Labeled lines in the retinotectal system: Markers for retinorecipient sublaminae and the retinal ganglion cell subsets that innervate them

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Introduction

Retinal ganglion cells (RGCs) are the sole conduits of information from the retina to the brain. Because many parameters of the visual scene are encoded and processed in the retina, RGCs carry multiple messages. Perhaps best studied is information about the location of a visual stimulus, conveyed by the retinotopic mapping of position in the visual field onto retinorecipient nuclei (McLaughlin and O’Leary, 2005). In addition, at each point in the visual world, different RGCs convey information about qualities such as motion, contrast and color (Rodieck, 1998). Physiologists have characterized RGCs tuned to these stimuli, and histologists have revealed distinct morphological features of some functionally defined RGC subsets (reviewed by Wasse and Boycott, 1991; Masland, 2001a,b). Little is known, however, about how such diversity arises, in part because few molecular markers are known that correspond to functionally or morphologically defined RGC subsets.

One important correlate of this nonretinotopic coding is the lamina-specific arborization of RGC processes. Dendrites of RGCs arborize in specific sublaminae of the retinal inner plexiform layer (IPL), where they receive synapses from distinct subsets of amacrine and bipolar cells. For example, dendrites of ON-center RGCs, which respond optimally when their receptive field centers are illuminated more brightly than surrounding areas, are generally restricted to IPL sublaminae adjacent to the ganglion cell layer. Conversely, dendrites of OFF-center RGCs are generally restricted to sublaminae adjacent to the inner nuclear layer (Wasse and Boycott, 1991). Within these ON- and OFF-zones, RGCs with dendrites in specific sublaminae are further distinguished by other receptive field properties, such as motion sensitivity (Roska and Werblin, 2001; Masland, 2001a,b).

Not only dendrites but also axons of RGCs exhibit striking laminar restrictions. In the optic tectum of the chick, for example, all RGC axons terminate in 4 retinorecipient laminae (RRLs), called stratum griseum et fibrosum superficiale (SGFS) B, C, D and F, and the arbor of each RGC is confined to a single RRL (van Gehuchten, 1892; LaVail and Cowan, 1971; Acheson et al., 1980; Yamagata and Sanes, 1995a; see Fig. 1a). Tectal cells integrate these inputs to generate unique responses to luminosity, motion and direction (Jassik-Gerschenfeld and Guichard, 1972; Frost, 1993; Luksch et al., 2004), which are then conveyed to higher brain centers (Luksch, 2003).

In that they collect information from multiple IPL sublaminae and deliver it to multiple RRLs, lamina-specified RGC subsets...
Fig. 1. Analysis of lamina-specified RGC subsets. (a) A schematic showing laminar targeting of retinal ganglion cell (RGC) axons in the tectum. All RGC axons that enter the tectum arborize in SGFS-B, C, D and F. Distinct subsets confine their arbors to just one of these 4 retinoreceptive laminae (RRLs). Panels b–f show double-label in situ hybridization to E16 retina; each row shows the separate channels (b–f and b′–f′) followed by the merged image (b″–f″). Most somatostatin I- (SOM I-) positive cells are SP-positive and therefore likely to be B-RGCs (b–b″). All somatostatin I-positive cells are AChR β2-negative and therefore unlikely to be F-RGCs (c–c″). Somatostatin II- (SOM II-) positive RGCs are SP-negative (d–d″) and AChR β2-positive (e–e″), and therefore likely to be F-RGCs. All somatostatin I-positive RGCs are NMB-positive (f–f″). Arrowheads indicate double-labeled RGCs. Panels g and h show control and enucleated E15 tecta immunostained for NMB. Immunoreactive axon-like profiles present in SGFS-A,B of control tecta (arrowheads) are absent from enucleated tecta, suggesting their retinal origin. NMB-positive cell bodies (asterisks) are found in both control and enucleated tecta. INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar in panel f is 100 μm for panels b–f; 15 μm for panels g, h.
comprise a set of parallel pathways from the retina to the brain. Molecular markers for these subsets, and for the tectal neurons they innervate, would therefore be valuable tools for tracing the connections of these subsets and elucidating the mechanisms that underlie their development. Here, we provide such markers. Our results not only define multiple retinotectal circuits but also reveal striking heterogeneity within both lamina-specified RGC subsets and their target RRLs. Moreover, making use of the new markers, we assess the relationship between the laminar choices that RGCs make in the IPL and those they make in the RRL, providing evidence that RGCs bring information from multiple IPL sublaminae to each RRL.

Results

The chicken optic tectum comprises 16 readily distinguishable laminae. RGC axons enter the optic tectum through the most superficial lamina, the stratum opticum (SO), then dive into the neuropil, arborizing in just 4 of the 16 laminae, SGFS-B, C, D and F, which we refer to here as RRLs (Fig. 1a). (These RRLs are called lamina 3, 4, 5 and 7, respectively, in an alternative nomenclature used by some authors; see Yamagata et al., 1995 for details.) The terminal arbor of each RGC is confined to a single RRL. (van Gehuchten, 1892; LaVail and Cowan, 1971; Ehrlich et al., 1987; Britto et al., 1992a, Yamagata and Sanes, 1995a,b). Within the RRLs, RGC axons form synapses on local interneurons as well as on dendrites of multipolar and pyramidal neurons with somata in at least two deeper layers, SGFS-I and stratum griseum centrale (SGC) (Reiner and Karten, 1982; Hunt and Brecha, 1984; Mey and Thanos, 1992; Karten et al., 1997; Luksch, 2003). We refer to RGCs that project to SGFS-B, -C, -D or -F as “B-RGCs”, “C-RGCs”, “D-RGCs” or “F-RGCs”, respectively.

To date, most studies of RGC subsets that project to specific RRLs have relied on two markers: substance P (SP), which selectively labels at least some B-RGCs, and the acetylcholine receptor (AChR) β2 subunit, which selectively labels at least some F-RGCs (Ehrlich et al., 1987; Keyser et al., 1988; Britto et al., 1992a,b, 1994; Yamagata and Sanes, 1995b). Here, we sought additional markers of RGC subsets that project to individual RRLs. To this end, we constructed and differentially screened libraries from putative B-RGCs (SP+/AChRβ2−, D-RGCs (SP−, AChRβ2−) and F-RGCs (SP−, AChRβ2+) (see Experimental methods). Candidate genes were initially tested by in situ hybridization to sections of E15–18 retina. Those expressed by discrete subsets of cells in the ganglion cell layer were then retested by double label in situ hybridization with a probe to the RGC marker, Thy-1. Genes expressed by RGCs were further analyzed for coexpression with SP and AChR β2. Examples are shown in Figs. 1b–f.

In parallel, we sought markers that distinguish individual RRLs from each other. Our starting point here was a series of monoclonal antibodies to tectum (Takahashi et al., 1999), along with probes generated during a previous search for markers that distinguish RRLs generally from nonretinorecipient laminae (Yamagata et al., 1995; Yamagata and Sanes, 2005).

Neuropeptide-containing subsets in the B circuit

B-RGCs

Neuromedin B. A cDNA encoding the neuropeptide neuromedin B (NMB) was isolated from a cDNA library generated from a single SP-positive B-RGC (see Experimental methods). Double-label in situ hybridization with a Thy-1 probe revealed that all NMB-positive cells in the ganglion cell layer were RGCs. Most (~60%) of the NMB-positive RGCs were SP-positive and none were AChRβ2-positive (Table 1), indicating that NMB is selectively expressed by B-RGCs. The SP-negative, NMB-positive RGCs might also project to SGFS-B, to other RRLs (SGFS-C, D, or F), or to non-tectal retinorecipient nuclei. As one test of these alternatives, we generated an antibody to NMB (see Experimental methods) and used it to stain tectum. Fine, varicose NMB-positive fibers were present in SGFS-A/B of control tecta (Fig. 1g). Such fibers were absent from enucleated tecta (Fig. 1h), suggesting that they arose from RGCs projecting to SGFS-B. No NMB-positive fibers were detected in deeper RRLs, indicating that NMB-expressing RGCs projecting to the tectum confine their arbors to SGFS-B (data not shown). These results also suggest that at least some RGCs projecting to SGFS-B are SP-negative.

Somatostatin I. The neuropeptide somatostatin I was extensively coexpressed with SP and NMB: all somatostatin I-positive RGCs were NMB-positive, and most (~70%) were SP-positive. In contrast, none were AChR β2-positive (Figs. 1b, c, f and Table 1). As was the case for NMB, antibodies to somatostatin stained fine fibers in SGFS-A and -B of control tecta but not in enucleated tecta (data not shown).

Together with previous work (Ehrlich et al., 1987; Yamagata and Sanes, 1995b), these results define a set of three neuropeptides – SP, NMB and somatostatin I—that mark overlapping populations of RGCs projecting to SGFS-B. Because overlap is only partial, we can distinguish at least 4 B-RGC subsets: NMB+ somatostatin I−SP−, NMB+ somatostatin I−SP+, NMB+ somatostatin I−SP+ and NMB+ somatostatin I+ SP+.

SGFS-B

Reelin. In a previous study, we described a monoclonal antibody, TB5, that selectively stained SGFS-A/B at E14, a time before these two laminae can be distinguished from each other (Takahashi et al., 1999; Fig. 2a). By E18, SGFS-A and -B are distinct; TB5 stained

| Table 1 |
| Markers of lamina-specified RGC subsets |
| Marker (X) | % X that is SP+ | % SP that is X+ | % X that is β2+ | % β2 that is X+ | % X that is Thy1+ |
| Substance P | ++++ | ++++ | 0 | 0 | 100 |
| Neuromedin B | ++++ | ++++ | 0 | 0 | 100 |
| Somatostatin I | ++ | ++ | 0 | 0 | 100 |
| Cadherin-7 | 0 | 0 | 0 | 0 | 100 |
| AChR β2 | 0 | 0 | ++++ | ++++ | 100 |
| Ezrin | 0 | 0 | ++++ | ++++ | 100 |
| Somatostatin II | 0 | 0 | ++++ | ++++ | 100 |
| Cadherin-11 | 0 | 0 | ++ | ++ | 80 |

Summary of results from fluorescent double in situ hybridization studies on E16 chick retina sections. Symbols denote the degree of overlap with substance P (SP, a marker for RGCs that project to RRL-B) and with AChR β2 subunit (β2, a marker for RGCs that project to RRL-F): 0 is <5%; + is 5–30%; ++ is 31–60%; +++ is 61–90%; ++++ is >90%. The last column shows the percentage of marker-positive cells in the ganglion cell layer that were positive for the RGC marker Thy-1. Results were averaged from 4–8 20× microscope fields (200× total magnification).
both laminae, with more intense labeling of SGFS-A than SGFS-B (Fig. 2b). SGFS-C-F and deeper non-RRLs were essentially TB5-negative at both stages. High-magnification micrographs suggested that most of the TB5 antigen was intracellular (cytoplasmic and/or membrane-associated) but that some was extracellular (data not shown).

To identify the antigen recognized by TB5, we prepared lysates from metabolically labeled tecta, immunoprecipitated material from the lysates and analyzed the precipitates. TB5 precipitated a ~400 kDa protein that was not recognized by a control antibody of the same subclass (Fig. 2c). Reelin, a well-characterized secreted protein of this approximate size, was already known to be present in superficial laminae of several brain areas, including tectum (D’Arcangelo et al., 1997; Bernier et al., 2000). We therefore asked whether an anti-reelin antibody (R4C11; Takeuchi-Tan and Ogawa, 2001) precipitated a similar protein, and found that it did (Fig. 2c). Moreover, R4C11 and TB5 stained tecta in a similar pattern (data not shown). To ask whether the TB5- and R4C11-reactive proteins were identical, we precipitated protein from tecta with TB5, then probed the precipitated material on Western blots with R4C11. R4C11 recognized the protein precipitated by TB5 (data not shown). As an additional test, we performed in situ hybridization, using a cDNA to chick reelin. Cells that expressed reelin were similar in size, shape and position to those stained by TB5 and R4C11 (Fig. 2d). Together, these results demonstrate that TB5 recognizes reelin and that reelin is concentrated in SGFS-A/B.

To characterize reelin-positive cells, we double-stained sections with TB5 plus markers for neurons (NeuN), for inhibitory interneurons (GABA) and for glia (glutamine synthetase; Linser and Moscona, 1981). All reelin-positive cells were NeuN- and
GABA-positive but none were glutamine synthetase-positive, indicating that reelin is selectively expressed by inhibitory interneurons in SGFS-A/B (Figs. 2e–h).

Neuromedin B. Antibodies to NMB labeled not only retinal axons but also neuronal somata in SGFS-A/B (Figs. 1g and 3a). In situ hybridization with a probe for NMB selectively labeled neurons in SGFS-A/B (Fig. 3b), indicating that neurons in this RRL express the NMB gene. Small numbers of NMB-positive cells were present in SGFS-D, but their density was <5% that in SGFS-A/B. Thus, NMB is a useful, albeit not completely specific marker of SGFS-A/B.

Substance P receptor. A subset of interneurons in SGFS-B expresses the SP receptor (SP-R; Fig. 3c, Yamagata and Sanes, 1995b), consistent with the selective expression of SP by RGCs that innervate SGFS-B (see above). We used double-label methods to determine the relationship of the reelin-, NMB- and SP receptor-positive neurons to each other. Reelin- and SP-receptor-positive neurons occupied distinct sublaminae, with reelin-positive cells superficial to the SP-R-positive cells (Fig. 3d). No neurons were both reelin- and SP-R-positive. Likewise, few if any NMB-positive cells were SP-R-positive (Fig. 3e). In contrast, most of the reelin-positive cells coexpressed NMB (Fig. 3f).

Fig. 3. NMB and SP receptor mark SGFS-B. Sections of E15 tectum were probed for neuromedin B (NMB) by immunostaining (a) and by in situ hybridization (b). Both methods show expression predominantly in SGFS-B (arrowheads). SP receptor (SP-R) immunoreactivity (c) is also concentrated in SGFS-B at E15. Double-label analysis shows that few SP-R-positive cells (d, e′) are reelin- or NMB-positive (arrowheads in panels d′, c), but that many reelin-positive cells are NMB-positive (f, f′). Most NMB- and reelin-positive cells are superficial to the SP-R-positive cells. Merged images including DAPI-labeled nuclei (blue) are shown in panels d″–f″. Abbreviations as in Fig. 2 legend. Scale bar in panel f is 40 μm for panels a–c; 20 μm for panels d–f.
Together, these results define five neuronal subtypes within SGFS-A/B. One, concentrated in the more superficial portion of the lamina, is reelin- and NMB-positive but SP-R-negative. A second, in the deeper portion of the lamina, is SP-R-positive but NMB- and reelin-negative. Smaller, superficial groups are NMB- or reelin-positive and SP-R-negative. The fifth group, which may be heterogeneous, expresses none of these three markers.

Cadherin-7-expressing synaptic partners in the C circuit

C-RGCs

A small fraction of RGCs, as well as many neurons of the inner nuclear layer, expressed cadherin-7, a Type II classical cadherin (Wohrn et al., 1998). None of the cadherin-7-positive RGCs cells expressed SP or AChR β2 (Figs. 4a,b, Table 1). This pattern suggested that cadherin-7 is a marker of C- and/or D-RGCs. Two lines of evidence supported the former possibility.

First, SGFS-C exhibited high levels of cadherin-7-immunoreactivity but SGFS-D did not, consistent with the idea that axons of cadherin-7-positive RGCs terminate in SGFS-C (Fig. 4g). Although interneurons intrinsic to SGFS-C also express cadherin-7 (see below), the intensity of cadherin-7-immunoreactivity was markedly reduced in tecta deprived of retinal input by early enucleation (Figs. 4e, f). This result suggests that cadherin-7 protein in SGFS-C is derived from retinal axons as well as from local sources.

Second, the thickness and cell density of SGFS-C, the target of C-RGCs, are higher in dorsal than in ventral tectum (Karten et al., 1997). Therefore, one would expect that the density of C-RGCs would be higher in ventral retina, which projects to dorsal tectum, than in dorsal retina, which projects to ventral tectum. Consistent with this prediction, the density of cadherin-7-positive RGCs was higher in ventral retina (~10% of RGCs) than in dorsal retina (~5% of RGCs; Figs. 4c, d). Likewise, the thickness of the cadherin-7-immunoreactive band in tectal SGFS-C was greater in dorsal than in ventral tectum (data not shown).

SGFS-C

Cadherin-7. The gene encoding cadherin-7 was expressed by a subset of cells in SGFS-C and cadherin-7 immunoreactivity was concentrated in the neuropil of this lamina (Figs. 4e, g; see also Miskevich et al., 1998). A few cells in deeper layers also contained cadherin-7 RNA, but little cadherin-7 immunoreactivity was detectable in these layers (Figs. 4e, g, h), suggesting that cadherin-7-positive neurons in deeper laminae extend processes either to SGFS-C or outside of the tectum. Thus, cadherin-7-positive RGC axons are likely to terminate on cadherin-7-positive neurons in SGFS-C.

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Fig. 4. Cadherin 7 marks SGFS-C and RGCs that project to it. Double-label in situ hybridization to E16 retina (a, b) demonstrates that all cadherin-7-positive RGCs are negative for both SP and AChR β2, suggesting they project neither to SGFS-B nor to SGFS-F. Density of cadherin-7-positive RGCs is higher in ventral (c) than in dorsal (d) tectum, as predicted for a population projecting to SGFS-C. Immunostaining demonstrates that cadherin-7 protein is concentrated in SGFS-C (e) and its levels are decreased by enucleation (f), suggesting it derives from both RGC axon terminals and tectal neurons. Sections of E15 tectum were probed for cadherin-7, by in situ hybridization (b) and immunostaining (g). Both methods show expression predominantly in SGFS-C. Parvalbumin immunoreactivity (i) is also concentrated in SGFS-C within the RRL at E15; it is also observed in a subset of cells in deeper layers. Double-label analysis (j, j′; merged image in j″) shows that many cells in SGFS-C are both cadherin-7- and parvalbumin-positive. Arrowheads show corresponding points. Abbreviations as in Figs. 1 and 2 legend. Scale bar in panel f is 80 μm for panels a, b, g–i; 160 μm for panels c, d, 40 μm for panels e, f; 20 μm for panel j.
Parvalbumin. Because calcium-binding proteins such as parvalbumin and calbindin mark specific subsets of interneurons in several brain areas (Baimbridge et al., 1992), we assessed their distribution in tectum. Within the RRL, parvalbumin-positive cells were selectively concentrated in SGFS-C (Fig. 4i). A subset of neurons in deeper non-RRLs was also parvalbumin-positive. Many parvalbumin-positive cells within SGFS-C coexpressed cadherin-7 (Fig. 4j).

Sublaminar distinctions in the D circuit

D-RGCs

Like cadherin-7, cadherin-11 was expressed by a subset of RGCs as well as by cells of the inner nuclear layer. No cadherin-11-positive RGCs coexpressed SP, but about 35% coexpressed AChR β2 and somatostatin II (Figs. 5a, b and Table 1). This result indicated that cadherin-11 is preferentially associated with F-RGCs (which account for about 10% of all RGCs) but is also expressed by RGCs that project elsewhere. In tectum, cadherin-11 immunoreactivity was concentrated in SGFS-D and -F; SGFS-E, which is not an RRL, was cadherin-11-negative (Fig. 5c). Immunoreactivity was absent from the RRL of enucleated tecta (Fig. 5d), indicating that it was derived in large part from retinal axons. Consistent with this conclusion, in situ hybridization of tectal sections with a cadherin-11 probe revealed expression only in a small population of cells in SGC (data not shown). Thus, our experiments suggest that cadherin-11-positive RGCs project to both SGFS-D and to SGFS-F. The cadherin-11-positive D-RGCs may be a subset of the large population of small RGCs known to project to this RRL (Karten et al., 1997); our screen did not, however, isolate markers that selectively label the majority of this population.

Detailed analysis revealed that, within SGFS-D, cadherin-11 was concentrated in the deeper half (Fig. 5c), suggesting that specific subsets of RGCs might be specified to project to the deeper or more superficial portion on this RRL. An alternative possibility was that retinal afferents in general ramify in deep but not superficial SGFS-D; in other words, that only deep SGFS-D is an RRL. To distinguish these alternatives, we stained tectal sections with antibodies to γ-catenin, which we showed previously to be a selective marker of RGC axons within the tectum (Miskevich et al., 1998). Quantitative analysis showed that retinal afferents are distributed broadly throughout SGFS-D, whereas the cadherin-11-positive subset of these afferents is concentrated in the deeper portion of SGFS-D (Figs. 5e-h). Thus, molecularly distinct groups of retinal axons project to superficial and deep portions of SGFS-D.

SGFS-D

Neuropilin-1. Antibody TB2, described by Takahashi et al. (1999), selectively stained neuropil in SGFS-D/F by E12; staining became restricted to SGFS-D by E15 as lamination patterns matured (Fig. 6a and data not shown). Initial studies indicated that TB2 recognized an extracellular epitope of a membrane-associated protein present on a subset of neurons (data not shown). These characteristics, and similarities between its distribution and that illustrated for the axonal guidance receptor, neuropilin-1 (Takagi et al., 1995), suggested that TB2 might recognize neuropilin-1. To test this possibility, we expressed neuropilin-1 in non-neuronal cells, and asked whether TB2 stained those cells. Indeed, TB2 stained neuropilin-1-transfected cells but not untransfected cells in the same cultures (Figs. 6b, b′). Thus, TB2 recognizes neuropilin-1.

In situ hybridization revealed two sites of neuropilin-1 expression within the tectum (Fig. 6d). First, neuropilin-1 was expressed by a subset of cells within SGFS-D; double-labeling with antibody to the neuron-specific antigen NeuN indicated that most of these cells were neurons (data not shown). Second, a subset of the large projection neurons in the superficial portion of SGC was neuropilin-1-positive. Many neurons in SGC have dendrites that arborize in SGFS-D, and most of these neurons have somata in the superficial portion of SGC (Karten et al., 1997). This correspondence suggests that some of the TB2-immunoreactive processes in SGFS-D are dendrites of neuropilin-1-expressing SGC neurons.
Fig. 6. Neuropilin-1, calbindin, ChAT and BEN mark SGFS-D and -F. Neuropilin-1 protein and RNA were detected in sections of E15 tectum using antibody TB2 (a) or in situ hybridization (d). TB2 immunoreactivity is confined to SGFS-D (a). Antibody TB2 stained cells transfected with a neuropilin-1 (b, b’) but not a neuropilin-2 (c, c’) expression vector, indicating that it is specific for neuropilin-1 (neuropilin ectodomains were fused to GFP; cells were counterstained with DAPI (blue)). Neuropilin-1 RNA is expressed by cells in SGFS-D but also in the deep portion of SGC (d), whereas neuropilin-2 RNA is broadly expressed (e). Calbindin immunoreactivity is concentrated in SGFS-D (f, j), while choline acetyltransferase (ChAT) immunoreactivity is concentrated in SGFS-F (g, k). Arrowheads indicate ChAT-positive somata. Double-label analysis shows that BEN is present in both SGFS-D and -F whereas neuropilin-1 is confined to SGFS-D (h, h’). Within the RRL, N-cadherin (i) and calbindin (j) are concentrated in SGFS-D while ChAT is concentrated in SGFS-F (k). Line scans below each micrograph in panels h–k show density of immunostaining as a function of laminar depth. Abbreviations as in Fig. 2 legend. Scale bar in panel i is 80 μm for panels a, d–g; 40 μm for panels b, c, h–j.
Interestingly, both neuropilin-1 immunoreactivity and neuropilin-expressing cells were concentrated in the lower portion of SGFS-D (Figs. 6a, d, h), corresponding to the area innervated by cadherin-11-positive D-RGCs (Fig. 5). Moreover, Karten et al. (1997) have shown that the neurons with somata in superficial SGC and dendrites in SGFS-D, which we infer are neuropilin-1-positive, are directly postsynaptic to retinal afferents and have dendritic arbors confined to the lower portion of SGFS-D. Together, these observations suggest that both pre- and postsynaptic elements in the D circuit can be subdivided into superficial and deep subgroups.

Neuropilin-2. Neuropilin-1 has one close homologue, neuropilin-2, which is also an axonal guidance receptor (Fujisawa, 2004). TB2 did not recognize neuropilin-2-transfected cells, indicating that it is specific for neuropilin-1 (Figs. 6c, c′). We used in situ hybridization to assess the possibility that neuropilin-2 might also mark a subset of RRLs. Neuronal-2 was expressed more broadly in tectum than was neuropilin-1; neuropilin-2-positive cells were present but not concentrated in the RRL with the highest density in the non-RRL, SGFS-G (Fig. 6e).

Calbindin. Antibodies to the calcium-binding protein calbindin (D28K) selectively labeled the superficial SO and SGFS-D at E18 and later (Fig. 6f). Immunoreactivity in SGFS-D was present both in a subset of neuronal somata and in neighboring processes (Fig. 6j). Calbindin-positive fibers and cells were concentrated in the deep portion of SGFS-D (Fig. 6j), corresponding to the region containing neuropilin-1-cells and cadherin-11-positive axons. Anti-calbindin labeling of the SO was consistent with reports of similar staining in the mammalian superior colliculus (Schmidt-Kastner et al., 1992; Gonzalez-Soriano et al., 2000).

BEN. The immunoglobulin superfamily adhesion molecule BEN (Pourquié et al., 1992), also called ALCAM, CD166, SC1, DMGRASP and JC7, is concentrated in SGFS-D and -F (Fig. 6h; Yamagata et al., 1995). In tecta doubly stained with anti-neuropilin-1 (TB2) and anti-BEN, extensive overlap is found in SGFS-D, supporting the molecular division of SGFS-D into molecularly distinct superficial and deep sublaminae (Figs. 6h, h′). Another adhesion molecule selectively expressed in the RNL, N-cadherin (Yamagata et al., 1995; Miskevich et al., 1998), is present throughout SGFS-D as well as in SGFS-B, -C and -F (Fig. 6i).

Multiple RGC markers in the F circuit

F-RGCs

Previous studies showed that AChR β2 and ezrin, a membrane-actin linker, are selectively expressed by F-RGCs (Britto et al., 1992b; Yamagata and Sanes, 1995b; Takahashi et al., 1999). A third marker, the neuropeptide somatostatin II (GenBank accession number: DQ279789, also called cortistatin) was isolated by screening a cDNA library generated from an AChR β2-positive F-RGC (see Experimental methods). Nearly all (≥90%) somatostatin II-positive RGCs were AChR β2-positive and nearly all (≥90%) AChR β2-positive RGCs were somatostatin II-positive, but no somatostatin II-positive RGCs were SP-positive (Figs. 1d, e and Table 1). Thus, somatostatin II joins AChR β2 and ezrin as a marker of F-RGCs.

As noted above, cadherin-11 is expressed by a subset of retinal axons that arborize in SGFS-F. The overlap of cadherin-11, AChRβ2 and somatostatin II RNAs in RGC somata (Fig. 5b) suggests that most or all cadherin-11-positive arbors in SGFS-F should also contain AChRβ2, but that some AChRβ2-positive arbors should be cadherin-11-negative. Double-stained sections validated these predictions. In particular, within SGFS-F, cadherin-11 and AChRβ2 were often colocalized, supporting the idea that individual F-RGCs express both genes (Fig. 5i).

SGFS-F

The acetylcholine-synthesizing enzyme, choline acetyltransferase (ChAT), is concentrated in SGFS-F (Sorensen et al., 1989; Medina and Reiner, 1994; Yamagata and Sanes, 1995b; Fig. 6g). Immunoreactivity is associated with dendrites of cholinergic neurons whose somata lie in deeper SGFS-F; the dendrites extend through SGFS-F and -G without branching, then arborize profusely in SGFS-D (LaVail and Cowan, 1971; Hunt and Brecha, 1984; Yamagata and Sanes, 1995b). Comparison of laminae labeled by anti-ChAT with those labeled by anti-neuropilin-1 or anti-calbindin emphasized the restriction of ChAT to SGFS-D (Figs. 6g, k). The different distributions of BEN and neuropilin-1 further emphasized the distinct molecular architectures of SGFS-D and -F: in tecta doubly stained for these two markers, extensive overlap is found in SGFS-D, while the neuropilin-1-negative, BEN-rich lamina corresponds precisely to SGFS-F.

Dendritic lamination patterns of B-, C- and F-RGCs

Subsets of RGCs not only restrict their axonal arbors to individual tectal RRLs, but also restrict their dendritic arbors to individual sublaminae within the retinal IPL (e.g., Ramón y Cajal, 1892; Masland, 2001a,b). How might laminar choices made by RGC axons and dendrites be related? At one extreme (Model 1 in Fig. 7a), choices might be coordinate, with all RGCs that project to a particular RRL receiving their inputs from one or a few particular IPL sublaminae. In this case, IPL sublaminae would be mapped onto RRLs. At the other extreme (Model 2 in Fig. 7a), axonal and dendritic choices might be specified separately, with RGCs projecting to a particular RRL receiving input from many different IPL sublaminae. In this case, each RRL would collect inputs from many IPL sublaminae.

To distinguish these and intermediate models, we biohistochemically labeled RGCs in a flat-mounted preparation to fill their dendritic arbors. We then sectioned the labeled retinae and performed fluorescent in situ hybridization with probes to genes that mark RGC subsets projecting to individual RRLs. IPL sublaminar assignments of filled RGC dendrites were made by dividing the IPL into five equal subdivisions, with sublamina 1 closest to the inner nuclear layer and sublamina 5 closest to the ganglion cell layer (Ramón y Cajal, 1892). We used this method previously to show that dendrites of most RGCs expressing the adhesion molecule sidekick-1 are confined to IPL sublamina 4 (Yamagata et al., 2002).

To mark B-, C- and F-RGCs, we used probes to SP, cadherin-7 and somatostatin II, respectively. These markers were chosen because they are the best-validated markers for the subsets in question; somatostatin II was used rather than AChR β2 because its signal is more robust, presumably reflecting its higher expression level. Examples are shown in Figs. 7b and c and data are summarized in Figs. 7d–f. Among the RGCs that expressed SP, and thus projected to SGFS-B, were individual cells with dendrites in IPL sublamina 1, 3, 4 and 5. Among the RGCs that expressed cadherin-7, and thus projected to SGFS-C, were individual cells with dendrites in IPL sublamina 2, 3 and 4. Among the RGCs that
expressed somatostatin II, and thus projected to SGFS-F, were individual cells with dendrites in IPL sublamina 3, 4 and 5. Dendrites in all RGC subsets we analyzed exhibited prominent arborization in IPL sublamina 4, for reasons we do not understand. However, dendritic arbors of SP-, Cadherin-7- or somatostatin II-positive RGCs within sublamina 4 were usually broader than those of sidekick-1-positive RGCs (see Yamagata et al., 2002), and in several cases represented part of a bi-stratified dendritic pattern (Figs. 7e, f). Our data, therefore, rule out Model 1 (Fig. 7a), suggesting that RGCs receiving information from multiple IPL sublaminae can form a convergent projection to a single RRL.

Discussion

Subsets of RGCs gather visual information in multiple sublaminae of the retinal IPL and transmit it to multiple populations of neurons within the four tectal RRLs, where it is processed further. Analysis of these parallel pathways develop and function has been hampered by lack of molecular markers. We therefore sought markers that distinguish individual tectal RRLs and the RGC subsets that innervate them. Our progress toward this goal is summarized in Fig. 8. We further asked whether each RRL receives direct input from just a single IPL sublamina or from multiple sublaminae. As shown in Fig. 7, the new RGC subset markers made it possible to answer this question.

Caveats

Assignment of markers to specific RRLs is relatively straightforward, but their assignment to lamina-specified RGC subsets is subject to several caveats. In principle, the best approach would be to fill subsets of RGCs retrogradely by injection into individual RRLs, then ask whether a marker is selectively expressed by one such subset. Unfortunately, however, the RRLs are so narrow that this approach is infeasible. Instead, assignments were based on coexpression with previously validated markers of B- and F-RGCs, SP and AChR β2, respectively. SP and AChR β2 had themselves been validated by showing that immunoreactivity is concentrated in appropriate RRLs and is lost following enucleation (Ehrlich et al., 1987; Britto et al., 1992a,b). These methods are not quantitative, however, so it is possible that not all RGCs labeled by either marker project to the specified RRL or that all RGCs projecting to the RRL are labeled by the marker. Our own enucleation data are subject to the same limitations. In addition, a minority of RGCs project to non-tectal targets (Britto et al., 1989; Tombol et al., 2004), and we have not taken these into account.
There are also caveats that apply to both RGC and RRL markers. One is that we considered only a limited developmental window, between E14 and E18. Although the retinotectal projection is relatively mature by E18 (Nakamura and O’Leary, 1989; Mey and Thanos, 1992; Yamagata and Sanes, 1995a,b), expression patterns may change subsequently. Second, we categorized expression as “present” or “absent,” ignoring quantitative variations in marker levels as well as low levels of expression that are difficult to distinguish from background but might be significant.

Potential functions for retinotectal markers

Our aim was to identify markers for RGCs and RRLs that will be useful for mapping connectivity and monitoring its development. It is possible, however, that some of the markers are themselves involved in the development or function of lamina-specific connections. Four sets of genes are particularly interesting in this context.

First, cadherin-7 and -11 mark non-overlapping RGC subsets that project to distinct RRLs. Cadherin-7-expressing RGCs project to SGFS-C, whose interneurons selectively express cadherin-7. In that many cadherins interact homophilically (Tepass et al., 2000), cadherin-7 might mediate lamina-specific connectivity. Such a mechanism would be consistent with a growing body of evidence that cadherins are expressed in restricted patterns and play roles in multiple aspects of neural development, including synapse formation (Redies and Takeichi, 1996; Inoue and Sanes, 1997; Wohrn et al., 1998, 1999; Benson et al., 2001; Treubert-Zimmermann et al., 2002; Takeichi and Abe, 2005). For cadherin-11, the situation is more complex. Cadherin-11-positive RGCs terminate in both SGFS-F and the lower part of SGFS-D, suggesting that laminar specification may involve categories beyond the conventional, histological divisions. Moreover, few cells in SGFS express cadherin-11, so cadherin-mediated interactions with target cells would likely be heterophilic, as has been reported for several Type II classical cadherins (Shimoyama et al., 2000).

Second, several molecules implicated in neuronal migration and axon guidance are concentrated in RGC subsets or in individual RRLs: reelin (SGFS-A/B), neuropilin-1 (SGFS-D), BEN (SGFS-D+F) and ezrin (F-RGCs). The best-studied role of...
reelin is in migration of neurons to proper laminae but recent studies have shown that reelin also regulates some aspects of synapse formation (Rice et al., 2001; Tissir and Goffinet, 2003). Neuronal-1 and BEN could also be involved in the formation of RGC terminal arbors and subsequent synaptogenesis (see references in Fujisawa, 2004; Weiner et al., 2004). The actin-binding protein ezrin has been shown to interact with the cytoplasmic domain of some transmembrane adhesion molecules (Dickson et al., 2002), suggesting a possible role for ezrin in the targeting of F-RGC axons.

Third, several neuropeptides are associated with lamina-specified RGC subsets, including SP, NMB and somatostatin I in B-RGCs and somatostatin II in F-RGCs. SP receptors are concentrated in SGFS-B, to which the SP-positive RGCs project, raising the possibility that somatostatin and NMB receptors might likewise be concentrated in individual RRLs. The best-characterized function of such neuropeptides is to modulate neurotransmission that, in the case of the retinotectal synapse, appears to be glutamatergic (Dye and Karten, 1996). Selective expression of neuropeptides and their receptors in distinct retinotectal circuits provides a means of shaping excitatory transmission to perform lamina-specific functions. In addition, neuropeptides could influence formation of projection patterns, as suggested by reports of roles for SP and somatostatin I in neuronal migration and axon guidance (De Felipe et al., 1995; Yacubova and Komuro, 2002).

Finally, parvalbumin and calbindin are also expressed by subsets of interneurons in a lamina-specific manner. These Ca2+-binding proteins are involved in intracellular Ca2+ homeostasis and regulation of neuronal excitability (Fierro et al., 1998). Differential expression of these proteins may influence on their unique electrophysiological profiles.

Convergence and divergence in the retinotectal projection

We combined intracellular filling and in situ hybridization (Yamagata et al., 2002) to ask how the laminar choices made by RGC dendrites and axons are correlated. We have followed Ramón y Cajal (1892) in dividing the IPL into five equal sublaminae, but evidence exists for ≥10 physiologically defined, sublaminae (Roska and Werblin, 2001). Because there are only 4 RRLs in the chick tectum, we expected some convergence of inputs from IPL sublaminae onto RRLs. Indeed, our results show that individual RRLs receive input from multiple IPL sublaminae (Fig. 7). Nonetheless, our results hint that some restrictions exist, suggesting that connectivity patterns are intermediate between the extreme sketched in Figs. 7a and b.

The multiplicity of RGC types

Approximately 10–15 RGC types have been identified in a number of vertebrate species, including chicks, using morphological and/or electrophysiological criteria (Masland, 2001a,b, 2004; Naito and Chen, 2004). An assumed correlate has been the existence of molecular markers that are selectively expressed by each type.

Our study has substantially increased the number of molecular markers for subsets of RGCs whose axons project to individual tectal RRLs. Analysis of these markers’ coexpression has produced two surprising results. First, few pairs of RGC subset markers (AChR β2 and somatostatin II being an exception) demonstrated complete overlap in their expression patterns. Second, few, if any, of the markers are expressed by the full complement of RGCs projecting to a given RRL. Rather, we find that the RGCs subsets projecting to individual RRLs are defined by clusters of markers expressed in partially overlapping, combinatorial patterns (Fig. 8). At present, we can molecularly define >20 RGC classes (Fig. 8 and data not shown). In that we examined only ~30 markers, it is very likely that further subdivisions within these molecularly defined RGC types exist.

This “fuzzy” grouping of molecularly defined RGC subsets raises the question of what is meant by a neuronal “type”. It is likely that morphologically defined RGC types represent important functional categories (Masland, 2004), but these morphological types may be molecularly heterogeneous. Such heterogeneity would be expected if, for example, each subtype defined by dendritic stratification pattern in the IPL is subdivided into multiple groups with distinct laminar preferences in the tectum (for example 10 IPL sublaminae ×5RRL subdivisions =50 types). Additional heterogeneity may be needed to specify the appropriate retinorecipient nucleus (in that some RGC axons do not project to tectum) as well as position in the retinotopic map (e.g., levels of ephrins and Eph kinases; McLaughlin and O’Leary, 2005). Thus, RGCs and, by implication, other neuronal types, are likely to be defined by combinatorial expression of multiple markers, which together specify or are regulated by their connections and influence or are influenced by their functions.

Experimental methods

Histology and immunostaining

Immunohistochemical staining and in situ hybridization were performed as described previously (Yamagata et al., 1995, 2002). To visualize the dendritic processes of lamina-specified RGCs, in situ hybridization was combined with biotin intracellular filling, as described in Yamagata et al. (2002). Briefly, tungsten particles (1.3 μm) coated with fluorescein-conjugated dextran (10,000 MW; Molecular Probes, Eugene, OR) were shot into pieces of E16 retina flat-mounted onto nitrocellulose filters (Millipore, Billerica, MA) using a Helios Gene Gun (BioRad, Hercules, CA). Retinas were incubated for 1 h to permit dye diffusion, then fixed, frozen and sectioned orthogonally at 20 μm in a cryostat. Sections were processed for fluorescent in situ hybridization and the dextran signal was enhanced using anti-fluorescein-peroxidase and fluorescein-tyramide (TSA Plus system; Perkin-Elmer Life Sciences, Wellesley, MA).

The NMB sequence was identified from a chicken cDNA (deposited in GenBank with accession number DQ279788); its predicted amino acid sequence is identical to those of human and mouse NMB. To generate antibodies to NMB, we immunized mice with the peptide GNLWATGHFMC coupled to keyhole limpet hemocyanin (United States Biologicals, Swampscott, MA). Hybridomas were generated and cloned by standard methods, and their supernatants were tested by ELISA. One monoclonal antibody (NMB1; IgG1 subclass) that reacted strongly with the immunizing peptide was characterized further. Staining of tissue by NMB1 was blocked by competition with the immunizing peptide, and immunoreactive somata corresponded to those labeled by in situ hybridization with a probe specific for NMB.

Other antibodies used in this study were as follows: TB2 and TB5 were described by Takahashi et al. (1999); anti-SP receptor (Shigemoto et al., 1993) was from Ryuichi Shigemoto (National Institute for Physiological Sciences, Okazaki, Japan); anti-NeuN and anti-calbindin 28 were from Chemicon (Temecula, CA); anti-glutamine synthetase (Linser and Moscona, 1981) was a gift from Paul Linser (University of Florida, Gainesville, FL); anti-reelin (Takeuchi-Tan and Ogawa, 2001) was from M. Ogawa (Brain Science Institute, RIKEN, Wako, Japan); anti-GABA and anti-parvalbumin were from Sigma (St. Louis, MO); anti-cadherin-7 (Nakagawa and Takeichi,
Immunoprecipitation

obtain longer cDNAs. Single-cell libraries were used to screen conventional cDNA libraries to double label fluorescent in situ hybridization with the pan-RGC marker, E16 retina, to select those that labeled subsets of cells in the ganglion libraries were rescreened with probes that had been enriched by a construct the library but not with probes from other cell types. An anti-AChRβ2 was picked that reacted with probes from the cell type used to

Transfection

Chicken neurophin-1 and neurophin-2 cDNA sequences were amplified using RT-PCR (Platinum Taq DNA polymerase High Fidelity, Invitrogen, Carlsbad, CA), and cloned into the pCMV EGFP-N1 vector (Clontech, Mountain View, CA) to generate neuropilin-green fluorescent protein (GFP) fusion proteins. Human embryonic kidney 293 cells were transfected using the Lipofectamine reagent (Invitrogen).

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