Morphology and laminar distribution of cortico-claustral neurons in different areas of the rabbit cerebral cortex


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SUMMARY

This study examines the morphology and laminar distribution of cortico-claustral neurons that were retrogradely labelled after single injections of biotinylated dextran amine in the caudal two thirds of the rabbit insular claustrum. Labelled neurons were studied in neocortical areas 17, 18, and A2, insular cortex and cingulate areas 24a,b and 29b,c,d. Most of the labelled neurons lay in layer VI for area 17 and in layer V for the remaining areas. Most of the labelled neurons were either normally oriented pyramidal cells or inverted neurons (spiny neurons with their major dendritic shaft oriented towards the white matter). Labelled inverted neurons were 85% for area 17 and under 30% for other isocortical areas. For the allocortex, they were less than 20%. For all areas, labelled inverted neurons lay chiefly in layer VI and normally oriented pyramidal neurons in layer V. The findings show that while neurons with inverted morphology are a minor but consistent source for axonal projections from the cortex to the claustrum, they are paramount in area 17. The findings also show that the laminar distribution of cortico-claustral cells is also area-specific.

Key Words: BDA - Dorsal claustrum - Nerve cells - Pyramidal - Polymorphic - Inverted - Spiny.

INTRODUCTION

The claustrum is a narrow grey shell underneath the cerebral pallium. Latero-medially, the claustrum is localized between the insular cortex and the putamen. Tiny sheets of white matter—the extreme and external capsules—separate the claustrum from these structures. The claustrum is divided into a dorsal and a ventral part: the insular and endopryiform claustra, respectively. The claustrum is connected with many cortical areas but its function is not fully understood. Most of these connections are reciprocal. Although the claustral territories for these connections partially overlap, it is possible to chart maps of cortico-related zones within the claustrum. The endopryiform claustrum is connected with the entorhinal cortex, parasubiculum, subiculum and parts of the amygdala in rats (Krettek and Price, 1977a; 1977b; Sherk, 1986), cats (Krettek and Price, 1977a; 1977b; Sherk, 1986; Witter et al., 1988) and monkeys (Pearson et al., 1982). The connections with high-order unimodal and polymodal sensory areas are distributed between the dorsal and ventral parts of the insular claustrum in cats (Sherk, 1986; Witter et al., 1988; Clascá et al., 1992) and monkeys (Pearson et al., 1982). In cats (LeVay and Sherk, 1981; Sherk, 1986; Witter et al., 1988; Clascá et al., 1992) and rabbits (Carman et al., 1964; Müller-Paschinger and Tömöl, 1989; Kowianski et al., 1996; 1998; Gutiérrez-Ibarluzea, 1998) the con-
nection with motor and unimodal sensory cortices tends to accumulate in the dorsal part of the insular claustrum, whereas the ventral part of the insular claustrum is connected with cingulate and other cortices. The cortico-related territories overlap even more in the rat claustrum (Minciacchi et al., 1985; Sadowski et al., 1997). The extensive connections with the cortex together with recent PET data (Hadjikhani and Roland, 1998) suggest that the claustrum may play a role in data processing between cortical areas.

Corticofugal pathways in mammals arise mostly from pyramidal neurons. These cells can be classified according to their efferent targets, which often correlate with their radial location in the cortex. Pyramidal neurons that lie in granular and supragranular layers form cortico-cortical connections. In contrast, infragranular pyramidal cells may project either to subcortical centres or to the cortex (reviewed in Jones, 1984). The morphology of a pyramidal cell class and its efferent target can also be correlated. For example, in layer VI of the cat visual cortex, the pyramidal cells that project to the claustrum differ from those that project to the lateral geniculate nucleus (Katz, 1987); in layer V of the rat visual cortex the callosal pyramidal neurons differ from those that project to the superior colliculus or the pons (Hallman et al., 1988; Hübener and Bolz, 1988). These anatomical differences may involve a specific combination of cell size, dendritic pattern, and local axonal collaterals.

Although pyramidal neurons are the principal morphologic type of projection neuron in the cortex, inverted neurons—that is, cells with their major dendritic shaft oriented towards the white matter—also project their axon out of the cortical grey matter. These neurons are spiny cells that lie preferentially in the infragranular layers; in contrast to other spiny cells of the infragranular layers, the inverted neurons characteristically furnish the cortex and other telencephalic centres (Bueno-López et al., 1991; Reblet et al., 1992).

The present study attempts to describe the morphology and laminar distribution across cortical areas of the neurons that furnish the insular claustrum. We chose the rabbit as a model because it has a more developed claustrum than the rat. The rabbit claustrum is comparable to that of the cat, dog, tree shrew, cow, sheep and goat (Sherk, 1986) and is classified within type II claustra (Sherk, 1986; Kowianski et al., 1999).

**Materials and Methods**

This study was performed on 6 healthy young-adult New Zealand rabbits (weight ≈2000 g). Animals were treated in accordance with European laws for experimental animal care (86/609/EEC). Each animal received an injection of biotinylated dextran amine (BDA) in the insular claustrum and the tissue was subsequently processed.

**Surgery**

Rabbits were anaesthetised using ketamine (35 mg/kg) and xylazine (5 mg/kg) and placed in a stereotaxic frame. The antero-posterior, medio-lateral and dorso-ventral coordinates for the injection were determined from a stereotaxic atlas (Shek et al., 1986) and pilot studies, and computed relative to bregma (with bregma and lambda in the same horizontal plane). The final stereotaxic coordinates for the injection were as follows: antero-posterior, 4 mm; medio-lateral, 8 mm; dorso-ventral, 4.5 mm (measured from skull surface). In order to administer the tracer injection to the claustrum, a small hole was made in the skull and a glass micropipette (5-10 μm in diameter) attached to a holder in the stereotaxic frame was passed through a buttonhole made in the dura mater and then through the underlying somatosensory cortex. BDA (Sigma), 7.5% in 0.1M phosphate buffer, pH 7.4, was injected iontophotically by passing positive, direct current (10μA) through the micropipette in pulses (7 sec on, 7 sec off) for 30 min.

**BDA immunobistochemical detection**

Fifteen days after surgery, rabbits were given an overdose of anaesthetic and perfused through the left cardiac ventricle with physiological saline serum followed by a fixative solution of 2% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, and 1% glutaraldehyde in 0.1M phosphate buffer, pH 7.4. The excess of aldehydes was subsequently washed out with phosphate buffer. Brains were then excised, cut into blocks and stored at 4°C in 30% phosphate-buffered sucrose for 24-48 hours. Serial sections (50 μm thick) were then obtained on a freezing microtome. The avidin-biotin-peroxidase method (ABC Elite Kit, Vector) was used for the demonstration of BDA in the sections (Rajakumar et al., 1993). Afterwards, sections were treated with 1% diaminobenzidine di-hydrochloride (DAB) for visualisation of peroxidase. Sections were then transferred to a fresh DAB solution containing H₂O₂. The BDA end product was intensified with 1% CoCl₂.

For each brain, sections were divided into two series. The first section of each pair was lightly counterstained with toluidine blue in order to determine the location of the injection site and the layer distribution of the cortico-claustral cells. Finally, sections were mounted on gelatinised slides, air-dried and coverslipped with DPX.
Data analysis

Sections were examined with a Nikon Optiphot II microscope, equipped with a drawing tube and Nomarski optics. The stage of the microscope was attached to a computer equipped with the Neuron Tracing System program (Eutectic Electronics, Raleigh, NC, USA), which facilitated both the differential and the numerical analyses of the cells. We first made outlines of the sections and drew 20 equally thick strata from the pia to the white matter in the cortex to facilitate cell counting. The strata were correlated with the cortical layers as identified in the Nissl-counterstained sections. We then plotted all the retrogradely-labelled neurons observed. In the drawings we counted only those neurons whose somata and dendrites were filled up with BDA end product in order to determine the morphology of cortico-claustral cells. Since there was no significant difference between the data obtained from each animal, the results were pooled (percentage test, $P = 0.05$, $Z = 2$).

RESULTS

General description

Tracer injections were localised to the caudal two-thirds of the insular claustrum (Fig. 1). Following these injections we identified retrogradely-labelled neurons and anterogradely-labelled axon terminals scattered throughout the cortex, including somatosensory and motor areas but not primary auditory area A1. Because the injection pipette crossed the somatosensory cortex on its way to the claustrum, the motor and somatosensory areas were excluded from this study in order to avoid false positive results due to tracer contamination of these areas. Retrogradely-labelled neurons were then studied in occipital visual areas 17 and 18, temporal secondary auditory area A2, insular cortex and ipsilateral cingulate areas 24a,b and 29b,c,d. In the other hemisphere, retrogradely-labelled neurons were only observed in area 29d. The area nomenclature of this study follows Bueno-López et al. (1991). The density of the cortical labelling was congruent with the dorso-ventral localisation of the tracer injection in the insular claustrum. Thus, the more ventrally placed the injection, the denser the labelling in secondary auditory area A2, cingulate and insular cortex.

As a group, the retrogradely-labelled neurons were seen to be a mixture of typically and inversely oriented cells whose somata was localised to layers V and VI in all areas but for a few neurons that were observed in layer II/III in the cingulate cortex. Except for area 17 (see below), most of the retrogradely-labelled neurons were typically oriented pyramidal cells, which were observed chiefly in layer V. The remaining retrogradely-labelled neurons were inverted neurons and were also found in layer V, although preferentially in layer VI (Figs. 2 and 3).

Fig. 1. Microphotographs of a BDA injection in the insular claustrum. (A) Low magnification. (B) Same injection as in (A) but magnified 15 times to show claustral cells and axons labelled next to the injection site. Bar = 100 μm.
Retrogradely-labelled cells with typically oriented morphology were pyramidal neurons with rounded or triangular somata (25–40 μm thick) and an apical dendrite ascending towards the pia. While ascending, this apical dendrite characteristically tapered to an end in layer I but sometimes in layer III. This apical dendrite was poorly branched. All the dendrites of labelled pyramidal neurons were spiny. As described in Bueno-López et al. (1991), inverted neurons form a heterogeneous category of spiny cells whose common feature is a major dendritic system oriented in the direction of the white matter. In brief, this cell category includes not only the inverted pyramids but also Cajal’s triangular, fusiform, stellate and other polymorphic or atypically oriented cells whose descending dendritic shaft is thicker than any other dendritic shaft of the same cell. Examples of all of these subtypes of inverted neurons were labelled following the injections of BDA in the insular claustrum. The correlation observed between the areal location and cell morphology of cortico-claustral cells across cortical areas is detailed in Table 1 and Fig. 4 and in the sections below.
Visual cortex
In area 17, cortico-claustral cells were retrogradely-labelled in layer Vb, but particularly in layer VIa (81%). Between eight-nine out of every ten of these labelled cells were inverted neurons. In layer V, almost half of the labelled cells were inverted neurons. In layer VI, inverted neurons were paramount (94%). The remaining labelled cells of area 17 were typically oriented pyramidal cells.

In area 18, the labelled cells were distributed almost equally between layer V (56%) and layer VI. One out four of these labelled cells was an inverted neuron. In layer V, inverted neurons constituted only 6% of the labelled cells. This incidence was 50% in layer VI.

Auditory cortex
No cortico-claustral cell was retrogradely-labelled in the primary auditory cortex. As in area 18, these labelled cells were distributed almost equally between layers V and VI in A2; slightly more in layer V. Three out of ten labelled cells were inverted neurons in A2. In this area, and again as in area 18, inverted neurons constituted 6% of the labelled cells in layer V, and 54% in layer VI.

Table 1. Cortico-claustral projection cells as retrogradely labelled following BDA injections in the insular claustrum. Cingulate corresponds to the addition of areas 24a,b and 29b,c,d. Inv indicates spiny neurons with a major dendritic shaft oriented towards the white matter (inverted neurons).

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**Area 17**

**Area 18**

**A2**

**Insular cortex**

**Cingulate cortex**

**Fig. 4.** Histograms showing the laminar distribution of cortico-claustral cells and the incidence of typically oriented pyramidal cells (light bars) and inverted neurons (black bars). Cingulate corresponds to the addition of areas 24a,b and 29b,c,d. Number of cells is shown in Table 1.

**Insular cortex**

Of the cortico-claustral neurons that were retrogradely labelled in the insular cortex, 59% were observed in layer V. The incidence of inverted neurons among these labelled cells decreased to only two out of ten cells. Inverted neurons were 4% in layer V and 29% in layer VI.

**Cingulate cortex**

In areas 24a,b and 29b,c,d of the cingulate cortex, a very few cortico-claustral cells were retrogradely labelled in layer II/III in addition to the cells labelled characteristically in layers V-VI. Of all these retrogradely-labelled cells, 62% were counted in layer V. The incidence of inverted
neurons among retrogradely-labelled cells was lowest in the cingulate cortex: only one out of ten labelled cells was an inverted neuron. No inverted neurons were found among the cortico-cortical cells that were labelled in layer II/III. The incidence of inverted neurons was 2.5% for layer V and 30% for layer VI.

**Discussion**

**Laminar distribution of cortico-cortical cells**

In the present study, BDA was injected in the caudal two-thirds of the insular cortex in order to trace retrogradely the laminar and areal distribution of cortico-cortical projection cells. Given its narrowness and curvature, it is difficult to administer tracer injections to the claustrum without contaminating the surrounding structures. This difficulty has certainly appeared in cortico-claustral projection cells. Axonal connections between the claustrum and cortex have been studied previously by injecting anterograde and retrograde tracers but mostly in the cortex (Minciacchi et al., 1985; Sherk, 1986; Kowianski et al., 1996; 1998; Sadowski et al., 1997). In the present study, cortico-claustral projection cells were part of layers V-VI for all labelled areas except for the cingulate cortex, where a few cortico-claustral cells were also labelled in layer II/III. In rabbits, the projection from the cingulate gyrus has been reported to arise from cells of layers V and VI (Bassett and Berger, 1981). In the cat, the input to the claustrum from prefrontal, secondary visual and secondary auditory areas is furnished mostly by layer VI cells (LeVay and Sherk, 1981; Clască et al., 1992), with a small contribution from cells situated deep in layers III and V (LeVay and Sherk, 1981). After careful observation of sections encompassing the injections of BDA, we believe that the structures surrounding the claustrum were not contaminated by any possible leak of the tracer. However, tracer leaking along the pipette track might be the cause of the supragranular labelling referred to above in this study and in previous studies (Bueno-López et al., 1991; LeVay and Sherk, 1981). As stated in the Results section, this was the reason for excluding the labelling observed in somatosensory and motor areas. For area 17, the overwhelming majority of the cortico-claustral cells was observed in layer VI in our study. This is consistent with previous studies in monkeys (Carey et al., 1980), cats (Olson and Graybiel, 1980; LeVay and Sherk, 1981; Reale and Imig, 1983; Katz, 1987) and rabbits (Bueno-López et al., 1991). As with monkeys (Pearson et al., 1982), cats (Reale and Imig, 1983), and rabbits (Bueno-López et al., 1991), no labelled cells were found in the primary auditory cortex.

Taken together, our findings show that the laminar distribution of cortico-claustral neurons is area-specific. Thus, in the distribution of cortico-claustral cells between layers V and VI, the prevalence of layer V decreased gradually from the insular cortex to areas A2, 18 and 17, and abruptly from the cingulate cortex to area 17. This decrease may reflect a gradient in the laminar distribution of cortico-claustral cells either along the dorso-ventral dimension of the cortex, or just from the allo- to neocortex, or both. Differences along the cortex have been described for a number of cell features, including the numerical density of neurons (Beaulieu, 1993) and the distribution of chemically-defined subtypes of neurons (Kondo et al., 1994).

**Morphology of cortico-claustral cells**

Another aim of the present study was to identify distinct morphologic types of cortico-claustral projection neurons. Our findings show that at least two categories of cortico-claustral cells were labelled among the cortical cells furnishing the claustrum, i.e., typically oriented pyramidal cells and inverted neurons. These findings confirm and extend previous observations after WGA-HRP injections in the claustrum (Bueno-López et al., 1991). Because of its sensitivity, WGA-HRP is widely used for the labelling of retrograde and anterograde axonal connections. A single injection of 0.02 μl of WGA-HRP can retrogradely label thousands of cells. The dendritic anatomy of WGA-HRP-labelled neurons, however, is not fully visible because of the defective filling of the dendritic branches and spines with the end product of the HRP reaction. BDA is a very sensitive tracer that is used mostly to demonstrate anterograde projections. However, BDA is also taken up by the axon terminals next to the tracer injection and then transported retrogradely to the parent cell. Following this retrograde transport, BDA overcomes the faulty dendritic repletion that is characteristic of HRP procedures and shows up the dendritic secondary branches, and the spines, of the projection cell almost completely. Another advantage of the use of BDA is the small size of the iontophoretic injection needed for tracer uptake. One disadvantage of the use of BDA is the low number of retrogradely labelled cells that this tracer yields in comparison with WGA-HRP and other massive retrograde tracers.

Typically oriented pyramidal cells have been reported as the main source for projections to the claustrum in the cat visual cortex (LeVay and Sherk, 1981; Katz, 1987; Clască et al., 1992) and the rabbit cortex (Bueno-López et al., 1991). In layer VI of cat area 17, these cells were described as pyramidal cells that are morphologically indistinguishable from those that project to the lateral geniculate nucleus, except that they seem to lack axon collaterals to layer IV (LeVay and
Sherk, 1981). Layer VI neurons, with a thick and long, oblique basal dendrite and a thin and tapering apical dendrite, furnish the claustrum in cat visual cortex (Katz, 1987). Other authors have described cortico-claustral neurons in areas of the cat cerebral cortex other than the visual area as small pyramidal or fusiform cells always situated in the upper part of layer VI (Clasca et al., 1992). Following inspection of cortico-claustral cells in the Figures of LeVay and Sherk (1981), Katz’s (1987) and Clasca et al. (1992), we believe that at least some of them may be inverted neurons.

**Correlation between the morphology and cortical location of cortico-claustral cells**

Our findings show that not only the laminar distribution but also the morphology of cortico-claustral cells is area-specific. For the cortico-claustral cells of layer VI, inverted neurons constituted the majority of the cells in area 17, half of the cells in the remaining neocortical areas and only one in three cells in the cingulate and insular cortices. For the cortico-claustral cells of layer V, inverted neurons were 50% in area 17 and no more than 7% in all other areas. Hence, an increase in the incidence of inverted cells among cortico-claustral cells was observed not only from layer V to VI but also again from the allocortex to the neocortex. In particular, area 17 was unique in the incidence of inverted neurons among cortico-claustral cells. This is consistent with a WGA-HRP study, in which 83% of the cortico-claustral cells and 7.5% of the cortico-cortical cells were observed to be inverted neurons for the infragranular layers of area 17 (Bueno-López et al., 1991). In contrast, and as also reported in the same study, 25% of the neurons projecting either to the claustrum or to the cortex but for the infragranular layers of areas 18 and A2 were inverted. When counted in Nissl preparations, inverted neurons are less than 15% of the total number of infragranular neurons in area 17 and 8.5% in area 18 (Bueno-López et al., 1991). In neocortical areas, inverted cells projecting to cortical areas lie chiefly at the border between layers V and VI, while inverted cells furnishing the claustrum do so mainly throughout layer Vla (Bueno-López et al., 1991). Although this by no means implies that layer VI cells project only to the claustrum or other cortical areas, inverted cells do not project to the pons (Bueno-López et al., 1991) or to the relay thalamic nucleus (Bueno-López et al., 1991; Einstein, 1996).

**Concluding remarks**

Supragranular pyramidal neurons are primarily involved in the transmission of information to other cortical areas, while infragranular pyramidal neurons are the main elements conveying information to subcortical structures (Jones, 1984). In addition to the supragranular pyramidal neurons, many classic pyramidal cells and inverted neurons in infragranular layers nevertheless convey information from associative and secondary to primary sensory areas (Bueno-López et al., 1991; Reblet et al., 1992). There, a particular subtype of pyramidal cell of layer VI projects its axon back to the relay nuclei of the thalamus. This feed-back pathway has recently been proposed as the way by which top-down signals may convey stored information to interact with bottom-up (data driven) signals to interpret sensory processes (Tomita et al., 1999). Although inverted neurons are involved in the projections from the infragranular layers of associative and secondary sensory areas to primary sensory areas, they do not furnish the sensory-specific thalamic nuclei (Bueno-López et al., 1991; Einstein, 1996). The area-specific incidence of inverted neurons among the cortico-claustral projection cells found in the present study suggests that this type of neuron may play an important role in transferring the cortical feedback signals, but to the claustrum rather than to the thalamus. The exception is the primary auditory cortex. This lacked any type of cortico-claustral neuron.

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