Substance P-, F8Famide-, and A18Famide-like immunoreactivity in the nervus terminalis and retina of goldfish, Carassius auratus

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Substance P-, F8Famide-, and A18Famide-like immunoreactivity in the nervus terminalis and retina of the goldfish *Carassius auratus*

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Abstract. We re-investigated the occurrence of substance P-like immunoreactivity in the retina of the goldfish *Carassius auratus* using antisera to substance P and other tachykinins. Most antisera labelled a previously described single class of mono-stratified amacrine cells arborizing in layer 3 of the inner plexiform layer. Preabsorption experiments showed that these amacrine cells contained at least one tachykinin-like peptide. One antiserum (INC 353) to substance P labelled not only these amacrine cells but also fibres in layer 1 of the inner plexiform layer and fibres in the optic nerve. These fibres were identified as retinopetal projections of the nervus terminalis, in part because of colocalized labelling with antisera against gonadotropin-releasing hormone and FMRFamide. Preabsorption experiments showed that the substance P-immunoreactive material in the nervus terminalis was not substance P or any other typical tachykinin. Labelling of the nervus terminalis with INC 353 was blocked by preabsorption with two bovine FMRFamide-like peptides, F8Famide and A18Famide, which contain a substance P(4–7)-like region. Antisera to F8Famide and A18Famide strongly labelled ganglia of the nervus terminalis and retinopetal fibres. We suggest that labelling of the nervus terminalis by antisera to substance P and FMRFamide occurs because of homologies between these antigens and a non-tachykinin, endogenous peptide that is similar to F8Famide and A18Famide.

Key words: Amacrine cell – F8Famide – FMRFamide-like immunoreactivity – Nervus terminalis – Neuropeptide FF – Retina – Substance P – Goldfish (Teleostei)

Introduction

The retina of teleost fish contains a wide variety of neuroactive peptides that are contained in amacrine cells and occasionally other neurons (reviewed by Stell 1985; Marshak 1992). In the goldfish, *Carassius auratus,* immunoreactivity to substance P (SP) is localized mainly to a single class of amacrine cells with neurites ramifying in the middle (layer 3) of the inner plexiform layer (IPL) (Brecha et al. 1981; Yazulla et al. 1985; Li et al. 1986). However, several lines of evidence suggest the presence of multiple tachykinin pathways or mechanisms within the cyprinid retina: 1) differential actions of exogenous substance P upon "on" and "off" ganglion cells (Glickman et al. 1982; Djamgoz et al. 1983) were not readily explained by known connections of the SP-immunoreactive amacrine cells (Yazulla et al. 1985); 2) Li et al. (1986) reported a novel second class of SP-immunoreactive amacrine cells with neurites in IPL layer 1 of goldfish; and 3) analyses of retinal extracts from carp (Eskay et al. 1981) and goldfish (Marshak et al. 1987) indicated the existence of two or more SP-immunoreactive substances. Although none of the SP-immunoreactive substances appeared to be identical to substance P, further attempts to characterize the SP-immunoreactive peptides in cyprinid retina have not been reported. As only SP-directed antisera had been used in previous work, we attempted to extend the analysis of multiple tachykinins by utilizing antisera to the other mammalian tachykinins (neurokinins A and B) and to two non-mammalian tachykinins (kassinin and physalaemin), as well as antisera to substance P. We discovered, surprisingly, that one SP antiserum labelled the retinopetal fibres of the goldfish nervus terminalis; this result was reported in an abstract (Stell et al. 1985).

The vertebrate nervus terminalis (NT), or cranial nerve 0, is comprised of bilateral ganglia and processes closely associated with the olfactory nerves and bulbs. In the goldfish, each NT ganglion is embedded within an olfactory nerve, and its processes extend anteriorly into the olfactory lamellae and epithelium, laterally into the olfactory bulb, and caudally through the olfactory tract

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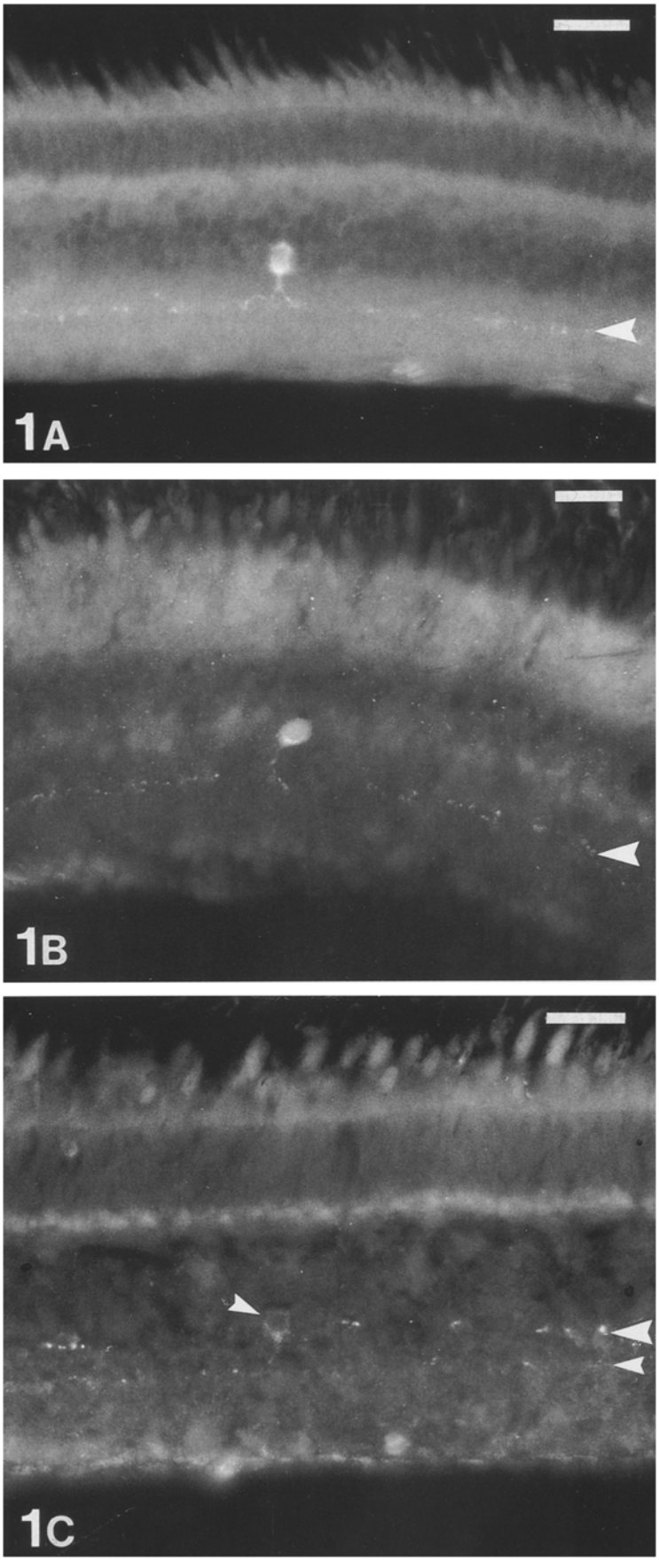

Fig. 1A–C. Vertical sections of goldfish retina treated with various tachykinin antisera. **A** Anti-substance P (SK-SP1) shows amacrine cells and neurites in layer 3 *(arrowhead)* of the inner plexiform layer. **B** Anti-kassinin (H2.3.84) shows a similar type of amacrine cell and neurites in layer 3 *(arrowhead).* **C** Anti-substance P (INC 353) shows fibres in layer 1 *(large arrowhead)* of the inner plexiform layer in addition to amacrine cells and neurites in layer 3 *(small arrowheads).* ×450 (**A, C**), ×400 (**B**). *Bars:* 25 μm

Table 1. Amacrine cell immunoreactivity following preabsorption of various tachykinin antisera with 10^{-4} or 10^{-6} M peptide (0, labelling completely blocked; 1, trace labelling; 2, labelling reduced; 3, labelling not different from control; NI, not investigated)

Antiserum		Preabsorbing peptide				
Antigen	Lot (Source)	Substance P	Physalaemin	Kassinin	Eledoisin	Bombesin
Substance P	R5 (Keen)	0	0	0	0	1
	SK-SP1 (Eskay)	0	0	0	0	1
	INC 353 (Incstar)	0	0	0	0	1
Neurokinin A	119-6 (Minamino)	1	1	0	0	2
Neurokinin B	122-6 (Minamino)	2	2	0	0	3
Kassinin	H2.3.84 (Shults)	3	3	0	0	NI

to innervate the ventral telencephalon, preoptic area, optic tectum, retina, and more posterior targets (Demski and Northcutt 1983; Springer 1983; von Bartheld and Meyer 1986; Kyle and Stell 1988). The goldfish nervus terminalis contains gonadotropin-releasing hormone (GnRH)-like immunoreactivity and is distinguished from other GnRH-immunoreactive cell populations in the brain by colocalization with substances that bind antibodies to the molluscan cardioactive peptide Phe-Met-Arg-Phe-amide (FMRFamide) (Stell et al. 1984; Kyle et al. 1985). Electron-microscopic studies of immunocytochemically labelled NT fibres have shown that the nervus terminalis contacts a variety of cells in the teleost retina, mostly in IPL layer 1, with an estimated 840 000 output synapses per retina in young goldfish (Kawamata et al. 1990). Target cells include dopaminergic interplexiform cells (Zucker and Dowling 1987; Ball et al. 1989); "off" amacrine cells (Ohtsuka et al. 1989); and glycine- and gamma aminobutyric acid-accumulating amacrine cells, bipolar cells, and photoreceptor cells (Ball et al. 1989). Although the application of GnRH and FMRFamide alters activity in retinal ganglion cells (Walker and Stell 1986) and horizontal cells (Umino and Dowling 1991), the role of the nervus terminalis in visual function is still obscure (Davis et al. 1988; Weiss and Meyer 1988; Owusu-Yaw et al. 1991). As SP immunoreactivity had not been reported at that time in the nervus terminalis of any vertebrate, we undertook an immunocytochemical characterization of the SP-like material in the goldfish nervus terminalis; preliminary conclusions have been reported briefly in two reviews (Stell 1985; Stell et al. 1987).

FMRFamide and a large family of closely related peptides have been identified in invertebrate nervous systems. In mammals, FMRFamide-like immunoreactivity has been demonstrated in the central nervous system of many species, and FMRFamide was found to exert diverse actions (reviewed by Raffa 1988). However, FMRFamide itself has not been identified in vertebrate tissues. Three related peptides, all containing an Arg-Phe-amide (RFamide) C-terminal dipeptide, have been identified in vertebrate brains: Leu-Pro-Leu-Arg-Phe-amide (LPLRFamide) from the chicken brain (Dockray et al. 1983) and Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-amide (morphine-modulating peptide, F8Famide, neuropeptide FF) and Ala-Gly-Glu-Gly-Leu-Ser-Ser-Pro-Phe-Trp-Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-amide (A18Famide, neuropeptide AF) from the bovine brain (Yang et al. 1985). We attempted to further characterize the FMRFamide-like material in the goldfish nervus terminalis by immunocytochemistry. We report here the presence of F8Famide- and A18Famide-like immunoreactivity in the goldfish nervus terminalis and suggest that both the FMRFamide-immunoreactivity and the SP-like immunoreactivity in the goldfish nervus terminalis can be explained by cross-reactivity of the antisera with one or more endogenous peptides similar to F8Famide and A18Famide.

Materials and methods

Animals. Goldfish *(Carassius auratus),* ranging from immature "feeders" to mature, breeding adults (3–10 cm standard length), were obtained from Aquatic Imports (Calgary, AB, Canada), Ozark Fisheries (Stoutland, Mo., USA), or local pet shops. Animals were kept in aerated water at about 20° C under fluorescent room lights (12 h light:12 h dark). No differences in results were noted as a result of age, sex, or reproductive state.

Fixation and sectioning. At least 2 h into the light phase, animals were anaesthetized in 0.1% tricaine methanesulfonate (MS222; Sigma Chemical Company, St. Louis, Mo., USA). Eyes were quickly removed and hemisected at the equator. The eye cups were fixed in Zamboni's fixative or 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight (about 16–18 h) at 4° C. When brain or olfactory bulbs were required, the dorsal cranium was removed, and the entire brain was fixed in situ for several hours. The brain and olfactory bulbs then were removed and placed in fresh fixative overnight. Tissues were transferred through several washes of cold 0.1 M phosphate buffer (pH 7.4) containing 3% sucrose and stored until use at 4° C in phosphate buffer containing 30% sucrose and 0.1% sodium azide. In later experiments, the washing steps in 3% sucrose-phosphate buffer were omitted. Vertical 15-µm-thick sections from eye cups were cut from the central retina near the optic nerve head and collected sequentially across a series of 2–6 gelatin-coated slides. Cryostat sections of brain were cut at thicknesses of 25–30 µm in either the horizontal or the frontal plane and collected on alternate slides. Slides were frozen until needed. For all experimental conditions described below, at least 20 sections from each of three fish were examined.

Immunocytochemistry. For all experiments, the indirect immunofluorescence procedure was used. Frozen slides were warmed to room temperature, and the sections were surrounded by a ring of rubber cement, which served to confine subsequently applied reagents over the sections. This rubber cement ring was removed before coverslipping. Slides were washed for 10 min each in two

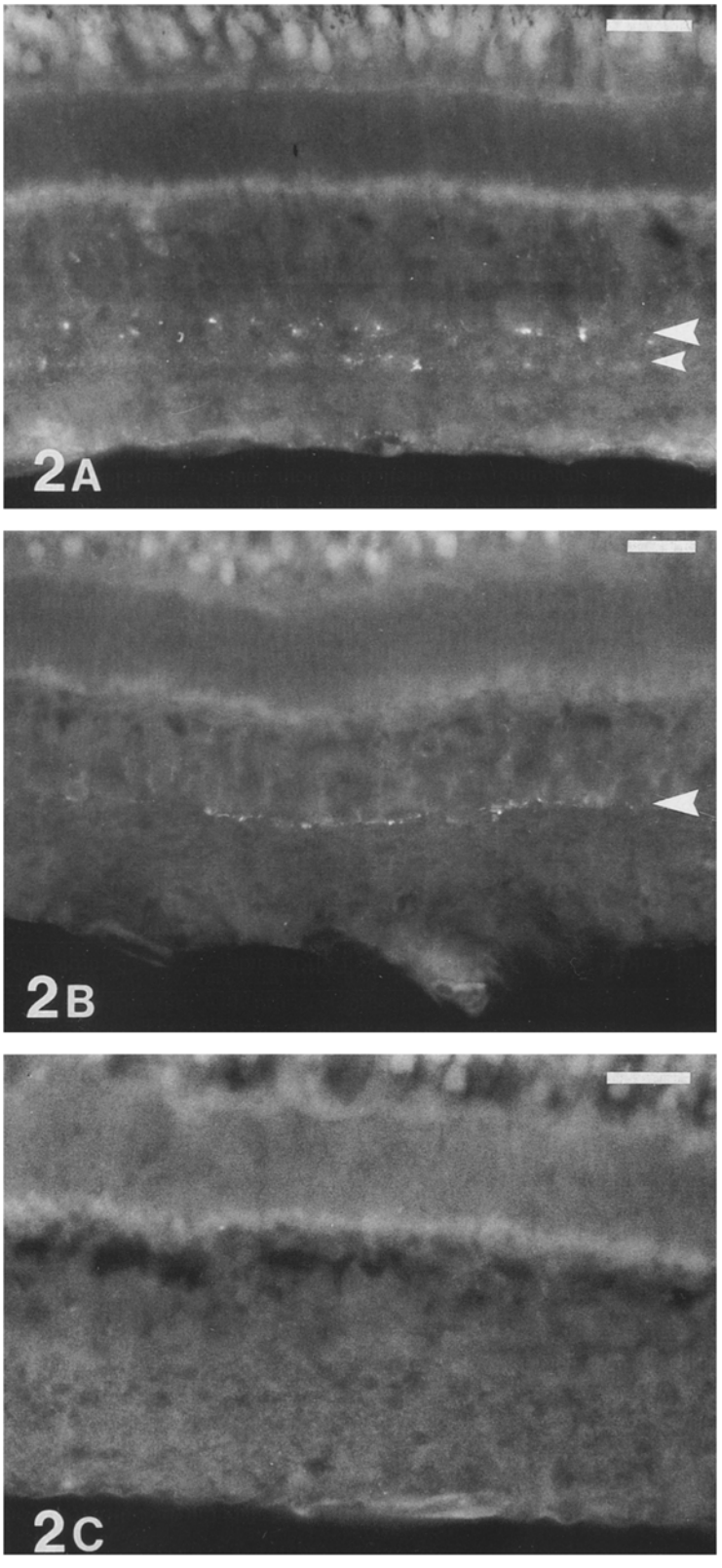

Fig. 2A–C. Vertical sections of goldfish retina after preabsorption of the substance P (INC 353) antiserum with fragments of substance P. **A** Control preabsorption (no peptide) shows fibres in both layer 1 *(large arrowhead)* and layer 3 *(small arrowhead)* of the inner plexiform layer. **B** Preabsorption with SP(7–11) shows fibres only in layer 1 *(arrowhead)*. **C** Preabsorption with SP(3–11) shows no immunoreactivity. ×450 (**A, C**), ×370 (**B**). *Bars:* 25 µm

changes of 0.05 M phosphate-buffered saline (PBS; pH 7.4). Sections were incubated in an air-tight, humidified chamber for 30 min with 1–3% normal goat serum diluted in PBS containing 0.3% Triton X-100, rinsed in PBS, and then incubated overnight at room temperature with primary antiserum diluted in PBS and Triton X-100. The following rabbit polyclonal primary antisera were used: 1) anti-substance P: INC 353, lots 8336022 and 8352022 (Incstar Corporation, Stillwater, Minn., USA), R5 (P. Keen), SK-SP1 (R. Eskay), SP77 and R140 (P. C. Emson), RRF12/10 (M. Reinecke), T531 (IAZ, Munich, Germany), 3-18-5 (G. P. Kozlowski), and SP-5 (L. H. Lazarus), as well as the monoclonal NC1/34HL (Pel-Freez Biologicals, Rogers, Ariz., USA); 2)

Table 2. Characterization of the substance P (INC 353) antiserum: immunoreactivity in the goldfish retina following preabsorption with 10^{-4} or 10^{-6} M peptide (0, labelling completely blocked; 1, trace labelling; 2, labelling reduced; 3, labelling not different from control; /, results at 10^{-6} M/10^{-4} M)

	Tachykinin and bombesin recognition controls				
	Substance P	Physalaemin	Kassinin	Eledoisin	Bombesin
Amacrine cells	0	0	0	0	1
Nervus terminalis fibres	0	3	3	3	3

	Substance P fragment specificity					
	SP(1–4)	SP(1–7)	SP(1–9)	SP(3–11)	SP(5–11)	SP(7–11)
Amacrine cells	3	3/0	2/0	0	0	0
Nervus terminalis fibres	3	0	0	0	2/0	3

	Cross-recognition controls				
	Substance P	Mammalian GnRH	Salmon GnRH	FMRFamide	Neurotensin
Amacrine cells	0	3	3	3	3
Nervus terminalis fibres	0	3	3	3	3

anti-neurokinin A: 119-6 ("anti-neuromedin L"; N. Minamino); 3) anti-neurokinin B: 122-6 ("anti-neuromedin K"; N. Minamino); 4) anti-kassinin: H2.3.84 (C. Shults); 5) anti-physalaemin: PS-1 (L. H. Lazarus); 6) anti-neurotensin: RRF6/7 and RRF5/7 (M. Reinecke), lot 26151 (Incstar Corporation), and N-2-11 (M. R. Brown); 7) anti-GnRH: GF4 and GF5 (N. M. Sherwood) and RIP4 (T. H. Magnus and W. K. Stell); 8) anti-gonadotropin-releasing hormone associated peptide (human): MC-1 (M. Culler) and 143, 913, and 1407 (R. P. Millar); 9) anti-FMRFamide: 231 and 232 (T. O'Donahue); 10) anti-F8Famide: #1 and #2 (H.-Y. Yang); 11) anti-A18Famide: #2 (H.-Y. Yang); 12) anti-avian pancreatic polypeptide: "Lance" (G. Pollack); 13) anti-peptide YY (porcine): 69D (L. Terenius); 14) anti-neuropeptide Y: 102B (L. Terenius); 15) anti-met-enkephalin: 7341 (J. Walsh); and 16) anti-leu-enkephalin: lot 47169 (Incstar Corporation). Optimal dilutions for fluorescence microscopy were determined to be 1:200–500 for most antisera. Exceptions were the NC1/3HL antiserum to substance P (used at 1:1000) and antisera to all RFamides (used at 1:2000). After two 10-min washes in PBS, sections were incubated for 60 min with goat anti-rabbit (or anti-rat for NC1/34HL) IgG conjugated to either fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) (Sigma Chemical Company or Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) diluted 1:20–50 in PBS and Triton X-100. After two 10-min washes in PBS, slides were coverslipped in 3:1 glycerine:water containing 4% n-propyl gallate, viewed with an epi-illumination fluorescence microscope fitted with the appropriate filters, and photographed.

Preabsorption experiments. The specificity of antisera for known peptides was assessed by adding the appropriate synthetic peptide at a final concentration of 10^{-4} to 10^{-8} M or an equivalent volume of PBS (control) to the diluted antiserum and allowing binding to occur overnight at 4° C. All peptides were obtained from Peninsula Laboratories (Belmont, Calif., USA), except for F8Famide and A18Famide which were gifts from Dr. H.-Y. Yang. The preincubated antisera were then used for immunocytochemical labelling as described above. The labelling intensities were ranked independently by at least two observers, and the ranks were compared to those of the controls in adjacent sections from the same eye.

Double-labelling experiments. To test for colocalization of peptides within the nervus terminalis, we performed modified double reciprocal labelling experiments, which used primary antisera raised in the same species. Sections of olfactory bulbs and nerves (containing the NT ganglia) or retina were incubated first in one primary antibody followed by FITC-conjugated goat anti-rabbit IgG and then in the other primary antibody followed by TRITC-conjugated goat anti-rabbit IgG; the primary antisera were applied in the reverse order in a parallel set of slides from the same section series. With this procedure, the reagents of the second labelling cycle were expected to interact with the first primary-secondary antibody complex. Non-colocalization of antigens would be indicated if cellular structures were labelled by the second primary antiserum but not the first. Colocalization of antigens would be suggested if all structures were labelled by both antisera, regardless of which antiserum was applied first. Controls included replacing one or the other of the primary antisera with saline and viewing each slide through the filter set designed for the other fluorophore.

Optic nerve-crush experiments. To distinguish retinopetal fibres from neuronal systems intrinsic to the retina, one optic nerve was crushed intraorbitally with fine forceps in six fish. Three weeks later, when GnRH-immunoreactive and FMRFamide-immunoreactive NT fibres were no longer detectable in the optic nerve-crushed retina (Muske et al. 1987; Owusu-Yaw 1990), sections from the nerve-crushed and intact retinas were processed for fluorescence immunocytochemistry.

Results

Substance P-like immunoreactivity

Most antisera to substance P (Fig. 1A) and those to neurokinins A and B and kassinin (Fig. 1B) labelled small, sparsely distributed cells in the inner nuclear layer (typically 2–5 cells per section). These putative amacrine cells usually had a single primary neurite and a fine arborization within IPL layer 3. Weak or no labelling was obtained with the SP antisera 3-18-5, SP-5, and R140 and the physalaemin antiserum PS-1. When selected antisera (anti-SP R5, SK-SP1, and INC 353, anti-neuroki-

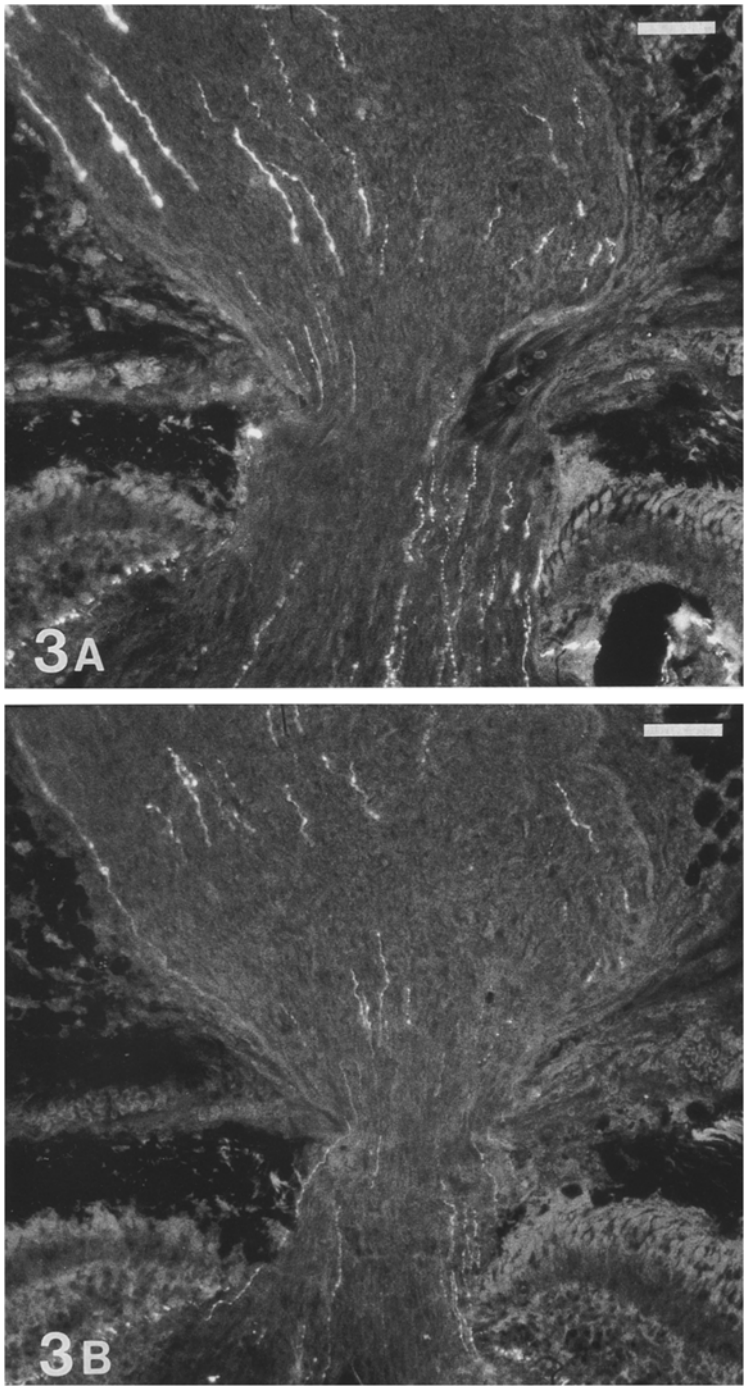

Fig. 3A, B. Adjacent vertical sections of goldfish optic nerve head showing efferent fibres of the nervus terminalis labelled with antiserum to F8Famide (**A**) or antiserum to gonadotropin-releasing hormone (**B**). ×200. *Bars:* 50 μm

nins A and B, and anti-kassinin) were preabsorbed with various tachykinin peptides, only kassinin and eledoisin completely blocked labelling by all antisera tested, while substance P and physalaemin completely blocked labelling by the three SP antisera (Table 1). Double-labelling experiments with substance P and neurotensin antisera failed to label any of the small amacrine cells arborizing in IPL layer 3 with one antiserum alone, regardless of which antiserum was applied first, indicating complete colocalization of SP- and neurotensin-like immunoreactivities in this population of amacrine cells.

A single antiserum to substance P, INC 353, regularly labelled not only the amacrine cells just described, but also fibres in IPL layer 1, the optic fibre layer, and the optic nerve (Figs. 1C, 5). The pattern of labelling in layer 1 was identical to that observed with antisera to GnRH and FMRFamide, which has been attributed to the retinopetal fibres of the nervus terminalis (Stell et al. 1984). We identified these SP (INC 353)-immunoreactive fibres in layer 1 as efferent fibres of the nervus terminalis by: 1) optic nerve-crush experiments, in which SP (INC 353)-immunoreactive fibres from layer 1 and

the optic nerve disappeared 2–3 weeks after surgery (labelling of the amacrine cells and fibres in layer 3 persisted, however, indicating that the SP immunoreactivity of the latter structures was intrinsic to the retina); 2) double-labelling studies, which failed to reveal any SP (INC 353)-immunoreactive fibres in IPL layer 1 or the optic nerve that were not also GnRH- or FMRFamide-immunoreactive; and 3) immunocytochemistry of consecutive sections of the olfactory nerves, which showed that the SP (INC 353) antiserum and antisera to GnRH and FMRFamide labelled the same cell population, namely the ganglion cells of the nervus terminalis.

Preabsorption experiments using the SP (INC 353) antiserum (Table 2, Fig. 2) showed that labelling of amacrine cells and layer-3 fibres was blocked completely by preabsorption with substance P, other tachykinins (such as physalaemin, eledoisin, and kassinin), or SP C-terminal fragments. On the other hand, labelling of the NT layer-1 fibres was blocked only by complete substance P or its fragments containing the mid-region amino acids 3–7. Heterologous preabsorptions showed that the SP (INC 353) antiserum did not recognize mammalian or salmon GnRH, FMRFamide, or neurotensin (Table 2).

F8Famide- and A18Famide-like immunoreactivity

Two antisera to F8Famide (Yang #1 and Yang #2) and one to A18Famide (Yang #2) robustly labelled fibres in IPL layer 1, the optic nerve layer, and the optic nerve (Fig. 3A); identical labelling was obtained with all three antisera. These fibres were identified as those from the nervus terminalis, because optic nerve crush abolished all labelling in retinas examined three weeks after surgery. As well, immunocytochemistry of adjacent sections showed that the F8Famide and A18Famide antisera labelled fibres within the optic nerve (Fig. 3) and cells within the olfactory nerve (i.e., the NT ganglion; Fig. 4) that were similar to those labelled with the SP (INC 353) antiserum and antisera to GnRH and FMRFamide.

To determine whether labelling with F8Famide occurs in other non-NT GnRH-immunoreactive cell populations in the goldfish brain, consecutive horizontal brain sections were incubated with anti-F8Famide (Yang #2) or anti-GnRH (GF5). As previously described (Kyle et al. 1985; Kah et al. 1986), GnRH-immunoreactive cell bodies were seen in 1) the NT ganglia embedded within the olfactory nerves; 2) a loose array of cells extending through the anterior telencephalon, lateral preoptic area (majority of cells), and hypothalamus; and 3) large, weakly labelling cells in the midbrain tegmentum. In contrast, F8Famide immunoreactivity was seen in the NT ganglia, but not in the preoptic or midbrain population of GnRH-immunoreactive cells.

Preabsorption experiments (Table 3) showed that NT labelling by the F8Famide and A18Famide antisera was blocked by both F8Famide and A18Famide, but not by FMRFamide or SP(1–7). Interestingly, NT labelling by both the FMRFamide and the SP (INC 353) antisera also was blocked by both F8Famide and A18Famide at preabsorbing concentrations of 10^{-5} M or less, as well as by the peptides against which the antisera were raised. Double-labelling experiments with the SP (INC 353) and F8Famide antisera failed to reveal any fibres in IPL layer 1 or the optic nerve that labelled with only one of these antisera, regardless of which antiserum was applied first.

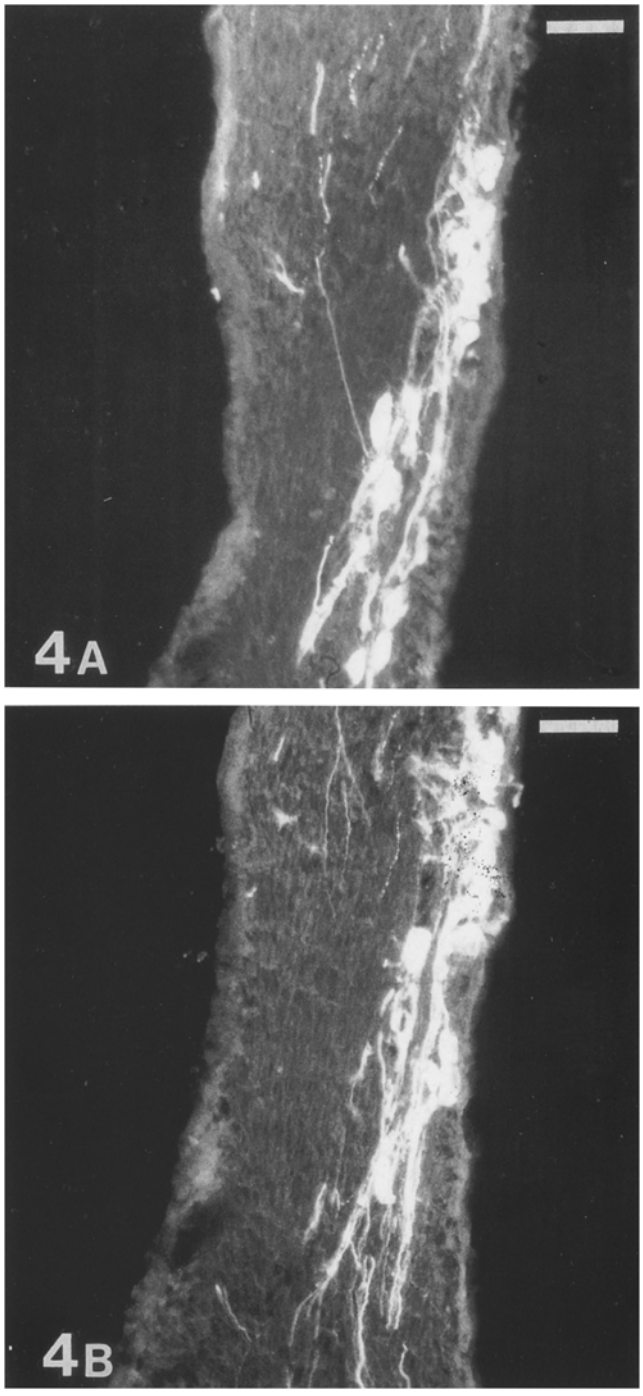

Fig. 4A, B. Adjacent horizontal sections through the goldfish olfactory nerve showing ganglion of the nervus terminalis labelled with antiserum to F8Famide (**A**) or antiserum to gonadotropin-releasing hormone (**B**). ×200. *Bars:* 50 μm

Table 3. Lowest concentration (from 10^{-8} to 10^{-4} M) at which a preabsorbing peptide completely blocked labelling of nervus terminalis fibres in the goldfish retina by various antisera (*, labelling not blocked completely by any peptide concentration used)

Antiserum		Preabsorbing peptide			
Antigen	Lot (Source)	F8Famide	A18Famide	FMRFamide	SP(1–7)
F8Famide	#1 (Yang)	10^{-5}	10^{-4}	*	*
	#2 (Yang)	10^{-5}	10^{-6}	*	*
A18Famide	#2 (Yang)	10^{-5}	10^{-6}	*	*
FMRFamide	231/232 (O'Donahue)	10^{-5}	10^{-5}	10^{-5}	*
Substance P	INC 353 (Incstar)	10^{-5}	10^{-6}	*	10^{-8}

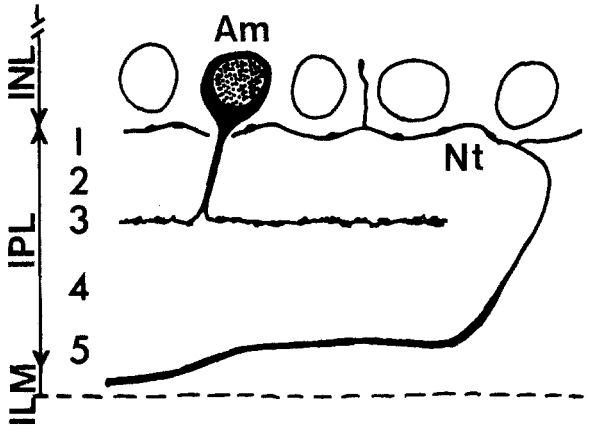

Fig. 5. Diagram of retinal structures labelled by the antisera used in this study. Amacrine cells (*Am*) with fibres in layer *3* of the inner plexiform layer (*IPL*) were labelled with antisera to neurotensin and a variety of tachykinins. Efferent fibres of the nervus terminalis (*Nt*) were labelled with antisera to gonadotropin-releasing hormone, FMRFamide, F8Famide, and A18Famide, as well as the INC 353 antiserum to substance P. *ILM* Inner limiting membrane; *INL* inner nuclear layer

Sections of goldfish retina also were incubated with antisera against peptides structurally similar to F8Famide and A18Famide. No labelling of NT retinopetal fibres was found with antisera to avian pancreatic polypeptide, peptide YY, neuropeptide Y, met-enkephalin, leu-enkephalin, or gonadotropin-releasing hormone associated peptide.

Fig. 5 summarizes the goldfish retinal staining patterns obtained with antisera used in this study.

Discussion

Amacrine cells

We confirmed the presence of SP-like immunoreactivity in a single type of small amacrine cell with fine neurites arborizing in IPL layer 3, corresponding to the single type described previously in goldfish (Brecha et al. 1981; Yazulla et al. 1985). We also confirmed the finding of Li et al. (1986) that neurotensin-like immunoreactivity is colocalized in this cell population (called "NT-1/SP-1" type in their study); however, we obtained no evidence with any substance P or tachykinin antisera for their "SP-2" type of amacrine cell that was immunoreactive for substance P alone and had arborizations in IPL layer 1. Li et al. (1986) suggested that the "SP-2" type contained an immunologically distinct form of substance P recognized only by the polyclonal RAG-3 antiserum used in their study and not by other SP antisera used in their own or previous work (Brecha et al. 1981; Yazulla et al. 1985; Yazulla and Studholme 1990). If the failure to label the "SP-2" cells with the relatively unspecific tachykinin antisera used in our study was not due to methodological differences, then in suggests that the "SP-2" cells contain an epitope recognized specifically by RAG-3, but do not contain substance P or any other typical tachykinin.

Tachykinins are characterized by a common C-terminal amino acid sequence of -Phe-X-Gly-Leu-Met-NH_2, where X is either a branched aliphatic amino acid (as in eledoisin, kassinin, and neurokinins A and B) or an aromatic amino acid (as in substance P and physalaemin). Preabsorption experiments showed that only kassinin and eledoisin blocked labelling of amacrine cells by all of the antisera used. Although the exact identity of an antigen can never be determined solely on the basis of immunocytochemical techniques, these results suggest that the immunoreactive material in the amacrine cells might be the aliphatic type of tachykinin. Such a tachykinin, "carassin", has been isolated from the goldfish brain. It shares 57% homology with the mammalian tachykinin neuropeptide γ, an extended form of neurokinin A (Conlon et al. 1991). Labelling of amacrine cells by the SP antisera used in our preabsorption studies was also blocked by substance P and physalaemin, as well as by kassinin and eledoisin. Hence, we cannot rule out the presence of a second amacrine cell tachykinin of the aromatic type. Multiple peaks of SP-immunoreactive material have been found after high performance liquid chromatography of carp and goldfish retinas, although the identity of these peaks remains to be established (Eskay et al. 1981; Marshak et al. 1987). If multiple tachykinins are present in goldfish amacrine cells, then they are likely to be colocalized, as the structure and distribution of cells labelled with antisera to neurokinins A and B and kassinin are apparently identical to those labelled with antisera to substance P. This might indicate a close affinity with mammals, in which the two preprotachykinins

that contain neurokinin A also contain substance P (reviewed by Escher and Regoli 1989).

Nervus terminalis

A single SP antiserum, INC 353, labelled not only the amacrine cells just described, but also the GnRH- and FMRFamide-immunoreactive NT retinopetal fibres in IPL layer 1, the optic nerve, and the NT ganglia in the olfactory nerves. Preabsorption studies utilizing this antiserum revealed two antibody subpopulations with different specificities. One subpopulation labelled amacrine cells and their fibres in IPL layer 3 and recognized all or part of the highly conserved tachykinin C-terminal pentapeptide, as shown by blocking with several tachykinins and the C-terminal part of substance P. The other antibody subpopulation labelled the nervus terminalis; it did not recognize the C-terminal part of substance P or other tachykinins but was specific for the internal sequence SP(3–7). Clearly, this SP-like material in the nervus terminalis is not substance P or any other typical tachykinin.

Where it has been examined, SP(3–7) immunoreactivity consistently overlaps FMRFamide immunoreactivity in 1) NT neurons of goldfish (this study); 2) the FMRFamide-immunoreactive subpopulation of NT cells in the dogfish *Squalus acanthias* (W. K. Stell, unpublished observations); and 3) retinopetal fibres in the amphibians *Rana catesbeiana* and *Xenopus laevis* (Uchiyama et al. 1988). This led us to speculate that SP(3–7) and FMRFamide immunoreactivity was due to a common molecule, perhaps a lower vertebrate form of FMRFamide (Stell et al. 1987). FMRFamide itself has not been identified in any vertebrate, but related molecules with an RFamide C-terminal dipeptide have been sequenced from chicken (Dockray et al. 1983) and bovine (Yang et al. 1985) brains. In goldfish retinal extracts, neither FMRFamide (Stell et al. 1987) nor chicken LPLRFamide (Muske et al. 1987) has been identified. However, our study found that antisera to the two bovine RFamides (F8Famide and A18Famide) robustly labelled the retinopetal fibres and ganglion cells of the nervus terminalis and that this immunoreactivity was colocalized with SP(3–7) -immunoreactivity.

Prompted by the sequence similarity of SP(4–7) (*Pro-Gln*-Gln-*Phe*) and the common C-terminal tetrapeptide of F8Famide and A18Famide (*Pro-Gln*-Arg-*Phe*-amide), we conducted cross-preabsorption studies and found that the NT-labelling antibodies in the SP (INC 353) and FMRFamide antisera recognized both F8Famide and A18Famide. The F8Famide and A18Famide antisera also recognized both bovine RFamides, but not FMRFamide or SP(1–7). The most parsimonious explanation of these results is that labelling of the goldfish nervus terminalis by antisera to F8Famide, A18Famide, and FMRFamide and by the SP (INC 353) antiserum is due to recognition of a single peptide class that contains an amino acid sequence similar to the common C-terminal tetrapeptide of the bovine RFamides.

Labelling of the nervus terminalis with the F8Famide, A18Famide, or FMRFamide antiserum is unlikely to be due to the presence of structurally related pancreatic polypeptide- or enkephalin-like material, as 1) the cell bodies of the goldfish nervus terminalis were only weakly reactive with an anti-bovine pancreatic polypeptide, which is known to cross-react with FMRFamide-like peptides (Muske et al. 1987), and did not label at all with antisera to avian pancreatic polypeptide, peptide YY, neuropeptide Y, met-enkephalin, or leu-enkephalin (this study); 2) the FMRFamide antiserum used here lacked affinity for the pancreatic polypeptide-like antigen present in a separate population of goldfish amacrine cells (Muske et al. 1987); and 3) labelling of rat brain cells with the F8Famide and A18Famide antisera used here was unaffected by preabsorption with met-enkephalin, met-enkephalin-Arg-Phe, peptide YY, or neuropeptide Y (Panula et al. 1987).

Human and rat GnRHs are synthesized as part of a larger precursor, which is extended C-terminally by a GnRH associated peptide, or GAP (Seeburg and Adelman 1984; Adelman et al. 1986). There is a sequence similarity between human and rat GAP(22–25) (Thr-*Gln-Arg-Phe* and *Pro-Gln*-Asn-*Phe,* respectively) and the bovine (and presumably goldfish) RFamide C-terminal tetrapeptide Pro-Gln-Arg-Phe-amide. Although the sequence of a corresponding GAP in goldfish remains to be established, it is unlikely that the F8Famide, A18Famide, or FMRFamide antiserum used here is cross-reacting with a GAP-like molecule in goldfish as 1) the GnRH (salmon form) precursors of the cichlid *Haplochromis burtoni* (Bond et al. 1991) and the Atlantic salmon *Salmo salar* (Klungland et al. 1992) have GAP sequences that differ substantially from their mammalian counterparts and contain no similarities to the bovine RFamide tetrapeptide; 2) FMRFamide-immunoreactive (Kyle et al. 1985) and F8Famide/A18Famide-immunoreactive (this study) substances are colocalized only in the GnRH-immunoreactive neurons of the nervus terminalis, but not in other GnRH-immunoreactive cell populations of the goldfish brain; and 3) antisera that recognize human GAP do not label the goldfish nervus terminalis (this study).

FMRFamide-like immunoreactivity has been reported in the NT or retinopetal fibres of fishes and amphibians in diverse taxonomic families and in a bird, suggesting that RFamide-like peptides are widespread in these systems in the lower vertebrates. Species investigated include (classification after Nelson 1984): cloudy dogfish (Scyliorhinidae; Chiba et al. 1991), spiny dogfish (Squalidae; Stell 1984), goldfish (Cyprinidae; Stell et al. 1984; Kyle et al. 1985; Muske et al. 1987; Bonn and König 1989b; this study), walking catfish (Clariidae; Rama Krishna and Subhedar 1992), a knifefish (Sternopygidae; Bonn and König 1989a), sockeye salmon (Salmonidae; Östhom et al. 1990), red-tailed goodeid (Goodeidae; Bonn and König 1988), black/green molly (Poeciliidae; Boer et al. 1980; Batten et al. 1990), threespine stickleback (Gasterosteidae; Ekström et al. 1988), whitespotted greenling (Hexagrammidae; Uchiyama 1990), white perch (Percichthyidae; Zucker and Dowling 1987), rainbow cichlid (Cichlidae; Rusoff and Hapner 1990a, b), Mexican axolotl (Ambystomatidae; Northcutt

and Muske 1991), African clawed frog (Pipidae; Uchiyama et al. 1988), tree frog (Hylidae; Muske and Moore 1988, bullfrog and leopard frog (Ranidae; Uchiyama et al. 1988; Wirsig-Wiechmann and Basinger 1988), and domestic chicken (Wirsig-Wiechmann 1990). Our results suggest that this FMRFamide-like immunoreactivity is due to the presence of F8Famide/A18amide-like peptides rather than to FMRFamide itself. Furthermore, reports of SP-like immunoreactivity in the nervus terminalis of the tench *Tinca tinca* (Alonso et al. 1989) and Mediterranean barbel *Barbus meridionalis* (Alonso et al. 1990) should be viewed with caution until the antiserum used has been completely characterized with regard to its possible cross-reactivity with F8Famide- and A18Famide-like peptides.

We conclude that neither of the two types of SP-immunoreactive structures in the goldfish retina contains authentic substance P. The first structure, a class of mono-stratified amacrine cells, probably contains a tachykinin-like peptide with an aliphatic amino acid in position 8 and possibly a colocalized second tachykinin that is more SP-like. The second structure, the retinopetal system of the nervus terminalis, probably contains one or more peptides with a C-terminus similar to that of the bovine peptides F8Famide and A18Famide. We suggest that the endogenous goldfish RFamides can cross-react with some SP antisera because of similarities in amino acid sequence with SP(4–7).

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