Sympathetic Reinnervation of the Rat Iris in Organ Culture

(nerve growth factor/norepinephrine uptake/fluorescence)

STEPHEN D. SILBERSTEIN*[‡], DAVID G. JOHNSON*, DAVID M. JACOBOWITZ[‡],
AND IRWIN J. KOPIN*

* Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20014; and

[‡] Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pa. 19104

Communicated by Seymour S. Kety, March 25, 1971

ABSTRACT Reinnervation of sympathetically denervated rat iris by superior cervical ganglion cells has been demonstrated to occur *in vitro*. Return of [³H]norepinephrine uptake by irises incubated in contact with ganglia was associated with the reappearance of nerve fibers containing catecholamines that were demonstrable by fluorescent histochemistry. The reinnervating neurons appeared to follow the same general pattern of innervation seen in the normal iris, but the density of the neural plexus was much greater. Nerve growth factor influenced the rate and extent of innervation of the iris but not of neuronal growth within the ganglion.

The dense sympathetic innervation of the thin rat iris makes it particularly suitable for histochemical localization of sympathetic neurons containing catecholamines (1), for study of uptake and release of radioactive norepinephrine (2), and for survival in artificial media *in vitro*. After denervation of tissues innervated with adrenergic nerves, reinnervation occurs by growth of new fibers from sympathetic ganglia. Sympathetic ganglia can also survive in artificial media, and the neurons continue to respond to acetylcholine stimulation for over a week after their isolation *in vitro* (3). We have found that sympathetic ganglia placed in apposition to irises in organ culture reinnervate these tissues and that nerve growth factor (NGF) influences the rate and extent of reinnervation.

METHODS

Organ cultures were prepared by the method of Trowell (4) and placed in a plexiglass culture chamber as described by Klein (5). Media consisted of a modification of BGJ_B (Grand Island Biological Co.) (6) supplemented with glucagon-free insulin (kindly supplied by Dr. R. Chance, Eli Lilly and Co., 5 mg/100 ml), lipid concentrate (7) dissolved in 100% ethanol and, in some preparations, NGF (100 U/ml) (Burroughs Wellcome & Co. Inc. USA), or equine anti-mouse nerve-growth-factor serum (1/500) (Burroughs Wellcome & Co. USA). Culture medium was changed every 48 hr.

Adult male Sprague-Dawley rats (175-200 g) were killed by cervical dislocation, and the superior cervical ganglia and anterior halves of the eyes were excised aseptically and immediately placed in ice-cold medium. The ganglia were dissected free of capsule, and the irises were removed from the eyes by blunt dissection under a microscope. Each iris was

stretched over a grid. In some preparations the iris was incubated with the superior cervical ganglion either overlapping one of its margins or adjacent to, but not touching, the iris. At the end of the incubation period the irises were separated from the ganglia under the dissecting microscope and stored in ice-cold medium.

Fluorescence Studies. The irises were rinsed in saline, mounted as stretch-preparations on glass slides, dried, treated with formaldehyde gas, and examined directly under the fluorescence microscope (1, 8). The ganglia were rinsed in saline, freeze-dried and treated with formaldehyde gas, embedded in paraffin, sectioned, and examined for fluorescence (8).

[³H]Norepinephrine Uptake. The irises were incubated for 30 min at 37°C in an atmosphere of 95% O₂-5% CO₂ in Krebs-Ringer bicarbonate solution supplemented with ascorbic acid (200 mg/l) containing 1×10^{-7} M [³H]D,L-norepinephrine-7 (New England Nuclear Corp., 8.76 Ci/mmol). After incubation, the tissue was washed in fresh Krebs-Ringer solution for 10 min.

After digestion of the tissue in NCS solubilizer (a solution of organic base dissolved in toluene; Amersham/Searle) total tritium, which was mostly [³H]norepinephrine (9, 10 and unpublished observations), was assayed by liquid scintillation spectrometry in 15 ml of a liquifluor (New England Nuclear Co.)-toluene (42:1000) mixture. Counting efficiencies were determined by internal standards and averaged 25%.

RESULTS

Freshly removed irises concentrate [³H]norepinephrine when placed in an incubation medium containing the labeled catecholamine (2, 9). Uptake of [³H]norepinephrine by the iris was almost completely abolished 24 hr after incubation in organ culture (Fig. 1).

Irises incubated in media containing NGF and in contact with superior cervical ganglia showed the same initial loss of [³H]norepinephrine uptake; however, on the second day in organ culture the uptake returned to over one-third the amount found in fresh irises and continued to increase. After 8 days, [³H]norepinephrine uptake was greater than that found in freshly removed irises (Fig. 1). Irises that were incubated alone (or in the presence of, but separated from, superior cervical ganglia) failed to recover the ability to take up [³H]norepinephrine.

Abbreviation: NGF, nerve growth factor.

[‡] Requests for reprints should be sent to Dr. Silberstein in Bethesda (Bldg. 10, rm. 2D-46).

FIG. 1. Uptake of [^3H]norepinephrine by rat iris incubated *in vitro* after different intervals in organ culture. Results expressed as % of uptake by freshly removed ganglia.

When NGF was omitted from the culture medium, uptake of [^3H]norepinephrine by the irises incubated with overlapping superior cervical ganglia was not as great as in the presence of NGF (Fig. 2). When antibody to NGF was added to the culture medium, recovery of [^3H]norepinephrine uptake was further reduced.

On microscopic examination, irises cultured for 24 hr showed a complete loss of fluorescent nerver fibers. Irises incubated in contact with superior cervical ganglia (in medium supplemented with NGF) showed the same initial loss of fluorescent nerves (Fig. 3: compare *b* to *a*). However, one or two small regions of diffuse intense fluorescence without neuronal integrity were observed in the dilator region (Fig. 3*b*). After two days in culture, there was a return of nerve processes with many fibers in the dilator and sphincter regions (Fig. 3*c*). The nerves appeared to radiate from the points of contact of the ganglion with the iris toward both the sphincter and ciliary processes. The contact region of outgrowth of nerves from the ganglion contained intense amorphous fluorescence. Reappearance of fluorescent fibers was not seen in the irises cultured alone or when they were not in contact with ganglia.

At the end of 4 days, about one-quarter of each iris (the quarter nearest to the point of contact with the ganglion) contained a dense network of fluorescent fibers (Fig. 3*d*). Smooth fluorescent nerve trunks and processes were observed to course radially and circularly in the dilator-muscle region. The fibers were mostly circularly oriented in the sphincter region. There appeared to be a greater number of fibers and intensity of fluorescence per unit area than in a normal iris (Fig. 3*a*).

After 7 or 8 days in culture, fluorescent fibers were evident over a larger region of the iris (from one-quarter to one-half in some preparations) (Fig. 3*e*). Some of the fine terminal nerve fibers had developed varicosities, but most were still relatively small (Fig. 4*a*). An abundant number of nerve fibers were found in the dilator and sphincter regions. This overgrowth of nerves increased the intensity of fluorescence.

NGF appeared to enhance the rate and extent of reinnervation. Eight irises cultured in the presence of NGF (7 or 8 days) all showed a dense plexus of nerves. Without NGF, four irises

FIG. 2. Uptake of [^3H]norepinephrine by rat iris incubated in organ culture for 6 days. Irises in contact with sympathetic ganglion were cultured in media containing: no additions (+O), added nerve growth factor (+NGF) or added antibody to NGF (+anti-NGF).

were not innervated, two had poor growth, and two showed a moderate nerve density compared to those cultured with NGF. However, the increased nerve growth within ganglia was not dependent upon the presence of NGF.

After 7 and 8 days in culture, with or without exogenous NGF, the number of intercellular nerve trunks and fibers in superior cervical ganglia had greatly increased. Many of the neuronal cell bodies showed a moderate intensity of fluorescence (Fig. 4*b*). As in freshly removed ganglia, about 5–10% of the cell bodies were intensely fluorescent (11). Dispersed clusters of chromaffin cells were also observed, some with fluorescent processes emanating from the cell bodies.

DISCUSSION

The reinnervation of sympathetically denervated tissues that occurs *in vivo* appears also to take place in organ culture. Uptake of [^3H]norepinephrine by the iris is related to the density of the sympathetic neurons in this organ. After 24 hr in organ culture, irises take up small quantities of labeled catecholamines. This is presumably a consequence of the complete denervation reflected by the loss of fibers that develop catecholamine fluorescence. The reinnervation of the iris by sympathetic nerves, which is first clearly observed at 48 hr, accounts for the higher uptake of labeled catecholamine in the iris incubated with ganglia.

After 24 hr, the initial outgrowth of adrenergic neurons appeared as amorphous fluorescent areas, but [^3H]norepinephrine uptake was not significantly greater than that of iris cultures in the absence of ganglia (Fig. 1). It was from the region of contact that subsequent neuronal growth occurred. After 2 days in culture, distinct adrenergic fibers were observed in the dilator and sphincter regions of the iris. At the same time the uptake of [^3H]norepinephrine returned to about one-third of control. After 4 days in culture, uptake was about two-thirds of normal but only one-third to one-half of the iris had been reinnervated. The fact that the density of the fluorescent processes in the reinnervated portions of

FIG. 3. Rat iris (magnification $\times 105$). (a) Normal iris, adrenergic ground plexus. Stretch-preparation taken directly from the rat eye. Sphincter region (S); dilator (D). (b) Iris, 24-hr culture. Catecholamine fluorescent region containing initial outgrowth of adrenergic elements from the superior cervical ganglion. (c) 48-hr culture. Growth of adrenergic nerves from initial outgrowth in the dilator region towards the sphincter (left) and ciliary processes (right). (d) 4-day culture. Dense network of fibers in sphincter and dilator muscles. (e) 7-day culture. Hyperinnervation of adrenergic nerves in the dilator and sphincter muscles.

the iris was greater than that found in freshly removed iris is consistent with the disproportionate increase in uptake compared with the area of reinnervation.

After 8 days in culture, the uptake of [^3H]norepinephrine was greater than that found in the normal iris, presumably as a

consequence of the hyperinnervation found on histochemical examination (Fig. 3e). The pattern of adrenergic ground plexus in irises reinnervated in organ culture was similar to that observed by Olson and Malmfors (10) in irises reinnervated from sympathetic ganglia transplanted to the anterior

FIG. 4. (a) Iris, 7-day culture. Dense plexus of mostly smooth nerve processes, preterminal nerve trunks, and some varicose terminal fibers ($\times 280$). (b) Rat superior cervical ganglion, 7-day culture (with NGF). Fluorescent cell bodies, varicose fibers, and smooth nerve trunks are observed ($\times 180$).

chamber of the rat eye. This suggests that the pattern of reinnervation is not random and may be determined by the target organ.

NGF appears to enhance the rate and extent of reinnervation. Irises cultured with ganglia for 6 days in the absence of NGF took up only about 60% as much labeled catecholamine as irises cultured in media containing added NGF and appeared to be much less densely innervated. Innervation in the absence of exogenous NGF may be dependent on endogenous NGF remaining in the iris or in the ganglion (Johnson *et al.*, in preparation). The further reduction of [^3H]norepinephrine uptake by the addition of specific antibody to NGF supports this view.

The development of an *in vitro* system of sympathetic reinnervation in a chemically defined medium may make possible the characterization of the mechanisms of nerve-tissue recognition and interaction and permits testing of the effects of drugs or toxins on the processes of reinnervation.

SDS was a Research Associate in Pharmacology-Toxicology of the National Institute of General Medical Sciences, Bethesda,

Md. 20014. DMJ was supported in part by USPHS Research Grant No. 1 RO1 NB-06707-05 and by National Institute of Neurological Diseases and Stroke Research Career Development Award No. 5-K3-NB-13,935-05.

1. Malmfors, T., *Acta Physiol. Scand., Suppl.*, 248 (1965).
2. Farnebo, L.-O., and B. Hamberger, *J. Pharmacol. Exp. Ther.*, **172**, 322 (1970).
3. Larrabee, M. G., *Fed. Proc.*, **29**, 1919 (1970).
4. Trowell, O. A., *Exp. Cell Res.*, **16**, 118 (1959).
5. Klein, D. C., and J. Weller, *In Vitro*, **6**, 197 (1970).
6. Biggers, J. P., R. B. L. Gwatkin, and S. Heyner, *Exp. Cell Res.*, **25**, 41 (1961).
7. Ling, C. T., G. O. Gey, and V. Richters, *Exp. Cell Res.*, **52**, 468 (1968).
8. Falck, B., and C. Owman, *Acta Univ. Lund Sect. 2.*, **7**, 1 (1965).
9. Jonsson, G., B. Hamberger, T. Malmfors, and C. Sachs, *Eur. J. Pharmacol.*, **8**, 58 (1969).
10. Olson, L., and T. Malmfors, *Acta Physiol. Scand. Suppl.*, 348 (1970).
11. Jacobowitz, D., *Fed. Proc.*, **29**, 1929 (1970).