Cholinergic Basal Forebrain Neurons Project to Cortical Microvessels in the Rat: Electron Microscopic Study with Anterogradely Transported *Phaseolus vulgaris* Leucoagglutinin and Choline Acetyltransferase Immunocytochemistry

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Physiological evidence indicates that central cholinergic pathways are involved in the regulation of cerebral cortical blood flow. We investigated the possible contribution of basal forebrain cholinergic neurons from the substantia innominata (SI) to the innervation of cortical microvessels. Basalo-cortical perivascular nerve terminals were detected by light and electron microscopic immunocytochemistry of anterogradely transported *Phaseolus vulgaris* leucoagglutinin (PHA-L) following its injection in the SI, and were compared to cortical perivascular cholinergic (immunoreactive for choline acetyltransferase (ChAT)) terminals. The basal forebrain origin of cholinergic terminals was evaluated after unilateral ibotenic acid lesion of the SI. PHA-L varicose fibers reached and surrounded microvessels in all cortical subdivisions examined. When studied at the ultrastructural level in the fronto-parietal and perirhinal cortices, perivascular PHA-L nerve terminals were located significantly closer to microvessels in the fronto-parietal than in the perirhinal cortex (respective average distance of 0.98 ± 0.09 and $1.34 \pm 0.07 \mu\text{m}$; $p < 0.01$). PHA-L and ChAT terminals in the fronto-parietal cortex compared very well in their perivascular distribution but not in the perirhinal cortex. In both cortices, perivascular PHA-L terminals were similar in size to, but engaged more frequently in synaptic contacts than their ChAT counterparts. Following SI lesion, the density of cortical ChAT terminals including those reaching microvessels decreased significantly (56 and 63%, respectively, $p < 0.005$) in the fronto-parietal cortex, while the cortical and perivascular denervations were much less pronounced (26%, not significant and 35%, $p < 0.05$, respectively) in the perirhinal cortex. These results indicate that basal forebrain neurons project preferentially to fronto-parietal cortical microvessels and further show that a significant proportion of these projections are cholinergic. In addition, the difference in distribution and/or

synaptic incidence between perivascular PHA-L and ChAT terminals suggested that noncholinergic SI neurons also contribute to these neurovascular associations, and more so in the perirhinal cortex, as further indicated by the lesion studies. Such finding corroborates recent physiological evidence for a functional innervation of the cortical microvascular bed by SI neurons, a role that might be relevant to the overall pathology of Alzheimer's dementia.

[Key words: *Phaseolus vulgaris* leucoagglutinin (PHA-L), choline acetyltransferase (ChAT) immunocytochemistry, acetylcholine, basal forebrain, substantia innominata, cerebral blood vessels, cerebral blood flow, ultrastructure]

The cholinergic innervation of the neocortex has been extensively studied and its main origin identified as the nucleus basalis of Meynert in primates or its equivalent, the substantia innominata (SI) in the rat (Divac et al., 1975; Mesulam et al., 1983; Rye et al., 1984; Woolf et al., 1991). Numerous studies have provided evidence for the involvement of these cholinergic neurons in cognitive functions and in processes of arousal and attention, with their degeneration being a landmark characteristic of Alzheimer's dementia (Coyle et al., 1983; Bartus et al., 1985). In the rat, electrical or chemical stimulation of the SI has further been shown to result in considerable increases in cerebral blood flow in various cortical areas but prominently so in the frontal and parietal cortices (Lacombe et al., 1987, 1989; Arnerić, 1989; Biesold et al., 1989; Adachi et al., 1990a,b; and for a recent review, see Sato and Sato, 1992). These blood flow changes are accompanied by a local release of acetylcholine (ACh) (Kurosawa et al., 1989) and involve muscarinic (Dauphin et al., 1991) and/or nicotinic (Linville et al., 1993b) ACh receptors. Moreover, they appear to be independent from cortical interneurons (Linville et al., 1993a) and from pial vessels dilatation (Adachi et al., 1992b). Altogether, these data strongly suggested that the flow changes can be elicited by a direct action of the SI neurons on the cortical microvessels in order to control, locally, blood supply to cortical areas.

Using a double-immunofluorescence approach, we recently showed that a subset of intracortical bipolar cholinergic neurons, also immunoreactive for vasoactive intestinal polypeptide (VIP), were engaged in neurovascular associations with cortical microvessels (Chédotal et al., 1994a). However, the origin (intracortical and/or basal forebrain) of the vast majority of perivascular cholinergic fibers could not be established. The cholinergic neurons from the SI would appear as the best candidates for this

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innervation as their lesioning has been shown to result in reductions in basal cortical blood flow (Gomi et al., 1991; Peruzzi et al., 1993). Yet, lesion of these neurons reportedly does not impede choline acetyltransferase (ChAT) activity associated with cortical microvessels (Galea et al., 1991), excluding somehow a direct projection to cortical microvessels. These findings are rather surprising in view of previous anatomical reports demonstrating intimate relationships between cholinergic nerve terminals and cortical blood vessels (Eckenstein and Baughman, 1984; Arnerić et al., 1988; Chédotal et al., 1994a,b) or documenting the loss of the perivascular neural plexus (believed to originate in part from the nbM) from brain cortical microvessels in Alzheimer's disease (Scheibel et al., 1987; de la Torre and Mussivand 1993).

The aim of the present study was thus to assess the possibility that SI neurons contribute to the cholinergic innervation of the cortical microcirculation. To this end, we performed microiontophoretic injections of the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L) in the SI and determined, at the ultrastructural level and in two cortical subdivisions exhibiting distinct vasomotor responses to SI stimulation (Vaucher et al., 1995), whether PHA-L-labeled nerve terminals originating from SI neurons reach intracortical microarterioles and capillaries. In order to infer cholinergic specificity to perivascular PHA-L nerve terminals, their surface area and distance from the blood vessel wall were compared to those of perivascular cholinergic terminals in the same cortical area. Furthermore, the effects of unilateral SI lesion on cortical perivascular cholinergic terminals were investigated. The results show that SI neurons project preferentially to fronto-parietal cortical microvessels and that the bulk of perivascular cholinergic projections in this area originates in the basal forebrain.

Some of these data have appeared in abstract form (Vaucher and Hamel, 1994).

Materials and Methods

Animals

Male Sprague-Dawley rats, weighing 300–320 gm (Charles River, Montréal, Canada), were housed in a temperature-controlled (21–25°C) room, under natural daylight conditions and had free access to food and water. All experiments were approved by the Animal Ethics Committee based on the guidelines of the Canadian Council on Animal Care.

Surgical procedures

PHA-L injection in the SI. Animals ($n = 3$) were anesthetized with a mixture of ketamine (35 mg/kg, i.m., Ayerst, Montréal, QC, Canada) and xylazine (Rompun, 2 mg/kg, i.m., Haver, Etobicoke, ON, Canada) and mounted in a Kopf stereotaxic 900 frame adjusted to the coordinate system of Paxinos and Watson (1986) except that the incisor bar was set at -5 mm. PHA-L (Vector Laboratories, Burlingame, CA) was microinfused in a 2.5% solution in 10 mM sodium-phosphate buffer (NaPBS, pH 8.0) through a glass micropipette with a tip diameter of 20–30 μ m. The pipette was stereotactically lowered in the left SI and four injection sites (see below) were performed in order to increase the volume of injection. The tracer was iontophoretically injected with a 8–10 μ A positive current delivered by a 7 sec-on/7sec-off pulse for 25 min (Gerfen and Sawchenko, 1984). Pipets were left in place for 5 min before removal from the brain in order to avoid diffusion and were changed between each injection. The coordinates of the four injection sites were as follows [anterioposteriority (AP) and laterality (L) from bregma, verticality (V) below the skull surface, in mm]: AP: -0.9 , L: -2.7 , V: -7.6 ; AP: -1.4 , L: -2.8 , V: -7.9 ; AP: -1.3 , L: -3.3 , V: -7.9 ; AP: -1.8 , L: -3.1 , V: -8.0 (Fig. 1). The tracer was allowed to migrate for 14 d before the animals were processed for PHA-L immunocytochemistry.

Ibotenic acid injection in the SI. Rats ($n = 5$) were anesthetized and stereotactically prepared as above. A glass micropipette with a tip di-

ameter of 50 μ m was inserted twice in the left SI at the following coordinates (mm): AP: -0.9 , -1.8 ; L: -2.7 , -2.9 ; V: -7.6 , -7.9 below the skull surface. Ibotenic acid (10 μ g in 1 μ l of PBS 10 mM, pH 7.4; Sigma, St. Louis, MO) was slowly injected (0.1 μ l/min, 5 min/site) through the micropipette connected to a 10 μ l Hamilton syringe (Hamilton 1801 NE, Switzerland) driven by a microperfusor (pump 22, Harvard Apparatus, MA). To avoid diffusion of the neurotoxin along the pipette's track, the pipette was left in the same position 4–5 min before its removal from the brain. The rats were allowed to survive 7–10 d after injection of the neurotoxin and then used for choline acetyltransferase (ChAT) immunocytochemistry.

Immunocytochemistry

PHA-L immunocytochemistry. Following deep anesthesia of the rats with Somnotol (0.1 mg/100 gm body weight), the brains were fixed by perfusion through the ascending aorta with 500 ml of 4% paraformaldehyde and 0.06% glutaraldehyde in 0.1 M NaPB (pH 7.2–7.4) followed by 1000 ml of 4% paraformaldehyde alone. The brains were removed and postfixed in the latter solution for 2 hr and then cut at the level of the fronto-parietal or perirhinal cortex (Paxinos and Watson, 1986) in coronal thick (40–60 μ m) sections on an Oxford Vibratome.

For light microscopic (LM) examination, sections were preincubated 1 hr at room temperature in 20 mM potassium phosphate-buffered saline (KPBS, pH 7.4) containing 0.25% Triton X-100, 0.2% gelatin, and 0.001% azide. Free-floating sections were then incubated for 48 hr at 4°C with a biotinylated goat anti-PHA-L antibody (1:250, Vector Laboratories, Burlingame, CA) followed by a 1 hr incubation with an avidin biotin complex (ABC Elite 1:50, Vector Labs). After each incubation step, rinses were carried out in KPBS containing 0.25% Triton (4×8 min). The final rinse was done in Tris HCl buffer (0.1 M, pH 7.4). The immunocytochemical reaction product was developed in 0.05% of 3,3'-diaminobenzidine tetrachloride (DAB, Sigma) containing 0.03% ammonium sulfate nickel and 0.005% H_2O_2 . Sections were mounted onto gelatin-coated slides, dehydrated, and coverslipped with Permount.

For electron microscopy (EM), sections were first cryoprotected by incubation (15 min) in a 0.1 M Tris-sodium metabisulfite solution (pH 7.4) containing 36% sucrose and 14% glycerol and then frozen (15 sec) in isopentane at -50°C . They were immediately thawed in NaPB and processed for immunocytochemistry as described above for LM, except that the solutions were devoid of Triton X-100. Sections were then rinsed in 0.1 M NaPB and postfixed for 1 hr in 2% osmium tetroxide NaPB solution containing 7% dextrose (pH 7.2). After being washed for 20 min in 0.1 M NaPB, sections were stained en bloc with 2% uranyl acetate before dehydration and flat embedding in Araldite 502 (JBEM Services, Canada) for 48 hr at 60°C. After observation under the LM, areas of the fronto-parietal or perirhinal cortex were selected and reembedded in blocks of araldite. Serial thin (pale gold; 90 nm) or semithin (2–3 μ m) sections were cut on a Reichert-Jung Ultracut microtome. Semithin sections were mounted onto slices and observed under a Leitz Aristoplan light microscope (see below). Thin sections were collected on copper grids, double stained with uranyl acetate and lead citrate, and then examined under a JEOL CX100II electron microscope.

ChAT immunocytochemistry. The detailed protocol for ChAT immunocytochemistry at the LM and EM level has been published elsewhere (Chédotal et al., 1994b). Briefly, rat brains ($n = 3$) were fixed by perfusion, vibratome-cut coronal sections obtained, cryoprotected, and freeze-thawed as described above for PHA-L immunocytochemistry. Sections were then incubated overnight with a monoclonal mouse anti-ChAT antibody (2 μ g/ml in NaPBS; kindly provided by Dr. B. K. Hartman), followed by a biotinylated horse anti-mouse IgG (1:200, 1 hr, Vector Laboratories), and the ABC complex (1 hr). The immunocytochemical product was developed with DAB-Ni+ and H_2O_2 . Tissue sections were then treated as above for embedding in Araldite and serial thin (perirhinal cortex) or 3 μ m-thick semithin (rats with SI lesion) sections were obtained for analysis. For EM study, Triton X-100 was omitted from all solutions.

LM analysis of PHA-L and ChAT perivascular nerve terminals

The extent of the cortical projection areas of PHA-L-labeled SI neurons was evaluated on low magnification camera lucida drawings from brain sections obtained from rats in which the injection site (see Fig. 1) was limited to but encompassed the full extent of the SI. PHA-L immunoreactive projections in the fronto-parietal and perirhinal cortices were selected for further analysis since blood flow in these two cortical areas

Figure 1. Location of the PHA-L injection sites reported in three coronal planes of the brain atlas at -0.92 , -1.4 , -1.8 mm from bregma. The precise coordinates of the four injection sites located throughout the substantia innominata are given in details in Material and Methods.

was respectively increased or unaltered by stimulation of the SI (Vaucher et al., 1995). At the LM level, a nerve terminal was considered perivascular when it could be seen to follow the contours of the blood vessel walls (in vibratome sections), or when it was directly apposed to and literally touched the vessel walls (in semithin sections), as determined on photomicrographs (for more detail, see Chédotal et al., 1994b). The size (lumen diameter) of randomly selected intracortical microvessels ($n = 35$) reached by SI terminals was measured on high magnification photomicrographs of semithin sections. On similar sections from rats with unilateral SI lesion, the amounts of total and perivascular ChAT terminals were counted in a fixed area (0.2 mm^2) of fronto-parietal and perirhinal cortices and compared by Student t test between lesioned and control sides. A p -value ≤ 0.05 was taken as significant. For further information, the percentage decrease of perivascular cholinergic terminals was compared to that of total cortical cholinergic terminals determined in the same cortical areas.

EM analysis of PHA-L and ChAT perivascular terminals

At the ultrastructural level, a nerve terminal was considered perivascular when it was located within a $3 \mu\text{m}$ perimeter around the vessel basal lamina. This perimeter was selected from previous ultrastructural studies of functional neurovascular systems in peripheral, cerebral (Lee, 1981; Dodge et al., 1994), and small intraparenchymal (Chédotal et al., 1994b; Cohen et al., 1995) blood vessels. It was defined as the maximal distance within which ACh released from neuronal elements such as axon terminals might functionally influence the microcirculation either directly or through interaction with intervening processes located closer to the vessel wall. The morphometric characteristics of perivascular PHA-L and ChAT terminals were compared in both cortical subdivisions. The size (expressed in surface area) of each perivascular nerve ending and its nearest distance to the outer basal lamina of the microvessel were measured with a Bioquant II program and a MTi65 camera.

This analysis was performed on electron photomicrographs (working magnification of $14,000\times$) for 91 and 130 PHA-L terminals in the fronto-parietal and perirhinal cortex, respectively, and 128 and 143 ChAT terminals in these same respective cortical areas. Data from ChAT terminals in the fronto-parietal cortex were taken from Chédotal et al. (1994b) since they were obtained in the same area and from the same experimental protocol. A comparison of the synaptic frequency of perivascular PHA-L or ChAT terminals determined in single thin sections in the fronto-parietal and perirhinal cortices was also performed. A labeled (PHA-L or ChAT) varicosity was found to establish a synaptic contact when a junctional complex on an adjacent cellular element was apparent. This could correspond to a restricted zone of parallel membrane apposition and slight enlargement of the space with or without a clear postsynaptic thickening. As an additional means of comparison between the two perivascular systems, the synaptic incidence, which gives a precise estimate for a given neurotransmitter system of the proportion of nerve terminals engaged in junctional contacts, was calculated for PHA-L and ChAT varicosities. The synaptic incidence for whole varicosities was extrapolated from the observed synaptic frequency in single thin sections, using the stereological formula of Beaudet and Sotelo (1981), which takes into account the average diameter of the varicosities, the length of their junctional complexes, and the thickness of the sections. A representative population of cortical microvessels reached by perivascular terminals was randomly selected ($n = 46$) under electron microscopic observation. They were photographed under appropriate magnification ($\times 2500$ – $10,000$) and their lumen diameter measured as described above for the sectional area of nerve endings.

Statistical comparisons between the topometric data of perivascular PHA-L and ChAT-immunolabeled nerve terminals in the various cortical areas were achieved by one-way ANOVA followed by the multiple comparison test of Tukey, a $p \leq 0.05$ was considered significant.

Figure 2. Camera lucida drawings of PHA-L immunoreactive fibers and labeled cell bodies (black stars) reconstructed in coronal sections from three injected rats. AA: anterior amygdala; BLA: basolateral amygdala; CPu: Caudate putamen; Ent: entorhinal cortex; ic: internal capsule; Ins: insular cortex; MeA: medial amygdala; mfb: medial forebrain bundle; PRh: perirhinal cortex.

Results

PHA-L injected cells and their perivascular projections

The area of labeled cell bodies after PHA-L injections was very similar amongst the different rats. It extended from the anterior to the posterior part of the SI in both the ventral and dorsal aspects (Fig. 1). In coronal thick sections, projection fibers immunoreactive for PHA-L could be followed for a long distance through the striatum and along the corpus callosum (not shown) before appearing in the cerebral cortex where they were fine and varicose for the most part. PHA-L fibers were distributed throughout the cortical layers in all cortical subdivisions, the majority being confined to layers IV and V in the frontal and parietal cortices. In contrast, a very dense plexus of PHA-L labeled fibers invaded the full extent of the insular, perirhinal, and entorhinal cortices. The density of projection fibers was greatest in the amygdala, entorhinal, perirhinal, and insular areas as compared to more moderately distributed PHA-L fibers in the fron-

tal, parietal, and temporal subdivisions of the cerebral cortex, with only sparse labeling in the cingulate cortex (Fig. 2).

PHA-L fibers surrounding penetrating arteries, local microarteries as well as smaller intraparenchymal blood vessels of varying sizes (lumen diameter ~5 to 68 μ m) were observed in all cortical areas examined (Fig. 3). These neurovascular associations were better defined in semithin sections (Fig. 4) where multiple PHA-L cortical varicosities were in direct apposition with both local capillaries (lumen diameter ~4 to 8 μ m, 75% of vessels) and larger microvessels including small arteries, microarterioles, and possibly veins (lumen diameter ~8–30 μ m, 25% of vessels). Some of these cortical microvessels received more than one PHA-L immunoreactive nerve terminal (Fig. 4).

Ultrastructural analysis of perivascular PHA-L fibers

Perivascular PHA-L varicosities were studied in detail at the ultrastructural level in the fronto-parietal (Fig. 5) and perirhinal

Figure 3. PHA-L immunoreactive fibers in thick sections can be seen to surround intracortical blood vessels or a small penetrating artery (*double arrows in B*) located in the perirhinal (*A*), parietal (*B*), and frontal (*C*) cortical areas. Scale bars: 50 μ m.

Figure 4. PHA-L immunolabeled varicosities visualized in a semithin section (2 μm) in the parietal cortex. Note the direct apposition of some varicosities to the blood vessel wall (arrows). Scale bar: 50 μm .

cortices (Fig. 6). Their respective repartition around the vessel wall showed that more than 60% of them were located within 1 μm from the vessel in the fronto-parietal cortex in contrast to only 36% in the perirhinal cortex, with PHA-L terminals located in the immediate vicinity (0–0.25 μm) of the microvessels also being about twice as numerous in the fronto-parietal cortex (Fig. 7A,B, Table 1). In this cortical subdivision, perivascular PHA-L terminals decreased sharply in number as the distance from the vessel basal lamina increased while in the perirhinal cortex, perivascular terminals were rather uniformly distributed around the blood vessels (Fig. 7B). Overall, perivascular PHA-L nerve terminals in the fronto-parietal cortex were located slightly but significantly closer ($p < 0.01$) from the blood vessels than those of the perirhinal cortex (Table 1). Varicosities located in the immediate vicinity ($< 0.25 \mu\text{m}$) of microvessels virtually never contacted them directly since an intervening glial leaflet was normally present at the site of apposition (Figs. 5, 6, 8). In no instance were synaptic junctions established with these perivascular astrocytes. However, in single thin sections, PHA-L perivascular nerve terminals in the fronto-parietal and perirhinal cortices were seen to establish junctional contacts, often symmetrical, with adjacent dendritic branches (Fig. 6, Table 1). When extrapolated to the whole volume, the calculated synaptic incidence indicated that 23 and 28% of the perivascular PHA-L

terminals established junctional contacts with adjacent neuronal elements in the fronto-parietal and perirhinal cortical subdivisions, respectively. The size of perivascular PHA-L terminals was similar in both cortical subdivisions (Table 1).

Comparison of perivascular PHA-L and ChAT terminals

The average size (surface area) of PHA-L perivascular nerve terminals in the fronto-parietal and perirhinal cortices was statistically similar to, although slightly larger than that of perivascular ChAT terminals measured in corresponding cortical area (Table 1). In both cortices, perivascular ChAT terminals were located at an average distance of about 0.9 μm from the local microvessels (Table 1). While the distance from intracortical vessels compared very well between PHA-L and ChAT varicosities in the fronto-parietal cortex, perivascular PHA-L terminals in the perirhinal cortex (Fig. 8) were located further away from microvessels than their ChAT counterparts ($p < 0.001$; Table 1). Also, the repartition of perivascular PHA-L and ChAT terminals around blood vessel walls was similar in the fronto-parietal cortex, with about 60% of them being located within 1 μm from the basal lamina but not in the perirhinal cortex where only those containing ChAT were considerably enriched in the vicinity of the microvessels (Figs. 5, 7, Table 1). The synaptic frequency of perivascular ChAT varicosities on single thin sec-

Figure 5. Perivascular PHA-L-immunolabeled terminals in the fronto-parietal cortex. *A*, This small varicosity is located in the neuropile at less than 1 μm from the basal lamina of the capillary like most (62%) PHA-L frontoparietal terminals. *B*, Terminals in the immediate vicinity of the vessel wall are generally separated from it by an astrocytic leaflet (*open arrows*). *C*, PHA-L can also be detected in axonal segments running along the vessel wall. Note the zonulae occludens (*z.o.*) between two endothelial cells. Scale bars: 1 μm .

tions was similar in the two cortical areas ($\sim 5\%$), but was slightly lower than that of their corresponding perivascular PHA-L terminals (Table 1). These contacts were generally small, symmetrical, and on dendritic branches and a synaptic incidence in the order of 10–15% was obtained for cholinergic terminals in both cortices (Table 1). The size of the vessels targeted by PHA-L nerve endings was similar to that reached by cholinergic terminals and accounted for local capillaries (lumen diameter between 4 to 8 μm) in 63% of cases with the remaining terminals approaching larger vessels (8–15 μm ; 24%) and small arteries (15–30 μm ; 13%).

Lesion of the substantia innominata

The efficacy of the lesion was evaluated by comparison of ChAT immunolabeled cell bodies area between the lesion and the con-

trol sides. On the lesioned side, neurons containing ChAT immunoreactivity had largely disappeared from the SI and the nucleus basalis magnocellularis while those in the horizontal limb of the diagonal band of Broca were consistently spared (Fig. 9). The density of ChAT terminals in the fronto-parietal and perirhinal cerebral cortices was reduced in the hemibrain ipsilateral to the lesion, but the denervation was much more pronounced in the fronto-parietal cortex (Table 2). Similarly, perivascular ChAT terminals decreased drastically on the lesion side in the fronto-parietal cortical subdivision but only partially in the perirhinal cortex (Table 2, Fig. 9). The results also indicated comparable decreases in perivascular and neuronal ChAT terminals within the same cortical area (Table 2). The quantitative analysis performed on semithin sections from the control sides further

Figure 7. Histograms of the distribution of perivascular PHA-L and ChAT immunostained terminals within the 3 μ m interval around intracortical microvessels in frontoparietal (A) or perirhinal (B and C) cortex. The abscissa is divided in intervals of 0.25 μ m at increasing distance from the vessel basal lamina. The bars represent the number of varicosities found in each 0.25 μ m interval and are expressed as percentage of total PHA-L or ChAT terminals counted in each cortical area.

showed that the proportion of perivascular cholinergic terminals in the fronto-parietal cortex was about twice that of the perirhinal cortex (Table 2).

Discussion

Technical considerations

Pilot experiments, in agreement with Gerfen and Sawchenko (1984), showed that single injections of PHA-L diffused in a

very small volume and labeled a limited number of SI neurons. The resulting labeling of cortical projection fibers was thus sparse and insufficient for detailed ultrastructural analysis. In contrast, the implementation of four injection sites that encompass the great majority of neurons from the anterior to the posterior aspect of the SI together with the addition of the freeze-thaw procedure allowed to detect dense PHA-L projection fibers in every subdivision of the cerebral cortex. This multisite injection procedure was found to be restricted to neurons located within the SI and to result in prominent labeling in most layers of the perirhinal-insular and entorhinal cortical area with a more circumscribed distribution of the fibers in layers IV and V of the fronto-parietal cortex. Such a heterogeneous distribution is supported by previous reports (Luiten et al., 1987) and suggests that our injection and detection procedures provided a fair estimate of SI projections to the cerebral cortex.

SI neurons project to cortical microvessels

When examined in detail under light and electron microscopy, a subset of the cortical projections of PHA-L-injected SI neurons were found to be perivascular. They reached cortical penetrating arteries, small microvessels and capillaries. The finding that at least 30% of vessels corresponded in size to small arteries and microarterioles strongly suggests that these perivascular terminals may exert significant effects on vasomotricity through small resistance vessels. This contention is further supported by the fact that intracortical small arteries and arterioles have been shown to dilate or constrict in response to several vasoactive neurotransmitters (Dacey et al., 1987; Edwards et al., 1991; Sagher et al., 1993). Furthermore, since perivascular projections were studied in single sections, it is obvious that on its full length a given blood vessel is reached by a much larger number of SI terminals, a situation that is likely to increase their impact on microvascular functions. Such a vasomotor role for SI neurons appears more prominent in the fronto-parietal cortex as the distribution of their perivascular terminals within the 3 μ m perimeter around blood vessels, clearly indicated an enrichment in the immediate vicinity (< 0.25 μ m) up to 1 μ m from the vessel wall. This close proximity might not suffice to confer a functional role to perivascular terminals; however, combined with the fact that such a preferential vascular association was not found for PHA-L terminals in the perirhinal cortex, an area that does not modify its blood flow in response to SI stimulation (Vaucher et al., 1995), it provides a strong morphological substrate for the well documented actions of SI neurons on fronto-parietal blood flow (see below, and for review, Sato and Sato, 1992). Interestingly, similar intimate anatomical relationships for perivascular terminals have been associated to the effective neurogenic control of vasodilator or vasoconstrictor nerves in peripheral (Dodge et al., 1994), cerebral (Lee, 1981; Dodge et al., 1994), and intraparenchymal blood vessels (Cohen et al., 1995). In line with the fact that capillaries are approximately 10 times more numerous than arterioles (McDonald and Rasmussen, 1977), the majority of perivascular terminals reached intracortical capillaries, an observation that might be indicative of a role

Figure 6. Perivascular PHA-L-immunolabeled terminals in the perirhinal cortex. A, The terminal and the axonal segment are contained within the 3 μ m perimeter around the vessel wall (dashed line). B, Two varicosities probably from the same fiber are reaching towards the capillary, only the closest varicosity is located within the 3 μ m perivascular perimeter. C–D, Perivascular PHA-L varicosities visualized in two serial electron photomicrographs are seen to establish symmetrical synaptic junctions with surrounding dendritic branches (curved arrows) and are separated from the vessel by a glial leaflet (open arrows). Scale bar: 1 μ m.

Table 1. Morphological features of perivascular PHA-L and ChAT nerve terminals in the cerebral cortex

	Fronto-parietal cortex		Perirhinal cortex	
	ChAT ^a	PHA-L	ChAT	PHA-L
<i>n</i>	128	91	143	130
Distance from vessel wall ($\mu\text{m} \pm \text{SEM}$)	0.86 ± 0.07	0.98 ± 0.09	0.93 ± 0.06	$1.34 \pm 0.07^*$
Surface area ($\mu\text{m}^2 \pm \text{SEM}$)	0.32 ± 0.02	0.38 ± 0.03	0.31 ± 0.02	0.34 ± 0.03
Terminals within $0.25 \mu\text{m}$ (%)	32	23	28	13
Terminals within $1 \mu\text{m}$ (%)	60	62	56	36
Synaptic frequency (%)	5	8	4.5	14
Synaptic incidence (%)	14	23	9	28

Results have been obtained as described in Materials and Methods following analysis on the number (*n*) of perivascular terminals as indicated.

^a Data taken from a previous study performed with identical procedures within the exact same area (Chédotal et al., 1994b).

* Significantly different from corresponding ChAT nerve terminals ($p < 0.001$), and from the ChAT ($p < 0.001$) or PHA-L ($p < 0.01$) terminals in the fronto-parietal cortex, as evaluated by ANOVA and the multiple comparison test of Tukey.

for SI neurons in blood–brain barrier and transport mechanisms. Nevertheless, it cannot be excluded that pericapillary terminals may also participate in flow regulation by delivering signals that could be integrated for transmission upstream to microarterioles and small arteries, as recently hypothesized in peripheral and microvascular beds (see Song and Tymml, 1993; Iadecola, 1993).

Perivascular basal forebrain terminals never established junctional contacts with the vascular or glial cells, although they could do so with adjacent dendrites. This observation is suggestive of a communication via a mode of volume transmission, as already established for the functional innervation of major cerebral arteries and small pial vessels (Lee, 1981; Dodge et al., 1994) as well as for various neurotransmitters within the cerebral cortex (see Descarries et al., 1991), including ACh (Umbriaco et al., 1994). This involves diffusion of the vasoactive substance in the extracellular space before it reaches its receptor(s) in neighboring vascular, glial, and/or neuronal targets. Whether or not perivascular astrocytes, which are an integral component of the functional microvascular unit, are the primary effector of PHA-L-labeled SI terminals that abutted on glial processes rather than on endothelial or smooth muscle cells, will remain to be established. In fact, a pivotal role for perivascular astrocytes in the neurogenic control of cortical blood flow and blood–brain barrier mechanisms has been suggested previously from several characteristics of these cells (Hertz, 1992), but also from morphological evidence of neuronal–glial–vascular interactions (Chédotal et al., 1994b). It cannot be excluded, however, that the attraction exerted by cortical microvessels including perivascular astrocytes on SI terminals relies on the presence of glial and/or vascular growth factors (see Chédotal et al., 1994b) and partly fulfils a role in their survival and maintenance.

Chemical nature of perivascular basalo-cortical projections

In the fronto-parietal cortex, perivascular basalo-cortical projections shared similarities in terms of average distance from and global distribution around cortical microvessels with previously characterized perivascular cholinergic terminals (Chédotal et al., 1994b). In the perirhinal cortex, in contrast, perivascular PHA-L terminals differed from their cholinergic counterparts in their overall associations with the local microvasculature, suggesting that they are not exclusively cholinergic. This interpretation is further substantiated by the rather modest perivascular cholinergic denervation observed in perirhinal as opposed to fronto-parietal cortex following SI lesion, and this in spite of the greater density of PHA-L-labeled SI projections in the perirhinal cortex.

Altogether, these findings first indicate that the cholinergic input to microvessels in the perirhinal cortex originates primarily from local interneurons (Eckenstein and Baughman, 1984; Galea et al., 1991; Chédotal et al., 1994a) while most perivascular cholinergic terminals in the fronto-parietal cortex are from the basal forebrain. Second, they suggest that a subset of PHA-L terminals studied in the present study originate from noncholinergic SI neurons, this contingent being more important in the perirhinal than the fronto-parietal cortex. Such conclusion agrees very well with previous studies claiming that while up to 80% of basalo-cortical projections in the fronto-parietal cortex are cholinergic, this proportion is at the most 40 to 60% in the perirhinal cortex (Rye et al., 1984; Woolf, 1991). The finding that perivascular PHA-L terminals tended to be slightly larger and more frequently engaged in synaptic contacts with neighboring dendrites than their cholinergic counterparts further supports their mixed chemical nature. The possibility exists that SI neurons containing γ -aminobutyric acid (GABA) (Freund and Gulyas, 1991; Gritti et al., 1993), a neuromediator with vasomotor properties (for review, see Lou et al., 1987), could contribute to the pool of basalo-cortical nerve terminals associated with microvessels. Interestingly, GABA varicosities in the cerebral cortex are highly junctional and appear larger than cholinergic terminals (Houser et al., 1984), two characteristics that could partly explain the differences between PHA-L and ChAT perivascular terminals observed in the present study.

Perivascular cholinergic basalo-cortical projections: functional correlates

Cholinergic nerve terminals in the immediate vicinity of cortical microvessels had been reported previously (Eckenstein and Baughman, 1984; Armstrong, 1986; Arnerić et al., 1988) and partly or totally attributed to intrinsic bipolar cortical cholinergic neurons (Eckenstein and Baughman, 1984; Galea et al., 1991; Chédotal et al., 1994a). Our present data not only demonstrate that in the fronto-parietal cortex, the majority of these perivascular cholinergic terminals originate in the basal forebrain but also that they are proportionally twice as numerous as their counterparts in the perirhinal cortex. These findings concur with previous physiological studies that showed that cholinergic SI neurons regulate cortical blood flow primarily in the fronto-parietal cortex (for review, see Sato and Sato, 1992), most likely through muscarinic receptors localized on cortical microvessels (Estrada et al., 1983; Garcia-Villalon et al., 1991; Linville and

Figure 8. Perivascular ChAT immunostained terminals in the perirhinal cortex. *A*, Low-magnification electron photomicrograph in which three ChAT varicosities can be seen around the blood vessel. In *A* and *B*, only a glial leaflet (*open arrows*) separates the terminals from the basal lamina of the vessel wall. *C*, The *black arrow* shows the presence of a thin pericytic extension. Scale bars: 1 μ m.

Hamel, 1995) and/or perivascular astrocytes (Van der Zee et al., 1993; Hösl and Hösl, 1993).

The fronto-parietal and perirhinal cortices are areas where blood flow is respectively increased (for review, see Sato and Sato, 1992) or unaffected by SI stimulation (Vaucher et al., 1995). Whether this functional difference could be explained by the greater distance of PHA-L terminals from microvessels in the perirhinal as opposed to the fronto-parietal cortex, the non-cholinergic nature of an important subset of these, or by the

smaller proportion of perivascular cholinergic terminals in the area, remains to be established. However, it is also possible that the final mediator of the cholinergic basalo-cortical vasodilatation is produced locally in cortical cells following ACh release, as recently suggested for the cerebellar cortical vasodilatation induced by parallel fiber activation (Li and Iadecola, 1994). For instance, a role for nitric oxide (NO) has been claimed in this vasodilatory pathway (Adachi et al., 1992a; Raszkievicz et al., 1992) and interactions between cortical perivascular cholinergic

Table 2. Effect of SI lesion on total and perivascular cholinergic terminals in the rat cerebral cortex

Cortical subdivision	Side	Total ChAT terminals	Decrease %	Perivascular ChAT terminals	Decrease %
Fronto-parietal	Control	448 ± 20		10.7 ± 1.5	
	Lesion	198 ± 33***	55 ± 7.8 (31–74)	4.0 ± 0.9**	63 ± 6.8 (47–75)
Perirhinal	Control	680 ± 50		8.4 ± 0.9	
	Lesion	501 ± 60	26 ± 7.9 (7–43)	6.1 ± 0.9*	35 ± 13.2 (0–50)

Data are expressed as mean ± SEM ($n = 5$) of the amount of ChAT-immunolabelled terminals counted in an area of 0.2 mm² of cortical surface in two individual semithin sections for each rat. The range of percent decrease among the rats is given within parentheses.

*** $p < 0.001$; ** $p < 0.005$, and * $p < 0.05$ (in this case $n = 4$) by Student's t test.

SI terminals and NO neurons have recently been identified (Vaucher et al., 1995) and could take place in order to elicit the proper vasodilatory response (for review, see Iadecola, 1993; Iadecola et al., 1994). Further functional and pharmacological studies will be required to support such hypothesis, but it is clear that within the 3 μ m perimeter around blood vessels, perivascular terminals interact with other neuronal elements that could lead to the release of the primary vasoactive effector(s). The role of perivascular SI projections in the cortical areas where blood flow is unaffected by SI stimulation, although not exclusive to these areas, might be more closely related to the regulation of the blood–brain barrier. Such a possibility is supported by the documented association of perivascular ChAT terminals with capillaries (Arnerić et al., 1988; Chédotal et al., 1994b, this study) and the presence of receptors for various neurotransmitters including ACh on endothelial cells (Spatz et al., 1989; Xu et al., 1992).

Relevance to Alzheimer disease

In humans, cholinergic innervation of the neocortex originates almost exclusively in the nucleus basalis of Meynert in the basal forebrain (Ramon-Moliner and Nauta, 1966; Mesulam and Geula, 1988). It has been suggested that such cholinergic nerve terminals associate with intracortical microvessels, thus providing an additional mechanism through which ACh could influence cortical activity (Mesulam et al., 1992). Compelling evidence has recently accumulated that indicates biochemical and morphological changes in brain microvasculature in Alzheimer disease (for review, see Kalaria, 1992). More specifically, a denervation microangiopathy together with microvascular pathological changes including basal lamina thickening, β -amyloid deposit, and multiple capillary distortion (Perlmuter and Chang Chui, 1990; Scheibel et al., 1987; Vinters et al., 1994; Buée et al., 1994) have been consistently documented. Whether these vascular and neuronal events occur independently or whether the neuronal degeneration is a consequence of vascular dysfunction (Scheibel et al., 1987; de la Torre and Mussivand, 1993) remains to be established. In view of our present findings and

the recent reports on the fate and plasticity of cerebrovascular innervation in aging (Andrews and Cowen, 1994), it is tempting to speculate that if perivascular cholinergic innervation of cortical microvessels degenerates in AD, this could most likely elicit abnormalities in cortical microperfusion and/or blood–brain barrier functions, two features well documented in AD (for review, Kalaria, 1992).

In conclusion, we have demonstrated the existence of projection fibers between basal forebrain neurons and cortical microarterioles and capillaries, and have shown that a large proportion of these terminals in the fronto-parietal cortex originate from cholinergic SI neurons. The topometric results together with those of selective SI lesion further suggest that noncholinergic SI neurons also contribute to the neurogenic input to cortical microvessels, and more so in the perirhinal subdivision. Further, the data suggest that the microvascular bed in the fronto-parietal cortex might be more closely influenced by basal forebrain neurons than that of the perirhinal cortex, in agreement with physiological blood flow studies following stimulation of SI neurons. It has been suggested that a denervation microangiopathy occurs in senile dementia of the Alzheimer's type (Scheibel et al., 1987). Our study potentially points to the involvement of such neurovascular cholinergic terminals originating from the basal forebrain in this microvascular denervation process. However, such conclusion awaits further studies in human brain tissue.

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Figure 9. Effects of substantia innominata lesion on the cholinergic innervation of cortical microvessels. In semithin sections (*upper four panels*), the density of cortical cholinergic nerve terminals including those associated with intrinsic microvessels is reduced on the lesioned (*A* and *C*) as compared to the control side (*B* and *D*) and more so in the fronto-parietal than in the perirhinal cortex (*upper and middle panels*, respectively; see Table 2 for quantitative analysis). Note that some perivascular terminals remain (*arrows in A and C*), and particularly in the perirhinal cortex following effective ibotenic acid lesion of ChAT-immunostained SI neurons (*E*). Control side (*F*). Scale bars: 50 μ m (*A–D*); 100 μ m (*E* and *F*).

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