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Claustrocingulate connections in the rabbit and rat — a stereological study

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Unbiased stereological methods were used for estimating the numerical density and the total number of claustral neurones projecting to the cingulate cortex in rabbit and rat.

In rat the numerical density of neurones projecting to the retrosplenial granular cortex (RSG) differed significantly from those projecting to the retrosplenial agranular (RSA) and cingulate (Cg) cortices while in rabbit the numerical densities of retrogradely labelled neurones in the claustrum following injections into various areas of the cerebral cortex did not differ significantly. The total number of retrogradely labelled neurones in the claustral limbic zones did not differ significantly in both species.

The quantitative analysis of claustral zones projecting to a different cingulate cortex area, both in rabbit and rat, reveals that each of these zones is rather homogeneous.

key words: claustrum, claustrocortical connections, limbic cortex, rat, retrograde transport, fluorescent tracers, stereology

INTRODUCTION

Claustrum is a telencephalic structure present in almost all mammals. In all of them, two parts can be easily distinguished: the dorsal (insular) claustrum situated close to the deep layers of the insular cortex and the ventral one, called either prepiriform claustrum or endopiriform nucleus localised below the rhinal fissure close to the piriform and entorhinal cortices [5,19,32]. Many studies have revealed that the insular claustrum has reciprocal connections with almost all areas of the cerebral cortex, and that these connections are organised topographically [14,18,20,28,31,32].

Although the quantitative analysis of cortical connections with the claustrum is scanty [23], the topographical organisation of the claustral cortico-related zones shows differences among species [1,4,7,14,31,32]. Contrary to the insular part, the endopiriform nucleus is connected only with the allocortical regions [8,12,22,38,39].

Although the organisation of the central nervous system in rabbit (*Lagomorphae*) may, in many aspects, resemble that of rat (*Rodentia*), some remarkable differences exist [13,15,32], thus the quantitative description of the claustrocingulate connections in these species may be useful for further comparative analysis of the structure and function of the claustrum. This is especially important in relation to the integrative role of the claustrum in the transmission of information between areas of the cerebral cortex and the limbic system.

MATERIALS AND METHODS

Fifteen adult Wistar rats (body weight 300–350 g) and ten New Zealand rabbits (body weight 2.2–4.0 kg) were used for the study. Animal care and treatment

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were in accordance with guidelines for laboratory animals outlined by National Institutes of Health as well as by the Local Committee of the Medical University of Gdansk. Rats were anaesthetised with intraperitoneal (i.p.) administration of fentanyl (0.01 mg/kg) and dehydrobenzperidol (0.2 mg/kg). Rabbits were given fentanyl (0.03 mg/kg) with dehydrobenzperidol (1.4 mg/kg i.p.) and ketamine (70 mg/kg), intramuscularly. In sterile conditions, the animals were fixed in a stereotaxic frame (Trend Wells Inc.®, USA), and after skin incision, a small craniectomy was made in selected cortical areas according to stereotaxic and cytoarchitectonic data [27,36,36,41]. A glass microcapillar, coated with silicon gel, was inserted into the cerebral cortex to a depth calculated using stereotaxic coordinates. A pressure injection was made using 5 ml Hamilton syringe at a rate of 0.05 μl/min. A total volume of 3 μl of 2% water solution of Fast Blue (FB; Sigma Chemical, USA) was used.

After a single injection, the microcapillar was held in place for at least 5 min to prevent tracer leakage. Then the incision was closed surgically, and an antibiotic was given to the animal. After survival time of 7 days for the rats and 10 days for the rabbits, the animals were anaesthetised with nembutal in a dose of 70 mg/kg (rats) or fentanyl (0.03 mg/kg i.p.) and thiopental in a dose of 80 mg/kg i.p. (rabbits). Under deep anaesthesia, the animals were perfused transcardially with 0.9% saline containing 10,000 units of heparin; 4% solution of paraformaldehyde in phosphate buffer (pH 7.4 and 4°); and 10% sucrose in phosphate buffer (pH 7.4 and 4°). After perfusion, the brains were removed immediately from the skull and cryoprotected in 30% solution of sucrose in phosphate buffer (pH 7.4 and 4°) overnight. Then, they were cut frontally with the cryostat JUNG 1800 (Leica, Germany) into 50-μm-thick sections. All sections were saved, mounted on slides, and air-dried. Every third pair of sections was stained with cresyl violet. Then, the sections were studied under a fluorescent microscope Leica *DMLS* (Germany) equipped with an UV filter system, providing an excitation wavelength of 365 nm.

The volume of the injection site into the cortex was estimated by means of the Cavallieri formula [11].

The optical dissector and fractionator was used for estimation of the density and the total number of neurones in the claustrum and retrogradely labelled neurones projecting to the limbic cortex. Due to the small coefficient of variability of the numerical density and total number of neurones in the insular claustrum (about 4.8% in the rat and 6.5% in the rabbit) the estimation of these parameters was based on a sample of five animals.

The estimations were based on a set of systematic random sample of test areas according to the method described by West et al. [37] as well as by Burwell and Amaral [2]. Only neurones with sharply delineated nuclei (for cresyl violet) or perikarya (for labelled neurones) were counted.

For each parameter the mean and 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.5.0 (Statsoft®; USA) and InStat (GraphPad Software, Inc; USA). Due to unequal variances the nonparametric analysis of variance (Kruskal-Wallis test) with post-hoc Dunn's test was used to compare results obtained for the experimental groups. The averaged distributions of the labelled cells for all projection zones were compared using Chi-square test [40].

RESULTS

As a limbic cortex (Fig. 4B) we took into consideration only the areas of the cerebral cortex receiving connections from the anterior thalamic nuclei according to the definition of Rose and Woolsey [29].

Following Paxinos and Watson [27], the rat limbic cortex is divided into the anterior cingulate region or cingulate cortex (Cg; area 24) and the posterior cingulate region (area 29) or retrosplenial cortex. The retrosplenial cortex contains two different fields: the retrosplenial granular (RSG) and retrosplenial agranular (RSA) cortices. Fifteen rats were divided into three groups: Cg, RSG and RSA (each group contained five animals).

Due to the lack of clear classification of the retrosplenial granular and agranular areas in the rabbit limbic cortex, we divided them into the cingulate (area 24 — Cg) and retrosplenial (area 29 — RS) cortices [29,36]. Ten rabbits received FB injections into Cg and RS (five animals in each group).

The size and the localisation of the tracer injections

In all cases, the injection site of the tracer encompassed only the cortical layers of the specific cortical area, without destruction of the neuronal fibres of white matter below (Fig. 4E). The volumes of infiltrated cortex varied from 3.8 to 16.3 mm^3 in the rabbit and from 0.066 to 0.578 mm^3 in the rat (Table 1, 3). The differences between groups were not significant due to large variances, and no corre-

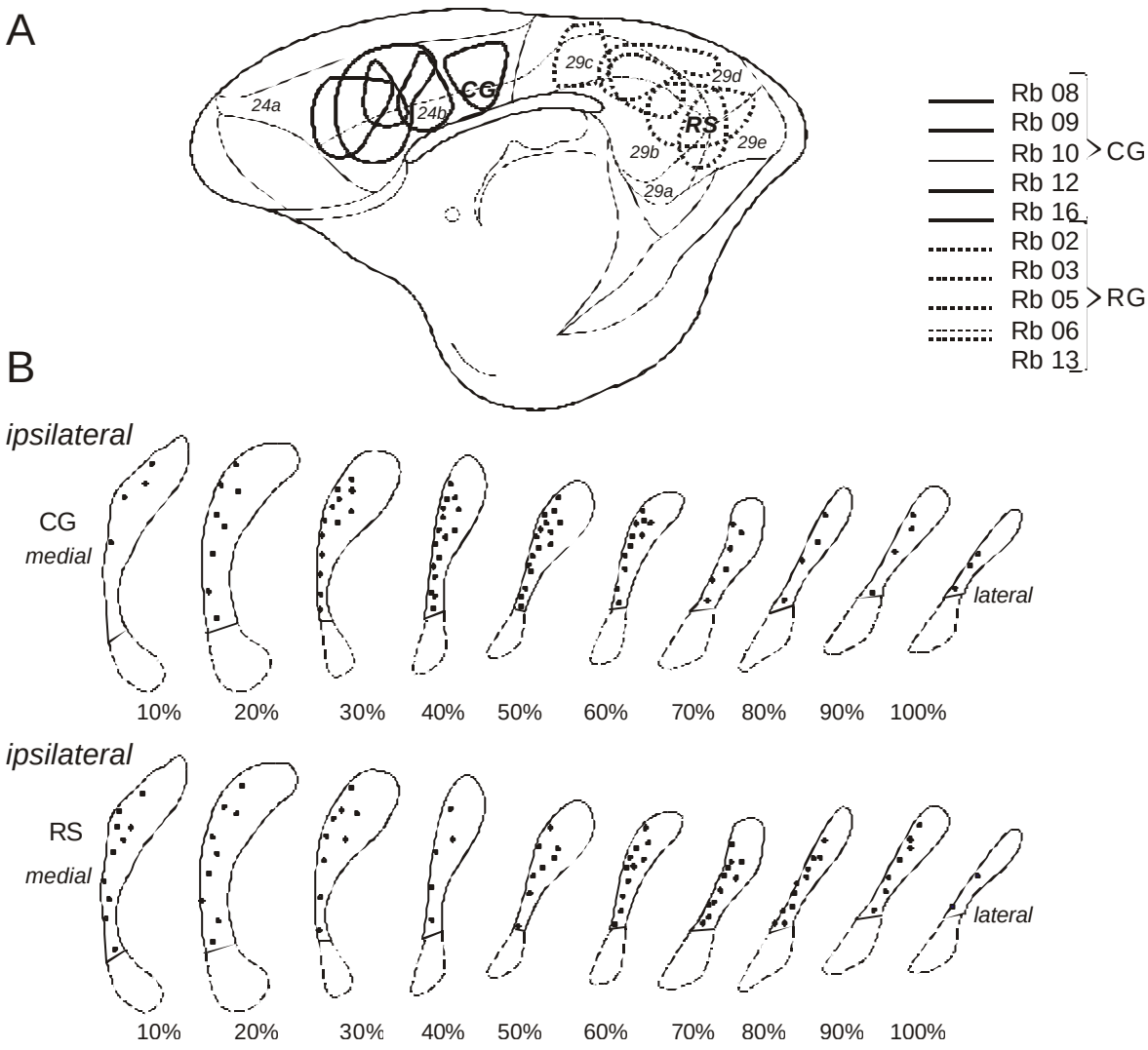

Figure 1. (A) Localisation of FB injections into the rabbit cingulate (CG) and retrosplenial (RS) cortices marked on a schematic view of the medial surface of the cerebral hemisphere; (B) distribution of labelled neurones in CG and RS zones of the claustrum; the distance between two consecutive schematic coronal sections of the claustrum is 10% of its total length. The stippled area represents the ventral claustrum

lation between the volume of infiltrated cortex and the number of retrogradely labelled neurones in the claustrum could be reported.

Quantitative comparison of the limbic projection zones of the rabbit

In all cases, after injections of the tracer into the selected cortical areas, retrogradely labelled neurones were present in the corresponding, topographically organised projection zones in the claustrum (Fig. 1, 4A, D). The labelled neurones covered the whole rostrocaudal extent of the claustrum. The highest percentage of labelled neurones for CG projection was observed in the anterior and central part of the nucleus and their number decreased gradually, whereas for RS projection – in the central and posterior parts of the structure (Fig. 3A). The distributions of labelled cells differed significantly.

The mean value of the numerical density of retrogradely labelled neurones in the claustrum after FB injections into CG was 13,558 ± 1,665 and into RS – 13,217 ± 2,376 per cubic millimetre (Table 1). The values did not show significant differences. The mean value of the numerical density of all neurones in the rabbit claustrum was 27,662 ± 1,105 per cubic millimetre (Table 2).

The total number of labelled neurones projecting to the cingulate cortex did not differ significant-

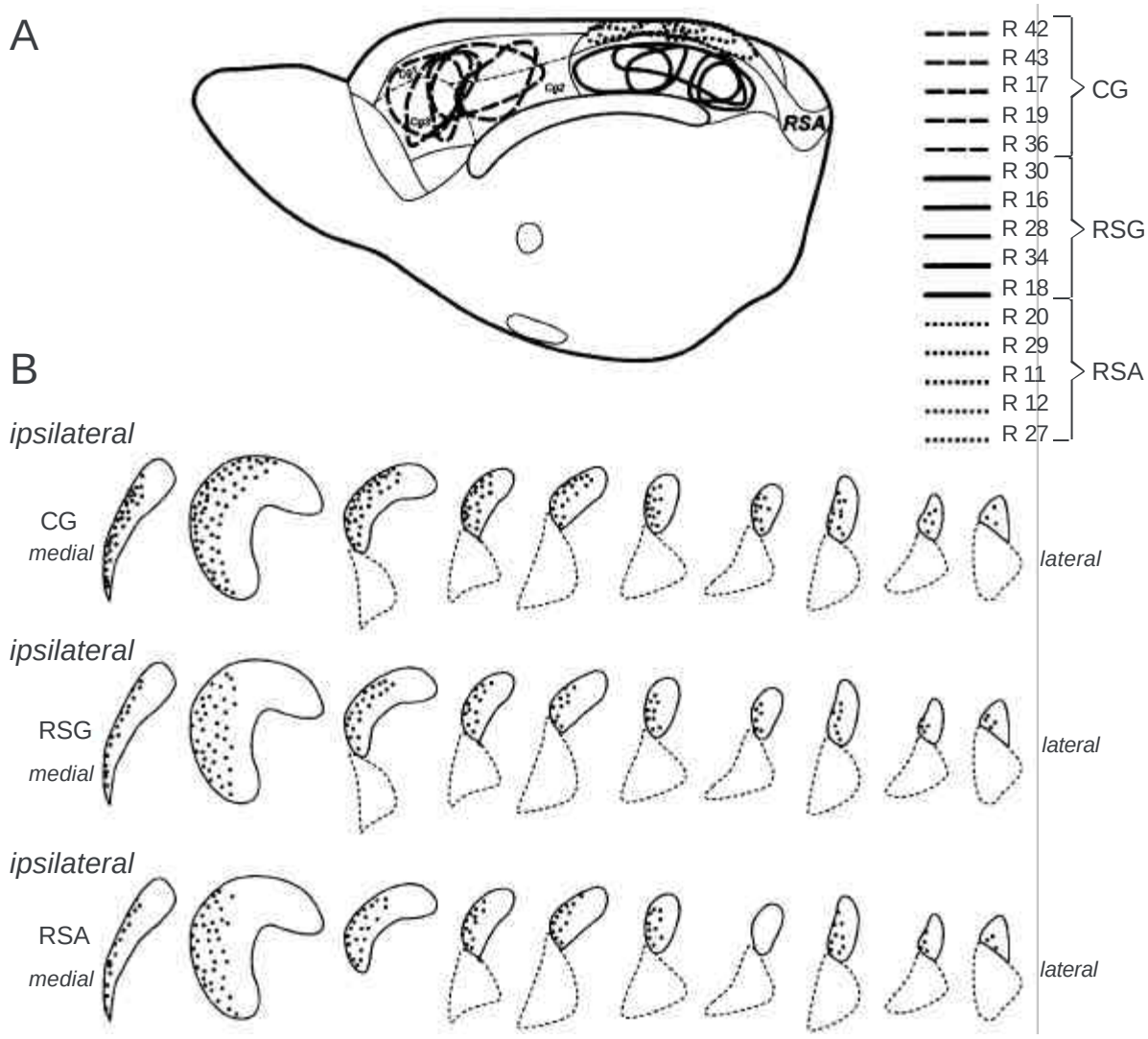

Figure 2. (A) Localisation of FB injections into the rat cingulate (Cg), retrosplenial granular (RSG) and retrosplenial agranular (RSA) cortices marked on a schematic view of the medial surface of the cerebral hemisphere; (B) distribution of labelled neurones in Cg, RSG and RSA zones of the claustrum; the distance between two consecutive schematic coronal sections of the claustrum is 10% of its total length. The stippled area represents the ventral claustrum

ly from those projecting to the retrosplenial cortex (86,231 ± 26,475 and 86,344 ± 45,175; respectively; Table 1).

Considering that the total number of neurones in the insular claustrum of the rabbit was 280,500 ± 18,120 on average (Table 2), the projections to CG and RSG constituted 31.07 and 31.11% of the total number of claustral neurones, respectively.

Quantitative comparison of the limbic projection zones of the rat

In the rat, following administration of FB into the three limbic cortical areas, labelled neurones were found throughout the whole rostrocaudal extent of the insular claustrum (Fig. 2, 4C, F). The highest percentage of labelled neurones for Cg, RSG and RSA projections were observed in the anterior and central parts of the nucleus and their number decreased gradually (Fig. 3B), although neurones projecting to RSA seemed to be distributed more equally; distributions of labelled neurones projecting to different cortical limbic areas along the anteroposterior axis of the claustrum did not differ significantly.

The mean value of the numerical density of retrogradely labelled neurones in the claustrum after FB injections into Cg was 15,639 ± 2,754, into RSG

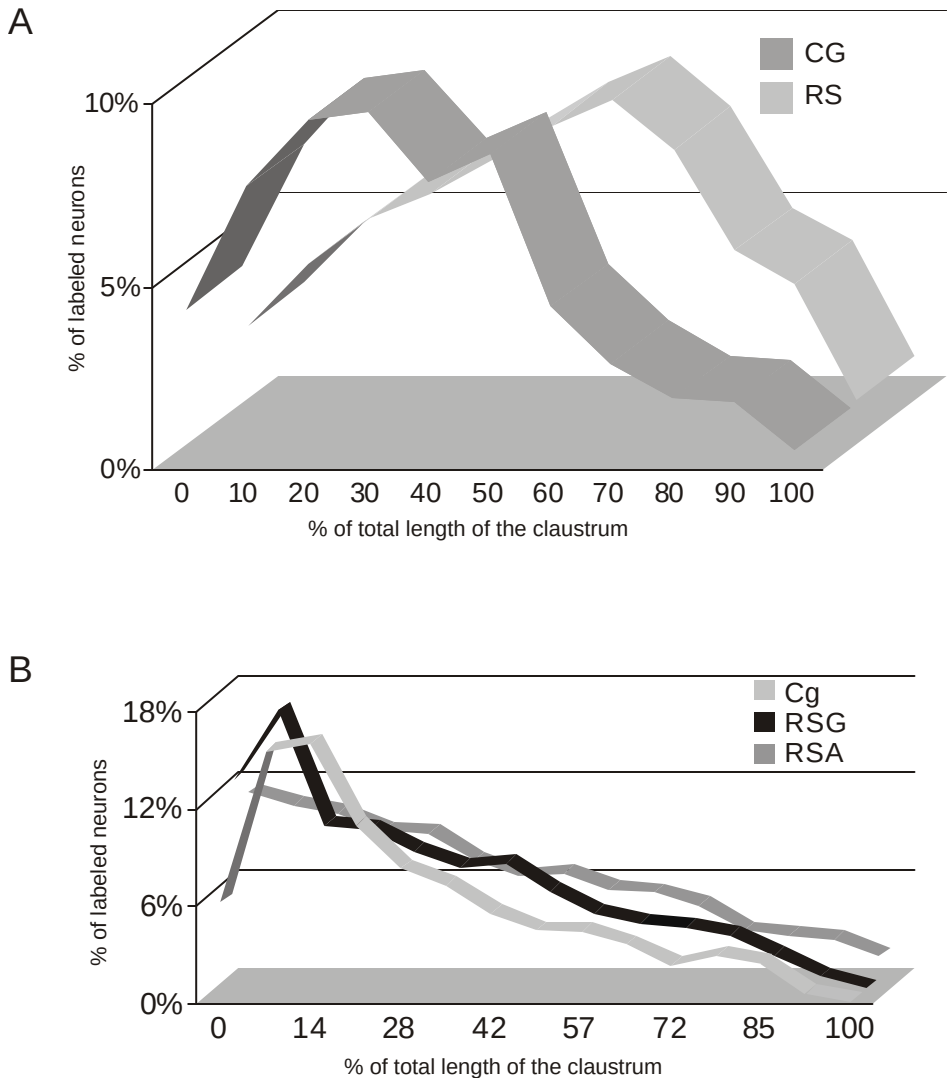

Figure 3. (A) Following administration of fluorescent tracers into CG labelled neurones were found mainly in the anterior and central part of the claustrum while after injections into RG — in greater number in the central and posterior part; the distribution in both groups showed significant differences; (B) Following administration of fluorescent tracers into Cg, RSG and RSA, the majority of labelled neurones were found in the anterior part of the claustrum and posteriorly their number decreased gradually; the distribution did not show any significant differences

— 21,485 ± 3,917 and into RSA — 13,346 ± 4,440 per cubic millimetre (Table 3). The values for Cg and RSA differed significantly from the value for RSG. The mean value of the numerical density of all neurones in the rat claustrum was 62,410 ± 1,800 per cubic millimetre (Table 4).

The total number of labelled neurones in rat insular claustrum following injections into the cingulate cortex (Cg) was 16,894 ± 4,140, into the retrosplenial granular cortex (RSG) it was 14,794 ± 2,810 and into the retrosplenial agranular cortex (RSA) it was 12,130 ± 2,090 (Table 3), with no significant differences.

The total number of neurones in the insular claustrum of the rat was 229,590 ± 11,200 on average (Table 4), so the percentage of labelled neurones projecting to the different limbic areas was 7.3% for Cg, 6.4% for RSG and 5.2% for RSA.

DISCUSSION

Any attempt to explain claustral functions must focus on the interrelations between this structure and different regions of the cerebral cortex. Since 1964, when for the first time degenerative methods showed the existence of topographically arranged connections between the claustrum and cortical re-

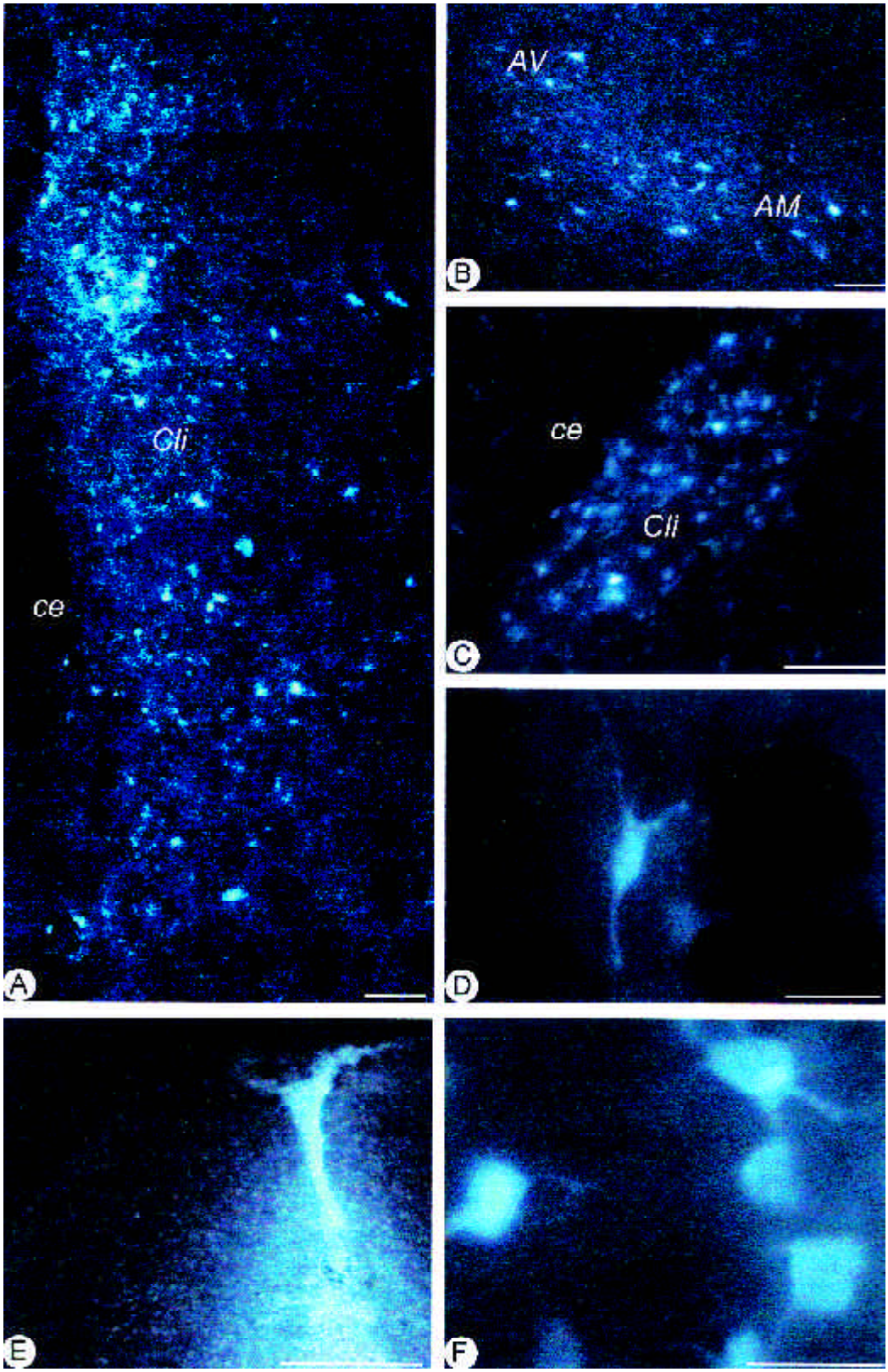

Figure 4. (A) Claustral projection zone to the cingulate cortex (CG) in the rabbit (FB, calibration bar — 100 μm); (B) Retrogradely labelled neurones in the rabbit anterior group of thalamic nuclei (calibration bar — 100 μm); (C) Claustral projection zone to the retrosplenial granular cortex (RSG) in the rat (FB, calibration bar — 250 μm); (D) FB-labelled neurones in the rabbit claustrum after injection into the retrosplenial cortex (calibration bar — 25 μm); (E) FB deposit in RSA on coronal section of the rat brain (calibration bar — 50 μm); (F) FB-labelled neurones in the rat claustrum after injection into the cingulate cortex (calibration bar — 25 μm). Abbreviations: AM — anteromedial nucleus of the thalamus, AV — anteroventral nucleus of the thalamus; ce — external capsule; Cli — insular claustrum

gions [3,26], the relations between both these structures have been studied intensively in rat, cat, rabbit, and monkey [14,18,20,28,31,32]. In general, the anterior part of the insular claustrum is mainly connected with the motor and prefrontal cortices, the central part with somatosensory areas while its posterior part with the visual and auditory cortices. However, the organisation of claustral cortico-related zones shows also differences depending on species [15,32]. The endopiriform nucleus has connections with the piriform and entorhinal cortices [6,22,39]. According to our previous study [21], there are also some differences in species concerning interrelations between the claustrum and cingulate cortex, how-

Table 1. The sizes of the cortical injections, the numerical densities and the total number of labelled neurones for the individual cases of the claustrocingulate projections in rabbit

CG	Size of injection [mm^3]	Numerical density of labelled neurones [N/mm^3]	Total number of labelled neurones [N]
Rb08	5.46	11,679	86,620
Rb09	16.32	13,294	57,370
Rb10	9.72	15,901	125,440
Rb12	12.18	12,530	94,500
Rb16	4.02	14,534	67,220
Mean value ± SD	9.54 ± 5.00	13,588 ± 1,665	86,230 ± 26,470
RS			
Rb02	6.48	12,971	103,500
Rb03	8.34	11,658	54,560
Rb05	10.02	13,131	44,160
Rb06	3.78	11,136	73,120
Rb13	9.66	17,187	156,370
Mean value ± SD	7.66 ± 2.57	13,217 ± 2,376	86,340 ± 45,170

Table 2. The numerical densities and the total number of neurones of the insular claustrum of rabbit

	Numerical density of neurones [N/mm^3]	Total number of claustral neurones [N]
Rb01	28,033	267,000
Rb03	27,065	294,000
Rb06	29,375	259,000
Rb09	27,344	279,000
Rb16	26,495	303,000
Mean value of total number ± SD	27,662 ± 1,105	280,500 ± 18,120

Table 3. The sizes of the cortical injections, the numerical densities and the total number of labelled neurones for the individual cases of the claustrocingulate projections in rat

CG	Size of injection [mm^3]	Numerical density of labelled neurones [N/mm^3]	Total number of labelled neurones [N]
R17	0.23	15,033	17,950
R19	0.284	14,026	18,180
R36	0.066	12,861	10,960
R42	0.105	20,030	15,180
R43	0.064	16,224	22,170
Mean value ± SD	0.149 ± 0.10	15,639 ± 2,754	16,890 ± 4,140
RSG			
R16	0.317	24,834	11,250
R18	0.243	19,015	13,360
R28	0.087	21,570	14,950
R30	0.084	25,671	15,560
R34	0.107	16,334	18,840
Mean value ± SD	0.168 ± 0.11	21,485 ± 3,917	14,790 ± 2,810
RSA			
R11	0.578	7,219	14,440
R12	0.494	13,781	13,310
R20	0.128	17,882	12,840
R27	0.259	10,772	9,190
R29	0.125	17,074	10,880
Mean value ± SD	0.316 ± 0.21	13,346 ± 4,440	12,130 ± 2,090

Table 4. The numerical densities and the total number of neurones of the insular claustrum of rat

	Numerical density of neurones [N/mm^3]	Total number of claustral neurones [N]
R11	59,891	236,400
R19	62,333	226,500
R23	64,858	228,700
R36	61,829	243,000
R43	63,148	213,300
Mean value of total number ± SD	62,410 ± 1,800	229,600 ± 11,200

ever, the general topographical arrangement can be observed. The claustral limbic zone generally occupies the medioventral part of the structure. The topography of this zone, both in rabbit and rat, has been studied and discussed previously [21].

The retrograde tracing techniques have often been used in investigations of connections between different areas of the nervous system. The limitation of the methods is the possible involvement of white matter fibres. With respect to the present findings, injections with any visible damage to the white matter were discarded. In the present experiments we were able to inject the tracer to a relatively small fragment of the selected cortical area, although we made an assumption that the input to the injection site reflected the input to the entire cortical region.

Only a few studies considered the number of projecting neurones by counting retrogradely labelled cells. Minciacchi et al. [23] performed quantitative analysis of multiple retrograde fluorescent labelled cells and revealed that the thalamic anterior intralaminar nuclei (AIN), compared to claustrum, contained a higher proportion of double-labelled neurones after injections into the primary somatosensory (S_1) and visual (V_1) cortices.

Burwell and Amaral [2] investigated the origin of cortical input to the rat perirhinal, postrhinal and enthorinal cortices by placing injections of the retrograde tracers at several locations within each of these areas. They used the same retrograde tracer (Fast Blue) and, similar to our quantitative technique, the fractionator sampling method, so the strengths and limitations of the method that was adopted for both studies were similar.

In the present study, for the characterisation of the claustral limbic zones we used in each experiment both the total number of retrogradely labelled cells and the density of labelling after injecting FB into the selected cingulate region. The density of the labelled cells provides quantitative description of the number of retrogradely labelled cells per unit area, which are located in the region, so it lets us make a comparison across the different claustral cingular zones. The group of five animals, with randomly selected injection sites, enabled the quantitative analysis of the claustrocingulate connections. As the volumes of infiltrated cortex did not differ significantly among studied groups of the same species, we may consider them as a relatively homogeneous group. That enables the comparison between the projections, but the quantitative data reported here will provide important background for investigations of synaptic efficiency and morphology in these regions. There was no significant difference between numerical densities of labelled neurones in CG and RS claustral zones of the rabbit. That may suggest (with some caution) no evident difference in the properties of the limbic projecting zones; that reflects their homogeneity. It has been found that limbic projecting zones in the rabbit overlap each other [21]. In the rat we observed a small preference of RSG projection, but in our opinion the difference does not reveal any significant hodological and functional implications and the three zones (Cg, RSG and RSA) overlap almost entirely [21].

The total number of retrogradely labelled neurones in the claustrum after injections of the tracer into the selected cortical areas does not reflect the exact number of projecting neurones as this parameter would require the tracer injection into the whole studied cortical area. However, the total number of labelled cells can provide information on the possible influence of the claustrum on the target area.

Due to the fact that only single claustral neurones send collaterals to various areas of the cingulate cortex [21] we can add the percentages of labelled neurones arising in various zones. As a result, we may conclude that in the rabbit about 60% of the total number of claustral neurones belong to the cingulate zone, while in the rat only 20%. The large difference can be partially explained by the big variance in volumes of the infiltrated cortex in these two species. However, we must stress that the role of the associative cortex increases, so the large percentage of labelled neurones in the claustrum of the rabbit in comparison to that of the rat may reflect the increase of the volume of the limbic cortex. That confirms the prior observations that the claustro-cortical loop is built up to counterbalance the cortical maps in some way [14,16,17,20,23,24,31,32].

Whereas numerous studies have emphasised the contribution of the hippocampus and amygdala to learning and memory, more recent observations have focused on the cortical regions that may play a role in this process: the retrosplenial and cingulate cortices [14,18,20,31,32,34]. Hirono et al. [10] suggested that damage of the posterior cingulate cortex may contribute to producing disorientation in time and place in patients with Alzheimer's disease. Valenstein et al. [35] found that lesions of the cingulum and retrosplenial cortex may cause amnesia by disruption of the thalamocortical portion of Papez circuit which is important for memory. Some pathological changes were also found in the limbic part of the claustrum in Alzheimer's disease [25,30,38].

It is clear that the claustrum is reciprocally connected with different areas of the neocortex but its functional interrelationships are not fully understood. Data obtained from various species and human suggest that the claustrum plays an important role in relations between the cingulate cortex and other areas of the cerebral cortex [9,18,23,33]. The quantitative analysis of the cingulate zones in the insular claustrum may form the basis for future research into the specific contributions of this structure to the limbic system.

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