Changes in cellular noradrenaline fluorescence in the denervated rat superior cervical ganglion

G. Krinke, K. Schnider and R. Hess[1]

Research Laboratories, Pharmaceuticals Division, Ciba-Geigy Ltd., CH-4002 Basle (Switzerland), 21 November 1977

Summary. The microfluorimetrical investigation showed that in the principal perikarya of the rat superior cervical ganglion, after inhibition of noradrenaline-synthesis and unilateral preganglionic axotomy, the fluorescence of newly synthesized noradrenaline was most intense at the periphery of the perikarya of the intact side.

By means of the formaldehyde-fluorescence method[2] for the detection of monoamines, it can be shown that the principal neurones of superior cervical ganglion of the rat display specific fluorescence due to their noradrenaline (NA) content. The intensity of this fluorescence can be taken as a measure of the NA concentration, as the 2 qualities are, within broad limits, proportional to each other[3].

Using small-spot microfluorimetry of the principal superior cervical perikarya, we found an inverse relation between the average intensity of fluorescence measured in the middle part of the cytoplasm and the estimated volume of the perikarya. Moreover, a significant decrease in average fluorescence intensity was demonstrated within the perikarya following postganglionic axotomy[4].

In experiments based on purely qualitative evaluations of fluorescence intensity[5-7], no effect of preganglionic axotomy on the fluorescence of the superior cervical ganglion cells was detectable. In the present study, an effect of preganglionic axotomy was demonstrated to occur when the axotomy was combined with inhibition of NA synthesis. The changes in NA concentration were recorded by microfluorimetry.

Materials and methods. Male Sprague-Dawley-derived rats, weighing 250–350 g, were used. The tyrosine-hydroxylase inhibitor H 44/68 (DL-α-methyl-p-tyrosine-methylester·HCl) was dissolved in 0.9% NaCl and injected i.p. in dose of 200 mg/kg. 2 h later, the left cervical trunk was transected just caudal to the superior cervical ganglion under ether anaesthesia. The success of the operation was checked by the occurrence of Horner's syndrome. The rats were sacrificed 2, 10 and 22 h after axotomy under light ether anaesthesia, and the superior cervical ganglia excised, frozen in liquid nitrogen, freeze-dried (the initial temperature of $-40\,°C$ being gradually raised to $+40\,°C$ over 5 days), treated with formaldehyde gas at 80 °C for 75 min and embedded under vacuum in paraffin. The formaldehyde gas was generated from paraformaldehyde powder, equilibrated at 50% relative humidity. Longitudinal sections of the ganglia, 5 µm in thickness, were examined in the Zeiss-Microfluorimeter, working with short-time excitation. For fluorescence epi-illumination an HBO 100 W/2 mercury lamp was used with a BG 12 filter, the approximate diameter of the illuminated field being 5 µm. The emission, limited by the barrier filter to 470–650 nm, was measured in a circular area 3.15 µm in diameter. Before each measurement, the perikarya were examined in phase contrast, and only those having a distinct nucleus with nucleolus were chosen. The 'small intensely fluorescent cells' (SIF cells) were purposely omitted. The measuring-field was oriented near to the nucleus in the 'central' cytoplasm and, in the same perikaryon, near the cell surface in the 'peripheral' cytoplasm. To avoid the effect of fading, the measurements of the 2 areas were made in alternate order of precedence. Usually 2 sections of each ganglion were examined, and in each of these 15–25 perikarya were measured. The intensity of fluorescence was expressed in arbitrary units (AU) and, to permit absolute comparisons, it was also related to that of standard solutions of NA in gelatin (figure 1, A and B). These NA standards were prepared according to Lichtensteiger[8] by addition of L-NA. HCl and an equimolar amount of ascorbic acid to a 2% gelatin solution. 4 samples of each NA concentration were processed together with the ganglia in all the experiments. After embedding in paraffin, the reference samples were cut into 5 µm sections (2 sections/sample) and for each section 10 values were obtained, using the same measuring-field as on the ganglia. The values for the perikarya and the NA standards were both corrected for background fluorescence by subtracting the average fluorescence intensity of corresponding uncondensed specimens. Regression curves of the fluorescence intensities of NA standards were calculated. The results showed that:

a) there was a significantly positive correlation between NA concentration and fluorescence intensity ($r = 0.81$–0.94)

b) the relation between the NA concentration and the fluorescence intensity was linear ($p < 0.05$) up to the highest concentration used ($27 \cdot 10^{-5}$ M)

The cell-size was estimated in a phase-contrast microscope by measuring the length (L) and width (W) of each perikaryon with the aid of an eye-piece micrometer. The ratio of these 2 parameters (L:W) was taken as an elongation[9] and their product (L·W) as a profile-field[10], the elongation being considered to represent the form and the profile-field the size of the perikarya.

Results. Superior cervical ganglia of untreated control rats were examined to determine the relation between the estimated size of the perikarya and the intensity of specific fluorescence in their central and peripheral cytoplasm. To test the homogeneity of the cell population, the perikarya were alloted to size-classes according to their profile-fields and the numbers of perikarya in each class compared. In addition, a dot-diagram was made, in which the length of the perikarya was related to their elongation. In both instances the diagrams reflected the distribution of a single cell-type. In the perikarya of uncondensed ganglia, the average non-specific fluorescence did not depend on the cell-size, and in all size classes it was slightly more intense in the central than in the peripheral cytoplasm. By contrast, the average intensity of specific fluorescence was inversely related to size of the perikarya. The periphery of the perikarya showed more intense fluorescence than the central cytoplasm in all but the smallest size-class, in which there was no obvious difference between the central and peripheral intensities (figure 2). On qualitative fluorescence-microscopic examination, a general decrease in the fluorescence intensity was observed 4 h after the administration of H 44/68, followed by an increase at later times (12 and 24 h). Although the pattern of distribution of fluorescence varied from one perikaryon to another, the fluorescence seemed particularly intense at the periphery (figure 1, C–E). After preganglionic axotomy on the left side, mere qualitative examination did not reveal any difference between the ganglia of the operated and the unoperated side. However, when the fluorescence was measured 24 h after the administration of the NA-synthesis inhibitor, the intensity was greatest in the peripheral area of the perikarya of the ganglia belonging to the right (unoperated) side (figure 3).

Discussion. According to present opinion, NA is stored in the perikaryon of adrenergic neurones, either in granular

form, bound to dense-core vesicles, or in free form, within the cytoplasmic matrix[11]. The granular appearance of the NA-specific fluorescence was shown to be associated with clusters of dense-core vesicles; diffuse fluorescence was considered to be due to free cytoplasmic NA[12–14]. Our quantitative results demonstrated a difference in the average fluorescence intensity between the central and the peripheral cytoplasm, reflecting an analogous difference in NA concentration between the 2 areas. The higher average NA concentration at the periphery of the perikarya was presumably due to the granular storage form, because NA bound to storage vesicles is more concentrated than in the diffuse form[15,16]. By means of high-resolution radioautography with ^{3}H-NA, Taxi[17] demonstrated clusters of labelled storage vesicles in the autonomic perikarya and their dendrites, mainly localized near the plasma membrane and associated with presynaptic zone-like formations. More recently, Richards and Tranzer[11] examined rat superior cervical ganglia by the electron-cytochemistry method and found storage ultrastructures dispersed individually throughout the perikaryon, but clustered around the cell periphery and in dendritic processes. After inhibition of NA-synthesis and unilateral preganglionic axotomy, our measurements revealed the greatest fluorescence intensity at the periphery of the perikarya of the intact side. This effect could be due to a disproportionate NA turnover in the denervated ganglia, resulting in a diminished content of newly synthesized NA. In fact, it is well known that the activity of the catecholamine-synthesizing enzyme tyrosine-hydroxylase depends on the existence of an intact transynaptic mechanism[18].

Although at present the absolute quantitation of catecholamine fluorescence *in situ* seems impossible, it is notewor-

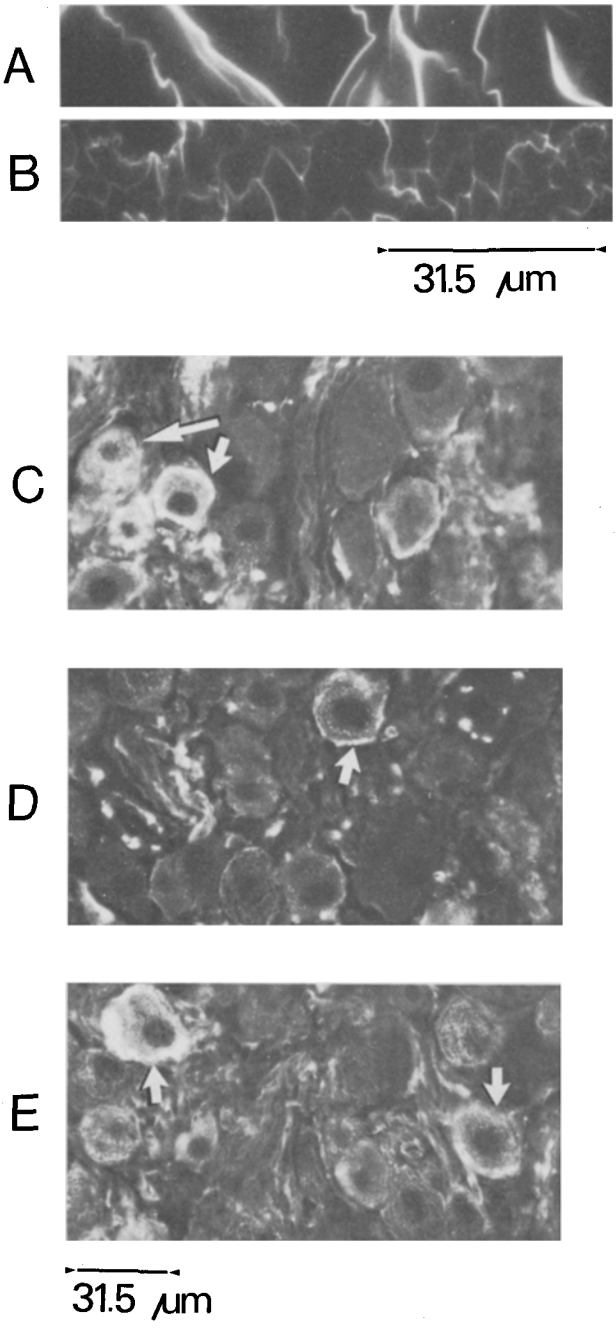

Fig. 1. Fluorescence microscopical image of samples processed according to formaldehyde-fluorescence method: *A* and *B* 2 examples of a standard NA solution in gelatin, × 100; *C–E* perikarya of the superior cervical ganglion: control (C), 4 (D) and 12 (E) h after the inhibition of NA-synthesis, × 40. An intense fluorescence is frequently seen at the cell periphery (short arrows), but also occurs near the nuclei (long arrow in C). 4 h after the inhibition of NA-synthesis there is a general decrease in the fluorescence intensity. (31.5 µm = 10fold measuring-field diameter).

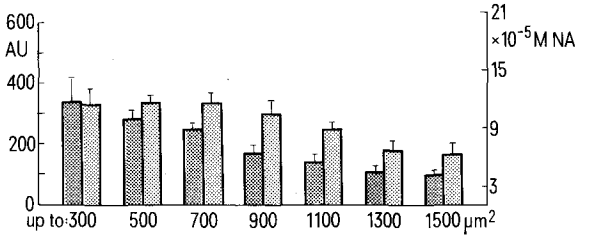

Fig. 2. Average intensity of specific fluorescence (+SEM) of 300 perikarya (3 left and 3 right control ganglia) arranged according to their profile-fields. For the inverse relation between the fluorescence intensity and the estimated size of the perikarya Spearman's rank-correlation coefficient was calculated, giving values of $R = -1$ ($p = 0.001$) for the perinuclear area and $R = -0.8571$ ($0.05 > p > 0.02$) for the peripheral area ▩ perinuclear area; ▨ peripheral area.

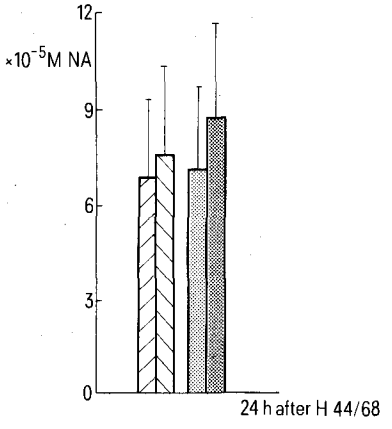

Fig. 3. Average intensity of specific fluorescence (+SEM) of perikarya of control ganglia and of ganglia denervated after the inhibition of NA-synthesis. 420 denervated and 420 control perikarya from 12 left and 12 right ganglia 24 h after H 44/68. The difference in central and peripheral fluorescence intensity is only significant in the right ganglia ($p < 0.01$; in the left ganglia $p > 0.1$; Wilcoxon test for paired differences). ▨ left perinuclear area; ▧ left peripheral area; ▨ right perinuclear area; ▩ right peripheral area.

thy that the average NA concentrations within the principal superior cervical perikarya observed in our experiments and those based on the gelatin standard do not deviate substantially from those surmised by other authors[16].

1 The authors are deeply indebted to Mr H. Petermann, and to Mr B. Rombach, Ciba-Geigy Ltd. for their mathematical support.

2 B. Falck, N.A. Hillarp, G. Thieme and A. Torp, J. Histochem. Cytochem. *10*, 348 (1962).
3 K.A. Norberg, M. Ritzen and U. Ungerstedt, Acta physiol. scand. *67*, 260 (1966).
4 G. Krinke, K. Schnider and R. Hess, Experientia *30*, 37 (1974).
5 B. Hamberger and K.A. Norberg, Experientia *19*, 580 (1963).
6 M. Härkönen, Acta physiol. scand. *63*, suppl. 237, 1 (1964).
7 O. Eränkö and M. Härkönen, Acta physiol. scand. *63*, 411 (1965).
8 W. Lichtensteiger, Prog. Histochem. Cytochem. *1*, 185 (1970).
9 M. Palkovits and J. Fischer, Karyometric Investigations 1, Akadémiai Kiado Budapest, 1968.
10 H. Adhami and H.P. Wald, Acta anat. *81*, 384 (1972).
11 J.G. Richards and J.P. Tranzer, J. Ultrastruct. Res. *53*, 204 (1975).
12 T. Hökfelt, Acta physiol. scand. *76*, 427 (1969).
13 L.S. Van Orden, J.P. Burke, M. Geyer and F.V. Lodoen, J. Pharmac. exp. Ther. *174*, 56 (1970).
14 O. Eränkö, Histochem. J. *4*, 213 (1972).
15 A. Dahlstroem, J. Haeggendal and T. Hoekfelt, Acta physiol. scand. *67*, 289 (1966).
16 G. Jonsson, Prog. Histochem. Cytochem. *2*, 299 (1971).
17 J. Taxi and C. Sotelo, Brain Res. *62*, 431 (1973).
18 H. Thoenen, R.A. Mueller and J. Axelrod, Nature *221*, 1264 (1969).

Giant pigment granules in dermal melanocytes of rat scrotal skin

M.E. Glimcher and G. Szabo[1]

Laboratory of Electron Microscopy, Harvard School of Dental Medicine, 188 Longwood Avenue, Boston (Massachusetts 02115, USA), 9 January 1978

Summary. Giant melanosomes, ellipsoidal (1.5 μm × 1.3 μm) or spherical (1.1 μm–1.4 μm in diameter) exist in the scrotal skin of the black pelted Long Evans rat. They are longer and wider than normal stage IV melanosomes (0.7 μm × 0.4 μm) found in these dermal melanocytes.

Giant pigment granules have been observed in the melanocytes of the skin of a number of mammalian species. Lutzner et al. reported the presence of giant pigment granules in the beige mouse[2] and in the Aleutian mink[3]. In humans, large accumulations of defective and large pigment granules have been found in patients with Chediak-Higashi Syndrome[4]; giant pigment granules (macromelanosomes) in the melanocytes and keratinocytes of the epidermis of patients with neurofibromatosis[5,6] and nevus spilus[7]. Here, we report for the 1st time the presence of giant melanosomes in the dermal melanocytes of a rodent. We feel it is necessary to report this single finding as no giant pigment granules have heretofore been found in dermal melanocytes nor in rodent skin.

Materials and methods. The effects of castration, testosterone replacement therapy, UV-light, and their combination, on scrotal skin pigmentation were studied using 51 black pelted, male Long-Evans rats, 50–160 days old (Blue Spruce Farms, Altamont, N.Y.). Periods of post-operative castration ranged from 11 to 109 days until sacrifice. Testosterone propionate (Sigma Chemical, St. Louis, Mo.) suspended in sesame oil was injected s.c. in the dorsum of the neck of 11–56-day post-operative castrates in dosages of 10^{-3}–10^{-7} g/100 g b.wt for up to 18 days. The UV-light source used was a Westinghouse FS-20 sunlamp with maximum output of 290–330 nm and peak at 310 nm. Daily dose ranged from 1.22×10^5 erg/cm^2 to 1.28×10^7 erg/cm^2 for exposures of 1–14 days.

The scrotal skin was excised and fixed in Ito-Karnovsky fixative[8,9], 5% glutaraldehyde, 4% formaldehyde, and 0.02% 2,4,6-trinitrophenol, for 2 h at 4 °C. The tissue was then washed in 0.1 M cacodylate buffer and post-fixed in 2% OsO_4 for 2 h at 4 °C. 1-μm-thick sections were stained with toluidine blue and photographed with a Zeiss Ultraphot II. Epon-embedded specimens were thin-sectioned using a Porter-Blum ultramicrotome, stained with uranyl acetate and lead citrate, and examined using an AEI Corinth C275 electron microscope.

Results and discussion. Giant melanosomes were observed in the course of an investigation in which the effect of castration and testosterone therapy on scrotal skin pigmentation was studied in black Long-Evans rats. Light and electron microscopic observation revealed the existence of giant pigment granules within the densely pigmented dermal melanocytes of 1 particular animal in the group which received daily testosterone replacement therapy (0.1 mg/100 g b.wt) for 10 days following 8 weeks of post-operative castration. These giant pigment granules are ellipsoidal (approx. 1.5 μm by 1.3 μm) or spherical (1.1 μm by 1.4 μm in diameter). Other melanosomes in dermal melanocytes of the same animal and of other animals were mainly in stage IV and approximately 0.7 μm by 0.4 μm.

The presence of normal melanosomes in stages II and III of formation in these dermal cells suggests that they are melanocytes and not macrophages. The histochemical tyrosinase reaction would clearly demonstrate this point.

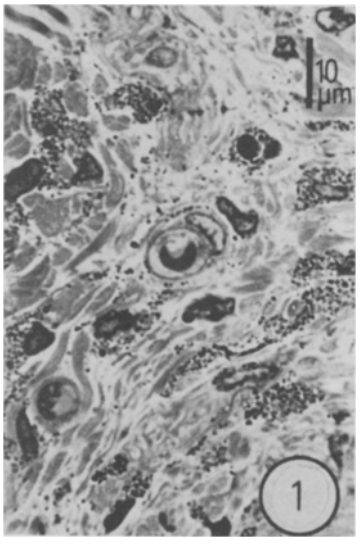

Fig. 1. Light micrograph of a 1-μm-thick Epon section stained with toluidine blue. Several giant pigment granules in the dermal cells. × 970.