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NEUROPHYSIN IN THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM

I. Production and Characterization of Monoclonal Antibodies[1]

YAKOV BEN-BARAK,*,[2] JAMES T. RUSSELL,*,‡ MARK H. WHITNALL,* KEIKO OZATO,§ AND HAROLD GAINER*,[3]

*Laboratory of Neurochemistry and Neuroimmunology, National Institute of Child Health and Human Development, National Institutes of Health, ‡Laboratory of Cell Biology, National Institute of Mental Health, and §Laboratory of Developmental and Molecular Immunity, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20205

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Abstract

Seven mouse monoclonal antibodies (IgGs) were produced against rat neurophysins (NPs). Three were specifically directed against vasopressin-associated NP (NP-AVP), and four were specific for oxytocin-associated NP (NP-OT). These specificities were observed in liquid phase assays, immunoblot, and immunoprecipitation experiments. Homozygous Brattleboro rat tissues and extracts, which do not contain vasopressin or NP-AVP, did not react with the anti-NP-AVP antibodies but reacted with high affinity to the anti-NP-OT antibodies. In immunoprecipitation assays the antibodies brought down the appropriate NPs as well as their precursor molecules synthesized *in vivo* with no detectable cross-reactivity. In solid phase assays where the antigens were presented in a different manner, there was a significant cross-reactivity of the anti-NP-AVP antibodies with NP-OT. The extent of this cross-reactivity in solid phase correlated with the cross-reactivities of the antibodies observed in immunocytochemical studies. These solid phase (and immunocytochemical) data demonstrated that liquid phase specificities and absorption controls of antibodies are inadequate to assess their immunocytochemical (solid phase) specificities. Posterior pituitary extracts from the mouse and frog, as well as purified NPs from the rat, cow, and human were studied for their cross-reactivities to two of the antibodies, PS 36 and PS 45. In liquid phase assays the anti-rat NP-OT antibody, PS 36, reacted only with rat and mouse NPs and did not cross-react with NPs from any of the other species. In contrast, the anti-rat NP-AVP antibody, PS 45, was cross-reactive across species lines including an NP-like antigen extracted from frog posterior pituitaries. Immunoblot staining with these antibodies showed heterogeneity of NP-AVP and NP-OT in the rat posterior pituitary. Analysis of the epitopes for PS 36 and PS 45 indicated the antigenic determinants were located near amino acid positions 80 to 81 in NP-OT and 75 to 86 in NP-AVP, respectively.

The neurophysins (NPs) are acidic proteins of $M_r = 10{,}000$, which are invariantly found associated with the neuropeptide hormones, oxytocin (OT) and vasopressin (AVP), in the hypothalamo-neurohypophysial system (Pickering and Jones, 1978; Seif and Robinson, 1978; Breslow, 1979; Cohen et al., 1979; Chaiken et al., 1983). They represent a family of highly homologous, disulfide-rich proteins, and specific NPs are associated with either AVP or OT in equimolar amounts in each neurosecretory (granulated) vesicle (NSV). Their principal function appears to be to act as "carrier" proteins for the peptide hormones, which they bind intravesicularly in a noncovalent complex. Upon secretion from nerve terminals in the posterior pituitary, they are co-released with the peptide hormones (Seif and Robinson, 1978). This intimate relationship between the NPs and the neuropeptides begins with the biosynthesis of the hormones. Vasopressin and the vasopressin-associated neurophysin (NP-AVP), and oxytocin and the oxytocin-associated neurophysin (NP-OT) are synthesized in separate neurons in the hypothalamus as part of common (prohormone) precursors (Brownstein et al., 1980; Ivell et al., 1983; Gainer, 1983). Because the neurons of the hypothalamo-neurohypophysial system are the best understood peptidergic neu-

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[2] Present address: Inter-Yeda Ltd., Kiryat Weizmann, Ness-Ziona 76110, Israel.

[3] To whom correspondence should be addressed, at National Institutes of Health, Building 36, Room 2A-Z1, Bethesda, MD 20205.

rons in the central nervous system, we were motivated to produce monoclonal antibodies to various molecular components (e.g., the NPs) in this system, in order to use these immunological "reagents" in a variety of cell biological studies.

Monoclonal antibodies are powerful tools for neurobiological studies (Barnstable, 1980; Zipser and McKay, 1981) and have many advantages, but also limitations (Edwards, 1981; Yelton and Scharff, 1981). The advantages allow for the production of homogeneous, reliable, and unlimited amounts of defined antibody reagents which can be used in *both* biochemical and morphological experiments. One of the limitations we were particularly concerned with was that, because of its homogeneity, a monoclonal antibody may not be able to distinguish between different molecules that bear similar antigenic determinants (see Edwards, 1981). Considerable confusion has surfaced in the neurobiological literature because of such cross-reactivities. For this reason, we have focused in this paper on the cross-reactivities of the monoclonal antibodies that we have produced. Antisera (polyclonal antibodies) against specific rat NPs have been produced, and these have shown low cross-reactivities in liquid phase radioimmunoassays (RIAs) (McPherson and Pickering, 1978; North et al., 1983). However, their use in solid phase assays and immunocytochemistry has not been reported, and it is possible that when they are evaluated in such assays their cross-reactivities will be unacceptable. Because of their polyclonal nature, it is difficult to evaluate the basis of the cross-reactivity in antisera, especially in solid phase assays such as immunocytochemistry.

In this paper we describe the production of seven mouse monoclonal antibodies reacting with rat NP and characterize their cross-reactivities by several types of biochemical and immunocytochemical assays. We were particularly interested in producing antibodies which would react with NP-OT and its precursor protein, but which would not cross-react with NP-AVP and its precursor. In the accompanying paper (Whitnall et al., 1985), a morphological study of the ontogeny of this neuronal system, based on the use of such characterized antibodies, is presented.

Materials and Methods

Preparation of the immunogen and immunization procedures

Our initial goal was to produce monoclonal antibodies directed against a variety of soluble proteins and peptides extracted from the posterior pituitary and the intermediate lobe of the rat. Therefore, BALB/c mice were immunized intraperitoneally with acid-soluble extracts of rat (Sprague-Dawley) neurointermediate lobes. The neurointermediate lobes were dissected on ice and homogenized in 0.1 N HCl, pH 1.3, and the 12,000 × *g* supernatant was collected and frozen. These extraction conditions preserve the structure and function of pituitary peptides. Prior to fusion, an immunization regimen was sought which would yield higher antibody titers against the immunizing solution. Sixteen mice were divided into four groups. The first group received 30 μg of protein/injection/mouse, and the second group was injected with 150 μg of protein/injection/mouse. The first injection was done in complete Freund's adjuvant. The injections were performed 2 weeks apart and consisted of the same quantity of antigen emulsified in incomplete Freund's adjuvant and phosphate-buffered saline (PBS), respectively. The third and fourth groups were immunized with posterior pituitary extract coupled to keyhole limpet hemocyanin (KLH), using glutaraldehyde as the coupling reagent. Twenty-five micrograms per injection per mouse (third group) and 100 μg/injection/mouse (fourth group) were injected using the same procedures as described above. Seven days following the third injection, sera were collected from the mice and assessment of their titers was done by enzyme-linked immunoabsorbent assay (ELISA) (see below) using whole uncoupled neurointermediate lobe extract as coating material. This initial screening of the sera showed that the mice injected with KLH-coupled posterior pituitary extracts had higher titers against the extract compared with mice injected with uncoupled material. Three mice with the highest serum titers were chosen from the KLH-coupled immunogen groups to receive intravenous injections of the immunogen 2 weeks following the third injection, and their spleen cells were used for fusion 4 days later.

Fusion and production of hybridomas

Spleen cells were fused with mouse nonproducer myeloma cells SP 2/0 AG14 (ratio 5:1), using polyethylene glycol M_r = 1000 according to published methods (Köhler and Milstein, 1975; Gefter et al., 1977; Kennett et al., 1980; Ozato et al., 1980; 1981). The cells were then placed into 1620 wells of 96-well flat bottom Falcon microtiter plates (2.2×10^5 cells/100 μl/well) in selective medium (Littlefield, 1964) containing hypoxanthine/aminopterin/thymidine (HAT medium). The wells were supplemented with 50 μl of fresh HAT medium every 4 to 5 days. Ten to 14 days after fusion, medium from wells with cell growth were collected for screening. Cells from positive wells were cloned by limiting dilution and expanded (Ozato et al., 1980; 1981). Two liters of culture supernatant were routinely harvested from each clone. Purification of antibodies from the culture supernatants was performed by affinity chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) (Ey et al., 1978) with elution by either 3 M guanidine chloride or buffers at pH 2.8 (0.2 M acetic acid). The purified antibodies were immediately neutralized and dialyzed against PBS/0.1% sodium azide, and culture supernatants and purified antibodies were stored either frozen (−20°C) or at 4°C in 0.1% azide. The clones are described by a PS (posterior pituitary-soluble antigen) and a number (Table I).

Screening strategy

The first stage of screening was to identify which of the culture fluids of growing hybrids contained antibodies specific to the hypothalamo-neurohypophysial system. This first involved a solid phase ELISA (Voller et al., 1976) test in which uncoupled neurointermediate lobe acid extracts (see above) were used to coat microtiter plate wells. Control wells were coated with anterior pituitary or cerebellar acid extracts. Those supernatants which were positive for the posterior pituitary extracts, but negative for the anterior pituitary and cerebellar extracts, were then used further in immunohistochemical screening tests. The immunohistochemical assay was performed to confirm the specificity of the antibody at this initial screening stage. Bouin's fixed whole brains and pituitaries from Sprague-Dawley rats were embedded in paraffin, and 6 μM parasagittal sections from brain and cross-sections from pituitary were made. The sections were deparaffinized, blocked by incubations in 10% normal goat serum in PBS (blocking solution) for 30 min, incubated in a culture supernatant diluted 1:2 in the blocking solution for 2 hr, washed in PBS, incubated in peroxidase-coupled goat anti-mouse IgG (N. L. Cappell Laboratories, Cochransville, PA) diluted 1:200 in blocking solution for 1 hr, washed in PBS,

TABLE I
Properties of anti-rat NP monoclonal antibodies

Antibody	Class[a]	Subclass[a]	K_{aff}[b]	Specificity[c]
			liters/mole	
PS 36	IgG[k]	2b	0.55×10^9	NP-OT
PS 38	IgG[k]	2b	0.48×10^9	NP-OT
PS 41	IgG[k]	2b	1.2×10^8	NP-AVP
PS 45	IgG[k]	3	0.25×10^{10}	NP-AVP
PS 46	IgG[k]	2b	0.50×10^8	NP-AVP
PS 60	IgG[k]	3	0.32×10^9	NP-OT
PS 67	IgG[k]	2b	0.59×10^9	NP-OT

[a] Antibody classes and subclasses were determined by Ouchterlony double diffusion tests, using a Miles Corp. mouse immunoglobulin subtype identification kit (data not shown).

[b] The affinity constants (K_{aff}) were determined by liquid phase RIAs using affinity-purified ^{125}I-rat NPs and unlabeled rat NPs. Similar data were obtained for PS 36, 38, 60, and 67 using affinity-purified NPs obtained from DI/DI rats (i.e., only NP-OT). The data were analyzed and K_{aff} was calculated by using a Fortran IV program (RIAPROG) developed at National Institutes of Health by V. B. Faden and D. Rodbard (data and calculations not shown).

[c] Specificity was determined under liquid phase assay conditions (e.g., Figs. 5 to 7).

and stained for peroxidase activity using 0.05% diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide for 6 min. Only those hybridomas whose supernatants selectively stained both the posterior pituitaries and cells and fibers of the hypothalamo-neurohypophysial system (e.g., see Fig. 1) were chosen for cloning.

Solid phase immunoassays

Solid phase assays were performed on 96-well PVS microtiter plates (Dynatech, Alexandria, VA). The wells in the plates were coated with 50 μl of either whole posterior pituitary extract (20 to 30 μg/ml) or purified NP (2 μg/ml) and incubated at room temperature for 2 hr or overnight at 4°C. The plates were washed in PBS, and the remaining free protein-binding sites were blocked by adding 100 μl of PBS containing 1% bovine serum albumin (BSA) (buffer I) for 30 min. The wells were washed again in PBS, and 50 μl of antibody culture supernatant or purified antibody solution diluted in buffer I were placed in the wells and incubated for 1 hr at room temperature. The wells were then washed free of excess antibody, and the antibody which bound to the well was measured by two methods. (*1*) A conventional ELISA technique (Voller et al., 1976) in which rabbit anti-mouse IgG (1:400 in PBS/1% BSA) and peroxidase-coupled goat anti-rabbit IgG (1:3200 in PBS/1% BSA) were applied sequentially (both reagents were obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN), and orthophenyldiamine (Sigma Chemical Co., St. Louis, MO) was used as the enzyme substrate. The absorbances of the individual wells were read at 492 nm with a Dynatech multiscan automated 96-well plate reader. (*2*) An indirect RIA in which ^{125}I-sheep anti-mouse IgG (New England Nuclear Corp., Boston, MA) was added to each well (~50,000 cpm/50 μl) and incubated for 1 hr. The washed wells were cut out of the plate and counted for their radioactivity in a Packard gamma counter (Packard Instrument Co., Inc., Downers Grove, IL). The RIA procedure had a markedly higher signal-to-background ratio, was about 10 times more sensitive than ELISA, and was preferentially used here in analytical studies. Furthermore, we found that 5% fetal calf serum in PBS was a superior blocking solution as compared to PBS/1% BSA; therefore, this blocking solution was used in most of the solid phase RIAs. Several mouse monoclonal antibodies were used as controls in the assays in this paper. These include 30-5-7, an anti-H-2 (IgG_{2a}) antibody (Ozato et al., 1980); 4-9-11, an anti-idiotype against an anti-H-2 (IgG_1) antibody (K. Ozato, unpublished observation), and HO-13-14, an anti-Thy 1.1 antibody (obtained from the American Type and Culture Collections, Rockville, MD).

Preparation of affinity-purified rat NPs

NP was affinity purified from both normal Sprague-Dawley rat posterior pituitaries (which contain both NP-OT and NP-AVP) and homozygous (DI/DI) Brattleboro rat posterior pituitaries (which contain only NP-OT; Sokol and Valtin, 1982). The Brattleboro rats were treated by four subcutaneous injections of desmopressin (Ferring Pharmaceuticals, Malmö, Sweden), 10 μg/injection/day in PBS, to control their polyuria and to maintain higher levels of NP in their pituitaries. In addition, the posterior pituitary extracts from the Brattleboro rats were assayed for NP-AVP (i.e., PS 41 immunoreactivity) before use in affinity purification procedures. By this prescreening procedure we were able to exclude any possible contamination due to heterozygous Brattleboro NP-AVP. The purification of NP from both types of animals was performed as described previously (Robinson et al., 1976a; Russell et al., 1980). Briefly, 0.1 N HCl-soluble posterior pituitary extracts were run on a G-75 column (Pharmacia) in 0.1 N HCl and 0.5 mg/ml of BSA, and the fractions of the 10,000-dalton range were pooled and passed through a lysine vasopressin-Sepharose affinity column in ammonium acetate solution (pH 5.7). The bound NPs were eluted by lowering the pH to 2.8 with formic acid, and the eluted material was lyophilized.

Preparation of ^{3}H-labeled rat NPs and ^{35}S-labeled rat NP precursors. [^{3}H]- or [^{35}S]cysteine was injected into the supraoptic nuclei (SONs) of anesthetized rats by previously described stereotaxic methods (Gainer et al., 1977). The rats were killed 24 hr after injection of [^{3}H] cysteine and 1 hr after injection of [^{35}S]cysteine. The posterior pituitaries were removed from the rats injected with [^{3}H]cysteine and were extracted for the ^{3}H-labeled NP which had been axonally transported from the injected SONs. The ^{3}H-labeled NPs were extracted by 0.1 N HCl and purified by lysine vasopressin-affinity chromatography (see above). The affinity-purified ^{3}H-NP was then separated into ^{3}H-NP-AVP and ^{3}H-NP-OT by high performance liquid chromatography using a mono Q column (Pharmacia), with a 0 to 1 M NaCl gradient in 20 mM bis-Tris-HCl (pH 6.4) buffer. The gradient duration was 60 min with a flow rate of 1 ml/min. The ^{35}S-labeled NP common precursors for AVP and NP-AVP (propressophysin, Pro-PP) and for OT and NP-OT (pro-oxyphysin, Pro-OP) were derived from micropunches of the SONs injected with [^{35}S]cysteine for 1 hr (Gainer et al., 1977). The 0.1 N HCl extracts of these micropunches were used to isolate the Pro-PP and Pro-OP prohormones (precursors) by previously published methods (Russell et al., 1980).

Purified NPs from other species

NPs were affinity purified from bovine (cow) posterior pituitary glands as described above for rat NPs. Cow NPI (cow NP-OT) and cow NPII (cow NP-AVP) were obtained from Dr. Irwin Chaiken (National Institutes of Health, Bethesda, MD). Separated human NPs, human NPIII and NPIV (NP-AVP, or nicotine-stimulated NP, NSN) and human NPIII (NP-OT, or estrogen-stimulated NP, ESN), were obtained from Dr. Joseph G. Verbalis (University of Pittsburgh, Pittsburgh, PA). The human NPs were separated by ampholyte displacement chromatography (Verbalis, 1983). The human NPII (NSN) samples contained 15% ESN, the human NPIII (ESN) contained 12% NSN, and the human NPIV (NSN) contained 19% ESN, as estimated by liquid phase RIA using specific antisera (J. G. Verbalis, personal communication).

Liquid phase RIA and immunoprecipitation procedures. Five micrograms of affinity-purified NP from normal rats (NP-AVP and NP-OT) or homozygous Brattleboro rats (NP-OT only) were iodinated using Na^{125}I (New England Nuclear) and the chloramine T method of Greenwood et al. (1970). These were used as the ^{125}I-labeled tracers in NP RIA procedures which have been described previously (Robinson et al., 1976b). Displacement curves and inhibition by cold ligands were obtained by using antibody supernatant concentrations which yielded 20 to 40% ^{125}I-NP binding. Separation between bound and free labeled antigen was effected by the addition of ethanol to a final concentration of 68%, followed by centrifugation, and the pelleted tracer was counted. Affinity constants were derived from displacement curves using a Fortran IV program (RIAPROG) developed at the National Institutes of Health by V. B. Faden and D. Rodbard. The Brattleboro rat NP was the source of ^{125}I-NP-OT. The ^{125}I-NP-AVP was obtained by incubating the ^{125}I-NP from normal rats for 4 hr at 4°C with saturating levels of PS 45 antibody and then incubating for 2 hr more in the presence of *Staphylococcus aureus* protein A-positive membrane (Boehringer-Mannheim). The antibody-antigen complex was then centrifuged at 12,000 × *g* for 4 min, and the pellet was washed three times with PBS and eluted in 0.1 N HCl. The eluted solution was neutralized and contained only ^{125}I-NP-AVP which was used as the tracer for this NP in binding studies.

Immunoprecipitations of the *in vivo* ^{3}H-labeled NPs and ^{35}S-labeled precursors were performed in PBS/1% BSA solution containing 1:3 dilutions of the monoclonal antibody supernatants. These immunoprecipitations were determined to be quantitative (or at saturating levels) when repeated immunoprecipitations of the same sample by additional antibody supernatants yielded no more bound radioactivity in the pellet. The labeled protein samples were incubated with antibody for 16 hr at 4°C, and then for 2 hr more in the presence of *S. aureus* membranes as described above. The immunoprecipitate was washed sequentially in 10 mM sodium phosphate, 1 mM EDTA, 1% Triton X-100 (pH 7.6) solution (two times); 100 mM NaCl, 20 mM NaH_2PO_4, 1 mM EDTA, 0.25% Triton X-100 (pH 7.6) solution (two times); and 10 mM NaH_2PO_4, 0.15 M NaCl (pH 7.6) solution (once) before elution of the labeled proteins in 0.1 M HCl.

Electrophoresis and immunoblot methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 11% acrylamide slab gels by conventional methods (Laemmli, 1970), and isoelectric focusing was done in 7.5% polyacrylamide gels as described previously (Russell et al., 1980). Ampholytes (Bio-Rad) used were in the pH ranges 4 to 6 and 3 to 10 in a ratio of 9:1, respectively. For electroblotting, the protein samples were applied across the whole width of the gel (either 1 mg of posterior pituitary protein extract or 250 μg of affinity-purified NP), and at the end of the electrophoretic run the separated proteins on the gel were transferred to nitrocellulose paper using a Bio-Rad Transblot apparatus and conventional procedures (Towbin et al., 1979). The nitrocellulose paper was cut into strips and each strip was stained according to the

methods of Matus et al. (1980) using 4-chloro-1-naphthol and hydrogen peroxide. Some strips were stained for total protein by amido black. Protein was estimated by the method of Lowry et al. (1951), with BSA as standard.

Light microscopic immunocytochemical methods

Male, 250-gm, heterozygous (control) and homozygous (AVP and NP-AVP-deficient) Brattleboro rats and Osborne-Mendel rats were obtained from the National Institutes of Health. Fixation was by perfusion through the heart with 4% paraformaldehyde, 0.2% picric acid, 0.1 M sodium cacodylate, pH 6. For NP staining, the brains could be stored up to several months in the original fixative. Fifty-micrometer coronal Vibratome sections were rinsed overnight in PBS at 4°C, then mounted on chrome-alum-coated slides, dried 15 min, dipped in acetone for 10 sec, and quickly transferred to PBS without being allowed to dry. Endogenous peroxidase activity was suppressed in 10% methanol with 3% hydrogen peroxide for 5 min, and the slides were then rinsed in PBS (three times for 1 min, two times for 5 min), followed by 0.25% Triton X-100 (5 min), PBS (5 min), 10% normal goat serum in PBS (NGS, 2 hr), and the primary antibody solution. The primary antibody solution consisted of hybridoma culture supernatants diluted in 10% NGS, 0.1% sodium azide. The slides were placed into primary antibody overnight at 4°C, rinsed in PBS (six times for 10 min), and incubated 4 hr at room temperature in affinity-purified goat anti-mouse IgG conjugated to peroxidase (Boehringer-Mannheim) diluted 1:40 in 10% NGS. The slides were then rinsed in Tris-buffered saline, pH 7.6 (six times for 10 min), and incubated for 60 min in the dark, in a peroxidase reaction solution consisting of 0.05% DAB (Sigma grade II), 0.0003% glucose oxidase (Worthington Biochemical Corp., Freehold, NJ; 157 units/mg; Itoh et al., 1979), 0.4% β-D-glucose, 0.04% ammonium chloride, 0.136% imidazole, and 0.08% nickel chloride (Hsu and Soban, 1982) in Tris-buffered saline, pH 7.6. The slides were then dehydrated in graded ethanols, cleared in xylene, and coverslipped with Permount.

Electron microscopic immunocytochemical methods

Pituitaries from male, 250-gm, heterozygous Brattleboro rats were immersion fixed in the fixative described above supplemented with 2% glutaraldehyde. The pituitaries were embedded in LR White resin (Ernest F. Fullam, Inc., Schenectady, NY), a hydrophilic mixture of acrylic monomers (for technical details about LR White, see Newman et al., 1983). Silver sections were picked up on nickel grids and stained as follows: 10 min with PBS; 10 min with 10% NGS in PBS; 10 min with monoclonal antibody diluted in 10% NGS; six 1-min rinses with PBS; 10 min with goat anti-mouse IgG-peroxidase conjugate (see above) at 1:40 in 10% NGS; 1 min with PBS (six times); 2 min with 0.05% DAB (Sigma grade II), with 0.01% hydrogen peroxide in PBS; 30 sec (two times) and 1 min (four times) with PBS; 30 sec with 0.5% osmium tetroxide in PBS; 30 sec (two times) and 1 min (four times) with H_2O. No uranyl or lead staining was used. Anti-H-2 monoclonal antibodies were used as negative controls as in the biochemical assays (see "*Solid phase assays*").

Results

In one of several fusion experiments, of a total of 1620 wells plated, 1319 wells (82%) showed significant cell growth 10 to 14 days after the fusion. Initial screening of the media from wells containing cell growth, by ELISA performed with acid-soluble posterior pituitary peptides as antigens, showed that 72 wells (5.5%) were above background in this assay (data not illustrated). The media from these positive wells were then screened by immunohistochemical staining of pituitary and brain sections (see "Materials and Methods"). Two major staining patterns were observed: media from 56 of the screened wells

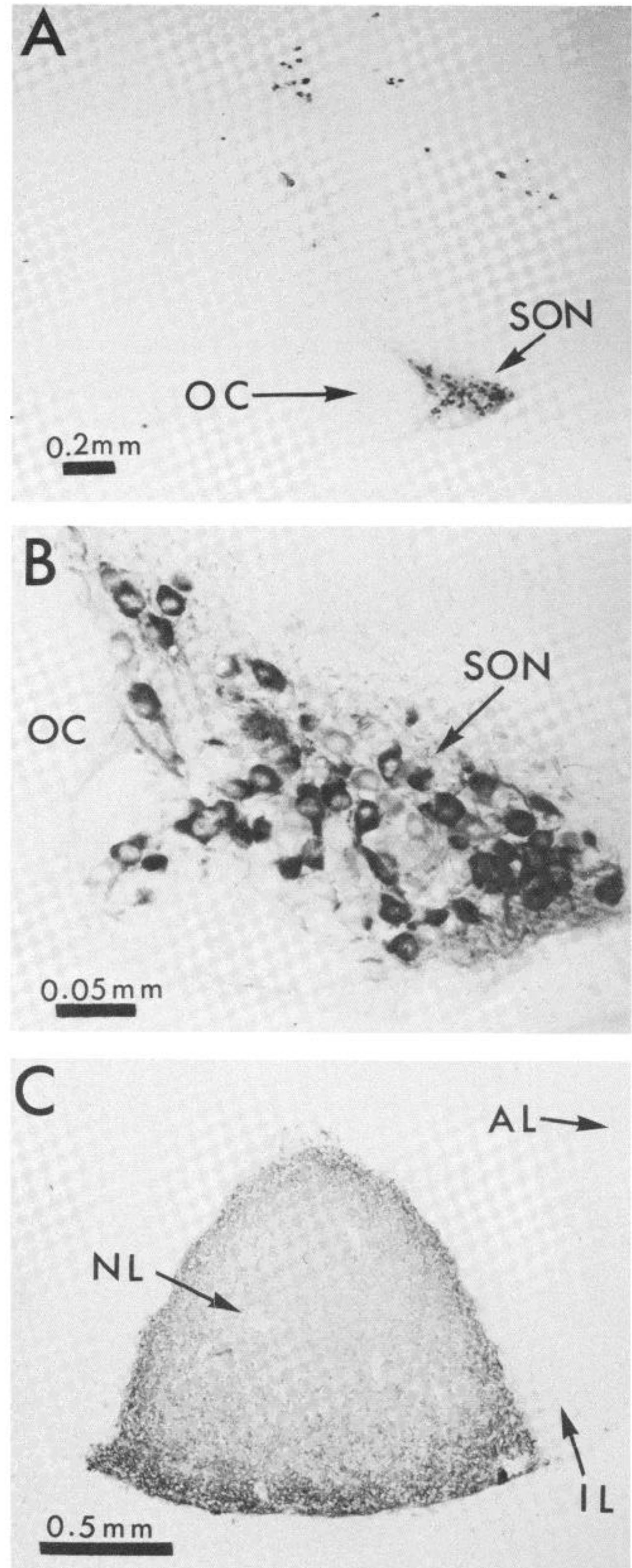

Figure 1

Figure 1. Immunohistochemical screening of monoclonal antibodies using 6-μm paraffin sections of rat hypothalamus and pituitary. Medium from one of the original fusion wells (PS 38) was used to stain both tissues (see "*Screening strategy,*" under "Materials and Methods"). *A*, Staining is shown in the supraoptic nucleus (*SON*) and dorsal accessory cells. *B*, Enlargement of the supraoptic nucleus shown in *A*. Staining is localized intracellularly and in the axons. *C*, The posterior pituitary (*NL*) is exclusively stained by this antibody. Note negligible background in the unstained areas of brain and pituitary. *AL*, anterior lobe; *IL*, intermediate lobe; *NL*, neural lobe; *OC*, optic chiasm.

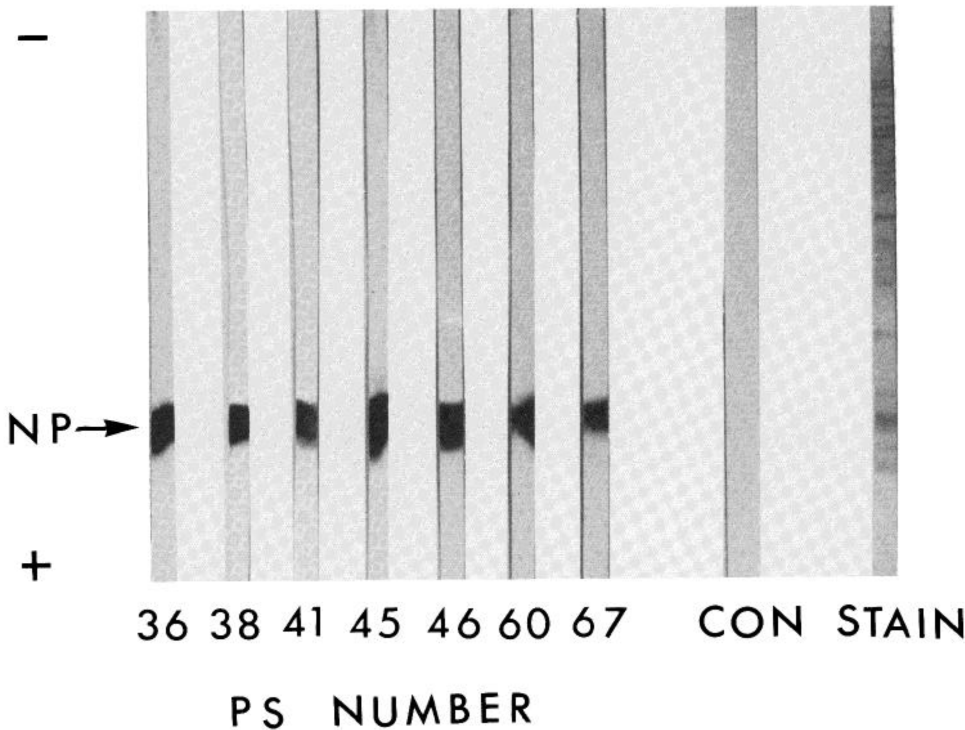

Figure 2. SDS-PAGE and immunoblots of rat posterior pituitary extracts. Only the staining of those antibodies which proved specific for the hypothalamo-neurohypophysial system in the immunohistochemical tests (e.g., Fig. 1) is depicted. Whole posterior pituitary extract was electrophoresed on an 11% SDS-polyacrylamide gel and was electroblotted onto nitrocellulose paper. Strips of the blotted paper were exposed to antibody culture supernatants and stained. The control (*CON*) lane did not have primary antibody solution. The protein pattern is shown in the *right lane* using amido black staining (*STAIN*). Each *lane* contains about 50 μg of protein of the posterior pituitary extracts. The *arrow* indicates the location of NP in the gel.

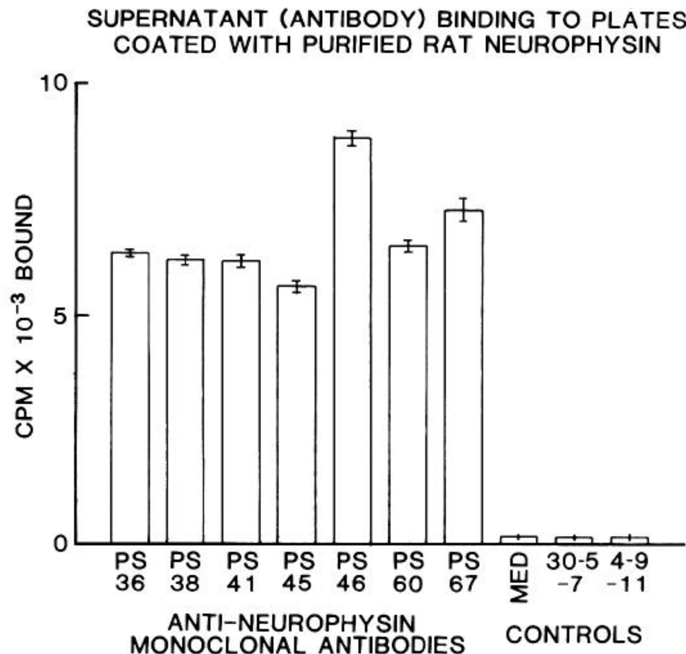

Figure 3. Solid phase RIAs of the anti-NP monoclonal antibodies. Plate wells were coated with purified rat NP. In control wells the primary antibody solution was substituted by HAT medium alone, or by anti-mouse H-2 monoclonals (30-5-7 or 4-9-11) (see "Materials and Methods"). Average values of six experiments ± SD are shown.

contained antibodies which stained glial and neuronal intracellular elements in the whole pituitary gland as well as in all brain areas, whereas media from 16 wells exclusively stained the posterior pituitary, the paraventricular nuclei (PVNs), and the SONs. An example of such staining from one of the 16 wells is shown in Figure 1. Of the 16 wells whose supernatants showed specificity for the hypothalamo-neurohypophysial system, we were able, following limiting dilution cloning, to establish and expand seven stable clones (Table I).

Identification of the antigens recognized by the monoclonal antibodies. In order to elucidate the identity of the antigen(s) recognized by the antibodies, we first determined the molecular weights of the antigen(s) by immunostaining SDS-PAGE transblots of the posterior pituitary soluble extract. Figure 2 illustrates that all of the antibodies stained a band of about M_r = 10,000 which co-migrated with NP, a major protein found in this system. We next performed RIAs with these monoclonal antibodies on microtiter plates in which the wells had been coated with affinity-purified NPs from normal rats. These data are illustrated in Figure 3. All of the PS monoclonal antibodies bound to the affinity-labeled NPs, whereas the control monoclonal antibodies (30-5-7 and 4-9-11, directed against mouse histocompatibility antigens, Ozato et al., 1980) did not significantly differ in binding from the HAT medium control.

Although the above solid phase immunoassays (Figs. 2 and 3) are valuable for rapid and simple evaluations of antibody specificity, a more precise and quantitative determination would be to use liquid phase RIAs (Berzofsky and Schechter, 1981). Figure 4 depicts such an analysis for two of the monoclonal antibodies (PS 36 and PS 45) in which ^{125}I-NP (from normal rats) was used as the tracer. The data in this figure show that unlabeled NP was highly effective in displacing the ^{125}I-NP from the PS 36 (Fig. 4*A*) and PS 45 (Fig. 4*B*) anti-

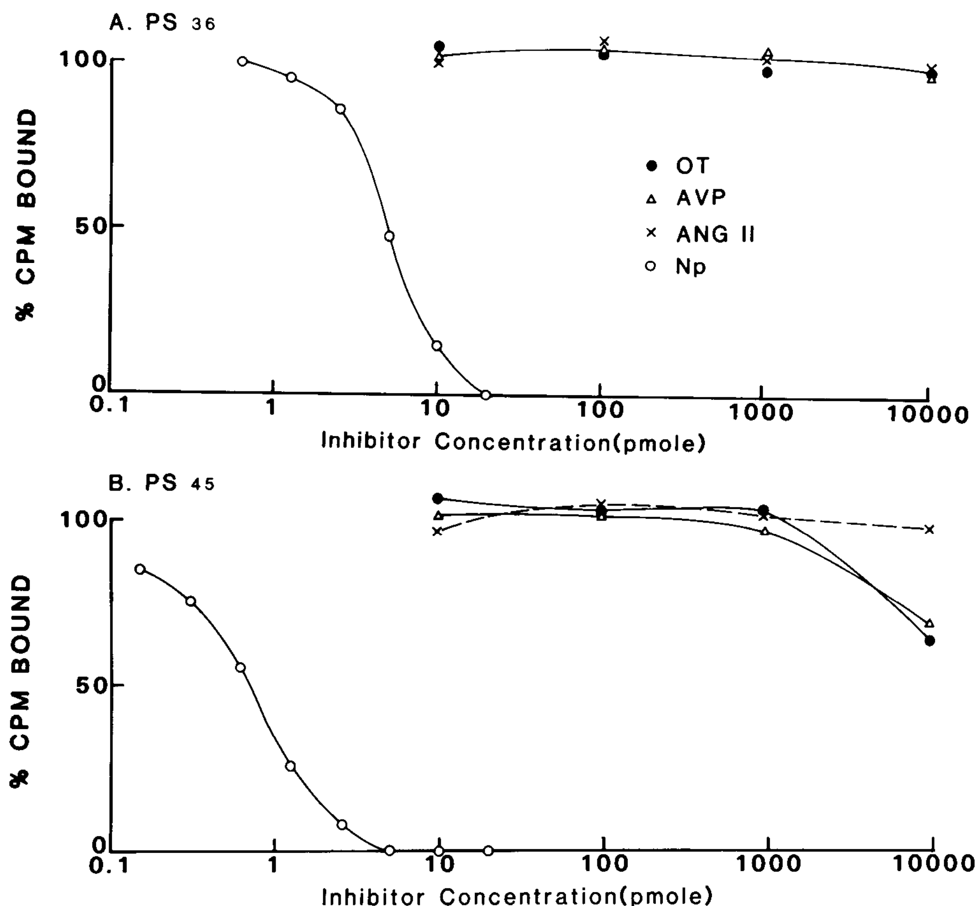

Figure 4. Liquid phase RIA using two representative anti-rat NP monoclonal antibodies (PS 36, *A*; and PS 45, *B*) and ^{125}I-rat NP. Displacement of ^{125}I-NP binding by rat NP, AVP, OT, and angiotensin II (*ANG II*) is shown. Note the low cross-reactivity of peptides other than NP. The *ordinate* shows percentage of counts per minute bound, where B_0 is 100%.

bodies. Antibody binding of the trace was completely displaced at less than 20 pmol/tube (200 ng/tube) of NP. In contrast, the highly abundant peptide hormones, AVP and OT, had little or no inhibitory effects on either antibody's ability to bind NP at concentrations as high as 10,000 pmol/tube. Other peptides which are known to be found in the rat neurointermediate lobe, i.e., dynorphin A and dynorphin B (100 pmol/tube), β-endorphin (100 pmol/tube), α-melanocyte-stimulating hormone (α-MSH, 1000 pmol/tube), and angiotensin II (10,000 pmol/tube), also did not affect antibody binding (data shown only for angiotensin II in Fig. 4). These data demonstrate that PS 36 and PS 45 are specific for NP. Even in the case of PS 45, where AVP and OT caused tracer displacement at high concentrations, the efficacy of NP relative to these peptides to produce 50% inhibition of binding was larger than 10,000:1. Similar experiments were done using the other PS monoclonal antibodies, with similar results (not illustrated). The affinity constants of the various antibodies for rat NP, derived from such displacement curves, are presented in Table I.

Specificities and cross-reactivities of the antibodies for the two NPs. The hypothalamo-neurohypophysial system of normal rats contains two distinct NPs, one associated with the cells that synthesize and secrete AVP (i.e., NP-AVP), and the other found in cells that produce OT (i.e., NP-OT). In the experiments described above (Figs. 2 to 4), no effort was made to distinguish between these two NPs. In the experiments to be described below, the specificities of the monoclonal antibodies for each type of NP were determined.

Affinity-purified NP from posterior pituitaries of normal rats was used as a source of both NP-AVP and NP-OT, whereas pure NP-OT was obtained from posterior pituitaries of homozygous (DI/DI) Brattleboro rats which do not contain either AVP or NP-AVP (Sokol and Valtin, 1982). Each of these NP preparations (5 μg each) was iodinated (see "Materials and Methods"). In these assays, varying concentrations of culture supernatants were incubated with fixed amounts of the labeled NP preparations, and the antibody-antigen complexes were precipitated (see "Materials and Methods") and counted. The results of these experiments are shown in Figure 5. The data indicate that the antibody specificities correspond to two groups. The first group includes PS 36, 38, 60, and 67, which precipitated only about 30% of the labeled normal NP but about 80% of the labeled NP-OT (from DI/DI rats) preparation. In contrast, the second group, which included PS 41, 45, and 46, completely failed to bind the DI/DI NP-OT even at the highest concentrations used, but reacted significantly with labeled NP from normal animals (40% bound by PS 45, which appeared to reach saturation levels). These experiments indicate that PS 41, 45, and 46 are highly specific for NP-AVP and do not cross-react with NP-OT, whereas PS 36, 38, 60, and 67 react with NP-OT exclusively. These conclusions are strongly supported by displacement experiments in which unlabeled NP-OT completely inhibited the binding of normal labeled NP by PS 36, 38, 60, and 67 but did not affect binding by PS 41, 45, and 46, even at concentrations of 2500 ng/tube (data not shown).

Figure 6*C* shows that when PS 36 and PS 45 are used together, at saturating concentrations (i.e., at the 10° dilutions shown in Fig. 5), 100% of the immunoprecipitable normal rat-labeled NP can be bound. Either of these monoclonal antibodies used alone can only bind about 50% of the total immunoprecipitable label (Fig. 6, *A* and *B*), which suggests that each of these antibodies recognizes a separate specific NP in the normal rat NP population. Similar data were obtained when PS 38, 60, or 67 were mixed with PS 45 (not illustrated). In contrast, mixtures of the anti-NP-OT antibodies (Fig. 6*B*) and anti-NP-

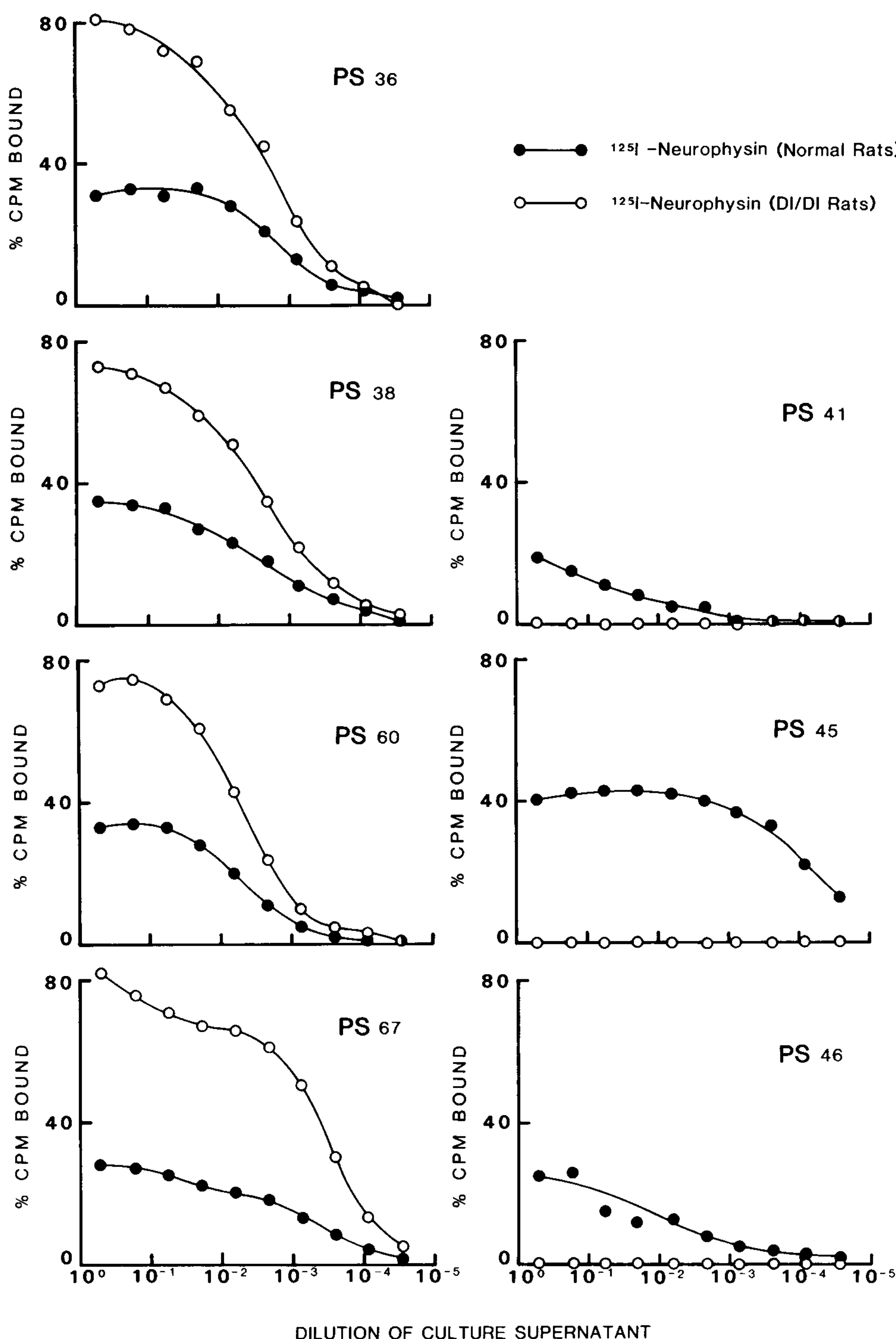

Figure 5. Liquid phase RIA of anti-NP monoclonal antibodies using normal ^{125}I-rat NP (containing NP-OT and NP-AVP) and DI/DI ^{125}I-rat NP (containing only NP-OT). Note that PS 36, 38, 60, and 67 react strongly with DI/DI ^{125}I-rat NP, whereas PS 41, 45, and 46 do not (*open circles*). Irrespective of specificity, all of the antibodies reacted with normal ^{125}I-rat NP; however, among the NP-AVP-specific NPs (PS 41, 45, and 46), only PS 45 reached saturation at the dilutions used. The *ordinate* shows percentage of counts per minute bound of total label in the incubation.

AVP antibodies (Fig. 6*A*) did not bind greater than 50% of the total labeled NP. The fact that PS 41 and PS 46 bound less labeled NP than did PS 45 (Fig. 6*A*) may be due, in part, to the lower titers of these antibodies in the supernatants (see counts per minute at 10° dilutions in Fig. 5). It is, however, apparent from the mix experiments in Figure 6*A* that the addition of PS 41 or PS 46 to PS 45 does not bind more NP label than do saturating levels of PS 45 alone.

The data in Figure 5 indicate that the anti-NP-AVP monoclonals do not cross-react with NP-OT, and the data in Figure 6 suggest a lack of cross-reactivity of the anti-NP-OT monoclonals with NP-AVP. Further evidence for the latter view is shown in Figure 7. In this experiment, putative ^{125}I-NP-AVP was isolated from the labeled normal rat NP by immunoprecipitation with PS 45 (see "Materials and Methods"). The PS 45-bound NP was eluted with 0.1 N HCl, neutralized, and then used as substrate for experiments in Figure 7. The data in Figure 7 show that this immunopurified ^{125}I-NP-AVP can be fully bound by PS 45. However, none of the anti-NP-OT antibodies (PS 36, 38, 60, 67) could bind this tracer (Fig. 7, *solid circles*), whereas all were fully capable of binding ^{125}I-NP-OT (Fig. 7, *open circles*). The conclusions of these studies are

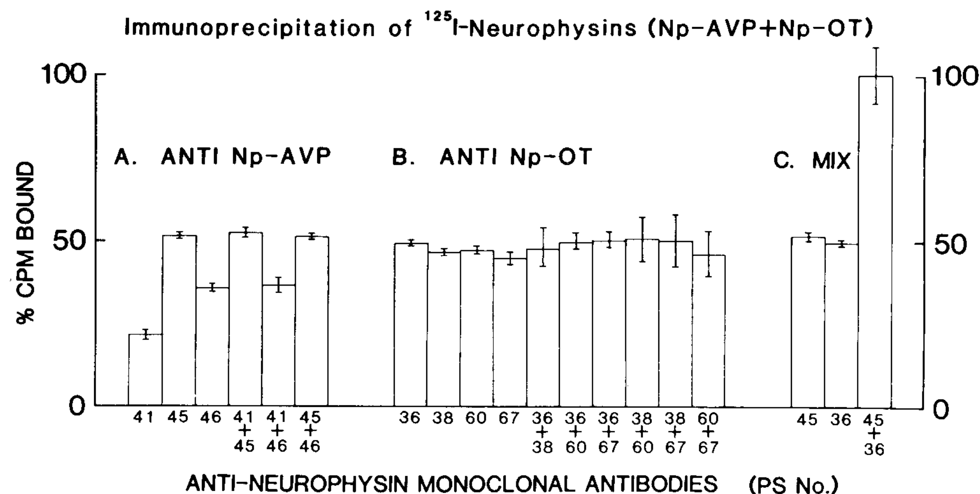

Figure 6. Immunoprecipitation of normal ^{125}I-rat NP (containing both labeled NP-OT and NP-AVP) by individual and mixed antibody supernatants. Concentrations of antibodies used correspond to the 10° dilutions shown in Figure 5. Note that the anti-NP-AVP (*A*) and anti-NP-OT (*B*) used alone or in pairs immunoprecipitated only around 50% of the total labeled NP, whereas the mixed antibodies (PS 36 and 45 in *C*) precipitated nearly 100%. The *ordinate* represents percentage of the total counts per minute in the incubate. $N = 6$ each, ± SD.

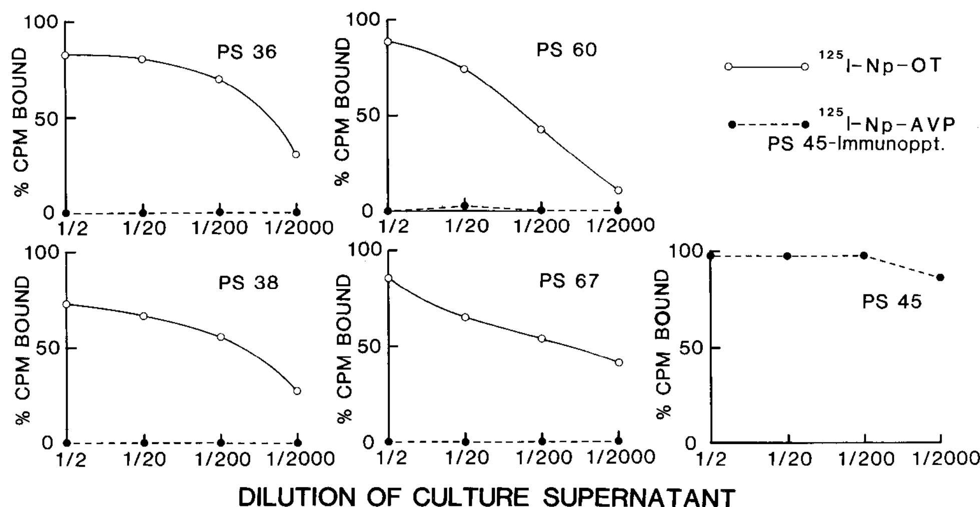

Figure 7. Evidence for the lack of significant cross-reactivity between PS 45-reactive ^{125}I-NP (i.e., NP-AVP) and anti-NP-OT-specific monoclonal antibodies in liquid phase (immunoprecipitation) assays. ^{125}I-NP-OT (○) and ^{125}I-NP-AVP (●) were used in immunoprecipitation experiments (see "Materials and Methods") with various monoclonal antibodies. The ^{125}I-NP-AVP reacted strongly with PS 45 but not detectably with PS 36, 38, 60, and 67. In contrast, the ^{125}I-NP-OT reacted well with PS 36, 38, 60, and 67 but not with PS 45 (data not shown here, but see relevant data in Fig. 5). Assay tubes contained either 305 cpm of ^{125}I-NP-AVP or 1623 cpm of ^{125}I-NP-OT.

that the monoclonal antibodies are either specific for NP-AVP (PS 41, 45, and 46) or for NP-OT (PS 36, 38, 60, and 67), with no detectable cross-reactivities under the liquid-phase assay conditions used here.

Cross-reactivities of the monoclonal antibodies in solid phase assays. Although the liquid phase assays described above are the methods of choice for cross-reactivity studies (see Berzofsky and Schechter, 1981; Berzofsky and Berkower, 1984), neurobiological studies often employ solid phase assays. Since immunological reagents (such as monoclonal antibodies) are often evaluated for their specificities under liquid phase assay conditions but are used in biological studies under solid phase conditions (e.g., immunocytochemistry), we show the data in Figure 8 as a cautionary point in this regard. Although PS 41, 45, and 46 showed absolutely no cross-reactivity for NP-OT in the above liquid phase assays (Figs. 5 to 7), significant cross-reactivity was observed with these antibodies to NP-OT from homozygous Brattleboro rats in solid phase assays (Fig. 8, *open circles*). Note that while the measurable cross-reactivities for PS 41 and PS 46 could be "diluted out" (Fig. 8, *A* and *C*), this was not possible for PS 45 (Fig. 8*B*). Similar data were obtained when purified rat NP antigens were used to coat the wells (data not shown).

Cross-reactivities of the monoclonal antibodies in immunocytochemistry. Several features of the morphology of the hypothalamo-neurohypophysial system of the rat served to facilitate our analysis of the antibody specificities to the NPs. These included the following. (*1*) The PVN is organized into discrete subnuclei, some areas consisting of nearly all OT-containing or nearly all AVP-containing magnocellular neurons (Vandesande and Dierickx, 1975; Hatton et al., 1976; Armstrong et al., 1980; Swanson and Kuypers, 1980; Rhodes et al., 1981; Sofroniew and Glasman, 1981; Silverman and Zimmerman, 1983; Swanson and Sawchenko, 1983). (*2*) A population of parvicellular neurons containing AVP and NP-AVP but no OT or NP-OT exists in the suprachiasmatic nucleus (SCN; Vandesande et al., 1975).

The anterior commissural nucleus (ACN) is a rostral extension of the PVN consisting of magnocellular neurons which contain OT and NP-OT but no AVP or NP-AVP (see above references). Hence, it represents a convenient location in which to test the immunocytochemical specificities of antibodies for NP-OT. The SCN represents a similar situation for NP-AVP, but consists of parvicellular neurons. Magnocellular neurons containing either NP-OT or NP-AVP are both found in the SON.

Figure 9 illustrates the staining of these nuclei using three representative anti-NP antibodies, i.e., PS 36, 41, and 45. Figure 9*A* shows the staining pattern for an anti-NP-OT monoclonal antibody (i.e., PS 36): the ACN and SON stained intensely, whereas the SCN remained unstained. This indicates that the PS 36 antibody showed little if any cross-reactivity with NP-AVP in these immunocytochemical experiments. Similar results were obtained with the other anti-NP-OT antibodies, PS 38, 60, and 67 (data not shown). The anti-NP-AVP antibody PS 41 clearly stained both the SON and the SCN but also slightly stained some cells in the ACN (Fig. 9*B*), indicating some cross-reactivity with NP-OT. A similar result was obtained using PS 46 (not illustrated). This result with PS 41

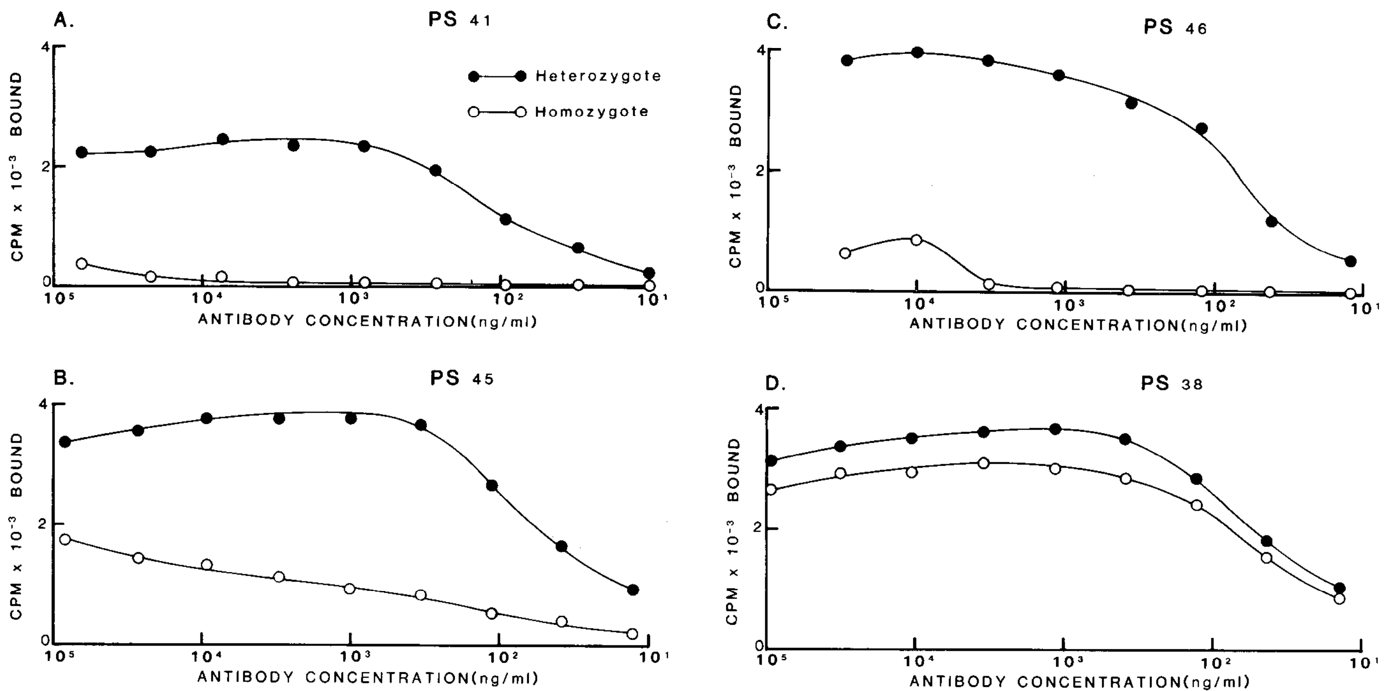

Figure 8. Solid phase RIAs of posterior pituitary extracts from homozygous (○) and heterozygous (●) Brattleboro rats. Wells of Dynatech plates were coated with 50 μl of the indicated extracts, 1 to 1.5 μg of total proteins/well, and incubated with Protein A-purified monoclonal antibodies (diluted with PBS and 1% BSA). Antibody binding was measured by the specific binding of ^{125}I-sheep anti-mouse IgG to the wells. Note that the anti-NP-AVP antibodies (i.e., *PS 41, 45,* and *46*) do show cross-reactivity with NP-OT antigens (○) in solid phase assays. Compare PS 45 data in this figure to the liquid phase assay in Figure 5.

was similar to that found in the solid phase assays (Fig. 8*A*); i.e., PS 41 is cross-reactive with NP-OT at high concentrations of antibody (or antigen, in the case of immunocytochemistry in Fig. 9*B*). That the behaviors of the antibodies in the solid phase biochemical assay are relevant to their behavior in immunocytochemistry is confirmed by the observations using PS 45 (Fig. 9*C*). PS 45 stained all of the nuclei (i.e., both OT and AVP neurons) very intensely (Fig. 9*C*), indicating strong cross-reactivity to NP-OT in the solid phase (see also Fig. 8*C*). As in the case of the biochemical assay (Fig. 8*C*), the cross-reactivity of PS 45 in immunocytochemistry could not be eliminated by dilution of the antibody (data not shown).

Immunocytochemical studies with the above three antibodies on the ultrastructural level were also performed. These studies represent an independent immunocytochemical test of antibody specificity since it is known that NP-AVP- and NP-OT-containing vesicles are located in separate endings in the posterior pituitary (see Morris et al., 1978; Castel et al., 1984). When ultrathin LR White sections of the posterior pituitary were stained with the anti-NP monoclonal antibodies, staining was present only over NSVs of about 160 nm in diameter which were located in subpopulations of axons, Herring bodies (swellings), and terminals (Fig. 10). Serial sections stained with either PS 36 (Fig. 10*A*) or PS 41 (Fig. 10*B*) showed staining in mutually exclusive populations of axons, swellings, and terminals, although PS 41 did display some cross-reactivity with the NSVs stained by PS 36. The reverse cross-reactivity, however, was not observed. All NSVs were stained by PS 45 (Fig. 10*C*), and no dilution of this antibody could be found at which one subpopulation of axons stained more intensely than the other. The staining of all axons dropped out between 1:1000 and 1:5000 (not shown). When mixtures of PS 36 and PS 41 were used, all of the NSVs stained in a manner similar to that shown for PS 45 in Figure 10*C* (data not shown). The control antibodies used at 1:10 dilutions resulted in no staining of the NSVs (Fig. 10*D*).

These immunocytochemical studies at both the light and electron microscopic levels indicate that antibody cross-reactivities in immunocytochemistry are better reflected by solid phase biochemical assays (as in Fig. 8) as opposed to liquid phase biochemical assays (as in Figs. 4 to 7).

Cross-reactivities of PS 36 and PS 45 with phylogenetically diverse NPs. Several properties of the anti NP-OT PS 36 and anti NP-AVP PS 45 antibodies were favorable for an evaluation of the epitopes which they recognized on rat NP. These included their high specificities in liquid phase assays (Figs. 4 to 7) and their relatively high affinity constants (Table I). In addition, the amino acid sequences of a variety of mammalian NPs are known (Pickering and Jones, 1978; Chauvet et al., 1981, 1983). By studying the cross-reactivities of these antibodies with phylogenetically diverse NPs of known structure, it was possible to correlate the antibody reactivity with the variations in NP amino acid sequence (see Table II and "Discussion").

Figure 11 illustrates liquid phase RIA data using PS 36 (Fig. 11*A*) and PS 45 (Fig. 11*B*) and phylogenetically diverse sources of the NPs. The *abscissa* in Figure 11 is expressed in nanograms of unlabeled protein per tube. In this RIA the competitive displacements of ^{125}I-labeled rat NP (containing both ^{125}I-NP-AVP and ^{125}I-NP-OT) by purified rat, bovine, and human NPs, as well as mouse and frog pituitary extracts, were compared. A qualitative analysis of the data in Figure 11*A* shows that PS 36 is quite reactive with rat and mouse NPs but does not appear to appreciably react with frog NP, cow NPI (cow NP-OT), or human NPIII (human NP-OT, or ESN) under these liquid phase conditions. It is possible that if higher concentrations (>1 μg/ml) of inhibitors were used in these studies, some cross-reactivity of PS 36 with frog, cow, or human NP would have

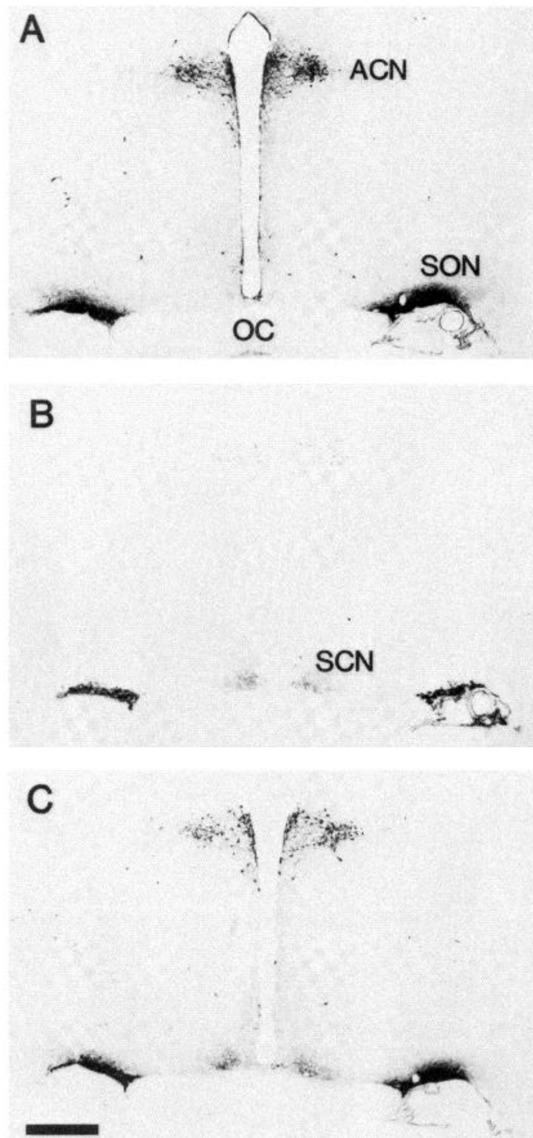

Figure 9. Immunostaining of 50-μm coronal Vibratome sections through the anterior commissural nucleus (*ACN*) (containing only NP-OT), the suprachiasmatic nucleus (*SCN*) (containing only NP-AVP), and the SON (containing both NPs) of heterozygous Brattleboro rat brain. *A*, Monoclonal antibody PS 36 (1:100) stains the ACN and SON, but not the SCN. *B*, Monoclonal antibody PS 41 (1:100) stains the SCN and SON, with faint cross-reactivity evident in some cells in the ACN. *C*, Monoclonal antibody PS 45 (1:100) stains all three nuclei. *A* is approximately midway between *B* and *C*; *B* and *C* are 500 μm apart. *OC*, optic chiasm. *Bar*, 500 μm.

been observed. In contrast, PS 45 is much more generally cross-reactive (Fig. 11*B*). The order of strength of reactivity of PS 45 with various NPs was rat NP ≡ mouse NP > cow NPII (cow NP-AVP) > human NPII, IV (two variants of NSN, human NP-AVP) ≡ frog NP > cow NPI (NP-OT) > human NPIII (human NP-OT, ESN). From the above, it can be seen that the PS 45 cross-reactivity is generally greater for NP-AVPs from other species as compared to NP-OTs, and, therefore, this antibody detects a conserved epitope which may be common to NP-AVPs across species lines. The apparent (but low) cross-reactivities of the NP-OTs from cows (cow NPI) and humans (human NPIII) may be, in part, due to a 10% contamination with NP-AVP molecules (see "Materials and Methods"). In this regard, it is interesting that the frog and pituitary extract cross-reacted with PS 45 (see "Discussion").

Heterogeneity of rat NPs. Isoelectric focusing of rat posterior pituitary extracts or affinity-purified rat NP reveals two major forms of NP: NP-OT, with a pI of 4.6, and NP-AVP, with a pI of 4.8 (Brownstein and Gainer, 1977; Russell et al., 1980). When such isoelectric focusing gels were electroblotted onto nitrocellulose paper and stained by PS 45 or PS 36 antibodies, many immunoreactive bands were revealed (Figs. 12 and 13). Figure 12 shows such immunostaining patterns for posterior pituitary extracts from homozygous (HOBB, DI/DI) and heterozygous (HEBB, DI/DI) Brattleboro rats. Although PS 45 fails to stain the HOBB pituitary extract (which does not contain NP-AVP), it stains considerably more than one band in the HEBB pituitary extract (which does contain NP-AVP). Thus, there appear to be several other forms of immunoreactive NP-AVP in HEBB rat pituitaries. This heterogeneity could be due to a slow proteolytic breakdown of the major form of NP-AVP during its long-term (>2 weeks) storage in the NSVs in the neural lobe. Similarly, the NP-OT, stained in both HOBB and HEBB extracts by PS 36, shows heterogeneity. It is of interest to note that at the dilutions of PS 45 used in this immunostaining of nitrocellulose blots, very little if any cross-reactivity of PS 45 for NP-OT (HOBB) could be detected.

Figure 13 shows immunoblot staining of affinity-purified normal rat NP (containing NP-AVP and NP-OT) by all of the monoclonal antibodies described in this paper. Although the high degree of heterogeneity in the stains using anti-NP-OT antibodies obscured their detailed patterns, it is clear that the major NP bands stained by the two classes of antibodies (Fig. 13, *large arrows*) are different and are consistent with previous observations that the major form of NP-AVP is more basic than the major form of NP-OT (Brownstein and Gainer, 1977).

The monoclonal antibodies react with the NP precursors. It was of importance to establish whether the anti-NP antibodies could recognize the NPs in their precursor forms, i.e., in the Pro-PP and Pro-OP prohormones (Russell et al., 1980). It was possible that the altered conformational structure of the precursor molecule might affect the specificity of the antibodies or render the antigenic determinant on the NPs inaccessible. In order to examine this question, radioactively labeled NPs and precursors were made by *in vivo* biosynthesis in rats (see "Materials and Methods") and isolated. The ^{35}S-labeled NP-AVP and NP-OT molecules synthesized in the SONs of living rats and transported to the posterior pituitary were then assayed for their reactivities to the antibodies by immunoprecipitation procedures. The data in Figure 14 show that these NPs synthesized *in vivo*, yielded the same specificity patterns as the iodinated NPs studied earlier (i.e., in Figs. 5 to 7).

Figure 15 shows the results of a similar set of immunoprecipitation experiments using ^{3}H-labeled NP precursors. The precursor containing NP-AVP, Pro-PP (Fig. 15*A*), was recognized by PS 41, 45, and 46, but not significantly by the anti-NP-OT antibodies (i.e., PS 38, 36, 60, and 67). The small apparent reactivity of the Pro-PP with the anti-NP-OT antibodies (compared to the HO-13-14 control) was probably due to the 10% contamination of the Pro-PP preparation by Pro-OP. In the case of the NP-OT precursor, Pro-OP (Fig. 15*B*), each of the anti-NP-OT antibodies could immunoprecipitate the Pro-OP, whereas the anti-NP-AVP antibodies were completely ineffective. Thus, all of the antibodies recognized their respective NPs in precursor form. Whereas the anti-NP-OT antibodies appear to be equally effective in precipitating NP-OT or Pro-OP (Figs. 6, 14, and 15), the effectiveness of the anti-NP-AVP antibodies differed, in the order PS 45 >> PS 46 > PS 41 (Figs. 6, 14, and 15). This is principally due to the lower apparent titers of the PS 41 and PS 46 antibodies in the culture supernatants (Fig. 5), and the lower affinity constants of these two antibodies (Table I). However, despite the lower effectiveness of the PS 41 and PS 46 antibodies in liquid phase assays (Fig. 6, 14, and

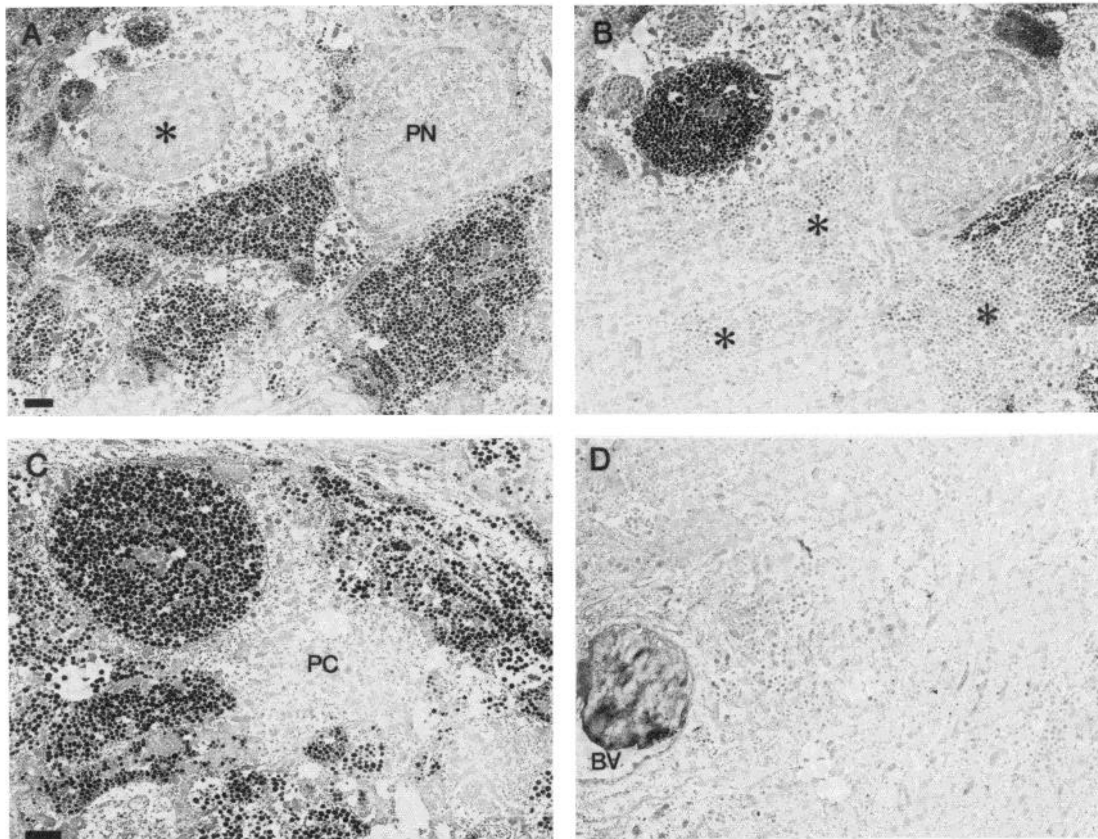

Figure 10. Immunostaining of ultrathin LR White sections of posterior pituitary of the heterozygous Brattleboro rat. *A* and *B* are adjacent serial sections stained with monoclonal antibody PS 36 (*A*, 1:1000) and monoclonal antibody PS 41 (*B*, 1:200). Monoclonal antibodies PS 36 and 41 stain all NSVs in mutually exclusive populations of axons, Herring bodies, and axon terminals. Some cross-reactivity of PS 41 to NSVs in the "unstained" axons, Herring bodies, and terminals is evident. *C*, Monoclonal antibody PS 45 (1:500) stains all NSVs in all axons, Herring bodies, and terminals. *D*, A control monoclonal antibody (directed against the mouse histocompatibility H-2 complex) at 1:10 produced no staining. *Asterisks*, unstained axons. *BV*, blood vessel; *PC*, pituicyte cytoplasm; *PN*, pituicyte (glial) nucleus. *Bars*, 1.0 μm. *B* and *D* are at the same magnification as *A*.

15), they appear to be comparable in effectiveness to PS 45 in solid phase assays (Fig. 8) but with lower cross-reactivities.

Discussion

In this paper we have described the production and characterization of seven mouse monoclonal antibodies against rat NP. Three were directed specifically against NP-AVP (PS 41, 45, and 46), and four were specifically against NP-OT (PS 36, 38, 60, and 67). The antibodies also recognized the antigens (NP) in their precursor forms with few, if any, changes in selectivity, indicating that the antigenic sites in the NPs were still accessible in the prohormones (Pro-OP and Pro-PP; Fig. 15). Although these antibodies fall into two classes with respect to the rat NPs, (i.e., anti-NP-AVP and anti-NP-OT), cross-reactivity studies using phylogenetically diverse NPs show that their antigenic determinants are not necessarily identical, even within classes. Only one of the antibodies (PS 67) was specifically directed against rat NP, whereas the others could react to varying degrees with NPs from other animal species (Fig. 11; unpublished data).

An unexpected result of this study was that even though our immunogen contained, in addition to NP, equimolar concentrations of AVP, OT, and the Pro-PP-derived glycopeptide, as well as substantial concentrations of β-endorphin, α-MSH, dynorphin, and leucine-enkephalin, we obtained monoclonal antibodies *only* to NP. We have no explanation for this apparent relative efficacy of NP as an immunogen. This could be related to a combination of factors: the relatively high concentration of NP in posterior pituitary extracts, its higher molecular weight (about 10,000) than the other peptides, and our KLH-coupling procedure. In a recent report, where mice were immunized with homogenized hypothalami (not using the KLH-coupling procedure), no monoclonal antibodies were obtained which were specifically directed against hypothalamic cells, although many "neuron-specific" antibodies were generated (Sternberger et al., 1982). In addition to the nature of our immunogen and the KLH-coupling procedure, our initial screening strategy, which employed both ELISA assays and immunohistochemistry, was helpful in defining which hybridoma supernatants contained antibodies specific against the hypothalamo-neurohypophysial system. The use of glutaraldehyde in the KLH-coupling procedure enhanced the probability that any specific antibodies which would be generated would be useful in electron microscopic immunocytochemical experiments (Whitnall et al., 1983) where glutaraldehyde would be the fixative of choice.

Epitope analysis based on NP amino acid sequences and antibody cross-reactivities. The cross-reactivity data shown in Figure 11 and the amino acid sequence variations in NPs shown in Table II permit a preliminary analysis of the epitopes recognized by PS 36 and PS 45. Figure 11 shows that: (*a*) PS 36 is relatively selective for rat (rodent) NP-OT as opposed to cow and human NP-OT (Fig. 11*A*), and (*b*) PS 45 appears to react with all NP-AVPs with varying affinities (Fig. 11*B*). In addition, no cross-reactivity was observed between PS 45 and PS 36 with rat NP-OT and NP-AVP, respectively, in liquid phase

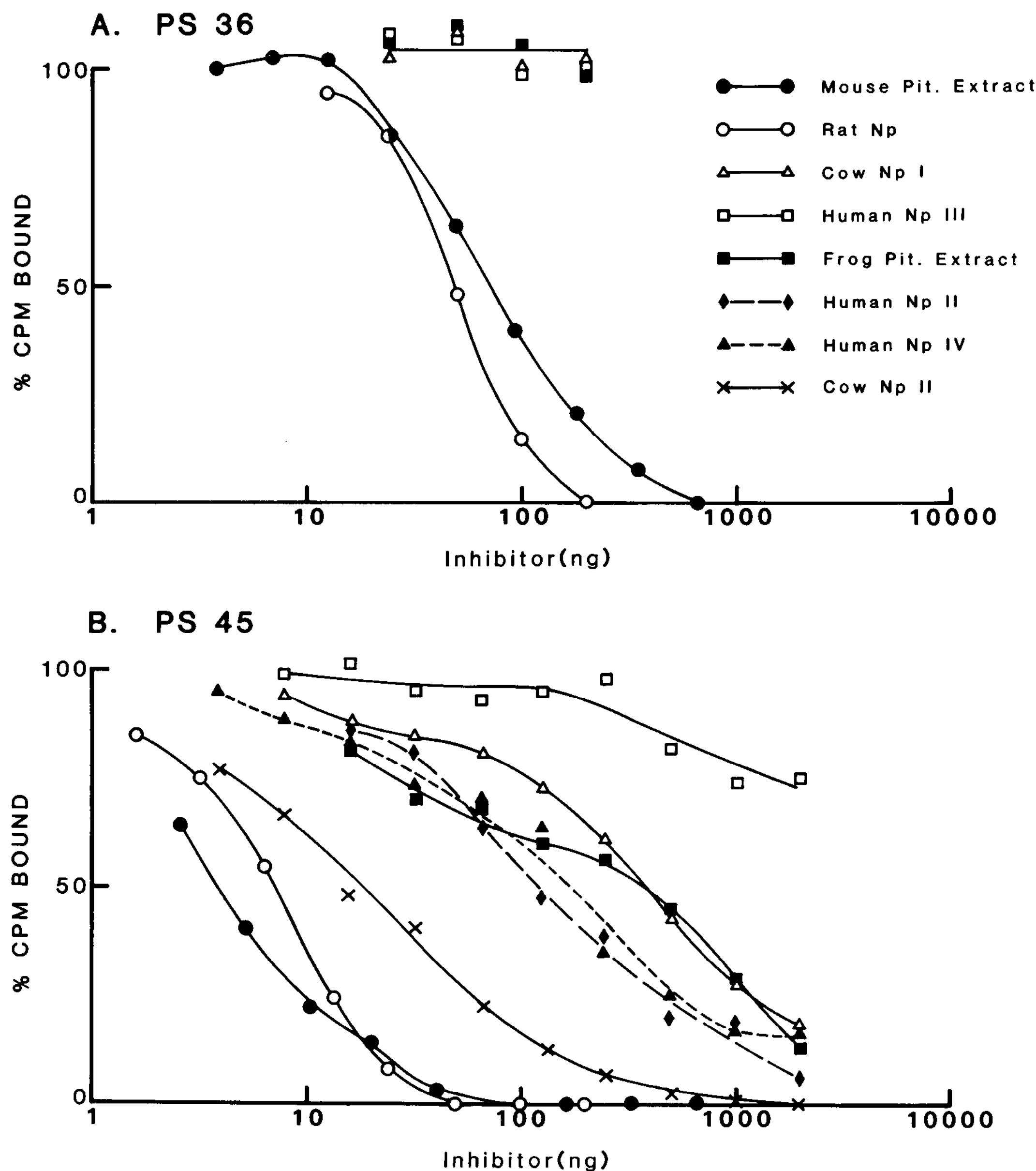

Figure 11. Cross-reactivity of phylogenetically diverse NPs and posterior pituitary extracts with PS 36 (*A*) and PS 45 (*B*) in liquid phase RIA. *A*, PS 36 (anti-NP-OT antibody) RIA; *B*, PS 45 (anti-NP-AVP) antibody) RIA. ^{125}I-NP from normal rats (containing both ^{125}I-NP-AVP and ^{125}I-NP-OT) was used as the tracer in these experiments. See "Materials and Methods" and the text for description of the procedures. The *abscissa* shows the amount of specific purified NP (in nanograms) which was used as the inhibitor. In the case of the mouse and frog pituitary extracts the nanogram value represents the amount of tissue protein used.

assays (see Fig. 5). Given these observations, the known amino acid sequence data in Table II can be used to evaluate the candidates for epitopes for these two antibodies. PS 36, for example, should be interacting at amino acid sites which differ between rat NP-OT and rat NP-AVP, but also where differences exist between rat NP-OT and cow and human NP-OT. These sites of difference are shown by rectangles in Table II.

We believe that position 80–81 is the most likely epitope locus for PS 36. The reasons are as follows: (*1*) the difference between all of the NP-AVPs and NP-OTs are most extreme in this position; i.e., position 80 contains a basic amino acid (Arg or His) in all NP-OTs as opposed to a hydrophobic amino acid (Val) in all NP-AVPs; (*2*) reductive alkylation of rat NP-OT blocks all of its reactivity with PS 36 (unpublished data), suggesting that a nearby cysteine is involved (e.g., position 79); and (*3*) trypsin cleavage of the rat NP-OT does not eliminate its reactivity with PS 36 (Table III), discounting positions 1 to 9 as participating in the epitope (Table II).

A similar analysis for the PS 45 antibody suggests that the amino acid positions 75 to 86 in rat NP-AVP contains the epitope. In this case, the cross-reactivity data imply some similarity between rat NP-AVP and cow and human NP-AVP, but differences between rat NP-AVP and rat NP-OT. The strong homologies in the central cores of NPs in general and the large differences between rat NP-AVP and cow NP-AVP from positions 89 to 95 remove these regions from consideration. This leaves the N-terminus, 1–9, and the C-terminus, 75–88 (Table II), as candidates. Similar experiments using trypsin cleavage (Table III) and reductive alkylation (unpublished data), as described for PS 36, were done for rat NP-AVP and PS 45, with similar results. The failure of trypsin to decrease rat NP reactivity with PS 45 would eliminate the 1 to 8 and 87

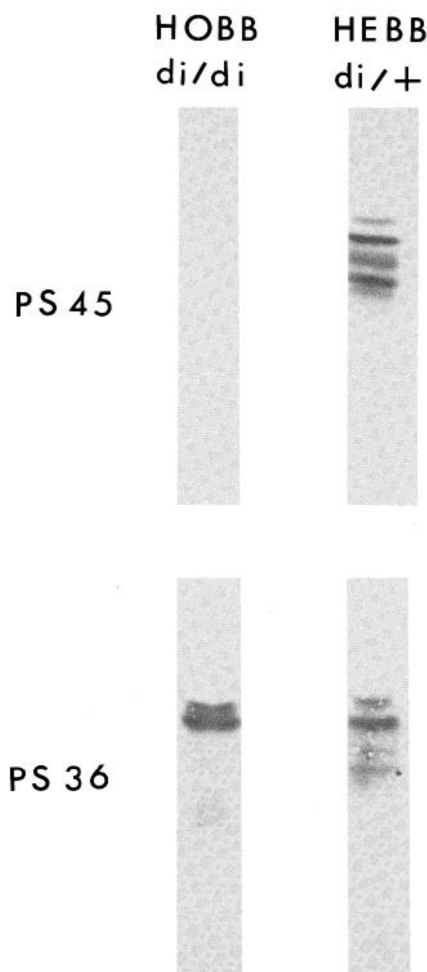

Figure 12. Isoelectric focusing and immunostaining by PS 45 and PS 36 of posterior pituitary extracts from homozygous (HOBB, DI/DI) and heterozygous (HEBB, DI/+) Brattleboro rats. Note the heterogeneity of immunostained bands, and the absence of PS 45-positive antigens in HOBB (DI/DI) pituitaries. The anode was at the top in these focusing runs. Dilution of culture supernatant used for staining was 10^{-1} (see Fig. 5), and total protein electrophoresed was 50 μg in each case.

to 89 positions as candidates (see Table II for tryptic cleavage sites), and the inhibition of reactivity by alkylation also suggests a nearby cysteine (e.g., cysteine 10 or cysteine 79). Position 9 in NP-AVP could be involved since all NP-AVPs have a Gln in this position in contrast to NP-OTs, which do not (see Table II). Since the trypsin experiments in Table III exclude positions 1 to 8 in NP-AVP, then the epitope would have to extend from position 9 into the central core. However, this whole region (i.e., positions 9 to 28) is invariant between rat, cow, and human NP-AVPs, and such an epitope locus could not explain the variations in cross-reactivities found for PS 45 and these NP-AVPs (Fig. 11). Hence, we believe that position 75 to 86 in NP-AVP is the most likely candidate for the epitope locus for PS 45. This locus would be near enough to the Ala-80 in rat NP-AVP to encompass the only change in cow and human NP-AVP (i.e., the change from Ala to Thr in position 81). This change would be consistent with the lower affinities of cow NP-AVP and human NP-AVP to PS 45 as compared to rat NP-AVP. Thus, it is most likely that PS 45 is recognizing rat NP-AVP near positions 75 to 86, centering on positions 79 to 81.

The above analysis cannot discount the possibility that the tertiary structure of NP may play a role in determining the epitope. Indeed, the complete loss of cross-reactivity after reductive alkylation of the cysteines in the NPs might suggest that this is the case. However, the gel electrophoresis-immunoblot studies were also done under reducing conditions and thereby produced abnormal folding of the molecules. Immunoreactivity was preserved under these conditions, suggesting that permanent modification of the cysteines (by alkylation) was necessary to prevent immunoreactivity with the antibodies.

Given the above caveats about this epitope analysis, we note that the 75 to 88 region represents a highly conserved amino acid sequence in the rat, cow, and human NP-AVPs (only two relatively conservative amino acid changes are made out of 14; Table II). Not only are the amino acid sequences in this region conserved, but the codons for these amino acids are as well. Among the 42 nucleotides coding for the amino acids in this region, only four differences are found between the rat and the cow (Schmale et al., 1983). In contrast, the codon changes between cow NP-OT and NP-AVP in this region involve 25 of the 42 nucleotides (Land et al., 1983). Thus, the 75 to 88 region appears to be highly conserved in NP-AVPs between mammalian species but is very divergent from NP-OT even in the same species. This contrasts from the central core of NP (positions 10 to 74), which is highly conserved in all forms of NP. Hence, the 75 to 88 region of the NP-AVP (and NP precursor) must have evolved independently of the central core, and it is intriguing that the antibody which recognizes this epitope (i.e., PS 45) is able to cross-react with an antigen (presumably NP) extracted from frog posterior pituitaries (Fig. 11*B*).

Cross-reactivities and specificities of the antibodies. Several lines of evidence in this paper point to the high specificities and low cross-reactivities of these antibodies to either rat NP-OT or rat NP-AVP (Figs. 4 to 7, 12, and 14). We found, however, that whereas this was the case in liquid phase assays, the situation in solid phase assays (Fig. 8) was quite different. As is illustrated by the case of PS 41 versus PS 45 (cf. Figs. 5 and 8*B*), the higher the affinity constant of the antibody, the greater was its cross-reactivity found in the solid phase assay. PS 41, which is about 25 times lower in affinity than PS 45 (Table I) and is a poorer antibody for the liquid phase RIA of NP-AVP, is a superior antibody for use in solid phase assays because of its lower cross-reactivity to NP-OT (Fig. 8).

We focused on this issue of cross-reactivity in solid phase assays because neurobiologists often use the same antibody reagents for both liquid phase RIA and immunocytochemical procedures. The immunocytochemical data we have presented (Figs. 9 and 10) clearly show that the antibodies used in this type of procedure are similar in their cross-reactivities to those found in solid phase biochemical assays (Fig. 8) and not liquid phase assays (Figs. 4 to 7). In principle, immunocytochemistry represents an extreme form of the solid phase assay in that the antigen (e.g., NP) is present in the NSV at very high concentration (i.e., around 84,000 molecules/NSV; see Morris et al., 1978). This effect of increased concentration in the solid phase for immunocytochemical assay as opposed to the liquid phase assay is expected on theoretical grounds (see Berzofsky and Berkower, 1984), in part due to an increase in the apparent affinity of the bivalent antibody for the antigen under these conditions. The high local concentration of antigen in solid phase assays (especially in ICC of antigens present in highly concentrated form in the cell) also causes an increase in apparent affinity due to a "retention effect" (Silhavy et al., 1975). Thus, even a monovalent antibody which has just detached from the substrate would have a higher probability of reassociating with an antigen molecule than of diffusing away from the substrate. As a consequence of the above, antibodies with modest liquid phase affinities can undergo essentially irreversible interactions with immobilized antigen in solid phase assays (Berzofsky and Berkower, 1984). Hence, a small cross-reactiv-

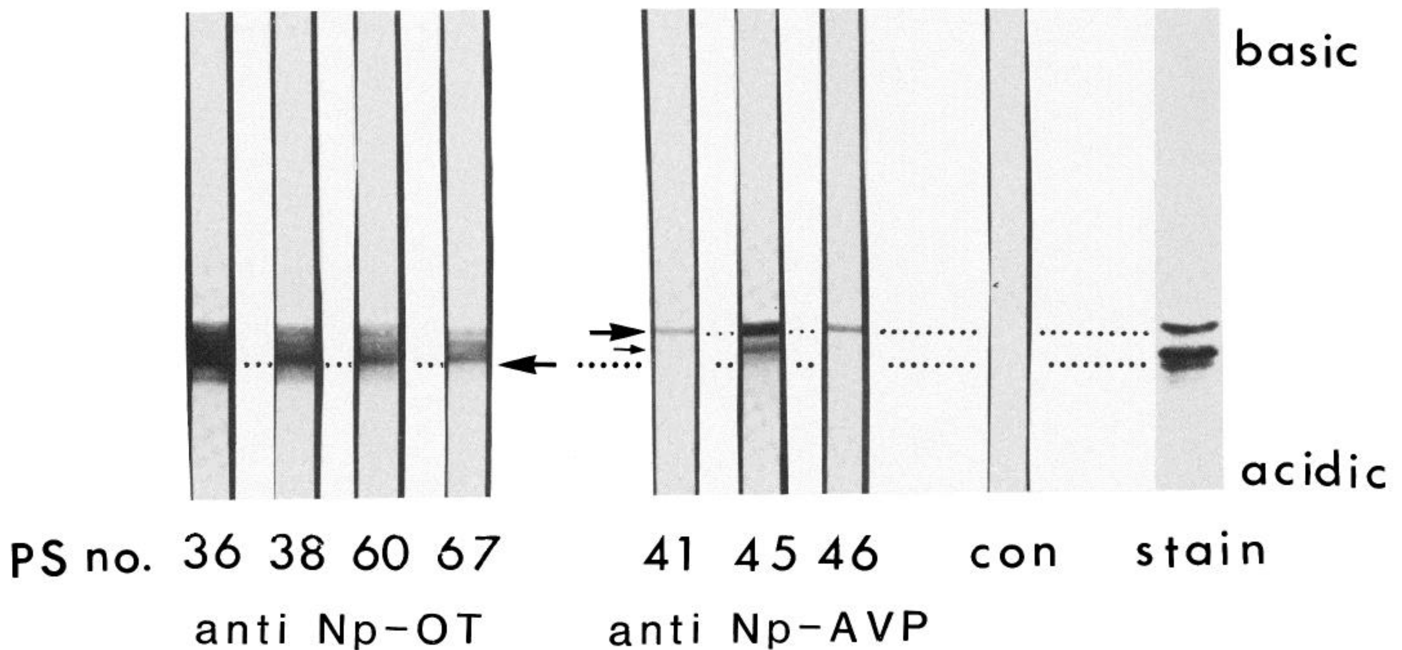

Figure 13. Isoelectric focusing (IEF) and immunostaining of affinity-purified NP from normal rat posterior pituitaries using various monoclonal antibodies at 10^{-1} dilutions (see Fig. 5). Note the heterogeneity of stained bands. Amido black stain is shown at the *right* (*stain*). Approximately 25 μg of affinity-purified NP were electrophoresed in each *lane*.

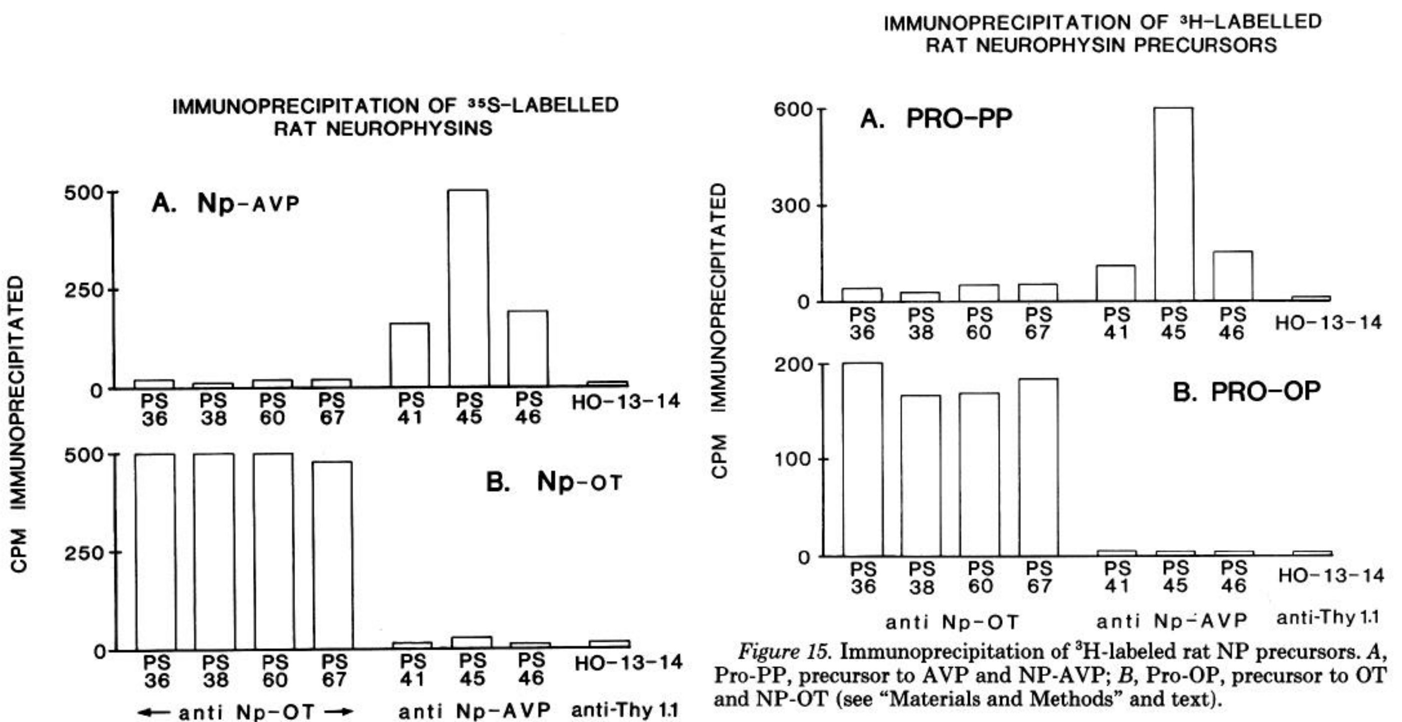

Figure 14. Immunoprecipitation of ^{35}S-labeled NPs synthesized *in vivo* and axonally transported to the rat neural lobe. The ^{35}S-labeled NPs were separated by ion exchange high pressure liquid chromatography into ^{35}S-NP-AVP (*A*) and ^{35}S-NP-OT (*B*) before immunoprecipitation (see "Materials and Methods"). Note the absence of measurable cross-reactivity, relative to control antibody (HO-13-14, anti-Thy 1.1 monoclonal antibody).

Figure 15. Immunoprecipitation of ^{3}H-labeled rat NP precursors. *A*, Pro-PP, precursor to AVP and NP-AVP; *B*, Pro-OP, precursor to OT and NP-OT (see "Materials and Methods" and text).

ity in liquid phase can become a significant problem in solid phase assays.

The discrepancy between liquid and solid phase affinities presents an obvious difficulty for absorption controls in immunocytochemistry. It has been noted that including an excess of antigen in the primary antibody solution does not always

TABLE II

Amino acid variations in NP sequences

The amino acids shown for each NP are those which show some variability between species in the highly homologous central core (B), and all of the N- and C-terminal amino acids. The rectangles illustrate the points of variability between rat NP-OT and NP-AVP (first pair), rat versus cow versus human NP-OT, etc. The asterisks next to the amino acid numbers designate sites where electrical charge changes are found between amino acids in rat NP-OT and NP-AVP, and the arrow (T) designates trypsin-sensitive sites in the native rat NP molecule. Sequence data were obtained from Chauvet et al. (1981, 1983) and Pickering and Jones (1978). Positions 11 to 29 and 37 to 68 in the central core have been omitted from this table since they are identical in all of the NPs in the species listed.

Amino Acid No.	Rat NP		NP-OT			NP-AVP		
	NP-OT	NP-AVP	Rat	Cow	Human	Rat	Cow	Human
1	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
2	Ala	Thr	Ala	Val	Ala	Thr	Met	Met
3	Leu	Ser	Leu	Leu	Pro	Ser	Ser	Ser
4	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp
5	Leu	Met	Leu	Leu	Leu	Met	Leu	Leu
6	Asp	Glu	Asp	Asp	Asp	Glu	Glu	Glu
7	Met	Leu	Met	Val	Val	Leu	Leu	Leu
8	Arg	Arg	Arg	Arg	Arg	Arg	Arg	Arg
	T →	← T						
9*	Lys	Gln	Lys	Thr	Lys	Gln	Gln	Gln
	T →							
B. Central core								
10	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys
29	Ala	Ala	Ala	Gly	Ala	Ala	Gly	Ala
30	Asp	Asp	Asp	Asp	Glu	Asp	Asp	Asp
36	Val	Leu	Val	Val	Val	Leu	Val	Val
69	Thr	Ala	Thr	Thr	Thr	Ala	Ala	Ala
73	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys
74	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys
C. COOH-terminus								
75	Ser	Ser	Ser	Ser	Ser	Ser	Asn	Asn
76*	Pro	Asp	Pro	Pro	Pro	Asp	Asp	Asp
77	Asp	Glu	Asp	Asp	Asp	Glu	Glu	Glu
78	Gly	Ser	Gly	Gly	Gly	Ser	Ser	Ser
79	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys
80*	Arg	Val	Arg	His	His	Val	Val	Val
81	Thr	Ala	Thr	Glu	Ala	Ala	Thr	Thr
82	Asp	Glu	Asp	Asp	Asp	Glu	Glu	Glu
83	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro
84*	Ala	Glu	Ala	Ala	Ala	Glu	Glu	Glu
85	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys
86*	Asp	Arg	Asp	Asp	Asp	Arg	Arg	Arg
		← T						
87*	Pro	Glu	Pro	Pro	Pro	Glu	Glu	Glu
88*	Glu	Gly	Glu	Glu	Glu	Gly	Gly	Gly
89	Ser	Phe	Ser	Ala	Ala	Phe	Val	Phe
90	Ala	Phe	Ala	Ala	Ser	Phe	Gly	His
91*	Phe	Arg	Phe	Phe	Phe	Arg	Phe	Phe
		← T						
92	Ser	Leu	Ser	Ser	Ser	Leu	Pro	Leu
93	Gln	Thr	Gln	Gln		Thr	Arg	
94							Arg	
95							Val	

block staining (Sternberger, 1979). That this may be the case, even for monoclonal antibodies, can be illustrated by considering the case of PS 45. PS 45 is an extraordinarily specific antibody for NP-AVP in liquid phase assays (Figs. 4 and 5) but cross-reacts strongly with NP-OT in solid phase assays (Fig. 8) and in immunocytochemistry (Figs. 9 and 10). Given this information, it is apparent that a conventional absorption control procedure using NP-OT would not absorb PS 45 and, hence, would not prevent this antibody from cross-reacting with NP-OT-containing cells in immunocytochemical studies. In contrast, conventional absorption procedures using NP-AVP would completely eliminate this antibody's reaction with both NP-AVP and NP-OT cells in immunocytochemical experiments. Therefore, these "controls" would lead to a completely *incorrect* conclusion, i.e., that the NP-OT cells contained NP-AVP! The inadequacy of absorption controls as criteria for proof for "molecular" identity or even "specific antigenic determinant" identity has also been demonstrated by Nigg et al. (1982).

We wish to emphasize the point that, although monoclonal antibodies are extremely valuable tools for neurobiologists, the evidence that they react "specifically" with biological antigens in a particular assay cannot be used as evidence for molecular identity (or even precursor relationships) in other assays, in

TABLE III

Effect of trypsin treatment of rat NP on immunoreactivity with antibodies

^{35}S-labeled NPs (NP-AVP or NP-OT) were exposed to trypsin (10 μg/ml) for 3 hr according to the methods of Breslow et al. (1982). The incubation was terminated by 0.5 mg/ml of TLCK, and the solution was used for immunoprecipitation studies (see "Materials and Methods").

Monoclonal Antibody	Counts per Minute Immunoprecipitated		
	Untreated NP (control)	Trypsin-treated NP	Percentage of Control
A. Anti-NP-AVP			
PS 41	548	544	99
PS 45	1180	1094	93
PS 46	579	546	94
B. Anti-NP-OT			
PS 36	717	828	116
PS 38	872	824	95
PS 60	587	718	122
PS 67	750	856	114

the absence of cross-reactivity and other physicochemical data. As a result of these considerations, we chose PS 41 for studying NP-AVP and its precursor, as opposed to PS 45 (despite its greater affinity for NP-AVP and its precursor in liquid phase assays), for use in our immunocytochemical studies on the ontogeny of specific NPs in the rat hypothalamo-neurohypophysial system (Whitnall et al., 1985).

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