a small amount for protein determination. All procedures were performed on ice.

19

20 *Determinations of T_4 and T_3 in serum and tissues*

21 It is known that serum interferes with the measurements of T_3 and T_4 by RIA. This is
22 dependent on the amount of serum used in the assay (more than 5 µl) and by the concentration of
23 these iodothyronines in serum (<1 µg/dl of T_4 and <150 ng/dl of T_3). Thus, extraction of the
24 iodothyronines prior to assay is required to measure T_3 and T_4, in a sensitive RIA, without
25 interference. The methods we used are given below and the results were confirmed by liquid
26 chromatography followed by tandem mass spectrometry (LC/MS/MS).

1.Extraction

All tissues and sera from E17, E18 and P0 mice, were extracted prior to measurement of T_4 and T_3 by a procedure reported in Ref. 1, with the modification described below. Measurement of T_4 in brain required further purification. With each extraction we prepared 3 blank tubes and 3 tracer tubes. Blank tubes contained all reagents except for sample and were used to check the assay background. The tracer tubes contained 1500-2000 cpm of high specific activity of [^{125}I]-labeled T_4 or T_3 added to the tissue homogenates and sera. These were used to assess the recovery of extracted iodothyronines in each experiment. Chloroform-methanol 2:1, containing 1 mM PTU, was used for the extraction twice. The final volume of chloroform-methanol extract was about 20 times the weight of tissue or serum volume. Then, 0.05% $CaCl_2$ was added to the chloroform-methanol extract to back-extract the iodothyronines into an aqueous phase, followed by one extraction with 0.05% $CaCl_2$-MeOH-chloroform (48:49:3). The pooled aqueous phases were evaporated in-vacuo and dissolved in corresponding RIA buffer. Aliquots of the samples were submitted to T_4 or T_3 measurement by RIAs (see RIA assays description below). Recovery after extraction was 75-85%.

The precision of measuring T_4 and T_3 by extraction and RIA assay was confirmed by LC/MS/MS in 20 samples. Serum samples with T_4 of 0.432 and 0.975 □g/dl using this extraction-RIA method gave 0.553 and 1.04 □g/dl when measured by LC/MS/MS. Sample with T_3 of 9.8 pg/mg protein (cerebral cortex), 11.7 pg/mg protein (liver) and 31.8 ng/dl (serum) by the extraction-RIA method had 7.8 pg/mg protein (cerebral cortex), 11.8 pg/mg protein (liver) and 33 ng/dl (serum), respectively, when measured by LC/MS/MS.

2. Purification

Because of the low T_4 content in cerebral cortex and rest of brain, large amount of tissue

extract was needed which required additional purification as described in Ref. 1. After extraction and back-extraction procedure described above, the pooled aqueous phase of iodothyronines was further purified with Bio-Rad AG 1X2 resin column (bed volume 1ml). The column was pre-treated with 70% acetic acid balanced with acetate buffer (AB), pH 7. After loading the sample onto the column, a series of washes followed: 2 ml pH 7 AB, 2 ml ethanol-0.1 mM PTU, 4 ml pH 7 AB, again 2 ml ethanol-PTU, 2 ml pH 7 AB, 2 ml pH 4 AB, 2 ml pH 3 AB, 2 ml 1% and 2 ml 35% acetic acid. All these washes were discarded. The iodothyronines were then eluted in six aliquots (0.5 ml each) of 70% acetic acid. Elution profiles were assessed by determining their [^{125}I]T_4 contents. The aliquots containing the largest amounts of the labeled tracers were pooled (usually the 2nd, 3rd and 4th fractions), and evaporated to dryness. The dried sample was dissolved in the RIA buffer and the T_4 content was measured by a sensitive T_4 RIA. Recovery after extraction and purification was 48-60%.

3. RIA assays

Serum total T_4 concentrations in P3, P7 and adult mice were measured by coated-tube RIA Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA) adapted for mouse using 25 µl serum. The limit of detection in serum was 0.25 µg/dl. The assay sensitivity is 125 pg T_4 per tube.

Total T_4 in sera from E17, E18 and P0 mice was measured by a sensitive RIA (Ref. 2) using a specific antibody against T_4, kindly provided by Maria Jesus Obregon (Instituto de Investigaciones Biomédicas, Madrid, Spain). The assay was performed on 5 µl serum extract as described above and in the original publication (Ref. 2). The assay sensitivity was 2.5 pg T_4 per tube and recovery was on the average 80%, thus the limit of detection in serum was 0.0625 µg/dl.

Tissue T_4 content was measured by a sensitive RIA, described in Ref. 2, after extraction and purification. Recovery was as indicated above.

Serum total T_3 in embryos, P0, P3 and P7 mice was measured by the sensitive RIA (Ref.2) after extraction. Specific antibody against T_3 was kindly provided by Maria Jesus Obregon. 8-13 □l of serum was used for extraction with an average recovery of 78%. The assay sensitivity was 1 pg T_3 per tube. T_3 concentration in all samples, regardless of genotypes, were lower than the limit of detection of 10 ng/dl. This was confirmed by LC/MS/MS in serum pools of mice from the same age and both genotypes.

Tissue T_3 concentration was measured by coated-tube RIA Kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA). Sample size was 50 □l. The limit of detection was 10 ng/dl. The assay sensitivity was 5 pg T_3 per tube. The precision of measurement was verified by LC/MS/MS as reported above.

Serum TSH and rT3 measurement

Total rT_3 was measured in 35 □l serum by RIA using reagents from Adaltis Italia (Rino, Italy). The limit of detection in serum was 1.25 ng/dl. TSH was measured in 50 □l of serum using a sensitive, heterologous, disequilibrium, double-antibody precipitation RIA (Ref. 3). The limit of detection was 10 mU/L. Due to high concentrations of rT_3 and TSH in serum of E17 to P3 mice, proper dilutions were made to preserve the serum and fit the values within the RIA standard curves. Thus, 2-6 □l serum were used in E17, E18 and P0 mice, whereas 10 □l serum were used in P3 mice for both TSH and rT_3 RIAs. The rT_3 RIA method was confirmed by LC/MS/MS. Serum samples with rT_3 of <2.5, 17.0, 32.3 and 22.8 ng/dl using the RIA method measured <0.08, 17.2, 26.7 and 20.7 ng/dl, respectively, by LC/MS/MS.

Measurements of iodothyronines by LC/MS/MS

Iodothyronines (T_4, T_3 and rT_3) were measured by liquid chromatography (LC, Shimadzu, Columbia, MD) followed by tandem mass spectrometry (MS/MS, API 4000 from

102 Applied Biosystems.) as follows. After addition of the internal standard $^{13}C_6$ –rT_3 and $^{13}C_6$-T_4
103 (Iso Sciences, King of Prussia, PA) to 50 or 100 µl serum sample the iodothyronines were
104 extracted with 4 volumes and again with 100 µl of EtOH : NH_4OH (98:2). The combined
105 supernatants were evaporated under a N_2 current at 37˚C. The residue was then reconstituted in
106 100 µl of MeOH : Water : HCOOH (55: 45: 0.1).

107 10 µl of reconstituted extract was injected into Kinetex C18-100A (2.6 µ, 30 x 3 mm)
108 (Phenomenex, Torrance, CA, US) column, protected by a Phenomenex C18-RP guard cartridge
109 and termostated in the column oven. Iodothyronines were chromatographed with 0.1 % formic
110 acid in deionized water, used as the aqueous mobile phase A, and 0.1 % formic acid in methanol,
111 as the organic mobile phase B. The column temperature was set at 45˚C and the flow rate was
112 0.4 ml/min. The LC gradient procedure and the initial mobile phase compositions were varied to
113 determine the optimal LC separation conditions.

114 The positive ion multiple reaction monitoring (MRM) mode was used for detection of the
115 iodothyronines. The MRM transition monitored was: m/z $777.8 > 731.9$ for T_4; m/z
116 $651.894 > 605.9$ for T_3; m/z $651.8 > 605.9$ for rT_3; m/z $657.8 > 611.9$ for $^{13}C_6$ –rT_3, and m/z
117 $783.8 > 737.9$ for $^{13}C_6$-T_4.

118 Standard curves were also constructed to assess linearity over the range of 5 to 200 ng/ml
119 for T_4, and 0.1 to 2.0 ng/ml for T_3, and for rT_3. Iodothyronines (Sigma-Aldrich, Milwaukee, WI)
120 were added to hormone deprived serum. A blank serum was from Pax8KO mice not treated with
121 T_4 or T_3 for at least 2 months.

122

123 *Protein measurement*

124 Protein concentration of tissues was measured by the Bradford method, using BSA as the
125 standard (Ref. 4). Values were used to express the tissue T_3 and T_4 content and D1 and D2
126 enzymatic activities.

127

128 *Extraction and measurement of tissue mRNA*

129 Total RNA was extracted using phenol/guanidine isothiocyanate (TRIZOL; Invitrogen,
130 Carlsbad, CA), and 2 µg was reverse transcribed using Superscript III ribonuclease H reverse
131 transcriptase kit (Invitrogen) in the presence of 100 ng random hexamers. Reactions for the
132 quantification of mRNAs by real-time quantitative PCR (qPCR) were performed in an ABI Prism
133 7000 sequence detection system (Applied Biosystems, Foster City, CA), using SYBR Green I as
134 detector dye. The oligonucleotide primers were designed to cross introns. Primer sequences used
135 for the qPCR of iodothyronine deiodinases (*Dio1* and *Dio3*), Thyroid hormone-responsive Spot14
136 (*Spot14)*, hairless (*Hr*), sonic hedgehog (*Shh*), semaphorin type 7a (*Sema7a*), Kruppel-like factor
137 (*Klf9*), L-aminoacid transporter type 1 (*Lat1)* and type 2 (*Lat2)*, solute carrier organic anion
138 transporter family, member 1C1(*Oatp1c1)*, member 3a1 (*Oatp3a1)*, member 1b2 (*Oatp1b2*),
139 member 1a1 (*Oatp1a1*), member 1a4 (*Oatp1a4*), Monocarboxylate transporter 10 (*Mct10)* and µ-
140 cristallin (*Crym*) mRNAs are provided in Supplemental table 1. Amplification of the
141 housekeeping gene RNA polymerase II (*RPII*) and 18S ribosomal RNA (*Rn 18s*) was used as
142 internal control for cortex and liver, respectively (Ref. 5).

143

144 *Transfer of Evans Blue across the placenta and the blood brain Barrier (BBB)*

145 To determine genotype differences in placental and BBB permeability, Evans Blue was
146 injected to pregnant dams and to newborn pups. On the 18 day of pregnancy, 4 µl/gm BW of 2%
147 Evans Blue was injected sc, and 16 hours later pups were removed and brain and liver collected.
148 Also P0 mice, immobilized on ice, were injected iv through the facial vein with the same amount
149 of Evans Blue and tissue were collected 2 hours later. Evans Blue concentration was measured by
150 OD at 320 nm and calculated against Evans Blue standard curve. The final result was expresses as
151 mg of Evans Blue per gram of tissue protein

152

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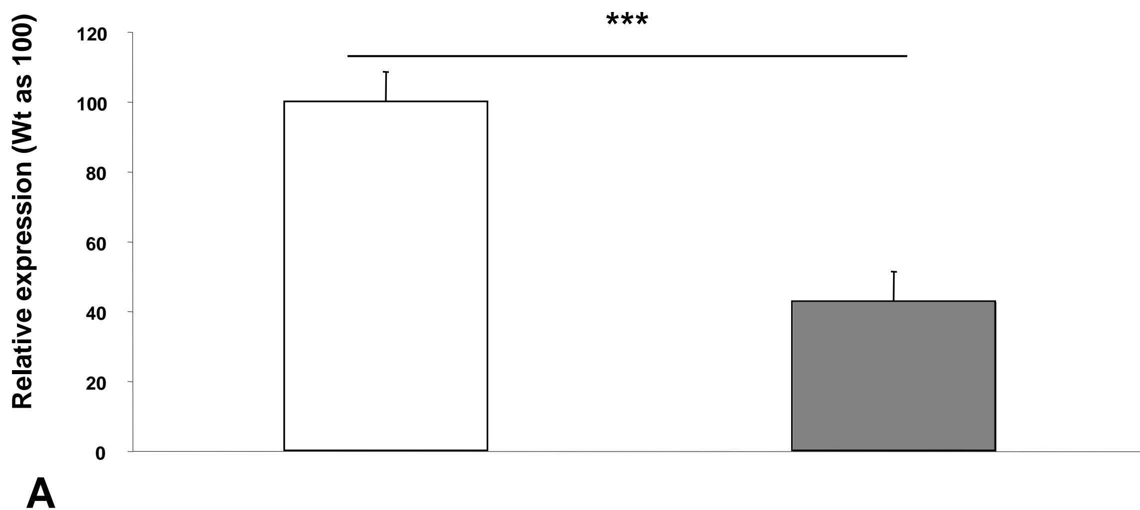
Gene	F Primer	R Primer
RPII	GCACCACGTCCAATGACAT	GTGCGGCTGCTTCCATAA
Rn 18s	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
Dio1	TGAACTTTGGCAGTTGCACC	CCGGATGTCCACGTTGTTCT
Dio3	CCGACCTGATGGCTTCCA	CGCGCCATGAACGGTGGTCA
Hr	CCAAGTCTGGGCCAAGTTTG	TGTCCTTGGTCCGATTGGAA
Shh	CGGCTGATGACTCAGAGGTG	ATGATGGCCGTCCTCATCCC
Sema7a	ACACACCGTGCTTTTCCATGA	CCTTTGTGGAGCCGATGTTC
Klf9	GGCTGTGGGAAAGTCTATGG	AAGGGCCGTTCACCTGTATG
Spot14	ATGCAAGTGCTAACGAAACGC	CCTGCCATTCCTCCCTTGG
Lat1	CTGCTGACACCTGTGCCATC	GGCTTCTTGAATCGGAGCC
Lat2	CCAGTGTGTTGGCCATGATC	TGCAACCGTTACCCCATAGAA
Oatp1c1	AATTCTAGTGTGGCCGGACTGA	CAGCAAGACAAGCCGACACAT
Oatp3a1	TCTGAGTGCGCCTTGATGC	AGAGAATCAGCGCCAGGTTG
Mct10	GGCCGCATTGCTGACTATTT	CAATGGGCGCCATGATAGA
Oatp1b2	CAGCAGTTTGGTCAGACAGCAT	GGTCAAGGTTAGGCCAGCAA
Crym	GTCCAGGCGTACAGTCACTA	AGCCTCCTGCACTGATGAAC

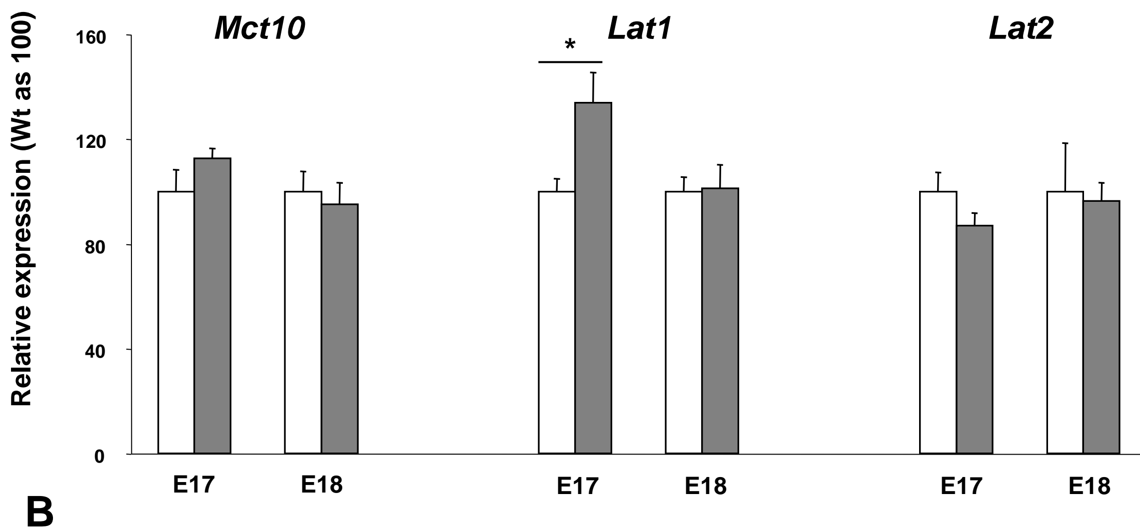
Supplemental table 1. Primers for qPCR.

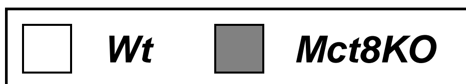
LEGEND OF FIGURES

Supplemental Figure 1. A) T_3 content in the placenta of *Mct8KO* and *Wt* mice at ages E17 and E18; B) Mct8 gene expression in the placenta in *Mct8KO* and *Wt* mice at age E17; and C) Gene expression of TH transporters *Mct10, Lat1, Lat2,* in the placenta of *Mct8KO* and *Wt* mice at ages E17 and E18. Results are expressed as mean ± SE *, $P < 0.05$; ***, $P < 0.001$. n = 8 for each group

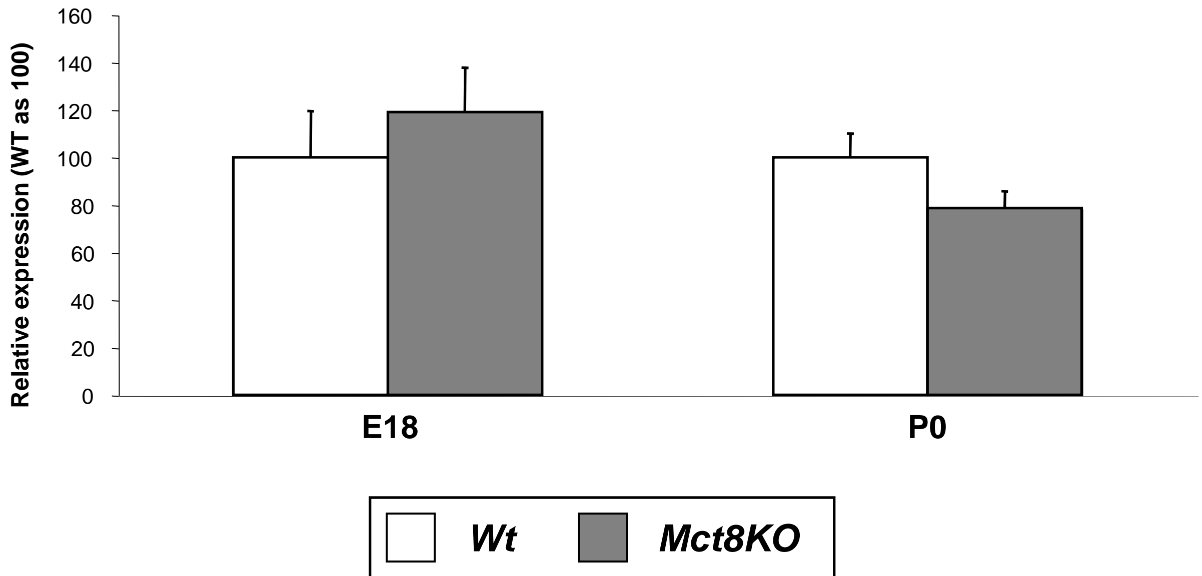
Supplemental Figure 2. *Crym* gene expression in cortex of Mct8KO and Wt mice at ages E18 and P0. Results are expressed as mean ± SE. n= 8 for each group







Supplemental Figure 1



Supplemental Figure 2