VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 59, No. 3, pp. 193–200
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ISSN 0015–5659
www.fm.viamedica.pl

The different patterns of maturation of the claustrocortical connections in a rabbit

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[Received 6 July 2000; Accepted 14 July 2000]

The quantitative analysis of the claustrocortical connections labeled with the fluorescent retrograde tracer Fluoro-Gold (FG) was conducted on 90 rabbits subdivided into the following age groups (P2, P7, P14, P21, P30, P60, P90, P120, P180). The equal volumes of retrograde fluorescent tracer FluoroGold (FG) were injected into the selected regions of the motor or somatosensory cortices. The volume of the dorsal part of the claustrum, total number of projecting neurons, numerical density and percentage distribution of projecting neurons were estimated by means of the unbiased stereological methods. The claustrocortical connections both with the motor and somatosensory areas in a rabbit are established in the postnatal life. The parts of the claustrum occupied by the motor and somatosensory projection zones as well as the morphology of the cortically projecting neurons do not reveal characteristic changes during the studied period. The significant decrease of the total number and numerical density of cortically projecting neurons as well as the increase of the claustral volume may reflect the process of adjustment of the claustrum to its modulatory function upon corresponding cortical areas. The intensity of the claustral connections with the motor and somatosensory cortices reveals significant difference during the studied period, being higher for the motor projection. It may be assumed that the claustrocortical connections established before birth undergo significant quantitative changes during postnatal development.

key words: claustrum, claustrocortical connections, rabbit, retrograde transport, fluorescent tracers, stereology

INTRODUCTION

Claustrum is a large cortico-related structure occurring in mammals, representing different levels of the phylogenetic development. In all species two principal parts of this structure can be distinguished: the insular claustrum (dorsal claustrum) and the ventral claustrum (prepiriform claustrum; endopiriform nucleus) [10,14,21,22]. Although, for many years the origin of claustrum was a subject of some controversies [4,5,11,12,17,26], according to the growing body of evidence its cortical origin is presently hardly to be questioned [1,2,8,24]. The development of the claustrum in the prenatal period in a rat was studied by means of the (^{3}H) thymidine autoradiography method [1]. According to presented results two parts of this structure are generated in different places and are originated in various periods of time. Neurons of the endopiriform nucleus are generated in the palliostriatal ventricular angle and this part originates on E14, E15, whereas cells of the dorsal claustrum are generated in the neocortical neuroepithelium and this part of the structure is originated later — on E15 and E16. On E20 the claustrum of the rat becomes visible as a separate structure. Between E20 and P14 significant morphological changes take place in the claustrum of the rat [15,16]. The

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stabilization of the total number of neurons, decrease of the neuronal density and increase of the volume of both parts of the structure are observed until the end of the second postnatal week. Additionally, only in the dorsal claustrum the loss of about 30% of neuronal population due to apoptosis is reported [16]. Both parts of the claustrum, characterized not only by various origins, reveal also differentiated pattern of connections — concerning mainly the neocortex in the case of dorsal claustrum and allocortex in the case of endopiriform nucleus. Because no information in the literature is available concerning the development of the claustrocortical connections, the aim of the following study was to assess the quantitative changes occurring in the claustral motor and somatosensory projections in the postnatal period in a rabbit. This was performed by means of the combination of the axonal retrograde transport and unbiased stereological methods.

MATERIAL AND METHODS

90 New Zealand rabbits of both sexes, divided into age groups (P2, P7, P14, P21, P30, P60, P90, P120, P180) were used. The care and treatment of the animals were in accordance with the guidelines for laboratory animals established by the National Institute of Health as well as by the local ethical committee. The animals were anaesthetized with Fentanyl (0.03 mg/kg i.p.), Dehydrobenzperidol (1.4 mg/kg i.p.) and Ketamine (70 mg/kg i.m.). In the sterile conditions, after placing in the stereotaxic frame and skin incision, a small craniectomy was performed. In all cases 2% water solution of the fluorescent retrograde tracer Fluoro-Gold (FG; Fluorochrome, USA) was used. The glass microcapillar was inserted into the cerebral cortex and the single pressure injection was made within the selected cortical areas, using the 5 μl Hamilton syringe at a rate of 50 nl/min. In each case the total volume of 700 nl of the tracer was injected into the motor or somatosensory cortex. After the injection the microcapillar was held in place for at least ten minutes to minimize the tracer leakage. The cortical regions were selected according to the stereotaxic coordinates [20,20]. The accuracy and extent of injections were verified in each animal by means of: (1) the cytoarchitectonic features of the rabbit cortex, described by Fleischhauer et al. [6] and (2) observation of labeled neurons in an appropriate thalamic nuclei.

After 5 days of survival the animals were deeply anesthetized with Fentanyl (0.03 mg/kg i.p.) and Thiopental (80 mg/kg i.p.) and perfused transcardially with 125 — 250 ml (depending on the age of animal) of 0.9% saline containing 10,000 units of Heparin, followed by 500 — 1,000 ml of 4% formalin in phosphate buffer (pH 7.4 and 4°C). Immediately after perfusion the brains were removed from the skull and dehydrated in 30% solution of sucrose in phosphate buffer (pH 7.4 and 4°C) for a night. Then they were cut with the cryostat Jung 1800 (Reichert, Germany) into 50-μm-thick coronal sections. Every second section was saved, mounted on a slide, air dried, and studied in a fluorescent microscope (Leica DMLS, Germany) equipped with the UV-filter system providing an excitation wavelength 365 nm.

Stereological study

The qualitative analysis of the claustrocortical projection into the motor and somatosensory cortices was performed by means of the following parameters: (1) volume of the dorsal part of the claustrum, (2) volume of the infiltrated cortex, (3) total number of retrogradely labeled neurons, (4) numerical density of retrogradely labeled neurons, (5) percentage distribution of retrogradely labeled neurons in the projection zone according to the rostro-caudal extent of the claustrum. The stereological analysis described by us elsewhere [9] was used to calculate the numerical density and the total number of neurons labeled with the retrograde tracer. The optical dissector was used to estimate the numerical density of labeled neurons. The optical fractionator method was applied simultaneously to the same set of sections stained with the fluorescent dye to acquire the unbiased total number of neurons [3,28]. For the evaluation of the distribution of labeled cells throughout the rostro-caudal extent of the claustrum in the consecutive sections the percentage values of both the section level in relation to the length of projection zone and the labeled cells in relation to all labeled cells were placed in diagrams as an abscissa and ordinate, respectively. The volume of the dorsal claustrum and volume of the infiltrated cortex were unbiasedly estimated by means of the Cavalieri formula [7]. For each parameter the mean and the standard error of the mean (mean ± SEM) were calculated. All calculations were performed in Excel 97 (Microsoft, USA). Statistical analysis was performed by means of the computer programs Statistica v. 5.5 (Statsoft; USA) and InStat (GraphPad Software, Inc; USA). The analysis of variance with post-hoc HSD test was used to compare results obtained for the experimental groups. The GLM module was used to define the homogenous groups.

RESULTS

In all animals the equal volume of retrograde fluorescent tracer was injected into the motor or somatosensory cortices and the volumes of infiltrated cortices did not differ significantly. In all studied groups of both projections the retrogradely labeled neurons in the claustrum were detected (Fig. 1, Fig. 5E, F). Since the earliest studied period the presence of motor and somatosensory projection zones can be reported. The shape and general topography of both projection zones are maintained during the whole studied period and do not reveal significant changes. The retrogradely labeled neurons in the claustrum are of different shapes (pirami-dal, multipolar, fusiform, triangular and oval) and volume, although the medium-sized neurons prevail (Fig. 5G, H).

Stereological analysis of the claustrocortical projections.

Volume of the dorsal claustrum. The volume of the dorsal part of the claustrum increases in the postnatal period until the end of the fourth week and stabilizes in the remaining age groups (Fig. 2; Fig. 5C; Table 1). After P30 the difference among groups is not significant. The relative increase in the claustral volume in the studied postnatal period is low and reaches the value of two times.

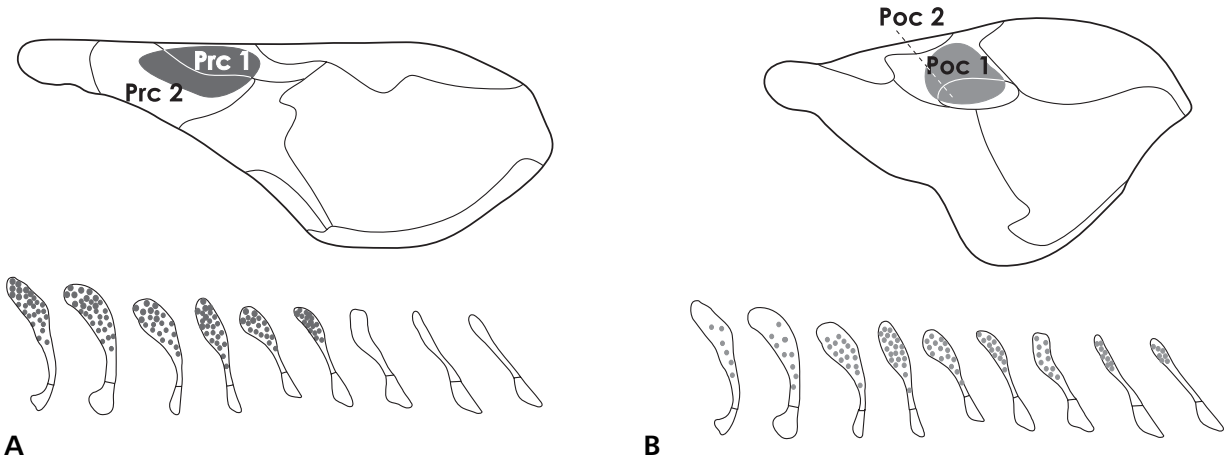


Figure 1. Schematic representation of the claustral A) motor and B) somatosensory projection zones on the coronal view of the structure. Top: site of the tracer injection into the motor cortex. Bottom: area occupied by the retrogradely labeled neurons in the claustrum after tracer injection. Abbreviations: Cl — dorsal (insular) claustrum; EDn — endopiriform nucleus (ventral claustrum); Poc 2 — medial postcentral region; Poc 3 — lateral postcentral region; Prc 1 — medial precentral region; Prc 2 — lateral precentral region.

Table 1. Total number of labeled neurons, numerical densities and volume of the dorsal claustrum in the age groups studied for the motor and somatosensory projections

Age group	Total number of labeled neurons [N]	Numerical density [n/mm^3]	Total number of labeled neurons [N]	Numerical density [n/mm^3]	Claustral volume [mm^3]
	Motor projection		Somatosensory projection		
P2	457707 ± 58069	135316 ± 23666	174440 ± 64120	62153 ± 11640	2,0 ± 0,1
P7	158480 ± 54183	46426 ± 6198	192640 ± 54226	41786 ± 8743	2,9 ± 0,3
P14	51200 ± 36800	45626 ± 19412	77840 ± 7280	33333 ± 952	3,4 ± 0,3
P21	89600 ± 30490	29718 ± 4849	69253 ± 22405	23947 ± 31	3,9 ± 0,4
P30	33600 ± 12084	28582 ± 2897	32000 ± 7200	21716 ± 4951	4,7 ± 0,3
P60	31600 ± 13717	39830 ± 1183	16133 ± 12574	29317 ± 4096	4,4 ± 0,3
P90	45173 ± 17832	35745 ± 1801	61040 ± 6630	18082 ± 1146	4,8 ± 0,3
P120	10080 ± 8960	30321 ± 6987	75413 ± 4344	31490 ± 2984	5,2 ± 0,5
P180	71260 ± 6732	34148 ± 8507	46853 ± 35991	21505 ± 1763	5,0 ± 0,2

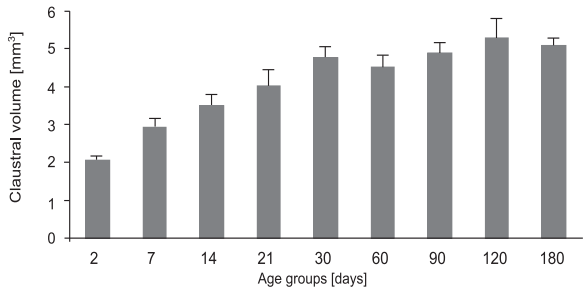


Figure 2. Mean values of the claustral volume in the studied age groups.

Total number of retrogradely labeled neurons. Analysis reveals that in the earliest period of postnatal development the total number of retrogradely labeled neurons is significantly higher than that in the later period. This can be observed both for the motor and somatosensory projection zones (Fig. 3). For the motor projection values observed in groups P2, P7 and P21 are significantly different from the rest of values in the remaining age groups. For the somatosensory projection the significantly different values are observed in age groups P2 and P7. No significant difference is observed in the total number of labeled neurons between the motor and somatosensory projections.

Numerical density of retrogradely labeled neurons. The highest values of numerical density of retrogradely labeled neurons are observed in the earliest age groups for the motor and somatosensory projections (Fig. 4). For both projections the values observed in P2 group are significantly different from the remaining values in the corresponding age groups. On the contrary to the total number of neurons, the mean values of the numerical density of labeled neurons for the motor projection are significantly different (higher) from the values for somatosensory projection.

Distribution of retrogradely labeled neurons. Analysis of this parameter reveals unequal distribution pattern of the projecting neurons within the studied zones in the claustrum. In all studied age groups of the motor projection the highest percentages of retrogradely labeled neurons within the projection zone are observed in its anterior half (Fig. 5A, B). For the somatosensory projection the highest values are localized more posteriorly — in the central and posterior third of the projection zone. Both projection zones reveal high degree of overlap along the antero-posterior extent of the claustrum.

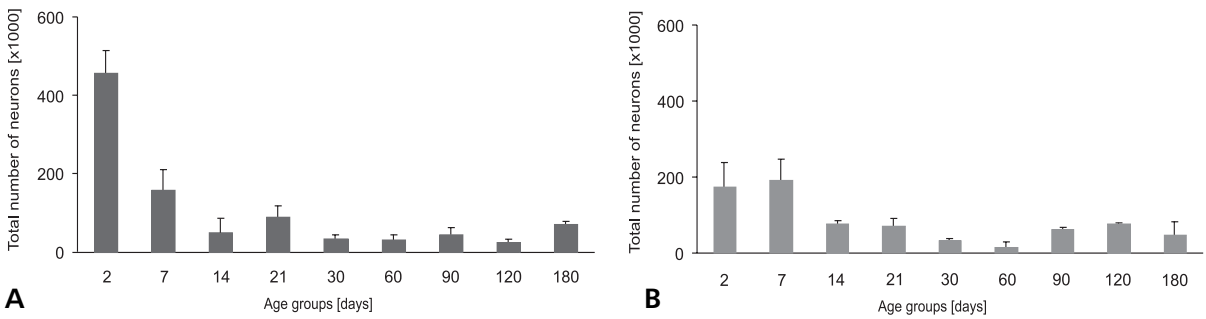


Figure 3. Mean values of the total number of claustral projecting neurons of the A) motor and B) somatosensory projections in the studied age groups. The values in P2, P7 and P21 groups for motor projection and in P2, P7 groups for somatosensory projection, differ significantly from the corresponding values of the other groups. There is no significant difference between the values of the total number of neurons in groups of motor and somatosensory projections.

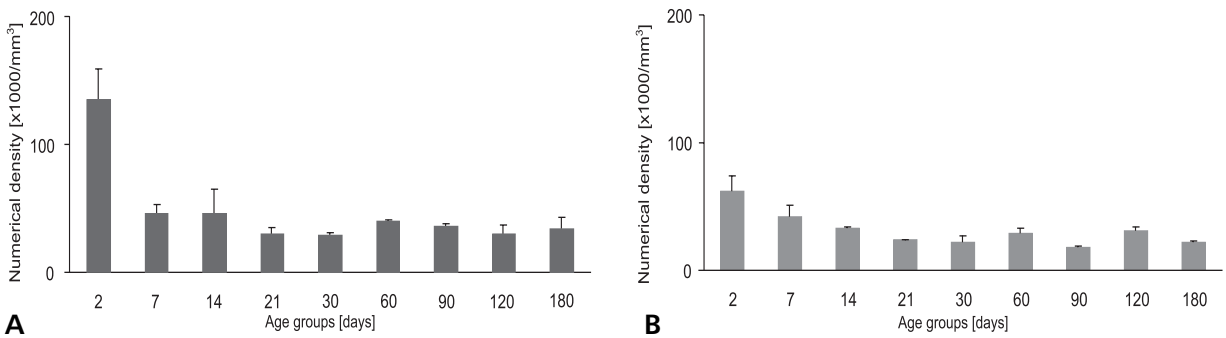


Figure 4. Mean values of the numerical density of projecting neurons of the A) motor and B) somatosensory projection zones in the studied age groups. The values in P2 groups differ significantly from the corresponding values in the other groups of both projections. There is a significant difference between the values of the numerical density in motor and somatosensory projections.

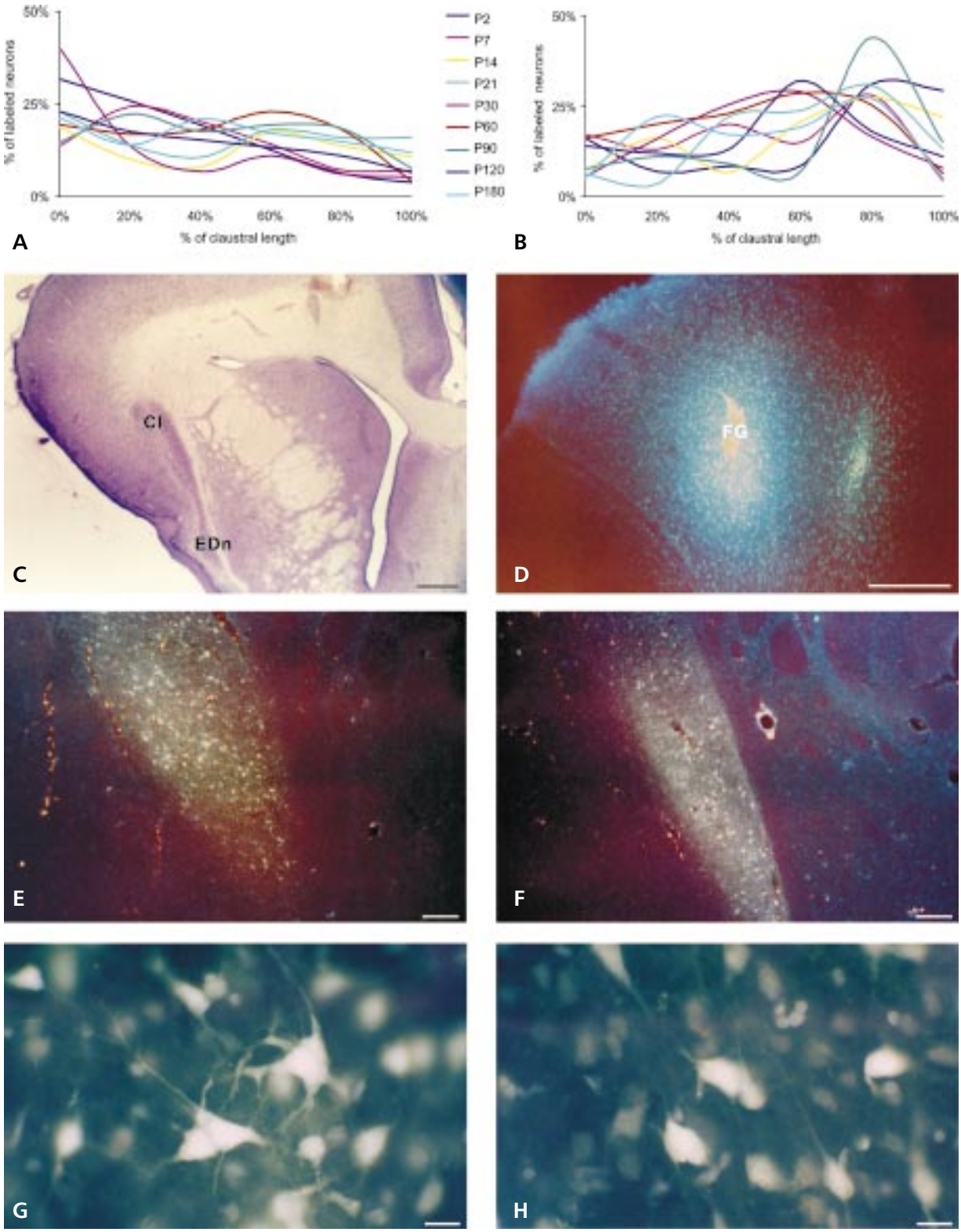


Figure 5. Distribution of the retrogradely labeled neurons for the A) motor and B) somatosensory projection zones in the representatives of the studied age groups. The distribution is presented as the ratio of retrogradely labeled neurons in the anteroposterior direction. C) In the rabbit two parts of the claustrum — dorsal (Cl) and ventral (endopiriform nucleus; EDn) are well separated. D) Site of the tracer (FG) injection into the frontal cortex. E) Retrogradely labeled neurons of the motor projection zone in the claustrum of the rabbit of P7 group. F) Retrogradely labeled neurons of the somatosensory projection zone in the claustrum of the rabbit of P7 group. G) Retrogradely labeled claustral projecting neurons of the motor and H) somatosensory projection zones in the rabbit of P120 group. (Scale bars: C = 1000 μm; D = 250 μm; E, F = 160 μm; G, H = 20 μm).

DISCUSSION

Methodological considerations

Combination of the rertrograde axonal transport and fractionator/dissector methods was applied for the quantitative analysis of the maturation of the claustrocortical connections in the rabbit. The obtained results reflect some general tendencies which take place in the structures of the central nervous system during its maturation, although they must be treated with some caution. In all experiments both pharmacological treatment as well as the duration of surgical procedure, postoperative treatment and survival time were similar in order to obtain repetitive results. In each studied case the same volume of tracer was injected to the motor or somatosensory cortical area, selected on the basis of the stereotactic coordinates and verified by the cytoarchitectonic analysis of the cortex surrounding the place of injection. Only the cases without the destruction of the subcortical structures were selected for the study. Unbiased stereological parameters were selected for the quantitative assessment of the claustrocortical projections. It must be firmly stressed that the value of the total number of retrogradely labeled neurons in the claustrum does not reflect the total number of all claustral neurons projecting into the motor or somatosensory cortical area, but it must be treated only as the indirect measure characterizing performed experiment with the injection of a definite volume of the tracer into the definite volume of the cerebral cortex in the animal of the same age. The comparable conditions of the experiments make this parameter, in our opinion, suitable for statistic analysis. The second parameter — numerical density of labeled neurons — characterizes more precisely the internal structure of the claustral projecting zone. This parameter is not as much influenced by the volume of the infiltrated cortex as the total number of retrogradely labeled neurons. So in our opinion it may better characterize the changes which take place in the projection zones independently from the development of the whole brain. In this respect changes of the numerical density may reflect first of all: changes of the total number of labeled neurons in various age groups, specific distribution of the labeled neurons for each studied projection, changes resulting from the increase of the neuropil volume and neuronal volume, and consequently from the increase of the claustral volume (estimated additionally). The distribution of labeled neurons along the antero-posterior extent of the claustrum may describe the structure of the studied projection zone. This characterizes the internal organization of the studied structure and may reflect the pattern of distribution/compartmentalization in it.

Quantitative changes of the claustrocortical connections during the postnatal period

The volume of the dorsal part of the claustrum increases significantly until the end of the fourth postnatal week. Then the phase of stabilization can be observed. The highest values of the total number and numerical density of retrogradely labeled neurons can be observed during the first week of postnatal development. This is characteristic for both the motor and somatosensory projection zones. Then, until the end of the third week for motor projection and the second week for somatosensory projection, the values of the total number of labeled neurons decrease and stabilize. Later no significant changes can be observed until the end of the studied period. The decrease of the numerical density for both studied projections finishes until the end of the first week. On the basis of these results it may be concluded that all significant changes are completed during the first month, but for each parameter the characteristic period of changes is different. The decrease of numerical density of labeled neurons may be the consequence of elimination of some projecting neurons and the increase of the claustral volume and the volume of neurons. As the total number of projecting neurons decreases nearly nine times and the volume of the dorsal part of the claustrum increases about two times, it may be suspected that the changes in the neuronal density are more closely related to the loss of projecting neurons, than to the increase of the structure volume. However, the quantitative study of the development of rabbit claustrum was not performed, the presented tendencies may be compared with the results obtained by Maciejewska et al. [16] in the rat. According to these results the total number and numerical density of all claustral neurons decrease significantly until P14. After this time both parameters stabilize in the rat. Decrease in the total number of neurons in the dorsal part of the claustrum is the result of apoptosis [16]. If the same mechanism is responsible for the reduction of the number of projecting neurons in the rabbit, it requires further studies. The decrease of the total number of labeled neurons may be also explained by the elimination of neurons, which do not develop the proper cortical connections or lose in competition for the neurotrophic factors [18,19,23]. The presented results may reflect some general tendencies in postnatal development, de-

scribed in the relationship to the other structures of the central nervous system [13,25,27]. It must be underlined that the claustrocortical connections both with the motor and somatosensory cortical areas in the rabbit are well established in the postnatal life. So, the qualitative formation of these connections takes place most probably during the prenatal life and to some extent precedes the quantitative changes observed by us in the studied period. The decrease of the total number and numerical density of cortically projecting neurons may reflect the process of adjustment of the claustrum to its modulatory function upon corresponding cortical areas. Comparison of the stereological parameters for the motor and somatosensory projections reveals some characteristic features. The intensity of the claustral connections with the motor and somatosensory cortices is differentiated during the studied period of postnatal life, being more numerous for the motor one. This observation is not confirmed as far as only the adult animals are taken into account [9]. It may be suggested that both motor and somatosensory cortical projections reveal differentiated dynamics during the developmental period, but finally, the so-called claustrocortical loop reveals balanced character in this species and no domination of the single projection can be reported.

ACKNOWLEDGEMENTS

This research was supported by the funds from State Committee of Scientific Research, Grant No PB 4.P05A.010.13.

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