The surface glycoprotein Thy-1 is excluded from growing axons during development: a study of the expression of Thy-1 during axogenesis in hippocampus and hindbrain

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Summary

Thy-1 is a developmentally regulated surface glycoprotein expressed on a number of tissues, including nerve where it is a major surface component of mature neurons. During neural development in the rat and mouse, expression of Thy-1 protein does not necessarily follow appearance of its mRNA, but additionally requires completion of the initial phase of axonal growth. Where there is a substantial lag phase between initial elongation and final axonal outgrowth into a terminal field (e.g. pontine projection to the cerebellum), Thy-1 protein appears at the cell body and dendrites of the neurons, but is excluded from their axons until the terminal phase of axonal growth is completed. In the more complex case of the vestibular ganglion neurons, whose axons project primarily to the vestibular nuclei in the brainstem before birth, and then 1-2 weeks later into the cerebellum, Thy-1 enters the proximal axonal regions where growth is completed, but not the distal growing ends. Thus complex controls govern the initial expression and distribution of Thy-1 so as to exclude it from growing regions of axons.

Key words: axogenesis, cerebellum, pons, post-transcriptional regulation

Introduction

Neurons are exceptional in the extent to which different areas of their surface are specialised to perform different tasks. Beyond the major division into a transmitting (axonal) and receiving (dendritic) compartment lie complex subdivisions: many axons project to several targets, and most dendrites receive inputs from multiple areas that form synapses differing in their location, morphology, chemistry and physiological effect. In development, each of these specialised areas matures at its own rate, determined not just by events intrinsic to the neuron but also by the other cells in its immediate environment (e.g. Ghosh et al. 1990). At the molecular level, therefore, one would expect the maturation of different parts of a neuron to proceed at different rates appropriate to the particular microenvironments contacted.

Such local modulation of the developmental appearance of the neuronal surface glycoprotein, Thy-1, appears to occur in brain. In the olfactory bulb, Thy-1 mRNA is expressed in all mitral cells at the same stage of differentiation, when their soma have migrated to their final position and begun to grow dendrites. Expression of Thy-1 protein, however, does not simply follow that of its mRNA, but requires some additional signal, which we suggested was related to the cessation of axonal growth (Xue et al. 1990).

We examine this possibility here by studying Thy-1 expression during development of four different axonal systems (Fig. 1). Two are projections to the granule cell layer of the cerebellum, chosen because this is one of the few areas of brain where the vast majority of local cells (the granule interneurons) express very low levels of Thy-1 (Morris et al. 1985a; Bolin and Rouse, 1986). The acquisition of Thy-1 by the terminals of these axons, as they sprout into the granule layer and mature over the period P5-28 (Altman, 1972; Hamori and Somogyi, 1983; Ito, 1984; Arsenio Nunes and Sotelo, 1985), can therefore be clearly followed. These fibres, collectively called mossy fibres because of their distinctively large terminals, come from diverse parts of the central and peripheral nervous systems. The first group that we have studied are those arising in the pons, axons whose only target is the cerebellar granule layer. These grow in two phases: initial elongation to cerebellar cortex in the perinatal period followed a week later by secondary sprouting into their terminal field. The second group are axons of the vestibular ganglion neurons. These are sensory neurons of the peripheral
Fig. 1. The four axonal systems studied. In the sagittal (upper) plane is shown the pontine projection to lobule VII of cerebellar cortex (these also project to most of the other vermal lobules and the cerebellar hemispheres; for the course and timing of growth of these axons, see Ito, 1984; Flumerfelt and Hrycyshyn, 1985; Payne and Bower, 1988); the projection of the vestibular ganglion (VG) axons to lobule IX (location of the superior vestibular nucleus (SVN) and of the two descending vestibular nuclei (DVN) are indicated; the fourth vestibular nucleus (the lateral) is not a major target for these axons; see: Brodal and Hoivik, 1964; Gacek, 1969; Morris et al. 1988); and the projection of hippocampal granule cells to the CA3 pyramids (see: Bliss et al. 1974, Stirling and Bliss, 1978; Amaral and Dent, 1981; Gaarskjaer, 1985). In the coronal plane is shown the projection of hippocampal granule cells to the CA3 pyramids (see: Bliss et al. 1974, Stirling and Bliss, 1978; Amaral and Dent, 1981; Gaarskjaer, 1985). In the coronal plane is shown the projection of hippocampal pyramidal neurons, ipsilaterally to other pyramidal neurons and the lateral septal nucleus (LSN), and contralaterally via the fimbria and ventral hippocampal fissure (VHC) to the other hippocampus (see: Fncke and Cowan, 1977; Zimmer and Haug, 1978; Swanson, 1978; Cowan et al. 1981; Frotscher et al. 1988; Buchhalter et al. 1990).

nervous system and, unlike the CNS neurons studied here, lack dendrites (Ballantyne and Engstrom, 1969). Early in neural development (E12–13 in rat), their axons grow into the brain where they bifurcate, one branch going to the two descending vestibular nuclei, the other to the superior vestibular nucleus and thence into parts of the cerebellar cortex (Fig. 1). Their major targets are the neurons of the three vestibular nuclei in the brain stem, which are contacted from E15 by terminal sprouts of the axons; numerous large terminals are formed on them by birth (Morris et al. 1988). The initial elongation of the axons also brings them, at E13–15, into the cerebellar cortex. Despite being the first axons to arrive here, they must wait for over 2 weeks for their terminal field, the granule cells, to mature. It was therefore of interest to see how this minor, and very late developing, arm of a complex axonal network would influence the appearance of Thy-1 on these cells.

Analysis of Thy-1 acquisition during axogenesis elsewhere in CNS is frustrated by the fact that Thy-1 is so ubiquitous a neuronal membrane component that its appearance on any one cell and its processes is obscured by the multitude of other similarly immunoreactive elements in its environment. Some analysis is possible in the hippocampus, however, where both the main projection neurons (pyramidal cells) and interneurons (granule cells) are clustered in distinct layers. The pyramidal cells show a complex pattern of projection, set up in late embryonic life (Fig. 1); their axons are the major components of the large fibre tract called the fimbria, and its extension in the midline, the ventral hippocampal commissure. The granule cell axons project, in the second postnatal week and later, to a single target in the ipsilateral hippocampus, the region immediately above the cell bodies of the large CA3 pyramids, but do not progress further around the pyramidal layer to contact the smaller CA1 pyramids (Fig. 1).

To identify the growth status of axons in our material, we have used immunostaining with antibodies to the growth-associated protein (GAP) 43 (Goslin et al. 1988) and to the microtubule-associated protein (MAP) 1x (Calvert and Anderton, 1985). The latter antibody (G10) detects an epitope on MAP1x which is present only on growing axons and is rapidly down-regulated on cessation of growth (Calvert et al. 1987; Woodhams et al. 1989; Garner et al. 1989; Sato-Yoshitake et al. 1989; Reiderer et al. 1990).

A feature of Thy-1 expression in non-neural tissues is the extent to which significant interspecies differences occur, even between mouse and rat (Morris, 1985). In this study of neuronal expression, we have therefore examined both rodent species, in order to distinguish between characteristics of a particular species and properties that might relate more generally to neural differentiation.

Materials and methods

The basic methodology for in situ hybridisation and immunohistochemistry, and much of the material, was identical to that already described (Xue et al. 1990). 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. Thy-1 mRNA was detected with a probe to the third exon of mouse Thy-1, which is 90 % homologous to the rat and gives equivalent signal in both species (Xue et
Thy-1 is excluded from growing axons

Results

Axonal projection from the pons to cerebellar lobule VII

At birth, fibres from many pontine neurons can be seen with G10 immunohistochemistry to have ascended the mouse brainstem laterally and entered the cerebellum, where they disperse to multiple locations including coursing medially in the vermis to enter, among others, dorsal lobule VII (Fig. 2B). As later (ventrally located; Adams et al. 1980) pontine neurons matured (P0–5) their axons also entered this tract. Although the pontine axons are in general the largest group of mossy fibres terminating in the cerebellar granule layer, in lobule VII in particular they constitute the vast majority of such axons (Batini et al. 1978; Kawamura and Hashikawa, 1981; Ito, 1984) and so it is here that we have focused our analysis.

Pontine neurons displayed a positive in situ hybridisation signal for Thy-1 mRNA from P0 (dorsal) to P5 (ventral) in the mouse (Fig. 3A–H). Thy-1 protein appeared on the surface membrane of their somata and dendrites from P5–8 (Fig. 3I–L), and by P12 was already of an intensity comparable to the adult.

The pontine axons (and other mossy fibres) form distinctively large synaptic terminals, called rosettes, readily seen in Thy-1 immunohistochemistry of adult cerebellum as intensely labelled structures interspersed within the granule cell layer (Fig. 4J–L). During postnatal development of lobule VII, the first, very weak, Thy-1 immunoreaction appeared at P5 on a few Purkinje cell bodies. By P12, there was no real internal granule layer, although medially of an intensity comparable to the adult. These still reacted with G10 antibody (Fig. 4E), although this staining was already decreasing in intensity by P14 and had disappeared entirely (Fig. 4H).

If these terminal sprouts of the pontine axons become Thy-1 immunoreactive a full week after their cell bodies do, what of their main shafts which grew to the cerebellar cortex in the first postnatal week? Their distal extent lies in the (at this stage presumptive) myelinated fibre layer of lobule VII, where they constitute 70% of the axons (Palkovits et al. 1972; Ito, 1984). The other components are the climbing fibres (at this stage Thy-1 negative, Morris et al. 1985b) and Purkinje cell axons. The latter became Thy-1 positive at the same time as the cell bodies and could be seen as the...
occasional Thy-1-labelled fibre in the myelinated fibre layer (eg Fig. 4D). The great majority of axons of the myelinated fibre layer became Thy-1 immunoreactive at the same time as the mossy fibre rosettes (Fig. 4J-L), suggesting that Thy-1 appears simultaneously on both the terminal region of the axon and its more proximal extent in the cerebellar cortex.

The pontine axons cannot be unambiguously identified more proximally in the peduncle, but nearer their cell bodies in the brainstem they cluster in laterally
Fig. 3. Developmental appearance of Thy-1 mRNA (A–H) and protein (I–L) in the mouse pons. Thy-1 mRNA, detected by autoradiography after *in situ* hybridisation with 35S-labelled riboprobe, is shown at low power in dark-field photographs at P0 (B; A is control incubated with sense probe), P5 (C), P12 (D) and P21 (E); the base of the pons is at the bottom of each photograph. Grains over individual cells are shown in bright-field photographs for P0 (F, these cells located dorsally in pontine nucleus), P5 (G) and P12 (H). Thy-1 protein is shown by immunoperoxidase labelling at P5 (I; high power DIC optics in J show labelling of surface membrane of soma and dendrite of a cell in I), P8 (K) and P12 (L). Scale bars are 100 μm in A (B–E same magnification), I, K and L, 10 μm in F (G, H same), and 5 μm in J.
Fig. 5. Molecular maturation of the fascicles of pontine axons in the mouse assessed with immunoperoxidase labelling for Thy-1 (first column), the G10 epitope (second column) and GAP43 (third column excluding L). Parasagittal sections from animals at P5 (A–C), P12 (D–F), P15 (G–I) and P21 (J–L; hatched area in K is shown at higher power in L where arrowheads indicate G10 immunoreactive axons; arrowheads in A–K point to the pial surface). Examples of fascicles of pontine axons visible after G10 staining shown with arrows, as are fascicles after Thy-1 staining at P12–21. Scale bars are 20 μm (A; B–K same) and 5 (L) μm.

Orientated fascicles and form a distinctive tract under the pial surface (brachium pontis; Fig. 2B). Their molecular maturation can be assessed by inspecting the superficial transverse fibres in the pons (Fig. 5). The deeper-lying fascicles started to become Thy-1 immunoreactive at P12, the more superficial by P21. G10 immunolabelling was already declining at P12, although even at P21 (but not P28) individual axons within the fascicles remained strongly immunoreactive (Fig. 5L). GAP43 antibodies labelled most of the pontine neuropil (including dendrites and axon fascicles) over this period (Fig. 5C,F,I).

Qualitatively the same pattern of Thy-1 acquisition occurred in the rat, but at a faster rate. Thy-1 mRNA appeared on the pontine neurons at E20–P2, and protein at the soma and dendrites at P0–2. Immunoreactivity of the axons, both near the cell bodies and at their terminals in the cerebellum, appeared from
Fig. 4. Maturation of mossy fibre terminals in the granule layer of the cerebellum, as seen with Thy-1 (A,D,G,J–M), G10 (B,E,H) and GAP43 (C,F,I) immunoperoxidase labelling of mouse lobule IX (M) and VII (others) at P5 (A–C), P12 (D–F), P15 (G), P14 (H,I), P21 (J), P28 (K) and P56 (L,M). Open arrow in A points to 2 Purkinje cells just becoming Thy-1 positive; arrowheads in other Thy-1 stained sections point to examples of synaptic rosettes, the (presumptive) myelinated fibre layer is denoted with an asterisk. Scale bar is 50 μm.
Fig. 6. Acquisition of Thy-1 by vestibular ganglion neurons and their axons. (A,B,B') E20 rat showing vestibular ganglion (arrowed, brain stem is immediately below the area photographed) and its axons projecting peripherally to the ampulla (right of photograph), immunolabelled with antibodies to parvalbumin (A), polyclonal antibodies to Thy-1 (B) and monoclonal OX7 antibodies to Thy-1.1 (B'). Even at low power ganglion cells and their axons can be seen strongly labelled in B, but not in B'. (C,D) Mouse vestibular ganglion at P6 (C) and P56 (D) labelled with polyclonal anti-Thy-1 antibodies; arrows indicate longitudinal profiles of axons (E,F) Mouse ganglion at P15, immunolabelled with monoclonal anti-Thy-1.2 (E) and polyclonal anti-Thy-1 (F) antibodies, axons (now myelinated) cut in cross-section. (G,H) Mouse P21 immunolabelled with the monoclonal anti-Thy-1.2 antibodies, showing in G the longitudinal profile of a labelled axon passing a poorly labelled vestibular neuronal cell body, and in H patches of immunolabelling clustered at the axonal poles of the cells (arrows). (I,J) Rat ganglion at P2 (I) and P5 (J) immunolabelled with monoclonal anti-Thy-1 antibodies. Arrow in I points to a neuron with weak, diffuse cytoplasmic labelling, and unstained axon and somatic surface membrane, typical of cells labelled with the monoclonal antibody at E18-20; other cells at this age are starting to show labelling of their axon and somatic surface membrane (K) Rat ganglion at P56, polyclonal anti-Thy-1 labelling. (L-N) Centrally directed rat vestibular axons at P2, immunolabelled with monoclonal anti-Thy-1.1 (L; arrow points to the single immunolabelled axon in these fascicles), polyclonal anti-Thy-1 (M) and anti-parvalbumin (N) antibodies. Scale bars are 250 \( \mu \)m in A (B same), and 10 \( \mu \)m in C (D,F,1-N same) and E (G,H same).

P12–21. The loss of G10 immunoreactivity from these axons also occurred earlier in this species, from P8–12.

Projection of vestibular ganglion axons to cerebellar lobule IX

This description of Thy-1 acquisition by the pontine axons in lobule VII applies to the mossy fibres in the other vermal lobules, except that it proceeded faster by 2–3 days in the most caudal and rostral lobules, where the granule cells themselves mature earliest (Altman, 1972). The majority of the mossy fibres of the most caudal lobules, IX and X, arise in the vestibular ganglion (Ito, 1984). The cell bodies and axons of this small ganglion can be identified by parvalbumin immunolabelling during development (Fig. 6A; Morris et al. 1988).

Vestibular ganglion neurons in the mouse showed detectable Thy-1 mRNA at E13 (Fig. 7D) and, by E15 (Fig. 7C), all cells gave a strongly positive signal which had not increased noticeably by birth (Fig. 7A,B). G10 immunoreactivity of vestibular axons declined postnatally, and had essentially disappeared at P6 (not shown) when Thy-1 immunolabelling was first seen on about 50% of these neurons. The actual staining pattern differed according to whether the polyclonal anti-Thy-1, or monoclonal anti-Thy-1.2, antibodies were used. With the polyclonal, some cell bodies and adjacent axons were positive at P6 (Fig. 6C; adult staining shown in Fig. 6D), six days before their terminals in the cerebellum. By P8, 50–80% of the cells were immunolabelled reasonably strongly, as were their centrally and peripherally directed axons. The proportion of labelled cells and axons increased thereafter: by P15 greater than 90% of the cells were Thy-1 positive, by P21 only very occasional cells were unlabelled; from P28 unlabelled cells or axons were not seen. When staining first appeared, it was predominantly on the surface membrane of soma and axon, and in a granular ring at the periphery of the somatic cytoplasm; cytoplasmic staining then spread more generally around the cell body and increased notably in intensity (Fig. 6C,D). From P28, all ganglionic neurons labelled with similar intensity.

The monoclonal anti-Thy-1.2 antibody gave a different picture. Part of this difference was simply quantitative – the polyclonal antibodies gave stronger staining. In addition, there was an important qualitative difference. At the earliest ages (P6–8), the monoclonal antibody gave very weak, diffuse labelling of somatic cytoplasm. Thereafter (including in the adult), it labelled axonal Thy-1 and a minor component of the cytoplasmic Thy-1, but failed to label the bulk of the cytoplasmic Thy-1 or that of the surface of the cell body (Fig. 6E–H). Where immunolabelling of the vestibular cell bodies was apparent, it was usually polarised towards the axon initial segment (Fig. 6H).

In the rat, ganglionic neurons first displayed Thy-1 immunolabelling with the polyclonal antibody at E18, and more strongly so at E20 (Fig. 6B). Labelling was of the axonal and somatic plasma membranes, with some additional cytoplasmic labelling. The axons still stained with the G10 antibody at this time, but this immunoreactivity declined sharply after birth. Thy-1 immunolabelling, however, spread to include virtually all ganglionic neurons and their axons at P2, and by P5 the acquisition of Thy-1 was complete. Cytoplasmic labelling increased in intensity relative to the surface membrane at P2–5, and remained a dominant feature of somatic staining in the adult (Fig. 6I–K). Immunolabelling with the monoclonal antibody gave a more complex picture only for the first few days of its appearance. At E18–20, weak cytoplasmic labelling was seen, but neither the somatic surface nor the axons were labelled (Fig. 6B and B'). At P2, some cells and axons showed labelling of their surface membrane (Fig. 6I–L–N), and by P5 (Fig. 6J) all cells showed immunolabelling which was indistinguishable from that of the polyclonal antibodies – strong surface and cytoplasmic labelling of somata, and of the axonal surface. The rosettes in the cerebellar granular layer of lobules IX and X were weakly Thy-1 labelled (with either antibody) at P12. The intensity of labelling increased in the third week of life, and by P21 was comparable to that of proximal regions of the axons.

The discrepancy in staining patterns between the polyclonal and monoclonal antibodies (also seen with the sensory neurons of the nearby trigeminal ganglion) requires the specificity of their reaction with the sections to be demonstrated. This was done in three
Fig. 7. Appearance of Thy-1 mRNA in vestibular ganglion and hippocampus. (A–D) Mouse vestibular ganglion, showing in situ hybridization signal in darkfield at P0 (A, light area at base of photo is bone), and at higher power in bright field at P0 (B), E15 (C) and E13 (D) (E–J) Dark-field photos of mouse (E,G,I) and rat (F,H,J) hippocampi at PO (E,F; pyramidal cells in layer marked Py), P5 (G,H) and P21 (I,J; DG, dentate granule cells; CA3, pyramidal field CA3). (K,L) Rat dentate granule cells, bright-field, at P5 (K) and P12 (L), showing the progression across the layer of mRNA-expressing cells. Scale bars are 50 μm (A, E (F same), and G (H–J same) and 10 μm for the bright-field photographs.
Thy-1 is excluded from growing axons

Fig. 8. Specificity of immunological reaction with vestibular ganglion. (A) SDS–PAGE analysis of pure Thy-1 (lane 1, Comassie blue staining of 12 μg of protein) and of immunoblots of adult rat pons (lanes 2,4) and vestibular ganglion (lane 3) labelled with polyclonal anti-Thy-1 (lanes 3,4) and mouse monoclonal OX7 anti-Thy-1.1 (lane 2) antibodies; lanes 5–9 are immunoblots of mouse vestibular ganglion (P8, lane 6; P56, lane 5) and pons (P8, lanes 7 and 8; P56, lane 9) labelled with rat monoclonal 30H12 anti-Thy-1 2 (lane 7) and polyclonal anti-Thy-1 antibodies (lanes 5,6,8,9). For the immunoblotting, 25 μg of protein was loaded per well, longer exposure times of the autoradiographic film have been used to bring up the weak bands. (B,C) P12 mouse vestibular ganglion cells (column B) and axons (column C) immunolabelled with polyclonal anti-Thy-1 antibodies, preincubated with 0, 7.5, 15 and 30 ng/ml of Thy-1. Very weak brown labelling was evident at 7.5 ng/ml of inhibiting Thy-1, with just a trace remaining at 15 ng/ml. At 30 ng/ml the sections were devoid of immunoperoxidase reaction. Nuclei were not counterstained on any of the sections used in this figure. Scale bar is 20 μm, applies throughout. (D) P21 mouse vestibular ganglion, unfixed cryostat sections, preincubated for 15 min with 0 or 150 μM of PI-PLC as indicated, immunolabelled with polyclonal anti-Thy-1 antibodies (two upper photos of cell bodies) or anti-N-CAM (two lower photos of cross-section of axons).

ways. Increasing dilutions of pure Thy-1 (Fig. 8A, lane 1) were preincubated with the polyclonal antibodies before they were applied to sections of mouse vestibular ganglion. Complete inhibition was observed down to very low levels of Thy-1 (15 ng/ml, or 1.2 fm), occurring equally on the cell bodies and axons (Fig. 8B,C). The second test was based on the fact that Thy-1 is anchored to the membrane by a phosphatidylinositol tail (Homans et al. 1988) and can be selectively removed by phosphatidylinositol-specific phospholipase C (PI-PLC; Low and Kincade, 1985). Treatment of unfixed sections of mouse vestibular ganglion with PI-PLC removed immunoreactivity for both the monoclonal (not shown) and polyclonal anti-Thy-1 antibodies (Fig. 8D, upper panels) without affecting staining (in this myelinated peripheral nerve, of the Schwann cell basement membrane (Martini and Schachner, 1986)) of another glycoprotein, N-CAM (Fig. 8D, lower panels). Finally, immunoblot analysis revealed no extra bands detected by the polyclonal antibody on this material (Fig. 8A, lanes 2–9).

**Thy-1 expression during hippocampal axogenesis**

Pyramidal neurons first showed detectable signal for Thy-1 mRNA after they had migrated to their definitive position and started to form their layer, at E16–17 in the mouse and E17 in the rat. The mRNA signal remained similar in intensity in the two species (Fig. 7E–J), and in the different hippocampal fields, during postnatal development.

The early immunohistochemical staining pattern of the hippocampal area was dominated by the presence of the entorhinal afferents, which in the mouse were immunolabelled for Thy-1 from P0 (Fig. 9A), and fibres from the subiculum which were Thy-1 positive from P5. The pyramidal neurons had already grown an extensive apical dendrite at P0 (Fig. 9A), and in addition basal dendrites by P5–8, when the vast majority of these cells were entirely Thy-1 negative in the mouse. The first Thy-1-positive cells were seen at P5 in lateral hippocampus (Fig. 9B; we have used silver–gold enhancement of the peroxidase product to demonstrate this) on the somatic and dendritic surface membranes of cells located on the basal (alvear) side of the layer. Some superficially located cells remained negative at P12 (Fig. 9C), although all appeared
Fig. 9. Development of Thy-1 immunoreactivity on hippocampal pyramid and granule cells, and their axons (A) Mouse hippocampus at P0. Thy-1 immunolabelling enhanced with silver-gold development labels (a dense black) only the entorhinal axons terminating on the distal ends of the pyramidal dendrites at the top of the photo, the course of some dendrites can be seen by virtue of the Nissl counterstain (e.g., arrowhead). (B) Mouse CA1 pyramidal cells at P5. Thy-1 immunolabelling with silver-gold enhancement, two cells at base of layer (arrowhead) have labelled apical dendrites, most pyramidal cells are unlabelled at this age. Immunolabelling above and below the pyramidal layer is due predominantly to afferent fibres, with contributions from branching dendrites of the few Thy-1 positive cells. (C) Mouse Thy-1 immunolabelling (without enhancement) of CA1 pyramids at P12, not all cells in layer are yet Thy-1 positive. (D) Rat CA3 pyramids at P8, most have strongly Thy-1-labelled somata and dendrites (no enhancement). (E,F) Adjacent coronal sections (phase-contrast photography) of mouse at P8 showing the area of the ventral hippocampal commissure (vhc, corpus callosum (cc) and longitudinal fibres of the dorsal fornix (df) are also shown) labelled for Thy-1 (enhanced immunohistochemistry, only occasional axons cut in cross-section in the fornix are positive) and G10 (F). (G,H) Thy-1 labelling of dentate granule cells at P12 in the mouse (G; arrowhead shows immunolabelled dendrites of a Thy-1-positive cell on the superficial face of the layer, most cells are unlabelled) and rat (H; most cells labelled). (I–L) Mouse P8 coronal sections showing CA3 (I,J) and CA1 (K,L) pyramidal fields labelled for Thy-1 (I,K; these sections have been silver-gold enhanced) and G10 (J,L). The granule cell axons grow above the somata of CA3 (stratum lucidum, demarcated by arrowheads) but not CA1 pyramids; so, stratum oriens; sm, stratum moleculare; sr, stratum radiatum, containing the entorhinal input. Scale bars are 10 μm (A–D, G,H) and 50 μm (E,F, I–L).

Thy-1 mRNA at P5 in both species, on the superficial intensity to the adult level, by P21.

In a gradient of maturation, both around the layer to the infrapyramidal blade, and across the layer to the deeper (hilar) face (Fig. 7K,L), being complete at P21. Within the layer, the least mature cells (small nucleus staining dark blue with the Nissl counterstain (Gaarskjaer, 1985)) were unlabelled for Thy-1 mRNA. As with pyramidal cells, substantial dendritic growth could be seen by the Nissl counterstain to have occurred before Thy-1 immunolabelling (of both soma and dendrites) appeared. This labelling followed a similar gradient, starting in the most mature regions at P5 and more generally by P12 (Fig. 9G) and being complete by P28. The granule axons run in a distinctive layer above the cell bodies of the CA3, but not CA1, pyramids. At P8 in the mouse these axons strongly labelled with G10 antibody, and conversely failed to be labelled with Thy-1 antibodies (Fig. 9I–L; the reciprocal staining by Thy-1 and G10 antibodies is particularly evident in the hippocampal layers). G10 labelling of granule axons declined in the third postnatal week, and the axons could be seen in coronal sections to be Thy-1 immunolabelled by P21.

As in hindbrain, in hippocampus also the expression of rat Thy-1, and loss of G10 immunoreactivity, followed a similar pattern, but with a faster tempo (e.g. Fig. 9D,H), than in the mouse.

Discussion

The pattern of Thy-1 expression in the four sets of neurons examined in this work can be summarised in five simple statements (see also Fig. 10) which apply equally to the rat and mouse.

1. Thy-1 mRNA expression is determined by somatic differentiation

The early developmental history of these neurons is quite diverse: the hippocampal pyramids migrate from primary germinal neuroepithelium of the lateral ventricle (Bayer, 1980); most of the hippocampal granule cells are generated in situ from a secondary neuroepithelium without migration (Cowan et al. 1981); the pontine neurons migrate from a secondary, precerebellar neuroepithelium (Altman and Bayer, 1987); and the vestibular ganglion neurons are not even derived from neural tube, but are placodal in origin (D’Amico-Martel and Noden, 1983; Altman and Bayer, 1982). Yet these, like the mitral cells of the olfactory bulb (Xue et al. 1990), all express Thy-1 mRNA at the same stage of development, when they have finished migration and begun dendritic growth (Fig. 10). This is a very basic developmental stage for neurons, and can be seen morphologically as an enlargement of both soma and nucleus, with the latter becoming round and much

labelled at P14. No difference was apparent in the rate of Thy-1 acquisition by fields CA1 or CA3.

Pyramidal axons cannot be seen clearly in Thy-1 immunohistochemistry at their proximal or distal ends, due to the abundance of other Thy-1-positive elements. However, they are major components of the axonal tract, the fimbria, where the first few Thy-1-immunoreactive fibres appeared at P5–8, although the majority did not acquire Thy-1 until the second and third postnatal week. (Fibres from the septum and early maturing subiculum also run in this tract, and the subicular fibres almost certainly constitute the earliest labelled axons seen here). The axons of the ventral hippocampal fissure were entirely Thy-1 negative for the first postnatal week (Fig. 9E) when they strongly immunolabelled with the G10 (Fig. 9F) and GAP43 (not shown) antibodies. The intensity of G10 immunolabelling of the commissure declined towards the end of the second week when, at P12–15, Thy-1 immunolabelling first became apparent. The area of immunoreaction had spread to the whole tract, and increased in intensity to the adult level, by P21.

Hippocampal granule cells first showed detectable Thy-1 mRNA at P5 in both species, on the superficially located cells of the suprapyramidal blade. This spread in a gradient of maturation, both around the layer to the infrapyramidal blade, and across the layer to the deeper (hilar) face (Fig. 7K,L), being complete at P21. Within the layer, the least mature cells (small nucleus staining dark blue with the Nissl counterstain (Gaarskjaer, 1985)) were unlabelled for Thy-1 mRNA. As with pyramidal cells, substantial dendritic growth could be seen by the Nissl counterstain to have occurred before Thy-1 immunolabelling (of both soma and dendrites) appeared. This labelling followed a similar gradient, starting in the most mature regions at P5 and more generally by P12 (Fig. 9G) and being complete by P28. The granule axons run in a distinctive layer above the cell bodies of the CA3, but not CA1, pyramids. At P8 in the mouse these axons strongly labelled with G10 antibody, and conversely failed to be labelled with Thy-1 antibodies (Fig. 9I–L; the reciprocal staining by Thy-1 and G10 antibodies is particularly evident in the hippocampal layers). G10 labelling of granule axons declined in the third postnatal week, and the axons could be seen in coronal sections to be Thy-1 immunolabelled by P21.

As in hindbrain, in hippocampus also the expression of rat Thy-1, and loss of G10 immunoreactivity, followed a similar pattern, but with a faster tempo (e.g. Fig. 9D,H), than in the mouse. Thus pyramidal neurons became Thy-1 positive at P0–2, their axons in the fimbria and ventral hippocampal fissure did so at P8–21. The granule cells were immunolabelled over the period P5–21, their axons above the CA3 pyramids acquired immunoreactivity over P5–21.
lighter stained with thionin. Part of this general differentiation of the neuronal soma involves the expression of Thy-1 mRNA.

(2) mRNA expression does not by itself lead to appearance of Thy-1 protein

For rat pontine neurons, or dentate granule cells in either species, appearance of Thy-1 mRNA and protein were closely linked, protein following its message within 24–48 h. However, for mouse vestibular ganglion neurons there was a delay of 2 weeks, even though the level of Thy-1 mRNA signal for most of this period was substantially higher than on the mouse pontine neurons when they were expressing Thy-1 protein (c.f. Fig. 7B,C with Fig. 3G,H). Rat and mouse hippocampal pyramids showed similar levels of mRNA signal, followed in 5 days in the rat, and 10 days in the mouse, by appearance of protein. Taken together with similar observations in the olfactory system (Xue et al. 1990), it is clear that some signal in addition to expression of its mRNA is required for expression of Thy-1 protein.

(3) Initial expression of Thy-1 protein is restricted to the dendritic compartment and follows cessation of primary axon elongation

The soma and dendrites of the pontine neurons became Thy-1 immunoreactive a full week before their axons immunolabelled (Fig. 10). A similar delay was evident in the case of the hippocampal pyramids and granule cells.

Although Thy-1 on its first appearance was restricted to the dendrite, there is no correlation between its time of appearance and dendritic growth. On some neurons (e.g. rat Purkinje cells, Morris et al. 1985a) Thy-1 is present at all stages on all developing dendrites; for olfactory mitral cells, Thy-1 is present on part of the growing dendritic tree (Xue et al. 1990); for hippocampal pyramidal cells, dendritic growth occurs for over a week, during which they receive their main afferent inputs (Zimmer and Haug, 1978), before Thy-1 is expressed.

However, appearance of Thy-1 protein closely follows completion of the initial phase of axonal growth (except for vestibular neurons, see below). In the rat, there is very little delay between the period of primary axon elongation and the appearance of Thy-1 protein. Rat pontine axons, for instance, reach the cerebellar cortex by birth (Payne and Bower, 1988); their cell bodies and dendrites become Thy-1 positive from P0 to P2. In the mouse, Thy-1 protein was detected on the cells studied 3–6 days later than in the rat (taking birth as the reference point). However, G10 immunoreactivity also disappeared from mouse axons a few days later than in the rat. Whether mouse axons actually grow more slowly (or start later), or the neurons respond molecularly more slowly to the completion of growth, is unclear. (The 2 day longer gestation of the rat could obviously contribute to, but not entirely account for, this difference. Most studies of the development of these fibre pathways have been done in the rat and not the mouse, so direct comparison of the timing of axonal growth in the two species is not possible).

A temporal relationship between completion of the initial phase of axon elongation, and appearance of Thy-1 protein, does not imply a causal relationship between the two, although it is worth noting an established mechanism exists whereby this could occur. When an axon reaches its terminal zone, further molecular maturation of the neuron is induced by factors such as NGF produced by the terminal field (Barde, 1989). Part of this maturation could be acquisition of competence to accumulate Thy-1 protein in dendrites.

(4) Thy-1 is only allowed into axons after they have completed both their initial elongation, and subsequent terminal sprouting

These two phases of axonal growth are quite distinct,
often in timing but also in the signals they respond to and substrates used (see e.g. Morris et al., 1988; O'Leary and Terashima, 1988; Ghosh et al., 1990). The appearance of Thy-1 on the pontine axons in the third and fourth postnatal weeks follows the formation and maturation of the mossy fibre terminals, which starts in the second week (Altman, 1972; Hamori and Somogyi, 1983; Arsenio Nunes and Sotelo, 1985). Indeed, although we have concentrated upon two of the mossy fibre projections to cerebellum, the point is perhaps best made by considering this group as a whole. They are a diverse group of fibres, arriving in the cerebellum from E12 (vestibular; Morris et al., 1988) to nearly two weeks later (part of the spinocerebellar group; Arsenio Nunes and Sotelo, 1985). Yet all acquire Thy-1 in their terminal sprouts (and the nearby distal region in the myelinated fibre layer) at the same time, dictated by the state of maturation of their targets, the late-developing granule cells, rather than by that of their cells of origin. The late acquisition of Thy-1 by the cerebellar climbing fibres, after they have finished terminal growth (Morris et al., 1985b), demonstrates the same effect. Although we cannot be as precise in identifying the acquisition of Thy-1 by the hippocampal axons, it is clear that this occurs some days after their cell bodies/dendrites display Thy-1 immunoreactivity, and after the axons have lost G10 immunoreactivity. It therefore seems probable that cessation of terminal growth also occurs with these before Thy-1 appears on their axons.

(5) Where axons grow at different times to multiple targets, Thy-1 is allowed into regions of completed growth, but remains excluded from growing regions

The growth of the vestibular axons into the granule layer of the cerebellum is a relatively small and late burst of terminal sprouting by axons that have principally terminated on the three vestibular nuclei of the brainstem two weeks earlier (Morris et al., 1988). In fact, their initial phase of axonal growth was substantially complete by E14–15 (Morris et al., 1988). The delay (in the rat, of 4–6 days; in the mouse, of more than 10 days) before Thy-1 protein became detectable on these cells was presumably because they lack dendrites, and therefore have no appropriate compartment in which to place Thy-1 until the axon becomes permissive. Nevertheless, the proximal regions of their axons became Thy-1 positive 6–12 days before their terminals in the cerebellum (Fig. 10). These axons follow a diffuse course across the brainstem (Morris et al., 1988), and the boundary between the Thy-1-positive and -negative areas cannot be determined. We suspect that within the brainstem they are Thy-1 positive, and only the growing cerebellar region is negative (Fig. 10). Such partitioning of Thy-1 along a shorter process, the growing mitral cell dendrite, has been demonstrated and occurs at defined cellular boundaries (Xue et al., 1990).

Specificity of the anti-Thy-1 antibodies

There was a discrepancy between the staining patterns of the monoclonal and polyclonal antibodies on the vestibular ganglion cells, especially in the mouse. We suggest the polyclonal antibodies reveal the true extent of Thy-1 on these cells, since their reaction is inhibited by pure Thy-1, removed by PI-PLC, and they stain no additional bands in western blots. The failure of the monoclonals to detect some somatic Thy-1 might be relatively trivial in origin. For instance, our current work shows that Thy-1 can be nicked by endogenous tissue proteases (two disulphide bonds hold the molecule together) and these forms are detected by the polyclonal, but not monoclonal, antibodies (B. Pliego Rivero, in preparation). If the sensory ganglia have an unusually high concentration of such proteases in their soma, then postmortem nicking of the Thy-1 would produce the observed staining pattern. Alternatively, it may demonstrate the presence of another molecule, intimately associated with Thy-1 at early stages of its biosynthesis, which masks the allele-determining residue 89 (Williams and Gagnon, 1982).

Implications for Thy-1 biosynthesis

In general, newly synthesised cell surface glycoproteins are carried internally to the axon terminal by rapid transport where they are incorporated into the plasma membrane (Grafstein and Forman, 1980; Forman, 1984). This model is compatible with the observed compartmentalisation of certain cell surface glycoproteins on growing axons, as described for instance for TAG-1 and L1 on developing commissural axons of rat spinal cord (Dodd et al., 1988), if the proximally located protein (in this case, TAG-1) is transiently expressed at an earlier stage than the distally located protein. However, it clearly cannot explain the situation seen here with Thy-1, where the glycoprotein on its first appearance is specifically excluded from the growing (distal) region of the axon. Given the lengths of the axons studied here, fast axonal transport would deposit Thy-1 at the growing tip within hours of its synthesis at the cell body. Our data require a model that can explain the initial vectorial phase of Thy-1 synthesis, and its developmental switch to include the axon; and a mechanism for allowing Thy-1 into proximal, but not the growing distal, axonal regions.

A key role in the initial vectorial synthesis of Thy-1 almost certainly follows its unusual mode of insertion into the plasma membrane, via a glycosphospholipid moiety (Homsans et al., 1988). The latter serves in epithelial cells to direct incorporation of such lipid-anchored proteins to the apical (rather than basolateral) surface (Lisanti et al., 1989). Hippocampal pyramidal neurons in culture show the same vectorial synthesis of viral glycoproteins as do epithelial cells, those proteins that are inserted into the apical epithelial surface being directed to the axonal surface in neurons (Dotti and Simons, 1990). These workers find Thy-1 appears only on the axons (and not dendrites) of rat hippocampal pyramids after two weeks in culture (equivalent to P11), so this endogenous glycosphospholipid-linked membrane protein shows the same vectorial insertion as the viral glycoproteins (Dotti et al., 1991). This encoding of axonal insertion of Thy-1 presum-
ably operates in vivo, and its activation could initiate access of Thy-1 to the axonal compartment. The earlier direction of Thy-1 to dendrites would presumably occur if, at this stage, newly synthesised Thy-1 retained its amino acid transmembrane tail (which is normally cleaved and replaced within seconds by the lipid anchor; Conzelmann et al. 1987). Such alternate forms of lipid-linked molecules are produced by alternative splicing giving rise to different mRNA species, or by association early in biosynthesis with another protein subunit that protects against cleavage of the original transmembrane polypeptide tail (see Kurosky and Ravetch, 1989). It would therefore be in keeping with known mechanisms of membrane protein biosynthesis if alternate membrane-anchored forms of Thy-1 resulted in dendritic and axonal insertion. We are currently examining this possibility, although alternative mechanisms, such as differential location of mRNA (as occurs with the dendritic cytoskeletal protein, MAP2 (Garner et al. 1988), and certain myelin proteins (Campagnoni and Macklin, 1988; Shiota et al. 1989; Gillespie et al. 1990)) are not excluded by our present observations.

Whatever the mechanism that establishes the initial separation of Thy-1 between dendrite and axon, how is this compartmentation maintained over the long time that we have observed? The glycoprophospholipid tail confers on Thy-1 the higher mobility in the plane of the membrane typical of a lipid (Ishihara et al. 1987), and it would be expected to diffuse along the axon unless some other mechanism acted to retard its progress. Since Thy-1 does not span the membrane, this barrier cannot be directly cytoskeletal; it could be an interaction with some other molecule, perhaps present on glial cells in the environment of more mature axons, as we have suggested to underlie the partitioning seen in growing mitral cell dendrites (Xue et al. 1990). If this is correct, then glial maturation would determine the progression of Thy-1 protein along the axon.

**Biological implication of this pattern of Thy-1 expression**

Although the molecular mechanisms underlying this biosynthetic partitioning of Thy-1 are unknown, their end result is clearly to exclude Thy-1 from regions of axonal growth. This in vivo observation is particularly interesting in the light of recent experimental evidence indicating that Thy-1 inhibits process outgrowth by neural cell lines (Mahanthappa and Patterson, 1989) especially on astrocytes (Morris et al. 1990). If this is a model for axonal (rather than dendritic) growth, then Thy-1 would be inhibitory for axogenesis. Since adult neurons do not prohibit Thy-1 from their axons, it will be interesting to assess whether this molecule is present on, and contributes to the failure of growth by, the abortive sprouts produced by lesioned axons in the astrocytic environment of adult CNS.

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