sen to de-emphasize differences between experimental groups. Our intention in using different stressed groups was to demonstrate the ubiquity of our phenomenon (which we have succeeded in doing) and its independence of the effects of ovarian hormones which are known to modulate food intake and body weight [G. N. Wade, *J. Comp. Physiol. Psychol.* **88**, 183 (1975)].
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Tracing Axons and Axon Collaterals of Spinal Neurons Using Intracellular Injection of Horseradish Peroxidase

Abstract. *Intracellular injection and subsequent histochemical localization of horseradish peroxidase have been used to stain the soma, dendrites, axons, and axon collaterals of spinocervical tract neurons and unidentified dorsal horn neurons in the cat. This technique may be used in combination with the intracellular injection of Procion yellow to demonstrate by light microscopy connections between physiologically typed vertebrate neurons.*

Techniques for staining neurons by the intracellular injection of dye through a microelectrode have made it possible to study morphological connections between physiologically identified neurons (*1*). Among the many substances that have been injected into nerve cells, cobaltous chloride and Procion yellow have provided the most complete picture of physiologically identified neurons. But, neither Procion yellow nor cobaltous chloride is ideal for studying the connections between vertebrate neurons. Their major disadvantage is that their rate of movement along axons appears to be positively related to the diameter of the processes (*2*). Although Procion yellow stains somata and dendrites, it has severe limitations for staining vertebrate axons and particularly axon collaterals (*3*, *4*).

To overcome the above difficulties we developed a technique in which we use orthograde axonal transport (*5*) followed by histochemical localization of intracellularly injected horseradish peroxidase (HRP). An advantage of using axonal transport is that there is evidence to suggest that substances are transported along axons at rates independent of the axonal diameter (*6*).

Experiments were performed on chloralose anesthetized cats (70 mg per kilogram of body weight) that were immobilized with gallamine triethiodide and given artificial respiration. Spinocervical tract neurons, or other dorsal horn neurons which received cutaneous inputs (*7*), were impaled with broken glass microelectrodes (*4*) that had been filled under pressure with 4 percent HRP (type VI, Sigma) in 0.05*M* tris-HCl buffer which contained 0.2 mole of KCl per liter (*p*H = 8.6). Successful penetrations were made with electrodes having a resistance of 5 to 20 megohm, which proved very stable for the passage of both depolarizing and hyperpolarizing currents. Horseradish peroxidase was passed from the microelectrodes by applying a depolarizing current of 20 to 40 na for 450 msec, every 600 msec. Neurons that received from 170 to 750 na/min were successfully filled with HRP. After the injection the animals survived for 4 to 9 hours.

Generally the soma-dendritic spike of impaled neurons decayed within the first minute after penetration. The presence of postsynaptic potentials from natural stimulation or electrical stimulation of cutaneous nerves or of the dorsal columns was used as an indication that the electrode was inside a cell. Postsynaptic potentials were usually negative during depolarizing pulses but positive during hyperpolarizing pulses. Furthermore, during hyperpolarizing pulses, spinocervical tract neurons showed an axon spike upon stimulation of the ipsilateral dorsolateral funiculus at the third cervical segment (*7*).

After several neurons were injected, the cats were perfused first with saline and then by 10 percent buffered formalin (*p*H 7.6) (*8*). The spinal cord was removed and stored for 3 days in cold (2°C) buffered formalin, washed in 0.1*M* phosphate buffer

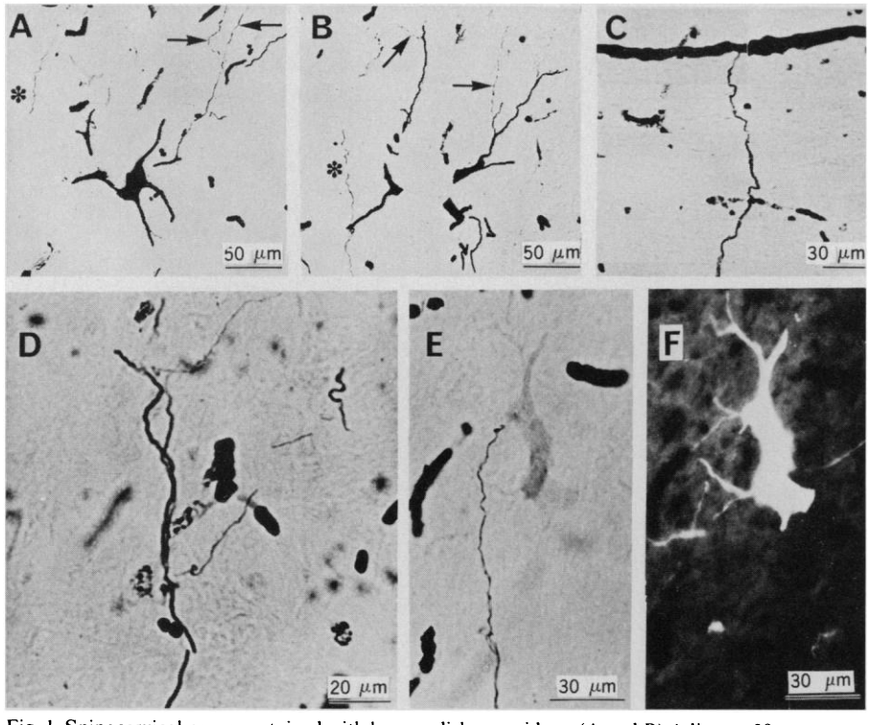

Fig. 1. Spinocervical neurons stained with horseradish peroxidase. (A and B) Adjacent 30-μm transverse sections through a spinocervical tract neuron (375 na/min). Before penetration, this neuron was excited by all hair types and by skin pressure on the lateral surface of the foot. Staining of fine dendrites is indicated by arrows, and the presence of axon collaterals is indicated by asterisks within the dendritic field of the neuron. (C) Axon of a spinocervical tract neuron running through the dorsolateral funiculus and dropping off a collateral 3.1 mm from the cell body. This plate is a montage built from ten adjacent, 30-μm sagittal sections. (D) Several fine dorsally directed collateral branches which could be traced back to the collateral shown in (C). These endings were situated in the gray matter 1.3 mm ventral to the parent axon. (E) A collateral from a spinocervical tract neuron ending on, or very close to, dorsal horn neuron injected with Procion yellow, as photographed with ordinary illumination. (F) The same neurons as in (E) but as photographed with fluorescent illumination. Dark bodies not associated with nervous elements are blood corpuscles stained by the incubation medium.

containing 5 percent sucrose, and sectioned serially at 30 to 50 μm on a freezing microtome. The portion of cord containing the cell bodies was cut transversely. In one cat 4 cm of ipsilateral cord rostral to this region was cut sagittally to facilitate the tracing of axons through the white matter. Sections were treated according to the method of Graybiel and Devor (*8*). In one cat, two dorsal horn neurons were injected with 5 percent Procion yellow (*4*) in order to ascertain whether this dye could survive the histochemical procedure required for the localization of HRP.

In three cats, we attempted to fill, with HRP, nine dorsal horn neurons from which we made stable intracellular recordings. Eight of these neurons were easily located histologically. The somata and dendrites were stained brown and were easily distinguished under ordinary illumination (Fig. 1, A and B). The dendritic trees of these neurons were well filled when compared with a sample of 24 dorsal horn neurons that we have successfully reconstructed after injecting Procion yellow (*4*). In fact, the dendritic trees of neurons filled with either HRP or Procion yellow show a degree of complexity which is at least equal to that shown in Golgi studies of dorsal horn neurons (*4, 9*).

A feature of neurons filled with HRP was the staining of relatively long lengths of axons. Axons were stained light brown and were easily distinguishable from any blood in the cord, which was stained a very dark brown (Fig. 1C) (*8*). In one animal we attempted to trace the axons of three spinocervical tract neurons and one unidentified dorsal horn neuron. The axons of the spinocervical tract neurons could be traced for 9 to 15 mm out through the gray matter and along the dorsolateral funiculus. The survival times after injection of HRP varied from 4 to 9 hours, which suggests that cellulofugal transportation occurred at a rate of 1.5 to 2.5 mm per hour. The most rostral millimeter of the stained portions of these axons was stained a faint brown. The unidentified neuron had two stained axons. One projected rostrally in the ipsilateral dorsolateral funiculus and the other projected across the cord and into the contralateral ventral funiculus. The former was stained for a distance of 10 mm, and the latter was followed rostrally for 4.3 mm in the ventral funiculus (this was the total extent of the contralateral cord examined). By comparison, in our sample of 22 spinocervical tract neurons filled with Procion yellow, only three axons could be traced more than 1 mm from the soma and none further than 2 mm.

Horseradish peroxidase also stained axon collaterals in both the spinocervical tract and in unidentified dorsal horn neurons. Collaterals were distinguishable from dendrites in that (i) they followed a more tortuous path, (ii) they often extended for relatively long distances (300 to 1300 μm) before branching, (iii) after the collaterals branched, daughter branches often followed approximately parallel pathways for up to 70 μm, and (iv) the collaterals generally showed little taper along their length. Before branching, collaterals had diameters varying from 1.4 to 2.0 μm but the finest collateral branches had diameters of less than 0.5 μm (Fig. 1, C and D). Occasionally we observed elliptical expanded regions (1.5 μm in diameter) that occurred periodically along the length of fine collaterals or large (2.5 to 4.0 μm in diameter) knobs on the ends of fine collaterals. These structures may be synaptic boutons similar to those reported in neurons stained by extracellular deposition of HRP (*5, 10*).

With one possible exception we have been unable to demonstrate any collaterals arising from 22 spinocervical tract neurons and two dorsal horn neurons in which the dendrites and somata were well filled with Procion yellow. In constrast, with HRP as an intracellular stain, we could demonstrate collaterals leaving the axons of spinocervical tract neurons, both within the gray matter and from the dorsolateral funiculus, at a distance of up to 6.2 mm from the soma. Collaterals leaving axons within the dorsolateral funiculus could be traced ventrally and medially through the ipsilateral gray matter to depths of up to 1.5 mm. These collaterals often reached depths of 1.3 mm before branching. After branching, they often projected several branches to more dorsal regions of the gray matter (Fig. 1D). Collaterals leaving axons close to their point of exit from the gray matter branched profusely and were often found within the dendritic field of the parent cell (Fig. 1, A and B). These collaterals were more difficult to reconstruct that those leaving axons in the dorsolateral funiculus. The accurate reconstruction of the collaterals of a neuron seems to be facilitated by minmizing the number of injected neurons.

Procion yellow appears to be unaffected by the HRP technique. In one experiment we found a collateral of a spinocervical tract neuron filled will HRP ending on, or extremely close to, a dorsal horn neuron filled with Procion yellow (Fig. 1, E and F). Both neurons received inputs from the dorsal surface of the toes. Although it is impossible by light microscopy to verify that the apparent contact between these two neurons is a synapse, we propose that the use of both Procion yellow and HRP can provide suggestive evidence for a synaptic contact between two neurons. The product of the histochemical reaction used to demonstrate HRP is electron opaque (*5, 10*); it is therefore conceivable that a combination of fluorescence and electron microscopy might be used to verify the synaptic nature of an apparent contact between neurons filled with HRP and Procion yellow (*11*).

With the use of the intracellular injection of HRP it is now possible to trace projections of a single, physiologically typed vertebrate neuron with a degree of detail hitherto impossible. Consequently, the numbers of identified projections of a specific neuron are no longer limited to the number of sites tested for antidromic excitation. Therefore, the intracellular injection of HRP may be useful for closing the gap between the anatomy and physiology of vertebrate nervous systems.

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8. The technique of A. M. Graybiel and M. Devor [*Brain Res.* **68**, 167 (1974)] was used with the following modifications and precautions. (i) Our incubation medium was made up immediately before use; (ii) sections were not counterstained; (iii) a nonfluorescing mounting medium enabled sections to be viewed with fluorescence illumination; and (iv) a good perfusion of the cord was found to be advantageous, as treatment of the tissue with the incubation medium results in intense staining of blood corpuscles (and thus small blood vessels) in the cord.
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