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Organization of Auditory Callosal Connections in Hypothyroid Adult Rats

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Abstract

Callosal connections were studied with tracers (horseradish peroxidase (HRP) and wheat germ agglutinin–horseradish peroxidase (WGA–HRP)) in normal rats and rats deprived of thyroid hormones with methimazole (Sigma) since embryonic day 14 and thyroidectomized at postnatal day 6. In hypothyroid rats, the auditory areas, in particular the primary auditory area, showed cytoarchitectonic changes including blurred lamination and decrease in the size of layer V pyramidal neurons. In control rats, callosally-projecting neurons were found between layers II and VI with a peak in layer III and upper layer IV. In hypothyroid rats, labelled neurons were found between layers IV and VI with two peaks corresponding to layer IV and upper layer V, and in upper layer VI. Quantitative analysis of radial distribution of callosally-projecting neurons confirmed their shift to infragranular layers in hypothyroid rats. Three-dimensional reconstructions showed a more continuous tangential distribution of callosally-projecting neurons in hypothyroid rats which may be due to the maintenance of a juvenile 'exuberant' pattern of projections. These changes in cortical connectivity may be relevant for understanding epilepsy and mental retardation associated with early hypothyroidism in humans and to clarify basic mechanisms of cortical development.

Introduction

Thyroid hormones (TH), 3,5,3′ triiodothyronine (T3) and thyroxine (T4), play an important role during brain development and their deficiency at early ages produces a generalized damage in the brain and particularly in the cytoarchitecture of the neocortex (Eayrs, 1955). Hypothyroidism in developing rats (H rats) increases the density of cortical cells, decreases soma size of cortical neurons, especially in layer V (Eayrs and Taylor, 1951) and density of axons (Eayrs and Horn, 1955).

Other changes observed in the cortex of H rats reflect alterations in the maturation of individual neurons. These may include changes in the length and branching of dendrites (Eayrs, 1955; Eayrs and Goodhead, 1959; Ruiz-Marcos and Ipiña, 1986), in the gene expression and synthesis of proteins (Nunez *et al.*, 1980; Gravel *et al.*, 1990; Muñoz *et al.*, 1991; Cerezo *et al.*, 1991) as well as in the number of microtubules of apical dendrites of pyramidal neurons and in their spatial arrangement (Berbel *et al.*, 1983), and in myelination of callosal axons (Gravel *et al.*, 1990; Berbel *et al.*, 1993). These changes, in addition to a reduction in the numbers of several types of receptors (e.g. cholinergic, beta-adrenergic and GABA-ergic) found in H rats (Legrand, 1984; Sandrini *et al.*, 1991), might be responsible for a delay in synaptogenesis (Cragg, 1970). In addition, early thyroid dysfunction affects auditory structures and functions (Trotter, 1960; Van Middlesworth and Norris, 1980; Ruiz-Marcos *et al.*, 1983; Uziel *et al.*, 1983; Prieto *et al.*, 1990a,b).

More relevant for the mental retardation characteristic of early hypothyroidism in humans could be changes in the pattern of cortical connections. In H rats, unlike in normal rats (C rats), callosally-projecting neurons were distributed continuously over areas 18a, 18b and 17 and over somatosensory areas (Gravel *et al.*, 1990).

In the present study, we have examined the organization of callosal auditory connections in H rats to obtain clues to better understand the role of TH in neocortical morphogenesis.

Materials and methods

Female Wistar rats were used; there were 5 C rats (3 from one litter and 2 from another) and 11 H rats (1 or 2 per litter from 8 litters). Litters were equated to 8 pups at postnatal day (P) 8 (2 days after thyroidectomy in H rats). C and H rats were killed at ages between P132 and P235 (Table 1).

Experimental hypothyroidism

In order to decrease the amounts of TH transferred from the mother and to inhibit the synthesis of TH by the fetal thyroid, pregnant rats were treated with 0.02% methimazole (MMI; Sigma) in the drinking water (Morreale de Escobar *et al.*, 1989). Treatment began at embryonic day (E) 14 (vaginal plug appeared at E0) and continued, after birth (= E21) until P9. From P9 onwards (3 days after thyroidectomy, see below), 1% calcium gluconate was added to the

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MMI solution and it was maintained until the death of rats. In addition to the MMI treatment, ether anaesthetized rats were thyroidectomized at P6 following a procedure already described (Zarrow *et al.*, 1964). H rats were kept with their mothers until killed to prevent premature death of rats. If weaned at P21, they usually died before P80, most probably because of undernutrition due to retarded dental development and difficulties with gnawing normal solid food.

The degree of hypothyroidism was assessed by the body weight curve. In addition, tissue homogenates from the temporal cortex of deeply anaesthetized littermate rats at different ages were processed for the determination of TH by specific radioimmunoassays adapted for rat samples (Obregón *et al.*, 1978; Morreale de Escobar *et al.*, 1985). TH was determined in 3 C rats (one at P153 and 2 at P232) and 7 H rats (one each at P10, P17, P25 and P30, and 3 at P153).

Tracer injection

C and H rats were anaesthetized with Imalgene 1000 (Rhône Mèrieux; 1.2 ml/kg i.p.) and were injected with horseradish peroxidase (HRP) alone or conjugated with wheat germ agglutinin (WGA). Tracers were injected between 4 and 5 mm from the bregma, between 7 and 10 mm from the sagittal sinus aiming at the primary auditory cortex (Te1; Krieg, 1946; Zilles, 1985; Zilles and Wree, 1985; Sally and Kelly, 1988; Herbert *et al.*, 1991; Figs 1 and 2) and between 900 and 1000 μm under the pial surface, using micropipettes (40 μm mean tip diameter) connected to a picopump. For injected volume, dilution of tracers and age at injection see Table 1.

Two days after HRP or WGA–HRP injections, rats were deeply anaesthetized and perfused with 1% paraformaldehyde, 0.5% glutaraldehyde, 0.002% $CaCl_2$ and 0.1 M sucrose in 0.1 M phosphate buffer (pH 7.4) for 30 min, followed by 10% buffered sucrose for 10 min. After perfusion, the brains were removed, immersed in 20 and 30% buffered sucrose until they sank, frozen and coronally cut at 60 μm. Floating sections were processed with tetramethylbenzidine (Mesulam, 1978), mounted on gelatin coated slides, quickly dehydrated in ethanol, cleared in xylol and coverslipped. Every sixth section was counterstained with 0.05% borax and 0.05% toluidine blue at 50°C.

TABLE 1. Summary of experiments

Animal code	Age at injection (days)	Concentration and type of tracers	Number of injections	Total injected volume (μl)
Control				
IR03	130	50% HRP	2	0.50
IR07	151	5% WGA–HRP	1	0.10
IR22	230	50% HRP	1	0.80
IR24	233	50% HRP	1	0.10
IR25	233	5% WGA–HRP	3	0.30
Hypothyroid				
IR04	151	50% HRP	2	0.50
IR09	149	5% WGA–HRP	1	0.10
IR10	149	5% WGA–HRP	1	0.10
IR11	155	10% WGA–HRP	1	0.10
IR12	155	10% WGA–HRP	1	0.10
IR13	131	50% HRP	1	0.10
IR14	131	50% HRP	1	0.10
IR15	147	50% HRP	1	0.50
IR16	147	5% WGA–HRP	4	0.50
IR19	149	50% HRP	4	0.60
IR21	151	50% HRP	3	0.90

Analysis of the material

Sections were studied and photographed using a Zeiss Axiophot photomicroscope. Quantitative analysis of distribution of callosally-projecting neurons was performed in 3 C and 6 H rats on the basis of optimal location and size of injections and quality of labelling (Fig. 2). The position of retrogradely labelled neurons in selected sections was plotted with a computer-linked microscope which consisted of an AT-486 computer equipped with a Screen Machine video board (Fast Electronic, Germany) connected to a Sony AVC-D7CE CCD video camera mounted on an Olympus BH-2 microscope. We used the computer program Neurograph (Microptic, Spain) for drawing, measurement and spatial representation of sections. Differences in measurements were assessed using Kruskal–Wallis non-parametric test for analysis of variance.

Distribution of neurons was represented in outlines of individual frontal sections and in 3-D reconstructions from serial non-consecutive sections. In addition, histograms of the fraction of labelled neurons at different depths in the cortex were generated according to the following modification of a previous method (Innocenti, 1980; Innocenti and Clarke, 1983). A line running half way between the pia and the white matter was generated and divided in 50 μm bins; neurons were projected onto it according to a minimal distance algorithm. The number of neurons per bin was represented as two vertical segments, the upper one indicated the number of neurons in layers II and III, and the lower one in layers IV–VI (Fig. 11).

Results

Body weight and concentrations of T4 and T3

Treatment with MMI and subsequent thyroidectomy reduced the body weight of H rats to about one-third of that of C rats (on average,

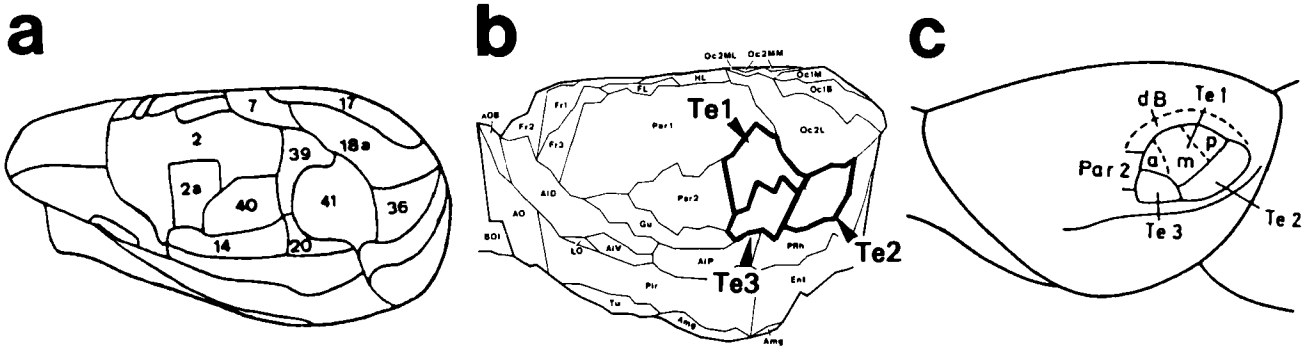


FIG. 1. Outlines of a lateral view of the left hemisphere of a rat brain with the position and extent of auditory cortical areas. (a) After Krieg (1946); (b) after Zilles (1985); (c) after Herbert *et al.* (1991). Note the overlap of areas 41, 36 and 20 with areas Te1, Te2 and Te3, respectively.

300 ± 54 g in C and 107 ± 20 g in H rats; Fig. 3a). During the early postnatal period (between P10 and P30), the concentrations of T4 and T3 in the brain of littermates of H rats were markedly reduced (on average, 0.13 ± 0.08 ng T4/g tissue weight and 0.34 ± 0.2 ng T3/g) as compared to C rats at P153–232 (see below). However, the concentrations of T4 and especially of T3 in the brain of littermates of H rats at the time of experiment (P153) were not markedly decreased. On average, we found 0.23 ± 0.16 ng T4/g tissue weight in H versus 1.72 ± 0.13 ng/g in C rats and 0.56 ± 0.05 ng T3/g in H versus 1.0 ± 0.06 ng/g in C rats (Fig. 3b).

Cytoarchitecture of the auditory cortex

In C rats, the cytoarchitecture of the auditory cortex from P130 onwards was similar to that described in previous studies (Krieg, 1946; Zilles and Wree, 1985; Games and Winer, 1988; Roger and Arnault, 1989; Fig. 4a). In particular, Krieg's area 41 (overlapping area Te1; Zilles, 1985; Zilles and Wree, 1985; Herbert *et al.*, 1991) because of its clear-cut layering stood out with respect to more ventral Krieg's areas 36 and 20 (partially corresponding to secondary auditory areas Te2 and Te3, respectively; Zilles, 1985; Zilles and Wree, 1985; Herbert *et al.*, 1991; Fig. 1). On the other hand, in H rats in the region topographically corresponding to Te1, the cortical grey matter was thinner, appeared to have somewhat higher cell density and its layering was far less clear (Fig. 4b). These features allowed two of the investigators to identify H rats from inspection in a double blind test.

A finer analysis of the material and measurements of the cortical layers in two Nissl-stained sections chosen at random from each rat revealed other differences between C and H rats. Layer I was thicker ($P < 0.001$) in H (151.4 ± 19.4 μm; 15.3% of the whole cortical thickness) than in C rats (113.9 ± 32.3 μm; 10.6%). Layers II and III were thinner ($P < 0.037$) in H (205.9 ± 20.3 μm; 20.8%) than in C rats (246.5 ± 25.8 μm; 23.1%). In H rats, the border between layers IV and V was very difficult to identify because the cell density was similar in both layers and in addition, layer V pyramids were smaller than in C rats and they were intermingled with small cells. However, scattered normal-looking large pyramidal neurons were still observed at the bottom of layer V. Together, layers IV and V were similar in thickness ($P > 0.69$) in H (334.8 ± 20.5 μm; 33.8%) and in C rats (3542.1 ± 34.2 μm; 32.9%). Layer VI was thinner ($P < 0.035$) in H (297.8 ± 18.6 μm; 30.1%) than in C rats (357.6 ± 43.1 μm; 33.4%; Fig. 4a,b).

Organization of callosal connections

Location of tracer injections

The cores of tracer injections, characterized by very dense extracellular deposits of HRP, varied between 0.3 and 1.8 mm in their tangential extent and involved all cortical layers. These cores were surrounded by halos of variable extent corresponding to zones of diffusion of the tracers. Based on cytoarchitectonic criteria, in C rats, injection sites proved to be in the caudal part of area Te1 and involved the surrounding areas to variable extents. Topographically corresponding parts of the cortex were injected in H rats except in three cases where injections were placed more caudally (IR11 and IR12) and laterally (IR15) presumably in Te2 and Te3, respectively (Fig. 2). The locations of the injections were controlled by analysis of the thalamus where retrograde labelled neurons were invariably found in various subdivisions of the medial geniculate body.

Radial distribution of labelled neurons

In C rats, the radial distribution of labelled neurons in the contralateral auditory cortex matched previous descriptions (Jacobson and

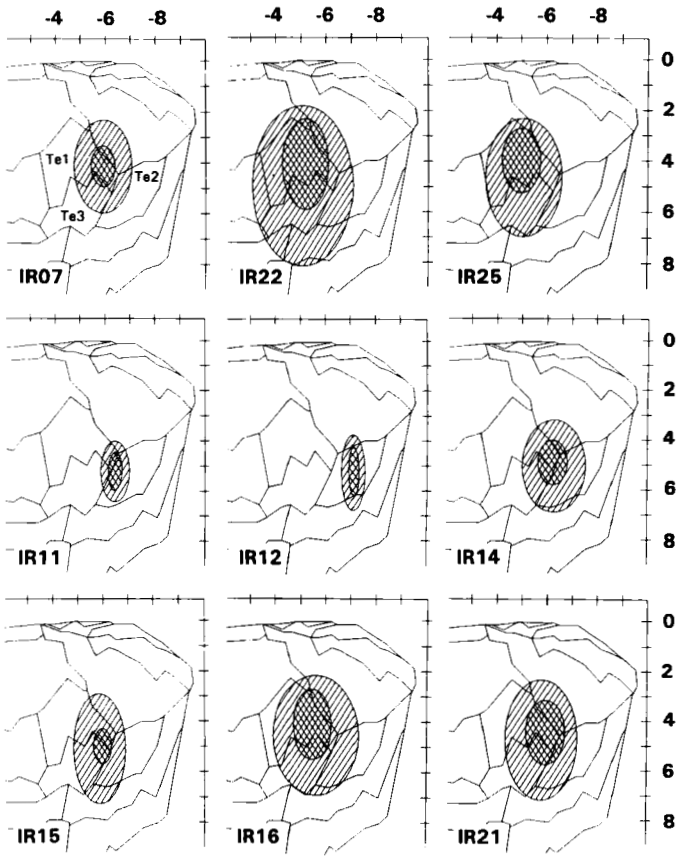


FIG. 2. Position and extent of tracer injections projected on a standard lateral view (Zilles, 1985) of the left hemisphere of C (IR07, IR22 and IR25) and H (IR11, IR12, IR14, IR15, IR16 and IR21) rats. Cross-hatched areas indicate the cores of the injections; hatched areas the halos of diffusion of the tracers. Zilles' stereotaxic coordinates are indicated in the upper and right axes. The codes of the animals are indicated in the lower left corners.

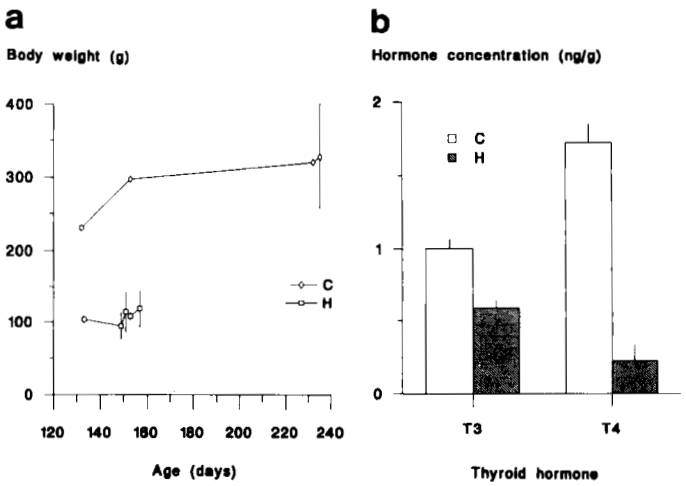


FIG. 3. (a) Evolution of body weight in C and H rats. In C rats, body weight increases markedly with age. In H rats, body weight increases slightly and remains well under C values. (b) Concentrations of T3 and T4 in the temporal cortex of C rats (one at P153 and two at P232) and H rats at P153. Levels of T3 and T4 are lower in H than in C rats.

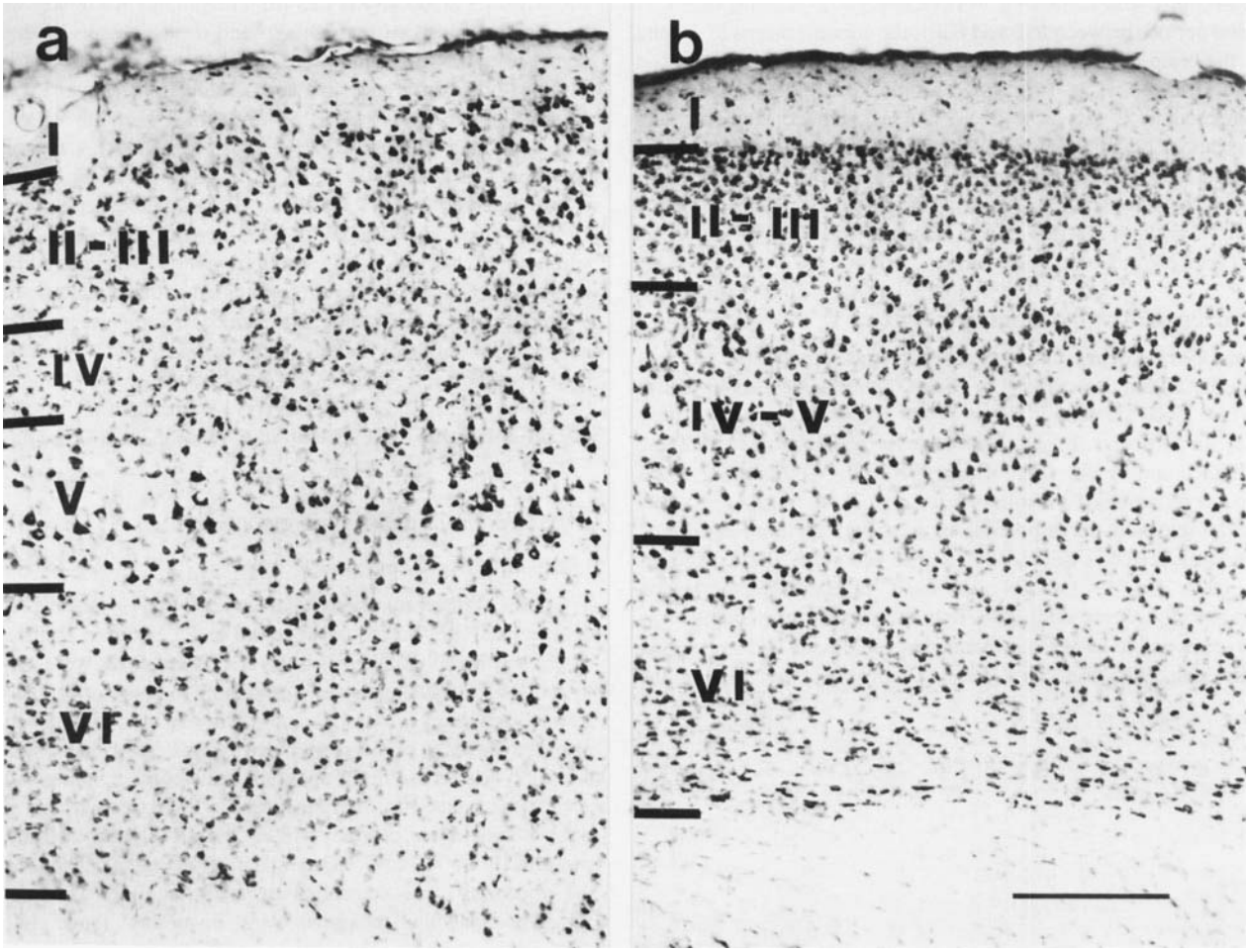


FIG. 4. Photomicrographs of Nissl-stained sections showing the cytoarchitecture of the auditory cortex in C (a) and H (b) rats. Note the thinner grey matter and the blurred cortical layering in H rats. H rats also show increased cell density in all layers, especially in layers IV and V. Scale bar 200 μm; same calibration for a and b.

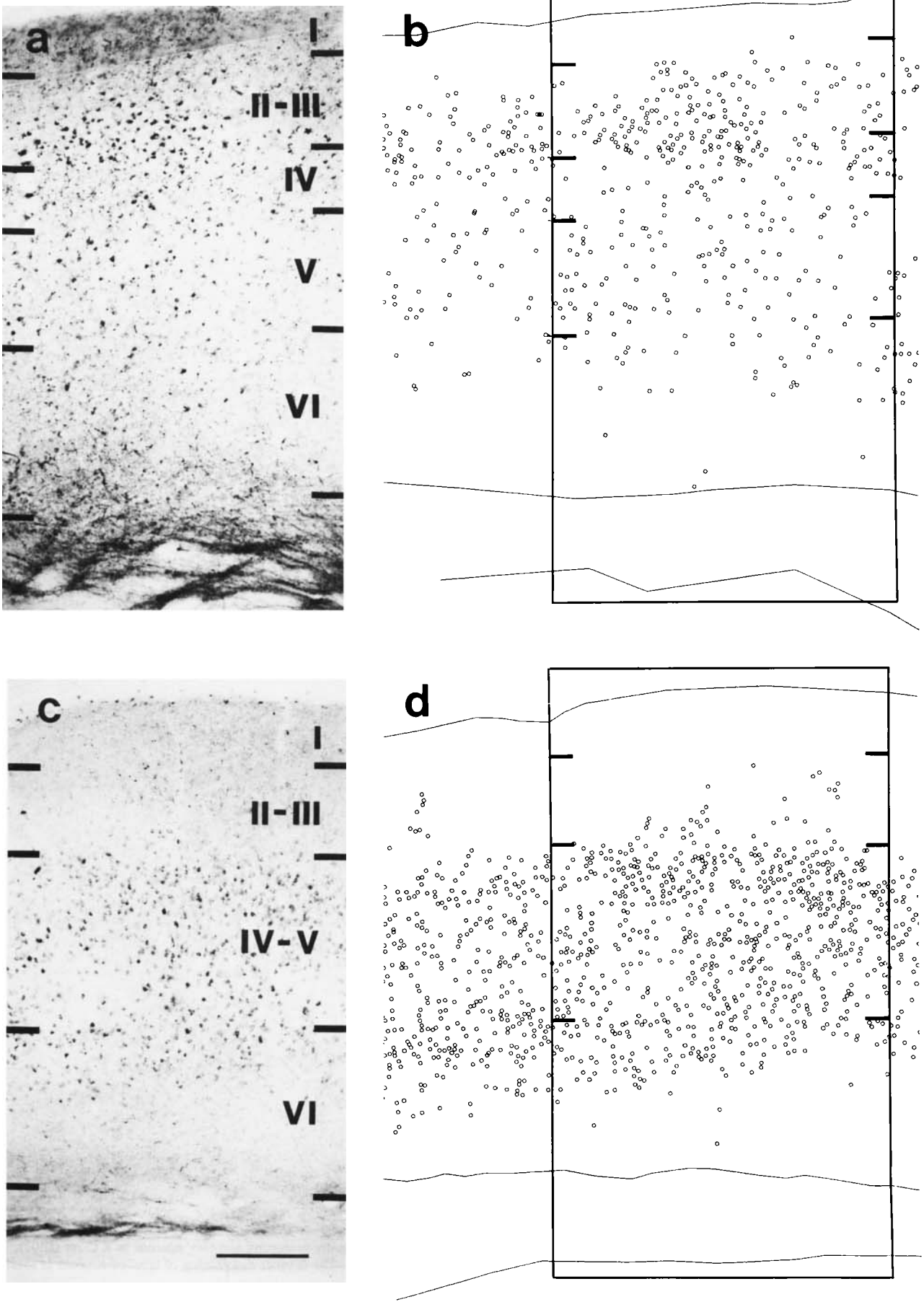
Trojanowski, 1974). Callosally-projecting neurons were found in layers II–VI, with the highest density in layer III and upper layer IV; a decreased density of labelled neurons in layer IV gave to the overall distribution a bilaminar appearance (Figs 5a,b, 6 and 7).

In contrast, in H rats, labelled neurons were found in layer IV, upper layer V, and in upper layer VI. A region of decreased density of labelled neurons in the deeper half of layer V gave to the distribution a somewhat trilaminar appearance. In layers II and III, only a few patches of scattered labelled neurons were found (Figs 5c,d, 8–10).

Quantitative analysis of the distribution revealed that, in C rats, in all subdivisions of the auditory cortex, layers IV–VI contained the highest fraction of labelled neurons while between 13.6 and 49.3% of them (31.6% on average) were in the supragranular layers II and III (Fig. 12a–c). The proportion of labelling in different layers varied among auditory areas depending on the location of the injection. In general, labelling was highest in areas homotopic to the injections (Figs 2, 11 and 12). Thus, in IR07 which was injected in Te1 at the border with Te2 (Fig. 2), the proportion of labelled neurons in supragranular layers was higher in Te1 (23.7%) and in Te2 (31.3%) than in Te3 (13.6%; Fig. 12). Similarly, in IR22 and IR25 which were injected in Te1 at the border with Te2 (Fig. 2), the fraction of labelled neurons in the supragranular layers was the lowest in Te2 (Fig. 12).

The density of labelled neurons was higher in H than in C rats; there were, on average, 904 neurons per section in H (Fig. 11d–f) versus 432 neurons per section in C rats (Fig. 12a–c). However, only between 0.5 (Te2 in IR16) and 8.4% (Te1 also in IR16; 3.9% on average) of labelled neurons were in supragranular layers II and III (Fig. 12). As in C rats, there was a tendency for a higher fraction of labelled neurons to be found in cortical layers at sites homotopic to the injection (Figs 2, 11 and 12).

FIG. 5. Photomicrographs (a,c) showing retrograde labelled neurons and borders between layers in C (IR25) and H (IR16) rats, respectively. Borders between layers were defined in each case by overlaying, using a camera lucida, the corresponding Nissl-stained adjacent section. In b and d, plots of retrograde-labelled neurons are shown; boxes correspond to areas shown in the photomicrographs. In C rats, an important proportion of labelled neurons are in supragranular layers II and III. On the contrary, in H rats, almost all labelled neurons are between layers IV and VI. Scale bar 200 μm; same calibration for a–d.


a
I
II-III
IV
V
VI
b
c
I
II-III
IV-V
VI
d

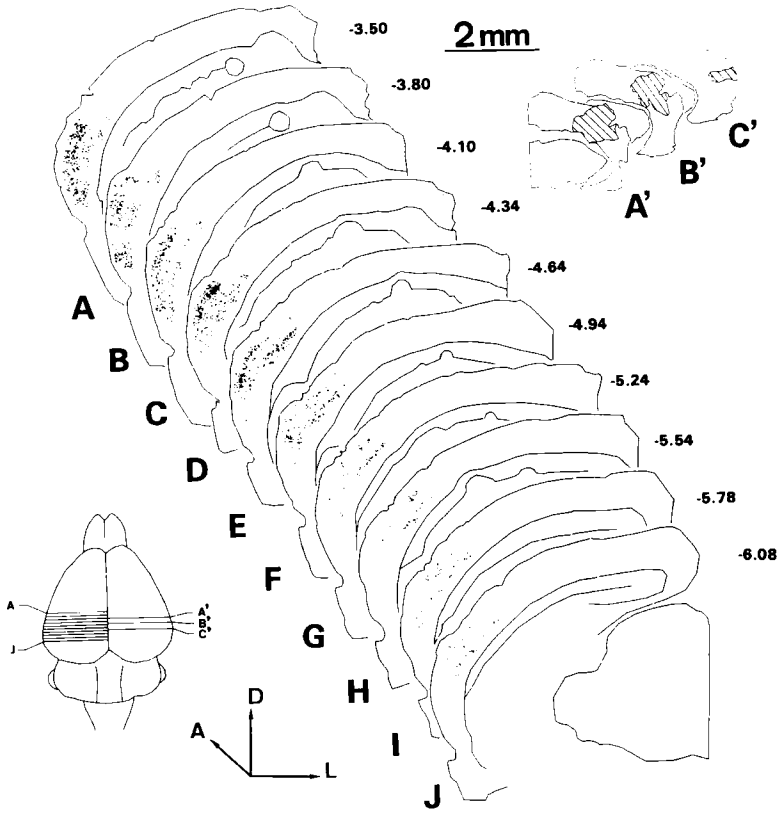


FIG. 6. Outlines of coronal sections (A−J) showing distribution of retrogradely labelled callosally-projecting auditory neurons in a C rat (IR22), following the WGA−HRP injections shown by sections (A′−C′) in the upper right inset (see also Fig. 2). Levels of sections (A−J) and (A′−C′) are shown in the brain figurine. Labelled neurons are distributed between layers II and VI with a peak-density in layers II and III. Numbers indicate anteroposterior stereotaxic coordinates after Paxinos and Watson (1986). Scale corresponds to sections A−J. Arrows indicate auditory (A), dorsal (D) and lateral (L) directions.

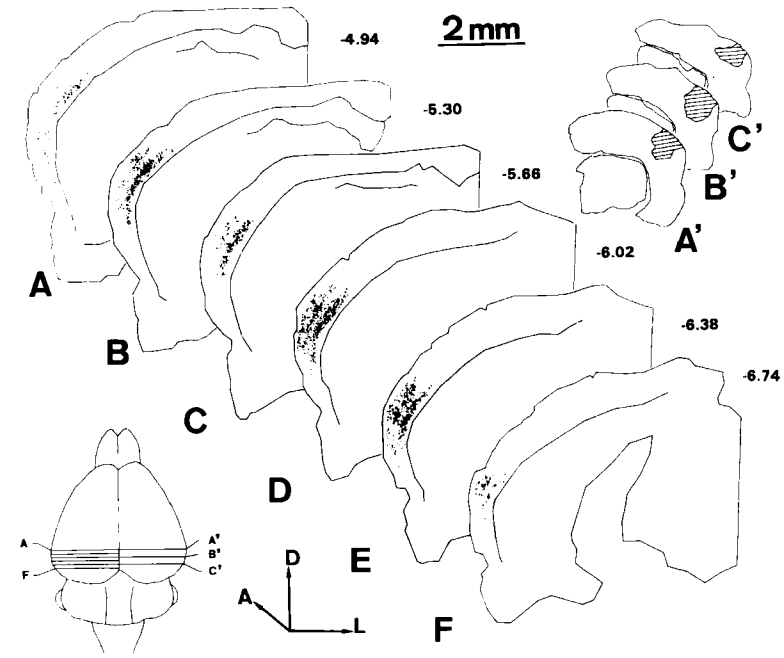


FIG. 7. Outlines of coronal sections (A−F) showing distribution of retrogradely labelled callosally-projecting auditory neurons in a C rat (IR07), following the WGA−HRP injection shown by sections (A′−C′) in the upper right inset (see also Fig. 2). The laminar distribution of labelled neurons is similar to that in Figure 6. Conventions are as in Figure 6.

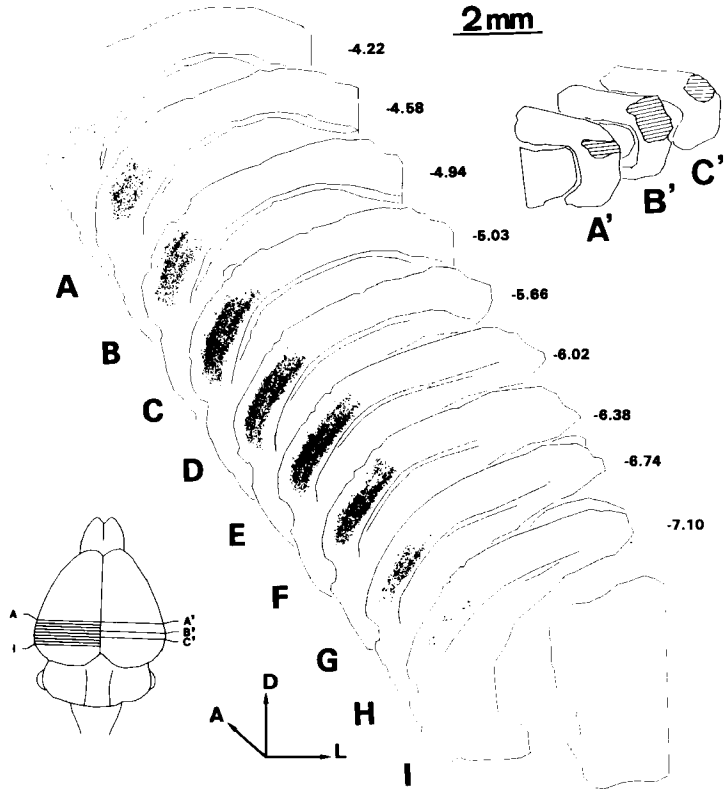


FIG. 8. Outlines of coronal sections (A–I) showing distribution of retrogradely labelled callosally-projecting auditory neurons in a H rat (IR16), following WGA–HRP injections shown by sections (A′–C′) in the upper right inset (see also Fig. 2). Labelled neurons are distributed between layers IV and VI with two peaks in the density; one in upper layer IV and V, the other in lower layer IV and V and upper layer VI. Conventions are as in Figure 6.

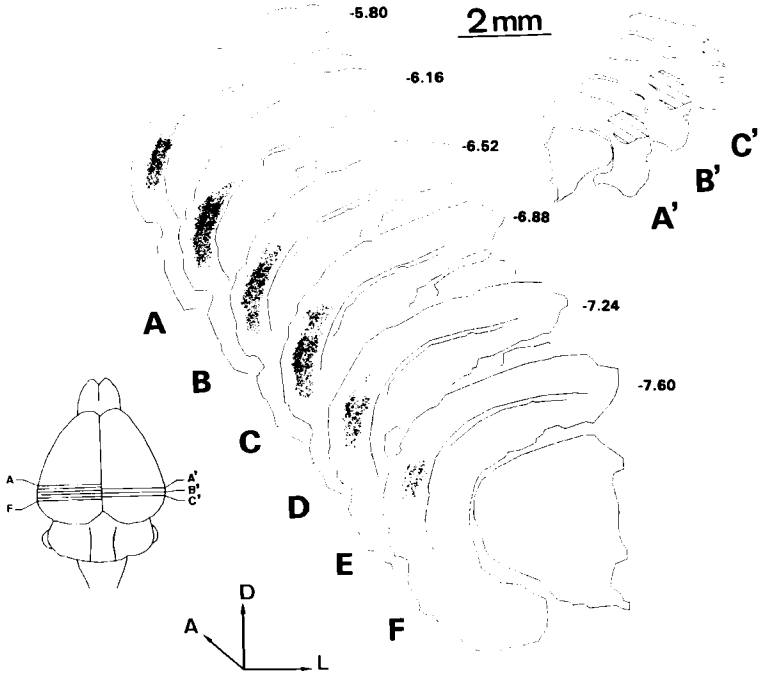


FIG. 9. Outlines of coronal sections (A–F) showing distribution of retrogradely labelled callosally-projecting auditory neurons in a H rat (IR14), following the HRP injection shown by sections (A′–C′) in the upper right inset (see also Fig. 2). The laminar distribution of labelled neurons (in layers IV, V and VI) is similar to that in Figure 8. Conventions are as in Figure 6.

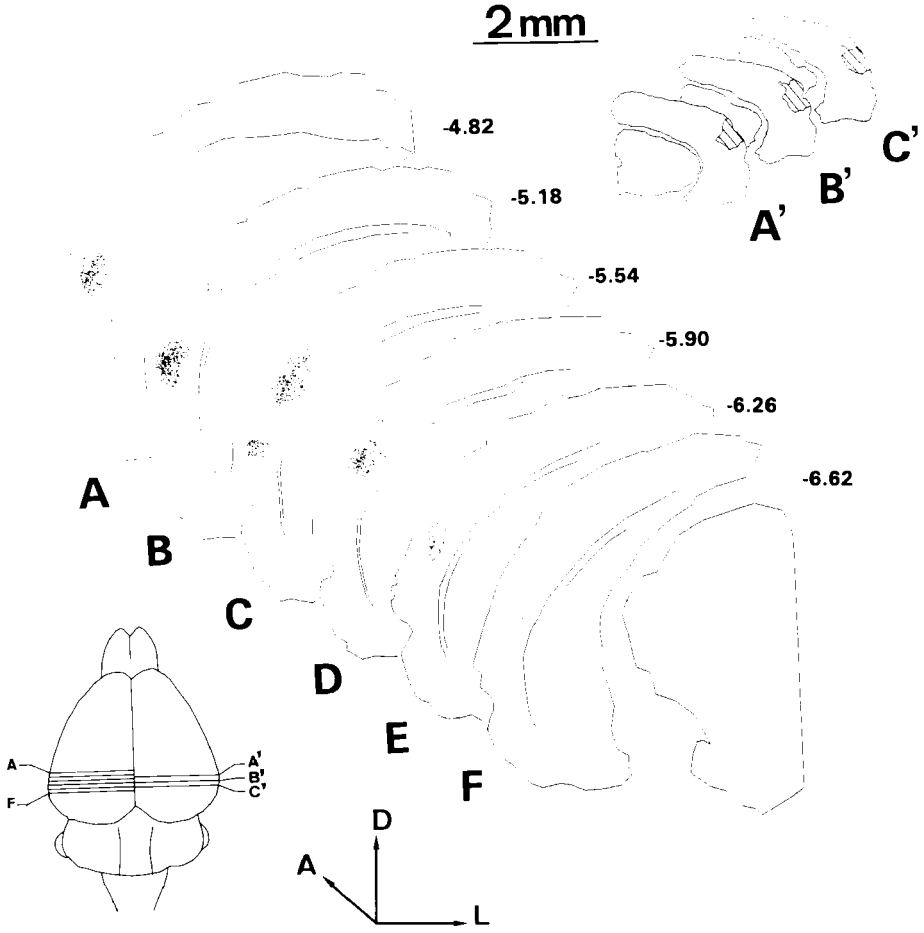


FIG. 10. Outlines of coronal sections (A–F) showing distribution of retrogradely labelled callosally-projecting auditory neurons in a H rat (IR11), following the WGA–HRP injection shown by sections (A′–C′) in the upper right inset (see also Fig. 2). The laminar distribution of labelled neurons (in layers IV, V and VI) is similar to that in Figures 8 and 9. Neurons are distributed into two patches: one rostral and ventral (A–D), the other caudal and dorsal (B–F). Conventions are as in Figure 6.

Tangential distribution of labelled neurons

In both C and H rats, the tangential distribution of labelled neurons depended on size and location of the injection. In most experiments, labelled neurons were found in all the subdivisions of the auditory cortex and usually extended to the surrounding areas; their density was highest at sites homotopic to the injection (Fig. 11).

In C rats, which received large injections, labelled neurons were tangentially distributed in a widespread zone which, in coronal sectors, appeared as a dorsoventrally running band. The tangential distribution of labelled neurons was uneven within this band due to the irregular alternation of patches, usually seen in supragranular layers, with high and low densities of labelled neurons (Figs 6, 7 and 11a–c). In infragranular layers, labelled neurons were tangentially distributed in a continuous band.

In H rats, with large injections (e.g. IR14, IR16 and IR21), labelled neurons were also tangentially distributed in a widespread zone which, in coronal sections, appeared as a dorsoventrally running band (Figs 8, 9 and 11d,e). Unlike in C rats, in the tangential direction, density of labelled neurons was homogeneous. The difference in the distribution between H and C rats is particularly clear in 3-D reconstructions (Fig. 13a,b).

The homogeneous distribution of labelled neurons in H rats does not imply the absence of topography in the connections between areas in the two hemispheres.

In two H rats that received small injections of tracer mainly localized in Te3 (IR11 and IR15; Fig. 2), labelled neurons showed a tangentially discontinuous distribution in two clusters between 400 and 1300 μm in diameter and separated dorsoventrally by a gap void of labelled neurons between 400 and 900 μm wide; both clusters extended rostrocaudally over 1–1.5 mm (Figs 10 and 11f) and occupied part of Te1 and of Te3.

In one C rat (IR25), where the injection site was probably restricted to Te1, no labelled neurons were found in Te2. In one H rat (IR12), in which the injection appeared to be restricted to Te2, no labelled neurons were found in Te1 and Te3 (Figs 2, 11 and 12). Thus, in C and H rats, callosal connections appear to be topographically organized both within corresponding and non-corresponding areas.

Anterograde labelling

In addition to neurons, terminating axons were also labelled and appeared in polarized light as a 'dusty' precipitate. Some of these axons are presumably of callosal origin, others may be retrogradely-labelled collaterals of callosally-projecting neurons. In C and H rats, the tangential distribution of labelled terminating axons overlapped with

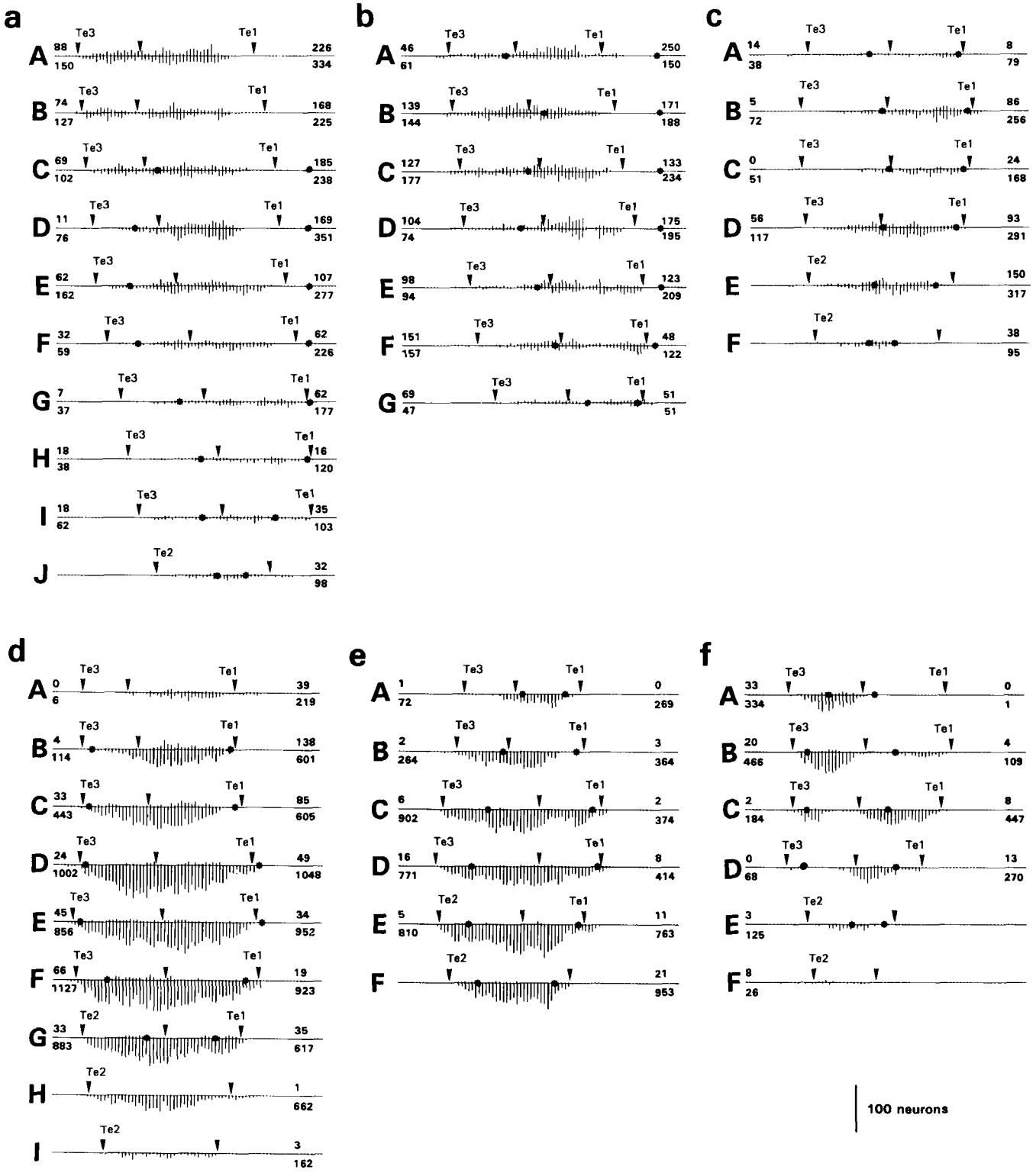


FIG. 11. Histograms showing the distribution of retrogradely labelled callosally-projecting neurons in sections shown in Figures 6–10. Each section is represented by an upright and an inverted histogram separated by a continuous line; they represent neurons in layers II and III and IV–VI, respectively. These histograms are separated by a horizontal line representing the border between layers III and IV. (a) Data from IR22 (shown in Fig. 6). (b) Data from IR25. (c) Data from IR07 (shown in Fig. 7). (d) Data from IR16 (shown in Fig. 8). (e) Data from IR14 (shown in Fig. 9). (f) Data from IR11 (shown in Fig. 10). Borders between auditory areas according to Zilles (1985) are indicated by arrowheads. Limits of injections (asterisks) measured from the contralateral hemisphere are overlayed on the zones of retrograde labelling. Numbers indicate the absolute number of labelled neurons in layers II and III and IV–VI for each cortical area. Height of histograms is proportional to number of neurons (calibration) in 50 μm wide radial bins of cortex.

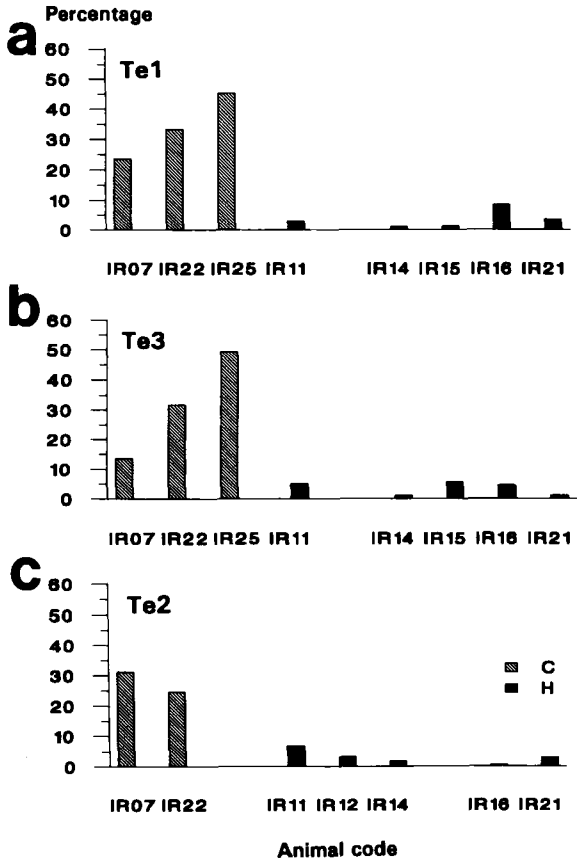


FIG. 12. Histograms representing retrogradely labelled callosally-projecting neurons in supragranular layers as percentage of the total number of labelled neurons in C (IR07, IR22 and IR25) and H rats (Ir11, IR12, IR14, IR15, IR16 and IR21). In all auditory cortical areas Te1 (a), Te3 (b) and Te2 (c) the percentage of supragranular neurons is higher in C than in H rats.

that of retrogradely-labelled neurons (Fig. 14a–d). In C rats, labelled axons were mostly distributed between layers III and VI with a peak in layer III although a narrow band of labelled axons in layer I was also found (Fig. 14b).

In H rats, labelled terminating axons were also found between layers I and VI but, differently from C rats, they were distributed in four bands. Two of these bands were thinner and narrower in their tangential extent and were located between layers I and III, in a region devoid of callosally-projecting neurons. The other two were thicker and broader in their tangential extent and were located in layer IV and at the bottom of layer V in correspondence with the bands of labelled neurons (Fig. 14c,d).

Discussion

The model of experimental hypothyroidism

Recent studies have proved that nuclear TH receptors are present in fetal brains at E14, reaching the normal fetal level (~30% of adults; Pérez-Castillo *et al.*, 1985) at E16. Cortical neurogenesis starts at E16 (Berry *et al.*, 1964). Therefore, to study the role of TH in cortical development it seems to be necessary to inhibit the fetal synthesis and maternal transfer of TH at early fetal ages (Morreale de Escobar *et al.*, 1987).

Other goitrogens, such as propylthiouracil, have been used to inhibit maternal transfer and fetal synthesis of TH (Rami and Rabié, 1988; Gravel and Hawkes, 1990; Gravel *et al.*, 1990) and the peripheral diodination of T4 (Geffner *et al.*, 1975; Saberi *et al.*, 1975). In this study, we used MMI because it seems to be a most efficient goitrogen (Marchant *et al.*, 1978). Rats treated with MMI and thyroidectomized 5 days after birth show an important reduction in the levels of TH in brain tissue (Muñoz *et al.*, 1991). Although it has been reported that MMI might have teratogenic effects (Van Dijke *et al.*, 1987) when administered at E14, all MMI-treated pregnant rats arrived at term and the number of pups per litter was similar to C rats.

Results from radioimmunoassays showed that TH levels in brain tissue were low in our H rats. These small amounts of TH present in H rats may have an exogenous origin such as food-pellets containing animal proteins or animal excrements. These variations in levels of TH, probably also present in injected rats, may reflect variations in the organization of their cortical connections. In fact, the number of retrogradely labelled neurons in layers II and III, although always very low compared to controls, varied across H rats possibly reflecting changes in the TH content.

It may be worth stressing that, in addition to TH, other hormones and hormone-dependent metabolites are unbalanced in H rats (Eayrs, 1959; Balázs, 1974; Balázs *et al.*, 1975; Morreale de Escobar *et al.*, 1983). TH is needed for growth hormone gene expression (Seo *et al.*, 1978) and synthesis (Hervás *et al.*, 1975). The decrease in growth hormone observed in H rats not only arrests body growth but also may be responsible for some degree of brain damage (Eayrs, 1959; Legrand, 1977).

Other developmental anomalies of H rats could be due to the concomitant undernutrition which not only affects normal development of the CNS (Balázs *et al.*, 1979) but also causes premature death of the rats. To maximally prevent undernutrition, our H rats were kept with their mothers all their lifetime and suckled long after P21 (see Materials and methods).

In spite of uncertainty as to what causes the abnormalities in H rats, the latter have been widely used as as model for congenital hypothyroidism (for a review, see Morreale de Escobar *et al.*, 1989). In this study, they provided a useful model for studying the development of cortical cytoarchitectonics and connectivity.

Experimental hypothyroidism as a model for studying cortical development

Cytoarchitectonic changes

The unsharp cortical layering in Te1 in H rats may be due, at least in part, to the decreased size of layer V neurons and the increased density of cells already noticed in previous studies of other areas (Eayrs, 1955). In addition, however, the absence of a clear-cut layer IV, the presence of grains in layer V and the lack of callosally-projecting neurons in layer III suggest that irregularities in neuronal migration may also have occurred. In the cerebellar cortex of H rats, granules migrate to the molecular layer without reaching their final location in the granuler layer (Nicholson and Altman, 1972; Legrand, 1979; Lauder, 1989). In addition, hypothyroidism might affect neuronal migration by interfering with the development of the astroglia as reported for the hippocampus (Rami and Rabié, 1988).

The increase in cell density may be due, as previously suggested (Eayrs, 1955), to a decrease in the volume of the neuropile caused by a decrease in the length and ramification of dendrites and in the density of axons. Nevertheless, a decreased rate of normal developmental cell death cannot be excluded at this time (for the notion of cell death in developing cortex, see Heumann *et al.*, 1978; Finlay and Slattery, 1983). Although the neocortex is decreased in both its tangential and

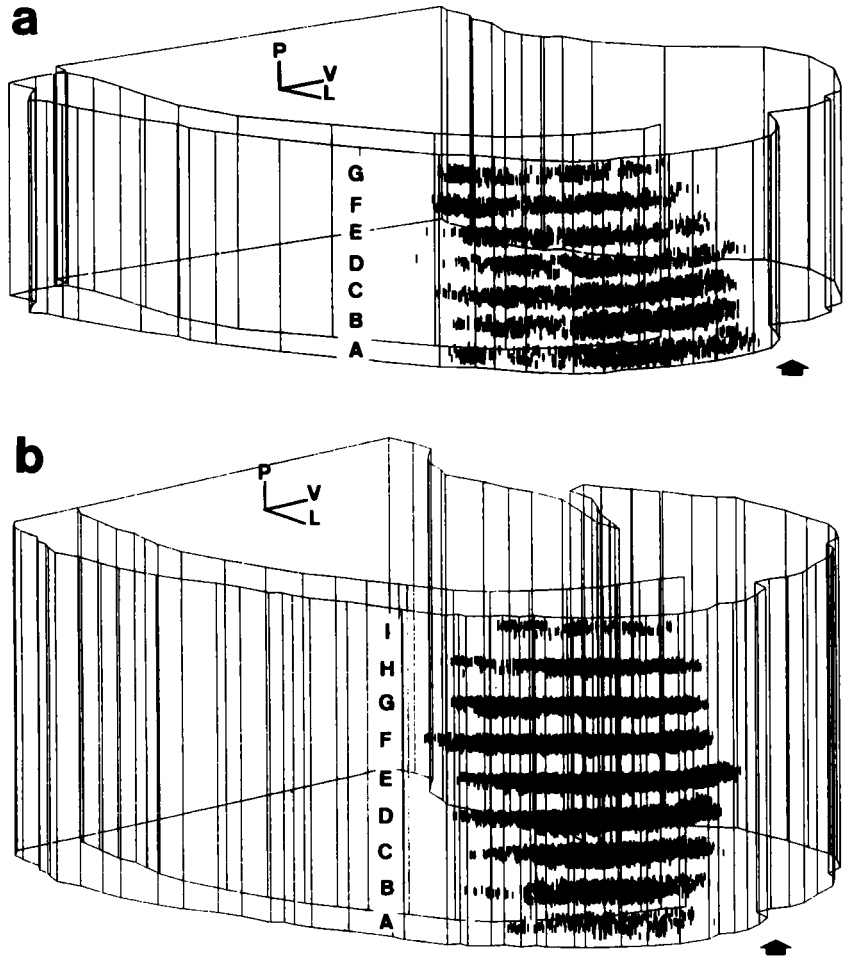


FIG. 13. Three-dimensional reconstruction, showing the tangential distribution of labelled neurons. (a) Data from IR25. (b) Data from IR16. Note the irregular patchy distribution of labelled neurons in C rats. In H rats, labelled neurons are distributed homogeneously. Arrow points to the rhinal sulcus. P, posterior; V, ventral; L, lateral.

radial extent, layer I is thicker in H than in C rats and this layer, in H rats, appeared to receive abnormal projections, some of which, presumably, were of callosal origin. Interestingly, the apical tuft of layer III pyramidal neurons is longer and more ramified in H than in C rats (Ruiz-Marcos and Ipiña, 1986).

Changes in connectivity

We found that neurons projecting to the contralateral auditory cortex acquired a deeper location and were more densely and evenly distributed in H than in C rats. In addition, terminating axons, at least partially of callosal origin, acquired an abnormal distribution in layers I–III of H rats.

No changes in the radial distribution of callosally-projecting neurons of the visual and somatosensory cortex were reported (Gravel and Hawkes, 1990). Our different results may reflect areal differences in cortical maturation. Alternatively, both the earlier onset of hypothyroidism (E14 versus E17 in Gravel and Hawkes' study) and a more severe hypothyroidism induced in the present study may have caused additional changes in the development of cortical connections.

Our findings that callosal projections originate from deeper layers in H than in C rats is interesting since the laminar origin of cortical projections is a particularly robust and reliable trait of cortical organization, related to the time a cortical neuron is born (McConnell and Kaznowski, 1991; McConnell, 1992). Changes in the radial orgin of cortical projections have been reported in several conditions. In the reeler mouse, projections to subcortical targets, normally originating in the infragranular layers V and VI, originate also from superficial layers; conversely, corticocortical projections, normally originating in layers II and III, originate from deep layers (Caviness, 1980). In these mutants, as in rats which were X-irradiated during neurogenesis (Jensen and Killackey, 1984) or in ferrets after heterochronic grafts (McConnell, 1988), neurons destined for a given layer can migrate abnormally and be 'trapped' in a different layer. However, they still form projections appropriate for their time of neurogenesis or serial position in a clone (for a review, see McConnell, 1991).

Similar mechanisms could explain the changes in the radial distribution of callosally-projecting neurons in our H rats. There are, however, alternative possible explanations which should be checked in further studies. Firstly, normally non-callosally-projecting neurons in deep cortical layers may have become competent to grow axons to the contralateral hemisphere in H rats. Secondly, transient projections from layers Vc and VIa (Ivy and Killackey, 1981) may be stabilized at the expense of other projections, possibly through a competitive mechanism. Nevertheless, our results seem to indicate that callosal connections are topographically organized between auditory areas. As in the cat (Rouiller *et al.*, 1991), in H rats and presumably in C

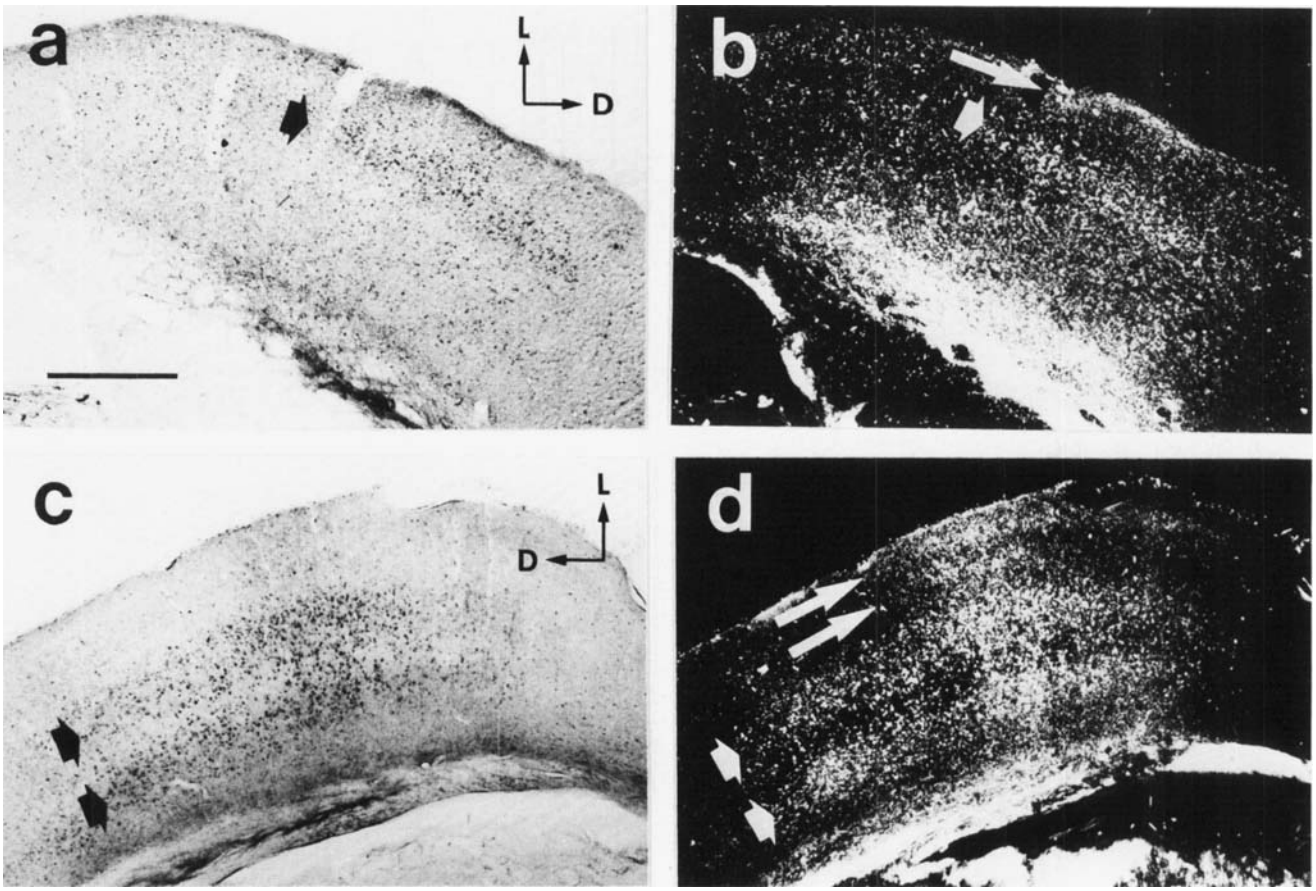


FIG. 14. Pairs of photomicrographs showing retrograde (a,c; bright field) and anterograde (b,d; polarized light) labelling in C (a,b; IR25) and H rats (c,d; IR16). In C rats (b), the peak of the anterograde labelling is found in layers II and III (thick arrow) matching the retrograde labelling (a). A narrow band of labelled axons (most of them presumably callosal) in upper layer I is also observed (long arrow). In H rats (d), two peaks of anterograde labelling are found in layer IV and upper layer V, and in upper layer VI (thick arrows) matching the retrograde labelling (c). Two narrow bands of anterogradely labelled axons (most of them presumably callosal) in layers I and II are also observed (long arrows). L, lateral; D, dorsal. Scale bar 500 μm; same calibration for a–d.

rats, caudal auditory areas may not communicate callosally with the primary auditory field.

In nearly all the areas and species studied thus far, the uneven distribution of callosally-projecting neurons was found to originate from a localized, selective loss of callosal projection (for a review, see Innocenti, 1991). Therefore, the even distribution of callosally-projecting neurons observed in the H rats results from the maintenance of exuberant callosal projections (Gravel and Hawkes, 1990).

If this interpretation is correct, it would have several interesting consequences. Firstly, it has been suggested that the development of callosal connections implies, at the level of individual axons, the transition from a juvenile–labile to either an adult–stable state or their elimination. The transition to the adult state was found to involve maturation of several cytoskeletal proteins (Figlewicz *et al.*, 1988; Guadaño-Ferraz *et al.*, 1990; Riederer *et al.*, 1990; Riederer and Innocenti, 1991) and the radial growth and myelination of at least a fraction of the axons (Berbel and Innocenti, 1988; Berbel *et al.*, 1989). In H animals, cytoskeletal maturation is delayed or does not occur (Nunez *et al.*, 1980; Cerezo *et al.*, 1991); the same is true for the radial growth of axons and myelination (Gravel *et al.*, 1990; Guadaño-Ferraz *et al.*, 1991; Berbel *et al.*, 1993). The H condition therefore seems to dissociate stabilization of juvenile axons from cytoskeletal maturation, radial growth and myelination which were previously thought to be necessarily linked. Alternatively, the adult H brain may maintain aspects of developmental plasticity.

In a very different condition, experimental microcortex, produced by deleting granular and infragranular layers in the cat, maintained transient projections from visual to auditory areas but not transient callosal projections (Innocenti and Berbel, 1991a,b). By modifying the target cortex, the H condition may, therefore, interfere with the mechanism of target recognition which presumably plays a role in the stabilization/elimination of developing cortical connections (Innocenti, 1991).

In a somewhat speculative vein it was suggested that developmental exuberance and the deletion of connections which takes place in development may be a mammalian counterpart of metamorphosis in amphibians and possibly in holometabolous insects (Gravel and Hawkes, 1990; Kind and Innocenti, 1990). In amphibians and insects, the onset of metamorphosis is a hormonally triggered event. In particular, in amphibians, the absence of TH prevents the onset of metamorphosis, maintaining the tadpole in a juvenile state. In these tadpoles, as in H rats, normal developmental changes including regressive events fail to occur (Kollros, 1981; Schönenberger and Escher, 1988).

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Abbreviations

C	control
E	embryonic day
H	hypothyroidism
HRP	horseradish peroxidase
MMI	methimazole
P	postnatal day
TH	thyroid hormones
WGA	wheatgerm agglutinin

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