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ANTIBODIES TO SMALL TRANSMITTER MOLECULES AND PEPTIDES: PRODUCTION AND APPLICATION OF ANTIBODIES TO DOPAMINE, SEROTONIN, GABA, VASOPRESSIN, VASOACTIVE INTESTINAL PEPTIDE, NEUROPEPTIDE Y, SOMATOSTATIN AND SUBSTANCE P

Ruud M. Buijs, Chris W. Pool, Joop J. Van Heerikhuize, Arja A. Sluiter, Paul J. Van der Sluis, Marja Ramkema, Tjitske P. Van der Woude and Eline Van der Beek

Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam, The Netherlands

ABSTRACT

In the present paper a procedure is decribed by which antibodies to small transmitter molecules can be obtained using glutaraldehyde as coupling reagent. This procedure was developed based on the condition that the immunogen used for the preparation of the antibody should, as much as possible, have the same identity as the transmitter molecule fixed in the tissue. In order to achieve this goal, aminergic, amino acid or peptidergic transmitters were conjugated to a protein carrier by using glutaraldehyde. The resulting antibodies could be used in an immunocytochemical procedure which allowed the detection of these transmitters after a single tissue fixation procedure with glutaraldehyde.

The development of specific staining techniques has contributed much to our knowledge of the neuroanatomy and the function of the central nervous system. Since the introduction of the Golgi staining technique, several other methods have been introduced to study neuronal structures and connections. When it was realized that in the central nervous system information is conveyed via the release of neurotransmitters, attention was focused on the development of techniques that would enable the localization of such compounds. This led to the development of, *e.g.* the Falck-Hillarp technique to demonstrate amines (7), the enzyme cytochemical techniques for the cholinergic system, and the Gomori staining for neurosecretory systems. The lack of general applicability or specificity of these techniques was overcome by the introduction of immunocytochemistry. The enormous potential of this technique for unravelling the organization of transmitter-specific neurons with their processes is based on the potency of antibodies to detect very small differences between molecules. Initially, the belief that it was impossible to raise specific antibodies against small transmitter molecules found broad support. The solution to this problem had already been hinted at by Landsteiner (11), who showed that small changes in molecules result in a

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Dedicated to Professor Yutaka Sano, on the occasion of his retirement.

change in the recognition of these molecules by an antibody, and, moreover, that it is possible to raise antibodies to small molecules. It took fifty years before these findings were applied to brain research. Geffard *et al.* (8, 9), Steinbusch and Verhofstad (16) and Storm-Mathisen *et al.* (17) introduced the use of antibodies to small transmitter molecules in neuroanatomy. Geffard *et al.* (8, 9) developed a technique which enabled the maintenance of the transmitter molecule identity throughout the entire immunocytochemical procedure, from the preparation of the immunogen to induce the antibody through the fixation and staining of the tissue and the specificity tests. This strategy combined with practical procedures, will be described in the present paper.

Preparation of the Immunogen

The outcome of an immunocytochemical staining procedure depends on the reaction of an antibody with an antigen. As has been discussed by Pool and Buijs (13), fixation occupies a central position in this process because it will change the conformation of the antigen and hence the specificity and affinity of the antibody. Therefore, especially in the case of small molecules, the immunogen used for the preparation of antibodies should as much as possible have the same identity as the molecule fixed in the tissue for the immunocytochemical procedure. Of course small molecules need to be coupled to a larger molecule in order to become immunogenic or to be detectable in tissue. Following this line of reasoning it is necessary to use the 'tissue fixation' for the preparation of these 'immune' conjugates. For that purpose it is desirable to use glutaraldehyde instead of formaline, for reasons of fixation speed and the formation of by-products as is discussed by Geffard *et al.* (8, 9) and Pool and Buijs (13). If glutaraldehyde is used as a conjugating agent, all molecules with a free NH_2 group can be coupled to a protein carrier. However, a number of other groups are also accessible to glutaraldehyde fixation (2, 10).

In order to obtain an optimum glutaraldehyde conjugate, a number of conditions must be met.

1) In order to obtain a sufficiently high antibody response at least 0.1-0.2 μmol hapten should be conjugated to 1 mg protein carrier.

2) A glutaraldehyde concentration as low as possible should be used. Usually a final concentration of 0.1% is sufficient and in order to avoid polymerisation a final concentration of 0.5% should not be exceeded. That the concentration of glutaraldehyde is of such importance can be explained by the fact that a large variety of molecular groups reacts with glutaraldehyde at a high concentration. At low concentrations glutaraldehyde will react first with primary NH_2 and E NH_2 groups of lysine. The fact that higher concentrations of glutaraldehyde during the preparation of the immunogen yield antibodies which result in a highly non-specific background staining in the tissue section indicates that such conjugates induce too many antibodies to glutaraldehyde linked groups.

3) For catecholamines, $Na_2S_2O_5$ or other reducing agents, such as ascorbic acid, should be added to the buffers after the coupling in order to avoid oxidation of the catecholamine-ring structure. Ascorbic acid, however, may also be used during coupling.

4) After coupling the immunogen should be reduced by means of $NaBH_4$ (12) in order to prevent the induction of antibodies to the charged C=N group of the immunogen (see for further details 13). Before the borohydride treatment the reaction mixture has a yellow colour, after reduction it ought to be blank, otherwise more borohydride should be added.

5) For each antigen the optimal glutaraldehyde coupling conditions should be determined by adding tritiated antigen in order to establish the optimal reaction time for the preparation of the immunogen. It should be noted that also each protein carrier has a different number of binding sites for glutaraldehyde resulting in a different number of antigens that may be coupled. Therefore separate binding curves should be made for each antigen and each protein carrier used.

Table 1 *Immunogen Preparation for Different Transmitters*

Hapten	Concentration hapten	Added concentration glutaraldehyde	Reaction time
DA	1.22 mg/ml	1%	30 min
5HT	2.5 mg/ml	1%	10 min
GABA	0.6 mg/ml	1%	30 min
Peptides	±5 mg/ml	1.5%	3-4 h

The following solutions should subsequently be added: 0.1 ml hapten, 0.8 ml thyroglobulin 2.5 mg/ml, 0.1 ml glutaraldehyde in phosphate buffer pH 6 (Table 1).

The reaction is stopped by adding approximately 5 mg $NaBH_4$. For peptides the reduction step with borohydride is unnecessary and may even lead to destruction of the hapten and the production of antibodies to unwanted groups. Thereafter the immunogen is dialyzed against 0.2% $Na_2S_2O_5$, buffer pH 6 at 4°C.

This strategy resulted in the production of the following antibodies: serotonin (5HT), dopamine (DA), gamma amino buteric acid (GABA), or peptides such as vasopressin (VP), somatostatin (SOM), vasoactive intestinal peptides (VIP), neuropeptide Y (NPY), substance P (SP).

Immunization

For immunization in rabbits, 1 ml of immunogen should be mixed thoroughly with 1 ml Freund complete adjuvant and injected subcutaneously and intramusculatory. For repeated immunizations, the second immunization is given after a week, followed by a biweekly immunization scheme using Freunds incomplete adjuvant. Heparinized blood should be taken after the 6th week and 1 week after the last immunization. Alternatively, for several haptens the immune response was investigated following a single immunization.

Initially it was investigated whether a single immunization with glutaraldehyde conjugated serotonin was sufficient to give an immune response. Therefore one animal received a single immunization with 40 μmol serotonin conjugated to 2 mg thyroglobulin. Three and 5 months later this animal received an additional immunization. Another animal received repetitive immunizations of the same immunogen. The course of the antibody titre, which could be followed as staining intensity on 100 pg serotonin spots fixed with glutaraldehyde on a nitrocellulose gelatin matrix, is indicated in Fig. 1.

This result indicated that repetitive immunizations are not necessary per se to obtain an immune response, but that a single immunization may be sufficient as well. Since peptides are much more expensive than amines or amino acids, repetitive immunization with peptides may be a costly operation. We thought it might be much more profitable to restrict ourselves to single immunizations. We therefore used a single immunization procedure for a number of peptides. For all peptides the same conjugation protocol was used. Immunizations were started against somatostatin (SOM), cholecystokinin (CCK), neuropeptide (NPY), atrial natriuretic factor (ANF), corticotrophin releasing factor (CRF), vasoactive intestinal peptide (VIP), substance P (SP). All immunizations resulted in the production of antibodies to the immunogens, although the titre of ANF and CRF was too low to allow an acceptable immunocytochemical detection in fixed tissue. With all other peptides the single immunization with 0.5 mg peptide resulted in the production of antibodies of sufficient titre to enable the immunocytochemical localization in glutaraldehyde fixed tissue (Figs. 3, 4, 7, 8).

Antibody Test Procedure

The specificity of the antibody should be tested in various ways, which is discussed

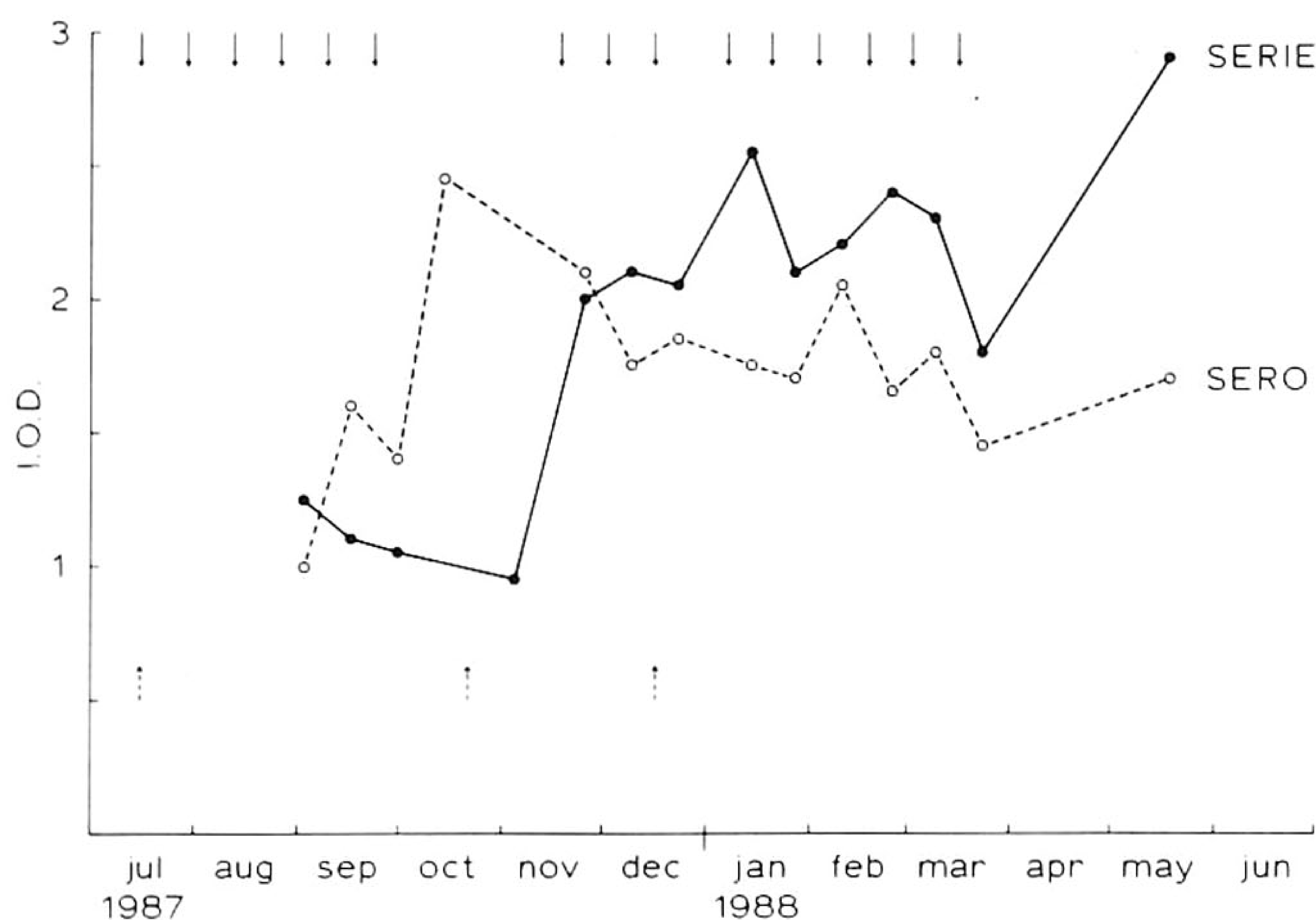


Fig. 1 Illustrates the staining intensity in integrated optical density of 100 pg serotonin at several different tissues after immunization. Immunization was started mid-July 1987. Rabbit Serie got several immunizations (upper arrows), the staining intensity of the bleeding one week after the booster is indicated by the dots. Rabbit Sero got three immunizations (lower arrows). The staining intensity of the bleedings of this animal is indicated by the open circles.

extensively by Pool and Buijs (13). To control for the titre and initial specificity a number of different procedures can be used in which testing of glutaraldehyde fixed antigen instead of free antigen is crucial. We have developed the following test systems in order to evaluate the potency and specificity of the antiserum.

Sheets of nitrocellulose membrane (with pore size 0.1 μm) are incubated for 1 h in a gelatin solution (2 g/l ; 0.5 ml/cm^2) at 40°C with gentle rocking, washed four times with water and air dried. Using this procedure the membranes retain reproducible amounts of gelatin (50 μg/cm^2). The amount of gelatin as well the pore size appear to be crucial since both influence fixation efficiency and immunocytochemical detection. One μl antigen solutions containing 5–100 pg/μl are applied to the nitrocellulose-gelatin matrix. Subsequently, a sheet of filter paper (Whatman 3 mm), which has been soaked in 5% glutaraldehyde in 0.1 M phosphate buffer pH 7.6, and then dried until a weight increase of $\pm$30% is measured, is put on the NC paper and pressblotted for 5 min (see for further details 18, 19). Thereafter the NC membranes, to which the antigens are fixed, undergo the same immunocytochemical staining procedure as the tissue sections. After DAB staining the optical density of the spots is measured and expressed as integrated optical density using an IBAS image analyzing system (Kontron).

The obvious advantage of this procedure is that the staining and fixation procedure for the evaluation of the specificity is identical to that used for the tissue. An example of such results is given in Fig. 2, which illustrates that the antibody to serotonin hardly shows any crossreaction with NA or DA and that the antibody to dopamine hardly crossreacts with 5HT or NA spots, albeit that the concentration of the tested compound is often much higher than the homologous one. These results show that the present procedure results in the production of highly specific and sensitive antibodies. In addition, in order to establish possibly unknown components, which may 'crossreact' with the antibody, the compo-

nents from a tissue homogenate can be separated into a gel by focusing techniques. This gel may be 'pressblotted' in a similar way on NC paper and subsequently be stained with the antibody under study. In this way it is possible to detect whether the pure component that reacts with the antibody is identical to the positive band in the preparation. Here too it can be established whether other bands in the gel react positively (18, 19).

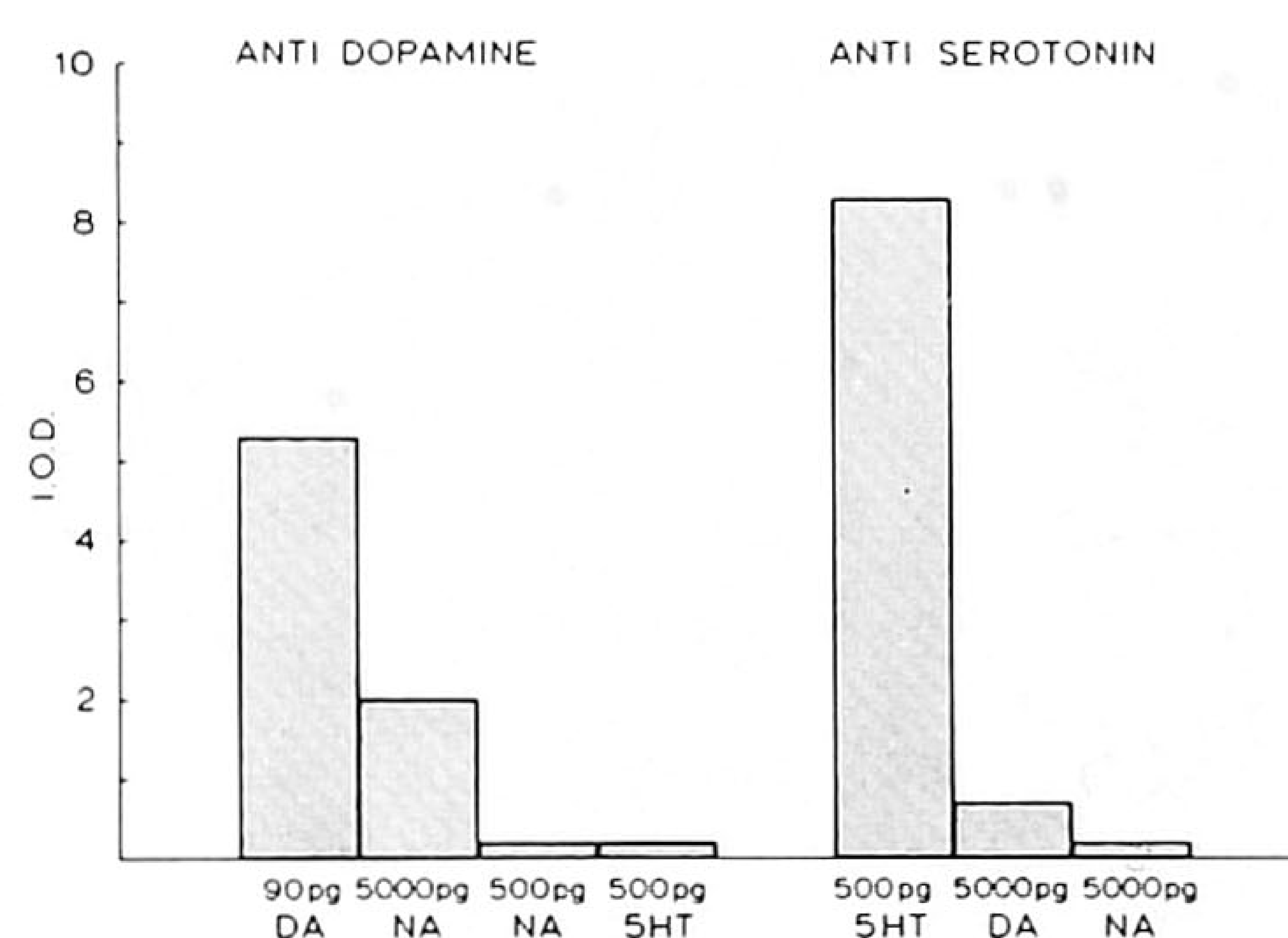


Fig. 2 Illustrates the staining intensity of spots with different concentrations of amines using an antibody against dopamine or serotonin. With the antidopamine only one spot of 5,000 pg noradrenaline rises above background level. With anti serotonin only 5,000 pg shows a detectable reaction.

Tissue Fixation

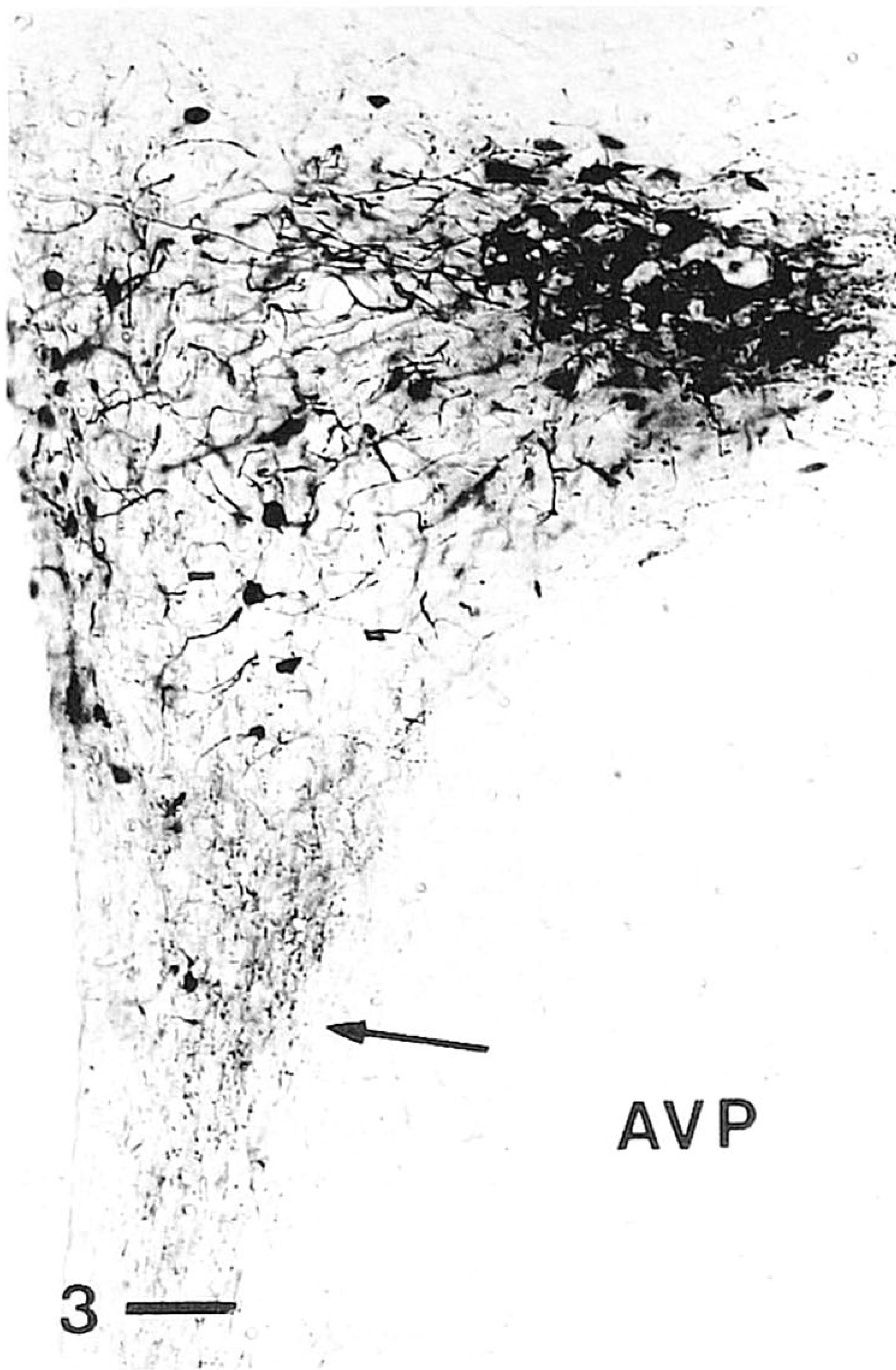
The fixation of small transmitter molecules in tissue yields considerable problems. In general, these molecules are highly diffusible. Sometimes they are released under hypoxic conditions. In order to ensure a rapid fixation, a pressure perfusion system is necessary via which first 50 ml saline and then the fixative can rapidly be introduced into the brain. As fixative a high concentration of glutaraldehyde is used, sometimes together with 1% paraformaldehyde to improve (ultra)structural preservation. The glutaraldehyde is diluted in phosphate buffer pH 7.4 or, if necessary, for the catecholamines, in citrate or acetate buffer pH 4 on account of their antioxidant properties. Ascorbic acid may be added for further protection of the catecholamine ring. Postfixation in the same fixative up to 2 h. Hereafter the catecholamine ring of dopamine should be protected by the addition of a reductant, usually 1% $Na_2S_2O_5$, to the washing buffer up to and including the first antibody incubation. Depending on the properties of the antigen tissue sections, should sometimes be reduced with $NaBH_4$ in order to obtain a better recognition of the antigen by the antibody (see Table 2).

Herewith, using one glutaraldehyde fixation procedure, it became possible to detect a large number of different antigens ranging from amines and amino acids to neuropeptides. As can be concluded from Table 2, in contrast to neuropeptides, which are readily visible after formalin fixation, it is im-

Table 2 *Indicates the Relative Recognition of the Antigen in Tissue Fixed with Different Fixatives*

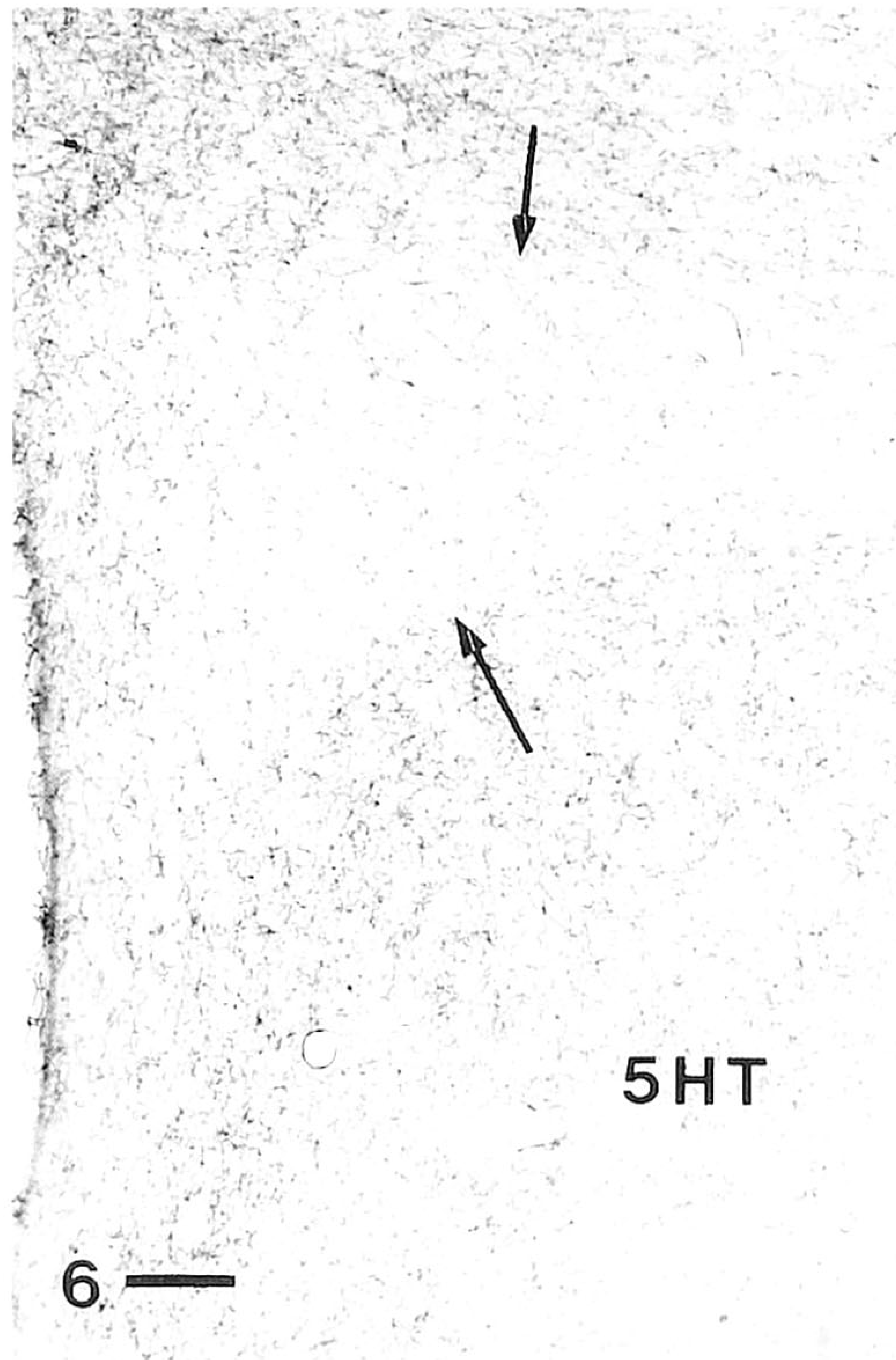
	Glutaraldehyde pH 4	Glutaraldehyde pH 7.2	Borohydride	Formalin
DA	+++	+++	N	–
5HT	+++	+++	Y	–
GABA	++	+++	N	–
Vasopressin	++	+++	N	+++
Substance P	+++	++	N	+++
VIP	++	+	Y	+++
NPY	++	+	Y	+++
Somatostatin	++	+	N	+++

The number of ++ indicates the staining intensity. –, no staining; +, weak staining; ++, fair; +++, intense. Under borohydride, Y and N indicate whether this treatment is necessary or not after glutaraldehyde fixation.


AVP
3

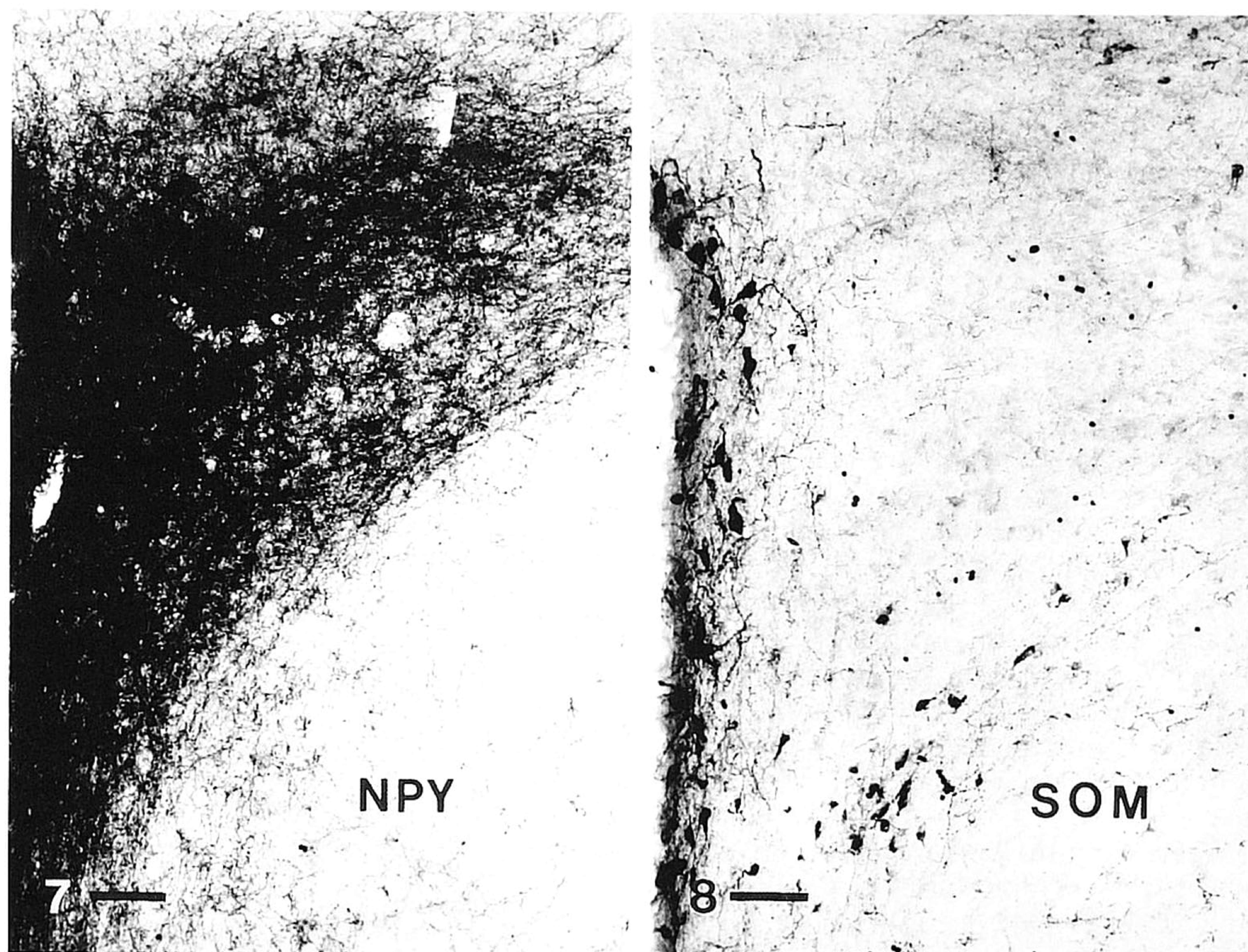
VIP
4

DA
5


5HT
6

possible to detect amines or amino acids with formalin fixation when the present antibodies are used. GABA and 5HT can be fixated with formalin, albeit at a much lower speed than with glutaraldehyde (13). This result can be explained by the fact that the antibody raised against glutaraldehyde conjugated antigen only recognizes this antigen when this is coupled with glutaraldehyde. The high affinity of glutaraldehyde for these amines, as compared to formalin, allows, however, the addition of small concentrations of formalin to the fixative without a notable reduction of the staining intensity.

That, in the case peptides, formalin fixation results in even better detection can be explained by the fact that the addition of the relatively small glutaraldehyde does not change the antigenic site to any considerable extent. The present antibodies are therefore not necessarily directed to the glutaraldehyde conjugated part of the molecule. This fixation probably exposes groups which are also accessible after formalin fixation. In addition, the strong glutaralde-



Figs. 3–8 Transverse sections in the region of the paraventricular nucleus of the hypothalamus (PVN) fixed with 5% glutaraldehyde in 0.1 M acetate buffer pH 4. Bar, 100 μm.

Fig. 3 illustrates arginine vasopressin staining, the arrow points to the fibres arising from the suprachiasmatic nucleus. A similar projection is indicated by the lower arrow in Fig. 4, which is stained with a vasoactive intestinal peptide antibody, the upper arrow indicates VIP fibres in the dorsal part of the PVN. Fig. 5 illustrates the dopaminergic innervation of the PVN, note the presence of DA positive cell bodies in the periventricular zone. Fig. 6 shows the serotonin innervation of the PVN, the arrows point to the relatively sparse innervation of the magnocellular part of this nucleus. Fig. 7 shows the dense innervation of neuropeptide Y in all areas of the PVN, while Fig. 8 shows the presence of somatostatin containing cell bodies in the periventricular zone and at the ventral and dorsal border of the PVN.

hyde fixation probably hinders the penetration of the antibodies sometimes resulting in a less intense staining than with formalin fixation. The opposite, that antibodies to formalin conjugated immunogens also detect the antigen in glutaraldehyde fixed tissue, is not necessarily true. The fact that sometimes the tissue needs to be reduced with borohydride for an optimum detection of the antigen and sometimes not, only indicates that at times the positive charge resulting from the double bond prevents the successful binding of the antibody to the antigen. If this reduction is not necessary, the antibody probably recognizes that part of the molecule which is not disturbed by the double band, or the antibody binding is not hindered by the presence of the double band.

As can be concluded from Table 2, fixation with glutaraldehyde at a low pH results in a much better detection of peptides than glutaraldehyde fixation at a high pH. This result can be explained by the fact that a large variety of molecular groups reacts with glutaraldehyde at pH 7, but that at pH 4 the affinity of glutaraldehyde for most of these groups is low except for primary NH_2 and E NH_2 groups of lysine (2, 10). By selecting the pH of the fixative one can therefore also select the groups that react with glutaraldehyde. Since for the preparation of the immunogen a much lower concentration of glutaraldehyde is used, the groups with the highest affinity, such as the primary NH_2 and the E NH_2 group of lysine, will be conjugated preferentially, which may explain this phenomenon.

Apart from the sensitivity and specificity of the present antibodies one of the big advantages is that, using a single fixation procedure, these antibodies allow the detection both amines, aminoacids and peptides in consecutive sections. An example of such staining is given in Figs. 3–8 which shows staining of the paraventricular nucleus of the hypothalamus for 6 different antigens after usage of one single fixation procedure.

Ultrastructural Localization

Usually preembedding staining gives satisfactory results for the ultrastructural localization of catecholamines (see *e.g.* 3, 4, 21, 22). For the localization of peptides it is sometimes necessary to use Triton X-100 because without the detergent often no staining can be observed in axonal terminals (see for further discussion 20). For some peptides OsO_4 postfixation may be used with subsequent postembedding staining on ultrathin sections (1, 6, 14, 20). This should be checked for each peptide separately. However, it is our experience that when it is possible to detect a peptide, using postembedding staining in a site where it is present at a high concentration, *e. g.* the median eminence or hypophysis, the concentration of peptides in axonal terminals in other brain sites is often too low to allow such a detection.

It has turned out that for GABA, as well as other amino acids, OsO_4 fixation does not destroy the antigenicity of the molecule, provided that OsO_4 is removed via etching before the immunocytochemical procedure (5, 15). The possibility to use OsO_4 for these antigens can be explained by the fact that these molecules cannot be oxidized and do not possess double bonds, and consequently will not be changed irreversibly by the fixation with OsO_4. Hence the perfect preservation of their recognition in a tissue section optimally fixated for ultrastructural preservation.

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