

UNRAVELING THE SEAT OF CONSCIOUSNESS: ANATOMICAL REDEFINITION
AND MOLECULAR CHARACTERIZATION OF THE CLAUSTRUM

By

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To my parents, Sue and Arun

and

To my wife, Mackenzie

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CHAPTER I

INTRODUCTION

Preface

In their seminal comprehensive study of comparative neuroanatomy, Ariëns Kappers, Huber, and Crosby wrote of “The Problem of the Claustrum” (1936, 1960). The “problem” they described detailed the controversy over the *morphology, ontogeny and phylogeny, hodology, and function* of what remains arguably the most enigmatic mammalian forebrain structure. A wealth of data has amassed on the claustrum since this description, providing particular insights into the origins, connections, and cellular morphological properties of this nucleus. Despite these advances, many fundamental aspects of the “problem” persist.

Nomenclature

Consistent with the mysterious nature of this nucleus, the nomenclature of the claustrum is unclear. This structure was originally named the “nucleus taeniaformis” by the French comparative anatomist Vicq d’Azyr around the turn of the 18th century, and would soon thereafter be renamed the “claustrum” by Burdach (Rae, 1954). “Claustrum” often collectively refers to both “dorsal claustrum,” otherwise known as the “insular claustrum” or “field 8” (Brodmann, 1909), and a structure that is ventrally contiguous called the “ventral claustrum,” otherwise known as the “endopiriform nucleus” (Loo, 1931), or “claustrum ventrale” (Drugat, 1966; Drugat et al., 1990, 1993). The endopiriform nucleus can further be subdivided into the dorsal endopiriform nucleus and the ventral endopiriform nucleus (Paxinos and Watson, 1997); the ventral endopiriform nucleus, which also is known as the “claustrum praefiriforme” (Brockhaus, 1940; Narkiewicz, 1964), is very poorly described.

We will use the terms claustrum and endopiriform nucleus to refer to these two structures, following current conventions.

Morphology

Macroscopic morphology. The claustrum is a long, band-like grey matter structure in the ventrolateral telencephalon of all therian mammals (marsupials and placentals), and arguably in monotremes (Kowiański et al., 1999; Butler et al., 2002; Ashwell et al., 2004). The therian mammals can be divided into two groups based on claustrum morphologies: species lacking an extreme capsule of white matter (hedgehog, bat, mouse, and rat), and species possessing an extreme capsule of white matter (guinea pig, rabbit, cow, carnivores, non-human primates, and human).

In species lacking an extreme capsule, the structural organization of the claustrum has been most heavily studied in the rat. Nonetheless, the structural boundaries of the claustrum in the rat are nebulous (and other extreme capsule-lacking species), in part because no claustrum-specific neuroanatomical marker has been identified. It is, therefore, not surprising that accounts of claustral borders for the rat vary between brain atlases (Paxinos and Watson, 2007; Swanson, 2004), as well as across various primary research sources (Bayer and Altman 1991; Druga et al., 1993; Kowianski et al., 1999; Krettek and Price, 1977; McKenna and Vertes, 2004; Sloniewski et al., 1986). Nevertheless, a consensus view of rat claustral anatomy as described in brain atlases has emerged. Paxinos and Watson do not cite a source for their definition, but Swanson cites Krettek and Price (1977, 1978). Krettek and Price (1977) define the claustrum as extending along the entire rostrocaudal length of the striatum, where it resides immediately adjacent to the medially-lying external capsule (EC). Interestingly, both the Paxinos and Swanson atlases extend the claustrum much further rostrally than the descriptions of Krettek and Price (1977), well into the frontal pole where it lies immediately ventrolateral to the forceps minor

(Fig. 1). The reason for this rostral extension is unclear. Recently, Paxinos and Watson (2007) noted in their atlas a new, dorsally-lying component to the claustrum, which they termed the “dorsal claustrum” (Fig. 1). This area has yet to be investigated by other authors, rendering its potential functional significance, or even the validity of its existence, uncertain.

In species possessing an extreme capsule, the structural organization of the claustrum is easier to define. The claustrum is defined as the thin strip of grey matter interposed between the striatum and the insular cortex (Fig. 2). Consistent with the definition of its name, meaning “hidden” or “enclosed space,” the claustrum is completely enveloped by the medially-lying EC and the laterally-lying extreme capsule of white matter. The border created by this surrounding white matter and the grey matter that is enclosed within defines the structural boundaries of the claustrum. In humans, as an example of a species possessing an extreme capsule, the claustrum is present along the entire rostrocaudal extent of the striatum (Jennes et al., 1995). Dorsoventrally, the claustrum extends along the entire medial face of the adjacent insula. Along this dorsoventral axis, the claustrum undulates slightly, following the contours of the insula. From an oblique angle, then, the claustrum appears as a wavy sheet of grey matter (Rae, 1954).

Morphological analyses of the human claustrum have been performed by several investigators (Dejerine, 1895; Macchi, 1947; Rae, 1954; Filimonoff, 1966; Morys et al., 1996), all of whom identified the endopiriform nucleus (*claustrum ventrale*). However, the human endopiriform nucleus differs from the claustrum by having a much more restricted anteroposterior extent, appearing only at mid-striatal levels. In other species with an extreme capsule, the endopiriform nucleus roughly shares the same rostrocaudal extent as the claustrum (Kowiański et al., 1999), appearing as a ventral extension of the claustrum. In the macaque, for instance, the endopiriform nucleus reportedly forms the ventral bulb-like mass extending from the claustrum. In species lacking an extreme capsule, the claustrum

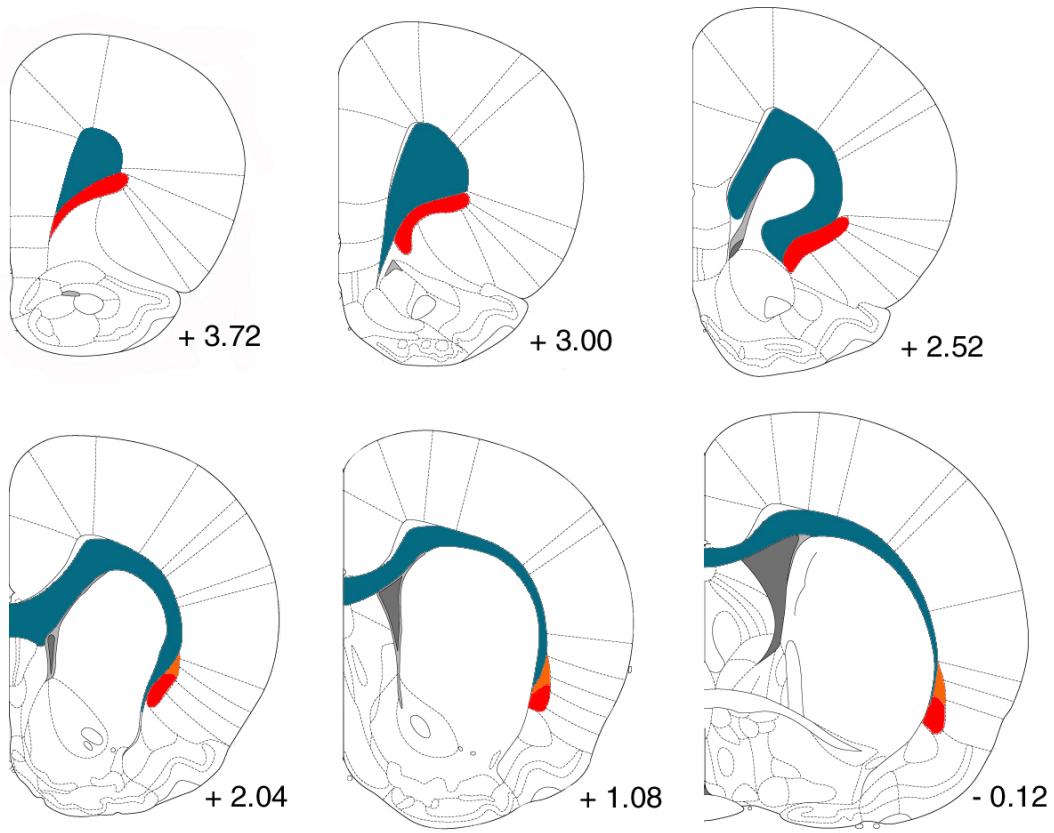


Figure 1. The current structural boundaries of the rat claustrum as shown in the brain atlas of Paxinos and Watson (2007). White matter structures are shaded blue, the claustrum is shaded red and the “dorsal claustrum” is shaded orange. Values represent distance in mm relative to bregma. Current definitions extend the claustrum from the forceps minor to the posterior end of the striatum. In addition, the claustrum is thought to abut white matter structures at all levels.

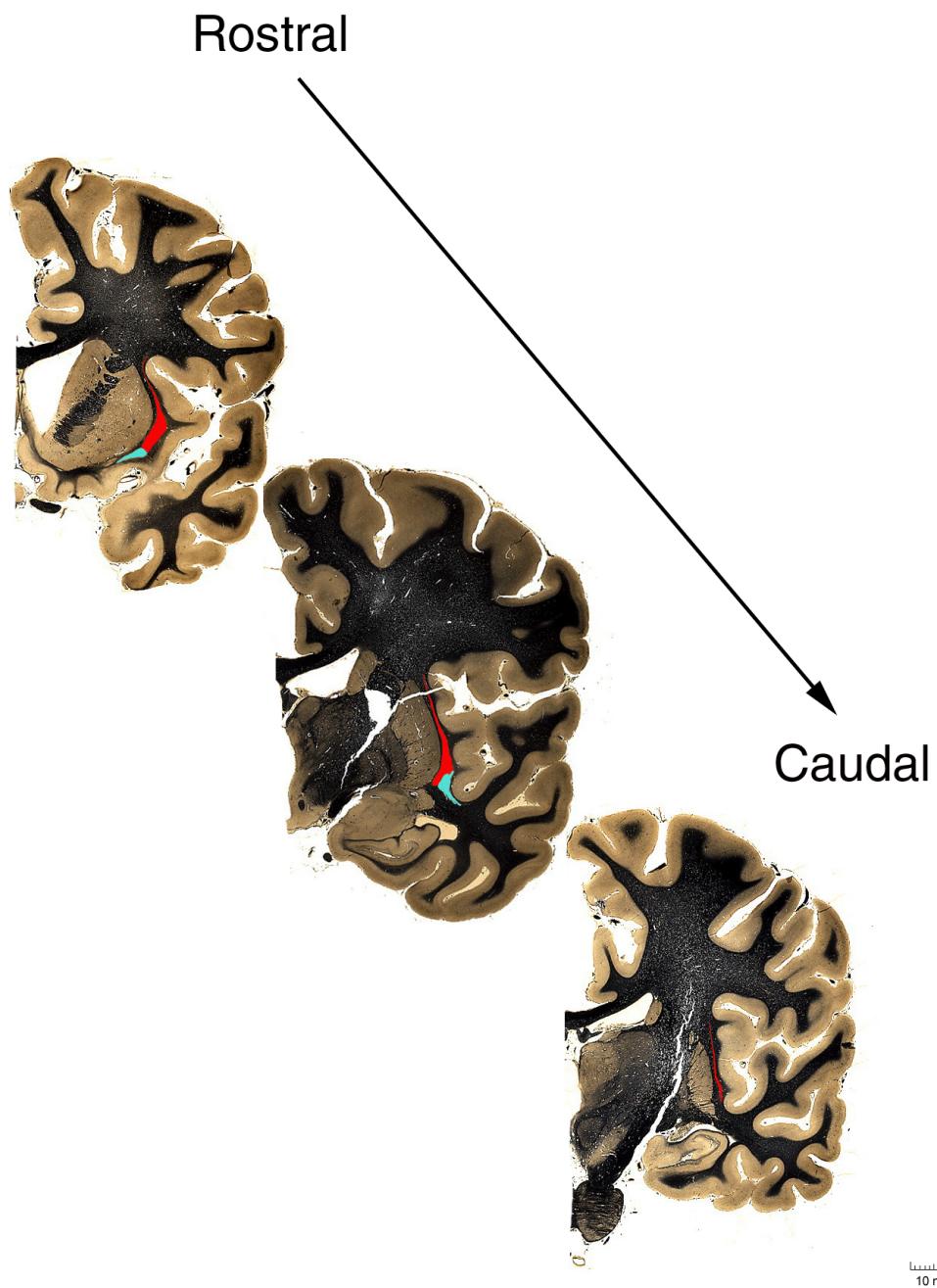


Figure 2. Coronal myelin-stained sections of human telencephalon depicting the structural boundaries of the claustrum. The claustrum is shaded in red, while the ill-defined endopiriform nucleus is shaded in blue. The claustrum extends the rostrocaudal length of the striatum (caudate nucleus [c] and putamen [p]), which lies medially. At all rostrocaudal levels, the claustrum is completely surrounded by white matter. The medially-lying EC resides between the striatum and the claustrum, while the laterally-lying extreme capsule is flanked by the claustrum and the insular cortex (ic). Modified from The Human Brain Atlas (Michigan State University, 2008).

and endopiriform nucleus are both ovoid, making the border between these structures easier to discern. In the rat, the endopiriform nucleus is present at all rostrocaudal levels of the claustrum, with the exception of the most anterior end. The structural definitions of the endopiriform nucleus in all species have been based solely upon cytoarchitectonic grounds (Kowiański et al., 1999). As such, contemporary neuroanatomical methods are required to validate these definitions. Uncovering neuroanatomical markers of the claustrum that clearly demarcate this structure from surrounding tissues, including the endopiriform nucleus, would be extremely useful.

Microscopic morphology. Following early investigations of the gross anatomy of the claustrum by Dejerine (1895) and others, Rae (1954) published a careful microscopic analysis in the human. Using silver impregnation studies, he found that the interface of capsular fibers and the grey matter body of the claustrum, which represents the structural boundaries of the claustrum, are not clearly demarcated. He found that the dense collection of cell bodies and fibers in the core of the claustrum gradually change toward the border with capsular fibers. Specifically, he observed that fusiform-shaped cells became more prevalent toward the perimeter of the claustrum. Instead of observing an abrupt transition of cell bodies to axons at the interface between grey and white matter, fusiform cell bodies were intermingled within white matter. Interestingly, both Meynert (1884) and Brodmann (1909) also observed the prevalence of the fusiform somata in the claustral perimeter and the insular cortex. Besides the heterogenous distribution of fusiform cells in the claustrum, Rae (1954) found a homogenous distribution of other cell types within the claustrum, including ovoid, triangular, and polygonal types.

More recent Golgi impregnation analysis of human tissue has defined two types of claustrum neurons, type I and type II (Braak and Braak, 1982). Golgi type I neurons comprise roughly 85% of all claustral neurons and are evenly distributed throughout the

body of the claustrum (Braak and Braak, 1982; Sherk, 1986; Spahn and Braak, 1986). They have spiny dendrites with axons projecting out of the claustrum, and have cell body diameters of 15-29 μm . This population represents the excitatory neurons that send projections to and receive projections from the isocortex. A combined neuronal tract tracer and *in situ* hybridization study demonstrated that the claustral projection neurons express the gene encoding the vesicular glutamate transporter 2 (Hur and Zaborsky, 2005). Because VGLUTs (1 and 2) are considered to be unambiguous markers of cells that use glutamate as an intercellular signaling molecule, it can reasonably be inferred that these claustral projection neurons are glutamatergic.

The less common Golgi type II neurons, comprising the remaining 15% of claustral cells, have cell body diameters of 10-15 μm , are aspiny, and have axons that do not project outside the body of the claustrum, as evidenced by human and cat studies (Levay and Sherk, 1981; Braak and Braak, 1982). Type II neurons are therefore thought to be interneurons. This suggestion is bolstered by the fact that retrograde tract tracing studies have found that these cells do not accumulate tracer, consistent with the fact that they are interneurons. These cells express three types of calcium-binding proteins: parvalbumin (PV), calbindin (CB), and calretinin (CR) (Drugat et al., 1993; Reynhout and Baizer, 1999). The rat claustrum is rich in PV-positive interneurons, but relatively poor in CB and CR-positive interneurons (Drugat et al., 1993; Paxinos et al., 1999). Immunocytochemical analysis reveals a dense cloud of PV-immunoreactive (-ir) neuropil in the rat claustrum, while a plexus of neuropil rich in CR-ir surrounds the claustrum in what appears as a “shell” around the nucleus. However, overlap exists between the PV-ir and the CR-ir plexuses.

Unlike the rat claustrum, the primate claustrum has a much more homogenous distribution of the interneuron populations, although the density between these populations varies (Reynhout and Baizer, 1999). Reynhout and Baizer (1999) found PV-ir neurons to be large, multipolar cells with smooth dendrites. In comparison, CR-ir cells are smaller,

have elongated somas, are bipolar, and exhibit beaded dendrites. The CB-ir neurons were shown to exist in three forms: a dense population with small cell bodies and winding dendrites, a second multipolar type not unlike the PV-positive neurons, and a third bipolar type resembling the CR-positive neurons. Similar cell types have been observed in several different species, including human (Brand, 1981; Mamos, 1984; Mamos et al., 1986, Levay and Sherk, 1981; Braak and Braak, 1982).

Not unlike the isocortex, then, the claustrum is composed of inhibitory-like interneurons and excitatory projection neurons. Unlike the cortex, the claustrum does not exhibit a layered organization. Moreover, the apical dendrites of the type I projection neurons are not oriented in any specific direction, and these neurons express VGLUT2, which is typically restricted to subcortical cells (Hur and Zaborsky, 2005). This suggests that the claustrum is a subcortical, or at least non-cortical, structure despite its physical apposition to and high connectivity (see *Hodology* with isocortex).

Ontogeny and Phylogeny

Ontogeny. Early in the 20th century, the ontogenetic and phylogenetic derivations of the claustrum were intensely contested by several comparative anatomists. Investigators agreed that the claustrum is of pallial derivation. However, a dispute arose over whether the claustrum should be considered a derivative of cortex or a subcortical (albeit pallial) structure. Holl (1899) viewed the claustrum as a doubling of the insular cortex, and Smith (1910, 1917) later independently concluded that the claustrum derived from the upturned aspect of the piriform cortex. This notion of a doubling of adjacent cortex was also supported by Brodmann (1909) and others who concluded that the claustrum is cortical in origin. De Vries shared this view, but stipulated that he did not believe that this necessarily meant that the claustrum was derived from cortex (Ariëns Kappers et al., 1960).

Carrying the isocortical derivation hypothesis further, Sonntag and Woollard (1925)

noted the resemblance of layer VI cells of the insular cortex and claustral cells in the aardvark. They concluded that the deepest layer of insular cortex is a “two-layered lamina multiformis” that is separated by the extreme capsule. Under this model, the superficial layer of this “lamina multiformis” is layer VI of insular cortex, with the deep layer being the claustrum. Similarly, Rose (1928) held that in mammals lacking an extreme capsule, the claustrum is the innermost extension of insular layer VI. In mammals possessing an extreme capsule, both Rose (1928) and Brodmann (1909) suggested that the claustrum is differentiated into an independent cortical layer, with what was termed insular layer VII representing the extreme capsule and layer VIII representing the claustrum. By this definition, the claustrum is cortical, but does not appear layered because it, itself, is a layer of insular cortex.

Standing in opposition to the notion that the claustrum is a cortical component, Landau (1919), and later Faul (1926), believed the claustrum to be subcortical, grouping it with striatal areas, though considering it not to be developmentally related to either striatum or cortex. Holmgren (1925) held a similar view but made the insightful assertion that the claustrum is a pallial structure not derived from cortex. He submitted that the claustrum derives from the ventricular surface, rather than as an infolding of the overlying insular cortex, and should be grouped along with the amygdaloid complex. His perspective was largely ignored, however, as the bulk of opinions regarded the claustrum as a component part of the insular cortex (Ariëns Kappers et al., 1960).

It would take 75 years of speculation and investigation before convincing evidence finally found Holmgren’s view of claustrum ontogeny to be correct. Performing an elegant analysis of pallial and subpallial genetic markers in the developing chicken and mouse brains, Puelles and colleagues (2000) demonstrated the existence of four distinct pallial regions in the developing telencephalon. In addition to the medial, dorsal, and lateral pallial areas previously identified, a new “ventral pallium” was also defined. Based on these

findings, Puelles and coworkers (2000) assigned the claustrum to the lateral pallium, along with the dorsal piriform cortex and basolateral amygdala. The new “ventral pallium” gives rise to the endopiriform nucleus, as well as other sites including the ventral piriform cortex, olfactory bulb, and lateral and intercalated nuclei of the amygdala. This view suggests that because the claustrum lacks a laminar organization and is derived from lateral pallium along with the basolateral amygdala, it should not be considered cortical.

If the claustrum is indeed not a cortical structure, and is derived separately from the endopiriform nucleus, one might predict that the birth date of claustral neurons differs from that of cells in endopiriform nucleus and isocortex. Bayer and Altman (1991) used tritiated thymidine birth-dating analysis to determine that claustral neurons primarily arise on embryonic day (E) 15 and 16, while endopiriform neurons are born earlier, on E14 and E15. Interestingly, cortical layer VI neurons are born at approximately E12.5, with the more superficial layers completing development by E15.5 (Valverde et al., 1989; Molyneaux et al., 2007). Despite the distinct birth-dating difference between the claustrum and the isocortex, Bayer and Altman (1991) showed that claustrum neurons are derived from the cortical epithelium. This finding is consistent with the lateral pallial derivation findings of Puelles and coworkers (2000), and the position held by Holmgren (1925). In contrast to the claustrum and isocortex, the endopiriform nucleus derives from the palliostriatal ventricular angle, a zone that straddles the border between the primordia of the basal ganglia and isocortex (Bayer and Altman, 1991). Further distinguishing the claustrum from the endopiriform nucleus, claustral neurons migrate ventrally along the axis of the EC where they populate in a caudal to rostral fashion over time. Endopiriform neurons form a gradient in the orthogonal axis to that of the claustrum, with older neurons populating ventrally, and younger neurons populating dorsally (Bayer and Altman, 1991). So, despite the lack of clear boundaries between the claustrum and the endopiriform nucleus, these structures appear to be developmentally distinct.

Phylogeny. The lateral pallium derivation of claustrum suggests comparability of this structure to the anterior part of the dorsal ventricular ridge in reptiles (DVR) (Striedter, 1997), an assumption that was also advanced by Holmgren (1925). Extending comparisons to the avian brains, the lateral pallium in birds gives rise to the mesopallium (formerly known as hyperstriatum ventrale); thus, aspects of this structure may be considered comparable to the claustrum from a developmental perspective. However, caution must be taken in considering the DVR/mesopallium as a true claustral homologue. The reptilian and avian pallia are not layered, but are rather organized as a grouping of nuclei. Since the claustrum and cortex (a layered pallium) only appear in mammalian brains, and because the claustrum and the isocortex are highly interconnected (see Hodology), it is possible that the claustrum co-emerged with isocortex as a necessary, but non-cortical, structure for a layered pallium. In this regard, the origins of the claustrum are linked to the origins of isocortex, the latter of which remains a matter of debate (Northcutt and Kaas, 1995).

Despite the existence of isocortex in all mammals, the presence of the claustrum in all mammals is not immune to controversy. An ongoing debate persists over the existence of a claustrum in the monotreme clade. Butler et al. (2002) and others (Abbie, 1940; Divac et al., 1987) reported the claustrum to be absent in both the platypus and the Australian short-beaked echidna. Although these authors logically used the rhinal fissure as a guide for honing their regional analysis, they restricted their analysis to amygdalar rostrocaudal levels. In all therian brains, only the caudal-most tail of the claustrum is present at these levels. Nevertheless, these findings would suggest that the claustrum arose in therian mammals only, countering the argument that the claustrum co-emerged with isocortex.

Ashwell et al. (2004) has since presented evidence for the existence of a claustrum in both the Australian short-beaked echidna and the platypus. If the claustrum is indeed

present in monotremes, then it is reasonable to argue that the claustrum was present in the extinct ancestral mammals, possibly including the cynodont therapsids. Another possible conclusion is that the claustrum was present in ancestral mammals, but secondarily lost in monotremes. Finally, the claustrum may have appeared in therian mammals as an independent derivation of the lateral pallium. In order to determine which of these possibilities is most correct, a tract tracer study testing the existence of a claustrum based on connections with isocortex is needed. However, because echidnas and the platypus are protected species, such a study is unlikely to be performed. An alternate solution would be to identify a neuroanatomical marker for the claustrum so that immunohistochemical analysis can be performed on archival monotreme tissue.

Hodology

Cortical connections. Among the first reports on claustral connectivity was Leonardo Bianchi's 1897 degeneration study performed in a cebus monkey (Bianchi, 1922). Bianchi noted a line of degeneration along the length of the claustrum following "mutilation of the external surface of the frontal lobe in front of the intermediate motor zone," suggesting that the claustrum is connected to this area of frontal cortex. Roughly forty years later, the debate over claustral connectivity, in addition to its ontogenetic and phylogenetic origins, was developing. By then some authors had reported that the claustrum was connected to the ventral thalamus, some argued for cortical connectivity, while Berkelbach van der Sprengel (1926) stood alone in thinking the claustrum to be connected contralaterally to the amygdala.

Through the mid 20th century, degeneration studies in rabbit, cat, and macaque (Narkiewicz, 1964, 1972; Carman et al., 1964; Druga, 1966, 1968; Kemp and Powell, 1970; Chadzypanagiotis and Narkiewicz, 1971) suggested that the claustrum is connected with all areas of isocortex (to be referred to as cortex, excluding from consideration other types

of cortices such as allocortex). A general feature that arose from these studies was that the claustrum is topographically organized, with rostral areas of cortex innervating rostral areas of the claustrum and caudal cortical sites projecting to the more caudal claustrum. Using tract tracing methods, these findings have been substantiated and extended by showing that the cortical projections to the claustrum are reciprocated (Sanides and Buchholtz, 1979; Olson and Graybiel, 1980; Irvine and Brugge, 1981; Levay and Sherk, 1981; Macchi et al., 1981; Pearson et al., 1982; Druga, 1984; Shameem et al., 1984; Carey and Neal, 1985; Minciucchi et al., 1985; Adinolfi and Levine, 1986; Sloniewski et al., 1986; Druga et al., 1990; Baizer et al., 1997; Sadowski et al., 1997; Künzle and Radtke-Schuller, 2001). Today it is generally accepted that the claustrum is reciprocally connected with all cortical sites (Sherk, 1986). The connections between the claustrum and cortices are primarily ipsilateral, although a weaker contralateral projection has been reported in several studies (Norita, 1977; Olson and Graybiel, 1980; Squatrito et al., 1980; Li et al., 1986).

Due to the technical difficulty of placing discrete injections of tract tracers into the claustrum, information on the laminar organization of cortical projections to the claustrum are limited. Levay (1986) and Olson and Graybiel (1980) discretely deposited an anterograde tracer into the claustrum of cat. They demonstrated that the claustrum projects to all layers, with the densest innervation to layers IV and VI of area 17 (primary visual cortex). Claustral axons synapse with spiny dendrites (of presumptive excitatory cells) in all layers, but in layer IV they also synapse onto aspiny dendrites (Levay, 1986). Projections from the cortex to claustrum appear to arise predominantly, if not exclusively, from pyramidal and fusiform cells of layer VI (Levay and Sherk, 1981; Olson and Graybiel, 1980). Approximately 3-4% of layer VI cells in the visual cortex project to the claustrum, and this population is distinct from neurons projecting to the lateral geniculate nucleus of the thalamus (Olson and Graybiel, 1980; Levay and Sherk, 1981). Electron microscopy studies have shown that cortical projections form asymmetric synapses onto spiny

(presumed excitatory) and aspiny (presumed inhibitory) cells of the claustrum (Levay and Sherk, 1981). Other cortical sites examined also display projections from layer VI; although a weak innervation to the claustrum from deep layer III of auditory area Ep is present in the cat, and projections from layers V and VI of the cingulate gyrus of the rabbit have been reported (Bassett and Berger, 1981; Levay and Sherk, 1981).

Several other studies have shown discrete zones in the claustrum project to and receive projections from different regions of cortex (Fig. 3). In the macaque, distinct cortical representations have been widely demonstrated. Discrete representations for S1 (Kemp and Powell, 1970; Jones et al., 1977; Pearson et al., 1982), motor cortex (Künzle, 1975; Pearson et al., 1982), frontal cortical areas 46 and 8 (Künzle and Akert, 1977; Pearson et al., 1982), V1 and temporal visual areas (Kemp and Powell, 1970; Turner et al., 1980; Dineen and Hendrickson, 1982), and area 22 have been reported (Jones and Powell, 1970; Turner et al., 1980; Mufson and Mesulam, 1982; Pearson et al., 1982). Two groups have noted that claustral cells rarely project to more than one cortical area (Macchi et al., 1983; Li et al., 1986).

Perhaps the most detailed demonstration of discrete claustral territories is the work done by Olson and Graybiel (1980). They used electrophysiological recordings from subregions of the cat claustrum following various sensory stimuli and found that the cortical representation for visual and tactile information within the claustrum maintained an orderly retinotopic and somatotopic organization. By injecting tracers into the claustral site from which they recorded, Olson and Graybiel (1980) found that discrete subdivisions within the claustrum receive projections from and send projections to cognate sensory cortices. These sensory representations are non-overlapping and restricted spatially along the dorsoventral axis of the claustrum, but extensively span the rostrocaudal axis.

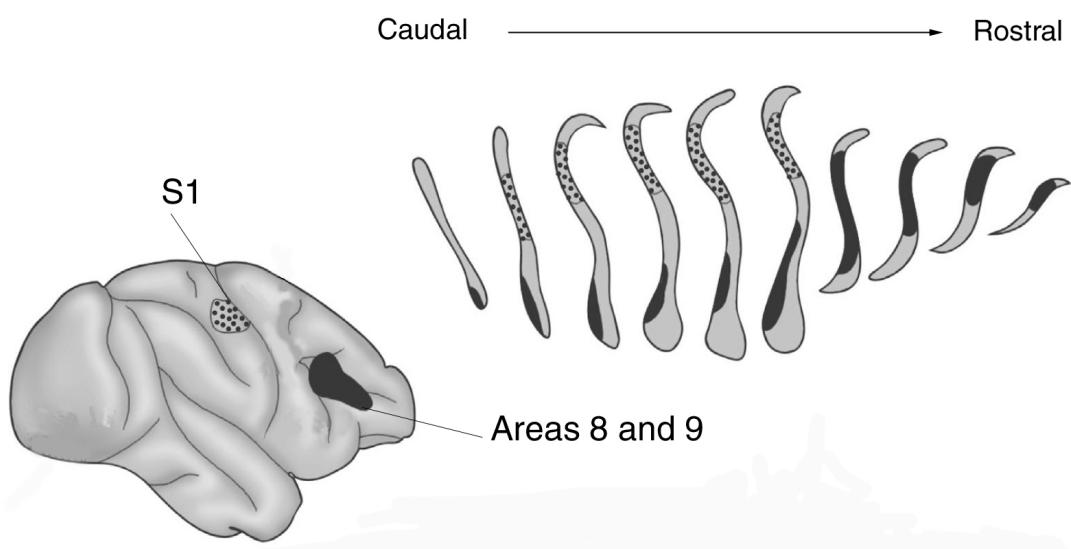


Figure 3. Isocortical areas are represented in discrete zones within the claustrum. Injection of a neuronal tract tracer with anterograde and retrograde properties (HRP) into S1 and areas 8 and 9 of macaque results in retrogradely-labeled cell bodies and axon fibers within dorsal and ventral regions of the claustrum, respectively. Modified from Pearson et al., 1982.

Studies in the rat have shown that the representations of primary auditory, visual, somatosensory, and motor cortices are also regionally distributed in the claustrum (Li et al., 1986; Sadowski et al., 1997). Primary auditory and visual cortical representations overlap in the ventral part of the claustrum, while somatosensory and motor cortical representations overlap in the dorsal part of the claustrum (Li et al., 1986; Sadowski et al., 1997). In contrast to the rat claustrum, the claustrum of felines and primates, which have a much elaborated cortex compared to rodents, is far more segregated in its zonal distribution.

Based on the widespread connectivity of claustrum with cortex, and the zones of cortical representation in the claustrum, it appears that the organization of the claustrum resembles that of the thalamus (Olson and Graybiel, 1980). Are there connections within the claustrum that link these cortical recipient and projection zones together? Following the discrete injections of HRP into the claustrum by Olson and Graybiel (1980) and later Levay (1986), these investigators reported no inter-zonal connections, but no further data exist to support or deny the existence of intra-zonal-connections (Sherk, 1986; Crick and Koch, 2005). As such, the consensus is that no connection between zones exists (Sherk, 1986; Crick and Koch, 2005).

Subcortical connections. In addition to the claustrum's reciprocal connections with cortex, modern tract tracing studies have suggested the presence of subcortical projections. Studies in the hedgehog, rat, cat, tree shrew, and macaque have reported claustral projections to the dorsal thalamic nuclei (Levay and Sherk, 1981; Carey and Neal, 1986; Dinopoulos et al., 1992; Erickson et al., 2004; McKenna and Vertes, 2004; Vertes and Hoover, 2008), striatum (Arikuni and Kubota, 1985), hippocampus (Amaral and Cowan, 1980), and hypothalamus (Levay and Sherk, 1981; Vertes, 1992; Yoshida et al., 2006). Interestingly, in many of these studies the retrogradely-labeled somata in the claustrum were seen to form a ring-like pattern around what appears to be the center of the body

of the claustrum. These findings are consistent with the segregation of the claustrum into a PV-ir rich “core” surrounded by a VGLUT2-rich “shell,” as proposed by Real et al. (2006). According to this interpretation, the “shell” may be connected to subcortical sites, while the “core” may be connected with cortex.

Immunohistochemical studies suggest that the claustrum in rats and cats receives a serotonergic innervation, presumably from the brainstem raphe nuclei (Baizer, 2001; Rahman and Baizer, 2007). This serotonergic input was reported to be evenly distributed across the entire claustrum. Consistent with these findings, five subtypes of serotonin receptors are known to be present within the claustrum, including 5-HT_{1A}, 5-HT_{1F}, 5-HT_{2A}, and 5-HT_{2C} receptors (Mengod et al., 1996; Pasqualetti et al., 1999; Pompeiano et al., 1994; Wright et al., 1995). The significance of this potential subcortical connection has yet to be experimentally elucidated.

Endopiriform nucleus. Another subcortical structure that has been reported to project to the claustrum is the endopiriform nucleus. Lipowska and colleagues (2000) found that the endopiriform nucleus in the rat and rabbit projects to the lateral aspects of the claustrum that border the EC and the insula. This connectivity pattern would again appear to be consistent with the notion of a “core” and “shell” organization of the claustrum. Taken together with other studies indicating such an arrangement, the “shell” of the claustrum projects to the thalamus, hypothalamus, and endopiriform nucleus. The claustral “core,” then, is allied with the cortex.

Despite originally being named the “ventral claustrum,” the connections of the endopiriform nucleus differ significantly from those of the claustrum. The endopiriform nucleus in rat is known to project to the perirhinal, entorhinal, insular, orbital, and prepiriform cortices, as well as pallial amygdala areas, olfactory tubercle, and most subdivisions of the hippocampus (Behan and Haberly, 1999; Lipowska et al., 2000; Wyss

et al., 1979; Wyss, 1981; Markowitsch et al., 1984; Wilhite et al., 1986; Witter et al., 1988, 1989). Many of these connections have also been seen in the cat, including connections with the subiculum, parasubiculum, hippocampus, and amygdala (Krettek and Price, 1977). Taken together, these findings, with the developmental data by Bayer and Altman (1991) and Puelles and coworkers (2000), indicate that the claustrum and the endopiriform nucleus are two distinct nuclei.

Insular cortex. Brodmann (1909), Loo (1931), Rae (1954) and others noted similarities between the insular cortex and the claustrum. The insular cortex, like the claustrum, has widespread connections with other parts of the brain. Studies have shown that the insula projects to or receives inputs from the nucleus of the solitary tract, olfactory bulb, amygdala, hippocampus, the parvicellular part of the posteromedial ventral thalamic nucleus, as well as the entorhinal, motor, primary and secondary somatosensory, anterior cingulate, prefrontal, orbitofrontal, primary auditory, auditory association, and visual association cortices (Augustine, 1985, 1996; Mesulam and Mufson, 1985; Nakashima et al., 2000; van der Kooy et al., 1984). While the claustrum and the insular cortex share many sites in their respective connectivity profiles, there has been no indication in the literature showing these profiles to be identical. Based on structural, developmental, and hodological lines of evidence the claustrum is not part of insular cortex.

Function

The final, and most puzzling, “problem” of the claustrum lies in its function. Relative to other prominent telencephalic structures such as the cortex, striatum, and the globus pallidus, knowledge of claustrum function is sorely lacking. Despite waves of interest in the claustrum over the last century, only a few nuggets of functional information and some controversial hypotheses on its functional attributes exist. Why has the function of the

claustrum proven to be so hard to unlock? The shape of the claustrum has made complete and discrete claustrum lesions impossible to achieve using conventional chemical or mechanical means. Clinical pathological correlation studies have yielded extraordinary information about the function of many brain sites, but no reports of claustral lesions have been reported in humans following cerebral hemorrhage or ischemia. This is likely due to the fact that both the claustrum and the insula receive their vascular supply from branches of the middle cerebral artery. There is, however, a report of bilateral claustral lesions in a 12-year-old girl suffering from severe, transitory encephalopathy (Sperner et al. 1996). Following a three week period of recurrent complex partial and myoclonic seizures, the patient was psychotic and had temporary loss of vision, hearing, and speech. Structural (MRI) studies indicated bilateral lesions of the claustrum, probably due to edema. Five weeks later, the patient had completely recovered, and the claustral “lesions” had resolved, along with all neurological deficits. There have been no similar cases reported. Without having the ability to generate reproducible, discrete lesions of the claustrum in animals, the functional roles of this nucleus remain unknown.

Multisensory integration. Drawing upon connectivity data, one can generate certain predictions of the functional attributes of the claustrum. Based on its bidirectional cortical connectivity, the claustrum has been proposed to function as a multisensory integrator: serving to bind information from disparate sensory cortices. Supporting this notion, Segundo and Machne (1956) and later Spector and coworkers (1974) found electrophysiological evidence for sensory convergence in the claustrum. Both groups recorded from claustral neurons in awake and anesthetized cats that were exposed to sensory stimuli of different modalities. They showed that 75% claustral cells responded to more than one sensory modality (Spector et al., 1974). The polymodal neurons responded to as few as two modalities, and to as many as six (touches, flashes, clicks, smells, vagal,

and tooth pulp stimulation). The most common convergences observed were somato-olfactory, somato-visceral, and somato-nocioceptive (Segundo and Machne, 1956). Notably, polymodal cells were distributed throughout the claustrum (Spector et al., 1974), and these cells displayed unique firing patterns for each type of modality-specific stimulus (Segundo and Machne, 1956).

In addition to sensory convergence, another physiological trait of claustral cells consistently found across functional studies is their quiescent nature. The spontaneous firing rate is quite low, usually only becoming activated following the presentation of a sensory stimulus (Segundo and Machne, 1956; Spector et al., 1974). This effect appears to be independent of the wakefulness of the animal. The meaning of this physiological quiescence has yet to be addressed.

If the claustrum is functioning as a multisensory integrator, how does it do so? The answer to this question is simply not known. However, two different theories for multisensory integration have been proposed, and it is possible to fit the claustrum into both theories. The first theory states that multisensory integration occurs in polymodal sites that only process specific sensory combinations; these types of cells have been reported in a variety of areas including arcuate sulcus, superior temporal sulcus, inferior and posterior parietal lobules, the amygdaloid complex, hippocampus, and the superior colliculus (Thompson et al., 1965; Ettlinger and Wilson, 1990). Because the claustrum has multisensory-responsive cells, the claustrum may serve to bind some types of sensory modalities. The second theory, proposed by Ettlinger and Wilson (1990), states that no one structure in brain executes the processes required for cross-modal performance. Instead, only a subcortical relay nucleus is required through which different sensory cortices can access each other in order to associate modalities; this subcortical relay nucleus was proposed to be the claustrum. In this way, the claustrum would somehow synchronize cortical areas to accomplish the feat of crossing modalities. Ettlinger and Wilson (1990) did

not state, however, how this may be accomplished or where the binding of multimodal information would occur.

In vivo functional imaging studies exploring multisensory integration largely support the second theory, which places the claustrum as the necessary subcortical relay nucleus. This support is due to a growing body of evidence showing activation of the claustrum/insula region in cross-modal matching tasks (Hörster et al., 1989; Lewis et al., 2000; Olson et al., 2002; Naghavi et al., 2007; Kavounoudias et al., 2008). A representative finding comes from Hadjikhani and Roland's (1998) positron emission tomography (PET) study that involved a task that had subjects attempting to identify objects in their hand (to which they were blind) to a matching object in their visual field (but out of reach) that was amongst a series of similar, but non-identically-shaped objects. They showed that visual and somatosensory cortices and the claustrum were activated during this task. Other studies using functional magnetic resonance imagery (fMRI) have gone on to show that a combination of the appropriate sensory cortices and the claustrum were activated during similar matching paradigms (Olson et al., 2002; Naghavi et al., 2007; Kavounoudias et al., 2008). Thus, a relay function for the claustrum is gaining support. Many questions still exist, however, including *how* the claustrum is functioning in this proposed relay role. Furthermore, these imaging studies do not address the question of where multisensory information is being bound exactly, i.e., what brain site decides what stimuli should and should not be bound?

Crick and Koch's hypothesis. In an attempt to address this last issue, Francis Crick and Cristoph Koch (2005) hypothesized that the claustrum is where sensory information is bound, functioning as a generator of the unified perception of a multitude of sensory stimuli in one's environment (conscious percepts). An explanatory example of this novel idea is the experience of holding a rose, where one perceives not only the color of the petals, but

also the fragrance, and the texture of the petals. Putting these stimuli together, one is able to recognize the object as a rose rather than experiencing each stimulus as a separate sensory entity. Crick and Koch argued that since almost all theories attempting to explain the neural correlate of such an experience (consciousness) require a “need to rapidly integrate and bind information in neurons that are situated across distinct cortical and thalamic regions” (also see also Bachmann, 2000; Llinas, 2001), that the claustrum may be perfectly suited to subserve such a function due to its unique feature of reciprocal connectivity with all areas of isocortex, its central positioning in brain, and its connections with the thalamus.

Crick and Koch (2005) went on to propose that the claustrum, through its broad connections with cortex and thalamic structures, receives the multimodal information that represents one’s sensory world, where it is then “*rapidly combined and bound in the claustrum*” (Crick and Koch, 2005). The binding of multisensory information in the claustrum thus underlies the unification of sensory experiences. They offered the analogy that if different areas of cortex were like players in an orchestra, they would be able to individually play with ease. However, once these players attempted an orchestral piece, they would grow increasingly out of synchrony without the help of a conductor, that being the claustrum. Thus, in an ‘aclaustral’ human, the different sensory stimuli of a basketball, for example (brown color, round shape, rubbery aroma, nubby texture, and the tinny reverberation as it bounces on the ground), may all be sensed, but the perception may only be of a random array of isolated stimuli, rather than as the unified object we readily recognize as a well-inflated, bouncing basketball.

Although converging lines of evidence are beginning to strongly point toward a multisensory integration function for the claustrum, how this role might tie into global brain function, or into Crick and Koch’s model, is completely unknown. Furthermore, without a behavioral correlate to claustrum dysfunction, investigators are left with very little

information from which to base future experiments. With the advancement of Crick and Koch's (2005) hypothesis, however, claustral researchers hopefully may now be armed with the inspiration to tackle what is proving to be one of the great mysteries of systems and cognitive neurosciences.

Summary

The claustrum is a grey matter nucleus that is arguably present in all mammals. In primates, it is surrounded by white matter, lying sandwiched between the striatum and the insular cortex. In the rat, the anatomical boundaries of the claustrum are debated, but it is generally thought that the claustrum ventrolaterally abuts the EC and forceps minor. The claustrum, like cortex, has excitatory projection neurons and interneurons. The expression pattern of transcription factors define the claustrum as a pallial structure, but since it is not layered, should not be considered a cortical structure, per se. Tract tracing studies have demonstrated that in addition to the widespread reciprocal connections with cortex, the claustrum receives inputs from and may project to some subcortical structures, including the dorsal thalamus, striatum, and lateral hypothalamus. Although the function of claustrum is unknown, evidence suggests a role in multisensory integration.

CHAPTER II

REDEFINITION OF CLAUSTRUM ANATOMY

Introduction

It is evident that many fundamental aspects of the “problem” of the claustrum remain, particularly regarding function. How can these issues be overcome? In an effort to understand any nucleus in brain, it is essential to have a clear understanding of form before function can be surmised. Unfortunately, despite more than a century of structural investigation into the claustrum, the complete picture of its anatomy remains as “hidden” as its name suggests. It is therefore imperative to first fully elucidate the structural boundaries of this nucleus. Only then is it possible to evaluate claustral connectivity and allow for a proper inference of functionality. Without this knowledge, any analysis of claustrum function will be stymied.

Why has the anatomy of the claustrum remained elusive while so many other brain structures have been defined in detail? The claustrum’s unusual shape, coupled with its juxtaposition to white matter structures and insular cortex in the rat have made it exceedingly difficult to target with discrete tract tracer injections. As a result, our *reliable* knowledge of rat claustral connectivity is based exclusively on tract tracer injections into non-claustral sites. Although a feasible approach, an accurate interpretation of results from such studies requires a full understanding of the claustrum’s anatomical boundaries. However, accounts of the anatomical boundaries of the rat claustrum vary widely (Sloniewski et al., 1986; Druga et al., 1993; McKenna and Vertes, 1994; Paxinos and Watson, 2007; Swanson, 2004).

Here, we carefully examine claustral anatomy using classical neuroanatomical techniques. Specifically, this study seeks to define the structural boundaries of the rat

claustrum through histochemical and immunohistochemical approaches, followed by an analysis of connectivity. The resultant data lead to a novel appreciation of the borders of the claustrum as well as the positioning of the claustrum in extended neural circuits. Consideration of the anatomical boundaries of the claustrum as revealed by these studies strongly suggests a new architecture of the claustrum across all therian mammalian brains.

Methods

Subjects. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 300-400 g were group-housed on a 12h light-dark cycle with lights on at 0700, and provided with food and water *ad libitum*. 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 Animal Care and Use Committee.

Histochemistry and immunohistochemistry. Rats were transcardially perfused with room temperature 0.1M sodium phosphate buffer, pH 7.3, followed by ice-cold 4% paraformaldehyde in phosphate buffer. Brains were postfixated in 4% paraformaldehyde overnight and cryoprotected in 0.1M phosphate buffer containing 30% sucrose. Using a sliding microtome, frozen coronal sections of forebrain were cut at 40 µm thickness. Sections were stored in a solution of phosphate buffer with 30% sucrose and 30% ethylene glycol at -20° C. Conventional immunoperoxidase (IP) or immunofluorescence (IF) protocols were used to reveal the localization of several proteins as previously described (Deutch et al., 1996). Primary antibodies used included mouse anti-parvalbumin (1:1000 IF; 1:3000 IP; Sigma-Aldrich Inc., St. Louis, MO), rabbit anti-parvalbumin (1:2000; SWANT, Bellinzona, Switzerland), rabbit anti-FluoroGold (1:3000 IF; Chemicon Inc., Temecula, CA), goat anti-cholera toxin b (1:5000; List Biological, Campbell, CA), mouse anti-crystallin mu (1:150; Novus Biologicals, Littleton, CO) and mouse anti-NeuN (1:1000; Chemicon).

Acetylcholinesterase (AChE) histochemistry was performed according to methods described in Tago et al. (1986) with no iso-OMPA pre-incubation. Cytochrome oxidase (CO) histochemistry was performed according to the procedure of Wong-Riley and Welt (1980). Primate (*Circopithicus aethiops*) tissue was obtained from frozen archival samples. Immunohistochemical protocols on the primate tissue follow the methods previously described (Deutch et al., 1996).

Neuronal tract tracing. Rats were deeply anesthetized with isoflurane and placed in a stereotaxic frame. The retrograde tracers FluoroGold (FG, Fluorochrome, Englewood, CO) and cholera toxin-B (CTb) were injected into various areas of cortex, thalamus and hypothalamus, including the prelimbic region of the prefrontal cortex (PFC), anterior cingulate cortex, primary motor cortex, primary somatosensory cortex, somatosensory association cortex, mediodorsal nucleus of the thalamus, ventral posterior complex of the thalamus, nucleus reuniens, and the lateral hypothalamus (LH). A glass pipette (20 µm tip diameter) was used to iontophoretically deposit a 3% solution of FG in 0.1M cacodylate (+ 2.5 µA, 7 sec on/7sec off for 10min). CTb was pressure injected at a volume of 50 nL.

Results

Rat histochemical and immunohistochemical analysis. Histochemical staining for CO resulted in a relatively discrete ovoid staining pattern in the region of the claustrum at striatal rostrocaudal levels. In contrast, CO histochemistry did not reveal any discrete discernible nucleus in what has been called the claustrum at levels rostral to the anterior pole of the striatum (Fig. 4). As such, it was not possible to define a body of the claustrum at levels anterior to the striatum. However, at striatal levels the claustrum (body of the claustrum) was apparent, although it was not contiguous with the medially lying EC, being separated from the white matter by a cellular zone (Fig. 4B). Histochemical staining for

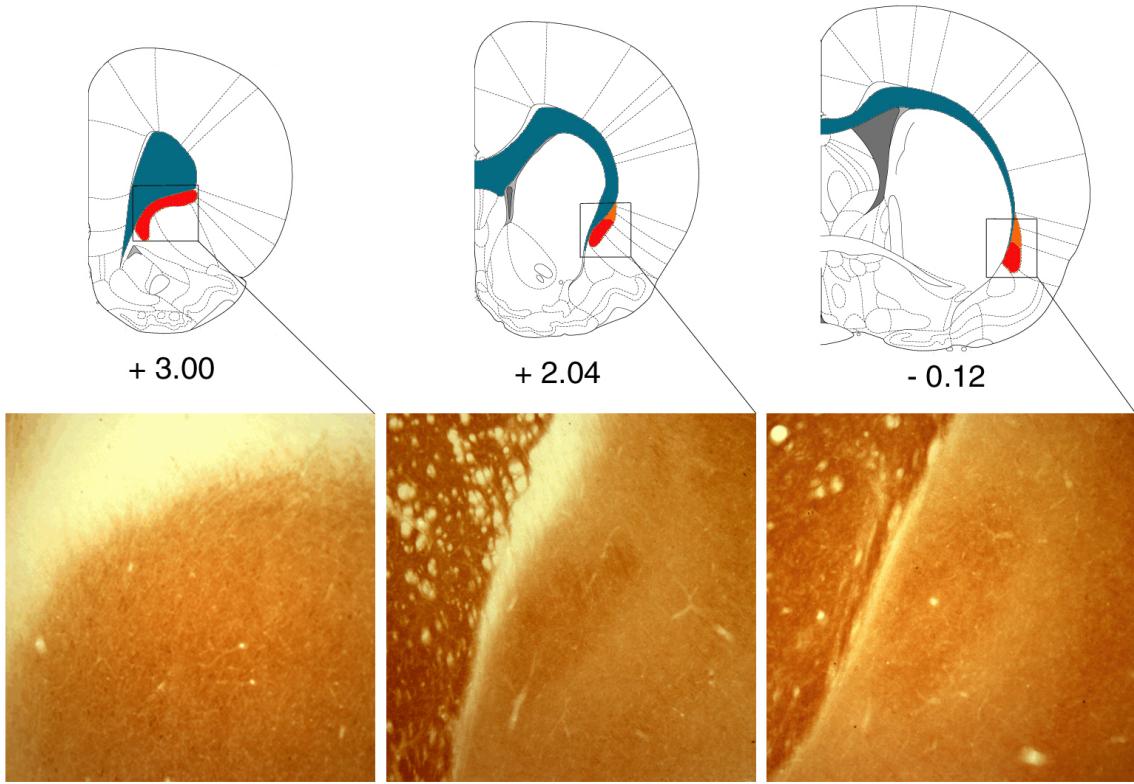


Figure 4. Cytochrome oxidase (CO) histochemistry reveals the structural boundaries of the claustrum at striatal levels only. Regions circumscribed in the atlas depictions represent the area of CO staining below. Values represent distance in mm relative to bregma. Anatomical boundaries consistent with current definitions are undetectable at levels rostral to the striatum (A). In contrast, at striatal levels CO histochemistry reveals a distinct claustrum. Unlike previous definitions, however, the claustrum (as seen particularly well at bregma + 2.04) appears to be rotated away from the medially lying EC.

AChE revealed a pattern of axons that surrounded a zone of AChE-poor staining that corresponded to the claustrum at striatal levels (Fig. 5D). Consistent with the CO histochemistry, AChE staining allowed for a visualization of a distinguishable claustrum at striatal but not more rostral levels (Fig. 5C).

In order to buttress our histochemical findings, we assessed the distribution of several proteins using immunohistochemical methods. PV-ir, the vesicular glutamate transporter (vGLUT) 3-ir, and the neuronal marker NeuN-ir all revealed a discernible body of the claustrum at striatal levels (Fig. 5), but did not delineate a claustral structure rostral to the striatum. Notably, at levels rostral to the striatum, AChE, PV-ir, NeuN-ir and vGLUT3-ir all showed a laminar pattern of staining paralleling the ventrolateral face of the forceps minor (Fig. 5).

All of the histochemical and immunohistochemical stains, including PV-ir, revealed that the claustrum was not immediately adjacent to the EC. Closer examination of AChE fibers revealed a distinct band of darkly-stained axons between the EC and the body of claustrum (Fig. 6A). This band of fibers appeared medial and dorsal to the claustrum and continues along the border with the EC streaming ventrally beyond the claustrum. The pattern of these AChE fibers coursing dorsally and ventrally to the claustrum and between the claustrum and the EC suggests a separation of the claustrum from the white matter. Consistent with this suggestion was the observation that NeuN-ir neurons were interposed between the neurons of the claustrum and the EC, albeit at lower density than that of the claustrum. These neurons were in the area occupied by the darkly stained AChE fibers (Fig. 6B). These observations suggest that the claustrum may be embedded within deep layers of the insular cortex. It should be noted that CO histochemistry and the distribution of PV-ir and vGLUT3-ir also indicate that the claustrum is separated from the EC (Fig. 5), and not adjacent to the EC as had been previously described.

To further characterize this finding, immunohistochemical staining for crystallin mu

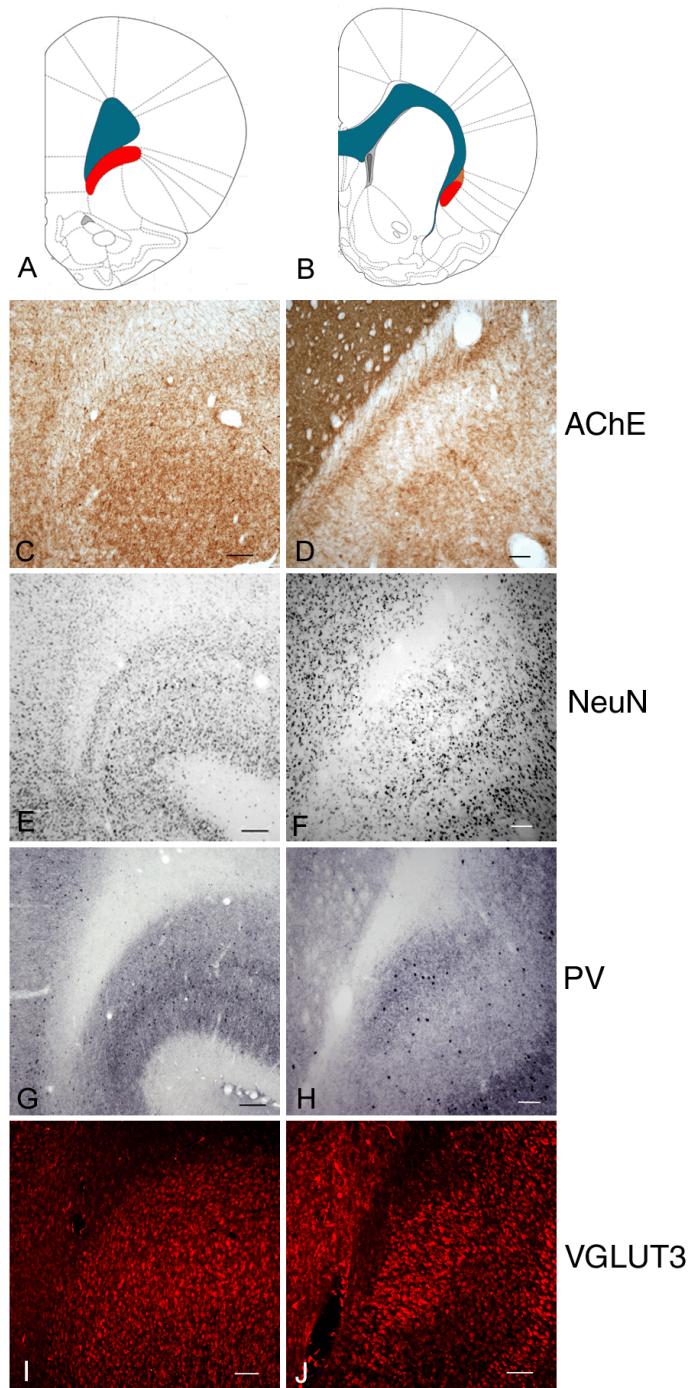


Figure 5. Further histochemical and immunohistochemical analyses reveal a distinguishable structural boundary of the claustrum at striatal levels, but not at more anterior levels. The atlas depiction in panel A represents the rostrocaudal level for panels C, E, G, and I. Panel B represents the rostrocaudal level for panels D, F, H, and J. Histochemical analysis for acetylcholine esterase (AChE) (C, D), as well as immunohistochemical analysis for the neuronal marker NeuN (E, F), the calcium-binding protein parvalbumin (G, H), and the vesicular glutamate transporter 3 (vGLUT3) (I, J) are consistent with the CO histochemical findings. Scale bars: (C, E, G, I) 200 μ m; (D, F, H, J) 100 μ m.

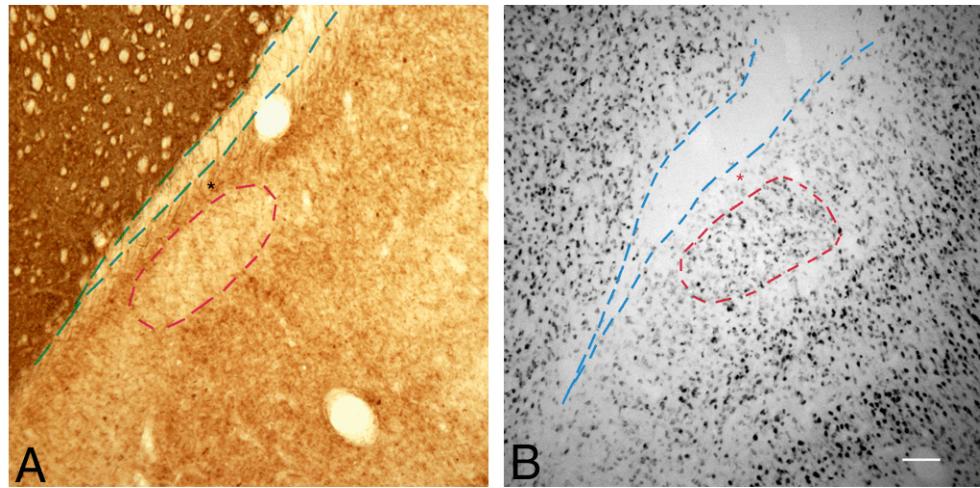


Figure 6. Histochemical and immunohistochemical analyses suggest the claustrum does not lie immediately adjacent to the EC. AChE histochemistry reveals an AChE-rich band of fibers (asterisk) that extends along the EC (outlined in blue) and appears to separate the claustrum (outlined in red) from white matter (A). NeuN immunohistochemistry on an adjacent section reveals that this AChE-rich region between the claustrum and the EC contains neuronal cell bodies (asterisk) (B). Scale bar: 100 μ m.

(Crym), a marker for layers deep V and VI, revealed a “ring” of Crym-ir cell bodies and neuropil surrounding a zone of Crym-absent staining (Fig. 7). Double staining for PV and Crym revealed that Crym-ir neatly encompasses the PV-defining body of the claustrum (Fig. 7), indicating that the claustrum is not immediately adjacent to white matter, but is embedded within insular cortex. Because PV resulted in the greatest distinction between claustrum and surrounding structures (Figs. 5, 7), it was used as a “marker” with which to define the claustrum for subsequent tract tracing analysis.

Rat neuronal tract tracing. To determine the relationship between the structural borders of claustrum as defined by PV localization, we injected the retrograde tract tracer FG into various cortical and subcortical sites. FG was first deposited into the medial prefrontal cortex (Fig. 8). The injection site was restricted to the prelimbic cortex and did not involve white matter structures. Retrograde labeling in the claustrum in this case showed numerous FG-filled cell bodies in the PV-ir claustrum at striatal levels, but not at more rostral (frontal cortical) levels (Fig. 9). A discrete iontophoretic deposit of FG in the cingulate cortex (24b) (Fig. 10) revealed a similar pattern of retrograde labeling seen after FG injection into prelimbic cortex (Fig. 11). Thus, retrogradely-labeled cells were not observed in the territory anterior to the striatum that was previously designated as the claustrum. FG injections into several other cortical regions, including including motor, somatosensory, and somatosensory association cortices (data not shown) all resulted in the same staining pattern: retrogradely labeled cells in the claustrum at striatal levels, but 1) not at more rostral (frontal cortical) levels, and 2) not in the zone between the claustrum and the EC.

Injections of FG into various subcortical sites revealed a converse staining pattern. We first injected FG into the dorsal thalamus, including the mediodorsal, central medial, intermediodorsal, and paraventricular thalamic nuclei (Fig. 12). At levels rostral to the

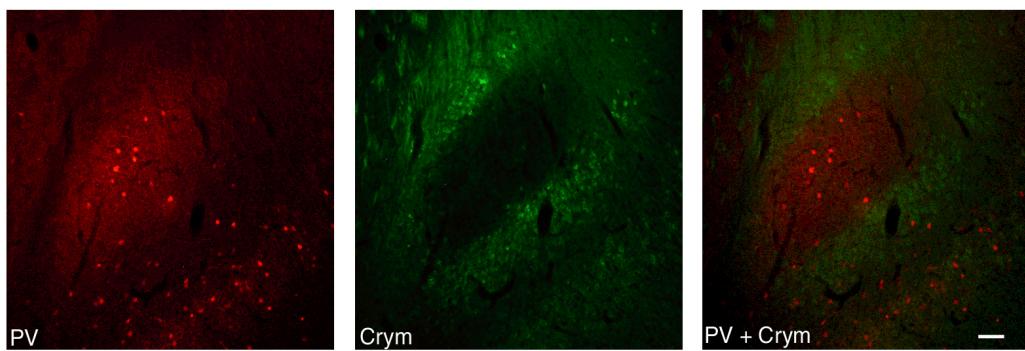


Figure 7. Immunohistochemistry reveals the claustrum to be embedded within layer VI of insular cortex. Parvalbumin (PV, red) immunofluorescence reveals the body of the claustrum. Immunohistochemical staining for Crystallin mu (Crym, green)-ir, a protein whose expression is restricted to deep layers of cortex, surrounds the PV-ir body of the claustrum (PV+Crym). Scale bar: 100 μ m.

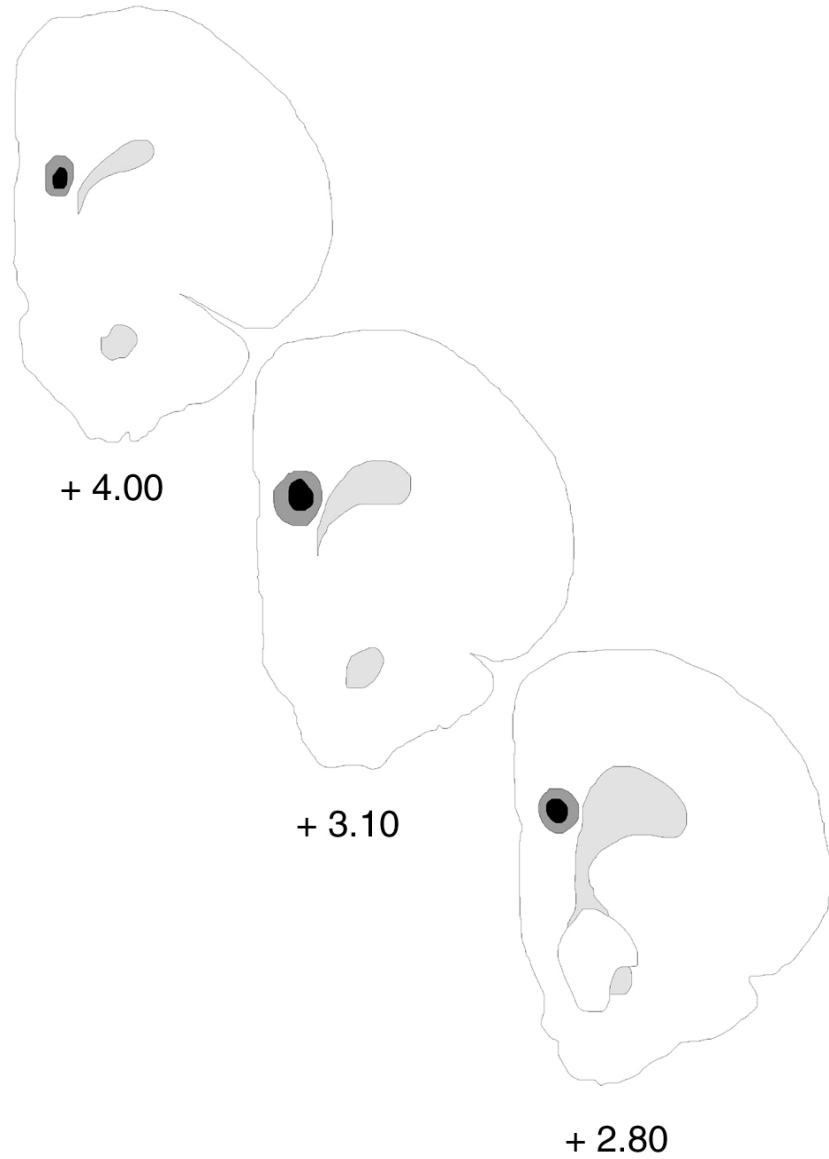


Figure 8. Reconstruction of an iontophoretic deposit of FluoroGold (FG) into the medial prefrontal cortex (prelimbic region, area 32), shown at three different rostrocaudal levels. Values are distances in mm relative to bregma. The core of the deposit is depicted in black, and the penumbra in gray. The injection primarily involves layer VI. White matter structures are shaded light gray.

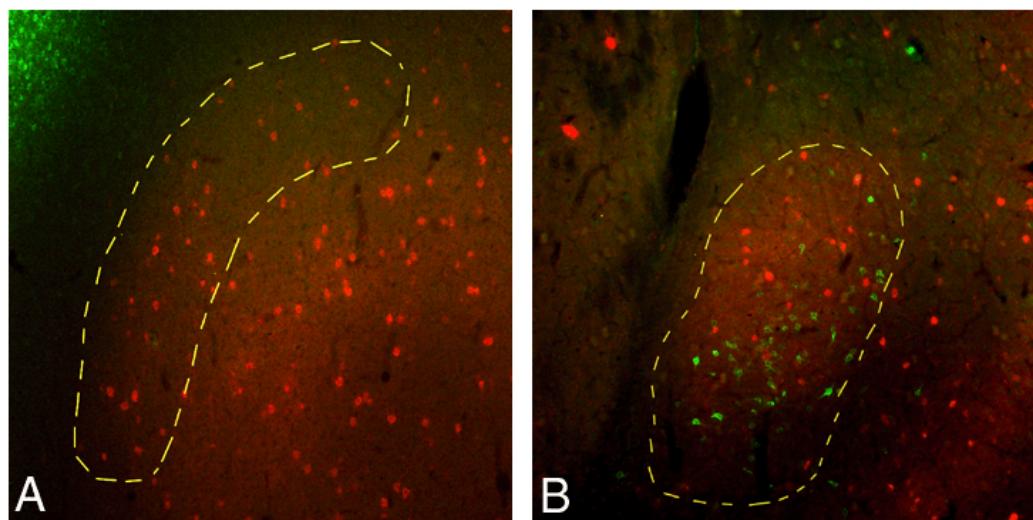


Figure 9. The region of the “claustrum” rostral to the striatum is hodologically distinct from the claustrum at striatal levels. The photomicrographs show double immunofluorescence for PV (red) and FG (green) from an animal injected with FG into the medial prefrontal cortex (Fig. 8). Rostral to the striatum (panel A) no FG-positive cell bodies are seen in the area previously defined as the claustrum (outlined in yellow). However, at striatal levels (panel B), numerous FG-positive cell bodies are in the PV-defining body of the claustrum.

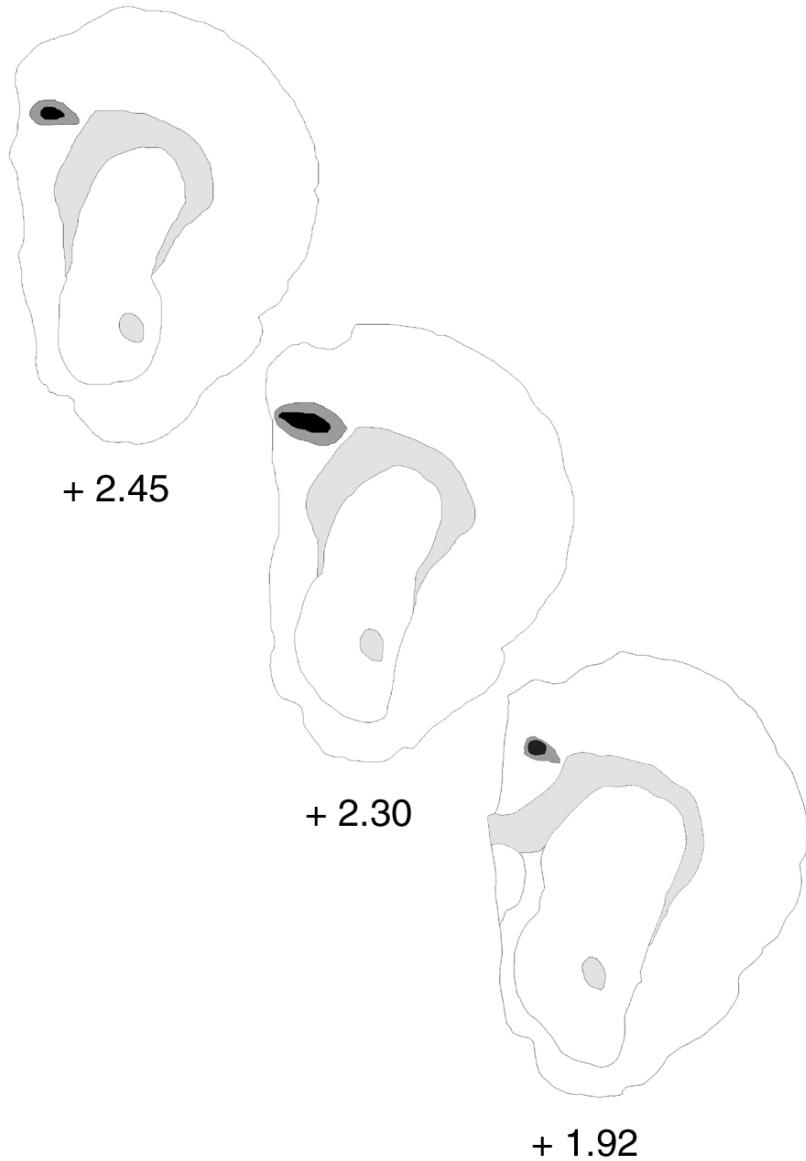


Figure 10. Reconstruction of an intophoretic deposit of FG into the pregenual anterior cingulate cortex (area 24b) shown at three different rostrocaudal levels. The injection spans layer II/III and layer V.

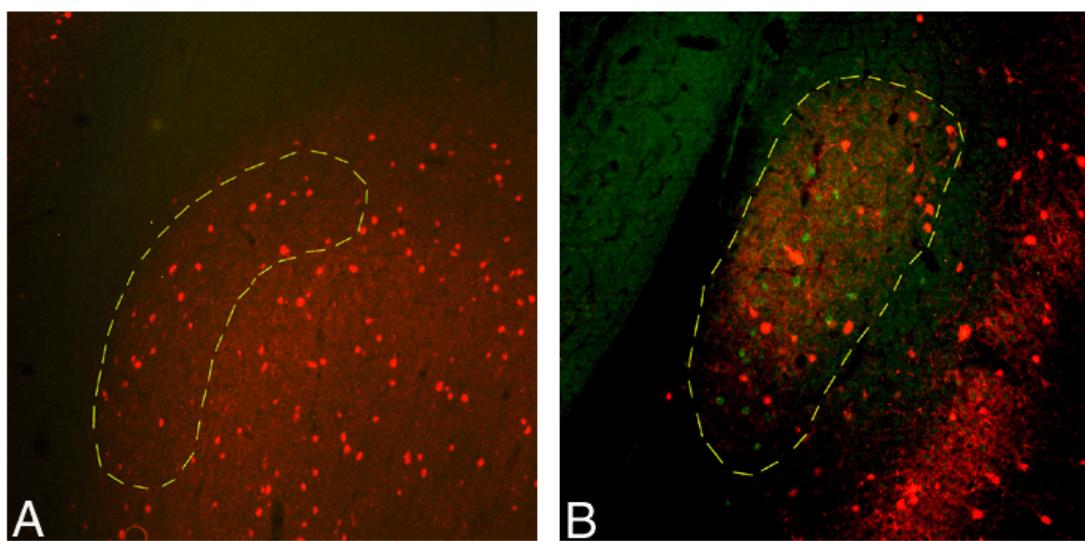


Figure 11. Injection of a retrograde tract tracer into the anterior cingulate cortex (see Fig. 10) reveals differences in connectivity between the area of the “claustrum” rostral to the striatum and the claustrum at striatal levels. Rostral to the striatum, no FG-positive cell bodies (green) are present in the area previously defined as the claustrum (outlined in yellow) (panel A). However, at striatal levels, numerous FG-positive cell bodies are in the PV-defining body of the claustrum (B).

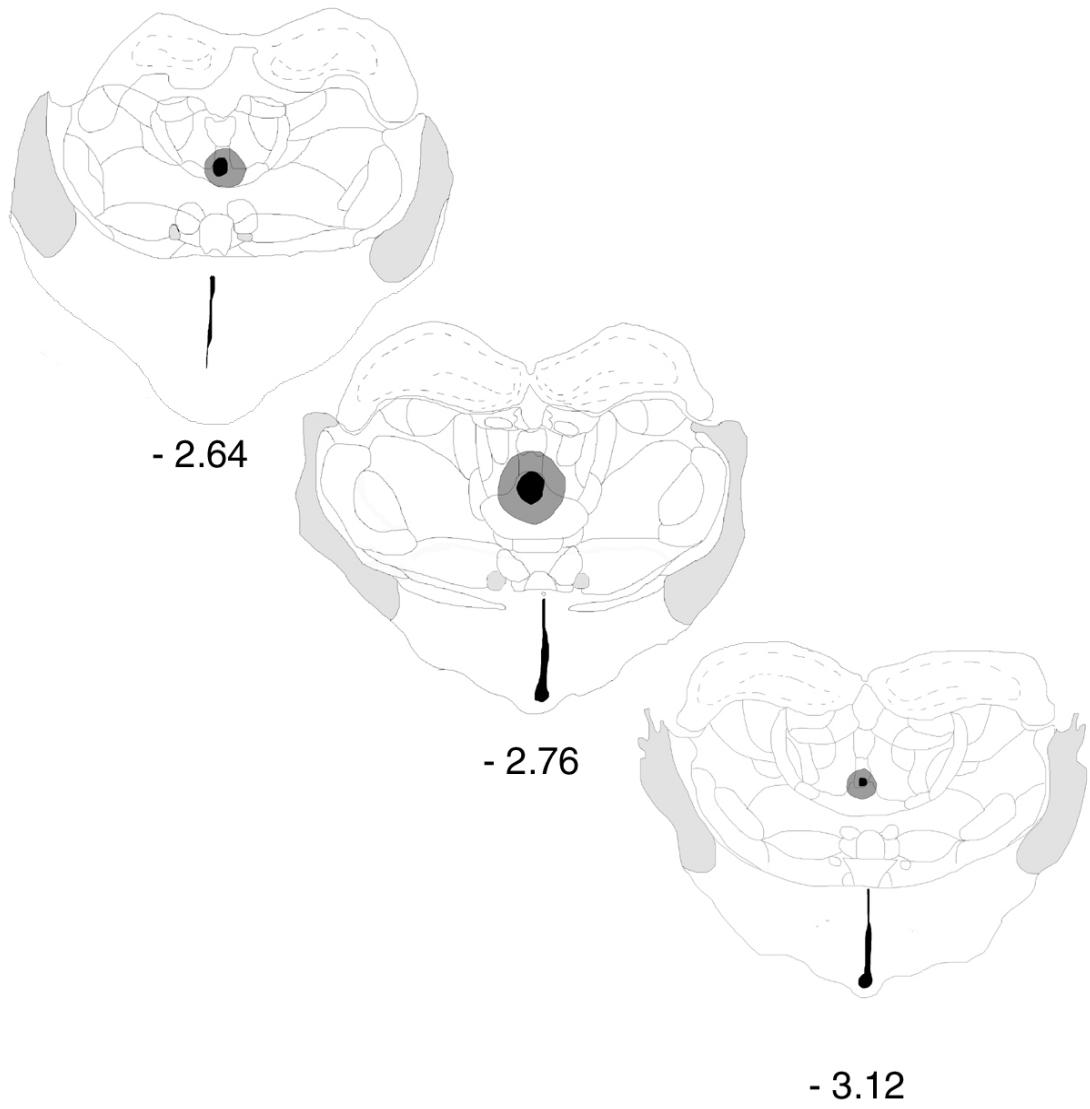


Figure 12. Reconstruction of an intophoretic deposit of FG into the central aspect of the dorsal thalamus shown at three different rostrocaudal levels. The injection involves aspects of the paraventricular, mediiodorsal, intermediodorsal, and central medial thalamic nuclei.

striatum, retrogradely-labeled cells were observed in the area previously defined as the claustrum, but no FG-positive cells were present within the claustrum at striatal levels. Retrogradely-labeled cells were, however, present within the laterally-lying layer VI of insular cortex and between the claustrum and the EC (Fig. 13), indicating that the claustrum is surrounded by layer VI of insular cortex. Interestingly, following a deposit of the retrograde tract tracer Ctb into the LH (Fig. 14), this same effect was seen. In this case, CTb-ir cells were present in the area rostral to the striatum previously defined as claustrum as well as completely surrounding (but not invading) the body of the claustrum at striatal levels (Fig. 15). Several other injections into various regions of the dorsal thalamus and LH also revealed this pattern of labeling (data not shown). These results further suggest that the claustrum is surrounded by insular cortex and that the territory rostral to the striatum in the convexity of the forceps minor (that which was previously described as claustrum) displays a pattern of connectivity consistent with deep layers of insular cortex, not the claustrum.

Vervet histochemical and immunohistochemical analysis. Since our phenotypic expression patterns and tract tracing data suggest that the rat claustrum is surrounded by layer VI of insular cortex, what does this imply for species where the claustrum is embedded within white matter, such as primates? Considering the accretion of white matter fibers that accompanies isocortical elaboration events through time, the existing EC widens, an internal capsule develops and an extreme capsule arises laterally around the body of the claustrum. Based on our findings, one might reasonably conclude that the relationship of EC-insular layer VI-claustrum is still present. Moreover, it would not be unreasonable to think that in species where the claustrum is embedded within white matter, that the extreme capsule, as it formed through time, did not perfectly parcel claustrum from insular layer VI. Thus, insular layer VI may also be surrounding the body of the claustrum in species

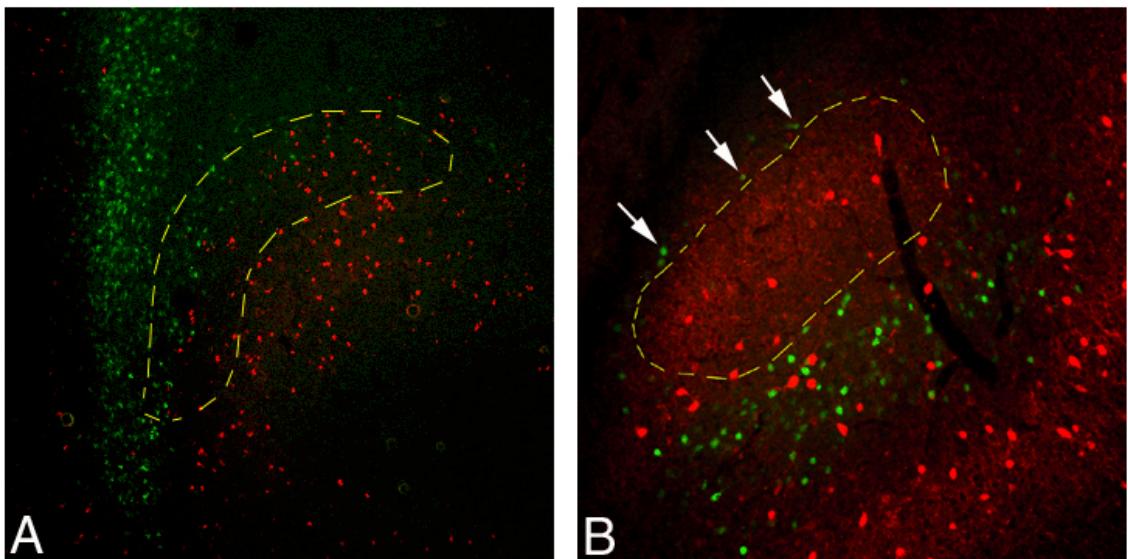


Figure 13. Injection of a retrograde tract tracer into the dorsal thalamus further distinguishes the rostral “claustrum” from the claustrum at striatal levels. Double PV (red) and FG (green) immunofluorescence from an animal injected with FG into the dorsal thalamus (Fig. 12). In panel A, rostral to the striatum, several FG-positive cell bodies are present in the area currently defined as the claustrum (outlined in yellow). However, at striatal levels (panel B), FG-positive cell bodies surround the PV-defining body of the claustrum. Notably, FG-positive cell bodies are present in the area of insular cortex (deep layer VI) between the claustrum and the EC (arrows).

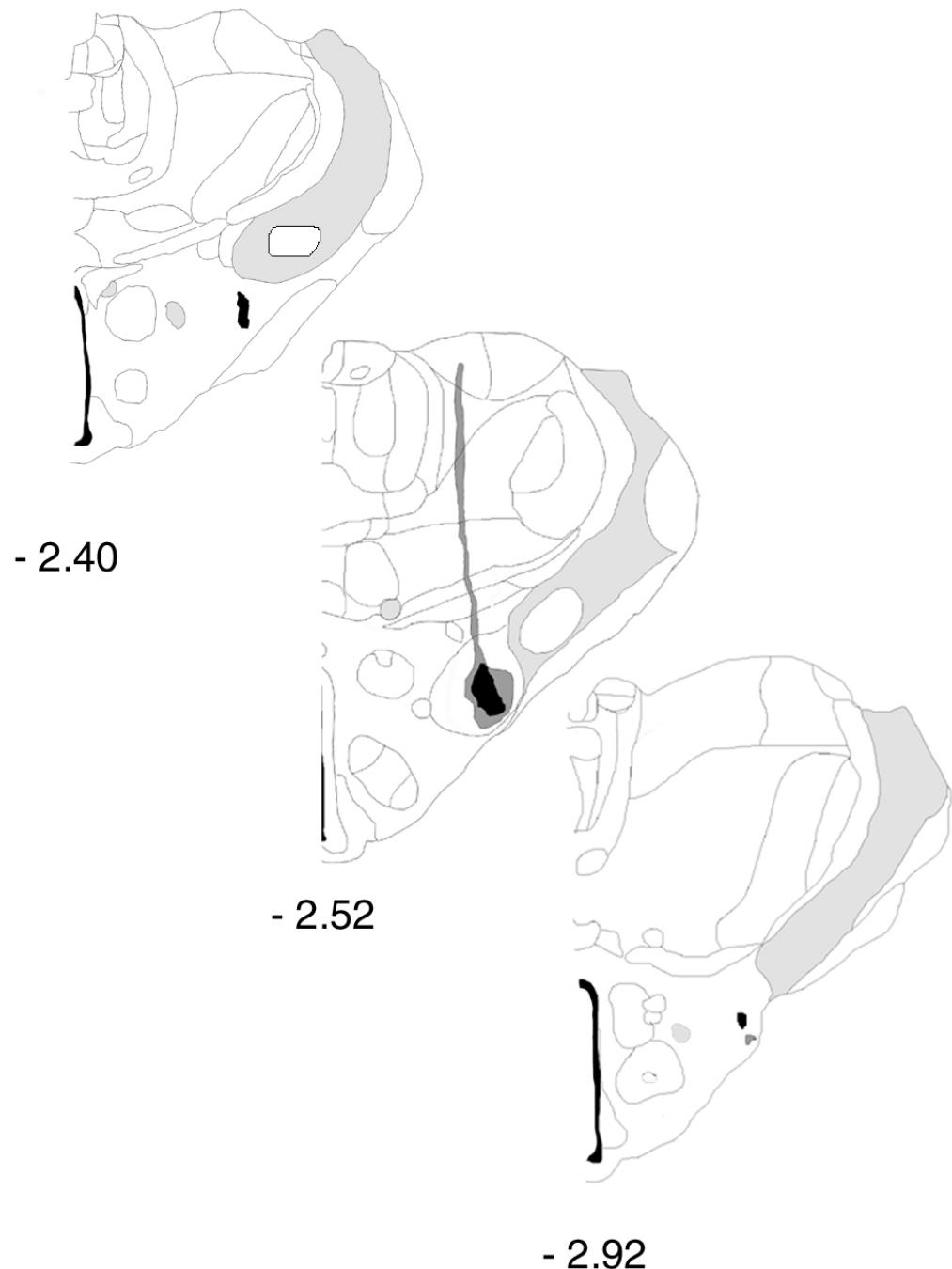


Figure 14. Reconstruction of a pressure injection of cholera toxin b (CTb) into the lateral hypothalamus shown at three different rostrocaudal levels.

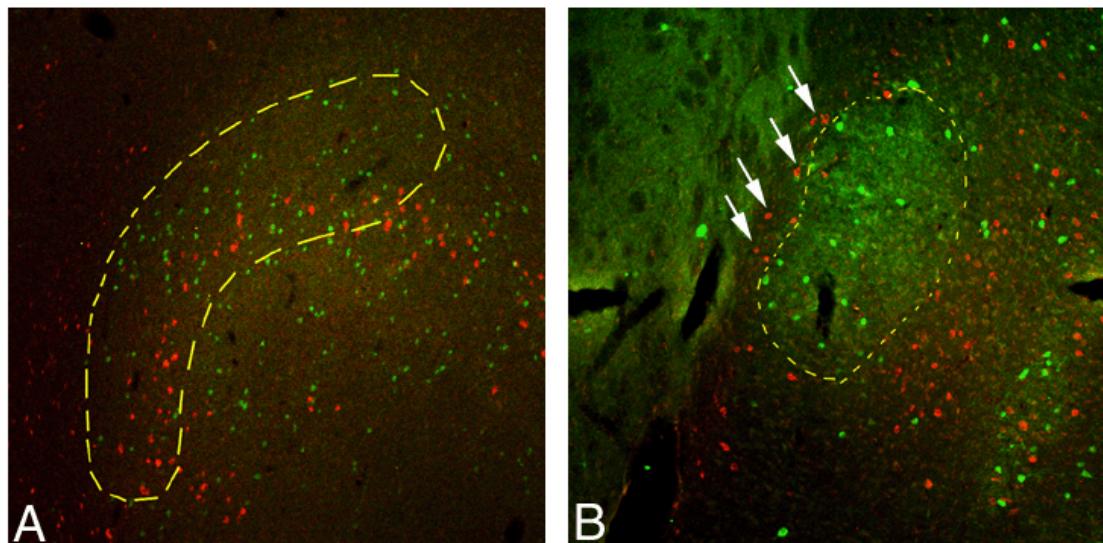


Figure 15. Injection of a retrograde tract tracer into the lateral hypothalamus (see Fig. 12) recapitulates the connectivity pattern of dorsal thalamic injections. Rostral to the striatum, CTb-positive cell bodies (red) are present in the area previously defined as the claustrum (outlined in yellow) (panel A). However, at striatal levels, CTb-positive cell bodies surround the PV-defining body of the claustrum (arrows) (panel B).

possessing an extreme capsule. We therefore examined this possibility in an old world monkey.

CO histochemistry of vervet forebrain tissue revealed the body of the claustrum distinctly from surrounding white matter (Fig. 16). AChE histochemistry showed that the border between the claustrum and the surrounding capsular fibers is much less defined, such that the claustrum appears to expand, particularly toward the neighboring striatum (Fig. 13). This external expanse of AChE fibers is particularly heavy on the medial aspect, as was seen in the rat claustrum (Fig. 13). This indicates that just as AChE fibers lie between the EC and the claustrum of the rat (insular layer VI), the same is true for the primate.

Immunohistochemical staining for PV revealed the body of the claustrum within the white matter of the vervet. Double staining for Crym and PV revealed a Crym-ir ring surrounding the PV-positive body of the claustrum (Fig. 17). This band of Crym-ir, which was particularly dense on the medial side of the claustrum, extended dorsally along the perimeters of the claustrum. As the claustrum extends dorsally and curves laterally, Crym-ir was intermingled with PV-ir cell bodies (data not shown). This indicates that some of what is currently described as the dorsal extension of the claustrum in the primate is actually insular layer VI.

Conclusions

Using classical neuroanatomical techniques, we demonstrate evidence for a significant shift in rat claustrum anatomical boundaries and, as a result, the connectivity pattern of this nucleus. We posit that the claustrum is limited to striatal anterior-posterior levels only and does not lie immediately adjacent to the EC, but is embedded within layer VI of the dysgranular and agranular insular cortices. A graphical depiction of this redefinition is detailed in our summary diagram (Fig. 18). According to this structural

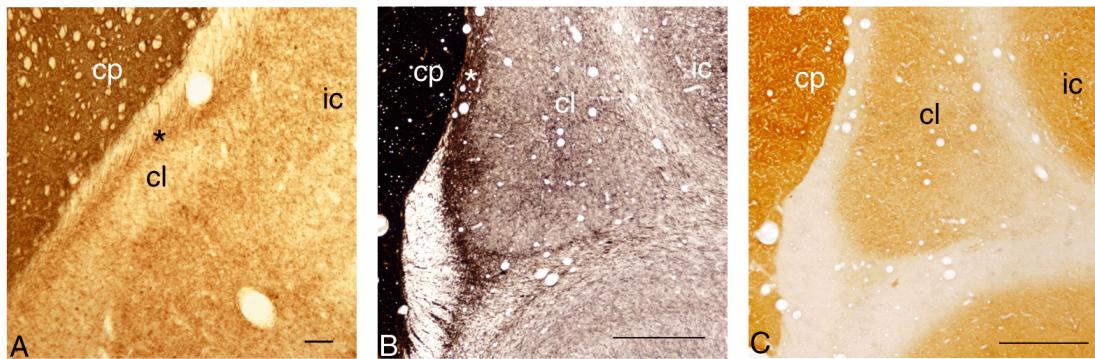


Figure 16. Histochemical analysis of macaque telencephalon suggests that the claustrum is surrounded by insular cortex. AChE histochemistry in the rat reveals that the area between the claustrum and the EC is richly invested with cholinesterase fibers (asterisk) (panel A). Similarly, AChE staining in the macaque reveals a band of fibers medial to the claustrum (asterisk) (panel B). CO staining in an adjacent section reveals the actual body of the claustrum is smaller than that suggested by AChE staining (C). Abbreviations: striatum (cp); claustrum (cl); insular cortex (ic). Scale bars: (A) 100 μ m; (B, C) 1 mm.

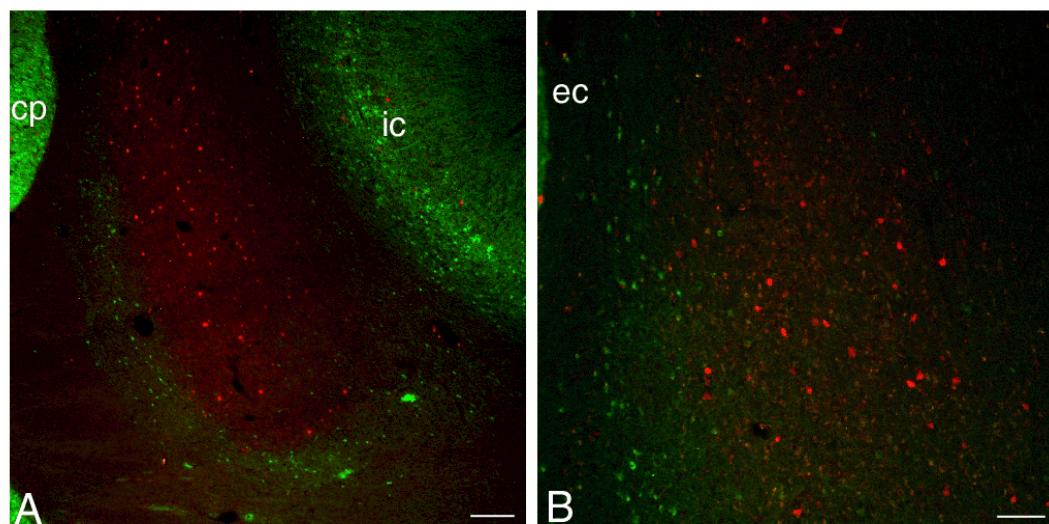


Figure 17. Immunohistochemistry reveals the claustrum in the vervet is surrounded by insular cortex. Double immunofluorescence for PV (red) and Crym (green) in the ventral aspect of the claustrum shows a band of Crym-positive neurons surrounding the PV-positive body of the claustrum (A). A magnified view of the dorsal aspect of the macaque claustrum reveals many Crym-ir insular cortex cells on the EC side of the claustrum (B). Abbreviations: striatum (cp); insular cortex (ic); EC (ec). Scale bars: (A) 200 μ m; (B) 100 μ m.

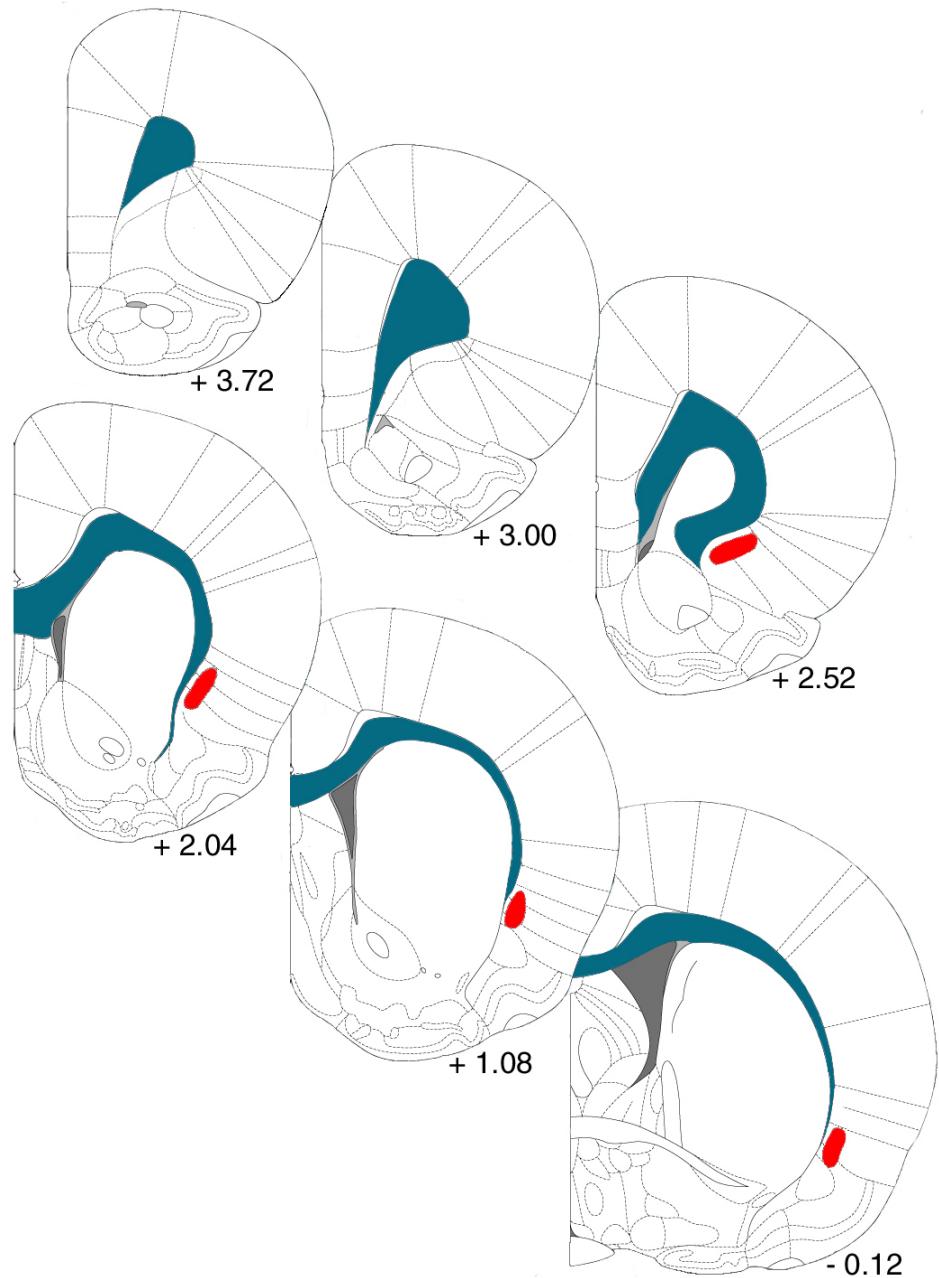


Figure 18. The revised view of the structural boundaries of the rat claustrom. White matter structures are shaded blue, the claustrom is shaded red. Values represent distance in mm relative to bregma. What was previously designated the claustrom ventrolateral to the forceps minor at levels anterior to the striatum ($\geq + 3.00$) has been removed, as this is insular cortex. Furthermore, at all striatal levels, the claustrom is embedded within the insular cortex, although it gradually migrates closer to the EC as it extends caudally. The “dorsal claustrom” as defined by Paxinos and Watson (2007) has been removed. Modified from Paxinos and Watson (2007).

redefinition, the claustrum is reciprocally connected to isocortical sites, and not connected to the dorsal thalamus or the lateral hypothalamus (Fig. 19). This finding overhauls the claustrum connectivity literature, which states that the claustrum innervates both of these regions. Our data indicate that many, if not all, of the subcortical connections once assigned to the claustrum should now be considered to arise from the deep layers of insular cortices.

Following tracer injection into the dorsal thalamus, retrogradely-labeled cells were seen abutting the forceps minor, a region currently defined as the claustrum. Lateral hypothalamic injections resulted in retrogradely-labeled cells not only immediately adjacent to the forceps minor, but uniformly removed ventrolaterally, suggesting a laminar organization. Since this area of the claustrum consistently shared retrograde labeling patterns with that of deep layers of insular cortex at striatal levels, we conclude that layers V and VI of insular cortices reside in this area, rather than the claustrum as is currently accepted. As such, this restructuring would represent a significant mediiodorsal extension of the insular cortex toward the forceps minor. Such an alteration significantly changes the known laminar organization of this region, and, therefore, warrants a thorough re-examination.

Recently, Paxinos and Watson (2007) suggested the presence of a “dorsal claustrum” that differs from the main body of the claustrum. We have not observed expression of any protein that delineates claustral boundaries in this dorsal zone. Moreover, the “dorsal claustrum” does not contain cells that are retrogradely-labeled from cortical sites, nor does it contain anterogradely-labeled fibers following BDA injections into PFC (data not shown). Because the “dorsal claustrum” was defined based upon histochemical observations (Paxinos and Watson, 2007), it is likely that the “dorsal claustrum” is a transitional zone where cortical layer VI begins to diverge as it envelops the body of the claustrum, with deep layer VI turning ventromedially and the more superficial part of layer

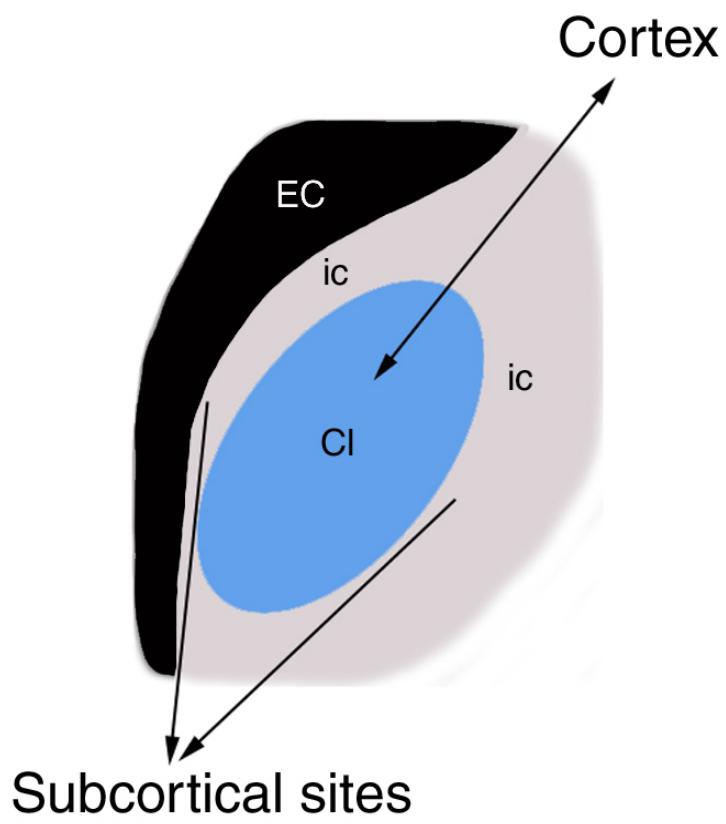


Figure 19. Summary diagram of the revised structural boundaries and connections of the claustrum. The external capsule (EC) is shaded black, the claustrum (cl) blue, and the insular cortex (ic) gray. The claustrum reciprocally connects to all cortical areas, while the surrounding insular cortex projects to subcortical sites, including the dorsal thalamus and the lateral hypothalamus.

VI coursing ventrolaterally. Thus, “dorsal claustrum” likely does not represent a unique nucleus.

Our findings in the green vervet indicate that the claustrum is surrounded by rudimentary insular layer VI matter. Since the green vervet claustrum is embedded within white matter, as it is in humans, this suggests that the claustrum in humans is surrounded by insular layer VI as well. Further supporting this idea, Crym *in situ* hybridization results in the Allen Brain Atlas (<http://www.brain-map.org>) show Crym-positive cell bodies surrounding what appears to be a body of the mouse claustrum. This observation bolsters our assertion that the Crym-ir neuropil we observed surrounding the claustrum in rat and green vervet arise from insular cortex. Our findings likely call for a redefinition of claustrum neuroanatomy in humans, as well as all other therian species.

CHAPTER III

CORTICAL REPRESENTATIONS IN THE RAT CLAUSTRUM

Introduction

The data presented in the previous chapter, including the revised definition of the claustrum and its connections, resolves several of the historical “problems” noted by Ariëns-Kappers and co-authors (1960) regarding the structural boundaries and connections of this structure. However, publications over the past 50 years have led to a new “problem,” that of cortical representations within the claustrum. Several studies have now shown that the claustral cortical connectivity is organized into modality-specific zones (Kemp and Powell, 1970; Künzle, 1975; Jones et al., 1977; Künzle and Akert, 1977; Olson and Graybiel, 1980; Dineen and Hendrickson, 1982; Pearson et al., 1982; Mufson and Mesulam, 1982; Tannè-Garièpy et al., 2002). Using electrophysiological approaches, Olson and Graybiel (1980) showed that the claustrum in cat is organized into sensory-specific zones that both send projections to and receive projections from specific sensory cortices. These findings raise an interesting question: if the claustrum is indeed functioning as a multisensory integrator, how is information bound within the claustrum if these sensory zones are non-overlapping?

Crick and Koch (2005) offered a solution to this problem by proposing that waves of activity might flow through the claustrum, subserving communication between zones of this structure. They hypothesized that an as yet unidentified interneuron population, dendro-dendritic chemical synapses, or gap junctions formed between claustral interneurons, may account for the intra-claustral communication necessary for the claustral subdivisions to associate with one another and ultimately allow for the integration of multimodal information. In this way, the claustrum organizes cortical inputs such that different sensory cortices are synchronized in order to bind multimodal information and

generate a conscious percept.

How does one begin to test this hypothesis? It has been well accepted that the cortex is arranged in a topographical manner, and that information is processed across these topographical regions in a hierarchical manner (Weller et al., 1984; Kaas, 1987; Felleman and Van Essen, 1991; Kaas and Garraghty, 1991). According to this organization, sensory information is first processed by primary sensory cortical areas, followed by secondary sensory, sensory association, and finally frontal “executive-level” cortical centers. Because analyses of the cortical representations across the hierarchy of cortical processing centers are very limited in the rat (Li et al., 1986; Sadowski et al., 1997), we examined regions in the rat brain representing the full hierarchy of cortical processing, including primary sensory, sensory association, and frontal cortices. This work uncovers a zonal distribution of cortical representations of sensory, association, and prefrontal cortices, but a uniform distribution of the anterior cingulate cortex representation within the claustrum of the rat. These results are considered in a functional context.

Methods

Subjects. Methods follow those described in Chapter II.

Immunohistochemistry. Methods follow those described in Chapter II.

Neuronal tract tracing. Methods follow those described in Chapter II.

Analysis. Anatomical boundaries of the claustrum were defined by the findings in the previous chapter (see Fig. 18). The distribution of retrogradely-labeled claustral neurons were charted and injection sites were reconstructed using NeuroLucida software (MicroBrightField Inc; Williston, VT).

Results

To assess the representation in the claustrum of cortical regions that span the hierarchy of information processing, we examined claustrocortical connections with primary sensory cortex, sensory association cortex, and higher order processing sites of the frontal cortices. Because all sensory zones within the claustrum, regardless of modality, receive projections from and send projections to the cognate area of cortex (Olson and Graybiel, 1980), we chose to examine the claustrocortical connections of just one sensory modality in the rat: somatosensation. As opposed to the visual system, which has been widely studied in several species, the rat sense of somatosensation is well developed. Thus, claustrocortical projections to the following regions were assessed: S1 somatosensory, lateral parietal association, medial prefrontal (prelimbic), and pregenual anterior cingulate cortices (area 24b).

Deposit of FG into the primary somatosensory cortex resulted in an injection site spanning the forelimb region, dysgranular zone, and a portion of the barrel field region (Fig. 20), involving layer II/III. S1 injections of FG consistently resulted in retrogradely-labeled cells that were restricted to the dorsal half of the claustrum (Fig. 20). We next examined retrograde labeling following injection of FG into the lateral parietal association cortex (Fig. 21), involving layers II/III and IV. This injection site was discretely localized to this region. Retrograde labeling was seen in the dorsal half of the claustrum at all rostrocaudal levels (Fig. 21).

In contrast to the S1 and lateral association cortex injections, iontophoretic deposit of FG into the medial prefrontal cortex (prelimbic region) resulted in FG-ir cells in the ventrolateral aspect of the claustrum at all rostrocaudal levels (Fig. 22). At rostral levels, the number of FG-ir cells was noticeably greater than that of S1 or lateral parietal association cortical injections (Fig. 22). The retrogradely-labeled cells at this level nearly spanned the entire ventrolateral half of the claustrum. More caudally, the number of FG-ir

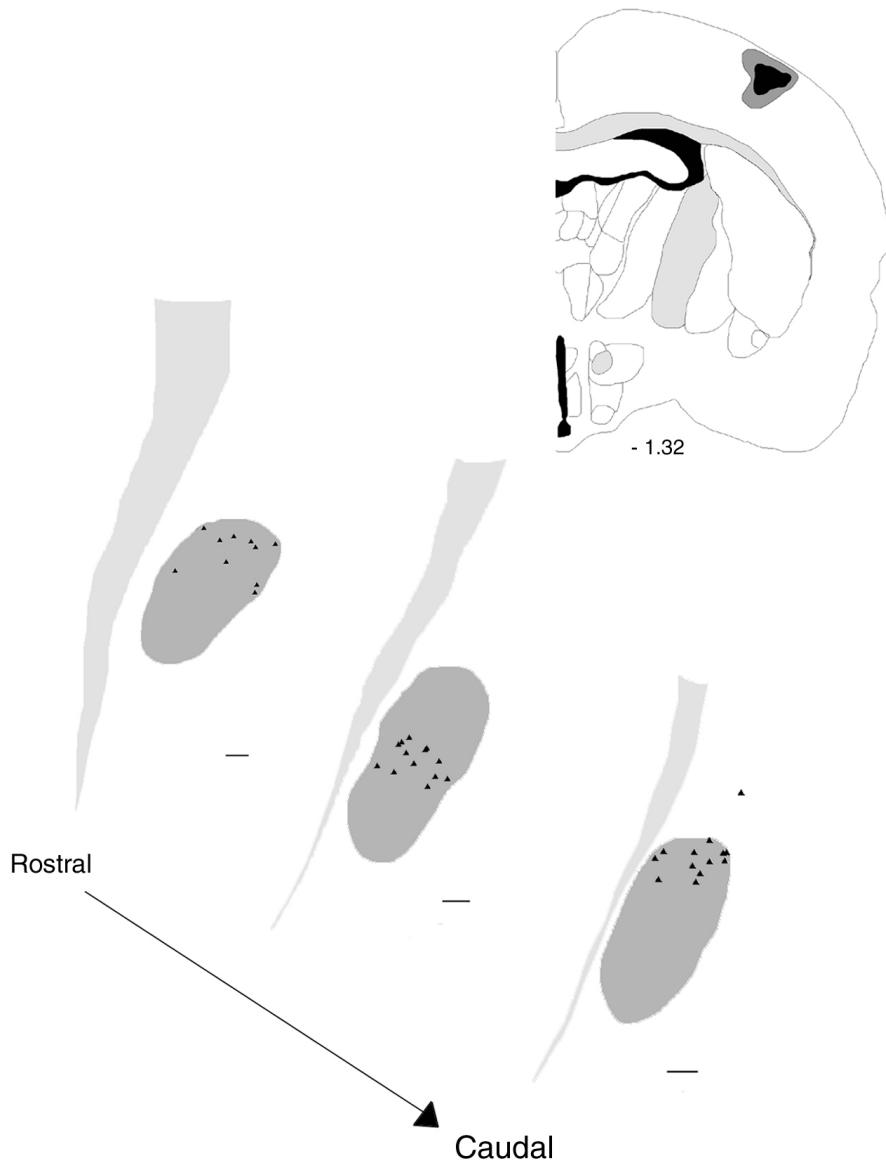


Figure 20. The S1 representation in the claustrum of rat is restricted dorsally. Reconstruction of a pressure injection deposit of FG into S1 (upper right). The core of the injection site is shaded black, the penumbra is shaded dark gray, while white matter structures are shaded light gray and ventricular lumen is shaded black. Chartings of retrograde labeling in the claustrum at three different rostrocaudal levels (lower left). FG-positive cells are present throughout the entire rostrocaudal extent of the claustrum. Scale bars: 100 μ m.

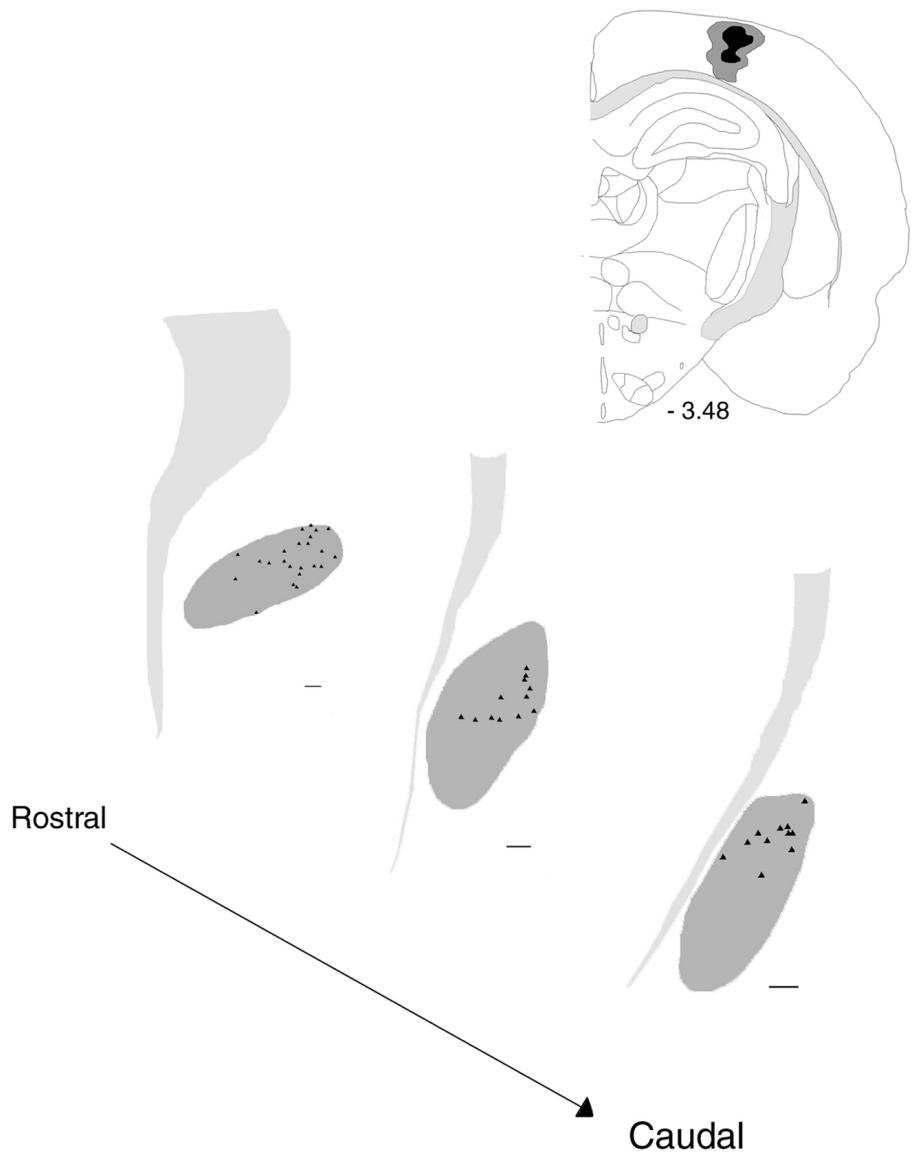


Figure 21. The lateral parietal association cortex (LPtA) representation in the claustrum of rat is also restricted dorsally. Reconstruction of a pressure injection deposit of FG into the LPtA (upper right). Chartings of retrograde labeling in the claustrum at three different rostrocaudal levels (lower left). FG-positive cells again are present throughout the entire rostrocaudal axis of the claustrum. Scale bars: 100 μ m.

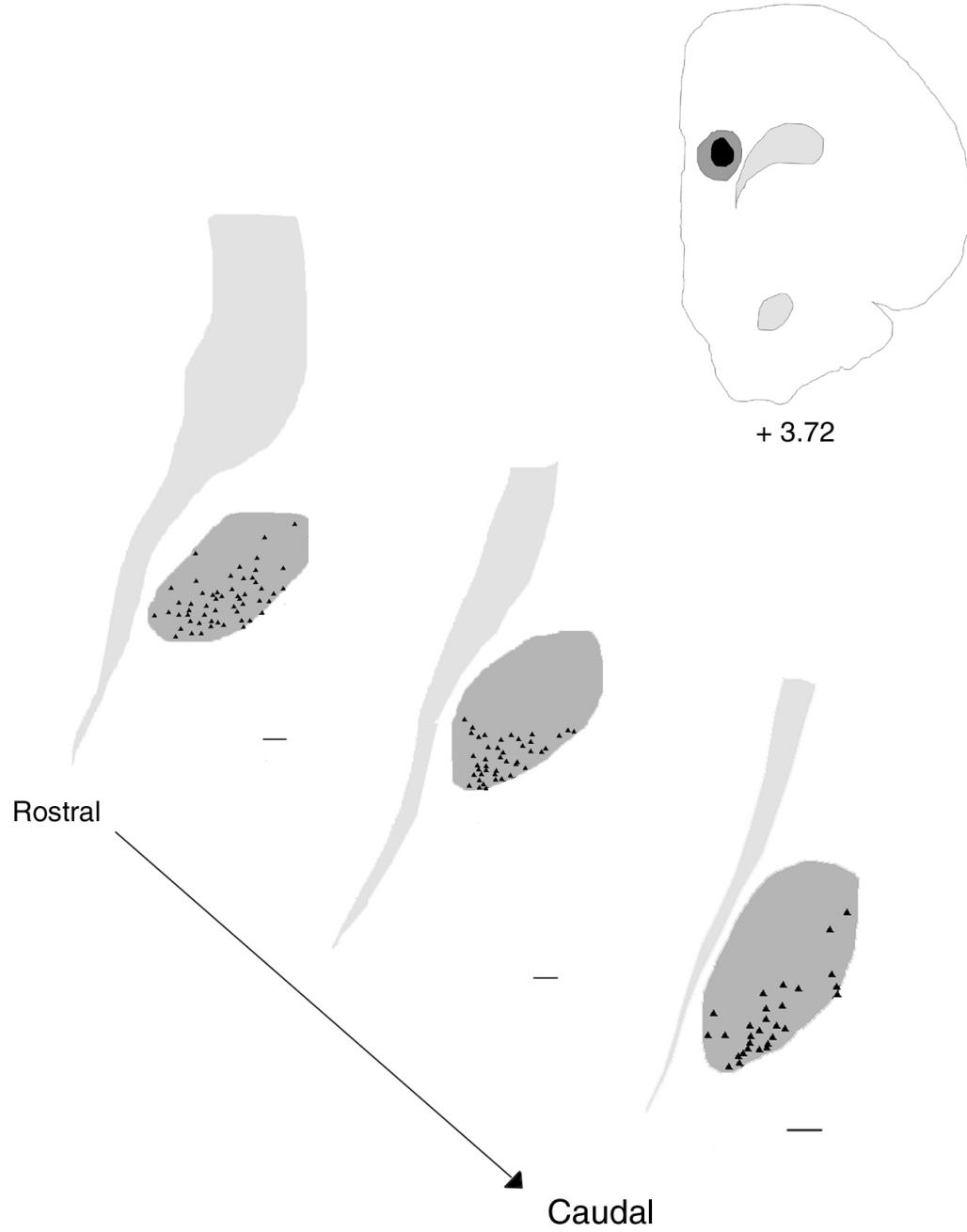


Figure 22. The medial prefrontal (prelimbic) cortex (mPFC) representation is restricted to the ventrolateral part of the claustrum. Reconstruction of an iontophoretic deposit of FG into the mPFC (upper right). Chartings of retrograde labeling in the claustrum at three different rostrocaudal levels (lower left). FG-positive cells are present throughout the entirety of the rostrocaudal extent of the claustrum, where they reside ventrolaterally. Scale bars: 100 μ m.

cells remained high relative to S1 and association cortical injection labeling, but the distribution of cells appeared more restricted ventrolaterally as compared to rostral levels (Fig. 22).

Unlike all previous sites examined, iontophoretic deposit of FG into the anterior cingulate cortex (area 24b) did not result in a zonal distribution of retrograde labeling within the claustrum (Fig. 23). Instead, FG-ir cells were distributed uniformly throughout the body of the claustrum at all rostrocaudal levels. The number of FG-ir cells was much greater in rostral levels as compared to more caudal sections (Fig. 23).

Conclusions

Here we show that primary somatosensory and lateral parietal association cortices have the most limited representations within the claustrum, and that these representations are restricted to the dorsal aspect of the claustrum. These findings are consistent with previous studies showing restricted zones of primary sensory cortical representations in the rat (Li et al., 1986; Sadowski et al., 1997). In particular, Sadowski and coworkers (1997) observed a representation of S1 cortex in the dorsal claustrum, consistent with our findings. In other species, restricted representations of primary sensory, association, and dorsolateral prefrontal cortices in the cat and monkey have also been demonstrated (Olson and Graybiel, 1980; Mufson and Mesulam, 1982; Pearson et al., 1982; Tanné-Gariépy et al., 2002). Pearson et al. (1982) showed that the dorsal aspect of the claustrum in primate harbors the S1 representation, while the representation of areas 8 and 9 is in the ventral-to-central portions of the claustrum, depending on the rostrocaudal level examined. Thus, these previous reports largely corroborate our findings with regard to these cortical areas.

To our knowledge, this is the first study in the rat examining the representation of the anterior cingulate cortex in the claustrum. We show that the representation of this area is uniformly distributed across the claustrum. This represents a novel finding as most, if not

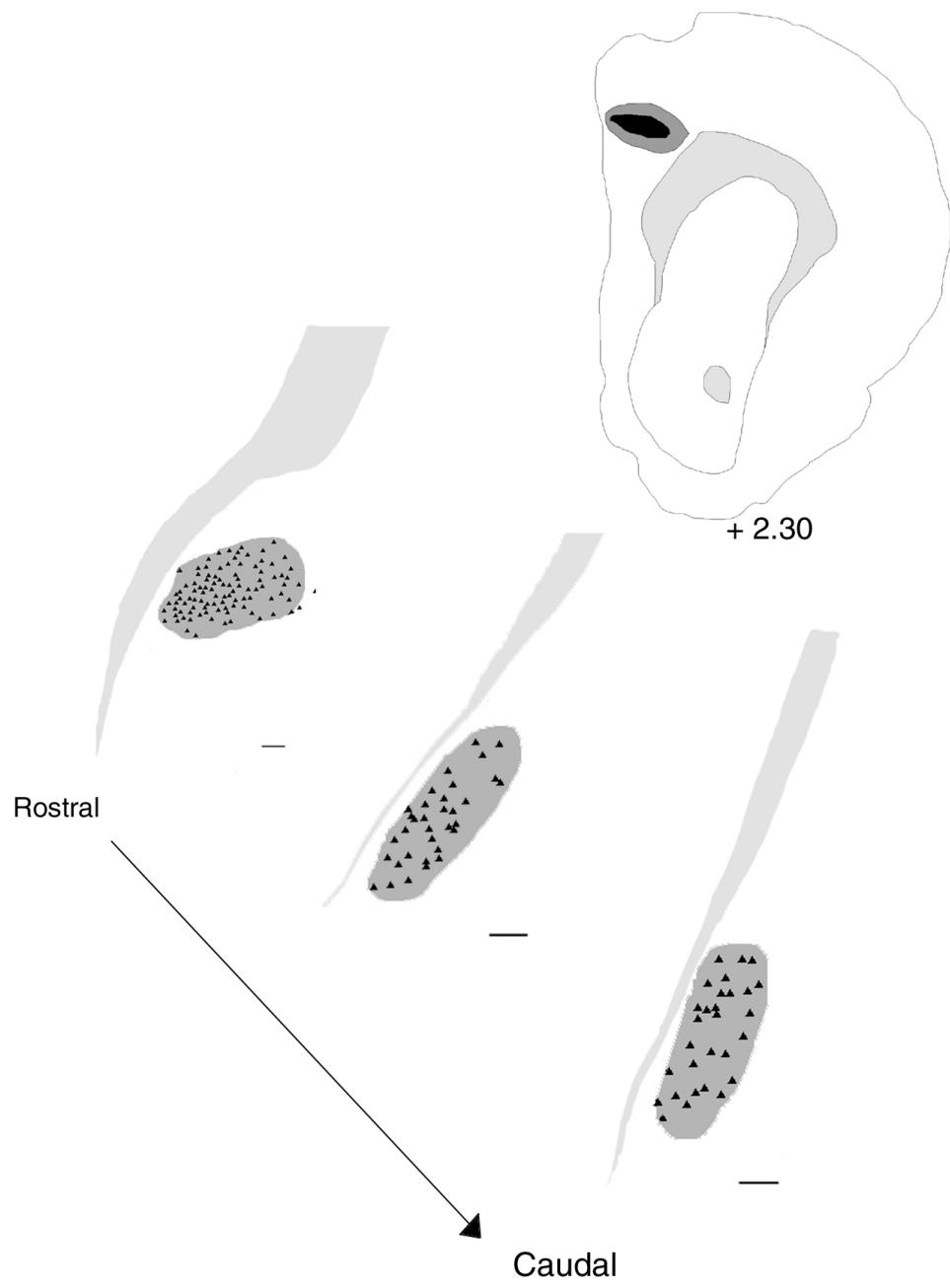


Figure 23. The anterior cingulate cortex representation in the claustrum of rat is relatively uniform throughout the claustrum. Reconstruction of an iontophoretic deposit of FG into the anterior cingulate (area 24b) (upper right). Chartings of retrograde labeling in the claustrum at three different rostrocaudal levels (lower left). FG-positive cells are present throughout the entirety of the rostrocaudal extent of the claustrum, where they are uniformly distributed. Scale bars: 100 μ m.

all, cortical representations examined to date in any species exhibit a restricted pattern of representation (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). The anterior cingulate cortex has been examined in the cat as well. Macchi and coworkers (1981) found that the distribution of the anterior cingulate representation, relative to all other cortical sites examined, is quite expansive. Markowitsch and colleagues (1984) also found that the cat anterior cingulate representation was widely distributed in the claustrum. In both cases, the distribution would not be considered as uniform as our findings would suggest. This discrepancy must, however, be viewed with caution because the definition of the structural boundaries of the cat claustrum used in these studies may have been inaccurate in light of the findings presented in Chapter II. A thorough mapping of the entire anterior cingulate cortex representation in the cat claustrum adhering to accurate claustral structural boundaries is needed to resolve these issues.

Perusal of the figures in this study might suggest that a laminar topography of claustrocortical projections exists. That is, that injections largely targeting layers II/III and IV result in retrograde labeling of the dorsal aspect of the claustrum, while injections into deeper layers result in retrograde-labeling in the ventral part of the claustrum. If all layers are targeted, as it would appear in the anterior cingulate injection site, then the distribution of retrogradely-labeled cells in the claustrum are uniformly distributed. Although the figures may loosely suggest such a conclusion, additional cases that were not presented involving injections into S1 and parietal association cortices spanning all layers reliably resulted in retrograde-labeling of the dorsal aspect of the claustrum. In addition, several cases of prefrontal cortical injections that varied in depth reliably resulted in labeling of the ventral aspect of the claustrum. Furthermore, Sadowski and colleagues (1997) showed that injections into rat S1 also resulted in labeling of the dorsal part of the claustrum, while Li and coworkers (1986) showed that injections involving all layers of primary motor and visual

cortices resulted in retrograde-labeling in the dorsal and ventral aspects of the claustrum, respectively. Therefore, our findings coupled with reports in the literature indicate that a laminar topography of claustrocortical projections does not exist.

In light of our data, conclusions can be drawn regarding sensory integration in the claustrum. If the Crick and Koch (2005) solution to this “binding” problem is true, that information is shared between subdivisions of the claustrum, the law of parsimony would suggest that the subdivisions within the claustrum would represent an unnecessary processing step. That is, if information is to be “bound” in the claustrum, why doesn’t input converge into already-overlapping cortical representations? Additionally, if the claustrum functions in the way that Crick and Koch predict, the claustrum is “above” executive cortical processing sites in the hierarchy of information processing (see Fig. 24). This means that the claustrum, through its receipt of connections from all areas of cortex, “decides” what sensory information should and should not be “bound.” This appears to be a terrific amount of processing for a structure that does not contain a laminar/columnar organization.

We propose that an alternate possibility to the Crick and Koch (2005) solution to the “binding” problem may be that the processing of sensory integration does not occur in the claustrum itself, but in a cortical site. In this scheme, our findings would suggest that the uniform representation of the anterior cingulate cortex, which spans all sensory subdivisions, allows for all sensory information to be channeled through the claustrum to this executive-level frontal cortical area for the purpose of “deciding” upon what information should or should not be bound. Once the appropriate binding combination for a particular set of multisensory inputs is “decided” upon in the anterior cingulate cortex, then information could be transmitted back through necessary sensory subdivisions within the claustrum in order to activate the appropriate sensory cortices for perception of the stimulus (Fig. 25).

This model also raises the possibility that the claustrum may represent a mechanism

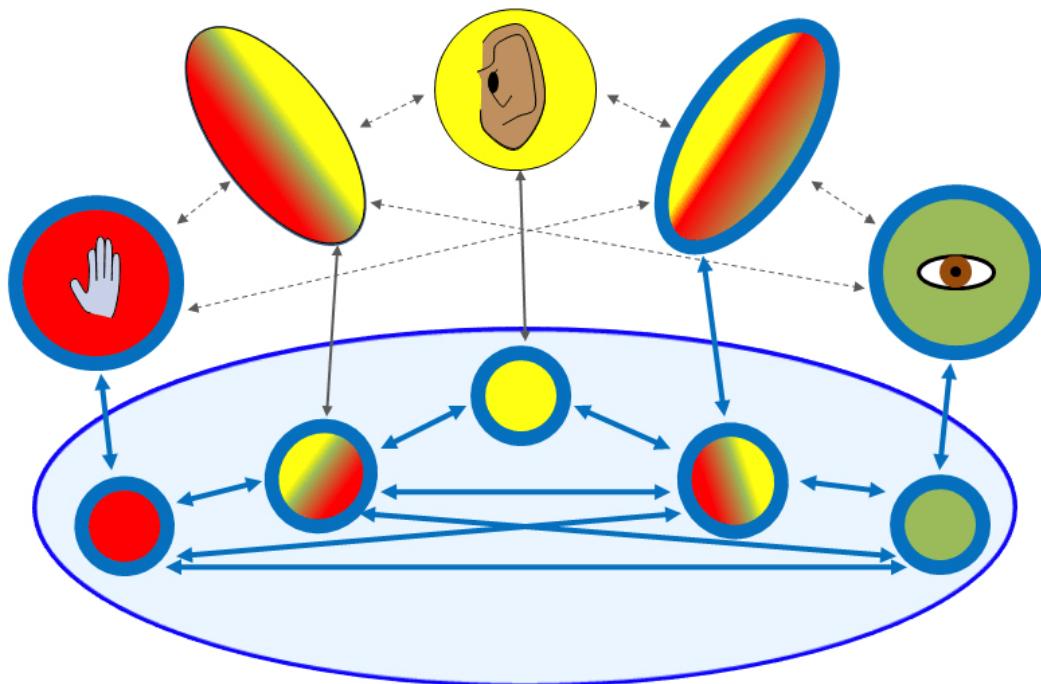


Figure 24. A diagrammatic representation of the Crick and Koch model of claustrum function. Sensory (hand, eye, ear) and association (rainbow shapes) cortices are represented on top and the claustrum is represented below (large light blue oval). Arrows indicate connectivity. Sensory subdivisions within the claustrum are color coded to reflect modality and are spatially segregated. Heavy blue lines indicate expected circuitry activation during a tactile-visual matching task. In this model, “waves” of activity flow through the claustrum during polymodal convergence in order to “decide” upon which sensory stimuli should be bound. The claustrum is also responsible for synchronizing the appropriate sensory cortices for the generation of the polymodal, conscious percept. Note that the zonal arrangement of the claustrum serves little purpose in this model.

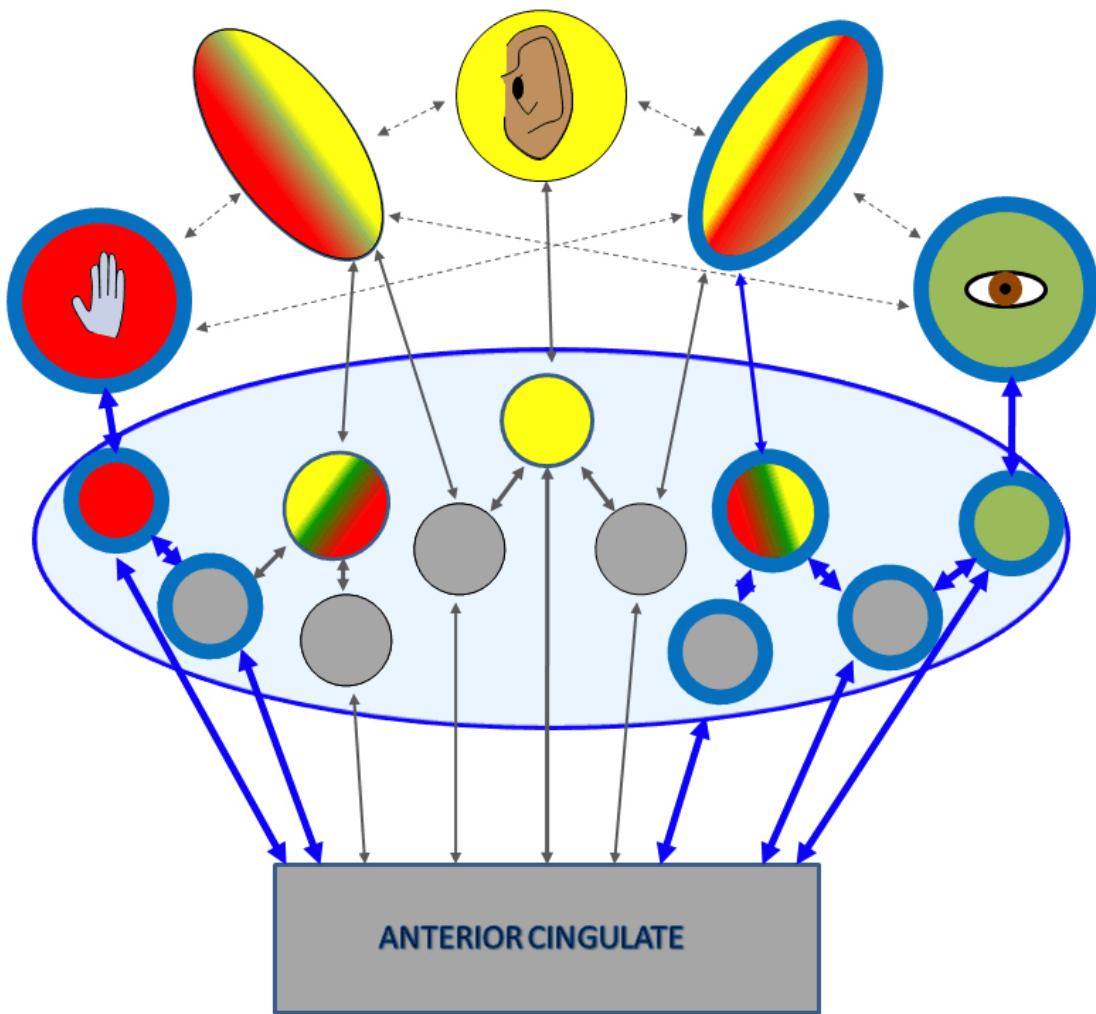


Figure 25. Our proposed model for claustral circuitry and function. The anterior cingulate cortex is depicted below the claustrum and gray circles in the claustrum represent cells projecting to the anterior cingulate cortex. Consistent with our findings and those of others, there are more anterior cingulate-projecting cells than any other type in the claustrum, and they are uniformly distributed. In addition, in fitting with the electrophysiological data, 75% of the cells are depicted as being multimodal. Heavy blue lines indicate expected circuitry activation during a tactile-visual matching task. Note that this model requires the zonal arrangement of the claustrum: the signal arising from the anterior cingulate cortex of what information should be bound, and/or what information should be attended to, is channeled through claustral subdivisions in order to synchronously activate (for multisensory integration) or synchronously prime (for attention) the cognate sensory cortices for perception of, or attention to, the stimuli.

for attentional allocation. Suppose that the uniform distribution of the anterior cingulate representation means that the claustrum supplies the anterior cingulate cortex with salient sensory information across all modalities. In this scenario, a signal derived from the anterior cingulate to sensory cortices via the claustrum could also serve to “prime” appropriate sensory cortices for the purpose of attending to specific, goal-directed stimuli. The zonal organization of cortical representation in the claustrum again becomes necessary in this context. Using this organization, the anterior cingulate cortex can compare the weight or salience of sensory inputs in order to “decide” what stimuli require attention and channel signals through the appropriate claustrum sensory subdivision to prime the appropriate sensory cortices. If the claustrum was not arranged into discrete zones, and these sensory subdivisions were intermixed, it would seem likely that the categorical allocation of attention to distinct sensory cortices would be blurred.

In opposition to the Crick and Koch (2005) hypothesis, then, we propose that the decision of what should be bound or what should be attended to is placed in the anterior cingulate cortex rather than the claustrum. Assigning this decision-making role to the anterior cingulate cortex is consistent with a large body of literature suggesting this area is involved in monitoring conflicts in information processing (Botvinick, 2007; Carter and van Veen, 2007). This model also takes advantage of, rather than being confounded by, the zonal distribution of cortical representations in the claustrum.

The findings presented in this chapter provide the first report of cortical representations in the claustrum based on the redefined structural boundaries for this nucleus. We show that the anterior cingulate cortex is uniformly represented within the claustrum. This finding represents a significant departure from claustrum dogma that states that all cortical representations in the claustrum are restricted to subdivisions. Our interpretation of this result offers a solution to the problem of sensory binding in the claustrum as elaborated by Olson and Graybiel (1980). We suggest that the claustrum may

not itself be integrating sensory information as Crick and Koch (2005) have suggested, but instead functioning as a component part of a broader sensory integration and/or attentional allocation mechanism involving a distributed network of brain sites including the anterior cingulate, sensory, and association cortices.

CHAPTER IV

IDENTIFICATION OF A NOVEL NEUROANATOMICAL MARKER FOR THE CLAUSTRUM

Introduction

The previous chapter provided a model of claustrum function. However, in order to test this model, or any other hypothesis of claustrum function, the ability to lesion or selectively inactivate the claustrum is required. Such a lesion has not yet been achieved because conventional lesion approaches have been stymied by the shape and size of the claustrum. Conventional methods do not permit the production of discrete mechanical or chemical lesions that do not involve fibers-of-passage or adjacent grey matter structures including the striatum, endopiriform nucleus, and insular cortex. Until such an “aclaustral” animal is generated, the contribution of claustral function to cognition and behavior will remain hidden.

How can the advances in our understanding of claustrum anatomy translate to a solution to the “problem” of claustrum function? As Crick and Koch (2005) suggested, the identification of proteins strongly expressed in claustrum but not surrounding structures is needed in order to generate a molecular lesion of this structure. The discovery of a protein marker whose expression pattern is discretely enriched within our newly-defined claustral borders would pave the way for molecular methods to selectively disrupt claustral function.

The identification of a discrete neuroanatomical marker for the claustrum would also allow for investigators to determine if the claustrum is present in the monotreme clade. Such a finding would settle the existing debate over the phylogenetic origins of the claustrum. Moreover, such a marker would be invaluable in delineating structural boundaries between the claustrum, insular layer VI, and the endopiriform nucleus in those species possessing an extreme capsule.

In order to uncover such a marker, we employed matrix assisted laser desorption ionization imaging mass spectrometry (MALDI-IMS). MALDI-IMS is an unbiased, discovery-based approach that reveals protein anatomical localization as well as relative protein expression information (Laurent et al., 2005; Schwartz et al., 2005; Chaurand et al., 2006; Cornett et al., 2007; Sinha et al., 2008; Andersson et al., 2008; Caldwell et al., 2008). Applying MALDI-IMS directly to rat forebrain sections, we discovered a novel protein marker, G protein gamma 2 subunit (GNG2), that is enriched in the claustrum not but adjacent territories.

Methods

Subjects. Adult male Sprague-Dawley rats were used as subjects.

MALDI-IMS. Briefly, rat brains were dissected and immediately frozen on powdered dry ice. Coronal sections were cut at 12 µm in the coronal plane on a cryostat. Sections were thaw mounted onto gold MALDI target plates and dried in a vacuum decissator for 30 min. Sections were then washed in 70% ethanol for 30 sec., followed by two washes in 95% ethanol for 30 sec. each. Excess liquid was drained and the sections were placed back in the vacuum decissator for an additional 10 min. Discrete 100 pl drops of sinapinic acid (SA) matrix (20 mg/ml SA in 50% acetonitrile and 0.3% TFA in water) were placed in a Cartesian array across the section using an acoustic picoliter droplet ejector (Portrait 630; Labcyte). Time was allowed between each matrix deposit iteration in order for the matrix to crystallize. Matrix-coated sections were analyzed on a MALDI-TOF instrument operating in linear mode (Autoflex, Bruker Datronics Inc., Billerica, MA). Each matrix spot was shot a total of 200 times by the laser. Data was analyzed with an imaging software tool (BioMap; <http://www.maldi-msi.org>). For further detail see Andersson et al., 2008.

Protein Identification. Freshly dissected claustra were frozen at -80 °C until processed. The tissue was cut into ~ 1 mm square pieces and transferred to an ice-chilled 1 mL DUALL® glass/glass homogenizer (Kontes, Vineland, NJ, USA). The tissue was homogenized in pre-chilled T-PER® tissue protein extraction reagent (50 mg wet weight tissue/1 mL T-PER®) by manual grinding for about 1-2 minutes. The homogenate was transferred to a 15 mL conical tube, placed on ice, and sonificated using a Branson Sonifier 450 Analog ultrasonic homogenizer (Danbury, CT) until foaming occurred (5-10 cycles) using 30% duty cycle and output control set to 3. The tissue homogenate was transferred to microcentrifuge tubes and centrifuged at 14,000 x g for 10 min at 4 °C in order to pellet cell/tissue debris. The supernatant was collected and stored at -80 °C until use. The concentration of total proteins in the supernatant was determined by Bradford assay according to the manufacturer instructions (Pierce; Rockford, IL), using a Molecular Devices SpectraMax® M2e microplate reader (Sunnyvale, Ca).

The tissue homogenate was mixed with an equal volume of 2:98 acetonitrile: water containing 0.1% TFA (v/v) and centrifuged (14,000 x g for 10 min at 4 °C). The supernatant was collected and 200 µL was injected onto a high-performance liquid chromatographic (HPLC) column to separate the proteins. Chromatography was carried out using a Waters Alliance HPLC system (Milford, MA, USA) configured with a 2690 separations module and a 2487 dual wavelength absorbance detector. Samples were fractionated on a Vydac (Hesperia, CA) 208TP5315 C8 reverse-phase column (5 µm particle size, 3.2 mm x 150 mm) at 40 °C. The analytical column was fitted with a Vydac 208GD52 C8 reversed phase guard column (5 µm, 2.1 mm x 10 mm). Mobile phase solvents A and B were 0.1% TFA (v/v) in water and 0.1% TFA (v/v) in acetonitrile, respectively. Proteins present in the homogenate were fractionated at a flow rate of 0.5 mL/min using linear gradients and the following program: 5% B for 10min, 5 to 25% B over 5 min, 25 to 60% B over 50 min, 60 to 95% B over 10 min and hold for 5 min. The mobile phase was ramped back to the initial

condition and the column allowed to re-equilibrate. Chromatographic effluent was monitored using ultraviolet (UV) detection at both 214 and 280 nm. Fractions were collected at 1 minute intervals (0.5 mL each) into a 96-well microplate and stored at -80 °C until use.

Prior to MALDI analyses, HPLC fractions in the microplate were dried in a vacuum centrifuge (SPD SpeedVac; ThermoSavant, Waltham, MA) and the resultant fractions were reconstituted in 30 µL 40:60 acetonitrile:water containing 0.1% TFA (v/v). Aliquots (~0.5 µL) of each fraction were robotically spotted using a SymBiot® XVI (Applied Biosystems, Foster City, CA) onto the MALDI target on top of the pre-spotted (~0.5 µL) SA matrix (20 mg/mL SA in 40:60 ACN: H₂O with 0.1% TFA (v/v)).

MALDI-MS analyses were performed using a Bruker Daltonics Autoflex III L200 mass spectrometer (Billerica, MA) in positive-ion linear acquisition mode under delayed extraction conditions. A mixture of standard proteins containing bovine insulin (Mr 5733.6), cytochrome C (horse heart, Mr 12360.2), apomyoglobin (horse, Mr 16951.5), and trypsinogen (bovine pancreas, calculated Mr 23981), along with SA matrix was spotted onto the MALDI target for external mass calibration. To achieve better mass accuracy (~200 ppm) by internal mass calibration, the standard protein mixture was co-mixed with matrix and HPLC fractions of interest. Spectra were evaluated using flexAnalysis software (Version 2.4; Bruker Daltonics).

Reconstituted HPLC fractions of interest from the MALDI analyses were transferred from the 96-well microplate to individual microcentrifuge tubes, after washing each well twice with 50µL 1:1 acetonitrile:water. Each sample and its washing were combined, taken to dryness, and stored at -80 °C prior to gel electrophoresis.

Dried fractions were resuspended in 14 µL 1:1 water: Novex® tricine sample buffer (2x) (Invitrogen) and the proteins separated on a precast 10-20% gradient Tricine gel (Invitrogen). Gels were fixed with 50% methanol, 10% acetic acid for 10 min and then stained overnight with Colloidal Blue (Invitrogen). The gels were destained for

approximately 7 hours with deionized water.

Stained protein bands were excised, cut into small pieces (1 mm), washed, and equilibrated with 150 µL 100 mM ammonium bicarbonate. The disulfide bonds of proteins present in the gel were reduced with 10 µL of 100 mM dithiothreitol (Sigma) at 50 °C for 15 min. The samples were allowed to cool and cysteine thiols alkylated at room temperature in the dark for 15 min by adding 10 µL of 500 mM iodoacetamide (Sigma) to produce the carbamidomethyl derivative of the reduced protein. The gel pieces were equilibrated twice with 100 µL 1:1 acetonitrile: 50 mM ammonium bicarbonate (aq) for 15 min. Gel pieces were then dehydrated in 100 µL acetonitrile for 10 min and dried under vacuum. The reduced and alkylated proteins in the gel were digested overnight at 37 °C with ~20 µL 25 mM ammonium bicarbonate containing 0.01 µg/µL sequencing grade trypsin.

LC-MS/MS analysis of peptides resulting from enzymatic digestion was performed using a Bruker Daltonics HCTultra PTM Discovery System ion-trap mass spectrometer equipped with a FAMOS™ model 920 autosampler (LC Packings-A DIONEX Company; Sunnyvale, CA) and Hewlett-Packard Series 1100 binary HPLC pump (Agilent Technologies, Inc.; Santa Clara, CA). Peptides were separated on a fused silica capillary column (100 µm x 11 cm) packed with C18 resin (Monitor C18, 5 µm; Column Engineering, ON, Canada) Mobile phase A was 0.1% formic acid (v/v), and mobile phase B was acetonitrile with 0.1% formic acid (v/v). Because the HPLC pumps were not accurate at nL/min flow rates, the pump was operated at 0.3 mL/min and the effluent split prior to the injection valve. The mobile phase flow rate at the exit end of the capillary column was measured to be 250 nL/min at a mobile phase composition of 25% B. Peptides were separated using linear gradients and the following program: 5% B for 10min, 5 to 27% B over 35 min, 27 to 50% B over 15 min, 50 to 95% B over 1 min and hold for 4 min. The mobile phase was ramped back to the initial condition and the column allowed to re-equilibrate.

Peptides eluted from the capillary tip were introduced into the nanoelectrospray source in positive-ion mode with an ion-transfer capillary voltage of approximately 1.5 kV. Nitrogen was used at a temperature of 150 °C and flow of 10 L/min. MS-MS spectra of peptides were acquired using data-dependent scanning whereby one full MS spectrum (375–1200 u) was followed by 4 MS/MS spectra of the 4 most intense ions from the full scan. The MS/MS spectra were recorded with a repeat of 2 spectra for each precursor mass prior to placing the ion mass on an exclusion list while previously analyzed precursors were dynamically excluded for one minute. Peptide sequences and protein coverage of the MS/MS data were determined using a Sequest algorithm and the Trans-Proteomic Pipeline (TPP), which utilizes PeptideProphet™ and ProteinProphet™ (Seattle Proteome Center, <http://tools.proteomecenter.org/TPP.php>). The TPP protein results were filtered by a ProteinProphet™ probability score of ≥ 0.8 and protein matches with less than two peptides identified were eliminated.

Coomassie (Bradford) protein assay kit and T-PER® tissue protein extraction reagent were obtained from Pierce (Rockford, IL). Sequencing grade trypsin was from Promega Corp. (Madison, WI). Molecular weight calibration standards included trifluoroacetic acid (TFA) , ammonium bicarbonate, iodoacetamide, dithiothreitol, and 3,5-dimethoxy-4-hydroxycinnamic acid (SA MALDI matrix) (Sigma-Aldrich, St. Louis, MO). Gel electrophoresis supplies were from Invitrogen (Carlsbad, CA). HPLC grade acetonitrile and Suprapur® (98-100%) formic acid were obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). A Milli-Q water purification system was used to generate water at $18.2 \text{ M}\Omega \text{ cm}^{-1}$; < 6ppb TOC.

Immunohistochemistry. Rats were transcardially perfused with room temperature 0.1 M phosphate buffer, pH 7.3, followed by ice-cold 4% paraformaldehyde in phosphate buffer. Brains were placed in a postfixative solution of 4% paraformaldehyde. Using a vibratome,

coronal sections of forebrain were cut at 40 μ m thickness. Sections were stored in a solution of phosphate buffer with 30% sucrose and 30% ethylene glycol at -20° C. Conventional immunofluorescence protocols were used to reveal the localization of GNG2 as previously described (Deutch et al., 1996). The primary antibody used was a rabbit anti-GNG2 (1:100; Sigma-Aldrich, Inc.).

Results

MALDI-IMS was applied directly to matrix-coated coronal sections through the rat frontal cortex at bregma (+3.00) and striatum (+2.04) in order to uncover proteins whose expression is restricted to the claustrum. A thorough search of the resulting mass spectrum revealed one protein species to be enriched in the claustrum at striatal levels (Fig. 26B, D). This claustral-specific protein species registered as a single peak of relatively high intensity with a mass-to-charge (m/z) ratio of 7,725 (Fig. 27).

Consistent with the anatomical definition of the claustrum presented in chapter I, very low expression levels of the m/z 7,725 protein were seen in the frontal cortical section, including in the area previously designated as the claustrum (Fig. 26A, C). Very low levels of expression of the m/z 7,725 protein were seen in insular and cingulate cortices, while little to no expression was observed in the striatum and white matter (Fig. 26). Overall, the pattern of expression of the m/z 7,725 protein corresponded well with the definition of the claustrum we presented in Chapter II, namely that the claustrum is present only at striatal levels where it is embedded in insular cortex. There was no m/z 7,725 protein expression within the “dorsal claustrum,” as defined by Paxinos and Watson (2007) (Fig. 1), consistent with our immunohistochemical and tract tracer data.

We identified the m/z 7,725 species by HPLC fractionation of claustral homogenate and subsequent MALDI verification of the m/z 7,725 protein. SDS-PAGE separation of the m/z 7,725-positive fractions resulted in a band in one fraction of roughly 7 kDa (Fig. 28).

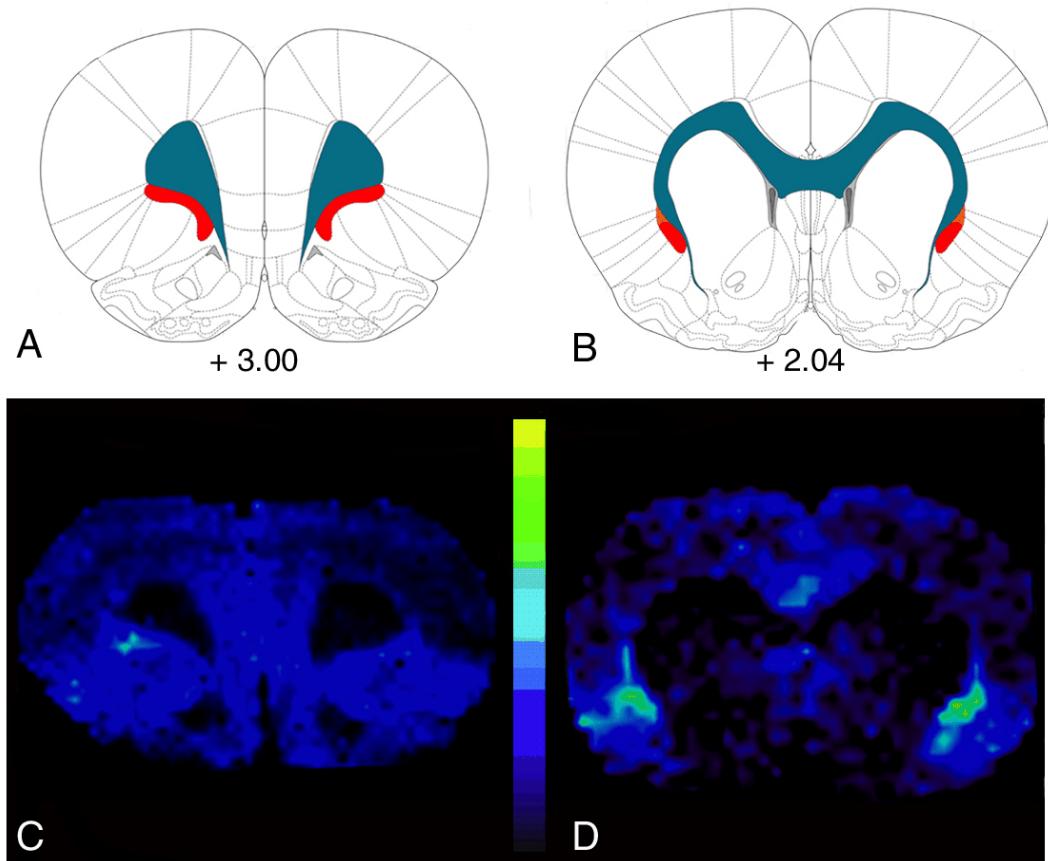


Figure 26. MALDI-IMS reveals a protein species enriched in the claustrum. Images acquired at two different rostrocaudal levels are depicted on the rat brain atlas plates from Paxinos and Watson (2007) (panels A and B). At striatal levels (panel B), an m/z 7,725 protein peak is enriched within the claustrum relative to surrounding structures (panel D). In the frontal cortical section, this protein species is not enriched in the region ventrolateral to the forceps minor (panel C).

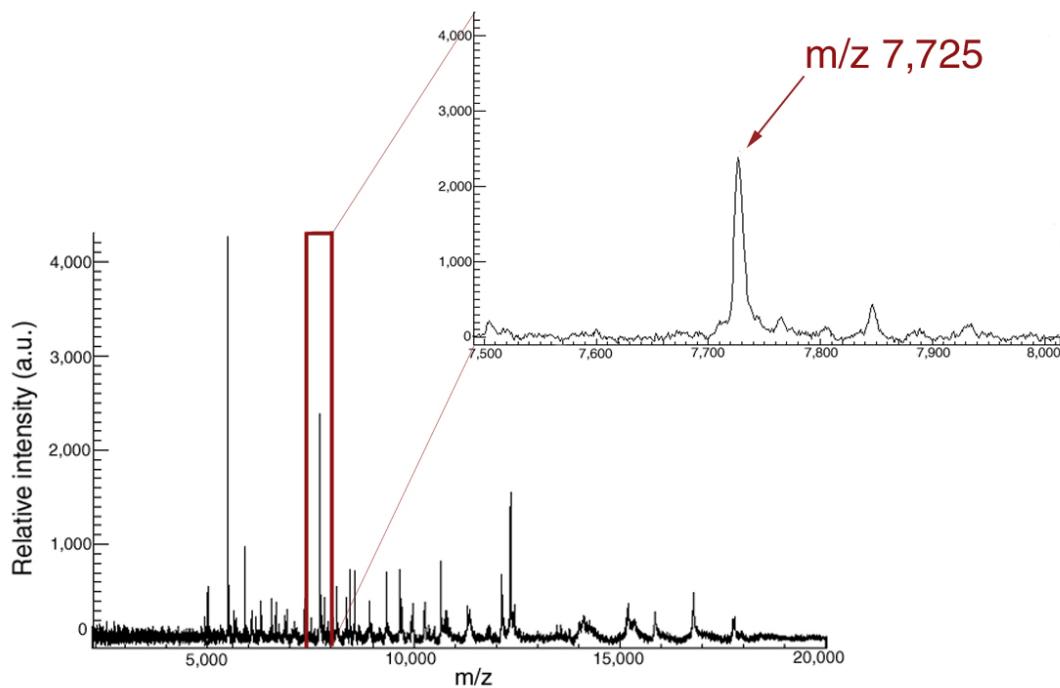


Figure 27. Mass spectrum of proteins and peptides in the rat claustrum as revealed by MALDI-IMS. The region boxed in red is magnified (upper right). A single peak of m/z 7,725 that was enriched in the claustrum was visualized in the MALDI-IMS images (fig. 26).

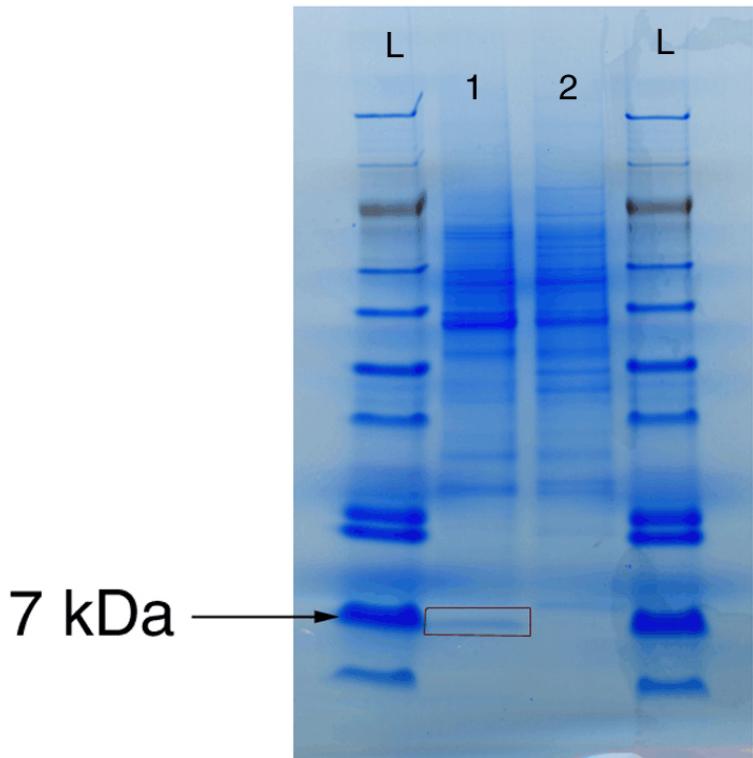


Figure 28. SDS-PAGE isolation of the m/z 7,725 protein species. Following fractionation of the claustral homogenate by HPLC, two fractions containing the m/z 7,725 species were identified by MALDI-MS. These fractions were separated by gel electrophoresis. A single band of ~7 kDa was seen in sample 1 (1), but was not observed in the second fraction (2). This band was excised (red box) for a subsequent in-gel trypsinization step. Abbreviations: (L) molecular weight ladder.

This 7 kDa band was excised and in-gel trypsinization was performed, followed by an LC-MS/MS mass fingerprint analysis. The mass fingerprint was searched against protein databases upon which a trypsinization algorithm had been applied. The database search revealed that the m/z 7,725 species matched to mouse GNG2 (Genbank accession: NP_034445) (Fig. 29). The rat full-length protein was not available in the databases searched. No other proteins were matched following the mass fingerprint analysis with the exception of keratins, which are common protein contaminants.

There was a small difference between the observed GNG2 mass and the mass predicted by the full-length sequence of rat GNG2. The observed mass of m/z 7,725 is 98 Da less than the predicted mass of 7,823 Da (Genbank Accession: EDL86207). However, this mass discrepancy can be accounted for by common post-translational modifications. An oxidation of two methionine residues within the sequence adds 16 Da each, for a total of 32 Da. Second, if the N-terminal methionine is lost, this would account for a loss of 131 Da. Taken together, these changes result in a mass of 7724 Da. The final dalton would come in the form of a protonation.

Immunohistochemical verification of GNG2 protein expression confirmed that this protein was abundantly expressed at striatal levels (Fig. 30), but not present at frontal levels (data not shown). Staining intensity was much less in the insular cortex, septum, and cingulate cortex, while very little expression was seen in other cortical areas, striatum, and white matter.

Conclusions

Using MALDI-IMS we discovered GNG2 as a novel and discrete neuroanatomical marker of the claustrum. Both MALDI-IMS and immunohistochemical analyses demonstrated that this protein is in register with the anatomical definition of the claustrum in Chapter II, thus validating the interpretation of our histochemical, immunohistochemical,

gi|54114974|ref|NP_034445 GNG2 guanine nucleotide binding protein (G protein), gamma 2

MASNNTASIA QARKLVEQLK MEANIDRIKV SKAAADLMAY CEAHAKEDPL LTPVPASENPFREKKFFCAI L
TASIA QARKLVEQLK MESIDR AAADLMAY CEAHAKEDPL LTPVPASENPFR

[NCBI ExPASy](#) [EBI](#) [PIR](#) [MIPs](#) [SGD](#)

Coverage: AA 80.3% (57 / 71 residues) Mass 61.3% (4795 / 7823 Da)

Figure 29. Alignment of peptides derived from trypsinization of the m/z 7,725 protein with the predicted trypsinized mouse full-length GNG2. The full-length rat GNG2 protein was unavailable in the databases used (underlined). In total, peptides of the m/z 7,725 species matched 57 of 72 amino acid residues, yielding a mass coverage of 61.3%. The rat protein contains a serine (red S) at residue 24.

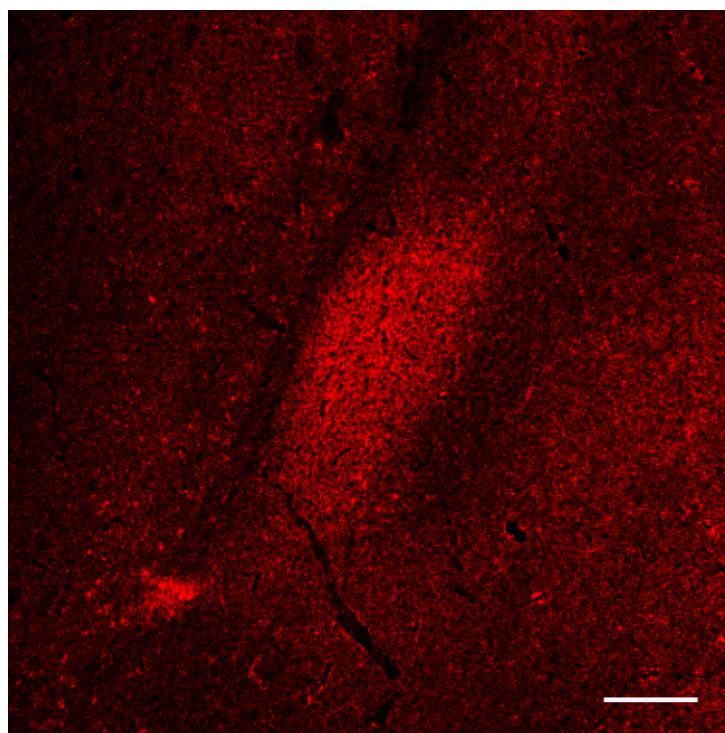


Figure 30. Immunohistochemical localization of GNG2 validates the MALDI-IMS data. GNG2-ir is enriched in neuropil in the claustrum relative to surrounding structures. Scale bar: 200 μ m.

and tract tracing data. Bolstering these findings, *in situ* hybridization for mouse GNG2 appeared restricted to the claustrum (Fig. 31). Low levels of GNG2 mRNA were also noted in the dorsal raphe nucleus. According to this, GNG2 is a claustrum-specific protein that is expressed by cells of the claustrum, rather than by axon terminals projecting to this structure.

The validation of claustrum anatomy using MALDI-IMS demonstrates the power of this technique. To our knowledge, this is the first demonstration of MALDI-IMS to aid in defining a neuroanatomical structure. Whereas previous neuroanatomical methods used to characterize structures relied upon targeted analysis of specific genes or proteins, MALDI-IMS now allows for an unbiased, discovery-based approach for the molecular characterization of brain sites. As such, MALDI-IMS represents the first technique to both *define* and *characterize* anatomical structures in brain. The spatial resolution offered by this technique, coupled with its detection sensitivity beyond the femtomole range for proteins and peptides in their post-translational state positions MALDI-IMS as a powerful tool for neuroanatomists in the post-genomic era.

The identification of GNG2 as a novel neuroanatomical marker for the claustrum represents a major advance toward a functional understanding of this nebulous nucleus. Because the expression analysis of GNG2 mRNA (Allen Brain Atlas) as well as protein is seen in claustral neurons, the application of molecular lesioning strategies targeting GNG2 should allow for the discrete ablation of the claustrum. This could be accomplished either through conditional knock-out or knock-down techniques, a toxin conjugated approach, or a combination of molecular genetics and toxin approaches. The means to test the hypotheses of claustral function as presented in Chapter III, as well as any other hypotheses, are now within reach.

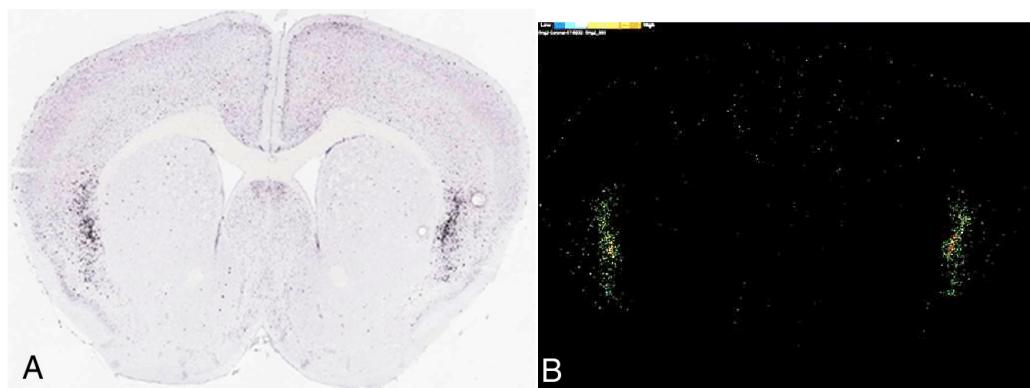


Figure 31. In situ hybridization analysis reveals GNG2 gene expression is restricted to the mouse claustrum (panels A, B). As in all Allen Brain Atlas sections, panel A is counterstained with a nissl stain, rendering the distribution of the dijoxijenine-positive cell bodies difficult to appreciate compared to nissl-positive cells. Panel B more clearly shows the expression GNG2. Images from Allen Brain Atlas.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

This work presents multiple lines of evidence supporting a redefinition of long-held structural boundaries of the claustrum in rat. In addition, our data similarly suggests that the anatomical boundaries of the claustrum require significant revision in all other therian mammals. Our findings also demonstrate that the representation of the anterior cingulate cortex in the claustrum is uniformly distributed. Our interpretation of these data lead us to propose a model of claustral function for multisensory integration and/or attentional allocation that provides a framework from which to base future functional studies of this nucleus. Finally, using a mass spectrometric imaging approach for defining neuroanatomical sites we discovered a novel protein marker of the claustrum, GNG2. The expression pattern of this protein is in register with our redefined anatomical boundaries of the claustrum, and should be useful in defining the structural boundaries of the claustrum in other mammalian brains. Moreover, because this protein is largely restricted in its expression to the claustrum, it may be exploited as a target for molecular disruption of this structure. These advances are discussed in light of the historical claustrum problems of *morphology, ontogeny and phylogeny, hodology, and function.*

Morphology

The histochemical and tract tracing studies presented here do much to settle the dispute over the structural boundaries of the claustrum in therian mammals. It is now possible to use a combination of immunohistochemical markers, such as Crym, PV and GNG2 to distinguish between claustral and insular layer VI in all therian mammalian brains. According to our data, in all species lacking an extreme capsule the claustrum would be

expected to be embedded in layer VI of insular cortex, and not immediately adjacent to the external capsule. In species with an extreme capsule (e.g. felines and primates) the claustrum would be expected to be surrounded by a band of insular layer VI cells. In all therian mammals, then, the structural boundaries of the claustrum should now be defined by the border between GNG2-ir, a marker of the claustrum, and Crym-ir, a layer VI marker.

Dorsal Claustrum. According to our findings, the “dorsal claustrum” as recently defined by Paxinos and Watson (2007) likely does not represent a distinct entity. In no case involving injection of a retrograde tract tracer into cortical areas were claustral neurons labeled in the so-called “dorsal claustrum.” Moreover, PV and GNG2 immunostaining did not reveal a region that resembled the “dorsal claustrum.” In addition, Crym-ir was present in this dorsal territory, consistent with this area actually being cortical layer VI. It seems likely that this region may be where deep layer VI and superficial layer VI bifurcate to envelop the claustrum. This bifurcation is evident upon close examination of AChE staining, a method heavily employed by Paxinos and Watson in creating the description of the “dorsal claustrum.”

Although the presence of a “dorsal claustrum” in the rat (Paxinos and Watson, 2007) may not be supported, on occasion a retrogradely labeled neuron following injection of tracer into cortical areas was noted dorsal to the body of the claustrum, however lateral to where Paxinos and Watson describe their “dorsal claustrum.” In addition, MALDI-IMS revealed a region dorsal and lateral to the claustrum that exhibited a weak signal for GNG2. This likely reflects a subtle dorsal extension of the claustrum deep into the bifurcation of the deep and superficial insular layer VI. Because claustral neurons migrate ventrally along the EC to form the body of the claustrum days after layer VI forms, claustrum neurons split insular layer VI medially and laterally as they arrive to populate the area. The remaining neurons lying dorsal to the body of the claustrum, then, are likely

residual neurons lying within the migrational path that claustrum neurons take during development.

As the cortex elaborated through evolution, the size of the claustrum expanded accordingly (Kowiański et al., 1999). If one examines the claustrum of several species, the expansion of the claustrum appears to have occurred dorsally. In rat, the claustrum appears as a nuclear mass, with a few eccentric cells lie dorsally. In squirrel, the claustrum, while still embedded in the insular cortex, is much extended dorsally in comparison to rat (data not shown). In primate brains, the claustrum is expanded dramatically along the dorsoventral domain. If one considers the migrational path of claustrum neurons in development, it is reasonable to conclude that where the claustrum has expanded dorsoventrally, the claustrum tapers off dorsally such that these dorsally-lying cells are intermingled with superficial and deep layer VI cells. Interestingly, this effect appears evident in sections stained for PV primate claustrum, where PV-ir is present with Crym-ir (data not shown). In order to definitively prove this possibility, neuronal tract tracer studies are required.

Structures surrounding the claustrum. To date, there has been no means of defining the border between the claustrum and the endopiriform nucleus. However, GNG2 expression at either the transcript or protein level now allows for this distinction to be made. The elucidation of the claustrum-endopiriform boundary may lead to changes in our knowledge of the anatomy of the endopiriform nucleus.

In addition to the possible redefinition of the endopiriform nucleus, this work offers new insights into the anatomy of the insular cortex. In the rat, at frontal cortical levels, the deep layers of insular cortex have been extended dorsomedially to abut the forceps minor. At striatal levels, deep layer VI should now be considered to be present between the claustrum and the EC. In mammals possessing an extreme capsule, layer VI of insular

cortex should also be considered to be present surrounding the body of the claustrum. This re-conceptualization not only significantly changes the perspective on insular inquiry in animal models, but also carries important implications for functional imaging studies that seek to assess insular or claustral activity in humans. This is of particular concern because current functional imaging methods already suffer from issues of spatial resolution.

The “core” and “shell” concept. Our demonstration that the rat claustrum is surrounded by insular layer VI in some ways is similar to the idea of “core” and “shell” subdivisions of the claustrum as proposed by Real and colleagues (2006). The “core” and “shell” concept is based on VGLUT2 and calretinin distribution, as well as the distribution of afferent fibers to the claustrum originating in the endopiriform nucleus (Lipowska et al., 2000). However, our data, using a phenotypic marker of layer VI cells (Crym), revealed that the “shell” of the claustrum is actually layer VI. This “shell” effect is evident in previous tract-tracing studies involving injections into thalamic and hypothalamic sites (Levay and Sherk, 1981; Carey and Neal, 1986; Dinopoulos et al., 1992; Vertes, 1992; Erickson et al., 2004; McKenna and Vertes, 2004; Yoshida et al., 2006; Vertes and Hoover, 2008), although these studies did not interpret their findings to reveal different compartments of the claustrum.

It is possible, however, to interpret our findings according to the “core” and “shell” arrangement. As such, the “shell” of the claustrum would be connected to thalamic and hypothalamic sites, while the “core” is restricted to reciprocal cortical connections. However, this interpretation falls short on several grounds. The “shell” of the claustrum, according to the literature and our findings, expresses Crym, is PV- and CO-poor, AChE-rich, receives connections from subcortical sites, and projects to subcortical sites. The entire layer VI of granular and agranular insular cortices also express Crym, are relatively PV- and CO-poor, AChE-rich, receive projections from subcortical sites, and projects to subcortical sites (Allen et al., 1991). In contrast, the “core” of the claustrum is PV- and CO-

rich, AChE-poor, and does not appear to receive inputs from any subcortical sites. These considerations also hold for primate species. In summary, these data point toward the “shell” of the claustrum being part of insular layer VI.

Ontogeny and Phylogeny

Ontogeny. What does the presence of insular layer VI cells populating the perimeter of the claustrum mean for the dispute concerning claustral ontogeny in the early 20th century? Rose (1928), Brodmann (1909), Smith (1910, 1917), and Holl (1899) all concluded that the claustrum is a fully differentiated, eighth layer of insular cortex (with the extreme capsule being layer VII). This was likely due to the observations of insular layer VI cells surrounding the claustrum. As a result, this “ring” of insular layer VI was likely misidentified as being claustral. Supporting this notion, Sonntag and Woollard (1925) found that claustral cells in the aardvark were strikingly similar to insular layer VI cells. In addition, Rae (1954) found an increasing prevalence of fusiform-shaped cell bodies toward the perimeter of the claustrum, which were actually intermingled within capsular fibers. These fusiform cells were also noted in the adjacent insular cortex. Considered in light of the present work, it appears that the spirited debate over claustral ontogeny in the early 20th century in part was based on the misidentification of layer VI cells as part of the claustrum.

Interpretation of thymidine birth-dating of claustral cells, as determined by Bayer and Altman (1991), needs to be reexamined in light of our data. Interestingly, Bayer and Altman did not include in their analysis the area of “claustrum” rostral to the anterior pole of the striatum. We can only surmise that this area was omitted because the birth date of these anterior cells differed from claustral cells present at striatal levels. Based on our data, one would predict that this rostral “claustrum” (now redefined as deep layers of insular cortex) would be generated at the same time as layers VI cells of insular cortex (E 12.5).

Phylogeny. The phylogeny of the claustrum remains unclear. A major issue is whether the claustrum is present in monotreme brains. However, with the identification of GNG2 as a discrete claustral marker, a resolution to this issue is now within reach. Assessing the distribution of GNG2 in the platypus and echidna brains likely would reveal the presence or absence of the claustrum in this clade. We suspect that such an analysis would indeed reveal a body of claustrum to be present, and therefore support the idea that the claustrum was present in ancestral mammalian species.

Our work has more clearly defined the hodology of the claustrum, showing that it is connected with cortex, but not subcortical sites as previously reported. Using these observations as a springboard, hodology now becomes a more reliable means of defining claustrum homologues in fish, amphibian, reptilian and avian brains. Based on the connections of the claustrum, a homologous structure to the claustrum in non-mammalian brains would be a telencephalic structure with only intra-telencephalic connections. However, there are significant problems in drawing comparisons between mammalian and non-mammalian brains. Chief among these, non-mammalian brains do not have a layered pallium. Because the claustrum, according to our work, appears to be reciprocally connected only with the layered pallium in mammals, employing hodological criteria alone will not be sufficient to define a claustrum in non-mammalian species.

The non-mammalian telencephalon, as previously mentioned, exhibits a nuclear rather than a layered organization. If one considers a complex, non-mammalian telencephalon, such as that of the pigeon, telencephalic sensory-recipient nuclear zones exist, and can be compared to sensory cortices in mammals (Reiner, 1995). Drawing on the principles of mammalian telencephalic organization, one might predict that an organizer of these sensory-recipient zones may be present (like the claustrum), where sensory input can be relayed for association amongst other structures. Based on the work previously described by Puelles and colleagues (2000), the candidate based on ontogeny for an avian

claustral homologue would be the mesopallium. It may therefore be of interest to examine the connections of this structure.

Of course, it is also entirely possible that another telencephalic structure not derived from the lateral pallium may function as a telencephalic “organizer,” a phenomenon that would represent homoplasy, rather than true homology. Interestingly, the central portion of the telencephalon (area DC, Northcutt and Braford, 1984) in the teleost *Xenomystus nigri* exhibits widespread reciprocal connections with several telencephalic areas (Mathur, 1998). Alternatively, it may not be possible to identify a structure that can be considered homologous or even homoplasious to the claustrum in non-mammalian brains.

Hodology

The redefinition of claustral structural boundaries not only accounts for much of the dispute over claustrum ontogeny through history, but it also accounts for the abundance of subcortical connection findings. This work shows that the claustrum is restricted to cortical connections, and that the thalamic and lateral hypothalamic connections once believed to be claustral are actually those of layer VI of insular cortex. Examination of existing anatomical studies reveals this distinction in several species. In the hedgehog, in which the claustrum is reported to lie sandwiched between the EC and the insular cortex, as it is in the rat, this same “ring” of insular layer VI cells can be seen. Injections of retrograde tracers into the dorsal thalamus of the hedgehog showed retrogradely labeled cell bodies encapsulating the apparent body of claustrum (Dinopoulos et al., 1992).

Through time, as species developed an extreme capsule following the elaboration of isocortex, the claustrum became enveloped by white matter. Prior to the complete encapsulation of the claustrum, this structure was partially bordered laterally by an inchoate extreme capsule in certain species. One example of this is the tree shrew. Injection of a retrograde tract tracer into the dorsal thalamus of this species results in labeled cells again

completely surrounding the claustrum notably on the lateral aspect of the claustrum where the extreme capsule borders this structure (Carey and Neal, 1986). In the macaque, in which the claustrum is completely enveloped in white matter, Erickson et al. (2004) revealed retrogradely-labeled cells surrounding the body of the claustrum again following injection of tracer into the dorsal thalamus. It should be noted that in these tracer studies, the authors interpreted the findings as a demonstration that the claustrum connects to the dorsal thalamus. However, in light of our findings, the retrogradely-labeled cells are likely insular layer VI neurons that populate the perimeter of the white matter-encapsulated claustrum. Interestingly, in the Erickson et al. (2004) study, a large number of retrogradely-labeled cells were observed in the dorsal extension of the macaque claustrum; our data on Crym localization suggests that this area of the “claustrum” is actually composed of an admixture of insular layer VI and claustral cells.

Despite these explanations, further outstanding issues regarding claustral connectivity must be resolved. Arikuni and Kubota (1985) showed that the claustrum is connected to the striatum, and Amaral and Cowan (1980) showed the claustrum to be connected to the hippocampus. Although we have not presented data on these areas, it is clear that the injection sites in these studies clearly involved white matter structures, clouding the interpretation of these results. It is worth noting that we have never observed retrogradely-labeled cells in the claustrum following injections of a retrograde tracer into the striatum (data not shown).

The findings by Lipowska and colleagues (2000) showing a projection from the endopiriform nucleus to the claustrum must also be explained. The restriction of these afferent fibers to the medial and lateral aspects of the claustrum would suggest that they are projecting to the surrounding layer VI cells of insular cortex. Taken together with the developmental data by Bayer and Altman (1991) and Puelles and colleages (2000), the endopiriform nucleus, by all accounts, appears be an all together distinct nucleus from the

claustrum. In light of this, the labeling of the endopiriform nucleus as “ventral claustrum” by Druga (1966) appears to be misleading. However, from a broader perspective, the claustrum and the endopiriform nucleus do appear to share certain principles of organization. The claustrum is a nuclear structure that connects to a wide array of *isocortical* sites. Similarly, the endopiriform nucleus is a nuclear structure that is connected to a wide array of *cortical or cortical-like* structures. In addition, both structures are centrally located within the telencephalon relative to the structures to which they connect. If the claustrum is considered a putative relay structure for isocortical areas and, thus, of sensory information as it relates to cognition, then it is reasonable to conclude that the endopiriform nucleus may be the claustral equivalent to limbic structures. The endopiriform nucleus, in this sense, would be a putative relay of limbic-associated cortical-like areas subserving affective information. Rather than interchangeably referring to the endopiriform nucleus as the “ventral claustrum,” perhaps a more accurate name for the endopiriform nucleus would be the “limbic claustrum.”

Finally, it has been suggested in the literature that the claustrum receives a serotonergic input (Wójcik et al., 2006; Baizer, 2001; Rahman and Baizer, 2007), suggesting that the claustrum receives a projection from the brainstem raphe nuclei. Consistent with this, Vertes (1991) reported labeled axon fibers to be present in the claustrum following injection of the anterograde neuronal tract tracer PHA-L into the rat dorsal raphe nucleus. However, upon examination of these results, the terminal labeling did not appear to be present within our definition of claustral boundaries. Further tract tracing and electron microscopy studies are required to validate this possible subcortical connection.

Our data on the representations of cortex in the claustrum extend previous studies in the rat. We showed that while primary sensory, association, and medial prefrontal cortical representations in the claustrum are restricted to limited subdivisions, the anterior

cingulate cortex is uniformly represented across the claustrum.

Our interpretation of these results provides a potential anatomical solution to the problem of information integration across claustral sensory subdivisions (Olson and Graybiel, 1980). Our work, however, is only a limited study of how different cortical areas are represented within the true structural boundaries of the claustrum. An analysis of representations of all cortical regions is required, including a replication of our anterior cingulate findings, using both retrograde and anterograde tract tracers. Nonetheless, our data provides insight into the potential function of the claustrum upon which future studies may be derived.

Function

We have seen how our data defining the claustral border shifts the known connections of the claustrum. Our data provide evidence that the claustrum is largely, if not exclusively, connected with the isocortex. This has important implications for claustral function. The proposed role for the claustrum in multisensory integration remains viable with the new hodological definition of the claustrum we have provided. That is, the claustrum may be serving as a cortical organizer to aid in the binding of multimodal information, as exemplified by claustral activation during tactile-visual matching tasks (Hadjikhani and Roland, 1998; Olson et al., 2002; Naghavi et al., 2007). Our data on the connections of the claustrum support our model (see Fig. 25) showing how the claustrum may function in multisensory integration. In addition, our data may be interpreted to support a role for the claustrum as a mechanism for allocation of attentional resources.

Multisensory integration. Our model for claustral function differs from the Crick and Koch (2005) model primarily by placing the processing power of deciding on the binding of sensory information with the cortex. We propose that information flows from an executive

decision-making cortical center (anterior cingulate cortex) to the claustrum, where the signal is channeled to the appropriate cortical subdivision. The cortical innervations arising from the various subdivisions of the claustrum in turn synchronously activate the appropriate sensory cortices to generate a percept of a multimodal stimulus. For example, if one sees and hears a musician playing a banjo, the visual and auditory input are “bound” somehow in the brain and one perceives the sound as emanating from the banjo. If however, one sees a musician playing a banjo and bird songs are heard instead, the brain must “check” the information to see if the visual and auditory inputs are compatible before making an assessment of whether or not the information should be bound. Without this “checking” mechanism, all sensory stimuli within close proximity in time or space would be bound.

According to Crick and Koch’s model, where binding occurs in the claustrum, one would expect the claustrum to be constantly active during conscious states. This is a reasonable expectation if the claustrum is continuously “binding” sensory information for the generation of conscious percepts. However, electrophysiological and functional imaging studies suggest otherwise. Segundo and Machne (1956) and Spector and colleagues (1974) independently showed that claustral cells are typically silent in awake and anesthetized preparations. In addition, fMRI studies suggest that the claustrum does not present as a site that is activated across a variety of functional imaging experiments. (Cavanna, 2007).

This selective activation of the claustrum *can* be explained in our model. We propose that the anterior cingulate cortex signals to the claustrum what sensory cortices require synchronization. Inherent in this information is those sensory cortices that should not be activated for synchronization. Based on this, one would predict that the claustrum would only be activated for stimuli that require binding, but not be activated for incongruent stimuli not requiring binding. Adding credence to this contention, Naghavi and colleagues (2007) performed a functional imaging study where activation in the claustrum was

compared between sets of sounds and pictures that were either conceptually related or not related. For example, a related set would be a picture of a car and the sound of a horn, and an unrelated set would be a picture of a car and a dog bark. They found that significant activation in the claustrum/insula region was only revealed during presentation of conceptually related sets as compared to the activation during presentation of unrelated sets. In our model, the greatest activation of the claustrum would be expected to be seen during conceptually related sets, and the lowest during unrelated (incongruent) sets. In this way, our model fits nicely with the findings of Naghavi and colleagues (2007).

Attentional allocation. Our data on cortical representations in the claustrum also suggests that the claustrum may function as a component of attentional allocation mechanisms. According to this model, multisensory integration is achieved either via the claustrum as described above or at the level of the cortex (via extensive cortico-cortical networks). If the former is the case, it will be important to establish whether these two functions can be dissociated into mutually exclusive processes. If they are mutually exclusive, this would imply that the claustrum subserves these functions as two parallel processes. Future studies examining this issue will require behavioral tasks sensitive to each process.

If multisensory integration is achieved at the level of the cortex, the claustrum receives the most salient information from sensory cortices for the purpose of supplying the anterior cingulate cortex with the necessary information to decide upon the appropriate sensory cortices to “prime” - via the claustrum - for attending to a particular set of stimuli. In this scenario, the claustrum would only be activated in response to the most salient sensory information. This suggestion fits well with electrophysiological findings demonstrating that claustral neurons are largely silent, responding only to sensory stimuli (Segundo and Machne, 1956; Spector et al., 1974). In addition to possibly conforming to physiological data, this model also may fit with the reports of claustrum activation during

multisensory integration tasks. For example, in the Hadjikhani and Roland (1998) study the observed claustral activation upon tactile-visual matching tasks may be due to the claustrum serving to “activate” somatosensory and visual primary and association cortices for the purpose of attending to the necessary visual and tactile stimuli.

This attentional allocation hypothesis of claustral function would predict that the claustrum is activated during unimodal or multimodal tasks. Why then is the claustrum only reported to be activated during execution of multisensory tasks (Hörster et al., 1989; Hadjikhani and Roland, 1998; Olson et al., 2002; Naghavi et al., 2007; Kavounoudias et al., 2008)? Our model predicts that if only one sensory modality requires attention, then only one sensory subdivision in the claustrum would be activated. If three sensory modalities require concurrent attention, then three claustral subdivisions would be activated. In this way, multisensory tasks would generate a greater level of claustral activity, and may explain why the BOLD signal in fMRI studies would only reach threshold for a statistically significant change during multimodal tasks.

Interestingly, the role for the claustrum in attentional allocation that we have explored is remarkably similar to Broadbent’s model for attention that was proposed in 1958 (Broadbent, 1958). According Broadbent’s model, sensory information is received and registered, presumably by cortex. The information then passes through an attentional “filter” where it is then sampled by “executive centers.” These executive centers then feed back to activate the appropriate sensory isocortices for attending to the stimuli at hand. A prime example of Broadbent’s model is the cocktail effect. This is where salient information, in the form of hearing one’s name in a din of voices at a cocktail party is sufficient to shift one’s attention away from the present conversation to the person who uttered one’s name. A role for the claustrum in attentional allocation is a novel concept, and whether the claustrum is an attentional filter needs to be explored experimentally.

Concluding Remarks

Aside from the alternate functions of the claustrum as proposed above, the results of the studies we have presented offer anatomical data that counters the hypothesis of claustral function proposed by Crick and Koch (2005). Their idea that the claustrum integrates multimodal information for the generation of conscious percepts is based upon the widespread interconnectivity that the claustrum exhibits with isocortex. In addition, their position relies upon evidence in the literature that states that the claustrum is connected to thalamic structures as well. Because our data demonstrates that the claustrum is not connected to the thalamus, a significant piece of the fabric of the Crick and Koch hypothesis is now unraveled. Furthermore, our observation that cells in the claustrum projecting to the anterior cingulate cortex are uniformly distributed across the entire claustrum leads us to an interpretation of possible claustral function that is contrary to the Crick and Koch hypothesis. Rather than placing the claustrum above cortical executive control centers (such as the anterior cingulate) in the hierarchy of information processing, our model suggests the claustrum subserves a distributed network of isocortical sites. Thus, our model is not incompatible with the subcortical relay model of multisensory integration as proposed by Ettlinger and Wilson (1990) and supported by others (Hadjikhani and Roland, 1998; Calvert, 2001; Olson et al., 2002). Our proposed role for the claustrum in sensory integration and/or attentional allocation *requires* the zonal organization of cortical representations for proper function, rather than being stymied by this arrangement. Further improving the stance of our model over that of Crick and Koch's, our model offers an explanation for the high threshold of claustral activation seen in electrophysiological and functional imaging studies. These data suggest that the Crick and Koch hypothesis that the claustrum is the seat of consciousness now appears largely untenable.

Future Directions

Structure. The claustrum remains an abyss of unanswered questions. Due to the new definition of claustral boundaries that we put forth, a comparative analysis of claustral boundaries in species across different orders of mammals, including (but not limited to) monotremes, rodents, marsupials, carnivores, prosimians, non-human primates and humans, would greatly support our findings. Using the distribution of Crym, PV, and GNG2, the structural boundaries of the claustrum can now be deduced in these species.

Our definition of structural boundaries also means that the connections of the claustrum need to be updated. Two pressing issues that need to be addressed are: is the claustrum really reciprocally connected to *all* areas of isocortex, and what is the true organization of cortical representations in the claustrum.

In order to reach the next level in claustrum research, it is essential to determine an internal circuit diagram of this structure. The model outlined in this work provides a basis from which to formulate hypotheses concerning intra-claustral connectivity. This model predicts that neurons of a specific sensory zone would not project to other primary sensory zones, but connections would be observed within sensory zones. To test this hypothesis, injections of Dil crystals into a known primary sensory subdivision of the claustrum could be made after retrograde tract tracer labeling of neurons projecting to other primary sensory cortices. Connections between subdivisions would be assessed.

The model proposed in this study also predicts that sensory information is conveyed to claustral neurons within subdivisions that project to the anterior cingulate cortex. In order to test this possibility, a transsynaptic viral neuronal tract tracer, such as pseudorabies virus (PRV) could be employed. According to our model, PRV injection into a primary sensory cortex would result in one of two outcomes. In both cases, retrograde filling of neurons in the corresponding claustral sensory subdivision would be observed. In the first possible outcome, due to the transsynaptic relay of PRV, so-called “second order” neurons would be

filled, which would include interneurons, and projection neurons connecting to frontal cortices. The second possible outcome is that “second order” labeling would be observed in neurons of frontal cortices that project to the primary sensory claustral subdivision under examination. Injection of PRV into the anterior cingulate cortex, which is uniformly represented in the claustrum, would either be expected to transsynaptically label claustral neurons projecting to primary sensory and association cortices, or directly transsynaptically label cortical neurons in primary sensory and association cortices.

Function. The discovery of GNG2 as a discrete marker of the claustrum opens a window to studies of the function of the claustrum. GNG2 can be used as a target to molecularly disrupt the claustrum. In order to avoid the developmental complications surrounding conventional knockout mice, an inducible knockout of GNG2, or a combined genetic and toxin-based approach is required. The regional specificity of the latter favors this approach over a conditional knockout.

Generating a transgenic mouse that expresses a diphtheria or tetanus toxin receptor under the GNG2 promoter would result in expression of these receptors in the claustrum. Because only a few other areas in brain express GNG2 (weakly in the medial septum and raphe nucleus) and since these other sites are far removed spatially from the claustrum, injection of diphtheria or tetanus toxin into the claustrum would selectively lesion this structure (Luquet et al., 2005). Before generation of these animals, we will need to determine in which types of cells GNG2 is expressed.

These approaches are within reason given the information presented in this work. An optimal scenario assessing claustrum function would involve subdivision-specific lesions of the claustrum. Through an identification of a protein that is expressed on claustral projection neurons, which may include GNG2, expressing a toxin under this promoter such that the toxin receptor is trafficked to axon terminals, injection of the toxin into primary

sensory cortical regions would result in primary sensory subdivision-specific lesions with the claustrum.

If these molecular disruptions of the claustrum could be generated, an assessment of behavior would be necessary to deduce claustral function. Based on Crick and Koch's hypothesis, and our model of claustrum function, the claustrum is either functioning as a multisensory integrator or an attentional allocation component. Testing the former would be difficult in a mouse model. However, Crick and Koch proposed a trace associative conditioning assay (Clark and Squire, 1998; Carter et al., 2003; Koch, 2004) in which a distracting stimulus is presented between the conditioned stimulus (CS) and unconditioned stimulus (US). The successful association of the CS with the US is proposed to test for the presence of awareness. Employing this task assumes that awareness and sensory integration are dependent mechanisms.

In order to test the theory that the claustrum functions as a necessary component of attentional allocation, an attention set-shifting paradigm may be appropriate (Birrell and Brown 2000; Colacicco et al., 2002). In this task, the animal is presented with a pair of bowls, one of which contains a food reward. Either an odor or a texture on the bowl cues the presence of the food reward and, within a single session, a series of discriminations are required. Theoretically, this task requires shifting attention from one sensory modality to another to gain access to the reward. Whether the claustrum is functioning as a multisensory integrator, an attentional allocation component, or a combination of both, the ability to lesion specific claustral subdivisions would greatly enhance the specificity of the behavioral tasks, and would, in turn, add weight to the interpretation of the results.

Given the widely distributed nature of its cortical projections and possible function in multisensory integration and/or attentional allocation, it is interesting to consider if claustral dysfunction is present in schizophrenia. If the claustrum is functioning as a component of multisensory integration mechanisms, dysfunction in this structure would

result in disorders of perception (hallucinations) and thought. Much as the thalamus has been implicated in the aberrant cortical activation thought to underlie hallucinations (Silbersweig et al., 1995, 1998; Weiss and Heckers, 1999; Shergill et al., 2000; Scruggs et al., 2003), the claustrum is also anatomically suited to generate such disruptions through its widespread cortical connectivity. If the claustrum is functioning in attentional allocation, dysfunction in this process would also fit with the symptoms of schizophrenia, as patients fair poorly in attentional set-shifting paradigms and attentional dysfunction has been posited as a central feature underlying many of the symptoms in the illness (Wynne et al., 1978; Morice, 1990; Goldberg and Weinberger, 1994; Donohoe and Robertson, 2003). Ultimately, the claustrum offers an important glimpse into the possible mechanisms underlying cognitive processes in the mammalian brain. Linking dysfunction in this fascinating structure to psychiatric disorders may well represent the future of claustrum research.

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