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Autoradiographic Studies With a Behaviorally Potent ^{3}H-ACTH$_{4-9}$ Analog in the Brain After Intraventricular Injection in Rats[1]

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REES, H. D., J. VERHOEF, A. WITTER, W. H. GISPEN AND D. DE WIED. *Autoradiographic studies with a behaviorally potent ^{3}H-ACTH $_{4-9}$ analog in the brain after intraventricular injection in rats.* BRAIN RES. BULL. 5(5) 509–514, 1980.—Autoradiographic studies aimed at identifying target cells in the brain for ACTH-like peptides were performed using (^{3}H-7-Phe)-4-Met(O_2),8-D-Lys, 9-Phe-ACTH$_{4-9}$, a behaviorally potent analog of ACTH$_{4-9}$. The ^{3}H-peptide was injected into the lateral ventricle of hypophysectomized rats that were sacrificed 5, 30, 60, 180, and 240 min later. Dry-mount autoradiograms of brain showed the highest density of silver grains in the ventricular lumen and choroid plexus. In addition, radioactivity penetrated brain tissue as far as 1000 μm from the ventricles, and was distributed predominantly over neuropil. Within 5 min after the injection, an intracellular concentration of radioactivity above background levels was observed in a small proportion of cells near the ventricles in the septum, caudate-putamen, preoptic area, hypothalamus, thalamus, amygdala, and hippocampus. The cellular labeling decreased in intensity at greater distances from the injection site and at longer survival intervals, and was no longer evident 4 hr after the injection. The labeled cells were usually small, dark, and often elongated, suggesting that ACTH peptides may act preferentially upon a morphologically distinct class of cells in the brain.

ACTH$_{4-9}$ Autoradiography Brain

PEPTIDES related to ACTH (adrenocorticotropic hormone) are known to act directly on the brain to influence the acquisition and maintenance of learned behaviors [3]. While virtually devoid of adrenal steroidogenic activity, a synthetic analog of ACTH$_{4-9}$ having three substitutions, 4-Met (O_2),8-D-Lys,9-Phe-ACTH$_{4-9}$ (Org 2766), was found to be 1000 times more potent than ACTH$_{4-10}$ in delaying the extinction of conditioned pole-jump avoidance responding [5], and to be orally active in reversing CO_2-induced amnesia [11]. This behaviorally potent analog was radioactively labeled with tritium for studies of its metabolic fate and distribution in the brain. Following intravenous, subcutaneous, or oral administration, the estimated concentration of intact peptide in the brain was on the order of 10^{-5} times the administered dose [14]. Although systemic administration resulted in rapid degradation and low brain uptake of the peptide, intraventricular administration produced relatively high levels of intact peptide in the brain [13]. Therefore the latter route was used for central distribution studies. These studies demonstrated that the septal area had the highest uptake of the ^{3}H-ACTH analog and that only in this area could the uptake be competitively displaced by pretreatment with peptides structurally and behaviorally related to the ACTH$_{4-9}$ analog [15].

To obtain higher resolution information about the distribution of ACTH$_{4-9}$ peptide uptake and binding sites in the brain, the present studies were performed using the dry-mount procedure [12] for the autoradiographic localization of diffusable substances.

METHOD

Animals and Surgery

Male Wistar albino rats, weighing 154–250 g, were obtained from TNO (Zeist, The Netherlands). Hypophysectomy was performed by the transauricular route, under light ether anesthesia. The effectiveness of surgery was verified at the end of the experiments by macroscopic inspection of the sella turcica.

Materials

(^{3}H-7-Phe)-4-Met(O_2),8-D-Lys,9-Phe-ACTH$_{4-9}$ (batch ^{3}H-OAIII, specific activity 24 mCi/μmol) was obtained from Organon B.V. (Oss, The Netherlands).

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Intraventricular Injection

Thirty min before the intracerebral injection, rats were anesthetized by intraperitoneal injection of 125 mg/100 g of urethane. Polypropylene cannulae (PP25) with inner diameter of 0.4 mm were attached to a propylene syringe and filled with artificial CSF, a small air bubble, and, at the tip, approximately 2 μl of artificial CSF containing 3.4 μCi of ^{3}H-ACTH$_{4-9}$ analog (equivalent to 0.11 μg peptide). To inject into the lateral ventricle, a hole was drilled in the skull 1.5 mm from the sagittal suture and 0 mm from bregma. Through this hole the cannula was lowered to a depth of 4.5 mm from the top of the skull, and fixed to the skull with dental acrylic anchored to screws. The ^{3}H-ACTH$_{4-9}$ analog was injected over a period of 10 sec and was followed by the injection of 2–3 μl of artificial CSF from the same cannula. At the end of the survival interval, the rat was killed by decapitation.

Survival intervals. Five days after hypophysectomy, rats were killed at the following times after the injection of the ^{3}H-ACTH analog: 5 min (n=2), 30 min (n=1), 1 hr (n=1), 3 hr (n=1), and 4 hr (n=1).

Pretreatment with ACTH$_{4-10}$. Beginning the day after hypophysectomy, rats were injected subcutaneously every other day for 3 weeks with either zinc phosphate vehicle (n=1) or 10 μg/100 g of ACTH$_{4-10}$ (n=1). The final treatment was given 2 hr before the intraventricular injection of the ^{3}H-ACTH$_{4-9}$ analog, and the rats were killed 30 min later.

Non-hypophysectomized rats. Experiments with intact rats were performed using a survival time of 5 min (n=2). To suppress pituitary secretion of ACTH, another rat was pretreated with dexamethasone (30 μg/100 g, SC), 4 hr before intraventricular injection of the ^{3}H-ACTH$_{4-9}$ analog, and killed 30 min later.

Autoradiographic Procedures

The brains were quickly removed, placed on tissue mounts, frozen in liquified propane at −180°C, and stored in liquid nitrogen until sectioning at 4 μm in a cryostat. Dry-mount autoradiograms were prepared by freeze-drying the frozen sections overnight, and mounting them on emulsion-coated (Kodak NTB 3) slides using pressure against a Teflon support. Some sections were thaw-mounted in the cryostat by being picked up under safelight conditions onto emulsion-coated slides at room temperature. These procedures have been described in detail by Stumpf and Sar [12]. After exposure for 2–4 weeks in dessicated boxes at −80°C, autoradiograms were developed in Kodak D-19 (1:1 dilution, 15°C) for 4 min, fixed, rinsed, stained with methyl green-pyronin (which stain DNA and RNA, respectively) or with toluidine blue (which stains RNA), air dried, and coverslipped.

RESULTS

Time Course

Five minutes. The density of silver grains was highest in the choroid plexus and lumen of the lateral, third, and fourth ventricles, and was relatively low in the ependymal cell layer. Grain density was high in brain tissue adjacent to the ventricles, where the grains were located primarily in the neuropil. In the portion of the septal area closest to the injection site, the perikarya of most large neurons were relatively free of radioactivity, while a very small percentage of cells exhibited an intracellular concentration of radioactivity up to 7 times higher than that of the surrounding neuropil. All of the results to be described here were obtained using the dry-mount procedure; cellular labeling above neuropil background was not observed in the thaw-mounted autoradiograms. Labeled cells were observed in the cerebral cortex near the cannula track (Fig. 1d), and near the lateral ventricle in the dorsal, lateral, triangular, and fimbrial septal nuclei (n.) both ipsilateral and contralateral to the injection (Fig. 1 a,c), and in the ipsilateral caudate-putamen (Fig. 1b), bed n. of the stria terminalis, medial and basolateral n. of the amygdala, and hippocampus. Labeled cells were also observed near the third ventricle, in the preoptic area (medial, suprachiasmatic and periventricular n.), and hypothalamus (periventricular n., anterior n., suprachiasmatic n., parvocellular and magnocellular paraventricular n., pars centralis of the ventromedial n., pars ventralis of the dorsomedial n., arcuate n., ventral and dorsal premamillary n., and medial mamillary n.), and in the periventricular n. of the thalamus. In all of these areas, the labeled cells were usually small (10–15 μm in length), often elongated, and had abundant cytoplasm that stained darkly with methyl green-pyronin or toluidine blue, indicating a relatively high content of Nissl substance. Silver grains appeared to be located over both the cytoplasm and nucleus. In addition to the above nuclei, the following fiber areas near the injection site or ventricles also contained labeled cells: superior fornix, fornix, commissure of the ventral fornix, fimbria of the hippocampus, and corpus callosum. These cells were presumably oligodendroglia, because of their location, small size, paucity of cytoplasm, and frequent arrangement in rows.

Thirty minutes. The distribution of labeled cells was similar to that seen after 5 min, with additional labeling of darkly stained cells in the n. of the diagonal band, and medial habenular n., and more extensive labeling of the ventral hippocampus, dentate gyrus, and overlying cerebral cortex near the lateral ventricle. In general, however, the intensity of cellular concentration of radioactivity was lower than that at five min (Figs. 1e, f and 2a–c).

One hour. A few small darkly stained cells were very weakly labeled in the dorsal and lateral septal nuclei, the preoptic area, and medial habenula. In the brain stem, large darkly stained neurons in the lateral cuneate n. near the fourth ventricle, and in the lateral reticular n. were occasionally labeled. In the cerebellum, the silver grain density was higher in the molecular than in the granular layer, and some Purkinje cells were labeled (Fig. 2d).

Three hours. The choroid plexus continued to exhibit heavy labeling, and, unlike earlier, radioactivity was concentrated in the ependyma of the lateral ventricle. In the lateral septum, caudate-putamen, medial habenula, arcuate n., and periventricular thalamic n., numerous cells, including both large and small neurons, were very weakly labeled. There was also weak labeling of glial cells in the fimbria of the hippocampus, commissure of the ventral fornix, and corpus callosum near the lateral ventricle ipsilateral to the injection. Cerebellar Purkinje cells and medium and large neurons in the lateral reticular n. were also weakly labeled.

Four hours. Silver grains were distributed diffusely in brain tissue near the ventricles but no cellular concentration of radioactivity was observed, except in cells of the choroid plexus and ependyma.

Pretreatment with ACTH$_{4-10}$

The pattern of autoradiographic labeling in the vehicle-injected 3-week hypophysectomized rat was similar to that described for the non-pretreated 5-day hypophysectomized

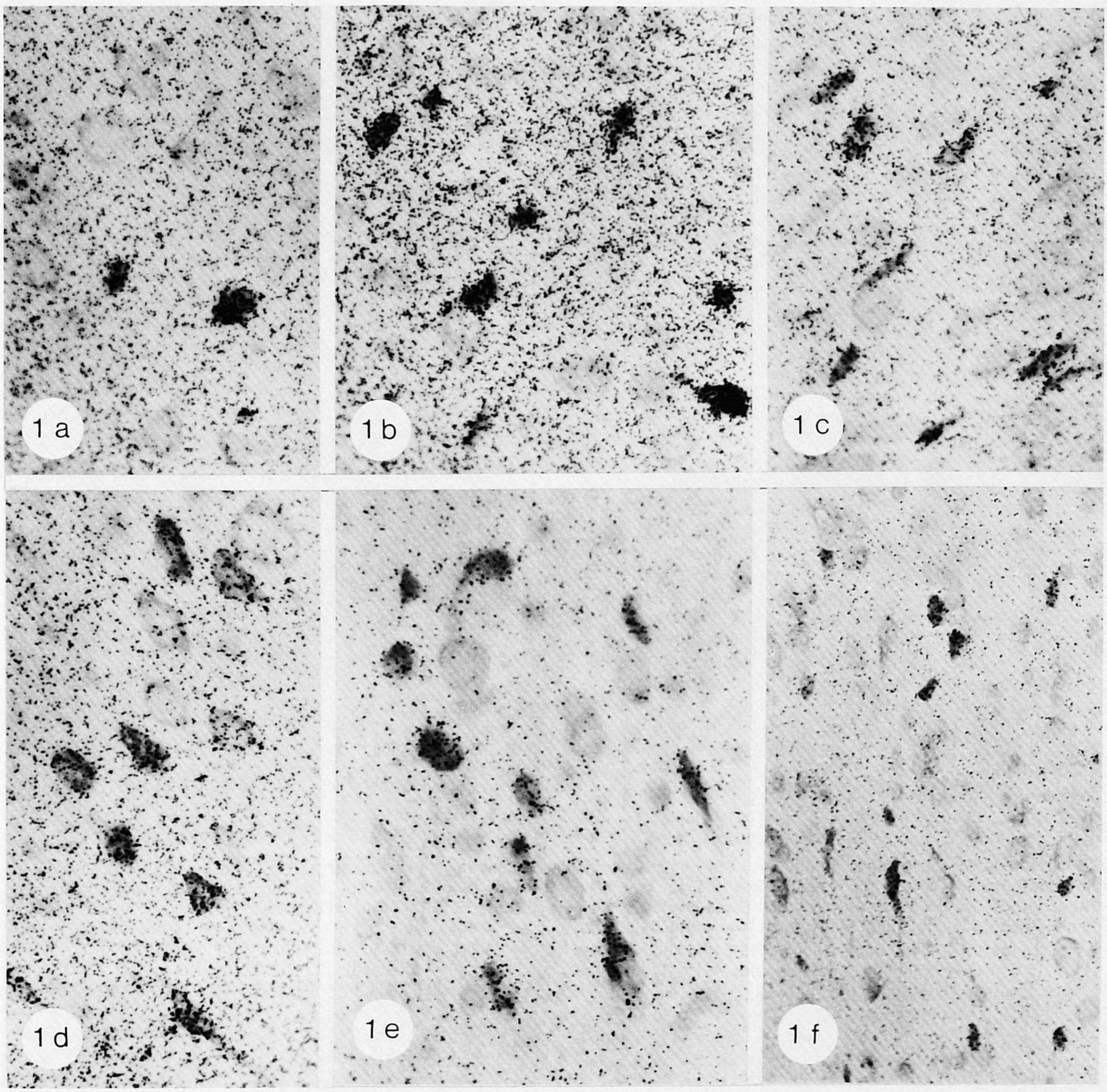

FIG. 1. Autoradiograms prepared 5 min (a–d) and 30 min (e–f) after intraventricular injection of the ^{3}H-ACTH$_{4-9}$ analog in hypophysectomized rats, showing cellular concentration of radioactivity in the ipsilateral dorsal septal n. (a), ipsilateral caudate-putamen (b), contralateral dorsal septal n. (c), cerebral cortex near the injection cannula tract (d), traingular septal n. (e), and horizontal limb of the n. of the diagonal band (f). Exposure time 28 days (a–d) and 14 days (e,f). Magnification ×615 (a–e), and ×385 (f).

rat killed after 30 min. In the rat pretreated with ACTH$_{4-10}$, the frequency and intensity of cellular labeling appeared to be slightly reduced, but quantitative comparisons were not possible. The density of silver grains was high in the choroid plexus, and weakly labeled cells, which were mostly small and darkly stained, were observed in the dorsal and triangular septal n. and caudate-putamen ipsilateral to the injection, n. of the diagonal band, medial and suprachiasmatic preoptic n., bed n. of the stria terminalis, hippocampus and dentate gyrus ipsilateral to the injection, cerebral cortex near the cannula track, and periaqueductal gray. Glia in the fimbria of the hippocampus were also labeled.

Non-hypophysectomized Rats

In rats killed 5 min after the injection of the ^{3}H-ACTH$_{4-9}$ analog, labeled cells were observed in the choroid plexus,

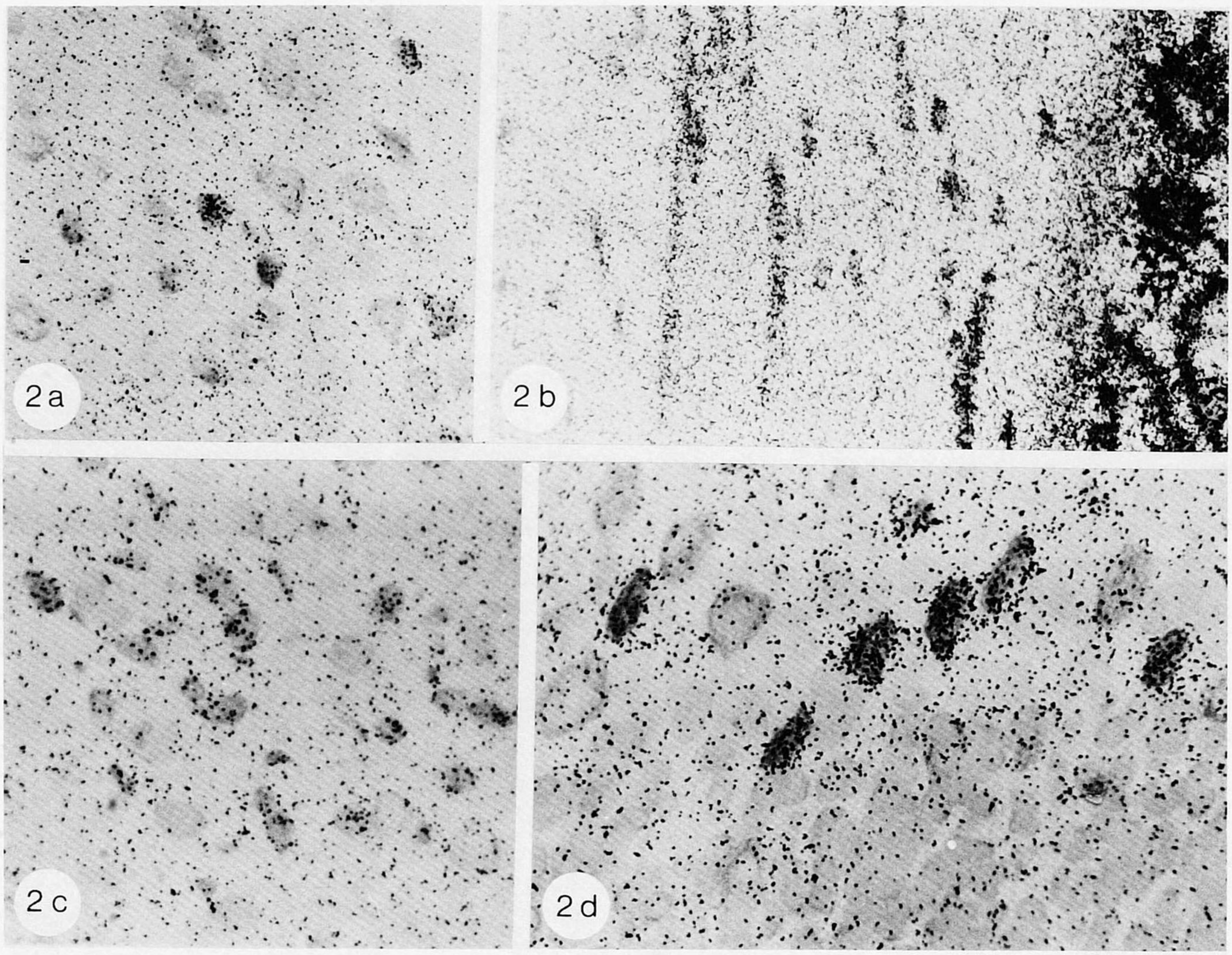

FIG. 2. Autoradiograms prepared 30 min (a–c) and 1 hr (d) after intraventricular injection of the ^{3}H-ACTH$_{4-9}$ analog in hypophysectomized rats, showing cellular concentration of radioactivity in the medial amygdaloid n. (a), choroid plexus of the lateral ventricle and oligodendroglia of the hippocampal fimbria (b), arcuate n. (c), and cerebellar Purkinje cells (d). Exposure time 28 days (a,b), 14 days (c), and 30 days (d). Magnification ×385 (a,b) and ×615 (c,d).

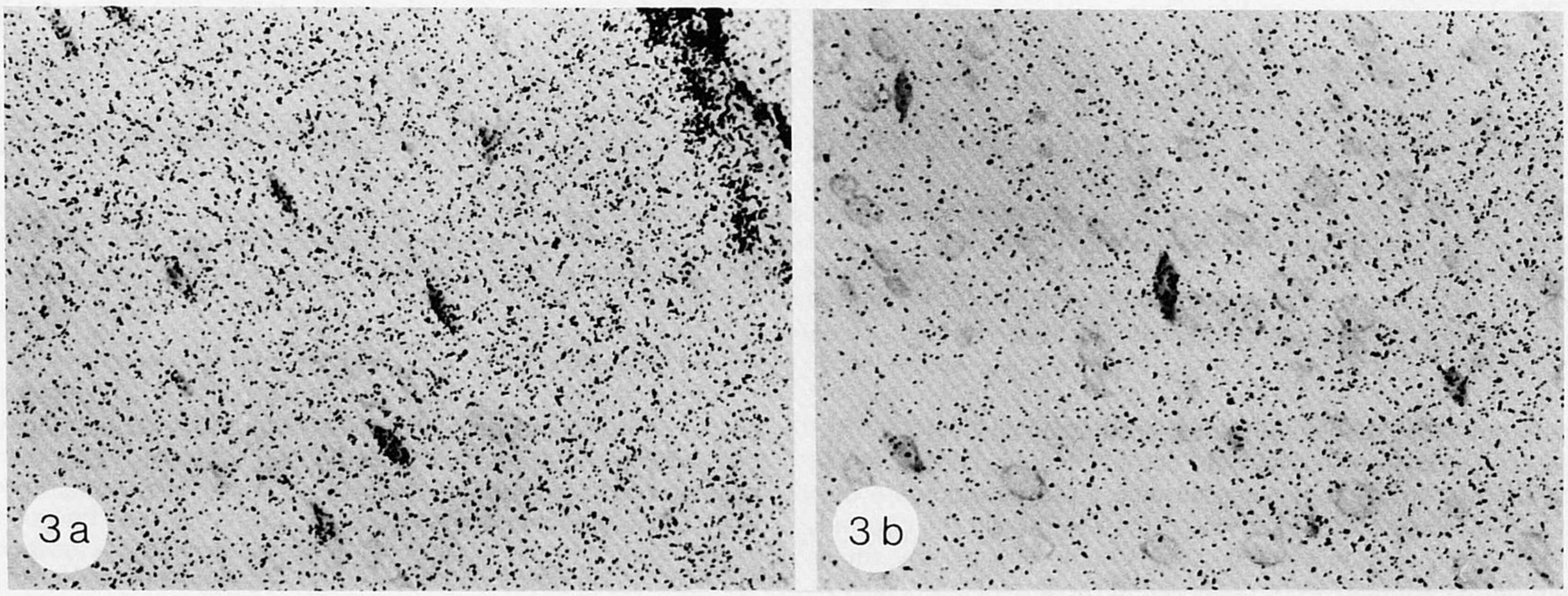

FIG. 3. Autroradiogram prepared 5 min after intraventricular injection of the ^{3}H-ACTH$_{4-9}$ analog in non-hypophysectomized rats, showing cellular concentration of radioactivity in (a) the contralateral dorsal septal n., with the lateral ventricle in the upper right corner, and (b) the ipsilateral caudate-putamen. Exposure time 14 days (a) and 28 days (b). Magnification ×385.

lateral septal n. (Fig. 3a), caudate-putamen (Fig. 3b), bed n. of the stria terminalis, medial and suprachiasmatic preoptic n., and cerebral cortex near the cannula track. The intensity of labeling in intact rats appeared to be somewhat less than that in 5-day hypophysectomized rats. The pattern of labeling in the dexamethasone-treated intact rat after a survival interval of 30 min was similar to that in the hypophysectomized rat after the same interval.

DISCUSSION

After injection of the ^{3}H-ACTH$_{4-9}$ analog into the lateral ventricle, radioactivity penetrated the brain to a maximum of about 1000 μm from the ventricular system, where it was distributed diffusely in the neuropil. Using light microscopic autoradiography it was not possible to distinguish whether the radioactivity in the neuropil was in extracellular fluid or associated with cellular processes. A small proportion of the autoradiographic silver grains were concentrated intracellularly suggesting internalization of the ACTH$_{4-9}$ peptide or its radioactive metabolites. Labeled cells were observed in regions near the ventricles in the septal area, caudate-putamen, preoptic area, hypothalamus, thalamus, hippocampus, amygdala, periaqueductal gray, and brain stem. The cellular labeling was dependent upon the extent of penetration of the peptide into brain tissue; it was most intense in areas closest to the injection site. Strong cellular labeling was evident at five min after the injection, and was no longer detectable four hr after the injection.

Data are available regarding the metabolism of the ^{3}H-ACTH$_{4-9}$ analog. Its half life was 38 min when incubated *in vitro* with rat brain extracts [16]. Verhoef, Palkovits and Witter [13] found that two hr after intraventricular injection of the ^{3}H-ACTH$_{4-9}$ analog, the major tritiated metabolite in brain, accounting for 34% of the recovered radioactivity, was tritiated water (which was removed from our tissue sections by lyophilization prior to autoradiographic exposure). The intact peptide contributed 40% of the remaining radioactivity, and the rest was distributed over at least four metabolites. Therefore, after the 5- and 30-min survival intervals used in the present experiments, the intact peptide may be expected to account for considerably more than 40% of the total visualized radioactivity. The chemical nature of the radioactivity autoradiographically localized in cell bodies may have been different from that of the total tissue radioactivity, of which it constituted a very small fraction.

It is unclear why a concentration of silver grains over some cells was observed in brain sections dry-mounted on photographic emulsion, but not in adjacent or nearby sections that were thaw-mounted. It is highly unlikely that the silver grains over cells were produced by pressure exerted against the emulsion during dry-mounting, because the intensity of the cellular labeling was a function of distance from the injection site and of exposure time. It is possible that radioactive material diffused out of cells during the thawing of the thaw-mounted sections. Alternatively, tritiated water may have been incompletely removed from the thaw-mounted sections, thereby elevating the amount of background radioactivity.

Whereas biochemical studies with the intraventricularly administered ^{3}H-ACTH$_{4-9}$ analog have measured the amount of radioactivity in whole tissue samples of various brain regions [13,15], the focus of the present autoradiographic study was the localization of cellular rather than whole tissue radioactivity. Nevertheless, some comparisons may be drawn between results from these two approaches. Autoradiographically labeled cells were found in most of the brain nuclei in which Verhoef, Palkovits, and Witter [13] reported a high uptake of radioactivity, although labeled cells were also found in a few areas reported to have low uptake (caudate-putamen, n. of the diagonal band, cerebellar cortex). Verhoef, Witter and de Wied [15] showed that hypophysectomy caused an increase in the amount of lyophylized radioactivity in many brain regions 2 hr after intraventricular injection of the ^{3}H-ACTH$_{4-9}$ analog, which was statistically significant in the septal area (26%) and preoptic area (30%). Furthermore, long-term substitution therapy of hypophysectomized rats with ACTH$_{4-10}$ (20 μg/rat every other day for 3 weeks) significantly decreased the uptake of radioactivity in the septum and medulla-pons by 29% and in the cerebellum by 23%, suggesting that 20–30% of the non-water radioactivity in these areas was specifically bound. However, the present autoradiographic study showed a persistence of a pattern of cellular labeling qualitatively similar to that in hypophysectomized rats despite normal levels of endogenous ACTH in intact rats or long-term pretreatment of hypophysectomized rats with non-radioactive ACTH$_{4-10}$ in a dose 140-fold higher than the ^{3}H-ACTH. Our failure to demonstrate competitive displacement may be interpreted as evidence that the visualized cellular silver grains do not represent binding to specific receptor sites.

The autoradiographic localization in rat brain of α-melanocyte stimulating hormone (MSH), which has the same amino acid sequence as ACTH$_{1-13}$, has been reported by Pelletier *et al.* [9]. Five min after the injection of ^{125}I-labeled MSH into the carotid artery, radioactivity was accumulated in the meninges, choroid plexus, and ependyma, and penetrated into brain tissue around blood vessels. Cellular localization of silver grains was observed in a few cells of the striatum and reticular nucleus of the thalamus. While the cells labeled after vascular administration of ^{125}I-MSH were much fewer in number than those labeled after intraventricular injection of ^{3}H-ACTH analog, those shown in the published autoradiograms of Pelletier *et al.* [9] appear to resemble the small dark cells labeled in the present study. These authors also reported the cellular localization of radioactivity 5 and 30 min after intraventricular, but not intracarotid, injection of ^{3}H-L-prolyl-L-leucyl-glycinamide, an MSH release-inhibiting factor with CNS effects [8]. Intracellular retention of radioactivity was observed in the lateral and medial septal nuclei, putamen, globus pallidus, induseum griseum, hippocampus, corpus callosum, and meninges, a distribution which partially overlaps that of cells labeled in the present study.

Peptide hormones such as ACTH are generally thought to act by binding to specific receptors on the surface of target cells [10]. Evidence that peptide hormones may also enter their target cells has recently been reported for epidermal growth factor [1], luteinizing hormone-releasing hormone and human chorionic gonadotropin [2]. Gorden *et al.* [4], using electron microscopic autoradiography, found that ^{125}I-insulin was initially associated with the plasma membrane of rat hepatocytes, but during 30 min of incubation the radioactivity underwent a progressive intracellular shift and association with lysosomes. Although the significance of the internalization of peptide hormones by target cells requires further research, several possible functions have been proposed [7].

An intriguing aspect of the present results is that within

the first 30 min after intraventricular injection of the ^{3}H-ACTH$_{4-9}$ analog, cellular labeling was limited to small (10–15 μm), frequently elongated, cells whose cytoplasm stained relatively intensely with Nissl stains. Because of their neuronal-like processes, these cells appeared to be neurons, although the staining techniques used did not distinguish with certainty between small neurons and glia. If the observed localization is related to the behavioral activity of ACTH peptides, it suggests that these peptides may act upon a topographically widespread but morphologically distinct class of cells in the brain.

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