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CHOLINERGIC BASAL FOREBRAIN PROJECTIONS TO NITRIC OXIDE SYNTHASE-CONTAINING NEURONS IN THE RAT CEREBRAL CORTEX

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Abstract—Stimulation of basal forebrain neurons elicits regional cerebral blood flow increases which are reportedly mediated by acetylcholine and nitric oxide. However, the modality of interaction between these two mediators remains unclear. Particularly, little is known about the source, i.e. endothelial, glial and/or neuronal, of the potent gaseous vasodilator nitric oxide. In the present study, we examined, by double immunocytochemical labelling of nitric oxide synthase and choline acteyltransferase at the light and electron microscopic level, the existence of morphological relationships between cortical nitric oxide synthase-containing neurons and cholinergic cells or nerve fibres. Using anterograde tract tracing and selective basal forebrain lesions, we further investigated the origin of the cholinergic input to cortical nitric oxide synthase neurons. The results confirm that cortical nitric oxide synthase-immunoreactive neurons are often associated with the local microvascular bed, show that intracortical neurons immunostained for nitric oxide synthase and choline acetyltransferase belong to two distinct neuronal populations and, further, that a subset of nitric oxide synthase-containing cell bodies and their proximal dendrites receive a cholinergic input which originates primarily from basalocortical projections.

Altogether, these findings suggest that cholinergic basal forebrain neurons could increase cortical blood flow partly via a local nitric oxide relay neuron whereby the freely diffusing gas would be the direct smooth muscle vasodilator agent. It is concluded that this interaction might contribute to the complex relationships between the basal forebrain and the cortical microcirculation, interactions which result in fine regulation of cortical perfusion. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: nitric oxide, anterograde transport, immunocytochemistry, basal forebrain, cortical microcirculation, substantia innominata.

Electrical and chemical stimulation of the basal forebrain (BF) elicits regional cerebral blood flow (CBF) increases which are particularly pronounced in the frontoparietal cortex.[29,45] These CBF changes are reportedly due to direct neurovascular interactions since they are not secondary to changes in local metabolism.[21,27,52] BF neurons provide the main cholinergic input to the neocortex[14,30,31,36] and, as recently demonstrated,[51] to the cortical microcirculation. They may thus affect directly the microvascular bed which is endowed with functional muscarinic acetylcholine (ACh) receptors.[16,20,34] However, evidence also suggests that intrinsic cortical neurons may contribute to the neurogenic control of the local microcirculation.[4,12] Indeed, autoradiographic and sampling studies have shown that the cerebrovascular responses to BF stimulation differ depending on the brain and cortical areas,[6,33,50] possibly reflecting differences in integrative mechanisms that locally regulate brain microperfusion.

Recent anatomical and pharmacological studies support the involvement of a relay neuron in the basalocortical vasodilatory pathway. Indeed, a majority of cholinergic nerve terminals in the vicinity of blood vessels were found to contact neuronal elements such as dendrites, cell somata and other axonal varicosities,[5,9,15] all of which represent potential sites for the release of the effective vasodilator. In this respect, the flow increases elicited by BF stimulation were found to be partly reduced following inhibition of nitric oxide synthase (NOS),[1,42] a finding which strongly indicated that nitric oxide (NO) is likely to be involved in the cortical vasodilatation. While Raszkiewicz and colleagues[42] favoured a neuronal rather than endothelial origin for the NO implicated in the basalocortical vasodilatation, its exact neuronal, glial and/or vascular origin still remains to be established. Considering the close association between cortical NOS neurons and the local microvascular bed,[17,25,43] and the proposal that NO might

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Abbreviations: ABC, avidin–biotin complex; ACh, acetylcholine; BF, basal forebrain; CBF, cerebral blood flow; ChAT, choline acetyltransferase; DAB, 3,3′-diaminobenzidine tetrachloride; IR, immunoreactive; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; PBS-G-T-A, sodium phosphate-buffered saline containing 0.25% Triton X-100, 0.2% gelatin and 0.001% azide; PHA-L, *Phaseolus vulgaris* leucoagglutinin; SI, substantia innominata.

be the factor that links local CBF to neuronal activation,[2,13,19,26,37] we investigated whether BF cholinergic neurons could influence the cortical microcirculation by interacting with local NOS neurons. To assess this issue, we performed double immunocytochemical labelling of NOS and choline acetyltransferase (ChAT, the synthesizing enzyme for ACh), at the light and electron microscopic level. Further, we determined if cortical NOS neurons receive cholinergic BF projections by means of anterograde tract tracing and selective lesions.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (n=11) weighing 300–320 g (Charles River, Montréal, Canada) were housed in a temperature-controlled (21–25°C) room, under natural daylight conditions and they 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

Phaseolus vulgaris *leucoagglutinin injection in the substantia innominata*. Animals (n=3) were anaesthetized 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). *Phaseolus vulgaris* leucoagglutinin (PHA-L, Vector Laboratories, Burlingame, CA, U.S.A.) was stereotaxically injected through a glass micropipette in four injection sites within the left substantia innominata (SI), as previously described in detail.[51] The coordinates of the four injection sites were as follows [anterioposteriority (AP) and laterality (L) from bregma, vertically (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. The tracer was iontophoretically microinfused in a 2.5% solution in 10 mM sodium phosphate-buffered saline (NaPBS, pH 8.0) with an 8–10 μA positive current delivered by a 7 s-on/7 s-off pulse for 25 min/site. The tracer was allowed to migrate for 14 days before the animals were processed for PHA-L and NOS double immunocytochemistry.

Ibotenic acid injection in the substantia innominata. Rats (n=5) were anaesthetized and stereotaxically prepared as above. Ibotenic acid (10 μg in 1 μl of PBS 10 mM, pH 7.4; Sigma, St Louis, MO, U.S.A.) was slowly injected (0.1 μl/min, 5 min/site) through a glass micropipette (tip diameter of 50 μm) in two sites within the left SI. The effectiveness of the lesion, as evaluated on alternate sections from the same rats, was previously demonstrated by its ability to induce a considerable decrease (∼55%, P<0.001) in cortical cholinergic terminals in the frontoparietal cortex.[51] The exact coordinates were (mm): AP: −0.9, −1.8; L: −2.7, −2.9; V: −7.6, −7.9, as defined above. The micropipette was connected to a 10 μl Hamilton syringe (Hamilton 1801 NE, Switzerland) driven by a microperfusor (pump 22, Harvard Apparatus, MA, U.S.A.). The rats were allowed to survive seven to 10 days and then used for NOS and ChAT double immunostaining.

Immunocytochemistry

Nitric oxide synthase immunocytochemistry. Following deep anaesthesia of the rats (n=3) with Somnotol (0.1 mg/100 g 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 NaPBS (pH 7.2–7.4) followed by 1000 ml of 4% paraformaldehyde alone. The brains were removed, post-fixed in the latter solution (2 h) and cryoprotected (30% sucrose) for two days. Serial coronal sections (30 μm thick) were obtained with a freezing microtome and alternate sections were preincubated (1 h, room temperature) in 0.1 M NaPBS containing 0.25% Triton X-100, 0.2% gelatin and 0.001% azide (PBS-G-T-A, pH 7.4) and then incubated overnight with a rabbit anti-NOS antibody (polyclonal antibody directed against a synthetic peptide from the C-terminal of the cloned rat cerebellar NOS protein; Euro-Diagnostica, Malmö, Sweden or Cedarlane Laboratories Ltd., Hornby, Ontario, Canada; 1:10,000 in PBS-G-T-A). They were subsequently incubated with a biotinylated goat anti-rabbit IgG (1:200, 1.5 h, Vector Laboratories), and the avidin–biotin complex (ABC Elite 1:50, Vector Laboratories). The immunocytochemical product was revealed with 0.05% of 3,3′-diaminobenzidine tetrachloride (DAB, Sigma) and 0.005% H_2O_2 and visualized as a pale brown precipitate. The reaction time was adjusted in order to obtain optimal labelling of cell bodies and proximal dendrites with few, if any, labelling of neuronal processes such as small dendrites and axon terminals. Between incubations, rinses were carried out in PBS containing 0.25% Triton (4 × 8 min), except for the rinse prior to chromogen development, which was in Tris–HCl buffer (0.1 M, pH 7.4). Sections were mounted onto gelatin-coated slides, dehydrated and coverslipped with Permount.

Phaseolus vulgaris *leucoagglutinin and nitric oxide synthase double immunolabelling*. Rats were prepared as above except that the brains were cut in thick (40–60 μm) sections on an Oxford Vibratome immediately after the post-fixation. Free-floating sections were preincubated (1 h, room temperature) in 20 mM potassium phosphate-buffered saline supplemented as above (KPBS-G-T-A). They were then incubated (48 h, 4°C) in a mixture of biotinylated goat anti-PHA-L (1:250, Vector Laboratories) and rabbit anti-NOS (1:10,000) antibodies in KPBS-G-T-A. After incubation with the ABC complex (1 h 15 min), labelling of PHA-L in nerve fibres and terminals was developed with DAB intensified with 0.03% ammonium sulphate nickel (DAB-Ni^+) in order to yield a dark brown precipitate. Neurons were then immunostained for NOS with DAB alone as above. The sections were either mounted onto microscope slides or embedded in Araldite and re-sectioned in semithin (3–4 μm) sections on a Reichert–Jung Ultracut microtome for light microscopy analysis (see below).

Choline acetyltransferase and nitric oxide synthase double immunolabelling. The 30-μm-thick frozen (alternate to those used for NOS immunostaining alone) or 60-μm-thick Vibratome (from ibotenic acid injected-rats) sections were used. They were incubated overnight in a mixture of a monoclonal mouse anti-ChAT ((2 μg/ml; ChAT-17 directed against purified rat brain ChAT,[11] fully characterized before for specificity[48] and generously provided to us by B.K. Hartman) and the rabbit anti-NOS (1:10,000) antibodies in PBS-G-T-A. Following incubation with a biotinylated horse anti-mouse IgG (1:200, 1.5 h, Vector Laboratories), and the ABC complex (1 h 15 min), the ChAT immunolabelling was developed in DAB-Ni^+ and H_2O_2 in order to obtain a dark brown labelling of the cell soma, dendritic arborizations as well as nerve terminals. While some sections immunostained only for ChAT were kept apart for quantitative analysis (see below), most sections were then stained for NOS cell bodies with DAB alone as described above. They were either mounted onto microscope slides or embedded in Araldite and re-sectioned in semithin (3–4 μm) sections for light microscopy analysis (see below).

For electron microscopy, 60-μm-thick Vibratome sections were cryoprotected in a sucrose–glycerol solution (15 min), frozen in isopentane at −50°C (15 s) and thawed.[51] They

were then processed for ChAT/NOS double immunolabelling as described above except that the solutions were devoid of Triton X-100. Tissue sections were then processed for electron microscopy (for details, see Ref. 51) and serial thin (pale gold; 90 nM) sections were collected on copper grids, double-stained with uranyl acetate and lead citrate and examined under a JEOL CX100II electron microscope.

Microscopic analysis

Light microscopic analysis of nitric oxide synthase, choline acetyltransferase/nitric oxide synthase and Phaseolus vulgaris *leucoagglutinin/nitric oxide synthase immunostaining*. Immunocytochemical protocols were developed in order to ensure virtually exclusive labelling of NOS cell bodies (detected as a pale brown precipitate) while PHA-L and ChAT nerve terminals as well as ChAT cell somata were intensely labelled (dark brown precipitate). This allowed for the examination of the putative innervation of cortical NOS by cholinergic BF projections. In cell perikarya, the two colours were easily distinguishable and, moreover, the precipitates had very distinct aspects, granular for NOS and uniform for ChAT, which provided an additional means to unequivocally discriminate between the two cell types (see Fig. 1). Moreover, all quantitative analyses were performed directly under the microscope in order to adjust focal plane and illumination to obtain optimal distinction between the two labels in thick sections, irrespective of the way neurons were sectioned or oriented within the cortical field. Due to the limitations in evaluating precisely the proximity between neural appositions and target neurons in thick sections, additional analyses were performed on double-immunolabelled (ChAT/NOS or PHA-L/NOS) semithin (3–4 μm) sections in order to unequivocally visualize neuronal elements abutting on NOS-immunoreactive (IR) cell bodies.

A first analysis was undertaken on 30-μm-thick sections immunostained for NOS alone or for ChAT/NOS, in order to establish if these two intracortical neuronal populations were distinct or co-localized. For this purpose, NOS-IR neurons were counted from the cingulate cortex up to the inferior border of the lateral orbital cortex (as determined by Zilles)[56] in consecutive mirror-image sections (six to eight/rat, n=3 rats, working magnification of $\times 250$) stained for NOS alone or double-stained for ChAT/NOS. The amount of NOS-IR cells under these two labelling conditions was determined by three independent observers, averaged (results shown in the text as $X \pm S.D.$) and then compared by Student t-test. $P \leq 0.05$ was taken as significant. Additional analyses were performed on PHA-L/NOS and ChAT/NOS double-labelled thick sections in order to evaluate the proportion of (i) NOS-IR neurons surrounded, touched or overlaid by SI projecting fibres immunoreactive for PHA-L; (ii) NOS-IR neurons associated with local microvessels, as determined by the direct apposition of their cell body or proximal neuronal processes on blood vessel walls and (iii) NOS-IR cell bodies contacted by ChAT-IR punctate structures. As a comparison, the proportion of ChAT-IR neurons associated with local blood vessels was determined in adjacent sections stained for ChAT alone. For this purpose, ChAT-IR neurons, including perivascular ones, were randomly counted in the same subdivisions of the cerebral cortex until a number similar to that of cortical NOS-IR neurons was obtained. Quantitative analyses were performed on semithin sections double-immunostained for (i) PHA-L/NOS in order to evaluate the proportion of NOS-IR neurons directly contacted by PHA-L immunoidentified basal forebrain SI terminals, and (ii) ChAT/NOS to compare the proportion of NOS-IR neurons receiving a cholinergic input on the contralateral (control) and ipsilateral (lesioned) sides of ibotenic acid-injected rats in the SI. Analyses were performed in three to four sections/rat (n=3 rats, working magnification of $\times 250$) and the data expressed as a percentage of the total number of NOS- or ChAT-IR neurons counted.

Electron microscopic analysis of choline acetyltransferase/nitric oxide synthase interactions. In the case of ChAT puncta apposed to NOS-IR neurons, the nature (axon terminal or dendrite) of the processes and of their somatic appositions (synaptic vs non-synaptic) was further investigated at the electron microscopic level. For this purpose, NOS-IR neurons endowed with ChAT-IR terminals were visualized and localized directly on the 60-μm-thick sections in Araldite. These were then embedded in capsules and trimmed in small blocks containing only one NOS-IR cell, or alternatively the position of the NOS-IR neurons was located in the blocks with reference landmarks such as blood vessels. Serial thin sections were then obtained and observed under a JEOL II electron microscope. The NOS-IR neurons were located under the electron microscope and examined for the presence of ChAT-IR terminals abutting on their somata or proximal dendrites.

RESULTS

Cortical nitric oxide synthase-immunoreactive neurons

Intrinsic cortical NOS neurons were found throughout the cortical mantle. They were generally large, round or stellate and distributed with no apparent organization with specific cortical layers. The pale brown DAB precipitate in NOS-IR cells was granular and could be detected only in the cytoplasm of the cell soma and proximal processes (Fig. 1A). This gave the NOS-IR neurons a characteristic dotted appearance which, together with their morphological features, made them easily distinguishable from the uniformly-stained cortical bipolar ChAT-IR neurons in the double-immunolabelled sections (Fig. 1B). The number of NOS-IR neurons from the cingulate to the inferior border of the lateral orbital cortex was comparable whether determined in sections immunostained for NOS alone or double-stained for ChAT and NOS (n=43.4 ± 4.8 versus 40.4 ± 6.0 cells, $X \pm S.D.$). As reported before[17,25,43] their cell soma, proximal dendrites or major large processes were frequently associated with local microvessels (Fig. 1). In the same cortical division as above, 57.4% (167 of 291 cells counted) of the NOS-IR neurons contacted local blood vessels, while this proportion was only 28.6% (95 of 332 cells counted) for the cortical ChAT-IR neurons.

Basal forebrain projections to cortical nitric oxide synthase neurons

In double immunostained thick PHA-L/NOS sections, basalocortical projections were highly varicose and darkly immunostained for the anterograde tracer PHA-L. PHA-L-immunostained varicose fibres in these rats were shown previously to distribute to deep as well as more superficial layers of the cortical mantle.[51] Irrespective of the cortical subdivision or layer, some NOS-IR neurons in the neuropil were often reached by a plexus of PHA-L fibres which also appeared to follow closely their proximal dendritic

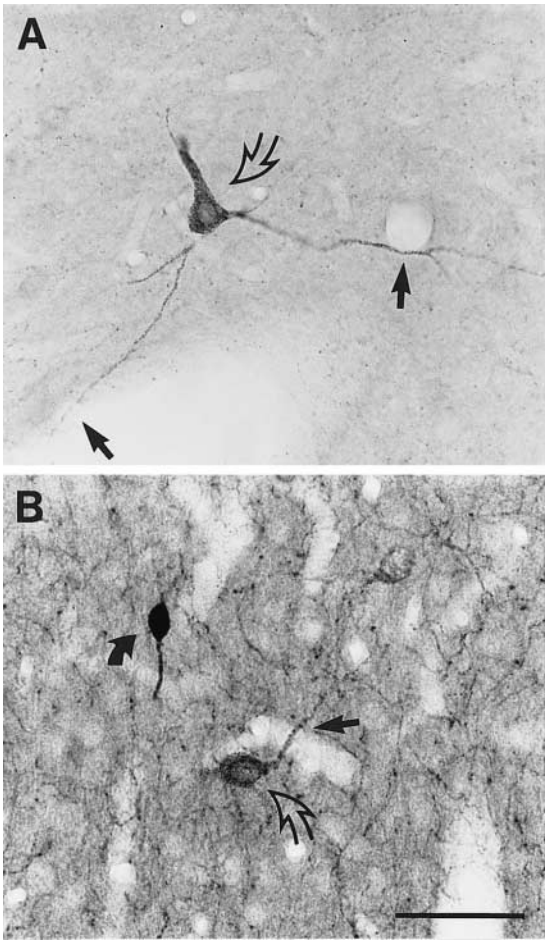

Fig. 1. Visualization of NOS neurons in rat cerebral cortex from sections immunostained for NOS alone (A) or double-stained for ChAT/NOS (B). NOS neurons (open arrow) stained with DAB (pale brown under microscopic observations) are large, generally round or stellate in shape and the immunocytochemical product has a dotted appearance. Their cell soma and/or proximal neural processes (black arrows) are often associated with blood vessels. In contrast, ChAT-immunostained neurons (curved black arrow, dark brown under microscopic observations) are small bipolar interneurons homogeneously and intensively stained with DAB-Ni^{+}. Note the virtual absence of nerve fibres immunostained for NOS (A) under our experimental conditions. Scale bar=50 μm.

processes (Fig. 2A–C). In fact, 38.9% (46 out of 118 cells counted) of NOS-IR neurons spanning from the cingulate to the inferior border of the lateral orbital cortex were approached by PHA-L fibres. These intimate associations between basalocortical terminals and NOS-IR neurons were confirmed in high resolution semithin sections in which 33.3% (25 out of 75 cells counted) of NOS-IR cell bodies were seen to be directly contacted by PHA-L puncta (Fig. 2D).

Cholinergic input to cortical nitric oxide synthase-immunoreactive neurons

In double stained sections for ChAT and NOS, numerous darkly-stained (dark brown) ChAT bipolar neurons and varicose fibres were visible throughout the cerebral cortex, with a few intermingled pale brown, large and mostly round NOS-IR neurons (Fig. 1B). These two populations of cortical neurons were often found in the vicinity of local blood vessels (Fig. 2E–G) and were occasionally seen to send proximal neuronal processes to neighbouring NOS-IR neurons (Fig. 2F) or ChAT-IR (Fig. 2G) neurons. ChAT-IR terminals frequently associated with NOS-IR perikarya and their proximal dendrites. The quantitative analysis in thick sections revealed that approximately half (53%, 165 out of 311) of the NOS-IR neurons in the cerebral cortex were reached by ChAT-IR punctate structures. As evaluated in semithin sections, lesion of the SI resulted in the virtual disappearance of this cholinergic input to cortical NOS-IR neurons (Fig. 2I), with only 22.6% (28 out of 124 cells counted) NOS-IR cells being contacted by ChAT-IR terminals, as compared to 60.4% (58 out of 96) on the control side (Fig. 2H), corresponding to more than 60% decrease.

When observed at the ultrastructural level, NOS-IR neurons were large cells and the immunostaining was characterized by the presence of reaction products associated with the membranes of the endoplasmic reticulum and Golgi complex, as well as a diffuse reaction in the cell cytoplasm. The ChAT-IR appositions to such NOS-IR neurons were found to correspond to axonal varicosities abutting directly on the cytoplasmic membrane of NOS-IR perikarya and proximal dendrites (Fig. 3). Some NOS-IR neurons followed across several serial thin sections received cholinergic input at multiple sites (Fig. 3). At the level of contact, junctional specializations were not observed.

DISCUSSION

All together, these results provide morphological evidence for cholinergic basal forebrain projections to NOS neurons in the rat cerebral cortex and strongly suggest that such anatomical relationships could be functionally relevant to blood flow regulation in the cerebral cortex.

Cholinergic input to cortical nitric oxide synthase neurons

The results show that intracortical NOS-IR and ChAT-IR neurons correspond to two distinct populations, easily recognized by their morphological features. This observation fully agrees with the study by Sugaya and McKinney,[46] which demonstrated by combination of *in situ* hybridization and immunocytochemistry techniques, that NOS mRNA-containing neurons were not immunostained for ChAT in the rat cerebral cortex. The cortical NOS-IR neurons were relatively sparse and distributed without apparent organization. Because of this characteristic, already reported in numerous studies

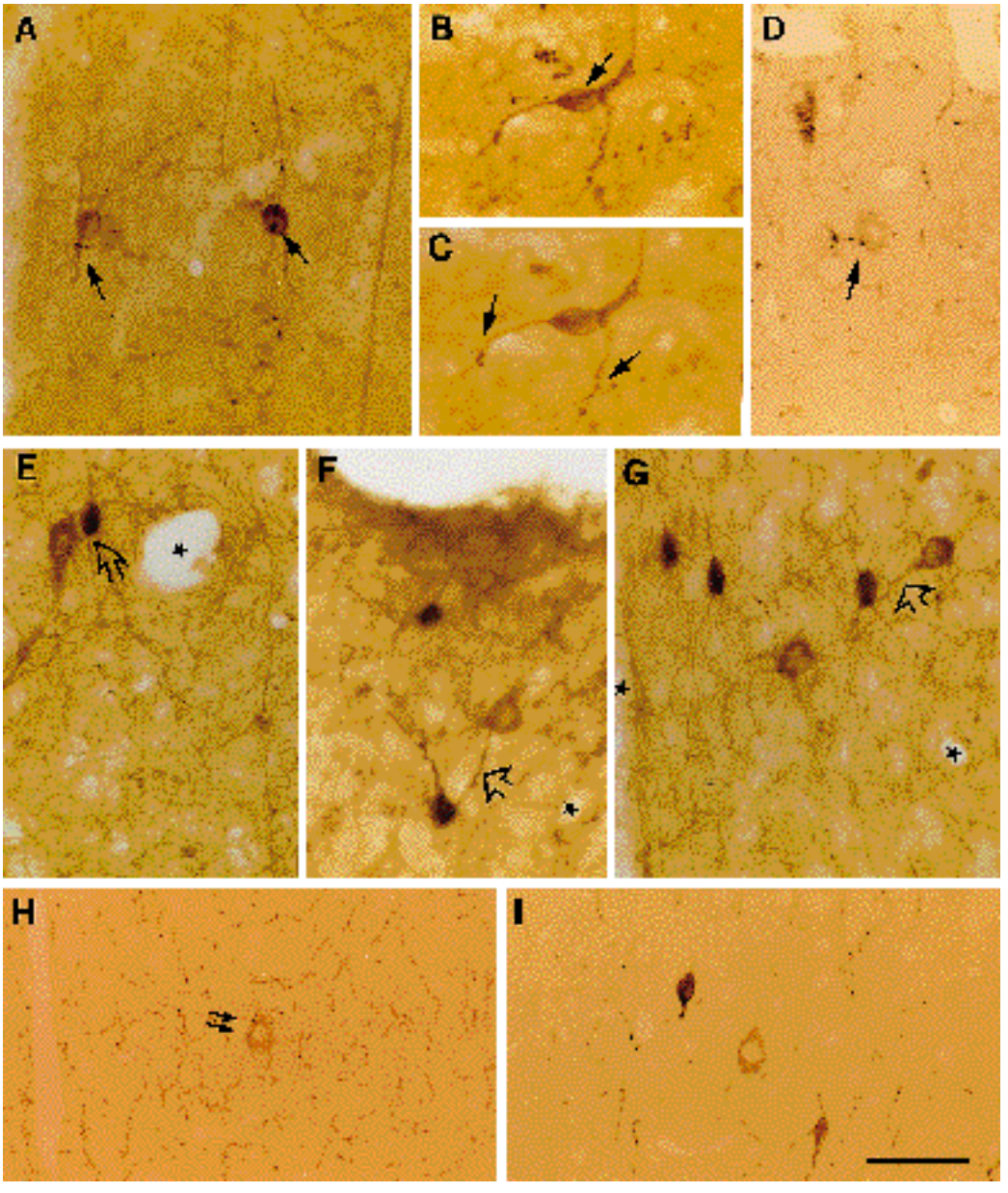

Fig. 2. Basalocortical and cortical input to NOS neurons in the frontoparietal cortex. (A) Basalocortical PHA-L-IR fibres (dark brown, arrows) reaching two NOS neurons (brown). (B, C) Two different plans showing PHA-L fibres (arrows) surrounding and running along the proximal dendritic processes of an NOS perikaryon. (D) In semithin sections, PHA-L varicosities (arrow) are seen to contact an NOS neuron. (E–G) Relationships between intracortical ChAT (dark brown) and NOS (pale brown) neurons as seen in 30-μm-thick sections. Note their close association (E, curved open arrow) in proximity to local blood vessels (stars) and the presence of cytoplasmic processes (open arrows) between the two cell types (stars). (H, I) In semithin sections, NOS-IR perikarya are often surrounded by numerous cholinergic varicosities (double arrows in H) which significantly disappear after SI lesion (I). Scale bar=50 μm.

using NOS immunocytochemistry or histochemistry for NADPH-diaphorase, a cofactor related to NOS,[8,46,47,53] Thomas and Pearce[47] termed these neurons the "solitary active cells". Solitary in distribution, they, or their axonal or dendritic processes, were found to exhibit associations with local blood vessels, in agreement with previous non-quantitative reports.[17,25,43] Moreover, the present data provide evidence that cortical NOS-IR neurons in the whole cortex, but also in the vicinity of blood vessels, are closely intermingled with bipolar ChAT-IR interneurons, which sometimes appear to extend proximal processes in their direction. This observation deserves further investigation as it may bear functional relevance, considering that these two neuronal populations contain vasodilatory substances.

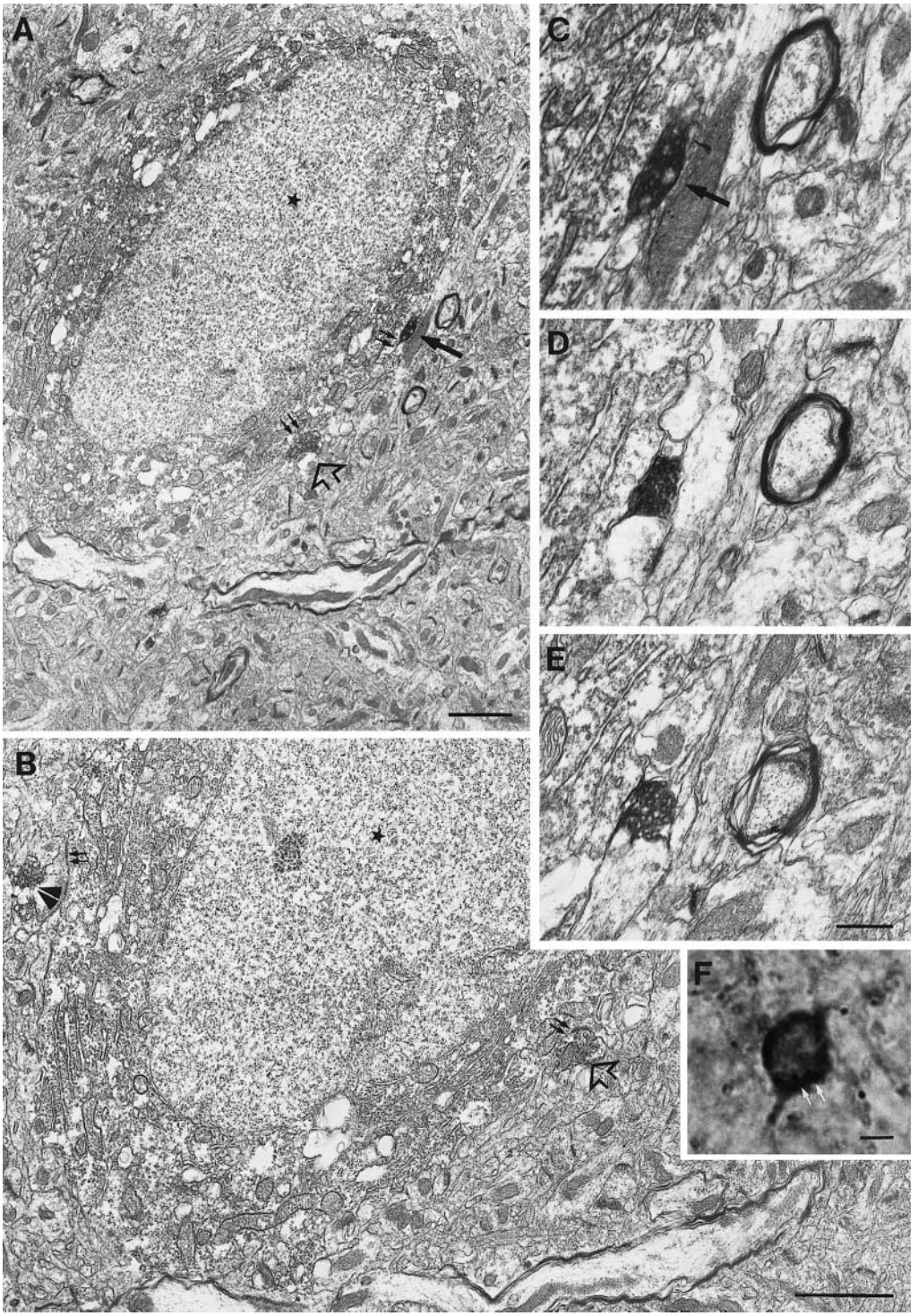

Fig. 3. (A, B) Electron micrographs from serial sections of a cortical NOS-IR neuron (star) which is surrounded by three ChAT-IR axonal varicosities, either directly apposed to (A, B, open and/or black arrows) or in proximity of (B, arrowheads) the cytoplasmic membrane (double arrows) of its cell soma. Note the presence of the same ChAT-IR varicosity (open arrows) on the two different levels of the NOS-IR neuron. Scale bars=2 μm. C is a higher magnification of one of the ChAT-IR nerve terminals (black arrow) seen in A which was followed in serial thin sections (D, E), and found to still directly contact the cell soma. No synaptic junctions were present at the site of apposition. Scale bar=0.5 μm. F is a photomicrograph of the same NOS-IR neuron innervated by ChAT-IR terminals (white arrows) as it appeared in the Araldite block before sectioning for electron microscopic analysis. Scale bar=10 μm.

Our results, however, clearly demonstrate that cortical NOS-IR neurons are contacted by cholinergic puncta and, further, axonal projections from the SI. The basalocortical projections, identified by the anterograde tracer PHA-L, were able to reach NOS-IR neurons, irrespective of the location in superficial or deep layers of the neocortex. We found that parts of these BF fibres are cholinergic since most ChAT-IR terminals surrounding NOS-IR neurons disappeared following lesion of the SI. They might even represent most of the BF projections to NOS-IR neurons as, reportedly, 80–90% of the basalocortical projections in the frontoparietal cortex are cholinergic.[44] Moreover, the lesion data are fully in line with the observation that BF cholinergic neurons provide the main source of cholinergic input to the neocortex,[14,30,31] including its microvascular bed.[51] However, other neurotransmitter(s) might be involved as the SI is a very heterogeneous nucleus.[44] It is most likely that the PHA-L-IR fibres surrounding NOS neurons are not exclusively cholinergic. In this respect, the possibility that some BF cholinergic projections also contain NOS, whose message is expressed in a subpopulation of SI ChAT-positive neurons,[46] is of considerable interest. Further investigation will be needed to clarify this issue.

Functional correlates

The cortical blood flow increases elicited by BF stimulation were found to be proportional to the cortical release of ACh,[28] potentiated and reduced by cholinergic agonists and antagonists, respectively,[12,32] and dependent on functional integrity of NOS for maximal cerebrovascular response.[1,42] Nevertheless, the exact source of NO in this cholinergic-mediated response has remained unclear. It has been considered unlikely that ACh released from nerve terminals would act on endothelial cells of intraparenchymal arteries to release NO, which would then exert its relaxing effect on smooth muscle cells. Both the diffusion distance and the physical barrier for ACh to reach the endothelium, together with the observation that endothelial-derived NO is more active in large arteries than in intraparenchymal microvessels,[18] favoured a neuronal origin of the NO involved in cortical vasodilatation elicited by BF neurons. The present findings provide an anatomical substrate for BF cholinergic terminals using NOS-containing neurons as a relay to regulate locally cortical CBF, and possibly other neural activities. The ultrastructural analysis revealed no junctional complexes between ChAT-IR terminals and NOS-IR perikarya or proximal dendrites. This would suggest a parasynaptic type of interaction whereby ACh would diffuse in tissue to reach and interact with selective and specific receptors on NOS neurons, a mechanism fully compatible with cholinergic neurotransmission within the cerebral cortex.[48] Moreover, the recent visualization of muscarinic receptors on cortical NOS neurons[39] strongly supports that the morphological interactions between ChAT-IR terminals and NOS-IR neurons reported here might be functional and able to generate the synthesis and diffusion of NO.[24]

There are a limited number of NOS neurons in the neocortex but their strategic association with blood vessels (this study and Ref. 25) together with the ability of NO to diffuse freely across cell membranes for relatively large distances ($\sim$100 μm, see Ref. 54), could contribute to extend the vascular effects elicited by BF stimulation to a larger segment of the microvascular bed. However, the short half life of NO as well as other local mediators would ensure that the vascular response remains restricted within a functional microcompartment of the cerebral cortex. Although previous functional[1,42] and morphological[39] observations, as well as the present findings, would support a functional relay through a cortical NOS neuron, other sources may also contribute to this cortical vasodilation induced by BF stimulation. A possible candidate would be the cholinergic basalocortical nerve fibres themselves, since a subset of BF cholinergic neurons, mostly located within the SI, also express NOS mRNA.[46] Although further studies would be needed to confirm the localization of both ChAT and NOS proteins within the same cortical nerve terminals, these two putative sources of cortical NO could possibly act in synergy to induce the potent neurogenic vasodilatory response. Another potential source is the astroglial cells which have been identified as a priviledged target for perivascular cholinergic nerve terminals.[9,51] Astrocytes contain a constitutive as well as an inducible form of NOS and can synthesize NO in response to various neuromediators.[40] They are endowed with muscarinic receptors[23,38,49] of specific subtypes[3,35] and their endfeet processes are known to surround most of the microvessel wall. Astrocytes have been involved in the induction and maintenance of the blood–brain barrier and, more recently, they have been identified as possible intermediates in the neurogenic control of brain microcirculation[9,22] and local metabolic activation.[41] It is thus conceivable that NO from astrocytic endfeet could diffuse to adjacent cerebral vessels and modulate their function.[7] Furthermore, a contribution of NO from endothelial sources has recently been suggested[55] in the BF-mediated cortical vasodilatation.

The participation of NO, possibly via a cortical relay neuron, in the BF-mediated cholinergic vasodilatation could be complementary to the direct neurovascular interactions whereby vasomotor tone in cortical microvessels would be modified via activation of specific subtypes of microvascular muscarinic acetylcholine receptors.[34] Interactions with other cortical interneurons, including those containing the vasodilators ACh and vasoactive intestinal polypeptide, which also locally contact the microcirculation,[10] are also possible.

CONCLUSIONS

The present results provide morphological support to physiological studies which suggested that NO is involved in the BF-mediated cortical vasodilatation.[1,42] They show that intracortical NOS neurons are a target for basalocortical ChAT nerve terminals, thus supporting that a local, neuronal source of NO could be involved in this neurovascular response. This situation would resemble the increase in cortical cerebellar blood flow elicited by activation of the parallel fibres which is, reportedly, mediated in part by NO produced locally in neurons of the molecular layer.[26] These findings, however, do not dispel a contribution from other neuronal, endothelial and/or glial sources of NO, neither from interactions between intracortical NOS-IR and ChAT-IR neurons.

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