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CYTOCHEMICAL LOCALIZATION OF DOPAMINERGIC NEURON CELL BODIES IN THE ARCUATE NUCLEUS

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Summary

A cytochemical technique for localizing biogenic amine was used to localize dopamine (DA) neurons in the arcuate nucleus (AN). The chromium (Cr) positive granules have a very irregular shape, are located mostly in the side of the neuron cell body that contains the Golgi apparatus. The neurons that possess this type of granule are relatively small and spindle shaped, with dimensions of approximately 30 x 12 microns. The size of dark granules in the cell bodies was decreased by reserpine, increased by Nialamide and significantly increased by L-Dopa or Nialamide plus L-Dopa. Biochemical analysis of DA in the arcuate nucleus shows parallel changes in the concentration in that structure in the same drugs treated animals. It is therefore likely that cells containing chromium-positive granules shown here are dopaminergic neurons of the arcuate nucleus.

Introduction

Norepinephrine (NE), dopamine (DA) and 5-hydroxytryptamine (5-HT) granules are known to react with potassium dichromate at pH 4.1 following fixation with glutaraldehyde[1,2]. This technique has been used with the electron microscopic localization of these biogenic amines in peripheral and central nervous tissues. This technique was currently applied to study the dopaminergic neurons in the arcuate nucleus since it has been shown with histofluorescence that the biogenic amine containing neurons of the arcuate nucleus are dopaminergic[3-5]. The fluorescence of dopaminergic neuron cell bodies in arcuate nucleus (A-12) can be reduced by reserpine as well as increased by administration of Nialamide plus L-Dopa[3]. The present study was done to demonstrate types of neurons in the arcuate nucleus, to show the configuration of the dopaminergic neurons in the arcuate nucleus using the technique of Wood[6].

Although fluorescent studies have been highly utilizable to show the presence of dopaminergic neurons, definite electron microscopic identification of these particular biogenic amine neurons have not been shown at the ultrastructural level. The previous work of Wood[6,7] indicates that such a procedure is highly feasible.

Materials and Methods

Sixteen adult male (175-320 g) Holtzman rats were divided randomly into 5 experimental groups and treated as follows:

1) Control group–untreated and uninjected, maintained in the same environment as those animals treated with drugs (3 rats).
2) Reserpine treated–reserpine 5 mg/kg, serpasil, CIBA Pharmaceutical Co., was administered intraperitoneally, 2 times, 12 hours apart. The rats were sacrificed 2 hours after the second injection (3 rats).
3) Nialamide treated–Nialamide, Pfizer, New York, N.Y., 500 mg/kg administered intraperitoneally; the animals were sacrificed 5 hours following injection (3 rats).
4) L-Dopa treated–L-Dopa, Sigma, St. Louis, MO, 100 mg/kg was administered i.p.; the rats were sacrificed 1 hour following injection (3 rats).
5) Nialamide plus L-Dopa treated–500 mg/kg Nialamide injection i.p was followed with L-Dopa injection 100 mg/kg i.p. (3 rats).

All animals, prior to sacrifice were anesthetised with Nembutal, 50 mg/kg i.p., then were treated as follows: The animals were perfused, via the ascending thoracic aorta with cold (4°C) 4% glutaraldehyde containing 1% sodium molybdate (G-Mo) in 0.2 M sodium cacodylate buffer (pH 7.2) at 60 mm mercury pressure. Perfusion was conducted until perfusate ran clear (approximately 30 minutes). The brain was removed, the median eminence and the hypothalamic regions were dissected free and divided into left and right sides. The brain tissues were immersed in the G-Mo solution for an additional 1½ hours (at 4°C). Then they were removed and immersed in 4% glutaraldehyde containing 2.5% potassium dichromate and 2% sodium sulphate in 0.2 M sodium cacodylate buffer (pH 4.1) for 2 hours at 4°C. Tissues were then stored in 4% paraformaldehyde in the sodium cacodylate buffer, pH 7.2 overnight in the refrigerator. The right half of the median eminence and the right arcuate nucleus were treated with 1% osmium tetroxide in the same sodium cacodylate buffer for 1 hour and the left sides were not osmicated; all tissues were dehydrated in sequence in ethanol, in propylene oxide and embedded in epon in the oven at 60°C for 48 hours. Blocks were trimmed to the area of the arcuate nucleus and the palisade layer of the median eminence by using the lateral recess of the third ventricle as a landmark (Fig. 1). Some median eminence blocks were sectioned coronally, and some in parasagittal sections as an approach to the arcuate nucleus. Ultrathin thick 0.5 micron and ultra-thin (500 Å) sections were made on an LKB ultratome III, placed on mesh grids and studied both osmicated and unosmicated, either stained or

unstained with lead citrate; electron microscopic observations and photography was done utilizing a JEM 100B Electron Microscope. Neuron cell bodies in the arcuate with characteristic DA inclusion were photographed at approximately the same magnification as routinely as possible in order that an adequate comparison of the cross-sectioned area of the cell body and the DA deposits could be determined.

Calculation of the area of the DA granules was accomplished as follows:

1) Each electron micrograph was photocopied.
2) Each photocopy of the micrograph was cut along the outlined granule using a pointed surgical blade on dental wax.
3) Cut out areas were weighed and the number of granules were noted, as was the magnification (X).
4) From given area (A) of photocopy paper and its weight (W), (K) value was established as A/W in mm squared per gram of the paper The K of the photocopy paper used was calculated to be 13,188.4 mm. squared per gram.
5) The actual average cross-sectional area of the granule in micron squared per granule was calculated by the formula:

$$A \text{ (micron squared per granule)} = \frac{KW.\ 10^6}{\text{number of granules.}\ X^2}$$

whereas,
W = total paper weight of the granules cut from the photocopy in grams,
K = mm squared per gram of the paper,
X = magnification of the photomicrograph.
The factor 10^6 arises from the fact that 1 mm^2 = 10^6 $micron^2$,
A = actual area of the granule in micron squared per granule.

A method of confirming that dense areas of the same tissue were positive for biogenic amines reaction and contained chromium (Cr) was previously reported[6, 7].

Another set of rats were treated with the same drugs and the same way as the group used for the electron microscopic study. Brains were removed after decapitation and arcuate nucleus (AN) and median eminence (ME) were dissected while in the frozen state (see Fig. 1) catecholamines of these brain samples were measured using combinations of techniques of Chang[8], Shellenberger and Gordon[9], and Anton and Sayre[10].

1. The arcuate nuclei and median eminence were rapidly removed from the rats, weighed, frozen in dry ice until used.
2. The brain areas were homogenized in 5 ml of 75% ethanol.
3. The homogenate was divided into two equal portions each of which was placed in 5 ml glass stoppered pyrex contrifuge tubes.
4. In one of the two tubes standard was added, (100 μl of the standard containing 1 μg/ml norepinephrine, and 2 μg/ml dopamine).

5. The tubes were briefly shaken in order to mix the standard solution with the homogenate, then centrifuged at 3000 rpm for 6 minutes.
6. The clear supernatant was transferred to a 5 ml centrifuge tube, 200 mg of alumina was added, and the pH was adjusted to 8 with 1N sodium acetate in 75% ethanol. About 0.9 ml of sodium acetate was needed for each tube (the preparation of the alumina; see Anton and Sayre[10]).
7. The tubes were shaken for 10 minutes and then left for 3 minutes to allow the alumina to settle down. In this step the catecholamines, norepinephrine and dopamine, are absorbed into the alumina, the serotonin remains in the supernatant.
8. The alumina was washed with 2 ml of cold deionized water and the tubes were shaken for 2 minutes. The alumina was allowed to settle for another 2 minutes. The supernatant was then aspirated off.
9. The catecholamines were eluted from the alumina with 0.2N acetic acid. To each tube 1.2 ml of 0.2N acetic acid was added. The tube was shaken for 10 minutes, then centrifuged at 3000 RPM for 2 minutes. The clear supernatant contained the norepinephrine and dopamine.
10. A 0.5 ml sample of the supernatant was transferred to the test tube for the assay procedure. Duplicate measurements were done for each sample, and the average fluorescence values were used for the calculation.

The procedure of Chang[8] was used for the fluorometric measurements. At the end, means of the biochemical data were calculated and the relationship to chromium positive granule size in AN were analysed.

Results

Gross behavioural changes were observed in drug-treated rats. The reserpine treated rats were lethargic and had severe diarrhea. Five hours after the injection of 500 mg/kg of Nialamide no alteration of the animal behaviour was observed; however, at rest they were hyperexcitable when startled. L-Dopa of 100 mg/kg i.p. slightly increase the resting activity of the rats and caused slight piloerection at 1 hour after injection. Nialamide plus L-Dopa treated animals, especially after the last L-Dopa injection, were hyperexcitable, exhibited piloerection, shivering and restlessness, hyperpyrexia, a high respiratory rate and showed evoked rigidity. These animals also exhibited convulsions, and were hyper-aggressive when confronted. A few animals, died a short time after L-Dopa administration, thus, the sacrifice time was 1 hour following the final L-Dopa injection.

The electron microscopic results are shown in Table 1. The majority of the arcuate nucleus (AN) micrographs were taken from tissues which were well perfused. Few of the pictures are shown in Figs. 2 and 3. Preliminary photography was done in thin sections of unosmicated tissue (before treating with osmium tetroxide solution). The granules stained by chromium are dark and irregular in shape, while other granules

in most cells remain unstained and have a more uniform homogeneous appearance (Fig. 4). The DA containing neuron cell bodies are smaller than most other neurons in the arcuate nucleus. Dimensions of the cell bodies are calculated to be about 12×30 microns and spindle-shaped. This type of neuron was seen near capillaries and the granules are located mostly in the side of the cell having the Golgi apparatus.

TABLE 1. SUMMARY OF DATA OF AVERAGE GRANULE CROSS-SECTION AREA IN ARCUATE NEURONS OF CONTROL, RESERPINE TREATED, NIALAMIDE TREATED AND NA + L-DOPA TREATED RATS.

The top horizontal column shows number of photomicrograph measured (N), and means ± standard error of mean (SEM). The lower four horizontal columns show the combination t-test among the groups, DF = degree of freedom, P-values and conclusion of the t-test, namely NS = not significantly different at 5% level; HS = highly significant different at 1% level.

	Control (C)	Reserpine (R) treated	Nialamide (Na) treated	L-Dopa (D) treated	Nialamide + L-Dopa (Na + D) treated
$\bar{X} \pm$ S.E.M.	N = 36	N = 30	N = 29	N = 23	N = 23
μ^2/granule	2.05 ± 0.14	1.77 ± 0.12	2.38 ± 0.22	2.60 ± 0.19	2.88 ± 0.23
t-test C vs		DF = 64 P = 0.1 − 0.05 NS	DF = 63 P = 0.1 − 0.05 NS	DF = 57 P = 0.01 − 0.005 HS	DF = 57 P < 0.0005 HS
t-test R vs			DF = 57 P = 0.01 − 0.005 HS	DF = 51 P < 0.0.005 HS	DF = 51 P < 0.0005 HS
t-test Na vs				DF = 50 P = 0.3 − 0.2 NS	DF = 50 P = 0.1 − 0.05 NS
t-test D vs					DF = 44 P = 0.2 − 0.1 NS

Electron dense areas are found in perikarya and axons, see Figs. 3 and 5. Data of granules in cross-sectional area from each photomicrograph and from each group of animals was extrapolated. Data of both cross-section and sagittal sections of the same drug-treated group were pooled when compared with other groups. The P values, degrees of freedom and conclusion of the t-test are shown in Table 1 and Fig. 6. The average granule cross-section area in the AN neurons of reserpine treated group is somewhat smaller than that of the control group, however, the difference is at the borderline of the 5% level of significance. Reserpine of this dose reduces the size of the DA granules in general. The average DA granule size of the reserpine treated animals, however, is significantly smaller than that of the Nialamide, L-Dopa and/or Nialamide plus L-Dopa treated group with the doses used.

The DA granule average cross-sectional area of the Nialamide treated animal is greater than the control; however, this increase is at the borderline of significance

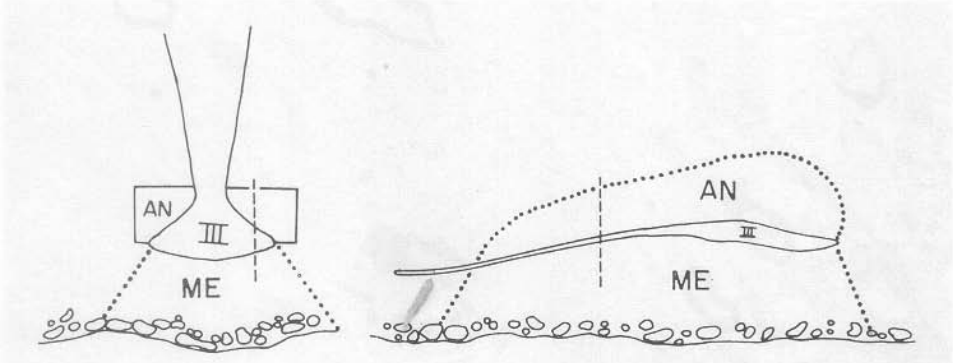

Fig. 1. Diagram of the rat brain tissue thick section. The left picture is the cross section of the arcuate nucleus (AN) and the median eminence (ME). The right picture is the parasagittal section of the same brain area. Note the relationship of the AN and ME with the third ventricle (III).

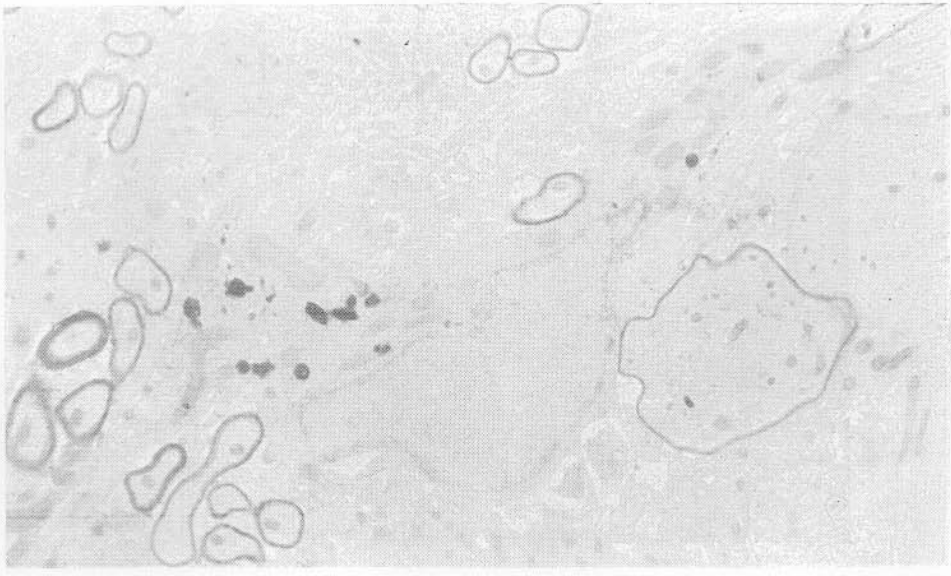

Fig. 2. Micrograph of arcuate nucleus (AN) representing suspected dopamine cell. Most chromium positive granules are irregular in shape. There are plently of mitochondria in the cell (× 2000).

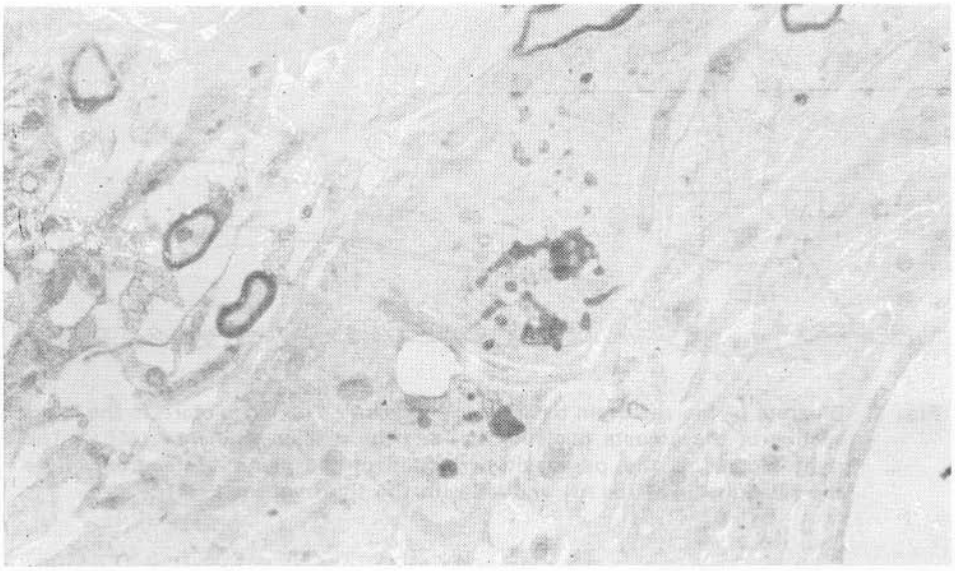

Fig. 3. Another representative of suspested dopamine cell in the arcuate nucleus. The section is tangential to the cell body. Chromium positive granules are very irregular in shape. Mitochondria are abundant in the cytoplasm (× 2500).

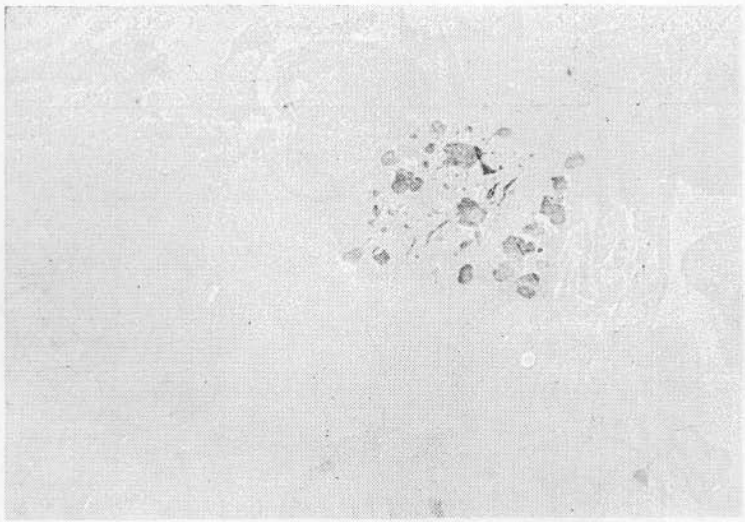

Fig. 4. Unosmicated tissue of arcuate nucleus. Granules of suspected dopamine neuron (right) become electron dense after treating the tissue with potassium dichromate, whereas smooth-shape granules of the other type of neuron (left) are not chromium positive (× 17,000).

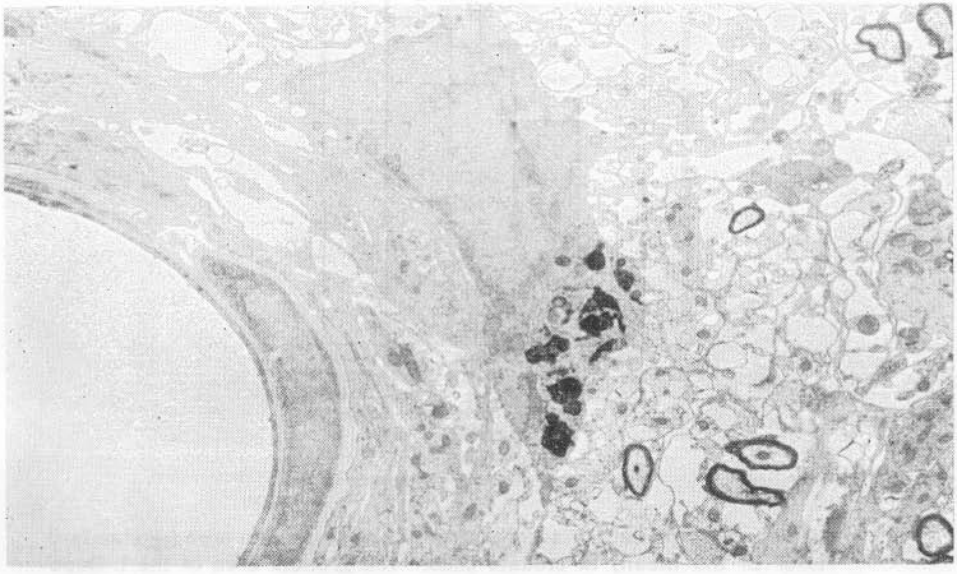

Fig. 5. Representative of arcuate neuron which has chromium positive granules. The cell body is spindle in shape. Granules are dark and very irregular. Most of these cells are near arterioles. It is likely to be dopamine containg neuron (× 5,000).

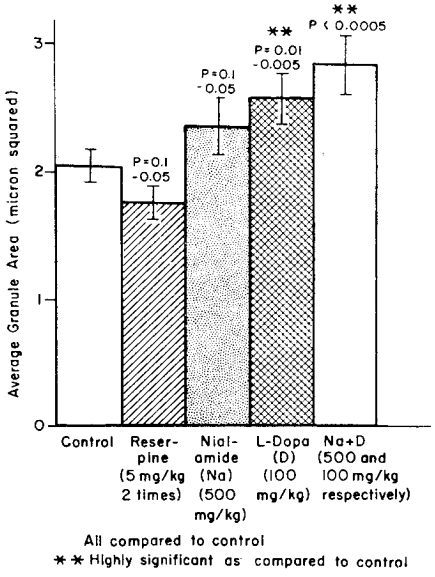

Fig. 6. Summary of the data of average granule cross-section area (± standard error of the mean) in arcuate cells. The granule area of the L-Dopa and Nialamide + L-Dopa treated rats are significantly larger than the control rats. The Nialamide, L-Dopa, L-Dopa + Nialamide treated rats have significantly larger granule area compared with reserpine treated rats. For other detail of comparisons see Table 1. NS = not signifcant difference. ** = highly significant difference at $P < 0.001$.

at the 5% level. The average cross-sectional area of the DA granule of Nialamide treated group is not significantly different from that of the L-Dopa or the Nialamide plus L-Dopa treated rats, although the mean of Nialamide treated rat is smaller.

L-Dopa treated rats have significantly larger cross-sectional area granules than either control or reserpine treated groups. The area is not significantly different from the Nialamide treated and the Nialamide plus L-Dopa treated rats, although the mean of the L-Dopa treated is larger than the Nialamide and smaller than the Nialamide plus L-Dopa treated animal.

L-Dopa plus Nialamide treated animals have much larger average DA granules cross-sectional areas than either control, reserpine, Nialamide or L-Dopa treated groups; however, it was only significantly larger than control and reserpine treated groups; see Table 1.

Actual average cross-sectional area in micron^2 per granule has been found to be smaller in higher magnification photographs than that of low magnification ones. This may well be due to the nonlinearity of the actual magnification or that the smaller granules tend to be photographed more, and the large granules are omitted from the area. Therefore, it is considered favorable to photograph all the anatomical structures at the same magnification when one wants to compare the size and/or surface area of the structure study.

Animals treated with identical drugs in the same way show reduction in DA and NE in both the arcuate nucleus (AN) and median eminence (ME) tissues, whereas Nialamide treatment does not alter DA and NE in ME and AN much (Fig. 7). L-Dopa treated rats as well as L-Dopa plus Nialamide treated rats show remarkable increase in NE and DA concentrations in both AN and ME.

Discussion

From data presented, the granules of the arcuate nucleus cells studied are electron-dense after treatment with potassium dichromate solution (Fig 4.) While other types of granules in most cells in the area, and even in the adjacent cells remain light, this indicates that these granules react with chromium. Similar granular localization has been noted in the arcuate nucleus of the Rhesus monkey by the same technique, and this has been shown to be chrome-positive from energy dispersive X-Ray analysis[6,7]. This has also been shown by many investigators that the area of the arcuate nucleus contains many dopaminergic neuron cell bodies[4,5]. While no NE or 5-HT cell bodies are observed there, the data show that the granule size is reduced by reserpine in this study, however, the reduction is at a 5–10% borderline of significance level. The increase in granule size with Nialamide is not significant at the 5% level; however L-Dopa and Nialamide plus L-Dopa increase the granule size significantly. All this seems to parallel with the properties of the DA neurons at A 12 in the arcuate nucleus when treated by similar drugs[3]. Change in the chromaffin positive granule size is not as dramatic as changes in fluorescence in previous reports; however, it is possible that there may be a change in number of the granule per cell or content of DA within the granules.

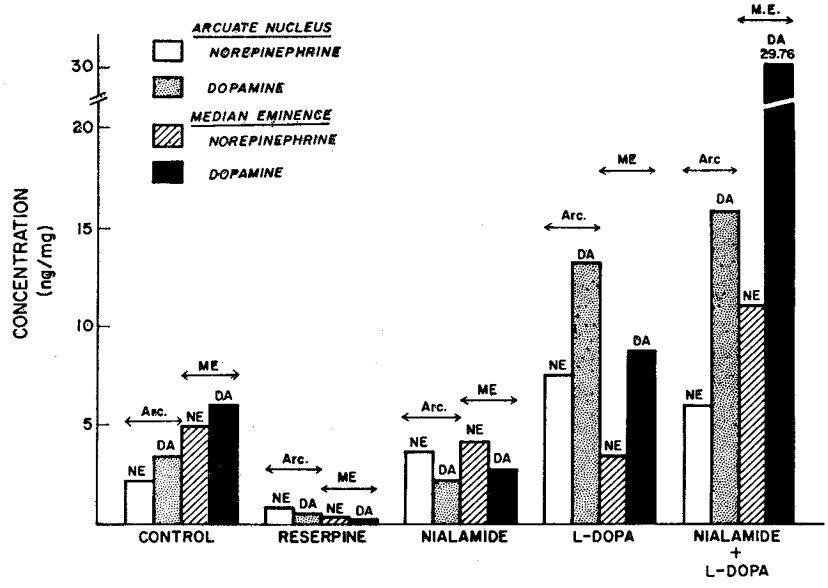

Fig. 7. Dopamine (DA) and norepinephrine (NE) concentrations in the tissues of arcuate nucleus (Arc.) and the median eminence (ME). Doses and injecting schedule of drugs used were the same as the previous electron microscopic studies. Data shown are triplicate measurements of pool tissues of five rats.

Chemical data on DA and NE concentration of AN and ME show their decrease and increase in the same directions as the fluorescence of biogenic amines[3]. Changes in DA concentration in the arcuate nucleus is likely to be changes in the cell bodies, since fluorescence microscopy[3-5] demonstrates that biogenic amine neurons in the arcuate nucleus are mainly dopaminergic. NE concentration changes in the arcuate nucleus and DA and NE concentration changes in the ME are likely to be in the nerve terminals[3-5].

Concentration changes of DA in the arcuate nucleus by drugs are parallel to changes in the size of chromium positive granules in the cell bodies of the arcuate neurons. The evidence presented here makes it seem highly probable that the granules under study are chrome positive and that they are the ones that are the dopamine containing organelles of the DA in neurons of AN. Further investigations on these cells are in progress.

Acknowledgement

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บทคัดย่อ

ได้ใช้เทคนิคทางไซโตเคมมิสตรีในการมองเห็นสารพวกไบโอจีนิกอะมีน เพื่อหาว่าเซลล์พวกไหนในอาคิวเอตนิวเคลียสของหนูเป็นเซลล์ที่มีดอปปามีนในระดับกล้องจุลทัศน์อีเล็คตรอน จากการทดลองพบว่าเซลล์พวกที่น่าจะเป็นดอปปามีนนิวรอนมีแกรนนูลที่ติดด้วยโครเมี่ยม แกรนนูลมีรูปทรงขรุขระ มักอยู่ทางด้านของตัวเซลล์ที่มีกอลจิแอปปาเรตัส เซลล์พวกนี้มีขนาดเล็กกว่าเซลล์ทั่วไปในอาคิวเอตนิวเคลียส และมีรูปกระสวยมีขนาดประมาณ 30 × 12 ไมครอน ขนาดของแกรนนูลของเซลล์พวกนี้ลดลงเมื่อฉีดหนูด้วยรีเซอร์บีนขนาดเพิ่มขึ้นเล็กน้อยเมื่อฉีดด้วยไนอะลามีด ขนาดเพิ่มขึ้นมากเมื่อฉีดด้วยแอลโดปา หรือฉีดด้วยแอลโดปาด้วยกันกับไนอะลามีด จากการวัดปริมาณสารดอปปามีนในอาคิวเอตนิวเคลียส พบว่าสารตัวนี้มีการลดความเข้มข้นโดยรีเซอร์บีนและเพิ่มความเข้มข้นโดยไนอะลามีด แอลโดปาหรือไนอะลามีดด้วยกันกับแอลโดปา ดังนั้นเซลล์ในอะคิวเอตนิวเคลียสที่มีคุณสมบัติดังกล่าวข้างต้น น่าจะเป็นเซลล์ประสาทที่มีดอปปามีนอยู่ข้างใน