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Substance P-like immunoreactive trigeminal ganglion cells supplying the cornea

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Summary. Trigeminal ganglion cells supplying the cornea were traced with intra-axonally transported horseradish peroxidase and, subsequently studied for the presence of substance P-like immunoreactivity. Approximately 0%–30% of trigeminal ganglion cells contained immunoreactive substance P. These cells were of a small size (15–50 μm in diameter) and were distributed throughout the ganglion. The ganglion cells supplying the cornea were of a relatively small size as well but were confined to the anteromedial part of the ganglion. Some of these cells were found to contain immunoreactive substance P.

Introduction

The avascular cornea is innervated by morphologically unspecialized sensory endings (Matsuda 1968; Hoyes and Barber 1976) and pain seems to be the predominant sensory modality evoked by thermal or mechanical stimulation of these nerves (see Belmonte and Giraldez 1981). Studies utilizing retrogradely transported tracers have indicated that the trigeminal neurons supplying the cornea comprise a segregated population of ganglion cells with somatotopically organized central endings (Marfurt 1981; Panneton and Burton 1981).

Substance P (SP) is a putative transmitter or modulator of nociceptive primary sensory neurons. The peptide is axonally transported toward both the central and peripheral endings (Cuello et al. 1978) of a population of small size ganglion cells (Hökfelt et al. 1975). Among several peripheral targets of the trigeminal nerve, the cornea has also been shown to be innervated by nerve fibers displaying SP-like immunoreactivity (SPLI) (Miller et al. 1981; Tervo et al. 1981). However, previous studies have failed to demonstrate the trigeminal ganglion cells, which probably give rise to corneal nerve fibers containing SP (Neuhuber et al. 1981).

In the present study, the presence of horseradish peroxidase (HRP) applied to the cornea was demonstrated in the trigeminal ganglion using both conventional HRP histochemistry and immunohistochemistry. The ganglia were also studied for the presence of neurons containing SPLI. By comparing opposed surfaces of paired consecutive sections, the first prepared for HRP and the second for SP immunohistochemistry, the possible SPLI content of corneal afferents was searched.

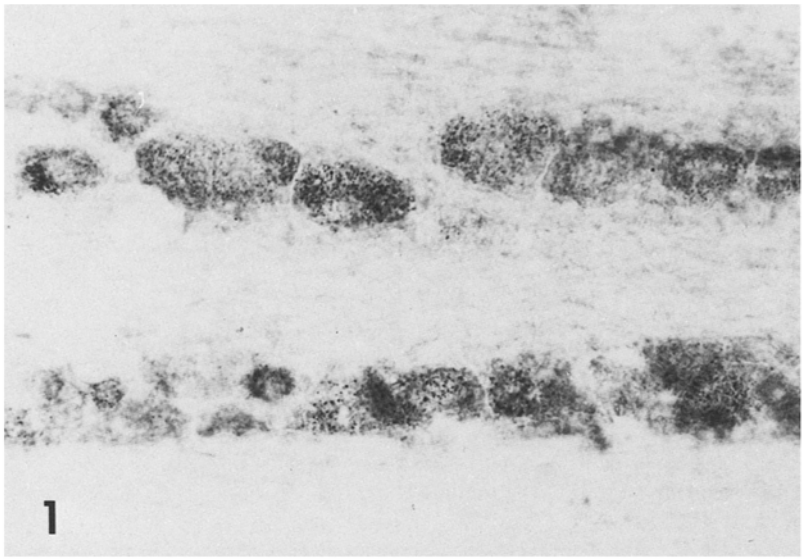
Material and methods

Ten Spraque-Dawley albino rats (200–250 g b.w.) of both sexes were used for immunohistochemical studies. The animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg b.w.). The right conreal epithelium was scratched with a needle. One μl of 30% HRP (Sigma Co., type VI) diluted in sterile saline was applied on the right cornea, avoiding leakage of the tracer outside the corneal surface. After 30 min the tracer was washed away and tarsoraphy was performed on both eyes. 12–24 h later the animals were perfused through the left heart ventricle, first with sterile saline at +22° C to wash out blood, and then with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, at +4° C. The trigeminal ganglia of both sides were excised and immersed in the same fixative for 2 h at +4° C. Subsequently, the tissues were immersed for 24 h in the phosphate buffer containing 5% sucrose.

Frozen sections 10 μm thick were prepared on gelatin coated slides, air dryed, and washed in 0.05 M phosphate buffered saline (PBS) for 30 min. Any possible endogenous peroxidase activity was destroyed by incubating the sections in 30% methanol and 0.5% hydrogen peroxidase (H_2O_2, Merck) for 30 min. Then the sections were washed in PBS for 30 min and subsequently in PBS containing 5% normal swine serum and 0.1% Triton-X for 60 min. The sections were prepared with the opposed surfaces of paired consecutive sections facing upward. The first section of each pair was incubated in the antiserum to HRP, diluted 1:100–1:500 and the second one to SP, diluted 1:100–1:500. Characteristics of the SP antiserum, raised in rabbits, have been described elsewhere (Stjernschantz et al. 1982). The peroxidase-antiperoxidase (PAP) method of Sternberger et al. (1970) was used. The incubation in the primary antiserum was carried out in humid chambers for 24 h at +4° C. Incubations in the secondary serum (diluted 1:50, DAKO as.) and in the PAP-solution (diluted 1:100, DAKO as.) were for 60 min at +22° C. All the solutions were in PBS containing 0.1% Triton-X and each incubation was followed by several washings over a period of 30 min. For demonstration of peroxidase activity, the sections were incubated for 5–20 min at +22° C in 0.1 M phosphate buffer, pH 7.2, containing 0.03% diaminobenzidine tetrahydrochloride (DAB, Fluka) and 0.01% H_2O_2. After rinsing, the sections were air dryed and mounted in glycerin-gelatin (Merck).

Immunohistochemical controls include: 1) Replacing of the specific antiserum with normal rabbit serum 2) Preabsorption of the primary antiserum with an excess (100 μg/ml) of synthetic SP (Sigma Co) for 12 h at +4° C.

An additional eight rats were used for conventional HRP histochemistry. They were treated as described above except that the fixation was carried out either with 1% glutaraldehyde and 2% formaldehyde (4 rats) or with 2% glutaraldehyde alone (4 rats). Following the rinsing, 40 μm frozen sections were prepared and incubated free floating, first for 20 min. in 0.03% DAB in 0.1 M



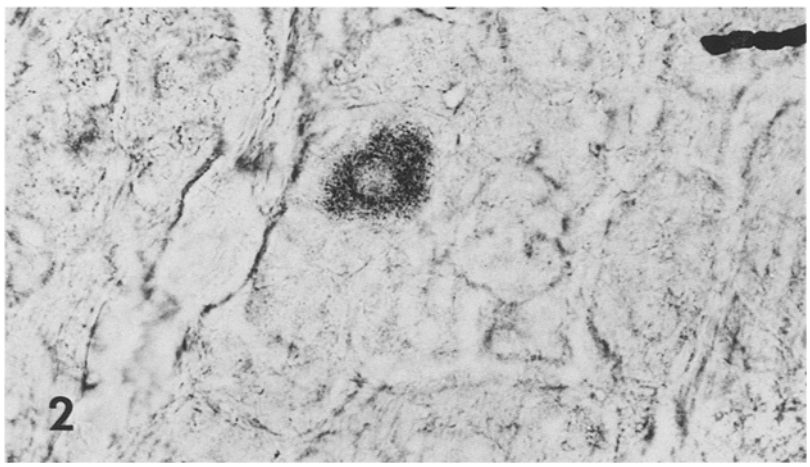


Fig. 1. Substance P-like immunoreactive cells in the ophthalmic division of rat trigeminal ganglion. Dark immunoreactive granules can be distinquished in the cytoplasm of these cells. Mag. × 400

Fig. 2. A HRP-labelled cell in the anteromedial part of trigeminal ganglion. The tracer was applied on the ipsilateral corneal surface and it was demonstrated by conventional histochemistry. Mag. × 400

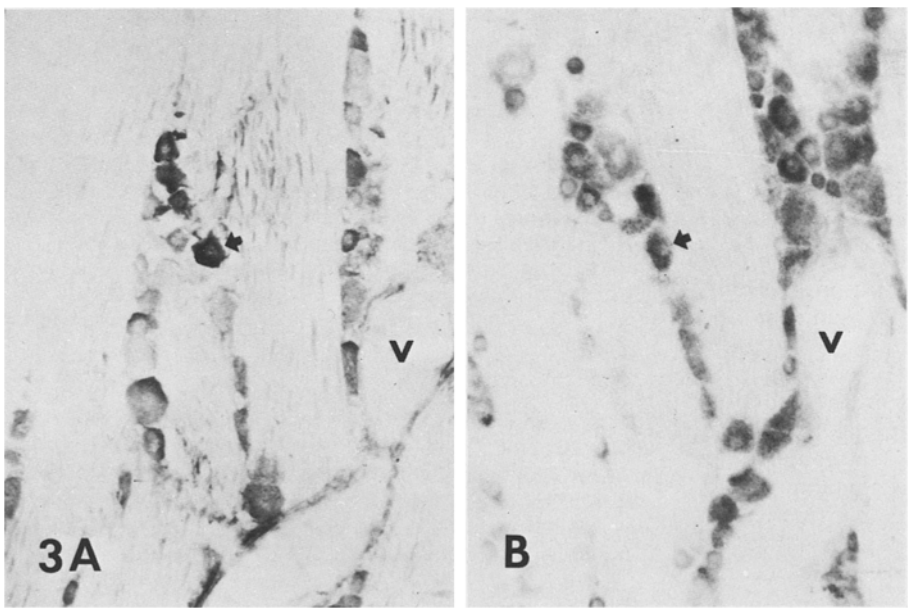


Fig. 3. Opposed cross section surfaces of paired consecutive sections demonstrating immunoreactivities for HRP (**A**) and substance P (**B**). Arrows indicate opposed cross section surfaces of a ganglion cell containing both immunoreactive HRP and substance P. *V* indicate a blood vessel. Mag. × 200

phosphate buffer, pH 7.4, at +22° C. Subsequently H_2O_2 was added to a final concentration of 0.01% and sections were incubated for an additional 20 min. The sections were then rinsed, air dryed on slides and mounted in glycerin-gelatin.

Results

Approximately 10%–30% of the ganglion cells displayed SPLI in cytoplasmic granules (Fig. 1). The nucleus was not stained. The size of the SPLI containing cells varied between 15 μm and 50 μm; and they were distributed throughout the ganglion with no somatotopical segregation. They appeared either isolated or in clusters of 2–4 ganglion cells. A similar pattern of SP immunoreactivity was observed in the ganglia, both contralateral and ipsilateral to the corneal application of HRP.

A similar distribution of HRP-labelled ganglion cells was obtained both with conventional and with immunohistochemical methods. In regard to the former method, only animals fixed with glutaraldehyde alone displayed HRP-labelled cells (Fig. 2): the addition of formaldehyde to the fixative completely abolished the reaction. Five of the ten animals used for immunohistochemical studies displayed HRP immunoreactive ganglion cells (Fig. 3A). With both HRP methods, the reaction product was restricted to the cytoplasm of the labelled cells. The HRP-labelled ganglion cells were segregated in the anteromedial part of the trigeminal ganglion, only ipsilateral to the HRP application. They were 20–50 μm in diameter and occasionally formed clusters comprising 2–3 cells.

By comparing the paired consecutive sections prepared for immunohistochemistry, it was possible to establish the opposed cross-section surfaces of certain HRP containing cells, some of which exhibited SPLI (Fig. 3). Only those cells which displayed a nucleus and which were of a larger size (40–50 μm) were included in the analysis.

Sections incubated with normal rabbit serum instead of the specific antiserum displayed only faint diffuse immunoreactivity in the cytoplasm and the nucleus of the ganglion cells. Preabsorption of the SP immunoserum with an excess of the antigen abolished the immunoreactivity.

Discussion

The combined use of retrogradely transported fluorescent dyes and immunohistochemistry is a useful tool for tracing defined pathways in the central nervous system (Kuypers et al. 1979; Hökfelt et al. 1979; Brann and Emson 1980; Sawchenko and Swanson 1981). However, such techniques have failed to demonstrate the presence of corneal afferents displaying SPLI (Neuhuber et al. 1981).

In the present study, the corneal afferents containing SPLI were revealed in the trigeminal ganglion by comparing the opposed cross-section surfaces of paired consecutive tissue sections: the first section was prepared for immunohistochemical demonstration of intra-axonally transported HRP and the second for SP immunohistochemistry. As the penetration of immunoreactivity into tissues is poor (Piekut and Casey 1983), it would appear that individual cells may exhibit immunoreactivity in both opposed surfaces of two tissue sections. To avoid false interpretion, only larger cells displaying a nucleus were considered as being potentially represented in both sections. Only a fraction of the HRP-labeled neurons were found to contain SPLI. This may, however, be due to certain methodological reasons, such as insensitivity of the immunohistochemical methods or failure to find appropriate pairs of cross-section surfaces for each cell.

Demonstration of HRP by conventional histochemistry in combination with immunohistochemistry is not recommended: The use of glutaraldehyde as a fixative is necessary to preserve HRP enzyme activity (Malmgren and Olsson 1978), whereas the immunohistochemical demonstration of SPLI requires formaldehyde fixation. The advantage of the immunohistochemical demonstration of both HRP and SP is that optimal fixation may be used.

The morphological appearance of the HRP-labeled corneal afferents in the anteromedial part of the ganglion, as demonstrated both with the conventional and immunohistochemical methods, was rather similar to that of the SPLI cells. That some of the corneal afferents were found to display SPLI is consistent with the finding that neurotomy of the ophthalmic trigeminal branch causes both disappearance of corneal SPLI nerve fibers (Miller et al. 1981) and a reduction in corneal SP content (Unger et al. 1981; Keen et al. 1982).

SP has been associated with transmission of nociceptive afferent input. However, more of the peptide is transported toward the peripheral than the central endings (Brimijoin et al. 1980; Harmer and Keen 1982), and stimulation of the trigeminal nerve causes release of SPLI also at its peripheral endings (Olgart et al. 1977; Bill et al. 1979). In the eye, SP is probably directly involved in the miotic response to nociceptive stimuli (Stjernschantz et al. 1979; Soloway et al. 1981), whereas its role in uveal antidromic vasodilatation seems to be questionable (Stjernschantz et al. 1981). However, apart from the afferent function, the significance of the presence of immunoreactive SP nerves in the avascular cornea is not clear. It seems possible that the appearance of SP in the peripheral branch of the corneal afferents may be only accidental, as the neuron may be unable to determinate the direction of the axonal transport of the peptide.

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