Expression of the neuronal surface glycoprotein Thy-1 is under post-transcriptional control, and is spatially regulated, in the developing olfactory system

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Summary

Expression of the neuronal cell surface glycoprotein Thy-1 has been studied during the development of the olfactory bulb in mice and rats, using in situ hybridisation and immunohistochemistry to follow the appearance of Thy-1 mRNA and protein, respectively. The mRNA was first detected 4 days before birth on all mitral cells, the main projection neuron of the bulb, as they formed a distinct layer and grew dendrites. At no stage was any spatial gradient of expression of Thy-1 mRNA evident around the mitral cell layer. Thy-1 protein, on the other hand, was first detectable 2 days later on a group of mitral cells immediately adjacent the point of entry of the olfactory nerve. The numbers of immunoreactive cells spread, over the next 7 days, to include all mitral cells, those located rostrally and laterally in the bulb being slowest to express Thy-1 protein. Thus there was a spatiotemporal gradient of expression of Thy-1 protein, which was not apparent in the earlier general expression of its mRNA, suggesting that some further inductive signal was required after transcription in order to get effective production of protein. Analysis of the growth of the mitral cell axons in the lateral olfactory tract suggested this signal was related to the cessation of axonogenesis, as Thy-1 immunoreactivity became detectable on these axons only when their expression of the transient epitope detected by the G10 antibody, present on microtubule-associated protein (MAP)1x only during axonal growth, declined.

For the first week after Thy-1 protein appeared on mitral cells, it was not distributed uniformly on their surface. Immunoreaction was relatively weak on the somatic surface, and the molecule appeared to be entirely excluded from the distal regions of its main dendrite, above the outer plexiform layer. Here the dendrite reaches up to the synaptic glomeruli formed with the incoming olfactory nerve axons. These distal regions of the dendritic shaft became immunoreactive only after the periglomerular cells had first begun to express Thy-1 protein in the glomeruli. Immunolabelling of the somatic membrane then increased, to give the adult pattern of uniform Thy-1 labelling of the neuronal membrane by the end of the second postnatal week. It is suggested that some of the molecular features of Thy-1, and anatomical features of the main bulb, could interact to produce this initial restriction of Thy-1 to particular parts of the mitral cell surface.

Despite the mitral cells of the accessory olfactory bulb developing earlier than those of the main bulb, both Thy-1 mRNA and protein appeared 5-7 days later on the cells of the accessory bulb. This may be related to the guidance role that the accessory axons are thought to play in respect of the later developing fibres of the main bulb.

Key words: neuronal differentiation, axonogenesis, dendrogenesis, olfactory system, Thy-1, glycoprotein, regulation, mouse, rat.

Introduction

The vertebrate central nervous system is formed by a complex developmental process in which intercellular interactions play a dominant role in specifying differentiation. This is seen for instance in the environmental determination of neuronal type in both the peripheral (Lumsden, 1989) and central (O'Leary, 1989; McConnell, 1989) nervous systems. One would therefore expect to find the expression of many differentiation-specific molecules of the neuronal surface controlled by such interactions. Surprisingly few studies appear to have addressed this possibility, a notable exception being the recent demonstration that the developmental
loss of functional laminin receptors on chick retinal ganglion cells is regulated by contact with their target cells, the optic tectum (Cohen et al. 1989).

Thy-1 is a major glycoprotein of the surface of mature neurons (Morris and Grosveld, 1989). As the simplest member of the immunoglobulin superfamily (Williams and Gagnon, 1982), it has generally been thought that it would participate in some binding function of the cell surface. Recently, it has been shown to bind tissue plasminogen activator with high affinity (Ware and Pittman, 1989; Pittman et al. 1989), and so its role may be associated with regulating the tissue remodelling events mediated by this extracellular protease (Saksela and Rifkind, 1988). In the cerebellum, Thy-1 appears upon completion of axonal growth (Morris and Beech, 1987), a timing compatible with a role in quenching protease activity associated with axonogenesis.

In order to gain further understanding of Thy-1 expression in relation to neural development, we have looked at the appearance of both Thy-1 mRNA and protein in the olfactory bulb. This is a region of unique plasticity, the only site in vertebrates where a set of axons (the primary olfactory fibres) grow continuously into brain in adult life (Graziadei and Monti Graziadei, 1978; Barber, 1981). The main synaptic units, the glomeruli, are formed over a protracted period, which commences early in embryonic brain (Hinds, 1972a, b; Hinds and Hinds, 1976; Doucette, 1989) and continues long after birth (LaMantia and Purves, 1989), indicating a previously unexpected continuity of growth by the central nervous system (CNS) components of the olfactory bulb. It was therefore of interest to know how the expression of a cell surface molecule like Thy-1 would be regulated in this system. This study benefits from the fact that the primary olfactory fibres are the only completely Thy-1-negative axonal tract in adult brain (Morris and Barber, 1983; Danciger et al. 1989), greatly simplifying immunohistochemical analysis of Thy-1-positive components, which can be difficult elsewhere in CNS where the presence of Thy-1 on all neuronal surfaces obscures its analysis on any one.

We have studied both the rat and mouse, since Thy-1 expression can vary between these species (Morris, 1985) and because different aspects of the development of the olfactory system have been studied in one or other, but not both. Our account, however, concentrates upon the mouse in which species we have an ongoing interest in modifying gene expression (e.g. Danciger et al. 1989).

Materials and methods

Animals and tissues

Pregnancies were dated from the appearance of a vaginal plug (embryonic day (E) 0), mice gave birth at E19, rats at E21. Day of birth was taken as postnatal day (P) 0. In the initial series, CBA×C57BL mice (the same F1 cross used for transgenic work (Kollias et al. 1987; Danciger et al. 1989) were examined at each embryonic day from E14 to birth; Wistar rats at each embryonic day from E17; and both species at P0, 2, 5, 8, 12, 21 and 56. Anaesthetised animals (minimum of 4 per time point) were decapitated, the brain removed quickly and bisected sagittally. One half was immediately frozen by covering with finely powdered dry ice before sectioning in the cryostat for in situ hybridisation. The other half was fixed by immersion in ice-cold acetic/alcohol, then processed through polyester wax for immunohistochemistry. In a second series for immunohistochemistry, mice were also taken at P0, 1, 2, 5, 8, 12, 14, 15, 21, 28 and 56, fixed by immersion in acetic alcohol and serial sections collected in either the sagittal or coronal planes as required.

In situ hybridisation

A 280 base pair BstEI-SacI fragment of the third exon of the mouse Thy-1.2 gene (Giguere et al. 1985), which is 89.9% homologous with the rat Thy-1.1 gene (Seki et al. 1985), was cloned into the pGEM4 vector (Promega). [35S]UTP-labelled RNA probes were generated in vitro and titred on sections of adult mouse and rat brain to determine a saturating concentration for in situ hybridisation, and the control (sense) strand used at the same concentration.

From a number of preliminary experiments in which both tissue preparation (immersion fixation before sectioning compared to post-fixation) and hybridisation conditions (to establish conditions of optimal stringency without loss of specific signal) were investigated, the following procedure was determined as most suitable for this study covering a broad developmental range. Sagittal 10 μm sections of brain were collected on gelatin–chrome alum-subbed slides, immediately dried under a fan for 1–2 min, then fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS), pH 7.4, for 0.5–2 h at room temperature. The slides were then washed twice in PBS, dehydrated in an ascending series of alcohol and stored dry at −20°C until use. From the complete series collected with each brain, occasional slides were thionin stained to determine the brain regions they contained. Four slides from each brain were then selected, containing the medial edge of the bulb rostrally and cerebellar mid-verniss caudally; medial bulb rostrally and lateral vermis with medial deep cerebellar nucleus caudally; lateral bulb rostrally and medial cerebellar hemisphere; and piriform cortex (lateral to the bulb) rostrally and lateral cerebellar hemisphere with the lateral deep nucleus caudally. Adjacent sections were also included as controls. These slides were brought to room temperature, rehydrated through a descending series of alcohol to PBS and treated with proteinase K and acetic anhydride (Wilkinson et al. 1987). The sections were then incubated overnight under a coverslip in a humidified chamber at 45°C (rat) or 50°C (mouse) with 25 μl of hybridisation mixture consisting of 50% formamide, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.3 M NaCl, 1× Denhart’s, 20 mM DTT, 500 μg ml−1 yeast RNA, 10% dextran sulphate and [35S]UTP-labelled RNA probe (1 ng, 106 disintegrations min−1). After hybridisation, sections were washed briefly in 4×SSC/0.1% 2-mercaptoethanol (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate) at room temperature, treated with 20 μg ml−1 RNase A in a solution of 10 mM Tris–HCl (pH 8.0)/1 mM EDTA/0.5 M NaCl for 30 min at 37°C and further washed in the same solution without RNase A for 30 min. Sections were washed once in 2×SSC at 45°C for 30 min and twice in 0.1×SSC at 60°C for 15 min each. Sections were finally dehydrated through 50%, 70% and 96% ethanol containing 0.3 M NH4OOCCH3, then 100% ethanol and air dried. Slides were dipped in a 50% aqueous solution of Ilford K2 emulsion,
exposed in sealed boxes at 4°C for 4–18 days before development, then counterstained with cresyl violet and coverslipped.

**Immunohistochemistry**

This was as previously described (Morris et al. 1985), modified slightly in that when sections were dewaxed the first solution was 96% ethanol containing 5% glacial acetic acid, rather than ethanol alone. Sections at the same level as those used for in situ hybridisation were chosen; in addition complete coronal series at P0, 2, 5 and 8 were also taken to enable Thy-1 appearance on the lateral olfactory tract to be better seen. Thy-1 in the rat was detected using the mouse monoclonal OX7 against the Thy-1.1 determinant (Mason and Williams, 1980; 1:250 dilution of ascitic fluid in PBS containing 1% bovine serum albumin (BSA)), in the mouse using the rat monoclonal 30H-12 detecting the Thy-1.2 determinant (Ledbetter and Herzenberg, 1979; 1:30 dilution of tissue culture supernatant in the same buffer). In addition, rabbit F(ab')2 antibodies to rat Thy-1 (Morris et al. 1985), which detect different determinants on the Thy-1 molecule, were also used; they gave an identical pattern of staining to the monoclonal antibodies at all ages tested (P2–P56) and are not described separately. The following primary antibodies were also used: mouse monoclonal G10 against an epitope present on MAP1x only during axonal growth (Calvert and Anderton, 1985; Calvert et al. 1987), used at a dilution of 1:1000 of ascitic fluid; rabbit IgG anti-rat N-CAM (Doherty et al. 1988) used at a dilution of 1:1000, to give alternative immunolabelling of a molecule on the mitral cell surface; and two separate rabbit antisera to rat MAP2, one affinity-purified antibodies to purified MAP2 (Brion et al. 1988), the other raised to a fusion protein (Calvert, unpublished); both, used at 1:200, gave identical staining of developing dendrites (Bernhardt and Matus, 1984; Tucker et al. 1988). Horseradish peroxidase (HRP)-coupled secondary antibodies used were rabbit anti-mouse IgG (Dakopatts; absorbed immediately prior to use with 5% normal rat serum to remove antibodies cross-reacting with rat Ig, and used at 1:40 in 1% BSA/PBS); sheep F(ab')2 anti-rat IgG (Amersham, 1:50; this did not cross-react with mouse Ig); and our own rat Ig-absorbed sheep F(ab')2 anti-rabbit F(ab')2. Peroxidase activity was developed with diaminobenzidine (0.1% in 100 mm sodium phosphate with 20 mm imidazole pH 6.0 containing 0.003% H2O2), and for some sections from younger animals this was then enhanced with silver/gold development (Morris, 1990). Sections were counterstained with thionin, or, after, silver/gold enhancement, with cresyl violet.

**Results**

In both mouse and rat brain, the earliest detectable Thy-1 mRNA was found 4 days before birth (E15 in mouse and E17 in rat) on mitral cells in the olfactory bulb, and on neurons of various early maturing (mostly motor) nuclei in brain stem. These two regions dominated the in situ hybridisation staining pattern until birth, as can be seen in the low power montage of mouse brain at P0 in Fig. 1.

**Spatiotemporal gradient of expression of Thy-1 protein but not mRNA by mitral cells**

At E15, the mouse mitral cells were seen as large cells whose nuclei counterstain pale blue, accumulating above the small, tightly packed, intensely blue subventricular cells. A low level of labelling of Thy-1 mRNA (2–10 grains per cell after 18 days exposure) was found only over the mitral cells (Fig. 2A; compare control section in B). No immunoreactive Thy-1 was seen (even with silver enhancement) at this age (Fig. 2E).

By the next day, the mitral cells were organised in a distinct layer in which all cells showed strong labelling for Thy-1 mRNA (Fig. 2C). No spatial gradient of labelling around the layer was apparent. Fig. 3A–D

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Fig. 1. Montage of dark-field photomicrographs of P0 mouse brain labelled by in situ hybridisation for Thy-1 mRNA. The autoradiographic signal appears as white grains. Scale bar is 1 mm. BS, brain stem; CB, cerebellum; CP, cortical plate; Hipp, hippocampus; OB, olfactory bulb.
shows a high-power view of grains over the mitral cells at different locations along the layer (at E18, when the mitral cells were more spread out, enabling the staining intensity of individual neurons to be better assessed). At E17, the first immunohistochemically detectable Thy-1 appeared on those mitral cells immediately facing the ventral site of entry of the olfactory nerve. This was barely detectable with conventional diaminobenzidine development of the peroxidase reaction (Fig. 2F) and so here (and elsewhere to show the first appearance of Thy-1 immunoreactivity) we have used a silver-gold enhancement which greatly amplifies the signal (Fig. 2G). By the next day Thy-1 immunoreactivity was additionally seen in a dorsal portion of the mitral cell layer (Fig. 2D) but was virtually absent from more caudally placed cells and particularly in the rostroventral face of the bulb. Over the next 6 days, the entire layer of mitral cells became Thy-1 immunoreactive, first caudally and then along the rostral face. In coronal sections, the dorsolateral region was also seen to be slow to acquire Thy-1 immunoreactivity (Fig. 5D). By P5, the layer was uniformly stained (Fig. 2H). Intensity of staining also increased over this time and, by P5 could be readily seen even at low power without silver enhancement.

Axons of the mitral cells in the lateral olfactory tract (see Fig. 7) similarly showed a gradient of expression of Thy-1 protein over this time. Immunoreactivity was first detectable on some axons in the bulb at E18 (Fig. 2D), and in the lateral olfactory tract by the next day (birth). This tract was readily identified throughout all but the most distal regions of its course from P0–5, since of all the growing fibre tracts in forebrain (seen with G10 immunohistochemistry in Fig. 4B) only this was labelled with Thy-1 antibodies (Fig. 4A). At the rear of the cortex, the entorhinal region also expressed Thy-1 protein in the late embryonic period, the only other area of forebrain to do so at this age.

Within the lateral olfactory tract, at all levels over the period P0–5 not all fibres were labelled. The deeper fibres, which had already started to downregulate their expression of the MAP1b epitope detected by G10 (compare Fig. 4C and D, and E and G), expressed Thy-1, and conversely the fibres adjacent the pial surface, lacking detectable Thy-1, showed intense immunoreactivity for G10. By P8, all fibres in the tract labelled for Thy-1 (and showed little G10 immunoreactivity).

At P0, the Thy-1-positive fibres coursed past the piriform cortex without entering it (Fig. 4C). The unlabelled area between the cortical cell bodies and the olfactory bulb fibres contained dendrites of the cortical neurons, as shown by MAP2 immunohistochemistry (Fig. 4E). Immunolabelling revealed the dendrites to be a very fine plexus at this age, contrasting with the large apical dendrites of nearby cortical pyramidal neurons, a distinction which remained even at P5 when the Thy-1-labelled fibres (Fig. 4F) had penetrated the dendritic arbors of the piriform cortical cells.

The olfactory tubercle develops relatively late, medial to the course of the lateral olfactory tract at the level of the rostral regions of the piriform cortex. Thy-1-immunolabelled fibres of the tract surrounded the tubercle at P2 and P5, and had penetrated it by P8 (Figs 4H–J).

Specialised spatial distribution of Thy-1 mRNA and protein at the soma

From its earliest appearance on mitral cells, the signal for Thy-1 mRNA was not evenly distributed around the nucleus, but rather lay predominantly on the apical side (Fig. 2A). As the intensity of the signal, and the mitral cell dendrites, developed around the perinatal period, this label clearly defined the lower regions of the mitral cell dendrites in the outer plexiform layer (e.g. Fig. 3A–D; this was more apparent late in the first postnatal week when the dendrites were better separated spatially). This mainly dendritic distribution of autoradiographic grains persisted at P12, although by P21 most of the grains were more uniformly distributed around the soma (Fig. 3E), and by P56 all mitral cells showed grains almost entirely restricted to the soma. In describing these grains in the outer plexiform layer as belonging to the mitral cells, they could not in the vast majority of cases be confused with labelling of the tufted cells. The latter could be seen in the outer plexiform layer to acquire Thy-1 mRNA from E18, as they differentiated to become a relatively large cell with a large, light blue-staining nucleus. Their autoradio-

Fig. 2. Early appearance of Thy-1 mRNA (A–C) and protein (D–H) in mouse olfactory bulb, demonstrated by in situ hybridisation and immunohistochemistry, respectively. (A) Bright-field photograph of grains over mitral cells in E15 bulb, autoradiograph exposed for 18 days; (B) adjacent section, treated identically except hybridised with the sense probe to provide a negative control; (C) low-power dark-field photograph of part of the bulb at E16, showing labelling of the row of mitral cells (arrow); 18 day exposure; (D) E18 bulb showing silver-gold enhanced immunohistochemical staining for Thy-1, predominantly of the mitral cell dendrites ventrally (large arrow) and rostromedially (medium arrow) and of their axons gathering in the inner plexiform layer (small arrows); (E) lack of Thy-1 immunohistochemical staining at E15 even with silver-gold enhancement; mitral cell bodies lie in the layer demarcated by the arrows; labelling at the top of the picture is of the meninges, and is seen with second antibody alone; (F) immunohistochemical staining for Thy-1 on mitral cell dendrites (below and to right of large arrow) at E17, diaminobenzidine staining only. To the left below the arrow the row of mitral cells continues (caudally) but has undetectable immunoreactivity; (G) adjacent section with silver-gold enhancement of the diaminobenzidine reaction product, showing labelling of dendrites (below and to right of large arrow) and mitral cell bodies (small arrows) in the region immediately adjacent the point of ingrowth of the olfactory nerve (top right of photo, ventral in the section); mitral cells on the left do not express Thy-1 even on their dendrites; (H) P5, immunohistochemical staining without enhancement, showing uniform labelling around the mitral cell layer. Scale bars are 20 μm in A (B same magnification), 200 μm in C, 500 μm in D (H same), 50 μm in E, and 100 μm in F (G same).
graphic labelling (illustrated at P21 in the mouse (Fig. 3E) and in adult mouse and rat (Fig. 3 G and l)) was always less than that of the mitral cells, from birth onwards being at the maximum about half that of the mitral cells.

Immunohistochemical staining of mitral cells for Thy-1 also showed preferential labelling in the processes compared to the soma during the period of cell growth. At its earliest appearance (E17), most mitral cells showed staining of their dendrites in the outer plexiform layer, and also of the cell bodies in the mitral layer (Fig. 2G). However, somatic staining rapidly became inconspicuous (Fig. 5A), so that from E18 to P5 Thy-1 labelling was of the mitral cell dendrites in the outer, and of their axons in the inner, plexiform layers, with apparently little somatic labelling in the intervening mitral layer. For comparison, immunostaining of cerebellar Purkinje cells during early dendritic growth, where the soma as well as the dendritic tree is clearly delineated by the staining, is shown in Fig. 5B. By P8, some mitral somata were again labelled by the antibodies (Fig. 5C), and from P21 the cell bodies of all mitral cells were immunostained with an intensity comparable to their dendrites.

How the glomeruli become Thy-1 immunoreactive
Glomerular structures are not fully differentiated from the outer plexiform layer at birth, but by P2 they can be clearly seen lying superficially to this layer, below the olfactory nerve layer. This is shown in Fig. 5D–F with Thy-1 staining, on adjacent sections with MAP2 staining of dendrites (Fig. 5G–I), and with G10 staining of growing axons (Fig. 5J–L). Two features of Thy-1 labelling of glomeruli are apparent: not all glomeruli were labelled; and even for the labelled glomeruli, the main apical dendrite extending from the outer plexiform layer to the glomeruli was not immunostained. The latter could not be an artefact of the plane of section, since these dendrites were clearly visible when labelled with MAP2 (Fig. 5H,1), and are even visible when weakly labelled with G10 (Fig. 5K, L; this chiefly labelled the primary olfactory fibres in the olfactory nerve layer and glomeruli, and axons (at this age, mostly of tufted cells) in the inner plexiform layer). Indeed, the apical dendrites were visible in the glomerular layer due to the thionin counterstain on the Thy-1-labelled sections (Fig. 5F). Single apical dendrites could be traced from the outer plexiform layer, where they were strongly Thy-1 Immunolabelled, to the glomerular layer where they were visible by virtue of the counterstain (Fig. 6C). Neither immunolabelling for the microtubule-associated proteins nor the counterstain gave a direct view of what staining for a surface molecule uniformly distributed on the mitral cell surface should look like. We therefore labelled adjacent sections for the neuronal surface glycoprotein N-CAM, which at P2 revealed the entire length of the apical process and the mitral cell soma (Fig. 6E; anti-N-CAM most heavily stained the primary olfactory axons at this age, and it is these that contribute to the heavy labelling of the glomeruli; the plane of focus chosen is optimal for showing the dendritic but not somatic immunolabelling).

In comparing Thy-1 labelled and unlabelled glomeruli at P2, a difference in neuronal development could be seen, perhaps most readily in the MAP2-stained sections (Fig. 5H,1). The Thy-1-positive glomeruli were in the more mature areas, where tufted cells were already in the outer plexiform layer and periglomerular cells were starting to invest the glomeruli with their dendrites (Figs 5H and 6A). In the region of the negative glomeruli, there were few tufted cells and especially no periglomerular cells (Fig. 51). The latter were the first glomerular elements to become Thy-1 positive, around their cell bodies and on their dendrites projecting into the glomeruli (Fig. 6B). In more strongly positive glomeruli, tufted cell dendrites and finally the apical process of the mitral cell (Fig. 6D) became Thy-1 immunoreactive, over the period P2–P12. In the mature mouse, the apical dendrites of mitral and tufted cells were the dominant Thy-1-immunolabelled elements of the glomerular layer (Fig. 6F). This same progression of glomerular staining, and immunolabelling ascending the apical dendrite, was seen in the rat (Fig. 6G, H) occurring over the same period.

Acquisition of Thy-1 by the interneurons
Despite the periglomerular cells having detectable Thy-1 immunoreactivity from P2 onwards, they showed very low levels of mRNA labelling which was first detectable at P5. Even in the adult, when the mitral cells had 40–80 autoradiographic grains per cell, the periglomerular cells had only 1–3 grains per cell associated with them in the mouse and 4–8 grains in the rat. The other main interneuron of the bulb, the granule cell, similarly had a low level of labelling which became apparent at P12 but remained less than 1 grain per cell even in the adult (Fig. 3E) and (compare with negative control Fig. 3F) for P21 in the mouse; rat granule layer can be seen below the mitral cells in Fig. 3H; the granule cell bodies are packed at high density (see Fig. 6I, J) so cumulatively contribute to detectable labelling despite the low signal from any one cell). Their acquisition of Thy-1 immunoreactivity was obscured by highly Thy-1-positive afferent fibres coursing around their cell bodies from P12 (see Fig. 6I, J for adult). The short axon cells occurring between the glomeruli, and within the granule cell layer, were not clearly evident in our material and will not be commented upon. No non-neuronal cells displayed detectable signal for Thy-1 mRNA or immunoreaction at any time.

Accessory olfactory bulb
The accessory olfactory bulb differed notably from the main bulb in its late onset of Thy-1 expression. In Fig. 5D, the neurons of the accessory bulb can be seen to be not labelled with the anti-Thy-1 antibodies which have labelled almost all of the mitral cells of the main bulb on this (mouse P2) section. In both the rat and mouse, RNA labelling was first observed over accessory bulb mitral cells the day before birth, at a level of 2–4 grains per cell (compare mitral cell labelling at the same
The expression of Thy-1 just described in the mouse was reactive Thy-1 was first detectable on the more rostrally of labelling of the mitral cells at the same age, and this autoradiographic exposure for 4 days for E-J. Scale bars cells below them. (H2) Frontal cortex on the same section. (A-D) Bright-field photographs of mitral cells at the same age, Fig. 3A-D). This labelling increased only slowly after birth (3–6 grains per cell for the more superficially lying cells at P2; the deeper lying cells had few grains) and the more mature cells had only 5–10 grains per cell at P5 when the main mitral cells had 20–30 grains. At P8–12 the accessory bulb cells also labelled at 20–30 grains per cell, which was approximately half the level of labelling of the mitral cells at the same age, and this ratio of lower labelling in the accessory bulb was maintained into adulthood. Very weakly immunoreactive Thy-1 was first detectable on the more rostrally located accessory mitral cells at P5 in the mouse, and more generally three days later. Immunoreaction was observed not only on the dendrites, but also around the cell bodies. By P12, the immunolabelling was of comparable intensity to that of the main bulb, which it thereafter resembled except that labelling of the outer plexiform layer was always distinctly weaker in the accessory bulb.

Differences between mouse and rat in Thy-1 expression
The expression of Thy-1 just described in the mouse was also observed in the rat, generally happening with the same time course when allowance is made for the 2 day longer gestation period in the rat.

There were however, two notable interspecies differences. First, Thy-1 immunoreactivity developed in the rat at a faster rate despite Thy-1 mRNA becoming detectable at the same age (allowing for the longer embryonic development of the rat). Thus Thy-1 immunoreactivity had spread all around the mitral cell layer of the main bulb by P0 medially and P2 at its lateral edge in the rat, at least 3 days earlier than in the mouse. Thy-1 immunoreaction appeared on the more mature cells of the accessory bulb at P2, again 3 days earlier than in the mouse. Moreover, Thy-1 immunoreaction, once it had appeared, quickly became easily detectable so that silver–gold enhancement was not required.

Second, immunohistochemical labelling of the areas dominated by the mitral, tufted and periglomerular cells (i.e. the mitral, outer plexiform and glomerular layers) was very intense in the rat, comparable to that of the fibres in the granule cell layer (Fig. 6J) or the neuropile of the nearby frontal cortex (not shown). In mice, staining of the same layers was distinctly weaker than that of the fibres in the granule cell layer or the neuropile of the frontal cortex (Fig. 6I). This difference was also observed in the mRNA signal, which was greater in the rat mitral cells (Fig. 3G1 and H1; labelling of the cortical neurons is shown for comparison (Fig. 3G2 and H2) although direct comparison of the labelling intensity between the two species is valid since the same probe was used at saturating concentration). Similarly the strength of the mRNA signal from the rat tufted (Fig. 3I) and periglomerular (Fig. 3J) cells was notably greater than that of their mouse counterparts (Fig. 3G1).

A final difference (although not of neural tissue) was also apparent in the perinatal period, when in the rat and not the mouse the pericytes of blood vessels were Thy-1 immunoreactive, as they are throughout the brain and peripheral nervous tissue in this species until the third week of life (Morris, 1985).

Discussion
This study of the developmental expression of Thy-1 in olfactory bulb demonstrates two remarkable features: expression of Thy-1 protein does not simply follow expression of its mRNA, but requires some further signal; and that when the protein is initially expressed it is spatially restricted on the cell surface.

Induction of expression of Thy-1 mRNA
The mitral cells of the main bulb showed detectable Thy-1 mRNA after they had emerged from the subventricular zone to start to form their definitive layer. It was associated with an obvious maturational change – the soma became bigger, with a large, pale-staining nucleus. From the studies of Hinds (1972a,b; Hinds and Ruffett, 1973; Hinds and Hinds, 1976; they and some other authors refer to the day of appearance of a vaginal plug as E1, and day of birth as P1; for simplicity, we have converted such dates to the convention we use in this discussion), it is clear that this phase occurs some time after axonal growth has started, at or shortly after dendritic growth has commenced. In fact, in the better histology present on sections processed for immunohistochemistry, it was apparent from the cresyl violet counterstain that many mitral cells had begun to grow dendrites at E15 when Thy-1 mRNA was first detected on them.

This expression of detectable Thy-1 mRNA at the time when a neuron has finished migration and begins dendritic growth, coinciding with a morphological maturation seen as enlargement of the cytoplasm and expansion of the nucleus to give one that distinctively stains a pale blue, is the general pattern we see in brain (Xue et al. unpublished data). It was also illustrated in this work by the tufted cells, although, as Pinching and
Fig. 4. Development of the lateral olfactory tract. (A) Frontal cortex of mouse at P0, enhanced immunohistochemical staining for Thy-1 labels only the lateral olfactory tract (arrow); (B) adjacent section stained with G10, lateral olfactory tract arrowed; (C–E) adjacent sections from P0 immunolabelled for Thy-1 (C), G10 (D) and MAP2 (E); (F, G) adjacent sections from P5 immunolabelled for Thy-1 (F) and G10 (G); (H–J) olfactory tubercle (large arrow) and piriform cortex (small arrow) after Thy-1 immunolabelling at P2 (H), P5 (I) and P8 (J). All are coronal sections of mouse brain, scale bars are 200 μm in A (B same), 50 μm in C (D, E same), 20 μm in F (G same) and 500 μm in H (I, J same).
Fig. 5. Thy-1 immunoreactivity is not uniformly present on mitral cell soma or dendrites. (A) Enhanced Thy-1 immunolabelling of mouse olfactory bulb at P0, showing labelling of the outer plexiform layer, some dendrites and axons leaving the mitral cells (arrows) but not noticeably of the mitral somata; (B) Thy-1 immunolabelling of cerebellar Purkinje neurons at P8, illustrating even labelling of somatic surface and dendrites; (C) Thy-1 immunolabelling of mouse olfactory bulb at P8, when some of the mitral cells (arrows) show somatic labelling; (D–L) mouse olfactory bulb at P2, adjacent coronal sections immunolabelled for Thy-1 (D–F), MAP2 (G–I) and G10 (J–L). In the low-power Thy-1 photograph, two heavily labelled glomeruli (large arrow) can be seen, which are shown at higher power in E. Thy-1-negative glomeruli (small arrow) are shown at higher power in F. The equivalent areas stained with the MAP antibodies are shown in (H,I) and (K,L). In D, mitral cells in the top left-hand corner (dorsomedial edge) of the layer show no Thy-1 immunolabelling, even in the outer plexiform layer; the unlabelled accessory olfactory bulb is denoted with an open arrow, the fibres of the accessory olfactory nerve projecting to it are visible (arrow) in J. Scale bars are 25 μm in A (B,C same), 250 μm in D (G,J same) and 50 μm in E (F,H,I,K,L same).
Fig. 6. Development of Thy-1 immunoreactivity on glomeruli. (A) MAP2 immunostaining of glomeruli, showing the dendritic shaft of periglomerular cells (arrows). P2 mouse; (B) periglomerular cell (arrow) seen with enhanced Thy-1 immunohistochemistry, mouse P2; (C) a large dendrite (arrow) passes from the outer plexiform layer, where it is immunolabelled for Thy-1, to the glomerular layer where it is not labelled by the antibodies; mouse P5; (D) Thy-1 immunolabelling of two mitral cell dendrites in the glomerular layer. mouse P2; (E) N-CAM immunolabelling of the entire mitral cell dendrite (arrow), mouse P2; (F) Thy-1 immunolabelling of mitral cell apical dendrites (arrows) in adult (P56) mouse olfactory bulb; (G,H) Thy-1 immunolabelling of rat olfactory bulb at P5 (G) and P56 (H), apical dendrites indicated by arrows; (I,J) Thy-1 immunolabelling of P56 mouse (I) and rat (J) bulb. Mitral cell layer indicated in each by arrows. In I, the heavily stained area on the left is frontal cortex. The staining intensity of the outer plexiform layer and glomeruli of the mouse should not be directed compared with that of the rat, since different antibodies were used; however, it is valid to compare their staining with that of nearby structures, such as the fibres of the granule layer and frontal cortex, which label with similar intensity in the rat and mouse. Scale bars are 25 μm for A (B, D same), 10 μm for C, 50 μm for E–H, and 250 μm for I, J.
Thy-1 expression in olfactory bulb

Fig. 7. Some features of the olfactory bulb and its projections in the lateral olfactory tract. The photograph is of the ventral surface of a P3 rat brain, showing olfactory bulbs (OB) and the approximate positions of the piriform cortex (PC), olfactory tubercle (OT), amygdala (A) and the entorhinal cortex (ERC). The drawing shows, on the right, the organisation of the main neuronal elements of the olfactory bulb. ONL, olfactory nerve layer, carrying the Thy-1 negative primary sensory fibres from the olfactory mucosa into the bulb; Glom, glomerular layer, site of the distinctively large synaptic glomeruli formed between these fibres and neurons of the bulb; OPL, outer plexiform layer, a dense neuropile dominated by dendrites of bulb neurons; ML, mitral cell layer, containing the cell bodies of the mitral cells, the main projection neuron of the bulb; IPL, inner plexiform layer, containing the axons of the mitral and tufted cells as they travel towards the lateral olfactory tract; GL, granule layer (much deeper than indicated) containing the main interneuron of the bulb, the granule cell (g); m, mitral cells, whose main apical dendrites ascend to the glomeruli and whose secondary dendrites ramify in the OPL; t, tufted cells, somewhat smaller projection neurons lying within the OPL with less extensive dendritic arborisations in the glomeruli; pg, periglomerular cells, small interneurons which are the main neuronal type in the glomerular layer. The lateral olfactory tract carries the mitral and tufted axons to their terminations, principally in the piriform cortex, but also in the anterior olfactory nucleus (AON), the olfactory tubercle (OT), amygdaloid nuclei (A) and parts of the entorhinal cortex (ERC). Based on drawings and descriptions in Pinching and Powell (1971), Shepherd (1972), Schwob and Price (1984) and Brunjes and Frazier (1986). Short axon neurons, found between the glomeruli and in the granule layer (Price and Powell, 1970; Pinching and Powell, 1971) have been omitted for simplicity since they are less common and are not evident in this study.

Powell (1971) observe, they are almost certainly late generated cells of the mitral lineage so this is perhaps not remarkable. Some periglomerular cells appear to express Thy-1 protein (at P2) before detectable message (at P5). It must be remembered that a small interneuron like a periglomerular cell sustains very little plasma membrane, probably more than 100-fold less than a large projection neuron like the mitral cell, and accordingly need have very little Thy-1 mRNA to supply a high density of Thy-1 on its surface. This also applies to the other main interneuron of the bulb, the granule cell, which even in the adult would probably have an undetectable level of Thy-1 mRNA except for the fact that the cells are clustered in rows, closely together, and so cumulatively display a detectable level of labelling.

What is the signal for induction of Thy-1 protein?
There was a spatiotemporal gradient of appearance of Thy-1 protein on the mitral cells in the bulb and hence on their axons in the lateral olfactory tract. Of the two, the latter is more informative because of the reciprocal relationship in the tract between expression of the transient MAP1x epitope recognised only on growing axons by G10 (Calvert and Anderton, 1987; Calvert et al. 1988), and Thy-1. The latter only appeared on the more mature axons which had already started to down-regulate their expression of the G10 epitope. We have previously shown, in a study of the cerebellum, that a similar reciprocal relationship holds for the expression of the cell surface molecule OX2, found only on growing axons, and Thy-1 (Morris and Beech, 1987). Could it be that the signal for the appearance of Thy-1 protein is the cessation of axonal growth? This is strongly supported by studies of the development of the tract, which have shown that mitral cell axons pass through the piriform cortex at E13 in the mouse (Hinds, 1972a), and fibres defining their full extension caudally in the lateral olfactory tract to the amygdala are present at the equivalent of a day later (E16) in the rat (Schwob and Price, 1984). The axons then send collaterals into their various terminal fields as the latter mature, in the perinatal period for the piriform cortex and amygdaloid...
nuclei, and late in the first postnatal week for late maturing structures such as the olfactory tubercle (Schwob and Price, 1984; Brunjes and Frazier, 1986; in each of these areas, there are topographical gradients of maturation, and cessation of new synapse formation does not completely stop until the end of the second postnatal week). Innervation of the piriform cortex and amygdala can be detected electrophysiologically at birth in the rat (Schwob et al. 1984). The onset of the appearance of Thy-1 protein that we observed, occurring for the mitral cells over the period E17 to P5 in the mouse and E19–P2 in the rat, is certainly compatible with it being linked to, or even slightly following, the cessation of axonal growth. It has been shown in the hamster that there is a topographically ordered entry of mitral cell axons into the olfactory tract, medially located mitral cells commencing axonogenesis before the lateral ones (Grafe and Leonard, 1982). We also observed a medial-to-lateral gradient in Thy-1 expression, although the late maturation of the most rostral cells does not appear to have been previously commented upon (nor looked for in the rat or mouse (Brunjes and Frazier, 1986)).

There is an obvious problem in identifying the cessation of axonal growth, which is that axons grow in multiple phases. The initial elongation of the mitral cell axons to the amygdala/entorhinal area is followed by later collateral growth into the synaptic target areas as these develop (Schwob and Price, 1984). The late progress of Thy-1-positive fibres into a late developing area, the tubercule, suggests that Thy-1 is excluded from the collaterals as they grow, even though the parent axons, and certainly their cell bodies at P5, are expressing this protein. This would suggest that a Thy-1-positive cell manages to exclude the presence of this molecule from areas of axonal growth, a proposition which can be more rigorously examined for axons in the pontine projection to the cerebellum (Xue et al. unpublished data), but which for mitral cells appears also to be the case with growth of their main apical dendrite (see below).

Thus we suggest that appearance of Thy-1 mRNA, although obviously necessary for expression of its protein, is not in itself sufficient, and a further signal related to the cessation of axonal growth is required. On arriving in their terminal zones, axons induce or receive target-dependent trophic factors which influence axon and cell survival and differentiation (Barde, 1989). Appearance of Thy-1 protein might be part of this response. Indeed, in PC12 cells Thy-1 expression is unusually sensitive to the action of one such trophic factor, NGF, responding at a lower factor concentration, and using different intracellular signalling, than the induction of another cell surface glycoprotein, N-CAM (Doherty et al. 1988).

**Spatial restriction of Thy-1 on the cell surface during dendritic growth**

When Thy-1 immunoreaction is first seen on mitral cells, it is on their dendrites in the outer, and axons in the inner, plexiform layers. Curiously, very little reaction is seen around the somatic membrane for the first two postnatal weeks, the main period of dendritic growth. It then becomes readily detectable at the cell body. We observe a similar progression with some other neurons, notably hippocampal pyramids (Xue et al. unpublished data), and not with others as we have illustrated here for Purkinje cells. N-CAM immunolabelling, also of the cell surface, did not show reduced somatic labelling, so it is unlikely this effect was due to trivial effects such as a relatively low density of plasma membrane around the young mitral cell soma compared to the dendrites. Therefore, the most likely explanation is that for a prolonged period during dendritic growth very little Thy-1 is present on the somatic plasma membrane.

The selective exclusion of Thy-1 from part of the mitral cell surface is even more strikingly seen in the case of the main apical dendrite, which is Thy-1 immunolabelled in the outer plexiform layer and not initially in the glomerular layer. There can be no doubt that the first labelling seen in the outer plexiform layer is that of mitral dendrites – at high power they can be readily traced back to their cells of origin, which in embryonic life are exclusively the mitral cells. In the perinatal period, the tufted cells also emerge as strongly Thy-1 immunoreactive, adding to the density of immunolabelled processes in the outer plexiform layer. There cannot be a contribution of afferent fibres to this immunolabelling, since the primary olfactory axons, which are here at this age (Hinds, 1972a), are Thy-1-negative. The first CNS fibres to reach the bulb do so around the time of birth, and are located well below the mitral cells, at the boundary between the subependymal zone and the internal granule layer (Schwob and Price, 1984). The centrifugal fibres seen in the glomerular layer in the adult (Pinching and Powell, 1972) must arrive later, and are too sparse to make a significant contribution to the staining pattern. The spatial restriction seen with Thy-1 staining of the apical dendrite is not seen with another surface molecule, N-CAM, and so cannot result from different densities of plasma membrane. The conclusion thus seems inescapable that the Thy-1-negative mitral and tufted cell dendrites seen in the glomerular layer arise from cells that have Thy-1-immunoreactive dendrites in the outer plexiform layer.

Thy-1 immunolabelling appears to be drawn up the apical dendritic shaft in response to glomerular differentiation, in which a key step is the emergence of Thy-1-expressing periglomerular cells. It should be noted that these cells are entirely restricted to the glomerular layer (Pinching and Powell, 1971), and, unlike the mitral and tufted cells, on expressing Thy-1 protein have no choice but to do so in this layer. Soon after they have established a Thy-1-positive environment in the glomeruli, the mitral and tufted dendrites also become Thy-1 immunolabelled in this layer.

Some properties of Thy-1 and olfactory bulb anatomy could interact to produce this remarkable pattern of expression on the mitral cell surface. Thy-1, along with the smallest form of N-CAM (He et al. 1986) differs from most other neuronal surface glycoproteins in
having its membrane-spanning polypeptide tail cleaved early in biosynthesis and replaced with a glycosylated phosphatidylinositol moiety which inserts into the membrane (Conzelmann et al. 1987; Homans et al. 1988). It has recently been shown in a kidney cell line that these lipid-linked proteins are selectively routed to the apical surface (Lisanti et al. 1988), and that the signal for this is associated with the glycosyl-phosphatidylinositol anchor (Lisanti et al. 1989). Thus it is possible that Thy-1 is selectively routed to parts of the plasma membrane, although it is difficult to see why it would not subsequently diffuse to other areas since its lipid tail confers upon it an unusually high mobility (Ishihara et al. 1987). This mobility may itself be the key to the selective distribution of Thy-1 on the membrane, if it is bound to some component in its environment which is itself restrained to particular tissue compartments. Tissue plasminogen activator, whose distribution in the olfactory system is unknown but whose mRNA is present at high levels in brain (Rickles and Strickland, 1988) is an obvious candidate (Ware and Pittman, 1989; Pittman et al. 1989), and an astrocytic receptor for Thy-1 has also been reported (Dreyer et al. 1989). If such a receptor were confined to the outer and inner plexiform layers (e.g. by being produced by glial cells with this distribution) then the high mobility of Thy-1 would serve to partition it into the region of its receptor. If the Thy-1 receptor involved is a soluble molecule like tissue plasminogen activator, the later appearance of Thy-1 on periglomerular cells would serve to trap the receptor within the glomerulus, and subsequently in the intervening space spanned by the apical dendrites.

In adult bulb, there is a diversity of glial types matching that of the neurons in the different layers of the bulb (Blanes, 1898; see Fig. 8). In particular, the outer plexiform layer is dominated by a stellate astrocyte (Fig. 8, cells D), smaller than that found in the glomerular layer, which also occurs rarely in the mitral layer from where its processes ramify in the internal and external plexiform layers. Clearly this could serve to restrict Thy-1 to these layers in the way we have suggested. When this lamination of glial types occurs in development is unknown, although it is clear that the glial distinction between the olfactory nerve layer and deeper regions of the bulb is set up as the primary olfactory fibres first invade the bulb (Doucette, 1989; Marin-Padilla and Amieva, 1989).

A similar restriction of the surface glycoproteins TAG1 and L1 to particular areas of rat spinal cord axons during development has been reported (Dodd et al. 1988). In this case, the domains occupied by the different molecules correlate with different phases of axonal growth in which these glycoproteins are suggested to play a guidance role. The restriction of Thy-1 to part of the growing dendritic surface does not occur generally. On many other neurons (e.g. rat Purkinje cells), it is present all over the dendritic surface at all phases of growth (Morris et al. 1985), whereas on others (e.g. hippocampal pyramidal cells) extensive dendritic growth occurs before Thy-1 appears (Xue et al. unpublished data). The functional significance, if any, of the restriction of Thy-1 on the growing mitral cell dendrite must relate to particular characteristics of the olfactory bulb. It is tempting to suggest this is related to the unique plasticity of this region, a suggestion that would...
fit with a role for Thy-1 in modulating the effect of extracellular proteases.

Could the changing patterns of Thy-1 immunoreactivity be an artefact of the immunohistochemical method used?

Having used immunohistochemistry to identify Thy-1 protein, it is pertinent to ask whether the epitopes recognised by our antibodies are on all forms of Thy-1. We have relied predominantly on two monoclonal antibodies each recognising different allelic forms of the molecule, determined by a single amino acid substitution at residue 89 (Williams and Gagnon, 1982). The main results have been confirmed by staining with a polyclonal antibody that recognises polypeptide epitopes distinct from the allelic ones (Morris, 1985). These are not affected by the only two known post-translational modifications of Thy-1, viz removal of its transient polypeptide transmembrane tail and its replacement early in biosynthesis by a glycoprophospholipid tail (Conzelmann et al., 1987), and glycosylation at Asn residues 23, 75 and 99 (see Morris, 1985). Phosphorylation, which can have a marked influence on the antigenicity of proteins (see, for instance, discussion of the relationship of the G10 MAP1x epitope with MAPs 1B and 5 (Sato-Yoshitke et al. 1989; Garner et al. 1989)), has not been observed to occur on Thy-1 (see e.g. Peyron et al. 1988). We have observed aldehyde fixation to selectively destroy the antigenicity of newly synthesised Thy-1 (Morris et al. 1983), but have not seen the simple precipitating fixative used here selectively impair Thy-1 immunoreaction in tissue. Thus, while it is impossible to exclude the possibility that the antibodies used fail to detect some, as yet unrecognised, form of Thy-1, this does seem unlikely.

Both in situ hybridisation and immunohistochemistry provide at the best semiquantitative information. In a separate paper (Xue and Morris, in preparation), we examine with quantitative methods the relationship between Thy-1 mRNA and protein levels in the cerebellum (where there is a delay before appearance of immunohistochemically detectable Thy-1) and cerebral cortex (where immunohistochemical appearance closely follows mRNA), and find they confirm the findings based on histological methods. Thus one of the substantive conclusions of this paper, that Thy-1 protein induction does not simply follow induction of its mRNA, can be independently confirmed using quantitative techniques.

Accessory olfactory bulb

Not only do the mitral cells of the accessory bulb cease mitosis and differentiate earlier than those of the main bulb (Hinds, 1968), they also send their axons caudally along the lateral olfactory tract earlier than do those of the main bulb (Schwob and Price, 1984). In fact, it has been suggested that they could act as pioneer fibres, guiding the caudal progress of the main mitral axons (Schwob and Price, 1984). These cells are the only exceptions we have noticed to the generalisations that Thy-1 mRNA is expressed as cells differentiate at the end of their migration, and protein is expressed soon after the cessation of axonal growth. Possibly the close association of their axons with those of the main bulb requires the accessory bulb neurons to delay expression of Thy-1 until after the main mitral axons have also finished growing. It will be interesting to look at other instances of pioneer and following fibres to see if the earlier fibres similarly have a delayed appearance of Thy-1.

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