DETECTION OF OXYTOCIN mRNA IN HYPERTONIC SALINE FOS-ACTIVATED PVN NEURONS: COMPARISON OF CHROMOGENS IN DUAL IMMUNOCYTOCHEMICAL AND IN SITU HYBRIDIZATION PROCEDURE

ZDENO PIRNIK, ALEXANDER KISS

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlarska 3, 833 06 Bratislava, Slovakia
E-mail:ueenpirn@ savba.sk

Objective. The aim of the present experiments was to elucidate: 1. the stability and usefulness of a 3,3´-diaminobenzidine tetrahydrochloride (DAB) chromogen intensified with nickel and cobalt (DAB-Ni-Co) in the dual immunocytochemical and *in situ* hybridization procedure using Fos-protein antibody and oxytocin mRNA (OXY mRNA) radiolabeled probe; 2. the susceptibility of the free floating and mounted cryostat sections, freshly prepared or stored for 24 month at –20 °C.

Methods. The dual staining procedure was tested on neurons of the hypothalamic paraventricular nucleus (PVN) activated by an intraperitoneal injection of hypertonic saline (HS, 1.5 M, 5 ml, 60 min). Two dual labeling procedures were compared: 1/ Fos-immunostaining with DAB alone and combined with OXY mRNA *in situ* hybridization and 2/ Fos-immunostaining with DAB-Ni-Co and combined with OXY mRNA *in situ* hybridization. In both experiments free floating and mounted cryostat sections, freshly prepared or stored for 24 month at –20 °C, were tested.

Results. HS strongly stimulated both the parvicellular and magnocellular population of PVN neurons followed by an extensive Fos-immunolabeling in many cell nuclei. The first staining sequence with Fos-DAB labeling resulted in a good staining quality on both the fresh and for 24 month stored mounted sections. Although the free floating sections during the *in situ* procedure showed the same staining properties as the mounted ones, with respect to their increased fragility on the end of the hybridization procedure, they were difficult to mount and stretch on poly-L-lysine coated slides. On the other hand, Fos-immunolabeling with DAB-Ni-Co exhibited improved staining density of the single DAB chromogen in the first staining sequence of the dual staining procedure. However, DAB-Ni-Co mixture showed up as an unstable chromogen complex which after completing the *in situ* hybridization process completely disappeared from each type of section.

Conclusions. The results of the present dual immunocytochemical-*in situ* hybridization staining utilizing Fos-antibody and OXY mRNA oligoprobe indicate that this procedure is applicable on free floating as well as mounted cryostat sections, freshly prepared or stored for 24 month at –20 °C. However, the dual procedure is only successful when the immunoproduct in the first sequence is visualized with an unintensified DAB and not with combined DAB-Ni-Co chromogen and when the histological sections in the second sequence are not processed as free floating but are attached to a poly-L-lysine coated microscopic glasses.

Key words: Fos immunohistochemistry – DAB and DAB-Ni-Co stainings - OXY mRNA *in situ* hybridization – Dual immunocytochemistry - *in situ* hybridization histochemistry - Rat

During the last few decades a number of different neuromorphological techniques have been introduced, mutually combined and extensively applied in the neurobiological research (GERFEN and

Sawchenko 1985; Kiss et al. 1988; Aguilera et al. 1995; Coolen et al. 1999). Immunolabeling of Fos-protein, a product of the immediate early gene c-fos, made possible to perform spatial demonstration of activated neurons, evaluation of morphological-functional relationships in the brain (Ceccatelli et al. 1989; Campeau et al. 1991; Duncan et al. 1993; Palkovits et al. 1997; Briski and Gillen 2001) and follow the activity of neurons with a single cell resolution (Staines et al. 1988). However, Fos immunohistochemistry itself can not reveal the phenotype character of the Fos-labeled neurons, since the standard double-labeling immunohistochemical technique is not successful when the basal content of the studied protein is too low or even undetectable i.e. corticotropin-releasing hormone peptide in the hypothalamic paraventricular nucleus (Kiss and Aguilera 1992, Kiss and Jezova 1998). Recently, however several immunohistochemical staining procedures were combined with *in situ* hybridization techniques. In these studies different methodical approaches have been tested and working conditions recomended including the order of the staining processes and the advantages and disadvantages of the substances applied (Young et al. 1986; Trembleau et al. 1988; Meister et al. 1990; Larsen et al. 1993; Fetissov et al. 1997; Cloez-Tayarani and Fillion 1997; Fetissov and Marsais 1999).

The present work was focused to compare the stainings properties of the single DAB chromogen with DAB accentuated with nickel and cobalt in the dual immunocytochemical-*in situ* hybridization procedure employing differently processed brain tissue. The aim of the study was to find out whether: 1. the intensified DAB-Ni-Co mixture might improve the quality of a single DAB Fos-immunostaining in the dual immunocytochemical-*in situ* hybridization procedure; 2. the quality of the immunocytochemical-*in situ* hybridization double-labeling might substantially be influenced by the character of the histological material used. In the experiments Fos-protein antibody was combined with oxytocin mRNA radiolabeled probe and processed on neurons of the PVN activated by i.p. injection of the hypertonic saline. In addition, the susceptibility of free floating and mounted cryostat sections, freshly prepared or stored for 24 month at –20 °C was tested.

Materials and Methods

Animals. Adult male Wistar rats weighing 200-250 g were used in the experiments. The animals were housed in a room with controlled light (14 h/day) and temperature (23 °C). They received regular rat chow (dry pellets) and tap water *ad libitum*. Principles of laboratory animal care and all procedures were approved by the Animal Care Committee of the IEE SAS Bratislava, Slovak Republic. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Treatments. Neurons of the hypothalamic paraventricular nucleus were stimulated by intraperitoneal injection of hypertonic saline (5 ml of 1.5 M) (Kiss and Aguilera, 1993). Normal saline controls (0.9 % NaCl) and HS treated rats were sacrificed 60 min after the injection. All the animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg i.p.) and perfused transcardially with 50 ml of ice-cold 0.9 % saline containing 150 μl of heparin (5000 IU/l) followed by 250 ml of 0.1 M phosphate buffer (PB, pH 7.4) containing 4 % paraformaldehyde, 0.1 % glutaraldehyde, and 15 % saturated picric acid. The brains were immediately removed and post-fixed overnight in the fresh fixative at 4 °C and then sunk in 15 % sucrose in PB for 48 h. Brain blocks were frozen at – 70 °C for 60 min and then frontally cut at –16 °C into alternate sets of 40 μm thick sections using a Reichert-Jung freezing microtome. This set of sections was collected in vials with cold PB. Another set of sections kept in a cryoprotectant solution (30 ml ethylene glycol, Fisher, 30 g sucrose, 1 g polyvinilpyrrolidone PVP-40 Sigma, adjusted to 100 ml with 0.1 M PB) at –20 °C for 24 month was also transfered into vials with cold PB.

Fos immunohistochemistry. Freshly prepared or for 24 month stored free floating brain sections were rinsed in 0.1 M glycine (pH 6.5, Sigma) for 60 min and washed in cold PB (3 x 10 min) followed by immersion in PB containing 3 % hydrogen peroxide for 60 min. After several washings in PB the sections were incubated with a polyclonal rabbit Fos-antiserum (a gift from Dr. Mikkelsen, Denmark, dilution 1:2000) in PB containing 4 % normal goat serum, 0.5 % Triton X-100, and 0.1 % sodium azide

for 48 h at 4 °C. Then the sections were several time rinsed in PB and subsequently incubated with a goat antirabbit IgG-HRP complex (1:500, Jackson ImmunoResearch, West Grove, PA) in 0.1 M PB containing 0.5 % Triton X-100 for 2 h at room temperature. Next PB rinsings (2 x 10 min) were followed by washing in 0.05 M acetate buffer (AB, pH 6). The visualization of the Fos was performed with a single DAB (Sigma) or with DAB intensified with density enhancers (0.5 g nickel ammonium sulfate and 0.5 g cobalt chloride, Sigma). In both cases 25 mg DAB was dissolved in 40 ml of AB and the chromogen reaction was running for 8 min in the presence of 40 µl of 30 % hydrogen peroxide. After several washings in AB, the DAB and DAB-Ni-Co stained fresh and stored sections were collected either as free floating in glass vials with AB and kept overnight at 4 °C or as mounted on the poly-L-lysine coated microscopic slides and left overnight at the room temperature.

A. OXY mRNA *in situ* hybridization of free floating Fos-labeled sections. In situ hybridization of Fos-immunolabeled (with DAB and DAB-Ni-Co) fresh or stored free floating sections was performed in 3 ml vials using 500 µl volume of solutions. The procedure started with acetylation for 15 min (with respect to the perfusion the fixation step was omited) in solution containing 0.1 M trietanolamine hydrochloride (TEA, pH 8.0), 0.25 % acetic anhydride and 0.42 % HCl dissolved in 0.1 % diethylpyrocarbonate-treated (DPCT) water. Thereafter the sections were dehydrated through 70 % and 80 % ethanol (1 min each) followed by 95 % and absolute ethanol (2 min each). Then the sections were transfered into 500 µl of hybridization solution containing 4 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 50 % formamide (Merck), 10 % dextran sulfate (Sigma), 1 x Denhardt's solution (Sigma), 250 mg/ml yeast tRNA (Sigma), 500 µg/ml sheared salmon sperm DNA (Stratagene, USA), 100 mM dithiothreitol (DTT, 1ml 5 M) (Sigma) and 12 x 10^6 dpm per 100 µl of the labeled probe oxytocin (48 oligonucleotides, 247-294) (a gift from Dr. G. Aguilera, NIH, USA) synthetized by Synthecell (Rockville, MD, USA). The probes were 3'-end–labeled with [35S]-dATP (1,200 Ci/mmol, NEN, DuPont, Boston, Ma, USA) using terminal deoxynucleotidyl transferase (Boehringer Mannheim GmbH, Vienna, Austria). The hybridization was carried out in closed vials for 24 h at 42 °C. After hybridization, the sections were washed in 1 x SSC at room temperature (RT) (5 min/wash, 3 washes in total) and then were removed in new vials with 1 x SSC. Then followed washing in 4 x SSC (5 min/wash, 2 washes in total at RT), 1 x SSC (30 min/wash, 2 washes in total at 50 °C), 0.1 x SSC (15 min/wash at 50 °C and 15 min/wash at RT). Finally the sections were mounted on poly-L-lysine coated slides, quickly rinsed in 70 % ethanol and air-dried.

B. OXY mRNA *in situ* hybridization of slide-mounted Fos-labeled sections. Slide-mounted Fos-labeled tissue sections (with DAB or DAB-Ni-Co) were brougth to room temperature, fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 10 min, washed in three changes of PB (5 min each), acetylated (15 min) with solution containing 0.25 % acetic anhydride, 0.1 M TEA (pH 8.0) and 0.42 % HCl dissolved in DPCT water**.** Then the slides were rinsed in PB (5 min), dehydrated through graded ethanol solutions 70 % and 80 % (1 min each), 95 % (2 min) and absolute ethanol (1 min) and air dried. The slides were hybridizated for 18 h at 42 °C with the same hybridization solution as mentioned above (in case A). Then followed washing in 4 x SSC (5 min/wash, 2 washes in total at RT), 1 x SSC (15 min/wash, 2 washes in total at 50 °C), 0.1 x SSC (30 min/wash at 50 °C and 30 min/wash at RT) and 70 % ethanol (2 min). Then the sections were air-dried.

Visualization. After hybridization the sections from each group were apposed to Hyperfilm β-Max autoradiography film (Amersham, Buckinghamshire, UK) at room temperature for 4 days to visualize target OXY mRNA expression in the PVN. Selected slides were then dipped in Kodak NTB2 emulsion diluted 3:1 with distilled water and stored at 4 °C for 5 days, developed with D19 developer and counterstained with thionin. Dipped slides were evaluated under a light microscope (Nikon Coolpix 990). The photomicrographs were taken through a digital camera (Nikon Eclipse 600) mounted on the microscope using Nomarski DIC optics and the pictures proceesed in a Photo-shop software computer program.

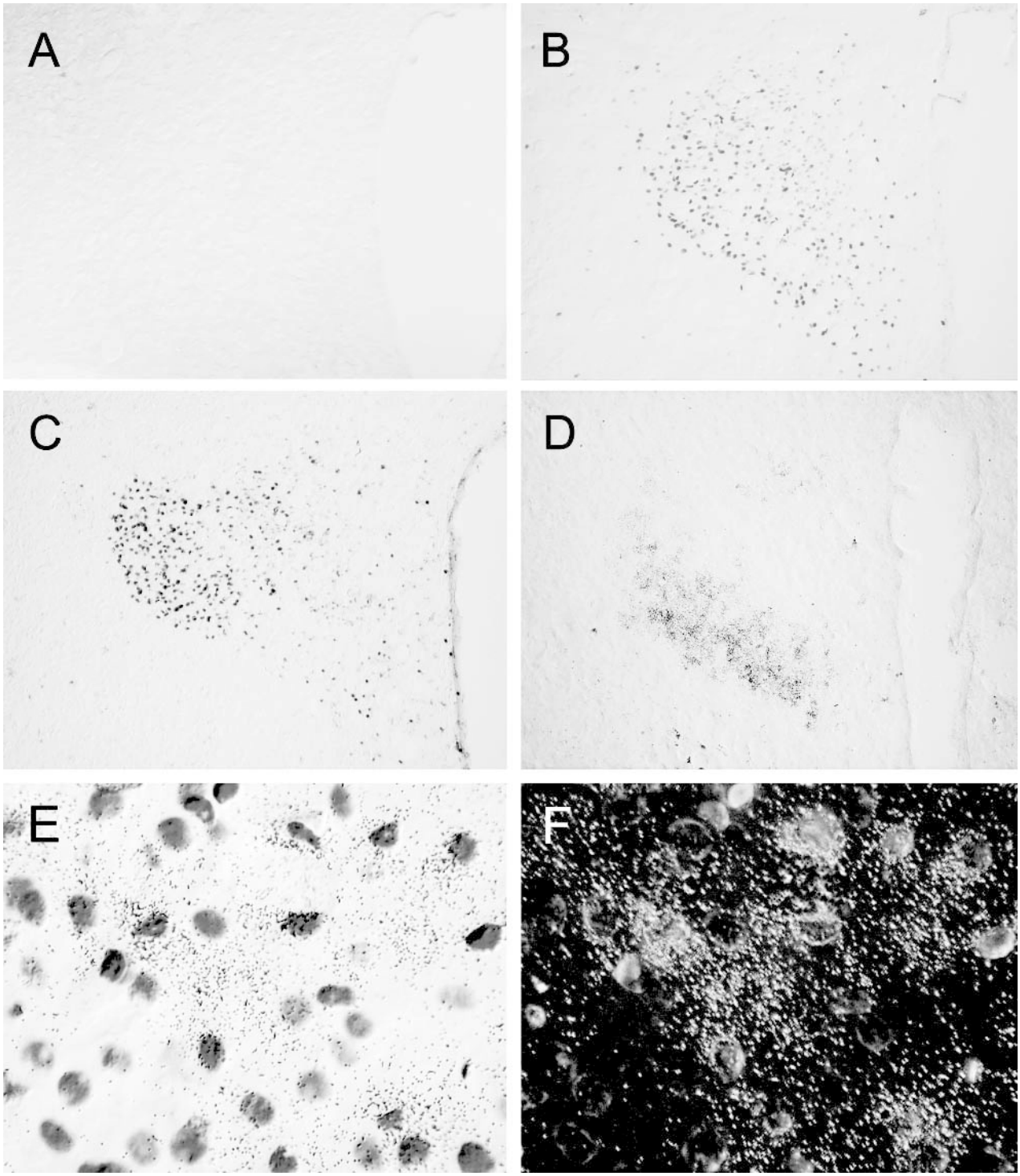

Fig. 1 Free floating cryostat sections immunoprocessed for Fos-peptide and mounted sections hybridized in situ for OXY mRNA in rats injected with HS. No Fos-labeling was seen in controls (A). Extensive Fos-labeling was observed in HS rats stained with DAB (B) as well as with DAB-Ni-Co complex (C) which increased the staining intensity of the single DAB. Disappearance of DAB-Ni-Co stained Fos nuclei from the PVN after completing the second sequence of the dual immuno-*in situ* hybridization procedure did not influence the OXY mRNA signal in the PVN represented by an accumulation of silver grains (D). Bright-field (E) and dark-field (F) microscopic demonstration of Fos-antibody and OXY mRNA after completing the dual immunohistochemical-*in situ* hybridization staining on a single cryostat section using Nomarski DIC optics.

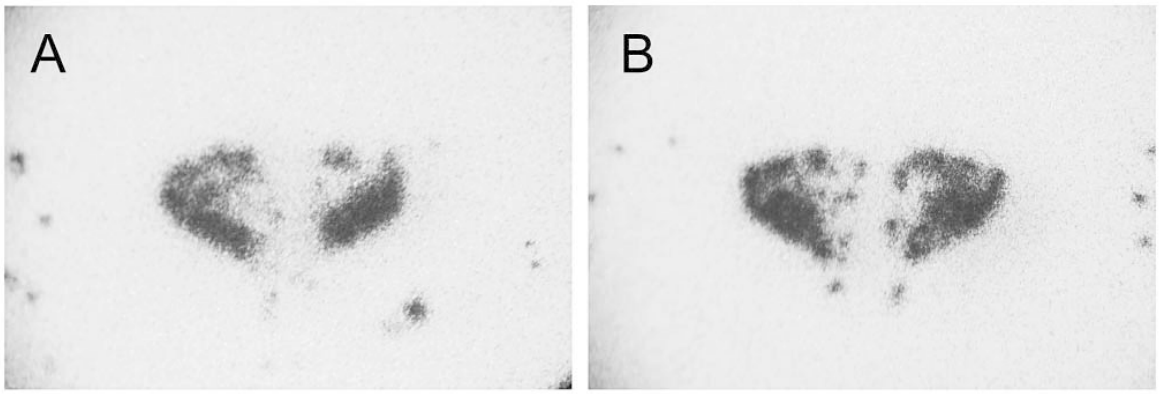

Fig. 2 Autoradiographic picture of the OXY mRNA distributed over the posterior magnocellular subdivision of the PVN 60 min after the i.p. injection of HS. Note that the radioactive signal developed on the β-Max autoradiography hyperfilm exhibites similar staining magnitude on both DAB Fos-positive (A) and DAB-Ni-Co Fos-negative (B) immunostained cryostat sections.

T a b l e 1
Demonstration of the presence or absence of the c-Fos and OXY mRNA signals during and after the processing of the dual immuno-*in situ* hybridization histochemistry by employing the DAB and DAB-Ni-Co chromogen stainings

stained with	type of section	Fos immuno-signal	OXY mRNA *in situ*-signal	Fos immuno-signal after *in situ*	Fos, final handling properties of sections
DAB	free floated	+	++	+	fragile
	mounted	+	++	+	OK
DAB-Ni-Co	free floated	++	++	–	fragile
	mounted	++	++	–	OK

Results

In comparison with controls, where no Fos-staining was present on the histological sections (Fig. 1A) the i.p. injection of HS induced activation of both parvicellular and magnocellular neurons in the PVN. This activation of neurons was followed by an extensive Fos-immunostaining with clear predominance in the posterior magnocellular subdivision of the PVN (Fig. 1B) and was recognizable in all types, i.e. freshly and long-lastingly stored, free floating as well as on poly-L-lysine coated microscopic glass mounted cryostat sections.

In the first sequence of the dual immuno-*in situ* hybridization procedure, i.e. immunocytochemical processing, the DAB reaction induced a red-brown color Fos-immunostaining (Fig. 1B), while DAB mixed with cobalt and nickel salts yielded from dark blue to black colored immunoprecipitaces (Fig. 1C) on each type of the histological section. In the second sequence of the dual staining, i.e. *in situ* hybridization procedure, the DAB immunolabeling of Fos remained unchanged while the intensity of DAB-Ni-Co labeled Fos-signal was considerably reduced on each type of the histological section and after completing the hybridization process even entirely eluted (Fig. 1D) from all types of the sections. However, despite the loose of Fos-labeling from the DAB-Ni-Co stained sections both the DAB and DAB-Ni-Co stainings exhibited OXY mRNA autoradiographic signal of a comparable density and magnitude in the PVN, which was well recognizable on the β-Max autoradiography hyperfilm (Fig. 2 A,B). When the DAB stained Fos-positive and DAB-Ni-Co stained Fos-eluted sections were dipped in an NTB2 Kodak emulsion an extensive precipitation of silver grains reflecting the OXY mRNA location in the PVN was detected on all types of histological sections (summarized in Tab. 1). The silver grains were densely accumulated along the lateral border of the posterior magnocellular subdivision of the PVN. In this area of the PVN the dual immuno-*in situ* stainings pro-

cess revealed dense concentration of silver grains in close vicinity with Fos-labeled magnocellular nuclei well recognizable at the level both the bright-field (Fig. 1E) and dark-field (Fig. 1F) microscopy. Finally, when the handling properties of the individual type of sections were compared the free floating sections displayed fragile consistence on the end of the dual procedure. Thus the diversely folded and dually labeled free floating sections were difficult to stretch them on the microscopic glasses.

Discussion

The present study revealed that in contrast to the single DAB staining, the DAB-Ni-Co complex is not enough stable for dual immunocytochemical and *in situ* hybridization staining procedure since after completing the Fos-immunostaining and OXY mRNA *in situ* hybridization procedure Fos-immunolabeling entirely disappeared from the histological sections. Furthermore, the results indicate that both freshly prepared or longlastingly stored cryostat sections are applicable in this technique, however, with respect to the high fragility of free floating sections on the end of the procedure, only mounted sections are reccomended for the second sequence of the dual staining.

It is generally approved that DAB chromogen produces a precipitate of yellow-red-brown color, which is stable for many years. However, in order to visualize the localization of multiple antigens *in situ*, reaction products of contrasting colors are required. DAB-nickel ammonium sulfate solution generating a blue-black reaction product to localize the first antigen and DAB alone generating an amber reaction product to localize the second antigen, have been considered as a stable reaction products providing excellent immunohistochemical color contrasts (Hancock 1984). However, our results indicate that while DAB-Ni complex is successfully applicable in the dual immunocytochemistry (Hancock 1984; Kiss et al. 1988), it is not suitable for dual immuno-*in situ* histochemical stainings, since it is not enough stable and therefore it is completely eluted from the immunostained Fos-labeled structures. The elution process appears to be continual and the prehybridization procedure diminishes already 50 percent of the Fos-density. Improvement in the intensity of DAB-Ni staining of Fos-labeled nuclei by adding a cobalt to the DAB-Ni mixture did not reverse the weak stability of the DAB-Ni-Co staining product. On the other hand, the inconstancy of the DAB-Ni-Co staining had no effect on the quality of the radioactive signal in the *in situ* hybridization sequence of the dual processing. However, important finding was that the lost Fos-immunoproduct in the first sequence of the dual staining process visualized by the DAB-Ni-Co complex instead a single DAB can not be after finishing the dual staining procedure substituted by any additional DAB-Fos-contraimmunolabeling. This finding is comparable with the problems in the creating of the immunohistochemical signal under the circumstances when the immuno-*in situ* hybridization dual processing is set up conversely, i.e. when the immunoprocessing is preceded by the *in situ* hybridization (Young et al. 1986; Fetissov and Marsais 1999; Krukoff et al. 1999a).

Furthermore, several other dual labeling techniques characterizing the phenotype of cells expressing neurotransmitters or specific neuroreceptors have been developed (Young et al. 1986; Cloez-Tayarani and Fillion 1997). However, when *in situ* hybridization was coupled with immunohistochemistry on the same section, a loss of *in situ* signal has also been observed (Young et al. 1986), which might be inconvenient when the rate of mRNA expression is too low. Some of the problems were preceded when the *in situ* hybridization labeling and immunohistochemistry were performed on adjacent paraffin-embedded sections (Cloez-Tayarani and Filiion 1997). In reality, the dual immuno-*in situ* hybridization labeling procedure tested in our conditions by prefering the immunoreaction in the first and the *in situ* hybridization in the second staining sequence, appears to be a very stable exhibiting no hybridization signal loose. In addition, the present dual technique of the simultaneous detection of proteins and mRNAs at the level of a single cryostate section preserves acceptable quality of the labeled structures and its implementation is fast and simple.

On the other hand, our results are in well accordance with works indicating that the DAB-Fos-immunohistolabeling is very stable chromogen suitable for visualization of Fos-protein in combination with mRNA probe of the known phenotype on the same section (Krukoff et al. 1999b). In addition, in this dual procedure we have demonstrated that the DAB

chromogen is applicable with great success for the visualization of Fos-protein not only on freshly prepared material but that also on long-lastingly stored cryostat sections. Finally, it should also be pointed out that the distribution of silver grains around the Fos-labeled neuronal nuclei in the magnocellular subdivision of the PVN reflecting the anatomical exactness of the OXY mRNA location after HS injection is consistent with the topography of the hypothalamic oxytocinergic neurons upon activation by osmotic stress (Giovannelli et al. 1992).

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Corresponding author: Mgs. ZDENO PIRNIK
Institute of Experimental Endocrinology
Slovak Academy of Sciences
Vlárska str. 3
833 06 Bratislava, Slovakia
Tel.:(42102)54772800
fax.:(42102)54774247
E-mail address: ueenpirn@savba.sk