Lack of Action of Exogenously Administered T3 on the Fetal Rat Brain Despite Expression of the Monocarboxylate Transporter 8

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Mutations of the monocarboxylate transporter 8 gene (MCT8, SLC16A2) cause the Allan-Herndon-Dudley syndrome, an X-linked syndrome of severe intellectual deficit and neurological impairment. Mct8 transports thyroid hormones (T4 and T3), and the Allan-Herndon-Dudley syndrome is likely caused by lack of T3 transport to neurons during critical periods of fetal brain development. To evaluate the role of Mct8 in thyroid hormone action in the fetal brain we administered T4 or T3 to thyroidectomized pregnant dams treated with methyl-mercapto-imidazol to produce maternal and fetal hypothyroidism. Gene expression was then measured in the fetal cerebral cortex. T4 increased Camk4, Sema3c, and Slc7a3 expression, but T3 was without effect. To investigate the cause for the lack of T3 action we analyzed the expression of organic anion transport polypeptide (Oatp14, Slco1c1), a T4 transporter, and Mct8 (Slc16a2), a T4 and T3 transporter, by confocal microscopy. Both proteins were present in the brain capillaries forming the blood-brain barrier and in the epithelial cells of the choroid plexus forming the blood-cerebrospinal fluid barrier. It is concluded that T4 from the maternal compartment influences gene expression in the fetal cerebral cortex, possibly after transport via organic anion transporter polypeptide and/or Mct8, and conversion to T3 in the astrocytes. On the other hand, T3 does not reach the target neurons despite the presence of Mct8. The data indicate that T4, through local deiodination, provides most T3 in the fetal rat brain. The role of Mct8 as a T3 transporter in the fetal rat brain is therefore uncertain. (Endocrinology 152: 1713–1721, 2011)

thyroid hormones T4 and T3 are important regulators of mammalian brain development (1–4). Their effects are largely mediated by the control of gene expression after the binding of the genomically active T3 to nuclear receptors. T3 is secreted by the thyroid gland, but it is also produced in target tissues by the 5’ deiodination of T4, a reaction catalyzed by type 2 deiodinase (D2) (5, 6). In some tissues, such as brain, developing cochlea, brown adipose tissue, and anterior pituitary, D2 plays an important role in providing T3 to the target cells. T4 and T3 are inactivated to rT3 and T2 by type 3 deiodinase (D3), which in the brain is expressed in neurons.

Cellular uptake of thyroid hormone requires the presence of plasma membrane transporters of several protein families (7). Mutations of one of these transporters, the monocarboxylate transporter 8 (MCT8, SLC16A2) cause a X-linked syndrome characterized by intellectual and neurological impairment from early infancy (8–12). In addition to the plasma membrane of target cells (13), Mct8 in rodents is expressed in the capillary endothelial cells

Abbreviations: BBB, Blood-brain barrier; BW, body weight; D2, type 2 deiodinase; D3, type 3 deiodinase; DAPI, 4’-6-diamidino-2-phenylindole; E, embryonic day; Glf, glial fibrillary acidic protein; Glut, glucose transporter; Mct8, monocarboxylate transporter 8; MMI, 2-mercapto-1-methylimidazole; OATP, organic anion transporter polypeptide (Slco1c1); T, thyroidectomized dams; TM, thyroidectomized dams given MMI.
forming the blood-brain barrier (BBB) and in the epithelial cells lining the choroid plexuses (14). While Mct8 transports T4 and T3, other transporters are more selective for T4. This is the case of the organic anion transporter polypeptide 14 (Oatp14 in rodents, OATP-F in humans, SLCO1C1). Oatp14 is also expressed in the brain capillaries and the choroid plexus (14, 15).

T3 therefore reaches the brain and the neural target cells via two routes. One is the direct access of T3 from the circulation via Mct8-mediated transport through the BBB. On the other hand, T3 is formed in astroglial cells by D2-mediated T4 deiodination (16, 17). The T3 formed in this pathway is then delivered to neurons and other neural target cells (17). In D2-deficient mice brain T3 concentrations are about half of normal, suggesting that each pathway contributes similarly to total T3 in the postnatal brain (18). We have recently shown subtle differences in the activity of the T3 from the circulation and that generated locally (19).

The fetal brain seems however to be largely dependent on T4 deiodination. In the second trimester human fetus there is a correlation between T3 concentration and D2 activity in several brain regions (20). In rats the administration of T4, but not T3, to pregnant dams increases the concentration of T3 in the brain of hypothyroid fetuses (21). The latter experiments indicated that the fetal brain depends critically on T4 for thyroid hormone action after its conversion to T3. We here extend these observations by showing that exogenously administered T4 to pregnant dams influences gene expression in the cerebral cortex of the fetuses, in contrast to T3 which had no effect. Because the main determinant for T3 entry into the brain appears to be the presence of Mct8 in the BBB (14, 22–24), we analyzed whether or not Mct8 is expressed in the fetal rat brain. The data indicate that the fetal brain responds to the administration of T4, but not to T3, despite Mct8 expression.

Materials and Methods

Animal handling

Female Wistar rats grown in our animal facilities and weighing 250–300 g were used. Protocols for animal handling were approved by the local institutional Animal Care Committee, following the rules of the European Union. Animals were under temperature- (22 ± 2 C) and light- (12-h light, 12-h dark cycle; lights on at 0700) controlled conditions and had free access to food and water. All surgical interventions were under anesthesia with a mixture of ketamine and medetomidine as described (25).

To induce maternal hypothyroidism the pregnant dams were thyroidectomized on embryonic day 10 (E10, the day of appearance of the vaginal plug was E0) sparing the parathyroid glands (T group). To induce maternal and fetal hypothyroidism, the T dams were given 0.02% 2-mercapto-1-methylimidazole (MMI, Sigma Chemical Co., St. Louis, MO) in the drinking water until E21 (TM group). T4 and T3 were separately administered to TM dams from E10 by constant infusion through osmotic pumps (Alzet 2ML2, delivering 5.0 µl/h, www.alzet.com) containing the hormone dissolved in 50% propylene glycol. The calculated daily doses infused, at the moment of implantation, were 5 µg T4/100 g body weight (BW) or 1.5 µg T3/100 g BW and were not corrected for increasing weight. The dams of the control (C) group were sham operated and implanted with osmotic pumps containing solvent (21). To analyze the relative effects of the maternal and the fetal thyroidal status, the following groups were compared: Control on E17 and E21 (C17 and C21); thyroidec- tomized dams on E10 and analyzed on E17 (T17); and thyroidectomized and MMI-treated dams from E10 and analyzed on E21 (TM21).

At the end of the experiment the dams were anesthesized and perfused as described after collection of blood (21). The fetuses were bled, separated from the placenta, and placed on ice. The livers and brains were removed. The brains were sagittally sectioned in halves. One half of the whole brain was used for T4 and T3 determinations. From the other half the cerebral cortices were dissected out for PCR assays. All tissues were frozen in dry ice after collection. Most assays were done using five to seven samples of each group. The fetal plasma and brain samples consisted of pooled material from three fetuses of each individual litter (21, 26). Fetal livers were processed individually. Thyroid hormones, TSH measurements, and real-time PCR were done as previously described (21, 26) with TaqMan probes (Applied Biosystems, Foster City, CA). Significance of differences between experimental groups was calculated by one-way ANOVA and the Tukey post hoc test using the GraphPad software (www.graphpad.com).

Immunofluorescence

Brains from E21 fetuses were fixed in 4% paraformaldehyde in 0.1 m phosphate buffer (pH 7.4) for 24 h at 4°C. They were then cryoprotected in 30% sucrose dissolved in 0.1M phosphate buffer (pH 7.4) for 24 h at 4°C. They were then cryoprotected in 30% sucrose dissolved in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde, frozen in dry ice, and 15-µm slices obtained in a cryostat. The slices were kept at −70°C until use. For immunofluorescence the slices were thawed, air-dried, washed in PBS, and incubated in methanol for 5 min at −20°C. After washing in PBS, the slices were blocked in PBS containing 0.1% triton X-100, 5% newborn goat serum, and 5% horse serum. The primary antibodies were diluted in the blocking solution, added to the slices, and incubated 16 h at 4°C. After three washings in PBS, the secondary antibodies were added and incubated in the dark for 1 h at room temperature. The slices were then washed in PBS and incubated with 4’,6-diamidino-2-phenylindole (DAPI), 0.1 µg/ml in PBS. Rabbit antibodies against Mct8 (XE045) and Oatp14 N terminus (XE066) were a generous gift of Dr. Lori Roberts (Xenoport, Santa Clara, CA) (14) and were used at 1:300 dilution. Goat anti-Glut1 (N-20, ref sc-1603) was from Santa Cruz Biotechnology (Santa Cruz, CA) and was used at 1:100 dilution. The secondary antibodies were donkey antigoat Alexa 488 (green) and goat antirabbit Alexa 546 (red) and were used at 1:2000 dilution. Omitting the first antibodies in the incubation reaction gave no signal.
Results

Our first goal was to analyze the relative activities of in vivo administered T4 and T3 on the fetal cerebral cortex. Instead of administering the hormones directly to the hypothyroid fetuses, they were given via subcutaneous infusion to pregnant dams. The dams had been previously thyroidectomized and treated with MMI to produce maternal and fetal hypothyroidism.

The outcome of treatment was checked by TSH and thyroid hormone measurements in the dams and fetuses (Fig. 1). The following groups were compared: control group (C) (i.e., euthyroid dams and fetuses), thyroidectomized dams (T) (i.e., hypothyroid dams and euthyroid fetuses), thyroidectomized and MMI-treated dams (TM) (i.e., hypothyroid dams and fetuses), TM dams treated with T4 (TM+T4), and TM dams treated with T3 (TM+T3). The T and TM dams had decreased circulating T4 and T3 and increased TSH. T4 treatment increased both T4 and T3. T3 treatment increased T3 to a similar level to that attained by T4 treatment. TSH was normalized by either treatment. Fetal TSH, on the other hand, was normal in the T group and increased in the TM group. T4 administration to the TM dams normalized fetal TSH, while T3 treatment had a modest effect.

In the liver, MMI treatment (TM dams) decreased T4 and T3 (Fig. 1). In fetuses from thyroidectomized dams, T4 remained at normal levels, and T3 was decreased by about 50%. A reduction of thyroid hormones in tissues of fetuses from thyroidectomized dams has been previously reported in some studies (27). T4 treatment increased fetal liver T4 and T3, without reaching normal control values. T3 treatment had no effect on liver T4 and increased T3 to the same level as after T4 treatment. The data indicated that similar amounts of T3 were available to the hypothyroid fetuses after T4 or T3 administration to the dams.

In the fetal brain, thyroidectomy caused a small decrease in T4, with no changes in T3, whereas additional treatment with MMI (TM dams) decreased T4 and T3 to around 30 and 10% of control concentrations. Treatment with T4 increased brain T4 without reaching C or T values. T4 treatment, however, increased T3 to the same values as C or T fetuses. T3 concentrations were also increased by T3 treatment but remained at about half the level reached with T4 treatment.

To analyze the relative activity of T4 and T3 treatment in the fetal brain we used as end points the expression of three of the genes recently shown to be altered by fetal hypothyroidism in the cerebral cortex and induced by T3 after addition to primary cortex neurons in culture (21, 26): Camk4 (Ca2+ and calmodulin-dependent protein kinase 4), Slc7a3 (encoding the cationic exchanger Solute carrier family 7 member 3), and Sema3c (or Semaphorin 3C).

In agreement with previous data (26) we show in Fig. 2 (left panels) that these genes are regulated primarily by the fetal thyroid hormones. On E17 (i.e., before onset of fetal thyroid secretion), there was no effect of maternal thyroidectomy (T17 vs. C17) on the expression of any of the genes analyzed. From E17 to E21 Camk4 and Sema3c
increased in control animals (C17 vs. C21). This increase can be attributed to the onset of function and progressive activity of the fetal thyroid gland taking place during this period. In E21 fetuses in which the thyroid gland was blocked by treatment with MMI (TM21 group), Camk4 and Sema3c remained at the E17 level. In contrast, Slc7a3 decreased slightly in control animals from E17 to E21, and hypothyroidism induced a greater decrease. These results show that fetal thyroid hormones regulate developmental changes in the expression of these genes, allowing an increase in the expression of Camk4 and Sema3c and a fine adjustment of Slc7a3.

The right panels of Fig. 2 show the expression of these genes on E21 after hormone administration to the dams.

Maternal thyroidectomy did not change the expression of the genes, in agreement with the primary role of the fetal hormones. Suppression of fetal thyroid secretion with MMI treatment decreased the expression of the three genes. Treatment with T4 was effective in normalizing gene expression, but T3 was without effect.

It is thought that D3 activity, which is high in fetal tissues, is an important modulator of T3 action in the developing brain (28, 29). Therefore we checked whether Dio3 expression in the cerebral cortex changed with the different treatments (Fig. 3). Although the mean levels after T3 treatment were higher, the changes were not significant.

Thyroid hormone action requires the presence of transporters in the plasma membrane of target cells (7). In the brain, transporters are required for T4 and T3 to cross the BBB (14, 22). We analyzed the expression of Mct8, Oatp14, Lat2 (Slc7a6), and Mct10 (Slc16a10) (Fig. 4). The data were normalized to the value obtained for Mct8 on E21 after correcting for 18S RNA. Mct8 mRNA content was higher during the prenatal stages than at postnatal d 15. Oatp14, Lat2, and Mct10 were also expressed in the fetal cortex and increased during the postnatal period. The amounts of Mct10 mRNA were extremely low in comparison with the other transporters. As a reference for gross cellular changes taking place in the cortex from the fetal to the postnatal period, we measured the expression of the glucose transporter Glut-1, expressed in the capillary endothelia, and the intermediate filament glial fibrillary acidic protein, Gfap, expressed in astrocytes. Glut-1 mRNA decreased slightly on E21 and then increased on P15. Gfap mRNA had a large increase on P15, reflecting the accumulation of astroglia taking place postnatally.

FIG. 2. Real-time PCR determination of transcript levels in the fetal cerebral cortex on E17 and E21. The experimental groups are as in Fig. 1. Left, Effect of maternal hypothyroidism (T) on E17 and of maternal and fetal hypothyroidism TM on E21. The number of samples was five in each group. Data are means ± se. One-way ANOVA for Camk4, F (3, 16) = 32.83, P = 0.0011; for Sema3c, F (3, 16) = 31.18, P < 0.0001; and for Slc7a3, F (3, 16) = 30.69, P < 0.0049. Right, Effect of T4 and T3 infusion on transcript levels on E21. The number of samples was six in each group. Data are means ± se. One-way ANOVA for Camk4, F (4, 25) = 6.40, P = 0.0011; for Sema3c, F (4, 25) = 9.20, P = 0.0001; and for Slc7a3, F (4, 25) = 8.86, P < 0.0049. Comparisons are as follows: different from C (a), different from T (b), and different from TM (c).

FIG. 3. Real-time PCR determination of Dio3 transcript levels in the fetal cerebral cortex on E21. The experimental groups are as in Fig. 1. Changes among the groups were not significant: F (4, 23) = 2.349, P = 0.084.
To analyze the expression and distribution of the Mct8 and Oatp14 proteins we used immunofluorescence and confocal microscopy. (Figs. 5 and 6). Figure 5 shows that Mct8 and Oatp14 were present in the cerebral cortex, and their expression was coincident with that of Glut-1. Both transporters were also present in the choroid plexus (Fig. 6). Oatp14 was observed in the epithelial cells forming the blood-cerebrospinal fluid barrier. The protein was mainly present in the apical and lateral borders, but it was also observed in the basal membrane of many cells. There was also some staining for Oatp14 inside the plexus. Interestingly, Oatp14 was also present in the ependymal cells lining the ventricle walls. Mct8 was present exclusively in the epithelial cells, mostly in the apical side, but also in the basal side of some cells. Expression in the choroid plexus exceeded by far that of the capillaries, so that the images had to be overexposed to observe the immunochemical signal from the vessels and from the choroid plexus in the same picture (Fig. 6C).

**Discussion**

Calvo *et al.* (21) administered increasing doses of T4 or T3 to MMI-treated pregnant dams, a model of maternal and fetal hypothyroidism similar to the one used in the present work. They found that T4 administration resulted in the presence of T3 in the fetal brain and prevented the increase of fetal TSH and fetal brain D2 activity induced by MMI treatment. In contrast, administration of T3 in comparatively higher doses did not increase brain T3 concentration over hypothyroid levels and had no effect on the fetal TSH or D2. The reason for the differences between T4 and T3 treatment was not the lack of placental transport, because both T4 and T3 were present in fetal plasma and tissues other than the brain after their administration to the dams. It was therefore proposed that the fetal brain was crucially dependent on T4. The lack of suitable T3 target genes precluded the demonstration that also systemic T4, but not T3, was active on fetal brain gene targets.

On the other hand, we have recently shown that fetal hypothyroidism affects cerebral cortex gene expression (19). Several of the genes affected by hypothyroidism were also increased by T3 in neuronal primary cultures, indicating a direct cellular response to T3. In the present work we have analyzed the expression of three of these genes to explore whether thyroid hormones from the maternal compartment were able to influence gene expression in the fetal cerebral cortex. The experimental set up was similar to that of Calvo *et al.* (21) and was based upon the use of thyroidectomy to achieve isolated maternal hypothyroidism and additional MMI treatment to induce maternal and fetal hypothyroidism. In agreement with the experimental set up, TSH determinations showed that thyroidectomy increased TSH in the maternal but not in the fetal blood, whereas MMI treatment increased TSH in both dams and fetuses.

T3 treatment suppressed maternal TSH but had little effect on fetal TSH. In contrast, T4 treatment was effective on both the maternal and the fetal TSH. The mechanism is not known, but the result agrees with previous work showing effects of exogenous T3 on fetal GH but not on fetal TSH (30). As in previous studies, the different effects of T4...
and T3 could not be explained by restricted T3 transport at the placental level. In fact, after T4 or T3 treatment similar amounts of T3 were found in fetal liver, expected to reflect circulating blood levels. Regulation of fetal TSH is independent on hypothalamic TRH until well after birth (31–33). Also, from the experiments cited above (30), the control by thyroid hormone is probably exerted at the pituitary level. It is likely that transporters play a role (34, 35), for example by facilitating the selective uptake of T4 into the D2-expressing cells in the fetal pituitary.

This experimental model allowed us to analyze the effect of the hormones from the maternal compartment on gene expression in the fetal cortex. The expression patterns of these genes are related to the onset of fetal thyroid gland function and, in agreement with previous data (26), they were not affected by isolated maternal hypothyroidism. Therefore, these genes are primarily regulated by the fetal thyroid hormones, without any direct or indirect effects of maternal hypothyroidism. These results allow us to discard that the effect of treatment on gene responses are attributable to a beneficial effect on the general thyroid status of the mother, or the fetus, rather than a direct effect on the fetal brain.

Gene expression in the hypothyroid fetal brain was normalized by T4, but not by T3, administration to the pregnant dams. The effect of T4 is most likely attributable to the T3 generated locally in the brain from the T4 precursor, because D2 activity increases in brain in the last days of gestation (36). Calvo et al. (21) observed that after administration of T4 to the pregnant dams, T3 accumulated in the fetal brain, but not after administration of T3. Although we found that T3 increased in the fetal brain after T3 administration, it was not enough to stimulate gene expression. The concentrations of T3 attained by T4 treatment were higher and resulted in normalization of gene expression. The reason for the quantitative differences between our results and those from Calvo et al. (21) are not clear to us, but the final conclusion, that the fetal brain is dependent on T4, and little or nothing on T3, remains the same.

Interestingly, the defective T3 accumulation and action in the fetal brain resembles the situation of mice deprived of the thyroid hormone transporter Mct8 (Mct8−/−) in which brain gene expression is less sensitive to exogenous T3 (22) and relies on D2-mediated T4 to T3 conversion (19). For this reason we analyzed the expression of thyroid hormone transporters in the fetal cortex by quantitative PCR and immunofluorescence. Mct8 has similar transport activities for T4 and T3, and also transports rT3 and T2. Oatp14 has a transport activity several fold higher for T4 and rT3 than for T3 (15, 37). Lat-2 was also studied because of its possible importance in the human fetal brain (38). By quantitative PCR we show that expression of Mct8 is more abundant in fetal cortex than in postnatal cortex. In humans Mct8 is also more abundant during early brain development (39). By quantitative PCR we show that expression of Mct8 is more abundant in fetal cortex than in postnatal cortex. 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agrees with the astrocytic expression. Therefore, it is likely that T4 from the circulation is transported via Oatp14 directly to the astrocytes where it undergoes D2-mediated conversion to T3. Mct8 did not overlap with aquaporin-4, suggesting that circulating T4 and T3 may be delivered to the extracellular fluid after Mct8-mediated transport. Similarly, blood glucose can access the neurons directly after transport to the extracellular fluid, or after transport into the astrocytes and conversion to lactate.

Once delivered to the extracellular fluid T4 and T3 will have direct access to neurons and serve as substrates for D3 (41). D3 is abundantly expressed in fetal brain and other tissues and decreases rapidly during the postnatal period (42–44). Its subcellular localization allows rapid degradation of T4 and T3 after they enter the D3-expressing cells (5). Therefore, D3 activity is another player in determining the relative contributions of systemic T3 vs. the T3 produced locally from T4. It is particularly important during development (28, 29) when D3 deletion increases target gene responses to T3 (45). In the human fetal brain, D3 activity in the cerebellum prevents the accumulation of T3 during mid-gestation, whereas in the cerebral cortex the increased T3 concentration during the second trimester is parallel to increased D2 activity (20). Given the presence of significant D2 activity in the rat fetal brain (36), which explains the present and previous data (21), most thyroid hormone action in the fetal brain would be attributable to the T3 of local origin (Fig. 7). As D3 expression decreases during further development, the contribution of systemic T3 would increase to reach the approximate contribution of about 50% to the T3 present in the brain (18). An additional possibility is that D3 is also expressed in the vascular endothelial cells, as shown previously in hemangiomas (46) and in the fetal microvessels of the placenta (47), but this would not explain a T3 selectivity.

The mechanism of transfer of T3 from the astrocytes to the neurons is not clear. Our previous data showed that Mct8 deficiency did not prevent the effect of T4 administration on the expression of target genes, despite the impairment of the effect of T3 (22). We also demonstrated that gene expression in the cerebral cortex of Mct8-deficient mice was compensated by D2 activity (19). Therefore, other mechanisms different from Mct8-mediated transport would supply T3 from the astrocytes to the neurons, at least in postnatal stages. Data extracted from the genomic database of mouse brain cells by Cahoy et al. (48) indicate that astrocytes express predominantly Oatp14 and much lower amounts of Lat-1 and Mct8 (Supplemental Fig. 1 published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org/). Neurons express Lat-1, Lat-2, and Mct8. Combinatorial expression of these transporters would allow the transfer of T3 from the astrocytes to different cellular subsets. We could not detect the Mct8 protein in the brain parenchyma in agreement with Roberts et al. (14). However this may be attributable to lack of resolution, and it could be that during fetal stages the expression of Mct8 in discrete cellular subsets is more important, relative to other transporters, for the uptake of T3 produced in the astrocytes. It would therefore be required to analyze the impact of Mct8 deletion on gene expression in the fetal mouse brain.

**FIG. 7.** Cartoon depicting the hypothetical fate of T4 and T3 in the fetal brain. T4 from the circulation crosses the BBB and reaches the astrocytes through Oatp14. Once in the astrocytes it is converted to T3 by D2 and is released to the neurons. T4 and T3 cross the BBB through Mct8 and are delivered to the interstitial fluid. It is possible that the topology of D3 in the neuronal membrane allows for easy inactivation of T4 and T3 with the production of rT3 and T2, which would then be released back to the circulation. Oatp14 and Mct8 are represented in black boxes. Other possible transporters are represented in gray boxes.

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