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Qualitative and quantitative differences in the motor and somatosensory cortical projections of the rat claustrum — combined retrograde transport and stereological studies

Przemysław Kowiański, Janusz Moryś, Marcin Sadowski, Jerzy Dziewiątkowski

Department of Anatomy and Neurobiology, Medical University of Gdańsk, Poland

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Using axonal retrograde tracing, combined with morphometric analysis, we compared the distribution and number of claustral neurons projecting to the motor and somatosensory cortical areas in the Wistar rat. Comparable volumes of the retrograde tracer Fluoro-Gold, were injected into the motor or somatosensory cortices. Injections into these areas resulted in labeling of neurons along the entire length of the claustrum. Neurons retrogradely labeled after injection into the motor cortex prevailed in the anterior part of the claustrum, whereas those projecting to the somatosensory cortex predominated in the central part.

The mean number of claustral neurons retrogradely labeled after tracer injections into the motor cortex significantly outnumbered that from the somatosensory cortical area ($p<0.01$). Similarly, the mean value of the numerical density of the retrogradely labeled neurons was significantly higher for the motor projection zone in the claustrum, than for the somatosensory projection zone ($p<0.001$). The contralateral claustral projections, both into the motor and somatosensory cortices, were considerably lower in number than the ipsilateral ones.

These findings indicate that: (1) the claustral projections to the various cortical regions seem to be differentiated (2) the distribution of claustral neurons projecting to the motor and somatosensory neocortical areas shows an anteroposterior gradient, (3) the claustrum of the rat appears to be more closely related to the motor than to the somatosensory system, (4) the rat claustrum seems to function more as a satellite than a relay structure in relationship to the cerebral cortex.

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

INTRODUCTION

The claustrum is a prominent subcortical structure present exclusively in the mammalian brain. Until the mid 60s it had remained an absolute "*terra nova*", both in terms of its connections and its function. Improved silver impregnation methods and methods based on anterograde and retrograde axonal tracing, introduced in the 70s and 80s, led to the discovery that the claustrum has numerous bidirectional connections with the various regions of the cerebral cortex. Studies, performed mostly on the rat, cat, rabbit and more rarely on monkey and man, proved that claustrocortical connections in these species are organized in a topographic way [5,7,8,10,11,14,17,20,22,23,28,29,37]. The neurons connected with the somatosensory cortex are located mainly in the central part of the claustrum, and according to the electrophysiological studies of Ol-

Address for correspondence: Janusz Moryś, MD, PhD Department of Anatomy and Neurobiology Medical University of Gdańsk ul. Dębinki 1, 80–211 Gdańsk, Poland, tel: +48 58 349 14 01, fax: +48 58 349 14 21, e-mail: jmorys@amg.gda.pl

son and Graybiel [27], show a somatotopic organization. LeVay and Sherk [14,15] succeeded in disclosing the retinotopic organization of the connections between the primary and secondary visual areas and the posterior part of the claustrum in the cat. Neurons related to the motor cortex occupy most of the anterior part of the claustrum, and according to the suggestion of Clasca et al. [4], may also be arranged in a somatotopic order. Apart from connections with the motor, somatosensory and visual cortices, the claustrum also projects to the auditory, cingulate, prefrontal, entorhinal, temporal, and parietal cortical areas [2,4,11,14,17,18,27,38,40]. Although the list of cortical areas connected with the claustrum is well known, the questions regarding differences in the claustral output to these fields remain unanswered. Do all cortical areas receive inputs from a similar number of claustral neurons? And most intriguing, do some cortical fields receive input from a significantly larger population of claustral neurons than others without respect to their size? To address these issues, we performed a series of experiments on the rat by means of retrograde axonal tracing combined with morphometric analysis.

MATERIAL AND METHODS

Surgery and histological procedure

Surgery was performed on 10 adult Wistar rats of both sexes (300–350 grams of body weight) obtained from our own animal colony. Animal care and treatment guidelines outlined by the local ethical committee were followed. For surgery, each rat was anesthetized with Nembutal (30 mg/kg) and fixed in the stereotaxic apparatus (Trend Wells Inc., USA). An aseptic neurosurgical protocol was followed to expose the cortical surface. After a sagittal skin incision, a small craniectomy was performed with a dental drill. The dura was cut and reflected to expose the motor (five animals) or somatosensory (five animals) cortices. A glass microcapillar was positioned stereotaxically, following the coordinates described by Zilles [42], and Zilles and Wree [41], and introduced into the cortex to a depth ranging from 1.5 mm (somatosensory cortex) to 2 mm (motor cortex). Three-percent aqueous solution of Fluoro-Gold (Fluorochrome Inc., USA) was massively injected in each case at a rate of 0.2 μl/min by means of Hamilton microsyringe attached to an electromechanical injection pump. After injection, the microcapillar was held in place for five minutes and then raised slowly to prevent leakage of the tracer. A total volume of 4 μl of the retrograde tracer was used in each animal. Following injection, the incision was surgically closed and 10,000 u/kg of penicillin was given prophylactically. After having survived for 48 hours, the animals were deeply anesthetized with an overdose of thiopental and perfused transcardially with 125 ml of physiological saline containing 2,500 units of heparin, followed by 500 ml of 4% formalin in cacodylate buffer (at pH 7.2 and 4°C) and 100 ml of 10% sucrose in cacodylate buffer (at pH 7.2 and 4°C). After removal from the skull, the brains were dehydrated overnight at 4°C in a 30% solution of sucrose in cacodylate buffer (at pH 7.2). Frontal 50-mm-thick serial sections were cut on a cryostat (Jung 1800, Leica, Germany). Every second section was saved, mounted on a slide, and air-dried. Every 12th section was stained with cresyl violet. The preparations were studied in a fluorescent microscope Leica DMLS (Germany) equipped with a UV-filter system providing an excitation wavelength of 365 nm. The accuracy and extent of the injections were verified by means of (1) the cytoarchitectonic features of the rat cortex, described by Zilles [42], (2) a stereotaxic atlas of the rat cortex [41,42], and (3) observation of labeled neurons in the appropriate thalamic nuclei.

Morphometric analysis

The following parameters were the subject of our analysis: (1) the total number of retrogradely labeled neurons in each projection zone, (2) the total number of neurons in the claustrum, (3) the numerical density of labeled neurons in each projection zone, (4) the numerical density of all neurons in the claustrum, (5) the ratio of the total number of labeled neurons for each projection zone to the total number of neurons in the claustrum, (6) the ratio of the numerical density of labeled neurons to the numerical density of all neurons in the claustrum, (7) the ratio of the number of labeled neurons on the consecutive sections to all labeled neurons throughout the fluorescently labeled zone.

The optical dissector method was used to estimate the numerical density of labeled neurons. Every twelfth section was taken according to sampling protocol [3,39]. The edges of the grid, mounted in the ocular under 10x magnification were used for the localization of the consecutive reference areas. Each second test area (0.2 mm x 0.2 mm) was chosen manually by the systematic random manner within the claustral border. Under 100 x magnification, one reference area (0.08 mm x 0.08 mm) was taken within the chosen test area. The neurons were counted within the dissector of the height equal 0.01 mm (controlled by the digital microcator Mitutoyo ID-C112B (Japan).

A 100x oil-immersion objective with a numerical aperture of 1.4 was used. Only neurons with sharply delineated nuclei or perikarya were counted, respectively.

The optical fractionator method was applied to the same set of sections labeled with the fluorescent dye to obtain an unbiased total number of retrogradely labeled neurons according to the procedure described by Burwell and Amaral [3]. Both methods were used for the estimation of the total number and numerical density of all neurons in the claustrum using the set of sections stained with cresyl violet.

In order to evaluate the distribution of labeled cells throughout the rostro-caudal extent of the labeled zone in the claustrum on the consecutive sections, the percentage values of both section levels in relation to the claustral length and the labeled cells on the section in relation to all labeled cells were placed in the diagrams as an abscissa and ordinate, respectively.

Statistical analysis

The mean and the standard deviation were calculated for each parameter. All calculations were made in Excel 97 (Microsoft, USA). Statistical analysis was performed by means of the computer program Statistica for Windows (Statsoft; USA). ANOVA was used to compare the results obtained for the experimental groups. The differences between the contra- and ipsilateral sides were checked by a Wilcoxon paired test.

RESULTS

Injections into the motor cortex

FG was injected into the motor cortex in five rats (Fig. 1B, 2A). In the ipsilateral claustrum (Fig. 1A), labeled neurons were found throughout the whole anteroposterior extent of the nucleus (Fig. 1C, E). The highest number of labeled neurons was found in the anterior part of the claustrum (Fig. 2A). In the central part the number of labeled neurons decreased caudally and disappeared gradually towards the posterior part. The distribution of labeled neurons in the contralateral claustrum was similar to that on the ipsilateral side, but the number of labeled neurons was significantly lower.

The mean value of the total number of neurons in the claustrum of animals in the studied group was estimated as 360,857 ± 43,948 (Table 1). There was no statistically significant difference between the mean values of the total number of claustral neurons in the animals of both studied groups. The mean value of the total number of labeled neurons in the ipsilateral claustrum was 110,926 ± 27,361 neurons, which corresponds to 31 ± 6% of the total claustral neurons. The mean value of the numerical density of the retrogradely labeled neurons for this projection was 62,635 ± 7,144 neurons per cubic mm, which accounts for 55 ± 3% of the numerical density of all neurons in the dorsal claustrum.

In the contralateral claustrum, the total number of labeled neurons was 21,636 ± 9,066 on average, whereas the numerical density of labeled neurons was estimated as 35,370 ± 11,444 per cubic mm (Table 2). The values calculated for the contralateral projection differ significantly from the corresponding values for the ipsilateral one (Wilcoxon paired test, $p<0.05$).

Injections into the somatosensory cortex

FG was injected into the primary somatosensory cortex of five rats (Fig. 2B). Labeled neurons were found throughout the whole anteroposterior extent of both ipsilateral (Fig. 1D,F) and contralateral claustrum. The highest number of labeled neurons was found in the central part of the claustrum, decreasing gradually towards anterior and posterior directions (Fig. 2B). In general, the distribution pattern of labeled neurons in the contralateral claustrum was similar to that seen on the ipsilateral side, although the number of neurons was significantly lower.

The mean value of the total number of labeled neurons in the ipsilateral claustrum was 59,424 ± ± 16,112 (Table 1). The mean value of the total number of the claustral neurons in this group of animals amounted to 332,472 ± 37 176, consequently 18 ± ± 7% of the total claustral neurons were labeled after tracer injections into the somatosensory cortex. The mean value of the numerical density of retrogradely labeled neurons for this projection zone was 36,171 ± 5,962 neurons per cubic mm, which constituted 34 ± 6% of the numerical density of all neurons in the dorsal part of the claustrum. The values of the total number of labeled neurons as well as the numerical density of retrogradely labeled neurons differ significantly between the motor and somatosensory projections ($p<0.01$).

In the contralateral claustrum, the total number of labeled neurons was 22,376 ± 9,323 on average, whereas the numerical density of labeled neurons was estimated as 24,505 ± 3,878 per cubic mm (Table 2). Both values differ significantly from the corresponding values for the ipsilateral projection ($p<0.05$; Wilcoxon paired test).

Additionally in the contralateral claustrum no significant difference between the motor and somatosensory projections in both the total number of labeled neurons and their numerical density could be observed.

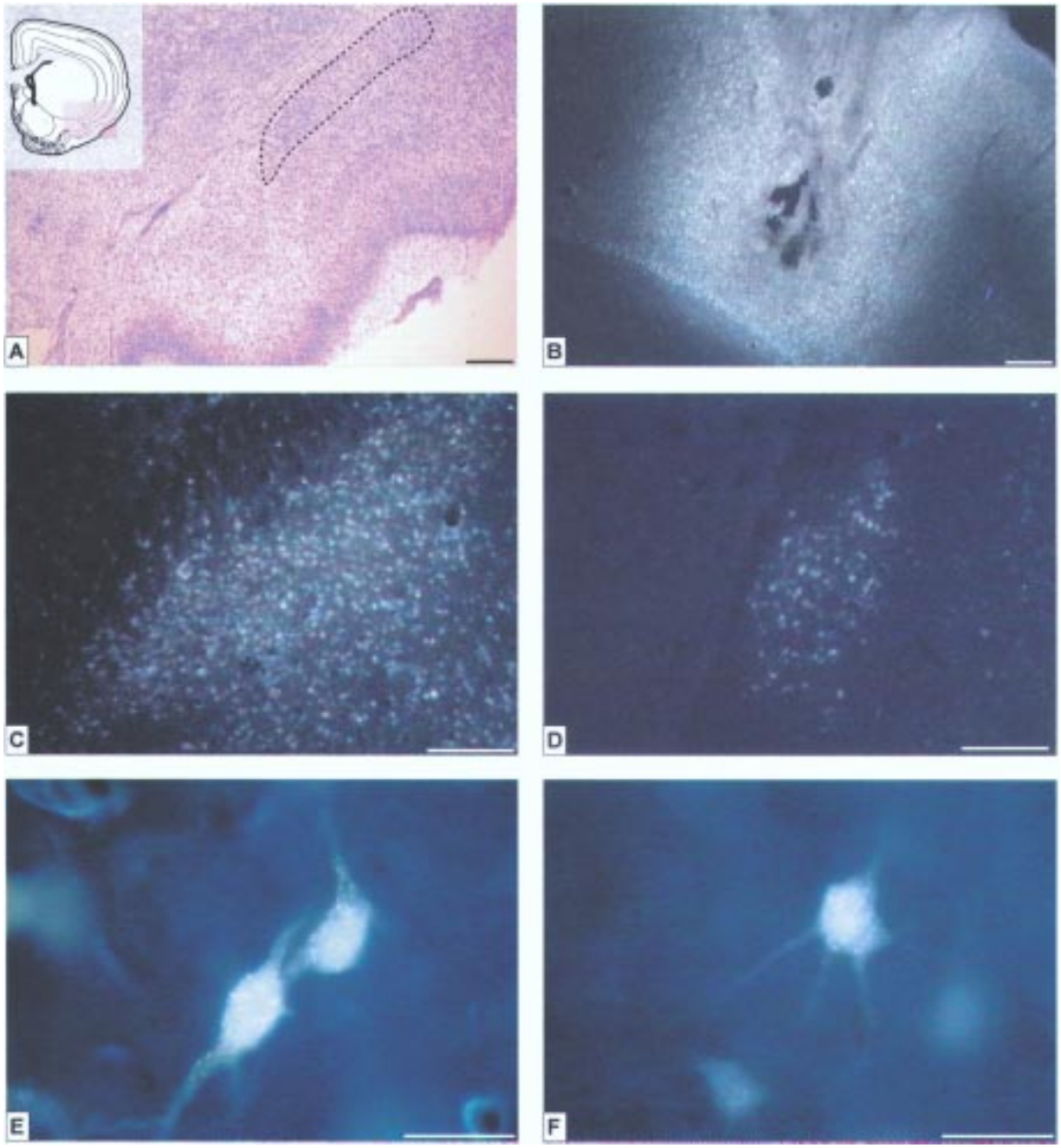

Figure 1. A) Cross-section through the anterior part of the rat claustrum (cresyl violet). B) Injection site into the motor cortex of the rat. C) Localization of the retrogradely labeled neurons in the anterior part of the claustrum after tracer (FG) injection into the motor cortex. D) Localization of projecting neurons of the somatosensory projection zone after tracer injection into the primary somatosensory cortex. E) Higher magnification of the labeled neurons of the motor projection zone. F) Higher magnification of the labeled neurons of the somatosensory projection zone. Scale bar — 200 μm (A-D); 25 μm (E,F).

DISCUSSION

Methodological considerations

The stereological approach to the study of the claustrocortical projections in the rat has not been applied yet. Before performing the present set of experiments, we tested various fluorescent tracers (including Fast Blue, Diamidino Yellow, Nuclear Yellow, Lucifer Yellow, and Fluoro-Gold) to find the one best suited to our purpose. It transpired that FG is superior to other tracers because it is easily soluble in water and evenly dispersed from the site of injec-

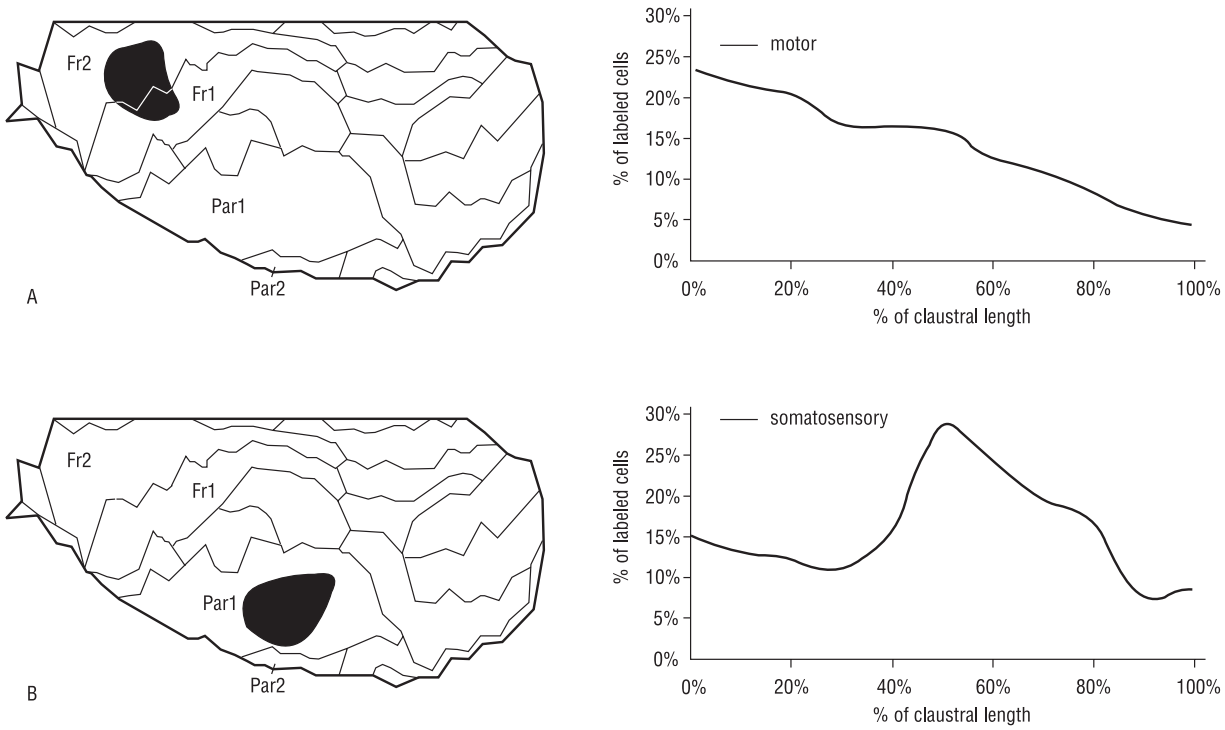

Figure 2. A) Schematic representation of the FG injection site into the motor cortex and distribution of the percentage of retrogradely labeled neurons in the anteroposterior direction of the claustrum for the motor projection in the representative case. B) Schematic representation of the FG injection site into the somatosensory cortex and distribution of the percentage of retrogradely labeled neurons in the anteroposterior direction of the claustrum for the somatosensory projection in the representative case. Abbreviations: Fr1, Fr2 — motor and premotor cortical areas, Par1, Par2 — primary and secondary somatosensory cortical areas.

Table 1. The total number and numerical density of labeled neurons in the claustrum on the ipsilateral side for the individual cases

Localization of injections	Total number of labeled neurons [N]	Total number of claustral neurons [N]	% of total number of neurons labeled with FG [%]	Numerical density of labeled neurons [N/mm^3]	Numerical density of claustral neurons [N/mm^3]	% of numerical density of neurons labeled with FG [N/mm^3]
Motor cortex						
R 45	112,675	352,765	32	63,290	112,965	56
R 46	102,720	417,600	25	61,143	114,474	53
R 47	87,840	343,680	26	55,455	105,294	53
R 48	156,960	387,840	40	74,318	122,424	61
R 49	94,435	302,400	31	58,967	108,621	54
Mean	**110,926 ± 27,361**	**360,857 ± 43,948**	**31 ± 6**	**62,635 ± 7,144**	**112,756 ± 6,505**	**55 ± 3**
Somatosensory cortex						
R 50	51,360	337,920	15	32,424	103,529	31
R 51	57,120	371,520	15	31,316	110,571	28
R 52	71,040	275,520	26	37,949	102,500	37
R 53	79,200	320,634	25	45,833	105,347	44
R 54	38,400	356,764	11	33,333	106,500	31
Mean	**59,424 ± 16,112**	**332,472 ± 37,176**	**18 ± 7**	**36,171 ± 5,962**	**105,690 ± 3,140**	**34 ± 6**

Table 2. The total number and numerical density of labeled neurons in the contralateral claustrum for the individual cases

Localization of injections	Total number of labeled neurons [N]	% of total number of neurons labeled with FG (%)	Numerical density of labeled neurons [N/mm^3]	% of numerical density of neurons labeled with FG [N/mm^3]
Motor cortex				
R 45	16,379	3	27,975	20
R 46	12,000	9	22,727	48
R 47	29,280	9	50,833	35
R 48	33,120	5	43,125	25
R 49	17,400	6	32,189	30
Mean	**21,636 ± 9,066**	**6 ± 2**	**35,370 ± 11,444**	**32 ± 11**
Somatosensory cortex				
R 50	25,074	7	19,427	19
R 51	17,684	5	29,447	27
R 52	24,960	9	24,762	24
R 53	34,560	11	26,667	25
R 54	9,600	3	22,222	21
Mean	**22,376 ± 9,323**	**7 ± 3**	**24,505 ± 3,878**	**23 ± 3**

tion. It is efficiently transported in the axons, it does not spread out of an axon, and its leakage from the neurons during histological processing is minimal [35]. Moreover, the great advantage of FG in morphometric analysis lies in the pattern of neuronal labeling. FG produces bright yellow labeling of perikarya and initial segments of dendrites, rendering identification of neurons easy, even when they contain only a small amount of the tracer.

Many factors may have an impact on the efficiency of retrograde transport and the number of labeled neurons. Some of them, including anesthetic protocol, concentration of fluorescent tracer, perfusion protocol, and histological procedure are relatively easy to control. Therefore, we maintained the same standards in all experiments in an attempt to make quantitative results as repeatable as possible. However, other factors cannot be controlled. Brain edema, tissue damage, and uneven tracer distribution in a target area may result in some differences in the number of labeled neurons after injections with similar amounts of tracer. These uncontrollable factors limit the evaluation of morphometric data and only reveal general tendencies and relationships.

The comparable volumes of the fluorescent retrograde tracer Fluoro-Gold (FG) were injected into the motor or somatosensory cortical fields in two groups of animals. A morphometric analysis was performed to determine the number of retrogradely labeled neurons in the claustrum. Due to the relatively small size of the rat brain a relatively large part of the cortical field under study could be covered with the fluorescent tracer, which should result in labeling of a considerable number of the claustral neurons projecting to this area.

Distribution and differences in the number of claustral neurons projecting to the motor and somatosensory areas

The claustral projection into the studied neocortical area shows two common features: (1) the presence of neurons projecting to the selected cortical area along the entire length of the claustrum and (2) domination of each part of the claustrum by projections to the different cortical fields. Neurons sending their axons to the motor cortex prevail in the anterior part of the claustrum, whereas the projection to the somatosensory cortex predominates in its central part.

Differences in the total number of neurons labeled after injection into the selected cortical fields indicate that claustral output to the studied cortical areas is not equal. The number of neurons labeled after injections into the motor cortex was significantly higher than the number of neurons labeled after injections of similar volume targeted into the somatosensory cortical field. The injections into the motor cortex resulted in an average labeling of 31 ± 6% of the claustral neurons, whereas the comparable

volumes of the tracer injected into the somatosensory cortex (which is about twice as large as the motor cortex) produced an average labeling of 18±7% of total neurons. These values do not provide an answer to the question about the whole number of projecting neurons into the motor or somatosensory cortices. This would require injection of the tracer exactly into the 100% of the cortex area of one distinguished cytoarchitectonic type, which is technically very difficult to achieve. However, the equal volume of the injected tracer and the repetitive conditions of the set of experiments enable us to suspect the unequal character of the claustral projection into the motor and somatosensory cortices. To confirm our statement, the numerical density of neurons projecting into the claustrum was studied in both projection zones. This parameter seems to be more independent of the volume of the infiltrated cortex and more closely associated with the structure of the projecting zone. Also comparison of the values of numerical density of labeled neurons for both studied projection zones reveals a statistically significant difference among them.

So far, there have been no quantitative data regarding the intensity of the claustrocortical connections. Sloniewski et al. [36] reported a similar rostrocaudal arrangement of claustroneocortical projections in the rat. However, they did not establish sharp borders between peaks of projection to motor, somatosensory, and visual cortical fields. This could result from differences in counting labeled neurons in the section. Sloniewski and colleagues [36] counted the total number of labeled neurons, whereas in this study we estimated the ratio of labeled neurons (FG) to all neurons (cresyl violet) in the section. However, what should be taken into account is that the total number of neurons in the cross-section of the claustrum diminishes from the anterior to the posterior part, and that the claustral projection zone is characterized by a percentage distribution of the labeled cells.

The anteroposterior arrangement of the claustrocortical projections in the rat [30] resembles the topography found in the cat and monkey. However, in the claustrum of carnivores and primates, the rostrocaudal extent of neuronal groups related to particular cortical areas appears to be much more confined [7,16,17,20,37]. As has been reported by various authors in the cat and monkey, the injection of fluorescent tracers or horseradish peroxidase into the motor or somatosensory areas resulted in labeling of neurons in the anterior two-thirds of the claustral length, with substantial overlap between these two populations [5,7,17,29,37].

The claustrum projects bilaterally to the motor and somatosensory areas. Neurons sending axons to all of these fields are present along the entire length of the claustrum, but there is a striking difference in the size of this projection: the number of claustral neurons projecting contralaterally is significantly lower than that projecting ipsilaterally, regardless of the target cortical field. In the light of these findings, the claustral impact on the contralateral hemisphere appears to be significantly weaker than on the ipsilateral one. The contralateral projections in the rat were also described by Sloniewski et al. [36] and Minciacchi et al. [21]. A multiple retrograde tracer study, made by Minciachi et al. [21] showed that some claustral neurons send axons to both ipsilateral and contralateral hemispheres, which indicates that the claustrum can simultaneously influence both hemispheres.

Electrical stimulation of the cortex results in the excitation of claustral neurons [15,27,34]. The character of the descending corticoclaustral projection has been confirmed in the electron microscopy studies. It was found that axons of cortical neurons make the asymmetric excitatory synapses on claustral neurons [9,12,13,25,26]. It was also found that glutamate is a putative neurotransmitter of these corticoclaustral connections [1]. Electrical stimulation of the claustrum results in the inhibition of cortical neurons [19,31-33], which suggests that corticoclaustral connections create a negative feedback mechanism. The inhibitory activity of the claustrocortical loop is regulated probably by a small number of GABA-ergic interneurons [6,24].

The numerous and topographically organized claustrocortical connections situate the claustrum in the position of a modulatory structure of the cortical activity, due to its recurrent inhibitory projection. Differences in the intensity of claustral projections into various neocortical areas suggest that the claustrum may have a stronger influence on the motor than on the somatosensory cortex. The significant prevalence of the ipsilateral over the contralateral connections confirms the role of the claustrum more as a satellite, than the relay structure upon the cerebral cortex.

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