An improved immunohistostaining procedure for peptides in human brain

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Received 18 July 1988; accepted 5 October 1988

Summary. Floating sections from human brains immersed for more than forty years in formalin, or from brains freshly fixed for a short time are treated by KMnO4-Pal's modified solutions to suppress the endogenous peroxidase activity before using the peroxidase-antiperoxidase method (PAP), or to remove the autofluorescence of lipofuscin, which is very intense in brains from old patients, before using the immunofluorescence method. Following this, immersion of sections in NaOH and H_2O_2 allows for the demasking of antigenic sites. These treatments enhance the immunolabelling considerably, with results comparable to those obtained with freshly fixed tissues, and facilitate the discrimination between specifically and unspecifically stained structures.
Key words. Neuropeptides; immunohistochemistry; human brain.

Neurochemical studies of the human central nervous system have increased in the last decade and have complemented the neuroanatomy of the normal and pathological brain [1]. In contrast to the situation in animal experimentation, a number of biological and practical aspects should be considered when using human material, apart from serum specificity problems [2]. All our neurochemical analyses on the human brain depend on postmortem tissue. Biological factors such as medication, neurological and psychiatric state, agonal state, time of day and date of death, age and sex are individual characteristics which can modify the central peptidergic content [3]. On the other hand, numerous studies have now established the comparative resistance of a broad range of biochemical components to postmortem change. Most of the peptides (substance P:SP, somatostatine:SOM, enkephalins:Enk, neuropeptide Y:NPY, cholecystokinin, vasopressin:AVP, oxytocin, neurotensine, neurophysins, DSIP) have been found to be stable up to 1 to 20 h after death [4]. Nevertheless, it is advisable to obtain brain material with the shortest possible delay after death when it is to be used for immunohistological peptidergic examinations.

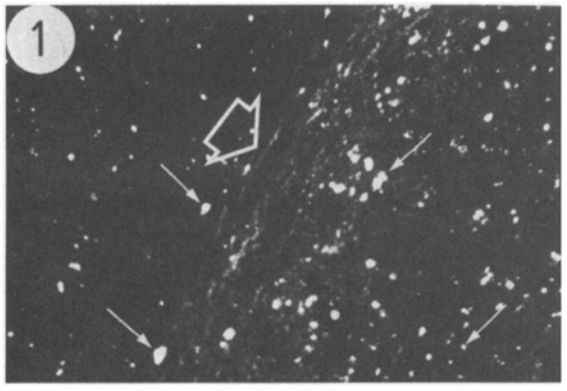
Despite the above-mentioned considerations, it seemed necessary to us to test and to modify the immunocytochemical methods using overfixed material stored for 40 or 50 years because we had at our disposal a very attractive collection of formalin-fixed brains and embedded blocks started in 1901. Here we present various improvements in immunohistochemical methods, made with the objective of obtaining comparable results for overfixed brains stored for 40 years and freshly fixed tissues.

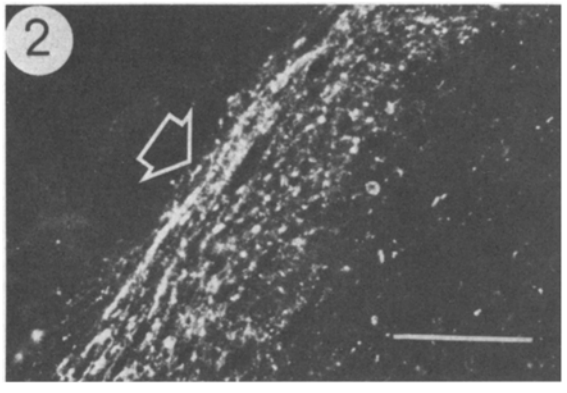
Material and methods. Twelve human brains chosen from our collection were used for this study. They were collected between 1943 and 1987 and immersed in a formalin fixative. Postmortem delay did not exceed 24 h except in one case (33 h). Patients' ages varied from 63 to 85. All were hospitalized for psychiatric disorders, 8 with degenerative dementia and 4 without neurological signs. A number of blocks from coronal sections were embedded in paraffin 20 years ago. One part of the remaining material was embedded in paraffin and the other part maintained in a fixative until sectioning. Dehydration and embedding were carried out according to the following time schedule; two changes of 70% and 90% ethanol for 1 day and three changes of 95% for at least 7 days each, then 4 changes of 100% ethanol for 4–5 days each (this schedule corresponds to the embedding of 1–2-cm thick brain sections). Following this, the tissue was immersed in three changes of toluene for 12 h each, followed by 3 × 4 h in pure paraplast at 60 °C. The tissue blocks in liquid paraplast were transferred into a heating vacuum incubator for 1 h to eliminate bubbles. After cooling, the blocks were then ready for sectioning. The sections were obtained using a standard microtome (30–40 µm) and stored. After deparaffinizing through a series of xylene (2 × 20 min) and alcohols (100%: 2 × 10 min; 95%: 2 × 10 min; 60%: 2 × 10 min) floating sections were rinsed 3 × in bidistilled H_2O. In addition, selected areas from immersed thick sections were cut with the Vibratome (Oxford Instr.) (50–100 µm).

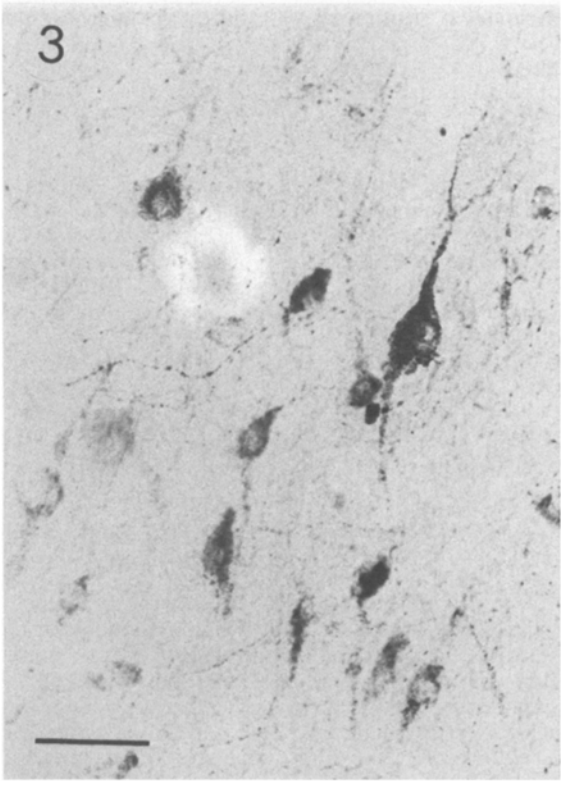
The following steps were the same for Vibratome sections and deparaffinized sections. They were treated first with $KMnO_4$ (0.25% in H_2O) for 20 min, washed 1 × in H_2O and immersed 2–3 min in a modified Pal's solution ($K_2S_2O_4$ 1% and oxalic acid 1% in H_2O) to bleach the sections and to suppress the endogenous peroxidase activity. They were then treated in NaOH (1 g) with 3 ml H_2O_2 (stock sol.: 30%) in 100 ml H_2O for 40 min. The sections were rinsed 5 × in phosphate buffered saline (PBS, 0.1 M, pH 7.4) and kept in PBS at 4 °C overnight. Afterwards, sections were incubated with antibodies diluted in PBS for 24 to 96 h at 4 °C. Several antibodies were tested; anti-SP (1:600), anti-NPY (1:1000), anti-SOM (1:1000), anti-DSIP (1:2000), anti-CLIP (1:10 000), anti-Enk (1:1000), anti-AVP (1:1000). After 3 washes in PBS, sections were incubated 1 h with IgG (1:100), rinsed 3 ×, incubated with PAP (1:100) for 1 h at 20 °C and rinsed 3 ×. 3'3-Diaminobenzidine was used as chromogen (0.05% + 0.01% H_2O_2 in PBS, 10–15 min). Twenty minutes' immersion of sections in 5% ammonium sulfate nickel (in Tris 0.05 M) slightly enhances the staining contrast, but this step can be omitted. Finally, sections were washed again in PBS and mounted on gelatinized slides, air-dried,dehydrated and mounted with Entellan.

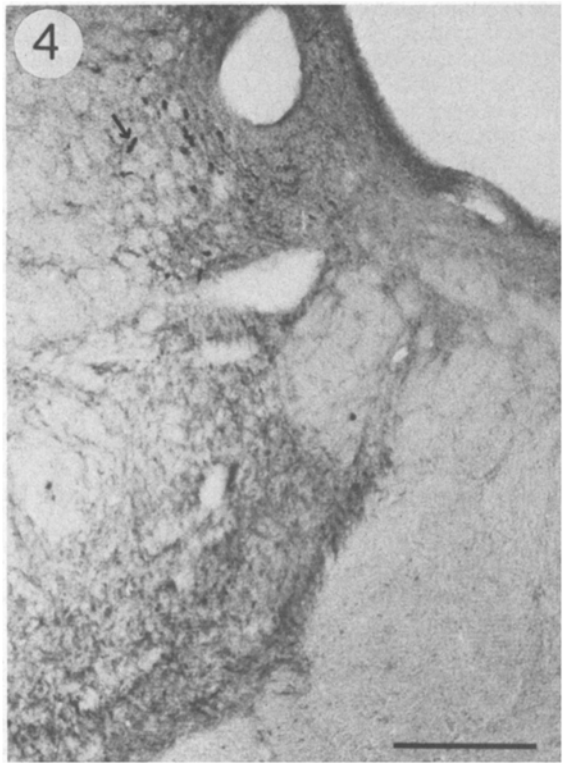
When using a immunohistofluorescence method with freshly fixed tissue (20 h), the same treatment but without NaOH-H_2O_2 immersion could be applied to sections cut on a cryostat (10 µm) and directly mounted on chrome-alum gelatinized coated slides. They were treated for 20 min and 3 min with $KMnO_4$ and Pal's solution respectively. After, the sections were rinsed with PBS (3 × 5 min), incubated with the antibody (1–24 h), and washed again with PBS (3 × 5 min). Finally, FITC-IgG (1:50) was applied during 1 h. After rinsing, sections were mounted in gelatine-PBS (3:1). Yellow lipofuscin autofluorescence disappeared and specific immunolabelling was markedly increased.

Results and discussion. Figure 2 shows the disappearance of lipofuscin autofluorescence and the enhancement of the SP-immunoreactivity compared to the adjacent section without treatment (fig. 1) in the human dorsal horn of the spinal cord. Immunofluorescence is more pronounced and it is easier to recognize labelled structures than it was before, when yellow lipofuscin autofluorescence masked the specific sites. Using the PAP method (fig. 3), hypothalamic VP-immunoreactive cells, like varicosities and fibers, contain a large quantity of reaction product. In the substantia nigra (fig. 5), the treatment washes out the melanin granules and abolishes the peroxidase activity of erythrocytes or the nonspecific endogenous peroxidase active sites. Without treatment on the adjacent section (fig. 4), SP-immunoreactivity appears pale









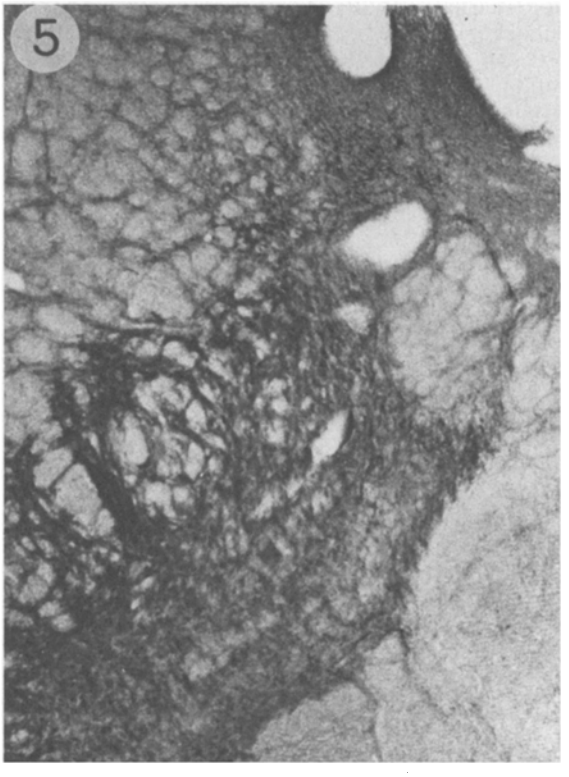


Figure 1. SP-immunoreactivity with immunofluorescence method in the dorsal horn of the human spinal cord without treatment. The non-specific autofluorescence of lipofuscin is indicated by small arrows and the pale SP-immunoreactivity by a large arrow.

Figure 2. Adjacent section to fig. 1 with $KMnO_4$-Pal's modified solution treatment. The immunoreactivity is enhanced (large arrow) and the non-specific fluorescence has disappeared. (scale bar: 200 μm)

Figure 3. VP-immunoreactive cells with PAP method in paraventricular nucleus after treatment (scale bar: 50 μm).

Figure 4. SP-immunoreactivity with PAP method in substantia nigra without treatment (arrow indicates melanocytes). Immunostaining appears pale and without contrast. (scale bar: 1 mm).

Figure 5. Adjacent section to fig. 4 with $KMnO_4$, Pal's modified solution, NaOH and H_2O_2 treatment.

and not well contrasted, often with several unspecific labelled structures (vessels, blood cells, melanin containing cells). The second step ($NaOH - H_2O_2$) allows the unmasking of the antigenic sites. The optimum time for $KMnO_4$ treatment was generally 20 min for thick Vibratome sections and also for deparaffinized sections. The same treatment can be applied on mounted cryostat sections (no longer than 20 min). Bleaching by Pal's solution for 2 min is often long enough to obtain 'white' sections. The time can be prolonged if necessary. For all tested antibodies except anti-Enk, this technique improves the immunostaining. These results confirm that these treatments are so efficient that they should be employed routinely when overfixed or fixed tissues are used in histochemical procedures.

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0014-4754/89/020159-03$1.50 + 0.20/0

The hypothalamic somatostatinergic pathways mediate feeding behavior in the rat [1]

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Received 10 August 1988; accepted 25 October 1988

Summary. The level of somatostatin in the hypothalamus was higher in satiated rats than in hungry rats. Elevating hypothalamic somatostatin levels by administering somatostatin into the hypothalamus produced a decrease in food intake, whereas lowering hypothalamic somatostatin levels by administering cysteamine into the peritoneal cavity produced an increase in food intake in rats.
Key words. Hypothalamus; somatostatin; anorexia; food intake; starvation; cysteamine.

Somatostatins (SS) constitute a family of related molecules including the originally identified SS (designated SS-14), SS-28 and still larger forms [2, 3]. SS-like immunoreactivity has been identified in brain regions outside those involved in the regulation of growth hormone release, which suggests that these peptides may function as a neurotransmitter within the central nervous system [4, 5]. Concerning ingestive behavior, many investigators [6, 7] suggested that SS may play a physiological role in control of food intake. For example, Aponte et al. [6] reported that intracerebroventricular infusion of SS resulted in a reduction in food intake in fed rats. Our recent results also demonstrated that the hypothalamus is the most sensitive site for SS-induced anorexia in rats [7]. Here, we have attempted to assess the SS levels of different brain regions both in satiated rats and in hungry rats. In addition, we assessed the effects on food intake in rats of elevating hypothalamic SS-levels by administering SS-14 into the hypothalamus, or depleting hypothalamic SS-levels by administering cysteamine into the peritoneal cavity. Such experiments could indicate whether feeding behavior might be related to the SS-levels in the rat hypothalamus.

Materials and methods. Experiments were performed on male Sprague-Dawley rats weighing between 270 and 310 g. The animals were fed with a dry powder chow that is commonly used for chickens (Taiwan Sugar Co.). They were housed individually in wire-mesh cages in a room maintained at 22 ± 1.0 °C with a 12 h: 12 h light-dark cycle. For administration of SS-14 (Sigma Chemical Co., Saint Louis, MO, USA) or normal saline into the lateral hypothalamus, a cannula guide tube with trocars was implanted, using the stereotaxic atlas and coordinates of Paxinos and Watson [8], in animals under pentobarbital sodium (60 mg/kg, i.p.) anesthesia. After two self-tapping screws had been attached to the calvarium of the parietal bones, the cannula guide tubes were inserted to the desired depth through the craniotomy holes. They were anchored with dental cement to the cranial surface, which had been scraped clean of perioteum. A period of 2 weeks was allowed to permit the animals to recover from surgery. At the time of injection, the cannula insert was connected to a 10-µl Hamilton microsyringe by PE 10 polyethylene tubing. The volume of injection down each cannula was 1.0 µl. The animals were trained to consume regular meals within a period of 2 h. Food intake, water intake and body weight were measured between 10.00 and 12.00 h each day in a lighted room. Dry powder chow was dispensed from a special spillage-reducing cup and water from a graduated cylinder with spouts.

In the first series of experiments, both the satiated rats (rats that had eaten a full meal) and the hungry rats (rats that had been starved for 24 h) were sacrificed for brain SS assay. Rats were decapitated and the cortex, corpus striatum, hypothalamus, lower brain stem and cervical spinal cord removed. Each brain tissue was homogenized at room temperature for 15 min. For each mg of brain tissues 2 ml of 0.2 N HCl was added for extraction [9]. The mixture was then heated at 95 °C for 12 min, followed by centrifugation at 20,000 × g at 4 °C for 20 min. The supernatant was stored in 1:10 dilutions at − 20 °C until assayed. The radioimmunoassay procedures for SS have been described previously [10].

In the second series of experiments, the effects on food intake of intrahypothalamic administration of normal saline or SS-14, and also of i.p. administration of cysteamine, were assessed in rats. At the end of the experiments, the animals were killed with an overdose of pentobarbital sodium and the cerebral circulation was perfused with 0.9% saline, followed by 10% (v/v) formalin solution. Later, sections of the fixed brain were cut at 40 µm and stained with thionin so that the stereotaxic coordinates of the cannulae could be verified.

Results and discussion. Table 1 contains a summary of the means and standard error values for SS-14 concentration of