Brain glucose utilization in mice with a targeted mutation in the thyroid hormone α or β receptor gene

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Brain glucose utilization is markedly depressed in adult rats made cretinous after birth. To ascertain which subtype of thyroid hormone (TH) receptors, TRα1 or TRβ, is involved in the regulation of glucose utilization during brain development, we used the 2-[^{14}C]deoxyglucose method in mice with a mutation in either their TRα or TRβ gene. A C insertion produced a frameshift mutation in their carboxyl terminus. These mutants lacked TH binding and transactivation activities and exhibited potent dominant negative activity. Glucose utilization in the homozygous TRβPV mutant mice and their wild-type siblings was almost identical in 19 brain regions, whereas it was markedly reduced in all brain regions of the heterozygous TRα1PV mice. These suggest that the α1 receptor mediates the TH effects in brain. Inasmuch as local cerebral glucose utilization is closely related to local synaptic activity, we also examined which thyroid hormone receptor is involved in the expression of synaptotagmin-related gene 1 (*Srg1*), a TH-positively regulated gene involved in the formation and function of synapses [Thompson, C. C. (1996) *J. Neurosci.* 16, 7832–7840]. Northern analysis showed that Srg1 expression was markedly reduced in the cerebellum of *TR$\alpha^{PV/+}$* mice but not *TR$\beta^{PV/PV}$* mice. These results show that the same receptor, TRα1, is involved in the regulation by TH of both glucose utilization and Srg1 expression.

cerebral glucose utilization | 2-[^{14}C]deoxyglucose | cretinism | synapse

Thyroid hormone is essential for normal postnatal growth and development of the nervous system (1–6). Neonatal hypothyroidism impairs development of virtually all tissues, but the most prominent deficits are observed in brain. Neuronal differentiation and axonal and dendritic outgrowth are delayed, and myelination is reduced. These morphological changes are accompanied by deficits in electrical, behavioral, and cognitive functions. In cretinism, for example, learning and memory are severely impaired. In rats, this array of functional deficits is, at least, partially prevented if thyroid replacement therapy is performed within the first 3–4 weeks of life.

Maturation of structure and function in the mammalian brain is normally accompanied by profound increases in local rates of glucose utilization (7, 8), but in rats made cretinous by radiothyroidectomy within the first 2 days after birth and studied later in adulthood, local cerebral glucose utilization (CMR_{glc}) in all brain regions examined was found to be depressed below values in euthyroid controls by 28–58% (9). The greatest decreases were found in the cerebral cortex and throughout the auditory system. Lesser changes were seen in hypothalamic regions, including those involved in the synthesis of thyrotrophin-releasing hormone (TRH). Numerous applications of the 2-[^{14}C]deoxyglucose method (10) have demonstrated a close correlation between local neural functional activity and local CMR_{glc} (11). The findings in cretinism suggest, therefore, an association between the structural, functional, and biochemical abnormalities and the widespread reductions in energy metabolism throughout the brain.

The action of the thyroid hormone, L-triiodothyronine (T3), is mediated by thyroid hormone receptors (TRs), which are ligand-dependent transcription factors (12, 13). Three ligand-activated nuclear TR isoforms have been identified, TRα1, TRβ1, and TRβ2, which are produced by alternative splicing of the primary transcripts of the TRα and TRβ genes, respectively. Recently, TRβ3 and TR$\Delta\beta$3 isoforms have been identified but have limited expression in the brain (14). Another spliced product of the TRα gene, TRα2 (15), resembles the viral v-*erb*A oncogene (16) because both molecules do not bind to thyroid hormone.

Each TR form has a unique developmental and tissue-specific expression (17, 18). They bind to specific DNA sequences known as thyroid hormone response elements (TRE) that are located in the promoter regions of the T3 target genes (19). A TRE has also been found in the first intron of the rat RC3/neurogranin gene (20). The TR–T3 complex binds to the TRE together with other coregulatory proteins that function as histone acetyltransferases and serve to remodel the chromatin structure and activate gene transcription (21, 22). In the absence of thyroid hormone, TR binds to TRE together with a complex of inhibitory coregulatory proteins that promote chromatin deacetylation and inhibition of gene transcription (21, 22). The TR–hormone complex activates most genes, although there are other genes, e.g., the thyroid-stimulating hormone (TSH) gene, that are repressed. Thus, the transcriptional activities of TR depend not only on the presence of thyroid hormone but also on the specific TRE involved. TRs bind to TRE as homodimers, TRα/TRβ heterodimers, or heterodimers with other members of the receptor superfamily (17).

Recently, two types of TR mutated mice, i.e., "knockout" and "knock-in," have been developed. In the knockout mice, TR genes are inactivated, and no receptor proteins are produced (23). In contrast, in the knock-in TRβPV mutant mice, TRβPV is produced, which is a protein that has totally lost T3-binding and transcriptional activities. TRβPV mutant mice were generated by a targeted mutation in the last 14 amino acids of the *TRβ* gene (24). The *TRβ* knockout mice show increased TSH and thyroid hormone production and impaired hearing (23). The phenotype of the TRβPV knock-in mice is consistent with that seen in patients with thyroid hormone resistance syndrome (24–26); they exhibit severe dysfunction of the pituitary–thyroid axis, impaired hearing, retarded growth, delayed bone maturation, and abnormal patterns in the expression of T3-targeted

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Abbreviations: T3, L-triiodothyronine; TH, thyroid hormone; TR, thyroid hormone receptor; TSH, thyroid-stimulating hormone; TRE, thyroid hormone response element; TRH, thyrotrophin-releasing hormone; *Srg1*, synaptotagmin-related gene 1; CMR_{glc}, cerebral glucose utilization.

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genes (24). More recently, mice with the same PV mutation targeted in the corresponding position of the *TRα* gene have been generated (TRα1PV mice). The phenotype of the heterozygous TRα1PV mice is clearly distinct from that of TRβPV mice and is characterized by dwarfism, increased mortality, and reduced fertility (unpublished data).

Inasmuch as structural and functional maturation and energy metabolism have been found to be markedly impaired when TH deficiency is established at early postnatal ages (9), we have examined which TR isotype, TRα1 or TRβ, mediates the developmental effects of TH on brain glucose utilization.

Materials and Methods

Generation and Characterization of Mutant Mice. The method for generating the TRβPV mutant mice has been described in detail elsewhere (24). The $TR\beta^{PV}$ gene, which was originally found in a thyroid hormone-resistant patient, has a C insertion at codon 448 in exon 10, resulting in a frameshift of the carboxyl-terminal 14 amino acids of TRβ (25). The mutant mice with the $TR\beta^{PV}$ gene have been found to express TRβPV mRNA in the cerebrum, cerebellum, and pituitary as well as in systemic organs (24). Furthermore, both heterozygotes ($TR\beta^{PV/+}$) and homozygotes ($TR\beta^{PV/PV}$) exhibit increased total L-thyroxine and TSH, enlarged thyroid glands, increased TSH-secreting cells in the pituitary, and retarded growth manifested as slow weight gain and delayed bone development. These abnormalities are more prominent in homozygotes.

The mouse with a targeted mutation in the *TRα* gene, which produces non-TH binding TRα1PV protein, was also generated by knock-in the same PV mutation in the corresponding position of the *TRα* gene (unpublished data).

Animal Preparation. All procedures performed on animals were in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee.

Local cerebral glucose utilization was determined in 4-week-old wild-type mice ($n = 3$) and in mice with either the $TR\alpha^{PV/+}$ ($n = 4$) or the $TR\beta^{PV/PV}$ gene ($n = 3$), as well as in 10-week-old wild-type mice ($n = 4$) or mice with the *TRβ* gene ($n = 3$) to assess the effects of the TRβ mutation in adult mice. All of the mice were maintained on a 12-h light/dark cycle, with humidity and temperature controlled at normal levels and allowed food and water ad libitum. On the day of the experiment, the mice were anesthetized with halothane (5% for induction, 1.0–1.5% for maintenance) in 70% N_2O/30% O_2, and 13-cm polyethylene catheters (PE10; Clay Adams, Parsippany, NJ) were inserted into the left femoral artery and vein. After the skin incision was sutured, a loose-fitting plaster cast was fitted to the lower torso and pelvis and taped to a plastic box to prevent locomotion. At least three hours were allowed for recovery from the anesthesia and surgery before initiation of the measurement of cerebral glucose utilization. Body temperature was maintained by warming of the environment with a heating lamp.

Physiological Variables. Mean arterial blood pressure was measured with a Digi-Med Blood Pressure Analyzer (MicroMed, Louisville, KY). Hematocrit was determined in arterial blood samples centrifuged in a Microfuge B (Beckman Instruments, Fullerton, CA). Arterial plasma glucose content was measured in a Beckman Glucose Analyzer 2 (Beckman Instruments).

Determination of Local Cerebral Glucose Utilization. Cerebral glucose utilization was determined by the quantitative autoradiographic 2-[^{14}C]deoxyglucose method as previously described (10), except for the withdrawal of smaller and less frequent blood samples to avoid excessive blood loss in such small animals.

Northern Blot Analysis. Total RNA (10 μg) was used for Northern blot analysis. After electrophoresis, RNA was transferred onto membranes (Hybond-N+, Amersham Pharmacia), which were hybridized with appropriate probes. cDNA probes for the human wild-type TRα1 and rat synaptotagmin-related gene 1 (*Srg1*) (27) were labeled with [α-^{32}P]dCTP in accordance with a random primer hexamer protocol. For quantification, the intensities of the mRNA bands were normalized against the intensities of glyceraldehyde-3-phosphate dehydrogenase mRNA. Thus, the blots were stripped and rehybridized with ^{32}P-labeled cDNA for glyceraldehyde-3-phosphate dehydrogenase. Quantification of the bands was done with a Molecular Dynamics PhosphorImager.

Statistical Analyses. Physiological variables and local CMR_{glc} in 19 structures of the brain of three groups of young mice—i.e., $TR\alpha^{PV/+}$, $TR\beta^{PV/PV}$, and wild-type mice—were compared by a one-way ANOVA followed by Dunnett's *t* test for multiple comparison against a single control group. Physiological variables and local CMR_{glc} in the adult mice with $TR\beta^{PV/PV}$ mutation and in the adult wild-type mice were compared by a non-paired *t* test.

Results

Expression of the *TRα1PV* mRNA in the Cerebellum and Cerebrum of *TRα1PV* Mice. $TR\beta^{PV}$ gene expression in the brain of the TRβPV mice has previously been described (24). Confirmation of the expression of the $TR\alpha^{PV}$ gene in the brain of $TR\alpha^{PV/+}$ mice was obtained by use of reverse transcriptase–PCR. With a primer pair of 5N and 3N (Fig. 1*A Top*) or 5N and 3PV (Fig. 1*A Middle*), cDNA fragments with sizes of 444 bp or 304 bp, representing the expression of the wild-type and mutant alleles, respectively, were found in the cerebellum and cerebrum (lanes 4 and 5 of Fig. 1*A Bottom*). Expression of the TRα1PV allele was further confirmed by Northern blot analysis with cDNA encoding human TRα1 as a probe (Fig. 1*B*). TRα1PV mRNA was expressed with a size of 1.8 kb (instead of 5.0 kb for the wild-type mice) only in the cerebellum and cerebrum of $TR\alpha^{PV/+}$ mice (lanes 3–4 and 2–3, respectively).

Physiological Variables. Kaneshige *et al.* (unpublished data) noted that $TR\alpha^{PV/+}$ mice are dwarfs, and in the present studies their body weights were less than half that of comparably young wild-type sibling mice ($P < 0.01$) (Table 1). The young mice with $TR\beta^{PV/PV}$ also exhibited slightly but statistically significant ($P < 0.01$) lower body weights than young control mice, but this difference is less prominent in adulthood (Table 1). Mean arterial blood pressure was lower in $TR\alpha^{PV/+}$ and $TR\beta^{PV/PV}$ mice than in young control mice ($P < 0.01$ and $P < 0.05$, respectively), particularly in the $TR\alpha^{PV/+}$ mice. The hematocrit was also slightly lower in the $TR\alpha^{PV/+}$ mice than in the young control mice ($P < 0.01$) (Table 1). There were no statistically significant differences in the physiological variables between the adult groups (Table 1).

Cerebral Glucose Utilization. Local cerebral glucose utilization in all 19 brain structures examined was statistically significantly ($P < 0.01$) lower in the young mice with $TR\alpha^{PV/+}$ than in the young control animals (Fig. 2). The reductions ranged from 57 to 72%. In contrast, young mice with $TR\beta^{PV/PV}$ showed no differences in local cerebral glucose utilization from that in young control mice (Fig. 3). There were also no significant differences in local cerebral glucose utilization between the adult $TR\beta^{PV/PV}$ and adult control groups.

Expression of Srg1 mRNA in the Cerebellum of $TR\beta^{PV/+}$, $TR\beta^{PV/PV}$, and $TR\alpha^{PV/+}$ Mice. Because local cerebral glucose utilization is closely related to neuronal functional activity in synaptic

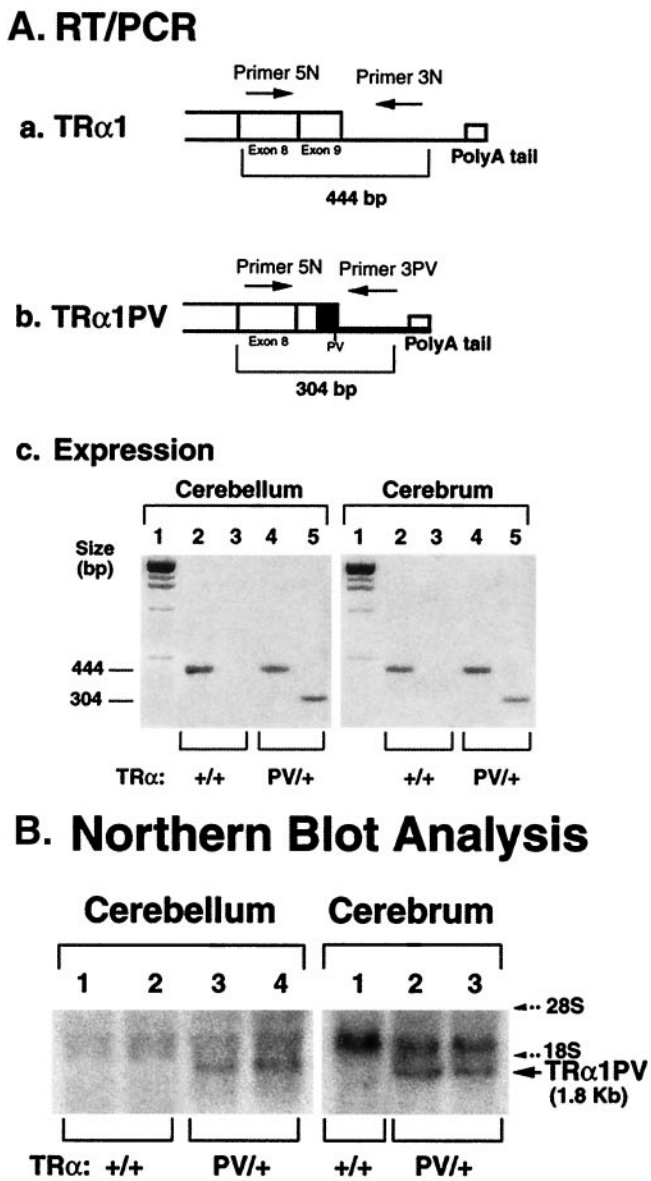

Fig. 1. Expression of the TRα1PV mRNA was analyzed by reverse transcriptase–PCR (*A*) and by Northern blot analysis (*B*). (*A*) RNA was isolated from the cerebellum and cerebrum. Reverse transcriptase–PCR was carried out with the primer pairs of 5N and 3N as shown (*Top*) and 5N and 3PV (*Middle*) for the wild-type TRα1 cDNA and TRα1PV cDNA, respectively. The 444-bp and 304-bp fragments represent the expression of the wild-type and mutant alleles, respectively, as shown (*Bottom*). The genotypes are marked. (*B*) RNA was prepared from the cerebellum and cerebrum of the $TR\alpha^{PV/+}$ mutant mice and their wild-type $TR\alpha^{+/+}$ siblings. Northern blot analysis was conducted with ^{32}P-labeled human TRα1 cDNA as a probe. The size of the mRNA is marked.

regions (47) and cretinous animals exhibit deficiencies in synaptic development and function, we also examined in the cerebellum of the $TR\alpha^{PV/+}$ and $TR\beta^{PV/PV}$ mice the expression of Srg1, a member of a family of proteins involved in the regulation of transmitter release (28). *Srg1* is a T3-positively regulated gene and is normally highly expressed in several regions of the rat brain (27, 29). Srg1 expression was reduced to 40% in the cerebellum of $TR\alpha^{PV/+}$ mice (Fig. 4*A*) but not at all in the $TR\beta^{PV/+}$ and $TR\beta^{PV/PV}$ mice as compared with the wild-type siblings (Fig. 4*B*). These results indicate that Srg1 is repressed selectively in the $TR\alpha^{PV/+}$ mice.

Discussion

Local Cerebral Glucose Utilization in $TR\beta^{PV/PV}$ and $TR\alpha^{PV/+}$ Mice. Mutagenesis of the two TR isoforms, TRα1 and TRβ, in mice have indicated that some effects of TH in tissues may be mediated exclusively by either one or the other and that some effects may require the participation of both simultaneously (23). For example, TRβ mediates both the TH negative feedback of TSH production in the pituitary and the activity of the auditory pathway (23, 30). Basal heart rate appears to be under control of TRα1, but TRβ mediates a TH-induced increase in rate (31, 32). Hypothyroidism, retarded growth, and postweaning lethality are seen in mice deficiency in both TRα1 and TRα2 (23, 33). Regulation of myosin isoform expression in mouse skeletal muscle requires the participation of both TRα1 and TRβ (34). In contrast, the two TH receptors mediate opposite effects on estrogen-stimulated sexual behaviors (35). Thus, the presence of each receptor isoform, their concentrations, and the TRα1/TRβ ratio are probably critical determinants of T3 action (23, 36).

The situation is even more complex in brain. There is a variety of possibilities that depend on which receptor is expressed in which of the many brain structures as well as on the stage of development. *In situ* hybridization and immunohistochemistry have provided information on the distribution of the two receptors in adult and developing rat and chicken brain (37–39). In the rat, the TRα1 receptor is present very early in development and is most abundant during fetal life; in fact, it has been shown to be present in stem cells (40). The TRα1 isoform is normally detectable in all regions of the hippocampus throughout brain development, whereas the TRβ isoform is expressed only in selected regions of this structure. TRβ increases with development in the caudate, nucleus accumbens, CA1 field of the hippocampus, and also in some other regions from birth to adulthood. Thus, the distribution of the TRβ receptor in brain is more restricted, and it is expressed preferentially at later developmental stages compared with the TRα1 isoform.

The present results confirm that TH is involved in the development of those processes in brain that regulate glycolysis or require the energy derived from the utilization of glucose. The data support that this involvement is mediated solely by the TRα1 and not by the TRβ. No changes in glucose utilization were found in any cerebral structures of the $TR\beta^{PV/PV}$ mice including structures of the auditory system, even though it has been reported that TRβ is essential for auditory function in both $TR\beta^{PV/PV}$ knock-in mice and *TRβ* knockout mice (23, 30, 41). In contrast, rates of glucose utilization were markedly decreased in all regions of the brain examined in the $TR\alpha^{PV/+}$ mice, including sensory and auditory cortex, thalamus, amygdala, hippocampus, and cerebellar cortex, regions known to be sensitive to TH deficiency. The reductions ranged from −57 to −72% and are comparable to the −36 to −57% reductions previously found in adult cretinous rats radiothyroidectomized just after birth (11). TH deficiency because of neonatal hypothyroidism has been reported to have more severe systemic effects than TR deficiency produced by double knockout mutation of the α and β isoforms (23, 42), possibly because of TR-independent actions of TH (43).

Isoform-Dependent Action of TR Gene Mutants on Local Cerebral Glucose Utilization. The precise mechanism by which the TRα1PV mutant receptor selectively interferes with the functions of wild-type TRs in brain is not clear. The lack of decreases in local cerebral glucose utilization in the $TR\beta^{PV/PV}$ mice may have been because of the overwhelming excess of TRα1 relative to TRβ in most neurons at early stages in brain development (37–40). Indeed, with a reporter system, mutant TRβPV and TRα1PV have been shown to inhibit the transcriptional activity of the

Table 1. Physiological variables before measurement of cerebral glucose utilization

Animal	Age, days	Body weight, g	Mean arterial blood pressure, mm Hg	Hematocrit, %	Arterial plasma glucose concentration, mM
Young mice					
Wild-type mice ($n = 3$)	32 ± 1	24 ± 0.4	96 ± 3	46 ± 1	12.3 ± 0.7
TR$\alpha^{PV/+}$ mice ($n = 4$)	29 ± 1	9 ± 1**	70 ± 3**	40 ± 0.4**	8.8 ± 1.2
TR$\beta^{PV/PV}$ mice ($n = 3$)	32 ± 1	17 ± 0.3**	84 ± 2*	44 ± 2	9.9 ± 0.9
Adult mice					
Wild-type mice ($n = 4$)	76 ± 3	26 ± 1	98 ± 5	44 ± 4	8.7 ± 0.2
TR$\beta^{PV/PV}$ mice ($n = 3$)	79 ± 3	26 ± 1	90 ± 5	39 ± 3	8.3 ± 0.7

The values are means ± SEM obtained in the number of animals indicated in parentheses. *, $P < 0.05$; **, $P < 0.01$.

wild-type TRs in cultured cells. The extent of the inhibition depended on the ratios of mutant/wild-type receptors (ref. 44 and unpublished data). It is reasonable to assume that the ratios of mutant/wild-type receptor proteins also play a critical role in the phenotypic consequences of mutant receptor genes *in vivo*. TRα1 is more abundantly expressed in most regions of the developing brain than TRβ (40). It has been shown that the level of TRβPV mRNA is similar to that of wild-type TRβ mRNA (24). Therefore, it is expected that the TRα1PV mutant receptor would be more abundantly expressed than TRβPV mutant receptor in the brain of mutant mice. Thus, in *TR$\beta^{PV/PV}$* mice, the ratio of TRβPV/TRα1 is not high enough to adequately interfere with the functions mediated by TRα1. On the other hand in TR$\alpha^{PV/+}$ mice, the TRα1PV/TRα1 ratio is high enough to interfere with the functions of TRα1, leading to the reduction of glucose utilization. We cannot, however, exclude other mechanisms that might explain the selective actions of the mutant TR isoforms on cerebral glucose utilization.

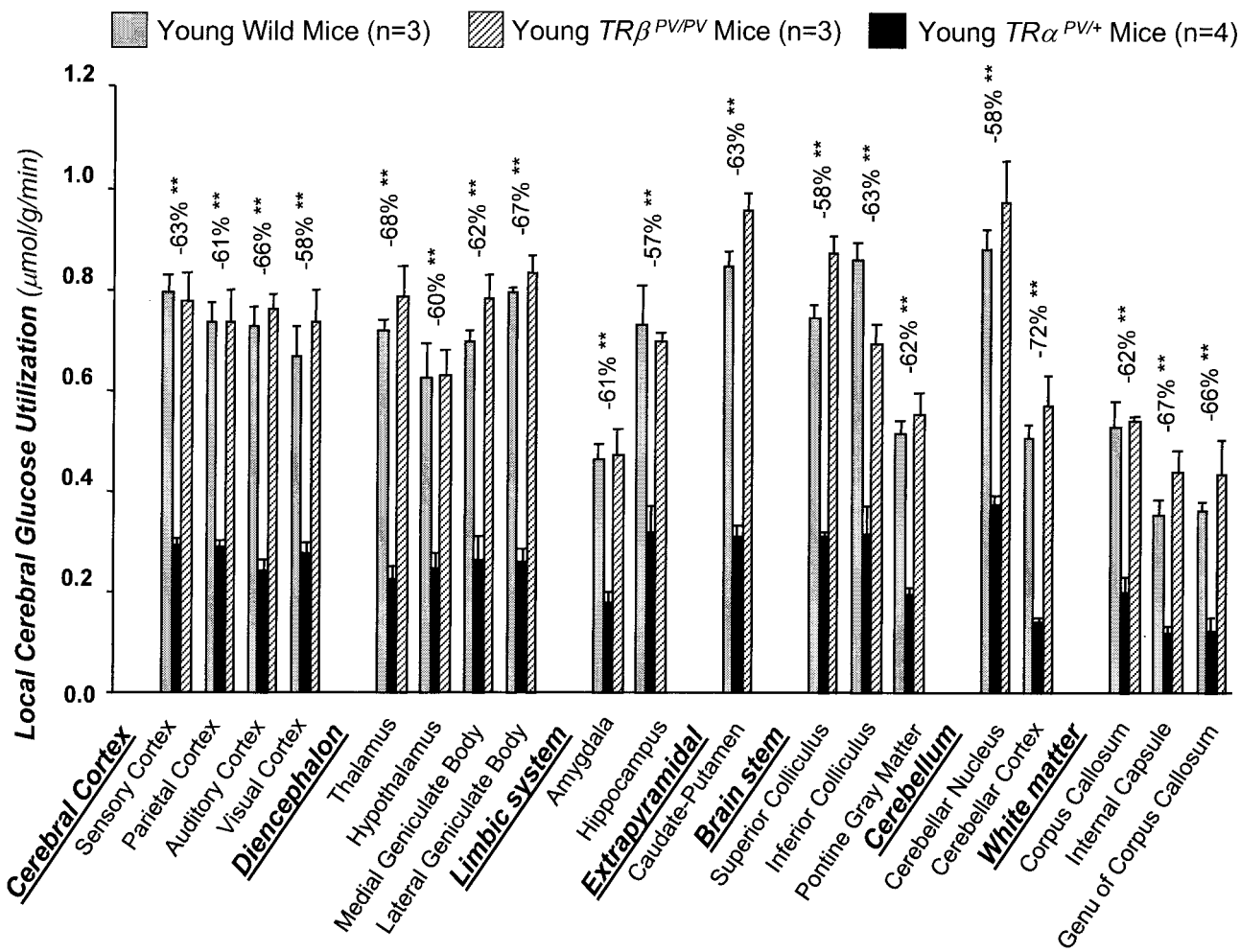

Fig. 2. Local cerebral glucose utilization (μmol/g per min) in 19 brain regions of young wild-type mice, young *TR$\beta^{PV/PV}$* mice, and young *TR$\alpha^{PV/+}$* mice. The percent decrease in glucose utilization measured in the *TR$\alpha^{PV/+}$* mice relative to their wild-type siblings is shown for each brain region.

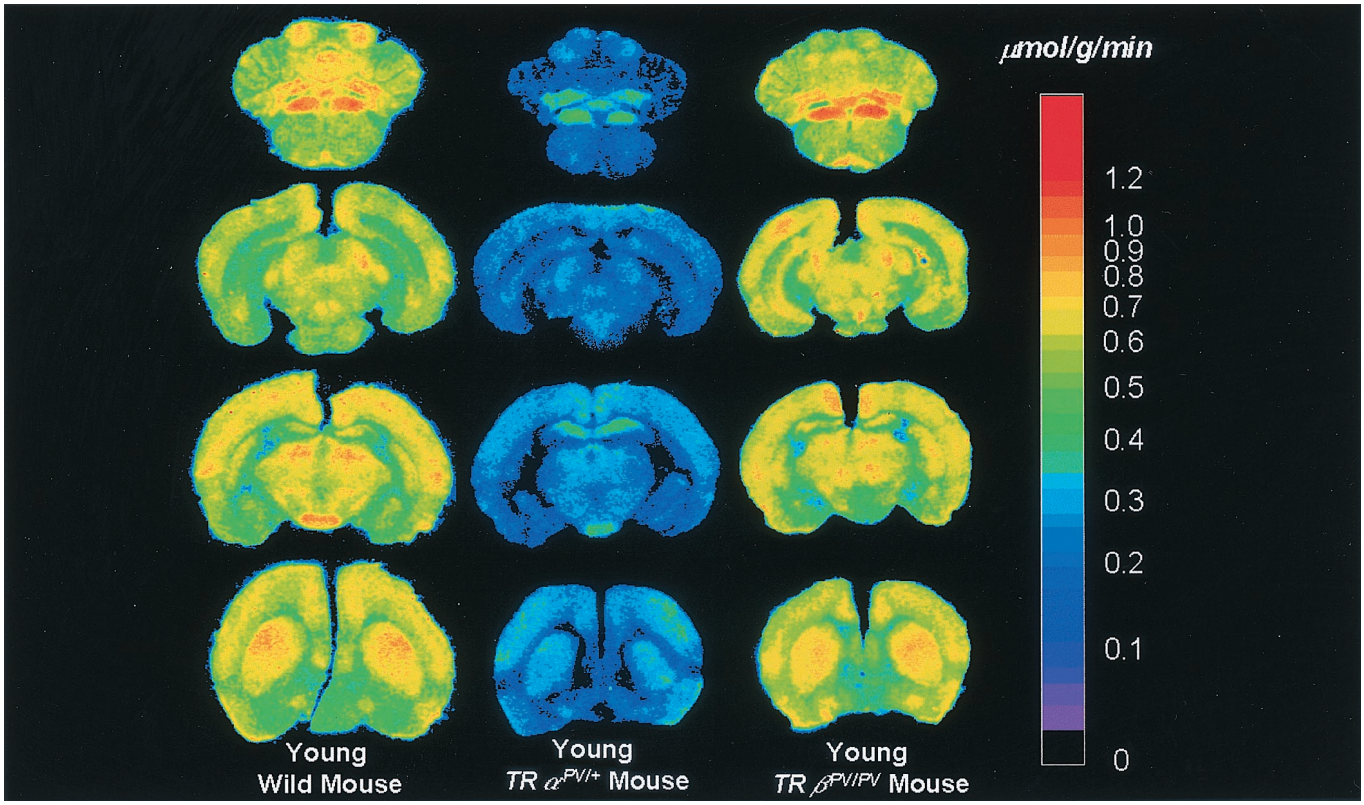

Fig. 3. Quantitative color-coded autoradiographs of representative brain sections from $TR\alpha^{PV/+}$, $TR\beta^{PV/PV}$, and wild-type mice. The local rates of glucose utilization are encoded in the color according to the calibrated color scale on the right side of the figure.

Relationship Between Changes in Local Glucose Utilization and Synaptic Activity. The major effects of neonatal hypothyroidism in brain are retarded outgrowth of neuronal processes, reduced neuropil and synaptic densities, and disturbances in the development of electrical activities (1, 2). Animals made thyroid hormone-deficient from birth have approximately half the number of synapses as euthyroid animals (45), and the size and packing density of dendritic spines are also reduced throughout the brain (46–48). The membrane-associated synaptic protein Srg1, which is believed to have a role in synaptogenesis and synaptic remodeling, has been reported to be under TH control during brain development (27, 29). Srg1 belongs to the synaptotagmin family of proteins that are involved in fusion of synaptic vesicles with synaptic membrane (28, 49), and triggering of neurotransmitter release at the synapse depends, at least in part, on Ca^{2+} and phospholipid binding to synaptotagmin I (28, 49). It is yet to be established, however, whether Srg1 also binds Ca^{2+} and functions as a calcium-dependent regulator of neurotransmitter release. TH also regulates the expression of other genes involved in formation and function of synapses, i.e., Krox-24 (50) and RC3/neurogranin (20), during development. Krox-24 is involved in modulation of neuronal connectivity and plasticity, and RC3/neurogranin, which binds calmodulin and is a substrate for protein kinase C, may also play a role in synaptic structure and/or function.

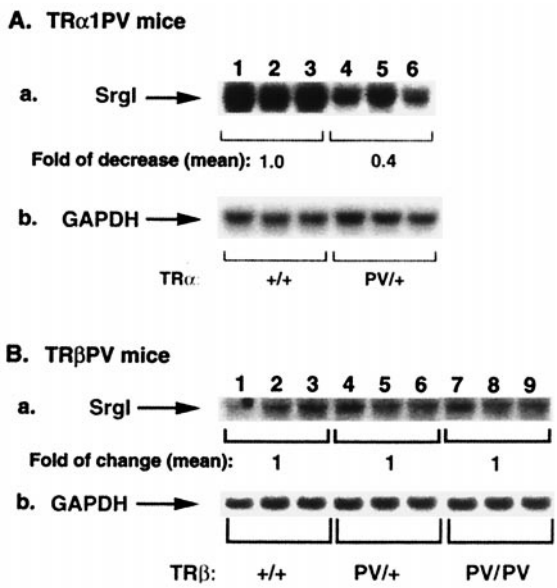

Fig. 4. Expression of Srg1 in the cerebellum of $TR\alpha^{PV/+}$ (*A*) and $TR\beta^{PV/+}$ and $TR\beta^{PV/PV}$ (*B*) ($n = 3$ for each group of 12-week-old male mice). RNA was isolated from the cerebellum. Northern blot analyses were carried out with quantification by a Molecular Dynamics PhosphorImager. Level of Srg1 expression was standardized by the level of glyceraldehyde-3-phosphate dehydrogenase mRNA obtained from the same sample.

Little is known about direct genetic influences of TH via the TRα1 receptor on enzymes involved in glucose metabolism. Several of the enzymatic activities in the glycolytic and oxidative pathways of glucose exhibit TH-dependent increases during brain development (51), but it is unlikely that all of these changes are mediated by direct TH regulation of their gene expression. The effects of TH on glucose metabolism might be indirect. Previous studies have shown that increases in glucose utilization associated with neuronal functional activation are linearly related to the frequency of action potentials in the afferent inputs, confined to the synapse-rich areas in the neuropil, and depen-

dent on Na^+,K^+-ATPase activity (52). Action potentials result from increased Na^+ influx and K^+ efflux in neurons, which then stimulate Na^+,K^+-ATPase activity to restore ionic gradients across the membrane to resting levels. The increased ATPase activity in turn stimulates glucose metabolism to maintain the ATP levels. It is noteworthy that brain Na^+,K^+-ATPase expression is regulated by TH at the gene level (53), particularly in developing brain (54). The reductions in glucose utilization throughout the brain found in the $TR\alpha^{PV/+}$ mice may, therefore, reflect lower Na^+,K^+-ATPase activity, which might result from loss of TH-dependent gene expression, decreased number of synapses in the neuropil, decreased synaptic activity, or any combination of these.

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