Published in final edited form as:

J Comp Neurol. 2018 October 15; 526(15): 2428–2443. doi:10.1002/cne.24502.

Input-output Organization of the Mouse Claustrum

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Abstract

Progress in determining the precise organization and function of the claustrum has been hindered by the difficulty in reliably targeting these neurons. To overcome this, we used a projection-based targeting strategy to selectively label claustrum principal neurons. Combined with adeno-associated virus (AAV) and monosynaptic rabies tracing techniques, we systematically examined the pre-synaptic input and axonal output of this structure. We found that claustrum neurons projecting to retrosplenial cortex (RSP) collateralize extensively to innervate a variety of higher-order cortical regions. No subcortical labeling was found, with the exception of sparse terminals in the basolateral amygdala (BLA). This pattern of output was similar to cingulate- and visual cortex-projecting claustrum neurons, suggesting a common targeting scheme among these projection-defined populations. Rabies virus tracing directly demonstrated widespread synaptic inputs to RSP-projecting claustrum neurons from both cortical and subcortical areas. The strongest inputs arose from classically defined limbic regions, including medial prefrontal cortex, anterior cingulate, basolateral amygdala, ventral hippocampus, and neuromodulatory systems such as the dorsal raphe and cholinergic basal forebrain. These results suggest that the claustrum may integrate information related to the emotional salience of stimuli and may globally modulate cortical state by broadcasting its output uniformly across a variety of higher cognitive centers.

Graphical Abstract Text

Output of mouse claustrum collateralizes broadly to reach a variety of higher-order frontal and retrohippocampal cortical structures as revealed by AAV tracing in projection-defined neurons. Claustrum input was demonstrated using monosynaptic rabies tracing and was found to arise predominately from classically defined limbic structures.

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Keywords

Clastrum; connectivity; AAV; rabies; retrograde tracing; frontal cortex; limbic; neuromodulatory; claustrum-cortical network; RRID:AB_2572212; RRID:AB_2079751; RRID:AB_141844

Introduction

The claustrum shares widespread connections with the cortex in a variety of mammalian species (Olson & Graybiel, 1980; LeVay & Sherk, 1981; Dinopoulos et al., 1992; Sadowski et al., 1997; Tanné-Gariépy et al., 2002; Smith & Alloway, 2014; Torgerson et al., 2014; Zingg et al., 2014; Reser et al., 2016; Wang et al., 2016; White et al., 2016; Atlan et al., 2016), and has been postulated to play an important role in processes ranging from attention (Goll et al., 2015; White et al., 2018) and salience detection (Remedios et al., 2010; Remedios et al., 2014; Kim et al., 2016), to the coordination of rapid eye movement (REM) sleep (Renouard et al., 2015; Billwiller et al., 2017; for review see Crick & Koch, 2005; Mathur, 2014; Brown et al., 2017). Progress in determining its precise function and anatomical organization, however, has been hindered by the difficulty in targeting claustrum neurons for experimental manipulation due to its small, irregular shape. In particular, functional studies recording from identified claustrum neurons *in vivo* remain scarce, and anatomical studies directly targeting the claustrum have led to conflicting results due to the challenge of confining neural tracer injections within the boundaries of the claustrum.

Recent developments in transgenic and viral tracing approaches in rodents, however, provide a path forward by facilitating experimental access to genetically-defined claustrum neurons. Toward this end, previous studies have sought to characterize claustrum-specific genetic

markers, such as Gng2, Gnb4, and Ntng2, and have led to the generation of several transgenic Cre-driver mouse lines that enable conditional access to claustrum cells (Mathur et al., 2009; Watakabe et al., 2014; Wang et al., 2016). These lines have been shown to provide relatively selective access to claustrum neurons, however sparse expression in neighboring cortical and endopiriform regions again requires careful targeting of viral injections and may result in non-specific labeling issues (Wang et al., 2016).

Alternatively, claustrum neurons may be defined based on their projection to a particular cortical region using injections of a retrogradely transported Cre-expressing virus. This approach has been reported using AAV6 (Kitanishi & Matsuo, 2017), however care must be taken in choosing a cortical region that receives input only from claustrum, but not overlying insular cortex, to avoid nonspecific labeling. In this study, we found that the posterior retrosplenial cortex (RSP) was ideally suited for this purpose as it receives dense input specifically from the claustrum, but not from surrounding structures, allowing for the precise targeting of claustrum cells following secondary injections of a Cre-dependent AAV vector.

Using this approach, we addressed several unresolved questions regarding the anatomical organization of the claustrum. Specifically, we aimed to (1) determine whether or not the claustrum projects subcortically, (2) examine the extent to which claustrum neurons collateralize broadly or form segregated, pathway-specific projections, and (3) directly reveal the presynaptic inputs to the claustrum, including those from previously unreported subcortical regions, using monosynaptic rabies virus mapping. Together, our results provide a more complete understanding of the claustrum network in the mouse brain, and provide an alternative approach for precisely targeting claustrum neurons that may be adapted for use in future functional studies.

Materials and Methods

Animal preparation and stereotaxic surgery

All experimental procedures used in this study were approved by the Animal Care and Use Committee at the University of Southern California. Male and female C57BL/6J and Ai14 Cre-dependent tdTomato reporter mice (Jackson Laboratories), and GAD67-GFP mice (from Dr. Yuchio Yanagawa, Brain Science Institute, RIKEN, Japan) aged 2–6 months were used in this study. Mice were group housed in a light controlled (12 hr light: 12 hr dark cycle) environment with ad libitum access to food and water.

Stereotaxic injections of viruses and neural tracers were carried out as previously described (Xiong et al., 2015). Mice were anesthetized initially in an induction chamber containing 5% isoflurane mixed with oxygen and then transferred to a stereotaxic frame equipped with a heating pad. Anesthesia was maintained throughout the procedure using continuous delivery of 2% isoflurane through a nose cone at a rate of 1.5 liters/min. The scalp was shaved and a small incision was made along the midline to expose the skull. After leveling the head relative to the stereotaxic frame, injection coordinates based on the Allen Reference Atlas (Dong, 2007) were used to mark the location on the skull directly above the target area and a small hole (0.5mm diameter) was drilled. The following coordinates were used to target RSP (3.9 mm posterior and 1.0 mm lateral to bregma and 0.4 mm ventral from the cortical

surface), ACA (0.5 mm anterior and 0.4 mm lateral to bregma and 0.9 mm ventral from the cortical surface), and V1 (4.0 mm posterior and 2.6 mm lateral to bregma and 0.4 mm ventral from the cortical surface). To target the CLA, a hole was drilled 0.1 to 0.7 mm anterior and 3.7 mm lateral to bregma, and the head was then rotated laterally 10° before lowering the pipette through the center of the hole to a depth of 2.4 mm below the cortical surface. Mice were injected in either the right or the left hemisphere, which resulted in identical, yet mirrored, patterns of labeling for both CLA input and output. Viruses or neural tracers were delivered through pulled glass micropipettes (inner diameter of tip: ~20 µm) using pressure injection via a micropump (World Precision Instruments). Total injection volume was 60 to 80 nl, at 15 nl/min. After withdrawing the micropipette, the scalp was sutured closed and animals were administered ketofen (5mg/kg) to minimize inflammation and discomfort. Animals were recovered from anesthesia on a heating pad and then returned to their home cage.

Histology

Following sufficient post-injection survival time, animals were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. Brains were extracted and post-fixed for 24 hours at 4°C in 4% paraformaldehyde and then sliced into 150 µm thick coronal sections using a vibratome (Leica, VT1000s). The sections were serially mounted onto glass slides and coverslipped. For some experiments, a fluorescent Nissl stain was added (Neurotrace 640, ThermoFisher, Cat# N21483, RRID:AB_2572212) to reveal cell body location and cytoarchitectural information. To label cholinergic neurons in the basal forebrain, brain sections were blocked with 10% donkey serum in PBS containing 0.1% Triton-X100 (PBST) for 1 hour, followed by overnight incubation at 4°C in PBST with goat anti-ChAT antibody (Millipore Cat# AB144P, RRID:AB_2079751; 1:1000 dilution). Sections were then washed with PBS 3 times and incubated in PBST with donkey anti-goat Alexa Fluor 647 conjugated secondary antibody (ThermoFisher Cat# A-21447, RRID:AB_141844; 1:200 dilution) for 2 hours at room temperature.

CTB labeling of claustrum projection neurons

To retrogradely label RSP-, V1-, and ACA-projecting CLA neurons, each region was injected in the same animal unilaterally with 80 nl of fluorescently conjugated Cholera toxin subunit B (CTB 488, 555, or 647, respectively; 0.5%; ThermoFisher). To compare the distribution of primary motor cortex (MOP; coordinates: 0.5 mm anterior and 1.5 mm lateral to bregma, and 0.5 mm ventral from the cortical surface)- and RSP-projecting CLA neurons, CTB 488 or CTB 555 was injected unilaterally (120 nl injection volume) into MOP and RSP, respectively. Mice were euthanized 7 days following injection to allow time for tracer transport and the claustrum was examined for the presence of CTB+ cell bodies.

AAV injections for mapping CLA output

To label the axonal output of projection-defined CLA neurons, 80 nl injections of AAVretro-hSyn- Cre-WPRE-hGH (see Tervo et al., 2017; 1.2×10^{14} GC/ml, custom ordered from ViGene Biosciences) were targeted to RSP, V1, or ACA in Ai14 or wild-type C57BL/6J mice to express Cre-recombinase in CLA neurons that project to each region, respectively. In the same surgical procedure, a second injection (60 nl) of AAV1-CAG-FLEX-eGFP-

WPRES-bGH (UPenn vector core, 1.7×10^{13} GC/ml) was targeted to the ipsilateral CLA for V1- and RSP-projecting CLA neurons. For ACA-projecting CLA neurons, iontophoretic injections of AAV1-CAG-FLEX-eGFP-WPRES-bGH (Stoelting, 5 μ A current, alternating 7 s on/off for 5 mins) were applied to restrict viral spread and minimize the potential labeling of adjacent Cre+ insular cortex neurons that also project to ACA. Animals were euthanized 3 weeks following injection to allow time for viral transport and transgene expression.

Monosynaptic rabies virus tracing

To label the presynaptic inputs to RSP-projecting CLA neurons, wild-type C57BL/6J mice were injected in RSP with 80 nl of AAVretro-hSyn-Cre-WPRES-hGH and in the same surgical procedure, the ipsilateral CLA was injected with 60 nl of a 1:1 mixture of AAV1-EF1a-DIO-HTB (Salk vector core, 5.7×10^{12} GC/ml, virus expresses histone-bound GFP, TVA receptor, and rabies glycoprotein) and AAV1-CAG-FLEX-RG (UNC vector core, 3.5×10^{13} GC/ml, virus expresses only rabies glycoprotein), which was added to boost rabies glycoprotein expression in starter cells and enhance the presynaptic transfer of rabies virus. Following 3 weeks, a second injection of pseudotyped, glycoprotein-deficient rabies virus (EnvA- G-mCherry rabies, Salk vector core, 8.6×10^7 GC/ml) was injected (80 nl) into the same location of the CLA. Animals were euthanized 1 week later and brains were extracted and examined for presynaptic, mCherry+ cell bodies. To examine the identity of presynaptic basal forebrain neurons, the same injection procedure was performed on GAD67-GFP mice (Tamamaki et al., 2003) and combined with immunostaining for cholinergic neurons (see Histology).

Imaging and quantification

All images were generated using a confocal microscope (Olympus FluoView FV1000). To quantify the number of double- and triple-labeled CTB+ CLA neurons projecting to ACA, V1, and RSP, one out of every two coronal sections was collected across the entire length of the CLA (from about +1.2 to -1.0 mm relative to bregma, 7 sections total, 150 μ m thick) and imaged in a single focal plane at 40X magnification. Individual CLA neurons were quantified manually and examined in separate fluorescent channels for the presence of two or more tracers for $n = 3$ mice total.

To quantify the density of axonal output for RSP-projecting CLA neurons, one out of every two coronal sections (150 μ m thick) were collected across the entire brain and mounted in serial order. Brain regions containing fluorescently labeled axons and terminal boutons were identified and scanned at 10X magnification at the same laser intensity. Using Photoshop, background was then subtracted from each image (thresholding to 4X greater than the mean background intensity) and images were rendered binary. For each brain region, at least five 300 $\times$ 300 μ m sample areas were then selected across multiple sections to generate an average pixel density corresponding to axonal fluorescence. Average values for each region were then expressed as a fraction of the total pixel density quantified for all regions in a given animal to generate a normalized projection density profile for $n = 3$ animals. To compare projection density profiles for ACA-, V1-, and RSP projecting CLA neurons, the same approach was applied, however only seven brain regions were selected for analysis and images were collected at higher magnification (40X). Average pixel density values for each

region were normalized by dividing by the total pixel density quantified for the 7 sampled regions. Normalized projection density profiles were then compared for $n = 3$ animals in each group.

To determine the number of cells that provide monosynaptic input to RSP-projecting CLA neurons, one out of every two coronal sections (150 μm thick) was collected across the entire brain and imaged at 4X magnification. Brain regions were delineated using the Allen Reference Atlas and mCherry+ cell bodies within each region were manually quantified and expressed as a fraction of the total cells quantified for a given animal. Normalized input profiles were then compared for $n = 4$ animals. To quantify the number of cholinergic and GAD67+ basal forebrain neurons that project to the CLA (Figure 6d-f), rabies labeled mCherry+ neurons were examined for co-expression of ChAT antibody or GAD67-GFP at 10X magnification for $n = 3$ animals. Co-labeled neurons were expressed as a percentage of the total number of rabies mCherry+ neurons in the basal forebrain for each animal.

Statistics

All statistical analyses were performed using GraphPad Prism 6. For comparison of ipsilateral and contralateral inputs to claustrum, percentages were first quantified from individual brain sections (at least 3 values for each region) for each of $n = 4$ animals and were determined to have a normal distribution using the Shapiro-Wilk normality test. Comparison of means was then performed by paired Student's t-test. Differences between data sets were considered significant if $p < 0.05$. To test for any significant differences in the overall projection pattern for each population of target-defined claustrum neurons, we performed two-way ANOVA analysis. Results for all histograms are expressed as mean $\pm$ 95% confidence interval. Lower case " n " always denotes the number of animals used in a set of experiments.

Results

Distribution and co-localization of RSP-projecting CLA neurons

The claustrum (CLA) has previously been shown to project to the retrosplenial cortex (RSP) in rodents using both retrograde and anterograde tracing approaches (Wang et al., 2016; White et al., 2016). However, the extent to which RSP-projecting claustrum neurons form a segregated population or colocalize with other projection-defined claustrum neurons remains uncertain. To directly test this, we injected equal volumes of cholera toxin subunit B (CTB) conjugated with Alexa 647, 488, or 555 into the anterior cingulate area (ACA), primary visual cortex (V1), and RSP, respectively, in the same animal to retrogradely label claustrum neurons that project to each of these regions (Figure 1a, Table 2). The ACA was selected since retrograde tracer injections here have been shown to strongly label claustrum neurons throughout the entire length of the structure, and the distribution of these cells has been confirmed to fall within the boundaries of the claustrum as defined by dense parvalbumin (PV) immunoreactivity and Gng2 expression (Mathur et al., 2009; White et al., 2016; White et al., 2018). Similarly, V1 was selected as it has been shown to receive claustrum input, however, some reports suggest that sensory cortical regions may receive segregated inputs from discrete parts of the claustrum (White et al., 2016; Kitanishi & Matsuo, 2017).

Interestingly, all three injections resulted in claustrum cell body labeling that was spatially overlapped and evenly distributed along the length of the structure (Figure 1c). However, approximately twice as many cells were observed projecting to the ACA compared to RSP or V1, suggesting a denser innervation profile for this structure (Figure 1d, Table 2). Occasionally, retrogradely labeled cells were found outside the claustrum in the surrounding insular cortex following injections in ACA, but not RSP or V1, making ACA a less attractive target for accessing claustrum neurons, despite its stronger connectivity.

To determine whether ACA-, V1-, and RSP-projecting claustrum neurons represent separate populations with unique output or rather collateralize to multiple targets, individual neurons were examined for the presence of two or more tracers using confocal imaging at 40X magnification (Figure 1b, bottom panels). For each population, a surprisingly large fraction of cells was found to be double-labeled, ranging from 23–57% for each combination, while the occurrence of triple-labeled cells ranged from 15–32% (Figure 1e). This suggests that claustrum neurons, including those projecting to RSP, may frequently collateralize to innervate multiple cortical regions. Taken together, these data reveal that RSP receives input from a population of uniformly distributed, broadly targeting claustrum neurons, and may therefore serve as a suitable target for capturing a representative population of claustrum cells using a retrograde viral tracing approach.

Output of projection-defined claustrum neurons

To directly examine the output of RSP-projecting claustrum neurons, a recently developed retrograde variant of AAV (AAVretro-hSyn-Cre; Tervo et al., 2016) was injected into the RSP in Ai14 Cre-dependent tdTomato reporter mice (Madisen et al., 2012). Robust labeling of Cre⁺/Tom⁺ neurons was observed throughout the length of the claustrum, but not in surrounding cortical or endopiriform regions (Figure 2b). Injection of a second, Cre-dependent GFP-expressing virus (AAV1-CAG-FLEX-GFP) within the CLA enabled selective expression of GFP in CLA neurons and characterization of their axonal output across the entire brain (Figure 2b-c). Interestingly, a dense pattern of innervation was observed throughout multiple cortical regions, including orbital and medial prefrontal cortex, ACA, and the retrohippocampal complex, in addition to the expected labeling in RSP (the primary target of these cells) (Figure 2c-d). This demonstrates that many RSP-projecting claustrum neurons collateralize extensively to reach a wide variety of cortical targets. In addition, relatively more diffuse labeling was observed throughout posterior parietal and visual cortical areas (Figure 2c), while little or no labeling was found in primary motor, somatosensory, or auditory areas (Figure 2d). Output was restricted almost entirely to the ipsilateral hemisphere. This projection pattern was similar to that previously characterized for two genetically-defined populations of claustrum neurons (Gnb4⁺ and Ntn2⁺; Wang et al., 2016), except that we did not observe axonal labeling in the insular or piriform cortices, nor in any subcortical structure outside of the basolateral amygdala (BLA), including the mediodorsal (MD) and parvocellular ventral posteromedial (VPMpc) nuclei of the thalamus, which receive input from the surrounding insular cortex (Shi & Cassell, 1998; Figure 2c). These results suggest that RSP-projecting claustrum neurons may comprise a subset of “generic” CLA principal cells that collateralize broadly and overlap substantially with other target-defined claustrum populations.

To further test this idea, we injected AAVretro-Cre in either ACA or V1 and AAV1-FLEX-GFP into the claustrum to directly compare the output of each of these populations with that of RSP-projecting neurons. In the case of ACA-projecting neurons, iontophoretic injections were applied in the claustrum to minimize the potential for labeling Cre⁺ neurons sparsely found in the overlying insular cortex. Qualitatively, the pattern of output across the entire cortex for ACA- and V1-projecting neurons matched that observed for the RSP-projecting population (Figure 3a), suggesting that each group branches substantially to innervate the same set of cortical structures. To more closely examine any targeting bias among these populations, we quantified and compared the relative axonal projection density in several different cortical regions for each case (Figure 3b). Similar densities were observed for each population (no significant difference revealed by two-way ANOVA), however ACA-projecting claustrum neurons showed a slight bias toward innervating frontal cortical regions (ORBvl, mPFC, ACA) relative to more posterior regions (RSP, ENTl, V1) when compared to RSP- and V1-projecting populations. These results demonstrate that, overall, these projection-defined claustrum neurons share a common pattern of cortical innervation and may represent largely overlapping populations, however subtle bias may exist in the relative density of innervation for different groups.

Monosynaptic input to RSP-projecting claustrum neurons

We next aimed to characterize the presynaptic input to the claustrum using monosynaptic rabies virus tracing and RSP-projecting claustrum cells as a representative starter population (Wickersham et al., 2007; Wall et al., 2010; Sun et al., 2014). RSP-projecting claustrum neurons were primed for uptake and monosynaptic transport of a pseudotyped mCherry-expressing rabies virus (EnvA- G-mCherry rabies) following injections of AAV1-DIO-TVA-GFP and AAV1-FLEX-RG into the claustrum (Figure 4a, see methods). Starter cells were identified based on their co-expression of GFP and mCherry (Figure 4b) while presynaptic neurons expressed only mCherry (Figure 4c-e). Serial sections were collected across the entire brain and imaged to reveal the distribution of claustrum inputs (summarized in Figure 5) and individual cell bodies were quantified within ipsi- and contralateral cortical and subcortical regions (Figure 6; Table 3).

Overall, most input to the claustrum arose from the cortex (Figure 6a), which accounted for about 84% of the total presynaptic cell population (Figure 6c). Among these inputs, higher-order cortical regions were most prominently labeled, including orbital, medial prefrontal, cingulate, temporal, and entorhinal areas (Figure 5 and Figure 6a). Cell bodies were found mostly in cortical layers 2/3 and 5 for each of these regions, however layer 6 was also labeled in temporal cortex (Figure 4d). Sparse labeling was also observed in the parietal cortex (layers 5 and 6), auditory cortex (layers 2/3 and 5), and in higher visual areas (layers 2/3, 5, and 6) (Figure 4d; Figure 5). Very little labeling was found specifically within primary visual cortex (VISp), however the few cells we did observe tended to be located within cortical layer 6 (Figure 5). This is consistent with previous findings in rat (Smith & Alloway, 2014) and in cat (LeVay & Sherk, 1983; Katz, 1987), though a far greater number of layer 6 CLA-projecting neurons are present in cat VISp, perhaps representing a species-specific difference. In addition, substantial intraclaustral connectivity was found in regions posterior to the starter population (Figure 4d, bottom right panel). Higher magnification

revealed many of these neurons exhibited dendritic spines, a morphological feature of CLA principal cells, suggesting these neurons may form a network of excitatory connections along the length of the structure. Finally, little or no labeling was observed in the somatosensory or motor cortex, or in the retrosplenial cortex (Figure 5; Figure 6a). Overall, the major sources of input to the claustrum appeared to be the same regions that were most heavily targeted by claustrum output, suggesting that these areas define a reciprocally connected claustrum-cortical network. A notable exception, however, is the retrosplenial cortex which receives dense input from, but does not project back to, the claustrum.

In addition, while nearly all claustrum-cortical output was found to target the ipsilateral cortex (Figure 2c), we found prominent inputs to the claustrum from contralateral cortical regions, suggesting an asymmetrical interhemispheric organization of the claustrum-cortical network. In particular, while most cortical input areas showed an ipsilateral bias in their projection to the claustrum, cell body labeling was approximately equal in both hemispheres of the medial prefrontal cortex and was about 30% greater in the contralateral cingulate cortex (Figure 6b). This is in line with previous studies that have reported bilateral asymmetry in cortical projections to the claustrum using retrograde (Smith & Alloway, 2010) and anterograde tracing methods (Smith & Alloway, 2014; Atlan et al., 2016; Wang et al., 2016). Together, these results suggest that cortical inputs to the claustrum are highly convergent, as a wide variety of cortical regions provide synaptic input to a specific set of projection-defined claustrum cells. Moreover, these inputs arise primarily from the same higher-order cortical regions that receive claustral output, thus defining a claustrum-cortical network that is largely reciprocal, but contains notable examples of asymmetry in its interhemispheric connections.

Finally, inputs to the claustrum from subcortical regions have not been systematically characterized. We therefore examined brain sections for any presynaptic cell body labeling in regions outside the cortex. Substantial labeling was found in the basolateral amygdala; supramammillary nucleus; paraventricular, anteromedial, and reuniens nuclei of the thalamus; and the CA1 region of the ventral hippocampus (Figure 4e, Figure 5, and Figure 6a). In addition, we found prominent input from neuromodulatory cell populations in the dorsal raphe (presumed serotonergic neurons), and the basal forebrain, which contains cortically projecting cholinergic neurons. To further confirm the identity of presynaptically labeled basal forebrain neurons, we performed monosynaptic rabies virus tracing in GAD67-GFP mice (Tamamaki et al., 2003) combined with immunostaining for choline acetyltransferase (ChAT), a marker for cholinergic neurons (Figure 6d-f). We found that approximately 65% of claustrum-projecting basal forebrain neurons are cholinergic, while ~20% of them are GAD67+. The remaining 15% may be either glutamatergic or GAD2+ inhibitory neurons. Together, these data suggest that, in addition to a prominent network of cortical inputs, claustrum neurons may be driven by a set of subcortical regions implicated in setting motivational and arousal states (summarized in Figure 7e).

Lastly, it is worth noting that very little interaction was observed between RSP-projecting CLA neurons and primary motor and somatosensory cortices in our input-output studies. This is similar to previous observations made by Wang et al. (2016), however other studies have suggested that a dorsal component of the CLA may be reciprocally connected with motor and somatosensory areas (Sadowski et al., 1997; Smith & Alloway, 2014; White et al.,

2016). To test if these motor cortex-projecting neurons overlap with RSP-projecting CLA neurons, we injected CTB 488 into the primary motor cortex (MOp) and CTB 555 into the RSP in the same animal and examined the claustrum for retrogradely labeled cells (Figure 7a-b). Interestingly, each injection gave rise to two largely segregated populations. RSP-projecting CLA neurons formed an oval-shaped cluster that extended the length of the structure, while MOp-projecting neurons formed a thinner cluster just dorsal to this region and were predominately located near the anterior half of the claustrum. The distribution of these MOp-projecting neurons roughly matched that of several known genetic markers for CLA and endopiriform (EP) cells, including *Gnb4*, *Ntng2*, and *Latexin* (Figure 7c; Watakabe et al., 2014; Wang et al., 2016), however they appeared to lie outside of the parvalbumin-enriched region used to define CLA in Mathur et al., (2009) which circumscribes cingulate- and RSP-projecting CLA populations (Mathur et al., 2009; White et al., 2016; Kim et al., 2016). Whether or not these MOp-projecting neurons co-express such genetic markers and should be considered part of a larger claustrum complex, or rather comprise deep layers of gustatory and visceral cortex as proposed by Wang et al. (2016), requires further investigation (see Figure 7d).

Discussion

In this study, we used a retrograde viral injection strategy to access CLA neurons that project to retrosplenial cortex. We found that injections of AAVretro-hSyn-Cre into the posterior-most parts of RSP in Ai14 mice robustly labeled neurons throughout the length of the CLA, but not in any surrounding structures. This enabled highly selective targeting of these CLA neurons with local injections of Cre-dependent AAV or pseudotyped rabies virus to establish their input-output profile. Using this approach, we (1) confirmed a lack of subcortical projections from CLA principal neurons, with the exception of sparse projections to BLA; (2) demonstrated extensive collateralization of CLA output, with similar targeting trends for three different projection-defined populations; (3) and showed for the first time the presynaptic sources of input to the CLA from across the entire brain using monosynaptic rabies tracing.

Several studies have systematically examined the output of the claustrum in rodents using anterograde or retrograde tracing techniques (Sadowski et al., 1997; Smith & Alloway, 2014; Wang et al., 2016; White et al., 2016). These studies characterized widespread projections to the ipsilateral cortex, and, in particular, emphasized strong innervation of higher cortical centers in prefrontal, cingulate, and retrohippocampal regions. Our results confirm these findings and clarify additional unresolved issues related to claustrum output. Specifically, some reports suggest the claustrum may project to subcortical regions such as the striatum, amygdala, thalamus, and hypothalamus (Carey & Neal, 1986; Dinopoulos et al., 1992; Wang et al., 2016). These results are inconsistent, however, and may be due to the spread of injections into the surrounding insular cortex. Using our projection-based approach to selectively target CLA neurons, we observed sparse termination in the BLA, however no other subcortical projections were identified. This suggests that CLA output is almost entirely restricted to the cortex, and previously observed thalamic and striatal projections may have arisen from labeled neurons in layers 5 and 6 of the insular cortex. In addition, we did not see any projections directly into, or from, the overlying insular cortex, as has been

noted in some previous studies (Wang et al., 2016; Kitanishi & Matsuo, 2017). This is in line with previous work in cat reporting such a lack of connections (Markowitsch et al., 1984), and suggests that the CLA functions separately from the insula, despite residing within its deepest layers. These observed differences in connectivity may be due to the enhanced selectivity in labeling CLA neurons, but not surrounding cell types, afforded by our projection-based targeting approach.

Despite extensive work characterizing claustrum output, the degree to which these neurons segregate to form target-specific output populations or collateralize to uniformly innervate the cortex remains unknown. This is important to resolve as some studies suggest the claustrum may be organized into modules with distinct input and output (Smith & Alloway, 2014; Atlan et al., 2016; White et al., 2016; Kitanishi & Matsuo, 2017), however recent findings based on the reconstruction of several individual claustrum neurons suggest that they are capable of collateralizing to a large number of cortical targets and may therefore broadly influence cortical function (Wang et al., 2017). Our multi-fluorescent retrograde tracing revealed that 23–57% of CLA neurons projecting to either V1, RSP, or ACA, also projected to another target, and a modest fraction (15–32%) projected to all three targets. The lower ends of these ranges corresponded to the ACA-projecting population, which consistently showed a lower fraction of co-labeling due to the comparatively larger (roughly two-fold greater) total population of CLA cells back-labeled with each injection (Figure 1c,d; Table 2). These co-labeling percentages are much higher than previously reported (White et al., 2016; Kitanishi & Matsuo, 2017), yet still likely represent an underestimate of the total number of CLA neurons that collateralize to multiple targets, given that each injection site samples only a small region of cortical space.

These data shed some light on the frequency of multi-targeting for CLA neurons, but still do not resolve whether projection-defined populations exhibit bias in their output profile and thus represent unique output channels from the claustrum. To address this, we examined the pattern of axonal output from three different target-defined CLA populations. Surprisingly, no significant difference in the cortical innervation pattern (as determined by two-way ANOVA) was observed for CLA neurons defined by their projection to ACA, RSP, or V1, though caution must be taken in interpreting these data given the high false discovery rate associated with smaller sample sizes ($n = 3$ in this case, see Colquhoun, 2014). In addition, each population innervated the full set of cortical targets previously described for two genetically-defined CLA populations (Gnb4+ and Ntng2+; Wang et al., 2016), suggesting that the output from each of these target-defined populations may be representative of the CLA as a whole, at least as it is defined here (i.e. within the region characterized by dense PV- and Gng2-immunoreactivity, which has been shown to circumscribe cingulate- and retrosplenial-projecting CLA populations; see Mathur et al., 2009; White et al., 2016; Kim et al., 2016; and Figure 7). Together, these data reveal that CLA principal neurons may frequently and indiscriminately collateralize to multiple targets, and their output may therefore serve to globally modulate processing across a wide variety of cortical areas. Given the laminar specificity of CLA output, especially in layers 1, 5a, and 6 of cingulate and medial prefrontal cortex, determining the specific post-synaptic cell classes that are innervated by the CLA, and the net effect of activation on local cortical processing, remains an important question to resolve (Jiang et al., 2013; Harris & Shepherd, 2015).

In addition, future studies may seek to clarify the existence of a more dorsally situated population of claustrum neurons that preferentially interacts with primary motor and somatosensory cortex, as suggested by previous work using anterograde and retrograde tracer injections in these regions (Smith & Alloway, 2010; Smith & Alloway, 2014; White et al., 2016). These neurons appear to be largely segregated from the ACA-, V1-, and RSP-projecting CLA neurons defined in this study, as axonal output from the latter populations was extremely sparse or absent in primary motor and somatosensory cortex (Figure 2c and Figure 3a), and dual-retrograde injections in RSP and primary motor cortex revealed mostly non-overlapping cell populations along the claustrum axis (Figure 7b). This population may therefore function in parallel with the more ventrally-defined CLA region in this study, perhaps analogous to the endopiriform nucleus which lies below the CLA and interacts specifically with olfactory-related cortical networks (Watson et al., 2017; Figure 7d). Whether or not these motor cortex-projecting neurons share genetic and morphological features of claustrum neurons, however, or rather belong to deep layers of overlying gustatory and visceral cortex requires further investigation (Wang et al., 2016).

Several studies have systematically characterized inputs to the claustrum in rodents using anterograde tracing (Atlan et al., 2016; Wang et al., 2016). This approach comes with some limitations, however, as the extent to which axons projecting to the claustrum form synaptic connections or merely pass through the structure remains uncertain, and the laminar distribution and morphological identity of the input neurons are not revealed. We therefore used monosynaptic rabies virus tracing to directly reveal this information and disambiguate the synaptic nature of the input. Using RSP-projecting CLA neurons as a starter population, we demonstrated widespread inputs from both cortical and subcortical regions. Most presynaptically labeled cells were found in the cortex (84% of total) and the greatest sources of input were often the same cortical regions that received the densest innervation from the CLA, including orbital, mPFC, cingulate, and entorhinal cortices, suggesting a reciprocal network among these higher order cortical regions and the CLA. An exception however, was retrosplenial cortex, which was found to receive strong input from CLA, but did not project back. Additionally, most input to the CLA arose from sources ipsilateral to the injection site, however about half of the input from mPFC and over 60% from ACA arose from the contralateral hemisphere. This is in agreement with previous observations reported with retrograde (Smith & Alloway, 2010) and anterograde tracing (Smith & Alloway, 2014; Atlan et al., 2016; Wang et al., 2016), however the functional significance of this bilateral asymmetry remains to be determined. It is interesting to note, as well, that such widespread presynaptic input, from nearly all of the potential sources implicated in previous studies (e.g. Wang et al., 2016), was observed for this projection-defined population of starter cells. This suggests that inputs to CLA neurons, in general, may be highly convergent and points to a more uniform, rather than modular, circuit organization for this structure in the mouse brain. Finally, it should be noted that this study reveals inputs specifically to excitatory principal neurons in CLA, so it remains to be determined if there are any differences in the sources or bias of inputs to local inhibitory cell-types within the CLA circuit.

Inputs to the CLA from subcortical regions have not been systematically reported. We therefore examined the entire brain for any sources of subcortical input to the CLA. Interestingly, we found prominent labeling in regions implicated in regulating the arousal

and the affective state of the animal, including the basolateral amygdala, CA1 region of the ventral hippocampus, paraventricular (PVT) and reuneins (RE) nuclei of the thalamus, lateral supramammillary nucleus (SUMI), and serotonergic and cholinergic inputs from the dorsal raphe and basal forebrain, respectively (Swanson, 2000; Vertes et al., 2015; Reppucci & Petrovich 2015; Hu 2016). Many of these regions have been shown to modulate their activity in response to rewarding or aversive stimuli or to cues that predict such outcomes (Hangya et al., 2015; Zhu et al., 2016; Burgos-Robles et al., 2017; Matias et al., 2017; Beyeler et al., 2018; Jimenez et al., 2018). The CLA may therefore integrate some of these response features and broadcast its output to the cortex to modulate processing under emotionally salient conditions. Taken together, our results provide a more complete understanding of the claustrum network in the mouse brain and provide an alternative approach for precisely targeting claustrum neurons that may be adapted for use in future functional studies.

Acknowledgments

This work was supported by grants to L.I.Z. from the U.S. National Institutes of Health (R01DC008983 and RF1MH114112). H.W.T. was supported by NIH grants (EY019049 and EY022478) and the Kirchgeessner Foundation. B.Z. was supported by NIH grant (F31DC015185). H.W.D. was supported by NIH grant (R01MH094360).

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Figure 1.

Co-localization of projection-defined claustrum neurons.

(a) Injection site locations in anterior cingulate (ACA, CTB 647, blue), primary visual cortex (V1, CTB 555, red), and retrosplenial cortex (RSP, CTB 488, green). Scale bar: 500 μ m.

(b) Retrograde labeling in claustrum (CLA). Bottom panels, 40X magnification of V1, RSP, and ACA projecting CLA neurons. Scale bars: 500 μ m, top panel; 50 μ m, bottom panels.

(c) Distribution of retrogradely labeled cells along the claustrum axis for each injection site. Values in mm relative to bregma. Scale bar: 250 μ m.

(d) Fraction of co-labeling for total population of CLA neurons quantified for all 3 animals. 12% of the entire population of cells project to all three injection sites (see Table 2 for cell counts for each animal).

(e) Quantification of co-labeling for V1, RSP, and ACA-projecting CLA neuron populations ($n = 3$ mice, error bar = 95% CI).

Figure 2.

Axonal output of RSP-projecting CLA neurons.

(a) Viral injection strategy for labeling CLA neurons.

(b) Example injection of AAVretro-Cre in RSP (top left panel) and AAV1-FLEX-GFP in CLA (bottom left panel) in an Ai14 tdTomato Cre-reporter mouse. Retrogradely labeled Cre + neurons (red) are found in CLA, but not surrounding brain structures, and co-express GFP (green, right panels). Scale bars: 500 μ m, left panels; 100 μ m, right panels.

(c) Overview of axonal projection pattern for RSP-projecting CLA neurons (right panel). No subcortical labeling was observed, except in the basolateral amygdala. Density of axonal termination was greatest in layers (L)2/3 and L5 of orbital cortex and L1, L5a, and L6 of cingulate, but was more diffuse in parietal and visual cortical areas (left panels). Scale bars: 1 mm, right panel; 250 μ m, left panels.

(d) Quantification of axonal fluorescence density expressed as a percentage of the total for all brain regions containing labeled axons ($n = 3$ animals, error bar = 95% CI).

Figure 3.

Comparison of output for different projection-defined CLA neurons.

(a) Claustrum injection sites (top panels, green) and example axonal labeling in several cortical regions for RSP-, ACA-, and V1-projecting claustrum populations.

(b) Quantification of fluorescent axon density expressed as a fraction of the total density found for 7 sampled areas for RSP-, ACA-, and V1-projecting claustrum populations. Two-way ANOVA showed no significant difference in overall targeting preference between each population ($n = 3$ animals for each group, error bar = 95% CI).

Figure 4.

Monosynaptic input to RSP-projecting CLA neurons.

- (a) Viral injection strategy for mapping pre-synaptic input to RSP-projecting CLA neurons.
- (b) Example injection site showing CLA starter cell population (yellow) co-expressing rabies glycoprotein (RG), TVA receptor, GFP and rabies-mCherry (red). Scale bar = 200 μ m.
- (c) Example images of presynaptic rabies-mCherry labeling (red) in orbital, medial prefrontal, cingulate, and entorhinal regions.
- (d) Example images of cortical input to claustrum. Labeled cells were most prominent in layers (L)2/3 and L5 of orbital and cingulate cortex (top panels), and L5 of parietal and entorhinal cortex (middle panels). More diffuse labeling was seen across deep layers of temporal cortex (bottom left panel). Presynaptic labeling was also observed within the claustrum in sections more posterior to the starter population (bottom right panel). Higher magnification confirmed many of these cells were principal neurons with dendritic spines (inset).
- (e) Examples images of subcortical input to the claustrum.

Figure 5. Summary of brain-wide input to RSP-projecting CLA neurons. Example labeling for one case plotted on corresponding coronal atlas sections. Red dots indicate location of retrogradely labeled cell bodies and yellow dots indicate extent of starter cell population. See table 1 for list of abbreviations.

Figure 6.

Quantification of presynaptic input to RSP-projecting CLA neurons.

(a) Number of cells projecting to CLA from ipsilateral (left) and contralateral (right) brain regions expressed as a percentage of the total number of labeled cells quantified for the entire brain for each case ($n = 4$ animals, error bar = 95% CI). See Table 3 for summary of cell counts.

(b) Quantification of bilateral asymmetry in the number of cells projecting to CLA from ipsilateral and contralateral cingulate, medial prefrontal cortex (mPFC), and all other cortical regions. Percentages reflect the number of cells quantified for ipsilateral or contralateral regions divided by the total number of cells quantified for that specific region for each case ($n = 4$ animals; error bar = 95% CI; n.s., not significant, $**p < 0.01$, $***p < 0.001$; paired t-test).

(c) Fraction of total cells projecting to CLA from cortical versus subcortical brain regions ($n = 4$ animals, error bar = 95% CI, $***p < 0.001$, paired t-test).

(d) Schematic of monosynaptic rabies virus injection strategy for labeling basal forebrain neurons that project to CLA in GAD67-GFP mice.

- (e) Retrograde labeling in basal forebrain (red). Cells co-localized with both GAD67-GFP inhibitory neurons (green) and cholinergic neurons (ChAT+ immunostaining, blue).
- (f) Quantification of co-localization with ChAT+ and GAD67+ neurons expressed as a percentage of total RAV+ neurons in the basal forebrain for each case ($n = 3$ mice, error bar = 95% CI, total cell counts for all 3 mice: 424 ChAT+/663 RAV+ cells, 137 GAD67+/663 RAV+ cells).

Figure 7.

Claustrium boundaries and summary of brain-wide connectivity.

- (a) Schematic of cholera toxin subunit B (CTB) injection sites in primary motor (MOp) and retrosplenial cortex (RSP). Values shown in mm relative to bregma.
- (b) Retrograde labeling in CLA (dashed circle) following injection of CTB 555 in RSP (red) and CTB 488 in MOp (green) in the same animal. MOp projecting cells cluster just dorsal to the CLA region defined by RSP-projecting cells. Values shown in mm relative to bregma. Scale bar 250 μ m.
- (c) Gene expression pattern for latexin, a known genetic marker for CLA and endopiriform (EPd) cell types (see Watakabe et al., 2014). Gene expression is enriched in a dorsal population of cells that may correspond to motor cortex projecting CLA neurons. In situ hybridization (ISH) data from the Allen Brain Atlas database (www.brain-map.org, Experiment #72340108).

(d) Schematic showing distinct network relationships for EPd and CLA as well as the potential inclusion of a dorsal CLA region that interacts specifically with somatosensory and motor cortex (see Smith & Alloway, 2014; Watson et al., 2017; but also see Mathur et al., 2009; Wang et al., 2016).

(e) Schematic representation of major inputs to CLA characterized in this study. Major inputs include medial prefrontal, cingulate, and retrohippocampal cortical regions (dark gray); and neuromodulatory (BF, DR), midline thalamic (PVT, RE), amygdalar (BLA), supramammillary (SUMI), and ventral hippocampal (CA1v) subcortical regions (light gray).

(f) Summary of claustrum output. Axonal projections are predominately restricted to ipsilateral cortex, except for a sparse subcortical projection to basolateral amygdala (BLA). Dense projections are found along midline cortical regions, including orbital, medial prefrontal, cingulate, and retrosplenial areas, as well as more laterally located temporal (TEa), entorhinal (ENT), and retrohippocampal (PRE, POST) regions. Substantial projections were also found diffusely throughout posterior parietal (PTLp) and visual (VIS) cortical regions.

Table 1.

List of anatomical abbreviations used in this study

ACAd	Anterior cingulate area, dorsal
ACAv	Anterior cingulate area, ventral
AId	Agranular insular area, dorsal
AIp	Agranular insular area, posterior
AIv	Agranular insular area, ventral
AMd	Anteromedial nucleus, dorsal
AMv	Anteromedial nucleus, ventral
AON	Anterior olfactory nucleus
AUD	Auditory cortex
BF	Basal forebrain
BLA	Basolateral amygdalar nucleus
BMA	Basomedial amygdalar nucleus
CA1	Hippocampal field CA1
CLA	Clastrum
CM	Central medial nucleus of the thalamus
DR	Dorsal raphe nucleus
ECT	Ectorhinal area
ENTl	Entorhinal area, lateral part
ENTm	Entorhinal area, medial part
ILA	Infralimbic area
IMD	Intermediodorsal nucleus of the thalamus
LA	Lateral amygdalar nucleus
LC	Locus coeruleus
MD	Mediodorsal nucleus of the thalamus
MOp	Primary motor cortex
MOs	Secondary motor cortex
NLOT	Nucleus of the lateral olfactory tract
ORBl	Orbital area, lateral part
ORBm	Orbital area, medial part
ORBvl	Orbital area, ventrolateral part
PAR	Parasubiculum
PERI	Perirhinal area
PF	Parafascicular nucleus
PIR	Piriform cortex
PL	Prelimbic area
POST	Postsubiculum
PRE	Presubiculum
PTLp	Posterior parietal cortex
PVT	Paraventricular nucleus of the thalamus
RSPagl	Retrosplenial cortex, agranular part

RSPd	Retrosplenial cortex, dorsal part
RSPv	Retrosplenial cortex, ventral part
SNC	Substantia nigra, compact part
SUBv	Subiculum, ventral part
SUMl	Supramammillary nucleus, lateral part
TEa	Temporal association area
TR	Postpiriform transition area
TTd	Taenia tecta, dorsal part
VISl	Lateral visual area
VISm	Medial visual area
VISp	Primary visual area
VTa	Ventral tegmental area

Table 2.

Summary of retrograde labeling in CLA following triple cortical injections of CTB

		Animal 1	Animal 2	Animal 3	Total
# of cells in CLA per injection location	ACA	280	211	343	834
	RSP	136	119	179	434
	VI	116	111	159	386
# of co-labeled cells in CLA	V1+RSP	67	44	79	190
	V1+ACA	53	58	80	191
	RSP+ACA	92	54	103	249
	Triple	45	27	50	122

CTB = cholera toxin subunit B; CLA = claustrum; ACA = anterior cingulate area; RSP = retrosplenial area; V1 = primary visual cortex

Table 3.

Number of retrogradely labeled neurons following monosynaptic rabies virus tracing of inputs to RSP-projecting CLA neurons

		Animal 1		Animal 2		Animal 3		Animal 4	
		Ipsi	Contra	Ipsi	Contra	Ipsi	Contra	Ipsi	Contra
Frontal Cortex(> +1.3 mm bregma)	ORBm	631	309	243	106	203	98	412	224
	ORBvl	597	71	360	94	323	68	511	83
	ORBI	450	93	258	43	203	35	318	68
	AI _d	242	22	101	9	153	16	229	15
	AI _v	183	15	39	8	64	11	138	14
	PL	299	255	204	154	149	141	273	193
	ILA	448	353	165	125	113	114	324	215
	MOs	125	31	103	53	80	25	109	39
	MO _p	52	26	42	4	15	8	38	18
Olfactory areas	AON	430	21	84	11	103	9	298	12
	TT _d	232	11	177	0	122	5	176	12
	PIR	126	0	39	0	28	0	59	0
	NLOT	24	0	7	0	13	0	18	0
Cortex (< +1.3 mm bregma)	ACAd	421	727	243	372	190	390	377	589
	ACAv	517	926	296	424	271	516	465	786
	MOs	271	315	85	85	62	71	183	198
	PTL _p	137	24	63	5	78	10	105	23
	VIS areas	30	0	12	0	15	0	19	0
	AUD areas	291	0	168	0	140	0	265	0
	TEa/ECT/PERI	899	15	430	0	490	0	777	9
	RSP (rostral)	42	8	8	5	22	3	35	5
	ENT _l	618	2	316	9	304	5	503	6
	ENT _m	10	0	14	0	9	0	13	0
	TR	89	0	64	0	48	0	78	0
Hippocampal areas	CA1	201	0	99	0	77	0	148	0
Ascending neuromodulatory systems	Basal forebrain	410	6	223	0	232	3	366	4
	VTA/SN _c	13	0	5	0	4	0	8	0
	LH	5	0	1	0	2	0	4	0
	Dorsal raphe	56	0	34	0	24	0	45	0
	LC	1	0	0	0	0	0	1	0
Thalamus	AM _d /AM _v	22	0	33	0	15	0	19	0
	Reuniens	101	0	35	0	23	0	66	0
	Intralaminar (PVT, CI, IMD)	279	0	187	0	152	0	235	0
	PF	24	0	9	0	13	0	23	0

		Animal 1		Animal 2		Animal 3		Animal 4	
		Ipsi	Contra	Ipsi	Contra	Ipsi	Contra	Ipsi	Contra
Mammillary areas	SUMI	44	0	20	0	24	0	36	0
Amygdala	LA	60	0	39	0	28	0	41	0
	BLA	412	51	204	26	218	16	285	35
	BMA	28	0	18	0	13	0	30	0
Total # of cells:		12101		5961		5567		9578	

ACAd = Anterior cingulate area, dorsal; ACAv = Anterior cingulate area, ventral; AId = Agranular insular area, dorsal; AIp = Agranular insular area, posterior; AIv = Agranular insular area, ventral; AMd = Anteromedial nucleus, dorsal; AMv = Anteromedial nucleus, ventral; AON = Anterior olfactory nucleus; AUD = Auditory cortex; BF = Basal forebrain; BLA = Basolateral amygdalar nucleus; BMA = Basomedial amygdalar nucleus; CA1 = Hippocampal field CA1; CLA = Claustrum; CM = Central medial nucleus of the thalamus; DR = Dorsal raphe nucleus; ECT = Ectorhinal area; ENTI = Entorhinal area, lateral part; ENTm = Entorhinal area, medial part; ILA = Infralimbic area; IMD = Intermediodorsal nucleus of the thalamus; LA = Lateral amygdalar nucleus; LC = Locus coeruleus; MD = Mediodorsal nucleus of the thalamus; MOp = Primary motor cortex; MOs = Secondary motor cortex; NLOT = Nucleus of the lateral olfactory tract; ORBl = Orbital area, lateral part; ORBm = Orbital area, medial part; ORBvl = Orbital area, ventrolateral part; PAR = Parasubiculum; PERI = Perirhinal area; PF = Parafascicular nucleus; PIR = Piriform cortex; PL = Prelimbic area; POST = Postsubiculum; PRE = Presubiculum; PTLp = Posterior parietal cortex; PVT = Paraventricular nucleus of the thalamus; RSPagl = Retrosplenial cortex, agranular part; RSPd = Retrosplenial cortex, dorsal part; RSPv = Retrosplenial cortex, ventral part; SNc = Substantia nigra, compact part; SUBv = Subiculum, ventral part; SUMI = Supramammillary nucleus, lateral part; TEa = Temporal association area; TR = Postpiriform transition area; TTd = Taenia tecta, dorsal part; VIS = visual cortex; VTA = Ventral tegmental area