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NADPH-diaphorase activity and neurovascular coupling in the rat cerebral cortex

Застосовуючи гістохімічний протокол вивчали розподіл НАДФН-діафоразореактивних (НАДФН-др) нейронів і нейронних відростків у корі мозку та їх взаємовідношення із паренхімальними судинами у нормальних дорослих щурів. Інтенсивно зафарбовані кортикальні інтернейрони, реактивні субкортикального походження аференти і мікросудини досліджували у світовому мікроскопі при малому (х 250) та великому (х 630) збільшеннях. НАДФН-др інтернейрони концентрувались у шарах 2–6 у первинній і вторинній (М1 і М2) моторних ділянках, однак домінуюча їх кількість (14 ± 0,8 у зрізі, P < 0,05) була знайдена у шарі 6. Чіткі накладання маркованих нейронів або аферентів на внутрішньокортикальні судини були знайдені в ділянках М1 і М2. Середня кількість мічених нейронів у слуховій (AuV), гранулярній і агранулярній (GI, AIP) ділянках острівкової кори становила 12,3±0,7, 18,5±1,0 та 23,3±1,7 одиниць на зріз відповідно. Численні варикозитети зафарбованих навколосудинних відростків, висхідних із основи переднього мозку, були виявлені у всіх шарах досліджуваних коркових ділянок, але домінуюча їх кількість відмічалась у шарах 1–3 фронтопарієтальної кори. Частота нейросудинного щеплення в різних ділянках кори мала таку послідовність: AuV (31,2 %, n=1040) > GI (18,0 %, n=640) > S1 (13,3 %, n=720) > M1 (6,3 %, n=1360). Велика кількість структурних асоціацій мічених клітин з мікросудинами у скроневій і острівковій корі вказує на те, що в цих ділянках кори НАДФН-др нейрони можуть бути залучені до регуляції регіонального кровотоку.

INTRODUCTION

A normal brain function strongly depends on continuous blood supply. Increased neuronal activity was found to correlate with the rise in regional cerebral blood flow (RCBF), known as functional hyperaemia. The tight coupling between RCBF and neuronal activity in cerebral cortex during behaviour or sensory interventions was demonstrated by brain imaging methods [14, 15, 26].

Nitric oxide (NO) has been recently identified as an important mediator of the neurovascular coupling process [8, 30]. Two main sources of neuronal NO were recognized in cerebral cortex. The first one is localized intracortically, and the second one is situated in the basal forebrain. Importantly, there are four groups (Ch1–Ch4) of the forebrain cholinergic neurons which contain NO synthase (NOS) and release NO from their terminals within the hippocampus, olfactory bulb and cortex [23, 30]. Immunohistochemical studies have demonstrated that the main centre of origin of cholinergic projections to the cortex may be identified as the substantia innominata and nucleus basalis of Meynert (SI–B complex) [7]. However, many cholinergic neurons are registered in the medial septal nucleus (MS), the ventral and horizontal nuclei of the diagonal band of Broca (VDB, HDB) [21], i.e., Ch1–Ch4, respectively [23]. It was found that nerve terminals (varicosities) of the basal forebrain origin have tight, but not direct, contacts with cortical microvessels, and noncholinergic SI–B neurons also contribute to the neurovascular associations [30, 31].

NOS-containing cells of origin of the ascending pathways to the cortex (cholinergic transmission), and local NO-generating perivascular interneurons within the cerebral cortex are implicated in several important brain functions including arousal, attention, cortical plasticity and memory. Abnormalities of the L-arginine-NO synthesis pathways and subsequent loss of NOS –containing neurons in the various structures of the basal forebrain and the cerebral cortex are associated with impairment of several important brain functions [9, 19, 30].

The cortical GABAergic interneurons (many of which are NO-generating cells) were identified as anatomical or functional targets for basal forebrain cholinergic and/or brainstem serotonergic projections [3]. Based on the results about the co-localization of GABA and NADPH-diaphorase (NADPH-d) in a subset of the cortical non-pyramidal neurons, or ACh and NADPH-d as coferment of NOS in the cortical afferent fibers, we suggest that the soma of these cells, its dendrites, and afferent fibers could be implicated in the structural associations with the cortical microvessels and arterioles. Parenchymal microvascular and neural (lamellar) architectures are well matched to the spatial and temporal requirements of neurovascular coupling in the cerebral cortex [3, 11]. Therefore, the aim of the present study was to compare the distribution of NADPH-d-reactive (NADPH-dr) cortical neurons and the nitrergic basal forebrain afferents within different layers of frontoparietal, temporal and insular cortices, and to study their structural associations with cortical vessels in rat. Some of the data have been published earlier [1, 34].

METHODS

Eight male Wistar rats weighing 250–350 g were used in the study. The handling of the animals was performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Perfusion. The animals were deeply anaesthetized (sodium pentobarbital 75 mg/kg i.p., Sigma, USA) and perfused through the ascending aorta with 200 ml phosphate-buffered saline (PBS) with heparin (25000 units/l) and sodium nitrite (0.2%) followed by 500 ml cold (8 °C) fixative solution containing 4% paraformaldehyde in 0.1 M PB at pH 7.4. The brains were quickly removed, postfixed in the same fixative overnight and cryoprotected in sucrose in PBS at 4 °C for 48 h. Frontal frozen sections of the brain (40-μm-thick) were cut on a freezing microtome. About 50 serial sections from each brain were collected in 10 wells with cold PBS, to be stained histochemically for NADPH-d that manifested NOS-containing neurons or nitrergic afferents in the cortex [4].

NADPH-diaphorase histochemistry. Sections were incubated in 0.1 M PB (pH 7.4) containing 0.3% Triton X-100, 0.2 mg/ml nitroblue tetrazolium (Sigma, USA), and 0.5 mg/ml β-NADPH tetra-sodium salt (Sigma, USA), at 37 °C for 1 h and additionally at room temperature for 12 h [33]. For intensification of the reaction, disodium salt of malic acid (Sigma, USA) 1.2 mg/ml was also added [18]. Mounted sections were cleared in xylene and cover-slipped with Entellan. NADPH-dr neurons and fibers were easily detected as light-blue structures, especially at high magnification (x250 and x630).

Data analysis. NADPH-dr cells were counted in the brain sections. Up to 3–5 stained sections were taken from each area of the cortex per rat and analyzed. A mean number ± S.E.M. of stained cells per section was counted in frontal, parietal, temporal and insular cortices, i.e., primary/secondary motor cortex (M1/M2), primary somatosensory cortex (S1), secondary auditory cortex (AuV) and the granular/agranular insular cortex (GI/AIP). The numbers of the stained neurons in the different areas were compared using two-

way statistical analysis of variance (ANOVA). Newman-Keuls' post hoc analysis was used when a significant difference was found. The factors of variation included two conditions (M1 and M2) areas and three frontal levels. Values of $P<0.05$ were considered statistically significant. The location of NADPH-dr cells and the fiber pathways in the basal forebrain and cortex were controlled according to the stereotaxic atlas [25] and an atlas of the regional and laminar distribution of cholinergic fibers in rat cerebral cortex [17].

RESULTS

Neocortex and Archicortex. In intact animals NADPH-d reactivity was detected in the frontal (motor primary/secondary area (M1/M2) and cingulate areas (Cg1 and Cg2)) and parietal (primary sensory area S1) cortices. As a rule, such activity was demonstrated in the cortical interneurons. In the M1 and M2 these interneurons were located at the rostral levels in layers 2–6. However, the predominant mean number of staining neurons was found in layer 6 of the M1 ($P<0,05$). In layer 6 of the M1 their mean number (14 ± 0.8 per section) was 3.5 times higher then that found in the layer in the M2 (4.0 ± 0.3) (Figs. 1, 2). Many reactive cells were additionally found within the subcortical white matter. The specific feature in distribution of NADPH-d reactivity in the studied regions of neocortex was distinct apposition of labelled neurones to the

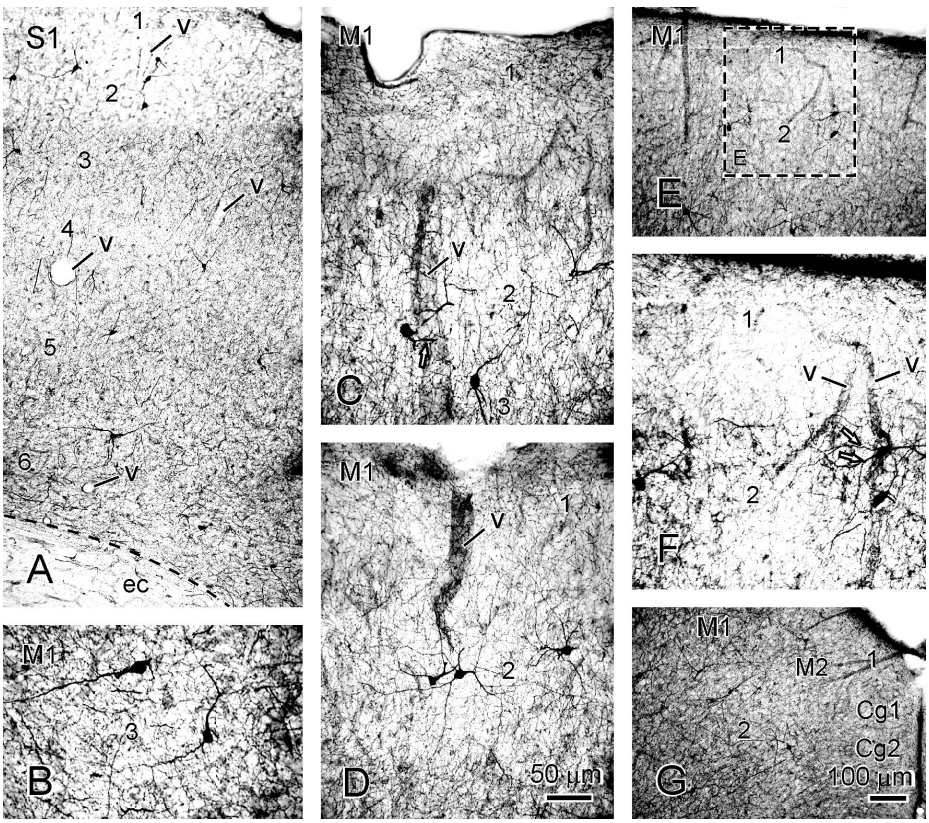

Fig. 1. Photomicrographs illustrating NADPH-diaphorase-reactive neurons and their structural associations with intraparenchymal microvessels in the primary somatosensory (S1) (A), primary and secondary (M1and M2) motor areas (B–F), primary and secondary (Cg1and Cg2) cingulate areas (G) of frontoparietal cortex. The boxed area in (E) denotes the site presented at higher resolution in (F), showing neurovascular associations (white arrows) in layer 2 of the M1 area. Note penetrating cortical microvessels (v) and reactive interneurons within layers 1–4, but not layers 5 and 6. Scale bars: 100 and 50 μm for (A, E, G) and (B, C, D, F), respectively

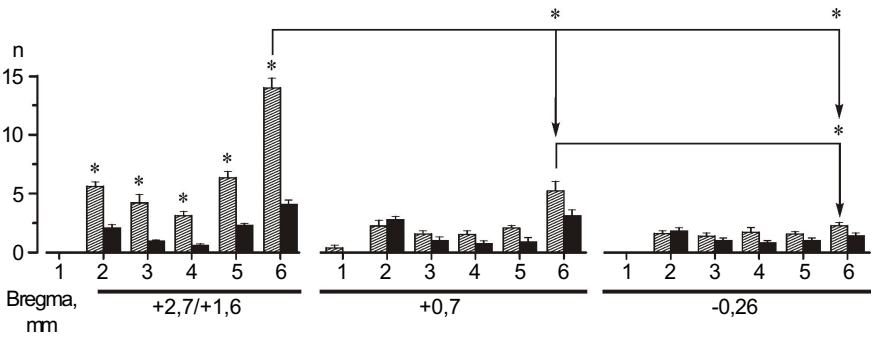

Fig. 2. Bar graphs of the mean number of NADPH-diaphorase-reactive neurons within different layers (1–6) in the motor cortex. Mean number ±S.E.M. of reactive cells per section (n) was defined in group of rats (n=8). The numbers below the bars indicate the antero-posterior distances to the bregma (mm) [25]. Asterisks that are placed on the column indicate a significant difference in the number of reactive cells in the primary vs. secondary motor cortex (hatched and black bars, respectively) for a given layer; or vs. given layer at caudal or rostral directions, when they are placed on the arrows (two-way ANOVA, $P<0,05$)

intraparenchymal vessels. The association between NADPH-dr neurons and microvessels was predominantly demonstrated in the layers 2 and 3 of motor cortex, where many arterioles and microvessels being registered (Fig. 1, C–F). Importantly, the revealed perivascular distribution of the stained neurons (soma and dendrites) was found in the immediate vicinity to intracortical vessels with different diameter ((arterioles (30–70 μm), microvessels (10–25 μm) and capillaries (5–10 μm)).

High number of NADPH-dr interneurons was found in temporal and especially insular cortices. For example, the number of them in the ventral auditory area (AuV), granular insular area (GI) and agranular insular area (AIP) reached 12.3 ± 0.7, 18.5 ± 1.0, and 23.3 ± 1.7 neurons per section, respectively ($P<0.05$). A lot of reactive bodies of cortical interneurons and their dendrites were closely associated with intraparenchymal microvessels (Fig. 3). Intensive NADPH-d reactivity in perivascular dendrites within layers 2–6 raises the possibility that they are the major source of NO released during neural activity in the AuV and GI cortical areas. In contrast to granular insular area, labelled interneurons in AIP are registered predominantly in layers 5 and 6. Comparative analysis of the number of detected neurovascular coupling in studied regions of the cerebral cortex showed the following order of frequency: AuV (31.2 %, n=1040) > GI (18.0 %, n=640) > S1 (13.3 %, n=720) > M1 (6.3 %, n=1360) > AIP (1.0 %, n=102) (see Figs. 1, 3).

Intracortical NADPH-diaphorase-reactive projections from the basal forebrain. In addition to NADPH-dr neuronal bodies and dendrites of the interneurons, the dense net of thin nitrergic fibers with numerous varicosities were also clearly detected in the parenchyma of the different areas of the cortex (see Fig. 4). Just as labelled dendrites of cortical interneurones, these fibers also demonstrated close associations with arterioles, microvessels and capillaries. Analysis shows that these reactive fibers have extracortical sources. They ascend from NADPH-dr neurons located in the SI–B complex and magnocellular preoptic nucleus (MCPO). Before appearing in the cerebral cortex they followed for a long distances through the lateral hypothalamus, anterior commissure (ac), internal capsula (ic), bed nucleus of the stria terminalis (BSTL), and then along the corpus callosum (see Fig. 4, F,G). The fine labelled fibers were distributed throughout the frontal, parietal and temporal cortices and the majority of them being distributed in layers 1–4 (Fig. 4, A–E). However, the densest plexus of NADPH-dr fibers was revealed predominantly in layer 1 within the full extent of

agranular insular area (Fig. 5). Only sparse NADPH-d reactivity was found in the limbic cortex (Cg1 and Cg2) (see Fig. 1, G). NADPH-dr fibers and their varicosities surrounding arterioles, microvessels and even capillaries of varying sizes were observed in all cortical areas examined (Figs. 4 and 5). We must note that in the frontoparietal cortex (M1, M2 and S1) such type of nitregic fibers were as a rule associated with arterioles and microvessels of layers 1–3 (see Fig. 4, A–E). It was impossible to reveal the direct contacts of reactive perivascular fibers with dendrites of the cortical neurons in the study. However, in electron microscopic investigations [31] it has been found that terminations (varicosities) of basal forebrain cholinergic afferents establish junctional contacts with glia, microvessels and simultaneously with adjacent neuronal elements (dendrites) in the frontoparietal cortex.

DISCUSSION

The morphological evidences presented in the study support the hypothesis that NOS-containing neurons participate in the mechanisms that match neural activity to the cerebral blood flow. There are the peripheral and central main sources of nitrergic innervation of the cerebral vessels. The peripheral source includes NOS-containing terminals from sphenopalatine ganglia that are projected to large cerebral vessels of the circle of Willis. The intracerebral and basal forebrain (SI–B complex and MCPO) NADPH-d/NOS-containing neurons are the cells of the central origin of nitrergic processes that are closely associated with intracere-

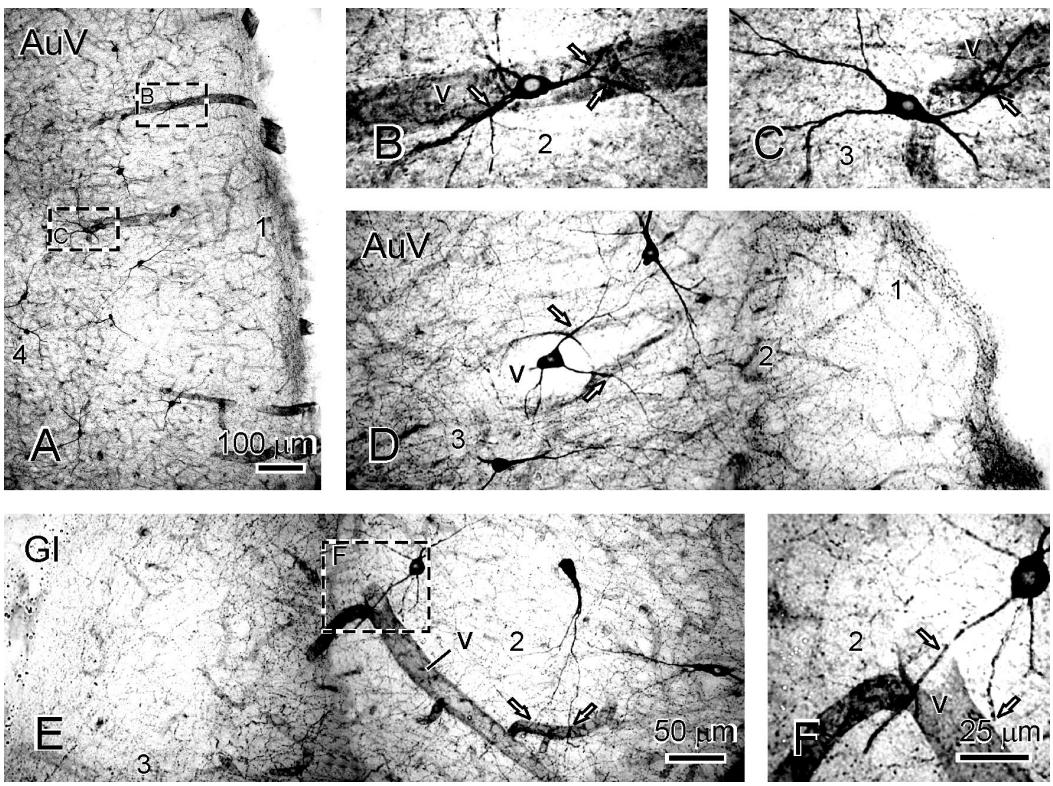

Fig. 3. NADPH-diaphorase reactivity in the ventral auditory area (AuV) (A–D) and granular insular area (GI) (E and F) of the temporo-insular cortex. Note the straight contacts of reactive neurons and their dendrites (white arrows) with microvessels in the cortex. The boxed area in A and E delimitate the areas presented at higher resolution in B, C and F, respectively. Scale bars: 100, 50 and 25 μm for (A), (D, E), and (B, C, D, F), respectively

brall arterioles and capillaries [12].

In the present study we found prominent NADPH-d positive fibers of the basal forebrain origin in layers 1–3 of the frontoparietal and temporal cortices, and in most layers of the insular (ins) cortical areas (see Figs. 4 and 5). However, our study demonstrates that compared to insular cortex frontoparietal and temporal cortices, which are phylogenetically new parts of the brain contain more inherent perivascular NADPH-dr neurons [27].

The basal forebrain ascending projections to gray matter of the cerebral cortex in 80 % were characterized as cholinergic terminals [17, 22, 23]. The data obtained indicate that majority of the intraparenchymal NADPH-dr terminals reached intracortical penetrating arterioles and small microvessels, and are in line with finding [10] that the basal forebrain cholinergic neurons, including NOS-containing neurons, participate in regulation of RCBF. Importantly, NOS-containing/cholinergic

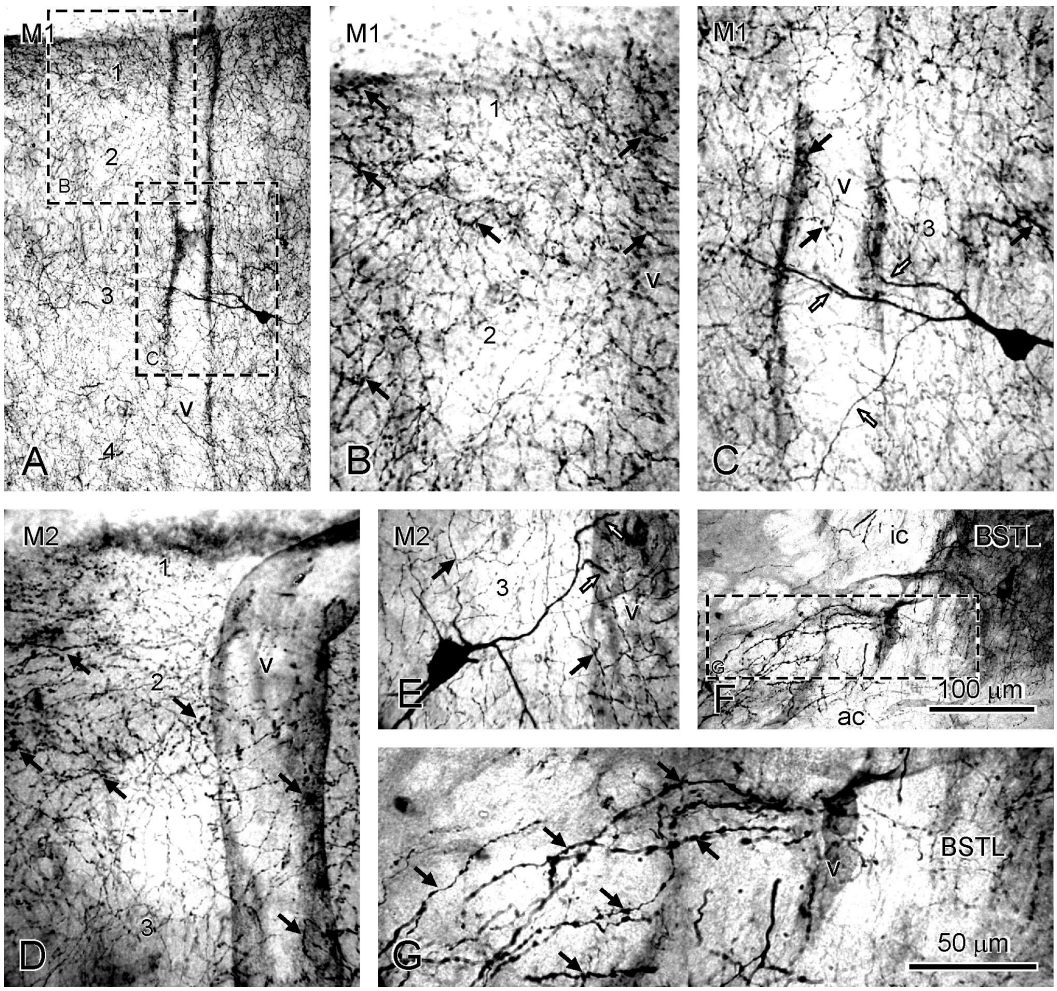

Fig. 4. NADPH-diaphorase-reactive perivascular neurons, dendrites and fibers (varicosities) within different layers (1–4) in the primary and secondary motor areas (M1 and M2) of the frontal cortex (A–E), and internal zone of the basal forebrain (F and G). Note the perivascular reactive neurons , staining varicosities (black arrows) and dendrites (white arrows) are opposed to microvessels (v) in the cortex (left side). The dashed boxes in A and F delimitate the areas presented at higher resolution in (B and C) and (G), respectively. BSTL, ac and ic, designate bed nucleus of the stria terminalis (lateral division), anterior comissure and internal capsule, respectively. Scale bars: 100 and 50 μm for (A, F) and (B–E, and G), respectively

neurons of the SI–B complex induce direct excitatory nicotinic or muscarinic effects to microvessels, and do not influence the cortical inherent NOS-containing/GABAergic interneurons [7, 27]. Moreover, there are also findings that NOS-containing neurons of basal forebrain that are noncholinergic synthesize amynobutyric acid (GABA), the second major neurotransmitter that also modifies cerebromicrovascular tone [32]. It has been recently shown that NOS-containing /GABA-synthesizing neurons have also the capacity to synthesize glutamate (Glu) as the third neurotransmitter [6, 24, 30].

Compared to basal forebrain, NOS-containing neurons within the islands of Calleja, that contribute to regulation of circulation in the limbic structures and basal ganglia, are also GABAergic but not cholinergic [28]. Predominant number of them express *c-fos* following fatiguing stimulation of neck muscle in rat [20].

The vasodilative responses of intraparenchymal vessels are independent of systemic blood pressure. However, cholinergic system seems to act cooperatively with other modulatory systems such as the noradrenergic ascending pathway originating in the locus coeruleus (LC) and the serotonergic ascending pathway originating in the dorsal raphe nucleus (DR) [3, 13, 16, 30]. Parenchymal small arterioles and microvessels have been

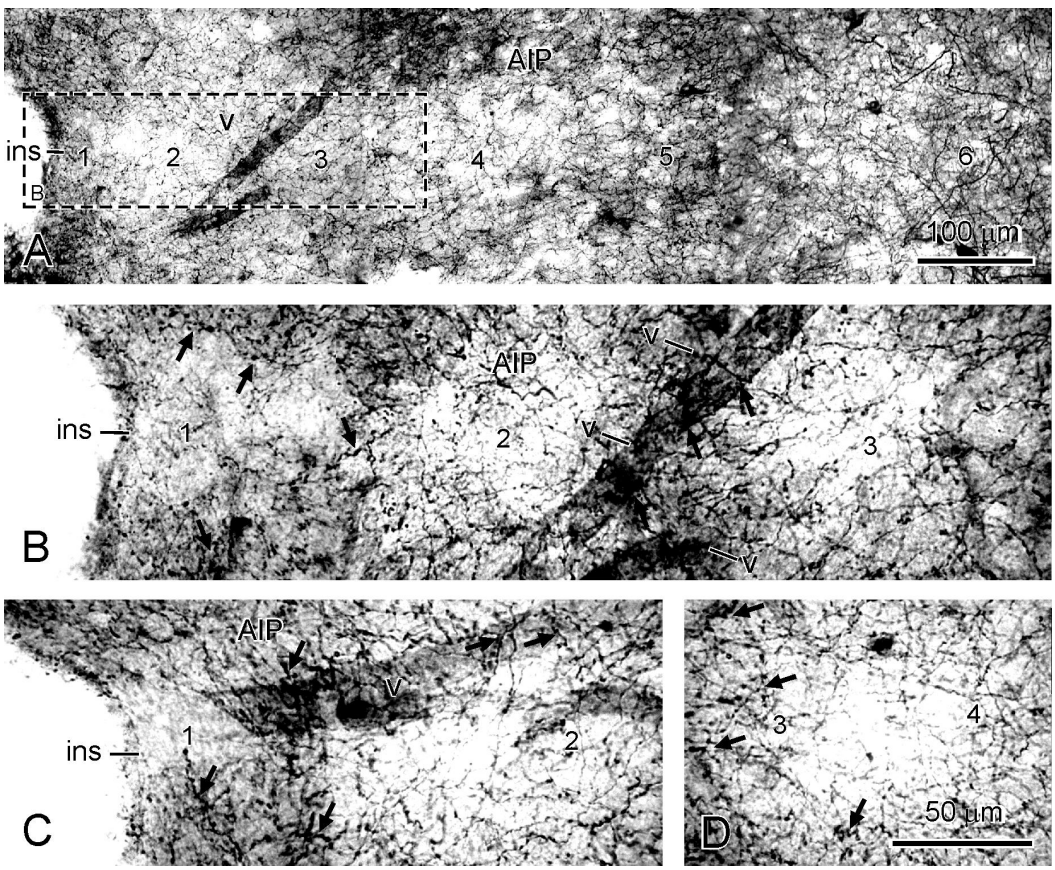

Fig. 5. NADPH-diaphorase-reactive fibers (varicosities) within different layers (1–6) in the agranular insular area, posterior part (AIP) of the insular cortex (ins). Note straight contacts of reactive varicosities (black arrows) with microvessels in the marginal layers 1 and 2 (A, B), but not in layers 3 and 4 (C). The boxed area in A denotes the site presented at higher resolution in B, showing the lateral part of the insular cortex (left side). Scale bars: 100 and 50 μm for (A) and (B, C and D), respectively

shown to dilate or constrict in response to several vasoactive modulatory transmitters and vasoactive peptides (VIP, SOM and NPY) and NO [3]. Recent evidence suggests that the vasodilator substances derived from astrocytes, which lie in close apposition to intraparenchymal arterioles, may be also involved in neurovascular coupling [16].

In conclusion, the data presented indicate that the activity of NADPH-d/NOS-containing perivascular fibers ascending from basal forebrain, local cortical NO-generating neurons and astrocytes may play an important role in mechanisms of neurovascular coupling in the limbic structures [2], and cerebral cortex [9, 34]. It was emphasized earlier that the regulation of RCBF in the cortex involves the coordinated interaction of the pyramidal neurons, interneurons, glia and vascular cells, and these neurological/neurovascular couplings are disrupted in aging or in pathological conditions as hypertension, stroke, Alzheimer disease [5, 29].

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NADPH-DIAPHORASE ACTIVITY AND NEUROVASCULAR COUPLING IN THE RAT CEREBRAL CORTEX

The distribution of NADPH-diaphorase-reactive (NADPH-dr) neurons and neuronal processes in the cerebral cortex and basal forebrain and their association with parenchymal vessels were studied in normal adult rats using NADPH-d histochemical protocol. The intensely stained cortical interneurons and reactive subcortically originating afferents, and stained microvessels were examined through a light microscope at law (x250) and high (x630) magnifications. NADPH-dr interneurons were concentrated in layers 2–6 of the M1 and M2 areas. However, clear predominance in their concentration (14 ± 0.8 $P<0.05$ per section) was found in layer 6. A mean number of labeled neurons in auditory (AuV), granular and agranular (GI, AIP) areas of the insular cortex was calculated to reach 12.3 ± 0.7, 18.5 ± 1.0 and 23.3 ± 1.7 units per section, respectively ($P<0,05$). The distinct apposition of labelled neurons to intracortical vessels was found in the M1, M2. The order of frequency of neurovascular coupling in different zones of the cerebral cortex was as following sequence: AuV (31.2 %, n=1040) > GI (18.0 %, n=640) > S1 (13.3 %, n=720) > M1 (6.3 %, n=1360). A large number of structural associations between labeled cells and vessels in the temporal and insular cortex indicate that NADPH-d-reactive interneurons can contribute to regulation of the cerebral regional blood flow in these areas.

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