Research report

Laminar redistribution of a glial subtype in the chick optic tectum

Frank Miskevich *

Department of Anatomy and Neurobiology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110, USA

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Abstract

Lamination is a central feature of structural organization and segregation within the central nervous system. Afferent fibers typically restrict their synapses to only one or a few specific laminae in the target region. Astroglial cells act as boundary markers for functional segregation of inputs in somatosensory cortex and the olfactory bulb and might also help to segregate particular connections in the neostriatum. This work presents evidence that a subset of astroglial cells expressing the carbohydrate recognized by tomato lectin are enriched in retinononrecipient laminae of the chick optic tectum. This segregation is dependent upon retinal innervation; enucleated chick tecta contain cells that bind tomato lectin but do not segregate into their normal laminae. These results suggest that tomato lectin positive astrocytes of the superficial chick tectum play a role in defining or restricting lamina specific connections of retinal axons.

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1. Introduction

Laminar organization of the central nervous system is a central feature of synaptic connectivity. Many afferent populations reach their target region of the brain and form synapses in only a restricted number of layers, thus allowing connections only between cells that either reside in or send processes to that lamina while excluding those in other laminae. The chick retinotectal projection provides an excellent model system for studying lamination. The tectum contains 16 distinct laminae, each with a different constellation of cell types [16] and defined projections from other regions of the brain [12].

The maturation of the retinal projection to the tectum is shown in Fig. 1. The retina projects from the eye through the optic chiasm and reaches the outermost layer of the tectum, the stratum opticum (SO) by E7–E8. Over the next several days retinal axons enter the most superficial neuropil, pause briefly, and then enter deeper layers of the superficial SGFS but never extend beyond the retinorecipient laminae. Each retinal axon projects to only a single retinorecipient lamina, and retinal ganglion cells expressing different neurotransmitters project to different layers [28,29]. By E18, retinal arbors have formed, and synapses in the retinorecipient layers are concentrating N-cadherin [19,30]. By E21 (P0) the retinotectal connections are mature enough to allow the chick to see. Molecular cues restricted to specific laminae are believed to form permissive and non-permissive areas of growth. In the chick optic tectum, for example, several cadherins, neurotransmitters, and a carbohydrate epitope are concentrated in retinorecipient laminae [19,30]. Blockade of N-cadherin or the epitope recognized by the lectin VVA impaired laminar specificity of retinal axons in vivo and in vitro [14]. Other proteins, including tenascin and an unidentified protein recognized by the sigma antibody, are specifically localized in the retinononrecipient layers of the superficial tectum [30], which is likely to be an inhibitory region for retinal axon arborization.

While some of the laminarily restricted antigens in the tectum have been localized to neurons, glia could also play a role in defining laminar specificity. Indeed, in other systems, glia seem to play a role in axonal guidance, particularly in olfactory glomeruli [9,27], somatosensory cortex [6] and the neostriatum [21]. Here I describe a chemically distinct subpopulation of astrocytes that becomes concentrated in the retinononrecipient laminae of the tectum. I show that the distribution of these astrocytes

* Department of Molecular, Cellular, and Developmental Biology, Yale University, PO Box 208103, New Haven, CT 06520, USA. E-mail: frank.miskevich@yale.edu

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is influenced by neuronal inputs and suggest that these glia may play an active role in restricting retinal arbors to their appropriate laminae.

2. Materials and methods

2.1. Immunohistochemistry

Chick tecta were dissected and fixed for 2 h in 4% paraformaldehyde in PBS buffer, incubated in 30% sucrose overnight, and quick frozen in OCT compound. Ten-micron sections were cut on a cryostat and collected on gelatin coated slides. Sections were then post-fixed 5 min with 4% paraformaldehyde in PBS, washed 3 times in PBS, and blocked in PBS +1% BSA. Sections were incubated in primary antibody overnight at 4°C. Slides were washed 3 times for 10 min in PBS and either preserved with 90% glycerol/PBS + paraphenylenediamine or incubated 2 h in secondary antibody and washed as above. Images were collected on a Zeiss Axiophot or digitally acquired using an Olympus confocal microscope. Lectins and antibodies used in these experiments were: (a) FITC labeled tomato lectin (fLEL, Vector Labs); (b) biotinylated VVA lectin (Sigma); (c) mouse monoclonal anti-NF-200 neuronal marker (Sigma); (d) mouse monoclonal anti-MAP2 neuronal marker (Sigma); (e) rabbit polyclonal R22 anti-GFAP astrocyte marker (R22, gift from L. Eng, Stanford); (f) rabbit polyclonal anti-vimentin glial marker (gift from R. Goldman, Northwestern); (g) mouse monoclonal S13 anti-myelin associated glycoprotein (MAG) oligodendrocyte marker (gift from D. Galileo, Medical College of Georgia); (h) rabbit polyclonal anti-glutamine synthetase and (i) rabbit polyclonal anti-carbonic anhydrase II astrocyte markers (gifts from P.J. Linser, University of Florida). Cy3 conjugated secondary antibody or rhodamine conjugated avidin were purchased from Cappel and Jackson Labs.

2.2. Enucleations

Fertilized chicken eggs were incubated until approximately E3 to E3.5 when the eye is becoming pigmented, corresponding to Hamburger and Hamilton stages 19–22 [11]. The top of the egg was then windowed and the covering membrane removed using fine forceps. The developing eye was carefully removed using an electrocautery device, avoiding major blood vessels in the alantois and embryonic forebrain. The egg was then resealed with scotch tape and replaced in the incubator until eggs set at the same time began hatching. Only embryos which maintained an apparently normal contralateral projection were used in this study. Tecta were then prepared, sectioned, and stained as described above.
composed of cell bodies and processes which, aside from their concentrated restriction to particular laminae, are very similar to those seen earlier in development (Fig. 2I). Deeper laminae which do not receive retinal inputs also contain tomato lectin positive cells, but these do not appear to change their distribution between E16 and P0 when the laminated pattern develops in the superficial SGFS (Fig. 2G and 2H).
3.2. Retinal innervation is required for tomato lectin positive cells to become laminated

The developmental restriction of tomato lectin cells and processes to retinononrecipient layers of the tectum at the time when retinal axons are actively expanding their arbors suggests that retinal inputs might be involved in this process. In order to test this hypothesis, one retina of a developing chick was surgically removed early in development, and the chick embryos were allowed to mature to hatching. In all cases studied, enucleated chick tecta failed to form tomato lectin positive bands in the superficial neuropil of the tectum. Several results indicated that the effect of enucleation was specifically to impair laminar restriction of the tomato lectin positive cells. First, tomato lectin positive cells remained and appeared relatively evenly distributed in the superficial SGFS (Fig. 3A and 3B). Thus, enucleation affected their distribution, not their formation or survival. Second, the lectin VVA maintained its restricted, laminated expression pattern in enucleated embryos, consistent with previous results (Fig. 3C; see [30]) and arguing against a general failure of lamination. Third, the ipsilateral tectum of the operated chick which received a normal retinal input showed tomato and VVA lectin staining that was indistinguishable from unoperated animals (Fig. 3D–3F). Finally, no differences were identified between enucleated and ipsilateral tecta in deeper layers of the tectum which contain tomato lectin positive cells but never encounter retinal axons (data not shown). Together, these experiments suggest that retinal input is required for the lamination of these cells and processes in the chick tectum.

3.3. Tomato lectin recognizes a subset of glial cells including some astrocytes

To identify the type of cell expressing the tomato lectin positive carbohydrate, tectal sections were double labeled

Fig. 3. Enucleation disrupts the lamination of tomato lectin positive cells. (A) Tomato lectin positive cells of enucleated P0 tecta are not distributed in a laminar pattern in the superficial SGFS. (B) High power micrograph emphasizing the non-laminated pattern of cells and processes. (C) Laminated staining of the lectin VVA in an enucleated tectum showing that lamination is not completely disrupted. (D) Ipsilateral control tectum showing the laminated pattern of tomato lectin staining at P0. (E) Higher power micrograph of laminated staining is indistinguishable from unoperated animals (see Fig. 2H). (F) VVA lectin stains SGFS-D in the ipsilateral control. Scale bar for panels A, C, D, and F is 30 μm and for panels B and D is 10 μm.
Fig. 4. Tomato lectin colocalizes with astroglial markers. Tomato lectin staining is shown in green and compared to (A) neurofilament staining in the superficial SGFS at E16, (B) MAP-2 staining at P0 in deep tectal layers, (C) vimentin staining of superficial SGFS at P0, (D) MAG staining of the stratum album centrale at P0, (E) GFAP staining of stratum album centrale at P0, (F) GS staining of superficial SGFS at E16, (G) CA2 staining in superficial SGFS at E16, and (H) CA2 staining in ependymal cells and deep tectal layers at P0. The only double positive staining is with the astrocyte markers GS and CA2. Scale bar for panels A, B, F, G and H is 10 μm, and for panels C–E is 15 μm.

with tomato lectin and a number of cell type specific markers; the results are shown in Fig. 4. Tomato lectin positive cells fail to co-label processes that are neurofilament (Fig. 4A) or MAP2 (Fig. 4B) positive, suggesting that this lectin does not recognize neurons. Similarly, tomato lectin did not colocalize with vimentin (radial glia, Fig. 4C), myelin associated glycoprotein (MAG, oligodendrocytes, Fig. 4D), or glial fibrillary acidic protein (GFAP, fibrous astrocytes, Fig. 4E). It did, however, colocalize with a subset of glutamine synthetase positive cells (GS, Fig. 4F). GS is a cytoplasmic marker for mature astrocytes in the chick tectum and positive astrocytes are found in all layers of the neuropil with no laminar pattern as previously reported [17]. The cells which colocalize GS and tomato lectin typically express lower levels of GS than other GS positive cells in the same field, but which are otherwise indistinguishable. In addition, GS labels almost all ependymal cells weakly, but these are tomato lectin negative (data not shown).

Carbonic anhydrase II (CA2) is another reported marker of chick astrocytes (see Ref. [17] for discussion and differences between chicks and mammals). Tomato lectin and CA2 also recognize overlapping populations of cells (Fig. 4G). These cells are found in both superficial and deep layers of the tectum. Interestingly, CA2 brightly stains the same subpopulation of ependymal cells that is recognized by tomato lectin but not GS (Fig. 4H and data not shown). Like GS, CA2 does not show any laminar specificity within retinononrecipient layers of the tectum ([17] and data not shown). While not all tomato lectin positive cells were positive for an astrocytic marker, based on the late developmental accumulation of the majority of tomato lectin positive cells and their colocalization with the astrocyte markers GS and CA2, it appears that tomato lectin labels a subpopulation of astrocytes in the chick tectum. Since glial maturation is not complete at hatching [17,18], it is possible that tomato lectin may stain both mature and immature cells.

4. Discussion

Lectins have been used for many years to specifically bind subsets of cells in nervous system, including a subset of astrocytes that surround whisker barrels in the somatosensory cortex [25]. Here, I identify a subset of glia in the developing chick brain which are positive for tomato lectin, a lectin specific for a subset of N-acetylglucosamine containing carbohydrates. The staining is specifically localized to the cell surface and is clearly visible on both the cell body and processes. By colocalization with two astrocytic markers of the chick, GS and CA2 [17], these cells were shown to include a specific subset of protoplasmic astrocytes. Tomato lectin positive cells in the ependymal layer are present at very early stages and could be a type of glial progenitor cell, some of which are known to be located in this layer [8]. Tomato lectin positive cells in the neuropil which are not CA2 or GS positive might be immature astroglia, but additional experiments would be needed to test this hypothesis.
Astrocytes are a heterogeneous, broadly expressed cell class in the central nervous system [10,17] and have already been shown to play significant roles in axon guidance and synapse formation [23,24]. This is true in several different regions of the developing brain. In the somatosensory cortex, astrocytes encircle whisker barrels and appear to inhibit axon growth outside of their defined area [6]. Glia surrounding whisker barrels fail to segregate or express particular inhibitory molecular cues including tenascin, chondroitin sulfate, keratin sulfate or the carbohydrate recognized by peanut lectin if afferent fibers are eliminated [26]. This is similar to what is seen in the lamination defect of tomato lectin positive cells in the enucleated chick tectum. The glial scaffold in the olfactory system is conserved from moths through mice [5,27], and the astrocytes appear to be essential to proper formation of glomeruli as mitotic inhibitors which selectively reduce the number of glia disrupts glomerular formation [22]. A similar situation is true in the neostriatum, where boundaries are functionally subdivided based on neurotransmitter phenotype and are dependent upon afferent interactions to form the glial boundaries [21].

Tomato lectin positive glial cells in the tectum may have a more complex and dynamic role. Unlike the cases above, these cells cannot provide an early boundary for retinal axon growth as incoming retinal axons must grow through retinononrecipient laminae in order to reach retinorecipient layers. Retinal axons enter the tectal neuropil at E12 well before tomato lectin positive cells stratify [28]. Inhibitory molecules such as a peanut lectin epitope, tenascin, chondroitin sulfate, and keratin sulfate that form glial boundaries in the cortex are broadly expressed in the chick tectum during retinotectal synapse formation, although tenascin does become restricted to retinononrecipient laminae after hatching (Ref. [30] and unpublished observations). Nevertheless, in vitro culture studies clearly show retinal axons preferentially select retinorecipient laminae for neurite outgrowth due to particular molecular cues [14,29].

This does not mean that there is no need to separate the individual retinorecipient tectal laminae. Although all retinal ganglion cells use glutamate as a neurotransmitter, retinal axons that terminate in different laminae contain additional distinct neurotransmitters [28] and synapse on specific postsynaptic targets [15]. This suggests that retinal axons that terminate in different layers are not functionally equivalent. It would be advantageous to provide a barrier between layers to prevent transmitter diffusion and arbor overlapping, an attribute already suggested for glial boundaries [24]. Tomato lectin positive cells require retinal input to become restricted to retinononrecipient laminae; this restriction takes place concurrently with retinal axon elaboration after laminae have been selected and axons have passed through the nonrecipient layers. Therefore, lectin positive cells probably do not play a causitive role in retinal axon lamination, but are induced to alter their distribution in response to retinal input and could play a role in restricting axon arborization to the recipient laminae or segregating functionally distinct retinal inputs.

Astrocyte properties are also known to be modified during the development of the visual system in other animals. In cats, injection of immature astrocytes into monocularly deprived adults allowed the formation of ocular dominance columns which is otherwise restricted to neonatal animals [20]. In addition, dark rearing of rats specifically reduced the number of glia in layer 5 of the visual cortex compared to controls [7]. Earlier in the visual pathway, the lateral geniculate nucleus of tree shrews becomes laminated after retinal axons innervate it [3] and fails to laminate in the absence of retinal axons [2,4]. Astrocyte processes become stratified before neurons in this structure, and have been suggested (though not proved) to play a role in forming the laminar pattern of neurons [13]. Tomato lectin positive glia of the chick also fail to become laminated in response to retinal input and superficial tectal laminae do not form normally [1], all consistent with the idea of visual inputs modifying astrocytes of target brain regions.

In summary, tomato lectin positive glia of the chick tectum become laminated in the superficial tectum in response to retinal signals. This type of glial boundary is reminiscent of astroglial boundaries in the whisker barrels of somatosensory cortex and glomerular formation in the olfactory bulb and suggests that these glia may be forming a boundary between functionally discrete retinal inputs.

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