Centrifugal fibres synapse on dopaminergic interplexiform cells in the teleost retina

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In teleost fish, centrifugal fibres originating in the olfactory bulb and containing FMRFamide-like and luteinizing hormone releasing hormone (LHRH)-like peptides project to the retina and terminate along the border of the inner nuclear and inner plexiform layers[1–3]. Using a novel simultaneous two-colour immunolabelling technique, we have found that these centrifugal fibres are often closely apposed to the dopaminergic interplexiform cells. Contacts between centrifugal fibres and dopaminergic interplexiform cells were observed by electron microscopy to be conventional type synaptic junctions. Since the dopaminergic interplexiform cells make synapses on horizontal and bipolar cells, providing an intraretinal centrifugal pathway for information flow from the inner to the outer plexiform layers[4–6], we conclude that every neuron in the teleost retina is potentially susceptible to central influences via these centrifugal fibres and dopaminergic interplexiform cells.

For light microscopy, centrifugal fibres in the retina of the white perch (*Roccus americanus*), and their cell bodies in the brain, were labelled with rabbit antisera directed to FMRFamide or [Trp^7, Leu^8]LHRH. Dopaminergic cells and their processes were labelled with a mouse monoclonal antibody directed to tyrosine hydroxylase (TOH; the rate limiting biosynthetic enzyme of dopamine). These antisera (FMRFamide at 1:1,000; TOH at 1:10,000) were applied simultaneously to 8-μm-thick cryostat sections of 4% paraformaldehyde fixed tissue mounted on gelatin coated slides. The peptide antisera were visualized using a fluorescein isothiocyanate (FITC) labelled goat anti-rabbit IgG secondary antibody (Boehringer Mannheim) while the TOH was visualized with phycoerythrin-labelled goat anti-mouse IgG (Biomeda). Both fluorochromes excite with blue light (450–490 nm) but have discrete emissions in the green and orange-red (long pass 520 nm). Using co-excitation of the two fluorochromes has allowed us to observe directly juxtapositions between centrifugal fibre varicosities with the cell bodies and processes of dopaminergic cells. Seeing both markers simultaneously greatly facilitates the screening of tissue for the relationships between the two.

For electron microscopy, a polyclonal rabbit anti-TOH antiserum (Eugene Tech, 1012) was used along with a peroxidase anti-peroxidase method that allows complete penetration of immunological reagents throughout a tissue block[6]. Following embedding in epon 812 and partial polymerization, 80-μm-thick sections were taken and mounted on glass slides. Selected sections containing a dopaminergic cell body were remounted with a cyanoacrylate adhesive onto blank epon blocks and thin sectioned. The unique morphological appearance of the stained elements was utilized to distinguish processes of centrifugal fibres from those of the dopaminergic cells. The former contained large (~100 nm), highly immunoreactive, large dense-core vesicles as well as small electron-lucent vesicles, whereas the latter had no large dense-core vesicles.

Centrifugal fibres originating from the nucleus olfactoretinalis run along the border of the inner plexiform and the inner nuclear layers. Fluorescence microscopy using our double labelling technique showed extensive clusters of FMRFamide-like immunoreactive fibres and varicosities juxtaposed to dopaminergic cell bodies (Fig. 3*a*). Additionally, centrifugal fibres surround the proximal processes of dopaminergic cells for about 50–75 μm from the cell body (Fig. 3*b*). In the goldfish retina, most if not all of the dopaminergic cells appear to be interplexiform cells[5], and our observations indicate that this is also the case in the white perch. More than 700 dopaminergic cells (in both central and peripheral retinal regions) were observed adjacent to peptide-containing centrifugal fibres. We conclude, therefore, that the dopaminergic cells and processes contacted by the centrifugal fibres are principally, if not exclus-

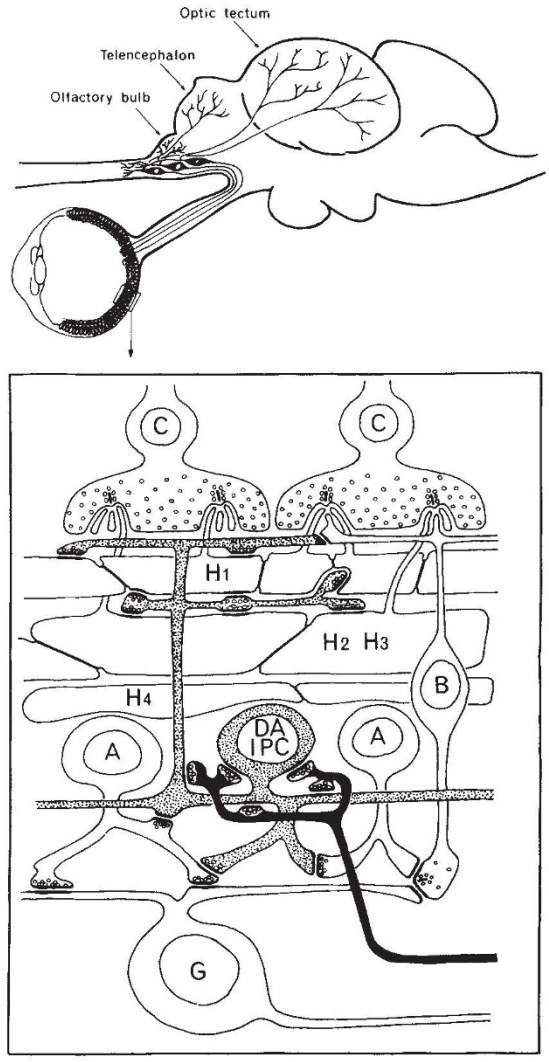

Fig. 1 A summary of the origins and synaptic connections of the olfactoretinalis-dopaminergic interplexiform cell (DA-IPC) system in the white perch. The cell bodies of the olfactoretinalis are located bilaterally near the midline at the base of the olfactory bulb where they send out processes toward the olfactory epithelium and bulb, as well as the telencephalon and optic tectum. Major axons decussate to the contralateral side where they enter the optic nerve and project out to the retina. In the retina, centrifugal fibres (solid black) ramify in the most distal inner plexiform layer and make extensive synaptic contacts onto the perikarya and proximal dendrites of dopaminergic interplexiform cells (stippled). Dopaminergic interplexiform cells, in turn, provide extensive synaptic output to cone horizontal cells (H1–H3) in the outer plexiform layer and some synaptic input to bipolar (B) cell dendrites in the outer plexiform layer and to amacrine (A) cells in the inner plexiform layer[5,6]. The interplexiform cells themselves also receive input from amacrine cells, and some amacrine cells are probably contacted by centrifugal fibres[12]. (C, cones; H4, rod horizontal cell; G, ganglion cell).

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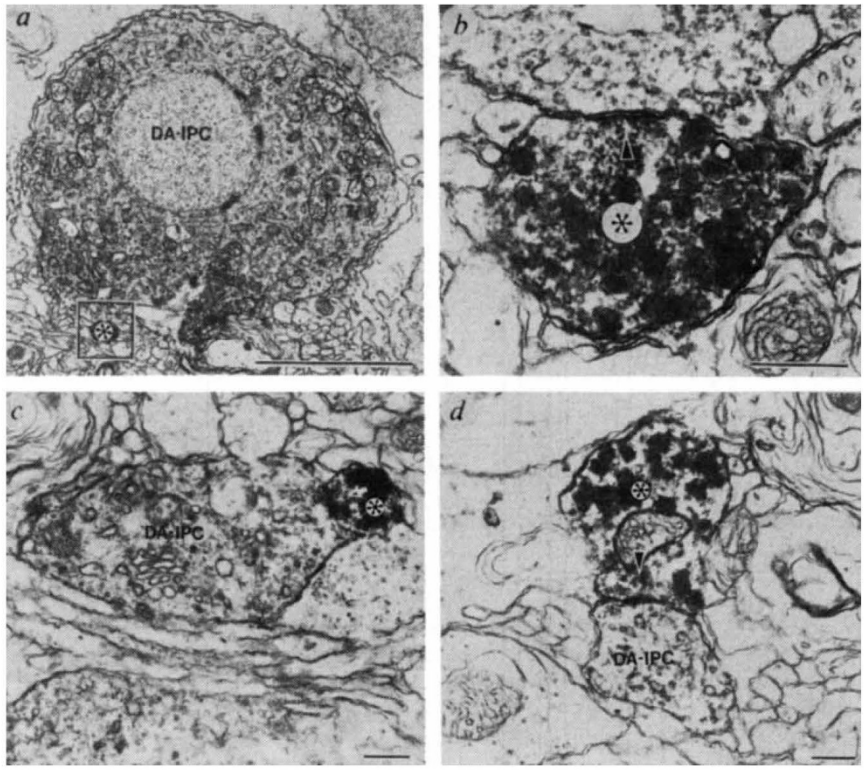

Fig. 2 Electron micrographs of the white perch retina double labelled using a PAP technique for FMRFamide and TOH. Labelled centrifugal processes are easily distinguished from dopaminergic interplexiform cells since the former contain many highly immunoreactive large dense-cored vesicles. *a*, Cross-section through the centre of a dopaminergic interplexiform perikaryon (DA-IPC) with a centrifugal process (*) contacting it at its most proximal border. *b*, At higher magnification and through several serial sections, this same process is seen making a conventional synaptic contact onto the cell body. *c*, A proximal dendrite (note characteristic organelles) from the same dopaminergic interplexiform cell is shown receiving a synaptic contact from a centrifugal fibre. *d*, A centrifugal process is seen synapsing onto an interplexiform cell dendrite located about 25 μm from the same cell body. In all of the synapses shown, note the distal location of the immunoreactive large dense-cored vesicles. Calibration bars: 5 μm (*a*); 0.5 μm (*b–d*).

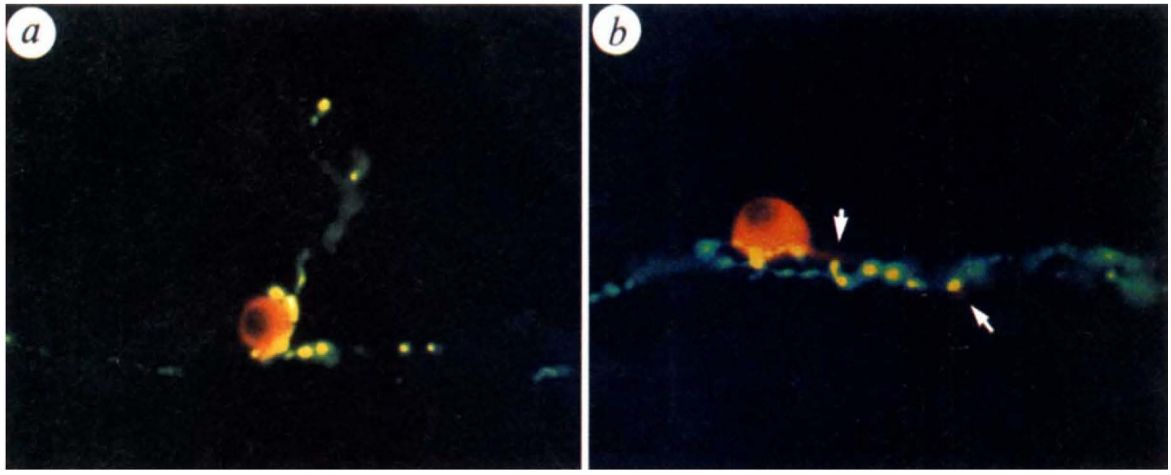

Fig. 3 Fluorescent micrographs of cryostat sections from white perch retina processed for simultaneous visualization of FMRFamide immunoreactive centrifugal fibres and TOH immunoreactive dopaminergic interplexiform cells. FITC- and phycoerythrin-labelled secondary antibodies were used; under blue excitation they resulted in apple-green centrifugal fibres and pumpkin-orange dopaminergic interplexiform cells and processes. Kodak Ektachrome P800/1600 was processed at push 1. *a*, Dopaminergic interplexiform cell perikaryon surrounded by a beaded string of centrifugal fibre varicosities. An extension of the centrifugal fibre is seen rising toward the outer plexiform layer. *b*, Dopaminergic interplexiform cell receiving extensive centrifugal contacts on its perikaryon and proximal dendrites. Both perikarya are about 12 μm in diameter.

ively, interplexiform cells. Centrifugal fibres occasionally ascend towards the outer plexiform layer. These ascending fibres invariably occur at the location of a dopaminergic interplexiform cell body, adjacent to where it extends a process towards the outer plexiform layer (Fig. 3*a*).

When visualized by electron microscopy, FMRF-amide-like immunoreactive processes showed both large dense-cored and small electron-lucent vesicles. These processes make conventional-type synapses, characterized by a cluster of small vesicles near the thickened presynaptic membrane (Fig. 2*b*,*c*,*d*). The large dense-cored vesicles tended to be distal to the presumed presynaptic site. When stained with anti-FMRFamide or with anti-[Trp^7,Leu^8]LHRH all large dense-cored vesicles were immunoreactive, whereas the small electron-lucent vesicles were always unstained. This indicates that both peptides co-localize within the large vesicles leaving a probable third neuroactive substance unidentified in the small vesicles.

Junctions between the centrifugal fibres and the cell perikarya and proximal processes of dopaminergic cells were frequently observed in double-labelled tissue, confirming the fluorescence microscopy observations. Figure 2*a* shows a section through a TOH-labelled dopaminergic cell perikaryon. A labelled centrifugal process is seen along its proximal border. Serial sections (Fig. 2*b*) revealed several synaptic junctions on the perikaryon. Additional centrifugal input was observed on a process from this same dopaminergic cell that was followed by serial sections (Fig. 2*c*,*d*). At least 13 conventional input synapses from centrifugal fibre terminals were observed in semi-serial sections

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through half of this cell body and its proximal dendritic tree. Centrifugal fibres did not appear to interact with dopaminergic interplexiform cells further than 75 μm from the cell body or into the proximal inner plexiform layer.

We also studied the distribution of FMRFamide and LHRH elsewhere in the white perch brain. Using either anti-FMRFamide or anti-[Trp^7,Leu^8]LHRH, bilateral clusters of large (25–35 μm diameter) cell bodies (35–50 per side) were visualized centred about the midline at the base of the olfactory bulb near the ventral surface. These clusters showed immunoreactivity for both peptides, but they were the only cells stained with the FMRFamide antibody in the white perch brain, indicating that they are likely to be the cells of origin of the centrifugal fibres. These cells send a profusion of processes throughout the olfactory bulb, telencephalon and optic tectum, and are themselves surrounded by varicosities, which are immunoreactive to FMRFamide. It appeared as if a single main axonal fibre projects from each cell body to enter the optic nerve.

We conclude that centrifugal fibres, originating from the olfactory system, interact with the retinal dopaminergic interplexiform cell system. This circuitry, summarized in Fig. 1, indicates that interplexiform cells represent the most distal reaches of the centrifugal fibre system in the fish retina. Thus, centrifugal influences can reach bipolar and horizontal cells via dopaminergic interplexiform cells and even the photoreceptors via feedback from cone horizontal cells[7]. It follows that every retinal neuron is potentially susceptible to central influences in the teleost. The physiological significance of this central input to the dopaminergic interplexiform cells is not clear. Present evidence suggests that the interplexiform pathway modulates light responsiveness and receptive field size of horizontal cells in the teleost outer plexiform layer[8–11].

We also show that centrifugal terminals within the retina probably contain two peptides that co-localize in the same large dense-cored vesicles. In these terminals, small electron-lucent vesicles are also observed that do not appear to be immunoreactive. Therefore, these centrifugal fibres may contain an as yet unidentified third neuroactive substance. The possibility of a third neuroactive substance being directly released at synapses onto dopaminergic interplexiform cells, in addition to the two peptides which have already been identified, suggests that the interaction between the centrifugal fibres and dopaminergic interplexiform cells is a complex one.

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1. Springer, A. D. *J. comp. Neurol.* **214**, 404–415 (1983).
2. Munz, H., Claas, B., Stumpf, W. E. & Jennes, L. *Cell Tissue Res.* **222**, 313–323 (1982).
3. Stell, W. K., Walker, S. E., Chohan, K. S. & Ball, A. K. *Proc. natn. Acad. Sci. U.S.A.* **81**, 940–944 (1984).
4. Dowling, J. E. & Ehinger, B. *Science* **188**, 270–273 (1975).
5. Dowling, J. E. & Ehinger, B. *Proc. R. Soc.* **B201**, 7–26 (1978).
6. Eldred, W. D., Zucker, C. L., Karten, H. J. & Yazulla, S. J. *Histochem. Cytochem.* **31**, 285–292 (1982).
7. Baylor, D. A., Fuortes, M. G. F. & O'Bryan, P. M. *J. Physiol., Lond.* **214**, 256–294 (1971).
8. Hedden, W. L. & Dowling, J. E. *Proc. R. Soc. B.* **201**, 27–55 (1978).
9. Teranishi, T., Negishi, K. & Kato, S. *J. Neurosci.* **4**, 1271–1280 (1984).
10. Piccolino, M., Neyton, J. & Gerschenfeld, H. M. *J. Neurosci.* **4**, 2477–2488 (1984).
11. Mangel, S. C. & Dowling, J. E. *Proc. R. Soc.* **B231**, 91–121 (1987).
12. Dowling, J. E. & Cowan, W. M. *Z. Zellforsch. mikrosk. Anat.* **71**, 14–28 (1966).

Non-responsiveness to a foot-and-mouth disease virus peptide overcome by addition of foreign helper T-cell determinants

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Study of the immune response to synthetic antigens has shown that uncoupled peptides can realize their potential as vaccines only if they contain domains that react with helper T-cell receptors and Ia antigens[1,2] in addition to antibody binding sites. Here we consider whether genetically restricted non-responsiveness to an uncoupled peptide could be overcome by synthesizing a peptide with an additional helper T-cell epitope from a different protein. We demonstrate that H-2^d mice, which are non-responders to the 141–160 VP1 peptide of foot-and-mouth disease virus (FMDV), can be converted into responders by immunization with peptides containing the FMDV sequence with defined 'foreign' helper T-cell determinants from ovalbumin or sperm whale myoglobin. Furthermore, the virus-neutralizing activity of the antibody raised against peptide was dependent on the determinant used. Thus, FMDV peptides with the added sequences 323–339 from ovalbumin and 132–148 from sperm-whale myoglobin elicited a high degree of neutralizing activity in B10.D2 mice. The sera from mice which received the peptide with the added sequence 105–121 from sperm whale myoglobin did not neutralize the virus, although they had high levels of anti-141–160 FMDV peptide activity. Our data indicate that the T-cell help given by the 'foreign' epitopes is B-cell clone specific. These results are likely to have important implications for the design of peptide vaccines.

A synthetic peptide comprising the 141–160 region of VP1 from FMDV elicits a neutralizing antibody response that protects guinea pigs against experimental infection[3,4]. The response to this peptide is not carrier-restricted[5,6], and the uncoupled peptide is immunogenic when presented in liposomes[6,7] or in incomplete Freund's adjuvant[7,8], provided that a non-natural cysteine is added to the carboxy terminus. These results led us to conclude[6] that this peptide not only contained an important site(s) that could elicit neutralizing antibodies against the virus (B-cell epitope), but also a site(s) capable of inducing helper T-cell activity[9] for this response (Th-cell epitope). This conclusion is supported by the stimulation of T-cell proliferation by the amino-acid sequence 150–160 of the peptide *in vitro*[7,8]. However, as this peptide can only contain a few Th-cell epitopes problems could arise in obtaining good immunity within an outbred population or in other species[8].

Indeed, the response of potential vaccine target species (cattle and pigs) to this free peptide is not as good as in guinea pigs (M.J.F., unpublished data), although the antibodies elicited by the peptide are qualitatively similar in all three species (E. J. Ouldridge, N. R. Parry & M.J.F., unpublished data). To overcome this genetic restriction of the immune response to the free peptide, we have investigated whether such non-responsiveness in a laboratory model could be modified by using a peptide with additional Th-cell epitopes. Control of FMD requires regular vaccination at intervals of 4–6 months to maintain large amounts of circulating antibody in the susceptible population, since natural B-cell or T-cell memory would be unable to provide protection in the event of severe virus challenge. Therefore, we considered the possibility of using 'foreign' Th-cell epitopes from non-FMDV proteins to modify the response to the FMDV peptide. Recently it has been shown that non-immunogenic B cell epitopes can be made immunogenic by conjugation with a 'natural'[10] or by co-polymerization with a 'foreign'[11] T cell epitope.

It was first necessary to determine whether the response to the 141–160 peptide was restricted in a genetically defined population of inbred mice. A range of congenic B10 mice,

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