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Different putative neuromodulators are present in the nerves which distribute to the teleost skeletal muscle

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Summary. The presence of putative neuromodulators in the nerve fibres was investigated in white skeletal muscle of two teleost fish not taxonomically correlated and showing different patterns of innervation (multiple versus focal innervation). Cryostat sections of epaxial, hypaxial and *adductor mandibulae* (AM) muscles of *Sparus aurata* and *Anguilla anguilla* were stained histochemically for reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase. Other sections were used for indirect immunohistochemistry (streptavidin-biotin and rhodamine immunofluorescence methods), employing antibodies specific for putative excitatory or inhibitory peptides, including CGRP, substance P, met-enkephalin, bombesin, and VIP. In addition, ultrastructural observations were performed in order to describe the morphology of the motor endplates. A strong immunoreactivity for CGRP and substance P was found in many nerve terminals. Met-enkephalin, bombesin and VIP immunoreactivities were less frequently observed. No immunoreactivity was observed to CCK, NPY or 5-HT. NADPH-diaphorase was identified in nerve fibres of the AM complex only of *A. anguilla*. Electron microscopy observations evidenced more than one type of synaptic vesicle in motor endplates. Some differences in putative neuromodulator distributions were observed in the two species and muscle complexes, which may be related to the different taxonomical position as well as the different pattern of innervation of white muscle fibres.

Key words: Teleosts, skeletal muscle, neuropeptides, NOS.

Introduction

The skeletal muscle of teleosts differs from that of higher vertebrates because the trunk musculature is myotomally arranged. Histochemically identifiable and functionally different fibres are segregated into homogeneous areas, which are completely distinct in the trunk musculature and very largely so in non-myomeric musculature such as adductor mandibulae (AM) complex (Scapolo et al., 1989). Slow red, fast white and possibly pink muscle fibres of the majority of teleost fish are all multiply innervated. Only in some (generally more "primitive") species is the motor innervation of pink and white muscle fibres focal (in the form of single endplates: Bone, 1964; Barker, 1968).

It is now known that co-release of a variety of neuroactive substances (mostly neuropeptides) can occur with acetylcholine and other neurotransmitters in cholinergic axons of mammalian species (Grinnell and Herrera, 1981). These substances may act as excitatory or inhibitory neurotransmitters and/or as neuromodulators. It has been shown that in mammals the neuromodulators may act either presynaptically or by regulating the number of post-synaptic receptors for acetylcholine. Mammalian motor endplates may contain morphologically different synaptic vesicles, which store neuromediators other than acetylcholine, especially neuropeptides (Matteoli et al., 1988).

As part of our on-going study on the phylogenetic distribution of neurotransmitters and neuromodulators in the motor endplates of vertebrates, we have studied their presence in two taxonomically unrelated teleost species: *Anguilla anguilla* (L.) and *Sparus aurata* (L.). The lateral as well as the non-myomeric muscles of the AM complex were studied in both species. Immunohistochemistry was used to identify putative neuromodulators in the motor nerves of white muscle, which is multiply innervated in *S. aurata* and focally innervated in *A. anguilla*. Fortunately, the primary structure of neuropeptides is generally highly conservative in vertebrates. This means that antibodies raised against mammalian forms of these peptides can be expected to identify their teleost homologues. In addition, histochemistry was used to identify the distribution of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase. In mammals this enzyme is correlated with nitric oxide

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synthase (NOS) activity (Dawson et al., 1991; Hope et al., 1991; Wolf, 1997), and there is evidence that NO acts as a neuromediator in the central nervous system (Garthwaite et al., 1989). Finally, electron microscopy was used to verify if morphologically different synaptic vesicles are present in fish motor endplates as they are in mammals (Matteoli et al., 1988).

Materials and methods

Adult *S.aurata* (n=6) and *A. anguilla* (n=4) fish (obtained from Maricoltura Italia, Monfalcone) were used for this study. Individuals of both sexes were collected. Body weight ranged between 200 and 1000 g for *S. aurata* and was about 400 g for *A. anguilla* ("silver eel" stage).

Fish were killed by an overdose of MS222 (Sandoz, Italy) anaesthesia at 10 a.m. Specimens of epaxial and hypaxial white lateral muscle and the white portion of the *adductor mandibulae* complex were collected for the histochemical and immunohistochemical analysis as well as for electron microscopy. Some samples of the gut were also collected from the same fish. Muscle and gut specimens were collected and processed as mentioned below immediately after the time of sacrifice.

Histochemistry

NADPH-diaphorase. Specimens were fixed overnight at 4 °C in 4% paraformaldehyde, rinsed several times in 0.1M phosphate-buffered saline (PBS) pH 7.4, then in 20% sucrose in the same buffer for 24 h at 4 °C, and snap-frozen in liquid nitrogen-cooled isopentane. Cryostat sections (20 μm) were picked up on gelatin-coated glass slides and incubated for 1 h at 37 °C in 0.1M PBS, pH 7.4, containing 0.15 mg/ml nitroblue tetrazolium (Sigma, Italy), 0.1% Triton X-100 and 1 mg/ml NADPH (Sigma), according to Scherer-Singler et al. (1983). The sections were then rinsed in PBS, dehydrated and mounted in Eukitt. The specificity of this stain was verified by excluding NADPH from the incubating medium, which abolished all activity. Positive controls included mammalian and fish gut samples.

Immunostaining

Specimens were fixed overnight at 4 °C in 4% paraformaldehyde, rinsed several times in 0.1M PBS, pH 7.4, cryoprotected in 20% sucrose in the same buffer for 24 h at 4 °C, and snap-frozen in liquid nitrogen-cooled isopentane. Some specimens were frozen without fixation. Cryostat sections (20 μm) were picked up on gelatin-coated glass slides. The sections obtained from the fixed specimens were treated with 0.05% bovine trypsin (Trypsin PCK cod.3740, Worthington Biochemical Corporation, USA) in Tris (hydroxymethyl-aminomethane)-HCl buffer 0.05M at pH 8.1 for 15 min at 37 °C (pH at this temperature was 7.8), in order to unmask the antigens and increase the immunostaining of the substances tested. Sections were then post-fixed for 20 min at room temperature in a Susa's liquid modified according to Stevens and Shaw (1982). Both fixed and unfixed sections were then incubated with the primary antisera indicated in Table 1.

The incubation with the primary antisera was carried out overnight at 4 °C in a moist chamber.

The antibody-antigen complexes were visualised using:

a) biotinylated swine anti-rabbit immunoglobulins (Dako, Italy) as secondary serum (dilution 1:600 for 30 min at room temperature), followed by StreptAB-Complex/HRP (horseradish peroxidase) (Dako) for 30 min at room temperature. Tris-buffered saline, (TBS: 0.05M Tris/HCl, 0.15M NaCl) pH 7.6 was used for dilutions and rinses throughout the whole procedure. The 3-amino-9-ethylcarbazole (AEC, Dako) was employed as a chromogen. Sections were mounted using an aqueous mounting media (Glycergel, Dako) and examined under an Olympus BX50 photomicroscope.

b) goat anti-rabbit immunoglobulins, labelled with tetramethyl-rhodamine isothiocyanate (TRITC) (Sigma). The incubation with secondary antibody was carried out at a dilution of 1:300 for 45 min at room temperature.

Table 1. Primary antisera tested, all raised in rabbits.

PRIMARY ANTISERA TESTED	SOURCE	CODE	OPTIMAL DILUTION
anti-human calcitonin gene-related peptide (CGRP)	Peninsula, UK	RAS 6009 N	1:500
anti-rat calcitonin gene-related peptide (CGRP)	Peninsula, UK	RIN 6006	1:500
anti-substance P	Peninsula, UK	IHC 7451	1:200
anti-synthetic methionine-enkephalin	Amersham, UK	RPN 1562	1:400
anti-bombesin[a]	Peninsula, UK	IHC 7113	1:200
anti-human, porcine and rat vasoactive intestinal peptide (VIP)	Peninsula, UK	IHC 7161	1:200
anti-cholecystokinin-octapeptide (CCK)	Amersham, UK	RPN 1592	1:200
anti-synthetic porcine neuropeptide Y (NPY)	Amersham, UK	RPN 1702	1:400
anti-serotonin (5-HT)	Peninsula, UK	61066	1:1000

[a]: the anti-bombesin serum used does not cross-react with substance P

Table 2. Comparative distribution of reactivity for NADPH-diaphorase and immunoreactivities in *S. aurata* and *A. anguilla*[a].

	Sparus aurata		*Anguilla anguilla*	
	Lateral muscle (white fibres)	Adductor mandibulae (white fibres)	Lateral muscle (white fibres)	Adductor mandibulae (white fibres)
NADPH-diaphorase	-	-	-	+
CGRP	+	-	-	+
Substance P	+	-	+	-
Met-enk	+	-	+	-
Bombesin	+	-	+	+
VIP	+	-	-	+
CCK	-	-	-	-
NPY	-	-	-	-
5-HT	-	-	-	-

[a]: + and - indicate respectively the presence and absence of reactivity.

All incubation solutions contained 0.1% Triton X-100. After several rinses in PBS, the sections were mounted in a mixture of glycerol-PBS (3:1). Sections were examined under the same Olympus BX50 microscope equipped for epifluorescence illumination.

The specificity of peptide immunostaining was verified: 1) by incubating sections with normal rabbit serum, instead of specific antisera and 2) by incubating sections with preabsorbed antiserum with the respective antigen (10-100 μg/ml). The preabsorption procedures were carried out overnight at 4 °C. Peptides were purchased from Sigma. The results of the controls were negative. As positive controls fish and mammalian gut samples were used.

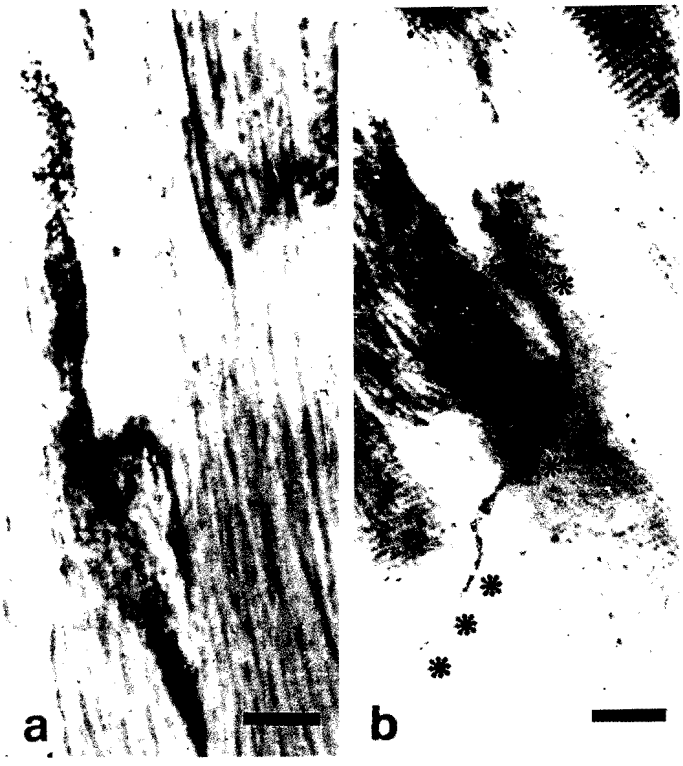


Fig. 1. Histochemical evaluation of NADPH-diaphorase activity in white skeletal muscle fibres of AM complex in *A. anguilla*. **a.** A reactive motor endplate is discernible. x 600. Scale bar: 16 μm. **b.** A solitary nerve fibre is seen (asterisks), with an evident granular reactivity. x 600. Scale bar: 16 μm.

Electron microscopy

Specimens were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, for 3 h at 4 °C. After several rinses in the same buffer, specimens were post-fixed in 1% osmium tetroxide for 1 h at 4 °C, dehydrated and embedded in EPON-araldite. Thin sections (60-70 nm) were obtained with an LKB IV Ultramicrotome, stained with uranyl acetate and lead citrate and examined under a Jeol 100SX electron microscope.

Results

The results obtained in the two species are summarised in Table 2.

Enzyme histochemistry

NADPH-diaphorase was only identifiable in neural structures of the AM complex of *A. anguilla*. These included both putative motor endplates (Fig. 1a) and solitary rare "en passant" nerve fibres (Fig. 1b).

Peptide immunostaining

Owing to the extremely thin nerve terminals, the streptavidin-biotin method gave a signal difficult to be discerned. The immunofluorescence method, on the contrary, provided an amplification of the signal and thus resulted in a better immunostaining.

As the antisera were raised against mammalian antigens, the results are referred to as "peptide-like" immunoreactivity (IR).

CGRP-like immunoreactivity was present in single, fine nerve fibres running close to white lateral muscle fibres in *S. aurata* (Fig. 2a,b), whereas in *A. anguilla* the CGRP-like-IR was present in AM complex white muscle

fibres (Fig. 2c,d). In both species the immunoreactive nerve fibres were rather numerous, even if unevenly distributed.

Substance P-like immunoreactivity was detected in the white lateral muscle fibres of both species. In *S. aurata* (Fig. 2e) a strong immunoreactivity was distributed along the length of some muscle fibres. In this case also, the immunoreactive nerve terminals ran close to muscle fibres.

Met-enkephalin-like immunoreactive nerve fibres were present contacting white muscle fibres in the lateral muscle of both *S. aurata* (Fig. 3a) and *A. anguilla*. In some cases aspects resembling motor endplates were observed (Fig. 3b).

Bombesin-like immunoreactivity was present in sparse nerve fibres contacting the white lateral muscle fibres in *S. aurata* (Fig. 3c). In *A. anguilla* the immunoreactivity was detectable in axons running along the white muscle fibres in both lateral and AM muscles.

Few nerve fibres showed a VIP-like immunoreactivity. Immunostaining was found in the white lateral muscle of *S. aurata* and in the AM complex of *A. anguilla* (Fig. 3d).

CCK-, NPY- and 5-HT-immunoreactivities were never observed in skeletal muscle, although they were found in the enteric nervous system and diffuse endocrine system of fish and mammalian gut.

Electron microscopy

In fish musculature the motor endplates were easily recognizable on the basis of their cytoplasmic feet containing a rich population of synaptic vesicles. In white muscle fibres of lateral muscle of *S. aurata* (Figs. 4a,b) and AM complex of *A. anguilla* the majority of synaptic vesicles were clear, with a subtle limiting membrane, others showed an electron-dense content and a thick membrane, others contained a dense core limited by an electron-lucent halo.

Discussion

The results in Table 2 show that the distributions of NADPH-diaphorase (which is possibly related to the synthesis of nitric oxide) and neuropeptides varied according to the species and muscles. CCK, NPY and 5-HT were never immunohistochemically found.

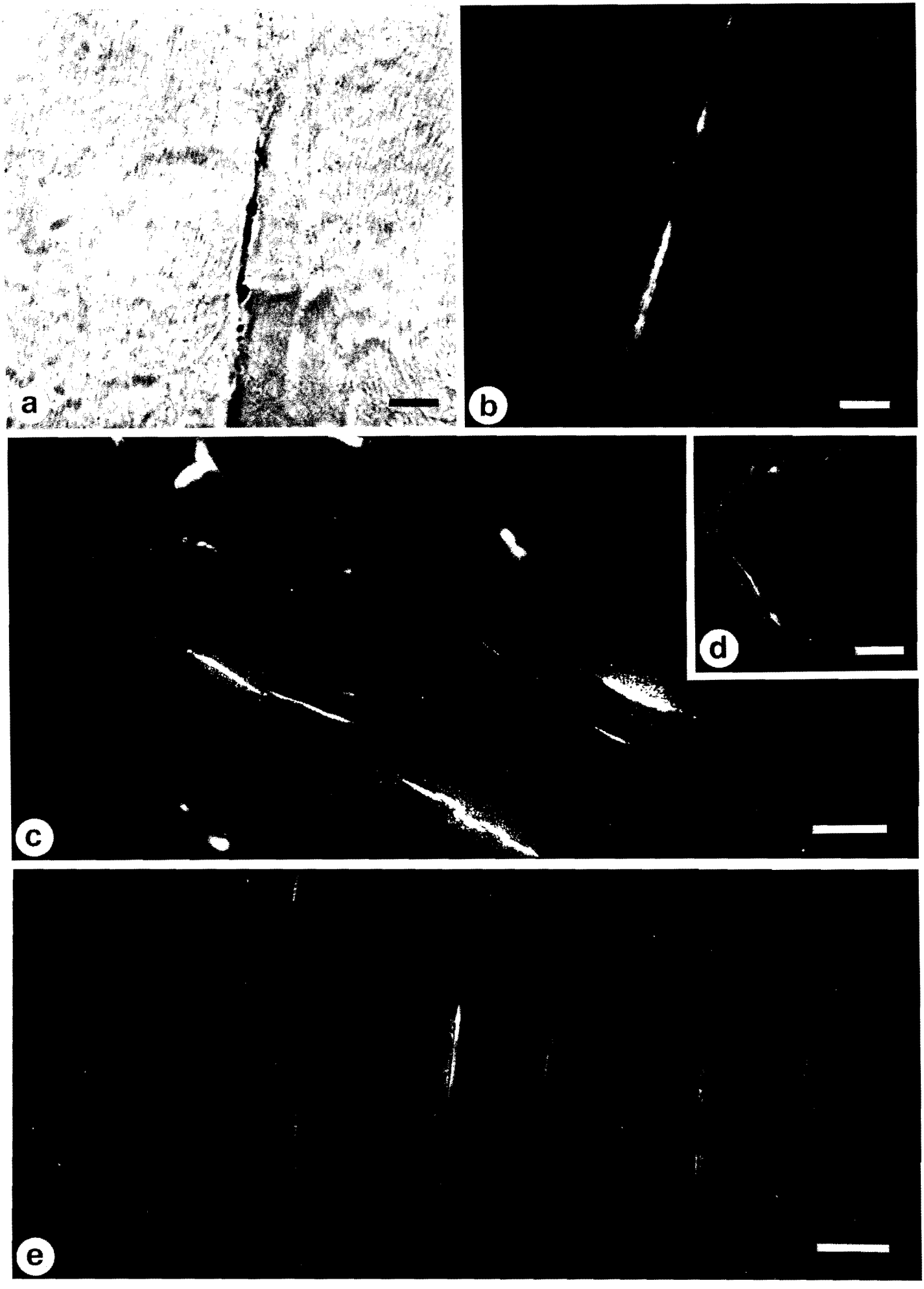
In the lateral muscle of *S. aurata*, but not in AM complex, components of the intramuscular nerve supply were shown to contain CGRP- and substance P-like peptides. By contrast, in *A. anguilla* CGRP-IR was found in AM only, and Substance P-IR in the lateral muscle only. The immunoreactive nerve fibres are single, fine and in close contact with white muscle fibres.

CGRP is present in motor neurons innervating mammalian skeletal muscles (Kimura et al., 1994) and it is released at neuromuscular junctions (Mora et al., 1989; Li and Dahlström, 1992; Rodrigo et al., 1994). In the motor nerves, CGRP is reputed to be released as a neuromodulator, which acts in regulating the number of ACh receptors (Ishida-Yamamoto and Tohyama, 1989; Csillik et al., 1993). CGRP differs slightly in its primary structure in mammalian, avian and amphibian (Andersen et al., 1992) species, but it is generally highly conserved in the evolutionary history of vertebrates. In fish CGRP has been identified by radioimmunoassay in the trout (Fouchereau-Peron et al., 1990).

Substance P is involved in neurotransmission in the mammalian nervous system, especially by primary afferents (Salt and Hill, 1983; Molander et al., 1987). In fish, Substance P and other tachykinins have been identified and characterised in a number of species (Conlon et al., 1991; Jenssen and Conlon, 1992; Moons et al., 1992). The structural similarity of these peptides to mammalian tachykinins is very high (Jenssen et al., 1993). An afferent significance of substance P-containing nerve fibres might be related to the local regulation of intensity and duration of muscle fibre contraction, in response to specific influences. This may be especially true in fish where reports of neuromuscular spindles are quite sporadic (Maeda et al., 1983), if not absent at all (Bone, 1964; Barker, 1968).

The opioid peptides leu- and met-enkephalins are known as endogenous ligands for vertebrate brain opiate receptors. Bombesin and other peptides sharing the C-terminal sequence of bombesin have been identified in amphibian skin and in mammalian brain and gut (Andersen et al., 1992). Bombesin-related peptides have been identified and characterised in the gut of a number of teleosts (Langer et al., 1979; Holmgren et al., 1982; Holmgren and Jönsson, 1988). VIP in vertebrates promotes both smooth muscle relaxation and a stimulation of fluid and electrolyte secretions in glandular complexes mediated by vasodilatation (Foskett et al., 1982; Thorndyke et al., 1989). Bombesin, VIP and met-enkephalin may share further functions as co-mediators in mammalian central and peripheral nervous systems, both in sensory and motor pathways (Hietanen et al., 1990; Edyvane et al., 1994; Keast and Chiam, 1994). We found a number of met-enkephalin-, bombesin- and VIP-like-immunoreactive nerve fibres in the white lateral muscle of *S. aurata*. In some cases, immunoreactive motor endplates were also seen.

Fig. 2. Immunohistochemical identification of CGRP-like and substance P-like immunoreactivities in white skeletal muscle fibres of *S. aurata* and *A. anguilla*. **a-d.** CGRP-like immunoreactivity. **a.** *S. aurata*, lateral muscle. A subtle nerve fibre is evident with the streptavidin-biotin method. x 800. Scale bar: 13 μm. **b.** *S. aurata*, lateral muscle. A strongly fluorescent subtle nerve fibre is evident. x 400. Scale bar: 25 μm. **c.** *A. anguilla*, AM complex. Muscle fibres appear supplied by numerous intensely stained nerve terminals. x 300 Scale bar: 50 μm. **d.** *A. anguilla*, AM complex. A detail of a transversally sectioned muscle fibre surrounded by a nerve terminal. x 600. Scale bar: 16 μm. **e.** Substance P-like immunoreactivity. *S.aurata*, lateral muscle. Rather numerous immunofluorescent nerve fibres are seen in close contact to muscle fibres. x 300. Scale bar: 50 μm.


a
b
c
d
e

Immunoreactivities to the same antisera were not detected in the AM complex of the same species. In *A. anguilla*, met-enkephalin- and bombesin-like immunoreactive nerves were seen in the lateral muscle, whereas bombesin- and VIP-like-immunoreactive terminals were observed in the AM complex.

These immunohistochemical results suggest that in fish, as in mammals, the somatomotor nervous system utilises a variable number of neuromediators with possibly different functional roles. In addition, our ultrastructural observations unequivocally show the presence of multiple populations of synaptic vesicles in

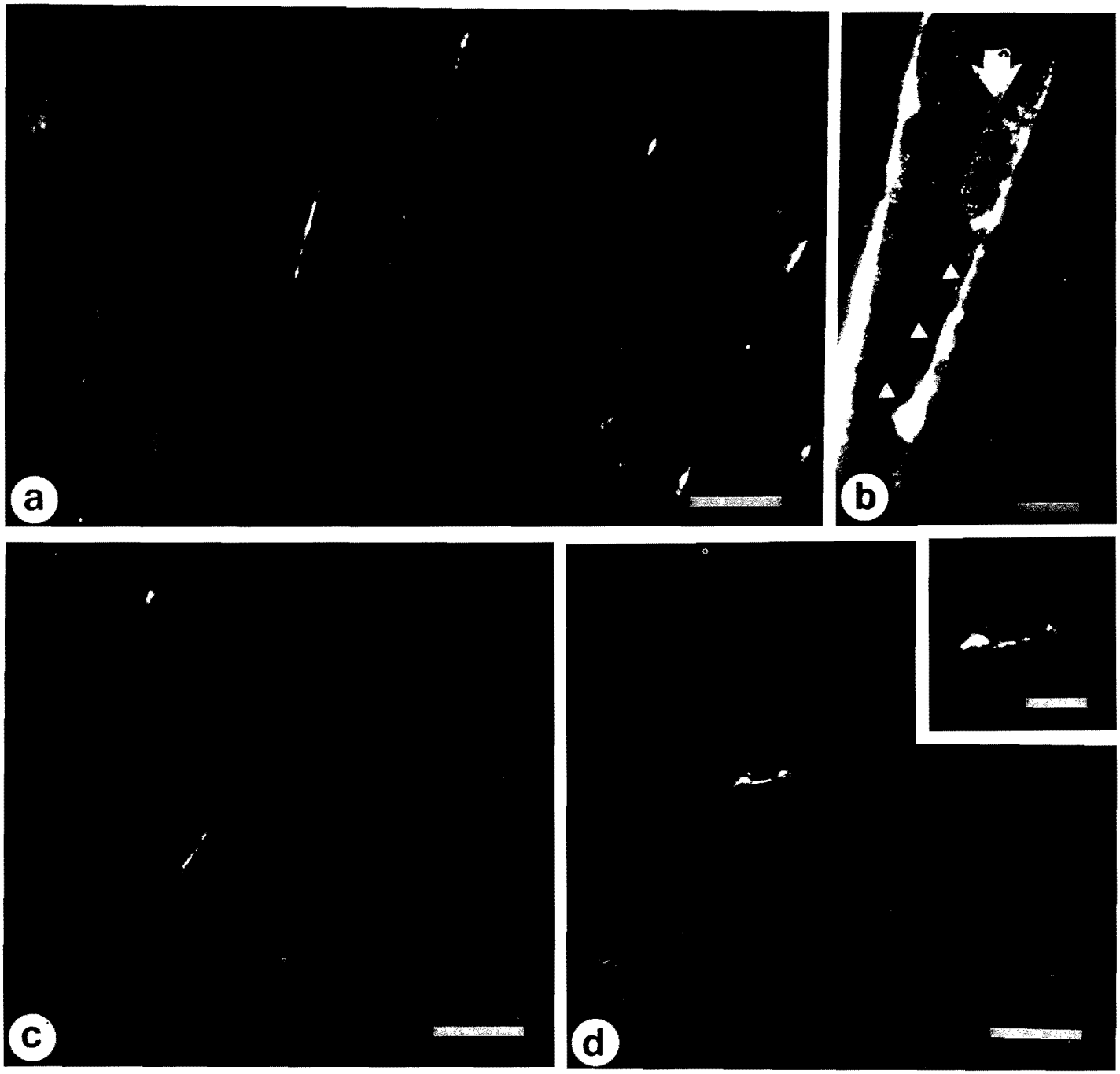


Fig. 3. a, b. *S. aurata*, lateral muscle, met-enkephalin-like immunoreactivity. **a.** Immunofluorescent nerve fibres are seen. x 300. Scale bar: 50 μm. **b.** Sub-terminal axons (triangle) and a motor endplate (arrow) are heavily immunostained. x 500. Scale bar: 20 μm. **c.** *S. aurata*, lateral muscle. Bombesin-like immunoreactivity is shown in nerve fibres. x 300. Scale bar: 50 μm. **d.** *A. anguilla*, AM complex. A VIP-like immunoreactive nerve ending is discernible. x 300. Scale bar: 50 μm. Insert: a higher magnification of the immunoreactive structure. x 400. Scale bar: 25 μm.

motor endplates of the lateral muscle of *S. aurata* and AM complex of *A. anguilla*. In mammalian endplates this aspect is correlated to the presence of more than one neurotransmitter or putative peptidic neuromediators (Matteoli et al., 1988).

We never detected any immunoreactivity to CCK-, NPY- and 5-HT-antisera, in either *S. aurata* or *A. anguilla*. On the contrary, these neuromodulators have been identified in some other fish (Jenssen and Conlon, 1992; Moons et al., 1992; Himick and Peter, 1994). A different primary structure of the peptides in the two species examined by us may explain these discrepancies. The absence of 5-HT-IR is likely to be related to the absence of this biogenic amine in the skeletal muscle of the species examined by us. By contrast, 5-HT is a common mediator in mammalian central and peripheral nervous systems (Steinbusch et al., 1978) and has been recently identified in the central nervous system in a teleost species (Ekstrom, 1994).

NADPH-diaphorase reactivity was observed only in the AM complex of *A. anguilla*. In addition to its function as a neuronal messenger in the central nervous system, nitric oxide was identified in the enteric nervous system of mammals (De Ridder and Weyns, 1994) and birds (Li et al., 1994), where it may act as a non-adrenergic non-cholinergic mediator (Sanders and Ward, 1992). Quite recently NOS has been identified in the goldfish Mauthner cell (Bell et al., 1997), where nitric oxide has been suggested to function both as an excitatory and inhibitory neuromodulator for skeletal muscles. It is also known that nitric oxide coexists with the classical neurotransmitter acetylcholine in mammalian central nervous system (Wetts and Vaughn, 1994) as well as with other neuromodulators, namely neuropeptides, in mammalian central and peripheral nervous systems (Ekblad et al., 1994; Keef et al., 1994; Tay and Moules, 1994; Burnett et al., 1995). A nitrergic innervation exists in mammalian skeletal muscles (Wörl et al., 1994). It has been suggested that NO may have a signalling function, possibly as a retrograde messenger, in developmental synapse elimination at the neuromuscular junction (Wang et al., 1995).

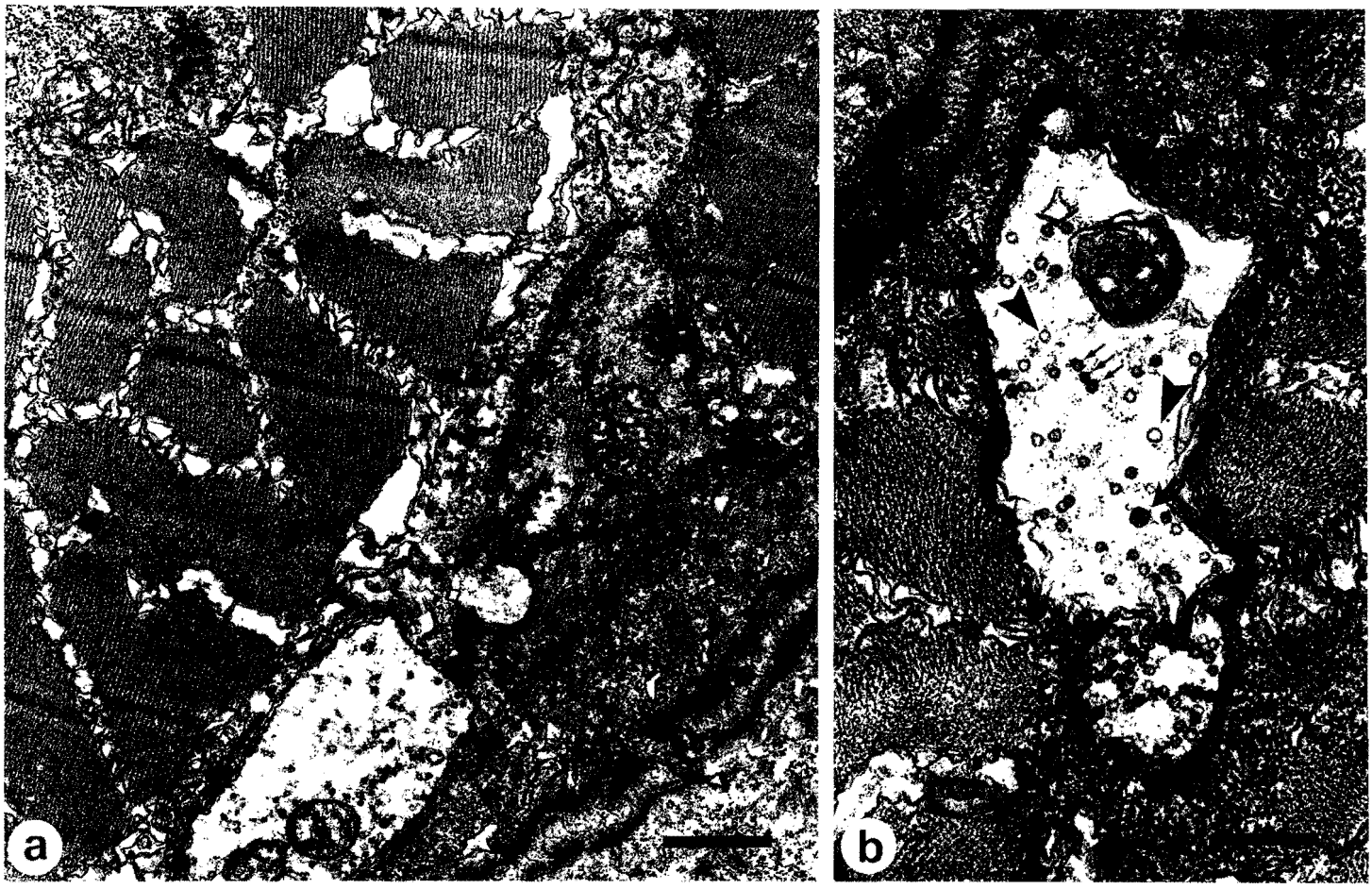


Fig. 4. a,b. *S. aurata*, thin sections of lateral muscle. A neuromuscular junction shows the presence of morphologically different synaptic vesicles. The majority of them are clear, with a subtle limiting membrane applied (arrowheads), others show an electron-dense content and a thick membrane (thin arrows), others contain a dense core limited by an electron-lucent halo (thick arrow). a, x 20,000; scale bar: 750 nm; b, x 37,500; scale bar: 400 nm.

We suggest that the white muscle fibres of the AM complex in *A.anguilla* receive a somatomotor innervation which utilises not only CGRP (and bombesin and VIP), but also NO as possible neuromodulator. This is in sharp contrast with the AM complex of *S. aurata*, which lacks either NO or the other neuromediators tested. On the contrary, the white lateral muscle of *A. anguilla* contains fewer putative neuromodulators than the same muscle of *S. aurata*, and thus taxonomic position per se cannot be the only explanation for this different pattern. This species-specific difference is possibly related to focal versus polyneuronal innervation. In addition, the trophic opportunism of *A. anguilla*, which consumes not only benthic invertebrates and fish but planktonic organisms also (Kennedy et al., 1992), may offer a further explanation. This opportunistic alimentary habit presumably needs different jaw movements when approaching different substrates. The large number of neuromodulators identified in AM complex of *A. anguilla* may sustain forms of synaptic plasticity in this species.

In conclusion, this work describes for the first time the presence and localisation of several neuromodulators in the somatomotor innervation of white myotomal and non-myotomal muscle in two different teleost species with different patterns of innervation. In many respects these results show similarities with the situation in muscle innervation of mammals. However, some species-specific differences were observed, which may be related to the different taxonomic position of the two fish examined and possibly to their different alimentary habits, as well as to the multiple versus focal innervation of white muscle fibres.

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