

AN AUTORADIOGRAPHIC ANALYSIS OF [³H]α-BUNGAROTOXIN
DISTRIBUTION IN THE RAT BRAIN
AFTER INTRAVENTRICULAR INJECTION

J. SILVER and R. B. BILLIAR. From the Department of Reproductive Biology, University Hospitals, Case Western Reserve University, Cleveland, Ohio 44106. Dr. Silver's present address is the Department of Neuroscience, The Children's Hospital Medical Center, Harvard University, Boston, Massachusetts 02115

Alpha-bungarotoxin (α-BuTX) had been found to block neuromuscular transmission by an antidepolarizing action (6). It has been suggested that α-BuTX binds irreversibly and with high specificity to the nicotinic-acetylcholine receptor of the post-synaptic membrane (6, 17), and that *d*-tubocurarine protects these receptors from the toxin (7, 19).

These conclusions have been substantiated by the autoradiographic findings that, in mice, ¹²⁵I-labeled α-BuTX accumulates within the motor end-plate region of various muscles (11, 17) in the same manner as radioactive curare (27). Here, we report the presence, in vivo, utilizing light microscope autoradiography, of a similar type of α-

BuTX binding in the hypothalamus and amygdala of the rat brain.

MATERIALS AND METHODS

α -Bungarotoxin was isolated from the lyophilized venom of *Bungarus multicinctus* (Miami Serpenterium Laboratories, Miami, Fla. and Sigma Chemical Company, St. Louis, Mo.) by a modification of the method of Lee et al. (18). The venom was first chromatographed on washed CM-Sephadex C-50, and fraction II (18) was isolated and further purified by CM-cellulose chromatography (18). Although Lee et al. (18) used 0.05 M NH_4OAc , pH 5.0, to elute two protein fractions, this buffer did not elute our fraction II from the CM-cellulose and the buffer concentration was increased to 0.1 M NH_4OAc , pH 6.0, which then resolved fraction II into two protein fractions. The second fraction (II-B) contained about 70% of the protein applied to the column and was lyophilized and desalted on Sephadex G-25 column equilibrated with 0.05 M NaH_2PO_4 , pH 7.5. 5 $\mu\text{g}/\text{ml}$ of fraction II-B reduced in vitro isometric contraction of the rat diaphragm (30-s interval stimulation of the phrenic nerve) to half-strength in 18–19 min and completely blocked contraction in about 30 min. These results indicate that fraction II-B had the properties of α -BuTX (6). Protein concentration was determined by optical density at 260 and 280 nm. Fraction II-B (25, 50, and 100 μg) was analyzed by disc electrophoresis (15% cross linking; stacked at pH 5.0, and run at pH 4.3, [alanine-acetate buffer]) and only one protein staining band was observed.

$[^3\text{H}]\alpha$ -Bungarotoxin ($[^3\text{H}]\alpha$ -BuTX) was prepared by the acetamidation method of Zull and Repke (30) for the preparation of tritiated polypeptides. The $[^3\text{H}]\text{acetamidino-}\alpha$ -bungarotoxin ($[^3\text{H}]\alpha$ -BuTX) (sp act $\sim 1.5 \mu\text{Ci}/\mu\text{g}$) was separated from unreacted [*methyl- ^3H*]acetimidate hydrochloride by gel filtration on Sephadex G-25. The $[^3\text{H}]\alpha$ -BuTX appeared radiochemically pure as indicated by disc electrophoresis (Fig. 1), and the $[^3\text{H}]\alpha$ -BuTX had the same electrophoretic mobility as the α -BuTX before acetamidation. In the rat phrenic nerve-diaphragm preparation, the $[^3\text{H}]\alpha$ -BuTX inhibited indirect-stimulated muscle contraction in the same time as the radioinert α -BuTX. Both $[^3\text{H}]\alpha$ -BuTX and α -BuTX were stored at -15°C in 0.05 M NaPO_4 buffer, pH 7.5.

Since α -BuTX does not readily pass the blood-brain barrier (17) and since our studies are primarily directed toward the hypothalamus, the $[^3\text{H}]\alpha$ -BuTX was injected intracranially. Female Holtzman rats (250–300 g) were placed in a stereotaxic instrument under ether anesthesia. With the use of bregma coordinates of Pellegrino and Cushman (21), 0.8 mm anterior, 7.5–8.0 mm ventral, and 0.0 mm lateral, a fine glass cannula was inserted into the third ventricle of the diencephalon in a region of the preoptic nuclei, and one of the following solutions was infused: (a) 7.0 μg of $[^3\text{H}]\alpha$ -BuTX (8.75×10^{-5} M) in 10.0 μl of (0.05 M; pH 7.4) NaH_2PO_4 buffer; (b) to test

for possible nicotinic specificity of toxin binding, 7.0 μg of *d*-tubocurarine chloride (1.0×10^{-3} M) in 10.0 μl of buffer followed 30 min later by the same solution as in (a); (c) to test for possible muscarinic specificity of toxin binding, 3.5 μg of atropine sulfate (1.0×10^{-3} M atropine) in 10.0 μl of buffer followed 30 min later by the same solution as in (a); (d) to test for nonspecific binding, uptake, or retrograde transport within cells, 1.6 μg of $[^3\text{H}]\text{parathyroid hormone } ([^3\text{H}]\text{PTH})$ (31) (2.0×10^{-5} M; 1.0 $\mu\text{Ci}/\mu\text{g}$) in 20.0 μl of (50.0 mM; pH 7.0) HOAC buffer; (e) to test for positive chemography (8), the same concentration as in (a) of radioinert α -BuTX (but no $[^3\text{H}]\alpha$ -BuTX) in 10.0 μl of sodium phosphate buffer. The cannula remained in place throughout the combined infusions in series (b) and (c). The various solutions were infused manually at an approximate rate of 1.0 $\mu\text{l}/\text{min}$. The injections of α -BuTX did not cause any overt symptoms.

The animals were anesthetized with ether and sacrificed 2, 12, or 24 h after injection by perfusion through the heart with, first, 10–20 ml of 0.9% saline and then 400 ml of 10% Formalin-0.9% saline fixative, both 37.0°C . The brains were placed in saline-Formalin solution for 1 wk and then processed routinely for paraffin embedding. Coronal sections were cut at 13 μm , mounted on glass slides, deparaffinized, and coated with Kodak NTB-2 nuclear tract, autoradiographic emulsion. The coated slides were stored in a light-proof container at 4°C for 4 wk, and then processed photographically, cover slipped, and examined under the phase contrast microscope.

RESULTS

$[^3\text{H}]\alpha$ -BuTX

2 h after stereotaxic infusion of $[^3\text{H}]\alpha$ -BuTX into the third ventricle (two animals), label was dispersed throughout the entire brain. Grain density was highest over brain tissue immediately surrounding the third ventricle and especially around the endothelium of blood vessels. Proceeding radially from the ventricle along its entire length, levels of grain density were progressively diminished. High concentrations of label were found overlying the upper portion of the ependymal zone which lines the third ventricle. In addition, a few isolated loci of dense label accumulation were scattered over the arcuate region of the hypothalamus.

12 h (two animals) and 24 h (three animals) after injection of $[^3\text{H}]\alpha$ -BuTX, the intensity of label overlying the greater part of the brain and around vascular endothelium was greatly decreased. No striking difference in labeling pattern was observed between the 12-h and 24-h animals. In several discrete areas the density of labeling was

markedly increased (Figs. 2-4). The most outstanding of these zones appeared as a series of contiguous clusters of intense radioactive label extending throughout the mediobasal region of the hypothalamus and the central nucleus of the amygdala. Thus, proceeding mediolaterally, accumulations of label were seen overlying (a) the ependymal zone on either side of the third ventricle, directly opposite and slightly dorsal to the medial boundary of the arcuate nucleus (Fig. 2), (b) the arcuate nucleus (Fig. 2), (c) the base of the tuber cinereum (Fig. 2), (d) the ventralmost aspect of the lateral hypothalamic area but immediately dorsal to the optic tract, (e) the supraoptic nucleus (Fig. 5), and (f) the central amygdaloid nucleus. In one animal at 24 h label was also present over the medial nuclei of the amygdala and habenula.

More rostrally, below the third ventricle, label appeared in the midline and was continuous with that spreading bilaterally along the base of the brain towards the supraoptic nuclei. Further forward, but continuous with more caudal labeled regions, the preoptic suprachiasmatic (Fig. 3) and

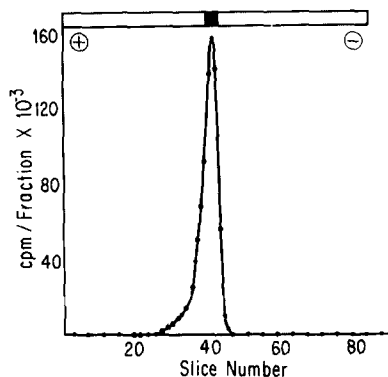


FIGURE 1 Disc electrophoresis of [^3H]acetamidino- α -bungarotoxin (869,000 cpm). The dark band at the top of the figure represents the location of the single protein-staining band. 1-mm thick slices were taken for quantification in a liquid scintillation spectrometer.

suprachiasmatic nuclei (Fig. 4) were heavily labeled. These dense labeling patterns were bilateral in all instances. In addition, throughout the uppermost wall and roof of the third ventricle, silver grains were concentrated over the internal glial cell layer of the ependymal region.

d-Tubocurarine Pretreatment Followed by [^3H]α-BuTX

2 h (one animal), 12 h (two animals), and 24 h (one animal) after the combined administration of first tubocurarine and then [^3H]α-BuTX, the pattern of diffuse labeling overlying the brain was similar to that after the injection of [^3H]α-BuTX alone (compare Fig. 6a and b). However, intense label accumulations over the central amygdaloid nucleus, basolateral hypothalamus, arcuate region, and supraoptic and suprachiasmatic nuclei were lacking. Label was still present at the upper ependymal zone of the third ventricle, but was diminished over the lower ependyma rostral and adjacent to the arcuate nucleus (compare Fig. 6a-c).

Atropine Pretreatment Followed by [^3H]α-BuTX

12 h (two animals) after the combined injection of first atropine and then [^3H]α-BuTX, the pattern of labeling over the brain was comparable to that seen 12 h after injection with [^3H]α-BuTX alone (Fig. 6a).

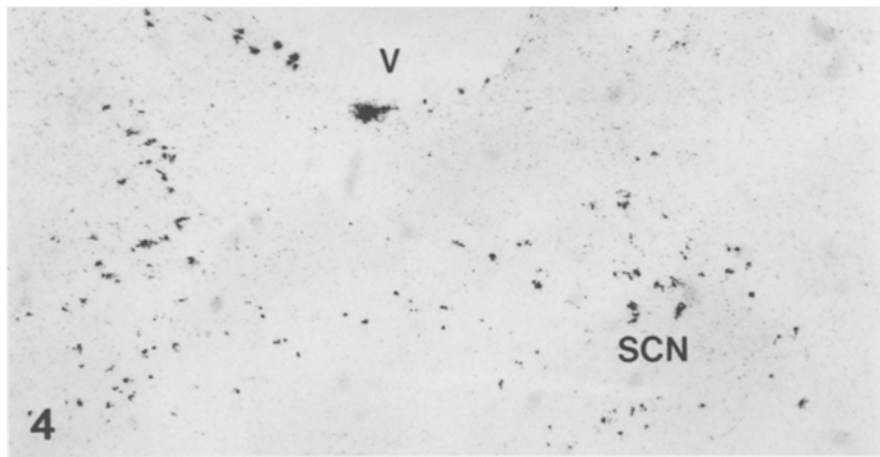
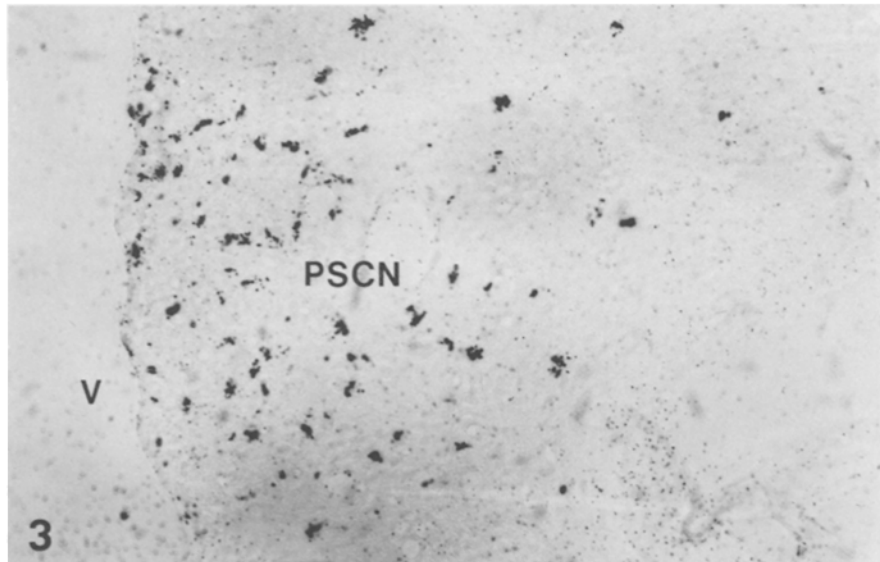
[^3H]PTH

2, 12, and 24 h (one animal per time period) after the injection of [^3H]PTH, the general distribution of diffuse label over the brain was similar to that seen after injection of [^3H]α-BuTX (Fig. 6c). At each time, PTH label was also present over the ependymal and adjacent glial layer of the roof and upper section of the third ventricle and around blood vessels. However, the heavily labeled areas of the lower ependymal region of the third ventri-

FIGURE 2 Light micrograph (unstained preparation) showing the distribution of [^3H]α-BuTX labeling over the basolateral hypothalamus and lower ependymal zone of the third ventricle (V) (indicated by arrow). LHA, lateral hypothalamic area; M, median eminence; A, arcuate nucleus. (24 h). ($\times 100$).

FIGURE 3 [^3H]α-BuTX labeling (24 h) over the preoptic suprachiasmatic nucleus (PSCN). V, ventricle. ($\times 300$).

FIGURE 4 [^3H]α-BuTX labeling (24 h) over the suprachiasmatic nuclei (SCN). V, ventricle. ($\times 200$).



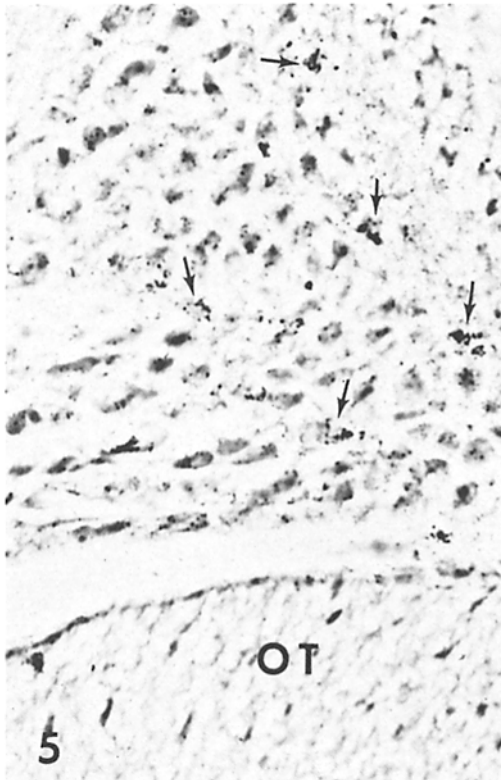


FIGURE 5 Light micrograph of the supraoptic nucleus (24 h) showing BuTX labeling (arrows). OT, optic tract. ($\times 400$).

cle, mediobasal, and basolateral hypothalamus, and central amygdaloid nucleus were absent.

NaH₂PO₂ Buffer + α -BuTX (Radioinert)

12 and 24 h (one animal per time period) after the injection of radioinert α -BuTX in buffer solution, the grain densities overlying the entire brain were at background levels.

DISCUSSION

Taken together, the various patterns of labeling with [3 H] α -BuTX alone and with competitors suggest the presence of a discrete and pharmacologically specific population of nicotinic-cholinergic neurons within the mediobasal and basolateral portions of the hypothalamus, and the central nucleus of the amygdala. Whether labeling in these regions is indicative of a pathway coursing horizontally between the arcuate region and the amygdala is unknown. However, a bundle of such course has been demonstrated by the use of vari-

ous classical anatomical techniques (25). Although the chemical nature of the 3 H or the concentrating mechanism in the hypothalamus is not presently known, α -BuTX is believed to bind specifically and essentially irreversibly to the cholinergic receptor at the end-plate region of the diaphragm muscle. Radioactive bungarotoxin, however, has also been demonstrated to be associated with non-end-plate elements of the diaphragm *in vitro* (23), but this appears to be related to the concentration of α -BuTX present in the incubation medium. After *in vivo* injection, however, most of the neurotoxin was associated with the end-plate region (23). In the present studies [3 H]PTH, a polypeptide with a molecular weight and a basic and acidic amino acid composition similar to that of [3 H] α -BuTX, did not result in intense labeling as observed with [3 H] α -BuTX. Also, tubocurarine, but not atropine, reduced [3 H] α -BuTX accumulation within the mediobasal hypothalamus and central amygdaloid nucleus, whereas neither drug had an appreciable effect on toxin labeling elsewhere in the brain. These data suggest the possibility that α -BuTX labeling over hypothalamic and amygdalar tissue may represent binding with high specificity to cholinergic membrane receptors, rather than nonspecific or possible axonal membrane (10) binding or general uptake within neurons. The retention of the labeling pattern for 24 h supports this suggestion. Other areas of dense label accumulation, such as the ependymal roof of the third ventricle and the endothelium of blood vessels, may represent sites of nonspecific toxin accumulation related to cerebrospinal fluid or blood-brain barriers (Fig. 6a-c) (4, 14).

Although *in vivo* and *in vitro* analyses of brain preparations with the use of α -neurotoxins and various other techniques have suggested the existence of cholinergic neuronal elements within and also distant from the hypothalamus and amygdala (1, 9, 10, 15, 16, 22, 24, 28), in the present study the limited diffusion of [3 H] α -BuTX from the third ventricle may prevent (or delay in animals at 2 h post-injection) autoradiographic labeling of peripherally located areas. Furthermore, recent studies on nerve-muscle systems have shown that the association, as well as the dissociation, reactions between α -bungarotoxin and "high affinity" ACh receptor sites may occur slowly (over many minutes or hours) (5, 12). If present centrally, comparably slow binding phenomena could also influence the temporal pattern of α -BuTX labeling. Other sites and methods of toxin injection

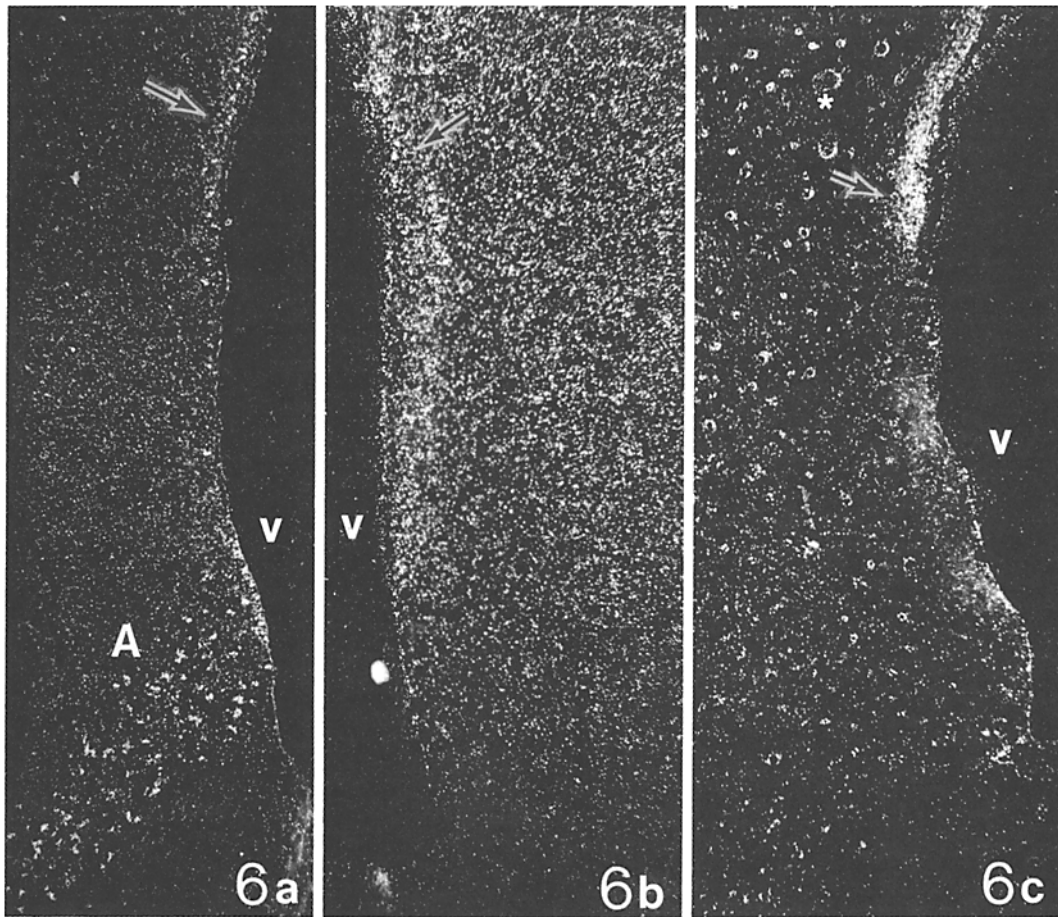


FIGURE 6 (a) Dark-field photomicrograph of the arcuate region of the hypothalamus in an animal which was injected with atropine and then 30 min later with $[^3\text{H}]\alpha\text{-BuTX}$ and sacrificed after 12 h. Note: (i) the persistence of heavy labeling over the arcuate region (A) and the adjacent portion of the lower ependymal wall, and (ii) the heavy labeling over the upper wall of the third ventricle (arrow). V, ventricle. ($\times 100$). (b) Dark-field photomicrograph of the arcuate region of the hypothalamus in an animal which was injected with tubocurarine and then 30 min later with $[^3\text{H}]\alpha\text{-BuTX}$ and sacrificed after 12 h. Note: (i) the absence of heavy labeling over the arcuate region and nearby ependyma; and (ii) the presence of labeling over the upper ependymal zone (arrow). V, ventricle. ($\times 100$). (c) Dark-field photomicrograph of the arcuate region of the hypothalamus in an animal which was injected with $[^3\text{H}]\text{PTH}$ alone and then sacrificed after 12 h. Note: (i) the absence of intense labeling over the arcuate region; and (ii) the accumulation of label over the roof of the third ventricle (arrow). Labeling of blood vessel endothelium is readily apparent in this section (e.g., above asterisk). V, ventricle. ($\times 100$).

may serve to elaborate additional cholinergic centers. In this regard Polz-Tejera et al. (22) have recently studied the *in vitro* binding of $^{125}\text{I}\text{-}\alpha\text{-BuTX}$ in the central nervous system of the rat by light microscope autoradiography and reported heavy labeling in the supraoptic nucleus and also the hippocampus. In that note (22), the presence or absence of labeling in other areas of the hypothalamus was not discussed.

A large portion of the $\alpha\text{-BuTX}$ label in the hypothalamus may overlie an anatomically and functionally well-defined neurosecretory region within the so-called "hypophysiotrophic area" of the diencephalon (13). This region, anatomically defined as a portion of the mediobasal hypothalamus immediately caudal to the preoptic area and rostral to the mammillary bodies, can maintain a pulsatile rhythm of high circulating concentrations

of luteinizing hormone even when deprived of its total afferent input (2, 13). It is premature to tell whether synapses involved in the release of neurohormones have been labeled by [³H]α-BuTX.

The α-BuTX labeling over the lower portion of the third ventricle may be of nerve cell processes rather than of ependymal cells. The differential effect of *d*-tubocurarine (but not atropine) pretreatment on the pattern of labeling over the upper and lower walls of the third ventricle, and the contrast of labeling between these two zones in [³H]PTH-injected animals, support this hypothesis (Fig. 6*a-c*). The position of labeling at the lower portion coincides with a region of contact between the third ventricle and a system of infundibular - cerebrospinal fluid - contacting (I - CSF) neurons (26). It has been suggested that, via the I-CSF neurons, luteinizing hormone-releasing hormone and other products may be released directly into the third ventricle and thereby transported within the CSF to the median eminence, where substances may be resorbed by tanyocyte processes (3, 20, 26, 29). It is noteworthy that the median eminence, the primary site of the ependymal tanyocytes, remained largely free of α-BuTX label.

SUMMARY

Purified α-bungarotoxin was isolated by chromatography and made radioactive with tritium ([³H]acetamidino-α-bungarotoxin). Infusions of [³H]α-bungarotoxin alone or preceded by tubocurarine or atropine were given into the third ventricle. 2, 12 or 24 h after injection the brains were prepared for autoradiography. Injections of α-bungarotoxin (radioinert) in buffer, or of [³H]parathyroid hormone in buffer, served as controls. The various patterns of labeling suggest the presence of nicotinic-cholinergic neurons within the arcuate and basolateral regions of the hypothalamus including the supraoptic and suprachiasmatic nuclei and, in addition, the central nucleus of the amygdala.

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Note Added in Proof: Recently, Gnodde and Schuiling (*Neuroendocrinology*, 1976, **20**:212-223) have shown that mecamylamine, a cholinergic nicotinic receptor blocking agent, when injected intraperitoneally, decreased plasma LH levels in ovariectomized rats, whereas scopolamine, a cholinergic muscarinic receptor blocking agent, had no effect on plasma LH levels.

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