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De Groot LJ, Chrousos G, Dungan K, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-.

Thyroid Hormones in Brain Development and Function

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Last Update: September 2, 2015.

ABSTRACT

Thyroid hormones are essential for brain developat through specific time windows influencing neurogenesis, neuronal migration, neuronal and glial cell differentiation, myelination, and synaptogenesis. The actions of thyroid hormones are mostly due to interaction of the active hormone T3 with nuclear receptors and regulation of gene expression. T4 and T3 also perform non-genomic actions. The genomically active T3 in brain derives in part from the circulation, and in part is formed locally by 5'-deiodination of T4, mediated by Dio2 in the astrocytes, in proportions that depend on the developmental stage. T4 and T3 are degraded by Dio3 present in neurons. Entry of T4 and T3 in brain is facilitated by specific transmembrane transporters, mainly the monocarboxylate transporter 8 (Mct8) and the organic anion transporter polypeptide 1c1 (Oatp1c1). In rodents Mct8 facilitates the transfer of T4 and T3 through the blood-brain barrier (BBB). Oatp1c1 transports T4 through the BBB and into to the astrocytes facilitating the generation of T3 in these cells. Primates have low amounts of OATP1C1 in the BBB, and depend of MCT8 for thyroid hormone transport. Therefore MCT8 mutations in humans cannot be compensated by T4 transport as in rodents. The thyroid hormone receptor subtypes TRα and TRβ are expressed throughout the brain from early development, and mediate overlapping actions on gene expression. However there are also TR-subtype specific actions. Dio3 for example is induced by T3 specifically through TRα1. In vivo T3 regulates gene expression during development from fetal stages, and in adult animals. A large number of genes are under direct and indirect regulation by thyroid hormone. In neural cells T3 may control around 5% of all expressed genes, and as much as one third of them may be regulated directly at the transcriptional level. Thyroid hormone deficiency during fetal and postnatal development may cause retarded brain maturation, intellectual deficits and in some cases neurological impairment. Thyroid hormone deficiency to the brain during development is caused by iodine deficiency, congenital hypothyroidism, and maternal hypothyroidism and hypothyroxinemia. The sydromes of Resistance to Thyroid Hormones due to receptor mutations, especially TRα, cause variable affectation of brain function. Mutations in the monocarboxylate transporter 8 cause a severe retardation of development and neurological impairment, likely due to deficient T4 and T3 transport to the brain. A challenge for the immediate future is a deep understanding of the pathophysiology of these syndromes, under the light of ample knowledge on thyroid hormone action in the brain, to allow designing appropriate therapies. For complete coverage of this and related areas in Endocrinology, please visit our free web-book, www.endotext.org.

INTRODUCTION

Thyroid hormones are essential for brain maturation, and for brain function throughout life. In adults, thyroid diseases can lead to various clinical manifestations (1,2). For example, hypothyroidism causes lethargy, hyporeflexia and poor motor coordination. Subclinical hypothyroidism is often associated with memory impairment. Hypothyroidism is also associated to bipolar affective disorders, depression, or loss of cognitive functions, especially in the elderly (3). Hyperthyroidism causes anxiety, irritability, and hyperreflexia. Both, hypothyroidism or hyperthyroidism can lead to mood disorders, dementia, confusion, and personality changes. Most of these disorders

are usually reversible with proper treatment, indicating that thyroid hormone alterations of adult onset do not leave permanent structural defects.

The actions of thyroid hormone during development are different, in the sense that they are required to perform certain actions during specific time windows. Thyroid hormone deficiency, even of short duration may lead to irreversible brain damage, the consequences of which depend not only on the severity, but also on the specific timing of onset and duration of the deficiency (4-8).

The rat has been the most widely used animal model in the sudy of thyroid physiology and in the actions of thyroid hormone in brain, and the use of knock out (KO) and knock in (KI) mice is providing new insights. When the results of animal studies are extrapolated to the human, it is important to realize that the timing of development in relation to birth among mammals presents substantial differences, even if the sequence of events is similar (9-11). Most brain growth occurs after birth in man and in rodents, but rats and mice are born with a less developed thyroid axis than humans. As a useful reference "the newborn rat may be compared with a human fetus in the second trimester of pregnancy, and the newborn human baby to a 6-10 day old rat" (12). For a recent and integrated view on biochemical aspects of thyroid hormone action on cerebral and cerebellar cortex development see (13), and for a neurodevelopmental and evolutionary perspective see (14).

The hypothyroid brain presents many structural defects (for an extensive review see (12): Increases in cell density in the cerebral cortex, due to reduction of the neuropil (15,16). Lower cell numbers in regions with significant postnatal cell acquisitions, for example the olfactory bulb and the granular layers of the hippocampus and cerebellum (16,17). Decreased number of GABAergic interneurons in the cerebellum with accumulation of neuronal precursors (18). Reduced number of parvalbumin interneurons in the cerebral cortex (19). Some specific cell types display stunted dendritic and / or axonal growth and maturation, for example cholinergic cells (20,21), Purkinje cells of the cerebellum (Fig 1) (22,23) and pyramidal cells of cortex layer V (24,25). Changes of dendritic spine number are also observed in the cortex and hippocampus after adult onset hypothyroidism, and are reversible with thyroxine treatment (26,27). These morphological changes are the consequence of altered biological processes as follows:

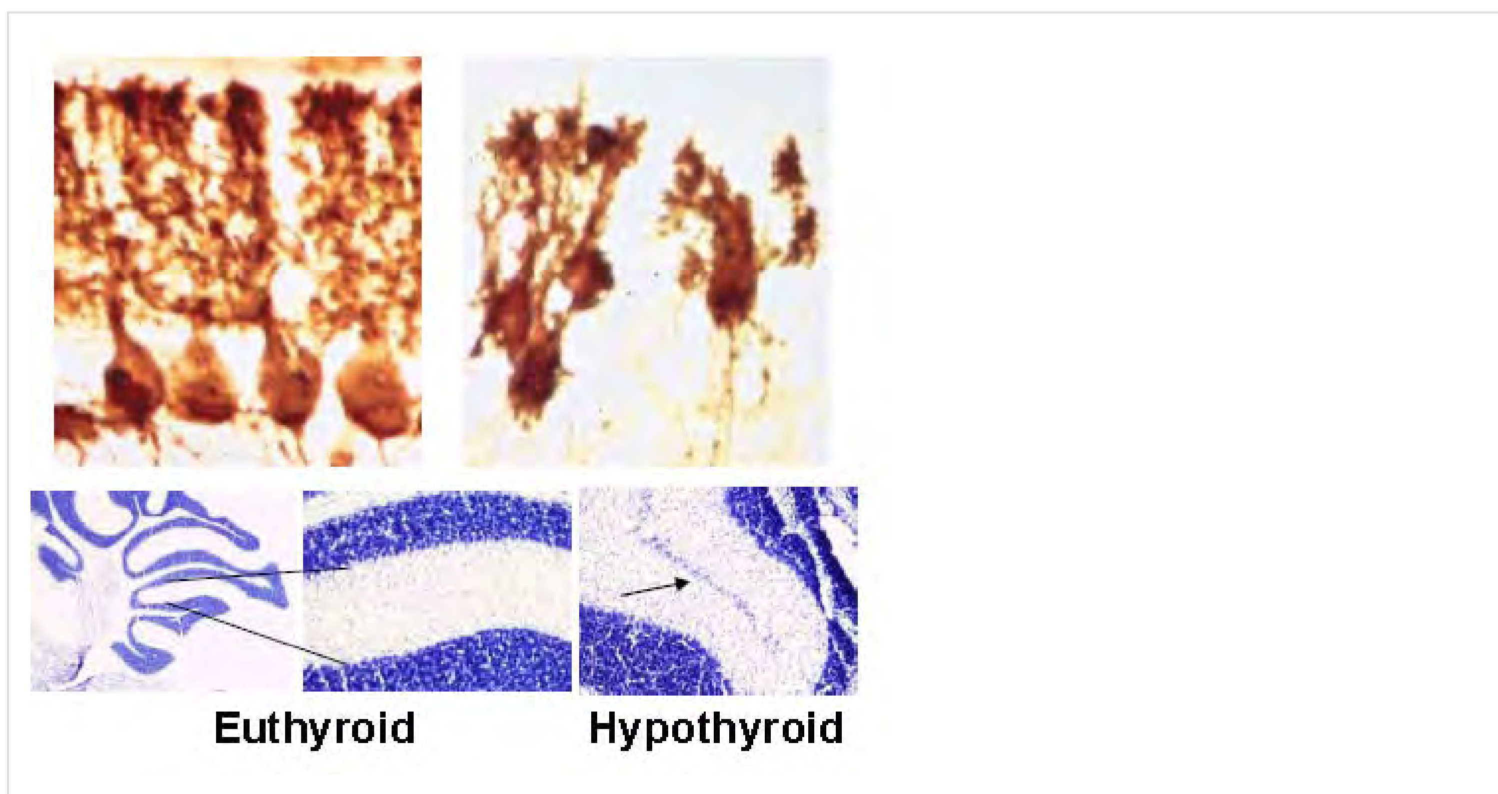


Fig 1

Postnatal morphological changes in the rodent cerebellum after neonatal hypothyroidism. Upper panel: Purkinje cells in a normal (left) and hypothyroid rat (right). Lower panel: persistence of the external granular layer (arrow) in a hypothyroid mouse cerebellum.

Neurogenesis

Thyroid hormones are involved mainly in late events of neural development, such as migration and terminal differentiation of neurons and glia. However a role of thyroid hormones on proliferation and differentiation of neural precursors in the embryonic neurogenic areas has been shown during tadpole premetamorphosis (28) or in relation to the effects of maternal thyroid hormones (29,30). Intriguingly, progenitor proliferation in the embryonic subventricular zone (SVZ) leading to neocortex expansion is under control of T4 through the integrin $\alpha_v\beta_3$ membrane receptor (31).

Several studies have demonstrated effects of thyroid hormone on neurogenesis in adult animals (32,33). Adult neurogenesis has been related to neuropsychiatric conditions, cognitive deficits and depression. It takes place mainly in two regions: the SVZ, and the subgranular zone (SGZ). The SVZ, located underneath the surface of the lateral ventricles, generates olfactory bulb interneurons in adult rodents. The SGZ is adjacent to the granular layer of the dentate gyrus, and generates granular neurons. Hypothyroidism depresses, and thyroid hormone administration stimulates, neurogenesis in these two areas (34-37). In the SVZ T3 increases differentiation of neuronal precursors (38) and increases the commitment of neural stem cells to migrating neuroblasts. This action correlates with repression of the *Sox2* gene (39). The effects of T3 on embryonic and adult neurogenesis appear to be mediated predominantly by TRa1 (28,29,39). However, effects through TRb have also been reported (40).

Another adult neurogenic niche may reside in the hypothalamic tanycytes, glial-like cells lining the walls of the 3rd ventricle (41). These cells have been linked to the central control of feeding, body weight, and energy balance, and have a very active thyroid hormone signalling system, as will be described below. In this area retinoic acid, formed locally, is an inhibitor of tanycyte proliferation. Interestingly Aldh1a1, one of the enzymes for the synthesis of retinoic acid, is strongly and synergistically induced by thyroid hormone and glucocorticoids (42), suggesting a mechanism by which T3 might affect neurogenesis in this area.

Cell Migration

Thyroid hormone exerts important influences on cell migration in the cerebral cortex, hippocampus and cerebellum. Among relevant possible mechanisms is an action on the radial glia. The radial glia derives from neuroepithelial cells that elongate as the embryonic brain epithelium thickens. These cells extend long processes to the cerebral wall, providing a scaffold that serves for cell migration (43,44). But they not only have a structural and supportive function. They are real stem cells that generate neurons and neuronal precursors, oligodendrocyte precursors, astrocytes and ependymal cells (45). Radial glia maturation in the fetal rat brain is delayed in the hippocampus of hypothyroid rats (46), and this may affect the migration of the neurons, but may also impair neurogenesis.

In the cerebral cortex, thyroid hormones are needed for the proper arrangement of the six layer pattern, formed by the timely migration of cells originated in the ventricular neuroepithelium. Deficiency of thyroid hormone during the period of cortical development leads to less defined cortical layers, due to disturbances of cell migration (47-49). One mechanism by which thyroid hormones may influence neuronal migration in the cerebral cortex is through the regulation of the expression of the *Reln* gene (Fig 2). The product of this gene, Reelin, is an extracellular matrix

protein produced by neurons located in layer I of the cerebral cortex, the Cajal-Retzius cells (50). Thyroid hormone regulates the expression of at least two genes expressed by these cells, *Reln*, and *Ptgds* (encoding Prostaglandin D2 synthase) (51,52). Reelin is essential for the orderly migration and the establishment of neocortical layers. Cajal-Retzius cells also have an important role in the migration of neuronal precursors in the hippocampus and in the establishment of synaptic connections (53).

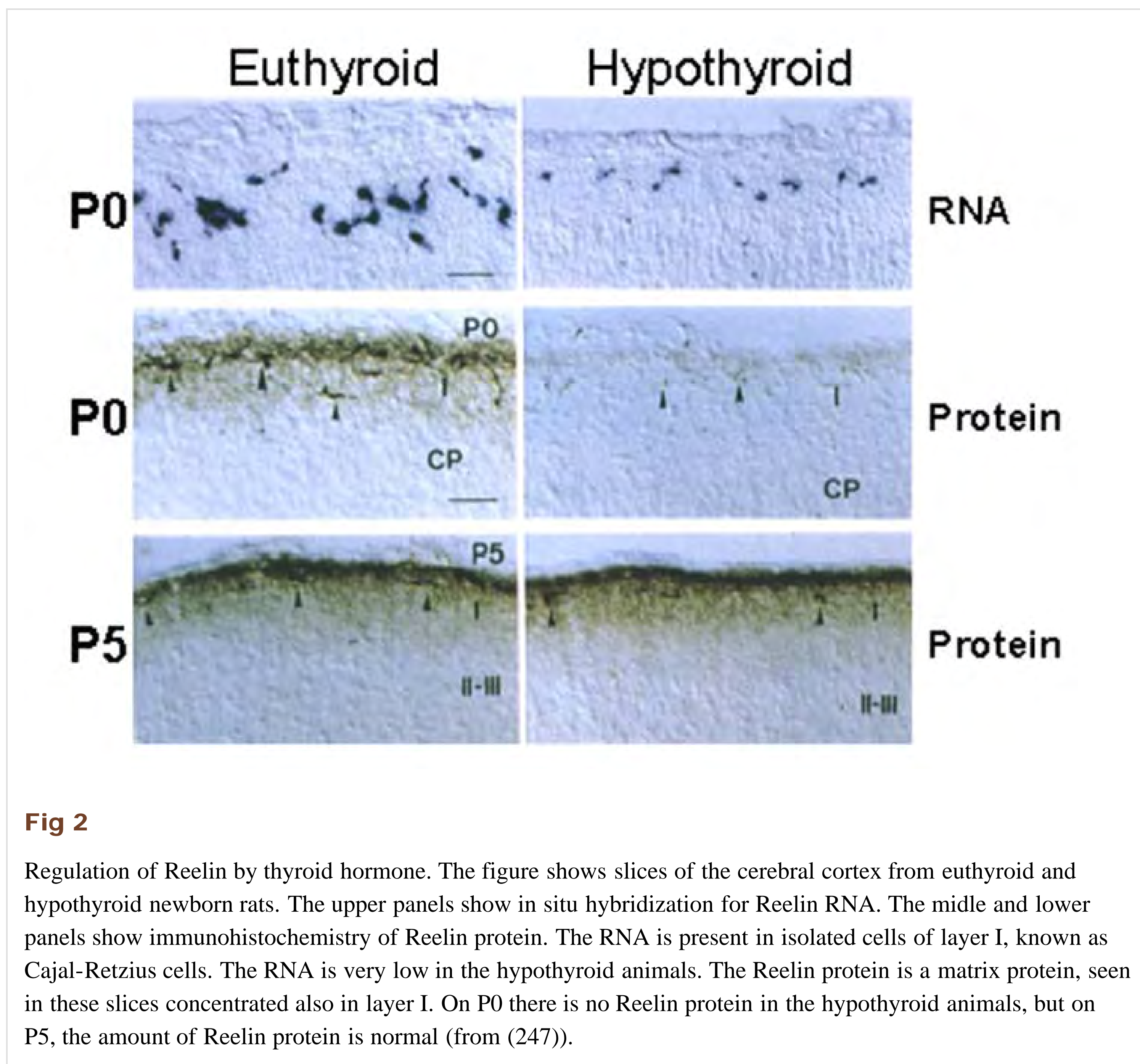


Fig 2

Regulation of Reelin by thyroid hormone. The figure shows slices of the cerebral cortex from euthyroid and hypothyroid newborn rats. The upper panels show in situ hybridization for Reelin RNA. The midle and lower panels show immunohistochemistry of Reelin protein. The RNA is present in isolated cells of layer I, known as Cajal-Retzius cells. The RNA is very low in the hypothyroid animals. The Reelin protein is a matrix protein, seen in these slices concentrated also in layer I. On P0 there is no Reelin protein in the hypothyroid animals, but on P5, the amount of Reelin protein is normal (from (247)).

Neuronal migration in the cerebral cortex is extremely sensitive to thyroid hormones, and even minor deficiencies are associated with migration defects. For example, transient maternal hypothyroidism in pregnant rats at embryonic days 12 to 15 caused significant misplacement of cells in the neocortex and hippocampus of the offspring when analyzed at 40 days of age, associated with audiogenic seizures (54). Moderate thyroid hormone deficiency during pregnancy caused neuronal ectopias in the corpus callosum (55).

The cerebellum in rodents is a sensitive target organ of thyroid hormones (56,57). Thyroid hormones are involved in the late phase of granular cell migration from the external germinal layer (EGL) to the internal granular layer (IGL). A characteristic feature of the hypothyroid cerebellum is a delay in the migration of granule cells so that the EGL persists beyond P20 (Fig 1) (58). This process takes place postnatally in rodents and is completed by P20, when the EGL disappears. Recognized cellular targets of thyroid hormone are the Purkinje cells and the Bergman glia (57). Proliferating granular cells migrate through the molecular layer, along the fibers of Bergmann glial cells, when they exit the cell cycle. The main factor for this control is Sonic Hedgehog (Shh), produced by the Purkinje cells. The encoding gene, *Shh* is a transcriptional target of T3 (59,42).

Myelination

Hypothyroidism causes delayed and poor deposition of myelin (60-62) whereas hyperthyroidism accelerates myelination (63). After prolonged neonatal hypothyroidism, the number of myelinated axons in adult rats is abnormally low in hypothyroid animals although most of the myelinated axons appear to have a normal thickness of the myelin sheath (Fig 3). Thyroid hormones exert important effects on differentiation of oligodendrocytes, the cells that produce myelin. During development, hypothyroidism delays oligodendrocyte differentiation and myelin gene expression, eventually becoming normal even in the absence of thyroid hormone treatment. However, the myelination defect remains in adult animals, although oligodendrocytes are not targets of thyroid hormones in the adult. It is likely that one additional factor mediating the effects of thyroid hormone on myelination is through effects on axonal maturation. Axonal maturation is impaired in hypothyroidism (21,49), and the lower diameter of axons in hypothyroid animals would prevent many axons from reaching the critical size to become myelinated (64).

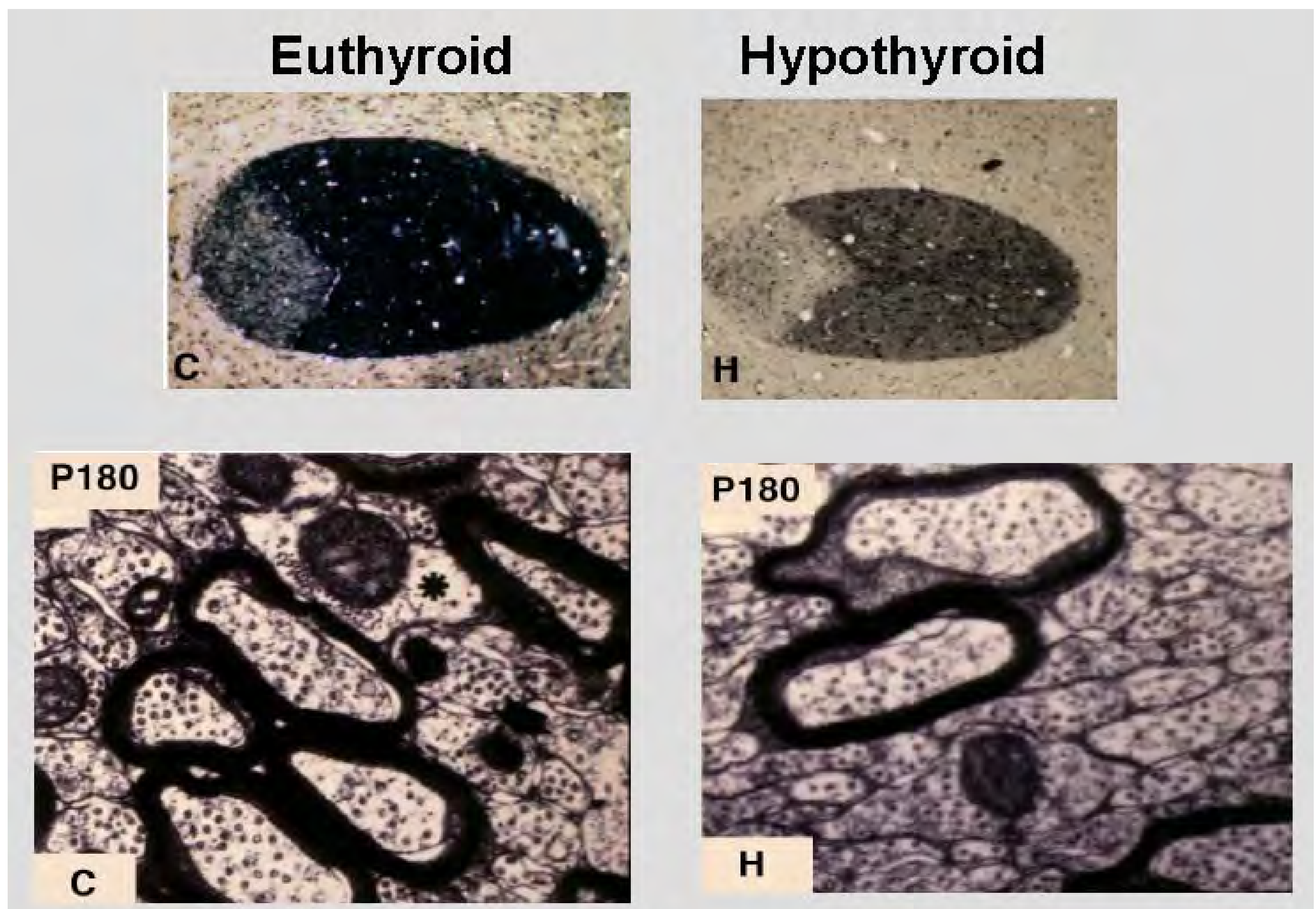


Fig 3

Myelination in the anterior commisure of euthyroid and hypothyroid rats. Hypothyroidism was produced during the neonatal period, and the rats were analyzed at 6 months of age. He aupper panels show transversal section of the anterior commisure stained for myelin. The lower panels show electro microscopy analysis. The number of myelinated axons is reduced in the hypothyroid rats in parallel to an increased number of small diameter axons. Those axons reaching a critical size have near normal myelin content, but still present structural defects (From (49)).

THYROID HORMONES IN THE BRAIN

The concentrations of T4 and T3 in the brain are controlled by very efficient regulatory mechanisms involving thyroidal secretion, transport to the brain, expression of deiodinases and, in the fetus, transplacental passage of thyroxine . T3 equilibrates rapidly between the plasma, liver, or kidney pools, whereas the brain pool equilibrates more slowly. If T3 alone is administered to adult hypothyroid rats as a constant infusion, the liver and kidney T3 concentrations are normalized with lower T3 doses than required by the brain. When T4 alone is administered, brain T3 is normalized at doses of T4 that result in relatively low T3 concentrations in plasma or liver (65,66). Administration of a constant infusion of T4 instead of T3 also maintains brain T3 concentration within a narrow physiological range, relatively independent of the T4 dose, thus avoiding T3 excess. These observations illustrate the importance of regulated local T4 deiodination in the control of T3 concentration in brain.

Sources of Thyroid Hormones for the Fetus

At least in the rat, the fetal brain is impermeable to T3, and all brain T3 derives from T4 (Fig 4). Maternal T4 crosses the placenta, and the fetal blood-brain barrier (BBB), generating T3 locally in the fetal brain. Maternal T3 also crosses the placenta and reaches most fetal tissues, but not the brain (67,68). The reason why the rat fetal brain is not permeable to T3 is unknown, and is not due to low expression of the Mct8 transporter (68). In fetal rats most brain T3 derives from T4, but in postnatal and adult rats, the brain T3 derives in part from the blood and in part from local T4 deiodination. In the absence of Dio2 (see next section) brain T3 is about 50% of normal (69), indicating that each source contribute nearly equally to the brain T3 content (70).

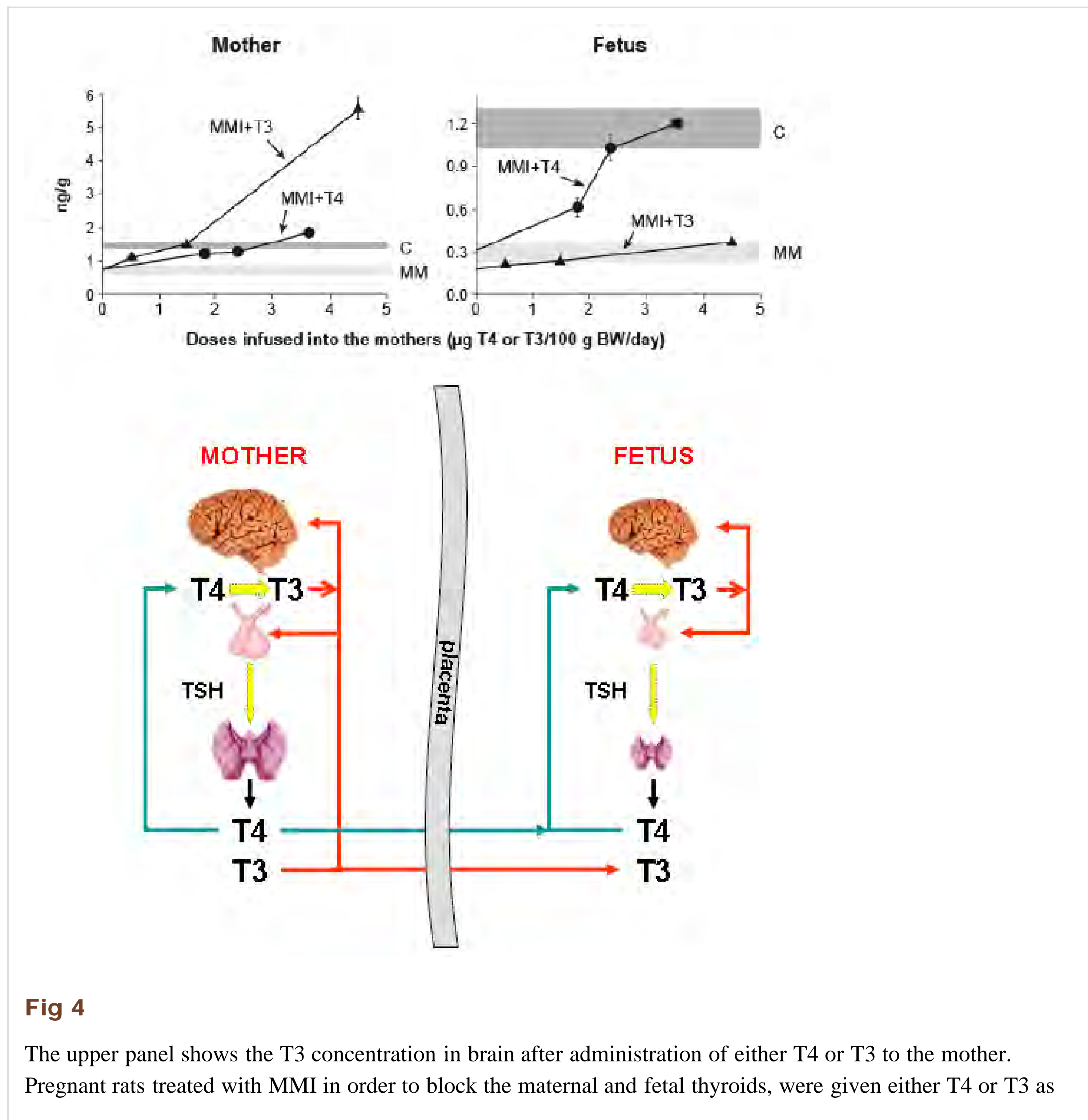


Fig 4

The upper panel shows the T3 concentration in brain after administration of either T4 or T3 to the mother. Pregnant rats treated with MMI in order to block the maternal and fetal thyroids, were given either T4 or T3 as

continuous infusion. T3 concentrations were measured in the brains of the mothers and the fetuses at term. In the mother, increasing doses of T3 led to a proportional accumulation of T3 in the brain. The normal T3 concentration was reached only by a narrow range of T3 doses. Administration of T4 led to a more flat accumulation of T3, and the normal T3 concentration was achieved through a wide range of T4 doses. On the other hand, ddministration of T3 failed to increase T3 concentration in the fetal brain, whereas administration of T4 efficiently normalized fetal brain T3 concentrations (From (67)). The cartoon in the lower panel offers an explanation for these results. In the mother, brain T3 derives partially from circulating T3 and from T4 deiodination. Circulating T4 and T3 in the fetus derive from the fetal thyroid gland and from the mother. However, brain T3 is exclusively a product of T4 deiodination.

Before onset of thyroid function the only source of fetal thyroid hormone is the maternal thyroid gland. Thyroid hormones are present in the rat embryos as early as 3 days after implantation and in the fetus well before onset of fetal thyroid gland function (71-76). During fetal development the proportion of hormone in the fetus originating in the fetal gland increases, and that of maternal origin decreases, but in the rat at term maternal T4 still accounts for about 17.5% of the fetal extra thyroidal thyroxine pool (77). In humans T4 is already present in the coelomic fluid bathing the yolk sack, as early as the 6th gestational week (78). In the fetal brain, T4 and T3 are present in significant amounts by the 10th week after conception (79). At term, about 30-50% of T4 present in neonates represents the maternal contribution (80).

Expression and Regional Distribution of Deiodinases

The predominant deiodinases present in brain are Dio2 and Dio3, products of the *Dio2* and *Dio3* genes respectively (81). Dio1 activity is prominent in cerebellum (82). Deiodinases are membrane-anchored proteins (81,83). Dio2 is localized to the endocplasmic reticulum with the catalytic site exposed to the lumen. Dio1 and Dio3 are anchored to the plasma membrane with the catalytic site exposed to the intracellular compartment. Early evidence suggested that Dio3 in the plasma membrane was oriented with its catalytic site exposed to the extracellular fluid (84) having easy access to extracellular iodothyronines. If this was so, then the high abundance of Dio3 in the fetal brain would provide a rapid mechanism of inactivation for the T3 entering directly the brain parenchima from the circulation, explaining the apparent impermeability of the fetal brain to T3 (68). However there is evidence that iodothyronines need to be internalized in the cell in order to act as Dio3 substrates (85), indicating that the catalytic site is oriented towards the interior of the cell or that there is a rapid internalization of Dio3 into endosomes (84).

Dio2 and *Dio3* are expressed in different cells, *Dio2* in astrocytes and *Dio3* in neurons. Astrocytes generate the active T3 from T4, whereas neurons degrade T4 and T3 to rT3 and T2, respectively. In some cellular contexts the balance between Dio2 and Dio3 activities is regulated by Shh, which increases Dio2 degradation and induces Dio3 expression (86,87). This significance of this pathway for the developing brain is unknown.

Type 2 Deiodinase in Brain

In *Dio2*-expressing tissues, such as brain, brown adipose tissue, and pituitary, 50% or more of T3 derives from local T4 deiodination (88,89). In the adult rat brain as much as 80% of nuclear bound T3 is formed locally from T4 (90). Dio2 activity in the rat fetal brain increases markedly by the end of pregnancy (91,92), with a parallel 18-fold increase of brain T3 despite little changes in the plasma (92). Dio2 activity increases in hypothyroidism, and is very sensitive to the administration of T4 (93). In iodine deficiency the increased Dio2 activity tends to maintain T3 concentrations normal despite greatly reduced T4 concentrations in plasma and brain (94). T4 inhbitis Dio2 activity by a non-genomic action at the post-translational level involving the actin cytoskeleton (95-97) and the ubiquitin-

proteasome pathway (98,99). Dio2 is also regulated at the mRNA level, but less importantly (100-103).

Strongest *Dio2* expression in the brain (Fig 5) occurs in the hypothalamic cells known as tanycytes (104), which were mentioned briefly in the section on Neurogenesis. These cells partially line the walls of the third ventricle (103-105) and extend long processes to the adjacent hypothalamus and the median eminence (106) ending in capillaries and axon terminals. The tanycytes express the Mct8 and Oatp1c1 transporters (see below) and therefore have the capacity to take up T4 and produce T3. T3 formed in tanycytes could be released to the CSF and reach other brain regions by diffusion, or it might be delivered to hypothalamic nuclei directly from the tanycytes processes. It is likely that T3 reaches in this way the paraventricular nucleus (PVN) where T3 regulates TRH production but does not express the *Dio2* gene (105). T3 produced by tanycytes could also participate in anterior pituitary regulation after delivery to the portal vessels (107). Activation of the *Dio2* gene in the basal hypothalamus is involved in photoperiods and seasonal breeding in birds and mammals (108,109).

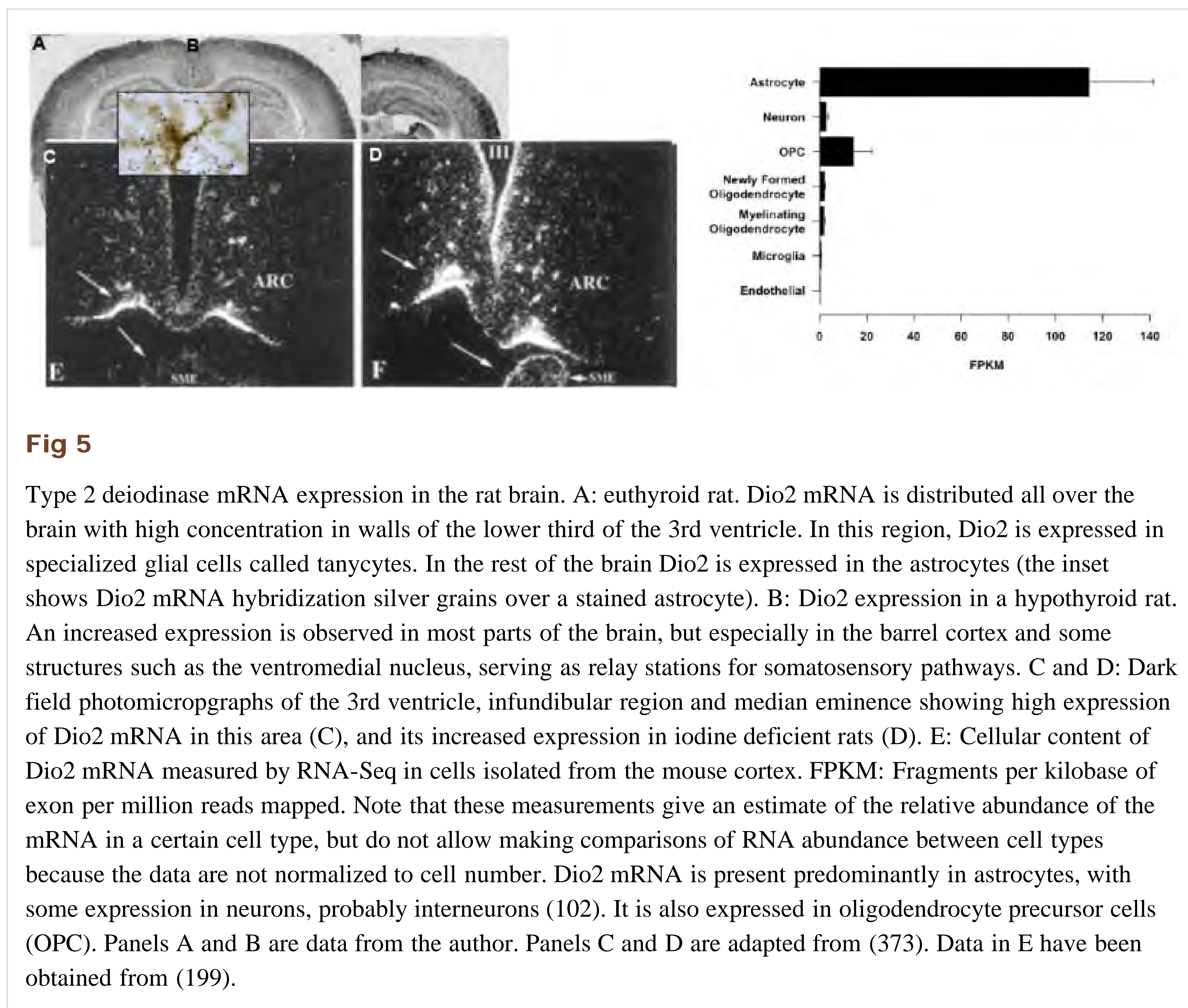


Fig 5

Type 2 deiodinase mRNA expression in the rat brain. A: euthyroid rat. Dio2 mRNA is distributed all over the brain with high concentration in walls of the lower third of the 3rd ventricle. In this region, Dio2 is expressed in specialized glial cells called tanycytes. In the rest of the brain Dio2 is expressed in the astrocytes (the inset shows Dio2 mRNA hybridization silver grains over a stained astrocyte). B: Dio2 expression in a hypothyroid rat. An increased expression is observed in most parts of the brain, but especially in the barrel cortex and some structures such as the ventromedial nucleus, serving as relay stations for somatosensory pathways. C and D: Dark field photomicropgraphs of the 3rd ventricle, infundibular region and median eminence showing high expression of Dio2 mRNA in this area (C), and its increased expression in iodine deficient rats (D). E: Cellular content of Dio2 mRNA measured by RNA-Seq in cells isolated from the mouse cortex. FPKM: Fragments per kilobase of exon per million reads mapped. Note that these measurements give an estimate of the relative abundance of the mRNA in a certain cell type, but do not allow making comparisons of RNA abundance between cell types because the data are not normalized to cell number. Dio2 mRNA is present predominantly in astrocytes, with some expression in neurons, probably interneurons (102). It is also expressed in oligodendrocyte precursor cells (OPC). Panels A and B are data from the author. Panels C and D are adapted from (373). Data in E have been obtained from (199).

Dio2 is expressed in astrocytes throughout the brain (102,105). Also the human *DIO2* is expressed in astrocytes and tanycytes (110). Actually *Dio2* has been included within one of the top 50 genes enriched in astrocytes (111). *Dio2*

is also present in oligodendrocyte precursor cells (Fig 5). Neurons in general express very ittle or no *Dio2* but some interneurons do, especially under hypothyroid conditions (102). *Dio2* KO mice (69) have reduced T3 concentrations in brain, similar to those of hypothyroid mice. The *Dio2* KO mice have minimal neurological impairment at young ages, and tests of locomotion, memory, anxiety, etc. are mostly normal. In contrast, adult *Dio2* KO mice manifest a severe motor impairment of central or muscle origin (82). In the earliest studies on *Dio2* knock out mice it was realized that expression of some T3 responsive genes, such as *Nrgn* (RC3/Neurogranin) was not altered in the absence of Dio2, despite having similar brain T3 concentrations as the hypothyroid mice. The cause for this discrepancy is not known, but the possibility of different roles for the T3 generated locally from T4, and the T3 reaching the neurons directly from the blood, is very intriguing. In fact, we have recently found that in the absence of Dio2, when all brain T3 derives from the circulation, expression of most positively regulated genes in the cerebral cortex is unaffected. In contrast, many negatively regulated genes show an expression level similar to hypothyroid animals. (112,113). It may be that more T3 is needed intracellularly for gene repression than for gene induction, and that the extra T3 needed is provided by Dio2 activity. Double *Dio1*/*Dio2* knock out mice also have minimal neurological phenotype (114).

Type 3 Deiodinase in Brain

Dio3 inactivates thyroid hormones through inner ring deiodination (114). Dio3 activity is highest in placenta and in fetal tissues and decreases after birth (91,115-117). In the human placenta, Dio3 activity is 200 times higher than Dio2 activity at all gestational ages (118,119). Although in cultured astrocytes *Dio3* expression can be induced by growth factors (120-122), *in vivo* it is expressed in neurons (123,124).

Dio3 plays an important role in the control of T3 concentration in developing tissues. For example, in metamorphosis Dio3 is negatively correlated with the responsiveness of tadpole tissues to thyroid hormone (125-128). In mammals placental Dio3 controls the transfer of maternal thyroid hormone to the fetus (129). *Dio3* expression is very high in the uterine implantation site and in the epithelial cells of the uterine lumen surrounding the fetal cavity (130). In the newborn rat brain discrete and intense expression of *Dio3* occurs in neurons located in areas involved in sexual differentiation of the brain (123,131), suggesting that these areas need to be protected from a possible interfering action of T3 during critical periods of sexual brain differentiation (132). The genomic response of the brain to T3 is amplified in the absence of Dio3 (113). T3 induces Dio3 at the transcriptional level, and the increased Dio3 activity reduces the effects of T3 on gene expression, and therefore protects the brain from excessive T3 (113). The effect of T3 on the Dio3 gene is selectively mediated through the TRa1 receptor subtype (133,42).

Dio3-deficient mice (134,135) have profound alterations in thyroid hormone economy, with greatly elevated thyroid hormone concentrations during the perinatal period. This hypermetabolic situation progresses into a state of central hypothyroidism, maintained through adulthood. In the brain, the expression of thyroid hormone target genes in the *Dio3* KO is elevated during the early postnatal period, and then decreases, in parallel to the thyroid state (136).

THYROID HORMONE TRANSPORT TO THE BRAIN

The Brain Barriers

The passage of substances from blood to brain is restricted by the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (blood-CSF) barrier (137) (Fig 6). The BBB is formed by the endothelial cells of brain capillaries which are apposed by tight junctions, so that substances must leave the circulation and enter the brain parenchyma through transcellular transport. Surrounding the brain capillaries are the astrocytic end-feet. It has been proposed that the astrocyte membranes do not cover completely the capillary surface, leaving patches of capillary wall in contact

with the interstitial fluid (138). Other studies show that the astrocytes cover completely the capillaries, leaving only narrow spaces for free diffusion (139). In any case T4 and T3 transported through the BBB could reach the astrocytes through their end-feet or be delivered directly to the interstitial fluid through the spaces between the astrocytes, as it happens for glucose (140).

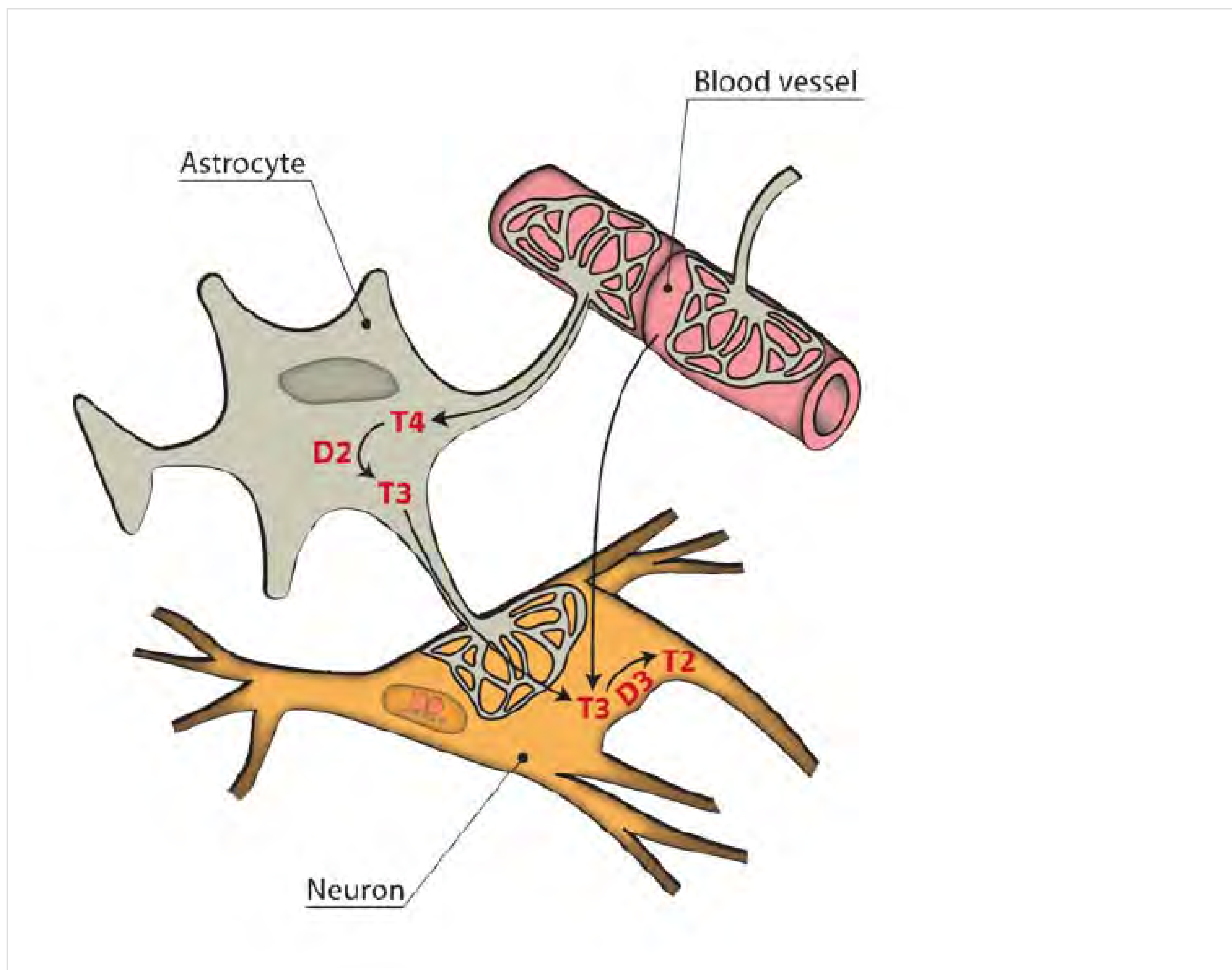


Fig 6

Entry of thyroid hormone to the brain through the blood-brain barrier (BBB). T3 can reach the target neurons via two ways. One is from the astrocyte, after 5'deiodination of the T4 taken up from the blood through the astrocyte end-feet. T3 can also reach the neurons directly from the extracellular matrix after crossing the BBB. In the neurons, T3 is degraded to T2 by D3. The end-feet of the astrocytes may be fenestrated, leaving part of the external capillary surface, in direct contact with the extracellular matrix.

The blood-CSF barrier is formed by the epithelial cells lining the ventricular side of the choroid plexus. Administration of labelled T4 or T3 leads to a rapid labelling of the choroid plexus and the appearance of the hormones in the CSF (141). Thyroid hormones are present in the CSF at less than ten times the concentration in serum (142-144), but the free hormone fraction is several fold higher in the CSF due to the low protein

concentration, so that the free hormone concentrations in the CSF (77 pM FT4 and 0.2 pM F T3) are of the same order as in serum (28 pM FT4 and 0.3 pM FT3) (145). It was suggested that transthyretin (TTR), the major protein of the CSF in many species, plays a role in T4 transport in the choroid plexus (146,147). However, the T4 transfer rate from plasma to tissue compartments, including the brain, is normal in transthyretin null-mutant mice (148,149).

The bulk of hormone that reaches most brain structures does it through the capillaries in the parenchyma. The fraction of brain thyroid hormone that is transported through the choroid plexus and the CSF has been estimated to be around 20% (150). Labelled T4 injected directly into the CSF accumulates mainly in the median eminence (151,152).

Cellular Transporters for Thyroid Hormone

The transport of thyroid hormone through the plasma cell membrane is facilitated by several classes of transporter proteins (153-159). The discovery that mutations in one of these transporters, the monocarboxylate transporter 8 (MCT8) cause a syndrome of severe neurological impairment and thyroid hormone abnormalities, provided a definitive proof for their pathophysiological relevance (160,161). The membrane transporters for thyroid hormones belong to several families including the monocarboxylate (MCT) transporters MCT8 and MCT10 (*SLC16A2* and *SLC16A10* genes), the organic anion transporter polypeptides (OATP, especially OATP1C1, *SLCO1C1* gene), the large neutral amino acid transporters (LAT-1 and LAT-2, products of the *SLC7A5* and *SLC7A8* genes), and the sodium/taurocholate cotransporting polypeptide (SLC10A1, or NTCP). MCT8 and MCT10 are specific for the transport of iodothyronines, with slightly higher affinity for T3 than for T4 (85,162). MCT10 is a transporter for aromatic amino acids (tryptophan, tyrosine, phenylalanine), and also mediates the influx and efflux of thyroid hormones. MCT10 appears to have little relevance for thyroid hormone transport in the brain (163).

The thyroid hormone transporters have a wide range of tissue distribution, with overlapping patterns of expression for most of them (153,164,159). In human and rodents MCT8 is strongly expressed in the endothelial cells of the brain microvessels forming the BBB, and in the apical border of the epithelial cells of choroid plexus (68,165). It is also expressed in the membrane of astrocytes, tanycytes, neurons, and oligodendrocyte precursors (159) (Fig 7). Oatp1c1 is a T4 transporter, and differential expression of Mct8 and Oatp1c1 is important to understand differential T4 and T3 transport through the BBB (Fig 7) (165,166). Mct8, which transports T4 and T3, is expressed in the endothelial cells of the BBB and in astrocytes, neurons, and oligodendrocyte precursors. It is also expressed in newly formed oligodendrocytes. Oatp1c1, a T4 transporter, is expressed preferentially in the the endothelial cells of the BBB and in the astrocytes. Mct8 and Oatp1c1 are also expressed in the choroid plexus (68,165). As mentioned earlier (Fig 5) *Dio2* is expressed mainly in astrocytes. The data on transporter and deiodinase expression, together with observations on T3 and T4 action on gene expression in trabsporter knock outs permits the anatomical and functional integration of transport and deiodination, as described in the next paragraph.

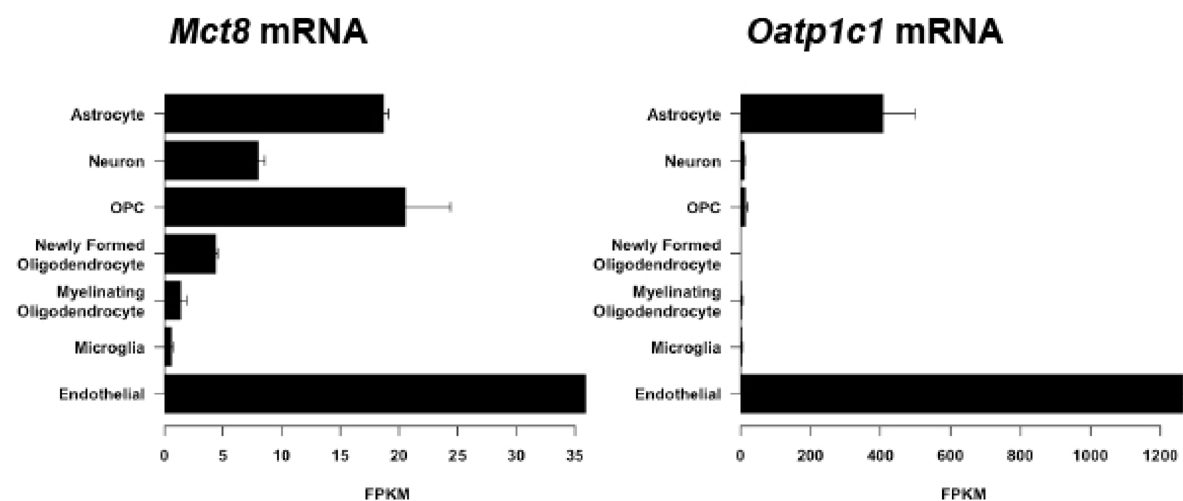


Fig 7

Content of Mct8 and Oatp1c1 mRNAs in native cells isolated from the postnatal mouse cerebral cortex, measured by RNA-Seq. The data are from the transcriptomic database of Zhang et al (199). Mct8 is expressed in astrocytes, neurons, oligodendrocyte precursor cells (OPC), and endothelial cells. It is also expressed in newly formed oligodendrocytes.Oatp1c1 is expressed in astrocytes and endothelial cells. FPKM: Fragments per kilobase of exon per million reads mapped. Note that these measurements give an estimate of the abundance of the mRNA in a certain cell type, but do not allow making comparisons of RNA abundance between cell types beceause the data have not been normalized by ell number.

Integration Between Transport and Deiodination in the Regulation of Thyroid Hormone Action in the Brain.

Specific expression of *Dio2* in astrocytes led to the hypothesis that in the brain T4 was converted to T3 in the astrocytes and then delivered to neurons in response to local needs (105). This hypothesis was later refined by the realization that Dio3 is present in the neurons (124), thereby providing a control of neuronal T3 through degradation of excess T3. It was later demonstrated in vitro using a co-culture assay that T3 produced in astrocytes could influence neuronal gene expression (167). Proof that this mechanism indeed operates in vivo was recently provided by studies on *Mct8* KO mice (166). These mice have a selective restriction of T3 accumulation in the brain, but T4 accumulation is normal (168,169). Furthernore, the lack of Mct8 impaired the action of exogenous T3 on brain gene expression, but not the action of T4, with the result that brain gene expression in the Mct8 KO is mostly normal (112). All the observations together permit to formulate the hypothesis that in the absence of Mct8 T4 crosses the BBB and the astrocytic membrane through Oatp1c1, producing T3 locally. Proof that the transporter is Oatp1c1 is that the Mct8 and Oatp1c1 double KO have a hypothyroid pattern of gene expression (170). Proof for the involvement of Dio2 is that also the Mct8 and Dio2 double KO produces a near hypothyroid pattern of gene expression (112). The astrocyte-neuron coupling involved in thyroid hormone transport, metabolism, and action is reminiscent of other forms of coupling between glia and neurons (140) such as glucose metabolism, or in the generation of glutamate and GABA. Another example of paracrine interaction is the cochlea, where Dio2 is present in the connective tissue (171) whereas the T3 receptor is expressed in the sensory epithelium and spiral ganglion (171-173).

THYROID HORMONE RECEPTORS IN THE BRAIN

A full description on thyroid hormone receptors (TR) can be found in the chapter on Cellular Actions of Thyroid Hormones by R. Sinha and P.M. Yen. A recent discussion on the relative roles of the TR isoforms, with special reference to the brain using mouse genetic models, has been published by Flamant and Gauthier (174). To understand the role of the receptors in the brain knowledge of their regional and developmental pattern of expression is needed. In rats the nuclear thyroid hormone receptor protein, measured by T3 binding assays, is present in brain at embryonic day 13.5-14 i.e., several days before onset of thyroid gland function (175) (Fig 8). The receptor increases subsequently and reaches a maximum on postnatal day 6 (176-178). Total brain receptor occupancy by the hormone increases in parallel with plasma and cytosol total and free T3 with a maximum of 50-60% on postnatal day 15 (179). At the mRNA level, all receptor isoforms are expressed in the brain. The predominant TR isoform is TRa1, widely distributed in the CNS from E14 to adulthood (180-182). From E19 to P0, TRa1 is present in the outer part of the cerebral cortex and hippocampal CA1 field. During the late fetal stage TRa1 becomes expressed in the piriform cortex, superior colliculus, and pyramidal layer of the hippocampus, and in the granular layer of dentate gyrus. In adult rats TRa1 expression is prominent in the cerebral cortex, cerebellum, hippocampus, striatum and olfactory bulb. The pattern of TRb1 expression during development is different than that of TRa1, with restricted low expression during the fetal period, and increasing during the postnatal period and through adulthood. TRb1 mRNA can be detected at E15.5 in the upper tegmental neuroepithelium. Between E17 and E20 only low levels are present in the brain, especially in the pyramidal layer of the hippocampus. On P0 a drastic increase occurs in the accumbens, striatum, and hippocampus. From around P7 TRb1 becomes also expressed in the cerebral cortex. The patterns of expression of TRb1 and TRb1 overlap (Fig 9), but in some cells, one of the isoforms is expressed preferentially. For example, in the cerebellum, differentiated granular cells express TRa1 while Purkinje cells express TRb1. Recent studies by Vennström and coworkers (183) have shown that TRa1 is expressed essentially in all postmitotic neurons, with the notable exception of Purkinje cells, and in a few glial cell types.

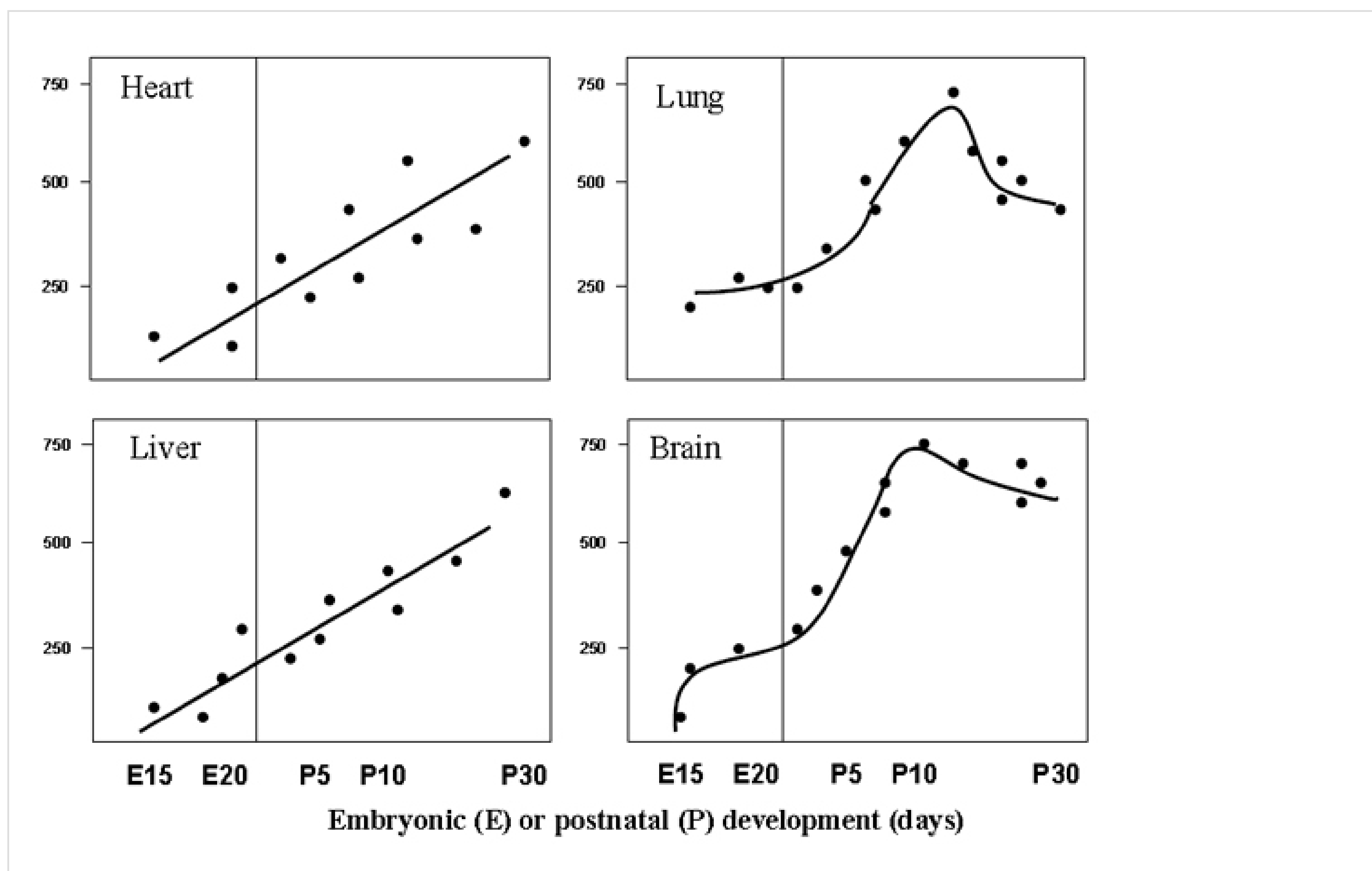


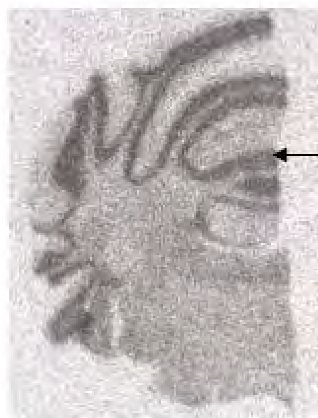
Fig 8

Ontogeny of T3 receptors in rat organs. Receptor concentration was measured by T3 binding assays. From (175).

TR expression in mouse brain

TRα1

TRβ1



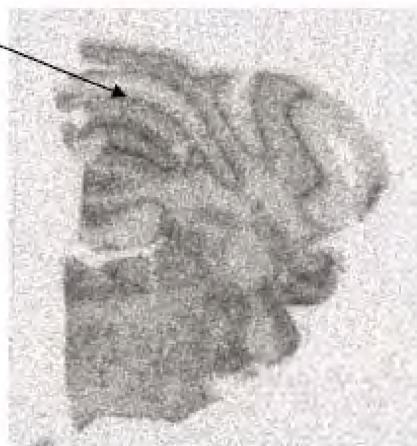


Fig 9

T3 receptor mRNA expression in the mouse brain, by in situ hybridization with TRα1 and TRβ1-specific probes. In the cerebrum (upper panels) there is an overlapping distribution of both receptor subtypes, with some differences in the hippocampus, amygdala ans hypothalamus. In the cerebellum (lower panel) TRα1 is expressed in the granular layer (GC), whereas TRβ1 is expressed in the Purkinje cell layer (PC).

As with the mRNA, the predominant receptor protein in the brain is TRa1, which accounts for about 70-80% of all receptor subtypes. In addition to TRa1 and TRb1, TRb2 isoform, initially considered to be pituitary-specific (184), was later detected by *in situ* hybridization in several regions of the brain such as the rostral caudate, the hippocampus and the hypothalamus (181). Actually the TRb2 protein contributes by about 10 % to the total receptor

protein in different tissues, including the brain (185). By immunohistochemistry the TRb2 protein was found widely distributed in the brain, in regions where the mRNA cannot be detected, such as layers II-VI of the cerebral cortex, and Purkinje cells of the cerebellum (186). Quantitative studies on the concentrations of TR mRNAs and proteins in different tissues were carried out by Ercan-Fang et al (Table 1) (187).

Table 1 TR isoform protein and mRNA concentrations in rat tissues

	Protein molecules/cell	mRNA molecules/cell
TR1		
Pituitary	2310	0.128
Liver	963	1.11
Brain	2360	16.4
Kidney	626	2.50
Heart	1300	2.70
TR1		
Pituitary	1470	0.873
Liver	3900	5.10
Brain	819	20.2
Kidney	578	13.5
Heart	1460	7.37
TR2		
Pituitary	5240	0.631
Liver	829	<0.00689
Brain	328	<0.00684
Kidney	178	<0.00501
Heart	506	<0.00453

The three TR isoform proteins are present in all tissues. This includes the TR2 isoform, despite that the corresponding mRNA is only detectable in pituitary. The concentrations of receptor protein were measured by specific immunoprecipitation of labelled T3 after incubation with tissue nuclear extract. The concentrations of mRNA were measured in northern blots using quantitative procedures. Data from (168).

In the human brain, the receptor protein, as measured by T3 binding assays is present at low levels in the fetus around the 10th week postconception (79) and the T3 receptor mRNAs can also be detected during the first trimester (188). The receptor protein concentration increases 10-fold from the 10th to the 16th-18th weeks postconception (Fig 10). During this time the brain gains in weight and DNA content by about 5 fold, so that the total brain T3 receptor content increases 500 times. This coincides with the period of active neuroblast proliferation (189). Of functional relevance, the ligand, T3 is also present in brain from the 10th week of gestation, at enough concentrations to result in about 25% occupancy of receptor (190). At the same time, in other organs such as liver and lung, even with higher receptor concentration, only T4 was present at 18th weeks. This indicates that during the second

trimester of pregnancy, the main source of T3 to the fetal brain is local deiodination of T4 mediated by Dio2. Kester et al (191) showed that T3 increases in the cerebral cortex during the second trimester, in parallel with increased Dio2 activity. Differential activity of deiodinases determines the amounts of T3 present in different regions of the brain. For example in the cerebellum high Dio3 activity keeps T3 concentrations low (Fig 11) at the same time that in the cortex Dio2 activity increases T3 concentration. These data are especially relevant in the context of the pathogenesis of neurological cretinism and for the consequences of maternal hypothyroxinemia on fetal brain development, as discussed in the last sections.

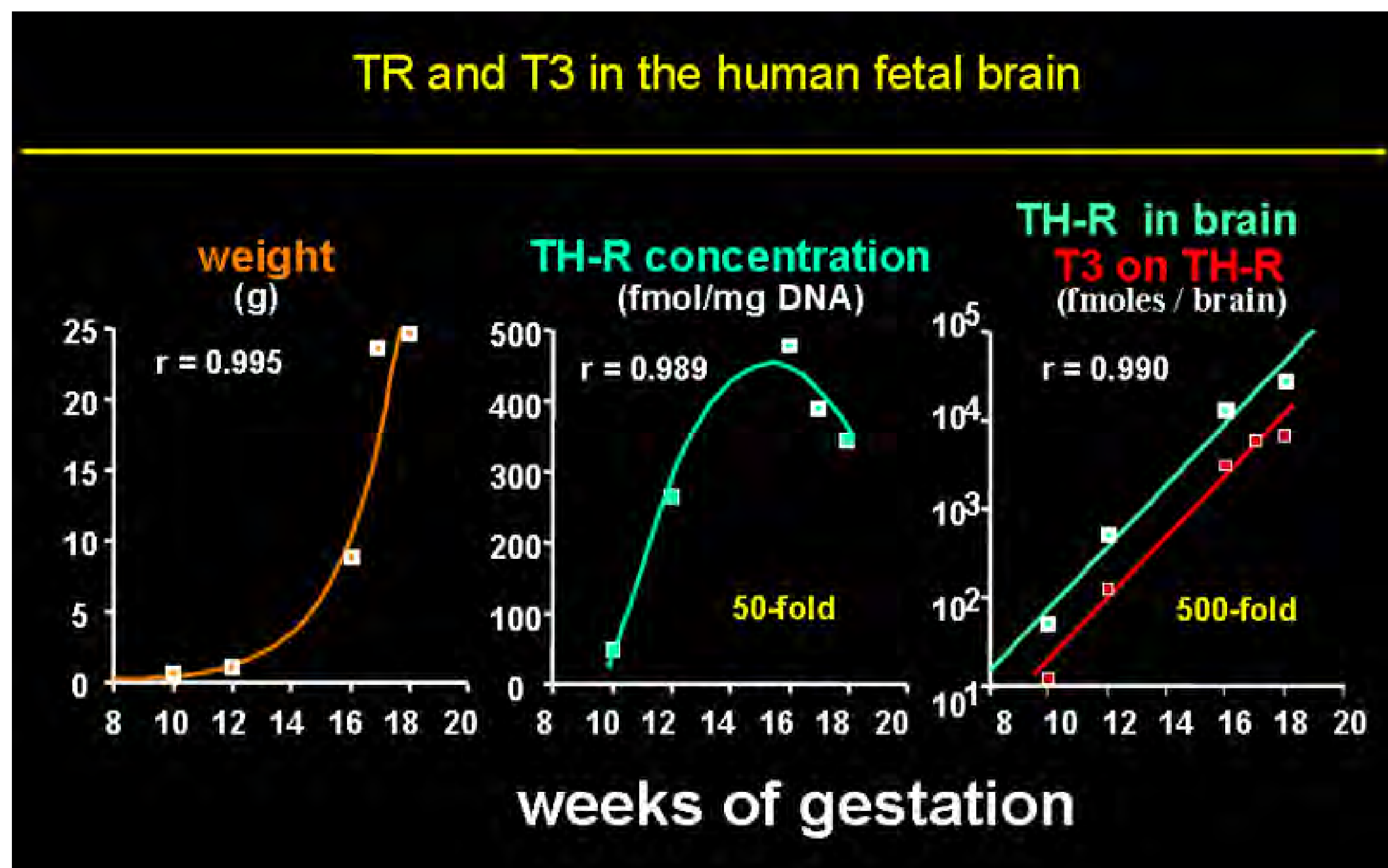


Fig 10

Ontogeny of T3 receptor in the human fetal brain, measured by T3 binding assays. During the period analyzed the brain increases exponentially in weight, due mainly to the wave of neuronal proliferation (left panel). The receptor concentration increases 50-fold from the 10th to the 16th week (middle panel). In addition despite the fact that the fetal thyroid glandis is still not functioning, T3 increases in concentration during the same period, and can be found in the receptor fraction. Accumulation of T3 reflects the increasing activity of Dio2 in the cerebral cortex, as shown in fig 10. From (79).

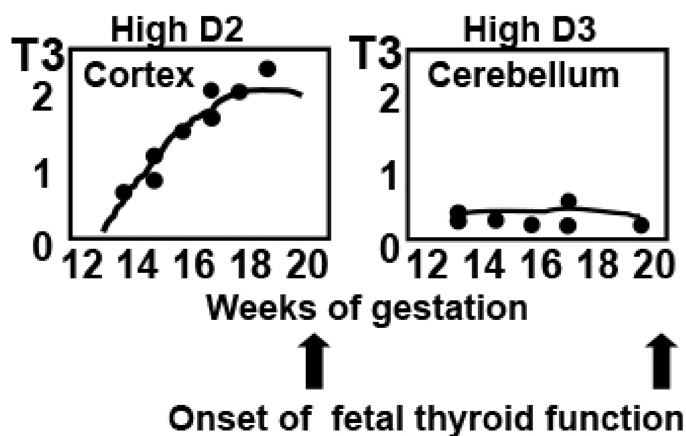


Fig 11

Concentrations of T3 in the fetal cerebral cortex and in the cerebellum. The cortex has high Dio2 activity, whereas the cerebellum has low Dio2 and high Dio3 activity. Accordingly, the coencentrations of T3 rise in the cerebral cortex and rfemain low in the cerebellum, reflecting different timing of T3 action. From (191).

In neural cell cultures, T3 receptors have been detected in neurons, astrocytes and oligodendrocytes (192,193). In dorsal root ganglia, sensory neurons and Schwann cells express T3 receptors (194) but the latter transiently (195). Studies *in vivo* have shown that TR isoforms colocalize with oligodendrocyte markers but not with astrocytic markers (196) and other studies have indicated that in primary culture, rat astrocytes do not express T3 receptor, but only the TRα2 isoform (197,198). As mentioned above, recent studies showed expression of the TRa1-encoding gene in some glial cell types (183). Transcriptomic studies of primary cells isolated from the cerebral cortex allow an estimation of the relative content of TRα1 and TRβ mRNAs. From a recent transcriptomic and splicing database (199) we can estimate the relative amounts of the TR subtypes and isoforms in different neural cells, astrocytes, neurons, oligodendrocyte precursors cells (OPC), newly fomed oligodendrocytes, myelinating oligodendrocytes, microglia, and endothelial cells (Fig 12). The raw data (Fig 12 left panel) shows the abundance of the mRNA products from the *Thra* and *Thrb* genes i.e., *TRα1*, *TRα2*, and *TRβ1*, *TRβ2* mRNAs respectively. We subtracted the contribution of *TRα2* in each cell type from the *Thra* transcripts data we use and arrived at the relative contents of *TRa1* and *TRb* in each cell type shown in the right panel of figure 12. All neural cells contain TRs, but TRα1 is more abundant in oligodendrocytes and neurons, and TRβ in astrocytes

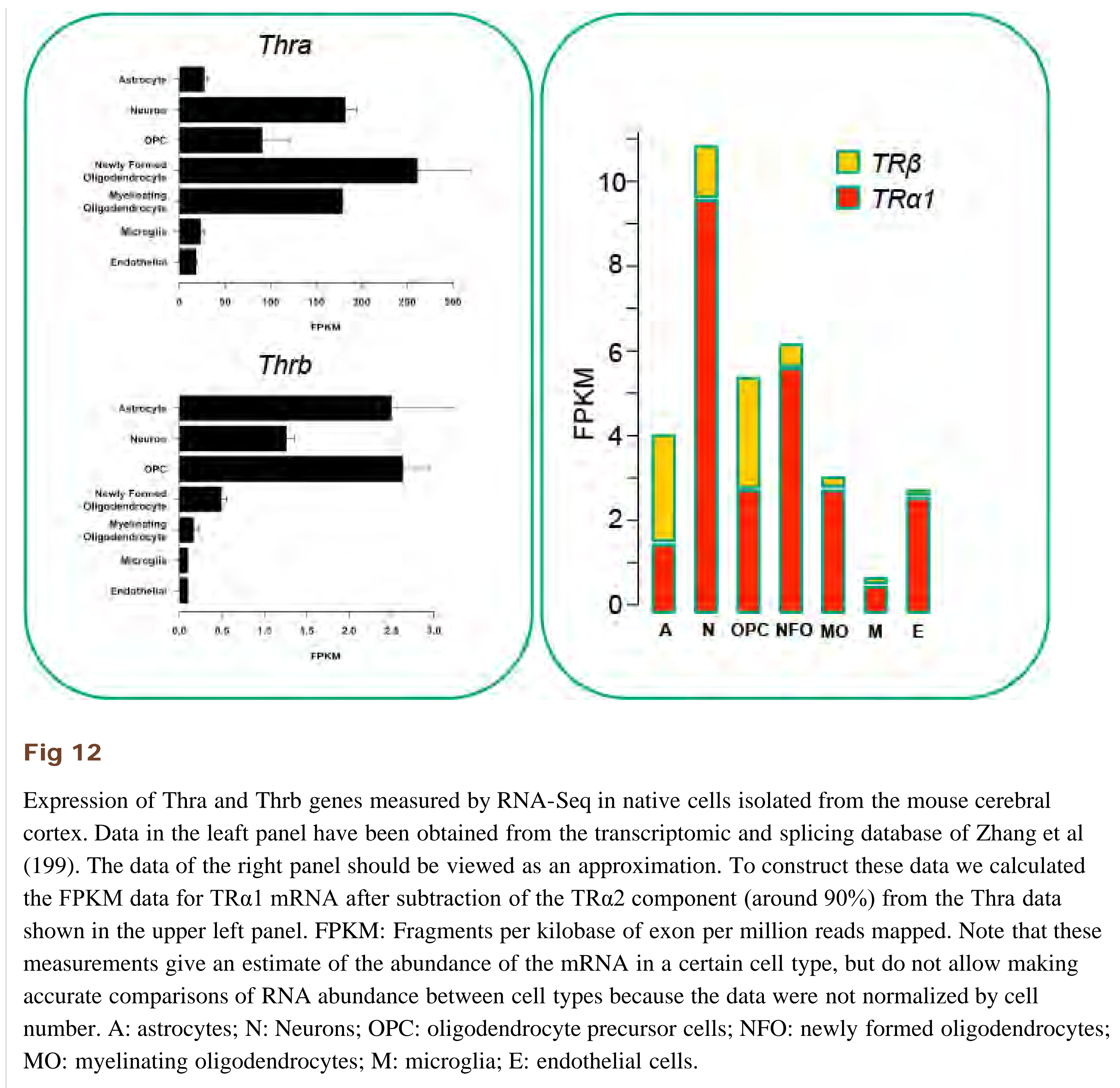


Fig 12

Expression of Thra and Thrb genes measured by RNA-Seq in native cells isolated from the mouse cerebral cortex. Data in the leaft panel have been obtained from the transcriptomic and splicing database of Zhang et al (199). The data of the right panel should be viewed as an approximation. To construct these data we calculated the FPKM data for TRα1 mRNA after subtraction of the TRα2 component (around 90%) from the Thra data shown in the upper left panel. FPKM: Fragments per kilobase of exon per million reads mapped. Note that these measurements give an estimate of the abundance of the mRNA in a certain cell type, but do not allow making accurate comparisons of RNA abundance between cell types because the data were not normalized by cell number. A: astrocytes; N: Neurons; OPC: oligodendrocyte precursor cells; NFO: newly formed oligodendrocytes; MO: myelinating oligodendrocytes; M: microglia; E: endothelial cells.

Both receptor types, TRα1 and TRβ mediate the actions of T3 in brain, but receptor-specific actions can be demonstrated on some genes (42,200,201). *Dio3* for example is selective of TRα1 (42,133), and other TRα- and TRβ-selective genes have been described (201).

MECHANISMS OF THYROID HORMONE ACTION IN THE BRAIN

Most effects of thyroid hormones on developmental processes are carried out through the control of gene expression by the binding of T3 to the nuclear receptors. Also non-genomic actions of T4 and T3 have also been demonstrated. T4 interacts with integrin $\alpha_V\beta_3$ and activates PI3K and MAPK pathways (202-204). We mentioned before the role of T4 in the expansion of progenitors in the neocortex, mediated through interaction with integrin $\alpha_V\beta_3$ (31). T3 interacts with a TRα variant located in the plasma membrane (205) and involved in cGMP signalling. T3 also

interacts with TRβ in the cytoplasm and mediates actions on maturation and plasticity of hippocampal pyramidal neurons through the regulation of PI3K and the Src family Lyn kinase (206). Another non-genomic action of T3 is to regulate the start and duration of the sensitive period of imprinting in chicks (207).

Many thyroid hormone-dependent genes have been identified mostly during the postnatal period. With few exceptions, the role of thyroid hormone is to accelerate the rate of gene expression changes during development. For example, myelin proteins accumulate rapidly in the brain during the first weeks of life in the rat, in parallel to an increased gene expression and RNA accumulation. The role of thyroid hormone is to control the timing and the rate of gene transcription so that accumulation of the gene products occurs at the required time and rate. In the absence of thyroid hormone, mRNA and protein accumulation slows down but their final concentrations in the tissue eventually attain a normal value (see an example of this phenomenon in figure 1). Another property of thyroid hormone regulation of gene expression in the brain is the regional specificity, even for genes regulated at the transcriptional level (208). Transcription of thyroid hormone target genes depends from the combinatorial activity of many transcription factors, and the relative importance of the TR may depend on the specific region and developmental period.

Many of the thyroid hormone-regulated genes identified during the postnatal period in the rat brain are sensitive to the hormone only during a time window that spans the first 2-3 weeks after birth (Fig 13). Most of these genes are not dependent of thyroid hormone in the fetal or in the adult brain. Based on these observations it was thought that the critical period of thyroid hormone sensitivity in the brain is limited to the first 2-3 postnatal weeks in the rat. However, this concept derives from the fact that most searches for thyroid hormone dependent genes in brain have been made during the postnatal period, at the peak of T3 receptor expression and occupancy. Although this period is probably the most sensitive to thyroid hormones in terms of the irreversible consequences of thyroid hormone deprivation, the rat brain is also sensitive, morphologically and functionally, and also in terms of control of gene expression in the fetus and in adult animals. Many genes have been identified regulated by thyroid hormone in brain, both by conventional Molecular Biology approaches, and by microarray analysis (112,209-215).

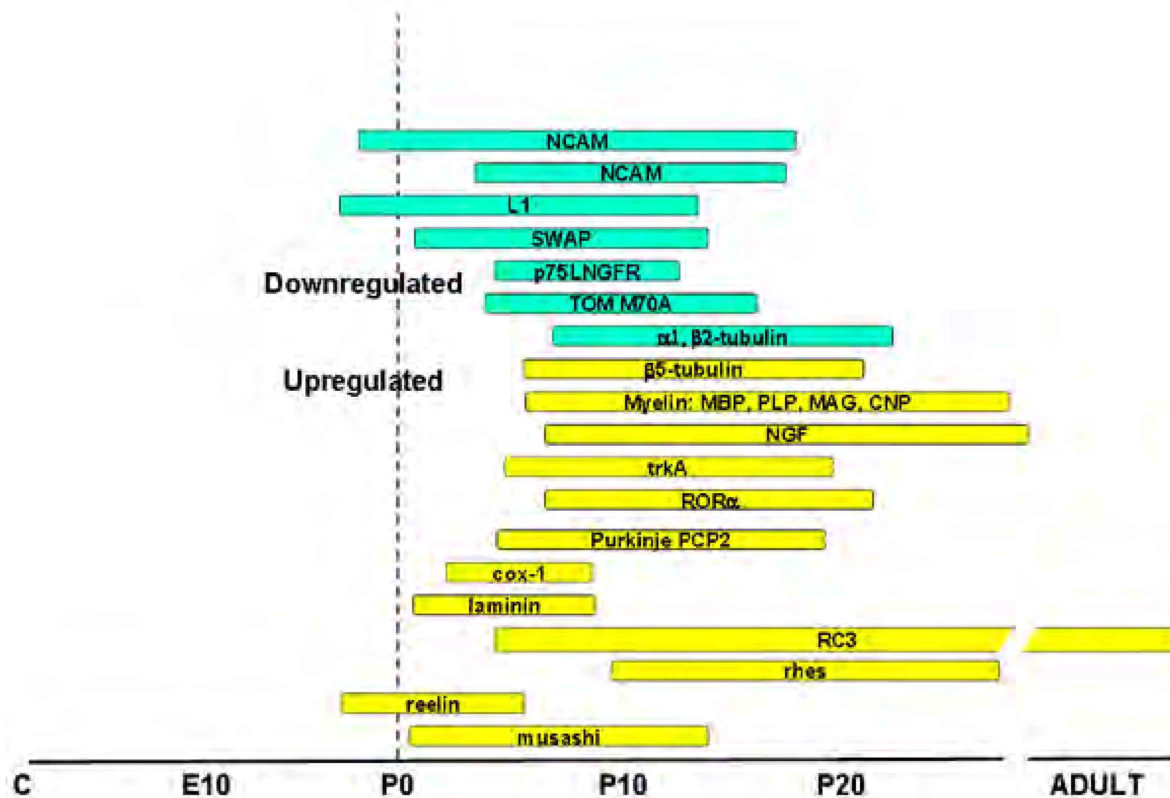


Fig 13

Windows of sensitivity for the regulation of brain gene expression by thyroid hormone during the postnatal period in the rat. A few examples of genes sensitive to thyroid hormone during the postnatal period is shown to illustrate the windows of sensitivity of the regulated genes. Other genes that are sensitive in the fetal period and in the adult are not shown in this figure. The windows of sensitivity of the myelin genes are different for each brain region, so that in the more caudal regions (for example brain stem and cerebellum) the window is earlier than in the frontal regions (cortex).

Chatonnet et al (216) have recently performed a detailed analysis of the published data on thyroid hormone action on gene expression in brain and in cultured cells and arrived at a shortened list of at least 37 genes likely targets of the TRs consistently found in different studies. In our own studies employing RNA-Seq of primary cerebrocortical cells (P. Gil-Ibañez, F. García-García, J. Dopazo, J. Bernal and B. Morte, Cerebral Cortex 2015, submitted), we found that 7.7% of expressed genes was positively or negatively regulated, almost in equal proportions, upon T3 addition. Up to 30% of these genes were still induced or repressed by T3 in the presence of cycloheximide, indicating an effect on transcription. Additionally, a large proportion of these genes contained a T3-responsive element as described by Chatonnet et al (201). It can be estimated that around 2.5% of all expressed genes in the cells were regulated transcriptionally by T3, and that at least 1% of all expressed genes were transcriptionally regulated by T3 thorugh a functional TRE. Examples of genes found regulated transcriptionally by T3 in several independent studies are *Shh*, *Hr*, *Klf9*, *Dbp*, *Gbp3*, and *Nrgn* (216).

There is a great diversity of genes regulatd by T3 in neural cells, and it is not possible to define a hierarchical relationship among the regulated genes. To introduce some order and meaning into this confusing topic it is convenient to examine the characteristics of gene regulation in different developmental periods and biological processes.

Regulation of Brain Gene Expression by Thyroid Hormone during Development and in the Adult

The Fetal Period

Early efforts to demonstrate an effect of thyroid hormones on fetal brain gene expression led to the conclusion that the fetal rat brain at term is not sensitive to thyroid hormones (217). However, regulation of several genes, were described in cultured fetal neurons (218) or in the brain in vivo (219-221). In the fetal cerebral cortex at the end of gestation thyroid hormone controls the expression of genes involved in biogenesis of the cytoskeleton, neuronal migration and growth, and branching of neurites. Interestingly, a large percentage of the thyroid hormone dependent genes were related to Camk4 signalling pathways (222) (223). *Camk4* was regulated directly by T3 in cultured primary neurons, confirming earlier results from other groups (224,225), but is not regulated by T3 in the postnatal rat brain in vivo ((217) and unpublished ovservations).

The Postnatal Period

Most actions of thyroid hormone on brain development have been studied during the postnatal period in the rat (Fig 13). As mentioned above, the first 2-3 weeks of postnatal life is the period of maximal sensitivity of the rat brain to thyroid hormone, in parallel to the postnatal increase in thyroid hormone secretion, brain Dio2 activity, and receptor and T3 concentrations. Important developmental events occur during this period: myelination, development of the cerebellum, the dentate gyrus, and the cochlea, proliferation and accumulation of glia cells, and terminal differentiation of neurons, synapse formation and axon and dendrite sprouting. The corresponding period in the human is from about the 20th week of gestation to the first 2 years. Practically all these events are influenced by thyroid hormone, and many genes involved in these processes whose expression is dependent on the thyroidal status have been identified.

The Adult Brain

In adult subjects thyroid hormones influence mood and behaviour, and thyroid dysfunction affects neurotransmitter systems (226) often leading to psychiatric disorders (227). High doses of T4 are effective in the treatment of bipolar depression (228,229). In the adult rat striatum, administration of a large single T3 dose leads up-regulation of 149 genes and down-regulation of 88 genes (209). Physiological doses of T3 given for several days to hypothyroid animals led to up-regulation of 18 genes, and down-regulation of just one gene. Therefore, acute large doses of thyroid hormone causes large changes in gene expression, with more modest changes with lower doses. Some of the regulated genes are related to circadian rythms and to wakefulness, with one of them (*Dbp* or D-site binding protein) proposed as a candidate gene in bipolar disorders (230), and likely to be regulated directly by TRα1 (215). Many other genes were involved in striatal physiology as components of several signalling pathways (Fig 14).

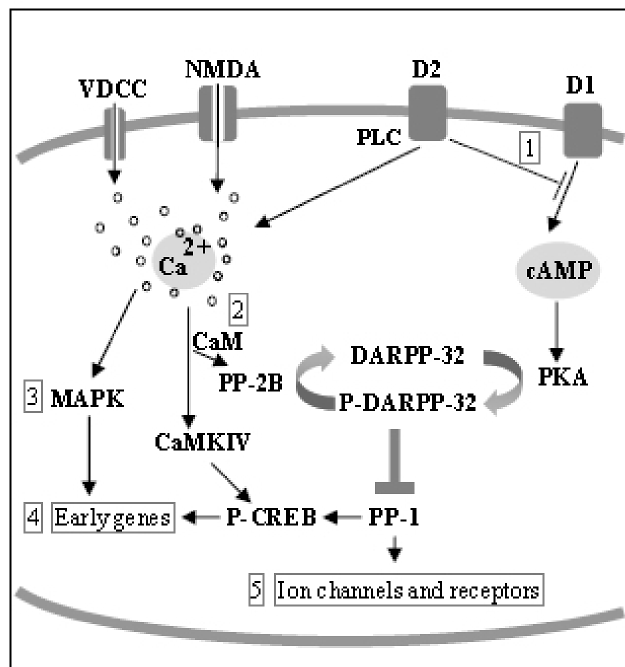


Fig 14

Regulation of gene expression by thyroid hormones in the adult striatum. Signaling pathways are schematically represented and the main groups of regulated genes shown in numerals. 1: G-protein coupled receptor signaling (Cnr1, Rgs9, Rasd2, Rasgrp1). 2: Ca2+/calmodulin pathway (Nrgn); MAPK pathways (Map2k3, Fos). 4: Early genes (Nr4a1, Arc, Dusp1, Egr1, Homer). 5: Ion channels (Scn4b). Abbreviations: VDCC, voltage dependent sodium channels, NMDA, N-methyl-D-aspartate, D1 and D2, dopamine receptors 1 and 2. From (209).

Regulation of genes involved in specific processes

Myelination

Neonatal hypothyroidism in the rat is associated with decreased myelin content, and reduced axon myelination. Thyroid hormone influences the expression of practically all myelin protein genes. The best characterized are those encoding the structural proteins (proteolipid protein (PLP), myelin basic protein (MBP), and myelin associated glycoprotein (MAG) (231). The period of thyroid hormone sensitivity for these genes in the rat brain extends from about the end of the first postnatal week up to the end of the first month, but the timing of regulation has a strong regional component, in parallel with the wave of myelination (232,233). Expression of myelin genes becomes normalized with time, even with continuous deprivation of thyroid hormones, but the number of myelinated axons remains lower than normal. The primary action of thyroid hormone on myelination is on oligodendrocyte differentiation (234). Thyroid hormone promotes differentiation of oligodendrocyte precursors in vitro (235). This effect is probably exerted through inhibition of the transcription factor E2F1 (236). The receptor involved in these actions of thyroid hormone is TRa1 (237,238), but effects thorugh TRβ have also been described (239), in agreement with the expression of *TRβ* in oligodendrocyte precursors (Fig 12).

Mitochondrial function.

An extensive review on thyroid hormone action on the mitochondria has been published (240). Mitochondria contain

truncated forms of TRα1 and RXRα, and thyroid hormone influences transcriptional activity of liver mitochondria (241,242). In the brain, the thyroidal status influences mitochondrial morphology and function (243,244) leading to changes in the expression of nuclear-encoded and mitochondrial-encoded mRNAs and proteins, such as 12S and 16S RNAs, cytochrome c oxidase subunits (244,245), NADH dehydrogenase subunit 3 (246), and a protein import receptor (51).

Cell Migration.

Thyroid hormone influences maturation of the radial glia, the path along which radial migration occurs in the cerebral cortex and the hippocampus (46). The mechanisms of thyroid hormone control of cellular migrations have not been defined in detail, but some of the molecules involved are regulated by thyroid hormone. Among them the extracellular protein Reelin, secreted by the Cajal-Retzius cells (53) of cortex and hippocampus, and by granular cells of the cerebellum, is under thyroid hormone control (247). The main function of Reelin is to stop migrating neurons, and it is essential for the inside-out pattern of cerebral cortex development. The protein Disabled1 (Dab1), a component of the Reelin signalling pathway is also under thyroid hormone regulation (53).

Thyroid hormones also regulate negatively the concentrations of other extracellular matrix proteins and adhesion molecules of multiple functions, including cell migration, neurite outgrowth, growth cone morphology, and axonal guidance and fasciculation, among them Tenascin C, laminin, L1, and NCAM (208,248-250). The concentrations of these proteins in neural tissue decrease during the postnatal period and thyroid hormones control the rate of this process.

Neural Cell Differentiation.

Thyroid hormone controls the expression of many genes encoding proteins with roles on terminal cell differentiation, such as cell cycle regulators, cytoskeletal proteins, neurotrophins and neurotrophin receptors and extracellular matrix proteins. Among the cell cycle regulators, E2F1, p53, cyclins and cyclin-dependent kinase inhibitors are regulated by thyroid hormone in cell culture (251-253). As already mentioned E2F1 is involved in oligodendrocyte differentiation (236).

Neural cell shape is determined by the cytoskeleton, which consists of microtubules (Tubulin), microfilaments (Actin), and intermediate filaments, specific for neurons (Neurofilaments), glia (Glial Fibrillary Acidic Protein), or maturing cells (Vimentin, Nestin). Tubulins α1 and α2 are downregulated by thyroid hormone, and tubulin β4 is upregulated (254,255). Microtubule associated proteins (MAPs) are also under thyroid hormone control at a posttranscriptional level. For example thyroid hormone regulates Map2 protein distribution in the Purkinje cell dendritic tree (256), and conversion of immature forms of Tau protein to mature forms of the TAU protein by alternative splicing of the Tau mRNA (257). The neurofilament genes *Nefh*, and *Nefm* are also under thyroid hormone control in the fetal and postnatal cerebral cortex (112,222).

Glial cells, in addition to radial glia and oligodendrocytes mentioned earlier, are also influenced by thyroid hormones: astrocytes (258,259) cerebellar Golgi epithelial cells (260), and microglia (261). As mentioned above, thyroid hormone influences the in vivo expression of astroglial genes such as those encoding Tenascin C, Laminin, and L1, which also have additional roles in neuronal migration and differentiation, and in axonal fasciculation. In vitro, the effect of T3 on astrocyte differentiation is blocked by β-adrenergic receptor antagonists (262).

Some of the effects of thyroid hormone on differentiation and survival might be mediated through control of neurotrophin expression. Interactions between thyroid hormone and NGF are important for the growth and maintenance of cholinergic neurons in the basal forebrain (20). Changes in NGF, TrkA and $p75^{NTR}$ after

hypothyroidism have been described (263). In the cerebellum thyroid hormone also controls the expression of NT-3 in vivo and in cultured cerebellar granule cells and it was suggested that the control of Purkinje cell differentiation by thyroid hormone is mediated through NT-3 produced by granule cells (264).

Genes Involved in Signalling Cascades

Many of the T3 target genes encode proteins involved in intracellular signalling. It is impossible to describe all of them. This topic awaits a unifying and simplified view of the physiological role of thyroid hormone at the cellular level, as more and more genes regulated by T3 are known (216). Here are cited only a few of the earliest examples to illustrate the diversity and pleiotropy of thyroid hormone effects on the brain. One of these proteins is RC3/Neurogranin (Nrgn) (208). Nrgn is a protein kinase C substrate that binds Calmodulin in the non-phosphorylated state and in the presence of low Ca^{2+} concentrations (265). Nrgn is involved in synaptic plasticity (266) by regulating the free calmodulin available and its distribution within the dendritic spines. *Nrgn* knock out mice display alterations of spatial memory (267). The mRNA and protein concentrations are under thyroid hormone control in developing rats, mice and goats, and the effect is mediated at the level of transcription through a response element located in the first intron of the gene (268).

Rhes (Ras homolog enriched in striatum), or Rasd2 (269,270) is striatal-enriched protein of the Ras family, with high homology with Dexras-1 (dexamethasone-inducible Ras protein) or Rasd1. These two proteins define a family within the Ras superfamily of small GTPases. Rhes regulates the AKT pathway, and mediates mTOR and dopamine signalling, and mutant-hungtintin toxicity, among other actions (271-273). One of the classical early known targets of thyroid hormone in the cerebellum, PCP-2 (Purkinje cell-specific protein 2, or L7) is involved in regulation of Gαi protein signalling (274). Thyroid hormone also regulates in vivo and in cultured cells the expression of *tubby*, a gene expressed in hypothalamic nuclei encoding a protein that also acts through G-protein signalling (275,276). Other genes involved in GPCR signalling are also under thyroid hormone control in the adult striatum (209).

Transcription Factors and Splicing Regulators

Regulation of proteins involved in transcription, and RNA stability and splicing is important for secondary influences in gene regulation. NGFI-A mRNA (Krox-24, Egr-1, Zif-268) is decreased in hypothyroid rats in several areas of the brain (277), and is induced by T3 at the promoter level in vivo (278). T3 regulates the krüppel-like factor 9 (Klf9) transcription factor formerly known as BTEB (basic transcription element binding protein), a member of the Sp1 family of transcription factors, in vivo and in cultured neuronal cells (42,200,279,280). Klf9 has been recently shown to mediate the actions of thyroid hormone in the loss of the regenerative capacity of Purkinje cells that occur during postnatal development (56). RORα, a member of the RZR/ROR family of orphan nuclear receptors is thyroid hormone dependent in the cerebellum. Disruption of the RORα gene is responsible leads to profound alterations in Purkinje cell growth and differentiation and granule cell migration (*staggerer* phenotype in mice) so that some of the actions of thyroid hormone on cerebellum could be mediated by this transcription factor (281). Also in the cerebellum, and other brain regions, the expression of the transcription factor Hairless is very sensitive to thyroid hormones (282). Hairless is a corepressor that heterodimerizes with the thyroid hormone receptor (283), so that in theory its induction would presumably tend to buffer other responses to thyroid hormone. Thyroid hormone also influences genes involved in RNA splicing, such as the mammalian homolog of the *Drosophila* splicing regulator *Suppressor-of-white-apricot* (SWAP) (284), and *Musashi-1*. Some of the posttranscriptional effects of thyroid hormone might be mediated through the control of RNA binding proteins controlling RNA stability.

Mechanisms of Gene Regulation by Thyroid Hormone

In some cases thyroid hormone responsive elements (TRE) were individually identified in the promoter or intronic regions of thyroid hormone dependent brain genes. Among these, myelin basic protein (285), the Purkinje cell specific gene (PCP2) (286), the calmodulin binding and PKC substrate RC3 (268), prostaglandin D2 synthetase (287,288) the transcription factor Hairless (282), the neuronal cell adhesion molecule (NCAM) (289) and the early response gene NGFI-A (278). More recently chromatin immunoprecipitation assays have identified TR binding sites in the developing mouse cerebellum (290) or cerebellar lines expressing TRα1 or TRβ1 (201). In the latter study only DR4 was found as TR binding sites, although in general there was poor correlation between the response to T3 and the presence of proximal TRE. Other targets are regulated at the levels of mRNA stability (acetylcholinesterase), protein translation (MAP2) (256) or mRNA splicing (Tau) (257). Regulation of splicing might be due to a primary action on the transcription of splicing regulators (291).

Interaction with Retinoic Acid and Glucocorticoids

Some actions of thyroid hormone may be due to interaction with other hormonal systems. For example, control of *Vdr* mRNA in the striatum may affect Vit D signalling (200). In primary cerebrocortical cultures T3 controls the expression of several enzymes involved in retinoic acid metabolism: the RA synthesizing emzymes Aldh1a1 and Aldh1a3, and the degrading enzyme Cp26b1 (42,216). The final effect on RA concentrations depends on the relative expression of each of the enzymes, which have developmental and regional variations, and on glucocorticoid signalling (Fig 15). *Aldh1a1* is up regulated by T3, preferentially through TRα1, and the effect of T3 is greatly potentiated by glucocorticoids. At the same time, T3 controls the expression of the glucocorticoid receptor, *Nr3c1*. *Aldh1a3* however is down regulated by T3, and *Cyp26b1* is up regulated by T3. Therefore, thorugh its actions on Aldh1a1, and especially in the presence of glucocorticoids, T3 will increment RA concentrations, whereas through Aldh1a3 and Cyp26b1 T3 will reduce RA concentrations. An example of a possible interaction between T3 and RA is in the control of the proliferation of the tanycyte stem cells as mentioned before in the section on Neurogenesis.

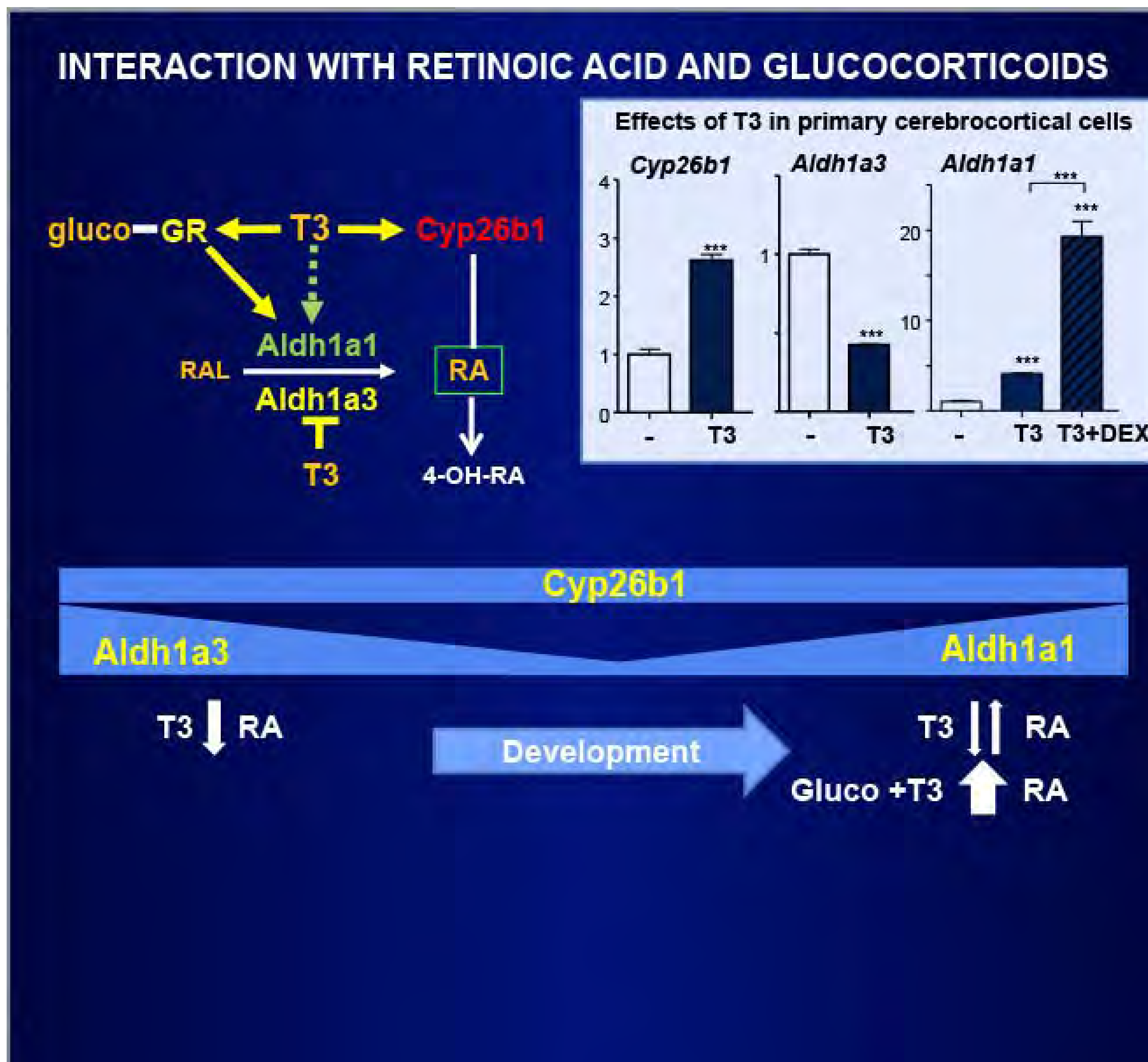


Fig 15.- I

nteractions between T3 and glucocorticoids in the regulation of retinoic acid (RA) concentrations. T3 controls the expression of two enzymes involved in RA syntesis from retinaldehyde (RAL), Aldh1a3, downregulated by T3, and Aldh1a1, upregulated by T3. Aldh1a1 is also regulated by glucocorticoids, and T3 and glucocorticoids have a synergistic action. Additionally the glucocoticoid receptor gene, Nr3c1 is upregulated by T3. T3 also upregulates the expression of Cyp26b1, which degrades RA to 4-OH-RA. The effects of T3 on Cyb26b1 and Aldh1a3 are at the transcriptional level. The net effect of T3 on RA depends on the specific region of the brain expressing Aldh1a1 or Aldh1a3, in relation to the expression of Cyp26b1 and the concentrations of glucocorticoids. Durng development Cyp26b1 remains relatively constant, whereas Aldh1a3 is highly expressed earlier in development and then decreases. Aldh1a1 follows the reverse pattern. Therefore, the next effect of T3 in early development will be to decrease RA concentrations, whereas in late development will either decrease or increase depending upon glucocorticoid signaling. From (42).

CLINICAL PROBLEMS RELATED TO TH ACTION ON THE BRAIN

The topics reviewd in this section are described more extensively in other sections of the Thyroid Disease Manager. Here I will only make a brief reference to how several conditions may affect development and function of the brain, in relation to previous sections of this chapter. The major causes of thyroid hormone deficiency during development are iodine deficiency, congenital disorders of the thyroid gland, and maternal and/or fetal hypothyroidism. In addition, the condition known as hypothyroxinemia, defined as low T4 in the presence of normal T3 and TSH, in the pregnant woman is suspected of a possible cause of developmental impairment. In the presence of a relatively normal supply of thyroid hormone, the action may be impaired due to tissue resistance. The syndromes of thyroid hormone resistance comprise transporter mutations, altered thyroid hormone deiodination due to SBP2 mutations, and mutations of the THRA and THRB genes (292-294). The consequences of thyroid hormone deficiency on brain maturation greatly depend on the specific stages of development affected (Fig 16).

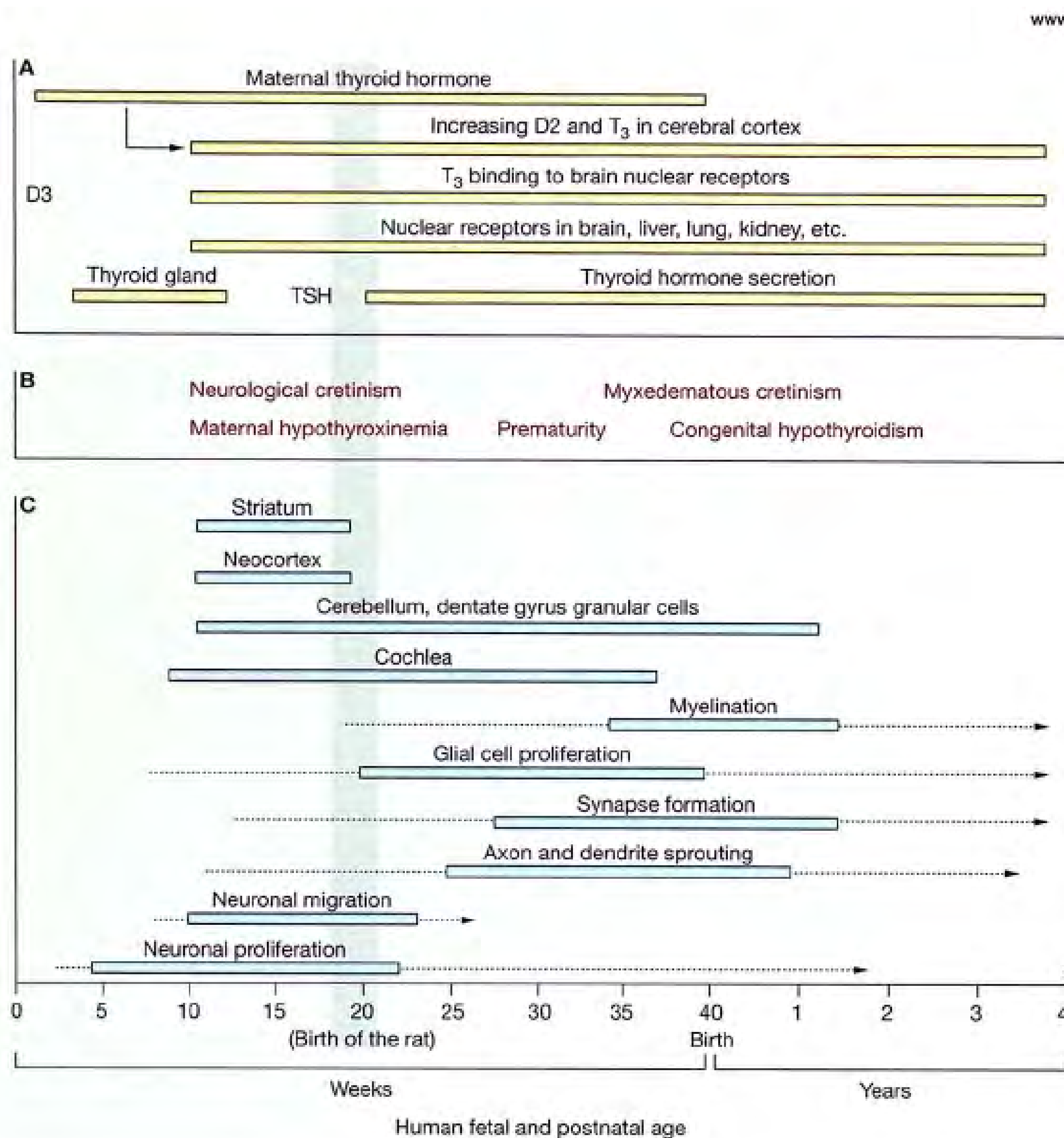


Fig 16

Relationship between human nervous system (C) and thyroid-related (A) developmental events, and the timing of insult of conditions due to thyroid hormone deficiency (B). For comparison with rat development, the equivalence for the birth of the rat is shown by a vertical blue line around postmenstrual week 20. From (6).

Iodine Deficiency Disorders. Endemic Cretinism

Daily adult needs of iodine are of the order of 150, and 250 µg during pregnancy and lactation (295,296). Iodine deficiency causes a wide spectrum of abnormalities collectively known as iodine deficiency disorders (297), with high incidence of abortions and stillbirths, increased perinatal and infant mortalities, neonatal goiter and hypothyroidism, and various degrees of psychomotor and mental defects, and cretinism.

Iodine deficiency has been, and still continues to be, endemic in many regions of the planet (http://www.iccidd.org). The consequence of severe iodine deficiency during development is the syndrome known as cretinism, of which two forms, Neurological and Mixedematous, were described by McCarrison (298) in the Himalayas and in Papua-New Guinea. In Neurological cretinism the thyroid gland is normal, and there are no signs of hypothyroidism. However, there is severe mental retardation, deaf mutism and a striato-pallidal disorder with spastic diplegia affecting the lower limbs (299). On the other hand, myxedematous cretins (300) are hypothyroid, with short stature, poor sexual development, and craniofacial abnormalities. They are also mentally retarded but not as severely as neurological cretins, and signs of neurological involvement are observed only in a minority of cases. In affected regions, usually a combination of the two forms is more frequently observed.

Until the 80's of last century, there were great controversies concerning the pathogenesis of each of these syndromes, and whether or not neurological cretinism was really due to thyroid hormone deficiency. Among other confusing reasons was the fact that early postnatal administration of iodine or of thyroid hormone was ineffective. It is clear today that the differences between the two forms of cretinism reflect different a different timing in the action, or lack of action, of thyroid hormones, according to developmental timing of expression of deiodinases, receptors, thyroid secretion, and maternal placental transfer of thyroid hormones. The clinical picture of neurological cretinism suggests damage to the striatum and the cerebral cortex during the second trimester of pregnancy, and it is due to the profound maternal hypothyroxinemia caused by severe iodine deficiency, during the first half of pregnancy. As described before (Fig 10), the T3 receptor is expressed from the second trimester, at a time when most T3 in fetal brain areas, such as the cortex depend on maternal T4, and local Dio2 activity (Fig 10). In contrast, Mixedematous cretinism is clearly due to failure of the fetal and infant thyroid gland during the last trimester of pregnancy and the postnatal period. In many cases destruction of the fetal gland is due to a combination of iodine deficiency and dietary goitrogens.

Congenital Hypothyroidism

Congenital failure of the thyroid gland occurs in about 1 in 3,000-4,000 newborns (for a review see (301). Neonatal screening and early thyroid hormone treatment have efficiently prevented its consequences. Causes for Congenital hypothyroidism are thyroid dysgenesis including ectopic thyroid gland and agenesia, and inborn errors of thyroid biosynthesis. In a small percentage of thyroid dysgenesis mutations of genes involved in thyroid embryogenesis have been described (302). The great majority of cases are nonetheless due to non Mendelian mechanisms, with the involvement of several genes (303). An isolated family with truncated TSH receptor has also been described (304).

Despite the systematic and early diagnosis and treatment (305) of Congenital hypothyroidism in the prevention of mental retardation some affected individuals may remain with minimal brain damage, indicated by learning disabilities and disturbance of fine motor coordination. The success of thyroid hormone treatment depends on the severity, onset, and duration of hypothyroidism. Children at risk of having neurological sequelae even with early treatment may require a substantially higher replacement dose of T4 than moderate cases (306). It is therefore important to identify these children by the estimation of bone maturation and plasma T4 levels at diagnosis.

Maternal Hypothyroidism and Hypothyroxinemia

Maternal thyroid hormones cross the placenta (307), and up to 50% of fetal blood T4 at term is of maternal origin (80). Therefore, in congenital hypothyroidism maternal hormones protect the fetal brain until birth, and even children with total thyroid agenesia do not suffer from severe neurological deficits as in neurological cretinism. The important role of maternal hormones is demonstrated in cases of severe maternal and fetal hypothyroidism. For example, in reported cases Pit-1 deficiency (308) or high titers of thyroid stimulation blocking antibodies (309,310) circulating thyroid hormones are very low in the mother and in the infant. The children suffer from permanent sensorioneural deafness and irreversible neuromotor development resembling neurological cretinism.

Maternal hypothyroidism, in the presence of a normal fetal thyroid, is also harmful for fetal brain development (311,312). High TSH in mothers during the second trimester of pregnancy is associated with a reduction of 4 points in the children IQ, and impaired attention, language, and visual-motor performance at 7-9 years. (312). Japanese children born from hypothyroid mothers who where treated after the 6th-16th week of gestation showed no signs of altered neurodevelopment (313). The authors argued against a major role of maternal T4 during early pregnancy. It is possible that iodine supply to the fetal thyroid is an important factor, considering the high iodine intake in Japan. At least in the rat, a good functioning of the the fetal thyroid gland is essential for cerebral cortex gene expression (68,222). The need for antenatal thyroid screening of pregnant women to prevent impaired cognitive development of children born from women with low thyroid hormone levels was examined by Lazarus et al (314) It was found that the cognitive development at 3 years of age of children born from mothers with low thyroid hormone and/or high thyroid-stimulating hormone (TSH), with or without T4 treatment, was not different from that of the control group.

But there is also evidence that not only frank hypothyroidism, but maternal hypothyroxinemia is associated with a higher incidence of neurological alterations in children (315,316), and increased incidence of Attention Deficit Hyperactivity Disorder (317). While the incidence of hypothyroidism in pregnant women is around 2.5%, hypothyroxinemia is much more prevalent, up to 30% (307,318,319), and it is usually due to mild iodine deficiency (320). In these situations, peripheral euthyroidism, and normal TSH levels, are maintained by several compensatory mechanisms, including preferential T3 secretion. For these reasons Iodine supplementation of women considering conception and during pregnancy and lactation is warranted (295). In rats Berbel et al have clearly shown that maternal T4 is needed for proper brain development (321). In pregnant women mild hypothyroxinemia due to iodine deficiency leads to altered neurocognitive performance of the progeny (322) but not if the hypothyroxinemia was corrected with iodine supplements during the first trimester.

Prematurity

Premature babies often present transient hypothyroxinemia lasting several weeks (323,324), in about 85% of cases. This is due interruption of maternal thyroid hormone supply, and immaturity of the fetal thyroid axis. The issue of whether the hypothyroxinemia of prematurity is “physiological”, or deserves thyroid hormone treatment has been thoroughly discussed. It is clear that circulating total T4 and FT4 concentrations are lower in preterm neonates than in fetuses of comparable age still in utero (325-327). Therefore, the hypothyroxinemia of prematurity may have clinical consequences (328) with increased risk of neurological impairment (329), risk of cerebral palsy (330) and white matter damage (331). In an experimental model of prematurity, Berbel et al (321) have shown that deprivation of maternal hormones similar to that suffered by premature babies leads to altered development of the neocortex and hippocampus, and that the defects were corrected by T4 treatment of the dams. Despite the importance of this topic, the issue of whether preterm babies should be treated with thyroid hormone is not settled (332-334).

Thyroid Hormone Transporter Mutations

Among the thyroid hormone transporters in the cell membrane (159), mutations have only been identified in MCT8, a 12 transmembrane protein encoded by *SLC16A2*, a gene located in chromosome X. They were first described by the groups of Refetoff (160) and De Visser (161) in children. Other cases were subsequently reported (335-337). Affected patients suffer from X-linked mental retardation, with severe developmental delay and neurological damage in addition to altered thyroid hormone levels in blood. Total and free T4 and rT3 are low, whereas total and free T3 are elevated. TSH is either normal or moderately elevated. The neurological syndrome consists of global developmental delay, lack of language development, profound mental deficiency, rotary nistagmus, impaired gaze and hearing, dystonic movements and severe proximal hypotonia with poor head control progressively developing into spastic quadriplegia. Some patients present paroxysmal dyskinesias (338). It was also recognized that the Allan-Herndon-Dudley syndrome (OMIM 300523) is due to MCT8 mutations (339). The neurological syndrome is likely due to defective T3 transport to neural cells during critical periods of fetal development (340-342), but the lesion responsibles for the clinical phenotype are not known. There are signs of hypomyelination by MR imaging, and the syndrome is sometimes included included within the leukoencephalopaties. About 5% of patients diagnosed of Pelizeaus-Merchbacher disease, a leukoencephalopaty usually due to *PLP1* mutations, have instead *MCT8* mutations (343).In a recent anatomical study of the brains of affected patiens (344), we found not only hypomyelination, but also retarded development of the cerebral cortex and the cerebellum, deficient neuronal differentiation with altered expression of neurofilaments, lack of parvalbumin interneurons in the cortex, and strongly diminished synatophysin, reflecting very poor synaptogenesis.

Mct8-deficient mice ($Mct8^{-/y}$) (168,169) have also, as expected, elevated circulating T3 and reduced T4 and rT3. However they have minimal, if any, brain impairment (345), with only modest reductions in the expression of T3 target genes. The knock out mice are only partial models of the disease, and have been useful to dissect the mechanisms leading to the unusual pattern of thyroid hormone concentrations in blood (346). Restricted access of T3 to neurons, where *Dio3* is expressed, impairs T3 degradation. The increased availability of T3 increases D1 activity in liver and kidney causing and increased rT3 degradation and T4 to T3 conversion, thereby reducing rT3 and T4 levels, and further contributing to increase T3. Additionally, thyroid gland secretion is altered because Mct8 is present in the basal membrane of the thyrocytes and plays an important role in thyroid hormone secretion (347,348). In the brain there is a reduction of T4 supply to the astrocytes which causes an increased Dio2 activity and increased production of T3 from T4. Therefore, there are two important features of this syndrome: one is the concomitant increase in the activity of the two deiodinases, D1 and D2. The second is the coexistence of “hyperthyroid” (liver) and “hypothyroid” (brain) tissues. Even within the brain, the presence of alternative transporters in some cells might perhaps expose these cells to excessive T3, although this remains as a possibility (345).

There is strong evidence that the main restriction to the entry of T3 to neurons is not at the level of the neuronal membranes, but at the BBB (165,166). Mct8, and other transporters, such as Oatp1c1 (349) are present in the membrane of capillary endothelial cells (see previous section). In the absence of Mct8, T4 is still transported due to its higher affinity for Oatp1c1, therefore contributing, through T4 to T3 production, to compensate the lack of T3 transport through the BBB. It has been postulated that reason why *Mct8* knock out mice do not show neurological impairment in contrast to patients, is due to compensation by a T4 transporter not be present in humans (165,345). Indeed, the adult and juvenile primate BBB contains MCT8 but little OATP1C1 (350). The OATP1 transporter is detected immunohistochemically in the human fetal brain capillaries (165) but in lower amounts than in rodents.

T3 receptor mutations

The majority of cases (85%) of the classical form of Resistance to Thyroid Hormone (RTH) are due to mutations in

the *THRB* gene (351). RTH patients may have learning disabilities, reduced IQ, and increased incidence of Attention Deficit and Hyperactivity Disorder. *Thrb* knock out mice, or knock in mice expressing receptor mutations are good models of RTH (352). In addition, the phenotypic analysis of these mice has revealed important roles of TRb in maturation of somatosensory systems. *Thrb* mutant mice have deafness and impaired color vision, due to altered maturation of cochlear hair cells, and retinal photoreceptors (353,354). Mice expressing a mutant TRb1 with strong dominant negative property had cerebellar abnormalities reminiscent of severe hypothyroidism, and neuromotor disability (355).

THRA mutations have recently been described in humans (356,357). The TRa1 mutant proteins display strong dominant negative activity. The patients suffer from growth retardation, delayed bone development, severe constipation, and mild cognitive deficits, with minimal alterations of circulating thyroid hormones: normal TSH, low T4/T3 ratios and strongly reduced rT3. T4 may be slightly reduced T4 or in the lower limit of normal range, and T3 may be slightly increased or in the upper limit of normal range. These alterations may be caused at least in part by deficient regulation of *Dio3* which is a TRa1-regulated gene (133).

Previously, extensive work was aimed at defining the phenotypes of mice with TRa1 inactivation or harboring a mutant TRa1 with the thought that it could help to identify patients (358). In agreement with this, expression of mutant TRa1 with dominant negative activity causes retarded growth (359), low cerebral glucose consumption (360), retarded brain development, and neuromotor impairment in developing animals (361-363), and a profound anxiety in adult animals, ameliorated by T3 treatment (362). The consequences of TRa1 mutations may also be heterogeneous depending on the mutation (364-366).

An important observation was that absence of TRa1 in mice was not equivalent to mutations of the same gene. Despite the fact that most actions of thyroid hormone in brain are mediated by TRa1, absence of this receptor does not lead to a “hypothyroid” brain. In other terms, receptor deletion is not equivalent to hormone deprivation. This is due to the intrinsic transcriptional activity of the unliganded receptor. In the absence of the hormone, as in hypothyroidism, the unliganded receptor (or aporeceptor) has hormone-independent activity, either repression or activation of transcription, leading to a disturbance of processes which are normally controlled by thyroid hormones (367). Therefore, to some extent it can be said that the hypothyroid phenotype is the consequence of unliganded receptor activity. In the absence of receptor, this activity is suppressed and hypothyroidism is not as detrimental as in its presence (368,369).

Since unliganded receptors have transcriptional activity, the question is if they have a have a physiological and developmental role in the absence of the hormone (367,370). The receptors are expressed somewhat before the onset of thyroid gland secretion, and therefore occupancy of receptors during development is dependent as explained above, from the maternal hormones and the activity of deiodinases. Dio2 activity increases in the human fetal cerebral cortex during the second trimester, and T3 concentration increases in parallel (191). During the same stages, the cerebellum expresses mainly Dio3 and T3 is concentration is kept very low. These findings suggest that at the same time of development, the liganded and unliganded receptors are involved in development of the cerebral cortex and the cerebellum respectively. Evidence for a role of unliganded receptors have been demonstrated in studies on amphibian metamorposis (371), or in the development of the inner ear in mice (372).

CONCLUSIONS

Thyroid hormone actions in the brain are extremely complex with a continuously changing landscape as development proceeds. Brain maturation involves a continuos change in cell composition so that the target organ of thyroid hormone is under constant change. This is reflected in the changing regulated gene network. Genes that are

transcriptional targets of T3 at a certain developmental time may be refractory at another time. The importance of thyroid hormone for the brain requires tightly controlled mechanisms of thyroid hormone delivery to the brain and the cellular interactions in the metabolism of thyroid hormones. Disruption of these mechanisms results in syndromes of profound neurological impairment. The challenge is to understand in detail the mechanisms of action of thyroid hormones at different stages of development and the pathophysiological states of the thyroid hormone action defects.

ACKNOWLEDGEMENTS

Work in the author's lab is supported by grants from the Plan Nacional de I+D+I, Spain SAF2011-25608, SAF2014-54919-R, and the Center for Biomedical Research on Rare Diseases (CIBERER), under the frame of E-Rare-2, the ERA-Net for Research on Rare Diseases. The author would like to acknowledge the contribution of the group members especially Drs Beatriz Morte and Ana Guadaño-Ferraz, through informal discussions on the topics presented in this chapter.

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Table 1 TR isoform protein and mRNA concentrations in rat tissues

	Protein molecules/cell	mRNA molecules/cell
TRα1		
Pituitary	2310	0.128
Liver	963	1.11
Brain	2360	16.4
Kidney	626	2.50
Heart	1300	2.70
TRβ1		

Pituitary	1470	0.873
Liver	3900	5.10
Brain	819	20.2
Kidney	578	13.5
Heart	1460	7.37
TRβ2		
Pituitary	5240	0.631
Liver	829	<0.00689
Brain	328	<0.00684
Kidney	178	<0.00501
Heart	506	<0.00453

The three TR isoform proteins are present in all tissues. This includes the TRβ2 isoform, despite that the corresponding mRNA is only detectable in pituitary. The concentrations of receptor protein were measured by specific immunoprecipitation of labelled T3 after incubation with tissue nuclear extract. The concentrations of mRNA were measured in northern blots using quantitative procedures. Data from (187).

Figure legends

Fig 1.-Postnatal morphological changes in the rodent cerebellum after neonatal hypothyroidism. Upper panel: Purkinje cells in a normal (left) and hypothyroid rat (right). Lower panel: persistence of the external granular layer (arrow) in a hypothyroid mouse cerebellum.

Fig 2.-Regulation of Reelin by thyroid hormone. The figure shows slices of the cerebral cortex from euthyroid and hypothyroid newborn rats. The upper panels show in situ hybridization for Reelin RNA. The midle and lower panels show immunohistochemistry of Reelin protein. The RNA is present in isolated cells of layer I, known as Cajal-Retzius cells. The RNA is very low in the hypothyroid animals. The Reelin protein is a matrix protein, seen in these slices concentrated also in layer I. On P0 there is no Reelin protein in the hypothyroid animals, but on P5, the amount of Reelin protein is normal (from (247)).

Fig 3.-Myelination in the anterior commisure of euthyroid and hypothyroid rats. Hypothyroidism was produced during the neonatal period, and the rats were analyzed at 6 months of age. He aupper panels show transversal section of the anterior commisure stained for myelin. The lower panels show electro microscopy analysis. The number of myelinated axons is reduced in the hypothyroid rats in parallel to an increased number of small diameter axons. Those axons reaching a critical size have near normal myelin content, but still present structural defects (From (49)).

Fig 4.- The upper panel shows the T3 concentration in brain after administration of either T4 or T3 to the mother. Pregnant rats treated with MMI in order to block the maternal and fetal thyroids, were given either T4 or T3 as continuous infusion. T3 concentrations were measured in the brains of the mothers and the fetuses at term. In the mother, increasing doses of T3 led to a proportional accumulation of T3 in the brain. The normal T3 concentration was reached only by a narrow range of T3 doses. Administration of T4 led to a more flat accumulation of T3, and the normal T3 concentration was achieved through a wide range of T4 doses. On the other hand, ddministration of T3 failed to increase T3 concentration in the fetal brain, whereas administration of T4 efficiently normalized fetal

brain T3 concentrations (From (67)). The cartoon in the lower panel offers an explanation for these results. In the mother, brain T3 derives partially from circulating T3 and from T4 deiodination. Circulating T4 and T3 in the fetus derive from the fetal thyroid gland and from the mother. However, brain T3 is exclusively a product of T4 deiodination.

Fig 5.- Type 2 deiodinase mRNA expression in the rat brain. A: euthyroid rat. Dio2 mRNA is distributed all over the brain with high concentration in walls of the lower third of the 3rd ventricle. In this region, Dio2 is expressed in specialized glial cells called tanycytes. In the rest of the brain Dio2 is expressed in the astrocytes (the inset shows Dio2 mRNA hybridization silver grains over a stained astrocyte). B: Dio2 expression in a hypothyroid rat. An increased expression is observed in most parts of the brain, but especially in the barrel cortex and some structures such as the ventromedial nucleus, serving as relay stations for somatosensory pathways. C and D: Dark field photomicropgraphs of the 3rd ventricle, infundibular region and median eminence showing high expression of Dio2 mRNA in this area (C), and its increased expression in iodine deficient rats (D). E: Cellular content of *Dio2* mRNA measured by RNA-Seq in cells isolated from the mouse cortex. FPKM: Fragments per kilobase of exon per million reads mapped. Note that these measurements give an estimate of the relative abundance of the mRNA in a certain cell type, but do not allow making comparisons of RNA abundance between cell types because the data are not normalized to cell number. *Dio2* mRNA is present predominantly in astrocytes, with some expression in neurons, probably interneurons (102). It is also expressed in oligodendrocyte precursor cells (OPC). Panels A and B are data from the author. Panels C and D are adapted from (373). Data in E have been obtained from (199).

Fig 6.- Entry of thyroid hormone to the brain through the blood-brain barrier (BBB). T3 can reach the target neurons via two ways. One is from the astrocyte, after 5'deiodination of the T4 taken up from the blood through the astrocyte end-feet. T3 can also reach the neurons directly from the extracellular matrix after crossing the BBB. In the neurons, T3 is degraded to T2 by D3. The end-feet of the astrocytes may be fenestrated, leaving part of the external capillary surface, in direct contact with the extracellular matrix.

Fig 7.- Content of *Mct8* and *Oatp1c1* mRNAs in native cells isolated from the postnatal mouse cerebral cortex, measured by RNA-Seq. The data are from the transcriptomic database of Zhang et al (199). Mct8 is expressed in astrocytes, neurons, oligodendrocyte precursor cells (OPC), and endothelial cells. It is also expressed in newly formed oligodendrocytes.Oatp1c1 is expressed in astrocytes and endothelial cells. FPKM: Fragments per kilobase of exon per million reads mapped. Note that these measurements give an estimate of the abundance of the mRNA in a certain cell type, but do not allow making comparisons of RNA abundance between cell types beceause the data have not been normalized by ell number.

Fig 8.- Ontogeny of T3 receptors in rat organs. Receptor concentration was measured by T3 binding assays. From (175).

Fig 9.- T3 receptor mRNA expression in the mouse brain, by in situ hybridization with TRα1 and TRβ1-specific probes. In the cerebrum (upper panels) there is an overlapping distribution of both receptor subtypes, with some differences in the hippocampus, amygdala ans hypothalamus. In the cerebellum (lower panel) TRα1 is expressed in the granular layer (GC), whereas TRβ1 is expressed in the Purkinje cell layer (PC).

Fig 10.- Ontogeny of T3 receptor in the human fetal brain, measured by T3 binding assays. During the period analyzed the brain increases exponentially in weight, due mainly to the wave of neuronal proliferation (left panel). The receptor concentration increases 50-fold from the 10th to the 16th week (middle panel). In addition despite the fact that the fetal thyroid glandis is still not functioning, T3 increases in concentration during the same period, and can be found in the receptor fraction. Accumulation of T3 reflects the increasing activity of Dio2 in the cerebral cortex, as shown in fig 10. From (79).

Fig 11.-. Concentrations of T3 in the fetal cerebral cortex and in the cerebellum. The cortex has high Dio2 activity, whereas the cerebellum has low Dio2 and high Dio3 activity. Accordingly, the coencentrations of T3 rise in the cerebral cortex and rfemain low in the cerebellum, reflecting different timing of T3 action. From (191).

Fig 12.- Expression of *Thra* and *Thrb* genes measured by RNA-Seq in native cells isolated from the mouse cerebral cortex. Data in the leaft panel have been obtained from the transcriptomic and splicing database of Zhang et al (199). The data of the right panel should be viewed as an approximation. To construct these data we calculated the FPKM data for *TRα1* mRNA after subtraction of the *TRα2* component (around 90%) from the *Thra* data shown in the upper left panel. FPKM: Fragments per kilobase of exon per million reads mapped. Note that these measurements give an estimate of the abundance of the mRNA in a certain cell type, but do not allow making accurate comparisons of RNA abundance between cell types because the data were not normalized by cell number. A: astrocytes; N: Neurons; OPC: oligodendrocyte precursor cells; NFO: newly formed oligodendrocytes; MO: myelinating oligodendrocytes; M: microglia; E: endothelial cells.

Fig 13: Windows of sensitivity for the regulation of brain gene expression by thyroid hormone during the postnatal period in the rat. A few examples of genes sensitive to thyroid hormone during the postnatal period is shown to illustrate the windows of sensitivity of the regulated genes. Other genes that are sensitive in the fetal period and in the adult are not shown in this figure. The windows of sensitivity of the myelin genes are different for each brain region, so that in the more caudal regions (for example brain stem and cerebellum) the window is earlier than in the frontal regions (cortex).

Fig 14: Regulation of gene expression by thyroid hormones in the adult striatum. Signaling pathways are schematically represented and the main groups of regulated genes shown in numerals. 1: G-protein coupled receptor signaling (*Cnr1*, *Rgs9*, *Rasd2, Rasgrp1*). 2: Ca2+/calmodulin pathway (*Nrgn*); MAPK pathways (*Map2k3*, *Fos*). 4: Early genes (*Nr4a1, Arc, Dusp1, Egr1, Homer*). 5: Ion channels (*Scn4b*). Abbreviations: VDCC, voltage dependent sodium channels, NMDA, N-methyl-D-aspartate, D1 and D2, dopamine receptors 1 and 2. From (209).

Fig 15.- Interactions between T3 and glucocorticoids in the regulation of retinoic acid (RA) concentrations. T3 controls the expression of two enzymes involved in RA syntesis from retinaldehyde (RAL), *Aldh1a3*, downregulated by T3, and *Aldh1a1*, upregulated by T3. *Aldh1a1* is also regulated by glucocorticoids, and T3 and glucocorticoids have a synergistic action. Additionally the glucocoticoid receptor gene, *Nr3c1* is upregulated by T3. T3 also upregulates the expression of *Cyp26b1*, which degrades RA to 4-OH-RA. The effects of T3 on *Cyb26b1* and *Aldh1a3* are at the transcriptional level. The net effect of T3 on RA depends on the specific region of the brain expressing *Aldh1a1* or *Aldh1a3*, in relation to the expression of *Cyp26b1* and the concentrations of glucocorticoids. Durng development *Cyp26b1* remains relatively constant, whereas *Aldh1a3* is highly expressed earlier in development and then decreases. *Aldh1a1* follows the reverse pattern. Therefore, the next effect of T3 in early development will be to decrease RA concentrations, whereas in late development will either decrease or increase depending upon glucocorticoid signaling. From (42).

Fig 16: Relationship between human nervous system (C) and thyroid-related (A) developmental events, and the timing of insult of conditions due to thyroid hormone deficiency (B). For comparison with rat development, the equivalence for the birth of the rat is shown by a vertical blue line around postmenstrual week 20. From (6).

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Bookshelf ID: NBK285549 PMID: 25905404