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Cortical Hierarchy Governs Rat Claustrocortical Circuit Organization

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Abstract

The claustrum is a telencephalic gray matter structure with various proposed functions, including sensory integration and attentional allocation. Underlying these concepts is the reciprocal connectivity of the claustrum with most, if not all, areas of the cortex. What remains to be elucidated to inform functional hypotheses further is whether a pattern exists in the strength of connectivity between a given cortical area and the claustrum. To this end, we performed a series of retrograde neuronal tract tracer injections into rat cortical areas along the cortical processing hierarchy, from primary sensory and motor to frontal cortices. We observed that the number of claustralcortical projections increased as a function of processing hierarchy; claustrum neurons projecting to primary sensory cortices were scant and restricted in distribution across the claustrum, whereas neurons projecting to the cingulate cortex were densely packed and more evenly distributed throughout the claustrum. This connectivity pattern suggests that the claustrum may preferentially subserve executive functions orchestrated by the cingulate cortex.

Keywords

claustrum; cingulate cortex; connectivity; retrograde; anterograde; RGD_737903; AB_10013220; AB_2314408; AB_2314412; AB_477329; AB_10000344; AB_10000345; AB_2313584; AB_2340602; AB_2341099; AB_2340806; AB_2340428; AB_2340433; AB_2337244; nif-0000-00110

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The claustrum is a prominent telencephalic structure lying medial to the insula and lateral to the striatum. It is reciprocally connected with many cortical areas (Riche and Lanoir, 1978; Sherk, 1986; Park et al., 2012; Torgerson et al., 2015), and this anatomical configuration has inspired two major functional hypotheses. The first hypothesis is that the claustrum functions as a multisensory integrator (Ettlinger and Wilson, 1990; Hadjikhani and Roland, 1998; Crick and Koch, 2005), whereas the second hypothesis states that the claustrum is a relay for cortical information subserving attentional allocation (Mathur, 2008, 2014; Goll et al., 2015). These two hypotheses predict two different patterns of claustrrocortical connectivity. For instance, the sensory integration hypothesis, which proposes that the claustrum integrates sensory information to form conscious percepts, predicts strong connectivity between sensory and motor cortices and the claustrum. In contrast, the attentional allocation hypothesis, which proposes that the claustrum transmits salient information to/from sensory association and executive cortices necessary for attention allocation, predicts that areas important for attention, such as the cingulate cortices, would be preferentially connected with the claustrum.

Our current understanding of claustrrocortical circuits is based on an accumulation of evidence from studies that were conducted in different species and that targeted only one of a few cortical sites in nonhuman primates, cats, or rats (Sanides and Buchholz, 1979; Olson and Graybiel, 1980; Irvine and Brugge, 1980; Levay and Sherk, 1981; Macchi et al., 1981; Pearson et al., 1982; Macchi et al., 1983; Shameem et al., 1984; Carey and Neal, 1985; Minciacchi et al., 1985; Adinolfi and Levine, 1986; Sloniewski et al., 1986; Druga et al., 1990; Baizer et al., 1997; Sadowski et al., 1997; Reser et al., 2014). Thus, an assessment of how the claustrum differentially connects with various cortical areas in a single species is required. Moreover, previous studies of claustral efferents have used overly inclusive definitions of the borders of the claustrum that led to the false assignment of retrogradely labeled cells that actually originate from insular cortex to the claustrum, thus limiting the interpretation of these studies (see Van de Werd and Uylings, 2008; Mathur et al., 2009). To address these issues, we have systematically examined the intraclaustral localization of cells projecting to cortical areas spanning the entire cortical hierarchy from primary sensory subdivisions to frontal cortical sites in the rat and defined claustral borders based on parvalbumin (PV) immunoreactivity, which is isomorphic with the expression pattern of the rodent claustral marker GNG2 (Mathur et al., 2009). The GNG2-based definition of the claustrum is corroborated by publicly available mouse gene expression data from the Allen Institute for Brain Science and human GNG2 protein expression (Lein et al., 2007; Pirone et al., 2012; Allen Mouse Brain Atlas, <http://mouse.brain-map.org>).

MATERIALS AND METHODS

Subjects

Thirty adult (8–24 weeks old) male Sprague-Dawley rats (Harlan, Indianapolis, IN; RRID:RGD_737903) weighing 300–400 g were group housed with food and water available ad libitum on a 12-hour light–dark cycle, with lights on at 0700. All studies were performed in accordance with the National Institutes of Health *Guide for care and use of laboratory*

animals and under the oversight of the Vanderbilt University and University of Maryland Animal Care and Use Committees.

Tract tracing

Rats were deeply anesthetized with isoflurane and placed in a stereotaxic frame. For retrograde tracing from individual brain regions, a 3% solution of Fluorogold (FG; Fluorochrome LLC, Englewood, CO) was iontophoretically deposited through glass pipettes with tip diameters of 20–30 μm , using pulsed +1.5 μA current 7 seconds on/off for 15 minutes); in two other rats, FG was delivered using +5.0 μA current. In other cases, 100–300 nl of a 3% FG solution, a 1% solution of cholera toxin B subunit (CTb), or a 1% CTb solution conjugated to AlexaFluor-555 (CTb-555; Molecular Probes, Carlsbad, CA) was pressure injected. A summary of the cortical areas targeted, the modes of tracer delivery, and the tracers used can be found in Table 1. For double retrograde injections, four rats received either 200-nl injections of FG and CTb or 200-nl injections of CTb-555 and CTb conjugated to AlexaFluor-488 (CTb-488; Molecular Probes) into two separate cortical locations. An additional three rats received an anterograde/retrograde double injection, which consisted of a volume of 200 nl of a 15% solution of 10,000 MW biotinylated dextran amine (BDA; Life Technologies, Grand Island, NY) pressure injected into the left anterior cingulate cortex (ACC) and 200 nl of FG or CTb-488 pressure injected into the right ACC.

Histochemistry and immunohistochemistry

Rats were transcardially perfused with room-temperature 0.1 M sodium phosphate buffer, pH 7.3, followed by ice-cold 4% paraformaldehyde in phosphate buffer. Brains were postfixed in 4% paraformaldehyde overnight and cryoprotected in 0.1 M phosphate buffer containing 30% sucrose. Frozen coronal sections were cut through the forebrain at 40–50 μm thickness. Sections were immediately used for histology or were stored at -20°C in a solution of 0.1 M phosphate buffer containing 30% sucrose and 30% ethylene glycol. Conventional or immunofluorescence protocols were used to reveal the localization of anterograde/retrograde tracers or proteins of interest, following previously described methods (Deutch et al., 1996). For animals receiving nonconjugated CTb injections, a goat anti-CTb antibody (List Biological, Campbell, CA; RRID:AB_10013220) was used. For animals receiving FG injections, two rabbit anti-FG antibodies were used, purchased from Fluorochrome LLC (RRID:AB_2314408) or EMD Millipore (Billerica, MA; RRID:AB_2314412). Three different antibodies against PV generated from different species were used: mouse (Sigma-Aldrich, St. Louis, MO; RRID:AB_477329), rabbit (SWant, Bellinzona, Switzerland; RRID: AB_10000344), or goat (SWant; RRID:AB_10000345). Information regarding the primary antibodies is listed in Table 2. Secondary antibodies generated in donkey and conjugated with fluorophores from Jackson ImmunoResearch (West Grove, PA) were used for immunofluorescence: anti-rabbit conjugated with AlexaFluor-488 (RRID:AB_2313584) or AMCA (RRID:AB_2340602) for FG and PV immunofluorescence, anti-mouse conjugated with AlexaFluor-488 (RRID:AB_2341099) or AMCA (RRID:AB_2340806) for PV immunofluorescence, and anti-goat conjugated with AlexaFluor-488 (RRID:AB_2340428) or AlexaFluor-594 (RRID:AB_2340433) for PV immunofluorescence. For animals receiving BDA anterograde tracer injections, a

streptavidin protein conjugated with Cy3 (Jackson ImmunoResearch; RRID:AB_2337244) was used at a 1:1,000 dilution.

Antibody characterization

The mouse anti-PV antibody from Sigma-Aldrich (catalog No. 3088) generated against purified frog muscle PV recognizes a protein of ~12 kDa on immunoblots and stains cortical interneurons with the characteristic morphology and localization of PV-containing interneurons (Martin et al., 2007); according to the manufacturer, this antibody does not recognize other members of the EF-hand calcium binding protein family. Both the rabbit and the goat anti-PV antibodies (SWant. Nos. PV 25 and PVG-214, respectively) were generated against PV purified from rat muscles (Kägi et al., 1987; Schwaller et al., 1999), recognize a single band of ~12 kDa in immunoblots (Celio and Heizmann, 1981; Schwaller et al., 1999), show no immunolabeling after preadsorption of the antibody with recombinant rat PV, and do not stain cells in PV knockout mice (SWant). Immunolabeling for injected CTb is completely blocked by preadsorption of excess cholera toxin B subunit (Stocker et al., 2006), and no CTb-labeled cells are seen in animals that have not received CTb injections (Pakan et al., 2008). Similarly, we did not observe any FG-like immunostaining when using the rabbit anti-FG antibody in animals not injected with FG (data not shown), and the cells labeled by the FG antibody also displayed their endogenous fluorescence when viewed with UV illumination. In parallel immunohistochemical reactions performed in the absence of each primary antibody used in this study resulted in a complete lack of immunostaining for the corresponding antigen.

Data analysis

The structural boundaries of the claustrum were defined by dense PV-immunoreactive (-ir) neuropil of the claustrum, which is isomorphic with the expression pattern of the claustrum-restricted protein GNG2, as previously described (Mathur et al., 2009). This anatomical definition of the claustrum excludes areas rostral to the genu of the corpus callosum and is consistent with the observed retrograde labeling from all cortical sites. All other structures are in reference to Paxinos and Watson (2007). Cells were charted in NeuroLucida (MicroBright-Field, Williston, VT; RRID:nif-0000-00110). The numbers of cells within the borders of the claustrum at three anteroposterior levels were then determined: 1) rostral claustrum at the level of the genu of the corpus callosum, 2) a midclaustrum level defined as roughly equidistant between the genu of the corpus callosum and the decussation of the anterior commissure, and 3) caudal claustrum at the level of the decussation of the anterior commissure. We also counted the numbers of retrogradely labeled cells between the PV-ir-defined claustrum and the pial surface. We restricted our analysis of retrogradely labeled cells in the claustrum ipsilateral to the retrograde tracer deposit because in all areas examined the number of retrogradely labeled cells was considerably greater in the ipsilateral claustrum. Photomicrographs were pseudocolored and were adjusted for brightness and contrast in Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

RESULTS

Retrogradely labeled cells in the claustrum arising from cortical tracer deposits were charted at rostral, middle, and caudal levels of the claustrum, which are defined by the PV-ir neuropil (Fig. 1A).

Primary sensory and motor cortices

Motor cortex (M1/M2)—FG deposits into M1 or M1/M2 just lateral to the cingulate cortex involved all cortical layers. Retrogradely labeled cell bodies were consistently present in the dorsolateral portion of the claustrum at all anteroposterior levels, with the number of FG-ir somata remaining relatively constant across the rostrocaudal extent of the claustrum (Fig. 1B,C). The retrogradely labeled cells were often located at the border between claustrum and surrounding insular cortex. Only small numbers of cells were present in the insula.

Visual cortex (V1/V2)—An FG injection into primary visual cortex involved V1 with relatively minor invasion into neighboring V2 (Fig. 1D). FG-ir cell bodies were scattered across the dorsal half of the claustrum at all anteroposterior levels, with a few FG-ir cells seen in ventral portions of the claustrum. Similarly, retrogradely labeled cells from a FG deposit into V2, which did not extend into V1 (Fig. 1E), were mostly observed in the dorsal claustrum. However, in contrast to the pattern of labeling observed after V1 injection, labeled cells from the V2 deposit were largely restricted to the posterior claustrum.

Somatosensory cortex (S1)—An FG deposit into S1 involved primarily the forelimb region of the somatosensory cortex, with some lateral extension into the barrelfield cortex (Fig. 1F). Retrogradely labeled cells were observed in the dorsal half of the claustrum at all levels, although labeling was more ventral at midclaustrum levels.

Auditory cortex (A1)—An FG injection into A1 cortex resulted in a discrete deposit of the tracer through all layers of A1, with only minor involvement of the surrounding secondary auditory cortex (Fig. 1G). Retrogradely labeled cells were restricted mainly to dorsal aspects of the claustrum, and labeling was less dense than for other sensory cortices.

Association cortices

Lateral parietal association cortex (LPA)—An FG deposit into the LPA spanned the entire cortical depth (Fig. 2A). FG-ir cells were localized to the dorsal half of the claustrum at anterior and posterior levels, although a few cells were noted slightly more ventrally. At midclaustrum levels, FG-ir cells were localized more ventrally relative to FG-ir cells at anterior and posterior portions of the claustrum.

Prelimbic and infralimbic cortices—Iontophoretic injection of FG into the dorsal prelimbic cortex (area 32), with slight invasion of the penumbra dorsally into the pregenual ACC (area 24b), resulted in FG-ir cells distributed evenly throughout the dorsoventral and mediolateral extent of the anterior pole of the claustrum (Fig. 2B). However, FG-ir cells were generally restricted to the ventrolateral claustrum at posterior levels. An iontophoretic

injection of FG into infralimbic cortex (area 25) with slight invasion dorsally into prelimbic cortex (area 32) resulted in sparse labeling of FG-ir cells in ventral portions of the claustrum at all rostrocaudal levels (Fig. 2C).

Anterior cingulate cortex (ACC)—CTb injection resulted in a tracer deposit that largely involved the anterior pregenual cingulate cortex, with some possible extension into the M2 motor cortex (see Fig. 3A); the deposit spanned layers I–V. Retrogradely labeled cells were nearly evenly distributed across the dorsoventral as well as anteroposterior extent of the claustrum, with the exception of the extreme ventral aspect of the posterior claustrum, where only few cells were evident. A more posteriorly targeted injection of FG into the most posterior aspect of the pregenual cingulate cortex (see Fig. 3B) resulted in an even and particularly dense distribution of FG-ir cells across all axes of the claustrum, with the possible exception of the dorsalmost tip of the structure at its posterior pole. In all cases in which tracer was deposited into the ACC, including the supragenual and pregenual cortices, retrogradely labeled cells were also observed just outside the dorsal and lateral borders of the claustrum, in the insular cortex.

Middle and posterior cingulate cortices—An injection of CTb into the cingulate cortex at the level of the genu of the corpus callosum (see Fig. 3C) spanned all layers of the midcingulate cortex at mid-striatal levels. Retrogradely labeled cells were distributed across the entire claustrum and notably dense at all anteroposterior levels examined. Another CTb injection at the level of the crossing of the anterior commissure (see Fig. 3D) resulted in retrogradely labeled cells across the entire claustrum, with the density of retrograde labeling uniform over the anteroposterior extent of the claustrum.

Retrosplenial cortex—For two rats, FG was deposited in the region of the retrosplenial cortex. In the first rat, the deposit was localized to the lateral dysgranular retrosplenial cortex (area 29d) without any significant invasion of medially adjacent area 29c but with a small extension of the penumbra laterally (Fig. 3E). In the second rat, the FG deposit involved the areas 29d and 29c, with extension into the V2 (Fig. 3F). FG-ir cells were observed throughout the body of the claustrum in both cases, with only the dorsal one-third of the anterior pole of claustrum lacking labeled cells, and the retrograde labeling in the claustrum was notably dense for the second rat (Fig. 3F, Table 3).

Dual retrograde tracer deposits into cortex

The relatively unrestricted distribution of retrogradely labeled cells in the claustrum following tracer deposit into cingulate cortices suggests that the claustral representations of these sites overlap with each other and with the segregated distributions of all other cortical sites that we examined. This overlap raises the possibility that claustral cells collateralize to innervate multiple aspects of cingulate cortices and/or multiple cortical sites. To test this, we first deposited different retrograde tracers into each of two distal sites along the cingulate cortices. In one rat, CTb-488 was deposited into pregenual cingulate cortex and CTb-555 was deposited into the lateral retrosplenial dysgranular cortex (Fig. 4A,B; see Supporting Figures for magenta-green copies of dual retrograde tracer deposits). As expected, immunofluorescence revealed retrogradely labeled cells positive for CTb-488 and CTb-555

that were uniformly distributed in the claustrum (Fig. 4C). However, among the observed retrogradely labeled claustrum cells, very few were double-labeled (Fig. 4D), suggesting that claustrum projection neurons do not collateralize to innervate different aspects of cingulate cortices. For another rat, CTb was deposited into pregenual cingulate cortex and FG was deposited into lateral retrosplenial dysgranular cortex. As with the first rat, we observed uniformly distributed retrogradely labeled cells in the claustrum, and very few were double labeled (4.1%). (For this rat, a reconstruction of single- and double-labeled cells as well as a tabulation of single- and double-labeled cells inside and outside the claustrum across all three anteroposterior levels of the claustrum can be found in Figure 6A and Table 4, respectively.)

Next, we deposited retrograde tracers into a cingulate cortex site and a cortical site with a restricted representation in the claustrum. Specifically, in one rat we deposited CTb-488 into the pregenual cingulate and CTb-555 into M1 (Fig. 5A,B, respectively). As expected, immunofluorescence revealed retrogradely labeled cells positive for CTb throughout the claustrum, whereas cells positive for FG were less prevalent and restricted to the dorsal claustrum (Fig. 5C). Among the observed retrogradely labeled claustrum cells, very few were double labeled (Fig. 5D), further suggesting that claustrum projection neurons rarely collateralize to innervate multiple cortical targets. For another rat, CTb was deposited into the pregenual cingulate and FG was deposited into M1. As with the first rat, very few of the retrogradely labeled cells were double labeled (0.7%). (For this rat, a reconstruction of single- and double-labeled cells as well as a tabulation of single- and double-labeled cells inside and outside the claustrum across all three anteroposterior levels of the claustrum can be found in Fig. 6B and Table 4, respectively).

Dual anterograde/retrograde tracer deposits into cingulate cortex

To determine whether the preferential relationship between the cingulate cortex and the claustrum observed is reciprocal and to determine the distribution of possible cinguloclaustal fibers, we deposited the anterograde tracer BDA and the retrograde tracer CTb-488 into the left and right cingulate slightly caudal to the genu of the corpus callosum, respectively, in two rats. Injections involved all layers of cingulate cortex (Fig. 7A,B), which is shown for a representative case. In the left claustrum, there was sparse labeling of axons and cell bodies (Fig. 7C); however, for the right claustrum, we observed a relatively dense plexus of anterogradely labeled axons, which overlapped with retrogradely labeled cells (Fig. 7D). Thus, projections from the cingulate to the claustrum are reciprocal and originate primarily from the contralateral hemisphere, whereas claustral projections target cortical areas of the ipsilateral hemisphere. This same pair of injections was performed in an additional rat, but FG was used as the retrograde tracer, and slight invasion laterally into M2 was observed for both tracers. Similar results were observed for this case (data not shown).

DISCUSSION

We found that claustral projections to most cortical regions, including sensory, motor, and certain association cortices, originate within discrete zones of the claustrum, consistent with previous reports. In contrast, claustral cells retrogradely labeled from the prelimbic

prefrontal cortex (area 32) were more widely distributed. Surprisingly, claustral cells projecting to the cingulate cortex were numerous and noticeably more dispersed throughout the claustrum, and this pattern was observed across the entire anteroposterior extent of the cingulate (i.e., from pregenual cingulate to retrosplenial cortex). We have summarized this connectivity pattern diagrammatically in Figure 8.

Despite the numerous claustrum projections to cingulate cortex that we observed, very few of these projections target multiple aspects of the cingulate or motor cortex, as evidenced by our double retrograde tracer injections. Finally, we observed dense reciprocal innervation of the claustrum from the cingulate, which preferentially targeted the contralateral claustrum, contrasting with claustrum projections to cingulate, which were primarily ipsilateral. This reciprocal connectivity pattern has been observed with other cortical areas and the claustrum (Alloway et al., 2009).

Importantly, this study is the first, to our knowledge, to examine claustrrocortical connectivity comprehensively across the cortical hierarchy and to use a nonarbitrary definition of the claustrum to examine claustral projections. Specifically, we defined the claustrum as the circumscribed, PV-enriched area of neuropil, which we have previously shown to be isomorphic with the claustral marker GNG2 (Mathur et al., 2009).

Claustral projections to “lower” order cortical regions originate from discrete zones within the claustrum without any noticeable topography

In agreement with previous studies of claustrrocortical projections, we found that retrograde tracer deposits into most cortical areas labeled cells clustered in relatively discrete zones of the claustrum (Olson and Graybiel, 1980; Levay and Sherk, 1981; Mufson and Mesulam, 1982; Pearson et al., 1982; Sherk, 1986; Li et al., 1986; Sadowski et al., 1997; Tanné-Gariépy et al., 2002). Although such a subregional pattern of localization of retrogradely labeled cells often suggests a topographical organization of projections, we were unable to discern any topography of claustrrocortical projections. Our inability to uncover a clear topographical organization of claustral projections onto cortical regions is consistent with previous studies in cat, monkey, and rat (Druga et al., 1982; Pearson et al., 1982; Carey and Neal, 1985), whereas other reports have suggested that a loose topographical order of claustr-cortical projections exists in cat (Macchi et al., 1981, 1983) and rat (Li et al., 1986; Sloniewski and Pilgram, 1984; Sadowski et al., 1997; Smith and Alloway, 2010). However, claims of topographical organization of claustrrocortical projections were often based on the patterns of retrograde labeling from only a few (two or three) cortical sites, not across the many sites examined in this study.

Claustral projections to the cingulate are numerous and distributed throughout the claustrum

Several reports have briefly commented on the presence of claustral projections to the rodent cingulate cortex. Li et al. (1986) found that claustral labeling after posterior cingulate injections of the rat was not dispersed across the entire claustrum but concentrated in the ventral half of the claustrum. Condé et al. (1995) and Hoover and Vertes (2007) both noted claustral inputs to the pregenual ACC of the rat, but the resolution of the chartings in these

studies was too low to discern the applied anatomical definition of the claustrum or whether neurons in discrete sectors of the claustrum, or the entire structure, give rise to the anterior cingulate projection. Vogt and Miller (1983) mentioned label in the dorsal part of the posterior claustrum after horseradish peroxidase injection into the posterior cingulate cortex, although Finch et al. (1984) offered very low-resolution chartings suggesting that there is a similar degree of retrograde labeling in the claustrum after horseradish peroxidase injections of the anterior and posterior cingulate. Van Groen and Wyss (1992) found that the claustrum projects to the retrosplenial cortex of the rat, but the pattern of claustral cells that innervated the retrosplenial cortex was not specified. To our knowledge, this is the first detailed study on claustral projections to the cingulate cortex. In contrast to the restricted intraclaustral distributions of retrogradely labeled projections to most cortical areas, we found that retrogradely labeled projections to, or immediately lateral to, cingulate cortex were distributed across the entire claustrum. This unrestricted distribution was observed across the anteroposterior extent of the cingulate cortex.

Reciprocal inputs from the cingulate are distributed throughout the claustrum and are predominantly contralateral

We observed dense terminal labeling in the contralateral claustrum after depositing anterograde tracers into cingulate cortex and labeling, albeit weaker, in the ipsilateral claustrum. The terminal labeling overlapped with the distribution of retrogradely labeled cells in the claustrum following retrograde tracer deposits into the cingulate of the opposite hemisphere. Thus, the preferential relationship between claustrum and cingulate appears to be reciprocal. Moreover, a specific pattern is apparent in which the cingulate preferentially targets the contralateral claustrum, whereas the claustrum preferentially targets the ipsilateral cingulate. This bias of cortical afferents to the contralateral claustrum and claustrum afferents to ipsilateral cortex has been observed by others for both the rat and the cat (Norita, 1977; Olson and Graybiel, 1980; Squatrito et al., 1980; Li et al., 1986; Colechio and Alloway, 2009; Smith and Alloway, 2010; Smith et al., 2012) but has yet to be substantiated in primates.

Functional implications

The function of the claustrum has remained enigmatic despite over a century of speculations and hypotheses. It has been proposed that the claustrum is a locus for multisensory integration (Ettinger and Wilson, 1990; Hadjikhani and Roland, 1998), possibly for the generation of conscious percepts (Crick and Koch, 2005), leading to the prediction that individual sensory representations in the claustrum would be numerous and exhibit substantial overlap. However, our anatomical data show that projections to individual sensory and motor cortices from the claustrum are relatively weak and that their distributions exhibit minimal, if any, overlap. This organization is in sharp contrast to claustral cells that innervate the cingulate cortex, which are dense and broadly distributed across the claustrum. Given the recent observation of excitatory intraclaustrum signaling (Orman, 2015) coupled with the finding that claustrum neurons fire selectively to salient sensory stimuli (Specter et al., 1974; Remedios et al., 2010), it is possible that cingulate cortices exert “top-down” control over other cortical areas via the claustrum, thus allocating attention to a given sensory modality (Mathur, 2008; Mathur, 2014). This allocation could, at the least, be

supported by contiguous expanses of claustrum, such as the “puddle” region (Baizer et al., 2014; Johnson et al., 2014). Such an arrangement would allow the claustrum to support higher order cognitive processes, such as attention and attentional control over actions (Mathur, 2008; Mathur, 2014), which are known functions of anterior portions of cingulate cortex (Muir et al., 1996; Shima and Tanji, 1998; Botvinick, 2007; Johnson et al., 2007; Hayden et al., 2011). Posterior regions of cingulate, such as retrosplenial cortex, on the other hand, are thought to process spatial information and support navigation. This is substantiated by the findings that the retrosplenial cortex possesses head-direction cells (Chen et al., 1994; Cho and Sharp, 2001) and that navigation using allocentric (external, spatial landmarks) and egocentric (internal, self-motion) cues are both disrupted following lesions to retrosplenial cortex (Cooper and Mizumori, 1999; Harker and Whishaw, 2002; Pothuizen et al., 2008). Taken together, the claustrum might be subserving multiple higher order cognitive functions.

The interhemispheric “all-access” relationship of the cingulate cortex and claustrum that we observed suggests that the claustrum itself might not be integrating sensory information but instead functioning as a component of a broader saliency detection network subserving higher order functions (e.g., attention) that involve widely distributed brain regions that include a large majority of the cortical mantle. Such a relationship has been hinted at with respect to the ferret claustrum and visual regions; parietal cortex is preferentially connected with the claustrum relative to primary and secondary visual cortices (Patzke et al., 2014). At the least, the striking claustrum-wide distribution and density of projections to cingulate observed in this study suggest that the claustrum supports cingulate cortex functionality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LITERATURE CITED

- Adinolfi AM, Levine MS. An autoradiographic study of the postnatal development of pericruciate cortical projections to the neostriatum and the claustrum of the cat. *Brain Res.* 1986; 389:99–108. [PubMed: 3948021]
- Alloway KD, Smith JB, Beauchemin KJ, Olson ML. Bilateral projections from rat MI whisker cortex to the neostriatum, thalamus, and claustrum: forebrain circuits for modulating whisking behavior. *J Comp Neurol.* 2009; 515:548–564. [PubMed: 19479997]
- Amiez C, Joseph JP, Procyk E. Anterior cingulate error-related activity is modulated by predicted reward. *Eur J Neurosci.* 2005; 21:3447–3452. [PubMed: 16026482]
- Baizer JS, Lock TM, Youakim M. Projections from the claustrum to the prelunate gyrus in the monkey. *Exp Brain Res.* 1997; 113:564–568. [PubMed: 9108221]

- Baizer JS, Sherwood CC, Noonan M, Hof PR. Comparative organization of the claustrum: what does structure tell us about function? *Front Sys Neurosci.* 2014; 8:117. doi: 10.3389/fnsys.2014.00117.
- Carey RG, Neal TL. The rat claustrum: afferent and efferent connections with visual cortex. *Brain Res.* 1985; 329:185–193. [PubMed: 3978440]
- Celio MR, Heizmann CW. Calcium-binding protein parvalbumin as a neuronal marker. *Nature.* 1981; 293:300–302. [PubMed: 7278987]
- Chen LL, Lin LH, Green EJ, Barnes CA, McNaughton BL. Head-direction cells in the rat posterior cortex. I. Anatomical distribution and behavioral modulation. *Exp Brain Res.* 1994; 101:8–23. [PubMed: 7843305]
- Cho J, Sharp PE. Head direction, place, and movement correlates for cells in the rat retrosplenial cortex. *Behav Neurosci.* 2001; 115:3–25. [PubMed: 11256450]
- Colechio EM, Alloway KD. Differential topography of the bilateral cortical projections to the whisker and forepaw regions in rat motor cortex. *Brain Struct Funct.* 2009; 213:423–439. [PubMed: 19672624]
- Condé F, Maire-Lepoivre E, Audinat E, Crépel F. Afferent connections of the medial frontal cortex of the rat. II. Cortical and subcortical afferents. *J Comp Neurol.* 1995; 352:567–593. [PubMed: 7722001]
- Cooper BG, Mizumori SJ. Retrosplenial cortex inactivation selectively impairs navigation in darkness. *Neuroreport.* 1999; 10:625–630. [PubMed: 10208601]
- Corbetta M, Miezin FM, Dobmeyer S, Shulman GL, Petersen SE. Selective and divided attention during visual discriminations of shape, color, and speed: functional anatomy by positron emission tomography. *J Neurosci.* 1991; 11:2383–2402. [PubMed: 1869921]
- Crick FC, Koch C. What is the function of the claustrum? *Philos Trans R Soc Lond B Biol Sci.* 2005; 360:1271–1279. [PubMed: 16147522]
- Deutch AY, Lewis DA, Whitehead RE, Elsworth JD, Iadarola MJ, Redmond DE Jr, Roth RH. Effects of D2 dopamine receptor antagonists on Fos protein expression in the striatal complex and entorhinal cortex of the nonhuman primate. *Synapse.* 1996; 23:182–191. [PubMed: 8807746]
- Druga R. Claustrum-neocortical connections in the cat and rat demonstrated by HRP tracing technique. *J Hirnforsch.* 1982; 23:191–202. [PubMed: 7108198]
- Druga R, Rokyta R, Benes V Jr. Claustrum-neocortical projections in the rhesus monkey (projections to area 6). *J Hirnforsch.* 1990; 31:487–494. [PubMed: 1701467]
- Ettlinger DJ, Wilson WA. Cross-modal performance: behavioral processes, phylogenetic considerations and neural mechanisms. *Behav Brain Res.* 1990; 40:169–192. [PubMed: 2178346]
- Finch DM, Derian EL, Babb TL. Afferent fibers to rat cingulate cortex. *Exp Neurol.* 1984; 83:468–485. [PubMed: 6199226]
- Goll Y, Atlan G, Citri A. Attention: the claustrum. *Trends Neurosci.* 2015; 38:486–495. [PubMed: 26116988]
- Hadjikhani N, Roland PE. Cross-modal transfer of information between the tactile and the visual representations in the human brain: a positron emission tomographic study. *J Neurosci.* 1998; 18:1072–1084. [PubMed: 9437027]
- Harker KT, Whishaw IQ. Impaired spatial performance in rats with retrosplenial lesions: Importance of the spatial problem and the rat strain in identifying lesion effects in a swimming pool. *J Neurosci.* 2002; 22:1155–1164. [PubMed: 11826144]
- Hoover WB, Vertes RP. Anatomical analysis of afferent projections to the medial frontal cortex in the rat. *Brain Struct Funct.* 2007; 212:149–179. [PubMed: 17717690]
- Irvine DR, Brugge JF. Afferent and efferent connections between the claustrum and parietal association cortex in cat: a horseradish peroxidase and autoradiographic study. *Neurosci Lett.* 1980; 20:5–10. [PubMed: 7052548]
- Johnson JI, Fenske BA, Jaswa AS, Morris JA. Exploitation of puddles for breakthroughs in claustrum research. *Front Syst Neurosci.* 2014; 8:78. doi: 10.3389/fnsys.2014.00078. [PubMed: 24860441]
- Kägi U, Berchtold MW, Heizmann CW. Ca²⁺-binding parvalbumin in rat testis. Characterization, localization, and expression during development. *J Biol Chem.* 1987; 262:7314–7320. [PubMed: 3294830]

- Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski MS, Brockway KS, Byrnes EJ, Chen L, Chen L, Chen TM, Chin MC, Chong J, Crook BE, Czaplinska A, Dang CN, Datta S, Dee NR, Desaki AL, Desta T, Diep E, Dolbeare TA, Donelan MJ, Dong HW, Dougherty JG, Duncan BJ, Ebbert AJ, Eichele G, Estin LK, Faber C, Facer BA, Fields R, Fischer SR, Fliiss TP, Frensley C, Gates SN, Glattfelder KJ, Halverson KR, Hart MR, Hohmann JG, Howell MP, Jeung DP, Johnson RA, Karr PT, Kawal R, Kidney JM, Knapik RH, Kuan CL, Lake JH, Laramée AR, Larsen KD, Lau C, Lemon TA, Liang AJ, Liu Y, Luong LT, Michaels J, Morgan JJ, Morgan RJ, Mortrud MT, Mosqueda NF, Ng LL, Ng R, Orta GJ, Overly CC, Pak TH, Parry SE, Pathak SD, Pearson OC, Puchalski RB, Riley ZL, Rockett HR, Rowland SA, Royall JJ, Ruiz MJ, Sarno NR, Schaffnit K, Shapovalova NV, Sivisay T, Slaughterbeck CR, Smith SC, Smith KA, Smith BI, Sodd AJ, Stewart NN, Stumpf KR, Sunkin SM, Sutram M, Tam A, Teemer CD, Thaller C, Thompson CL, Varnam LR, Visel A, Whitlock RM, Wohnoutka PE, Wolkey CK, Wong VY, Wood M, Yaylaoglu MB, Young RC, Youngstrom BL, Yuan XF, Zhang B, Zwingman TA, Jones AR. Genome-wide atlas of gene expression in the adult mouse brain. *Nature*. 2007; 445:168–176. [PubMed: 17151600]
- LeVay S, Sherk H. The visual claustrum of the cat. I. Structure and connections. *J Neurosci*. 1981; 9:956–980. [PubMed: 6169810]
- Li ZK, Takada M, Hattori T. Topographic organization and collateralization of claustrorocortical projections in the rat. *Brain Res Bull*. 1986; 17:529–532. [PubMed: 3779451]
- Macchi G, Bentivoglio M, Minciacchi D, Molinari M. The organization of the claustroneocortical projections in the cat studied by means of the HRP retrograde axonal transport. *J Comp Neurol*. 1981; 195:681–695. [PubMed: 6161950]
- Macchi G, Bentivoglio M, Minciacchi D, Molinari M. Claustroneocortical projections studied in the cat by means of multiple retrograde fluorescent tracing. *J Comp Neurol*. 1983; 215:121–134. [PubMed: 6853768]
- Martin LJ, Liu Z, Chen K, Price AC, Pan Y, Swaby JA, Golden WC. Motor neuron degeneration in amyotrophic lateral sclerosis mutant superoxide dismutase-1 transgenic mice: mechanisms of mitochondriopathy and cell death. *J Comp Neurol*. 2007; 500:20–46. [PubMed: 17099894]
- Mathur, BN. Vanderbilt University, PhD dissertation, Neuroscience. 2008. Unraveling the seat of consciousness: anatomical redefinition and molecular characterization of the claustrum.
- Mathur BN. The claustrum in review. *Front Syst Neurosci*. 2014; 8:48. [PubMed: 24772070]
- Mathur BN, Caprioli RM, Deutch AY. Proteomic analysis illuminates a novel structural definition of the claustrum and insula. *Cereb Cortex*. 2009; 19:2372–2379. [PubMed: 19168664]
- Minciacchi D, Granato A, Barbaresi P. Organization of claustrorocortical projections to the primary somatosensory area of primates. *Brain Res*. 1991; 553:309–312. [PubMed: 1718545]
- Mufson EJ, Mesulam MM. Insula of the old world monkey. II: Afferent cortical input and comments on the claustrum. *J Comp Neurol*. 1982; 212:23–37. [PubMed: 7174906]
- Norita M. Demonstration of bilateral claustrorocortical connections in the cat with the method of retrograde axonal transport of horseradish peroxidase. *Arch Histol Jpn*. 1977; 40:1–10. [PubMed: 69423]
- Olson CR, Graybiel AM. Sensory maps in the claustrum of the cat. *Nature*. 1980; 288:479–481. [PubMed: 7442793]
- Orman R. Claustrum: a case for directional, excitatory, intrinsic connectivity in the rat. *J Physiol Sci*. 2015 doi: 10.1007/s12576-015-0391-6.
- Pakan JM, Graham DJ, Iwaniuk AN, Wylie DR. Differential projections from the vestibular nuclei to the flocculus and uvula-nodulus in pigeons (*Columba livia*). *J Comp Neurol*. 2008; 508:402–417. [PubMed: 18335537]
- Pardo JV, Pardo PJ, Janer KW, Raichle ME. The anterior cingulate cortex mediates processing selection in the Stroop attentional conflict paradigm. *Proc Natl Acad Sci U S A*. 1990; 87:256–259. [PubMed: 2296583]
- Park S, Tyszka JM, Allman JM. The claustrum and insula in *Microcebus murinus*: a high resolution diffusion imaging study. *Front Neuroanat*. 2012; 6:21. doi: 10.3389/fnana.2012.00021. [PubMed: 22707933]

- Passetti F, Chudasama Y, Robbins TW. The frontal cortex of the rat and visual attentional performance: dissociable functions of distinct medial prefrontal subregions. *Cereb Cortex*. 2002; 12:1254–1268. [PubMed: 12427677]
- Patzke N, Innocenti GM, Manger PR. The claustrum of the ferret: afferent and efferent connections to lower and higher order visual cortical areas. *Front Syst Neurosci*. 2014; 8:31. [PubMed: 24616671]
- Paxinos, G., Watson, C. The rat brain in stereotaxic coordinates. Elsevier; London: 2007.
- Pearson RC, Brodal P, Gatter KC, Powell TP. The organization of the connections between the cortex and the claustrum in the monkey. *Brain Res*. 1982; 234:435–441. [PubMed: 6800568]
- Pirone A, Cozzi B, Edelstein L, Peruffo A, Lenzi C, Quilici F, Antonini R, Castagna M. Topography of Gng2- and netrinG2-expression suggests an insular origin of the human claustrum. *PLoS One*. 2012; 7:e44745. doi: 10.1371/journal.pone.0044745. [PubMed: 22957104]
- Pothuizen HH, Aggleton JP, Vann SD. Do rats with retrosplenial cortex lesions lack direction? *Eur J Neurosci*. 2008; 28:2486–2498. [PubMed: 19032585]
- Procyk E, Tanaka YL, Joseph JP. Anterior cingulate activity during routine and non-routine sequential behaviors in macaques. *Nat Neurosci*. 2000; 3:502–508. [PubMed: 10769392]
- Quilodran R, Rothé M, Procyk E. Behavioral shifts and action valuation in the anterior cingulate cortex. *Neuron*. 2008; 57:314–325. [PubMed: 18215627]
- Remedios R, Logothetis NK, Kayser C. Unimodal responses prevail within the multisensory claustrum. *J Neurosci*. 2010; 30:12902–12907. [PubMed: 20881109]
- Reser DH, Richardson KE, Montibeller MO, Zhao S, Chan JM, Soares JG, Chaplin TA, Gattass R, Rosa MG. Claustrum projections to prefrontal cortex in the capuchin monkey (*Cebus paella*). *Front Syst Neurosci*. 2014; 8:123. doi: 10.3389/fnsys.2014.00123. [PubMed: 25071475]
- Riche D, Lanot J. Some claustrum-cortical connections in the cat and baboon as studied by retrograde horseradish peroxidase transport. *J Comp Neurol*. 1978; 177:435–444. [PubMed: 412882]
- Sadowski M, Morys J, Jakubowska-Sadowska K, Narkiewicz O. Rat's claustrum shows two main cortico-related zones. *Brain Res*. 1997; 756:147–152. [PubMed: 9187325]
- Sanides D, Buchholz CS. Identification of the projection from the visual cortex to the claustrum by anterograde axonal transport in the cat. *Exp Brain Res*. 1979; 34:197–200. [PubMed: 83241]
- Schwaller B, Brückner G, Celio MR, Härtig W. A polyclonal goat antiserum against the calcium-binding protein calretinin is a versatile tool for various immunochemical techniques. *J Neurosci Methods*. 1999; 92:137–144. [PubMed: 10595711]
- Shameem N, Sanderson K, Dreher B. Claustral afferents to the rat's visual cortex. *Neurosci Lett*. 1984; 49:247–252. [PubMed: 6493606]
- Sherk, H. The claustrum and the cerebral cortex. In: Jones, EG., Peters, A., editors. *Cerebral cortex*. 5th ed. Plenum Press; New York: 1986. p. 467-499.
- Shima K, Tanji J. Role for cingulate motor area cells in voluntary movement selection based on reward. *Science*. 1998; 282:1335–1338. [PubMed: 9812901]
- Sloniewski P, Pilgram C. Claustrum-neocortical connections in the rat as demonstrated by retrograde tracing with Lucifer yellow. *Neurosci Lett*. 1984; 49:29–32. [PubMed: 6436755]
- Sloniewski P, Usunoff KG, Pilgram CH. Retrograde transport of fluorescent tracers reveals extensive ipsi- and contralateral claustrum-cortical connections in the rat. *J Comp Neurol*. 1986; 246:467–477. [PubMed: 2422230]
- Smith JB, Alloway KD. Functional specificity of claustrum connections in the rat: interhemispheric communication between specific parts of motor cortex. *J Neurosci*. 2010
- Smith JB, Alloway KD. Interhemispheric claustral circuits coordinate sensory and motor cortical areas that regulate exploratory behaviors. *Front Syst Neurosci*. 2014; 19 doi: 10.3389/fnsys.2014.0009330:16832-16844.
- Smith JB, Radhakrishnan H, Alloway KD. Rat claustrum coordinates but does not integrate somatosensory and motor cortical information. *J Neurosci*. 2012; 32:8583–8588. [PubMed: 22723699]
- Spector I, Hassmannova J, Albe-Fessard D. Sensory properties of single neurons of cat's claustrum. *Brain Res*. 1974; 66:39–65.

- Squatrino S, Battaglini PP, Galletti C, Riva Sansaverino E. Projections from the visual cortex to the contralateral claustrum of the cat revealed by an anterograde axonal transport method. *Neurosci Lett*. 1980; 19:271–275. [PubMed: 6189028]
- Stocker SD, Simmons JR, Stornetta RL, Toney GM, Guyenet PG. Water deprivation activates a glutamatergic projection from the hypothalamic paraventricular nucleus to the rostral ventrolateral medulla. *J Comp Neurol*. 2006; 494:673–685. [PubMed: 16374796]
- Tanné-Gariépy J, Boussaoud D, Rouiller EM. Projections of the claustrum to the primary motor, premotor, and prefrontal cortices in the macaque monkey. *J Comp Neurol*. 2002; 454:140–157. [PubMed: 12412139]
- Torgerson CM, Irimia A, Goh SYM, Van Horn JD. The DTI connectivity of the human claustrum. *Hum Brain Mapp*. 2015; 36:827–838. [PubMed: 25339630]
- Van De Werd HJ, Uylings HB. The rat orbital and agranular insular prefrontal cortical areas: a cytoarchitectonic and chemoarchitectonic study. *Brain Struct Funct*. 2008; 212:387–401. [PubMed: 18183420]
- Van Groen T, Wyss JM. Connections of the retrosplenial dysgranular cortex in the rat. *J Comp Neurol*. 1992; 315:200–216. [PubMed: 1545009]
- Vogt BA, Miller MW. Cortical connections between rat cingulate cortex and visual, motor, and postsubicular cortices. *J Comp Neurol*. 1983; 216:192–210. [PubMed: 6863602]

Figure 1.

Retrograde tract tracer deposits into motor and primary sensory cortices results in a restricted pattern of labeling within the claustrum. Black shading indicates tracer deposit core; dark gray shading indicates the penumbra of less dense labeling surrounding the core of the tracer deposit; light gray shading indicates the location of white matter structures. Dark gray in the claustrum reconstructions indicates white matter of the external capsule; light gray indicates the body of the claustrum as defined by parvalbumin (PV) immunoreactivity; black dots represent retrogradely labeled cell bodies. Claustral reconstructions proceed anterior to posterior, from left to right, as represented diagrammatically in the top row of **A**, with black boxes indicating approximate location of claustrum reconstructions. Photomicrographs of representative PV immunoreactivity, defining the boundaries of the claustrum, are given in the bottom row of **A**. Reconstruction of tracer deposit site, approximate anterior/posterior level of tracer deposit, and resultant labeling in and around the claustrum is shown for primary motor cortex (M1; **B**), posterior M1/secondary motor cortex (M2; **C**), primary visual cortex (V1; **D**), secondary visual cortex (V2, **E**), forelimb region of the primary somatosensory cortex (S1; **F**), and primary auditory cortex (A1; **G**). A/P, anterior/posterior; EC, external capsule; CL, claustrum; CTX, cortex. Scale bars=200 μ m.

Figure 2.

Retrograde tract tracer deposits into lateral parietal association cortex and medial prefrontal regions result in labeling restricted to dorsal and ventral portions of the claustrum, respectively. Reconstruction of retrograde labeling in the claustrum and surrounding insular cortex following deposits into lateral parietal association cortex (**A**), prelimbic part of the medial prefrontal cortex (**B**), and infralimbic cortex of the medial prefrontal cortex (**C**). A/P, anterior/posterior.

Figure 3.

Retrograde tract tracer deposits into different aspects of the cingulate cortex reveal a much wider pattern of labeling in the claustrum than in other sensory, motor, and association cortices. Reconstruction of claustral and insular cortical labeling following deposit into the pregenual anterior cingulate cortex (ACC; **A**), a more posterior level of pregenual ACC (**B**), middle cingulate cortex (**C**), posterior cingulate cortex (**D**), retrosplenial dysgranular cingulate cortex (**E**), and a more posterior aspect of retrosplenial dysgranular cortex (**F**). A/P, anterior/posterior.

Figure 4.

Photomicrographs illustrating minimal double labeling in the claustrum after dual retrograde tract tracer deposits into posterior cingulate and ACC. Tracer deposits of cholera toxin B subunit conjugated to AlexaFluor-488 (CTb-488) into the right ACC (**A**) and CTb conjugated to AlexaFluor-555 into the right posterior cingulate cortex with invasion into the retrosplenial dysgranular cortex (**B**). **C**: CTb-555 (red), CTb-488 (green) and double-labeled CTb-555+CTb488 (yellow) neurons from the right claustrum. **D**: Boxed area from **C** showing an example of a double-labeled cell (arrowhead). A/P, anterior/posterior. See Supporting Figure 1 for magenta-green copy. Scale bars=1mm in A,B; 150 μ m in C; 50 μ m in D.

Figure 5.

Photomicrographs illustrating minimal double labeling in the claustrum after dual retrograde tract tracer deposits into ACC and M1. Tracer deposits of cholera toxin B subunit conjugated to AlexaFluor-488 (CTb-488) into the right ACC (**A**) and CTb conjugated to AlexaFluor-555 into M1 (**B**). **C**: CTb-555 (red), CTb-488 (green), and double-labeled CTb-555+CTb488 (yellow) neurons from the right claustrum. **D**: Boxed area from **C** showing examples of double-labeled cells (arrowheads). A/P, anterior/posterior. See Supporting Figure 2 for magenta-green copy. Scale bars=1mm in A,B; 150 μ m in C; 50 μ m in D.

Figure 6.

Dual retrograde tract tracer deposits of the cortex reveal few double-labeled cells in the claustrum. **A:** Top: Reconstructions of a cholera toxin B subunit (CTb) deposit into the ACC and FluoroGold (FG) deposit of M1. Bottom: Retrograde labeling in the area of the claustrum. Few double CTb+FG-labeled cells were seen (stars). **B:** Top: Reconstructions of a CTb deposit into the ACC and an FG deposit into a region that straddles the posterior cingulate and retrosplenial dysgranular cortex. Bottom: Again, few double-labeled cells were seen. A/P, anterior/posterior.

Figure 7.

Anterograde/retrograde tract tracers deposited contralaterally into the cingulate cortex reveals a unilateral convergence of fibers and cell bodies in the claustrum. Injection sites for the anterograde tracer deposit of BDA into the left cingulate (**A**) and retrograde tracer deposit of CTb-488 into the right cingulate (**B**) slightly caudal to the genu of the corpus callosum. Photomicrographs illustrating a section of the left (**C**) and right (**D**) claustrum lying roughly equidistant between the genu of the corpus callosum and the decussation of the anterior commissure. BDA-ir (red) fibers and CTb-488-labeled (green) cell bodies were seen predominantly in the right claustrum (**D**), indicating projections from the cingulate to the claustrum originate primarily from the contralateral hemisphere, whereas projections from the claustrum to the cingulate originate primarily from the ipsilateral hemisphere. A/P, anterior/posterior. See Supporting Figure 3 for magenta-green copy. Scale bars=1mm in A,B; 150 μ m in C,D.

Figure 8.

Cortical hierarchy determines the distribution of claustrum projection neurons. Claustral projections to sensory and motor cortices are sparse and exhibit a restricted distribution within the claustrum, whereas projections to prelimbic cortex (area 32) are numerous and exhibit a more widespread distribution. Claustral projections to all aspects of cingulate cortices are numerous and distributed throughout the extent of the claustrum.

TABLE 1Targeted Cortical Areas, Injection Locations, Delivery Method, and Retrograde Tracer Used¹

Targeted area (n)	Injection location	Mode	Tracer
A1 (1)	A1	Pressure	FG
ACC (7)	ACC	Iontophoresis	FG
	ACC	Pressure	CTb
	ACC	Pressure	CTb
	ACC	Pressure	FG
	ACC	Pressure	CTb
	ACC	Pressure	CTb
	ACC/M2	Pressure	CTb
CC (2)	CC	Pressure	CTb-555
	CC	Pressure	CTb-555
LPA (1)	LPA	Pressure	FG
M1 (2)	M1	Pressure	FG
	M1	Pressure	FG
M1/M2 (2)	M1/M2	Pressure	CTb
	M1/M2	Pressure	FG
IL-PFC (1)	IL-PFC	Iontophoresis	FG
PL-PFC (1)	PL-PFC	Iontophoresis	FG
RSD (3)	RSD	Pressure	FG
	RSD/CC	Pressure	FG
	RSD/MPA	Iontophoresis	FG
S1 (1)	S1	Pressure	FG
V1 (1)	V1	Pressure	FG
V2 (1)	V2	Pressure	FG

¹ A1, primary auditory cortex; ACC, anterior cingulate cortex; CC, cingulate cortices (middle or posterior); CTb, cholera toxin B subunit; CTb-555, cholera toxin B subunit conjugated to AlexaFluor-555; FG, Fluorogold; IL-PFC, infralimbic cortex (area 25); LPA, lateral parietal association cortex; M1, primary motor cortex; M2, secondary motor cortex; MPA, medial parietal association cortex; PL-PFC, prelimbic cortex (area 32); RSD, retrosplenial dysgranular cortex; S1, primary somatosensory cortex; V1, primary visual cortex; V2, secondary visual cortex.

TABLE 2Primary Antibody Information[/]

Antigen	Immunogen	Source, manufacturer; RRID	Dilution
PV	Frog muscle PV	Mouse, polyclonal; Sigma Aldrich P3088; AB_477329	1:1,000 (IF) 1:3,000 (IP)
PV	Rat muscle PV	Rabbit, polyclonal; SWant PV 25; AB_10000344	1:2,000 (IF)
PV	Rat muscle PV	Goat, polyclonal; SWant PVG-214; AB_10000345	1:5,000 (IF)
CTb	CTb	Goat, polyclonal; List Biological 703; AB_10013220	1:5,000 (IF)
FG	FG	Rabbit, polyclonal; Fluorochrome LLC; AB_2314408	1:3,000 (IF)
FG	FG	Rabbit, polyclonal; EMD Millipore 153; AB_2314412	1:10,000 (IF)

[/] CTb, cholera toxin B subunit; FG, Fluorogold; IF, immunofluorescence; IP, immunoperoxidase; PV, parvalbumin.

TABLE 3Percentages of Retrogradely Labeled Cells Inside and Outside the Claustrum for Various Cortical Injections¹

Targeted area (n)	Region	Percentage labeled cells inside claustrum			Percentage labeled cells outside claustrum			No. labeled cells	
		Rostral	Middle	Caudal	Rostral	Middle	Caudal	In	Out
A1 (1)	A1	11.1	55.6	33.3	0.0	0.0	0.0	9	0
ACC (7)	ACC	55.4	28.0	16.6	71.4	14.3	14.3	175	7
	ACC	49.3	34.2	16.5	68.0	22.1	9.9	552	172
	ACC	58.3	30.8	10.9	79.3	13.8	6.9	276	58
	ACC	36.1	39.3	24.6	90.0	0.0	10.0	61	10
	ACC	47.6	35.5	16.9	63.6	30.7	5.7	349	140
	ACC	46.0	37.6	16.4	76.1	19.4	4.5	165	67
	ACC/M2	23.5	47.1	29.4	100.0	0.0	0.0	17	2
CC (2)	CC	36.6	42.0	21.4	16.7	33.3	50.0	205	12
	CC	37.0	37.0	26.0	33.3	33.3	33.3	100	3
LPA (1)	LPA	50.0	26.1	23.9	0.0	0.0	0.0	46	0
M1 (2)	M1	31.8	27.3	40.9	54.5	27.3	18.2	22	11
	M1	25.0	40.0	35.0	61.5	38.5	0.0	20	13
M1/M2 (2)	M1/M2	49.8	36.3	13.9	61.4	28.6	10.0	273	70
	M1/M2	19.0	42.9	38.1	90.0	10.0	0.0	21	10
IL-PFC (1)	IL-PFC	22.2	27.8	50.0	96.0	0.0	4.0	18	25
PL-PFC (1)	PL-PFC	42.9	39.9	17.2	73.3	16.7	10.0	203	30
RSD (3)	RSD	47.0	27.2	25.8	52.6	42.1	5.3	423	19
	RSD/CC	32.8	32.2	35.0	100.0	0.0	0.0	143	29
	RSD/MPA	33.3	30.2	36.5	0.0	0.0	100.0	96	2
S1 (1)	S1	28.6	37.1	34.3	0.0	0.0	100.0	35	1
V1 (1)	V1	31.2	39.0	29.8	66.7	33.3	0.0	77	6
V2 (1)	V2	26.3	26.3	47.4	100.0	0.0	0.0	38	1

¹ A1, primary auditory cortex; ACC, anterior cingulate cortex; CC, cingulate cortices (middle or posterior); IL-PFC, infralimbic cortex (area 25); LPA, lateral parietal association cortex; M1, primary motor cortex; M2, secondary motor cortex; MPA, medial parietal association cortex; PL-PFC, prelimbic cortex (area 32); RSD, retrosplenial dysgranular cortex; S1, primary somatosensory cortex; V1, primary visual cortex; V2, secondary visual cortex.

TABLE 4

Singly and Doubly Retrogradely Labeled Cells Inside and Outside the Claustrum for Double Cortical Retrograde Tracer Injections¹

	FG	CTb	FG+CTb	Percentage FG	Percentage CTb	Percentage double
N=1	M1	ACC				
Rostral, in	14	415	3	3.3	96.7	0.7
Middle, in	14	171	1	7.6	92.4	0.5
Caudal, in	5	99	1	4.8	95.2	1.0
Rostral, out	13	38	0	25.5	74.5	0.0
Middle, out	13	8	0	61.9	38.1	0.0
Caudal, out	2	4	0	33.3	66.7	0.0
N=1	RSD	ACC				
Rostral, in	124	154	14	44.6	55.4	5.0
Middle, in	52	169	11	23.5	76.5	5.0
Caudal, in	42	40	2	51.2	48.8	2.4
Rostral, out	4	1	0	80.0	20.0	0.0
Middle, out	0	7	0	0.0	100.0	0.0
Caudal, out	1	6	0	14.3	85.7	0.0

¹ ACC, anterior cingulate cortex; CTb, cholera toxin B subunit; FG, Fluorogold; M1, primary motor cortex; RSD, retrosplenial dysgranular cortex.