Three brainstem nuclei of the rat share cytoplasmic Fos-like immunoreactivity

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SUMMARY

We investigated the neuronal origin of Fos-like immunopositive nervous fibers recently observed in our laboratory. Two different anti-Fos antisera, from Genosys and from Calbiochem, were used. They were tested in both non-stimulated and noxious-stimulated rats. A strong and Golgi-like immunoreactivity in the cytoplasmic compartment of neurons was detected only when using anti-Fos from Genosys. Cytoplasmic labelled neurons were localized at the level of nucleus O, central gray *pars alpha,* and nucleus raphe pontis. This non-nuclear labelling remained constant irrespective of whether the noxious stimulation was applied or not. These results indicate that the neurons of these three brainstem nuclei share the property of being labelled by the Genosys polyclonal anti-Fos antibody, suggesting a closer relationship among them than reported to date.

Key Words: Nucleus O – Central gray pars alpha – Nucleus raphe pontis – Fos – Genosys – Rat

INTRODUCTION

Fos is the 380-amino-acid protein product of the proto-oncogene c-*fos*. It binds to DNA and regulates the transcription of nearby genes (Distel and Spiegelman, 1990; Ransone and Verma, 1990; Sheng and Greenberg, 1990). Since Fos expression can be induced by a wide variety of extracellular stimuli (for a review see Morgan and Curran, 1991 and Herrera and Robertson, 1996), Fos has been proposed to act as a third messenger that links extracellular signals to long-term alterations in gene expression (Curran et al., 1990). Despite basal Fos expression being relatively high in several structures of the central nervous system (Herdegen et al., 1995; Valverde-Navarro et al., 1996), it has been widely used as a cellular marker in neuronal pathway tracing (Sagar et al., 1988; Dragunow and Faull, 1989).

Immunocytochemical labelling to Fos protein locates in the nucleus of neurons. Labelled cells appear as stained, rounded or eliptic dots at light microscopy (Smith and Day, 1993). The nuclear location of Fos reaction has been confirmed by electron microscopy associated with the euchromatin (Sambucetti and Curran, 1986; Mugnaini et al., 1989). No reference has been found indicating Fos-like immunoreactivity outside the neuronal nuclei. In previous studies, however, we have observed the existence of fibers specifically labelled when an anti-Fos antibody from Genosys was used. Far from being a background staining, Fos-positive fibers and terminals were identified in specific nuclei of the central nervous system (Olucha et al., 1995). Thus, we decided to identify the cell bodies from which labelled fibers should arise.

The aim of this paper is to study the distribution of cytoplasmic labelled cells after using the polyclonal anti-Fos antibody from Genosys. Moreover, we wished to clarify whether this non-nuclear Fos-like labelling changes after stimulation and whether it is visible using another anti-Fos antiserum different to that from Genosys.

MATERIALS AND METHODS

Animals and Fos induction

Experiments were performed on adult male Sprague-Dawley rats weighing between 300-350 g (Animal Facility, Valencia University). Animals

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were randomly assigned to the stimulated or to the non-stimulated group. The formalin test (Dubuisson and Dennis, 1977; Porro and Cavazzuti, 1993) was chosen as the stimulation procedure (n=7), since pain is a widely used method to induce Fos expression (for a review see Harris, 1998). Noxious stimulation was applied in conscious animals since anaesthesia has been shown to induce Fos expression (Takayama et al., 1994). Non-stimulated animals (n=7) were included as a control group. All experimental procedures were approved by the Animal Care and Use Committee of the University of Valencia.

Perfusion

Directly from the facility (control group) or one hour after noxious stimulation (stimulated group), rats were deeply anaesthetized with an overdose of sodium pentobarbital (120 mg/kg, i.p.). They were then perfused transcardially with 150 ml of heparinized (15000 UI/l) saline, followed by 500 ml of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were removed, postfixed overnight in the same fixative at 4ºC, and cryoprotected with 30% sucrose in phosphate-buffered saline (PBS, 0.01M, pH 7.4, 24h). Brains were cut on a cryostat (40 µm coronal sections) and sections were collected in six equivalent series.

Primary antisera

Two different polyclonal antisera against a conserved region of the *N*-terminal mouse and human Fos protein were used to perform immunocytochemistry; one from Calbiochem raised in rabbit against Fos residues 4 to 17 (1:10000) and the other from Genosys, raised in sheep against residues 2 to 17 (1:1000).

Immunocytochemistry

Free-floating tissue sections were processed for Fos-like immunocytochemistry at room temperature, unless otherwise indicated, according to the following protocol. Endogenous peroxidase activity was inhibited by washing sections for 30 minutes in 1% hydrogen peroxide (H_2O_2) in 0.01M PBS. Sections were then incubated for 2 hours in 3% bovine serum albumin (BSA) to avoid the unwanted unspecific interactions before incubation in primary antibodies against Fos protein. Primary antibodies were used diluted 1:10000 (rabbit anti-Fos, Calbiochem) and 1:1000 (sheep anti-Fos, Genosys) in a PBS solution with 0.3% BSA and 0.1% Triton X-100. After 48 hours, sections were incubated for 2 hours in the appropriate secondary biotinylated antibody: goat anti-rabbit or rabbit anti-sheep. The avidin-biotin-peroxidase technique was then applied for one hour. Finally, the reaction was visualized using diaminobenzidine as chromogen, diluted in a Tris-buffered saline (TBS, 0.05M, pH 8.00) solution with 0.01% H_2O_2, enhanced with 0.1% $NiNH_4SO_4$, until optimal staining intensity was achieved. The reaction was then terminated by adding excess TBS. Sections were mounted onto chrome alum gelatin-coated slides and air dried before being dehydrated via a graded ethanol series, cleared in xylene and coverslipped with Merckoglass. Sections of each series were studied under brightfield microscopy (Nikon Eclipse E600). All labelled neurons were plotted with the aid of a camera lucida drawing tube (Zeiss Axioskop).

Two extra non-stimulated rats were perfused with the same fixative solution with the addition of 0.1% glutaraldehyde. The brains of these animals were not frozen and coronal sections of 40µm thickness were obtained with a vibratome. After the pre-embedding immunocytochemical detection of the Fos protein, following the same procedure mentioned above, sections were placed in 2% osmium tetroxide in 0.1M phosphate buffer (pH 7,4) for 2 hours, dehydrated in ethanol and embedded between two smooth sheets of plastic in a drop of Araldite. The embedded sections were examined under a light microscope to identify cytoplasmic Fos-like immunoreactivity. Selected areas were cut from the section and glued onto the base of an Araldite cylinder. Semithin 2 µm sections were obtained with an ultramicrotome and counterstained with toluidine blue. These sections were used for qualitative observations at light microscopical level.

To establish the specificity of the primary antibody, omission of the Fos antiserum from the immunostaining procedure was tested, no neuronal labelling being obtained.

RESULTS

Nuclear labelling

Fos nuclear labelling was observed when both Calbiochem and Genosys polyclonal c-*fos* antisera were used. The distribution pattern of Fos nuclear labelling was in accordance with previous studies, both in the control group and after noxious stimulation. The formalin test resulted in the activation of a large number of hindbrain and forebrain areas (Lantéri-Minet et al., 1994), whereas the control animals displayed Fos-like immunoreactive nuclei in a significantly lower number of areas of the central nervous system (Herdegen et al., 1995). No significant differences were observed when Calbiochem or Genosys anti-Fos were used. A detailed description of the Fos nuclear labelling distribution was outside the scope of this paper.

Cytoplasmic labelling

In both the control and stimulated groups, cytoplasmic labelling was observed only when

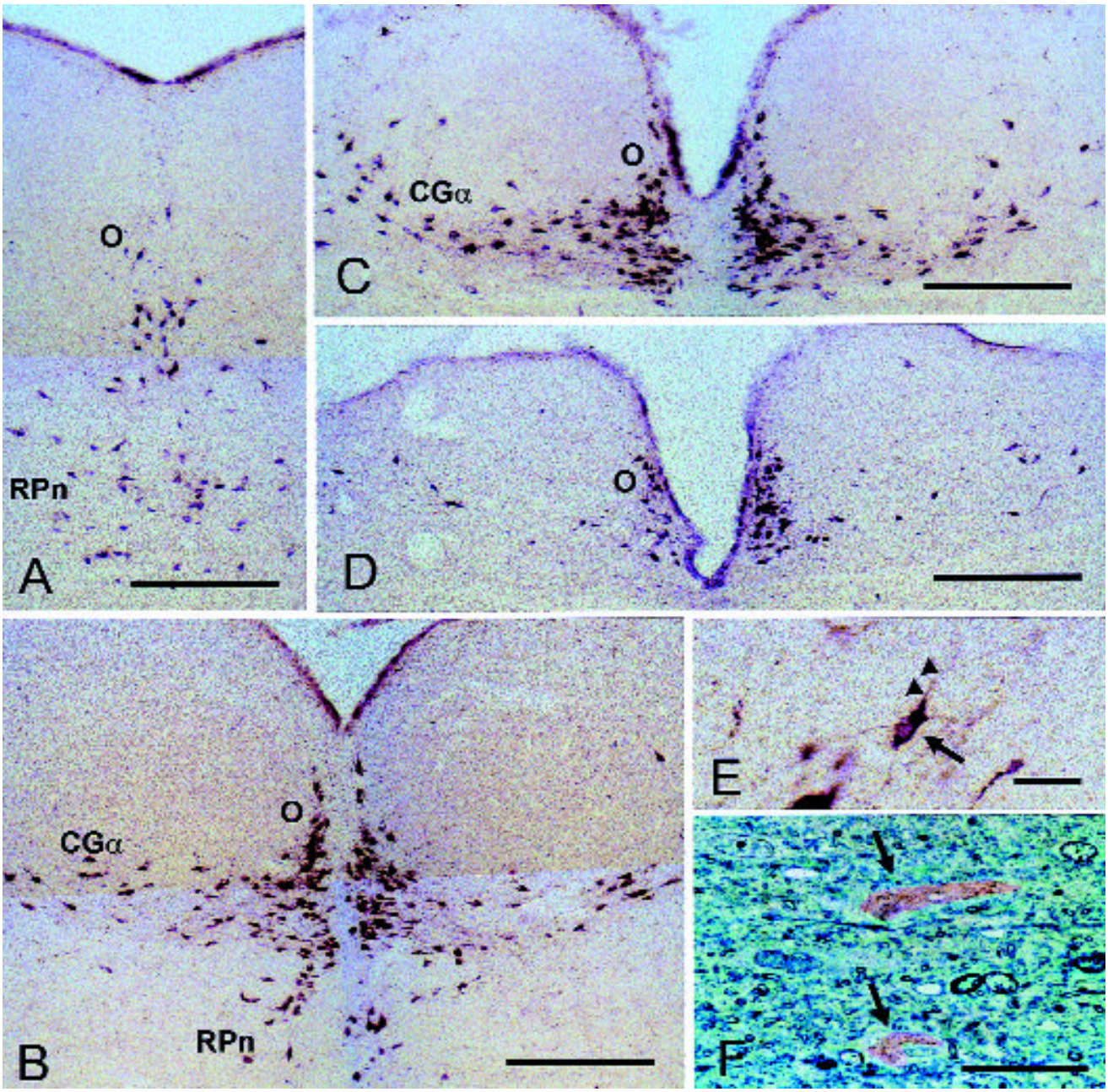

Fig. 1.- Low-power photomicrographs showing cytoplasmic Fos-like immunoreactivity. **A**: At the level of the nucleus raphe pontis and rostral-most portion of the nucleus O; **B** and **C**: at the level of the nucleus O and central gray *pars alpha*, and **D**: at the caudal-most portion of the nucleus O. **E**: High-power photomicrograph at central gray *pars alpha* showing a labelled neuron. The cytoplasmic compartment (arrow) and processes (arrowheads) are immunopositive whereas the nucleus is immunonegative. **F**: Semithin photomicrograph showing the immunoproduct (brown) located in the cytoplasm but not inside the nucleus of two neurons (arrows). A-D, Scale bar corresponds to 300 µm. E and F, Scale bar corresponds to 30 µm.

Genosys polyclonal sheep anti-Fos was used. This non-nuclear labelling was present in the perikarya, primary dendrites and fibers, leaving the nucleus free. Many fibers were labelled throughout the brain when the Genosys anti-Fos antiserum was used. A preliminary description of their distribution can be found in Olucha et al. (1995).

Neurons undoubtedly exhibiting Fos-like immunoreactivity in the cytoplasm, but not into the nucleus, were located mainly in the ventral part of the caudal pontine tegmentum, immediately dorsal to the medial longitudinal fasciculus and ventromedial to the posterodorsal tegmental nucleus. This area includes the nucleus O (Fig. 1B-D) and central gray *pars alpha* (Fig. 1B-C), from Bregma -9.68 mm to Bregma -10.04 mm, according to Paxinos and Watson (1986). In our study, cytoplasmic labelled neurons also extended to the rostral-most nucleus raphe pontis, at Bregma -9.30 mm and -9.68 mm (Fig. 1A-B). No cytoplasmic labelled neurons were observed in the surrounding pontinemesencephalic nuclei, posterodorsal tegmental nucleus, dorsal tegmental nucleus (central and pericentral divisions) and laterodorsal tegmental nucleus (Fig. 2). The medulla oblongata was also free of labelling.

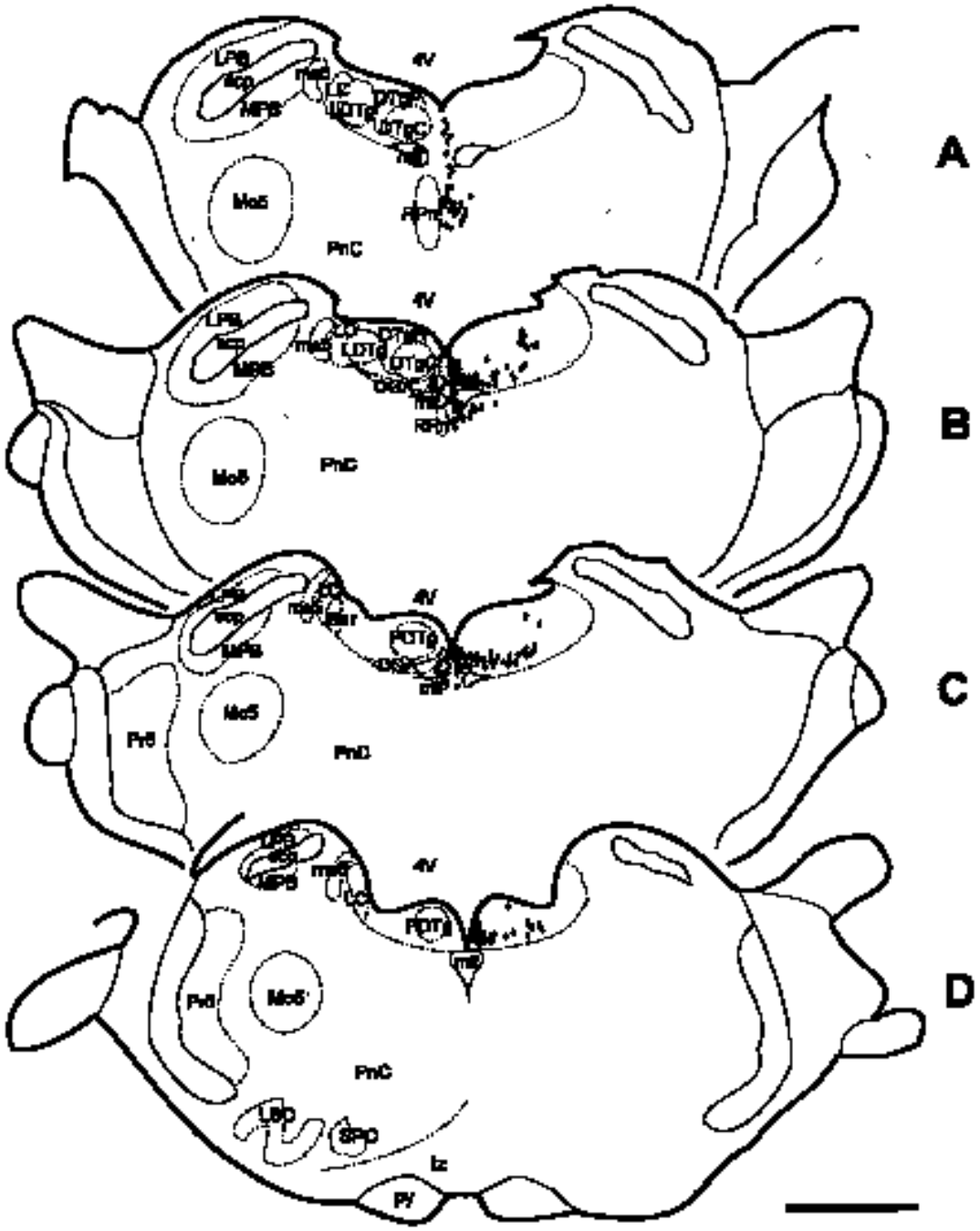

Fig. 2.- Schematic drawing showing non-nuclear Fos-like immunoreactive cells in coronal planes of the lower brain stem of the rat when the Genosys antibody to Fos was used. Right half shows labelled cells in the nucleus raphe pontis, the nucleus O and central gray *pars alpha*. In the left half, the distribution of immunoreactivity has been omitted. (**A**) Bregma -9.30 mm. (**B**) Bregma -9.68 mm. (**C**) Bregma -9.80 mm. (**D**) Bregma -10.04 mm., according to the atlas of Paxinos and Watson (1986). Scale bar corresponds to 1000 µm.

No double labelled (nucleus and cytoplasm) cells were observed. Neurons from the three brainstem structures displaying a Golgi-like labelled cytoplasm showed an immunonegative nucleus. The non-nuclear immunoreactive product was visualized not only in the perikarya but also in primary dendrites. Labelled neurons displayed a fusiform, triangular and even rounded morphology (Fig. 1E).

Study of the immunopositive cells with the immersion objective allowed us to confirm that labelled cells were indeed neurons, not glial cells. Study of the semithin sections also confirmed that the immunoreactive product was localized within the cytoplasmic compartment of the neurons, leaving the nucleus free of immunoreactivity (Fig. 1F).

Discussion

To our knowledge, here we report for the first time a non-nuclear Fos-like immunoreactivity. Fos, the protein product of the proto-oncogen c-*fos*, is known to be located in the nucleus of neurons, bound to DNA (Sambucetti and Curran,

1986; Mugnaini et al., 1989), and to regulate the transcription of nearby genes (Distel and Spiegelman, 1990; Ransone and Verma, 1990; Sheng and Greenberg, 1990). However, our findings reveal cytoplasmic anti-Fos labelling mainly in the neurons of the nucleus O, central gray *pars alpha* and nucleus raphe pontis when the Genosys polyclonal sheep anti-Fos (residues 2 to 17) antiserum was used. It completely disappeared, however, when a similar, but not identical, rabbit anti-Fos (amino acids 4 to 17) was used.

The main defining property of the immediate early genes, such as the c-*fos* proto-oncogene, is that they can be transiently and rapidly induced by a variety of stimuli such as pain, light, sound, etc. (Morgan and Curran, 1991; Herrera and Robertson, 1996) This feature has favored the notion that the immunocytochemical detection of Fos can be used as a cellular marker of neuronal activation (Sagar et al., 1988; Dragunow and Faull, 1989; Bullit, 1990; Harris, 1998). However, this non-nuclear Fos-positive labelling remains constant in both control and stimulated groups. This lack of fluctuation following external stimulation, together with the extra-nuclear location, suggests that cytoplasmic labelling does not correspond to the nuclear phosphoprotein Fos. However, whether this labelling identifies a different protein with a common sequence of aminoacids corresponding to residues 2 to 17 of the nuclear Fos, or whether it is due to a cross-reaction derived from the Genosys method of raising and purifying the anti-Fos antiserum cannot be determined from the present data.

If the first assumption is correct, since the labelled protein is readily detectable by using a specific Fos amino acid sequence antiserum, this sequence of residues should be common to both the Fos protein and the labelled non-nuclear and non-inducible protein. Nonetheless, we searched different protein and DNA sequence data banks (FASTA, SSEARCH, BLAST and PepSearch) for the Genosys immunogen sequence MFSGFNADYEASSSRC (Fos residues 2 to 17) and it was not identified in any protein, except Fos or Fos-related proteins. We would be faced with a non-identified protein. In such a case, it would be very interesting to run a Western blot, cut out the corresponding band on the gel, and sequence it, attempting to characterize the protein.

In favor of the second possibility, we found that the Calbiochem antigenic sequence (Fos residues 4 to 17) is almost the same as that from Genosys, except for the lack of the two first amino acids (Fos residues 2 to 17). This small difference between the immunogens could also point to the complex resulting from the binding of the synthetic Fos peptide to the carrier and/or the host splicing during antiserum production as the bases for their differing immunoreactivity. As mentioned above, to determine whether this non-nuclear labelling is due to a Fos (or Fos-related) protein or to a new unexpected protein, blocking the immunocytochemical labelling with a corresponding peptide, or performing a Western blot of the pontine region with the same antibodies (Genosys and Calbiochem) to reveal a protein migrating at a size specific for the Genosys antiserum would be the next steps to be carried out.

We found no reference to any cytoplasmic labelling restricted to these three brainstem nuclei. The most comprehensive report is that of Paxinos and Butcher (1986). These authors attribute the term nucleus O to Meessen and Olszewski (1949) concerning a paired nucleus identified in the ventral part of the caudal pontine central gray. This nucleus is directly above the medial longitudinal fasciculus and ventromedial to the posterodorsal tegmental nucleus. In the same paper, they define the central gray *pars alpha* as a differentiated part of the central gray lateral to the nucleus O. Later, however, there has been no agreement on this point. Some authors have failed to distinguish the nucleus O from central gray *pars alpha*, giving both nuclei the term nucleus O (Takagi et al., 1980; Kubota et al., 1983), or dorsal tegmental nucleus *pars ventromedialis* (Morest, 1961; Cowan et al., 1964), whereas others have followed Messen and Olszewski´s nomenclature (Petrovicky, 1973; Tohyama et al., 1978; Riley and Moore, 1981; Paxinos and Butcher, 1986). Both nuclei are clearly identified in Nissl-stained sections, the nucleus O cells being larger and more densely stained, as well as in acetylcholinesterase-stained sections (Paxinos and Butcher, 1986). Cells of both nuclei also contain neurotensin (Jennes et al., 1982) and cholecystokinin (Kubota et al., 1983). Nevertheless, mRNA for a metabotropic glutamate receptor, mGluR3, was seen only in the nucleus O, but not in the central gray *pars alpha* or in the nucleus raphe pontis (Ohishi et al., 1993). The lack of serotonin does not permit their inclusion in the dorsal raphe, contrary to what some authors have considered (Jennes et al., 1982).

Regarding the connections of these nuclei, Vertes (1988) observed that injection of WGA-HRP into the medial septum and vertical limb of the diagonal band nucleus resulted in dense retrograde cell labelling in the nucleus raphe pontis, central gray *pars alpha*, as well as in the nucleus O, named nucleus incertus in accordance with Berman´s terminology in the cat (Berman, 1968).

More recently, nuclear expression of the c-*fos* proto-oncogene has been reported in the nucleus O and central gray *pars alpha* following peripheral injection of chemical irritants administered under brief halothane anaesthesia

(Lanteri-Minet et al., 1994; Bon et al., 1996). In all cases, the anti-Fos antibodies used were generated by the authors or by a colleague, respectively. Induction of Fos in these two nuclei was attributed to the anaesthesia rather than to noxious stimulation.

Concluding remarks

The strength and high degree of specificity of the cytoplasmic Fos labelling found in our sections renders the idea of gaining further insight into this putative system very attractive. As mentioned above, the following step would be to isolate and characterize the cytoplasmic antigen marked with the Genosys sheep anti-Fos and to raise specific antiserum as well. Further, the distribution pattern of the Genosys Fos-like immunoreactive fibers could yield many data on related structures and may hence be of functional significance.

Our results show that the neurons of these three pontine nuclei share the property of being labelled by the Genosys polyclonal anti-Fos antibody. Regardless of the fact that Genosys extra-nuclear Fos-like labelling belongs to a novel protein or a well-known cross-reacted protein, the important finding of this work is the demonstration that the previously observed Genosys polyclonal anti-Fos labelled fibers and terminals (Olucha et al., 1995) arise from a small number of neurons grouped within three pontine nuclei: the nucleus O, central gray *pars alpha*, and nucleus of the raphe pontis, suggesting a closer relationship among them than anatomical or physiological studies have thus far reported.

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Abbreviations

4V	fourth ventricle
Bar	Barrington´s nucleus
BSA	bovine serum albumine
CGA	central gray *pars alpha*
DTgC	dorsal tegmental nucleus, central
DTgP	dorsal tegmental nucleus, pericentral
LC	locus coeruleus
LDTg	laterodorsal tegmental nucleus
LPB	lateral parabrachial nucleus
LSO	lateral superior olive
me5	mesencephalic trigeminal tract
mlf	medial longitudinal fasciculus
Mo5	motor trigeminal nucleus
MPB	medial parabrachial nucleus
O	nucleus O
PBS	phosphate-buffered saline
PDTg	posterodorsal tegmental nucleus
PnC	pontine reticular nucleus, caudal
Pr5	principal sensorial trigeminal nucleus
py	pyramidal tract
RPn	raphe pontis nucleus
scp	superior cerebellar peduncle
SPO	superior paraolivary nucleus
TBS	tris-buffered saline
tz	trapezoid body

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