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The dopaminergic innervation as observed by immunohistochemistry using anti-dopamine serum in the rat cerebral cortex [1]

M. Yoshida, M. Sakai[a], K. Kani[a], I. Nagatsu[a] and M. Tanaka

Department of Pharmacology, Kurume University School of Medicine, Kurume 830 (Japan), and [a] Department of Anatomy, School of Medicine, Fujita-Gakuen Health University, Toyoake 470-11 (Japan)
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Summary. By using an antiserum raised against dopamine bound to bovine serum albumin, thinner dopamine-labeled nerve terminals were visualized immunohistochemically within neocortical areas, in addition to well-documented dopaminergic innervation into the prefrontal and limbic cortices.
Key words. Dopamine; cerebral neocortex; immunohistochemistry.

The cortical dopaminergic innervations of the rat was thought for a long time to be localized exclusively within the prefrontal and limbic cortices[2,4,8,9]. However, new dopaminergic terminal fields were recently discovered in some neocortical areas situated along a dorsal sagittal strip, using fluorescence histochemistry after pharmacological manipulation[3]. In the present study, we have reexamined the distribution of cortical dopaminergic innervation, by means of immunohistochemistry using antiserum raised against dopamine in untreated rat brain. Particular emphasis was placed on the investigation of the dopaminergic input into the remaining neocortical areas.

Materials and methods. Preparation of antiserum: Production of the antiserum to dopamine (DA) was principally based upon the method of Geffard et al.[5,6]. Briefly, DA hydrochloride (42.4 mg) was coupled to bovine serum albumin (BSA, 40 mg) by adding glutaraldehyde (GA) (0.25 mmol) in 10 ml of 0.1 M phosphate buffer (PB), pH 7.4. While stirring, 1 ml of $NaBH_4$ (10 mM) was added to the solution. After incubation for 20 min, the solution was dialyzed against PB for 2 days, and centrifuged. The supernatant (0.5 mg/ml protein) was used for immunization. This DA-GA-BSA conjugate was mixed with complete Freund's adjuvant and injected in rabbits at intervals of two weeks. The antiserum was obtained after 4 months.

Tissue preparation and immunohistochemical staining: Male Wistar rats weighing 150–400 g were anesthetized with Nembutal and the blood was washed out with about 50 ml of phosphate buffered saline (PBS), and subsequently with 450–900 ml of 5% GA in 0.01 M cacodylate buffer, pH 5–7. After perfusion, the brain was removed and sliced into 3–4-mm thick blocks. After being placed in the same fixative, for 1–3 h, the tissue blocks were sectioned into 40-µm-thick sections on a microslicer (DTK-1000, DSK). These sections were first incubated in 1% $NaBH_4$ in PBS for

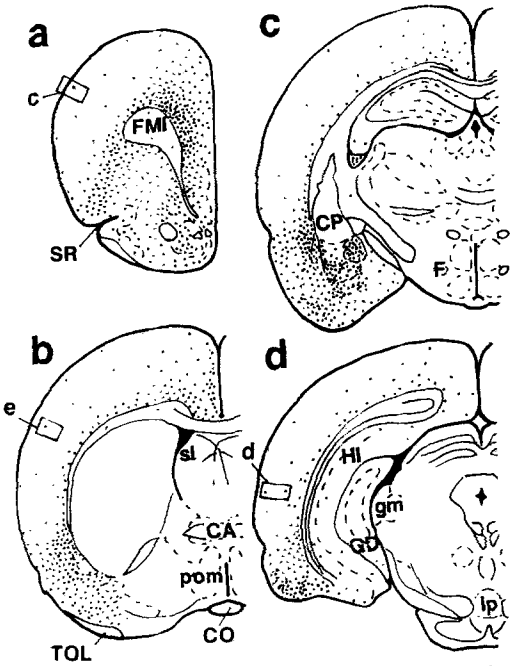

Figure 1. The schematic drawings of dopamine-immunoreactive nerve terminals on frontal sections of the rat cerebral cortex. Note that dense-labeled terminals represented as dots were localized mainly in the prefrontal and limbic cortices, but sparse dopamine-labeled terminals were found throughout the remaining neocortical areas. Rectangles c, d and e indicate the approximate position of the photographs in figure 2. CA, anterior commissure; CO, optic chiasma; CP, nucleus caudatus-putamen; F, fornicus; FMI, forceps minor corpus callosum; GD, dentate gyrus; gm, medial geniculate nucleus; HI, hippocampus; ip, interpenduncular nucleus; pom, medial preoptic area; sl, lateral septal nucleus; SR, rhinal sulcus; TOL, lateral olfactory tract.

30 min, then in 10% normal sheep serum for 60 min. They were further processed for the peroxidase-antiperoxidase (PAP) immunohistochemical method[11]. At first, sections were incubated with antiserum against DA (1:2000–5000) in 0.05 M Tris-buffered saline (TBS), pH 7.6 containing 1% normal sheep serum and 0.3% Triton X-100 for 24–48 h. Sections were then washed and incubated with goat anti-rabbit IgG (Miles, 1:100) for 2–5 h. Following washes, they were incubated with PAP complex (DAKO, 1:100) for 2 h. The sections were finally reacted with diaminobenzidine (0.4 mg/ml) and 0.03% hydrogen peroxide in 0.05 M Tris-buffer, pH 7.6 for about 10 min, and they were mounted on slides, dehydrated and covered with a coverslip. Characterization of antiserum: to evaluate the specificity of the antiserum, several control tests were performed. Many neuronal elements (presumably containing DA) were stained with the antiserum (fig. 2a) or with the serum preabsorbed with BSA, but none were seen in sections incubated with the serum preabsorbed with antigen (DA-GA-BSA) (fig. 2b). In order to examine the cross-reactivities with several related substances such as noradrenaline (NA), adrenaline (AD), serotonin (5-HT), histamine (HA), tyramine (TA), octopamine (OA), L-3,4-dihydroxyphenylalanine (L-DOPA) and 5-hydroxytryptophan (5-HTP), an enzyme-linked immunosorbent assay (ELISA) was performed. This ELISA system was successfully modified for the detection of small molecules as described previously[10,13–15]. Cross-reactivities towards these compounds were determined from the absorbance (OD 492 nm). Our DA antiserum scarcely cross-reacts with NA (< 0.1%), and does not react with L-DOPA, AD, 5-HT, TA, OA nor 5-HTP in our ELISA system.

Results and discussion. DA-immunoreactive terminals were schematically drawn in figure 1, and they were found more or less throughout the whole cerebral cortex. They were represented as fine, sinuous fibers with irregularly spaced varicosities. A dense DA-labeled nerve plexus was located in the anteromedial, anterior cingulate, suprarhinal, pyriform and entorhinal cortices, which confirms the previous findings by histochemical studies[2,4,8,9,12]. Thinner DA-labeled terminals, in turn, were noticed within the neocortical areas such as the dorso-lateral frontal cortex (fig. 2c), the parieto-temporal cortex (figs 2d and 2e) and the occipital cortex (fig. 2f). Remaining neocortical fields also received sparse dopaminergic innervations. These neocortical DA-labeled

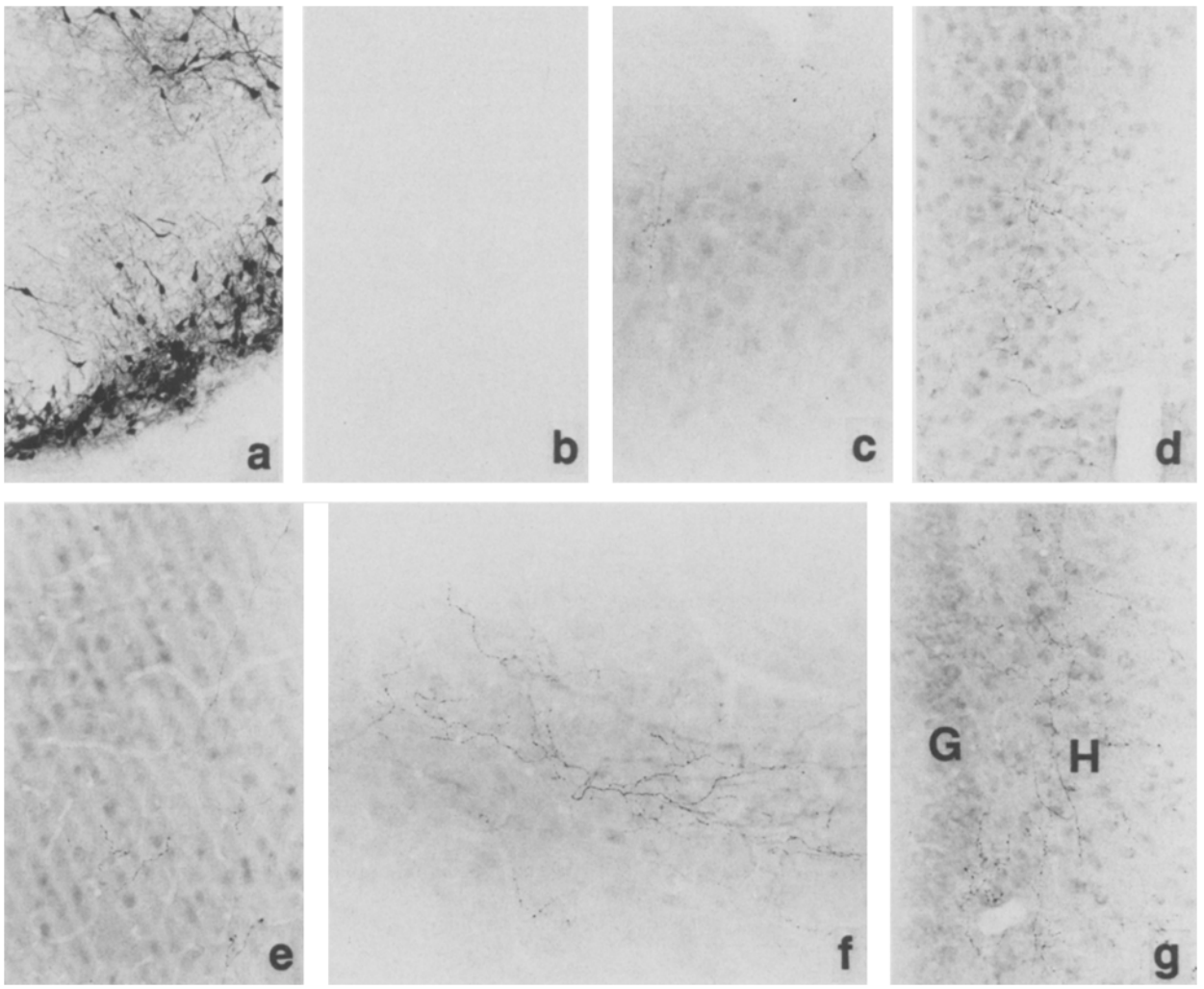

Figure 2. Photomicrographs of dopamine-immunoreactive neuronal structures in the rat central nervous system. *a* and *b* Substantia nigra, frontal section, × 75. *a* Many neurons of the substantia nigra were labeled with the antiserum; *b* no labeling was seen in the section incubated with the antiserum preabsorbed with antigen (dopamine-GA-BSA). *c–f* Cerebral cortex. Thinner dopamine-labeled innervation observed in *c* the dorso-lateral frontal cortex (area 10, × 300), *d* the temporal cortex (area 41, × 150) and *e* the parietal cortex (area 2, × 150) as indicated in the rectangle in figure 1. *f* Deep occipital cortex (area 17), sagittal section, right side is rostral. × 150 *g* Dopamine-labeled nerve plexus observed in the temporal part of the dentate gyrus. G, granule cells; H, dentate hilus. × 150.

fibers were predominantly seen in its deep cortical layers (layer V–VI) where they followed a rostro-caudal course towards the occipital cortex (fig. 2f). DA-labeled terminals were also demonstrable within the hippocampal formation especially in its temporal part (fig. 2g).

The specificity of immunoreactions visualized by using our DA antiserum was controlled by several absorption tests and ELISA. In the ELISA method, we used many related compounds bound onto poly-L-lysine (MW 300,000) via GA, as fixed antigens. Under these conditions, the strongest cross-reaction was revealed only toward NA (< 0.1%). The antiserum preabsorbed with NA-GA-BSA conjugate was then applied alternatively for staining, in order to exclude possible labeling in noradrenergic systems, but no alteration in staining was confirmed even after this treatment. From these procedures, we believe that our antiserum revealed a highly specific immunoreaction to DA in GA-fixed tissue.

After the destruction of the noradrenergic system, dense fluorescent (presumably dopaminergic) terminals could be visualized in the prefrontal (anteromedial, suprarhinal and anterior cingulate) and limbic (pyriform and entorhinal) cortices. The detailed organization of these dopaminergic systems has been well documented in many studies[2, 4, 8, 9, 12]. Few systematic surveys, however, have been performed on the distribution of dopaminergic input into the remaining cortical structures. Recent study, using both fluorescence histochemical and tyrosine hydroxylase-immunohistochemical techniques combined with pharmacological manipulations, reported the presence of dopaminergic terminals within restricted neocortical fields (sensorimotor, visual and retrosplenial cortices)[3]. Our immunohistochemical study confirms the above observations, and we have also detected additional dopaminergic terminals within the dorso-lateral frontal cortex (Krieg's area 10), the parieto-temporal cortex (area 1, 2, 40, 41 and 20) and some areas of the occipital cortex (area 17 and 18a)[7].

Though these neocortical dopaminergic innervations show a markedly lower density than the prefrontal or limbic dopaminergic input, it is suggested that dopamine might be involved in synaptic transmission at neocortical sites.

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The use of iodinated density gradient media for the isolation of rod outer segments

R. A. Delgadillo, R. M. Smets, Q. R. Yang, D. Vanden Berghe and A. Neetens

Institute of Experimental Ophthalmology and Laboratory of Microbiology, University of Antwerp, UIA, B-2610 Antwerp (Belgium)
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Summary. Sucrose, nycodenz, metrizamide and a mixture of equal volumes of sucrose and metrizamide were used as density gradient media for the isolation of retinal rod outer segments. The high osmolarity of sucrose had a strongly negative effect on the nature of the rod outer segments, whereas they were much better preserved using iodinated density gradient media such as nycodenz and metrizamide for their isolation.
Key words. Retina; rod outer segments; density gradient; iodinated density gradient media; sucrose; nycodenz; metrizamide; electron microscopy.

Rod photoreceptor cells of the mammalian retina process light to initiate neurotransmission; light induces shedding of the rod outer segments (ROS). One of the basic functions of the retinal pigment epithelium cells is the phagocytosis of the continuously shed ROS during the visual cycle[1, 2]. A technical problem currently being investigated is the isolation of rod outer segments, in order to study phagocytosis in vitro. Generally ROS are isolated by using sucrose density gradient media[3–6]. In our experience, ROS were strongly osmotically influenced in sucrose containing media. In order to avoid this, we tried iodinated density gradient media such as metrizamide and nycodenz, which are described as being less osmotic than sucrose[7].

Materials and methods. Light adapted cattle eyes collected at the local slaughterhouse were kept for 4–5 h at 4 °C before use. The retinas were carefully removed from the eye cup using blunt forceps. 10 retinas were incubated in 100 ml EDTA-buffer (NaCl 137 mmol, EDTA 1.08 mmol, $Na_2HPO_4 - 12\,H_2O$, 7.90 mmol, $NaH_2PO_4 - H_2O$, 1.28 mmol, KCl 2.7 mmol, phenol red 0.01 mmol, pH 7.4) at 4 °C for 15 min. This suspension was shaken by hand for 1 min and centrifuged at 45 × g for 5 min. The supernatant