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INOSITOL 1,4,5-TRISPHOSPHATE RECEPTORS: IMMUNOHISTOCHEMICAL LOCALIZATION TO DISCRETE AREAS OF RAT CENTRAL NERVOUS SYSTEM

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Abstract—The second messenger inositol 1,4,5-trisphosphate triggers the release of intracellular Ca^{2+} stores upon binding to the inositol 1,4,5-trisphosphate receptor protein, a calcium channel that has been purified and molecularly cloned. To clarify the roles of inositol 1,4,5-trisphosphate receptor in the central nervous system, we have examined in detail the distribution of inositol 1,4,5-trisphosphate receptors in the rat brain and spinal cord using immunohistochemical methods. Inositol 1,4,5-trisphosphate receptors are present in neuronal cells, fibers and terminals in a wide distribution of areas throughout the central nervous system. These include a number of areas not previously reported, such as the olfactory bulb, thalamic nuclei and dorsal horn of the spinal cord. In addition, we have noted a strikingly high density of inositol 1,4,5-trisphosphate receptors in circumventricular organs and neuroendocrine structures such as the area postrema, choroid plexus, subcommissural organ, pineal gland and pituitary.

The distribution of inositol 1,4,5-trisphosphate receptors in discrete structures throughout the central nervous system, including interconnected neuronal systems and neuroendocrine and circumventricular organ structures, presumably reflects the importance of Ca^{2+} release mediated by the phosphoinositide second messenger system in control of diverse physiological processes.

Inositol 1,4,5-trisphosphate (InsP_3) derives from the phosphoinositide second messenger system and triggers the release of intracellular calcium (Ca^{2+}) following binding to its receptor protein. The InsP_3 receptor (InsP_3R) has been purified³⁵ as a protein of c. 1,000,000 mol. wt, comprising four identical subunits which have been molecularly cloned in mouse,⁸ rat^{18,19} and human.²⁹ Reconstitution of the InsP_3R in liposomes demonstrates that the protein comprises the Ca^{2+} channel as well as the InsP_3 recognition site.⁷

Initial studies by autoradiography revealed discrete localizations of InsP_3 in the brain, with extremely high densities in Purkinje cells of the cerebellum and substantial levels in the hippocampus, as well as other areas.^{39,40} The limitations of autoradiography precluded localization at a cellular level. Intracellular localizations of InsP_3R at the electron microscopic level in Purkinje cells of the cerebellum reveal a concentration in subcomponents of the endoplasmic reticulum.^{19,30,31} In T-lymphocytes, InsP_3 influence Ca^{2+} conductance in plasma membrane,⁹ while biochemical and immunohistochemical evidence indicates a localization of InsP_3R protein to the

plasma membrane of these cells.¹⁵ In olfactory cilia immunohistochemical studies localize InsP_3R to plasma membrane.²⁸ To clarify the types of synaptic interactions that influence the InsP_3R system, it would be desirable to localize neurons, cells and fibers enriched for InsP_3R by immunohistochemistry at the light microscopic level. Localizations to neuronal processes and terminals are evident in photoreceptor and bipolar cells of the retina.²⁴ Very recently, limited immunohistochemical mapping for InsP_3R has been reported in developing and adult mouse brain.²² In the present study we report a detailed immunohistochemical mapping of InsP_3R in rat brain, showing an association of the receptor with cells, fibers and terminals in a number of discrete areas, as well as unique localizations to structures of the circumventricular organ system.

EXPERIMENTAL PROCEDURES

Purification of inositol 1,4,5-trisphosphate receptor

InsP_3R , used for affinity purification of anti- InsP_3R antibodies, for preadsorption controls and for immunization of a goat, was purified to homogeneity from crude rat cerebellar membranes that were solubilized as previously described^{7,35} with 1% Triton X-100 or 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate. InsP_3R was then purified using heparin-agarose affinity chromatography as described,^{7,35} followed by affinity chromatography on InsP_3 -agarose.²⁶ Fractions from the heparin-agarose column containing InsP_3R were diluted to a NaCl concentration below 250 mM and incubated batchwise with InsP_3 -agarose for 30 min. The InsP_3 -agarose was then poured into a column and washed with 0.1%

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Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediamine tetra-acetate; InsP_3 , inositol 1,4,5-trisphosphate; InsP_3R , inositol 1,4,5-trisphosphate receptor; NGS, normal goat serum; NRS, normal rabbit serum; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

Triton X-100 and 250 mM NaCl in homogenization buffer and then eluted with 1 mM phytic acid (InsP₆; Sigma) in the same solution. All steps were carried out at 4 °C.

Preparation of affinity-purified antibodies

Antibodies against InsP₃R purified from rat cerebellum were raised in New Zealand White rabbits (Harlan Sprague-Dawley, Indianapolis, IN) as previously described.²⁴ Antibodies were also produced in a goat (mixed Toggenberg/Neuberg, Cocalico Biologicals, Reamstown, PA). The goat was initially immunized (day 0) with 500 µg purified InsP₃R in Freund's complete adjuvant by s.c. injection in the inguinal and axillary regions. The goat was subsequently boosted by i.m. injection in the hindquarters and s.c. injection at multiple sites on the back with 250 µg InsP₃R in Freund's incomplete adjuvant on days 14, 21, 35, 60 and 88 following the initial injection. The goat was bled on days 67, 74, 81 and 98, and serum was prepared and stored at -20 °C until use. To decrease the concentration of interfering antibodies present in the serum before affinity purification of specific anti-InsP₃R antibodies, crude

antiserum from rabbit or goat was adsorbed by sequential overnight batch incubations at 4 °C with two affinity matrices. The first consisted of c. 20 mg protein extracted from crude brain membranes using high pH (NaOH extract) according to Jay *et al.*¹³ and immobilized on CNBr-activated Sepharose. After incubation of the serum with this material, the serum was collected and incubated with the second adsorption matrix, which consisted of solubilized cerebellar membrane extracts that had been treated with heparin-agarose (i.e. with the InsP₃R already removed) and immobilized on CNBr-activated Sepharose.²⁴ Anti-InsP₃R antibodies were purified from the treated serum using purified InsP₃R immobilized on CNBr-activated Sepharose (InsP₃R-Sepharose). Approximately 1 mg of InsP₃R was immobilized on 7 ml of resin. The antiserum was incubated batchwise with InsP₃R-Sepharose overnight at 4 °C. The InsP₃R-Sepharose was then poured into a column and washed extensively with 1% Triton X-100 in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, 1.5% NaCl) followed by TBS alone. Specific antibodies were then eluted using 4 M MgCl₂. Elution was monitored by reading absorbance

Abbreviations used in the figures

A	anterior lobe of the pituitary	LMol	lacunosum moleculare layer
aca	anterior commissure, anterior part	LP	lateral posterior thalamic nucleus
Acb	nucleus accumbens	LR	lateral reticular nucleus
aci	anterior commissure intrabulbar part	LSI	lateral septal nucleus
AD	anterior dorsal thalamic nucleus	MD	mediodorsal thalamic nucleus
AO	accessory olfactory tract	ME	median eminence
AP	area postrema	Med	medial fastigial cerebellar nucleus
Aq	cerebral aqueduct	mfb	medial forebrain bundle
AVDM	anteroventral thalamic nucleus, dorsomedial part	MG	medial geniculate
AVVL	anteroventral thalamic nucleus, ventrolateral part	MM	medial mammillary nucleus
BST	bed nucleus of the stria terminalis	Mol	molecular layer of the cerebellum
BSTL	bed nucleus of the stria terminalis	Mtr	mitral cell layer of the olfactory bulb
CA1-CA4	fields of Ammon's horn	OB	olfactory bulb
cb	cerebellum	Oc	occipital cortex
Cg	cingulate cortex	opt	optic chiasm
CG	central gray	Or	oriens of the hippocampus
ChP	choroid plexus	P	posterior lobe of the pituitary
Cl	claustrum	PA	preabsorbed antibody
CPu	caudate-putamen	Par	parietal cortex
cu	cuneate fasciculus	PDT	posterodorsal tegmental nucleus
DC, DCo	dorsal cochlear nucleus	Pi	pineal gland
DG	dentate gyrus	Pir	piriform cortex
DH	dorsal horn	Pk	Purkinje cells of the cerebellum
dif	dorsolateral fasciculus	Pn	pontine nuclei
DM	dorsomedial hypothalamic nucleus, compact part	Py	stratum pyramidale of the hippocampus
DR	dorsal raphe	Rad	stratum radiatum of the hippocampus
EP	entopeduncular nucleus	SC	superior colliculus
EPL	external plexiform layer of the olfactory bulb	SCO	subcommisural organ
Fr	frontal cortex	SFO	subfornical organ
Gl	glomerular cell layer of the olfactory bulb	SG	substantia gelatinosa
GN	trigeminal nerve	sm	stria medullaris
GP	globus pallidus	SNC	substantia nigra compacta
Gr	granule cell layer of the cerebellum	SNr	SNR substantia nigra reticulata
I	intermediate lobe of the pituitary	SO	supraoptic hypothalamic nucleus
I-VI	laminae of the cerebral cortex	Sol	solitary tract nucleus
IC	inferior colliculus	Sp5	spinal tract of the trigeminal nerve
ic	internal capsule	SUM	supramammillary nucleus
ICj	islands of Calleja	Te	temporal cortex
IntA	interpositus cerebellar nucleus, anterior part	Tu	olfactory tubercle
IntP	interpositus cerebellar nucleus, posterior part	VCA	ventral cochlear nucleus
IO	inferior olive	VH	ventral horn of the spinal cord
IP	interpeduncular nucleus	VL	ventrolateral thalamic nuclei
LA	lateral amygdaloid nucleus	VP	ventral pallidum
LC	locus coeruleus	VTA	ventral tegmental area
LD	laterodorsal thalamic nucleus	ZI	zona incerta
LDVL	laterodorsal thalamic nucleus, ventrolateral part	3V	third ventricle
LG	lateral geniculate	5N	trigeminal nucleus
LM	lateral mammillary nucleus	12	hypoglossal nerve

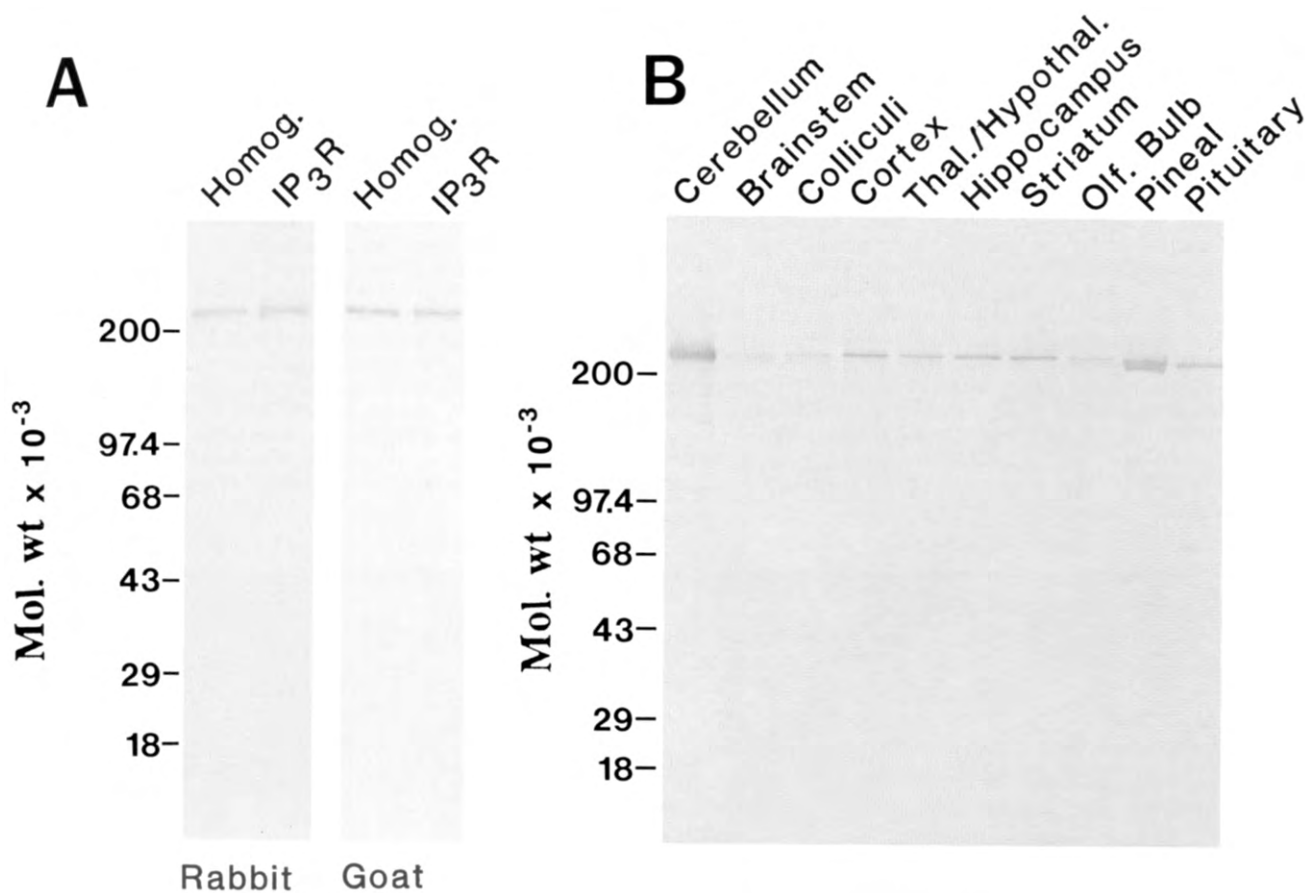


Fig. 1. Immunoblot characterization of affinity-purified anti-InsP₃R antibodies and distribution of immunoreactivity in rat brain. (A) A crude homogenate of rat cerebellum (Homog.; 25 μ g) or purified InsP₃R (IP₃R; 1 μ g) was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The blots were then stained with affinity-purified anti-InsP₃R antibodies from rabbit (left; 1:1000 dilution) or goat (right; 1:2000 dilution) as described in Experimental Procedures. (B) Crude membranes were prepared from various regions of rat brain and pineal and pituitary glands, as described in Experimental Procedures. The membrane proteins were resolved by SDS-PAGE (50 μ g/lane) and transferred to polyvinylidene difluoride membranes. The blots were then stained with affinity-purified rabbit anti-InsP₃R antibodies (1:1000 dilution), as described in Experimental Procedures. The relative molecular masses of the molecular weight markers ($M_r \times 10^{-3}$) were as follows: myosin, 200; phosphorylase b, 97.4; BSA, 68, ovalbumin, 43, carbonic anhydrase, 29; beta-lactoglobulin, 18.

at 280 nm, and fractions containing protein were pooled and dialysed against 10 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1 mM EDTA and 0.05% NaN₃ for 3–4 h followed by dialysis against two to three changes of 20% sucrose in the same buffer. The affinity-purified antibodies were aliquoted and stored at -70°C until use. Affinity-purified antibodies were used in all experiments.

Preparation of membranes

Male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) were decapitated, and tissue was rapidly removed and homogenized with a Polytron (Brinkmann) in 8–10 vols of 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.4 mM phenylmethylsulfonyl fluoride, 4.8 μ g/ml antipain, 9.6 mg/ml leupeptin, 9.6 mg/ml aprotinin, 5 μ g/ml chymostatin and 5 μ g/ml pepstatin A (homogenization buffer). Homogenization and all subsequent steps were carried out at 4°C . Homogenates were centrifuged for 20 min at 40,000 g and the supernatants discarded. Pellets were resuspended in the same buffer and assayed for protein using the Coomassie Blue assay from Pierce.

Sodium dodecyl sulfate electrophoresis and immunoblot analysis

Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 1.5-mm-thick 4–16% gradient gels using the system of Laemmli¹⁶ and the separated proteins were transferred to Immobilon-P membranes (Millipore) according to Towbin *et al.*³⁷ Blots were blocked with 3% bovine serum albumin (BSA) in TBS for 1 h before overnight incubation with affinity-purified antibodies in 3% BSA in TBS. After this incubation, blots were washed three times for 10 min each in 3% BSA in TBS and then incubated with horseradish peroxidase-linked goat anti-rabbit secondary antibody (1:1500 dilution; Boehringer Mannheim) for 1 h at room temperature in the same solution. After three washes of 10 min each in TBS, the blots were developed using H₂O₂ and 4-chloro-1-naphthol as the substrates.

Preparation of tissue and immunocytochemistry

Rats were deeply anesthetized using sodium pentobarbital and perfused with 50 ml of 50 mM sodium phosphate (pH 7.4)–0.9% NaCl (PBS) followed by 200–300 ml freshly

dissolved paraformaldehyde in 100 mM sodium phosphate (pH 7.4). Tissue was then removed by careful dissection and further fixed by incubation in the same solution for 4–18 h. Tissue was then cryoprotected by incubating in 10% sucrose in 100 mM sodium phosphate buffer (pH 7.4) until the tissue sank, followed by sinking in 30% sucrose in the same buffer. Alternatively, tissue was cryoprotected by sinking in 20% glycerol in the same buffer. After freezing the tissue with dry ice, sections (40 μ m) were cut into TBS using a sliding microtome. Sections were used fresh or stored at -20°C in an antifreeze solution [10% (w/v) polyvinylpyrrolidone (average mol. wt 40,000), 30% (w/v) sucrose, 10% (v/v) ethylene glycol, 50 mM sodium phosphate, pH 7.4] before use. Sections were permeabilized before immunocytochemistry by incubation in 0.3% Triton X-100 in TBS for 30 min. Sections to be stained with rabbit anti-InsP₃R antibodies were blocked by incubation in 5% normal rabbit serum (NRS) in TBS for 30 min. The sections were then incubated overnight at 4°C with gentle shaking in primary antiserum in 1.5% normal goat serum (NGS) (rabbit antibody) or

1.5% (goat antibody) in TBS containing 0.1% Triton X-100 and 0.05% NaN₃. The affinity-purified anti-InsP₃R antibodies from rabbit or goat were used at a concentration of *c.* 2 $\mu\text{g/ml}$ concentration.

After the primary incubation, the sections were washed three times in TBS for 10 min each. The sections were then incubated with the appropriate biotin-conjugated secondary antibody (goat anti-rabbit or rabbit anti-goat; 1:200 dilution; Vector Laboratories) for 1 h at room temperature in 1.5% normal serum from the same species as the secondary antibody in TBS containing 0.1% Triton X-100. After three washes in TBS, the sections were next incubated with an avidin-biotin-peroxidase complex (Vector Elite, 1:50 dilution; Vector Laboratories) in TBS for 45 min at room temperature. The sections were again washed three times for 10 min each in TBS and were then developed with a substrate solution consisting of 0.1% H₂O₂ and 0.5 mg/ml diaminobenzidine in TBS. Sections were rinsed with TBS and mounted on subbed glass slides. After dehydration, the sections were coverslipped with Permount (Fisher).

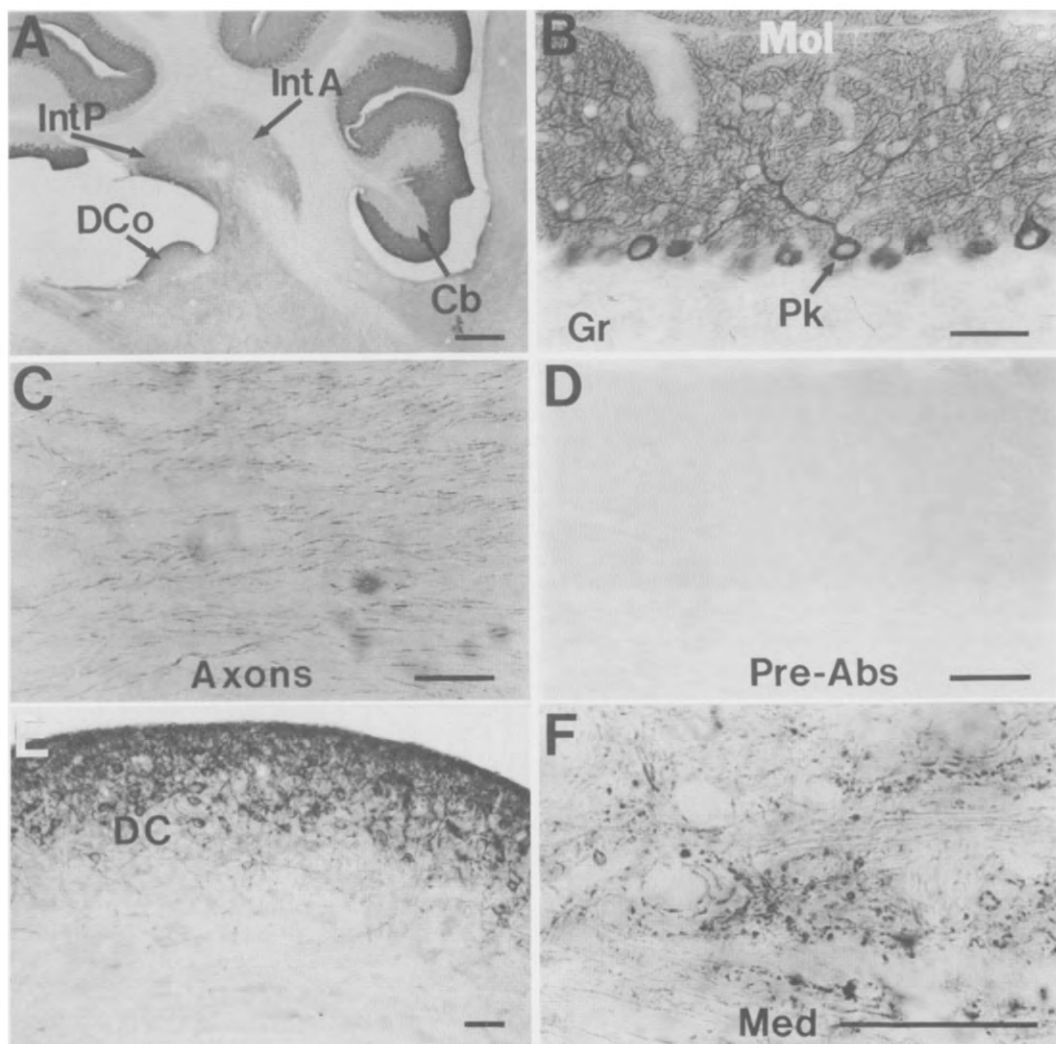


Fig. 2. Distribution of InsP₃R immunoreactivity within the cerebellum and dorsal cochlear nucleus. Note the intense labeling of InsP₃R within the molecular layer of the cerebellum and the immunostaining of Purkinje cell axons (C) which course to innervate deep cerebellar nuclei. Note the absence of staining in D, in which anti-InsP₃R antibodies were preabsorbed with InsP₃R antigen. E Shows the staining pattern of InsP₃R in the dorsal cochlear nuclei. F Shows an example of InsP₃R immunoreactivity in the medial cerebellar nuclei. Note the punctate appearance of staining. Scale bars = 500 μm (A); 50 μm (B–F).

RESULTS

Characterization of immunoreactive inositol 1,4,5-trisphosphate receptor

We used affinity-purified antibodies derived from polyclonal antisera raised in rabbits and goats (Fig. 1). Both antisera display high affinity for InsP₃R. Specificity of the antisera is evident in western blots displaying single 260,000 mol. wt bands in rat cerebellar homogenates (Fig. 1A). Western blots demonstrate striking regional variations in InsP₃R densities, as previously reported,²² with the highest concentration by far in the cerebellum. Strikingly, the pineal gland displays InsP₃R levels higher than any brain region other than the cerebellum. Among the other brain areas, the corpus striatum and cerebral cortex are relatively high, while the olfactory bulb and colliculi are substantially lower. Pituitary InsP₃R densities are lower than pineal but substantially higher than cortex or corpus striatum. All immunohistochemical studies have employed affinity-purified rabbit or goat antisera with identical results obtained with both.

Inositol 1,4,5-trisphosphate receptor localization to cells, fibers and terminals in the cerebellum and dorsal cochlear nucleus

As previously observed,^{22,30} the cerebellum at low power displays high densities of InsP₃R in the molecular layer with prominent discrete concentrations in Purkinje cell bodies (Fig. 2). The dorsal cochlear nucleus displays similar high concentrations of InsP₃R as noted previously by Mignery *et al.*¹⁹ (Fig. 2A, E). The interpositus deep cerebellar nucleus stains heavily for InsP₃R, with greater intensity in the posterior portion. Cell bodies and processes of Purkinje cells stain prominently (Fig. 2A, B), with no staining evident in sections utilizing antibodies preadsorbed with InsP₃R protein (Fig. 2D). Axons in cerebellar white matter also stain, as do punctate terminals about cells in the lateral and medial deeper cerebellar nuclei, as well as the other deep cerebellar nuclei (Fig. 2C, F).

The dorsal cochlear nucleus displays structural homologies to the cerebellum, with the cartwheel cells functionally resembling Purkinje cells.^{2,21}

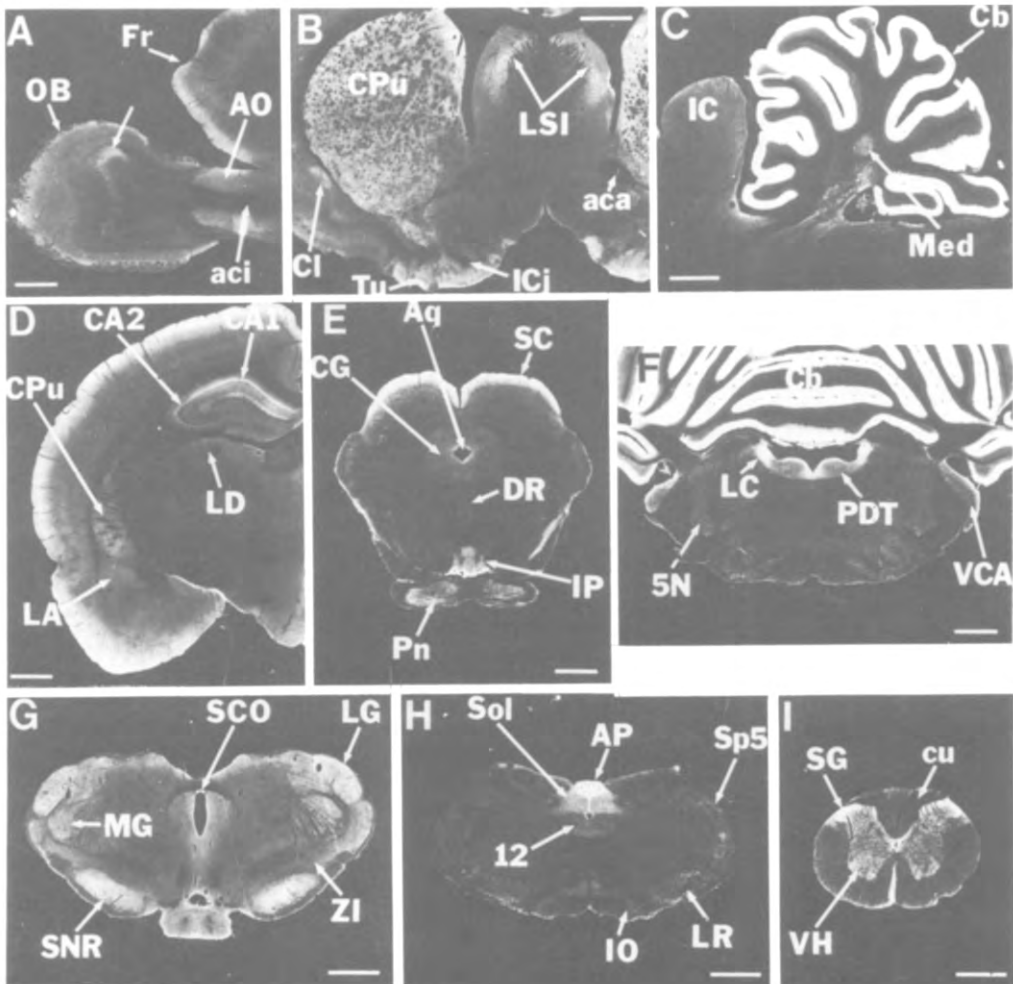


Fig. 3. Low-power dark-field photomicrographs illustrating the overall distribution of InsP₃R immunoreactivity in rat brain. The unlabeled arrow indicates an area which stained non-specifically in the olfactory bulb. Scale bars = 500 μ m.

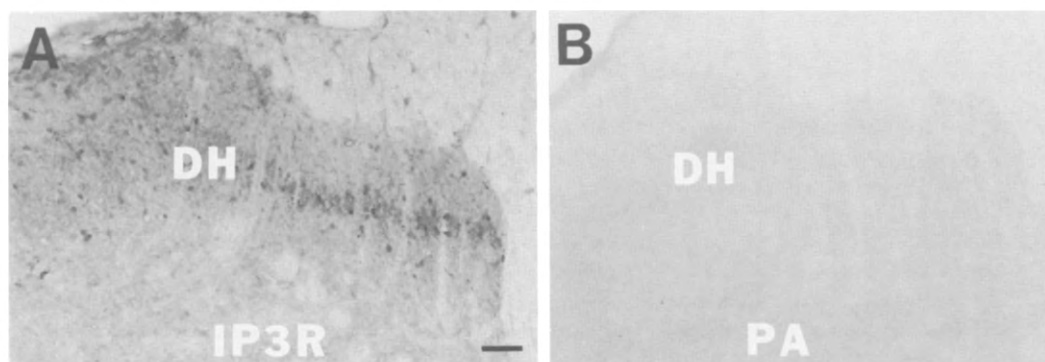


Fig. 4. Bright-field photomicrographs of InsP_3 (IP_3R) immunoreactivity within the spinal cord. Note the intense staining within the dorsal horn and the absence of staining after preabsorption with InsP_3R antigen. Scale bar = $50\ \mu\text{m}$.

Interestingly, in the dorsal cochlear nucleus cartwheel cells are intensely stained, comparable to the staining of cerebellar Purkinje cells (Fig. 2E).

Inositol 1,4,5-trisphosphate receptor in diverse brain regions

Use of high-affinity antisera has enabled us to detect InsP_3R in a number of discrete areas where it has not been previously observed. For instance, our initial autoradiographic studies^{39,40} and recent immunohistochemical investigations²² failed to detect InsP_3R in the olfactory bulb. We now observe substantial staining in the glomerular layer of the bulb (Fig. 3A). Autoradiographic studies had not identified InsP_3R in the dorsal spinal cord, whereas

our immunohistochemical staining reveals high intensities in the substantia gelatinosa with moderate levels in the ventral horn (Figs 3I, 4). Very little characterization of InsP_3R in the brainstem has been reported previously. We demonstrate very intense labeling of InsP_3R in the area postrema and fairly high levels in the closely adjacent nucleus of the solitary tract (Fig. 3H). Other brain stem regions with high concentrations of InsP_3R not previously noted are the locus coeruleus (Fig. 3F), the interpeduncular nucleus (Fig. 3E) and the pontine nuclei (Fig. 3E). As previously observed in autoradiographic investigations,⁴⁰ the substantia nigra reticulata has high densities of InsP_3R , as do the caudate-putamen, olfactory tubercle and molecular layer of the cerebellum (Fig. 3).

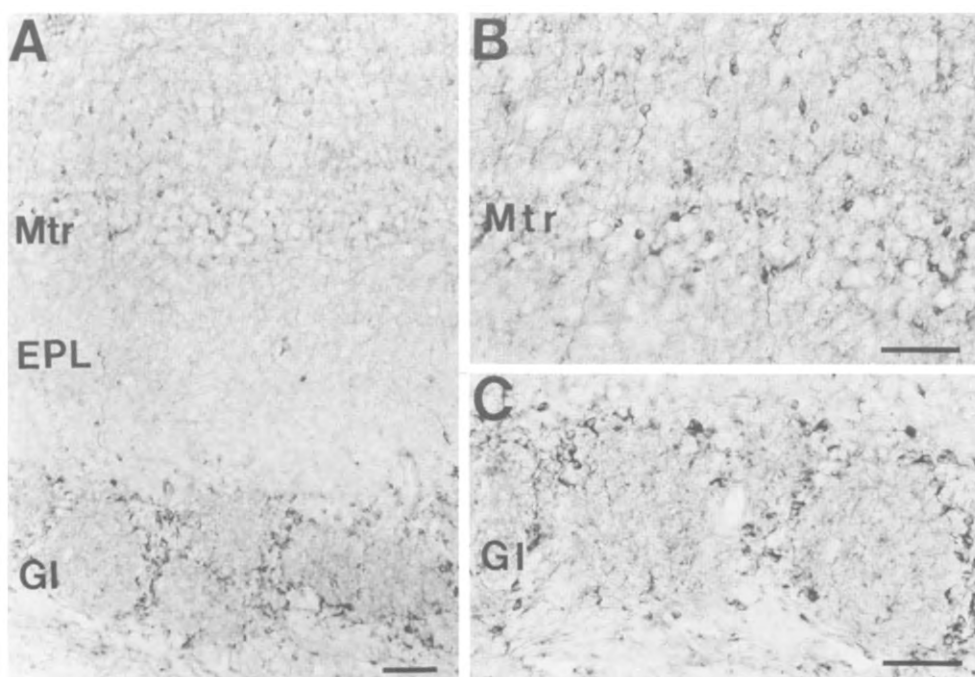


Fig. 5. InsP_3R immunoreactivity within the olfactory bulb. Note the absence of staining within mitral cells (B) and the intense staining of periglomerular cells (C). Scale bars = $50\ \mu\text{m}$.

Forebrain

InsP₃R densities are discretely localized in the olfactory bulb, and most concentrated in periglomerular cells and their processes (Fig. 5). In the mitral cell layer, InsP₃R occurs in granule cells, while only weak immunoreactivity is occasionally observed in the closely adjacent mitral cells. In the internal granule cell layer, InsP₃R stains intensely in granule cells (Fig. 5).

In the basal ganglia, InsP₃R occurs in substantial densities in the caudate-putamen, nucleus accumbens and olfactory tubercle, with highest concentrations in the olfactory tubercle (Fig. 6). Within the caudate, InsP₃R occurs exclusively in medium spiny neurons and appears to be associated with all such neurons,

while very low levels of immunoreactivity are selectively localized in fibers of the adjacent globus pallidus (Fig. 6B). Similarly, in the accumbens, InsP₃R occurs in medium spiny neurons as well as their processes (Fig. 6A). In the ventral tegmental nucleus, which projects to the nucleus accumbens, InsP₃R is highly concentrated in cell bodies of these projection neurons, which are predominantly dopamine containing⁶ (Fig. 6D). Similarly, the pars compacta of the substantia nigra displays InsP₃R immunoreactivity in the presumably dopamine-containing cells (Fig. 6C). Very intense staining in neuropil is apparent in the pars reticulata of the nigra (Fig. 6C). The occurrence of InsP₃R in fiber tracts is highlighted by intense staining in fibers of the internal capsule (Fig. 6E).

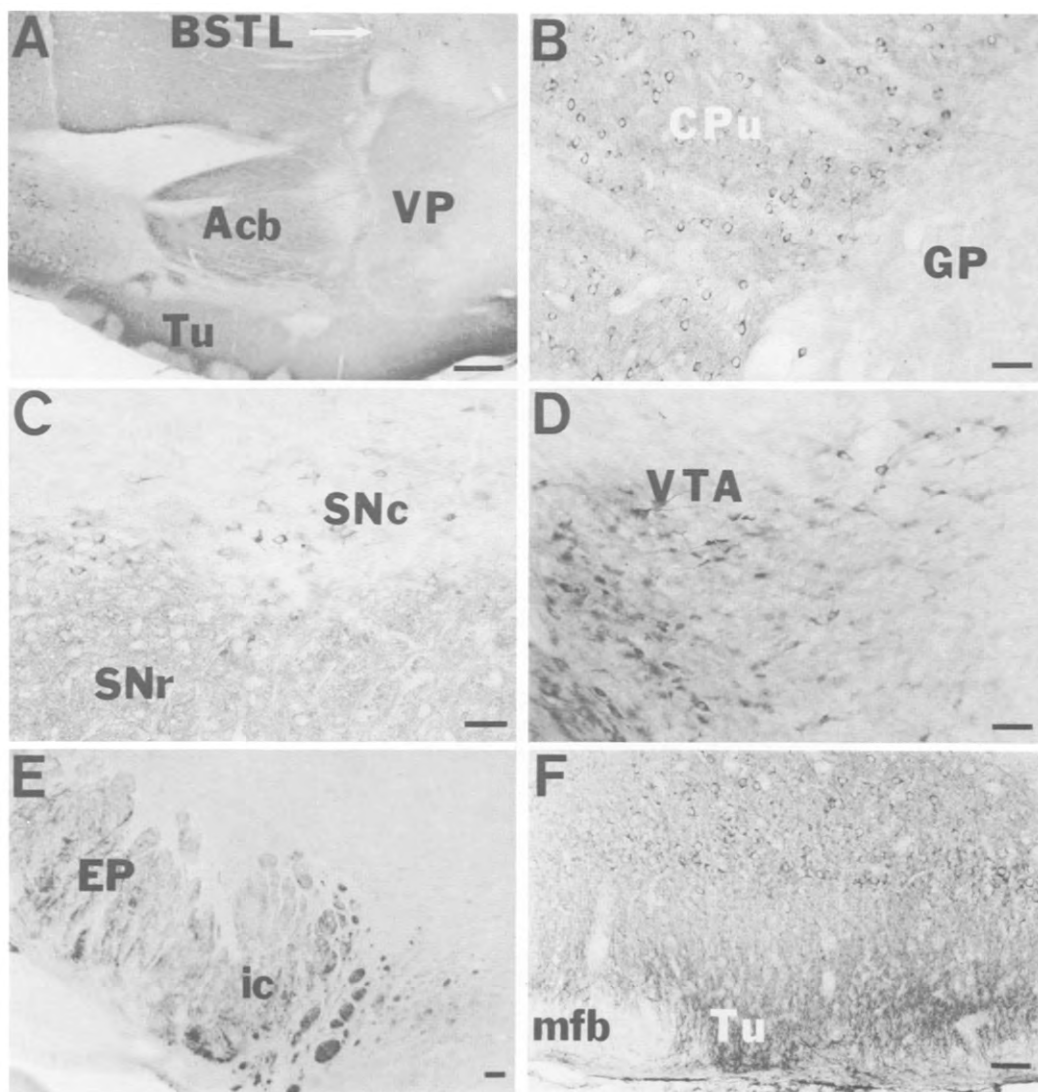


Fig. 6. Photomicrographs of InsP₃R immunoreactivity in the nigrostriatal pathway. In B, note the intense staining of the medium spiny neurons and the moderate staining in the adjacent neuropil. C shows the distribution of InsP₃R in the substantia nigra. Note the cellular staining in the substantia nigra compacta and neuropil staining in the substantia nigra reticulata. E shows InsP₃R immunostaining in the nerve fiber of the internal capsule (ic). Scale bars = 500 μ m (A); 50 μ m (B–F).

In the cerebral cortex, in the occipital, temporal and parietal areas, intense InsP_3R immunoreactivity is apparent, particularly in cells in layers II, V and VI (Fig. 7). Notable staining is evident in the large pyramidal cells of layer V, as well as the non-pyramidal cells of layer II. The non-pyramidal cells of layer VI in the parietal cortex, which project to the thalamus, also display substantial immunoreactivity. In the cingulate cortex, staining is evident in superficial neuropil and deep cells (Fig. 7C).

In the hippocampus, InsP_3R occurs in numerous structures (Fig. 8). Pyramidal cells are labeled throughout the hippocampus, but most intensely in CA1. Processes of these pyramidal cells are stained in the stratum radiatum and stratum lacunosum-moleculare. Granule cells of the dentate gyrus also stain (Fig. 8E). All areas that project to the hippocampus are rich in InsP_3R , specifically cells in the lateral septal nucleus (Fig. 8D), as well as in the entorhinal

cortex (data not shown). Terminals of the perforant pathway, which arises from the entorhinal cortex, stain notably for InsP_3R just superficial to stratum lacunosum-moleculare. Cells in the nucleus of the stria terminalis, which receive prominent projections from the hippocampus, also stain (Fig. 8F).

The mammillary bodies, which receive prominent projections from the hippocampus, display InsP_3R immunoreactivity with high levels in cells in the medial portion of the lateral nucleus, whereas the lateral portion of the lateral nucleus and the medial nuclei are almost devoid (Fig. 9A, B). Fibers coursing into the medial portion of the lateral subdivision are greatly enriched in InsP_3R and could conceivably reflect the hippocampal projections.

Thalamus, hypothalamus and circumventricular organs

Nakanishi *et al.*²² failed to detect InsP_3R immunoreactivity in the thalamus. We observe

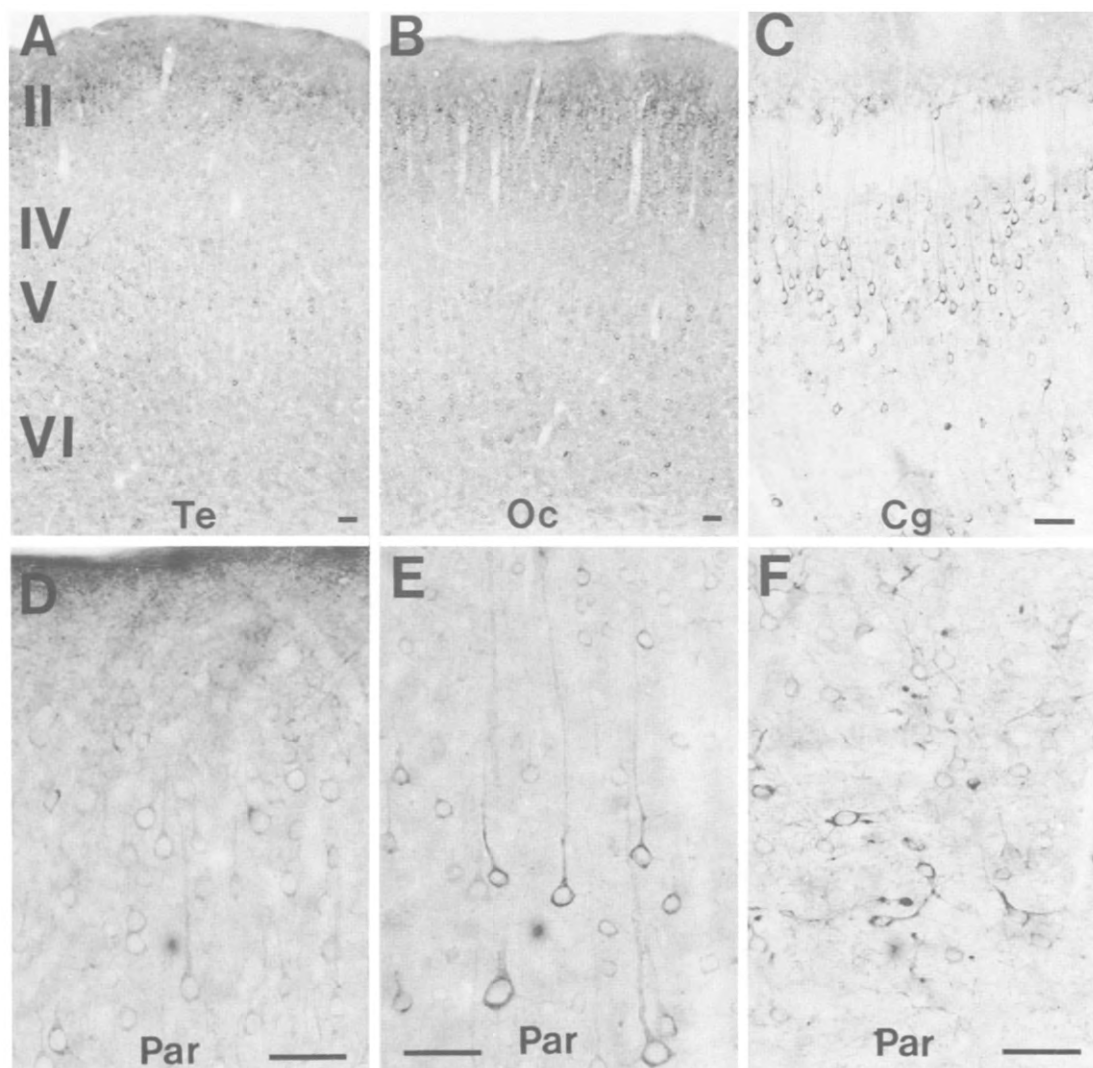


Fig. 7. Distribution of InsP_3R in the cortex of rat brain. Note the intense labeling of pyramidal cells throughout the cortex. Scale bars = 50 μm .

moderate levels of staining in many thalamic nuclei (Fig. 10; Table 1). Typical subnuclei depicted are the ventrolateral and laterodorsal nuclei (Fig. 10B, C), with InsP₃R enriched in cells in the laterodorsal nucleus, with high concentrations of InsP₃R in the larger projection neurons. By contrast, the anterodorsal nucleus of the thalamus displays negligible immunoreactivity and the dorsomedial portion of the anteroventral thalamic nucleus displays only faint immunoreactivity (Fig. 10A).

Some of the most striking InsP₃R localizations in the brain are evident in neuroendocrine structures. In the pituitary, substantial levels are present in discrete cell populations in the anterior lobe, with negligible levels in the intermediate lobe and immunoreactivity occurring in the fiber groups of the posterior pituitary (Fig. 11). The supraoptic nucleus displays InsP₃R in large cell bodies (Fig. 11A), whereas the magnocellu-

lar portion of the paraventricular nucleus is essentially devoid of InsP₃R. Both supraoptic and paraventricular nuclei project vasopressin neurons to the posterior pituitary, whereas only the supraoptic contains oxytocin cells. The selective association of InsP₃R with cells in the supraoptic nucleus and fibers in the posterior pituitary suggests an association with oxytocin.

The median eminence is enriched in fibers with InsP₃R (Fig. 11B). Cells in the arcuate nucleus, which projects to the median eminence, also contain InsP₃R with highest densities in cells of the ventromedial arcuate and somewhat lesser levels in ventral lateral groups of cells (Fig. 11B). The ventromedial arcuate is rich in somatostatin- and neuropeptide Y-containing neurons, which might reflect those containing InsP₃R, whereas the ventral lateral arcuate possesses high densities of growth hormone-releasing factor neurons.¹¹

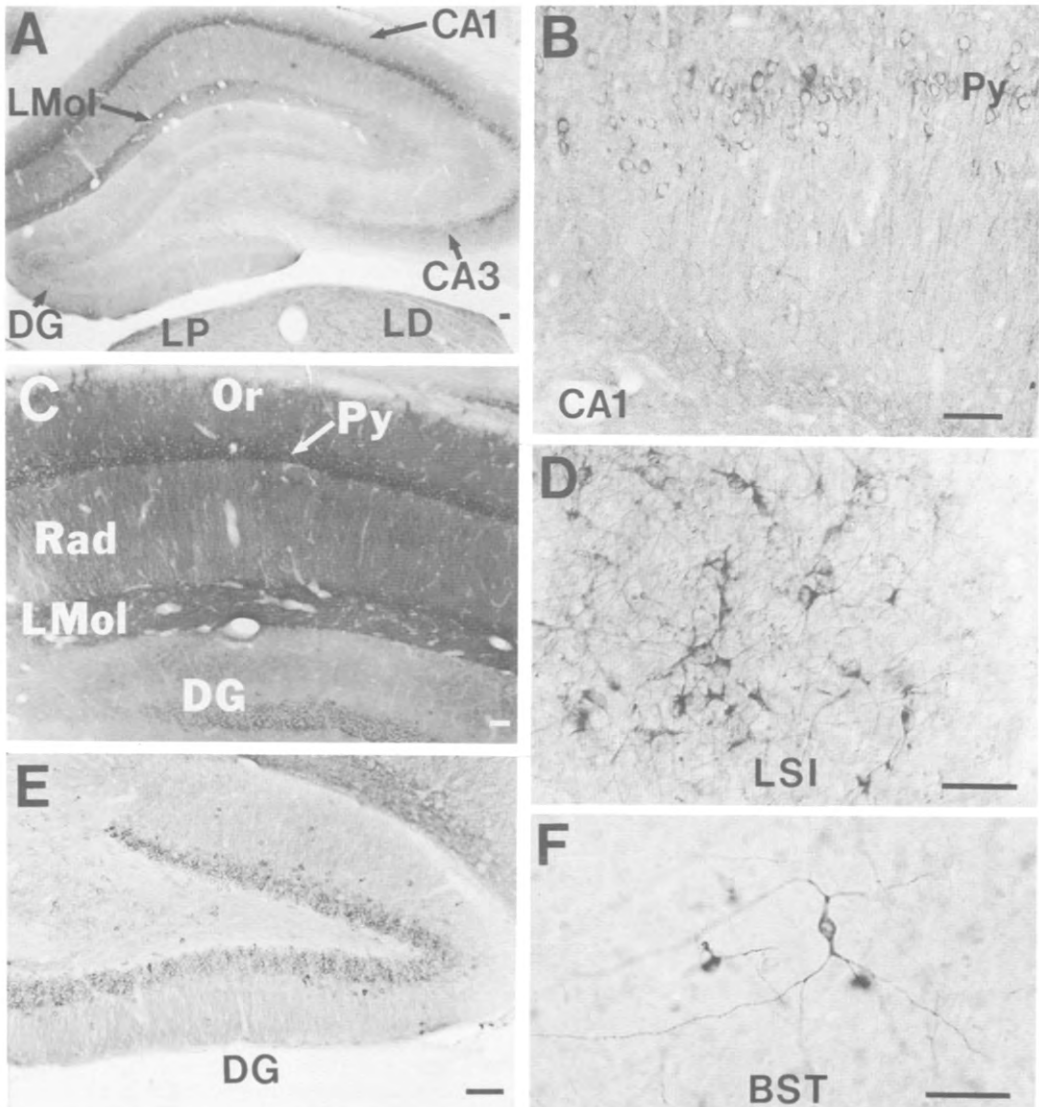


Fig. 8. Bright-field photomicrographs of InsP₃R in the hippocampal pathway. Scale bars = 50 μ m.

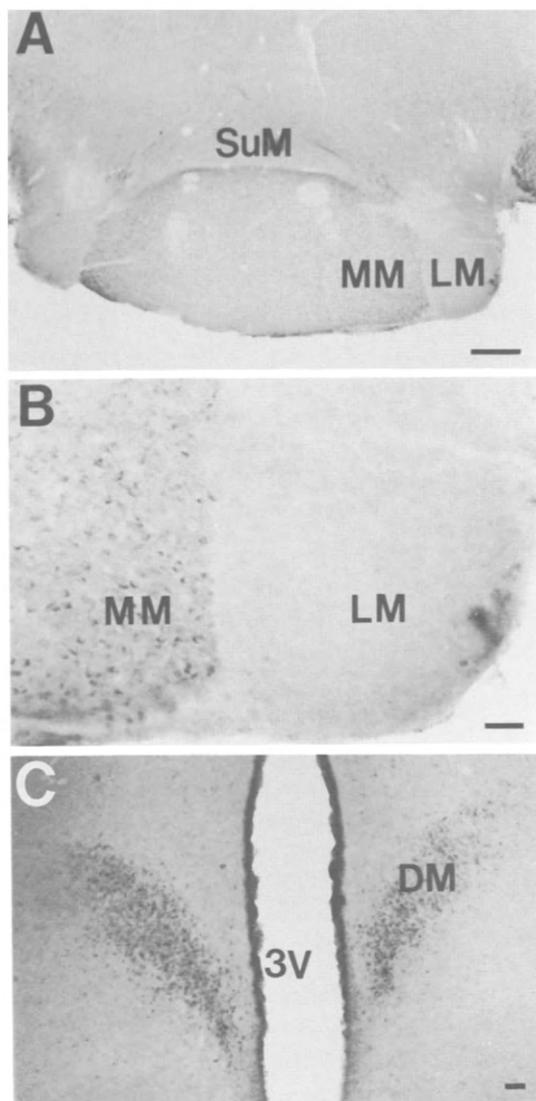


Fig. 9. Distribution of InsP_3R immunoreactivity within the mammillary nuclei and dorsal medial hypothalamic nucleus. Scale bars = 50 μm .

The circumventricular organs are also greatly enriched in InsP_3R (Fig. 12). In the subcommissural organ (Fig. 12A), InsP_3R is concentrated in the ependymal cells, which secrete glycoproteins into the third ventricle.¹² InsP_3R is similarly concentrated in the presumably secretory cells of the choroid plexus. In the subfornical organ, intensely stained InsP_3R fibers are localized at the ventricular surface. The dorsal medial nucleus of the hypothalamus, which projects to the subfornical organ,³⁶ contains a high density of discretely localized InsP_3R (Fig. 9C). Under low-power (Fig. 3II) and high-power magnification (Fig. 12B), the area postrema displays intense InsP_3R staining cell bodies at the ventricular border with dendrites projecting into the nucleus of the solitary tract.

Extremely high densities of IP_3R are apparent in the pineal gland, with levels about the same as in the nearby molecular layer of the cerebellum (Fig. 12E). Within the pineal gland, IP_3R densities are most evident in pinealocytes (Fig. 12F).

Comparative localizations of inositol 1,4,5-trisphosphate receptor messenger RNA and protein

mRNA occurs only in cell bodies of neurons making a particular protein, while the protein itself can occur throughout the neuron, so that comparing localizations of protein mRNA can shed light on the disposition of the protein being investigated. For instance, in the substantia nigra, the pars reticulata is greatly enriched in InsP_3R protein but displays

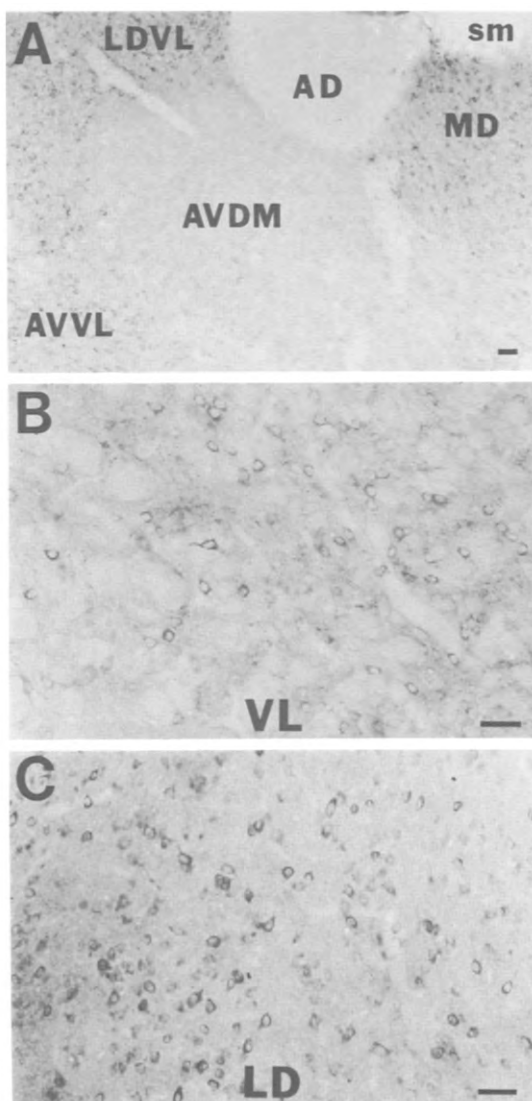


Fig. 10. Bright-field photomicrographs of InsP_3R immunoreactivity within the thalamic nuclei. High-power views of the staining pattern of InsP_3R in the ventrolateral and laterodorsal nuclei are shown in B and C, respectively. Scale bars = 50 μm .

Table 1. Relative densities of inositol 1,4,5-trisphosphate receptor immunoreactivity

Region	Relative density		Region	Relative density	
	Cells	Neuropil		Cells	Neuropil
Olfactory bulb			Hypothalamus		
Olfactory nerve	0	0	Anterior hypothalamic area	1	0
External plexiform layer	1	2	Periventricular nucleus	2	2
Internal granular layer	1	1	Lateral hypothalamic area	0	0
Glomerular layer	2	2	Dorsomedial compact nucleus	3	1
Mitral cell layer	0	1	Ventromedial nucleus	2	1
Anterior olfactory nucleus	3	2	Supraoptic nucleus	3	1
Olfactory tubercle	4	4	Paraventricular nucleus	1	1
Amygdala			Arcuate nucleus	2	2
Lateral nucleus	2	2	Mammillary nucleus	2	1
Basolateral nucleus	3	3	Pituitary		
Basomedial nucleus	3	3	Anterior lobe	4	0
Anterior area	2	2	Posterior lobe	0	2
Central nucleus	3	3	Cerebral cortex		
Medial nucleus	3	3	Neocortex		
Basal ganglia			layer I	0	4
Caudate-putamen	4	4	layers II-VI	3	3
Globus pallidus	0	2	Cingulate cortex		
Accumbens nucleus	4	4	anterior	3	3
Clastrum	4	4	posterior	3	3
Ventral pallidum	0	3	Entorhinal cortex	3	3
Septal area			Piriform cortex	4	4
Lateral septal nucleus	4	3	Cerebellum		
Bed nucleus stria terminalis	3	1	Molecular layer	0	6
Hippocampal formation			Granular layer	1	1
Hippocampus			Deep cerebellar nuclei	0	4
pyramidal cell layer CA1	4	4	Purkinje cell layer	6	—
stratum oriens CA1	0	3	Midbrain		
pyramidal cell layer CA3	2	2	Raphe nucleus	1	0
stratum oriens CA3	0	2	Superior colliculus,		
stratum radiatum CA1	0	3	superficial gray layer	3	2
stratum radiatum CA3	0	1	Central gray	1	1
Dentate gyrus			Red nucleus	0	0
Granular cell layer	2	0	Substantia nigra		
Molecular layer	1	2	compacta	3	2
Hilus	1	0	reticulata	0	3
Subiculum	3	3	Interpeduncular nucleus	3	1
Habenula			Inferior colliculus		
Medial nucleus	0	1	dorsomedial	1	1
Lateral nucleus	0	0	ventrolateral	1	1
Pineal	5	5	Ventral tegmental area	3	2
Thalamus			Pons		
Ventroposterior nucleus,			Pontine nucleus	1	2
lateral and medial	2	1	Locus coeruleus	3	2
Mediodorsal nucleus	2	1	Parabrachial nucleus	1	1
Laterodorsal nucleus	2	1	Medulla oblongata		
Posterior nuclear group	1	1	Nucleus of the solitary tract	1	2
Paratenial nucleus	2	1	Inferior olive	1	1
Reuniens nucleus	1	1	Nucleus of the spinal tract of		
Ventral lateral geniculate nucleus	2	1	the trigeminal nerve	1	1
Dorsal lateral geniculate nucleus	2	1	Spinal tract of the trigeminal nerve	1	1
Medial geniculate nucleus	2	1	Dorsal cochlear nucleus	4	1
Anterior pretectal area	1	0	Ventral cochlear nucleus	1	1
Reticular thalamic nucleus	2	1	Spinal cord		
Rhomboid nucleus	2	1	Substantia gelatinosa	0	3
Gelatinous nucleus	1	1	Dorsal horn	2	2
Paraventricular nucleus	3	1	Ventral horn	2	2
Parafascicular nucleus	1	1	White matter	1	0
Ventrolateral nucleus	2	1	White matter tracts		
Anterodorsal nucleus	0	0	Corpus callosum	0	1
Anteroventral nucleus			Internal capsule	0	3
Dorsomedial nucleus	1	1	Fornix	0	2
Ventrolateral nucleus	3	1	Anterior commissure	0	0
Anteromedial	3	1	Optic tract	0	0
Zona incerta	0	0	Superior cerebellar peduncle	0	1
Stria medullaris	0	0	Medial lemniscus	0	0

Relative densities of InsP₃R immunoreactivity in various areas of the rat CNS are shown. In this table, the highest density of staining (in cerebellar Purkinje cell and molecular layers) was arbitrarily assigned a value of 6 and the relative densities or other regions were estimated by two independent observers by visual inspection of a large number of representative sections from a total of more than five rats.

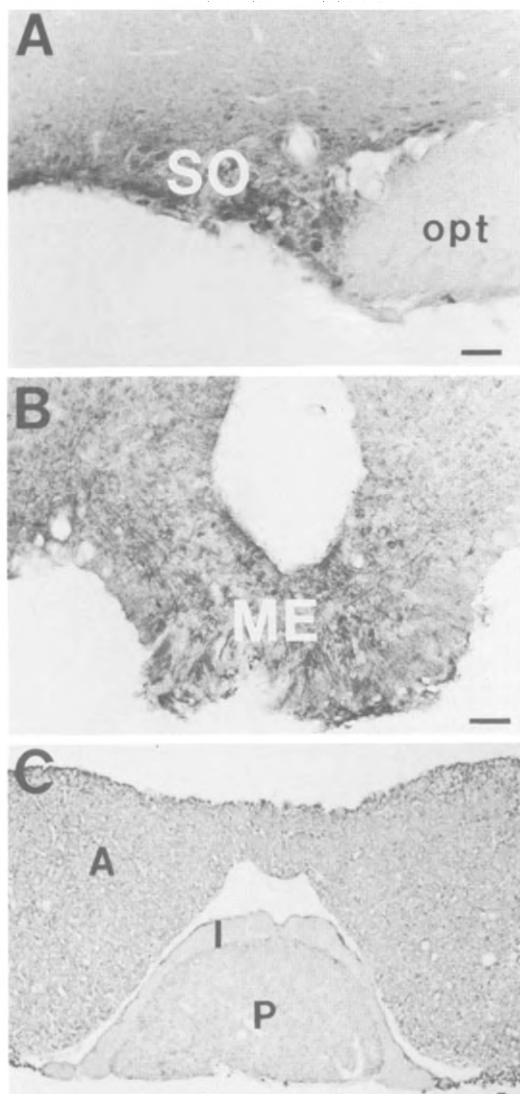


Fig. 11. Distribution of InsP_3R immunoreactivity within the pituitary hypothalamic axis. Scale bars = 50 μm .

negligible mRNA levels by *in situ* hybridization (Fig. 13). This fits with evidence from lesion studies that InsP_3R in the pars reticulata occurs in terminals of the descending striatonigral pathway.⁴⁰ By contrast, substantial InsP_3R mRNA is evident in the pars compacta, which contains low levels of immunoreactivity, suggesting that most InsP_3R protein formed by the compacta cells is transported into dendrites or axons.

In the hippocampus, InsP_3R mRNA is evident in pyramidal cells of all layers, with highest levels in CA1. Immunoreactivity also occurs in the pyramidal cells, as well as in the basal and apical dendritic areas. Immunoreactivity lies in a dense band, reflecting terminals of the perforant pathway, consistent with the absence of mRNA in this layer (Fig. 13).

The dentate gyrus contains substantial mRNA in the granule cells, and high levels of InsP_3R

immunoreactivity also occur in these cells (Fig. 13). These granule cells project mossy fibers to CA3. Interestingly, no InsP_3R immunoreactivity is evident in the mossy fiber projection area in CA3, suggesting that InsP_3R synthesized in the dentate granule cells functions primarily in the dendrites of these cells, where staining is pronounced.

In the caudate, InsP_3R mRNA is distributed homogeneously, consistent with its occurrence in medium spiny neurons throughout the caudate. The even more homogeneous appearance of immunoreactivity fits with the localization of InsP_3R in fibers as well as cell bodies. In the olfactory tubercle, mRNA is concentrated in cell bodies, whereas InsP_3R protein is evident in more superficial layers corresponding to dendrites of these cells. The endopyriform nucleus and the claustrum contain substantial levels of both InsP_3R and mRNA and protein (Fig. 13).

DISCUSSION

A number of interesting themes are apparent from the diverse localizations of InsP_3R . Earlier studies had focused primarily on the concentration of the receptor in endoplasmic reticulum in cell bodies such as Purkinje cells.^{19,30,31} Throughout the brain we find high densities of the receptor in neuronal fibers and terminals as well. Earlier we reported high concentrations of InsP_3R in terminals of retinal photoreceptors and bipolar cells.²⁴ In these sites the phosphoinositide system might be involved in synaptic transmission associated with axo-axonic synapses. Alternatively, nerve terminal depolarization, which triggers calcium influx, might activate the phosphoinositide system to elicit intracellular release of calcium via InsP_3R .

A number of interconnected neuronal systems are enriched in InsP_3R and their various components. For instance, most of the neuronal systems of the hippocampus have high densities of InsP_3R . Entorhinal neurons enriched in InsP_3R project to granule cells of the dentate gyrus, which contain InsP_3R , which in turn project mossy fibers to pyramidal cells of CA3 and CA4 that contain InsP_3R , which themselves project Schaffer collaterals to InsP_3R -enriched CA1 pyramidal cells.

Particularly striking is the high density of InsP_3R in circumventricular organs and neuroendocrine structures. The choroid plexus and subcommissural organ secrete into the ventricular fluid. The phosphoinositide system has been associated with glandular secretion in the periphery. Indeed, InsP_3R disposition was first characterized in the blowfly salivary gland,^{4,5} and extensive studies of InsP_3R release of calcium have been conducted in the lacrimal gland.^{3,20} Both exocrine and endocrine pancreas possess robust phosphoinositide systems.³² In pancreatic beta cells, the phosphoinositide system may be involved in insulin release.^{23,27} Recently, we have demonstrated InsP_3R localized to endoplasmic reticulum and other

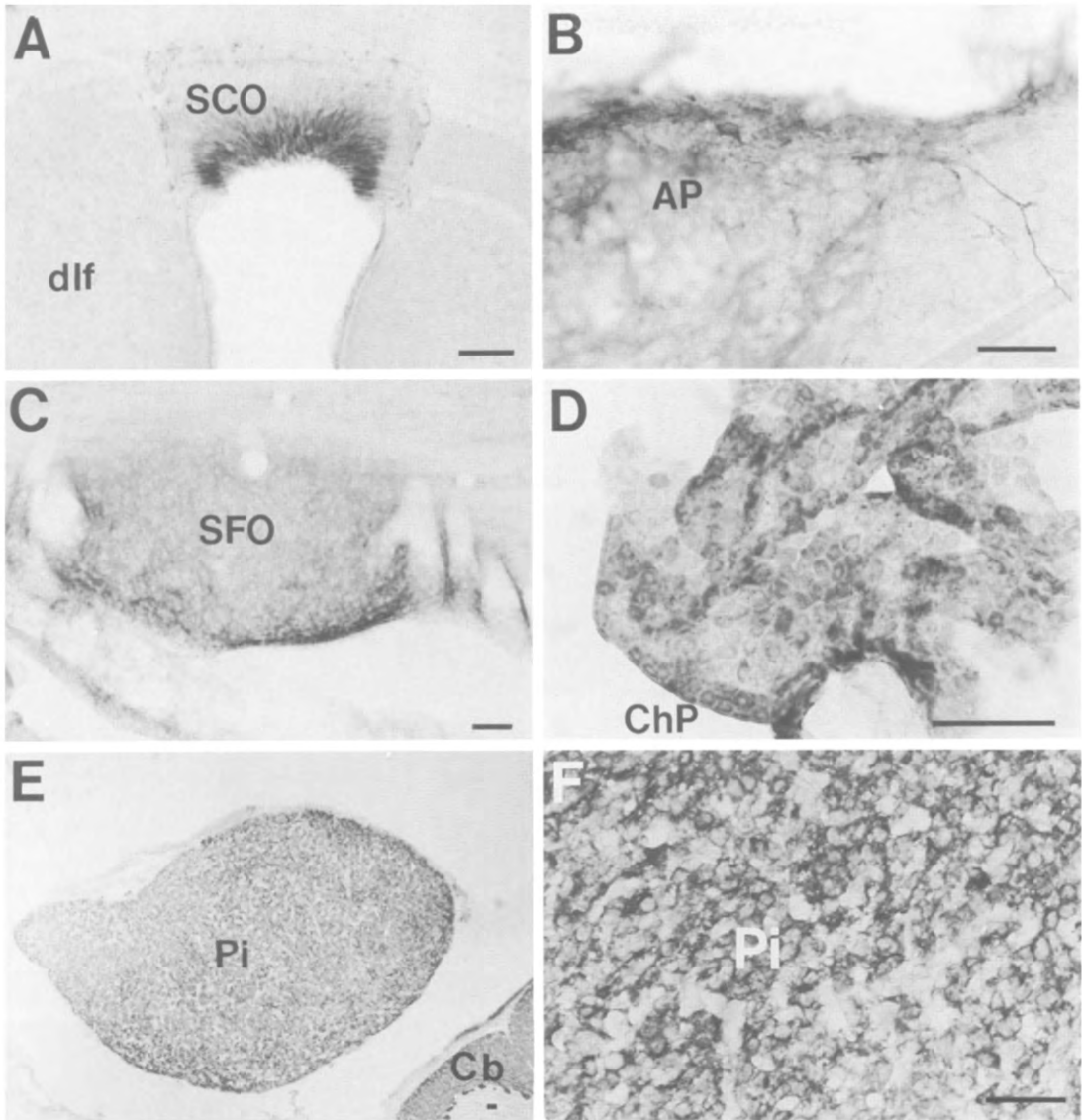


Fig. 12. Photomicrographs of InsP₃R immunoreactivity in circumventricular organs throughout rat brain. Scale bar = 50 μ m.

structures of the pancreas.³³ Thus, the InsP₃R may serve a similar role in these circumventricular organs.

High concentrations of InsP₃R in circumventricular organs may reflect substantial densities of receptors associated with the phosphoinositide system. For instance, the choroid plexus and the area postrema are highly enriched in vasopressin V-1 receptors, which act through the phosphoinositide system.²⁵ Similarly, the subfornical and subcommissural organs and the area postrema are enriched in angiotensin II receptors, which act through the phosphoinositide system.^{10,17} The choroid plexus also has high densities of 5-HT_{1C} serotonin receptors that act by turning on phosphoinositide synthesis.¹ Accordingly, InsP₃R may be involved with signal transduction in these structures.

The exact role of InsP₃R in the pituitary is unclear. The selective localization of InsP₃R in the supraoptic but not the paraventricular nucleus suggests that InsP₃R is contained in oxytocin neurons, but colocalization studies will be required to delineate this possibility. Similarly, we do not yet have direct evidence as to the subtypes of cells in the anterior pituitary that are enriched in InsP₃R.

The pineal gland possesses the highest density of InsP₃R of any structures examined except for the cerebellum. The innervation of the pineal gland in the rat derives mainly from sympathetic fibers whose cell bodies are in the superior cervical ganglion.¹⁴ Besides norepinephrine, these neurons also contain neuropeptide Y. The pineal gland is greatly enriched in adenyl cyclase, as well as both beta and alpha₁ adrenoceptors.³⁴ The extremely high density of

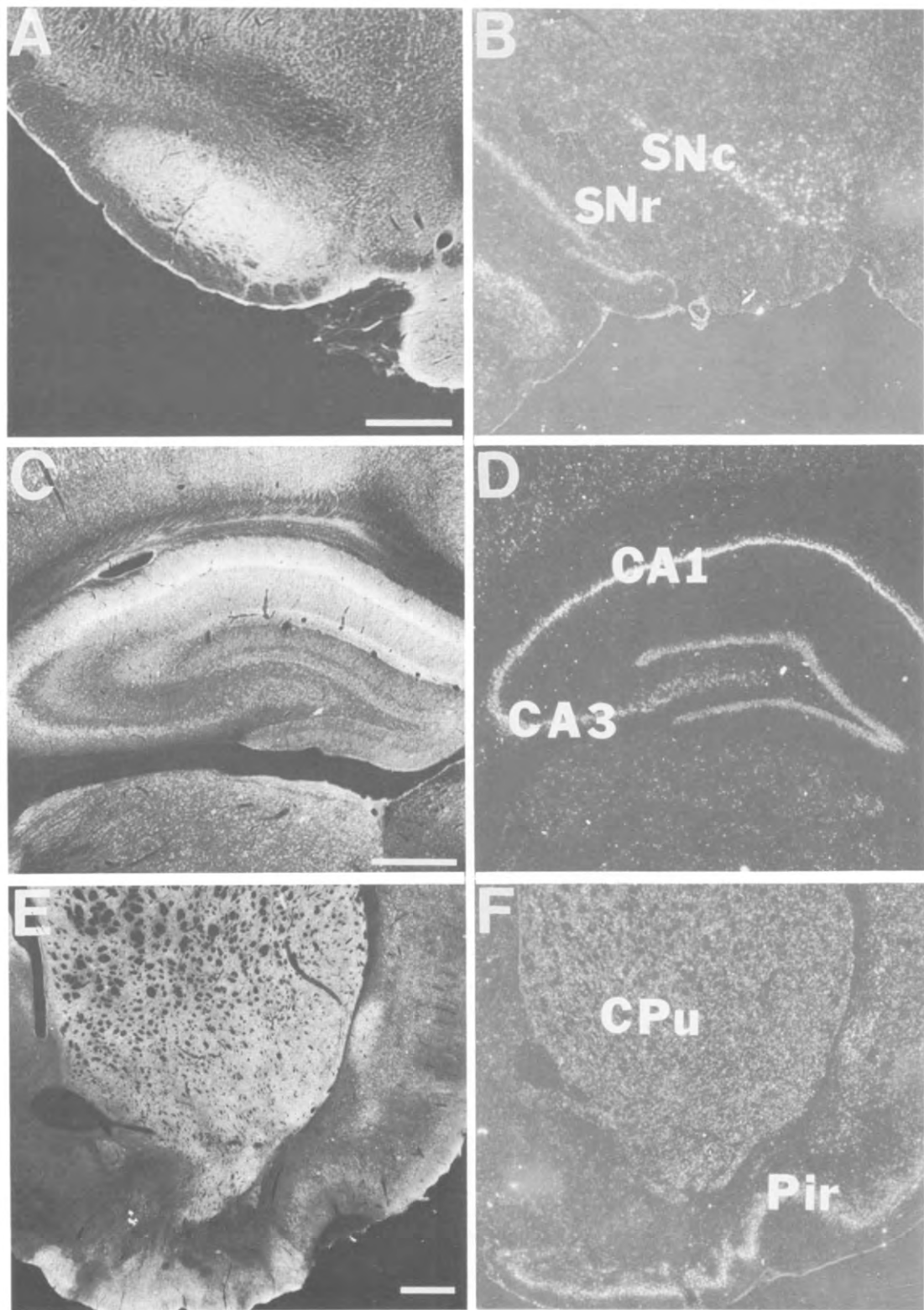


Fig. 13. Comparison between InsP₃R immunoreactivity (A, C and E) and mRNA (B, D and F) within the substantia nigra, hippocampus and striatum. Scale bars = 500 μm.

InsP₃R in pinealocytes suggests that the phosphoinositide system is at least as important in mediating sympathetic regulation of the pineal gland as the cyclic AMP system.

Our results differ in comparison to previous results, mainly in that we detected significant levels of InsP₃R in areas where it was not previously detected; for example, in the thalamus, olfactory bulb and spinal

cord. These differences probably reflect the higher sensitivity or our immunostaining technique, using affinity-purified polyclonal antibodies, in comparison to the previously used autoradiographic detection of bound ligand^{39,40} and immunohistochemical staining using monoclonal antibodies.²² Although InsP₃ binds to its receptor with nanomolar affinity, its rate of dissociation is extremely rapid,⁴¹ possibly hampering autoradiographic detection. In comparison to monoclonal antibodies, polyclonal antibodies are typically higher in affinity.

Although InsP₃R has not been previously demonstrated in the spinal cord, our results showing InsP₃R in the dorsal horn are consistent with previous results of physiological studies showing release of intracellular Ca²⁺ in dorsal horn neurons by substance P, a known activator of phosphoinositide turnover.³⁸

CONCLUSIONS

InsP₃R immunoreactivity is present in widespread areas of the CNS and is mainly confined to neurons.

A number of interconnected neuronal systems are enriched in InsP₃R and immunoreactivity is present in neuronal cell bodies, dendrites, axons and terminals in various areas of the CNS. In addition, strikingly high densities of InsP₃R are present in a number of circumventricular and neuroendocrine structures, including the area postrema, choroid plexus, subcommissural organ, pineal gland and pituitary. These results suggest important roles for the Ca²⁺ release arm of the phosphoinositide second messenger system in control of diverse physiological processes.

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