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Autoradiographic Distribution of ^{125}I Calcitonin Gene-Related Peptide Binding Sites in the Rat Central Nervous System

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SKOFITSCH, G. AND D. M. JACOBOWITZ. *Autoradiographic distribution of ^{125}I calcitonin gene-related peptide binding sites in the rat central nervous system.* PEPTIDES **6**(5) 975–986, 1985.—Using autoradiographic method and ^{125}I-Tyr° rat CGRP as a ligand, receptor binding sites were demonstrated in the rat central nervous system. Saturation studies and Scatchard analysis of CGRP-binding to slide mounted tissue sections containing primarily cerebellum showed a single class of receptors with a dissociation constant of 0.96 nM and a B_{max} of 76.4 fmol/mg protein. ^{125}I-Tyr° rat CGRP binding sites were demonstrated throughout the rat central nervous system. Dense binding was observed in the telencephalon (medial prefrontal, insular and outer layers of the temporal cortex, nucleus accumbens, fundus striatum, central and inferior lateral amygdaloid nuclei, most caudal caudate putamen, organum vasculosum laminae terminalis, subfornical organ), the diencephalon (anterior hypothalamic, suprachiasmatic, arcuate, paraventricular, dorsomedial, periventricular, reuniens, rhomboid, lateral thalamic pretectalis and habenula nuclei, zona incerta), in the mesencephalon (superficial layers of the superior colliculus, central nucleus of the geniculate body, inferior colliculus, nucleus of the fifth nerve, locus coeruleus, nucleus of the mesencephalic tract, the dorsal tegmental nucleus, superior olive), in the molecular layer of the cerebellum, in the medulla oblongata (inferior olive, nucleus tractus solitarii, nucleus commissuralis, nuclei of the tenth and twelfth nerves, the prepositus hypoglossal and the gracilis nuclei, dorsomedial part of the spinal trigeminal tract), in the dorsal gray matter of the spinal cord (laminae I–VI) and the confines of the central canal. Moderate receptor densities were found in the septal area, the "head" of the anterior caudate nucleus, medial amygdaloid and bed nucleus of the stria terminalis, the pyramidal layers of the hippocampus and dentate gyri, medial preoptic area, ventromedial nucleus, lateral hypothalamic and ventrolateral thalamic area, central gray, reticular part of the substantia nigra, parvocellular reticular nucleus, Purkinje cell layer of the cerebellum, nucleus of the spinal trigeminal tract and gracile fasciculus of the spinal cord. The discrete distribution of CGRP-like binding sites in a variety of sensory systems of the brain and spinal cord as well as in thalamic and hypothalamic areas suggests a widespread involvement of CGRP in a variety of brain functions.

Calcitonin gene-related peptide CGRP Autoradiography CNS

ALTERNATE processing of the rat and human calcitonin genes resulted in a novel peptide, the calcitonin gene-related peptide (CGRP) [1, 26, 27, 33]. This peptide was recently isolated and characterized [18,19]. The 37 amino acid human CGRP was found to differ from rat CGRP in four amino acids. CGRP-like immunoreactivity was shown to be widely distributed in the central and peripheral nervous system of man, rat and other species by immunohistochemistry [4, 12, 25, 27, 31, 36, 37] and radioimmunoassay [2, 27, 30, 32, 36].

Using membrane binding assays, receptor sites for ^{125}I-human and rat CGRP were identified in a variety of post-mortem human and rat brain areas [5,35]. In the present paper the overall autoradiographic distribution of ^{125}I-rat CGRP binding sites in rat brain sections is demonstrated.

METHOD

Four male Sprague Dawley rats were perfused via the ascending aorta with 150 ml of ice cold 50 mM phosphate buffered saline (PBS) containing 0.5% sodium nitrite and 10% sucrose. The brain and the cervical spinal cord were removed quickly, cut into 8 mm slices, frozen on dry ice and cut into serial 20 μm sections in a cryostat. The sections were thaw mounted on chrom-alum coated slides, dried under a stream of cold air and frozen at −20°C until they were used for autoradiography.

In vitro labelling was performed according to the general principles described previously [39], with minor modifications. No prior fixation was used and the slides were placed in cassettes with large sheets of film [21,24]. Sections which contained primarily cerebellum were used for time course and saturation curve studies. To displace endogenous ligands slides were preincubated at room temperature in 50 mM Tris-HCl buffer (pH 7.7) containing 5 mM $MgCl_2$, 2 mM EGTA twice for 15 min. Thereafter sections were incubated in the same solvent containing 1% (w/v) bovine serum albumin, 0.05% leupeptin, 0.001% pepstatin A and various con-

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ABBREVIATIONS USED IN FIGURES

a	nucleus accumbens
ac	nucleus amygdaloideus centralis
al	nucleus amygdaloideus lateralis
ali	nucleus amygdaloideus lateralis inferior
c	nucleus caudatus
CA	commissura anterior
CAI	capsula interna
CCA	corpus callosum
ccgm	nucleus centralis corporis geniculati medialis
CER	cerebellum
CFV	commissura fornicis ventralis
CO	chiasma opticum
cp	nucleus caudatus putamen
CP	commissura posterior
dh	dorsal horn of the spinal cord
DP	decussatio pyramidis
F	fornix
FLM	fasciculus longitudinalis medialis
FMT	fasciculus mamillothalamicus
FPVT	fibrae periventricularis thalami
FR	fasciculus retroflexus
FST	fundus striati
gp	globus pallidus
gr	nucleus gracilis
HI	hippocampus
hab	habenula
hcp	horn of the caudatus putamen
ic	colliculus inferior
inc	insular cortex
io	nucleus olivaris inferior
lc	locus coeruleus
na	nucleus arcuatus
nco	nucleus commissuralis
ndm	nucleus dorsomedialis hypothalami
nha	nucleus hypothalamicus anterior
nhp	nucleus hypothalamicus posterior
npV	nucleus sensorius principalis nervi trigemini
ntd	nucleus tegmenti dorsalis (Gudden)
ntdl	nucleus tegmenti dorsalis lateralis
ntm	nucleus tractus mesencephali
ntV	nucleus tractus spinalis nervi trigemini
ntVd	nucleus tractus spinalis nervi trigemini, pars dorsomedialis
nvm	nucleus ventromedialis hypothalami
nV	nucleus originis nervi trigemini
nXII	nucleus originis nervi hypoglossi
os	nucleus olivaris superior
OVLT	organum vasculosum laminae terminalis
P	tractus corticospinalis
p	nucleus pretectalis
PCM	pedunculus cerebellaris medius
PCS	pedunculus cerebellaris superior
PFC	prefrontal cortex
ph	nucleus prepositus hypoglossi
pom	nucleus preopticus medialis
pv	nucleus periventricularis thalami
pvn	nucleus paraventricularis
py	pyramidal layer of the hippocampus
rd	nucleus raphe dorsalis
re	nucleus reuniens
rh	nucleus rhomboideus
sa	septal area
sdi	nucleus dorsalis septi, pars intermedia
SFO	organum subfornicale
SGC	substantia grisea centralis
sgs	stratum griseum superficiale colliculi superioris
snr	substantia nigra zona reticularis
tec	temporal cortex
tl	nucleus lateralis thalami
TO	tractus opticus
V	end of third ventricle
vp	ventral pallidum
zi	zona incerta
VII	nervus facialis

centrations of ^{125}I-Tyr° rat CGRP (Peninsula Labs, San Carlos, CA; Lot No. 007444) with a specific activity of 1400 Ci/mmol. For saturation curve and Scatchard plot studies concentrations of 0.05 to 2.9 nM of iodinated ligand were used. The autoradiographic localization of receptors was done at a concentration of 0.69 nM iodinated ligand. To determine the amount of nonspecific binding cold synthetic rat CGRP was added in a concentration of 1 μM.

To determine the stability of the radioactive ligand, tissue sections were incubated in 0.69 nM ^{125}I-Tyr° CGRP and an aliquot of the incubation solution was analyzed by high performance liquid chromatography (HPLC) at various times. The HPLC-system consisted of 2 Waters 6000 A pumps, a Waters U6K injector with a variable sample loop holding up to 2 ml, a Waters 720 system controller, a Waters 440 absorbance detector set at 280 nm, and a Spectroflow monitor SF 770 (Schoeffel Instruments) absorbance detector set at 210 nm. The solid phase consisted of a Waters μBondapak C_{18} column. The mobile phase was a linear gradient of solution A (0.1% trifluoroacetic acid in distilled water) and solution B (Acetonitrile) starting with an isocratic initial flow of 80% A and 20% B for 3 min and changing during the following 50 min to 30% A and 70% B. The constant flow rate was 1 ml/min. The column outflow was collected with an LKB 2111 Multirac Sampler in 1 min fractions in polystyrene tubes.

For binding experiments the slides containing tissue sections of different brain areas were incubated with the iodinated ligand for 90 min at room temperature, rinsed quickly in 50 mM phosphate buffered saline containing 1% (w/v) bovine serum albumin and further washed in this solution on ice twice for 10 min each. After a final rinse in distilled water the sections were rapidly dried under a stream of warm dry air. Autoradiograms were developed using Kodak X-Omat XAR-5 film after 4–7 days of exposure at 4°C. Tissue sections were stained with 0.1% thionin. Binding for saturation curves and time course was determined by scraping off the dried sections and counting in a gamma counter. Thereafter the material was rehydrated with 0.1 N HCl (1 ml), homogenized by sonication and the amount of protein analyzed [17].

RESULTS

The rate of association of 0.69 nM ^{125}I-Tyr° rat CGRP to slide-mounted tissue sections containing primarily cerebellum revealed that after 90 min specific binding had almost been completed (Fig. 1). HPLC analysis showed that following 100 min of incubation of rat brain slices at room temperature no major breakdown of the radioactive ligand had occurred, whereas after 180 min almost half of the specific activity was lost. Thus for further experiments a 90 min incubation time was chosen.

Saturation studies and Scatchard analysis of ^{125}I-Tyr° rat

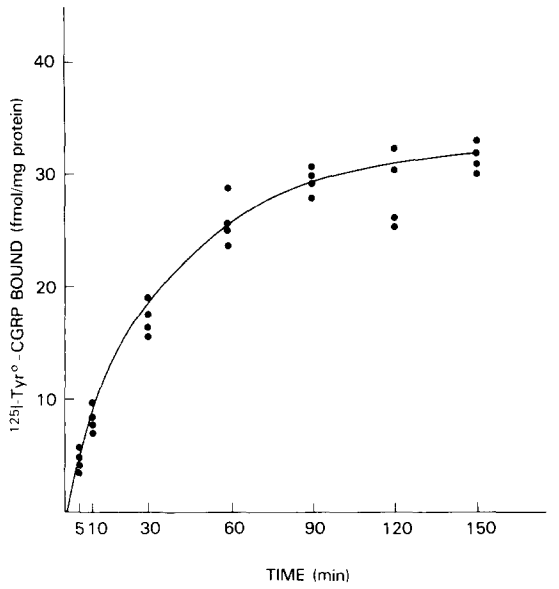

FIG. 1. Association of ^{125}I-Tyr°-rat CGRP to rat tissue sections. Twenty μm sections containing mainly cerebellum were incubated in 0.69 nM ^{125}I-Tyr°-rat CGRP for various times. After 90 min specific binding is almost completed.

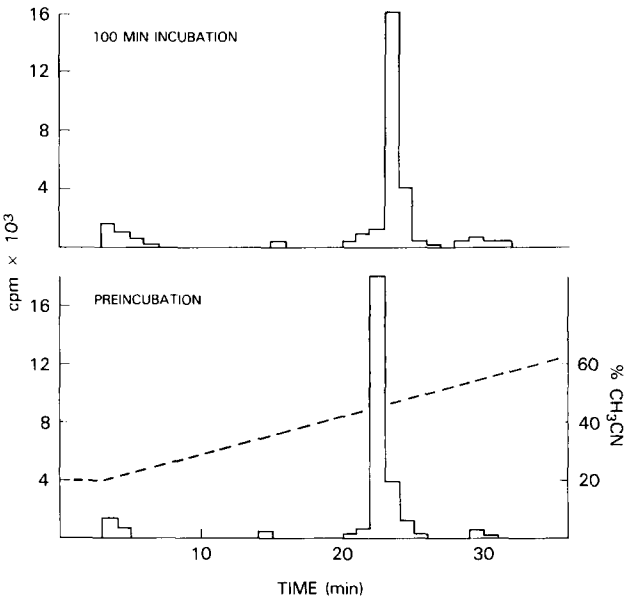

FIG. 2. HPLC analysis of aliquots of the incubation solution prior (preincubation) and after 100 min of incubation of rat brain sections showing less than 5% breakdown of the ^{125}I-Tyr°-CGRP ligand at room temperature. In the bottom panel the gradient characteristics of the HPLC system are indicated as a broken line.

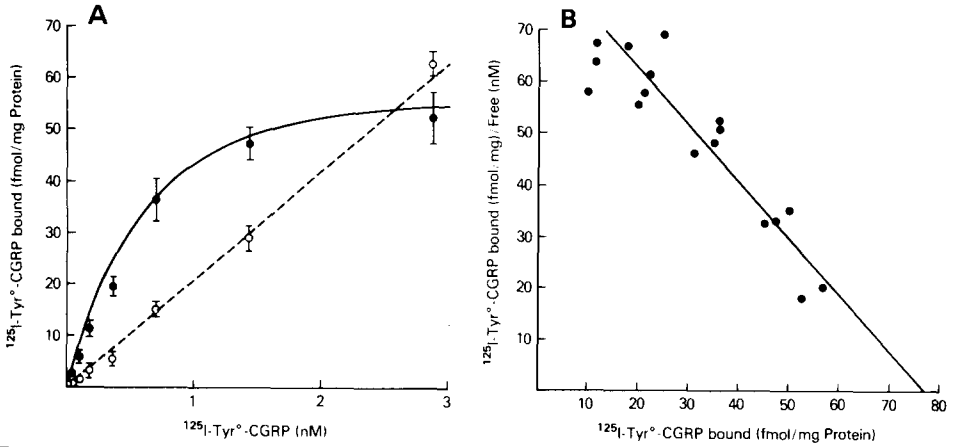

FIG. 3. Saturation curve and Scatchard analysis of ^{125}I-Tyr°-rat CGRP binding to tissue sections containing mainly cerebellum. Twenty μm sections were incubated with increasing concentrations of ^{125}I-Tyr°-rat CGRP. Nonspecific binding was assessed by addition of 1 μM unlabeled synthetic rat CGRP (open symbols). Specific binding (filled circles) appeared saturable (A). Experiments were performed twice in quadruplicates. Scatchard analysis (B) revealed that in sections containing mainly cerebellum ^{125}I-Try°-CGRP bound to one class of receptors (R=0.94) with an equilibrium dissociation constant (K_D) of 0.96±0.36 nM (mean±S.D.) and a maximal number of binding sites (B max) of 76.38±13.3 fmol/mg protein (mean±S.D.).

CGRP binding to slide-mounted tissue sections containing primarily cerebellum showed a single class of receptors as the Scatchard plot was strictly linear (regression coefficient R=0.94) with a dissociation constant of 0.96 nM and a maximal number of binding sites of 76.38 fmol/mg protein (Fig. 3).

The autoradiographic localization of receptors was performed at a concentration of 0.69 nM ^{125}I-Tyr° rat CGRP and revealed high densities of CGRP-receptors with an acceptable background (samples are shown in Fig. 4). The distribution of ^{125}I-Tyr°-rat CGRP binding sites is demonstrated in Figs. 4–8. For easier identification of structures the slides were stained with thionin at the end of the experiment and are shown on the right side of the panels. It is understood that the use of the term "receptor" refers to "binding sites."

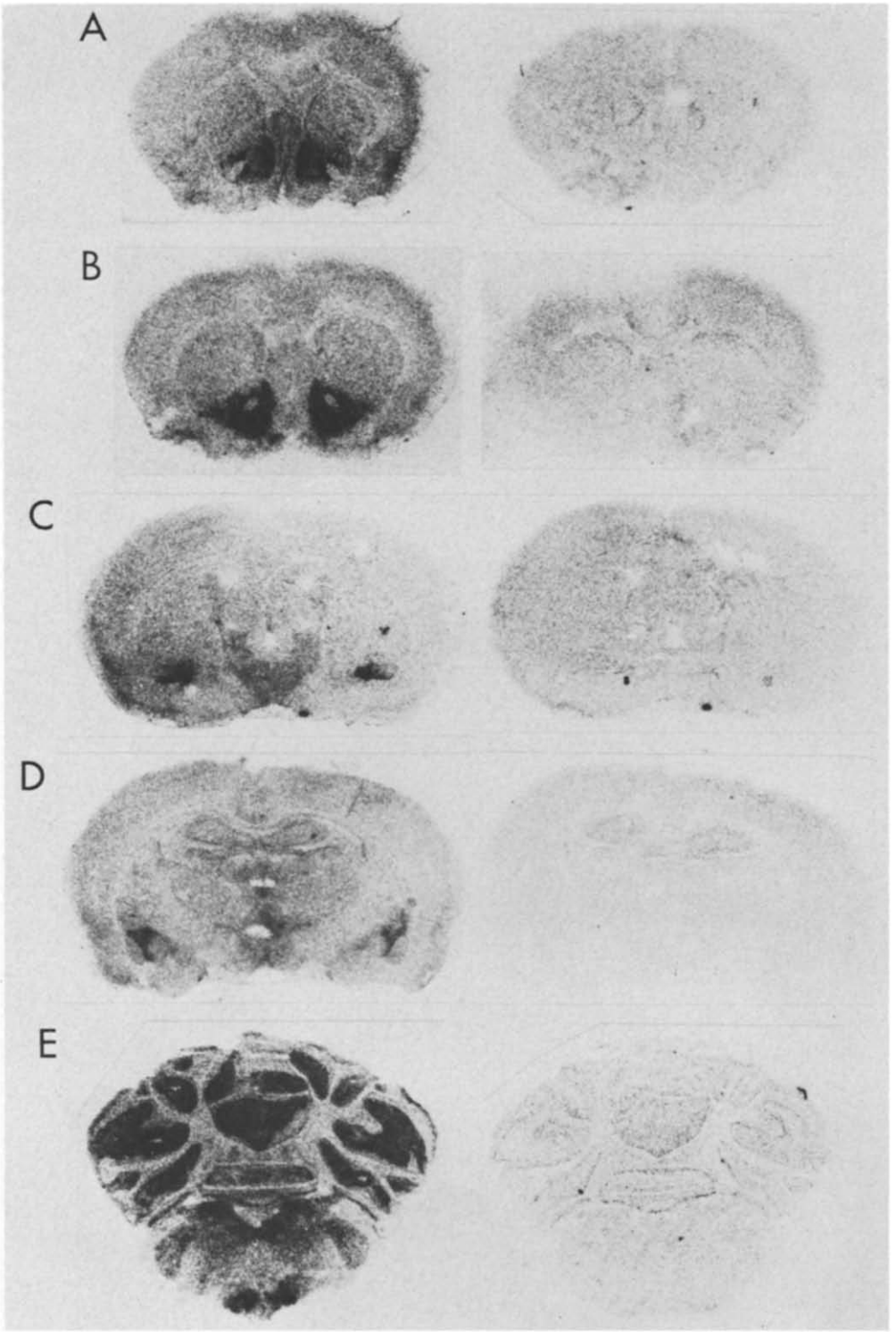

FIG. 4. Autoradiographs showing ^{125}I-Tyr°-rat CGRP binding sites in the rat brain. Sections were incubated in 0.69 nM ^{125}I-Tyr°-rat CGRP for 90 min (left panels). As a control adjacent sections were incubated in 0.69 nM ^{125}I-Try°-rat CGRP plus 1 μM unlabeled synthetic rat CGRP for 90 min (right panels). Sections were exposed to the same film for 4 days. Control sections revealed a negligible background. Approximate coordinates to the intraaural plane according to König and Klippel [12]. (A) 9200, (B) A8620, (C) A6860, (D) A5340, (E) P5500.

Telencephalon

A high density of CGRP receptors was found in the medial prefrontal cortex (Fig. 5 A), basal insular cortex (Figs. 5–7) and the outer layers of the temporal cortex (Fig. 8 B–D). The mid portion of the nucleus accumbens (Fig. 5 C,D) was densley labeled whereas its anterior part (Fig. 5 B) showed high densities in small dispersed patches. The septal area contained low to moderate densities of CGRP receptors especially in the dorsal septal nuclei (Fig. 5 C,D). The dorsomedial part (head) of the anterior caudate nucleus also contained a moderate density of receptors (Fig. 5 D). The fundus striatum (the ventral part of the nucleus caudatus putamen dorsal and ventral to the lateral projections of the anterior commissure, see [22]) contained a high density of CGRP receptors (Figs. 6,7) which in its caudal extensions seem to be continuous with the central amygdaloid nucleus (Fig. 7) as well as the lateral amygdaloid nucleus (Figs. 7, 8 A) and the most caudal parts of the caudate putamen (Fig. 7 D), all of which are very dense in CGRP receptors.

The medial amygdaloid nucleus contained moderate accumulations of receptors (Fig. 7). The organum vasculosum laminae terminalis (OVLT) contained dense accumulations of CGRP binding. The dorsal and ventral bed nuclei of the stria terminalis contained a moderate number of receptors (Fig. 6 B,C) except for the most lateral part of the anterior dorsal nucleus interstitialis striae terminalis which showed a dense band of CGRP receptors in close apposition to the internal capsule (Fig. 6 B). The pyramidal layers of the hippocampus and the dentate gyri contained moderate densities of receptors (Figs. 6 D, 7). The subfornical organ showed a moderate to dense accumulation of receptors (Fig. 6 C).

Diencephalon

CGRP receptor accumulations were noted in the preoptic, thalamic and hypothalamic areas. The medial preoptic nucleus contained a moderate receptor density (Fig. 6 B). In the hypothalamus the anterior, periventricular and suprachiasmatic nuclei, the paraventricular, arcuate and dorsomedial nuclei as well as the zona incerta contained a high density of CGRP receptors whereas the ventromedial nucleus and the lateral hypothalamic and medial forebrain bundle area contained low numbers of receptors (Figs. 6 C,D, 7). The posterior arcuate nucleus was very dense in receptors (Fig. 7 A); the posterior hypothalamic nucleus showed a moderate density of receptors.

The lateral thalamic nucleus, the thalamic periventricular nucleus, the pretectalis, rhomboid and reuniens nuclei contained dense accumulations of CGRP receptors (Fig. 7) as did the habenula (Fig. 7 D). The ventrolateral thalamus showed a medium density of CGRP receptors (Fig. 7). The anterior part of the central gray contained moderate receptors whereas in its dorsal aspects the fibrae periventriculares thalami were dense in CGRP receptors (Fig. 8 A). The medial mamillary body contained sparse receptors (Fig. 8 B).

Mesencephalon

The mesencephalon contained the highest density of CGRP receptors in the superficial layer of the superior colliculus (stratum griseum superficiale colliculi superioris; Fig. 8 B,C). The deeper layers, the central gray and the reticular part of the substantia nigra contained a moderate number of CGRP receptors (Fig. 8 B,C). The central nucleus of the geniculate body was found to be very dense in CGRP receptors as was the dorsal raphe (Fig. 8 B,C). More caudally the inferior colliculus, the nucleus of the fifth nerve, the principal and dorsal nucleus of the fifth nerve, the locus coeruleus, the nucleus tractus mesencephali, the nucleus tegmenti dorsalis and its lateral part contained dense receptor accumulations (Figs. 8 D, 9 A). The superior olive and the parvocellular reticular nucleus contained a moderate to dense number of binding sites (Fig. 9 A).

Cerebellum

Dense accumulations of CGRP receptors were located in the major portion of the molecular layer of the cerebellum. Low to moderate densities of receptors were found in the Purkinje cell layers (Figs. 8 D, 9 A–C).

Medulla Oblongata

The inferior olive, the nucleus tractus solitarii, the nucleus commissuralis, the nuclei of the tenth and twelfth nerves, the prepositus hypoglossal and the dorsal aspects of the gracilis nucleus contained dense accumulations of CGRP receptors (Fig. 9 B,C). The nucleus of the spinal trigeminal tract and the parvocellular reticular nucleus contained a moderate number of receptors (Fig. 9 A–C). The dorsomedial portion of the spinal trigeminal tract (Fig. 9 A) contained dense binding sites.

Spinal Cord

The dorsal gray matter of the spinal cord (laminae I–VI) and the confines of the central canal showed a dense receptor accumulation. Moderate receptors were found in the gracile fasciculus (Fig. 9 D). The ventral horn (laminae VII–IX) showed low to moderate receptors.

DISCUSSION

Saturable receptor binding with high affinity, HPLC-analysis of the incubation solution and the striking similarity of the distribution of receptors in some discrete areas with previously reported CGRP-like immunoreactivity [27,31], indicates that in fact we showed the distribution of authentic CGRP receptors rather than that of breakdown products of the ligand. Our results are also in agreement with previous membrane binding studies which showed binding sites in the cortex, cerebellum, midbrain, pons medula, hypothalamus and spinal cord of the rat [5]. Recently CGRP receptors were also shown in the human cerebellum and spinal cord using autoradiography, in addition to membrane binding from a variety of brain areas [35].

A comparison of the distribution of CGRP binding sites to the localization of CGRF fibers and perikarya revealed that most of the areas that contained CGRP positive nerve fibers also contained appreciable binding sites with the exception of the tractus of the spinal trigeminal nerve which contained a low density of binding sites. This is understandable in view of the fact that axons would not be expected to contain receptors to the peptide contained within these tracts. Two prominent regions that showed a striking lack of concordance between CGRP nerves and peptide binding were the cerebellum and superior colliculus which contained no visible nerve fibers but very dense receptor binding. Likewise the dorsal portion of the hippocampus-dentate gyrus contained only rare fibers, while binding was observed in the pyramidal layer of Ammons horn and the granular layer of the dentate gyrus. However, very low amounts of CGRP

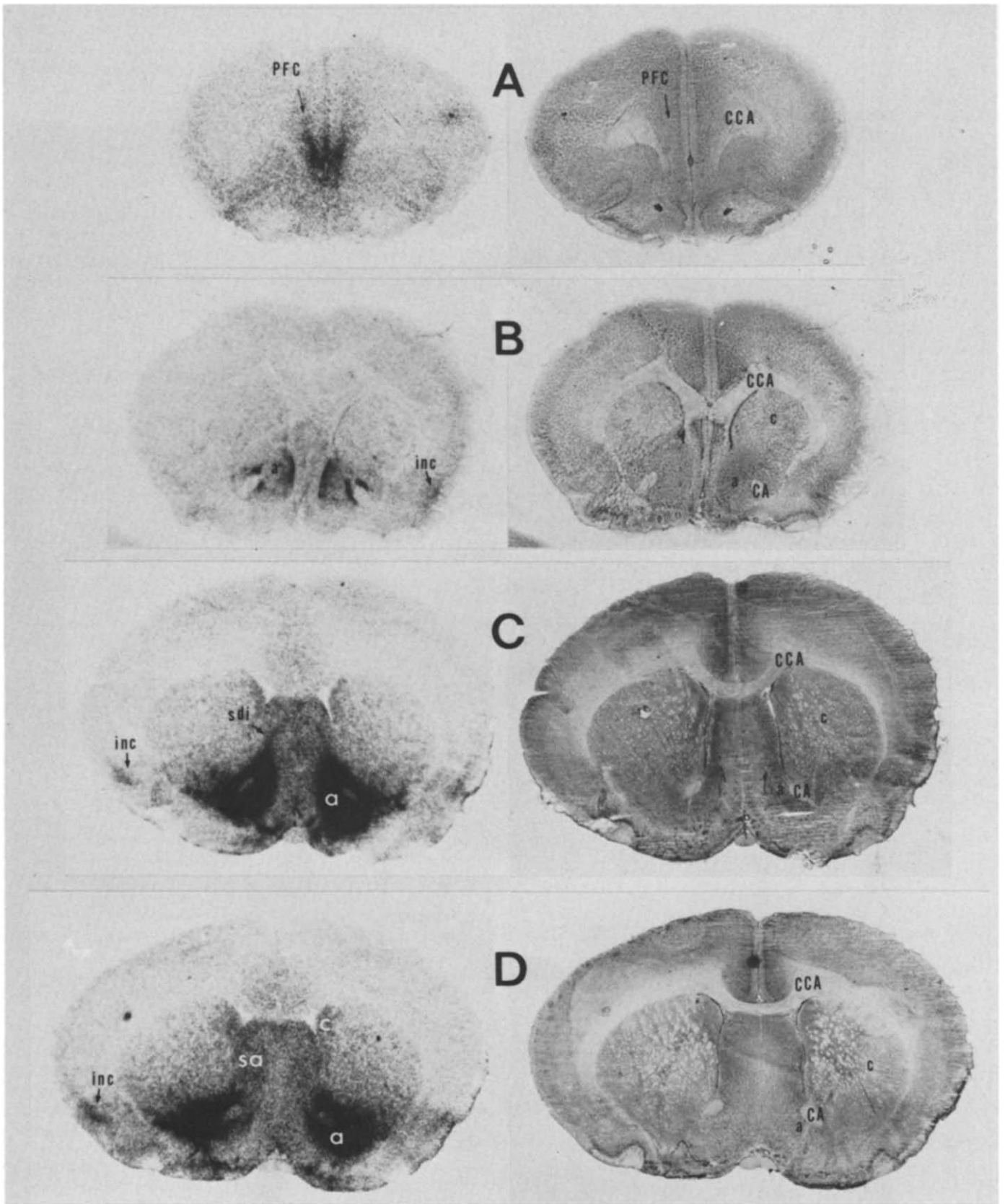

FIG. 5. (A) A10300, (B) A9200, (C) A8620, (D) A8380.

could be measured by RIA in the hippocampus. Regions such as the nucleus accumbens, preoptic medial nucleus and amygdala lateralis which contain only trace amounts of immunoreactive fibers and very low concentrations of CGRP [32], had very dense receptor binding sites. Generally, with few exceptions, there is a relationship between the distribution of CGRP fibers and CGRP receptor binding sites.

Discrepancies of the distribution of peptide receptors and immunoreactivity are a commonly known phenomenon [29,38]. The reasons for the lack of concordance between receptors and immunoreactive fibers may be due to one or more factors: (a) the receptor could function where the peptide concentration is low but the receptor concentration is high due to increased synaptic efficiency as suggested by Shultz *et al.* [29]; (b) there may be several receptor types, some of which may not recognize the iodinated ligand. Iodination may slightly distort the molecule which subsequently cannot bind to certain receptor types; (c) nerve fibers may actually be present but not visualized by immunocytochemical procedures. It may be possible that there is a population of very fine fibers with peptides not firmly bound so that the peptide is released when the anesthetized animal is perfused with paraformaldehyde solution (immunohistochemistry) or physiological saline (receptor autoradiography). Such a labile terminal field of fibers might also release peptides following decapitation of the awake animal thereby resulting in low concentrations of the peptide measured in discrete areas; (d) receptor areas with little or no peptidergic fibers (e.g., cerebellum, superior colliculus, nucleus accumbens) may be responsive to circulating peptides which eminate from the periphery (and pass through the blood-brain barrier), or from diffusion of peptide from adjacent innervated areas. For example, in the present study, dense receptor binding was observed in the lateral amygdaloid nucleus which is in close proximity to the densely innervated central amygdaloid nucleus.

In the present study there appears to be a direct rostrocaudal continuity in the receptor localization of the nucleus accumbens with the fundus striatum—ventral pallidum and caudal caudate-putamen, amygdala centralis and lateralis. It is interesting to speculate that this receptor complex may constitute a "functional unit," although it is not an anatomically defined unit. Functionally the nucleus accumbens and the caudate-putamen are concerned with motor activity [8,23] and the amygdala has been linked with somatic-endocrine function [11,34]. It is also interesting that the mid-portion of this "functional unit" is well innervated with CGRP-containing fibers [31], while the rostral and caudal regions (nucleus accumbens and amygdala lateralis, respectively) contain sparse or no fibers.

Interestingly an equivalent competition of salmon- (but not human-) calcitonin and CGRP on CNS receptors was reported [5,35]. Furthermore, it was suggested that CGRP might be an endogenous ligand for calcitonin receptors in the central nervous system and concluded that calcitonin and CGRP may interact on the same receptor site. A detailed comparison of previously published autoradiographic studies of calcitonin-binding sites [7,20] could not be made. However, similarities of localization of binding sites in large areas could be seen. Calcitonin and CGRP receptor sites were observed in the ventral portion of the caudate nucleus, the nucleus accumbens, the preoptic, hypothalamic, medial amygdala, mesencephalic and metencephalic areas. Major differences are seen in all cortical areas, thalamus and cerebellum where we found dense CGRP-binding but no calcitonin-binding was observed [7,20]. The central function of CGRP and its receptors is unknown at present. Some evidence for a central action of CGRP originates from the finding that after intracerebral administration of CGRP gastric acid secretion is inhibited [15,16].

Recently CGRP was shown to coexist with substance P in sensory neurons of the spinal trigeminal area, the trigeminal ganglion, the superficial layers of the spinal cord and the dorsal root ganglia [4, 14, 30, 37]. Furthermore it was demonstrated that both substance P and CGRP [25,30] are sensitive to capsaicin treatment which causes selective degeneration of a population of primary sensory neurons [9, 10, 28]. This implies that CGRP, as well as substance P, might be involved in the transformation or modulation of peripheral nociceptive messages as well as in cardiovascular reflexes. Involvement of CGRP in central blood pressure control is supported by the observation that CGRP receptors and nerves are present in the anteroventral third ventricle area (AV3V) which seems to be important for the development of central hypertension [3,6].

From a functional standpoint it is noteworthy that receptor binding sites were also observed in the auditory system [superior olive, inferior colliculus, medial geniculate body, nucleus of the lateral lemniscus and a small portion of the temporal lobe cortex (auditory cortex)], the somesthetic system (dorsal horn of the spinal cord, gracilus and cuneate nuclei), the cerebellar afferent pathway (via the inferior olive to the cerebellum), the gustatory pathway [nucleus tractus solitarius, nucleus parabrachialis (pontine taste area), amygdala, medioventral posterior nucleus of the thalamus and the insular cortex], and efferent pathways from the cortex transmitting signals through the trigeminal sensory nucleus, nucleus tractus solitarius, reticular formation, and superior colliculus.

The significance of CGRP receptors in multisynaptic pathways is not clear. The binding technology as used in this study does not reveal detailed receptor localization, i.e., cell body and/or neuronal (or glial) fibers. Therefore information as to actual pathway localization of receptor sites is not available. Lesion studies of various pathways should clarify this point.

ACKNOWLEDGEMENTS

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FACING AND FOLLOWING PAGES

FIGS. 5–9. Distribution of CGRP receptors in rat brain. Autoradiographs were produced by apposing coronal 20 μm sections previously incubated in 0.69 nM ^{125}I-Try°-rat CGRP for 90 min, against Kodak X-OmAT XAR-5 film for 4–7 days (left panels). After exposing the slides were stained with thionin for histological examination (right panels). Approximate coordinates are given anterior or posterior to the intraaural plane according to König and Klippel [12] and Skofitsch and Jacobowitz [27]. The atlas of Skofitsch and Jacobowitz provides the detailed immunocytochemical localization of CGRP-like immunoreactive neurons.

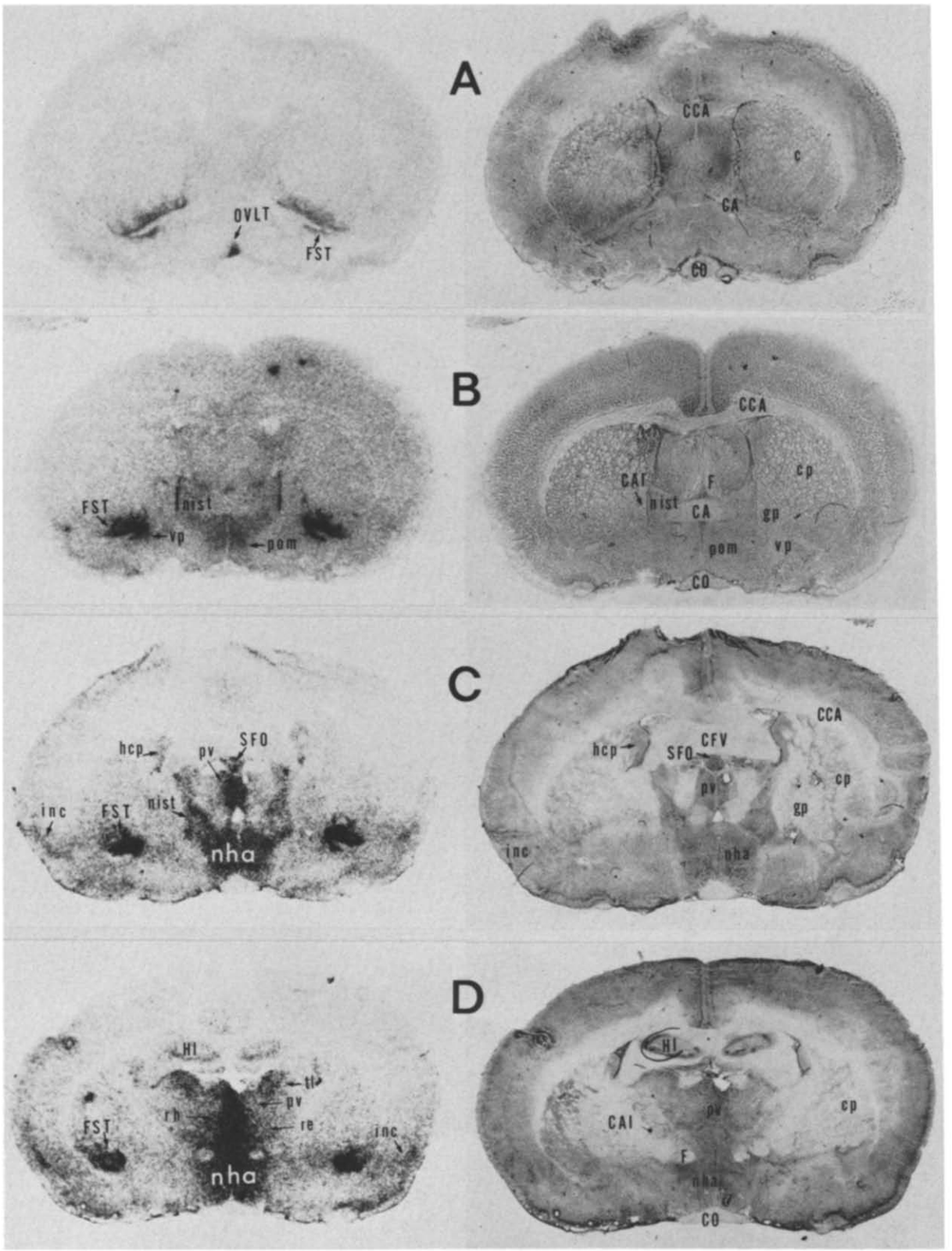

FIG. 6. (A) A7500, (B) A6860, (C) A6400-A6300, (D) A6200-A5150.

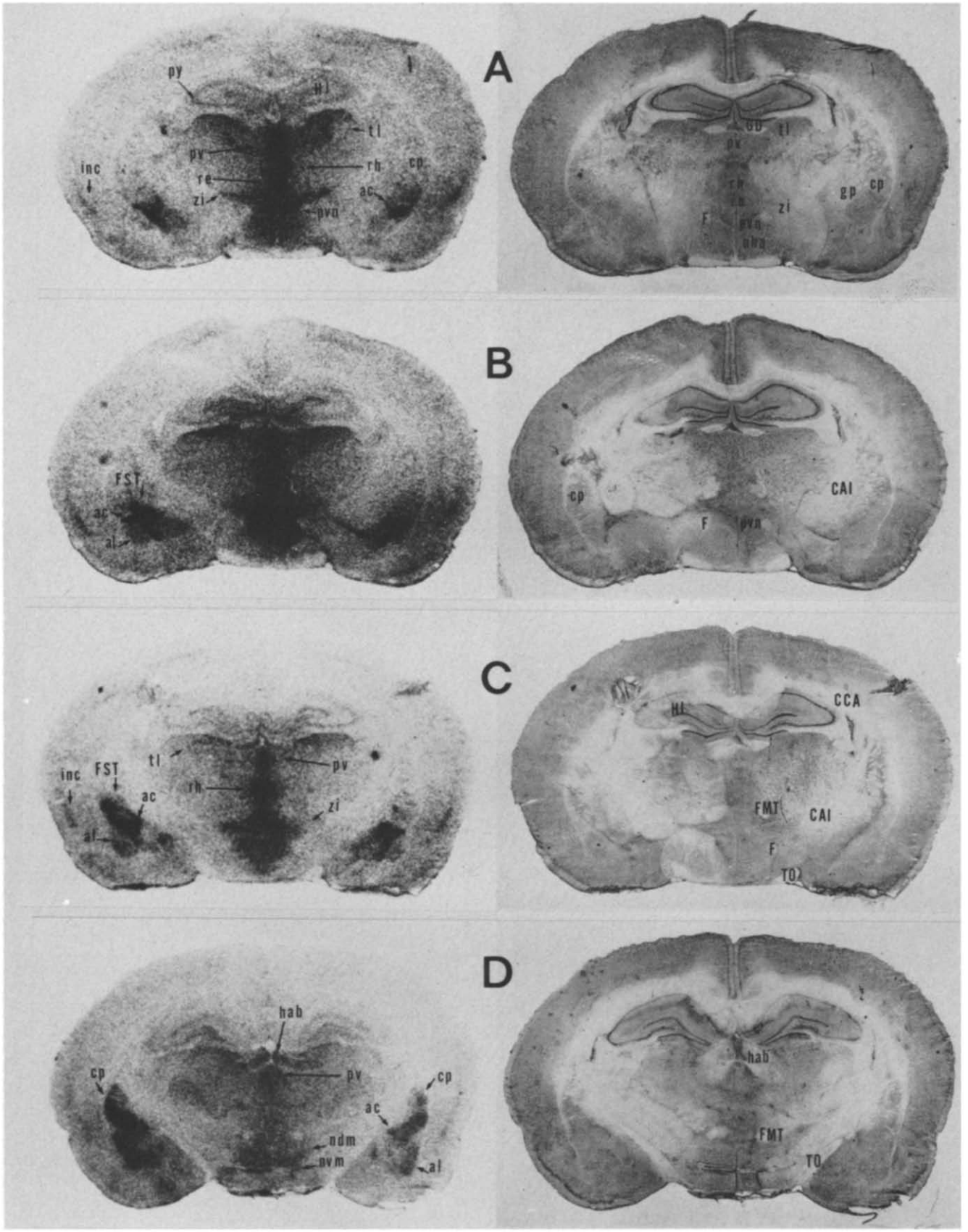

FIG. 7. (A) A5700-A5100, (B) A5600-A4900, (C) A5300-A4600, (D) A4380-A4110.

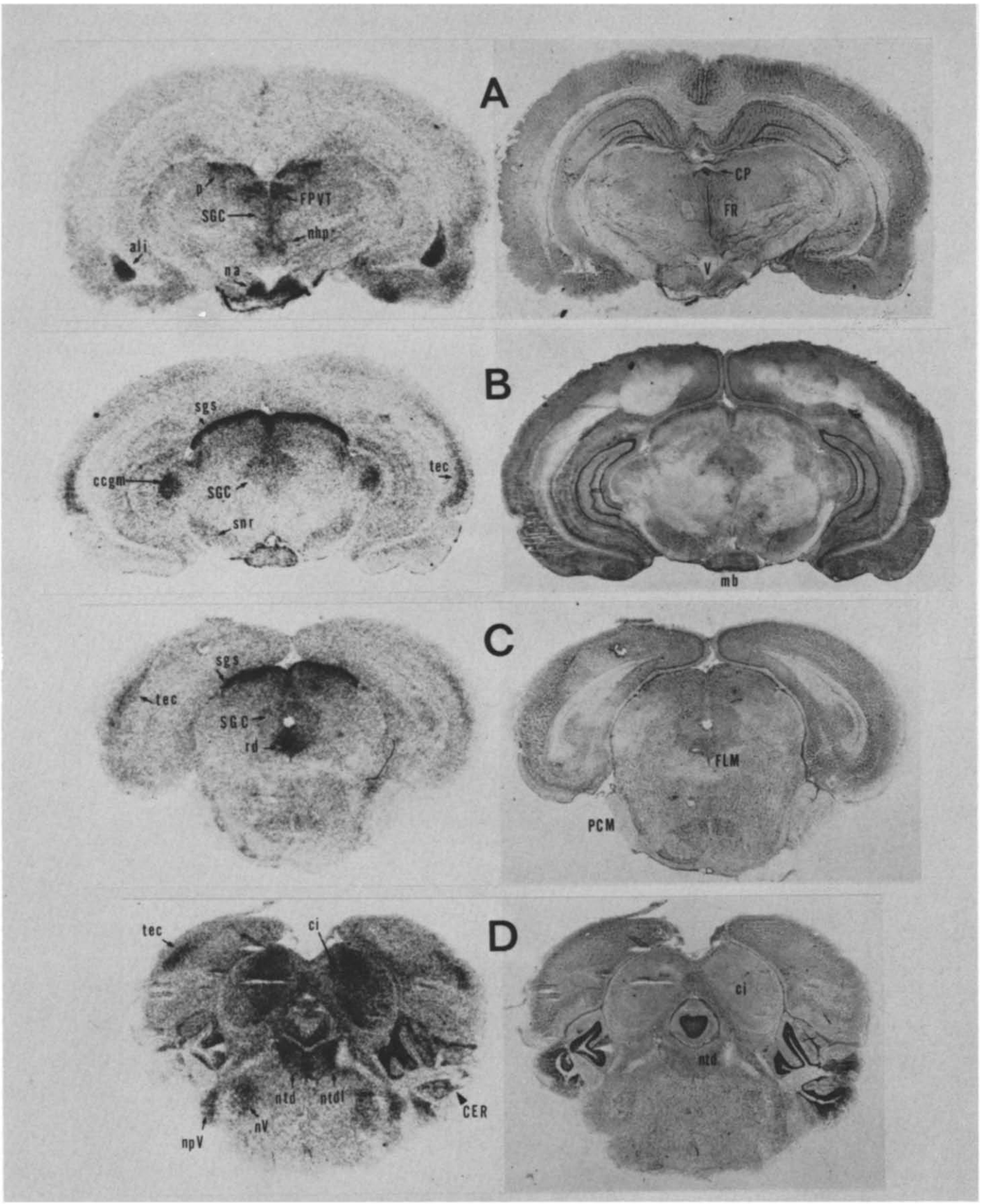

FIG. 8. (A) A3200-A3000, (B) A2200-A2000, (C) A500, (D) P1500-P2000.

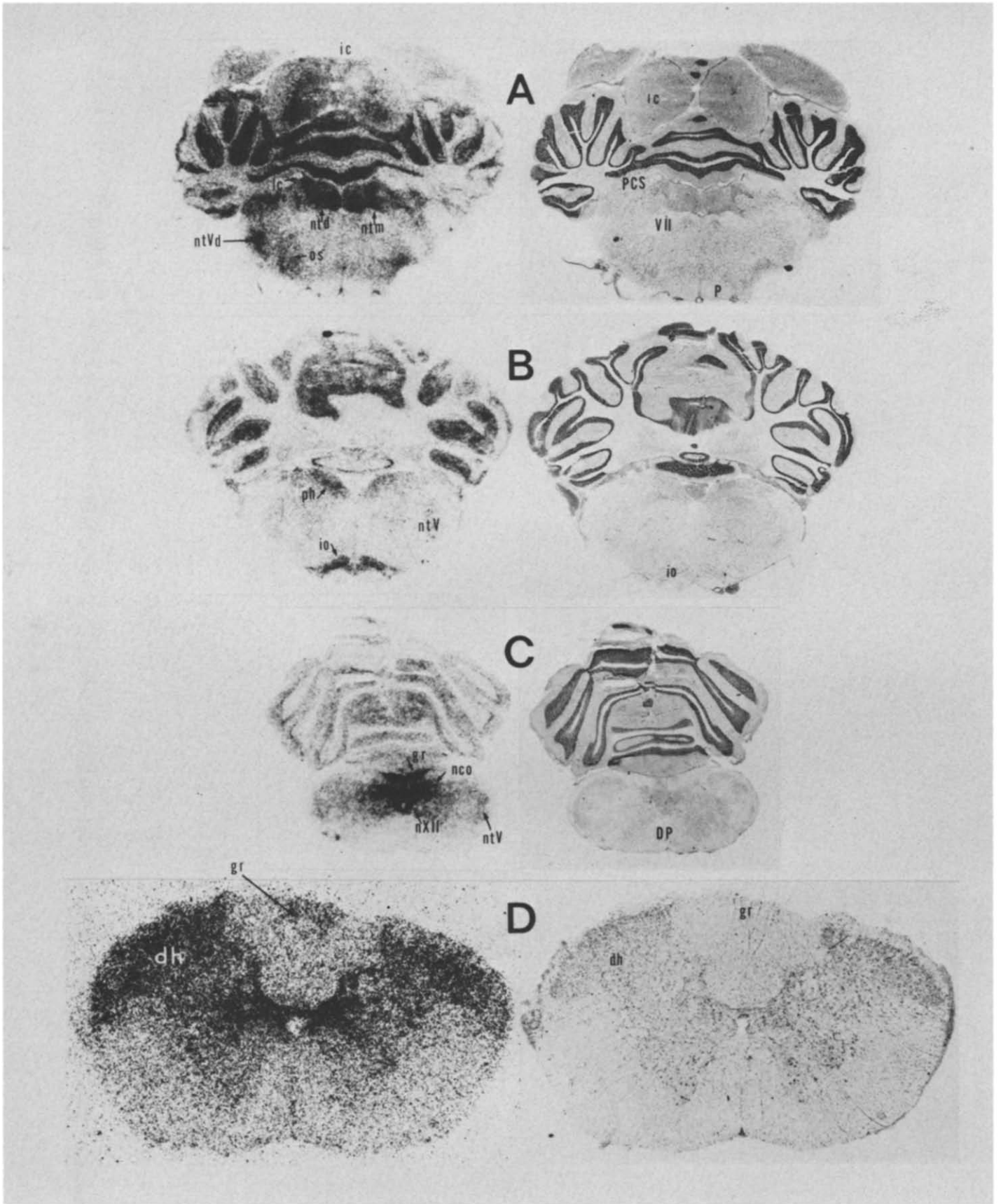

FIG. 9. (A) P1800-P3400, (B) P4400-P6500, (C) P7200, (D) spinal cord level C1.

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