Some Mammalian Retinae Lack FMRF-Amide-Like Immunoreactive Efferents

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The retinae of a monkey, cat, rat and mouse were examined for FMRF-amide immunoreactivity, a marker for efferent fibers in the retinae of lower vertebrates. No FMRF-amide immunoreactive retinal fibers were found despite their presence in the rat CNS. Therefore, we conclude that efferent fibers, if present in mammalian retinae, are immunologically different from efferent fibers in lower vertebrates. Invest Ophthalmol Vis Sci 30:791–794, 1989

Centrifugal fibers that innervate the retina exist in lower vertebrates, including amphibians,1–3 fish,4–9 birds10,11 and reptiles12–16 as well as in cyclostomes.16 These fibers (often called retinal efferents) have been demonstrated both physiologically and anatomically; they appear to have a variety of different sources in different species, and often have multiple sources in a given species, especially in fish.7,14 In general, the centrifugal fibers terminate in the inner plexiform layer of the retina near the cell bodies of amacrine cells.8,11,17 The function of these fibers is not known.

The evidence for centrifugal innervation of the retina is much more controversial in mammals. Cajal17 stated that he occasionally saw efferents terminating on amacrine cells in the dog retina.17 Later attempts to demonstrate these fibers have met with varying degrees of success; for a review of earlier work see Itaya.18 Recent anatomical evidence exists both for efferents18–23 and against them.24

In each of the species of frogs, fish and lizards that have been studied, some of the efferents to the retina have an internal antigen that reacts immunologically with antisera prepared against the molluscan cardioexcitatory peptide, FMRF-amide (fish, frog, lizard—Rusoff, unpublished observations). These fibers are designated FMRF-amide immunoreactive (FMRF-ir). The role of this antigenic molecule in the vertebrate retina is not yet clear; however, some cells in the goldfish retina do change their response properties after superfusion with FMRF-amide.24 This result suggests that the antigenic molecule is either FMRF-amide or has a region whose configuration strongly resembles that of FMRF-amide.

FMRF-amide-like peptides are also present in the mammalian central nervous system.29–34 They have been studied extensively in the rat and can be demonstrated particularly in the hypothalamus, brainstem and spinal cord, using either immunocytochemistry29–31 or radioimmunoassay.32 It is unlikely that the antigenic molecule is simply FMRF-amide30–33; isolated mammalian peptides which cross-react with antiserum to FMRF-amide are all larger peptides,33 but their carboxyl terminals all are very similar to FMRF-amide. The carboxyl end appears to be the region that the antiserum recognizes, and only this end is necessary to elicit a response, as FMRF-amide alone iontophoresed onto cells in the rat brainstem is excitatory.34

Since FMRF immunoreactivity has been found in some retinal efferents in each of the lower vertebrates which has been examined and is present in the mammalian central nervous system, we decided to search for efferents in mammalian retinae containing FMRF-amide immunoreactivity.

Materials and Methods

All procedures described herein conform to the ARVO Resolution on the Use of Animals in Research. We examined the retinae of five different species of mammals: monkey, cat, rat and mouse. The monkey was a rhesus macaque, the cat was a domestic short hair, the rat was a Wistar and the mouse was a C57BL/6J. The monkey retina was obtained through the kindness of Dr. John B. A. Kistler of the Department of Psychology, Montana State University. The monkey was anesthetized with sodium pentobarbital (Nembutal) and underwent a standard unilateral enucleation. The retinae were harvested and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2–3 days before use. The other species were obtained from commercial sources and, except for the rat, were housed in the Animal Facility of the Department of Biology, Montana State University. The rats were housed in a light-controlled environment (14L:10D) and given food and water ad libitum. The cats were housed in a light-controlled environment (12L:12D) and fed a commercial chow diet. The monkeys and cats were housed in large enclosures and had the opportunity to exercise. The monkeys were fed a special diet of monkey chow and the cats were fed a commercial cat food. The mice were housed in standard animal cages in a light-controlled environment (12L:12D) and were given food and water ad libitum.

Preparation of Antisera

Three syngeneic rabbits were immunized with 100 µg of FMRF-amide conjugated to keyhole limpet hemocyanin in complete Freund’s adjuvant (CFA) given subcutaneously at three sites on the back of the rabbit. The rabbits were boosted with 50 µg of FMRF-amide conjugated to keyhole limpet hemocyanin in incomplete Freund’s adjuvant (IFA) given subcutaneously at three sites on the back of the rabbit on days 14 and 28. Blood was collected from the ear vein of the rabbits daily for antibody levels to be determined by ELISA. After 35 days, the rabbits were bled and the sera were assayed for antibody levels to FMRF-amide by ELISA. The antisera were affinity-purified on a column of FMRF-amide conjugated to sepharose 4B. The rats were perfused, and the eyes were enucleated. The retinae were fixed in 4% paraformaldehyde in PBS for 2–3 days before use. The retinae were then dissected and mounted on glass slides. The slides were then stored at 4°C until use.

Immunocytochemistry

The slides were incubated in 1% normal goat serum (NGS) in PBS for 1 h at room temperature. The slides were then incubated with the primary antibody at a concentration of 1:100 in 1% NGS in PBS for 3 h at room temperature. The slides were then incubated in biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at a concentration of 1:200 in 1% NGS in PBS for 1 h at room temperature. The slides were then incubated in avidin-biotin-peroxidase complex (Vector Laboratories) at a concentration of 1:100 in 1% NGS in PBS for 1 h at room temperature. The slides were then incubated in 1% 3,3-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris buffer (pH 7.6) for 3 min at room temperature. The slides were then rinsed in distilled water and mounted in Permount (Fisher Scientific).
Fig. 1. (A) Photograph of a section of the retina of an adult rainbow cichlid fish taken midway between the optic disc and the peripheral margin of the retina. The arrow indicates an efferent axon near the amacrine cells in the inner nuclear layer. This axon is immunoreactive for FMRF-amide. Scale line indicates 25 \( \mu \text{m} \). (B) Photograph of a section from the posterior pituitary of a rat. The arrows point to FMRF-ir cells. The arrowhead points to a cell which is not immunoreactive. Scale line indicates 10 \( \mu \text{m} \). (C) Photograph of a section from the dorsal retina of a monkey taken midway between the optic disc and the peripheral margin of the retina. This section received the same treatment as the section shown in (A); however, no immunoreactive fibers are present. Scale line indicates 25 \( \mu \text{m} \). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. (D) Photograph of a section from the retina of the same rat whose pituitary is shown in (B). This section received the same treatment as the section shown in (B); however, no immunoreactive fibers are present. Scale line indicates 25 \( \mu \text{m} \). Abbreviations are as in (C).
mammals: a primate, *Macaca nemestrina*, a domestic cat, a mouse with a pigmented coat and eyes (C57B1/6), an albino mouse (Balb/cByJ), and an albino rat (Holtzman). The primate eye was fixed by perfusion with 4% paraformaldehyde containing lysine and periodate. The rat was perfused with 4% paraformaldehyde in 0.1 M phosphate buffer with 3% sucrose; its retina and brain were then further fixed by immersion in fixative. The other mammalian retinai and retinai from rainbow cichlid fish (*Herotilapia multispinosa*) used as positive controls were fixed by immersion in the same fixative. All tissues were then washed in phosphate buffer and cryo-protected in 30% sucrose in phosphate buffer. They were then immersed in O.C.T. compound (Tissue-tek, Naperville, IL), frozen and sectioned at 10 or 20 μm in a Frigocut cryostat (Reichert, West Germany). The entire retina of each mouse eye and one rat eye was used; the portion of the cat retina centered on the optic disc and fully surrounding it was used; and two portions of the primate retina were used—that tissue peripheral to the fovea and that immediately superior to the fovea, extending from the optic disc to the peripheral margin. The fish retinae and pituitary from the rat brain that were used as tissue controls were sectioned in an identical manner. As each section was cut, it was placed on a gelatin-subbed slide. The slides were stored in the refrigerator for 24 hr or less before being brought up to room temperature to react the tissue. The slides were then bathed in each of the solutions required to reveal FMRF-amide immunoreactivity in the tissue, using the peroxidase-antiperoxidase procedure of Sternberger.35 All washes were phosphate-buffered saline (PBS) plus 0.3% Triton-X 100, unless otherwise stated. Sections were incubated in wash plus 10% DMSO for 30 min, washed, incubated in normal goat serum for 1 hr, and incubated overnight in the antibody to FMRF-amide (Immunonuclear, Stillwater, MN and/or Cambridge Research Biologicals, New York, NY) at room temperature. The antibodies from the two sources were usually mixed, with that from Immunonuclear used at a dilution of 1:1000 and that from Cambridge used at 1:5000. This mixture yielded optimal staining versus background on the fish retinae run with each mammalian retina as positive controls. Negative control slides were incubated with normal rabbit serum at a dilution of 1:100. Slides were then washed, incubated with goat anti-rabbit serum for 1 hr, washed, incubated with peroxidase-anti-peroxidase complex (DAKO, Carpenteria, CA) for 1 hr, washed, washed in 0.1 M phosphate buffer, and reacted to reveal the peroxidase using diaminobenzidine as the chromogen and glucose-glucose oxidase as the hydrogen peroxide source.36 The tissue was counterstained with cresyl violet, dehydrated, cleared with xylene and coverslipped with permount.

**Results**

All fish retinae run as positive controls contained FMRF-amide immunoreactive fibers crossing the inner plexiform layer and terminating near the amacrine cell bodies in the inner nuclear layer (Fig. 1A). As reported by others,30 the posterior, but not the anterior, pituitary of the rat brain contained numerous FMRF-ir cells and fibers. Figure 1B shows two cells in the posterior pituitary densely filled with the reaction product (arrows). A neighboring cell that is not immunoreactive is indicated by the arrowhead for comparison. However, over 100 sections from each mammalian retina were examined and none of them contained any FMRF-ir fibers. Figure 1C and D show representative sections from the primate and rat retinai, respectively, in which no immunoreactive fibers are visible. Portions of the optic nerve head were included in some of the sections from the retinae of the mice and the cat and no immunoreactive fibers were seen in these optic nerves either.

**Discussion**

We conclude that centrifugal fibers, if present in the primate, cat, rat or mouse retinae, do not contain detectable levels of FMRF-amide immunoreactivity. If there are FMRF-amide-like peptides in these retinae, they are present either in extremely small amounts or have different cross-reactivities from the FMRF-amide-like peptides found both in the retinae of cold-blooded vertebrates and in the mammalian CNS. These results do not show that there are no centrifugal fibers in these retinae. However, our ability to easily demonstrate FMRF-ir cells and fibers in rat CNS and FMRF-ir efferents in the retinae of lower vertebrates but not in these mammalian retinae strongly suggests that any efferent fibers present in the mammalian retina differ in their internal chemicals from one major group of retinal efferents present in most cold-blooded vertebrates.

**Key words:** mammals, retina, efferents, FMRF-amide immunoreactivity

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