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The innervation of the adrenal gland
I. The source of pre- and postganglionic nerve fibres to the rat adrenal gland*

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INTRODUCTION

It is now accepted that the chromaffin cells of the adrenal medulla are innervated by preganglionic sympathetic neurons present in the thoracolumbar segments of the spinal cord (Young, 1939; Coupland, 1965*a*). The fibres of these preganglionic neurons form part of the splanchnic nerves in the mammal and these nerves are thought to be cholinergic (Feldberg, Minz & Tsudzimura, 1934; Coupland, 1965*a*, *b*; Lewis & Shute, 1969).

However, several studies have shown the presence of both cholinergic and adrenergic nerve terminals as well as myelinated and unmyelinated fibres in the adrenal medulla (Unsicker, 1973; Coupland 1972, 1984). Prentice & Wood (1974, 1975) demonstrated adrenergic fibres and terminals with bouton *en passage* configuration adjacent to noradrenergic cells in the adrenal medulla of the cat.

Earlier evidence of a postganglionic adrenergic innervation to the adrenal chromaffin cells was reported by Young (1933), in which he described postganglionic fibres ending in relation to chromaffin cells in the dogfish *Scyliorhinus canicula*. Furthermore, Swinyard (1937) observed up to 30% undegenerated fibres in the adrenal medulla after section of the last ten thoracic and first two lumbar spinal nerves. He suggested that these remaining fibres were postganglionic. Celler & Schramm (1981) provided physiological evidence for postganglionic fibres running in the splanchnic nerve to the rat adrenal medulla.

Despite the above evidence, only the distribution of the preganglionic cell bodies innervating the adrenal medulla have been studied using retrograde fibre techniques (Ellison & Clark, 1975; Schramm, Adair, Stribling & Gray, 1975; Holets & Elde, 1982).

Therefore, it was decided, firstly, to search for a postganglionic sympathetic supply to the adrenal medulla and, secondly, to confirm the source of the preganglionic input to the gland, using the retrograde fluorescent tracer Fast Blue.

MATERIALS AND METHODS

Ten male adult Wistar rats (250 g) were used in this study. They were kept under standard laboratory conditions with food and water *ad libitum*.

The animals were anaesthetised with an intraperitoneal injection of sodium pentobarbitone of 60 mg/kg body weight. The area of skin around the thoracic spine

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was then shaved of fur and swabbed with 70% ethanol. A midsagittal incision was made in the skin from the fifth thoracic vertebra to the fifth lumbar vertebra and the skin was reflected laterally. The left kidney was then exposed retroperitoneally through a paramedian incision extending from the twelfth thoracic to the fourth lumbar vertebra. The retroperitoneal fat above the kidney was retracted exposing the left adrenal gland. Care was taken not to compromise the blood and nerve supply of the gland. The posterior approach was used to minimise the trauma to the animal and also to avoid entering the peritoneum.

A 2% aqueous suspension of Fast Blue (Dr Illing Makromolekulare Chemie, Gross-Umstadt, FRG) was slowly pressure injected (5 μl) into the adrenal medulla over a period of 15 minutes via a glass micropipette (tip diameter 20 μm) coupled to a 25 μl Hamilton syringe. The micropipette was held in a specially constructed holder and fitted into a stereotaxic frame (David Kopf Instruments). The micropipette was advanced using a hydraulic drive (Clark Electricals, UK).

In all injections the micropipette remained in the gland for 5 minutes after completion of delivery and was then slowly withdrawn over a further period of 10 minutes to allow tracer to drain from the tip and be taken up by the tissues, thus preventing the escape of the tracer by backflow along the track created by the micropipette. The entry point of the micropipette in the capsule was then gently cauterised electrically. Care was taken to avoid spilling of the tracer and any animal with a suspected spill was rejected. No evidence of leakage of tracer from the penetration point was seen since, in any of the animals processed, subsequent examination of the surface of the gland and adjacent tissues showed them to be fluorescence-free.

The gland was returned to its original position and the posterior abdominal wall sutured. The animal was allowed to recover and returned to the animal house.

After five days the rats were anaesthetised as previously and perfused transcardially in the following sequence: prewash (containing 3% dextran 70 and 1% procaine hydrochloride) at body temperature for 1 minute and at a pressure of 120 mmHg; fixative (10% depolymerized paraformaldehyde in 0·1 M phosphate buffer, pH 7·3) at room temperature at a pressure of 120 mmHg for 20 minutes. Fixation was terminated with 0·1 M phosphate buffer containing 10% sucrose (w/v) perfused for a further 10 minutes.

The paravertebral and prevertebral ganglia as well as the thoracic and upper lumbar segments of the spinal cord were dissected out. The location and course of the splanchnic nerve and associated pre- and paravertebral ganglia can be seen in Figure 7. The ganglia were embedded in fresh ox liver and 20 μm frozen sections cut on the cryostat (Bright Instruments).

The longitudinal extent of each spinal segment was defined by estimating the midpoint between the point of entry of the corresponding ventral roots and the adjacent ventral roots. The segmental borders were marked by a needle track and 20 μm serial sections were cut either longitudinally (dorsoventral direction) or transversely.

The sections were picked up from the knife edge with chrome alum gelatin-coated slides, air dried and examined systematically at low magnification ($\times$25 oil) using a Leitz Ortholux microscope fitted with Ploempack fluorescent system using excitation wavelengths of 360 and 390 nm.

The morphology, location and number of retrogradely labelled neurons were noted. To avoid the possibility of double counting, only those cells where the nucleus was visible were registered. Fluorescent photomicrographs of labelled cells were taken on Kodak Ektachrome 200 ASA film at $\times$25 and $\times$63.

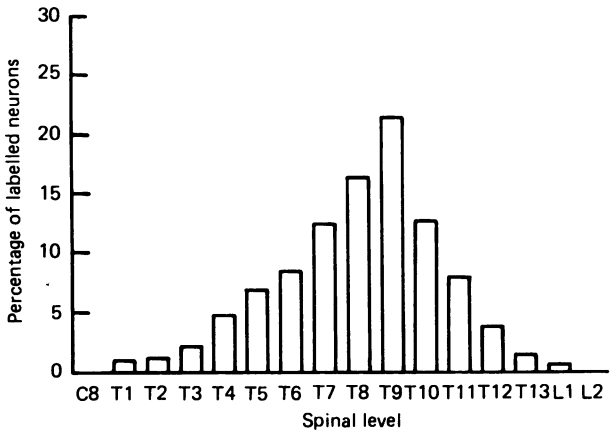


Fig. 1. Percentage contribution of retrogradely labelled neurons in the spinal cord after injection of Fast Blue into the left adrenal gland.

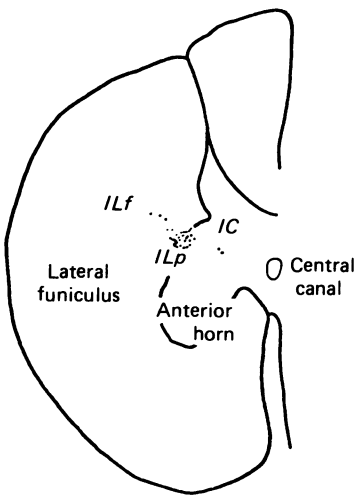
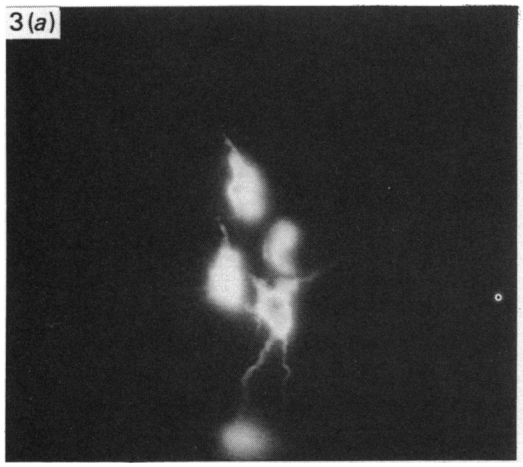


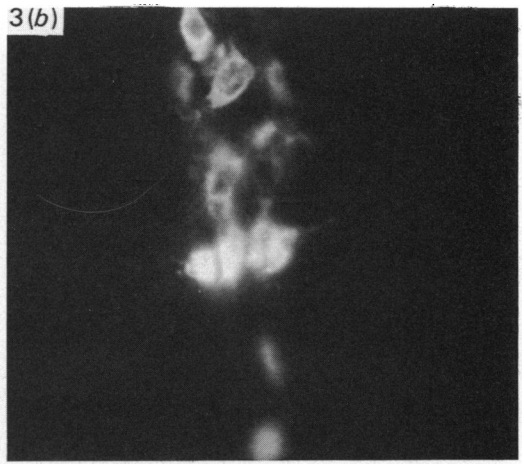
Fig. 2. Schematic diagram to illustrate the distribution of labelled neurons in the ILf, ILp and IC nuclei of the spinal cord. Transverse section.

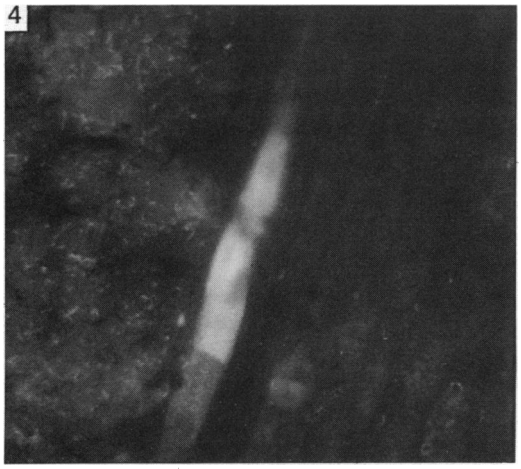
A series of five animals was used to establish the extent of spread of the tracer within the adrenal gland. At 1 and 3 hours following the injection of tracer, the animals were perfusion-fixed as above and 20 μm serial sections cut from the excised glands.

RESULTS

Examination of the Fast Blue-injected adrenal glands after 1 hour, 3 hours and 5 days showed that the focal point of tracer deposition was the medulla, which in all cases showed fluorescence throughout its extent. However, it was evident that in some cases the tracer had spread from the injection site into the inner cortex and occasionally adjacent to the micropipette track, as far as the inner part of the zona glomerulosa. This was particularly evident in the glands examined after 1 hour and 3 hours. Examination of the glands after 5 days revealed a more discrete medullary distribution of tracer. No fluorescence was found either on the inner surface of the







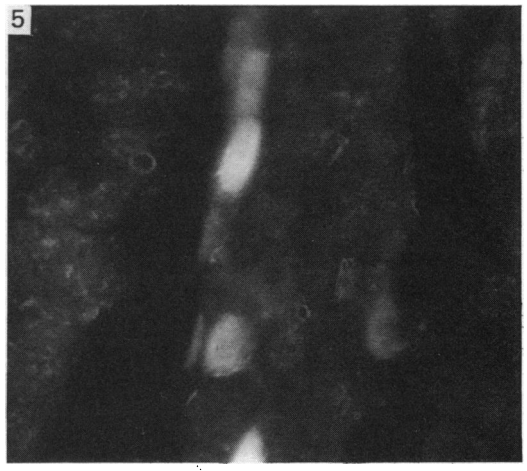


Fig. 3 (*a–b*). Horizontal sections through the spinal cord showing clusters of Fast Blue-labelled cells in the ILp nucleus. In (*a*) note the axon processes running in a longitudinal direction. In (*b*) note the reduction in the number of labelled neurons rostrocaudally. × 300.

Fig. 4. A group of three Fast Blue-labelled paravertebral ganglion cells in the left sympathetic chain. × 300.

Fig. 5. Fast Blue-labelled cells in the suprarenal ganglion. × 300.

capsule or on the external surface of the gland at any of the time intervals examined.

To test whether small amounts of tracer which may have entered the adrenal blood vessels could result in spurious labelling of neurons, 5 μm of tracer was injected into the circulation via the tail vein. No labelled cell bodies were identified in any of the tissues (brain, spinal cord and peripheral autonomic nervous system) examined.

Distribution of Fast Blue-labelled cells

Spinal cord

Retrogradely labelled cells were found in the spinal cord from T1 to L1 segments (Fig. 1). Labelling was always ipsilateral to the side of the injection. No labelled cells

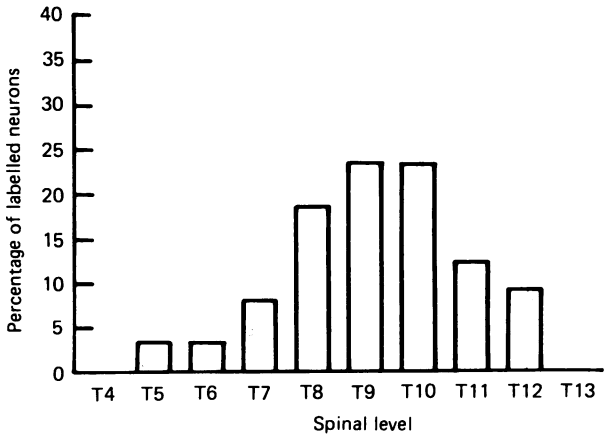


Fig. 6. Percentage contribution of Fast Blue-labelled neurons in the ganglia of the left paravertebral sympathetic chain.

were found in the cervical, lower lumbar or sacral levels of the spinal cord. Sixty three percent of the total labelled cells were found within segments T7 to T10, the highest percentage occurring in the ninth thoracic segment (Fig. 1). The total number of labelled cells within a spinal cord ranged from 543 to 798 ($\bar{x}$ = 698·4, S.E. = 27·2). The labelled cells were present in three regions of the intermediate zone as defined by Petras & Cummings (1972). Approximately 95% of the fluorescent cell bodies were present in the nucleus intermediolateralis pars principalis (ILp), 4% in the nucleus intermediolateralis pars funicularis (ILf) and 1% in the nucleus intercalatus (IC) (Fig. 2). No labelled cells were found in the central autonomic nucleus.

Labelled cells in the lateral funiculus were mostly spindle-shaped with their long axis running in the mediolateral direction. In parasagittal sections they appeared spherical. Their processes ran transversely making contact with cells in the intermediolateral column. The cells of the nucleus intercalatus lie medial to the intermediolateral column. This cell group contained both spindle-shaped and spherical cells with processes projecting towards the intermediolateral cell column and the central canal.

The cells in the intermediolateral zone did not form a continuous column within the cord, they appeared as clusters and, within a segment, the number of labelled cells decreased as one moved in a rostral to caudal direction from mid-segment (Fig. 3*b*). The labelled cells were either bipolar or multipolar. The majority of the cells in the intermediolateral cell column were bipolar and their long axis was approximately double the length of the short axis (22 × 8 μm). In the longitudinal sections, the axonal processes of labelled cells could be seen. Although many of the processes extended in several directions, the majority ran longitudinally in the cord along the junctional region between the lateral horn and lateral funiculus (Fig. 3*a*).

Sympathetic chain

Retrogradely labelled cells were found in the paravertebral sympathetic ganglia extending from T5 to T12 vertebrae inclusive. No labelled cells were found in other paravertebral ganglia. The stellate ganglion was taken as the first thoracic ganglion for the purposes of reference.

The distribution of labelled ganglion cells in the left sympathetic chain, after injection of Fast Blue into the left adrenal medulla, can be seen in Figure 6. Most of

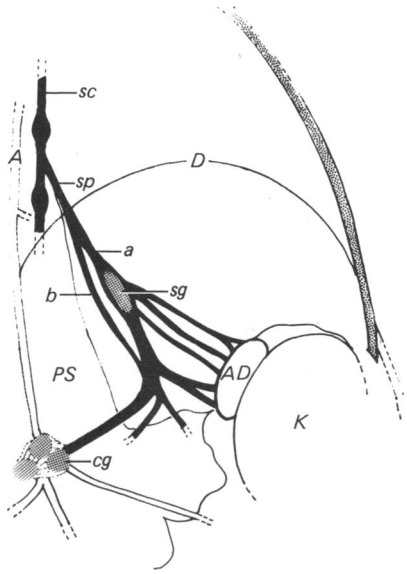


Fig. 7. A diagram to show the course of the greater splanchnic nerve innervating the left adrenal gland. *K*, left kidney; *AD*, left adrenal gland, *A*, aorta; *D*, diaphragm; *PS*, psoas muscle; *sp*, greater splanchnic nerve; *a*, anterior and *b*, posterior divisions of the splanchnic nerve; *sg*, suprarenal ganglion; *cg*, coeliac ganglia and plexus; *sc*, sympathetic chain.

the retrogradely labelled postganglionic cell bodies were found in the ganglia between the level of the eighth thoracic and the tenth thoracic vertebra. A total of 55–80 ($\bar{x}$ = 68, S.E. = 2·5) cells were labelled in the left sympathetic chain. Labelled cells did not appear to have a zonal distribution in the ganglia. The retrogradely labelled ganglion cell bodies were usually spherical (20 × 20 μm) but an occasional fusiform cell (25 × 20 μm) was labelled. The processes of these ganglion cells were rarely seen and they could be followed only for a very short distance. Retrogradely labelled postganglionic cell bodies in a paravertebral ganglion, at the level of the sixth thoracic vertebra, are illustrated in Figure 4.

Suprarenal ganglion

The labelled cells found in the paravertebral ganglion were morphologically similar to labelled cells in the ganglia of the sympathetic chain (Fig. 5). Between 14 and 28 ($\bar{x}$ = 21, S.E. = 1·7) labelled cells were found in each suprarenal ganglion. No labelled cells were found in the coeliac ganglia.

Labelled postganglionic cells in the pre- and paravertebral ganglia accounted for 11·4% of the total number of labelled neurons found within the spinal cord, sympathetic and suprarenal ganglia, following the injection of tracer into the adrenal medulla.

DISCUSSION

Conclusive evidence is presented to show that the adrenal gland and especially the adrenal medulla in the rat receives both pre- and postganglionic sympathetic innervation. This is the first report to our knowledge to demonstrate the ganglionic source, using a retrograde tracer, of postganglionic sympathetic nerves supplying the adrenal gland.

Retrograde axonal transport of Fast Blue was found to be particularly suited to studying the distribution of neurons projecting to the adrenal gland, permitting fast and reliable identification of labelled cells. Particular care was taken to prevent leakage of tracer from the injection site. On the rare occasions when this did occur the animal was rejected. The possibility of labelling of neurons by the intravascular injection of tracer was excluded by control experiments, but it is likely that the relatively uniform labelling of the medulla noted was in part a consequence of entry of tracer into the medullary blood capillaries (Coupland & Selby, 1976). If the labelling were due to a more general vascular transport one would have expected bilateral labelling of the cord. In the current work labelling was exclusively ipsilateral.

Examination of the deposition site of Fast Blue in the adrenal glands at 1 hour, 3 hours and 5 days after injection indicated that the majority of nerve terminals exposed to the tracer would be situated within the adrenal medulla. However, owing to the subsequent spread of tracer within the gland away from the deposition site and the persistence of tracer along the deeper part of the micropipette track, it must be concluded that some nerve terminals as far peripherally as the zona glomerulosa may also have been exposed to small amounts of tracer.

The majority of labelled preganglionic cell bodies were present in spinal segments T1 to T10 with the greatest contribution (22·8%) arising from T9. This agrees well with the results of Holets & Elde (1982). However, Schramm *et al.* (1975) found that the left adrenal gland received the greatest contribution from T8 and the right gland from T9, although they found a similar overall distribution of labelled cells. Both these results refer to the rat adrenal gland. No labelled cell bodies were found in the contralateral intermediate horn, indicating that the innervation is purely ipsilateral.

The majority of labelled preganglionic cells were found in the nucleus intermediolateralis pars principalis (ILp) of the spinal cord. This agrees well with Neuhuber, Sandoz & Fryscak (1986), who showed that the majority of fibres carried in the splanchnic nerve arise from cells in the ILp and ILf. Since it is to this region that supraspinal control centres would be likely to project, it is interesting to note that Seybold & Elde (1984) showed that A_2-adrenergic and serotonergic receptors were more concentrated over neurons in this region than over those in adjacent regions of the intermediolateral horn. These cells have also been reported to be associated with nerve endings containing 5-HT and Substance P-like immunoreactivity (Appel, Wessendorf & Elde, 1986).

The distribution of labelled postganglionic cells in the sympathetic chain was restricted to paravertebral ganglia from T5 to T12. It is interesting to note that the greatest contribution came from ganglia at T9 and T10 vertebral levels and hence closely follows the distribution of labelled preganglionic neurons. It was unexpected to find that the only prevertebral ganglion to contain cells projecting to the adrenal medulla was the suprarenal ganglion. Hence, in the rat, the ganglia of the coeliac plexus do not project to the adrenal gland.

So far as preganglionic fibres are concerned, there is good evidence that these are destined entirely or mainly for the medulla where the majority synapse with chromaffin cells. The distribution of postganglionic axons which enter the adrenal gland is less completely understood. Prentice & Wood (1975) have suggested that in the cat, as evidenced by the uptake of the false transmitter 5-hydroxydopamine, postganglionic adrenergic fibres innervate chromaffin cells. However, in the mouse there is no evidence of adrenergic innervation of the adrenal medulla (Kent & Coupland, 1981) and the majority of adrenergic nerve fibres labelled by both 3H-noradrenaline and 3H-adrenaline lie in the zona glomerulosa. No evidence of

adrenergic innervation of the adrenal medulla has been obtained in the rat (Coupland, unpublished). Recently however, Kleitmann & Holzwarth (1985) have reported on the distribution of catecholamine nerves in the rat adrenal cortex using both fluorometric methods and electron microscopy in the normal and chemically sympathectomised animals. They observed nerve fibres and varicosities in the proximity of blood vessels and parenchymal cells of the zona glomerulosa. These were not observed after neonatal chemical sympathectomy by 6-hydroxydopamine but persisted – in part at least – after section of the greater splanchnic nerve. This later observation is in keeping with the present finding of labelled postganglionic neurons within the suprarenal ganglion. The axons of these cells are distributed to the adrenal gland.

Some of the labelled postganglionic neurons may of course have contained other transmitters and in this regard, it is important to note that Hökfelt, Lundberg, Schultzberg & Farenkrug (1981) and Holzwarth (1984) described the distribution of vasoactive intestinal peptide in both rat adrenal cortex and medulla.

Therefore it may be concluded that the preganglionic fibres predominantly innervate chromaffin cells of the adrenal medulla and that the postganglionic fibres may predominantly innervate structures within the adrenal cortex. Further work using localised iontophoretic injections of tracer into the adrenal gland is in progress to clarify this issue.

SUMMARY

The sympathetic innervation of the rat adrenal medulla was studied using the fluorescent tracer Fast Blue.

Labelled preganglionic cell bodies were located in the intermediolateral horn of the spinal cord at segments T1 and L1; the greatest number was found in T9. The ILp nucleus contained 95%, the ILf nucleus 4%, and the IC nucleus 1% of the total number of labelled preganglionic cells.

Labelled postganglionic cell bodies were found in the sympathetic ganglia at levels T4 and T12; the maximum number were located in ganglia at T9 and T10. In addition, labelled cells were found in the suprarenal ganglion. No labelled cells were found in the ganglia of the coeliac plexus.

The number of labelled preganglionic cells in the spinal cord accounted for 88·6%, the labelled cells in the sympathetic chain for 8·7% and those in the suprarenal ganglia for 2·7% of the total number of labelled cells found.

The detailed distribution within the gland of postganglionic axons has yet to be determined, but it is thought that some are destined for the adrenal cortex.

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