

A Moderate and Transient Deficiency of Maternal Thyroid Function at the Beginning of Fetal Neocortico genesis Alters Neuronal Migration

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Epidemiological studies and case reports show that even a relatively minor degree of maternal hypothyroxinemia during the first half of gestation is potentially dangerous for optimal fetal neurodevelopment. Our experimental approach was designed to result in a mild and transient period of maternal hypothyroxinemia at the beginning of corticogenesis. Normal rat dams received the goitrogen 2-mercapto-1-methylimidazole for only 3 d, from embryonic d 12 (E12) to E15. Maternal thyroid hormones decreased transiently to 70% of normal serum values, without clinical signs of hypothyroidism. Dams were injected daily with 5-bromo-2'-deoxyuridine (BrdU) during 3 d, from E14–E16 or E17–E19. Their pups were tested for audiogenic seizure susceptibility 39 d after birth (P39) and killed at P40. Cells that had incorporated BrdU were identified by immunocytochemistry, and quantified: numer-

ous heterotopic cells were found, whether labeled at E14–E16 or E17–E19, that were identified as neurons. The cytoarchitecture and the radial distribution of BrdU-labeled neurons was significantly affected in the somatosensory cortex and hippocampus of 83% of the pups. The radial distribution of γ -aminobutyric acidergic neurons was, however, normal. The infusion of dams with T_4 between E13 and E15 avoided these alterations, which were not prevented when the T_4 infusion was delayed to E15–E18. In total, 52% of the pups born to the goitrogen-treated dams responded to an acoustic stimulus with wild runs, followed in some by seizures. When extrapolated to man, these results stress the need for prevention of hypothyroxinemia before midpregnancy, however moderate, and whichever the underlying cause. (*Endocrinology* 145: 4037–4047, 2004)

NEOCORTICOGENESIS IS AN early process in the development of the central nervous system (CNS) that begins by the fifth week of gestation in humans (1, 2) and by embryonic d 11 (E11) in rats (3). During this process, radially and tangentially migrating neurons are described (4–6), which give rise to glutamatergic (excitatory) and to γ -aminobutyric acidergic (GABAergic; inhibitory) neurons, respectively. The maternal thyroid is then the only source of T_4 and T_3 for the brain of the fetus because its thyroid gland does not start contributing to fetal requirements until midgestation in man, and E17.5–E18 in rats. Therefore, the amount of maternal T_4 that the fetus receives early in pregnancy will determine thyroid hormone action in its brain because it depends on maternal T_4 for its intracellular supply of the active form of the hormone, T_3 . Maternal hypothyroxinemia during this period of brain development may result in poor fetal neurodevelopment, a possibility that is strongly supported by an increasing number of epidemiological studies

and case reports, which have been recently reviewed (7–10). The term hypothyroxinemia indicates that, whether or not TSH is increased above normal values, as in primary hypothyroidism, T_4 or free T_4 (FT_4) is low compared with values usually found at the same stage of pregnancy in normal women with an adequate iodine intake (7, 8).

The most severe CNS brain damage, that of the neurological cretin, is the result of early and profound maternal hypothyroxinemia, caused by severe iodine deficiency (ID). This condition impairs the adequate production and secretion of T_4 by the maternal thyroid. As discussed more extensively elsewhere (7, 11), the degree of the neurological damage is related to the severity of the maternal hypothyroxinemia and not to her circulating T_3 or TSH levels, which are normal. Even more important, as a public health problem, is that in ID areas the mental development of the noncretin population is also affected. Both neurological cretinism and the mental impairment of the general population can only be prevented before midgestation.

We have recently described what we believe is the first direct experimental evidence of a potentially irreversible damage to the fetal brain caused by early maternal hypothyroxinemia, without hypothyroidism (11). Pups born to severely iodine-deficient dams showed alterations in the cytoarchitecture of the somatosensory cortex and hippocampus, and in the radial neuronal migration into the neocortex, which had been caused by events taking place before onset of active fetal thyroid secretion. Such alterations may well be

Abbreviations: Ab, Polyclonal antibody; BrdU, 5-bromo-2'-deoxyuridine; CNS, central nervous system; CNP, 2',3'-cyclic nucleotide 3'-phosphodiesterase; DAB, 3,3'-diaminobenzidine; E, embryonic day; FT_4 , free T_4 ; GABAergic, γ -aminobutyric acidergic; ID, iodine deficiency; IQ, intelligence quotient; ir, immunoreactive; MMI, 2-mercapto-1-methylimidazole or methimazole; mAb, monoclonal antibody; nNOS, neuronal nitric oxide synthase; NeuN, neuronal nuclei; PB, phosphate buffer; P, postnatal age; TR, thyroid hormone receptor.

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causally related to the neurodevelopmental handicaps described in neurological cretins and the general population of areas with severe ID. The study, however, did not directly address some of the questions raised by an increasing number of reports, which strongly suggest that milder degrees of maternal hypothyroxinemia, caused by moderate ID, or by mild thyroid failure from other causes, increase the risk of bearing children with an intelligence quotient (IQ) of 85 or less, with a frequency that may be 150–200 times that of children born with congenital hypothyroidism (7).

Some of these questions could not be answered previously because of the experimental design that had been used (11). The dams were severely hypothyroxinemic before and throughout pregnancy and lactation, and therefore we could not define: 1) a window of special sensitivity of the developing brain to the decrease in maternal T_4 ; 2) the degree of maternal hypothyroxinemia required to observe alterations of neuronal migration in the progeny; 3) the timing after which normalization of the maternal hypothyroxinemia might no longer prevent fetal CNS damage; and 4) whether the alteration of the migratory phase of cells labeled with 5-bromo-2'-deoxyuridine (BrdU) at E17–E19, was a consequence of derangement of the previous phase. It also appeared interesting to determine whether, or not, the tangential neuronal migration was also affected, for which different subtypes of GABAergic neurons were studied.

To answer this type of question, we have here restricted both the degree and the duration of the maternal thyroid dysfunction, by treating normal dams with a goitrogen for only 3 d at the beginning of neocortico-genesis.

Materials and Methods

Animals and treatments

Wistar rats were housed in temperature-controlled (22–24 C) animal quarters, with automatic light and darkness cycles of 14 and 10 h, under veterinary control according to European Community guidelines and after approval by the Ethics Committee of our Institutions.

Young adult females, weighing approximately 200 g, were mated with normal males on E0. The main experimental series are described in Fig. 1, with the number of animals studied in each group for the different endpoints¹ and consisted of: one group of normal rats and three groups of dams that drank 0.02% 2-mercapto-1-methylimidazole (methimazole, MMI) from E12–E15. One group received MMI treatment alone (3dMMI₁₂), and the other two groups were infused with T_4 , again for 3 d, from E13–E16 (3dMMI₁₂ + T_{4-13}) and from E15–E18 (3dMMI₁₂ + T_{4-15}); the *subscripts* indicate the day of onset of treatments (Fig. 1). Onset of T_4 infusion was delayed for 1 d after onset of MMI, considering that its effects on circulating hormone levels were likely to be more rapid

¹ The number of animals used per group for the different endpoints in normal (a), 3dMMI₁₂ (b), 3dMMI₁₂ + T_{4-13} (c) and 3dMMI₁₂ + T_{4-15} (d) groups was as follows: Number of dams for sampling for hormone determinations of pregnant dams at E15: a (n = 5); b (n = 7); c (n = 4); and d (n = 8). Number of dams for hormone determination at E19: a (n = 9); b, (n = 8); c (n = 4); and d (n = 8). Number of pups for hormone determinations at P40: a (n = 13); b (n = 15); c (n = 11); and d (n = 15). Number of pups per litter used for audiogenic seizure susceptibility studies: a, 46 pups of nine litters (46/9); b, 38/5; c, 32/4; and d, 55/10. The number of dams used for morphological studies was: a (n = 6); b (n = 12); c (n = 12); and d (n = 4). Number of pups per litter used for quantitative immunohistochemistry, except for 3dBrdU₁₇ subgroups, were: a, eight pups of two litters (8/2); b, 11/3; c, 11/3; d, 10/3. Number of pups per litter used for quantitative analysis of 3dBrdU₁₇ subgroups, were: a, seven pups of two litters (7/2); b, 9/3; c, 9/3.

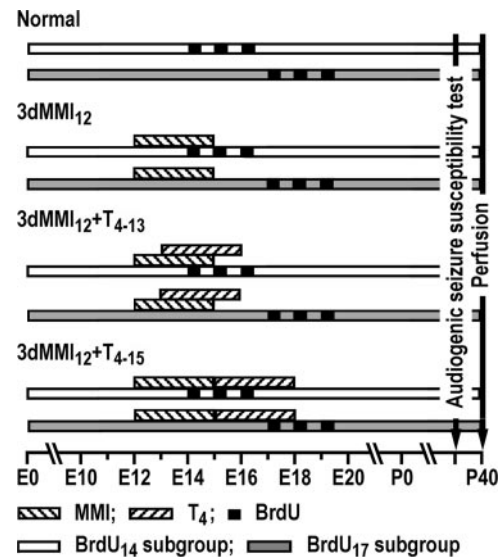


FIG. 1. Shows the four main groups of rats and the different treatments applied. The BrdU₁₄ and BrdU₁₇ subgroups are indicated by white and gray bars, respectively. The length of MMI and T_4 treatments are indicated by lined bars. The day of each BrdU injection is indicated by a black square. Behavioral tests were performed at P39, and the rats were perfused at P40.

than those of the oral administration of MMI. A pilot experiment (12) was carried out infusing for 3 d a dose of 2.4 μ g T_4 /100 g body weight/d that is adequate to maintain euthyroidism of thyroidectomized adult rats (13). This dose increased circulating T_4 and T_3 about 4-fold. Thus, for the present study we infused 0.6 μ g T_4 /100 g body weight/d. The solution was prepared as previously described (14) and infused using osmotic minipumps (ALZET, model 2001, Alza Corp., Palo Alto, CA), placed under the dorsal skin (13).

The pregnant rats of all the above groups were injected ip for 3 d with BrdU, 20 mg/kg body weight/d, dissolved in physiological saline. Half of each group of dams was injected on E14, E15, and E16 (3dBrdU₁₄ subgroups), and the other half at E17, E18, and E19 (3dBrdU₁₇ subgroups). There was no 3dBrdU₁₇ subgroup of 3dMMI₁₂ + T_{4-15} dams (Fig. 1; see footnote¹).

Blood (~0.5 ml) was obtained under slight ether anesthesia from the jugular vein from dams of the four main experimental groups on the mornings of E15 and E19. The plasma was spun off and kept at –20 C, for the determination of T_4 , T_3 , and TSH concentrations.

In all cases, both onset and withdrawal of treatments were carried out between 0800 and 1000 h.

At 2–3 d of postnatal age (P2–P3) each litter was culled to eight pups. At P39, pups from the four groups were tested for their audiogenic seizure susceptibility, and at P40 all pups were weighed, anesthetized, bled, and perfused with 50 ml saline followed by 200 ml 4% paraformaldehyde and 0.1 M sucrose in 10 mM phosphate buffer (PB, pH 7.3–7.4), containing 0.002% $CaCl_2$. The brains were postfixed by immersion in PB at room temperature for 4 h, and stored at 4 C in PB containing 0.1 M sucrose.

Immunohistochemistry

The brain was coronally sectioned using a Vibratome PELCO 101 (Ted Pella Inc., Redding, CA) in eight parallel series of 100 μ m-thick sections. Sections containing the primary somatosensory cortex (taken at –2.0 to –4.0 mm from bregma) and the mediocaudal portion of the hippocampal formation and the primary auditory cortex (both, at –3.8 to –4.3 mm from bregma) were collected in PB saline. One series was immunostained with anti-BrdU monoclonal antibody (mAb; 1:40), biotinylated horse antimouse antibody (Ab; 1:150), Vectastain ABC kit (Vector, Burlingame, CA; 1:200), and 0.05% 3,3'-diaminobenzidine (DAB) (15). These dilutions were the same whenever these four reagents were used. An adjacent series was stained with cresyl violet. Six series were double

immunostained. Two of them were double immunostained for fluorescence, starting with sheep anti-BrdU Ab (10 $\mu\text{g}/\text{ml}$), biotinylated rabbit antisheep Ab (5 $\mu\text{g}/\text{ml}$), and NeutrAvidin (Molecular Probes Europe, Leiden, The Netherlands), Rhodamine Red (Molecular Probes Europe) conjugate (1 $\mu\text{g}/\text{ml}$). One of these two series was then immunostained with antineuronal nuclei (NeuN) mAb (1:400), a marker for neurons; the other with anti-2',3'-cyclic nucleotide 3' phosphodiesterase (CNP) mAb (5 $\mu\text{g}/\text{ml}$), a marker for oligodendrocytes. They were then incubated with biotinylated horse antimouse Ab and Avidin (Molecular Probes Europe), BODIPY FL (Molecular Probes Europe) conjugate (1 $\mu\text{g}/\text{ml}$). The remaining four series were immunostained for conventional light microscopy with markers of different subtypes of GABAergic neurons (16–19). In two series; the sections were first processed with rabbit anticalretinin Ab (1:2000) or rabbit antineuronal nitric oxide synthase (nNOS) Ab (1:100), which was followed with biotinylated goat antirabbit Ab (1:150), ABC kit, and DAB. These sections were then double-immunostained with anti-BrdU mAb, followed with biotinylated horse antimouse Ab, ABC kit and DAB, intensified with 0.1% ammonium nickel sulfate (20). The remaining two series were first processed with sheep anti-BrdU Ab (10 $\mu\text{g}/\text{ml}$), followed with biotinylated rabbit antisheep Ab (5 $\mu\text{g}/\text{ml}$), ABC kit and DAB, intensified with 0.1% ammonium nickel sulfate (20). One series was immunostained with antiparvalbumin mAb (1:1000), the other, with anticalbindin D-28K mAb (1:2000). They were then immunostained with biotinylated horse antimouse Ab, ABC kit and DAB. Immunostained sections were mounted on gelatinized slides, air dried during 24 h, dehydrated in ethanol, cleared in xylol, and coverslipped.

For quantitative analysis, we have studied five to 11 pups of two to three litters of dams from the subgroups of the four main experimental groups,¹ and also from 3dMMI₁₀ and 3dMMI₁₁ dams (see *Results*). In each instance, cells immunoreactive to BrdU, parvalbumin, calbindin D-28K, calretinin, and nNOS were counted in a total of nine to 12 probes in the primary somatosensory cortex (one to two probes per pup); the mean values corresponding to each pup were later used for statistical evaluation of the results. Probes 600- μm wide were placed over the primary somatosensory cortex and spanned from layer I to the subcortical white matter, as previously detailed (11). In all cases, the borders between layers and strata were placed at the same relative depth, measured from adjacent cresyl violet-stained sections. The relative frequencies of immunoreactive (ir) cells in each layer were averaged across probes and animals. We plotted only BrdU-ir cells that showed intensely and homogeneously labeled nuclei, or diffusely labeled nuclei of lesser intensity, but with clumped chromatin. These cells have entered the last S-phase of their cell cycle during uptake of the injected BrdU and correspond to types 1 and 2, respectively, of Takahashi *et al.* (21).

Drugs and reagents

BrdU was from Roche Molecular Biochemicals España (Barcelona, Spain). T₄, MMI, DAB, and cresyl violet were obtained from Sigma-Aldrich (St. Louis, MO). Anti-BrdU mAb was from Amersham Pharmacia Biotech Ltd. (Little Chalfont, Buckinghamshire, UK). Sheep anti-BrdU and biotinylated rabbit antisheep Abs were from Abcam Ltd. (Cambridge, UK). Horse antimouse and goat antirabbit biotinylated Abs, Anti-NeuN, and anti-CNP mAb were from Chemicon International Inc. (Temecula, CA). Antiparvalbumin and anticalbindin D-28K mAbs and rabbit anticalretinin Ab were from Swant (Bellinzona, Switzerland). Rabbit anti-nNOS Ab was obtained from Zymed Laboratories Inc. (San Francisco, CA).

Determination of serum T₃, T₄, and TSH

Their concentrations were determined by very sensitive and highly specific RIAs for T₄ and T₃ (22) and TSH (23). TSH values are expressed as weight equivalents of the National Institute of Diabetes and Digestive and Kidney Diseases rat TSH-RP-3 reference preparation.

Audiogenic seizure susceptibility

At P39, pups were exposed to the electric doorbell used by van Middlesworth (24, 25) to study audiogenic seizure susceptibility of rat pups born from iodine-deficient or goitrogen-treated dams. Rats were individually placed in a plastic cage. The electric bell was placed 20 cm

from the bottom of the cage, so that the intensity of the sound reaching the rat was 95–100 dB. Once the bell was switched on, stimulation was maintained for 90 sec, unless the animal responded with a tonic-clonic seizure, when rapidly interrupted. The animals were observed for the onset of wild runs, or wild runs followed by seizures, and the onset of each type of reaction was timed. Rats were returned to their initial cages until they were killed for the immunohistochemical studies. The dams used for this part of the study were from the same strain previously used to assess audiogenic seizure susceptibility in pups from iodine-deficient dams (14).

Statistical analysis

Plots and counts of ir cells were obtained using the Neurograph system (Microptic, Barcelona, Spain). The Systat statistical software (Systat Inc., Evanston, IL) was used for two-way ANOVAs of cell density, factors being layers and experimental groups. Significant layers-groups interactions ($P < 0.001$) were found in the primary somatosensory cortex the primary somatosensory cortex both for the 3dBrDU₁₄ and 3dBrDU₁₇ subgroups. One-way ANOVAs of ir-cell densities were then used to identify layers that were affected by the experimental condition, followed by Tukey's test to identify significant differences ($P \leq 0.05$) between means.

The SPSS statistical package (SPSS Inc., Chicago, IL) was used for detection of differences between concentrations of hormones, number of pups/litter, and body weight, with one-way ANOVA and the protected least significant difference test for multiple comparisons, after validation of the homogeneity of variances by the Bartlett-Box *F* test; for the audiogenic seizure susceptibility variables using the Mann-Whitney non-parametric test for frequencies with which the pups responded with wild runs, or wild runs followed by seizures. The Kaplan-Meier survival analysis was applied to the timing of onset of wild runs or seizures. The test calculates the percentage of pups that survive the 90-sec acoustic challenge without a response.

Results

Effects of different treatments on thyroid function

At E15 (Table 1), maternal plasma T₄ concentration of the 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₅ dams decreased significantly ($P < 0.05$) to approximately 70% of values of normal dams. On the contrary, the plasma T₄ of the 3dMMI₁₂ + T₄₋₁₃ dams was higher than that of 3dMMI₁₂ and normal dams. Plasma T₃ changed similarly, decreasing in both 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₅ dams; T₃ in 3dMMI₁₂ + T₄₋₁₃ dams was higher than that of 3dMMI₁₂ dams and not different from normal. Although mean plasma TSH values were higher in 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₅ dams compared with those of the control group differences among these three groups were not statistically significant. This lack of a rapid and commensurate increase of circulating TSH in the 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₃ dams, despite the decrease in circulating T₄ and T₃, supports the concept of hypothyroxinemia (7), namely, that low T₄ and T₃ concentrations are not necessarily accompanied by an increased TSH. TSH was lower in the 3dMMI₁₂ + T₄₋₁₃ dams than in dams from the other three groups.

At E19 (Table 1), plasma T₄ values of the 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₃ dams were no longer different from that of normal ones, whereas the mean value was higher in the 3dMMI₁₂ + T₄₋₁₅ dams, although the difference was not statistically significant. There were no differences among groups in plasma T₃. Plasma TSH was the same as that of normal dams for all groups except for the lower value of the 3dMMI₁₂ + T₄₋₁₅ dams.

No differences were found among experimental groups at

TABLE 1. Circulating T₄, T₃, and TSH in dams from the experimental groups at E15 and E19

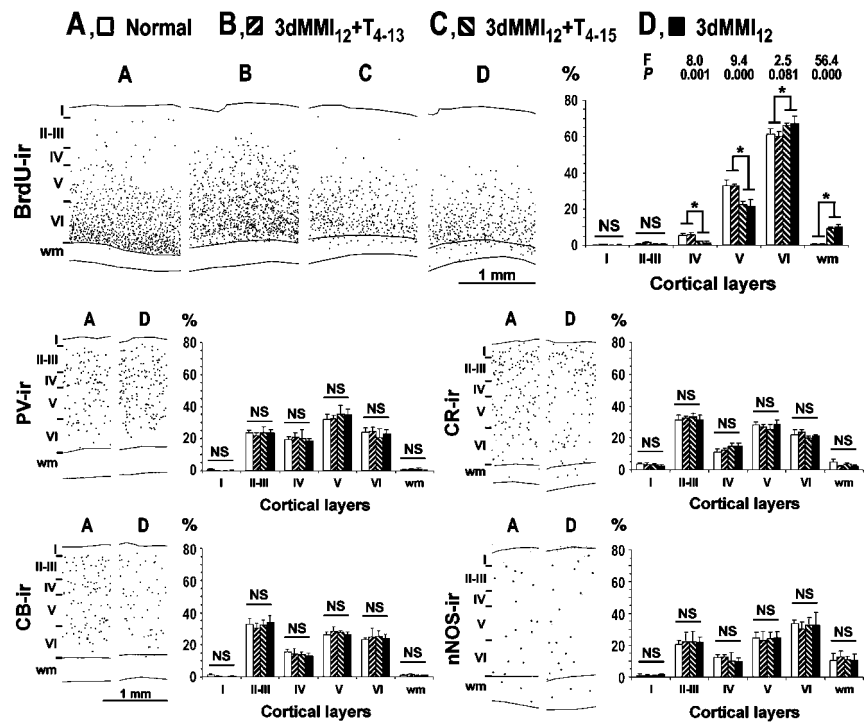
	Pregnant dams at E15			Pregnant dams at E19		
	T ₄	T ₃	TSH	T ₄	T ₃	TSH
Normal	15.90 ± 1.89	0.37 ± 0.03	0.91 ± 0.45	13.40 ± 1.19	0.38 ± 0.04	0.47 ± 0.08
3dMMI ₁₂ + T ₄₋₁₃	21.00 ± 1.27 ^a	0.40 ± 0.02	0.53 ± 0.11	9.50 ± 0.77	0.27 ± 0.03	0.69 ± 0.13
3dMMI ₁₂ + T ₄₋₁₅	10.50 ± 0.02 ^{a,b}	0.22 ± 0.02 ^{a,b}	1.00 ± 0.03	16.50 ± 1.43	0.32 ± 0.03	0.32 ± 0.03
3dMMI ₁₂	11.60 ± 0.67 ^{a,b}	0.29 ± 0.02 ^{a,b}	1.44 ± 0.38 ^b	12.70 ± 0.95	0.35 ± 0.05	0.63 ± 0.14

Data represent the mean value ± SEM. The TSH values are expressed as weight equivalents of the NIDDK-Rat TSH-RP-3 reference preparation. All units shown are nanograms per milliliter.

^a A statistically significant difference compared with normal rats.

^b A statistically significant difference compared with 3dMMI₁₂ + T₄₋₁₃ rats.

FIG. 2. Plots of cells from normal (A), 3dMMI₁₂ + T₄₋₁₃ (B), 3dMMI₁₂ + T₄₋₁₅ (C), and 3dMMI₁₂ (D) pups at P40, that are ir to BrdU (BrdU-ir; 3dBrdU₁₄ subgroups), to parvalbumin (PV-ir), to calretinin (CR-ir), to nNOS (nNOS-ir) and heavily ir to calbindin D-28K (CB-ir). Histograms (right side of the plots) show results of their quantification in different cortical layers of the primary somatosensory cortex. The borders between layers are indicated by horizontal lines. Asterisks indicate a statistically significant difference between pups (normal and 3dMMI₁₂ + T₄₋₁₃ vs. 3dMMI₁₂ + T₄₋₁₅ and 3dMMI₁₂), as revealed by the F and P values. The degrees of freedom were 3, 36. NS, Not statistically significant differences. Same magnification for PV-ir, CR-ir, nNOS, and CB-ir plots.



P0 in the number of pups born to the dams (9.4 ± 1.03), or at P40 in their body weight (131.9 ± 0.90 g), plasma T₄ (31.2 ± 1.0 ng/ml) and T₃ (0.97 ± 0.02 ng/ml), or TSH (0.48 ± 0.02 ng/ml).

Distribution of cells

The radial distribution of BrdU-ir nuclei in the primary somatosensory cortex was abnormal in many pups of the 3dBrdU₁₄ subgroups of 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₅ dams, with heterotopic BrdU-ir cells at locations different from those corresponding to their birth date (Fig. 2). In these pups, the percentage of BrdU-ir cells decreased in layers IV and V, and increased in layer VI and subcortical white matter (see P values in Fig. 2), whereas no differences were found between 3dMMI₁₂ + T₄₋₁₃ and normal pups. These alterations in radial migration, revealed by the presence of heterotopic neurons in the subcortical white matter, were milder in two of 12 of the 3dMMI₁₂ and one of nine of the 3dMMI₁₂ + T₄₋₁₅ pups. They were never observed in the pups from normal dams. Heterotopic BrdU-ir cells were also found in the subcortical white matter of the auditory cortex (not

shown). On visual inspection, their number appeared similar to that found in the primary somatosensory cortex.

Alterations in the radial distribution of BrdU-ir cells from the 3dBrdU₁₇ subgroups of normal, 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₃ pups were also detected (Fig. 3). Compared with pups from the normal dams, the proportion of BrdU-ir cells in pups from the 3dMMI₁₂ dams decreased in layers II–III and IV, and increased in layers V, VI, and in the subcortical white matter. Such alterations were not observed in pups born to 3dMMI₁₂ + T₄₋₁₃ mothers.

Two additional groups of two dams each were also treated for 3 d with MMI, but starting at E10 and E11, and injected with BrdU as described. All pups from these 3dMMI₁₀ and 3dMMI₁₁ dams showed a radial distribution of BrdU-ir cells comparable to that illustrated in Figs. 1 and 2 for 3dMMI₁₂ pups.

In contrast with the radial distribution of the BrdU-ir cells, the radial distribution of neurons immunoreactive to parvalbumin, calbindin D-28K, calretinin, and nNOS was similar in all groups to that of pups from normal dams (Fig. 2). Two types of calbindin D-28K-ir neurons (heavily and lightly

labeled) were observed according to the intensity of the immunostaining. Heavily labeled calbindin D-28K-ir neurons showed a morphology corresponding to local circuit neurons, whereas most of the lightly stained calbindin D-28K-ir neurons resembled pyramidal cells, mostly located in layers II-III. In the four main experimental groups, the parvalbumin-ir and calbindin D-28K-ir neurons were mostly found in cortical layers II-III to VI. The radial distribution of parvalbumin-ir neurons was very uniform with a moderate high density in layer V (Fig. 2). The proportion of heavily labeled calbindin D-28K-ir neurons was similar in layers II-III, V,

and VI with a decrease in layer IV (Fig. 2). Calretinin- and nNOS-ir neurons were also found in layers II-III to VI, with the proportion being lower in layer IV. In contrast to parvalbumin- and heavily labeled calbindin D-28K-ir neurons, a small but significant number of calretinin- and nNOS-ir neurons were observed in layer I and in the subcortical white matter (Fig. 2).

Cytoarchitectonic changes

In addition to an abnormal migration of BrdU-ir cells, we also observed changes in cresyl violet-stained coronal sections obtained from pups of the 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₅ dams, both as regards the cytoarchitecture of the barrel cortex of the primary somatosensory cortex and of the hippocampus, whereas these features were normal in the 3dMMI₁₂ + T₄₋₁₃ pups (Fig. 4, A and B). Although not shown, changes observed in pups from the 3dMMI₁₀ and 3dMMI₁₁ dams were again comparable to those from the 3dMMI₁₂ dams. The major features in the normal pups were typical barrels in layer IV, and easily distinguishable borders between layers. In contrast, these features are less prominent in the progeny of 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₅ dams (Fig. 4, C and D). These changes were not seen in the 3dMMI₁₂ + T₄₋₁₃ pups (Fig. 4B). No statistically significant differences were found in the thickness of the neocortex among the four groups (on average, 1740 ± 67 μm). Again, changes in all the pups from the 3dMMI₁₀ and 3dMMI₁₁ dams (not shown) were comparable to those from the 3dMMI₁₂ dams.

In the hippocampus, as in the barrel cortex, the gross cytoarchitecture of 3dMMI₁₂ + T₄₋₁₃ pups (Fig. 4F) was similar to that of normal pups (Fig. 4E). In the hippocampal CA1, the pyramidal cell layer showed clear-cut borders with the adjacent strata. In contrast, in the hippocampal CA1 of

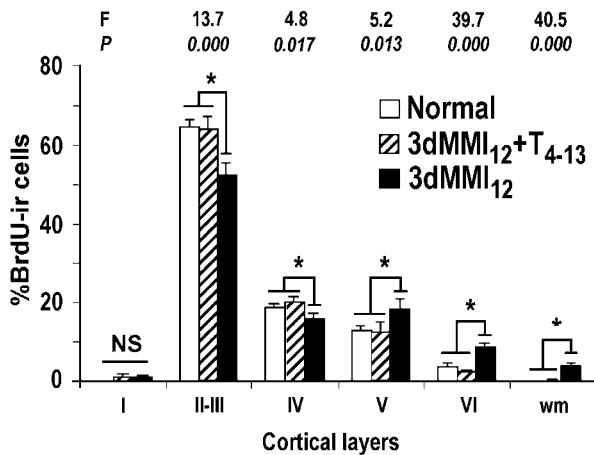
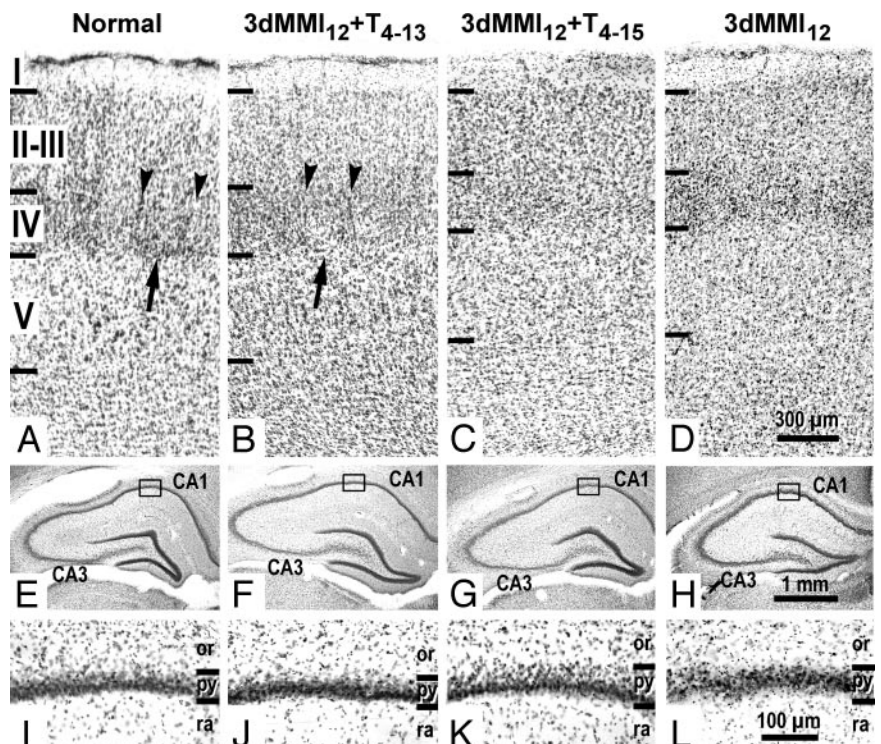


FIG. 3. Histogram showing the percentages of cells ir to BrdU (BrdU-ir) in different layers of the primary somatosensory cortex of pups at P40 from 3dBrdU₁₇ subgroups of normal, 3dMMI₁₂ + T₄₋₁₃ and 3dMMI₁₂ dams. Asterisks indicate a statistically significant difference between normal (or 3dMMI₁₂ + T₄₋₁₃) and 3dMMI₁₂ pups, as revealed by the F and P values. The degrees of freedom were 2, 22. NS, Not statistically significant differences.

FIG. 4. Photomicrographs of cresyl violet-stained coronal sections showing the cytoarchitecture of the barrel cortex of the primary somatosensory cortex (A–D) and hippocampus (E–L) in normal, 3dMMI₁₂ + T₄₋₁₃, 3dMMI₁₂ + T₄₋₁₅ and 3dMMI₁₂ pups at P40. Borders between layers (*horizontal lines*) are clear-cut in normal (A) and 3dMMI₁₂ + T₄₋₁₃ (B) pups, whereas they are more blurred in 3dMMI₁₂ + T₄₋₁₅ (C) and 3dMMI₁₂ (D) animals. In layer IV of normal and 3dMMI₁₂ + T₄₋₁₃ pups, barrels (*arrows*) are normal and well-defined and demarcated by septae (*arrowheads*). In contrast, barrels in layer IV of 3dMMI₁₂ + T₄₋₁₅ and 3dMMI₁₂ pups are not seen. In I–L panels are enlargements of the insets outlined in E–H. In the hippocampus, the differences between groups are not prominent at low magnification (compare E and F with G and H). However, at a higher magnification, the border between strata pyramidal (py) and oriens (or) in the hippocampal CA1 of normal and 3dMMI₁₂ + T₄₋₁₃ pups is more clear-cut than in 3dMMI₁₂ + T₄₋₁₅ and 3dMMI₁₂ pups (compare I and J with K and L). ra, Stratum radiatum. Same magnification for A–D, E–H, and I–L.



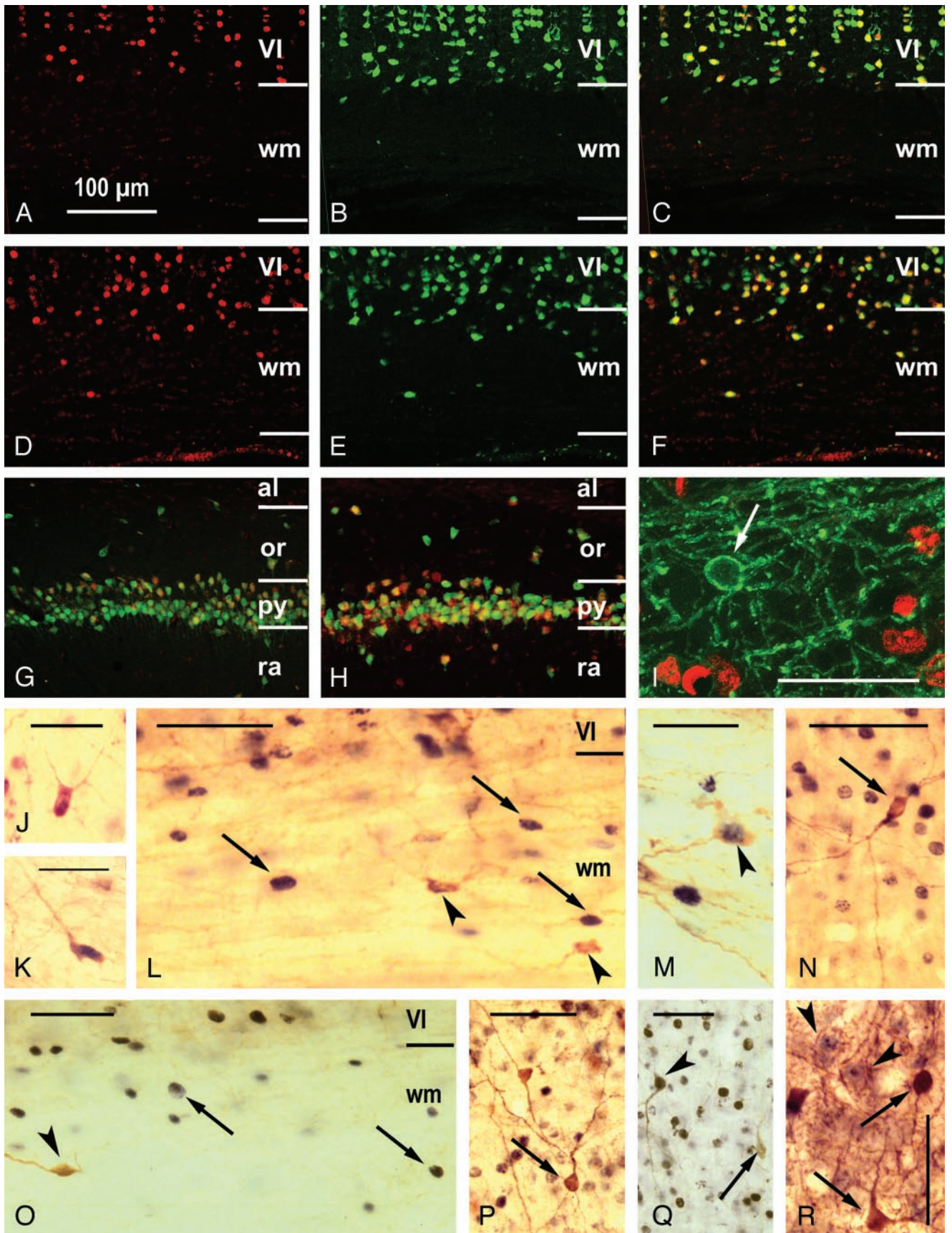
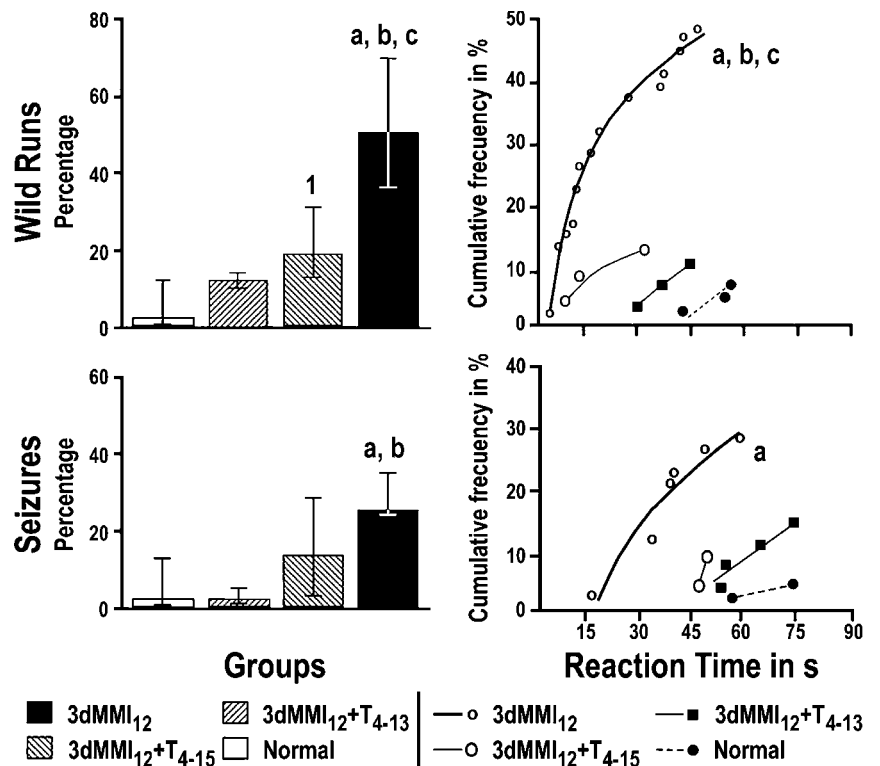


FIG. 6. Responses of normal, 3dMMI₁₂ + T₄₋₁₃, 3dMMI₁₂ + T₄₋₁₅ and 3dMMI₁₂ and 3dMMI₁₂ pups to an acoustic stimulus. Panels on the *left* correspond to the proportion (median with 25th and 75th percentiles) of pups responding with wild runs and with wild runs followed by a seizure, respectively. Panels on the *right* represent the cumulative frequency of pups from the same groups that respond with wild runs alone or followed by a seizure, respectively, at the intervals after onset of the stimulus that are shown in the abscissa. a, Indicates a statistically significant difference compared with normal, b compared with 3dMMI₁₂ + T₄₋₁₃ and c compared with 3dMMI₁₂ + T₄₋₁₅ group.



the 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₅ progeny, the border of the pyramidal layer with the strata oriens was more blurred (Fig. 4, K–L). The same was observed in pups from the 3dMMI₁₀ and 3dMMI₁₁ dams.

Double-immunolabeling

Double-labeling for BrdU and NeuN showed that all BrdU-ir cells that were located in heterotopic positions were neurons, whether found in the subcortical white matter (Fig. 5, C and F) or in the hippocampal stratum oriens and the hippocampal alveus (Fig. 5, G and H). This result was further confirmed by the absence of double-labeling for BrdU and cyclic nucleotide CNP. Double-labeling for CNP was only occasionally observed (Fig. 5I) for those BrdU-ir cells that were classified as type 3 after Takahasi *et al.* (21).

Both in the 3dBrdU₁₄ and 3dBrdU₁₇ subgroups, approximately 10% of cells that were ir to parvalbumin, calretinin, calbindin D-28K, and nNOS were also ir to BrdU (see examples in Fig. 5, J, K, L, and P). However, most of the

heterotopic neurons in the subcortical white matter that stained with the above markers were BrdU-negative (Fig. 5, L and O); only in a few cases were cells double-labeled for BrdU and nNOS observed (Fig. 5, K and M). Other features of immunostaining with these markers such as size and shape of the cells were similar to those of normal pups (Fig. 5, N, P, and Q). In 3dMMI₁₂ pups, perisomatic parvalbumin-ir terminals were prominent around the soma of pyramids in layers II–III and Va and similar to found in normal pups (Fig. 5R).

Audiogenic seizure susceptibility

Acoustic stimulation showed that there were significant differences between the pups born to normal and 3dMMI₁₂ dams in the proportion of pups that responded with wild runs alone, or with wild runs followed by a seizure (Fig. 6). The time required by the 3dMMI₁₂ pups to respond (Fig. 6) was also much shorter. The results obtained in the 3dMMI₁₂ + T₄₋₁₃ and 3dMMI₁₂ + T₄₋₁₅ pups differed from those of the

FIG. 5. Confocal photomicrographs showing BrdU and NeuN immunostaining in the primary somatosensory cortex (A–F) and the hippocampal CA1 (G and H) of normal (A, B, C, and G) and 3dMMI₁₂ (D, E, F, and H) pups at P40 (3dBrdU₁₄ subgroups). Single-labeling to BrdU (A and D; *red*) and to NeuN (B and E; *green*), and double-labeling (C, F, G, and H; *yellow*) are shown. Note the absence of ir cells in the subcortical white matter (wm) of the primary somatosensory cortex and alveus (al) of the hippocampal CA1 in normal pups. In the 3dMMI₁₂ pups, all cells in wm ir to BrdU (D; *red*) are neurons (F; *yellow*). Heterotopic neurons (*yellow*) were also found in the al of 3dMMI₁₂ pups (H). Panel I shows a BrdU-negative cell ir to CNP that is an oligodendrocyte (*arrow*; *green*), as well as nuclei ir to BrdU (*red*) in layer VI of a 3dMMI₁₂ pup (3dBrdU₁₄ subgroup). Light microscope photomicrographs are shown of the hippocampal CA1 (J) and the primary somatosensory cortex (K–R) of 3dMMI₁₂ pups of the 3dBrdU₁₄ (J–L, O, and Q) and 3dBrdU₁₇ (M, N, P, and R) subgroups. Panels J–N show BrdU and nNOS immunostaining. Single-labeling to BrdU in wm (*dark blue nuclei*; *arrows* in L); single-labeling to nNOS (*brown cytoplasm*) in wm (*arrowheads* in L) and in layer II–III (*arrow* in N); and double-labeled neurons in wm (K and M) and the stratum oriens of the hippocampal CA1 (J) are shown. Panels O–Q show BrdU and calretinin immunostaining. Single labeling to BrdU (*dark blue nuclei*) in the wm (*arrows* in O); single labeling to calretinin (*brown cytoplasm*) in the wm (*arrowhead* in O) and in layers V (*arrow* in P) and II–III (*arrow* in Q); and a double-labeled neuron in layer II–III (*arrowhead* in Q) are shown. Panel R shows BrdU and parvalbumin immunostaining. Single parvalbumin-ir neurons in layer V (*arrows*) and some perisomatic parvalbumin-ir boutons on the soma of two pyramidal BrdU-ir neurons are indicated (*arrowheads*). Same magnification for A–H. Scale bar in I–R, 50 μm.

3dMMI₁₂ group, either in the proportion of animals responding with wild runs or seizures, in the length of time required for the response, or in both. The frequency of wild runs and seizures was similar in pups from both groups of T₄-infused 3dMMI₁₂ dams, and was between those observed for the normal and the 3dMMI₁₂ pups. There was a difference, however, in the time needed for the wild runs to start: the pups from the 3dMMI₁₂ + T₄₋₁₅ responded as quickly as the 3dMMI₁₂ pups, whereas those from the 3dMMI₁₂ + T₄₋₁₃ group required a more prolonged stimulation, comparable with that needed by the few pups from normal dams that reacted to the acoustic stimulus (Fig. 6).

Discussion

The experimental design

The 3 d of treatment with MMI resulted in a decrease of maternal circulating T₄ and T₃, which reverted to normal after goitrogen withdrawal. The decrease was not only transient, but also quite moderate when compared with the hypothyroxinemia of the iodine-deficient dams, with undetectable T₄ levels (<2.5 ng/ml), but normal circulating T₃ (11). On the last day of the present 3 d of treatment with MMI, circulating T₄ and T₃ had only decreased to about 70% of normal values. Moreover, there were no appreciable clinical effects of this treatment, as shown by the normal size of the litters and the body weight of the pups. It also did not affect the parameters of thyroid function of the progeny that were quite normal whichever the treatments of the dam. The MMI-treated dams will therefore be referred to as being hypothyroxinemic (7, 11) because there was no clinical or subclinical biochemical evidence of hypothyroidism, such as an increase in circulating TSH.² Present results also confirm that an increased TSH should not be systematically used as sole predictor of primary thyroid function clinically and that conditions exist where there may be thyroid hormone insufficiency without the expected increase in circulating TSH. The previous observation (11) that histochemical and cytoarchitectonic alterations were similar in pups from both LID groups, despite the very different plasma T₃ values of their mothers, is consistent with our present contention that the decrease to about 70% of circulating T₃ in the 3dMMI₁₂ dams plays a lesser role than their hypothyroxinemia.

The present experimental design also included dams that were infused with T₄ during, or after, the 3-d treatment with MMI. The decrease of the maternal T₄ and T₃ levels was indeed avoided by infusion of T₄ during this 3 d of treatment with MMI, showing that the neurodevelopmental changes found in their pups are not caused by MMI *per se*. They also show that the changes persist if the T₄ infusion is delayed.

The relatively mild degree of transient hypothyroxinemia in pregnant rats, induced with the present design at the beginning of fetal corticogenesis, was enough to alter the organization of the neocortex in most of their pups, as revealed by an abnormal migration and cytoarchitecture, and to result in a functional deficit, as documented by an in-

creased frequency of abnormal responses to an acoustic stimulus. These are, to our knowledge, novel findings. Thus the present experimental design has contributed to answering points 1–4 in the introductory section, permitted further insight into the type of neuronal migration that is affected, and posed new challenges for further research, especially as regards possible functional consequences of a relatively mild degree of transient maternal hypothyroxinemia.

Effects on histogenesis and cytoarchitecture

The alterations of the distribution of BrdU-ir cells among different layers of the primary somatosensory cortex and the hippocampal CA1, and the changes in the cytoarchitecture of these cortical areas found in the progeny of the 3dBrdU₁₄ and 3dBrdU₁₇ subgroups of the 3dMMI₁₂ dams are qualitatively quite similar to those observed in pups born to iodine-deficient dams that were severely hypothyroxinemic because of marked, and chronic, ID (11). Another similarity between the pups of the iodine-deficient and the 3dMMI₁₂ dams is that the heterotopic BrdU-ir cells found in the subcortical white matter are neurons. There might, however, be a slight quantitative difference: all pups from iodine-deficient dams were affected, whereas some of the 3dMMI₁₂ pups (<15%) were not. Although there were only two litters per group, all pups from the 3dMMI₁₀ and 3dMMI₁₁ dams were abnormal, and this suggests that E12, the only day on MMI common to 3dMMI₁₀, 3dMMI₁₁ and 3dMMI₁₂ dams, might be especially sensitive to maternal hypothyroxinemia with respect to cytoarchitecture and neuronal migration into the neocortex and hippocampus.

The present evidence that even a mild and transient decrease of maternal thyroid hormone insufficiency during early pregnancy effectively alters early brain development is in conceptual agreement with the findings described by others (27–30), who used different experimental designs and studied different end-points. They have shown that severe maternal hypothyroidism, induced by surgical thyroidectomy or MMI treatment starting 2 wk before mating, alters the expression of different cerebral genes at gestation d 16 (equivalent to our E15), and that their expression responds within hours to single injections of T₄ into the dam. These observations increase the likelihood that the alterations in neuronal migration and cytoarchitecture described here are the consequence of inadequate and/or untimely expression of one or more cerebral genes that are thyroid hormone sensitive during this developmental period (31). The unique features of the present model might be quite useful for the identification of genes specifically involved in the radial migration of neurons into the cortex, which are involved in processes such as neuron-glia and neuron-substrate adhesion or signal molecules that might guide juvenile neurons to their final destination (32).

The present study presents additional information with respect to the study of iodine-deficient pups (11) regarding the possible effects of this early maternal hypothyroxinemia on radial migration of neurons generated in the cortical ventricular zone *vs.* the tangential and radial migration of cells originated in the medial ganglionic eminence and their final radial positioning (4–6). These two populations of migrating

² This lack of an increase in circulating TSH is in conceptual agreement with previous observations (26) showing that in pregnant rats an increase in circulating TSH is both delayed with respect to a decrease in T₄ and T₃ and less marked than expected.

neurons will give rise to glutamatergic (excitatory) and to GABAergic (inhibitory) neurons, respectively. Present results confirm alteration in the radial migration of the former, and exclude that of the latter, because the radial distribution of cells ir to parvalbumin, calbindin D-28K, calretinin, and nNOS was not affected in 3dMMI₁₂ pups. This, however, does not exclude the possibility that the tangential migration of GABAergic neurons might also be abnormal in 3dMMI₁₂ pups, despite their normal radial distribution.

Another important issue has been clarified with the present experimental design, which could not be adequately answered in iodine-deficient pups (11). The abnormal distribution of cells incorporating BrdU at E17–E19, as well as the cytoarchitectural alterations described here, are the result of the previous transient exposure to MMI. It would appear that, once the initial radial neuronal migration is altered, other phases of neurodevelopment might also be affected.

Functional and behavioral alterations

The abnormalities in cell migration and the subtle changes in cytoarchitectonic organization found in the progeny of 3dMMI-treated dams indicate that the normal process of brain maturation and, consequently, the establishment of normal brain functions, are likely to be impaired. Indeed, progeny of severely hypothyroid mothers, caused by chronic MMI treatment, with altered neuronal migration and deranged barrel formation, present alterations of GABAergic (33), callosal (15, 34) and thalamo-cortical connections (20). In addition, alterations of behavior and biogenic amine metabolism have been also observed in euthyroid pups of hypothyroid pregnant rats (35). The increased audiogenic seizure susceptibility reported here for the 3dMMI₁₂ pups supports the possibility that inhibitory circuits are altered. In the 3dMMI₁₂ pups, about 20% of glutamatergic neurons are in abnormal locations, as shown by their concomitant BrdU labeling. Therefore, although the radial distribution of GABAergic neurons is similar between normal and 3dMMI₁₂ pups, many of these heterotopic neurons, mostly located in layers Vb and VI are most probably abnormally innervated by GABAergic axons because in these layers we have not observed perisomatic parvalbumin-ir basquet terminals, similar to those found in layers II–III and Va.

We tried the present crude behavioral test because we had applied it before to the progeny of iodine-deficient dams (14), following the procedure initially described for such animals by van Middlesworth (24) and later applied to the progeny of goitrogen-treated dams (25). Such studies disclosed a relationship between audiogenic seizure susceptibility, hearing loss and a derangement in the development of the organ of Corti. The latter was known to be a postnatal event in rodents and treatment of the dams with goitrogens was usually started from a few days before birth up to P14–P21 (25). Such observations, therefore, did not suggest that early maternal hypothyroxinemia might also result in similar behavioral defects. But it was also known that expression of the $\beta 1$ and $\beta 2$ thyroid hormone receptors (TR) transcripts appear very early in the auditory system, at E12.5, and are restricted to that portion of the inner ear that gives rise to the cochlea (36). The later finding of audiogenic seizure susceptibility

and hearing loss in $\beta 1$ and $\beta 2$ TR-deficient mice (37) prompted us to test the possible influence of maternal hypothyroxinemia, at about E12, on the audiogenic responses of their pups. They were indeed found to be abnormal in many of the pups. We wish to remark, however, that this finding might not be reproduced in rat strains that are genetically resistant to audiogenic seizure susceptibility or less susceptible to audiogenic seizure (38) than ours (14).

Epilepsy is a multifactorial disease in which not only central and peripheral circuitry seems to be involved but also the alteration of glutamatergic (excitatory) and GABAergic (inhibitory) cortical neuronal systems (39). In fact, selective alterations in GABA_A receptor subtypes in human temporal lobe epilepsy have been recently reported (40). However, these comments do not necessarily imply that the behavioral alterations observed in 3dMMI₁₂ and 3dMMI₁₂ + T₄₋₁₅ are causally related to the abnormal radial migration of neurons: differences in the effects of T₄ infusion on behavior compared with those on corticogenesis suggest different underlying mechanisms.

Possible clinical implications for adequate human development

In humans, neurogenesis in the neocortex begins by E35–E42 and ends by E140, whereas neural radial migration begins by E56 and ends by E168 (1, 2). Thus, neurogenesis and migration takes place in a total of about 125 d, from wk 5–6 to wk 24 after conception. In the rat, the same events occur in about 10 d, between E11 and E21 (3). Therefore, a period of time of 3 d in rats might correspond roughly to a period of 37–38 d in humans. Extending these speculations somewhat further, the present critical period of sensitivity to maternal hypothyroxinemia would correspond roughly from late first trimester of pregnancy up to midgestation. Thus, our results strongly suggest that abnormal neuronal migrations could be found in the progeny of pregnant mothers that have been transiently and moderately hypothyroxinemic/hypothyroid at the beginning of fetal neocorticalogenesis, even if only during as short a period of time as 5–6 wk. This hypothesis cannot be assessed for ethical reasons, but a deficit of T₄ for the fetus during such a period of time may alter normal migration of cortical neurons and the cytoarchitectonic organization of the neocortex and hippocampus. These alterations would become irreversible if T₄ treatment were delayed. The subtle changes in migration and cytoarchitecture described here may be an underlying cause of the decreased mental development and neurological alterations described in children born in areas of mild and moderate ID (9, 41) or in children born from mothers whose hypothyroxinemia early in pregnancy might be due to other causes (7, 42–44). Vermiglio *et al.* (45) have recently reported the results of a more than 10 yr follow-up of 16 children, euthyroid since birth, born to European mildly iodine-deficient mothers, compared with 11 age- and sex-paired children born to iodine-sufficient women from a nearby control village. Thyroid function of the women had been assessed clinically and biochemically at three time points during gestation, with the mean FT₄ of the iodine-deficient

group being significantly lower than that of the controls at 8 wk (13.9 ± 2.0 vs. 15.7 ± 1.7 pmol/liter) and 13 wk (13.0 ± 1.7 vs. 14.7 ± 1.7 pmol/liter), whereas T_3 and TSH were not different. There was a difference of 18.1 points in the mean IQ, in favor of the controls, with a correlation between maternal FT_4 before midgestation and the IQ of the offspring. Moreover, almost 70% (11/16) of the children born to the moderately iodine-deficient mothers, and none of the children of the control group, had attention deficit hyperactivity disorders. This abnormally high frequency of attention deficit hyperactivity disorders is surprisingly similar to that reported for the syndrome of generalized resistance to thyroid hormone, which is frequently the consequence of mutations in $\beta 1$ and $\beta 2$ TR isoforms (46, 47). In both conditions, the underlying cause of the disorder may well be an inadequate availability of receptor-bound T_3 during early brain development.

In conclusion

Present experimental results are the first to show that a moderate and transient maternal hypothyroxinemia during a period roughly corresponding in man from the end of the second month up to midgestation, causes irreversible alterations of brain development. These findings should contribute significantly to our awareness of the increasing number of reports on the poorer mental development of children born to mothers who have been hypothyroxinemic before midgestation, often only moderately so, especially if this occurs at the beginning of cortical neurogenesis and no palliative T_4 therapy is applied to prevent the possible damage. As pointed out by Utiger (48), until 5 yr ago there was a general consensus that thyroid hormone was necessary for adequate brain development already during the third trimester of pregnancy, but its role until the middle trimester was not generally recognized. Attention was drawn, however, to the possible damage caused by maternal thyroid hormone insufficiency during early development, especially because environmental conditions, such as ID, were increasingly detected in women of child-bearing age, even in the United States. Present experimental results fully support that thyroid hormone of maternal origin is necessary in adequate amounts for normal brain development before and during the middle trimester.

A condition of transient maternal hypothyroxinemia is not likely to be detected early in pregnancy because it may not result in clinical or even subclinical hypothyroidism. T_4 treatment of the latter condition is being strongly recommended (49) because the increased TSH indicates that thyroidal hormone production is already inadequate for that person, even if circulating concentrations are within the very broad normal range. Present results suggest that this recommendation ought to be extended to maternal hypothyroxinemia because production of T_4 may already be inadequate, although not sufficient to trigger an increase in circulating TSH, physiologically suppressed during the first trimester of pregnancy (8). This condition appears to be quite frequent, even in Western societies and, as shown here, is potentially damaging to the devel-

oping brain. For this reason, our present study strongly supports our previous conclusions (7, 11, 50) regarding the need to implement mass screening of pregnant women early in gestation, at the beginning of neocortical neurogenesis. For the sake of the unborn child, these programs should be based primarily on first trimester parameters related to circulating FT_4 , and not only on the detection of antithyroid Ab positivity and increased serum TSH, as previously proposed for the sake of the mother (51).

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