

# Heterogeneous Distribution of the cAMP Receptor Protein RII in the Nervous System: Evidence for Its Intracellular Accumulation on Microtubules, Microtubule-organizing Centers, and in the Area of the Golgi Complex

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**Abstract.** The cellular and subcellular distribution of the regulatory subunit RII of cAMP-dependent protein kinase was studied by light and electron microscopy immunocytochemistry in tissue sections from rat brain and in primary cultures of brain cells. RII immunoreactivity was present in most neurons, although at variable concentration. In addition, RII was also detectable in other cell types including glia, neuroepithelial cells, and cells of mesenchymal origin. In the cell cytoplasm, RII immunoreactivity was concentrated at certain sites. An accumulation of RII immunoreactivity was found in all RII-positive cells at the Golgi area, precisely at a region directly adjacent to one of the two major faces of the Golgi complex. RII was also highly concentrated in some microtubule-rich cell processes such as cilia and neuronal dendrites, but was below detectability in most axons. In neurons, its concentration in dendrites is consistent with the previ-

ously demonstrated high affinity interaction between RII and the dendritic microtubule-associated protein 2. In addition, RII was accumulated at basal bodies of cilia and at centrosomes, i.e., sites known to act as microtubule organizers. RII-labeled centrosomes, however, were visible only in cells where the Golgi complex had a pericentrosomal organization, and not in cells where the Golgi complex was perinuclear such as in neurons and glia in situ. We hypothesize that centrosomal RII is bound to the pericentriolar microtubule-organizing material and that this material remains associated with the *trans* region of the Golgi complex when the latter is no longer associated with the centrosome.

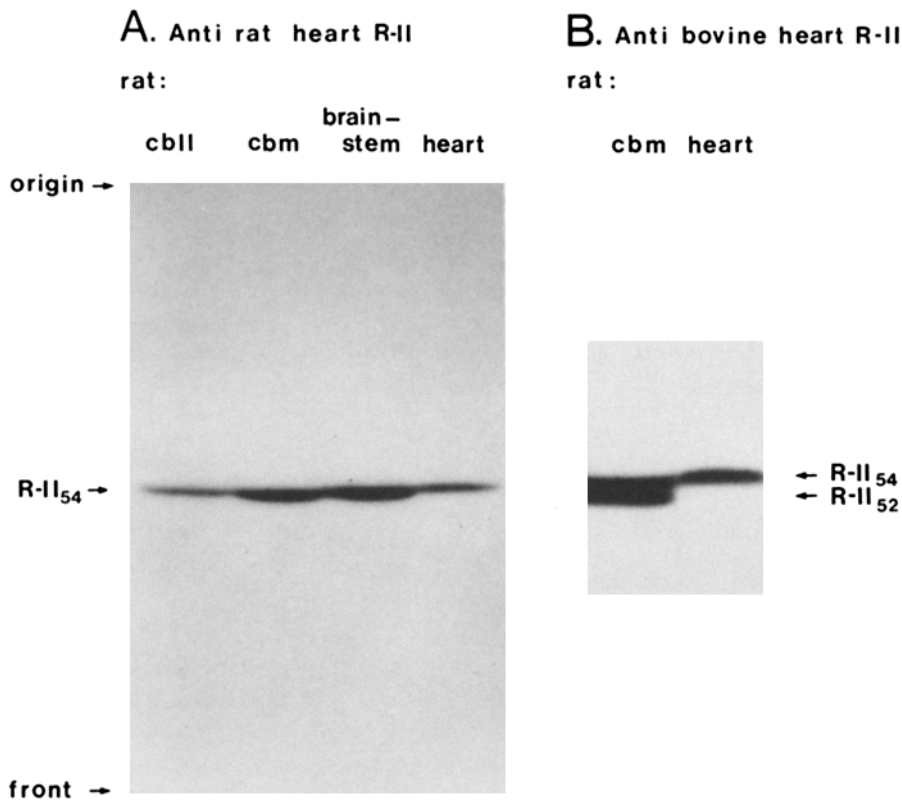
Our results suggest a key but not obligatory role of cAMP in the Golgi-centrosomal area, the headquarters of cell polarity, mobility and intracellular traffic, and in the function of a subpopulation of microtubules.

THE second messenger effects of cAMP are mediated by its binding to two cAMP receptor proteins, RI and RII,<sup>1</sup> so named because they have been discovered as the regulatory subunits of the type I and type II cAMP-dependent protein kinases, respectively (33, 42, 54, 55, 57, 80). Binding of cAMP to RI or RII promotes the dissociation, and thereby the activation, of the catalytic subunit (C) which is essentially identical in both enzymes (33, 42). RI, RII, and C, as well as a variety of substrate proteins for C, are present at high concentrations in the nervous system where cAMP plays a key modulatory role (54, 55).

1. *Abbreviations used in this paper:* C, catalytic subunit of cAMP-dependent protein kinase; Golgi antiserum, rabbit antiserum directed against the 135-kD marker protein of the Golgi complex; MAP2, microtubule-associated protein 2; RI and RII, regulatory subunits of type I and type II cAMP-dependent protein kinases, respectively; RII-IgGs, rabbit antibodies directed against rat RII; WGA, wheat germ agglutinin.

In the brain, as in some other tissues, a fraction of type II cAMP-dependent protein kinase appears to be bound to insoluble components of the cell cytoplasm since, after tissue homogenization, a significant proportion of it is recovered in particulate fractions (14, 28, 47, 48, 63, 66, 75, 80). Binding of the kinase to particulate material is thought to be mediated by RII because C, but not RII, can be released into the soluble phase by cAMP (14, 71, 75).

Over the last few years, we and others have been interested in identifying the individual protein and subcellular structures with which RII interacts in the nervous system. Several different approaches have demonstrated that RII can bind a number of brain proteins in addition to the C subunit (28, 37, 45, 46, 52, 65, 71, 75). The best characterized of these proteins is the microtubule-associated protein 2 (MAP2) (45, 52, 71, 75). MAP2 is highly concentrated in neurons (21) and appears to bind RII to microtubules, particularly to microtu-



**Figure 1.** (A) Monospecificity of the affinity purified antibody against rat heart RII. Proteins of whole homogenates (200  $\mu$ g) were separated on an 8% SDS polyacrylamide gel, transferred to nitrocellulose, and radioimmuno-labeled using the antibody (40  $\mu$ g) and  $^{125}$ I-protein A. Only one band of  $M_r$  54,000, the RII subunit, was recognized. *cbll*, cerebellum; *cbm*, cerebrum. (B) Use of a different antiserum (rabbit antiserum raised against bovine heart RII) to demonstrate that rat brain contains two forms of RII ( $M_r$  54,000 and 52,000), only one of which was recognized by the antibody used for the radioimmunolabeling shown in A and for immunocytochemistry. Radioimmunolabeling was performed as described for A (antiserum dilution was 1:300).

bules located in perikarya and dendrites, the cell regions to which MAP2 is restricted (2, 21, 52). Like MAP2 some other RII-binding proteins appear to be microtubule-associated (45).

Binding proteins for RII may serve to confine C to particular sites, thereby defining the substrates more susceptible to phosphorylation by C. Alternatively, we have suggested (45, 46, 52) that RII may be a multifunctional regulatory protein for many proteins other than C. Some recent reports support this hypothesis (12, 40, 41). The example of calmodulin illustrates how a second messenger-binding protein can regulate, via interactions of variable affinities, a variety of enzymes and proteins (11, 50).

In the present study we have taken a complementary approach to the biochemical studies mentioned above for investigating the sites of interaction of RII in the rat nervous system. Antibodies against RII were used to identify, by immunocytochemistry, the localization of endogenous RII. We have found that RII is present in all cell types of the central nervous system and that in the neuronal population its concentration is highly variable. Moreover, as suggested by the previous biochemical data (37, 45, 65, 71), we found that RII is not homogeneously distributed in the cytoplasm.

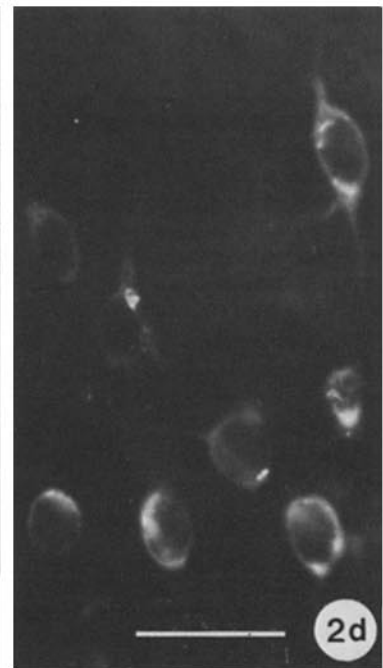
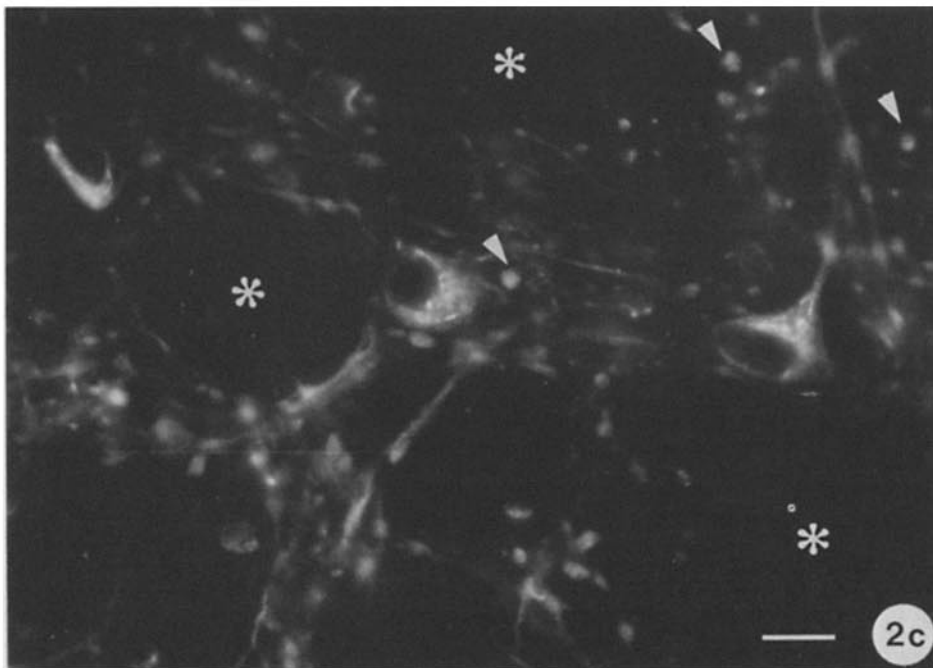
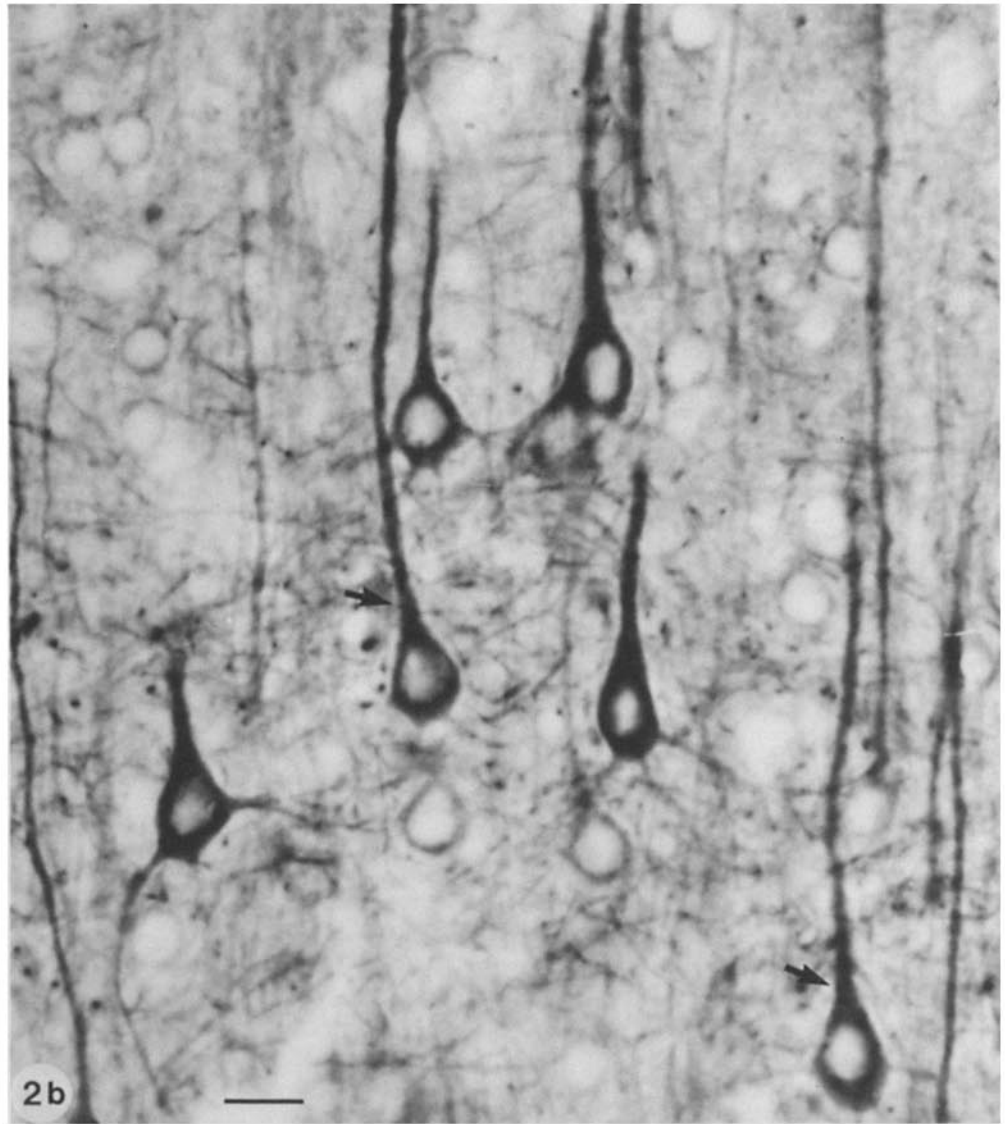
Rather, it is concentrated in defined areas, such as in some microtubule-rich cell processes (dendrites of neurons and cilia), at microtubule-organizing centers (centrosomes and basal bodies of cilia), and in the area of the Golgi complex. Shortly before this manuscript was submitted, a paper was published describing the subcellular distribution of RII in cultured fibroblasts and epithelial cells (56). This paper also reported RII to be concentrated at centrosomes and the Golgi complex, but the precise localization of RII in the Golgi complex was not determined in that study. Some of our present results have been presented previously in a preliminary form (22, 23).

## Materials and Methods

### Purification of Rat Heart RII

RII was purified from 500 rat hearts according to published procedures (15, 47) with the following modifications. RII was eluted from the cAMP-Sepharose column by a phosphate buffer (1 M EDTA, 10 mM  $\beta$ -mercaptoethanol, and 20 U/ml Trasylol [Bayer, West Germany] in 10 mM K-phosphate, pH 6.8) containing 8 M urea. After dialysis against phosphate buffer, RII and some of its degradation products were separated on a DEAE-cellulose column (1  $\times$  6 cm) preequilibrated in phosphate buffer. RII was eluted with a gradient of 0–0.4 M NaCl in phosphate buffer (total elution volume 400

**Figure 2.** Localization of RII immunoreactivity in frozen sections of rat brain. (a, c, and d) Immunofluorescence; (b) immunoperoxidase. (a and b) Layer V of the cerebral cortex. The intensity of RII-immunostain varies dramatically from neuron to neuron. Some large pyramidal cells (arrows) are heavily stained. In positive cells, both perikaryon and dendritic trees are stained. In the immunofluorescence micrograph (a) very bright immunoreactive particles in the perikaryal region are visible. (c) Ventral pallidum. In this region all neuronal perikarya and the varicose dendrites exhibit an intense immunoreactivity. Brightly stained elongated particles forming a network around cell nuclei can be seen. Asterisks label tracts of white matter containing bundles of unstained axons. Arrowheads point to cross-sectioned dendrites. (d) Endopiriform nucleus. Heavily immunoreactive particles around the cell nuclei are visible. Bars, 25  $\mu$ m.



ml), and 5-ml fractions were collected. Fractions containing RII were pooled and concentrated. The yield of RII was ~1 mg, and the protein appeared to be homogeneous when analyzed by SDS polyacrylamide gel electrophoresis.

### Preparation of Rabbit Antibodies (IgGs) Directed against Rat Heart RII

For the preparation of an antiserum against rat heart RII, rabbits were immunized as described (47) except that 50 µg of rat heart RII was used for the initial immunization and 20 µg for subsequent booster injections. Antibodies specific for RII (RII IgGs) were purified from the serum by affinity chromatography (16) using antigen immobilized on Sepharose 4B (Pharmacia, Uppsala, Sweden).

### Other Antibodies

The preparation and characterization of a rabbit antiserum directed against bovine heart RII has been previously reported (47). Polyclonal rabbit antisera directed against the 135-kD marker protein of the Golgi apparatus (Golgi antiserum) (7, 49) and against MAP2, were kind gifts of Dr. D. Louvard (Paris, France) and Dr. R. Vallee (Worcester Foundation, Shrewsbury, MA), respectively. Rabbit IgGs directed against bovine succinate-cytochrome C reductase and against bovine cytochrome C oxidase (10) were a kind gift of Dr. C. Montecucco (Padova, Italy).

### Characterization of Antibodies

Antibody specificity was tested by an immunoblot procedure against proteins contained in whole tissue homogenates. Tissues were homogenized in 4 vol of 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM EDTA, 10 mM β-mercaptoethanol, and 50 U/ml Trasylol. Homogenate proteins were separated on an 8% SDS polyacrylamide gel, transferred to a nitrocellulose sheet, and radioimmunolabeled as described (46) using RII-IgGs followed by <sup>125</sup>I-protein A (Amersham International, Amersham, UK). Some immunological properties of the rabbit serum directed against rat heart RII before affinity purification have been reported previously (44).

### Cell Cultures

Cell cultures were prepared as already described (25, 26). Briefly, the rostral mesencephalon and the striatum from 13- and 15-d-old Swiss mouse embryos, respectively, were dissociated mechanically in phosphate-buffered saline (PBS) and plated on 18-mm polyornithine (1.5 mg/ml) (Sigma Chemical Co., St. Louis, MO) coated coverslips. Cells were grown for various periods of time in a medium consisting of minimal essential medium and nutrient mixture F12 (1:1) (Gibco, Paisley, UK) enriched with glutamine ( $2 \times 10^{-3}$  M), glucose ( $3.3 \times 10^{-2}$  M), sodium bicarbonate ( $0.3 \times 10^{-2}$  M), and containing 10% heat-inactivated fetal calf serum (Flow Laboratories, Settimo Milanese, Italy). After 5–15 d in culture the cells were processed for immunocytochemistry.

### Light Microscopy Immunocytochemistry and Cytochemistry

**Tissue Sections.** Sprague-Dawley albino rats, 175–250 g, were anesthetized and transcardially perfused as described (20). 4% freshly prepared formaldehyde or 3% formaldehyde-0.25% glutaraldehyde were used as fixatives. Frozen sections (10–20-µm thick) were prepared and immunostained either on glass slides or in suspension as described (21). Affinity purified RII IgGs were used at a concentration of 0.1 mg/ml for immunofluorescence or 0.01

mg/ml for immunoperoxidase. As controls nonimmune rabbit IgGs (Cappel Laboratories, Cochranville, PA) were used at the same concentration. Polyclonal rabbit sera and immune sera were used diluted 1:20 for immunofluorescence and 1:50 for immunoperoxidase. Secondary antibodies were goat anti-rabbit IgGs conjugated to rhodamine (Cappel Laboratories) or sheep anti-rabbit F(ab)<sub>2</sub> conjugated to peroxidase (Pasteur Institute, Paris, France). In some cases (Fig. 7 c), sections stained in suspension by the immunoperoxidase procedure were flat-embedded in Epon and semi-thin-sectioned, rather than being mounted as such on glass slides.

**Cell Cultures.** Cells grown on coverslips were briefly rinsed with phosphate buffer and subsequently fixed for ~1 h at ice-temperature with 4% formaldehyde in phosphate buffer. Immunofluorescence was performed using the same procedure used for tissue sections on glass slides.

**Double Labeling.** In some cases tissue sections or cell cultures previously stained for RII by immunorhodamine were overlaid (15 min) with fluorescein-conjugated wheat germ agglutinin (WGA) (Vector Laboratories, Inc., Burlingame, CA) diluted 1:700 in PBS and then washed with PBS to remove unbound lectin.

**Photography.** Light microscopy observation was performed with a Zeiss photomicroscope III equipped with epifluorescence and planapo objectives. Bright field pictures were taken with Panatomic X films and developed with Microdol X or D76 (1:1); fluorescence pictures were taken with Technical Pan 2415 film and developed with undiluted D19. All films and chemicals were from Kodak.

### Electron Microscopy Immunocytochemistry

Frozen tissue sections were prepared and stained in suspension by immunoperoxidase as described for light microscopy with the exception that 3% formaldehyde-0.25% glutaraldehyde were used as fixatives and that Triton X-100 was omitted from all incubation and washing media. Omission of Triton X-100 reduced antibodies penetration into the tissue, but a satisfactory labeling was observed in the most superficial portions of the sections. Immunostained frozen sections were then flat-embedded in Epon. After Epon polymerization, regions of interest were identified by light microscopy, dissected out, re-embedded in larger Epon blocks, and thin sectioned. These sections were counterstained with uranyl acetate and examined with a Philips 301 electron microscope.

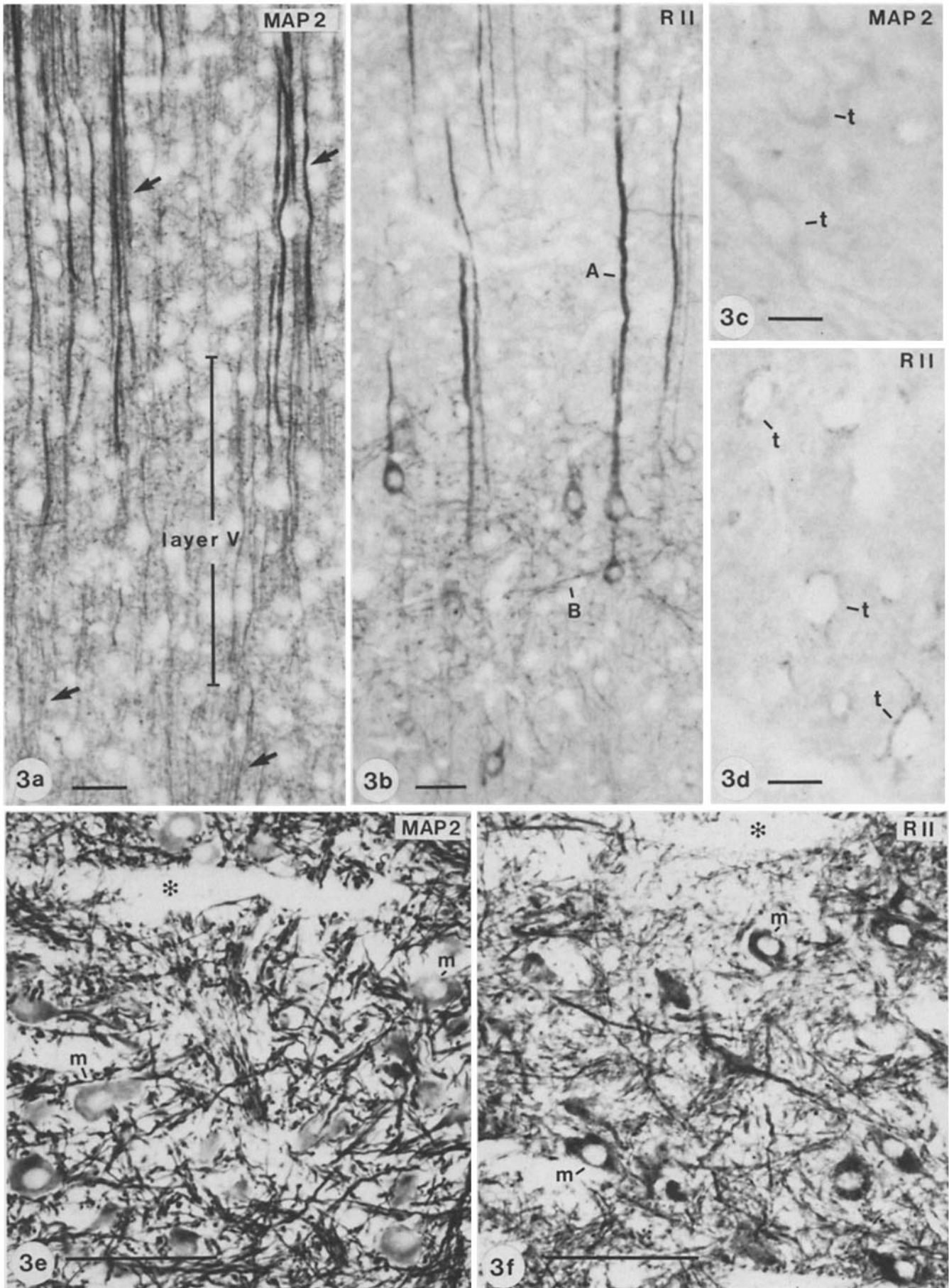
## Results

### Characterization of RII-IgGs

The monospecificity of the RII-IgGs when tested against rat tissues is shown in Fig. 1 a. Essentially identical results were obtained with mouse tissues (not shown). RII-IgGs recognized only one protein of  $M_r$  54,000<sup>2</sup> in either homogenates of rat heart or of three major regions of rat brain (cerebrum, cerebellum, brain stem). This protein, which co-migrates with purified rat heart RII (44), has been previously shown by a variety of criteria (44) to represent RII. In the brain, however, it appears to represent only a fraction of the total RII. From previous work it is known that whereas the heart contains primarily one form of RII ( $M_r$  54,000), the brain contains two major isoforms ( $M_r$  54,000 and 52,000)

2. Molecular weights given refer to the dephospho-form of the molecules.

**Figure 3.** Comparison between MAP2 (a, c, and e) and RII immunoreactivity (b, d, and f) in dendritic trees of neurons. Immunoperoxidase. (a and b) Adjacent sections of the cerebral cortex (pial surface is at top). All pyramidal neurons contain high concentrations of MAP2 in their dendritic trees. Apical dendrites, which have different diameters depending upon the level of the cortex in which their perikarya are located, are collected in bundles (arrows). The largest apical dendrites originate from layer V pyramidal neurons. Only some pyramidal cells of layer V are intensely RII-positive. In these cells the high level of RII immunoreactivity in perikarya is paralleled by high levels of RII in dendrites. A, apical dendrites; B, basal dendrites. (c and d) Adjacent sections of the thalamus. Only low levels of MAP2 are present in the dendrites of the thalamic neurons shown in the figures (t). An RII-positive perikaryal network is visible in these neurons, but RII is undetectable in dendrites. (e and f) Adjacent sections of a motor nucleus in the brain stem. Motor neurons (m) contain high levels of both MAP2 and RII. RII is concentrated both in perikarya and dendrites. Asterisks, unstained bundles of axons. Bars: (a and b) 50 µm; (c and d) 25 µm; (e and f) 100 µm.



(29, 38a, 39, 47, 85). Both brain isoforms were labeled (although with a low affinity) by an antiserum previously raised by us against bovine heart RII (47) (Fig. 1 *b*). The low affinity of this latter antiserum for rat and mouse RII prevented its use for immunocytochemistry (52). Since the IgGs used in this study selectively recognize one subtype of brain RII, and since they do not recognize RI, our morphological results demonstrate some major target sites for cAMP which represent only one subset of all cAMP target sites.

### Immunocytochemistry of Tissue Sections

Various regions of the central nervous system were examined by light microscopy immunoperoxidase or immunofluorescence, or by both procedures. RII immunoreactivity was found to have a very widespread distribution. This is in agreement with previous biochemical reports (77, 78). In all brain regions RII immunoreactivity was present in neurons as well as in non-neuronal cells. The bulk of RII was, however, concentrated in the grey matter. A detailed description of the localization of RII in different cell types follows. In all cases the specificity of the immunostain described has been carefully assessed by comparison with staining patterns obtained with non-immune IgGs or with a variety of other antibodies.

**Neurons.** In sections stained either by immunofluorescence or by immunoperoxidase the overall staining intensity varied from neuron to neuron. Such heterogeneity is illustrated dramatically in Figs. 2, *a* and *b*, and 3 *b* which show fields of layer V of the cerebral cortex. It is clear that while some neurons contain a very high concentration of RII, others exhibit only a barely detectable immunoreactivity. Immunostaining of adjacent sections for MAP2, a cytoplasmic antigen that is present at roughly constant concentration in all pyramidal cells of the cerebral cortex (21), ruled out that the neuronal heterogeneity observed with RII-IgGs was due to artifactual reasons (Fig. 3, *a* and *b*).

The large pyramidal neurons of layer V were among the neurons that contained the highest level of RII. Other heavily stained cells included some hippocampal pyramidal cells (CA4 and CA3 region), neurons of the medial habenula, Golgi cells of the cerebellum, and the multipolar neurons of the spinal cord, brain stem (Figs. 3 *f* and 4, *a*, *c*, and *d*), mesencephalon, hypothalamus, globus pallidus (Fig. 2 *c*), and basal forebrain (not shown). In the great majority of neurons at least a very low level of immunoreactivity was detectable. Apparently devoid of immunoreactivity were Purkinje cells of the cerebellum (not shown). It is of interest that Purkinje cells contain high levels of the cGMP receptor protein, cGMP-dependent protein kinase (20). In all brain regions that contain distinct, well defined, neuronal types (subset of cells with similar morphology and similar synaptic connec-

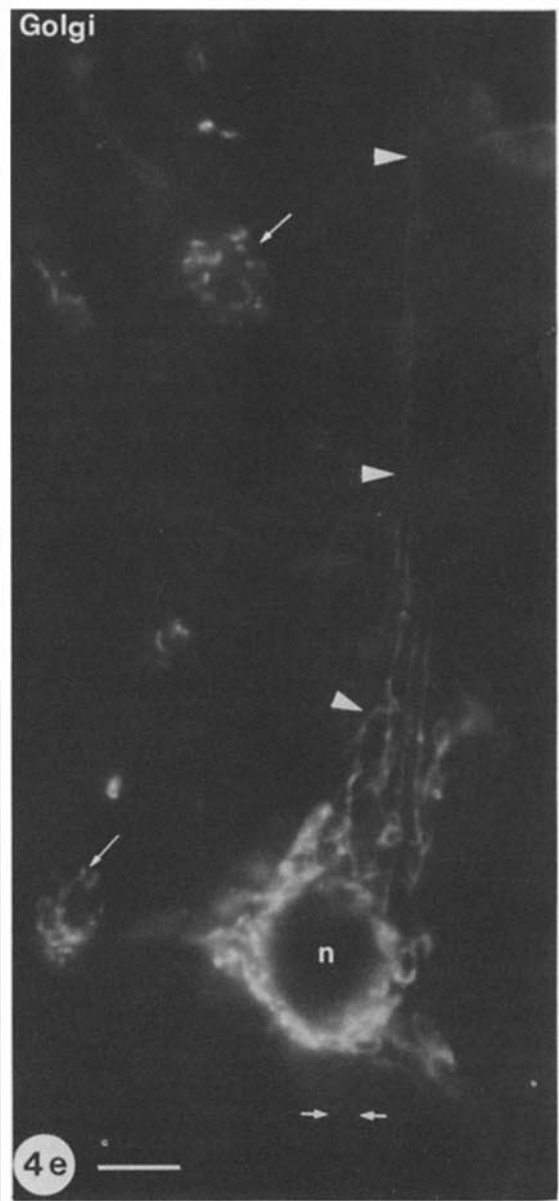
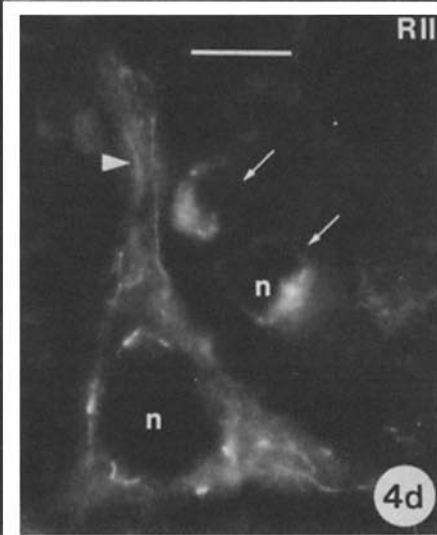
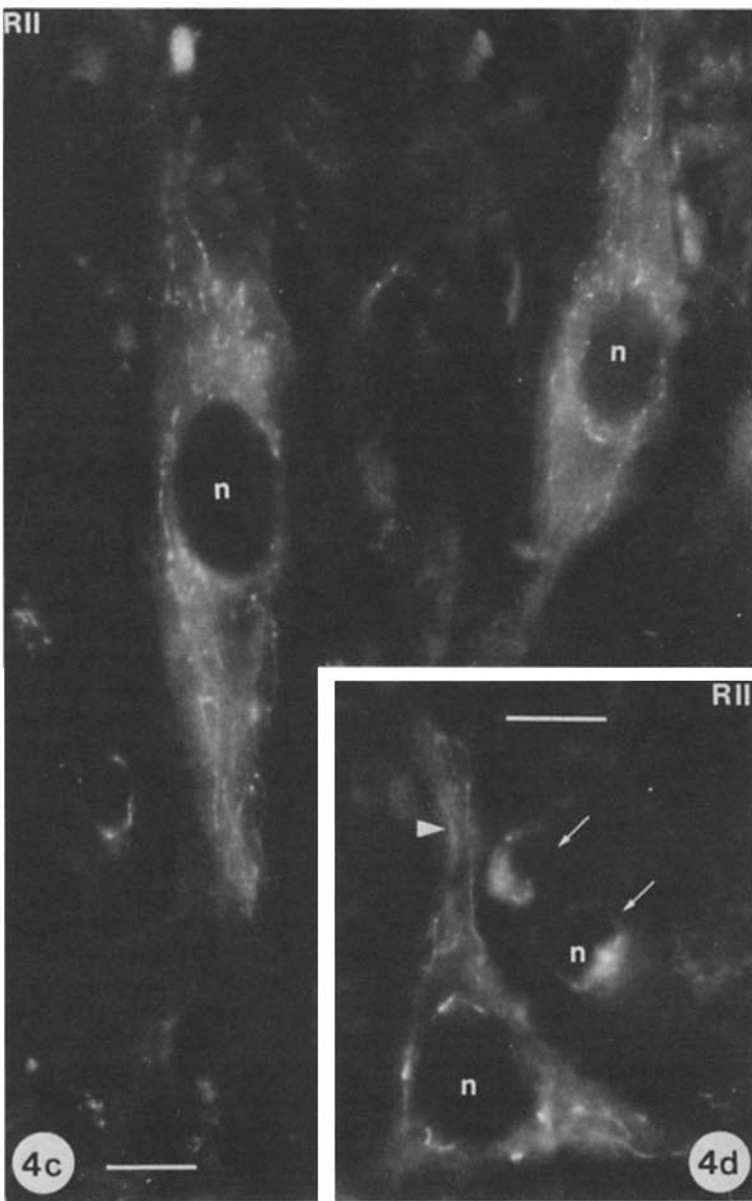
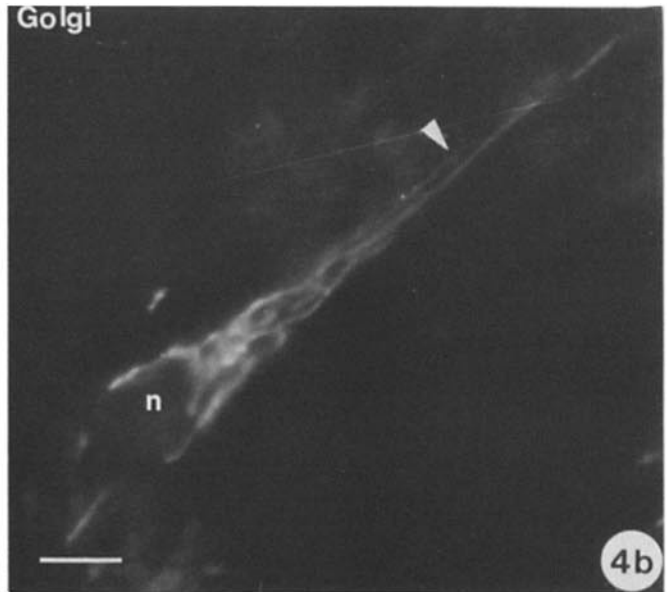
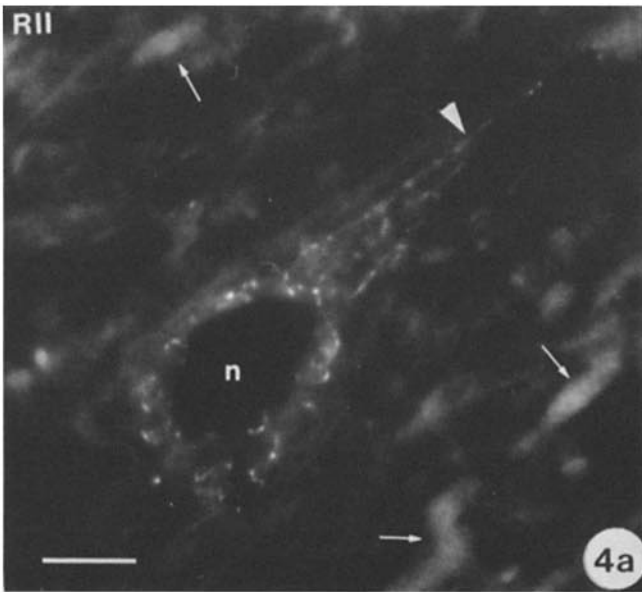
tions), the intensity of RII immunostain was very similar in all cells of a given subset.

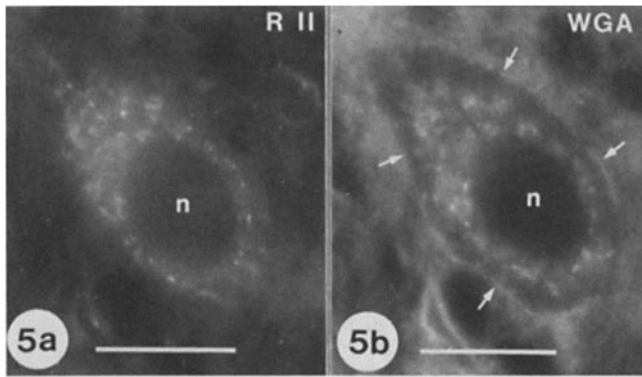
In the cytoplasm of RII-positive neurons, RII appeared to be concentrated at specific cellular sites. In the perikaryal-dendritic region two overlapping patterns of immunoreactivity could be seen (Fig. 2, *a*, *c*, and *d*; Fig. 4, *a*, *c*, and *d*). One was a diffuse immunoreactivity that, when intense, could also be detected in the most distal dendritic branches. The other was a brighter, particulate, perikaryal staining, which in most cases was perinuclear and, in cells with large dendrites, penetrated for some distance the proximal dendritic segments. The two patterns were clearly visible in sections processed by immunorhodamine. In those processed by immunoperoxidase the particulate perikaryal staining was often obscured by saturating levels of the diffuse stain (compare Fig. 2 *a* and 2 *b*). High power observation revealed that the bright perikaryal stain was due to convoluted, apparently interconnected threads, which, in general, formed a network around the nuclei (Fig. 4, *a*, *c*, and *d*). Occasionally, some threads emerged from the network and took a rectilinear course to penetrate proximal dendritic segments (Fig. 4, *a*, *c*, and *d*).

A comparative analysis of neurons of various brain regions indicated that the extent to which the particulate perinuclear stain was accompanied by the diffuse dendritic stain correlated with the presence of MAP2 in dendrites. In cells with MAP2-rich dendritic trees, such as cortical pyramidal cells or multipolar neurons of the brain stem (21), the perinuclear stain was accompanied by a conspicuous dendritic stain (Fig. 3, *a*, *b*, *e*, and *f*). In contrast, in other neurons (for example most thalamic neurons) which have very little MAP2 in their dendrites (21), only the perinuclear stain could be seen (Fig. 3, *c* and *d*). Cells that did not exhibit the particulate perinuclear stain also did not exhibit any dendritic stain even if they had MAP2-rich dendritic trees.

Some of the features of the immunoreactive perikaryal network were reminiscent of the Golgi complex in neurons as revealed by the classical Golgi silver stain (35). To establish a relationship between this network and the Golgi complex, we immunostained adjacent sections of brain tissue with RII-IgGs and with an antiserum directed against a marker of the Golgi complex (kind gift of Daniel Louvard). This antiserum (Golgi antiserum), which specifically recognizes a 135-kD intrinsic membrane protein of the Golgi complex (7, 49), produced clear images of neuronal Golgi complexes (Fig. 4, *b* and *e*). The similarity between Golgi immunoreactivity and RII-positive perinuclear particles was striking even though some subtle differences (such as a more discontinuous appearance of RII-positive particles) were noticeable. This similarity was observed in all neurons despite the remarkable variability of the shape of the Golgi complex. We excluded that RII-positive threads were elongated mitochon-

**Figure 4.** Immunofluorescence of frozen sections: comparison between the distribution of particulate RII immunoreactivity (*a*, *c*, and *d*) and the morphology of the Golgi complex (*b* and *e*) in perikarya and large proximal dendrites of neurons in the reticular formation. In all micrographs, the threadlike appearance of the RII- and Golgi-immunoreactivity is visible. The apparently interconnected threads penetrate large dendrites for some distance (*arrowheads*). The two staining patterns are highly similar even though RII-stained filaments are more irregularly stained than Golgi filaments. In *a* dendrites containing diffuse immunoreactivity are also visible (*arrows*). Small arrows in *d* and *e* point to Golgi-like (*d*) and Golgi (*e*) stain in small cells adjacent to the large neurons. The ones in *d* are probably glial cells. A pair of small opposing short arrows (below the large neuron in *e*) point to a process that excludes Golgi elements. This is probably the axon hillock. *n*, cell nuclei. Bars, 10  $\mu$ m.





**Figure 5.** Comparison of the morphology of the perikaryal RII-immunoreactive network with the morphology of the Golgi complex as visualized by WGA binding. Frozen section of brain stem double-labeled with rhodamine-conjugated RII-IgGs (a) and with fluorescein-conjugated WGA (b). A large neuron is shown in the pair of photographs (n, cell nucleus). The RII-positive network is very similar (not identical) to the network stained by WGA. WGA, in addition, stains a variety of other structures in the neuropile which surround the neuron. WGA staining of axons and axon terminals at the neuronal surface outlines its profile (arrows). Bars, 20  $\mu$ m.

dria by immunostaining sections for mitochondrial markers (succinate-cytochrome C reductase and cytochrome C oxidase) (10). Mitochondria also appeared as linear, wavy, or convoluted threads, but their distribution in the neuronal cytoplasm was different from that of RII-positive particles (not shown).

A precise comparison between the staining patterns obtained with antibodies to the 135-kD protein and to RII could not be made since both antibodies had been raised in rabbits. However, the similarity of the RII-positive perikaryal network with the network of Golgi complex was further confirmed by counterstaining with fluorescein-conjugated WGA sections previously stained for RII by immunorhodamine. WGA has been shown to be a cytochemical marker for the Golgi complex (68). The two staining patterns were found to be very similar in the Golgi region (Fig. 5).

To establish in which subregion of the Golgi area RII was localized, we analyzed at the electron microscopic level the distribution of the reaction product in peroxidase-labeled sections. As shown in Fig. 6, the major Golgi cisternae were devoid of stain. Reaction product was concentrated instead at a region rich in small vesicles adjacent to one of the two major faces of the Golgi complex (Fig. 6). The low resolving power of peroxidase immunocytochemistry did not permit a precise identification of the subcellular structures to which RII was bound.

In contrast to the often intense perikaryal-dendritic staining, RII immunoreactivity was in general not detectable in axons. Lack of labeling on tracts of white matter is evident in Fig. 2 c and 3 f. A moderate level of immunostain, however, was often visible in axon terminals, in particular in regions that are innervated by neurons containing high levels of RII in their perikarya (manuscript in preparation).

Cell nuclei were always RII negative under our experimental conditions.

**Glia and Connective Tissue Cells.** RII immunoreactivity was detectable also in glial cells and in connective tissue cells

of the meninges and of the vasculature (not shown). We did not attempt to establish whether all glial cells were stained, but RII-positive glial cells included oligodendrocytes, at least some astrocytes, and the Bergman glia of the cerebellum. In all these non-neuronal cells RII immunoreactivity was concentrated on particles clustered in proximity of the nucleus. Cell processes were not stained above background. As in the case of neurons, the morphology and distribution of RII-immunoreactive particles were always very similar to the morphology and distribution of elements of the Golgi complex. This was shown at the light microscopic level by double labeling the same sections with RII-IgGs and with WGA, and by immunostaining adjacent sections with RII-IgGs and with Golgi antiserum, respectively (not shown). Both RII-positive particles and Golgi elements totally surrounded the nucleus in glial cells, while they were in general restricted primarily to one side of the nucleus in most connective tissue cells (e.g., endothelial cells and fibroblasts). In the latter cells a small "hot spot" of RII immunoreactivity in the middle of the Golgi area was also visible.

Electron microscopy immunoperoxidase confirmed that also in non-neuronal cells, RII immunoreactivity concentrated in the Golgi region was confined to one side of the Golgi (not shown). In addition it showed that the "hot spot" of immunoreactivity represented RII concentrated in the centrosomal area (Fig. 8 a).

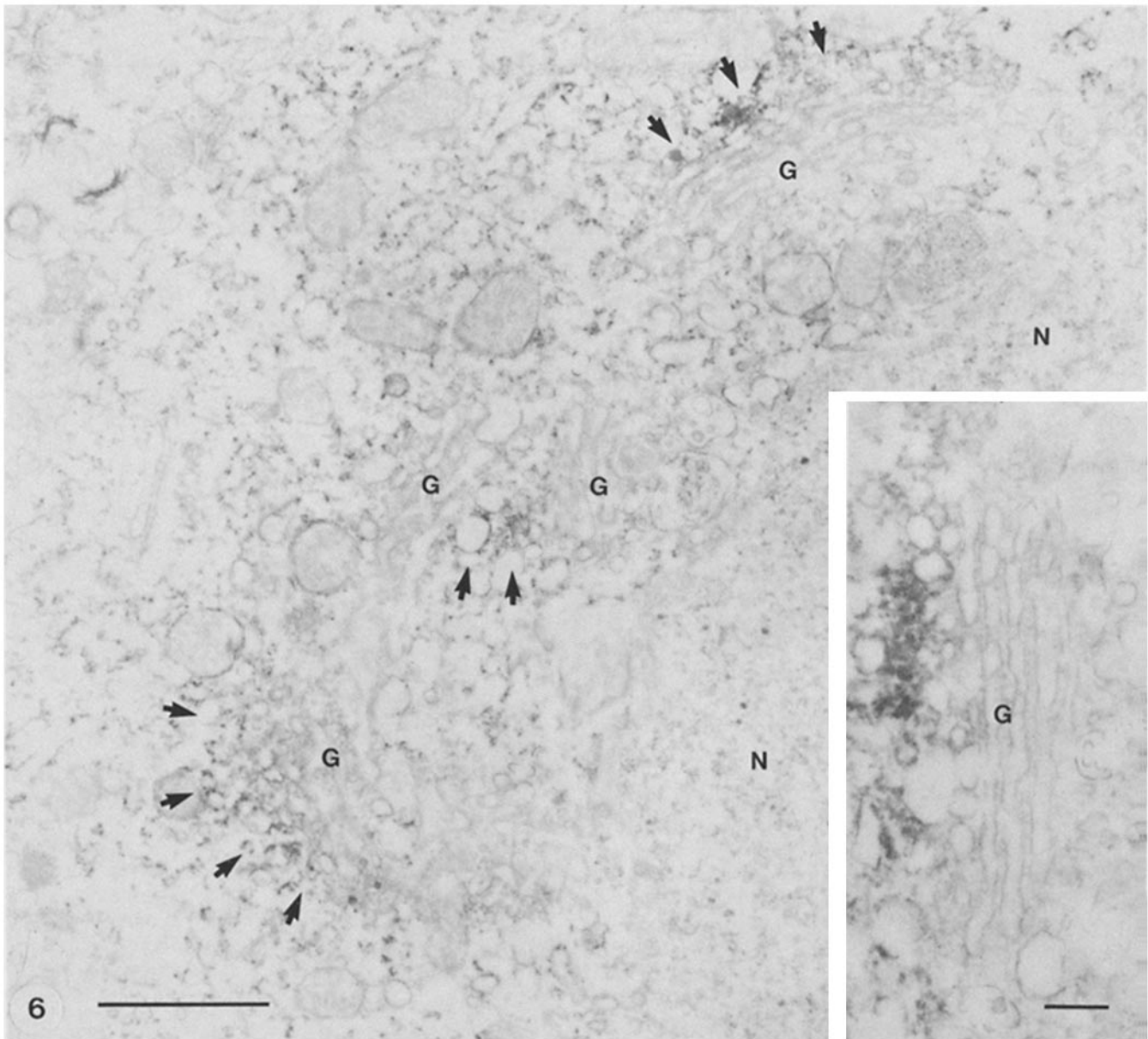
**Neuroepithelial Cells.** The ependymal epithelium and the epithelium of the choroid plexus in the brain, as well as the photoreceptor layer and the pigmented epithelium in the retina, all represent adult derivatives of the neuroepithelium that lines the embryonic ventricular cavities. A characteristic of some of the polarized cells of these neuroepithelia is that they bear cilia (ependymal cells and some cells of the choroid plexus) or cilia-derived structures (cones and rods of photoreceptors [4]) at their apical pole. In all ciliated neuroepithelial cells, a prominent accumulation of RII was seen at the basal body region of cilia and in cilia themselves. This localization was demonstrated by light microscopy (Fig. 7, a-d) and was confirmed by electron microscopic analysis of peroxidase-stained sections (Fig. 8 b). In photoreceptors, RII was concentrated on the inner segments (Fig. 7 f) which contain the equivalent of a basal body with its microtubular rootlets (4). In all neuroepithelial cells, the Golgi area (when not concealed by the basal body labeling, as in ciliated ependymal cells [Fig. 7, d and e]) was also clearly immunostained (Fig. 7, b and f).

### **Immunocytochemistry of Primary Brain Cultures**

The cellular and subcellular distribution of RII was also investigated by immunofluorescence in primary cultures of the nervous system. Two types of mouse brain cultures, previously characterized (25, 26), were examined after various times in vitro: striatal cell cultures and co-cultures of striatal and mesencephalic cells. The young cultures (5 d in vitro) (Fig. 9 a) contain primarily neurons and a few astroblasts. In older cultures (15 d in vitro) (Fig. 9, b-d) the number of astrocytes has increased and a few astrocytes are multinucleated.

RII immunoreactivity was detectable in both neurons and astrocytes. In neurons its overall concentration appeared variable and in general more intense in co-cultures containing mesencephalic neurons. This correlated with the fact that



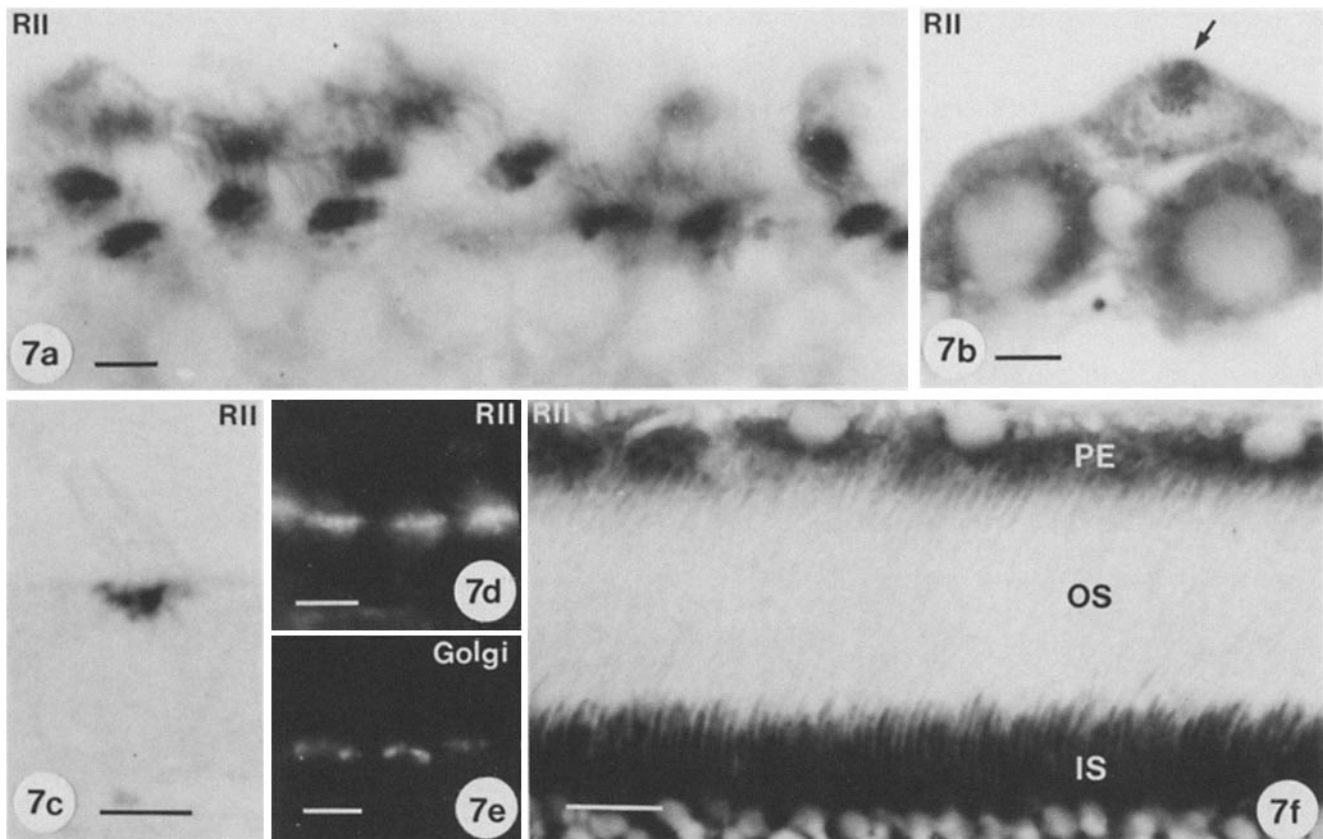


**Figure 6.** Localization of RII in the area of the Golgi complex of a cortical pyramidal neuron. Electron microscopy immunoperoxidase. Peroxidase reaction product, which is sparsely present throughout the cytoplasm, is highly concentrated at a region adjacent to one of the two major faces of the Golgi complex (arrows). Golgi cisternae are devoid of immunostain. The inset shows a portion of the Golgi complex at higher magnification. *N*, nucleus; *G*, Golgi elements. Bars, 1  $\mu\text{m}$ . Bar in inset, 0.2  $\mu\text{m}$ .

mesencephalic cells of adult animals also exhibited a greater level of RII immunoreactivity than the predominant population of striatal cells (not shown). In both neurons and glial cells, RII was highly concentrated on threadlike particles that appeared to originate, and almost to radiate, from an even brighter spot of immunoreactivity localized in proximity of the nucleus (Fig. 9, *a-c*). In multinucleated astrocytes, this spot (a double spot in a few cases such as in Fig. 9 *c*) was particularly prominent. Quite often, in astrocytes, the immunoreactive threads at some distance from the bright spot took a parallel course (Fig. 9, *b* and *c*). As in the case of cells in situ, these threadlike particles probably represented immunoreactivity associated with the Golgi complex. Their shape and localization were found to be very similar to that of Golgi elements. This was determined both by coun-

terstaining the same cells with labeled WGA (not shown) or by immunostaining sister cultures for the 135-kD Golgi marker (compare Fig. 9 *c* and 9 *d*). The central spots, however, were stained only by RII-IgGs. Considering the well known association of the *trans*-face of the Golgi complex with the centrosome (31, 62, 64) and the asterlike geometry of particles irradiating from these spots, they probably represent the centrosomes. The larger size of these spots in multinucleated cells are likely to reflect the coalescence of centrosomal material from more than one cell.

Apart from the Golgi-centrosomal stain, RII immunoreactivity in cultured glial cells was, in general, at the limit of detectability (Fig. 9, *b* and *c*). At most a very faint punctate pattern could be seen throughout the cytoplasm. In cultured neurons a diffuse fluorescence (with superimposed sparse



**Figure 7.** Distribution of RII immunoreactivity in neuroepithelial cells. (a, b, c, and f) Immunoperoxidase; (d and e) immunofluorescence. (a) Frozen section showing the ependymal epithelium. RII immunoreactivity in ependymal cells is concentrated at the apical pole where the basal bodies of cilia are located. Some immunoreactivity is detectable also in cilia. (b) Three cells of the epithelium that line the choroid plexus are shown. The top cell bears a cluster of cilia (arrow) and their immunoreactive basal bodies are seen *en face*. The intense perinuclear stain in the two bottom cells of the field co-localizes with a highly developed perinuclear Golgi complex (not shown). (c) Semi-thin Epon section (1  $\mu\text{m}$ ) showing at high resolution staining of the basal body region of an ependymal cell. (d and e) Adjacent sections of ependymal cells stained for RII and for the Golgi complex, showing that the Golgi complex is localized close to basal bodies. (f) Frozen section of the photoreceptor layer of the retina. The field includes the inner (IS) and outer (OS) segments of photoreceptors and the pigmented epithelium (PE). RII immunoreactivity is highly concentrated in the inner segments and is undetectable in outer segments. The immunoreactivity visible at the top of outer segments is not due to outer segments themselves (which appear as negative images) but to cells of the pigmented epithelium that are stained at their apical Golgi region. Bars: (a-c) 5  $\mu\text{m}$ ; (d-f) 10  $\mu\text{m}$ .

puncta) was visible both in perikarya and cell processes (Fig. 9 a). The diffuse fluorescence probably represented RII bound to MAP2 since a similar fluorescence distribution was observed in cultured neurons immunostained for MAP2 (not shown).

## Discussion

In this study we have used immunocytochemistry to investigate in the nervous system the cellular and subcellular distribution of RII, one of the major cAMP receptor proteins. Sites where cAMP receptor proteins are concentrated are likely to be sites where cAMP plays an important regulatory role. Thus, their demonstration is important not only to complement information obtained by other approaches about cAMP functions, but also to identify previously unknown sites of action of cAMP. A previous immunocytochemical study on the distribution of RII in the nervous system did not lead to conclusive results (17).

It is important to note that our present work only identifies

a subset of cAMP target sites since (a) RI sites have not been studied, (b) the localization of only one of the two major isoforms of brain RII was studied, and (c) immunocytochemistry reveals sites of high antigen concentration and not those where the antigen concentration is below a certain threshold.

## Cellular Distribution

In the nervous system RII was found to have a very widespread distribution in all cell types. This finding was anticipated since the type II cAMP-dependent protein kinase has been found in a large number of tissues and therefore in many different cell types (13, 32, 48, 79, 82). The concentration of RII, however, was found to be quite variable from neuron to neuron. It is of interest that phosphoproteins such as MAP2 and synapsin I, which are thought to be physiological substrates for the type II cAMP-dependent protein kinase (67, 71, 81), have a much more even distribution in the total neuronal population (18, 21).

Since RII levels appeared similar in cells with similar mor-

phology and connections, major variations in levels of RII are probably not related to the functional state of cells at the time of fixation. Rather, they may be related to their specific properties and connections. Other protein kinases, or protein kinase subunits, also appear to have an uneven distribution in the neuronal population (20, 30, 34, 59, and our unpublished observations). Since different synaptic inputs are known to activate distinct second messenger systems, the same phosphoproteins might be regulated by distinct second messengers in different neurons. In fact several phosphoproteins (including synapsin I and MAP2) can be phosphorylated at the same or at distinct sites by more than one kinase (1, 38, 72). Additional mechanisms to produce the same change in a brain phosphoprotein by distinct second messengers have been proposed (54, 55).

### Subcellular Distribution of RII

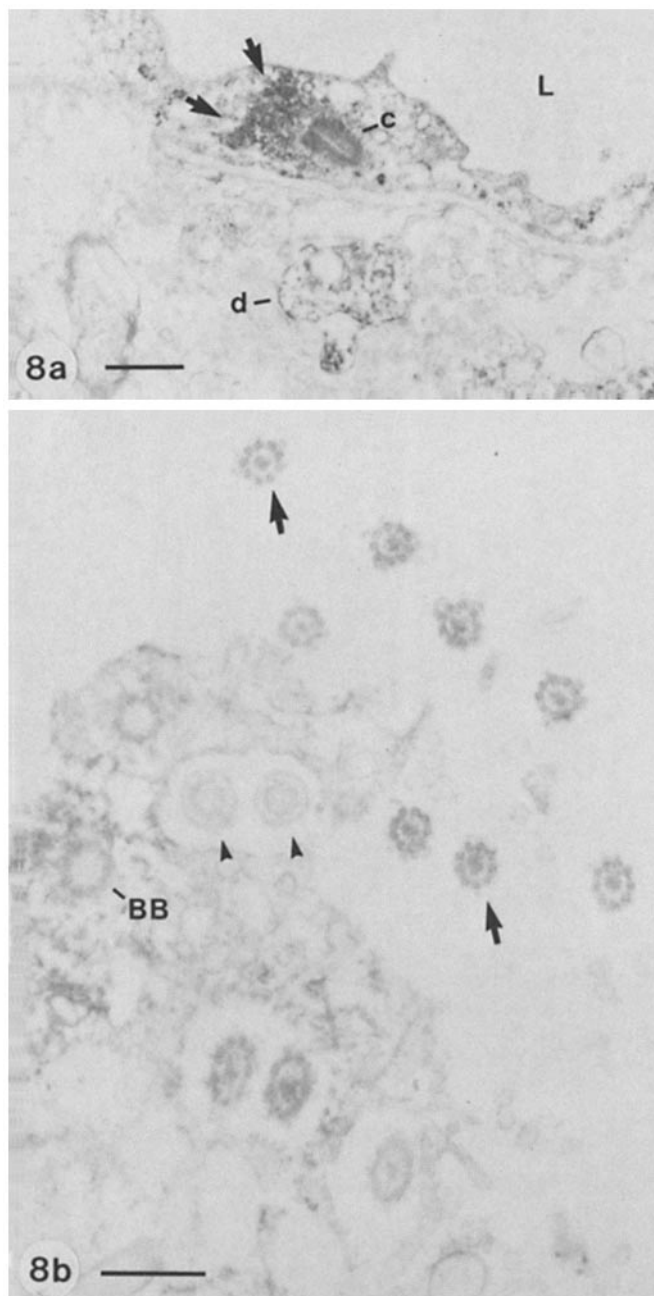
**Neuronal Dendrites and Other Microtubule-rich Cell Processes.** The selective accumulation of RII in dendrites and not in axons is consistent with previous data indicating a high affinity interaction between RII and MAP2 (45, 52, 71, 75), a microtubule-associated protein selectively concentrated on dendritic microtubules (2, 21, 52). MAP2 and RII co-purify regardless of the methods used to purify either MAP2 (71, 75) or RII (45). In addition, RII binds to MAP2 when RII is used in overlays of gel blots (45) or of frozen brain sections (52).

Even though individual neuronal microtubules are not resolved by light microscopy in frozen sections (21), two observations suggest that dendritic RII may, to a large extent, represent RII that is bound to MAP2 and, in turn, to microtubules. One is that the Golgi-like pattern of immunoreactivity is accompanied by a dendritic stain only in neurons that have MAP2-rich dendritic trees. The other is that peculiar proximo-distal changes in the distribution of MAP2 observed in some dendrites (21) coincide with the distribution of RII (unpublished observations).

RII was also detected in motile cilia which are operated by a microtubule apparatus. Furthermore, it was particularly concentrated in inner segments of photoreceptors. The inner segments of cones and rods contain microtubules and, in some animal species, have been shown to undergo contraction-elongation cycles which are regulated by cAMP (8).

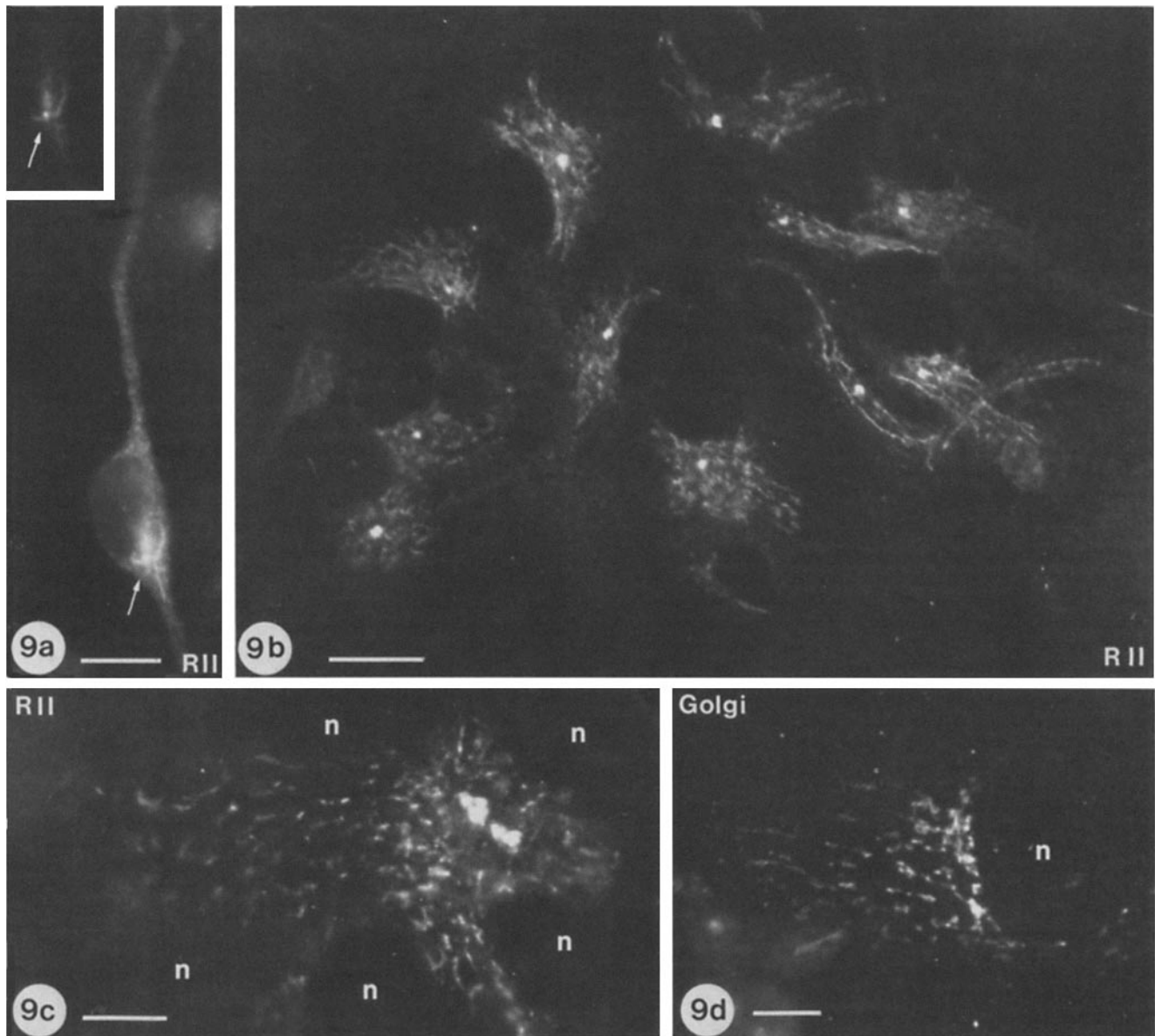
A localization of RII on microtubules of the mitotic spindle has been reported by others (6, 56).

**The Golgi Area.** In all cells that express a detectable amount of RII, a particulate pattern of stain is visible in the Golgi area. The relationship between this particulate pattern and the Golgi complex was demonstrated by (a) the striking similarity of the light microscopy staining patterns for RII and for the 135-kD Golgi marker protein (7, 49) in a variety of cell types that display remarkable differences in the geometry of the Golgi complex, (b) the quite similar fluorescent patterns obtained by double staining with RII-IgGs and with WGA, and (c) results of electron microscopy immunocytochemistry. Electron microscopy, in addition, revealed that RII was concentrated in a cytoplasmic region adjacent to one of the two major faces of the Golgi complex. This was probably the *trans*-face since RII was also concentrated on centrosomes (normally located at the *trans*-Golgi region [31]) and since the RII-positive side was that away from the nucleus in Golgi complexes closely apposed to the nuclear envelope



**Figure 8.** Localization of RII at centrosomes, cilia, and basal bodies by electron microscopy immunoperoxidase. (a) Cerebral cortex capillary. Presence of peroxidase reaction product on a centriole (c) and on pericentriolar material (arrows) in the cytoplasm of an endothelial cell. d, Immunoreactive dendrite; L, capillary lumen. (b) Apical region of an ependymal cell. The reaction product is concentrated on basal bodies (BB) and in the surrounding cytoplasm as well as in cilia (arrows). Ciliary stain, however, is only visible in those cilia that have been stripped of their plasmalemma probably as a result of manipulations preceding immunostaining. Arrowheads point to two unstained cilia surrounded by an intact sealed plasmalemma. Bars, 0.5  $\mu$ m.

(70) (unpublished observations). Subtle differences in the morphology of the Golgi complex as visualized by RII-IgGs or by antibodies to the 135-kD protein and by WGA were probably due to the fact that the latter two cytochemical



**Figure 9.** Localization of RII immunoreactivity at the Golgi-centrosomal area in cultured neurons and astrocytes. Immunofluorescence. *a-c* are from cultures immunostained for RII, *d* from a culture immunostained for the 135-kD Golgi marker protein. (*a*) Neuron (5 d in vitro). A moderate level of immunofluorescence is visible throughout the cell cytoplasm. In addition brightly fluorescent elongated particles radiate from an even more intensely fluorescent spot (*arrow*) localized in close proximity of the nucleus. This spot is better seen in the inset which shows a darker print of the perikaryal region of the same neuron. (*b-d*) Astrocytes (15 d in vitro). In each cell, RII is concentrated at a small spot and on threadlike particles which originate from such a spot. In the multinucleated astrocyte shown in *c*, two instead of one spot are visible and their size is larger than in mononucleated cells. *d* shows that the morphology of Golgi elements, as visualized by immunostain for the 135-kD protein, is very similar to the morphology of RII-immunoreactive threadlike particles (compare *c* with *d*). The central spot, however, is visible only after staining for RII. *n*, cell nuclei. Bars: (*a*) 10  $\mu\text{m}$ ; (*b*) 25  $\mu\text{m}$ ; (*c* and *d*) 10  $\mu\text{m}$ .

probes stain some of the Golgi cisternae and recognize luminal rather than cytoplasmic antigenic sites (7, 49).

**Centrosomes and Centrosome-related Structures.** RII was concentrated at the basal body region of cilia and at centrosomes, i.e., at two related cellular regions, which are known to act as nucleating centers for microtubules (53, 73). It is of interest that recently phosphoproteins concentrated at microtubule-organizing centers have been described (76).

Labeled centrosomes were visible in connective tissue cells in situ and in neurons and glial cells in culture. All these

cells appeared to have a pericentrosomal Golgi complex. In contrast, centrosomes were not visible in neurons and glia in situ despite the known presence of centrioles in these cells (60). Most neurons and cells in situ appeared to have a perinuclear Golgi complex.

We hypothesize that centrosomal RII is bound to the pericentriolar material that acts as the microtubular organizer, and that in adult neurons and glial cells in situ this material is not associated with centrioles. It has been shown recently that the microtubule-organizing material is not al-

ways bound to centrioles (9, 69). In particular, Tassin and co-workers have shown that microtubule-organizing material, which is pericentriolar in myoblasts, dissociates from the centriole when myoblasts fuse to form myotubes (69). They also found that such redistribution parallels a redistribution of the Golgi complex, which in myoblasts radiates from centrioles, whereas in myotubes is perinuclear (70). A similar redistribution of microtubule-organizing material might take place during the differentiation of neurons and glial cells.

**Nerve Terminals.** Some RII immunoreactivity has been found in nerve terminals, in particular in regions innervated by RII-rich neurons. This localization, which is consistent with the presence of substrates for cAMP-dependent protein kinase in nerve terminals (18, 19, 54), will be discussed elsewhere.

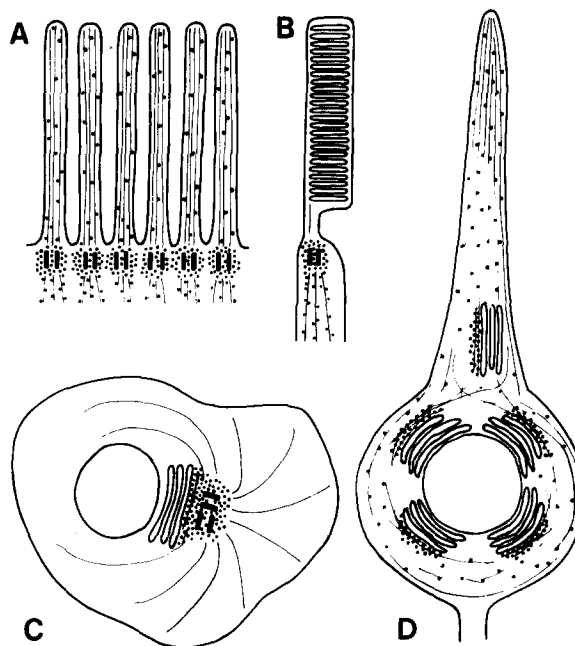
### **The Localizations of RII on Microtubules, Microtubule-Organizing Centers, and at the Golgi Complex May Be Related**

An important structural and probably functional relationship exists between microtubule-organizing centers, microtubules, and the Golgi apparatus. Microtubule-organizing centers act as nucleating centers for the microtubular cytoskeleton (53, 73). In addition, the integrity of the Golgi apparatus depends upon the integrity of the microtubular network, and clear morphological evidence of a close structural relationship between the Golgi complex (in particular the *trans*-Golgi region), microtubules, and microtubule-organizing centers has been provided (43, 62, 64, 83, 84). Thus, although other interpretations are possible, we speculate that the localization of RII on these various organelles are related (Fig. 10).

More than one RII-binding protein is likely to mediate the various RII localizations since the only well-characterized RII-binding protein, MAP2, co-localizes with only a subfraction of RII (21 and this study). MAP2, however, might be a member of a family of RII-binding proteins which alone, or in association with other proteins, play a role in binding subpopulations of microtubules to other components (cytoskeletal elements or vesicular organelles) of the cytoplasm. In neurons the microtubule subpopulation involved would be primarily the perikaryal and dendritic subpopulation, since both MAP2 (2, 21) and Golgi elements are present in perikarya and dendrites but not axons.

Some evidence suggests that MAP2 might cross-link microtubules to neurofilaments or to other cytoskeletal elements (3, 74). Other RII binding proteins might participate in the association of microtubules with other cytoskeletal elements of cilia, with components of microtubule-organizing centers, and with elements of the Golgi apparatus, in particular with elements of the *trans*-Golgi network (36).

The idea that the various localizations of RII reported in this study may be, totally or in part, mediated by proteins interacting with microtubules, is supported by our previous biochemical observation that, like MAP2, other brain RII-binding proteins also appear to bind microtubules. It is of interest that these proteins and MAP2 are also substrates for C (45, 67, 72). MAP2, which has more than 20 phosphorylation sites, can be phosphorylated at as much as 13 sites by C (72). Thus these RII-binding proteins might themselves be the target for the regulatory actions of cAMP. Furthermore these proteins might be directly regulated by the cAMP-RII



**Figure 10.** Schematic drawing summarizing the various subcellular localizations of RII described in the present paper and illustrating the idea that these localizations might be explained by an interaction of RII with subpopulations of microtubules and with microtubule-organizing centers. RII is represented by little dots; microtubules by lines. Sites where RII is concentrated include: cilia and their basal body region in ependymal cells (A); basal body and inner segments of photoreceptors (B); *trans*-Golgi area and pericentriolar material in cells with pericentrosomal Golgi (C); *trans*-Golgi area in cells with perinuclear Golgi, such as neurons in situ (D); neuronal perikarya and dendrites (D). The latter localization of RII is probably due, to a large extent, to RII bound to microtubules via MAP2. Neither RII nor MAP2 are concentrated on axonal microtubules which are not indicated in the scheme (the axon hillock is schematized as an open process at the base of the neuron).

complex independently of C in the same way as certain enzymes can be regulated by the  $Ca^{2+}$ -calmodulin complex (11, 50).

Regulation of the proteins that link microtubules to other cellular elements seems a particularly efficient way by which the function of microtubules either as components of the cell cytoskeleton, or as tracks for organelle translocation, can be regulated. Microtubule-associated RII binding proteins, however, might also serve to concentrate C in proximity of other physiological substrate proteins.

### **Concluding Remarks**

A role of cAMP in microtubule function has been suggested for many years (5, 67, 74). The present study shows that the intracellular distribution of one of the major cAMP-receptor proteins, RII, is compatible with this idea.

Our study points to a primary role of cAMP at the *trans*-Golgi-centrosomal area. This region has a key function in cell structure and polarity, in cell mobility, and in intracellular vesicle traffic (27, 31, 36, 51). Any of these cellular characteristics and properties, therefore, may potentially be regulated by cAMP. cAMP might also regulate in some indirect way (for example via regulating vesicle traffic) processes that take place inside the lumen of the *trans*-Golgi vesicles. Fi-

nally cAMP could perhaps control the rearrangement of the Golgi-centrosomal area that takes place during mitosis (7) or during certain steps of differentiation (70). Other organelle rearrangements that occur during mitosis are thought to be at least partially regulated by protein phosphorylation (58). It is also of interest that pp60<sup>v-src</sup>, a protein kinase thought to be involved in cell transformation, also appears to be localized in the Golgi-centrosomal area (61).

Much more work is needed to understand precisely which cellular functions are regulated via cAMP-RII at the sites we have identified in this study. It should also be determined which second messenger systems cooperate or substitute for cAMP-RII in neurons that contain only very low levels of RII. These may include Ca<sup>2+</sup>-calmodulin since calmodulin also has been found to be associated with microtubules (24) and since some proteins that bind RII may also bind calmodulin (37, 65).

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