

Cerebrovascular Projections from the Sphenopalatine and Otic Ganglia to the Middle Cerebral Artery of the Cat

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SUMMARY The location of the postganglionic parasympathetic cell bodies projecting to cerebral arteries is unknown. Using axonal tracing techniques, we examined whether the sphenopalatine ganglia (associated with the seventh cranial nerve) and otic ganglia (associated with ninth cranial nerve) contain perikarya which send axons to the feline middle cerebral artery (MCA). The tracers horseradish peroxidase (HRP: 3 cats) or wheat germ agglutinin (WGA: 6 cats) were applied to the MCA in a slow release polymeric system. Three days later the SPG, otic ganglia, and rete mirabile were harvested bilaterally and processed for tracer by the TMB method (HRP) or immunohistochemistry (WGA). In a given animal, approximately equal numbers of cells containing axonal tracer were found in both SPG. Labeled fibers occasionally could be seen extending into the vidian nerve. Positive cells were also found in the otic ganglia and in the walls of the internal rete mirabile. These results provide the first identification of parasympathetic cell bodies projecting to cerebral blood vessels.

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BOTH CHOROBSKI AND PENFIELD¹ and Cobb and Finesinger² in 1932 described a vasodilator pathway to pial arteries via the seventh, ninth, and tenth cranial nerves. Figure 1 presents in schematic fashion the gross anatomy of the mammalian seventh cranial nerve/greater superficial petrosal nerve (GSPN)/sphenopalatine ganglion (SPG)/otic ganglion complex.^{3,4} In 1970 Ruskell⁵ performed GSPN neurectomies in monkeys and found no degeneration in the deep petrosal nerves, suggesting minimal numbers of direct fibers from the GSPN to the carotid artery, but he also found some "minor changes" in the vidian nerve following damage to the SPG, which suggests some degree (albeit small) of retrograde projection from the SPG toward the brain. These combined physiological and anatomical data prompted the current investigations. In this report we seek to demonstrate for the first time the location of parasympathetic cell bodies associated with the seventh and ninth cranial nerves which project to the middle cerebral artery.

Materials and Methods

Operative Protocol

Adult mongrel cats (2–5 kg) were anesthetized with initial doses of ketamine hydrochloride (100 mg IP) and sodium pentobarbital (4–5 mg/kg IP) and supple-

mented with either ketamine (25 mg IM doses) or inhalation anesthesia (0.5% halothane, 2 l/min nitrous oxide, 2 l/min oxygen) as needed. The animals received preoperative (gentamicin 4–6 mg IM, penicillin G benzathine suspension 300,000–600,000 units IM) and in some cases postoperative (ampicillin 50 mg PO b.i.d.) antibiotics.

Using an operating microscope, the lateral skull was exposed and an extensive pterional craniectomy was performed. The dura was opened to expose the middle cerebral artery.⁶ Applications of various axonal tracer molecules were then performed using the method of Mayberg et al.^{7,8} For experiments using horseradish peroxidase (HRP), 3–5 mg of HRP (Type VI, P-8375, Sigma Chemical Company) and 0.1–0.3 mg HRP conjugated to *Triticum vulgaris* agglutinin (WGA) (L-9008, Sigma) were mixed into 14–16% (w/w) polyvinyl alcohol. For experiments using WGA, approximately 5 mg of the lectin (L-2101, E-Y Laboratories Inc.) was added to the polymer instead. The polymer containing the tracer was then put into a silicone rubber "boat" (with inner dimensions of approximately 1.5 × 5 × 8 mm) and the edges of the boat covered with white petrolatum to prevent lateral diffusion of the tracer in the subdural space. The boat was placed over M1 and M2 and anchored to the dura by a suture.

After 65–72 hours the animals were reanesthetized, perfused over 1–2 minutes with 500 ml 0.9% saline containing 1000 units of sodium heparin, and then perfused with either 1 liter 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for HRP histochemistry or 500 ml 5% (v/v) acrolein in the same buffer for WGA.^{9,10} The brain and bilateral trigeminal ganglia and sphenopalatine (pterygopalatine) ganglia were harvested from all the animals. The internal rete mirabile was harvested and processed bilaterally from two WGA animals, and the otic ganglia bilaterally from the last three WGA animals.

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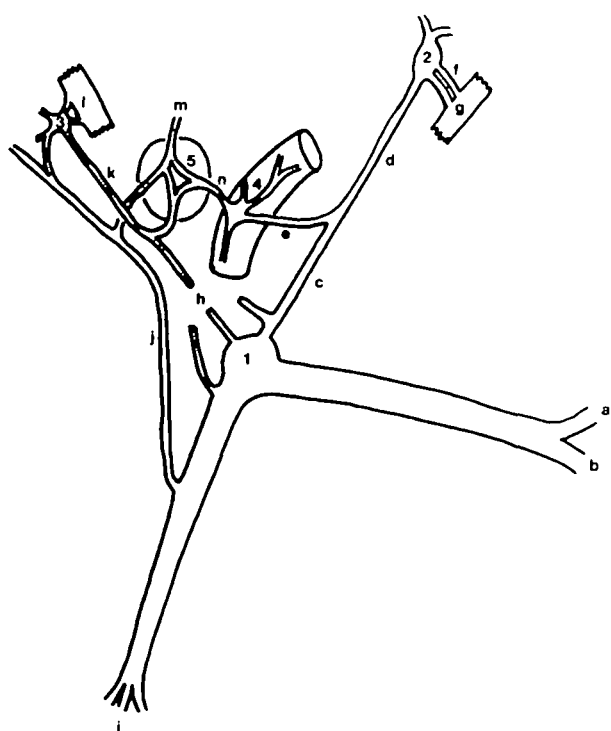


FIGURE 1. Schematic representation of the mammalian seventh cranial nerve, or facial nerve, and its connections. 1: Geniculate ganglion. 2: Sphenopalatine, or pterygopalatine, ganglion. 3: Otic ganglion. 4: Carotid plexus. 5: Tympanic plexus. a: Nervus intermedius. b: Motor root of seventh cranial nerve. c: Greater superficial petrosal nerve. d: Vidian nerve, or nerve of the pterygoid canal. e: Deep petrosal nerve. f: Sphenopalatine nerve. g: Maxillary nerve (second division of fifth cranial, or trigeminal, nerve). h: Geniculotympanic nerve, with variable position. i: Peripheral motor branches of seventh cranial nerve. j: Chorda tympani. k: Lesser superficial petrosal nerve. l: Mandibular nerve (third division of fifth cranial nerve). m: Tympanic nerve (from ninth cranial nerve). n: Caroticotympanic nerve.

The ganglia, rete, and tissue blocks were placed into 10% sucrose (w/v) in 0.1 M phosphate buffer pH 7.4 (PB) for 5–8 hours, and then transferred to 20% sucrose in 0.1 M PB for at least 8 hours prior to further processing.

HRP Processing and Analysis

The tissues were embedded in a medium consisting of 3% (w/v) edible gelatin (type B, Atlantic Gelatin, General Foods Corp.) and 30% (w/v) bovine serum albumin (fraction V, A-9647, Sigma) solidified by the addition of 37% formaldehyde (1:10, v/v). The blocks were left at 4 degrees Centigrade for 24 hours and then were serially sectioned at 30 microns with a microtome in a cryostat at –15 degrees Centigrade. The tetramethyl benzidine (TMB) technique of Mesulam^{11, 12} was used to process the tissue for HRP histochemistry. The sections were then mounted on gelatin-coated slides; half of the sections were counterstained with neutral red. Perfused cats without HRP application provided control samples.

In order better to present the distribution and orientation of the cells within the SPG, a *camera lucida* was used to draw all the sections and labelled neurons in one pair of SPG. These data were then manually digitized and entered into the 3-D Graphics Program of Bill Budge (California Pacific Computer Co.) for computer processing and reproduction as pairs of two-dimensional images of the three-dimensional reconstruction of the labelled cells within the ganglia as viewed from two closely separated points; therefore each “stereo pair” is suitable for visual fusion into a three-dimensional image.

WGA Processing and Analysis

Immunocytochemical processing for WGA and VIP was performed as described by Liu-Chen et al.⁹ In the final four cats all the sections were retained and divided into two or three sequential series for immunohistochemistry using WGA and VIP antisera and control serum; cells in the ganglia of the first two cats were not quantitated. Slides were incubated with primary antisera to WGA (anti-WGA rabbit IgG fraction, AL-2101, E-Y Laboratories Inc.) or to VIP (raised in rabbit, 39H2T, Immunonuclear Corp.) at a dilution of 1:4000 for 36–48 hours at 4 degrees Centigrade. Control slides were incubated with either normal goat or rabbit serum at the same dilution. Other controls were slides of SPG incubated with VIP antisera to which 2 micrograms/ml synthetic VIP had been added, or of known WGA labelled trigeminal ganglia incubated with WGA antisera to which 2 micrograms/ml WGA had been added.

The three slide series for each SPG, otic ganglion, and internal rete were reacted with WGA and VIP antisera and control serum. The primary antisera and biotinylated reagents were used for repeated processing of tissues.¹³ We have found these solutions stable for at least 3 months when stored at 4 degrees Centigrade.

Results

Controls

Sphenopalatine, otic, and trigeminal ganglion neurons did not display intrinsic peroxidase activity. The neurons of the trigeminal, sphenopalatine, and otic ganglia and internal rete did not react with normal rabbit or goat antisera in the experiments in which polymer alone or polymer with tracer was applied to the MCA. The specific staining of known WGA-labelled trigeminal ganglia was lost when excess free WGA was preincubated with the primary antisera. Similarly, the specific reaction of the VIP antiserum with SPG neurons^{14–16} was lost when synthetic VIP was included. Postmortem examination of the application sites showed that the boats had remained in good positions, and examinations of the underlying cortex revealed the limited local diffusion of the tracers into the cortex (usually approximately 3 mm⁸). In animals in which the tracer was not applied directly over the middle cerebral artery, no tracer could be found in the

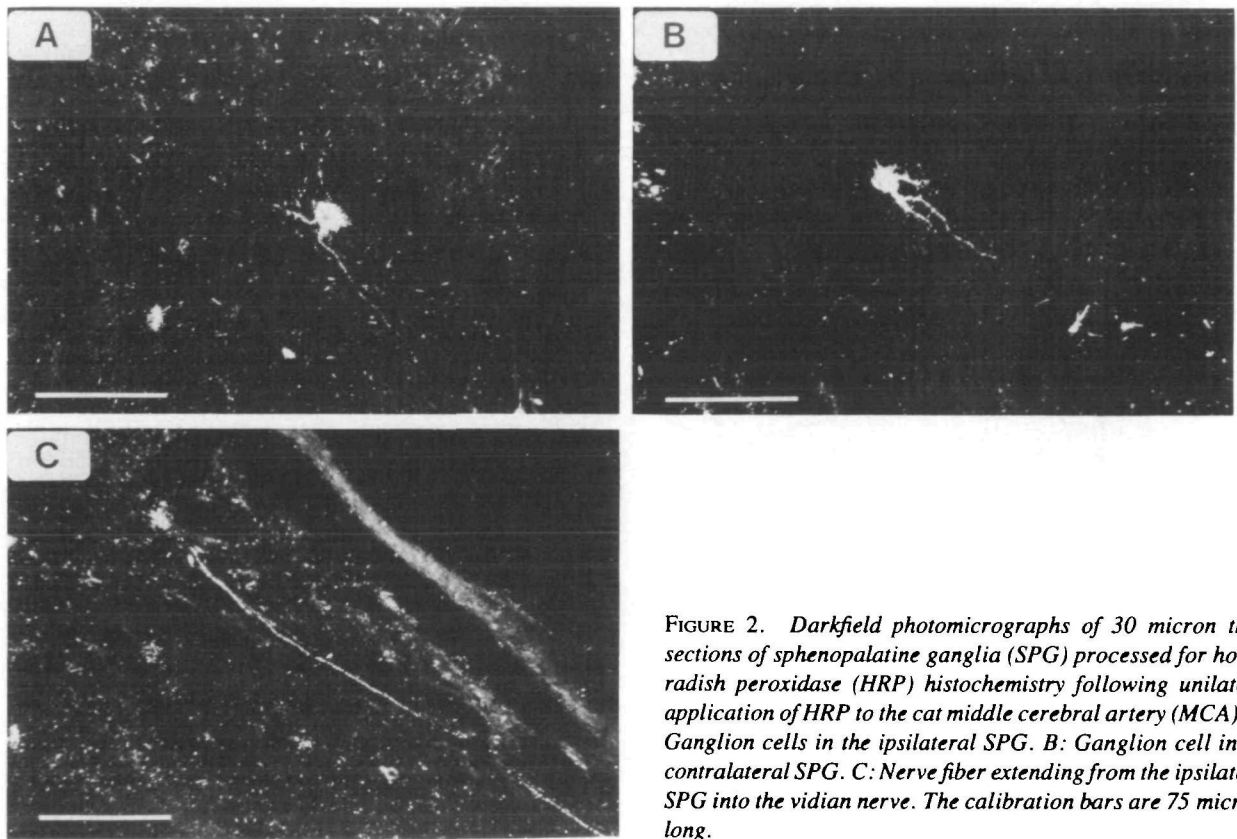


FIGURE 2. Darkfield photomicrographs of 30 micron thick sections of sphenopalatine ganglia (SPG) processed for horse-radish peroxidase (HRP) histochemistry following unilateral application of HRP to the cat middle cerebral artery (MCA). A: Ganglion cells in the ipsilateral SPG. B: Ganglion cell in the contralateral SPG. C: Nerve fiber extending from the ipsilateral SPG into the vidian nerve. The calibration bars are 75 microns long.

trigeminal ganglia; the unilateral presence of tracer in the ipsilateral trigeminal ganglion demonstrated that an effective local application with subsequent transport had occurred in all other cats.^{7, 17}

Sphenopalatine Ganglia

The SPG varied considerably in shape but usually were elongated with dimensions of approximately $2.5 \times 1.0 \times 1.0$ mm. Three cats underwent HRP applications. There were approximately equal numbers of tracer-labelled cells bilaterally. Five SPG were counted for labelled cells. There were 66 total peroxidase-positive neurons in three ipsilateral ganglia (3, 9, and 54) and 45 in two ganglia on the contralateral side (3 and 42). Neuronal perikarya containing label had an average diameter of 23 microns (range 11–38 microns, standard deviation 6 microns, 79 cells counted). Figure 2 shows representative labelled cells and processes.

It was possible to follow positive nerve fibers out into the vidian nerve bilaterally, in one case for 2.5 mm. Cell processes tended to be oriented toward the entrance of the vidian nerve into the ganglion as well. Cells are clustered around the fiber tracts in the posterior portion of the ganglion entered by the vidian nerve (fig. 3).

Six cats underwent WGA applications, with the same qualitative results of equivalent labelling of both ipsilateral and contralateral SPG. However, approximately twice as many cells were labelled. A total of 269 were found to be WGA-IR in 5 ganglia. 134 were

seen in two ipsilateral ganglia (104 and 30) and 135 in three contralateral ganglia (95, 37, and 3). Most labelled cells were concentrated near the entrance of the vidian nerve, similar to the results using HRP.

Otic Ganglia

Six otic ganglia from three cats were examined. They were spheroidal and 0.5–0.9 mm in diameter. The ipsilateral ganglia contained a total of 74 WGA-IR neurons (6, 67, and 1; fig. 4). Only a total of 3 WGA-IR neurons were found in contralateral ganglia (3, 0, and 0, corresponding to the ipsilateral listed above).

Internal Rete Mirabile

Preliminary investigations of the internal rete mirabile from 2 cats have shown 9 individual multipolar cells found in the adventitia of the ipsilateral rete which were WGA-IR but not VIP-IR (fig. 5a). Several clusters of VIP-IR ganglion cells were seen adjacent to these blood vessels; none of these cells were WGA-IR, however (fig. 5b). We have not observed individual neuronal cells which are immunoreactive for both WGA and VIP on adjacent sections in the internal rete. There also are VIP-IR fibers at the adventitia/media junction in these vessels (fig. 6).

Discussion

Over half a century ago electrical stimulation studies had suggested the presence of a vasodilator pathway from the seventh nerve to the pial vasculature. Chor-



FIGURE 3. Computer-generated stereo pair reconstructions of ipsilateral (A) and contralateral (B) SPG showing the shapes and positions of HRP-labelled cells and processes following unilateral application of HRP to the cat MCA. The ganglia are viewed from above; the bottom of the figures is anatomically caudal and represents the point where the vidian nerve enters the ganglia. The calibration bars are 250 microns long.

obski and Penfield¹ demonstrated that in cats and monkeys stimulation of the distal cut end of either the seventh nerve or greater superficial petrosal nerve caused the dilation of pial arteries (which were examined only ipsilaterally), and that the dilation produced by seventh nerve stimulation was blocked by sectioning the ipsilateral GSPN. Similar results were obtained by Cobb and Finesinger;² they too examined only ipsilateral vessels. Vasodilation stimulated via the ninth and tenth cranial nerves was also observed by these groups. Chorobski and Penfield further observed that little degeneration of the perivascular nervous plexus occurred following chronic sectioning of either the facial nerve or GSPN. They concluded that there was a vasodilatory pathway to the cerebral vessels running in the facial nerve, through the GSPN, and then via the deep petrosal nerve to the pericarotid plexus, and that this innervation was interrupted by synapses which occurred just before reaching the internal carotid artery (fig. 1).

The HRP and WGA tracer results presented here confirm and extend these physiological observations.

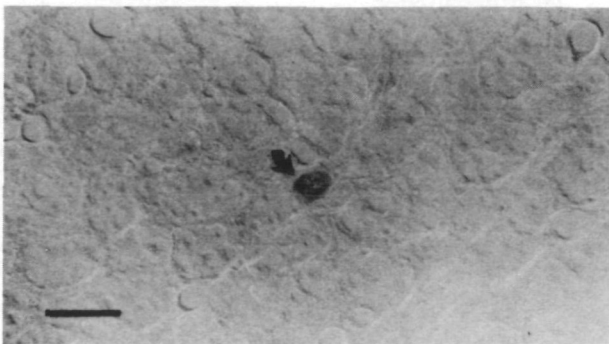


FIGURE 4. Photomicrograph with Nomarski optics of an 8 micron thick section of otic ganglion processed for WGA immunocytochemistry following unilateral WGA application to the MCA. The arrow indicates a WGA-IR ganglion cell. Calibration bar is 25 microns long.

The conclusion that a synapse separated the GSPN from the perivascular nerve terminals seems correct, but at least some of those synapses probably occur in the SPG itself. Tracer molecules applied around the middle cerebral artery are transported to neuronal cell soma in bilateral SPG; the absolute number of labelled cells varied widely from animal to animal and may reflect both individual variation in the animals themselves or different degrees of tracer uptake by nerve fibers variably traumatized by the vessel exposure and tracer implantation. Such variability is well described in the literature.^{7, 17} The neuronal shapes seen with the HRP technique are identical to those drawn by Carpenter in 1912 from histologic stains of SPG.¹⁸ Relatively few cells become labelled by retrograde transport; given approximately 12,000 ganglion cells per cat SPG,¹⁴ only 0.4% contain WGA. The projection to the otic ganglia seems to be more unilateral. Again, the specifically labelled cells are ganglion cells.¹⁸⁻²¹ In these studies the otic ganglion was harvested from the ventrolateral aspect of the mandibular nerve at the point it divides into the lingual and inferior alveolar nerves.²² The ganglia without labelled cells may be an artifactual result from incomplete sampling, since it also has been reported that there can be two or more accessory ganglia united by a plexus on the ventral surfaces of the mandibular nerve and maxillary artery.²³ Nevertheless, this variation may be real, since there are physiologic data on carotid vasodilation apparently induced by the seventh nerve which show a variable contribution also from the ninth and tenth nerves²⁴ which more probably would be mediated via the otic than the sphenopalatine ganglia.^{3, 4}

In the SPG, labelled cell processes extend into the vidian nerve; these presumably then project via the deep petrosal nerve to the internal carotid and thence out to the middle cerebral artery. The communication from the otic ganglion to the pial vessels may be through the pathways illustrated in figure 1 (lesser superficial petrosal nerve to tympanic plexus and then via the caroticotympanic nerve to the pericarotid plex-

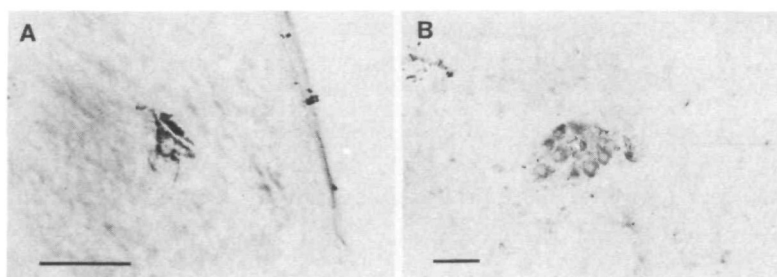
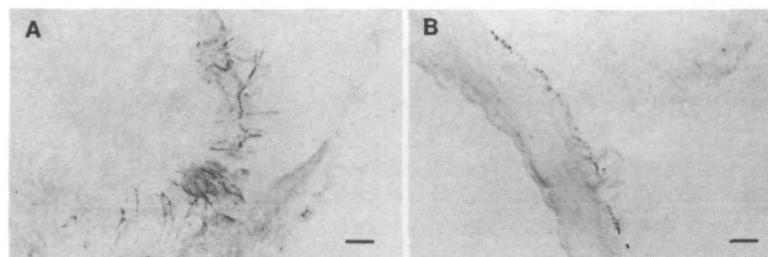


FIGURE 5. Photomicrographs with Nomarski optics of 10 micron thick sections of internal rete mirabile ipsilateral to WGA application to the MCA. Calibration bars are 25 microns long. A: Section processed for WGA demonstrating an adventitial WGA-IR multipolar cell which was not VIP-IR on the adjacent section. B: Section processed for VIP demonstrating a microganglion whose cells are VIP-IR but not WGA-IR on the adjacent section.

FIGURE 6. Photomicrographs with Nomarski optics of VIP-IR fibers at the adventitia-media junction in 10 micron thick sections of the internal rete mirabile. Calibration bars are 10 microns long. A: En face section. B: Transverse section.



us) or by direct communications to the cavernous sinus.²³ It is unlikely that the tracer seen in SPG and otic ganglia has a transsynaptic origin; internalization of WGA-HRP conjugate by neurons seems to peak at 3–24 hours²⁵ and subsequent retrograde transport occurs at about 108 mm/day.²⁶ The occasional neuronally-shaped WGA-IR cells seen in the adventitia of the ipsilateral rete may correspond to the neuronal cell bodies seen along intracranial vessels by Chorobski and Penfield,¹ Borodula and Pletchkova,²⁷ Vasquez and Purves,²⁸ and Cervos-Navarro and Artigas.²⁹

It is known that the SPG provides postganglionic VIP-containing fibers to the nasal mucosa in cats;^{14, 15, 30} monoaminergic neurons are not found in the SPG (or the otic ganglion),³¹ and 98.5% of SPG neurons are VIP-IR.¹⁴ The SPG therefore seemed a logical possible source for intracranial perivascular VIP fibers,^{32–40} but chronic unilateral extirpation of the SPG is reported not to affect the amount or distribution of VIP fibers (two cats, examined by Edvinsson et al.³⁵ However, this result reflects the bilateral projections of the SPG and the presence of VIP-IR fibers from other sources projecting to pial vessels.³⁴ In preliminary studies we have found 92 of 269 WGA-IR SPG neurons also to be VIP-IR (34%), and 26 of 74 WGA-IR otic neurons (35%) to be VIP-IR as well (Walters, Gillespie, and Moskowitz, unpublished data). These figures are minimum ones, since in the central portions of the ganglia it frequently is not possible to obtain adequate landmarks to be certain of identifying the same cell on adjacent sections. Also, the animals were not treated with colchicine, which tends to enhance the otherwise somewhat variable positivity of the cells with the VIP antiserum.¹⁶

These preliminary data therefore indicate that at least some of the VIP fibers along the middle cerebral artery originate from neurons in the sphenopalatine and otic ganglia. Some projections from cortical neurons³⁴ are possible; also, it recently has been suggested

that VIP-IR ganglion cells in the external rete and cavernous plexus may be an important source of the intracranial VIP perivascular fibers.^{41, 42} Although WGA-IR cells were found in the internal rete mirabile, they were not also VIP-IR in the 2 cats presented here.

Baecker et al.⁴³ describe VIP-IR nerves adjoining AV-anastomoses in the tongue, and mammalian cutaneous arteriovenous shunts whose dilation is unaffected by atropine or antihistamines^{44, 45} appear to be involved in thermoregulatory control, particularly cold vasodilation.⁴⁶ Interestingly, stimulation of the facial nerve or GSPN in the cat causes surface vasodilation of the face⁴⁷ and a decrease in carotid resistance.²⁴ Perhaps the cerebrovascular projection through the seventh nerve described here is involved in temperature homeostasis, as was suggested by Gibbins et al.;^{41, 42} VIP is a powerful vasodilator of cerebral blood vessels.^{48–52} If this were the case one would expect a system to have evolved to stabilize total blood flow while redistributing its components, which is indeed what is observed when CBF is examined during facial nerve or GSPN stimulation.⁵³

Other possibilities exist as well. This pathway may be important in vascular permeability^{54, 55} or in modulation of inflammation and immune system responses^{56, 57} as postulated for substance P-immunoreactive trigeminovascular fibers.^{58, 59} It is hoped that further studies on these projections from the SPG and otic ganglia will provide data to enable a better understanding of their functional role.

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Endothelial Dependent Relaxation Demonstrated In Vivo in Cerebral Arterioles

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SUMMARY The endothelium of mouse pial arterioles was injured *in situ* with a light/dye technique. The response of the arterioles to acetylcholine or to bradykinin was compared before and after the injury. All vessels failed to dilate after injury. In fact the predominant response now became constriction. The injured vessels were still capable of dilating to papaverine. Uninjured vessels continued to dilate to acetylcholine or bradykinin. The data show that relaxation of pial arterioles to acetylcholine or bradykinin is dependent on a normal endothelium. This is in keeping with demonstrations by others that an endothelial dependent relaxing factor or factors is(are) the mediator of the dilation to either acetylcholine or bradykinin. The present demonstration of such endothelial dependence is important because in contrast with the bulk of the literature it deals with *in vivo*, rather than *in vitro* data, and with microcirculation rather than large vessels. It is also important because it concerns brain circulation. The data suggests that endothelial injury, known to occur in a wide variety of pathologic states, could enhance vasospastic potential by eliminating dilating influences and/or converting them to constricting forces.

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SEVERAL YEARS AGO, Furchgott and coworkers^{1,2} demonstrated that the relaxation of large arteries to acetylcholine (ACh) was really mediated by some substance released from endothelium. The substance was called endothelial relaxing factor (EDRF). EDRF has now been demonstrated by many workers.^{1,4} Its chemical nature has not yet been identified, but it is extremely labile.^{1,5} Among candidates for EDRF have been free radicals, carbonyl compounds, products of lipoxygenase activity^{1,4} and substances produced by the action cytochrome P-450.⁶ In addition to ACh a number of vasoactive substances have also been found to trigger EDRF. Use of pharmacologic probes suggests that there may be more than one EDRF, and thus relaxation by two different endothelial dependent dilators may be mediated by two different EDRF's.^{1,9} However whether the actions of a vasoactive agent are

mediated by EDRF depends upon the species of animal, and the vascular bed.¹⁻³

Endothelial dependence was originally demonstrated *in vitro* by comparing responses of vessels with and without endothelium. The endothelium was totally removed by some mechanical abrasion. Some workers have questioned the physiologic significance of EDRF, at least insofar as ACh liberated from perivascular nerves, is concerned. They express doubt that ACh could diffuse all the way through adventitia and muscularis to initiate endothelial dependent relaxation,³ or they suggest that the ACh passing through the vessel wall would first trigger an effect mediated by muscarinic receptors on the muscle itself.⁷ This effect is constriction, not relaxation, and is observed in arteries from which endothelium has been removed.^{1,2} However ACh applied externally to smaller vessels (arterioles) *in situ*, definitely produces relaxation.^{8,9} Recently, endothelial injury of such arterioles *in vivo* has been shown to result in conversion of the relaxation to constriction.¹⁰

Such *in vivo* studies of microvessels and endothelial dependent relaxation are rare. Confirmation of such

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