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Catecholaminergic innervation of the rat adrenal cortex

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Summary. The zona glomerulosa of the rat adrenal gland is innervated by catecholaminergic nerves. Using histofluorescence techniques, we observed catecholaminergic plexuses surrounding adrenal capsular and subcapsular blood vessels. Individual varicose nerve fibers that branched off these plexuses were distributed among adrenal glomerulosa cells. This innervation was permanently eliminated after neonatal sympathectomy with guanethidine or 6-hydroxydopamine, but was not affected by ligation of the splanchnic nerve or extirpation of the suprarenal ganglion. At the ultrastructural level, axonal varicosities were commonly observed in close proximity to glomerulosa cells and blood vessels. Nerve fibers and varicosities were found to contain small (30–60 nm) clear vesicles as well as large (60–110 nm) and small (30–60 nm) dense-cored vesicles. In tissue fixed for the dichromate reaction with or without pretreatment with the false transmitter 5-hydroxydopamine, many nerve terminals contained numerous small dense-cored vesicles which are thought to contain catecholamines. These results establish the anatomical substrate for the catecholaminergic innervation of the rat adrenal cortex.

Key words: Catecholamine – Adrenal cortex – Sympathetic innervation – Splanchnic nerve – Innervation – Rat

There is a growing body of evidence that neural activity can directly affect endocrine organs classically considered to be entirely controlled by hormonal feedback mechanisms (Potter 1981). In particular, in the adrenal cortex, the autonomic nervous system directly modulates cell proliferation and may affect hormone synthesis. The neural basis of compensatory adrenal growth has been clearly demonstrated (Dallman et al. 1977) but the anatomical substrate mediating this response is largely unknown. Furthermore, while dopamine is present in the adrenal cortex and has been implicated in the control of aldosterone secretion (Aguilera et al. 1984), its release from adrenal cortical nerve fibers remains controversial. Innervation of the adrenal cortex has been demonstrated in a number of species with a variety of techniques (Unsicker 1971; Migally 1979; Robinson et al. 1977; Varndell et al. 1984; Holzwarth 1984). Only Robinson et al. (1977), studying the adrenals of sheep, described catecholaminergic innervation in detail.

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Because of evidence for sympathetic nervous mediation of cell proliferation during the compensatory adrenal cortical growth response to unilateral adrenalectomy (Kleitman and Holzwarth 1985), the present study was undertaken to investigate the catecholaminergic innervation of the adrenal cortex of the rat. We examined the distribution and source of this innervation, and the fine-structural relationship of these nerves to adrenal cortical cells.

Materials and methods

Animals

Forty-five male Sprague-Dawley rats (150–300 g, Holtzman) were housed in pairs on a 12:12 light-dark schedule with food and water ad libitum. For the sympathectomy study, pregnant Sprague-Dawley rats were purchased and pups were raised with their mothers until weaned at day 21.

Histofluorescence of catecholamines

Catecholamines were demonstrated using two induced histofluorescence techniques, the sucrose-potassium phosphate-glyoxylic acid (SPG) method (de la Torre 1980) and the aqueous aldehyde (FAGLU) method (Furness et al. 1978). To enhance visualization of catecholamines, the monoamine oxidase inhibitor, pargyline (Sigma, 400 mg/kg i.p.) was administered 1–4 h before sacrifice.

In brief, for SPG, rats were decapitated, and the adrenal and surrounding tissues were quickly frozen. Twelve to 20 μm sections were cut at −30° C, and dipped in SPG solution (1% glyoxylic acid in 0.2 M sucrose and 0.24 M KH_2PO_4, pH 7.4). The sections were dried, covered with mineral oil and heated to 95° C for 2.5 min. For FAGLU, anesthetized rats were perfused with FAGLU solution (4% paraformaldehyde-1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0). Adrenals and surrounding tissue were sectioned at −20° C. Ten-μm sections were floated on FAGLU at room temperature then mounted on slides and dried.

Surgical manipulations

To determine whether catecholaminergic nerves observed in the splanchnic nerve contribute to the innervation of the adrenal cortex, the left splanchnic nerve of a group of rats was ligated just proximal to the suprarenal ganglion. Complete ligation distal to this ganglion is unfeasible be-

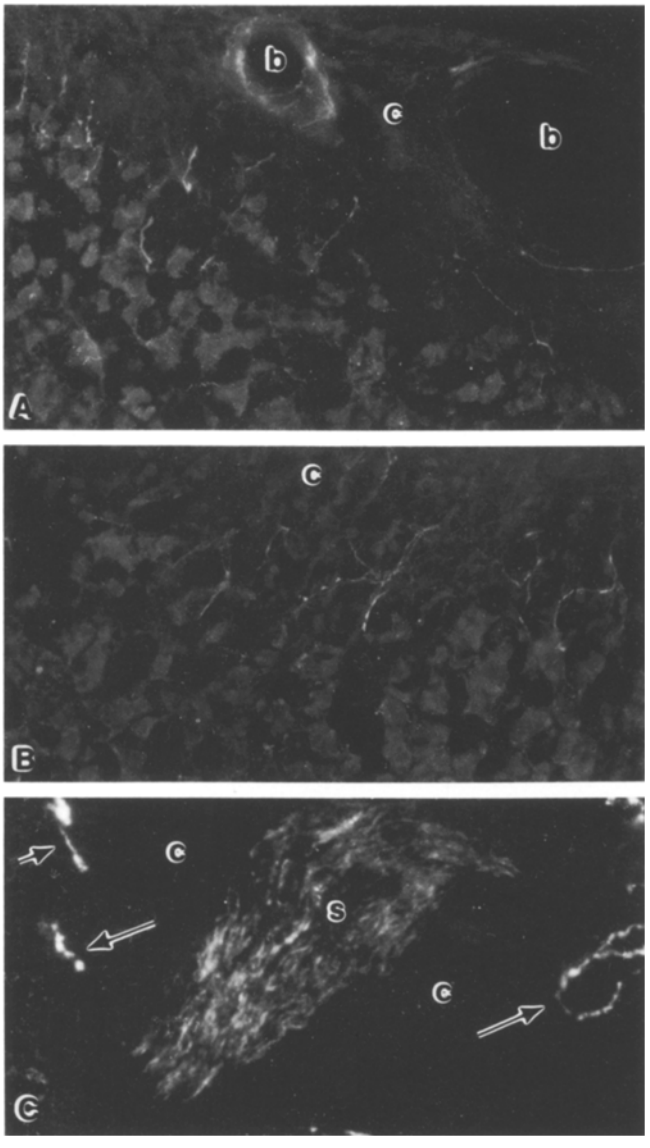


Fig. 1 A–C. Catecholaminergic nerve fibers in the zona glomerulosa and capsule of normal rat adrenal cortex. **A** Fluorescent fibers surround two capsular blood vessels (*b*) seen in cross section. Individual varicose fibers enter the cortex below the capsule (*c*) and course along glomerulosa cells. **B** Individual catecholaminergic fibers enter the adrenal cortex directly through the capsule (*c*), not associated with adrenal arteries observed in this or adjacent sections. **A, B** FAGLU histofluorescence. ×300. **C** Fluorescent fibers in the splanchnic nerve (*s*) as the nerve penetrates the adrenal capsule (*c*), en route to the medulla. Splanchnic nerve fibers are unbranched and not varicose in contrast to the varicose vascular fibers found beneath the capsule and outside the adrenal (*arrows*). SPG histofluorescence. ×300

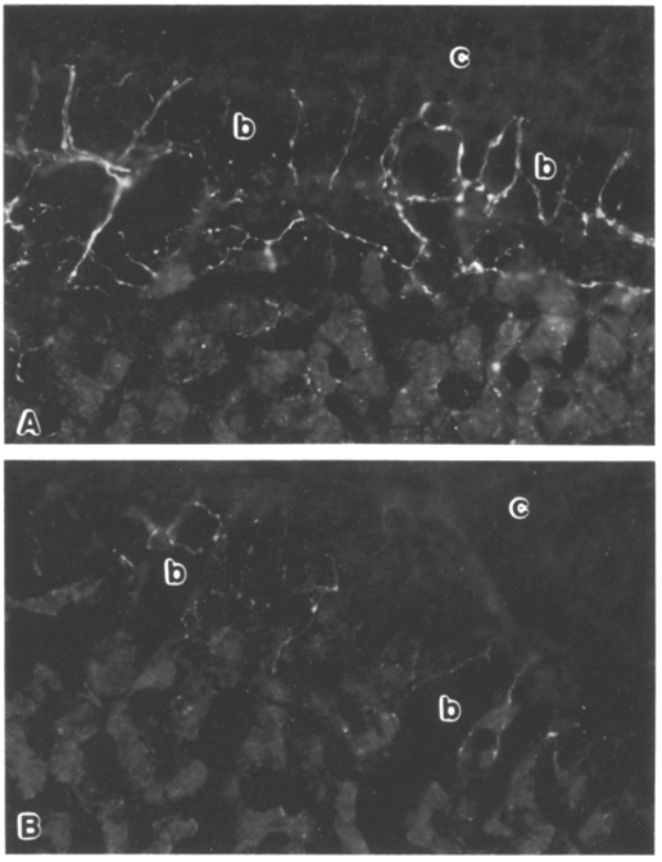


Fig. 2 A, B. Disruption of the splanchnic nerve did not affect the distribution of fluorescent fibers in the adrenal cortex or along adrenal blood vessels. **A** Two days after ligation of the splanchnic nerve, a plexus of fibers is shown running along a subcapsular (*c* capsule) blood vessel (*b*), cut longitudinally. Varicose fibers disperse from this plexus into the adjacent zona glomerulosa. **B** Two days after extirpation of the suprarenal ganglion, fibers were still observed among glomerulosa cells. FAGLU histofluorescence. ×300

cause the splanchnic nerve separates into many small branches. Therefore, to test whether the suprarenal ganglion was the source of the fluorescent fibers observed in the adrenal cortex, we extirpated the left suprarenal ganglion in another group of rats. Two or four days after surgery, both adrenals from each animal were prepared with FAGLU. Alternate sections were stained with cresyl violet to verify the ligation or extirpation.

Sympathectomy

In conjunction with studies on the role of sympathetic nerves in compensatory adrenal cortical growth (Kleitman and Holzwarth 1985) we tested whether the catecholaminergic nerves observed in the adrenal cortex were sensitive to chemical sympathectomy. Every other day for the first two weeks of life, rats were injected subcutaneously with guanethidine (ismelin monosulfate, CIBA-Geigy; 20 mg/kg in saline), 6-hydroxydopamine (6-OHDA, Sigma; 50 mg/kg in 10 μM HCl containing 10% ascorbic acid), or vehicle solutions. At 19 and 40 days of age, rats were prepared for fluorescence microscopy with FAGLU. To assess the efficacy of the drugs, the superior cervical ganglia were examined with standard histological methods.

Electron microscopy

The zona glomerulosa was examined ultrastructurally to assess the distribution of nerve fibers in relation to parenchymal cells. Visualization of catecholamines was enhanced by fixation in the presence of chromium salts (Tranzer and Richards 1976) and injections of the false transmitter, 5-hydroxydopamine (5-OHDA, Tranzer and Thoenen 1967).

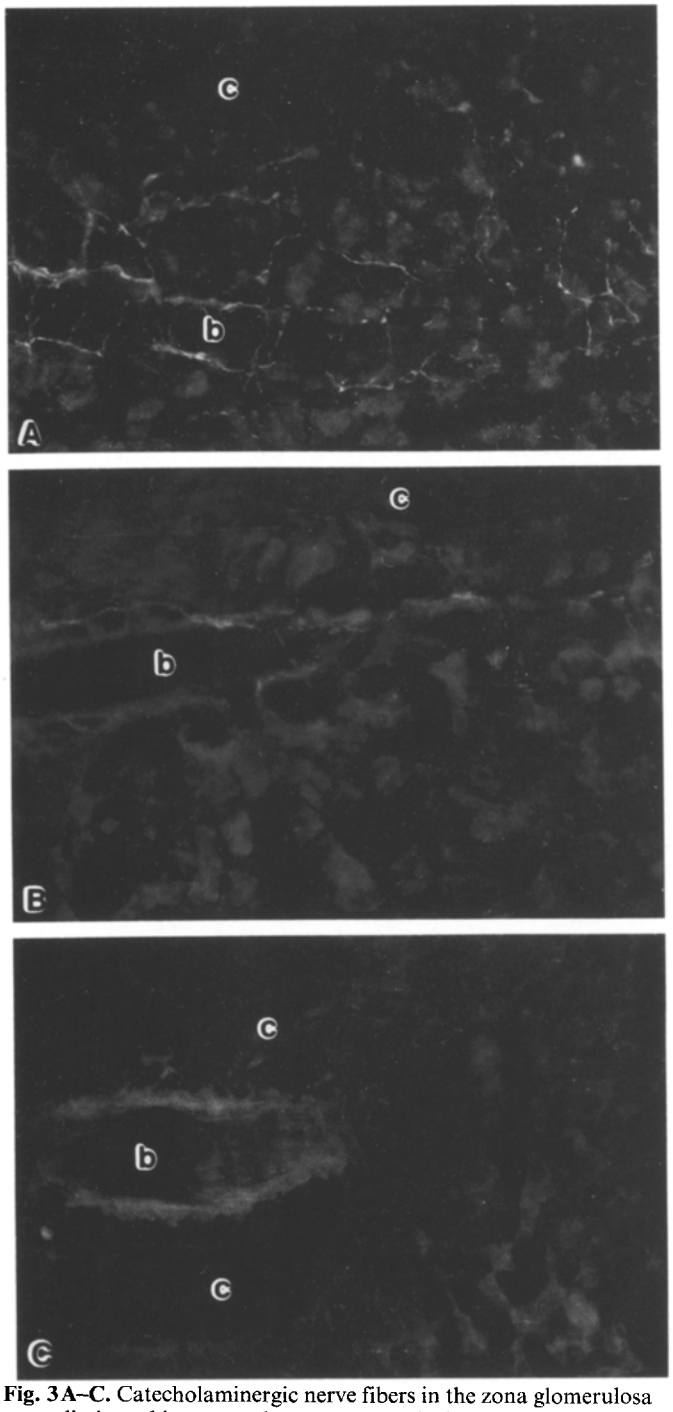


Fig. 3A–C. Catecholaminergic nerve fibers in the zona glomerulosa were eliminated by sympathectomy. **A** In the intact adrenal cortex, fluorescent fibers are shown along a blood vessel (*b*) and distributing among the surrounding glomerulosa cells; *c* capsule. **B** After guanethidine sympathectomy, innervation of blood vessels was drastically reduced and no fibers were seen among adrenal cortical cells. **C** After 6-OHDA sympathectomy, the adrenal capsule, blood vessels and cortex were devoid of sympathetic fibers. Only background fluorescence is apparent in capsular blood vessels and adrenal cells. 40-day-old rats, FAGLU histofluorescence. × 300

Standard fixation. Anesthetized rats were perfused with cold 2% glutaraldehyde-2% paraformaldehyde in 0.1 M phosphate buffer. Adrenals were postfixed at 4° C for 1 h, blocked and stored overnight in 0.2 M sucrose in 0.1 M phosphate buffer, pH 7.2. The tissue was then fixed with cold 1% OsO_4 and embedded in Spurrs low-viscosity medium. Thin sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall 1965).

Dichromate reaction. The procedure was as described above with the following exceptions: The primary fixative was cold 2.5% glutaraldehyde-1.5% paraformaldehyde in 0.1 M chromate-dichromate buffer, pH 7.2, and tissue was stored overnight in chromate buffer, pH 6.2. Some rats in this group were injected with 5-OHDA (Regis, 100 mg/kg, i.p.) 2 h before sacrifice.

Results

Catecholamine histofluorescence

Normal rats. We observed a network of green-fluorescent fibers in the zona glomerulosa of the adrenal cortex. Plexuses of fibers surrounded major capsular and subcapsular blood vessels; individual varicose fibers branched off and dispersed in the zona glomerulosa (Fig. 1A), appearing to course around groups of glomerulosa cells. Some fibers were also seen entering the zona glomerulosa individually through the capsule, apparently unassociated with any blood vessel in the plane of section or in adjacent sections (Fig. 1B). The fluorescent fibers were unevenly distributed around the circumference of the adrenal, being densest in areas where adrenal arteries enter the gland.

There were qualitative and quantitative differences in the results obtained with the two histofluorescence techniques. Although the intensity of fluorescence in fibers observed using SPG was greater, the results with FAGLU were generally superior because with it we observed a greater number and slightly wider distribution of fluorescent fibers. With both techniques, the fluorescent fibers were restricted to the capsule and zona glomerulosa. In only a few instances did we observe individual fibers coursing through the zona fasciculata, apparently en route to the adrenal medulla. These fibers were not varicose, and were never observed in tissue prepared by the FAGLU method. No individual fibers appeared to be associated with blood vessels or cortical tissue within the zona fasciculata or reticularis.

In addition to fibers entering along blood vessels, some fluorescent fibers were observed within the splanchnic nerve both proximal and distal to the suprarenal ganglion, through which the splanchnic nerve passes before entering the adrenal (Fig. 1C). These fibers were not varicose and did not appear to branch into adrenal cortical tissue. Nonetheless, fibers in the splanchnic nerve from the catecholaminergic cells of the suprarenal or other ganglia had to be considered as the possible source of fluorescent fibers in the adrenal cortex. To determine whether splanchnic catecholaminergic nerves contribute to the innervtion of the adrenal cortex, we ligated the left splanchnic nerve just proximal to the suprarenal ganglion in one group of rats, and extirpated this ganglion in another group.

Splanchnic interruption. Up to 4 days after the ligation, the extent and distribution of catecholaminergic fibers in the adrenal cortex were not substantially changed on either the ligated or unligated sides relative to unoperated controls (Fig. 2A). Histological examination of the site of ligation verified that the nerve was completely disrupted. Fluores-

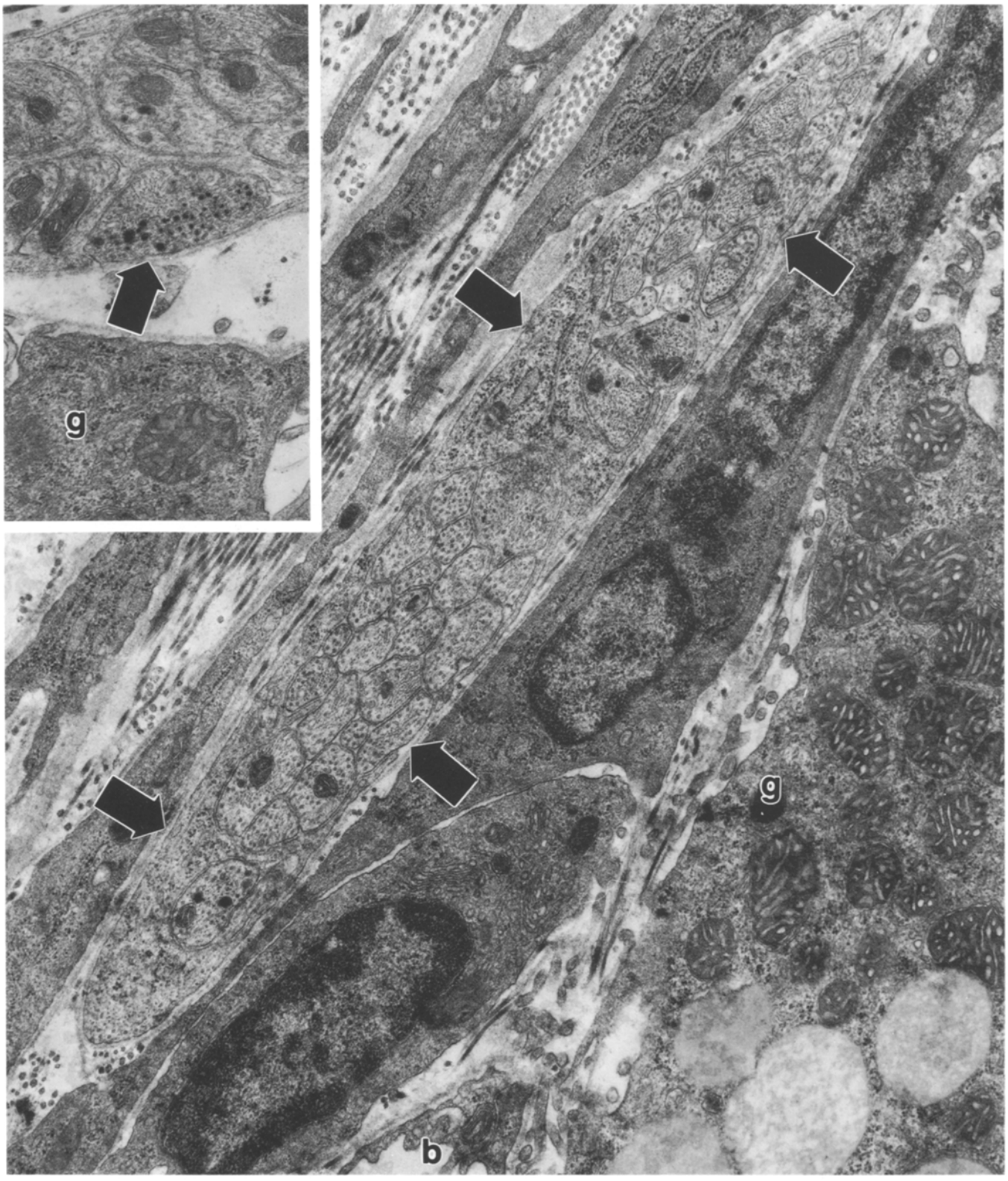
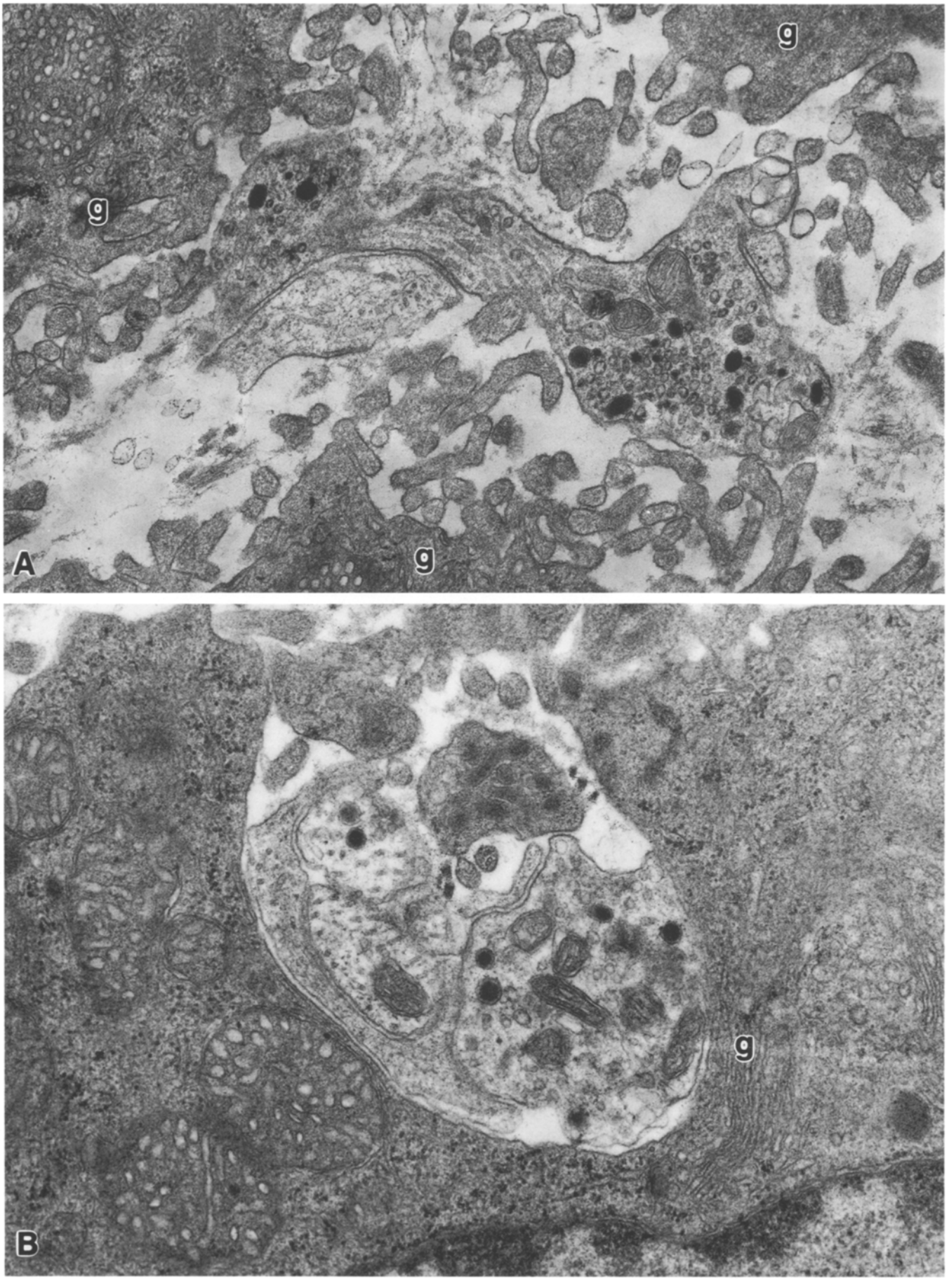


Fig. 4. A large nerve bundle (*arrows*) located beneath the adrenal capsule. Axons enclosed in a Schwann cell sheath, surrounded by basal lamina, contain microtubules, neurofilaments and mitochondria; *b* blood vessel; *g* glomerulosa cell. 5-OHDA injected, chromate fixation. ×22000. *Inset:* An aggregation of small dense-cored vesicles in a varicosity at the periphery of another subcapsular bundle (*arrow*). This varicosity is not ensheathed by a Schwann cell, but is separated from a glomerulosa cell (*g*) by 2 basal laminae. 5-OHDA injected, chromate fixation. ×26000

Fig. 5A, B. Axons in close apposition to adrenal cortical cells (*g*) and their filopodia. **A** Two boutons en passant along a single axon are filled with large and small dense-cored vesicles as well as clear vesicles. The larger varicosity is surrounded by numerous filopodia. The smaller varicosity abuts on a cortical cell. 5-OHDA injected, chromate fixation. ×38000. **B** Axons in a small nerve fascicle appear to be enveloped by a glomerulosa cell (*g*). The largest varicosity is directly apposed to the glomerulosa cell and appears to be a terminal site as it contains an aggregation of small clear vesicles as well as several large dense-cored vesicles and mitochondria. Standard fixation. ×40000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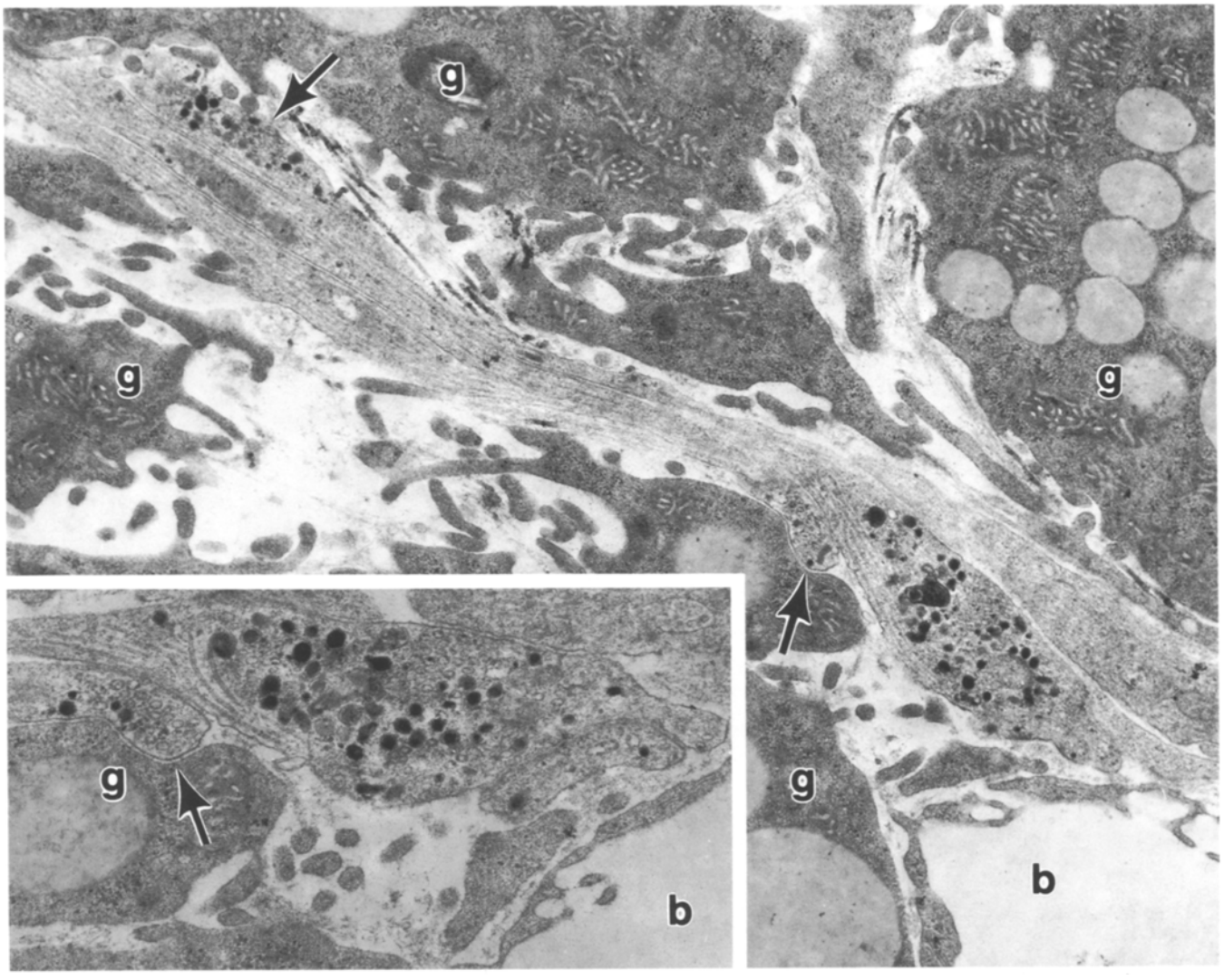

Fig. 6. A nerve fascicle coursing among several glomerulosa cells and a fenestrated capillary (*b*); note that no vascular smooth muscle is apparent. Three varicosities are shown: one in close proximity to glomerulosa cell filopodia (*top arrow*), another in close apposition to a glomerulosa cell (*inset, arrow*), and the third (*inset*) seems to be directed toward the capillary. Filopodia are also present near the latter varicosity. 5-OHDA injected, chromate fixation. ×20000; *inset*: (adjacent section) ×30000

cent fibers in the splanchnic nerve were observed both proximal and distal to the site of the ligation and the suprarenal ganglion cells remained fluorescent after the ligations. Both two and four days after extirpation of the suprarenal ganglion, histofluorescent fibers were still observed in the zona glomerulosa (Fig. 2B). Therefore, splanchnic fibers are not a major source of catecholaminergic fibers in the adrenal cortex.

Sympathectomized rats. Sympathectomy induced by neonatal administration of either guanethidine or 6-OHDA eliminated most catecholaminergic fibers from the adrenal cortex by 19 days of age, and no evidence of recovery was seen up to 40 days (Fig. 3). A few of the fibers on adrenal arteries remained after guanethidine sympathectomy, but no individual fibers were observed in the adrenal cortical tissue (Fig. 3B). After 6-OHDA treatment, virtually no fibers were visible in the adrenal cortex, or along blood vessels (Fig. 3C). Interestingly, the fluorescent fibers within the splanchnic nerve were still evident after these treatments. Unfortunately, the suprarenal ganglion was not examined in these animals. However, the superior cervical ganglion, examined to assess the effectiveness of the sympathectomy, was essentially devoid of fluorescent cells after guanethidine treatment. The 6-OHDA sympathectomy reduced superior cervical ganglion size and cell number to approximately 15% of control, however, the remaining ganglion cells appeared normal and were catecholaminergic.

Electron microscopy

Ultrastructurally, nerve bundles consisting of 5 to 50 axons surrounded by basal laminae were observed in and deep to the adrenal capsule (Fig. 4). Axons in these bundles were usually unmyelinated and sheathed by Schwann cells. The axons contained microtubules, neurofilaments, mitochondria, a few large dense-cored vesicles (60–110 nm) and, at the periphery of the bundles, some fibers contained aggregations of small clear or dense-cored vesicles (30–60 nm, Fig. 4B). Many more boutons containing aggregations of small dense-cored vesicles were observed in tissue from animals injected with 5-OHDA and processed with chromium salts.

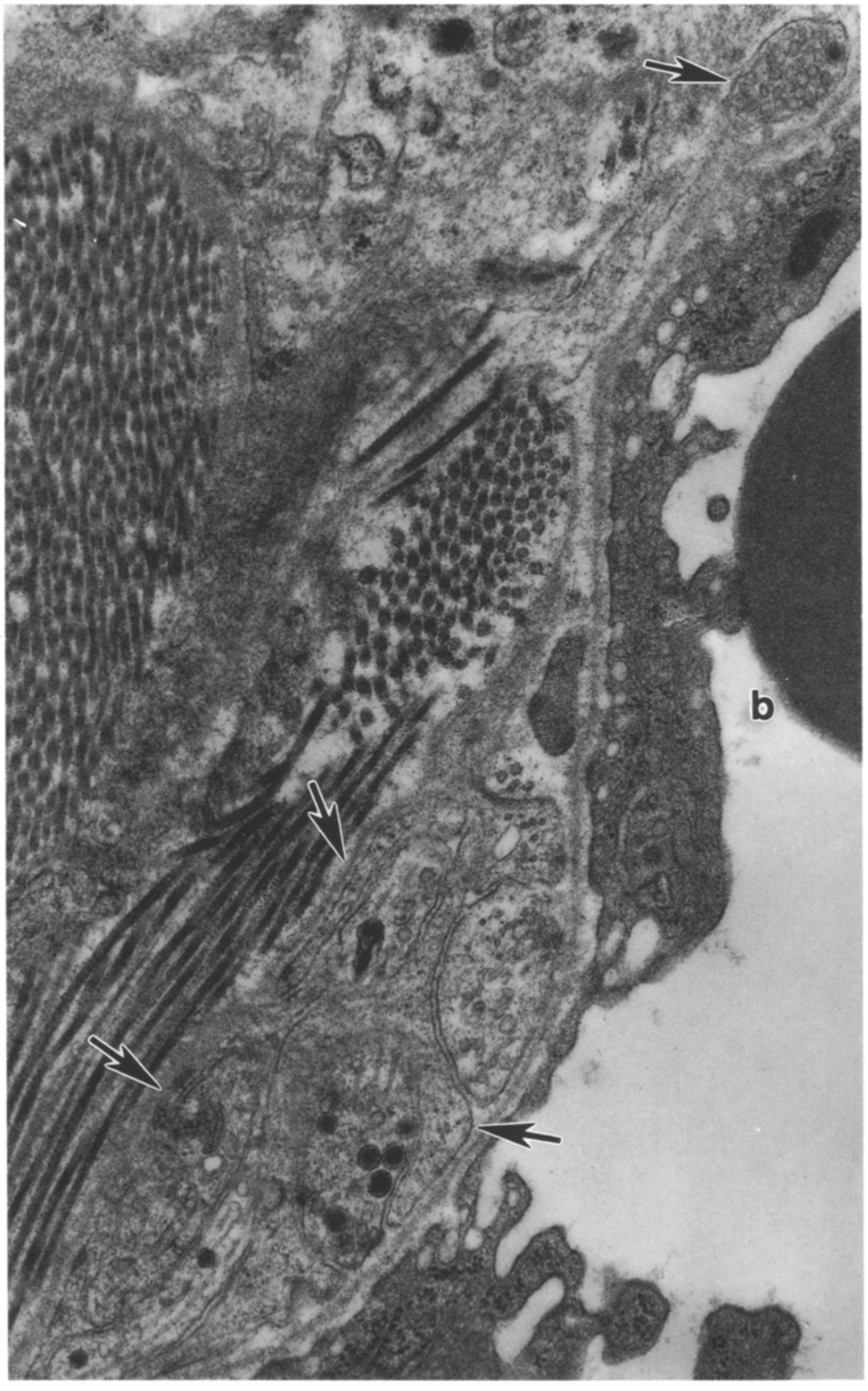

Fig. 7. Nerve fascicle (*arrows*) adjacent to a subcapsular blood vessel (*b*). Note the bouton containing predominantly small clear vesicles (*top arrow*), separated from the endothelial cell by basal lamina. Standard fixation. × 35000

We consistently observed individual fibers and small nerve fascicles in the zona glomerulosa. Individual fibers were located close (as little as 10 nm) to adrenal cells or to their numerous pseudopodia (Figs. 5, 6). Putative terminal areas (boutons en passant) were observed, identified by aggregations of vesicles in dilated regions of the fibers (Fig. 5). These boutons were also found between zona glomerulosa cells and blood vessels (Fig. 6). Many small nerve fascicles were associated with fenestrated blood vessels. Although the vessels were traced in several serial sections and appeared to be devoid of smooth muscle cells, vesicle accumulations were observed in adjacent nerve fibers, separated from the endothelial cells only by basal laminae (Fig. 7).

Based on the combinations of large, small, clear or dense-cored vesicles observed, there appeared to be several types of adrenal cortical nerves. Small dense-cored vesicles

have been shown to be the site of catecholamine storage (Thureson-Klein 1983), suggesting that fibers containing these correspond to fluorescent fibers seen in the zona glomerulosa at the light microscopic level. Some varicosities contained no small dense-cored vesicles, even after 5-OHDA treatment, and were presumably not catecholaminergic.

Discussion

Histofluorescent catecholaminergic fibers enter the adrenal cortex in vascular plexuses, branch into the zona glomerulosa, and disperse among adrenal cortical cells. Ultrastructural evidence confirmed that catecholaminergic nerve boutons are in direct apposition to cortical cells. The catecholaminergic fibers observed in the splanchnic nerve appear not to contribute to the adrenal cortical innervation. These findings are consistent with the observation that regenerated adrenal cortices also contain catecholaminergic fibers despite the absence of the adrenal medulla and its splanchnic innervation (M.A. Holzwarth, M.C. Berkelhamer and N. Kleitman, unpublished).

The appearance of sympathetic nerves in the adrenal cortex is reminiscent of classical descriptions of autonomic innervation whereby a small number of nerves can have a widespread influence. Both sympathetic control of blood flow entering the adrenal cortex and a paracrine signal are potential mechanisms by which a relatively sparse local innervation could affect many cells. Furthermore, because many adrenal cortical cells contact their neighbors with nexuses (Friend and Gilula 1972), neurotransmitters released into the extracellular space near the processes of several parenchymal cells may affect these cells as well as the many cells which they contact.

While the function of sympathetic innervation of the adrenal cortex is not known, catecholaminergic nerves have been implicated in the control of growth and function of adrenal glomerulosa cells. Compensatory adrenal cortical growth, has been clearly shown to be neurally mediated (Dallman et al. 1977), and is sensitive to chemical sympathectomy (Kleitman and Holzwarth 1985). Cell division occurs primarily in the zona glomerulosa during compensatory growth (Reiter and Pizzarello 1966; M.A. Holzwarth unpublished). Furthermore, cells in this zone contain β-receptors which induce steroid synthesis when stimulated by the adrenergic agonist isoproterenol in vitro (Shima et al. 1984). In addition, aldosterone secretion, which is specific to the zona glomerulosa, appears to be inhibited chronically by dopamine, although it remains controversial whether the dopamine originates from nerves in the adrenal cortex (Kvetnansky et al. 1979; Lacković and Relja 1984).

Direct innervation of the adrenal cortex has not been well established despite evidence for neural modulation of adrenal function and anatomical reports of adrenal cortical nerve fibers in several species. While nerve fibers in proximity to adrenal cortical cells had been demonstrated at the ultrastructural level (Unsicker 1971; Migally 1979), the transmitters involved were still largely a matter of speculation. Robinson et al. (1977) demonstrated catecholaminergic nerves in the adrenal glomerulosa of the sheep, a network of vasoactive-intestinal peptide- (VIP)-immunoreactive nerve fibers in the rat adrenal cortex was reported (Holzwarth 1984), but no clear demonstration of catecholaminergic innervation of the rat adrenal cortex existed. The present report extends the description of catecholaminergic nerves in the adrenal glomerulosa to the rat, the species most often used in studies of the role of the nervous system on adrenal cortical growth and function.

It is important to note that species differences do exist in the autonomic innervation of other organs, for example, rats have a sparser hepatic innervation than other species (Metz and Forssmann 1980). Innervation of the adrenal cortex may also be relatively limited in the rat. Migally (1979) observed nerve fibers in the zona reticularis of the mouse adrenal and we have observed more catecholaminergic fibers in the zona glomerulosa of the guinea pig than the rat, as well as fibers in the zona reticularis (M.A. Holzwarth and N. Kleitman, unpublished).

With the methods used in the present study we cannot specify which biogenic amine is present, but we can infer from other results its probable identity. Immunoreactivity to neither serotonin (Holzwarth et al. 1984) nor phenylethanolamine-N-methyltransferase (PNMT) (Holzwarth and Brownfield 1983) were observed in the adrenal cortex, making it unlikely that histofluorescent fibers contained serotonin or epinephrine. Furthermore, immunocytochemical evidence (Varndell et al. 1984) and receptor-binding studies (Shima et al. 1984) suggest that norepinephrine is a neurotransmitter in the zona glomerulosa. The presence of dopamine and dopamine receptors have also been demonstrated in the adrenal cortex (Kvetnansky et al. 1979; Lacković and Relja 1984) but as yet there is no evidence for the localization of dopamine as a tranmitter within nerve terminals. Contrary to the findings of the present study, the dopamine content of the adrenal cortex was shown to decrease with splanchnic ligation (Lacković et al. 1981) but was not decreased by chronic guanethidine treatments (Kvetnansky et al. 1979). Thus norepinephrine seems likely to be the transmitter of the nerve fibers observed in the present study.

Ultrastructurally, a variety of vesicle types were observed, even within individual fibers. Catecholamines are reported to be localized in the small dense-cored vesicles in the rat (Thureson-Klein 1983). While our observation of some boutons with both small clear and dense-cored vesicles, even in tissue prepared to maximize the stability of catecholamines, may represent a failure to preserve all of the amines in these fibers, it more likely represents the co-distribution of several transmitters within single nerve fibers. Co-distribution of norepinephrine with enkephalins (Varndell et al. 1984) in the adrenal glomerulosa may explain the present observations. Although VIP is also found in the zona glomerulosa, its distribution is more extensive and homogeneous than that observed for catecholamines (Holzwarth 1984). This, together with the observation that VIP-immunoreactive fibers are unaffected by sympathectomy, argues that these represent a separate population from the catecholaminergic fibers (M.A. Holzwarth and N. Kleitman, unpublished).

Although the description of catecholaminergic nerve fibers and their relation to adrenal parenchymal cells was a major goal of the present work, it is important to note that many catecholaminergic nerves were observed along blood vessels in the adrenal capsule and zona glomerulosa but not along the sinusoids of the zona fasciculata or reticularis. This limited distribution suggests that the sympathetic nervous system regulates blood flow upon entry into the adrenal gland. The classical description of this type of con-

trol is the innervation of smooth muscle surrounding vessels no smaller than precapillary arterioles (Vanhoutte et al. 1981). The present observation that many nerves in the zona glomerulosa are directly apposed to fenestrated endothelial cells was unexpected. It is possible that the effect of neural activity is transferred to nearby smooth muscle through the endothelial cells, or that nerves have a direct effect on the endothelial cells that they contact (Bevan and Duckles 1975; Raichle et al. 1975). Alternatively, these nerves could serve a neurohormonal role as a source of locally circulating catecholamines or of peptides such as VIP, neuropeptide-Y, or enkephalin.

In summary, we have observed along the blood vessels and dispersed among the cells of the zona glomerulosa of the rat adrenal cortex fluorescent catecholaminergic nerves that are sensitive to sympathectomy but not to disruption of the splanchnic nerve. Ultrastructural evidence supports light-microscopic observations that fibers that enter along blood vessels also innervate parenchymal cells. The proximity of varicosities to zona glomerulosa cells suggests that they influence adrenal cell function directly.

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