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Interhemispheric connections between the infralimbic and entorhinal cortices: The endopiriform nucleus has limbic connections that parallel the sensory and motor connections of the claustrum

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Abstract

We have previously shown that the claustrum is part of an interhemispheric circuit that interconnects somesthetic-motor and visual-motor cortical regions. The role of the claustrum in processing limbic information, however, is poorly understood. Some evidence suggests that the dorsal endopiriform nucleus (Dn), which lies immediately ventral to the claustrum, has connections with limbic cortical areas and should be considered part of a claustrum-Dn complex. To determine whether Dn has similar patterns of cortical connections as the claustrum, we used anterograde and retrograde tracing techniques to elucidate the connectivity of Dn. Following injections of retrograde tracers into Dn, labeled neurons appeared bilaterally in the infralimbic (IL) cortex and ipsilaterally in the entorhinal and piriform cortices. Anterograde tracer injections in Dn revealed labeled terminals in the same cortical regions, but only in the ipsilateral hemisphere. These tracer injections also revealed extensive longitudinal projections throughout the rostrocaudal extent of the nucleus. Dual retrograde tracer injections into IL and lateral entorhinal cortex (LEn) revealed intermingling of labeled neurons in ipsilateral Dn, including many double-labeled neurons. In other experiments, anterograde and retrograde tracers were separately injected into IL of each hemisphere of the same animal. This revealed an interhemispheric circuit in which IL projects bilaterally to Dn, with the densest terminal labeling appearing in the contralateral hemisphere around retrogradely-labeled neurons that project to IL in that hemisphere. By showing that Dn and claustrum have parallel sets of connections, these results suggest that

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CONFLICT OF INTEREST STATEMENT

None of the authors report any conflict of interest.

DEn and claustrum perform similar functions in processing limbic and sensorimotor information, respectively.

Graphical Abstract

Using neuroanatomical tracing techniques, the authors show that the dorsal endopiriform nucleus has similar patterns of intra- and inter-hemispheric connectivity with limbic cortices as the claustrum does with sensory and motor cortices. Thus, these two adjacent, highly similar nuclei likely serve similar computational strategies for the interhemispheric coordination of cortical information.

Keywords

claustrum; neuroanatomical tracing; endopiriform nucleus; cortex; limbic system; interhemispheric

INTRODUCTION

The claustrum has a multitude of long-range connections that indicate it might be critical for coordinating widely-separated cortical regions for the purpose of binding multimodal sensory and motor information into conscious percepts (Crick & Koch, 2005). More recently, it has been suggested that claustral connectivity provides gain control over its cortical targets to facilitate shifts in the allocation of attention, both within and across modalities (Mathur, 2014; Goll et al., 2015). Consistent with these hypothetical views, our tracing studies in the rat have established that the claustrum has unique connections that could enable the interhemispheric transfer of information between multiple cortical areas in the somesthetic-motor and visual-motor systems (Smith & Alloway, 2010; 2014; Smith et al., 2012). Very little is known, however, about the relationship between the claustrum and the limbic system.

In cats and primates, the ventral claustrum is reciprocally connected with limbic regions such as the amygdala, hippocampus, and the olfactory, entorhinal, and prefrontal cortices (Markowitsch et al., 1984; Room et al., 1985; Insausti et al., 1987; Witter et al., 1988). In rodents, these limbic areas are connected with a brain region lying ventral to the claustrum: the endopiriform nucleus (Wyss, 1981; Condé et al., 1995; Sobreviela et al., 1996; Vertes, 2004; Druga, 2014; Bienvenu et al., 2015).

There is considerable ambiguity regarding the relationship between the claustrum and the DEn. Whether the DEn in rodents and other animals has a homologous structure in the human brain is unclear because DEn has not been delineated in the human brain. Substantial confusion has arisen on this issue because of differences in nomenclature. The 'nucleus endopiriformis' was originally described as being continuous with the dorsal claustrum, but separate from piriform cortex (Loo, 1931), and this view was shared by other researchers (O'Leary, 1937; Scalia, 1966; Ebner, 1967; Martin & Hamel, 1967). Some researchers, however, have interchangeably used the terms 'ventral claustrum' and 'endopiriform nucleus' to describe the same structure (Markowitsch et al., 1984; Eid et al., 1996; Kowia ski et al., 1999; Majak et al., 2000, 2002). Other names used to describe this brain region include the claustrum praepyriforme (Brockhaus, 1940; Narkiewicz, 1964), the claustrum ventrale (Druga, 1966), the claustrum-endopiriform nucleus (Butler & Molnár, 2002; Striedter et al., 1998), the endopiriform claustrum (Reblet et al., 2002), and the claustroramygdalar complex (Puelles et al., 2000; Molnár & Butler, 2002a; Medina et al., 2004). Furthermore, Krettek & Price (1977) distinguished the dorsal and ventral sectors of the endopiriform nucleus.

These differences in nomenclature have prompted debate as to whether the endopiriform nucleus is functionally and anatomically separate from the claustrum. Cytoarchitectural analysis indicates that cell density is greater in the claustrum than in the endopiriform nucleus, suggesting they are separate nuclei (Loo 1931). This view is corroborated by work showing that these two brain regions have divergent neurogenetic gradients and that cells in each region originate at different developmental time points (Bayer & Altman, 1991). Furthermore, neocortical areas have connections with the dorsal claustrum, whereas allocortical regions are connected with the ventral claustrum and dorsal endopiriform nucleus (Dinopoulos et al., 1992; Kowia ski et al., 1999).

Some evidence, however, suggests that the claustrum and endopiriform nucleus represent two adjacent components of a single brain structure. Many peptides and gene expression profiles are continuous throughout both the claustrum and endopiriform nucleus, and these patterns are distinguishable from the overlying insular cortex (Kowia ski et al., 2001; 2004; Johnson et al., 2014; Watakabe et al., 2012; 2014). Based on these observations and other data, some investigators consider the claustrum and endopiriform nucleus to be claustroramygdaloid derivatives of a common embryonic field in the pallium (Molnár & Butler, 2002b; Reblet et al., 2002).

To assess the anatomical connectivity of the DEn and its similarity to the circuit connections of the claustrum, we injected a mixture of anterograde and retrograde tracers into the endopiriform nucleus. We observed a pattern of interhemispheric limbic circuit connections with the endopiriform nucleus that resemble the interhemispheric circuit connections of the claustrum (Smith & Alloway 2010). When different retrograde tracers were placed in multiple cortical targets of the DEn, we observed a substantial number of double-labeled neurons that send divergent projections to separate limbic cortical areas, much like the claustral neurons that project both to sensory and motor cortical areas (Smith et al., 2012; Smith & Alloway, 2014). These and other findings suggest that the DEn and claustrum could

perform similar computational functions in processing limbic and sensorimotor information, respectively.

METHODS

Experiments were performed on 14 adult male Long-Evans rats (Charles River Laboratories) weighing 300-550 g. All methods followed NIH guidelines and were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University.

Surgery

Rats were anesthetized by an intramuscular (IM) injection of ketamine HCL (40 mg/kg) and xylazine (12 mg/kg). Before intubating the trachea through the oral cavity with a 14-gauge catheter (Smiths Medical), animals received intramuscular injections of atropine methyl nitrate (0.5 mg/kg) to decrease bronchial secretions, dexamethasone sodium phosphate (5 mg/kg) to reduce brain swelling, and enrofloxacin (2.5 mg/kg) to prevent bacterial infection. Rats were then placed in a stereotaxic frame and ventilated with oxygen. The animal's heart rate, respiratory rate, end-tidal carbon dioxide, and blood oxygen were monitored during surgery (Surgivet capnograph, model V90041). A heated water blanket was placed underneath the animal, and a homeothermic blanket set at 37 °C was placed over the animal regulated by a rectal probe (Harvard apparatus). Corneal drying was prevented by application of ophthalmic ointment on the eyes. Bupivacaine (2.5 mg/kg) was injected along the incision path before exposing the cranium. Craniotomies were made over the dorsal endopiriform nucleus (1.0-0.5 mm rostral, 3.8-4.4 mm lateral), infralimbic cortex (2.7-3.2 mm rostral, 0.3-0.9 mm lateral), and entorhinal cortex (6.0-6.4 mm caudal, 7.6-8.0 mm lateral, 10° from midline) relative to bregma as indicated by a rat atlas (Paxinos & Watson, 2007). When the animal metabolized the ketamine/xylazine cocktail and started to show small whisker excursions, 1% isoflurane was introduced into the ventilation system.

Tracer Injections

To visualize anterograde connections, a 10% solution of Fluoro-Ruby (FR; D- 1817, Invitrogen) in 0.01 M PBS and a 10% solution of biotinylated dextran amine (BDA; D-7135, Invitrogen) in 0.01 M PBS were used. Retrograde connections were detected with either a 2% solution of Fluoro-Gold (FG; H-22845, Fluoro-Chrome) in 0.9% saline or a 2% solution of True Blue (TB; T-1323, Invitrogen) in dimethyl sulfoxide. A mixture of BDA and FG was often used to visualize anterograde and retrograde labeling from the same ejection site. Solutions of BDA, FG, and BDA/FG were iontophoretically expelled from a micropipette (30-40 µm tip diameter). Positive current pulses of 2-5 µA were applied for 30-60 min (7 s on-off duty-cycle) at each ejection site. Tracer leakage during iontophoretic ejections was prevented by applying a retention current (-10 µA) while the pipette was inserted or withdrawn from the brain. Suspensions of FR and TB were pressure injected with a 2 µL Hamilton microsyringe outfitted with a pulled glass micropipette in volumes ranging from 500-700 nL. Following tracer injections, the skin was sutured and treated with antibiotic ointment. Additional injections of atropine, dexamethasone, and enrofloxacin were given before taking the animal off of the ventilation system. Animals were individually housed for 7-10 days before being sacrificed.

Histology and immunohistochemistry

Animals were overdosed with an IM injection of ketamine (60 mg/kg) and xylazine (18 mg/kg) before being transcardially perfused with physiological saline with lidocaine and heparin, 4% paraformaldehyde (PFA), and 4% PFA with 10% sucrose. Brains were extracted and placed in 4% PFA with 30% sucrose at 4 °C for 2-3 days. The olfactory bulbs and cerebellum were removed, and a shallow slit was made lateral to the cerebral longitudinal fissure in the left hemisphere, to produce a fiduciary mark to distinguish between hemispheres. The brain was sectioned coronally (60 µm) using a freezing microtome and stored in 0.1 M PBS. A 1:2 or 1:3 series of sections were processed and mounted on gelcoated glass slides in serial order. The first series of sections were processed for thionin to reveal cytoarchitecture. The second series was used for fluorescent labeling and did not undergo any histological processing. For brains that contained BDA, a third series was processed for the tracer as described before (Smith & Alloway, 2014). Briefly, sections were agitated in 0.30% H₂O₂ to reduce endogenous peroxidases and cell membranes were permeabilized with 0.3% Triton X-100 in 0.1 M PBS (pH 7.40). Sections were then incubated for 2-4 h in an avidin-biotin horseradish peroxidase solution (Vector Novocastra Laboratories, Burlingame, CA). To visualize the BDA tracer, sections underwent a secondary reaction in a 0.1 M Tris buffer (pH 7.2) for 8-10 min with 0.05% diaminobenzidine, 0.0005% H₂O₂, 0.04% NiCl₂, and 0.04% CoCl₂.

Anatomical analysis

An Olympus BH-2 microscope equipped with a TRITC filter (41002; Chroma Technologies) and a UV filter (51004v2; Chroma Technologies) was used to visualize FR-labeled terminals and FG-/TB-labeled soma, respectively. The BDA-labeled terminals were visualized under brightfield. Varicosities appearing along the anterogradely labeled terminals were plotted because they are likely to represent en passant synapses (Kincaid & Wilson, 1996; Meng et al., 2004, Voigt et al., 1993). For retrograde labeling, only cells with one or more dendrites were plotted.

Photomicrographs were acquired with a Retiga EX CCD digital camera (Q-imaging) mounted to the BH-2 Olympus microscope. High-resolution fluorescent images were either acquired with a Leica DMi8 inverted microscope using a 20x objective or an Olympus FV-1000 laser-scanning confocal imaging system using a 40x objective. For TB and FG imaging, sections were scanned sequentially to demonstrate both single and double-labeled neurons. Sections were then merged to produce a composite image since both fluorophores have overlapping excitation spectra.

RESULTS

In our initial comparison of the claustrum and endopiriform nuclei, we surveyed the literature for reports that described the neurochemical markers in each region. Next, we looked at rat sections processed for cytochrome oxidase (CO), acetylcholinesterase (AChE), and Nissl substance, in our own tissue and in an atlas (Paxinos & Watson, 2007). We then compared the gross morphology of the claustrum in the rat and human brains.

To determine whether the endopiriform region has connections that resemble those described for the claustrum (Smith & Alloway, 2010; 2014), we performed a series of tracer injections in 14 rats split into three groups. In the first group, a mixture of anterograde and retrograde tracers was deposited directly into the DEn (n=4). In the second group, two retrograde tracers were injected into the same animal: one in infralimbic (IL) cortex and another in entorhinal (Ent) cortex (n=5) of the same hemisphere. The third group received an anterograde injection in IL of one hemisphere coupled with a retrograde tracer in IL of the other hemisphere (n=5).

Comparative cytoarchitecture and chemoarchitecture of the claustrum-endopiriform complex

To determine the degree of neurochemical similarity in the claustrum and DEn, we reviewed studies that characterized the proteins, genes, and other neurochemical markers in both of these structures. A list of substances common to both the claustrum and DEn appears in Table 1. Shared substances include nitric oxide synthase (NOS), cholecystokinin (CCK), neuropeptide Y, calretinin, latexin, and the κ , μ , and δ opioid receptors. In fact, whereas a total of 24 identified markers were observed in both DEn and the claustrum, only 17 markers were exclusive to one region or the other (Table 2). Substances uniquely observed in DEn but not in claustrum include vasopressin, GLUT 1 and 4, and glucocorticoid receptors. Based on this analysis, the evidence indicates a greater number of similarities than differences in the neurochemical composition of the DEn and claustrum.

Our next goal was to identify the claustrum and endopiriform nucleus using traditional histological techniques. This allowed us to visualize the geometry of the claustrum and DEn in rodent tissue, and then compare the rodent shape with that of the human claustrum (Figure 1). CO reactivity in rats revealed a densely-labeled core region in the claustrum with sparse reactivity in the dorsal claustrum and endopiriform nucleus (Figure 1A). The DEn is clearly identifiable in Nissl stained sections as being flanked medially by the white matter of the external capsule and laterally by the sparsely packed deep layer of the piriform cortex (Figure 1B), which appropriately corresponds to the definition in the rat brain atlas (Paxinos & Watson, 2007). As previously reported (Loo, 1931), Nissl staining reveals dense cell packing in the ventral claustrum, but cell packing is notably less dense in the DEn (Figure 1B). In contrast, cell distribution throughout the dorsoventral extent of the human claustrum appears uniform (Figure 1C). Figure 1D illustrates the dorsoventral outlines of the claustrum-endopiriform complex processed for CO, Nissl, and AChE in the rat, which are compared with the outline of the claustrum in a Nissl-processed section from the human brain. From these outlines, it is apparent that the shape of the human claustrum is qualitatively similar to the shape of the rodent claustrum-endopiriform complex. Hence, in both species these regions are characterized by a long, narrow dorsoventral extent with a mediolateral expansion of the ventral region, which represents the endopiriform in the rat.

Long-range connections of the endopiriform nucleus

To characterize the extrinsic and intrinsic connections of the DEn, a mixture of anterograde (BDA) and retrograde (FG) tracers was injected into the DEn as shown in Figure 2. In this case, the BDA injection was localized to the dorsal portion of the endopiriform nucleus, near

the rhinal fissure (Figure 2B), and almost no BDA diffused into the surrounding tissue. The corresponding FG injection was visualized in adjacent sections and was centered in DEn, with slight diffusion into the caudate-putamen, the ventral claustrum, and the deep layers of piriform cortex (Figure 2C).

A unique feature of the claustrum is the long-range intrinsic connectivity throughout its rostrocaudal extent (Smith & Alloway, 2010). To determine whether DEn contains similar long-range interconnections, the rostral (Figure 3A1), intermediate (Figure 3C1), and caudal (Figure 3E1) sectors of the DEn were examined for labeled terminals and soma produced by the DEn injection shown in Figure 2. Dense BDA-labeled terminals and fibers were seen rostral and caudal to the injection site (Figure 3A2,C2,E2), demonstrating longitudinal fibers coursing throughout the entire DEn. In adjacent sections, magnified views of the DEn under fluorescence revealed dense packing of FG-labeled neurons in the same regions that show BDA labeling (Figure 3B,D,F), indicating that these longitudinal projections are reciprocal. The interconnectivity seen throughout the rostrocaudal extent of the DEn corroborates an earlier study that injected rostral, middle, and caudal sectors of the endopiriform nucleus and observed labeling throughout the DEn (Behan & Haberly, 1999).

In addition to the dense intrinsic connectivity of the DEn, dense labeling from both anterograde and retrograde tracers were observed in the infralimbic (IL) and lateral entorhinal (LEnt) cortical areas, which is consistent with previous findings reported in cats, rats, and primates (Druga, 2014). In addition, labeled terminals and cell bodies were also observed in the ipsilateral amygdaloid complex, as well as the orbital, piriform, and insular cortices as previously reported (Behan & Haberly, 1999; Majak & Mory, 2007).

We previously reported that the claustrum receives bilateral inputs from frontal cortex, but projects mainly to ipsilateral cortical areas (Smith & Alloway, 2010; 2014). A similar interhemispheric pattern was also observed among DEn connections in the present study. To analyze this issue in greater detail, we examined the anterograde and retrograde labeling in IL cortex in each hemisphere (Figure 4) produced by the DEn injection shown in Figure 2. Inspection of BDA-labeled terminals in IL revealed only ipsilateral labeling (Figure 4B), with the densest projections to the deep layers of IL (Figure 4B,C). In contrast, retrogradely-labeled neurons were observed in both hemispheres, predominantly in the superficial and intermediate layers of IL (Figure 4D,E,F), but layer 6 was devoid of labeling and is consistent with our previous retrograde injections in claustrum (Smith & Alloway, 2010; 2014). Figure 4G shows the distribution of BDA and FG labeling across multiple sections of frontal cortex from an injection into the DEn, with the majority of labeling in IL. The same pattern of labeling was observed in all animals that received FG/BDA injections in DEn. This pattern of interhemispheric connectivity with cortex is qualitatively similar to the labeling observed in sensorimotor cortex following injections into the claustrum.

We also analyzed labeling in LEnt produced by the FG/BDA injections shown in Figure 2. The entorhinal cortex was found to have reciprocal connections with the DEn (Figure 5), but these connections were only present in the ipsilateral hemisphere. Neurons labeled by FG appeared throughout most layers of LEnt, but spared layer 1 and deep layer 6 (Figure 5F,G). Thin labeled fibers with en passant boutons terminated mainly in the deep layers of LEnt

cortex (Figure 5A,G). The density of this labeling appeared weaker than in IL cortex, and consisted of thin fibers with small boutons (Figure 5C). Similar axonal and terminal morphology was observed in the sensorimotor cortical regions following anterograde tracers in cat claustrum (LeVay & Sherk 1981; da Costa et al., 2010), and this terminal morphology is consistent with connections that have a modulatory influence on cortical neurons (Sherman & Guillery, 1998). Both the anterograde and retrograde connections of the DEn are similar to the connections between rat claustrum and sensorimotor cortex (Smith et al, 2012).

Divergent projections from the endopiriform nucleus

Our previous work demonstrates that individual claustral neurons have divergent axonal projections that innervate modality-related cortical areas (Smith et al., 2012; Smith & Alloway 2014). It is not known, however, whether DEn neurons also send diverging projections to multiple cortical targets in the limbic system.

To address this issue, we injected different retrograde tracers in IL and LEnt of the same hemisphere in each animal. As shown in Figure 6, we injected the retrograde tracers FG and true blue (TB) into IL and LEnt cortical regions, respectively. These paired tracer injections revealed overlapping populations of FG- and TB- labeled neurons in DEn, including many double-labeled neurons (Figure 6F). This indicates that DEn projection neurons have axonal collaterals that innervate both IL and LEnt. Digital reconstructions of the labeled neurons in DEn showed extensive overlap (white bins in Figure 6G). This pattern of connectivity is similar to the pattern we observed in the claustrum following paired injections of retrograde tracers in somatosensory and motor cortex (Smith et al., 2012), or in visual cortex and the frontal eye fields (Smith & Alloway, 2014).

Interhemispheric cortico-endopiriform-cortical loop

Another unique aspect of claustral connectivity is the interhemispheric cortico-claustrum-cortical loop that connects homotopic regions of frontal cortex (Smith & Alloway, 2010; 2014). However, it is not known whether DEn is part of a similar interhemispheric loop that interconnects limbic-related prefrontal regions such as IL cortex.

To determine if a DEn-based interhemispheric loop exists, we injected a retrograde tracer (FG) into IL of one hemisphere and an anterograde tracer (FR) into IL of the opposite hemisphere for each animal. As shown in Figure 7, these injections revealed an interhemispheric cortico-endopiriform-cortical loop that connected IL cortex in each hemisphere. The anterograde injection in the left IL cortex (Figure 7A) produced dense bilateral terminal labeling in the striatum and nucleus accumbens (Figure 7E). Bilateral anterograde labeling also appeared in DEn and was much denser on the contralateral (Figure 7F) than on the ipsilateral side (Figure 7D). Following the retrograde tracer injection in the right IL cortex (Figure 7C), dense neuronal labeling was observed only in the ipsilateral DEn. The retrogradely-labeled neurons were in close apposition to the anterogradely-labeled terminal projections from the FR injection in the left IL. Overlap analysis of anterogradely-labeled terminals with retrogradely-labeled neurons revealed strong overlap in DEn

ipsilateral to the retrograde tracer injection (white bins in Figure 7E), and this overlapping pattern appeared throughout the rostrocaudal extent of DEN in all of the injected rats.

DISCUSSION

We used different combinations of anterograde and retrograde tracing techniques to characterize the connections of DEN with the IL and ENt cortical areas, which are part of the limbic system. Among several new findings, our results demonstrate that DEN is part of an interhemispheric circuit that interconnects the IL cortical areas in both hemispheres. We also found that individual neurons in DEN are double-labeled after injecting different retrograde tracers into the IL and ENt cortical regions of the same animal. This is significant because divergent projections from DEN enable simultaneous transmission of information to both of these cortical targets. Our inspection of anterogradely-labeled projections from DEN indicates that the terminal arbors consist of thin axons with small boutons, a morphology that is associated with neuronal projections that exert a slow, modulatory influence on their postsynaptic targets. A laminar analysis of cortical neurons projecting to endopiriform revealed that cortico-endopiriform neurons originate in layers 2 through 5, but not layer 6, consistent with our previous findings for the claustrum. In addition to these new results, we also confirmed a previous study that reported intrinsic connections within DEN that extend throughout the entire rostrocaudal length of this structure (Behan & Haberly, 1999).

Parallel sets of connections were previously observed when we used the same tracing approach to characterize the connectivity of the claustrum with respect to the somesthetic-motor and visual-motor cortical areas (Smith & Alloway, 2010; 2014; Smith et al., 2012). In those studies, we discovered that the claustrum is an intermediate structure in an interhemispheric loop that interconnects corresponding motor cortical areas in both hemispheres. We also observed double-labeled claustral neurons when pairs of retrograde tracers were placed in modality-related sensory and motor cortical regions of the same hemisphere. Furthermore, we demonstrated the presence of long-range intrinsic connections that course through most of the rostrocaudal length of the claustrum.

Collectively, these studies indicate that DEN has circuit connections with the limbic system that parallel the somesthetic-motor and visual-motor circuit connections of the claustrum. Given the high degree of similarity among the circuit connections of the DEN and claustrum, this connectivity suggests that claustrum and DEN perform similar functions, but have different sets of inputs and outputs. When considered with respect to their neurochemical similarities and other data, it is reasonable to consider the DEN as the limbic component of a larger claustrum-DEN complex.

Longitudinal connections of DEN and the claustrum

We have shown that the claustrum has intra-nuclear, longitudinal projections that extend along its entire rostro-caudal length (Smith & Alloway, 2010). Using anterograde tracers, similar connectivity was previously observed for DEN (Behan & Haberly, 1999; Zhang et al. 2001), and our results confirm the presence of these longitudinal projections within DEN (Figure 3). Following injections of both anterograde and retrograde tracers into the same central location of DEN, we observed dense terminal projections throughout the entire length

of DEn. In these same cases, we also observed extremely dense populations of retrogradely-labeled neurons throughout DEn. These findings suggest that every part of DEn is interconnected, but the neuronal subtypes that form these interconnections remain unknown. Depending on the functional nature of these intrinsic connections, they may either synchronize activity throughout the nucleus or process information in some other way. It is interesting to note that our previous injections in claustrum revealed no labeling in DEn (Smith & Alloway, 2010). Similarly, our DEn injections in the present study revealed no labeling in the claustrum. These findings indicate that long-range connectivity within the claustrum and DEn extends rostrocaudally but not along the dorsoventral axis. Sensory maps within the claustrum are imbued from the topography of the cortical projections terminating in different areas along the dorso-ventral axis of the claustrum (reviewed in Druga, 2014). As a result, in rodents and primates, a general topography exists wherein the claustrum is organized into somatosensory, auditory, and visual zones, arranged dorsal to ventral, respectively (Pearson et al., 1982; Sadowski et al., 1997; Smith & Alloway, 2014). The fact that intrinsic interconnections within claustrum appear exclusively along the rostrocaudal axis is consistent with the segregation of auditory and visual regions in different dorsoventral domains, and with the lack of multimodal sensory responses in the primate claustrum (Remedios et al., 2010).

Interhemispheric circuit connections of DEn and the claustrum

The claustrum is involved in a unique interhemispheric circuit linking regions of motor cortex near the midline (Smith & Alloway, 2010; 2014). Specifically, a region of motor cortex (e.g. frontal eye fields in cingulate cortex) in one hemisphere sends dense projections to the contralateral claustrum where they terminate around neurons that project to the corresponding motor cortical area. This interhemispheric claustral pattern was also observed for motor cortical areas involved in regulating whisker movements (medial agranular cortex). This is significant because both of these motor cortical areas are involved in regulating bilaterally-coordinated movements.

In the present study, we observed an identical, interhemispheric cortico-endopiriform-cortical circuit linking IL cortices in each hemisphere (Figure 7). Thus, DEn has connections that could facilitate the interhemispheric transfer of limbic related information, just as claustrum facilitates the interhemispheric transfer of information related to somatomotor and visuomotor processing. We have hypothesized that this pattern of interhemispheric connectivity supports direct corticocortical communication between modality-related cortical regions, possibly providing the bihemispheric coherence necessary for the bilateral coordination of motor output in the case of somatomotor or visuomotor circuits (Smith & Alloway, 2014). An alternative hypothesis is that these interhemispheric connections coordinate widely separated modality-related cortical areas to produce a more cohesive and unified neural representation of sensorimotor and limbic-related information. This second hypothesis is more tractable in light of similar circuits existing for DEn and limbic cortex, which are not responsible for direct motor output but are involved in processing information related to motivation and visceral sensations.

Divergent projections from DEn and the claustrum

Many claustrum neurons have bifurcating projections that innervate modality-related cortical regions (e.g. frontal eye fields and visual cortex) that are separated by large distances but share direct cortico-cortical projections (Minciacchi et al., 1985; Li et al., 1986; Sadowski et al., 1997; Jakubowaska-Sadowska et al., 1998; Smith et al., 2012; Smith & Alloway, 2014). Using a dual retrograde tracer approach (Figure 6), injections in IL and LEnt revealed double-labeled neurons in DEn of the ipsilateral hemisphere. This pattern is identical to the claustral neurons with collateral projections that innervate both parietal (sensory) and frontal (motor) cortical areas that are interconnected by corticocortical projections. Hence, both claustrum and DEn are capable of modulating activity in modality related cortical regions. This pattern of connectivity may coordinate the activity of distantly separated cortical areas within and across hemispheres (Minciacchi et al., 1985), potentially synchronizing cortical areas to enhance communication fidelity and refine the temporal resolution of neuronal representations of stimuli or, possibly, to bind related sensory information into a single percept (Singer, 1999).

Laminar origin of cortical projections to claustrum-DEn complex

Early tracing studies on the claustrum in cats revealed the origin of corticoclaustral projection neurons in layer 6 of visual cortex (LeVay and Sherk, 1981). However, our recent tracing studies in rats revealed that corticoclaustral projection neurons originate from layers 2 through 5 in both motor cortex and secondary visual cortex, and layer 6 is specifically devoid of labeling (Smith & Alloway, 2010; 2014). In our current study, retrograde tracer injections into DEn revealed retrogradely-labeled neurons in layers 2-5 of both IL and LEnt, a similar pattern compared to our previous claustrum injections. Recent reviews on the claustrum have focused on the older tracing data in cat visual cortex and ignored this new opposing data in rodent (Smythies et al., 2012; Druga et al., 2014; Goll et al., 2015). Our results suggest that these hypotheses should be revisited to incorporate these new findings on the origin of cortical inputs to the claustrum-DEn complex.

Topographic organization of the claustrum-DEn complex

As shown in the circuit diagram in Figure 9, a topography exists along the dorsoventral axis of the claustrum-DEn complex. Early tracing studies in rodents revealed the existence of a crude topographic organization along the dorsoventral axis of the claustrum but with some overlap (Sloniewski et al., 1986; Li et al., 1986). Later this topography was refined to reveal a dorsal somesthetic-motor and ventral visuoauditory zones (Sadowski et al., 1997). More recently, our work revealed a dorsoventral topography related to different motor representations in frontal cortex (Smith & Alloway, 2010; 2014). Accordingly, we found that retrograde tracer injections in the whisker representation of primary somatosensory cortex were intermingled with neurons labeled by retrograde tracer injections in the whisker representation of primary motor cortex. Both populations of neurons, which included double-labeled cells, were observed in the intermediate dorsal-ventral levels of the claustrum. By contrast, the most ventral region of the claustrum contains neuronal populations that project to both the frontal eye fields and visual cortex. Our current results extend this principle of dorsoventral topography by demonstrating that DEn, which is

immediately ventral to the claustrum, projects to both IL in frontal cortex and LEnt in parietal cortex.

Is the DEn a limbic extension of the claustrum?

Debate exists in the literature regarding the relationship between the claustrum and DEn. In a rodent brain atlas, the DEn is shown as a discrete nucleus that is separate from the claustrum (Paxinos & Watson, 2007). However, primate brain atlases do not delineate the DEn or its homologue (Snider & Lee, 1962; Haines, 2011). Current hypotheses suggest that the DEn is homologous to the ventral region of the primate claustrum, largely based on connectivity with limbic cortical structures (Druga, 2014). In rodents, our data suggest that these two structures are probably not continuous parts of a single entity, but should be considered as distinct parts of a larger complex. As discussed earlier, both the DEn and claustrum have common patterns of connectivity: (1) intra-nuclear, longitudinal projections, (2) bifurcating intrahemispheric projections that innervate modality-related cortical areas, (3) laminar origin of cortical afferents from layers 2 through 5, and (4) interhemispheric cortico-claustrum-cortical or cortico-endopiriform-cortical loops. These parallel patterns of connectivity suggest similar computational functions. However, whereas the claustrum processes sensorimotor and visuomotor information, the DEn is concerned with processing limbic information. This pattern of connectivity is similar to dorsoventral topography observed in primates and cats (Markowitsch et al., 1984; Room et al., 1985; Insausti et al., 1987; Witter et al., 1988).

Our literature review of the neurochemical structure of the claustrum and DEn in rodents identified many similarities, including 24 enzymes, peptides, proteins, and receptors. However, we also identified 17 neurochemicals that were present only in the DEn. Furthermore, cytoarchitectural differences in cell packing density exist (Loo, 1931). No such analysis to our knowledge has been performed in the primate claustrum, making us unable to make conclusive statements of homology between primate ventral claustrum and rodent DEn. In light of the similarities in patterns of connectivity and some neurochemistry, but tempered by other differences in neurochemistry and cytoarchitecture, we contend that these structures should be referred to as the claustrum-endopiriform complex, in which the DEn is the limbic component.

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Figure 1.

Geometrical shape of the rodent claustrum-endopiriform complex is similar to the human claustrum. **A:** Cytochrome oxidase (CO) processed coronal section of rat brain at the level of the claustrum. Arrows identify the CO dense region of the claustrum (CLA). **B:** Nissl stained coronal section of rat brain showing the claustrum-endopiriform complex. Arrows refer to the dorsal and ventral regions of the claustrum (dCLA and vCLA, respectively), and wedges refer to the dorsal endopiriform nucleus (DEn). **C:** Nissl stained coronal section of human brain. Arrows identify the location of the claustrum. **D:** Outline of the claustrum-endopiriform complex in rat from sections stained for CO, acetylcholinesterase (AChE), and Nissl compared to the outline of the claustrum in human. Human claustrum section adapted with permission from <http://www.brains.rad.msu.edu> supported by the US National Science Foundation.

Figure 2.

Mixed anterograde/retrograde tracer injection into dorsal endopiriform nucleus. **A:** Coronal section of rat brain processed for Nissl, showing the location of the endopiriform nucleus. Number in upper right corner denotes distance from bregma in millimeters. **B:** Anterograde tracer injection of biotinylated dextran amine (BDA) in DEn in a section adjacent to the one shown in panel A. Location corresponds to inset in panel A. **C:** Retrograde injection of Fluorogold (FG) into DEn from section adjacent to the one shown in panel B. Scale bars = 1mm in A, 500 μ m in B.

Figure 3.

Tracer injections in DEn reveal extensive longitudinal projections throughout the entire rostrocaudal extent of the nucleus. **A:** Nissl stained section showing location of claustrum (CLA) and DEn. **A':** BDA-labeled terminals and neurons in DEn, rostral to the injection site shown in Figure 2B. Number in upper right represents distance rostral to bregma in millimeters. **B:** FG-labeled neurons in DEn from injection site shown in Figure 2C, panel from inset in A'. **C,C',D:** Anterograde (BDA) and retrograde (FG) labeling in DEn, caudal to the injection site shown in Figure 2. Arrow in C and C' denote blood vessel marked with an asterisk in D. **E,E',F:** Anterograde (BDA) and retrograde (FG) labeling in DEn, caudal to panels C-D. Arrow in E and E' denote blood vessel marked with an asterisk in F. Abbreviations: anterior commissure (ac); basolateral complex of the amygdala (BLA); hippocampal cornu ammonis area 1 (CA1); rhinal fissure (rf). Scale bars = 500 μ m in A; 100 μ m in B. Numbers denote distance from bregma in millimeters.

Figure 4. Endopiriform nucleus receives bilateral inputs from infralimbic cortex (IL) and projects back to ipsilateral IL. **A:** Nissl stained coronal section showing location of IL in the same case injected with BDA/FG shown in Figure 2. Number in upper right represents distance rostral to bregma in millimeters. **B:** Photomicrograph of BDA-labeled terminals in IL from inset region shown in A are exclusively ipsilateral to the injection site. **C:** High magnification image of BDA-labeled terminals in IL ipsilateral to the injection site. **D,E,F:** Retrogradely-labeled neurons in IL cortex in the left (panel D) and right (panel F) hemispheres. **G:** Plot reconstructions of BDA-labeled terminals (top row) and FG-labeled neurons (bottom row) for three sections through IL cortex. Abbreviations: forceps minor of the corpus callosum (fmi); rhinal fissure (rf). Scale bars = 2 mm in A; 500 μ m in B; 100 μ m in C; 500 μ m in D, 1 mm in E; 1mm in G. Numbers denote distance from bregma in millimeters.

Figure 5.

DEn nucleus is reciprocally connected to ipsilateral lateral entorhinal cortex (LEnt). **A:** Brightfield image of section showing BDA labeling in LEnt. **B:** Higher magnification image of BDA-labeled terminals from inset in A reveals thin fibers with small terminals. **C:** Higher magnification image of BDA-labeled terminals from inset in B. **D:** Nissl stained coronal section showing location of LEnt. **E:** Fluorescent image of adjacent section showing FG-labeling in LEnt. **F:** Higher magnification image of retrogradely labeled neurons in LEnt from inset in E. **G:** Plot reconstructions of BDA-labeled terminals (top row) and FG-labeled neurons (bottom row) for three sections of LEnt cortex. Abbreviations: Hippocampal cornu ammonis area 1 (CA1); rhinal fissure (rf). Scale bars = 500 μ m in A; 100 μ m in B; 20 μ m in C; 100 μ m in F; 1mm in G. Numbers denote distance from bregma in millimeters.

Figure 6.

Dual retrograde tracer injections into IL and LEnt cortices reveal double-labeled neurons in DEn. **A,D:** Fluorogold (FG) injection into IL. FG injection site shown in D from inset in panel A. **B,E:** True blue (TB) injection into LEnt cortex. TB injection site shown in E from inset in panel B. Arrowhead in B indicates blood vessel noted by asterisk in E. **C,F:** Intermingling of FG- and TB- labeled neurons, as well as double-labeled neurons, in ipsilateral LEnt cortex. Inset in panel C shows location in DEn of image in panel F acquired from an adjacent section processed for fluorescence. Asterisk in F indicates blood vessel indicated by arrowhead in C. Red arrowhead = FG labeled neuron; blue arrowhead = TB labeled neuron, white arrowhead = FG/TB double-labeled neurons. **G:** Digital reconstruction (center) and overlap analysis (left and right panels) of FG and TB labeling in a representative coronal section. Right panel shows ipsilateral overlapping bins (white) containing TB (blue) and FG (yellow) labeled neurons in DEn ipsilateral to the injection sites. Left panel shows a relative lack of labeling in contralateral hemisphere. Bin size = 50 μ^2 . Abbreviations: anterior pretectal nucleus (APT); claustrum (CLA); external capsule (ec); nucleus accumbens (NAc); neostriatum (ns); prelimbic cortex (PrL); rhinal fissure (rf). Scale bars = 1 mm in A; 500 μ m in C, 250 μ m in D; 25 μ m in F; 500 μ m in G (left); 1 mm in G (center). Numbers in upper right corners denote distance from bregma in millimeters.

Figure 7.

IL cortex in the left hemisphere projects to the contralateral DEn and terminates around neurons that project to IL in the right hemisphere. **A:** Anterograde injection of Fluororuby (FR) into the left hemisphere IL cortex, corresponding to left inset in panel B. **B:** Low magnification image of Nissl stained section showing location of bilateral tracer injections in IL. **C:** Retrograde injection of Fluorogold (FG) into right hemisphere IL cortex, corresponding to right inset in panel B. **D:** Confocal image of left hemisphere DEn showing FR-labeled terminals but no FG-labeled neurons. Image corresponds to left blue arrow in E. **E:** Overlap analysis of tracer labeling in coronal section. Red bins contain FR-labeled terminals, gold bins contain FG-labeled neurons, and white bins represent overlapping bins that contain at least one of each. Bin size = $50\mu\text{m}^2$. **F:** Confocal image of right hemisphere DEn shows intermingling of FR-labeled terminals and FG-labeled neurons. Image corresponds to right blue arrow in E. Abbreviations: external capsule (ec); forceps minor of the corpus callosum (fmi); neostriatum (ns); rhinal fissure (rf). Scale bars = $250\mu\text{m}$ in A; 1mm in B; $50\mu\text{m}$ in D; 1mm in E. Numbers in upper right corners denote distance from bregma in millimeters.

Figure 8.

Circuit diagram summarizing known interhemispheric connectivity of the claustrum-endopiriform complex. The DEn comprises a limbic extension of the interhemispheric loops seen in the dorsally situated claustrum. Line widths depict strength of anatomical connection. Circles denote divergent axonal projections that innervate modality-related cortical areas in the ipsilateral hemisphere. Color denotes modality: pink – sensorimotor forelimb; green – sensorimotor whisker; red – visuomotor; blue – infralimbic.

TABLE 1

Summary of neurochemical similarities between claustrum and dorsal endopiriform nucleus.

Substance	Type	Reference
Carboxypeptidase E	Enzyme	MacCumber et al., 1990
Nitric oxide synthase (NOS)	Enzyme	Guirado et al., 2003; Rodrigo et al., 1994; Vincent et al., 1992
Cholecystokinin (CCK)	Peptide	Schiffmann & Vanderhaeghen, 1991; Ingram et al., 1989; Savasta et al., 1988
Neuropeptide Y	Peptide	Kowia ski et al., 2001, 2004; Reuss et al., 1990
Nociceptin	Peptide	Florin et al., 2000
Urotensin II receptor (GPR14)	Peptide	Jégou et al., 2006
Brain-derived neurotrophic factor (BDNF)	Protein	Conner et al., 1997
Calbindin D-28K	Protein	Druga et al., 1993; Celio, 1990; Wójcik et al., 2004; Real et al., 2003
Calretinin	Protein	Druga et al., 2015; Wójcik et al., 2004; Real et al., 2003
Latexin	Protein	Arimatsu & Ishida, 1998; Arimatsu et al., 2009
Phosphatase inhibitor-1	Protein	Sakagami et al., 1994; Gustafson et al., 1991
Parvalbumin	Protein	Druga et al., 1993; Celio, 1990; Wójcik et al., 2004; Real et al., 2003; Pirone et al., 2015
α 2B Adrenoceptor	Receptor	Winzer-Serhan & Leslie, 1997
Dopamine receptor (D1)	Receptor	Dubois et al., 1986; Savasta et al., 1986; Wamsley et al., 1989
Dopamine receptor (D2)	Receptor	Weiner & Brann, 1989; Weiner et al., 1991
GFR α -1	Receptor	Burazin & Gundlach, 1999
NMDA Receptor Subunit 1	Receptor	Sato et al., 1995
Neuropeptide S receptor	Receptor	Clark et al., 2011; Xu et al., 2007
Neurotensin receptor	Receptor	Alexander & Leeman, 1998
κ 1 Opioid receptor	Receptor	Mansour et al., 1994a, 1994b, 1996; DePaoli et al., 1994; Gackenhaimer et al., 2005; Unterwald et al., 1991
μ Opioid receptor	Receptor	Mansour et al., 1994a, 1994c, 1995; Kitchen et al., 1997
δ Opioid receptor	Receptor	Mansour et al., 1994a
Opioid receptor-like (ORL1) Receptor	Receptor	Neal et al., 1999
Somatostatin receptor subtype 2 (SSTR-2)	Receptor	Helboe et al., 1999; Señarís et al., 1994; Perez et al., 1994; Breder et al., 1992

TABLE 2

Summary of neurochemical differences between claustrum and dorsal endopiriform nucleus.

Substance	Type	Endopiriform	Clastrum	Citation
Argininosuccinate synthetase	Enzyme	Yes	No	Nakamura et al., 1991
Motopsin	Enzyme	Yes	No	Gschwend et al., 1997; Iijima et al., 1999
c-kit receptor ligand	Ligand	Yes	No	Hirota et al., 1992
Atrial and C-type natriuretic peptide	Peptide	Yes	No	Langub et al., 1995
Nestafin-1	Peptide	Yes	No	Goebel et al., 2009
Neuropeptides B and W	Peptide	Yes	No	Jackson et al., 2006
Pancreatic polypeptide	Peptide	Yes	No	Whitcomb et al., 1997
Preproatrial natriuretic peptide	Peptide	Yes	No	Gundlach & Knobe, 1992
Vasopressin	Peptide	Yes	No	Szot et al., 1990
Angiotensinogen	Protein	Yes	No	Thomas & Sernia, 1988
GLUT1	Protein	Yes	No	Arluison et al., 2004; Schmitt et al., 1996
GLUT4	Protein	Yes	No	Choeiri et al., 2002; Messari et al., 1998
α 2A Adrenoceptors	Receptor	Yes	No	Uhlén et al., 1997
Glucocorticoid receptor	Receptor	Yes	No	Aronsson et al., 1988
Insulin-like growth factor-1	Receptor	Yes	No	Matsuo et al., 1989; Araujo et al., 1989
mGluR3	Receptor	Yes	No	Ohishi et al., 1993
Neurokinin-1	Receptor	Yes	No	Dam et al., 1990

TABLE 3

Summary of tracer injections.

Case	Left Hemisphere		Right Hemisphere	
	Brain Region	Tracer	Brain Region	Tracer
FA2	IL	FR	IL	FG
TI27	IL	FR	IL	FG
TI29			DEn	FG
TI30	DEn	FR	DEn	FG/BDA
TI40			DEn	BDA
TI44			IL	FG
			LEnt	TB
TI45	IL	FR	IL	FG
			LEnt	TB
TI46	IL	FR	IL	FG
			LEnt	TB
TI47	IL	FR	IL	FG
			LEnt	TB
TI48	IL	FR	IL	FG
			LEnt	TB
TI49			DEn	FG/BDA
TI50	IL	FR	IL	FG
TI51	IL	FR	IL	FG
TI52	IL	FR	IL	FG

Brain regions: infralimbic cortex (IL); lateral entorhinal cortex (LEnt); dorsal endopiriform nucleus (DEn) Tracers: Fluororuby (FR) – anterograde; Fluorogold (FG) – retrograde; Biotinylated dextran amine (BDA) – anterograde; True Blue (TB) – retrograde