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A polyclonal antibody against mammalian FOS can be used as a cytoplasmic neuronal activity marker in a teleost fish

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Abstract

A polyclonal antibody raised against a conserved region of a mammalian FOS sequence was tested for its use as an activity marker in the rainbow trout. The FOS-like expression in the trout is entirely cytoplasmic and appears in a Nissl-like pattern. The reaction is specifically induced by both orthodromic and antidromic electrical stimuli and during motor responses evoked by natural stimulation, although some positive neurons are found at locations that are not obviously related to the presented stimuli. Following spinal nerve stimulation, antidromically activated motoneurons were found to be positive in the ipsilateral spinal cord. Orthodromic driving of spinal moto- and interneurons by stimulation of the medial longitudinal fasciculus (MLF) in the hindbrain evoked FOS-like immunoreactivity throughout the motor column in the spinal cord, but not in regions lying caudal to a lesion of the MLF-axons. Evoking about 25 startle responses by natural auditory stimulation gives FOS-like immunoreactivity in the Mauthner cell, which initiates the response, whereas positive Mauthner cells were never observed in control fish. The stimulation protocols that were used strongly activated the stimulated cells and so the observed FOS-like immunoreactivity might be related to an increase protein synthesis needed to restore their depleted transmitter levels.

Keywords: Activity marker; FOS-like immunohistochemistry; Orthodromic stimulation; Antidromic stimulation; Mauthner cell; (Rainbow trout)

1. Introduction

The immunohistochemical (IHC) detection of the c-FOS protein formed by the immediate early gene *c-fos* has gained considerable interest during the last few years as a way of marking neuronal activity (Morgan and Curran, 1991; Sagar and Sharp, 1993), as in most systems this substance is specifically expressed by those neurons that respond to the presented stimulus. FOS IHC has been used in many mammalian systems to map pathways activated by a certain stimulus (Wan et al., 1992) or to differentiate between the cells activated in a particular nucleus in relation to various stimuli (Keay and Bandler, 1993; Lima et al., 1993; Sheng et al., 1993). The major advantage over techniques tradi-

tionally used for this purpose (e.g., 2-deoxyglucose) (Duncan et al., 1993; Reimer, 1993; Sallaz and Jourdan, 1993) is the cellular resolution provided by FOS IHC; the major disadvantage is that not all neurons respond to activation with an increase in *c-fos* expression and some cell groups express the FOS protein without a known relation to the stimulus that has been presented.

Techniques with cellular resolution have proved to be especially useful when applied in lower vertebrates and invertebrates, because their cells are mostly much larger and the number underlying a certain act is much smaller than is the case in mammals. The use of FOS IHC as an activity marker in vertebrates other than mammals and birds (e.g., Ambalavanar et al., 1993), has thus far not been reported. Although FOS has been shown biochemically to be present in non-neuronal cells in the carp (Stein-Izsak et al., 1987), and its expression becomes elevated from 1 day following optic nerve crush, i.e., coincident with nerve regeneration, it could not be detected in a similar experiment by IHC

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in glial or regenerating neuronal cells in the goldfish (Herdegen et al., 1993). The *c-fos* gene is known to be present in *Xenopus* and its sequence shows a strong homology to that of mammals (Kindy and Verma, 1990), but again there has been no report on FOS IHC in amphibians in relation to neuronal activity.

In this paper we report on the use of a polyclonal antibody, raised against a conserved region of a mammalian FOS-protein, as an activity marker in a teleost fish. The nature of the protein recognized in fish by the antibody is not yet known, but for convenience we will refer to the reaction product as 'FOS-like'. We show that the FOS-like reaction product relates to activity by using several natural and electrical stimuli that are known to activate a limited and defined number of neurons.

2. Materials and Methods

Experiments were done on 16 rainbow trout (*Oncorhynchus mykiss* Walbaum) ranging from 20.5 to 48 cm fork length, obtained from a local hatchery and kept in the laboratory in large tanks with continuously refreshed water at a temperature of 14 °C. Each fish was transferred to an isolated tank 1 week prior to the experiment, so that experiments could be done on naive fish and disturbances other than those intended could be avoided. All experimental procedures were performed under the terms of Dutch legislation and approved by a local committee.

2.1. Stimulation

FOS-like expression was studied in fish stimulated in 1 of 3 ways: (1) spinal motoneurons were antidromically activated by stimulating their axons in the spinal nerve of one segment ($n = 4$ fish); (2) the medial longitudinal fasciculus (MLF) was stimulated in the hindbrain so as to drive spinal inter- and moto-neurons synaptically via the descending axons ($n = 1$) and, (3) the Mauthner cell in the hindbrain was activated ($n = 7$) by evoking a series of startle responses (Eaton et al., 1981). The control situation for Exp. 1 was provided by other spinal segments (about 5 cm away). For Exp. 2, a control situation was obtained by making a spinal transection at the midbody level to prevent MLF-input to more caudal levels. The controls for the Mauthner-stimulated fish (Exp. 3) were passive, naive fish ($n = 4$). In all experiments, to avoid FOS-like expression while a fish was being caught (e.g., during struggling), before handling it was anaesthetized in the tank where the experiments were done with MS-222 ($\pm 60 \text{ mg} \cdot \text{l}^{-1}$; Sandoz, Basle) brought to pH 7 with NaHCO_3 .

(1) During spinal nerve stimulation, each fish was additionally anaesthetized by injecting $20 \text{ mg} \cdot \text{kg}^{-1}$

alphaxalone ('Saffan', Glaxovet, UK) i.p., every 90 min throughout the experiment. The fish was placed on crushed ice and aerated fresh water was run continually over its gills. The spinal nerve was exposed at anal levels and the medial ramus was placed on silver hook electrodes. To eliminate unilateral FOS-like induction as a result of the surgery, a bilateral incision was made, whereas electrical stimulation was given to one side only. A stimulus pulse of 0.2 ms duration, at a repetition interval of 2 s, was gradually increased in strength until a local muscle contraction was observed (10–20 V stimulator output), whereupon the stimulus was turned off and the fish was paralyzed by an i.p. injection of curare (tubocurarine chloride 1%, Multi-Pharma, Netherlands). When all gill movements had stopped, stimulation was resumed at a strength 5 V above the threshold for muscle contraction and maintained for 1 h.

(2) The MLF was stimulated with a bipolar Teflon-coated tungsten electrode (0.5 mm bare tip) inserted via the 4th ventricle after the skull had been opened in a fish anaesthetized as described above. The potential evoked in the spinal cord was recorded with a silver ball surface electrode (3.5 k Ω), 2.5 cm rostral to the dorsal fin. A complete transection of the spinal cord was made with fine scissors 5 mm rostral to the dorsal fin. After surgery was completed, the fish was paralyzed with curare and the MLF stimulated with 500 cycles of 3 s interval pulses at an amplitude that evoked a gross potential in the cord.

(3) Startle responses were evoked by delivering a hard blow to the side of the aquarium. Each fish was given sufficient stimuli so as to evoke 25–50 responses, depending on the habituation to the stimulus, over a period of approximately 30 min. It was then left for 90 min before being anaesthetized with MS-222.

At the completion of an experiment, immediately following stimulation in Exps. 1 and 2 and after 90 min in Exp. 3, each fish received an overdose of 'Saffan' (0.6–1 ml) and 0.5–1 ml heparin (Leo pharmaceuticals, Netherlands) and was then perfused through the heart with a cold (4°C) teleost Ringer's solution containing 0.1% procaine (pH 7.4) and subsequently with 10°C 4% para-formaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain and/or spinal cord were dissected and post-fixed for 2–4 h in the same fixative, immersed in 30% phosphate-buffered sucrose for at least 12 h, cut on a freezing microtome at 40 μm and collected in cold 0.05 M Tris-buffered 0.9% saline (pH 7.4, TBS).

2.2. Immunohistochemistry

All antibody concentrations were varied over a wide range, but only the optimal concentrations are given here.

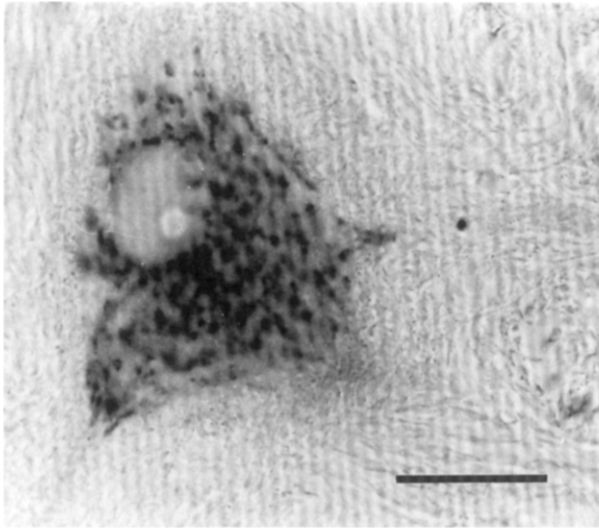


Fig. 1. Photomicrograph of a FOS-like immunoreactive neuron in the reticular formation in the brain of the trout induced by evoking startle responses (Exp. 3). All the FOS-like reaction-product is in the cytoplasm, appears in a Nissl-like fashion and extends into the base of the primary dendrites. Scale bar: 57 μm .

The collected sections were rinsed in TBS and pre-incubated for 1 h with 10% normal rabbit serum (NRS; Nordic, Netherlands) and 0.1% Tween-20 (all dilutions

were made in TBS), followed by a 44 h incubation at 4°C in a 1:10,000 polyclonal sheep anti-FOS (OA-11-823; Cambridge Research Biochemicals, UK) solution, containing 4% NRS, 0.1% Tween-20, 0.01% sodium azide, 0.05 mM phenylmethylsulfonyl fluoride and centrifuged before use for 30 min at 15,000 rpm. After rinsing with TBS + 0.2% NRS (+0.1% Tween-20 at alternating steps), the sections were incubated for 16 h at 4°C in a 1:50 solution of rabbit anti-sheep (Nordic; same diluent and treatment as the first incubation). The sections were rinsed in TBS (alternating steps + Tween-20) and incubated for 2–2.5 h at 4°C in a 1:400 solution of sheep peroxidase anti-peroxidase (PAP; Nordic) with 4% NRS and 0.1% Tween-20 (30 min at 15,000 rpm). After rinsing steps in TBS with NRS and Tween-20 (alternating), the sections were rinsed in 0.05 M Tris-buffer (pH 7.6) and stained with a nickel-intensified diaminobenzidine (DAB; Sigma) solution (1.14 mM NH_4NiSO_4 , 0.4 mM DAB, 0.01% H_2O_2 ; pH 8.0) for 5–15 min at 4°C. The sections were mounted on chrome-alum-coated slides and coverslipped with Entellan (Merck).

As a control for the staining specificity we omitted the primary antibody from the incubation medium for a few sections in every experiment and in those cases the sections were always completely devoid of staining.

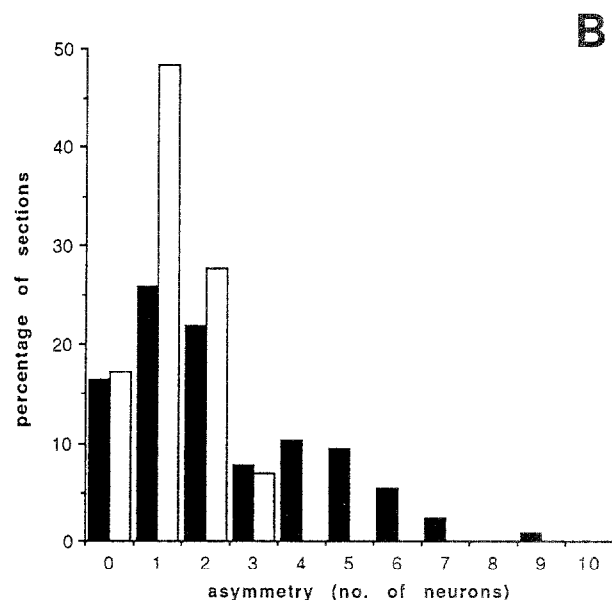
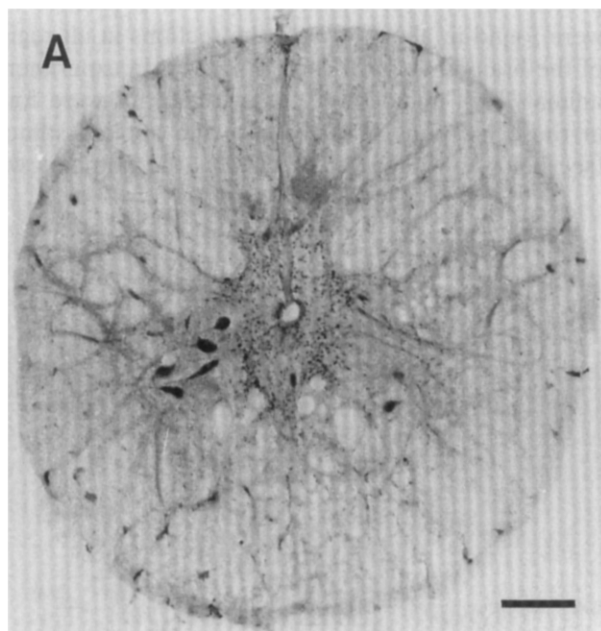


Fig. 2. FOS-like immunoreactivity in spinal motoneurons following unilateral antidromic activation. The basal FOS-like expression in the spinal cord is relatively high, but unilateral nerve stimulation results in an increase in the number of positive motoneurons on one side. A: photomicrograph of the antidromically activated motoneurons on the left side. Scale bar: 178 μm . B: Unilateral antidromic activation leads to asymmetry in the distribution of positive motoneurons over both sides of the spinal cord, whereas in unstimulated segments (basal expression level) this distribution is symmetrical in most sections. Asymmetry is given in the graph as the difference in motoneuron number between the left and right sides of each section of the cord. A large asymmetry (4–9 more neurons on one side) occurs only in the stimulated segment (filled bars). Symmetry (0 asymmetry), or a small asymmetry (1–3 cells more on one side) is found in both the stimulated and the control segments (open bars). The vertical axis gives the number of sections, as a percentage of the total number counted, in which each asymmetry value was found.

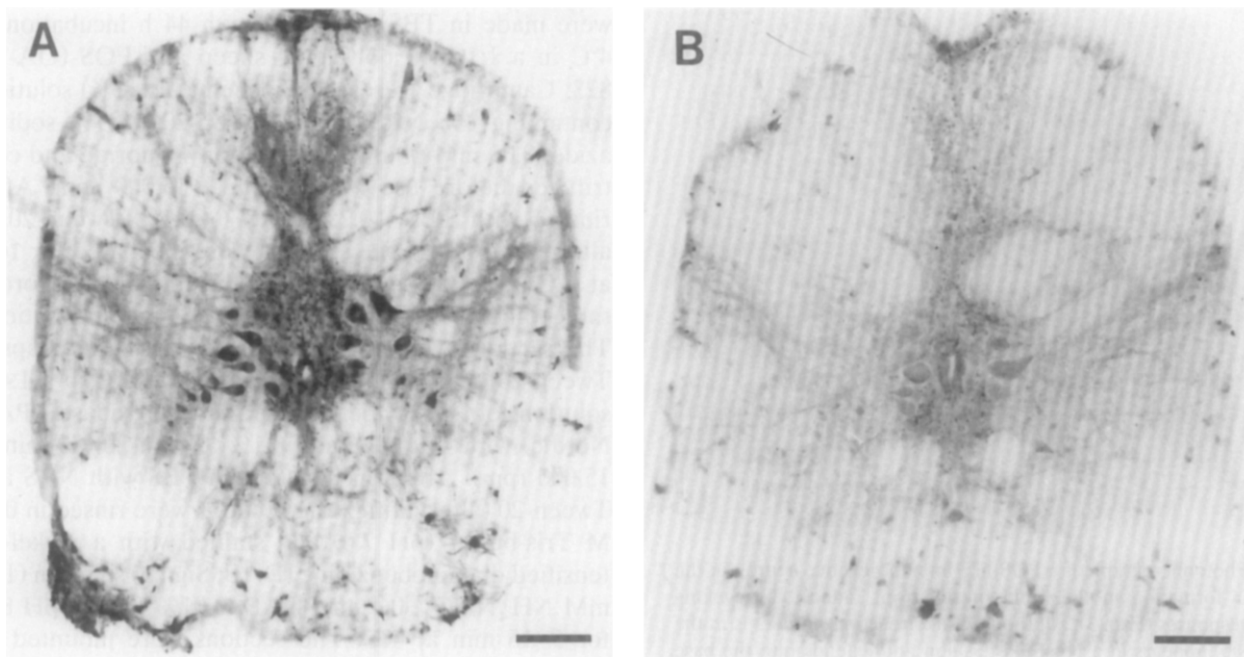


Fig. 3. Photomicrographs of 40 μm transverse sections of the spinal cord in a trout that was stimulated in the MLF. Section (A) is taken approximately 20 mm rostral to a spinal transection that prevented the MLF-input from reaching the level of section (B); approximately 40 mm caudal to the transection. Moto- and interneurons that are immunopositive in (A) can also be recognized in (B), where they are clearly negative. Some positive interneurons can be seen outside the grey matter, but these do not relate to MLF-stimulation as they appear in equal numbers both rostral and caudal of the transection. Scale bars: 160 and 123 μm , respectively.

3. Results

3.1. General appearance

In both experimental and control animals, neurons were found to immunoreact positively for a FOS-like

protein. In control animals only 1 or 2 positive cells were found in about 30% of the sections in all regions of the brain and spinal cord. However, in experimental animals high numbers of positive neurons were found at those locations that relate to the applied stimulus (see below), whereas at other sites the numbers were

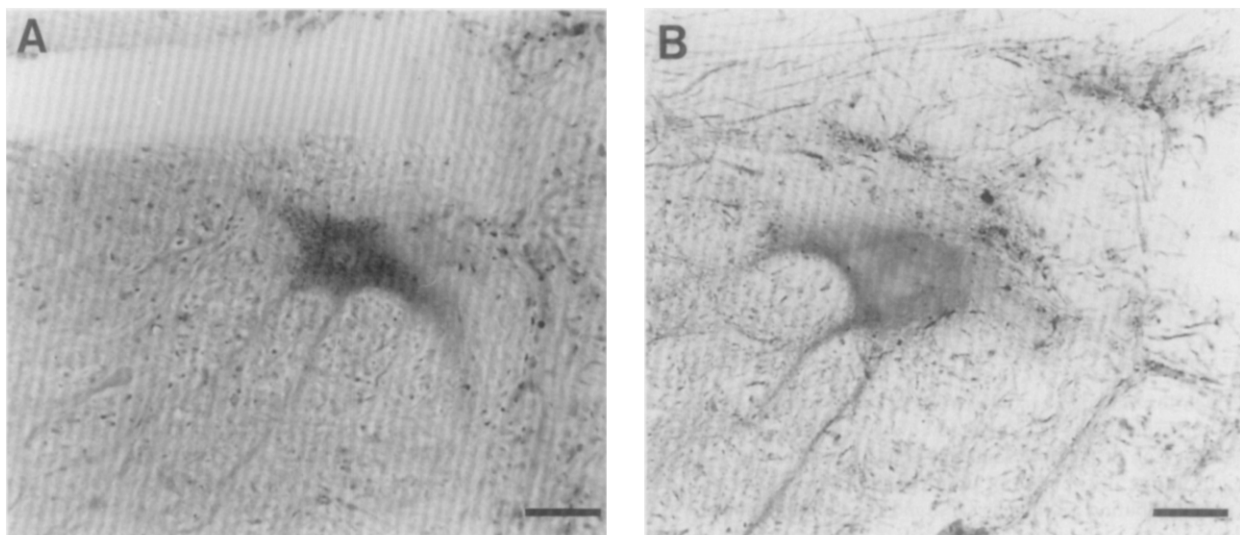


Fig. 4. A: positive Mauthner cell in the medulla after 50 startle responses had been evoked. B: negative Mauthner cell in a naive control fish. Scale bars: 57 μm .

similar to the controls. The FOS-like reaction is distinct, but is located entirely outside the cell nucleus (Fig. 1). Nissl body-like positive lumps are found throughout the cytoplasm, including the base of the primary dendrites, and sometimes a perinuclear rim of slightly higher density can be seen. None of these observations was time-related. FOS-like expression was never observed inside the nucleus.

Although not all positive cells react with the same intensity, the distinction between positive and negative neurons is easily made as negative cells never contain the dark Nissl-like granules although a slight aspecific background staining may be present (see Figs. 3B and 4B). A general increase of positive glial cells can be seen in all stimulated fish in association with FOS-like neurons, but not in control situations.

3.2. Spinal nerve stimulation

In the spinal nerve-stimulated fish, higher numbers of FOS-like positive motoneurons were found in the spinal cord than in controls. In the stimulated segment, antidromically activated motoneurons were present in large numbers on the side ipsilateral to the stimulated root (Fig. 2A). The positions of these positive neurons correspond to those that innervate the slow oxidative muscle (Fetcho, 1986), which is consistent with the applied stimulus. Positive motoneurons were also seen in unstimulated segments and contralaterally (the control areas) and so the effect of antidromic activation of the motoneurons can be best seen when the number of positive cells is expressed in terms of asymmetry between the two halves of the cord. Fig. 2B shows that a substantial asymmetric distribution is present only in the stimulated segment (filled bars), indicating a relationship to the stimulation.

3.3. Orthodromic stimulation

During MLF stimulation a clear synaptic potential complex could be recorded from the surface of the spinal cord rostral to the transection which indicates that moto- and inter-neurons were synaptically driven from the brainstem. Following MLF stimulation many immunopositive neurons were observed in all spinal cord segments rostral to the spinal transection (Fig. 3A), but very few positive cells could be found more caudally (Fig. 3B). Nearly all of the positive neurons rostral to the transection were lying in the motor column, at positions corresponding to those of the motoneurons and pre-motor interneurons. A few, mainly small cells were found outside the motor column.

Caudal to the transection, small numbers of all cell types were found to be positive, scattered throughout the cord, but positive large motoneurons were never

observed. The number of positive small neurons outside the motor column was not appreciably different from that rostral to the transection (compare Fig. 3A and B). We therefore do not consider these small cells to be activated by MLF stimulation.

3.4. Natural stimulation

In the third experimental configuration, FOS-like positive Mauthner cells (Fig. 4A) were found only in those fish in which startle responses had been evoked. Other positive cells were also found in these fish (such as the cell in Fig. 1), but none of these are as yet known to relate to the stimulus with the same certainty as for the Mauthner cell and therefore they will not be discussed in this context.

In the naive, unstimulated control fish, FOS-like positive cells were also found throughout the brain and spinal cord, but in low numbers at all locations. The Mauthner cells were negative in all control fish (Fig. 4B).

4. Discussion

The experiments described in this paper show that FOS-like expression in the trout, as detected immunohistochemically, can be reliably obtained in neurons that are related to the presented stimuli, suggesting that it is activity-specific. Variable, but lower numbers FOS-like positive cells are found also in cell groups that do not relate directly to the stimulus, as well as in control situations. This basal expression level of c-FOS imposes some limitations on the use of the technique, such that it can only be used in those situations where a clear and quantifiable control situation can be established.

In the present experiments the relation between stimulus presentation and FOS-like expression is very clear for the Mauthner cell and the MLF-stimulated animals. The antidromic stimulation experiments gave less clear-cut results, but they can also be considered as activity-specific. Sensory fibres are also stimulated in the medial ramus of the spinal nerve and these, via the dorsal root, will activate contralateral interneurons. The moto- and inter-neurons form an intricate network in the spinal cord (Grillner et al., 1990) and this probably accounts for the elevated numbers of positive neurons in the motor column at control locations.

Stimulation of the MLF is known to drive synaptically all spinal moto- and interneurons (Shapovalov, 1975). Caudal to the spinal transection such synaptic drive is absent and the number of positive moto- and inter-neurons does not exceed the numbers found in unstimulated fish.

Evoking startle responses activates many neurons in the brain and cord which relate to different components of the response. Only one cell is known to be invariably activated during the response, namely the Mauthner cell (Eaton et al., 1981) and therefore we have restricted ourselves to the analysis of just that cell.

A positive reaction in glial cells, as observed in the present experiments following stimulation, has been reported for mammals, but then only in response to injury (Dragunow and Hughes, 1993).

4.1. Cytoplasmic reaction product

The general appearance of the FOS-like positive cells in the trout is strikingly different from that reported so far in other species using the same antibody. In other studies on mammalian and avian species where the OA-11-823 antibody has been used, nuclear staining is mostly observed, although FOS-like staining in the cytoplasm has sometimes been reported (e.g., Cenci et al., 1992). In the trout the immunoreactivity was seen only in the cytoplasm, suggesting that either the protein here is not FOS, or the *c-fos* protein upon entering the nucleus in the trout cannot be reached by the antibody.

The antibody is known to be capable of recognizing a conserved region in the proteins formed by several members of the *c-fos* family, such as *fos B* and *fra-1* (Zerial et al., 1989), and possibly it is one of these that has been recognized in this study. In mammals FOS is certainly present in the cytoplasm following *c-fos* expression, but the expression is transient (Fort et al., 1987) and the protein seems to be rapidly taken up into the nucleus (Morgan and Curran, 1991). Although the metabolism of fish is slower and hence the turnover rate of FOS would be expected to be somewhat lower, this difference would be insufficient to account for the total absence of nuclear FOS in the longest surviving fish in this study (more than 3 h after the first stimulus). Interspecific differences in the structure of the protein would also seem possible, although the *c-fos* gene shows a strong homology between species (Kindy and Verma, 1990), as do other fish genes in relation to their mammalian counterparts (van Beneden et al., 1986; Molven et al., 1991; Oxtoby and Jowett, 1993; Schreiber-Agus et al., 1993).

A difference in the amino acid sequence outside the conserved region would not prevent the antibody from binding to its antigenic site, but could lead to a different tertiary structure of the FOS protein. In such a case, upon binding to other inducible nuclear proteins (dimerization) or to the AP-1 target sequence in the nucleus (Morgan and Curran, 1991), a change in the tertiary folding structure might block the antigenic site

from the antibody. Such a mechanism, combined with a slower cytoplasmic-nuclear turnover, could account for an exclusive cytoplasmic reaction product in the trout.

The inability of the antibody to penetrate the nuclear envelope has to be excluded as the nucleus must at least sometimes be in the plane of sectioning in our preparations, but a nuclear signal was never observed.

4.2. Transcription factors in teleosts

Several inducible transcription factors have been shown to be present and active in teleost fishes. Some of these play a role in development or differentiation, such as MYC (van Beneden et al., 1986; Schreiber-Agus et al., 1993), WNT-1 (Molven et al., 1991) and KROX-20 (Oxtoby and Jowett, 1993). Optic nerve regeneration is capable of inducing FOS, MYC (Stein-Izsak et al., 1987), JUN and CREB (Herdegen et al., 1993) over a time course that corresponds with the expression of regeneration-associated proteins, but in the latter, immunohistochemical study, no nuclear labelling for FOS, FOS B or KROX-24 could be detected. The JUN and CREB proteins showed an intracellular distribution that is similar (i.e., nuclear) to that of mammals.

In the present study we have obtained an exclusively cytoplasmic FOS-like reaction, which, relative to mammalian preparations, must be denoted as persistent. We have not tested for other transcription factors and therefore do not know whether the observed increase in FOS-like immunoreactivity following neuronal activity is associated with an increase in other transcription factors. A relatively persistent expression has also been found for JUN and CREB in the goldfish (Herdegen et al., 1993).

At present we do not know which factor induces the FOS-like reactivity in the activated neurons nor which genes are the target of the FOS-like protein. The gene encoding for the FOS-like product is most likely activated via depolarization rather than via a transsynaptic mechanism, as a similar reaction is found after antidromic activation (Exp. 1). The stimuli we have used are powerful and probably lead to complete transmitter-depletion. Activation of genes encoding for transmitter substances, or for proteins used in transmitter transport to the terminals might be a role for the protein that we have observed following stimulation in the present study.

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