QUANTITATIVE HISTOCHEMISTRY OF RIGHT-LEFT ASYMMETRIES IN THE DENSITY OF SULFIDE-SILVER STAINABLE FIBRES IN THE RAT CEREBRAL CORTEX

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Received for publication November 29, 1990 and in revised form February 13, 1991

The existence of possible right-left asymmetries in sulfide-silver stainable fibre systems in the rat frontal, parietal and occipital cortices was assessed using the neo-Timm histochemical technique associated with microdensitometry. In the cerebral cortex sulfide-silver stainable fibres are localized in the neuropil of laminae I-III and V. The density of sulfide-silver stainable fibres which is parallel to the density of zinc-containing presynaptic structures, was the highest in the right frontal cortex and the lowest in the right occipital cortex. In the frontal cortex sulfide-silver staining is stronger in the right hemisphere than in the left ($P < 0.001$). In the parietal cortex, values of the density of sulfide-silver stainable fibres did not show significant right-left differences. In the occipital cortex the density of sulfide-silver staining was higher in the left hemisphere than in the right ($P < 0.001$). The functional significance of the above right-left asymmetries in the density of sulfide-silver stainable fibres in the rat cerebral cortex cannot be established on the basis of our findings alone. However, our study indicates the sensitivity of specific histochemical techniques in assessing right-left asymmetries in cerebral cortex microanatomy.

The concept of the existence of side differences for certain brain functions (the so-called cerebral dominance) as well as of structural and biochemical asymmetries between right and left brain hemispheres was rapidly accepted in the beginning of the second half of the last century (for a review see 11). It was probably a work of Wigan, published in 1844 (28) which first reported the structural asymmetries between the right and left hemispheres in the human brain. Now we know that the existence of differences between the right and left hemispheres is a characteristic shared by the human species with several other mammalian and non-mammalian species (1, 8, 11, 12, 15, 22).

In the rat brain it has been demonstrated that the neocortex of the right hemisphere is thicker than that

of the left hemisphere (8). Moreover, the right hemisphere is larger and heavier than the left one (15), and the cross sectional area of the neocortex is greater for the right than for the left hemisphere (16). Rat brain is a rather useful model for cerebral cortical morphological studies. In fact, different from the human, monkey, cat and dog cerebral cortex, the rat neocortex is rather smooth and shows a clear division between the gray and white matter. Moreover, it is not difficult to obtain uniform cortical samples in different animals by using subcortical landmarks for measurements. In spite of this, the existence of anatomical-functional correlations in rat cerebral cortex asymmetry has not been so far established (16).

To contribute to a better knowledge of the topic we analyzed in the present study the existence of right-left asymmetries in the density of sulfide-silver stainable fibre systems in the rat cerebral cortex. In the rat cerebral cortex sulfide-silver stainable fibres are localized in the neuropil of cortical laminae I-III and

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V (for a review see 13). Their density reflects the density of zinc-containing cortical synaptic boutons (13, 17).

MATERIALS AND METHODS

a) Animals and tissue treatment.

Male Sprague-Dawley rats (Charles River, Italy) weighing 270–320 gr were used. Animals were kept under standardized humidity, temperature and lighting conditions on a constant 07:00 a.m. to 07:00 p.m. light-dark cycle. They had free access to water and laboratory chow.

Sulfide-silver stainable fibres were demonstrated using the so called neo-Timm's technique for the histochemical detection of zinc tissue stores (for ref. see 10). Briefly, rats were anaesthetized with ether and perfused through the left ventricle with 0.9% NaCl solution containing heparin (2 mg/kg) and polyvinylpyrrolidone (10%). This first solution was replaced by a second one of 0.1% Na_2S for 60 min. At the end of perfusion, the cranium was opened, the brain was quickly removed and divided into the two hemispheres. Each hemisphere was placed in a cryoprotectant medium (OCT, Ames, U.S.A.) and frozen in a dry ice-acetone mixture. Serial parasagittal (from the frontal to the occipital pole) 10 μm thick brain sections were mounted on gelatine-coated microscope slides and air dried. Sections were then incubated in darkness in a solution containing arabic gum (20%), citric acid (5%), hydroquinone (2%) and silver lactate (10%). Section's were washed free of gelatine, dehydrated, mounted in Entellan and viewed using a Zeiss II photomicroscope.

b) Microdensitometry.

The density of sulfide-silver stainable fibres in the neuropil of laminae I-III of frontal, parietal and occipital cortices was assessed by microdensitometry according to the protocol standardized in our laboratory for the quantitative analysis of histochemically reactive tissue zinc stores in brain (21, 23). The intensity of sulfide-silver staining in the right and left hemisphere was measured independently at the level of the frontal (prefrontal cortex, areas Cg 1–3), parietal (FL and Par 1 areas) and occipital (OC1M and OC2M areas) cortices identified and delimited according to the criteria of Zilles and Wree (29), in 8 consecutive sections of the right or left hemisphere per animal ($n=16$) using a Leitz MPV II microphotometer. The instrument was calibrated taking as "zero" the value of microscope slides areas without brain tissue. Measurements were randomly performed on 25 different areas of the neuropil of frontal, parietal or occipital cortices per

hemisphere and per animal using a $\times 40/0.95$ planapochromatic objective and a $\times 10$ ocular. Measurements were performed on a circular area of 10 μm in diameter in the centre of an ocular and delineated by a measuring diaphragm.

c) Statistics.

Differences in the intensity of sulfide staining between the 3 cortical areas of each hemisphere and between right and left hemispheres were evaluated statistically using the microdensitometric value of the density of white matter of corpus callosum (area without positive reaction) as covariate. The values obtained in different cortical areas of single animals were tested as values of dependent variables. The data were examined using programs 3D and 1V of the BMDP (using a main-frame NAS6660 with release 5.0 of VM/SP) to perform analysis of variance (ANOVA) and covariance (ANOCOVA).

RESULTS

A dense dark-brown staining was seen in the cerebral cortex in both right and left hemisphere. The staining was localized in the neuropil of laminae I-III and V of cerebral cortex (Fig. 1). Staining in the upper layers (laminae I-III) produced a rather thick band running homogeneously from the frontal to the occipital poles of the cerebral cortex.

Microdensitometric analysis of sulfide-silver stainable fibres in laminae I-III of the areas of cerebral cortex examined demonstrated the highest density of sulfide-silver staining in the right frontal cortex and the lowest in the right occipital cortex (Table 1). In the right hemisphere the highest density of sulfide-silver staining was noticeable in the frontal cortex, followed in descending order by the parietal cortex (-20.8% , $P<0.001$) and by the occipital cortex (-29.2% , $P<0.001$ vs frontal and -10.5% , $P<0.05$ vs parietal cortex) (Table 1 and Figs. 2–3). In the left hemisphere, the highest density of sulfide-silver staining was seen in the occipital cortex followed in descending order by the frontal and the parietal cortex (Fig. 2). However, as summarized in Table 1, the differences in the values of the density of sulfide-silver staining did not reach statistical significance between the three cerebral cortex areas investigated in the left hemisphere. Although sulfide-silver technique stained in the cerebral cortex both nerve cell bodies and the neuropil, the most consistent staining occurred in the neuropil where the right-left asymmetries were observed (Fig. 3).

Right-left asymmetries in the density of sulfide-silver stainable fibres in the rat cerebral cortex are

TABLE 1. *Microdensitometry of the intensity of sulfide-silver staining in the neuropil of laminae I-III of the rat right and left cerebral cortex*

Area	RIGHT (n=16)	LEFT (n=16)
FRONTAL CORTEX	74.93 $\pm$ 2.47	63.70 $\pm$ 2.59 (a)
PARIETAL CORTEX	59.32 $\pm$ 2.95 (a)	60.03 $\pm$ 3.09
OCCIPITAL CORTEX	53.04 $\pm$ 2.85 (a, b)	66.93 $\pm$ 2.99 (c)

The values expressed as means $\pm$ S.E. represent the intensity of sulfide-silver staining in the different areas of the rat cerebral cortex and are reported in arbitrary units. The number of animals used for microdensitometry is indicated by "n". For further details on microdensitometry see materials and methods.

a= $P<0.001$ vs. right frontal cortex

b= $P<0.05$ vs. right parietal cortex

c= $P<0.001$ vs. right occipital cortex

FIG. 1. Parasagittal section of the rat left cerebral hemisphere stained with the neo-Timm technique. Note the strong reactivity of the hippocampus (H) and of the olfactory bulb H. In the cerebral cortex sulfide-silver staining is located in the neuropil of laminae I-III (*) and in lesser amounts in lamina V (o). CC=corpus callosum; F=frontal cortex; P=parietal cortex; OC=occipital cortex; D=diencephalon; M=mesencephalon; C=cerebellum. $\times 10$

shown in Table 1. In the frontal cortex sulfide-silver staining is stronger in the right hemisphere by 16.3% than in the left one ($P<0.001$) (Fig. 2). No asymmetries in the density of sulfide-silver staining were seen between right and left parietal cortices. In the occipital cortex sulfide-silver staining is stronger in the left hemisphere than in the right one by 19.5% ($P<0.001$) (Figs. 2, 3).

DISCUSSION

Increasing evidence seems to suggest a functional role for heavy metals in general and for zinc in particular in brain. The strongest evidence has been accumulated for the hippocampus where zinc is contained in very high concentrations in the glutamatergic intrahippocampal pathway of mossy fibres. In the hippocampus it has been suggested that zinc has a role in the release and/or storage of the neurotransmitter glutamic acid (2, 3, 5, 13). Moreover, in the brain

FIG. 2. Sections of rat right (pictures A and C) and left (pictures B and D) frontal (A, B) and occipital (C, D) cortices stained with the neo-Timm technique. Sulfide-silver staining is localized in the neuropil of laminae I-III (u) and in lamina V (l) of the cerebral cortex. Note the different intensity of staining in the various cerebral cortex areas investigated in the 2 hemispheres. $\times 140$

zinc is related with several events which may be of neurochemical significance, such as catecholamine levels, enkephaline receptor binding, and concentration of circulating corticosteroids (4, 19, 20).

Investigation of zinc-containing neurons and

nerve pathways has been possible due to the development of appropriate histochemical techniques like the original Timm's technique, proposed in 1958 for the demonstration of heavy metals in brain (25, 26) and by the subsequent modifications of this technique, allow-

FIG. 3. Higher magnification of Fig. 2 showing that sulfide-silver staining is localized primarily in the neuropil (n) of laminae I-III of the cerebral cortex. The presence of zinc-containing nerve cell bodies is also noticeable. The different pictures correspond to the same shown in Fig. 2. $\times 250$

ing the rather specific demonstration of zinc stores in brain sections (6, 7, 10, 13, 14). Among the modifications of the original Timm's technique, the neo-Timm method, in which the concentration of Na_2S was reduced to 0.1% and silver lactate was replaced by silver

nitrate, is the most selective for the visualization of zinc but not of heavy metals tissue stores (10). Now we know of the existence of zinc containing neurons that probably represent a chemospecific neuron type in the central nervous system (for a review see 10).

Moreover, there is good evidence that zinc-containing neurons are able to take up, store and release zinc. Finally, an increasing number of putative zinc-sensitive target or receptor sites has been identified in the brain (9, 10).

Due to the association of zinc-containing neurons with limbic and cerebrocortical circuitry and their connection with excitatory aminoacid transmitters like glutamic acid, these chemospecific neurons were considered to have a possible role in cognitive and mnemonic processes (10). In view of this we investigated in the present study whether cerebral cortex sulfide-silver stainable fibres, which represent predominantly associational fibres arising from local cortical interneurons (for references see 10) show right-left asymmetries.

The analysis of the possible interhemispheric asymmetries was done using the highly sensitive and reproducible neo-Timm's technique (10) associated with microdensitometry. Previous electron microscope investigations have demonstrated that in the cerebral cortex zinc stained with the neo-Timm's technique is associated with presynaptic boutons and that the density at the light microscopic levels reflects the density of Timm-positive presynaptic structures (17). This correspondence allows us to hypothesize the existence of an asymmetry between right and left hemisphere in the frontal and occipital cortices in terms of sulfide-silver stainable fibres and probably of synaptic boutons. On the basis of the above findings we could conclude that besides the anatomical asymmetries in the rat cerebral cortex mentioned in the introduction and the neurochemical asymmetries in noradrenaline concentrations, gamma aminobutyric acid turnover and synaptosomal uptake of serotonin, noradrenaline and choline (18, 24, 27), zinc-containing neurons within cerebrocortical circuitry also show some degree of right-left asymmetries. Since the functional role of zinc chemospecific neurons in the cerebral cortex is still unknown, it is difficult to speculate about the significance of right-left asymmetries described above. In view of the localization of zinc containing sulfide-silver positive structures in associational fibres (10), it is possible that these asymmetries reflect differences in the density of these fibres in the cortical areas investigated. Anyway, the sensitivity of appropriate histochemical technique in assessing asymmetries in the rat cerebral cortex was demonstrated in the present study.

ACKNOWLEDGEMENTS

The present study was supported by a grant from

the Italian National Research Council (C.N.R.) (Progetto finalizzato invecchiamento, sottoprogetto 2, obiettivo 1). The kind help of Dr. W. L. Collier in the critical revision of the manuscript is gratefully acknowledged.

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