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Molecular Basis of Thyroid Hormone-Dependent Brain Development*

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I. Introduction

SOME 108 yr have passed since a committee of the Clinical Society of London stressed the important role of the thyroid gland in assuring normal brain development (1). Members of that committee drew this conclusion from the observation that patients with both sporadic and endemic cretinism clearly showed evidence of mental retardation. Subsequent clinical experience emphasized the importance of making the diagnosis of congenital hypothyroidism and initiating appropriate replacement treatment as soon as possible after birth. With the development of sensitive thyroid function screening tests (2–4) it became possible to identify congenital hypothyroidism at birth and to start treatment without delay (5). However, the persistence of iodine deficiency in many areas of the developing world still presents an important problem and has raised the possibility that even minimal degrees of undetected hypothyroidism in infants born in such iodine-deficient regions lead to suboptimal intellectual development (6, 7). Further, an understanding of the role of thyroid hormone in terminal brain differentiation may provide insights into the mechanisms by which other metabolic and nutritional factors influence this process. These considerations have stimulated renewed interest in the molecular basis of thyroid hormone-dependent brain development.

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II. Developmental Schedules

A. Species specificity

Although studies of thyroid hormone effects on brain development have employed several mammalian species, the most intensive and detailed studies have focused on the neonatal rat (8, 9). Any effort to compare these studies and to apply them clinically must take into account the conspicuous interspecies differences in the stages of brain development occurring before and after birth. The rat is an altricial species, born with a relatively undeveloped brain and with the thyroid-pituitary-hypothalamic axis not yet fully matured. The sheep, in contrast, is a precocial animal born with a relatively advanced state of brain maturation. Development of the ovine thyroid-pituitary axis is nearly complete at birth (10). The human child at birth is somewhat intermediate between the rat and sheep in that it has a relatively immature central nervous system but a more fully developed thyroid hormone axis. The contrasting patterns of development in these species have been extensively discussed by Fisher and Polk (11) and Stein *et al.* (12). The importance of such differences in developmental schedules is illustrated by the fact that the majority of experimental studies in the rat model have analyzed the effect of thyroid hormone deprivation in the early neonatal period, which corresponds to the late intrauterine phase of development in sheep and humans. Whereas there is evidence of a role for thyroid hormone in brain maturation during the intrauterine period in human (13–15) and sheep (16, 17), this is not yet clearly established in the rat. Thyroid hormone appears to regulate those processes associated with terminal brain differentiation such as dendritic and axonal growth, synaptogenesis, neuronal migration, and myelination.

B. Developmental studies in the rat

The first systematic studies of the effects of hypothyroidism on brain development in rats were reported by Eayrs and his colleagues (18–21). The distinguishing feature of the cerebral cortex in hypothyroid rats is the retarded development of the neuropil. In the hypothyroid rat, peripheral and central neuronal cell bodies are smaller and more tightly packed. Disorders of process growth and synaptogenesis are manifested by diminished axonal and dendritic outgrowth, elongation and branching, and alterations in the number and distribution of dendritic spines. Other groups have advanced biochemical evidence of deficient development of the central nervous system in the absence of thyroid hormone, including reduced myelination (22–24) and delayed expression of specific enzymes (25–27). These deficiencies are observed in neurons of the cerebral cortex, the visual and auditory cortex, hippocampus, and cerebellum. The affected areas can be related to the various deficits in learning and motor skills consistently observed in hypothyroid animals (12). In the cerebellum, thyroid hormone deprivation results in delayed proliferation and migration of granule cells from the external to the internal granular layer (28, 29), stunting of the dendritic arborization of the Purkinje cells (30, 31), and diminished axonal myelination (24, 32). Consistent with the finding of delayed cellular migration and neuronal differentiation, Faivre *et al.* (33) reported a dramatic decrease in microtubule numbers in Purkinje cells. Aniello and colleagues (34) also demonstrated a delay in the transition from immature to mature forms of the microtubule protein, tau, and in the developmental expression patterns of various tubulin isotype mRNAs (35). The developmental retardation of the rat brain resulting from thyroid hormone deficiency can be reversed if administration of thyroid hormone is begun before the end of the second week after birth (9, 36). The greater the delay in hormone replacement beyond this time the less is the chance of recovery of normal development. These age-dependent responses to thyroid hormone deprivation differ sharply from the reversible metabolic effects of thyroid hormone that occur in other tissues of the rat, such as stimulation of oxygen consumption (37) or hepatic lipogenic enzyme activities (38). These are reversible regardless of the length of the preceding hypothyroid state or age of the animal. Of additional interest is the finding that both adult and neonatal rat brains fail to respond to thyroid hormone with an increase in oxygen consumption (39).

One of the reasons for the popularity of the rat model is the relative ease with which hypothyroidism can be established in the dam, the intrauterine fetuses, and the neonates. Administration of either methimazole or propylthiouracil in drinking water or food will block the oxidation of iodide to iodine and consequently block the formation of T_4 and T_3 in the maternal thyroid. Since these drugs readily pass the placental barrier and are also transmitted to the suckling pups in the mother's milk, the fetus and neonate also become profoundly hypothyroid.

III. Thyroid Hormone Action

A. Sources of T_3 and T_4

The generally accepted model of thyroid hormone action assumes that T_3 is the primary hormone and that the principal function of T_4 is to serve as a precursor of T_3 in the deiodination of T_4 by iodothyronine deiodinases (40). The hypothesis holds that T_3 is bound to nuclear receptors (T_3R) with greater affinity than T_4 and that an interaction of the hormone with the receptor initiates a cascade of nuclear events that results in the augmentation or inhibition of expression of those genes to which the T_3-T_3R complex binds. One of the benefits derived from the peripheral conversion of T_4 to T_3 is that the slower fractional turnover of T_4 compared with that of T_3 helps to stabilize the level of circulating T_3. The level of serum T_4 plays an additional role in thyroid hormone homeostasis. In states characterized by low concentrations of serum T_4, such as that resulting from iodine deficiency, type II iodothyronine 5′-deiodinase activity rises in brain, resulting in enhanced conversion of T_4 to T_3, and, thus partially compensating for the diminished serum (41).

The interrelation of T_4 and T_3 in mediating the effects of thyroid hormone has assumed particular importance in the case of brain development. The brain is especially rich in type II iodothyronine 5′-deiodinase (41). The demonstration that augmented type II 5′-deiodinase activity in brains of hypothyroid animals serves to preserve the level of intracellular T_3 strongly suggests that the interaction of T_3 with specific nuclear receptors is a critical step in mediating thyroid hormone action in brain. However, when the level of plasma T_3 is raised sufficiently, the nuclear receptors can be readily saturated (42), a finding that demonstrates that T_4 is not an obligatory precursor in the generation of brain nuclear T_3. In the adult rat, van Doorn *et al.* (43) estimated that as much as 65% of T_3 in the cerebral cortex and 50% in cerebellum may be generated by conversion of T_4 to T_3. However, Crantz *et al.* (44) had earlier reported that even higher proportions of nuclear T_3 (cerebrum, >80%; cerebellum, 67%) were generated as a result of local production. Ruiz de Ona and colleagues (45) demonstrated in the rat that whereas fetal brain T_4 levels rose in parallel with plasma T_4 during the latter days of gestation, T_3 in fetal brain rose 18-fold, six times more than the 2-fold change in plasma T_3. This is also a period of increasing type II 5′-deiodinase activity in brain (45). It is likely, therefore, that also in fetal brain the bulk of T_3 is the product of local monodeiodination of T_4.

B. The role of maternal thyroid hormone in fetal brain development: direct or indirect?

An important related issue is the role of maternal hormone in fetal brain development. Complicating this issue is the variable extent of placental permeability to thyroid hormones among the species studied. Such differences result from structural features of the placentas as well as variability in concentrations of placental iodothyronine deiodinase activities (10, 46, 47). Early studies suggested that maternal hormone could not cross the placenta. However, more recent evidence (48, 49) has clearly demonstrated transplacental transport of thyroid hormones in rat. T_4 and T_3, all derived

from the mother by transplacental passage, have been detected in rat embryos before the start of secretion of the fetal thyroid on embryonic day 17 (48, 50, 51). In contrast, there is only very limited transfer of maternal hormone to the fetus in sheep (10). A recent study by Vulsma *et al.* (52) suggested that in human fetuses with congenital hypothyroidism, maternal-fetal transfer of T_4 may result in fetal plasma T_4 levels 25–50% of those in normal infants. Since type II 5′-deiodinase activity in brain increases in response to lowered concentrations of T_4 (53, 54), these levels of T_4 might suffice as substrate to maintain normal or near normal T_3 concentrations in brain but not in other tissues (55). This would account for the finding that most of these children will have normal intellectual development if treated promptly at birth.

In a recent review of the literature Porterfield and Hendrich (56) pointed out that brain development in the rat pup at birth is equivalent to that observed in the human fetus at 5 to 6 months gestation, and the rat at postnatal day 10 is comparable to the human baby at birth. Care should, therefore, be exercised in applying findings with respect to the role of maternal hormone in fetal brain development in one species to any other.

In the rat, Dussault and Coulombe (57) initially estimated that placental transfer of maternal T_4 is less than 1% of the fetal T_4 production rate. The more recent studies of Morreale de Escobar and her colleagues (48, 58), however, argue that physiologically significant amounts of T_4 and T_3 are in fact transferred to the fetus from the earliest stages of gestation. As much as 20% of fetal T_4 derives from transplacental transfer even after the fetal thyroid has begun to secrete hormone on fetal day 17. By sensitive RIA, Obregon *et al.* (48) found measurable levels of T_4 and T_3 in rat embryotrophoblasts as early as days 9 to 12, whereas the fetal thyroid first secretes T_4 about day 17. Hormone concentrations in the fetus up to day 17 were below detectable levels if the mother had been thyroidectomized.

What remains uncertain is the developmental role of the maternal transmission of thyroid hormone. Studies by Morreale de Escobar and her colleagues (45, 59) showed that despite low levels of plasma hormone in thyroidectomized dams, both T_4 and T_3 levels were normal from day 17 to day 22 of gestation in the brains and plasma of fetuses with normally functioning thyroids. Although one might expect that failure of maternal to fetal hormone transfer would elicit an increase in fetal pituitary TSH secretion (59a), TSH levels near term were also unaffected by maternal hypothyroidism (49, 59). However, others have reported evidence of increased fetal TSH (60, 61). An increase in TSH would suggest that the fetal thyroid is stimulated to increase hormonal secretion to compensate for loss of the maternal contribution. Consistent with the lack of effect of maternal hypothyroidism on T_3 and T_4 levels in the fetal brain was the absence of detectable change in type II 5′-deiodinase activity, which is already sensitive to T_4 levels at this time (45).

These results suggest that loss of the maternal contribution of hormone may not affect brain maturation during this stage of development as long as the fetal thyroid is functioning normally. Nevertheless, these fetuses had lower body and brain weights and reduced brain protein and DNA concentrations (50). Bonet and Herrera (61), however, have shown that retardation of fetal body and brain growth was only apparent if maternal hypothyroidism is present during the first half of pregnancy when the dam is undergoing marked metabolic changes associated with the onset of pregnancy. No such effect was noted if hypothyroidism was induced during the second half of pregnancy. In a series of papers, Porterfield and Hendrich (62–64) examined the effect of maternal thyroidectomy on various biochemical parameters in the fetal brain. They found that administration of GH to the thyroidectomized dams during the last days of gestation generally mitigated any of the observed deficiencies in brain growth, galactolipid accumulation, or carbohydrate utilization. They also found that GH treatment of the hypothyroid dams prevented the behavioral deficits otherwise observed in their progeny (65) and concluded "... the major factor responsible for the CNS developmental abnormalities (in progeny of hypothyroid dams) is the altered flow of nutrients or substrate to the fetus." These findings thus raise the basic question as to whether the adverse effects of maternal hypothyroidism result from a reduction in maternally transmitted T_4 to the fetus or whether the effects of maternal hypothyroidism result indirectly from the untoward effects of hypothyroidism on one or more gestational processes.

A report by Narayanan and Narayanan (66) indicating that maternal hypothyroidism retards the appearance of the mesencephalic nucleus of the fifth cranial nerve on gestational days 9 to 11 has yet to be confirmed. If these studies were confirmed, one would have at hand a potentially interesting model with which to further investigate the possible direct role of thyroid hormone action in early brain development. Such studies should be accompanied by efforts to define at a molecular level specific genes that are directly regulated by thyroid hormone during early stages of neural development. This would allow a distinction to be made between direct effects of deprivation of maternally derived thyroid hormone and indirect consequences resulting from the metabolic and nutritional deficiencies associated with maternal hypothyroidism.

Consistent with evidence of very limited placental transfer of thyroid hormone in the sheep (10), Potter *et al.* (67) found that at 50 days of gestation, before onset of fetal thyroid gland function, fetuses carried by thyroidectomized ewes exhibited normal body and brain weights. Yet, despite normal fetal plasma hormone levels throughout the remainder of gestation, there was a transient reduction in the rate of brain growth in midgestation and a lower rate of body weight gain throughout the latter half of gestation. Histological examination of the brain at late gestational stages, however, found no developmental or structural defects associated with the reduced brain weight. The findings in sheep, therefore, support the thesis that maternal hypothyroidism may indirectly result in delayed growth of the fetal carcass and brain, this despite normal fetal thyroid hormone levels. Normal differentiation of the brain will, nevertheless, be achieved so long as normal fetal thyroid function is present.

More recently, several additional reports have pointed to potential effects of fetal hypothyroidism on aspects of brain development. Das and Paul (68) found the β-adrenergic receptor concentration to be decreased in cerebral astrocytes. The level of the short form of the Gαs protein was reduced

(69), although most other G proteins were unaffected. Vega-Nunez *et al.* (70) observed a decrease in the mitochondrial 16S rRNA in the fetal brain but not of other mitochondrial RNAs. Since the mitochondrial genome is transcribed as a single transcript, this raises a question as to the possible role of thyroid hormone in processing of the separate RNAs. However, since in each of these studies, maternal as well as fetal hypothyroidism was induced by treatment with goitrogens, some uncertainty exists as to whether these effects are due directly to fetal hormone deficiency or to maternal hypothyroidism.

C. Intracerebral transport

There has been considerable interest in the mechanisms involved in intracerebral transport of T_4. The plasma T_4-binding protein transthyretin also is the major binding protein in cerebrospinal fluid (71). Synthesized in the choroid plexus, transthyretin has been postulated to facilitate the transport of T_4 across the blood-brain barrier to the brain (72, 73). Mendel *et al.* (74), however, have challenged this thesis by showing normal brain distribution of T_4 in rats injected with EMD 21388, a drug that blocks the binding of T_4 to transthyretin. More recently, Palha and colleagues (75) examined the effect of deletion of the transthyretin gene in mice and found, consistent with the free hormone hypothesis (76), that plasma free T_4 and T_3 were unchanged. Moreover, the brain type II 5′-deiodinase activity was unaltered, a finding also pointing to the euthyroid status of the brain. Thus, transthyretin does not appear to be essential for transport of thyroid hormone into the brain or other tissues.

IV. Molecular Actions of Thyroid Hormone in the Developing Brain

A. Nuclear receptors for thyroid hormone in brain

Most recent efforts to define the mechanism by which thyroid hormone facilitates brain development are based on the assumption that thyroid hormone action proceeds along a nuclear pathway generally similar to that operating in other tissues. The possibility that thyroid hormone action may also proceed by extranuclear routes, however, continues to be explored (77). We will consider one of these proposals (78, 79) subsequently.

Specific nuclear receptors for T_3 in adult rat brain were first identified by *in vivo* saturation analysis after the intravenous injection of ^{125}I-labeled T_3 together with increasing concentrations of unlabeled T_3 (80). Subsequently, *in vitro* techniques were developed to detect and quantitate receptors by analyzing T_3 binding in isolated whole nuclei or in nuclear extracts (81–83). These made it possible to detect receptors in the brains of fetal rats (39), sheep (84), and humans (85). The recognition that these sites exhibited high affinity and specificity for biologically active thyroid hormone analogs (86) strongly suggested that these structures represented the site of initiation of thyroid hormone action.

Studies by Schwartz and Oppenheimer (39) identified similar sites in the brains of late gestational rats. They noted a transient surge in concentration as plasma T_3 levels rise shortly after birth. Perez-Castillo *et al.* (87) observed that T_3 receptors were present in rat brain as early as day 14 of gestation, several days before the onset of fetal thyroid function. Bradley *et al.* (88) and Mellstrom *et al.* (89), using the more sensitive technique of *in situ* hybridization, were able to demonstrate the presence of early but very localized expression of TRβ in the fetal brain. Both TRβ1 and β2 were detected as early as embryonic day 12.5 in the portion of the otic vesicle that gives rise to the cochlea. It is of interest, however, that reports from several groups (90–92) indicated that the critical period for thyroid hormone action in cochlear development, both anatomic and functional, is limited to the perinatal period from gestational day 18 to postnatal day 5. Thus, these observations further strengthen the inference that the simple presence of TR at any given time in gestation cannot be assumed to connote a concurrent function.

In the sheep, nuclear receptors are evident by 50 days of gestation and are, as in the rat, constant until birth (84, 93) (term, 150 days). Very low levels of nuclear T_3 receptors were measured in human fetal brains during the 10th week of gestation but by the 16th week concentrations increase by about 10-fold (85). Thus, in both the human and sheep fetus, nuclear receptors are already present before the onset of fetal thyroidal secretion of hormone.

B. Thyroid hormone receptor isoforms and their tissue distribution

The affinity of T_3, T_4, and their analogs for the nuclear receptors in all tissues examined parallels their biological potency when due account is taken of differential metabolism (86) and the intensity of the biological reaction elicited is limited by the occupation of the receptors (94). It is clear that T_3 receptors present in fetal, neonatal, and adult rat brain do not differ with regard to their relative affinity for thyroid hormone analogs from the receptors identified in other tissues (87, 95). These observations were initially greeted with surprise since adult brain does not demonstrate responses to the administration of thyroid hormone typically observed in other tissues (37, 39, 96). The nature and mechanisms of the biochemical response of adult brain to altered levels of thyroid hormone are still poorly understood.

An important development in our understanding of thyroid hormone action was the cloning of the two genes coding for structurally related but nonetheless distinctive receptor proteins, designated T_3 receptor-α ($T_3R\alpha$) and T_3 receptor-β ($T_3R\beta$), situated in the human genome on chromosomes 17 and 3, respectively (Table 1) (reviewed in Refs. 77, 97, and 98). Alternate splicing of the initial transcript of the α-gene generates TRα1, which binds T_3, and two closely-related isoforms, collectively designated as TRα2, which do not bind T_3 and the function of which remain obscure. Some investigators have suggested that these receptor variants may compete with the bona-fide receptors and thus attenuate the physiological effects of T_3 (99, 100). Two isoforms also arise from the $T_3R\beta$ gene, $T_3R\beta$1, the mRNA of which is widely distributed in rat tissues, and $T_3R\beta$2, the mRNA of which is highly concentrated in the pituitary. The T_3 nuclear receptors belong to a class of closely related nuclear transacting factors that includes the receptors for the steroid hormones, vitamin

TABLE 1. Concentration of receptor isoforms and mRNA: estimation of the number of receptor isoform mRNA and protein molecules per cell in the adult rat

Tissue	α_1 Receptor			β_1 Receptor			β_2 Receptor	
	Protein	mRNA	Protein / mRNA	Protein	mRNA	Protein / mRNA	Protein	mRNA
Liver	624	1.0	624	3408	4.8	710	816	nm[a]
Cerebrum	1920	15.4	125	912	19.0	48	336	nm
Kidney	480	2.4	200	480	126.0	4	192	nm
Heart	1248	2.3	543	1152	6.9	167	480	nm

[a] Below level of detectability by Northern analysis. Calculations are based on data from Refs. 102 and 110. [Derived from Ref. 77.]

D, retinoic acid, and *cis*-retinoic acid. In common with these, the T_3Rs are characterized by a DNA-binding domain containing two zinc fingers, a carboxyl terminal segment that contains the ligand-binding domain and transactivation domains, and a distinctive amino-terminus whose function remains poorly defined.

Recent estimates of the content of the various T_3R isoform mRNAs and proteins in rat brain and other tissues are summarized in Table 1. Recently, low levels of $T_3R\beta2$ mRNA have also been identified in brain (101, 102). The level of functional receptor protein can be estimated from the difference in T_3 binding capacity in nuclear extracts before and after immunoprecipitation of the individual T_3R isoforms with specific IgG (103). In the case of TRβ2, the level of its mRNA was virtually unmeasurable, yet approximately 10% of the total nuclear binding capacity in brain is accounted for by this isoform. This was also found to be the case in liver, heart, and kidney (102). The $T_3R\beta2$ protein was easily observed to concentrate in nuclei of all these tissues by immunohistochemical analysis (102, 104). It appears possible that the half-time of disappearance of the mRNA is substantially shorter than the corresponding receptor protein. This would result in a situation where a majority of cells could display the receptor protein but the mRNA would be present at any time in only a minority of these cells (105).

C. Interactions of ligand, receptor, and DNA

The past decade has witnessed an extraordinary explosion of insight into the interactions of T_3 and its receptor with other nuclear proteins and specific sequences of DNA. The nature of these interactions are generally believed to constitute the molecular mechanism by which the hormone regulates gene expression. Extensive reviews of this process have recently appeared (77, 106). Briefly, the thyroid hormone receptor binds to DNA sequences generically designated as thyroid hormone response elements (TREs), in the regulatory region of target genes. The TRE characteristically consists of two DNA hexamers, termed half-sites, with the consensus sequence AGGTCA. Often the half-sites form a direct repeat separated by four bases although the orientation of the half-sites to each other and their spacing may vary considerably (106). Although T_3R may occupy the two half-sites as homodimers, available evidence suggests that the T_3R may preferentially form heterodimers on the TRE with receptors for *cis*-retinoic acid or other proteins. The potential for a given DNA sequence to act as a TRE can be verified by demonstrating that it confers responsivity to T_3 regulation upon a reporter gene in transient transfections. More recent investigations have shown that nuclear proteins may influence the expression of target genes by binding to the receptor without themselves binding directly to the DNA (107–109). Since such proteins may function in a positive or negative fashion, they have been designated as "co-activators" and "co-repressors."

D. Ontogeny of thyroid hormone receptor isoforms in brain

The identification of the various receptor isoforms prompted efforts to define the developmental pattern in the appearance of each in brain. Of interest was the finding that Northern analysis failed to show $T_3R\beta$ gene expression in fetal brain (110). Immunoprecipitation studies also failed to show evidence of $T_3R\beta1$ or $T_3R\beta2$ binding activity and indicated that $T_3R\alpha1$ accounted for the total binding capacity in fetal brain (103, 111). As indicated earlier, the work of Bradley *et al.* (88, 112) and Mellstrom and colleagues (89) makes it likely that some TRβ is present early in restricted segments of the brain. A dramatic 40-fold rise in $T_3R\beta1$ mRNA concentration begins at the time of birth (Fig. 1) and reaches maximal levels by postnatal day 10 (110, 113). In contrast, the levels of $T_3R\alpha1$ and $T_3R\alpha2$ mRNA, already high in the prenatal state, increase only transiently by a factor of 2 in the first days after birth. The $T_3R\alpha1$ and $T_3R\alpha2$ mRNA levels subsequently fall to adult levels by postnatal day 15. More recent studies showed that approximately 60% of the total T_3 nuclear binding capacity in the adult rat brain is due to $T_3R\alpha1$, 30% to $T_3R\beta1$, and the remaining 10% to $T_3R\beta2$ (Table 1) (102).

The early neonatal rise in $T_3R\beta1$ is of special interest inasmuch as this rise coincides with the neonatal surge in the level of serum T_3 and accounts for the postnatal rise in total T_3 binding capacity. The simultaneous increase of the ligand and its receptors suggest that both phenomena could be a reflection of a coordinated developmental program. A similar pattern of concurrent increases in the levels of thyroid hormone and of $T_3R\beta1$ have been observed in the chick (114) and amphibian tadpole (115, 116).

The nearly simultaneous rise in $T_3R\beta1$ and serum T_3 raised the possibility that the increase in T_3 *per se* was responsible for the induction of $T_3R\beta1$. Studies by Brown and colleagues earlier noted a similarly coordinated surge in $T_3R\beta$ and serum T_3 levels in tadpoles during normal metamorphosis and precocious administration of T_3 to the tadpoles induced a rise in the $T_3R\beta$ mRNA (115). Competence, the ability of the young tadpole to prematurely metamorphose in response to T_3, correlates with induction of the $T_3R\beta$ mRNA (116). Ranjan *et al.* (117) have more recently demonstrated the presence of

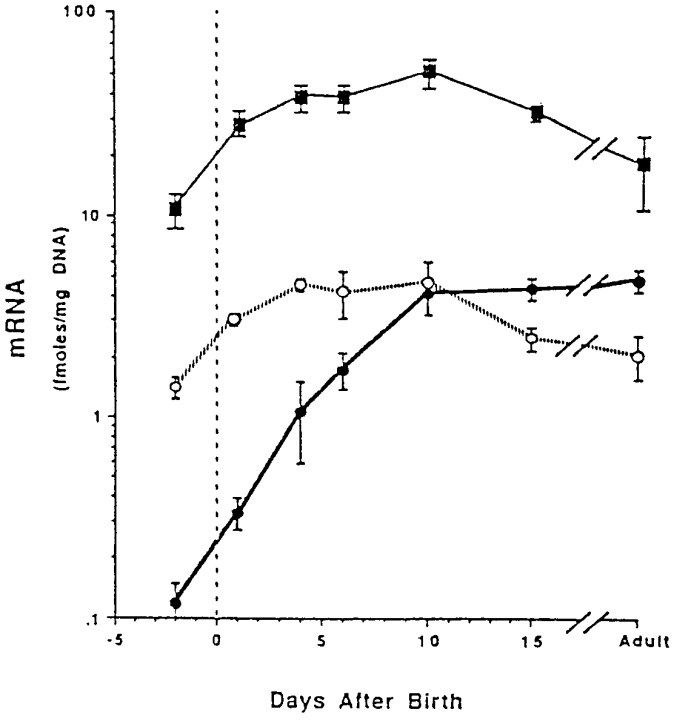

FIG. 1. Thyroid hormone receptor mRNA levels in the developing rat brain. Total RNA was extracted from rat brains at the indicated times, from 19 days of gestation to 2-month-old young adults, and analyzed by Northern blots and solution hybridization. *Open circles*, T3Rα1; *closed circles*, T3Rβ1; and *closed squares*, T3Rα2. [Reprinted with permission from K. A. Strait *et al.*: *J Biol Chem* 265:10514–10521, 1990 (110).]

a TRE in the promoter of the amphibian $T_3R\beta A$ gene. Presumably T_3 induces the expression of this gene through an interaction with $T_3R\alpha 1$. However, in the neonatal rat, a surge in TRβ1 mRNA identical to that in normal pups was demonstrated in pups rendered completely hypothyroid (110). Presumably, in the rat the rise in $T_3R\beta$ mRNA is governed by other developmental factors.

E. Search for T_3-responsive genes in the neonatal rat brain

Despite the abundant data indicating the importance of thyroid hormone in brain development and the presence of all T_3-receptor isoforms in brain, there was and continues to be a surprising lack of information of the specific brain genes regulated by T_3. Muñoz and colleagues (118) have tested the effect of hypothyroidism on a series of mRNAs in the developing brain. One of these, RC3 or neurogranin, has been extensively investigated. This mRNA is of neuronal origin and begins to accumulate between the 5th to 7th postnatal days and reaches maximum levels by day 12. In hypothyroid rat pups, the rate of accumulation was blunted and even at 30 days of age, the mRNA concentration was about half that in normal controls (119). The mechanism of T_3 regulation of this mRNA is unclear since to date a functional hormone response element has not been identified in the promoter region of the gene (120). Two genes, glial fibrillary acidic protein and glutamine synthetase, expressed in astrocytes were unaffected by the change in thyroid state, as was a series of unidentified brain-specific mRNAs. In contrast, levels of mRNA for myelin-associated genes specifically expressed in oligodendroglia, including myelin basic protein (MBP), myelin-associated glycoprotein (MAG), and proteolipid protein (PLP), were all reduced by at least 50% in the hypothyroid brains. Although the concentrations of microtubule-associated proteins (MAP), MAP-1, MAP-2, and tau, are affected by altered thyroid state, the mRNA levels do not change perceptibly (34, 121–123). However, Aniello *et al.* (34) have demonstrated that thyroid hormone can, directly or indirectly, regulate the timing of the splicing mechanism responsible for replacement of juvenile tau mRNA variants with the adult varieties. Some differences are evident among studies with respect to thyroid hormone regulation of nerve growth factor. Whereas all investigators observe changes in the NGF protein levels (124, 125), thyroid hormone is not consistently seen to affect the mRNA concentrations (118, 125, 126).

Thyroid hormone deprivation in neonatal rats was long known to result in a diminished rate of myelin production and the concentration of each of its component proteins, an impairment that can be reversed by thyroid hormone administration (reviewed in Refs. 8 and 9). Studies by Farsetti and colleagues (127, 128) have demonstrated that the gene for myelin basic protein is directly regulated by thyroid hormone. These investigators identified a sequence in the promoter of the mouse MBP gene at position −186 to −169 that was effective in the hormonal transactivation of a reporter gene in NIH3T3 cells. Thyroid hormone also regulates the mRNA levels of other myelin proteins, PLP, MAG, and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (Cnp) (129, 130). However, whereas Tosic *et al.* (129) reported that the hormone increased transcriptional activity of the MAG gene, Rodriguez-Pena and colleagues (130) found no difference in transcriptional rate in normal, hypo-, or hyperthyroid rats and attributed the hormonal effect to stabilization of the newly transcribed mRNA. Bogazzi *et al.* (131) have also described a sequence in the promoter region of the PLP gene that can be activated by thyroid hormone in transient transfection assays.

In an effort to identify potential T_3 target genes, our laboratory examined, by Northern analysis, total RNA from whole brain genes expressed prominently in cerebellar Purkinje cells. Studies by Nordquist *et al.* (132) had already identified three such genes that were also developmentally regulated. The differentiation of cerebellar Purkinje cells is known to be thyroid hormone-dependent and to occur in the early neonatal period (Fig. 2) (30, 31). The genes examined included calbindin, the myo-inositol-triphosphate (IP-3) receptor, and Purkinje cell protein-2 (Pcp-2). The latter codes for a protein of unknown function that is expressed exclusively in Purkinje cells in cerebellum and in the bipolar cells of the retina (132).

The developmental expression pattern of these neuronal genes in neonatal euthyroid and hypothyroid rats was compared with that for the myelin basic protein gene (133). The results of the experiment, illustrated in Fig. 3, show a similar pattern for each of the four genes studied. Hypothyroidism resulted in a substantial delay in the rise of each of the four mRNAs. However, by days 30–40 the same level of gene expression was attained as in the hypothyroid pups supple-

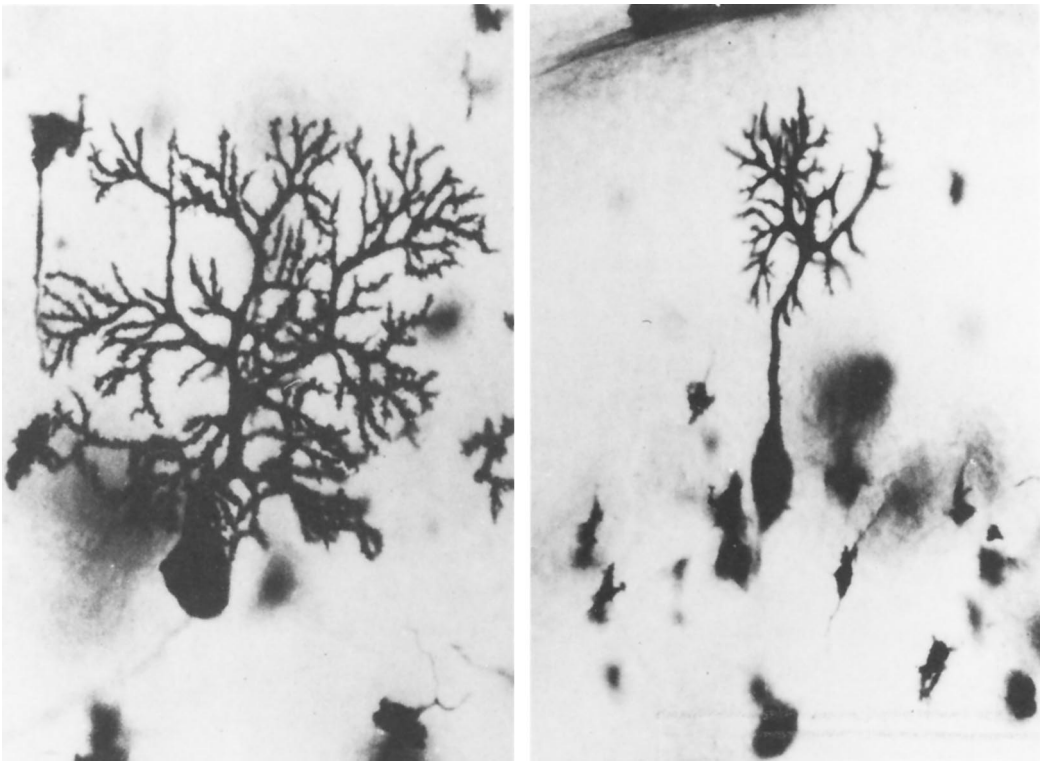

FIG. 2. Morphology of the developing cerebellar Purkinje cell in normal 14-day-old rats (*left*) and in rats made hypothyroid with propylthiouracil from day 18 of gestation (*right*). Note the marked hypoplasia of the dendritic tree in the hypothyroid rat. [Reprinted with permission from J. Legrand: *Thyroid Hormone Metabolism*. Marcel Dekker, 1986 (9).]

mented with T_3 despite maintenance of the hypothyroid state. These observations underscore the importance of timing in normal brain development since the profound deficit noted in Purkinje cell maturation (30, 31) in hypothyroid pups occurs despite ultimate normalization of the expression of these genes.

The data presented in Fig. 3 suggest that gene expression in response to T_3 is characterized by three phases: Phase 1, a refractory state that exists throughout the prenatal period in the rat; Phase 2, the T_3 responsive period from postnatal day 3 to postnatal day 20; and Phase 3, starting after day 20 during which gene expression is independent of T_3 regulation. This may not, however, be typical of all genes regulated by T_3.

Other experiments have attempted to define the mechanisms by which thyroid hormone regulates the expression of these genes. Studies in our laboratory have examined the gene coding for Pcp-2. Since expression of this gene in brain is confined to Purkinje cells, Pcp-2 appeared to be suitable as a model of neuronal as contrasted to glial gene expression. Initial transfection assays using constructs with the native Pcp-2 promoter showed only weak T_3-induced augmentation of gene expression (133). Subsequent studies, however, demonstrated that a potential TRE sequence consisting of three half-sites ligated to a heterologous promoter conferred T_3 responsivity on the reporter gene (134). Further, a sequence of 68 bp immediately 3′ of the TRE appeared to play an important role in silencing the response of the gene to T_3 (Fig. 4). Excision of the 68-bp sequence from the native Pcp-2 promoter resulted in robust T_3-mediated regulation of gene expression (135). More recent studies suggest this sequence may bind an inhibitory nucleoprotein that serves to restrain premature gene regulation by T_3 in the late fetal stages (135). Since the sequence did not result in attenuation of basal transcription, we have designated the 68-bp sequence as a response silencer region.

The availability of primary cultures of oligodendrocytes has also made it possible to examine the molecular basis of hormonal regulation of the myelin basic protein gene in defined culture conditions (136). When the precursors of oligodendroglia, O-2A cells, are cultured in the presence of thyroid hormone, they undergo differentiation and begin to express the MBP gene. In the absence of thyroid hormone, the increase in expression of MBP is delayed by 2–3 days in culture. MBP mRNA levels eventually achieve the same values as those attained in the presence of T_3. The pattern of appearance of T_3R isoforms also simulates that observed in whole brain. High levels of TRα1 mRNA are already present in the undifferentiated O-2A cell. The capacity of the cultured cell to respond to T_3 with enhanced MBP gene expression coincides with the appearance of $T_3R\beta1$ mRNA on day 2 of culture. Transactivation studies in the cultured oligodendrocytes confirmed that the T_3 effect is mediated by the TRE first characterized by Farsetti *et al.* (128). This model thus replicates, in a striking fashion, the pattern of response of MBP observed in the brain *in vivo*. During phase 1 in culture, the O-2A cell does not synthesize MBP nor is the MBP gene responsive to T_3. During differentiation (phase 2), T_3 accelerates the rate of expression of the MBP gene. In phase 3, the mature oli-

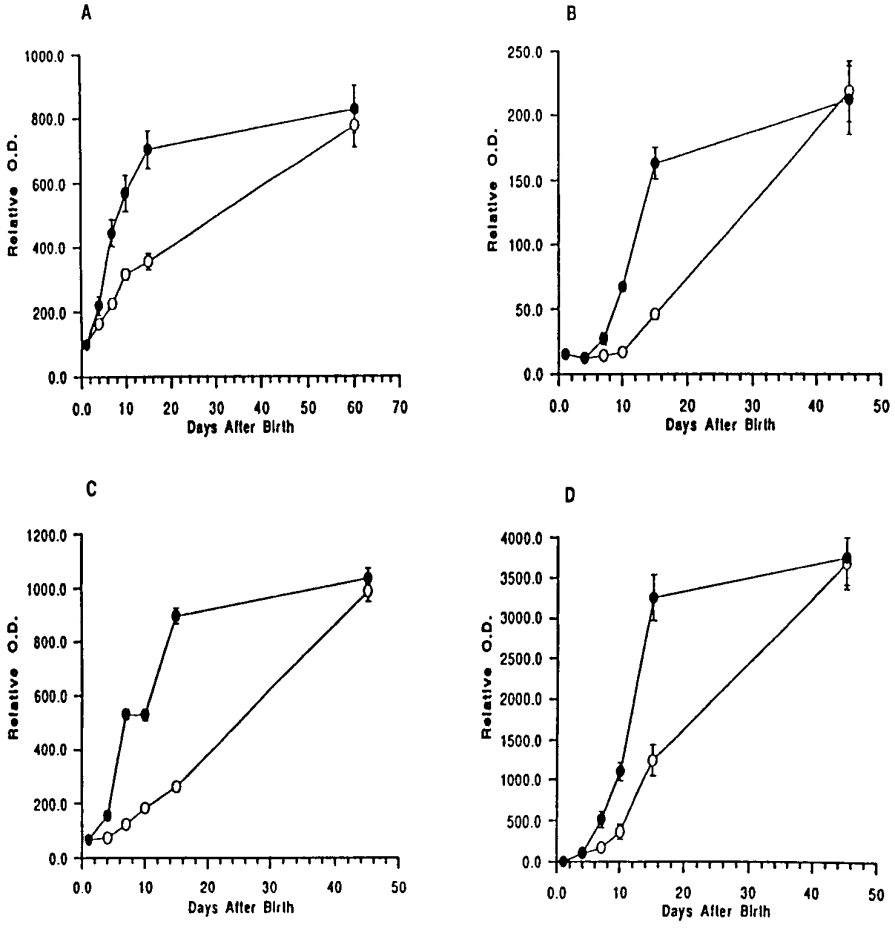

FIG. 3. Response of four brain mRNAs to hypothyroidism in the neonatal rat. A, Calbindin; B, IP3 receptor; C, PCP-2; and D, myelin basic protein. PCP-2 is expressed exclusively in cerebellar Purkinje cells and myelin basic protein, exclusively in oligodendroglia that are widely distributed throughout the central nervous system. Calbindin and IP3 receptor both are preferentially but not exclusively expressed in cerebellum. Measurements were made in aliquots of total RNA from whole brain. *Open circles*, hypothyroid animals; *closed circles*, hypothyroid pups treated with 0.1 μg T_3/day from birth. The T_3-treated hypothyroid pups showed a nearly identical pattern of expression to that seen in untreated euthyroid pups. [Reprinted with permission from K. A. Strait *et al.*: *Mol Endocrinol* 6:1874–1880, 1992 (133). © The Endocrine Society.]

godendrocyte no longer responds to T_3 but MBP synthesis occurs at maximal rates independent of the presence or absence of the hormone. Perhaps, as in the case of other brain genes, the role of T_3 in the maturing oligodendrocyte in culture is to augment the expression of target genes at a predetermined time point in the developmental schedule. Based on preliminary data (see below), we postulate that the appropriate time point at which T_3 acts is determined by the disappearance of suppressor factors that in the fetal state inhibit either basal gene expression or T_3-regulation (Fig. 5).

F. Regulation of gene expression by T_3 through indirect molecular pathways?

The striking similarity in the developmental expression patterns exhibited by the four genes illustrated in Fig. 3 raises the possibility that the regulatory mechanism is identical for each. However, an examination in our laboratory of 1100 bp of the calbindin upstream region has failed to provide evidence for a functional TRE as determined by failure to demonstrate T_3 regulation in transient transfection experiments (D. J. Carlson, K. A. Strait, H. L. Schwaltz, and J. H. Oppenheimer, unpublished data). Although there is precedent for the existence of a TRE this far removed from the start site of transcription (137), these findings raise the possibility that the expression of this gene is mediated by an intermediate agent. The lag time between individual components of the reaction mechanism may be too short to allow recognition of separate pathways. Lindholm *et al.* (138) have presented evidence that T_3 acts only indirectly on Purkinje cell differentiation and calbindin synthesis by causing increased secretion of neurotrophin-3 from the cerebellar granule cells. However, contrary to these findings, Alvarez-Dolado *et al.* (125) failed to observe an effect of hypothyroidism on neurotrophin-3 mRNA concentrations in cerebellum.

The study of the expression of the MBP and Pcp-2 genes suggests that the basic interaction of T_3 with target genes in brain is similar to the action of thyroid hormone in other tissues. However, the identity and function of the direct target genes of thyroid hormone in brain remain largely unknown.

G. Extranuclear actions of T_4?

There continues to be interest in the possibility that thyroid hormone exerts extranuclear effects. In general, evidence supporting this hypothesis appears sparse (77). In the case of the brain, however, there is one action of thyroid hormone that clearly appears to be independent of nuclear mechanisms, regulation of the type II iodothyronine 5′-deiodinase activity (41). This enzyme plays an important role in defending the brain against the effects of

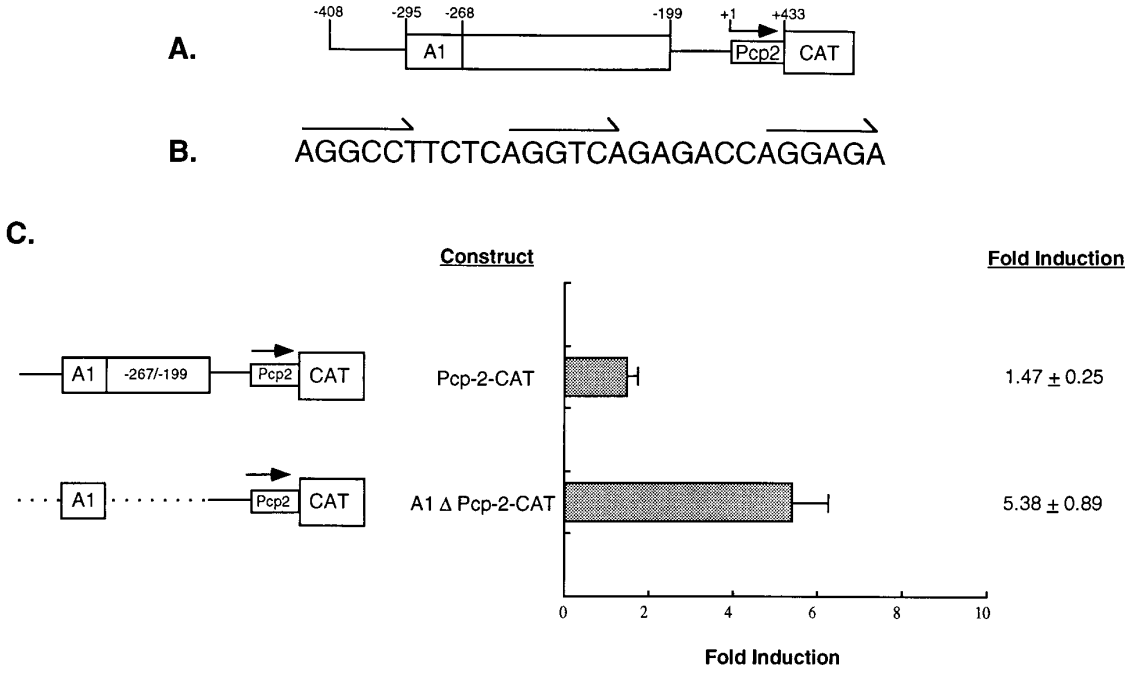

FIG. 4. Localization of the thyroid hormone response element (TRE, −295/−268) and response silencing region (RSR, −267/−199) in the Pcp-2 gene. A, Schematic representation of the Pcp-2 promoter region ligated to the CAT reporter gene. B, Sequence of the potential TRE, A1, at −295/−268 bases upstream of the Pcp-2 start site of transcription. The exact positions and orientations of the three half-sites are indicated. C, In transfection assays a construct containing the entire Pcp-2 promoter ligated to a CAT reporter (Pcp-2-CAT) shows minimal response to T_3. The construct from which the RSR (−267/−199) was deleted (A1 Δ Pcp-2-CAT) responded to T_3 with a several fold greater transcription rate. Fold induction is defined as the transcription rate in the presence of T_3 relative to that in the absence of T_3.

iodine deficiency and hypothyroidism. Studies by Leonard and his colleagues of the regulation of this enzyme by T_4 both *in vivo* (53, 54) and in cultured astrocytes (78) demonstrated that the action of the hormone is quite rapid and is not blocked by cycloheximide or actinomycin D. T_4 regulation was disrupted by cytochalasin B (139), suggesting that down-regulation of enzyme activity by T_4 required an intact actin cytoskeleton. Their studies also showed that T_4 acted to maintain the polymerization state of the actin. They propose that in the presence of T_4, type II 5′-deiodinase binds to F actin, after which the enzyme is internalized by way of the microfilamentous cytoskeleton and targeted to an endosomal pool (79, 140). Since cellular migration, neurite outgrowth, and dendritic spine formation are dependent on interactions of the actin cytoskeleton with other cellular proteins (141, 142), Leonard and colleagues suggest that the action of T_4 on the actin cytoskeleton may also influence these aspects of neuronal differentiation and, consequently, cell-cell interactions in brain. Actin polymerization in the cerebellum is reduced in the hypothyroid rat and restored by T_4 treatment; however, there are contradictory reports (143, 144) as to whether the kinetics of response to hormone *in vivo* are consistent with those observed in cell culture. Further efforts are required to establish that similar mechanisms may be operating *in vivo* and *in vitro*.

V. Theories and Speculations

Despite the efforts of many investigators, our understanding of how thyroid hormone regulates normal brain development remains fragmentary and provisional. Nevertheless, it may be possible to make some educated guesses. Given the widespread distribution of thyroid hormone receptor isoforms in vertebrate brains and the lack of compelling evidence to the contrary, it appears reasonable as a first approximation to posit that the initial action of thyroid hormone in stimulating brain development is mediated through an interaction of the T_3-receptor complex with specific gene targets. However, one is struck by the paucity of direct target genes that have been identified in brain to date and by the existence of genes, such as calbindin and RC3, the expression of which is augmented by T_3 but for which no conventional TRE has been demonstrated. It appears entirely possible that T_3 regulates the expression of such genes indirectly through regulation of the synthesis of rapidly turning over transacting nucleoproteins.

The role of TRα1 in the prenatal rat central nervous system remains unclear. It is possible that TRα1 may have a ligand-independent function in early brain development (145). The dramatic increase in circulating T_3 in the early neonate results from augmented pituitary TSH production, increased thyroidal T_4 secretion, and increased peripheral T_4 monodeiodination. The almost simultaneous rise in serum T_3 and the level of brain TRβ1 in contrast to the near constancy of TRα1 originally suggested to us that the T_3Rβ1 isoform mediated the developmental effects of T_3. In recent collaborative studies with Forrest *et al.*, who developed a T_3Rβ null strain of mice (146), we observed no significant differences between mutant and wild type mice with respect to the developmental expression of the Pcp-2 and MBP genes (147). Forrest *et*

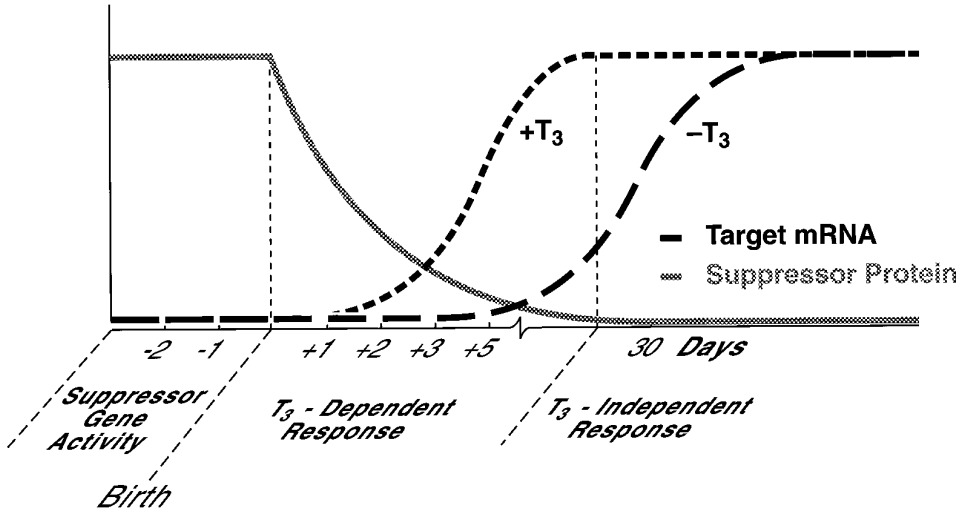

FIG. 5. Proposed model of the transient responsivity of brain genes to thyroid hormone during brain development in the neonatal rat. During the prenatal period expression of the target gene and its response to T_3 is suppressed due to the presence of nuclear protein(s) *(solid gray curve)*. The concentration of this protein declines after birth allowing both basal expression ($-T_3$) and regulation by T3 ($+T_3$) to occur. Although delayed, at about 1 month of age gene expression reaches normal adult levels even in the absence of thyroid hormone. The factors driving gene expression in this period are undefined.

al. (146) had earlier demonstrated that the absence of the $T_3R\beta$ did not result in deficiencies in most behavioral or neuroanatomical parameters. Clearly, in the mouse the $T_3R\beta1$ does not appear to be essential in mediating the postnatal stimulation of the two target genes examined, Pcp2 and MBP, or of brain morphogenesis. These conclusions are consistent with the clinical observations that patients with the thyroid hormone resistance syndrome due to homozygous deletion of the $T_3R\beta$ gene demonstrate normal mental development (148). However, the coordination of the rise in T_3 and $T_3R\beta1$ could represent an effort to increase the number of available receptors for the optimal development of specialized systems such as the cochlear-vestibular apparatus. The studies by Forrest *et al.* (149) demonstrated that although the absence of $T_3R\beta$ did not affect cochlear morphogenesis, these animals do suffer a major deficiency in auditory function. This is also consistent with the presence of deaf-mutism found in patients with thyroid hormone resistance due to deletion of the $T_3R\beta$ gene (150).

Although it is clear that the postnatal surge in thyroid hormone concentration in the rat is essential for normal brain development, it was not known whether premature increase in T_3 would result in early expression of responsive genes or other evidence of precocious development. We recently observed that injection of large doses of T_4 into pregnant rats on gestational day 15, sufficient to raise the fetal brain T_3 to adult levels for the remainder of pregnancy failed to elicit premature expression of the Pcp-2, calmodulin kinase IV, and myelin basic protein genes (151). Nor were there detectable changes at gestational day 21 in morphological development of the cerebellum in the fetuses with increased brain T_3 levels or in fetuses made hypothyroid by methimazole treatment from day 14 of gestation. These findings suggest that yet-to-be defined cellular processes render the cells responsive to thyroid hormone action in the postnatal period. Moreover, in rat fetuses treated with excess T_3 or with methimazole only in the last 6 days of gestation, there was no effect on brain weight, DNA, RNA, or protein content (151). However, it is important to emphasize again the importance of the differences in the developmental patterns of rat, sheep, and human. As pointed out previously, the T_3-sensitive postnatal period of brain development in the rat occurs during intrauterine gestation in humans and sheep. Thus, alterations in the thyroid status of the human fetus during the latter half of pregnancy may have important developmental ramifications. In the sheep, thyroidectomy *in utero* leads to major deficiencies in morphogenesis (16, 17).

Barres *et al.* (152) have pointed out that proliferating oligodendrocyte precursors, O-2A cells, require antecedent development before they are susceptible to the differentiating effects of T_3. In the case of the O-2A precursors, sensitivity to T_3 is established only after the cells undergo at least several cycles of division. The changes we have observed in the developing brain may well fit an analogous pattern.

The nature of the developmental factors that allow target genes to become sensitive to the action of T_3 remains unclear. One could postulate the developmental expression of a cofactor required for thyroid hormone effectiveness. Alternatively, suppressor mechanisms could operate in the fetal brain to prevent thyroid hormone-induced effects. Preliminary studies have identified one of these suppressor proteins as the orphan receptor, COUF-TF. Gel shift studies in our laboratory (158) have revealed nuclear proteins that bind to the upstream promoter region of the T_3 target genes, Pcp-2 and MBP. These proteins rapidly disappear at about the same time that these genes become sensitive to the action of thyroid hormone.

The mechanism responsible for the later ligand-independent expression of genes that are responsive to T_3 early in the postnatal period remains unclear. Studies of the MBP promoter transfected into cultured oligodendrocytes showed that the ligand-independent effect is abolished when the TRE is mutated (136). This raises the possibility that the late-phase MBP gene may be induced by the unliganded receptor or another transacting factor capable of binding to the TRE.

In this context, it is of interest to contrast these findings with the role of thyroid hormone in amphibian metamorphosis (reviewed in Ref. 153). In the rat, differentiation, however defective, continues despite delayed growth in the absence of T_3. Moreover, there is no evidence for a premature response of brain target genes to the early administration of T_3 (151). To the contrary, Gudernatsch (154, 155) first showed

that premature administration of thyroid hormone to tadpoles caused them to undergo early metamorphosis. Although thyroidectomy blocks metamorphosis, tadpoles continue to grow to an extremely large size (156, 157). It, therefore, appears that there is a fundamental difference in the mechanisms by which T_3 affects amphibian metamorphosis and those regulated by T_3 in mammalian brain.

VI. Conclusions

Our understanding of the effect of thyroid hormone on brain development at the molecular level is still rudimentary and clearly not proportionate to the importance of the subject. The evidence reviewed here does not allow us to determine with confidence whether the adverse effects of maternal hypothyroidism on fetal development are mediated directly by loss of the maternal hormone contribution to the fetus, indirectly by metabolic impairment of gestation, or both. Resolution of this issue will require additional evidence at a molecular level either demonstrating a direct action of the hormone on the fetal brain or additional evidence supporting the suggestion that the observed effects of maternal hypothyroidism on fetal development are explained by impaired gestation. Preliminary findings in the rat raise the possibility that suppressor proteins may block response to thyroid hormone during the intrauterine period, and that such suppressor proteins decrease shortly after birth. In the rat, by postnatal day 20 to 30, those brain genes that are transiently regulated by T_3 in the early postnatal period may function quite independently of T_3. The mechanism underlying T_3-independent gene regulation is unknown but appears, at least in the case of MBP, to require a functional TRE.

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