Functional properties, topological organization and sexual dimorphism of claustrum neurons projecting to anterior cingulate cortex

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\textbf{ABSTRACT}

\textbf{Objective:} To define the physiological properties of neurons projecting from the claustrum to the anterior cingulate cortex (ACC).

\textbf{Design:} To identify the claustrum in live slices, we used a transgenic mouse line that expresses yellow fluorescent protein (YFP)-tagged Volvox channelrhodopsin-1 at high levels within the claustrum. Claustrum cells projecting to the ACC were identified by retrograde labelling. Whole-cell patch-clamp recordings from labelled claustrum neurons were used to characterize the intrinsic electrical properties of these neurons. Cells were classified according to their intrinsic electrical properties, based on a previous classification scheme.

\textbf{Results:} Labelled neurons were found in the claustrum but not the insular cortex. Four types of ACC-projecting neurons were identified based on action potential adaptation and waveform: strongly adapting (SA) cell types 2, 3 and 4, and moderately adapting (MA) cell type 2. Labelled cells were predominantly SA4 in the anterior (44%) and posterior (63%) claustrum, while MA2 predominated (77%) in the central claustrum. The male anterior claustrum showed a bias toward SA3 cells (53%) while the female anterior claustrum showed a bias toward SA3 cells (76%).

\textbf{Conclusions:} There is ipsilateral dominance for ACC-projecting claustrum neurons, with the intrinsic properties of these neurons varying along the anterior–posterior axis. Sexual dimorphism was observed in ACC-projecting claustrum cells. Our results are consistent with the hypothesis that the claustrum serves as a link between the insular cortex and the ACC.

\section*{Introduction}

The claustrum is an enigmatic brain region of unknown function. Multiple functions have been hypothesized [1–6], largely based on the observation that the claustrum has widespread and reciprocal connections with most cortical regions [1,4,7–11]. In both humans [12,13] and rodents [14–19], the most abundant connections are found between the claustrum and the anterior cingulate cortex (ACC).

The ACC is part of a salience network (SN) that serves to switch activation of brain states from an inwardly focused default mode network to an externally responsive central executive network [20–23]. In human studies, the ACC has been observed to co-activate with the insular cortex in functional magnetic resonance imaging (fMRI)–blood-oxygen-level-dependent (BOLD) images during many conditions, including hunger, pleasurable touch [24], establishment and maintenance of a task set [25,26], induction of sadness [27] and visceral pain [28]. A weakly functioning SN is correlated with schizophrenia and delusional states in humans [29], conditions in which the claustrum has also been implicated [5,6]. While the SN was first described in humans [20–23], a putative SN has also been observed in rodents [30].

Because of the limited resolution of fMRI, the claustrum and insular cortex are not easily distinguished during functional imaging either in humans or in rodents. Indeed, it is not uncommon for the claustrum to be included in functional descriptions of the insular cortex in human [15], rodent [30] and monkey studies [31]. The rodent insular cortex is located posterior to the somatosensory cortex and lateral to the external capsule and claustrum. It consists of a granular area (gustatory and visceral cortex) and an agranular area (dorsal, ventral and posterior). As in humans [15], the mouse insular cortex receives input from the claustrum and perirhinal and ectorhinal cortices [32], and sends projections to the claustrum and somatosensory and motor cortices [17,32] but barely to the ACC [15,16].

Strong structural connections between ACC and insular cortex have neither been described in the human literature nor detected in rodent studies [14–
channelrhodopsin (ChR) mice of to examine YFP

Materials and methods

Animal surgery

All procedures were performed according to the guidelines of the A*STAR Biological Resource Centre Institutional Animal Care and Use Committee (IACUC). Adult Thy1-Volvox channelrhodopsin (ChR) mice of both sexes between 1 and 2 months of age were used in the patch-clamp experiments (line 012348, Jackson Labs, Bar Harbor, ME, USA). Anesthesia was induced by intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). Craniotomies were made at the right ACC: anteroposterior 0.73 ± 0.01 mm (mean ± SEM) from Bregma, lateromedial 0.20 ± 0.01 mm, dorsoventral 1.36 ± 0.02 mm. Injections of retrograde beads (250 nl; Lumafluor, Durham, NC, USA) were delivered at a rate of 5 nl/min. Mice were at least 28 days old at the time of bead injection and were used for experiments at least 10 days after surgery. Twice-daily doses of enrofloxacin (5 mg/kg) and buprenorphine (5 mg/kg) were delivered to the animal postoperatively for 3 days.

Brain-slice preparation

Mice were anesthetized with isoflurane and coronal brain slices (250 µm) were prepared using a vibratome (Leica) in ice-cold cutting solution (in mM): 240 sucrose, 2.5 KCl, 4 MgCl₂, 1.25 Na₃HPO₄, 26 NaHCO₃, 10 glucose and 1 CaCl₂ (osmolarity 356 ± 2 mOsm). Slices were incubated for 1 h at 34°C in oxygenated extracellular solution containing (in mM, pH 7.4, osmolarity 310 ± 3 mOsm): 126 NaCl, 24 NaHCO₃, 1 NaH₂PO₄, 2.5 KCl, 2.5 CaCl₂, 2 MgCl₂, 10 glucose and 0.4 ascorbic acid. Slices were kept for no longer than 6 h after the brain was sliced. All recordings were carried out at room temperature (24–27°C).

Patch-clamp recording

Whole-cell patch-clamp recordings were performed in claustrum slices submerged in a chamber continuously perfused with extracellular solution oxygenated with 95% CO₂ and 5% O₂. Bead-labelled neurons were visualized using a halogen lamp and observed through an mTomato filter in an upright microscope (Nikon F1 Eclipse, Tokyo, Japan). Borosilicate glass pipettes (5–9 MΩ) were filled with internal solution containing (in mM): 130 K-gluconate, 10 KOH, 2.5 MgCl₂, 2 NaCl, 10 HEPES, 0.5 EGTA, 4 Na₂ATP, 19.5 sucrose and 0.4 Na₃GTP (pH 7.3, osmolarity, 295 mOsm). Data were acquired with a Multiclamp 700B amplifier (Molecular Devices) with Digidata 1440A interface (Molecular Devices, Sunnyvale, CA, USA), digitized at 100 kHz and filtered with a 10 kHz low-pass Bessel filter.

Immunohistochemistry

Adult C57BL6 mice of both sexes between 1 and 2 months of age were used for immunohistochemistry and cell-counting experiments (n = 8). Anesthesia was induced by intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). Whole-animal perfusion fixation was performed with perfusion of 4% paraformaldehyde (PFA)–phosphate-buffered saline (PBS) solution through the right ventricle for 15 min. The brain was then removed from the mouse and fixed in 4% PFA-PBS solution for 24 h. The brain was subsequently cryoprotected in 30% sucrose–PBS solution for at least 48 h. Then, 80 µm thick brain slices were sectioned and stained with primary antibody [parvalbumin (PV), rabbit anti-PV, 1:500, Life Technologies; yellow fluorescent protein (YFP), anti-goat anti-chicken, 1:500, Life Technologies] and cell-counting experiments (n = 8).

Imaging and image processing

During whole-cell patch-clamp experiments, live slices were first imaged with a macroscope (Olympus MVX-10, Tokyo, Japan) (see Fig. 1(A)) to examine YFP...
expression in the claustrum. The macroscope was also used to confirm the site of injection by establishing that retrograde beads were located in the ACC (see Fig. 2(A)). Higher-resolution YFP fluorescence images were obtained using a confocal fluorescence microscope, either an Olympus Fluoview FVMP-E-RS (see Fig. 2(B)) or a Carl Zeiss (Oberkochen, Germany) Axioskop 2 (see Fig. 2(C)). Images of PV and YFP immunofluorescence (see Fig. 1(B,C)) were obtained using a two-photon microscope (Olympus Fluoview FVMP-E-RS), with optical sections acquired at 1 μm intervals.

Fluorescence images were processed using ImageJ software (https://imagej.nih.gov/ij/download.html). Forty Z stack images (1 μm per step) were processed to obtain a maximum-intensity volume rendering using Image J. The average fluorescence of five regions in the insular cortex (50 μm by 50 μm) was obtained and the mean fluorescence intensity and standard deviation (SD) were calculated. The image was subsequently smoothed by Gaussian filtering (three times) and a fluorescence threshold was set at the mean plus 3 SD. The final processed image was used for determining the claustrum region in the slice.

**Cell-type analysis**

Neurons were classified first by adaptation of their action potential (AP) discharge frequency and subsequently by the AP waveform. This classification scheme was developed by Tang et al. [36]. In brief, a series of step currents (1 s duration) with 15 pA increments were injected into the cell and the responses were analysed in Clampfit 10.3 software. Cells were first separated by spike-frequency adaptation, with a reduction in AP frequency of more than 20 Hz being classified as strongly adapting (SA) and cells with less than 20 Hz adaptation classified as mildly adapting (MA). The AP waveform was subsequently used for classifying SA cell types: bursting cells were classified as SA2, while non-bursting cells were further separated by their afterhyperpolarizing potential (AHP) properties into SA3 and SA4 (see Fig. 3 for examples).

**Statistical tests**

The Student’s t-test and one-way analysis of variance (ANOVA) were used for analyses that had quantitative variables (in Figs 1–3). Where data were categorical, the Pearson chi-squared test for independence was used (in Figs 4 and 5). To perform the chi-squared test, the pooled distribution of cells at all locations was compared against the distribution of cells at the anterior, middle and posterior locations. The difference between the pooled cell-type distribution and the distribution of cells examined at each location was then tested for statistical significance.

**Results**

Our goal was to characterize the physiological properties of claustrum neurons in the claustrum. To achieve this goal, we first developed a procedure that allowed us to identify the claustrum in living brain slices. We then used this procedure, in combination with retrograde labelling of ACC-projecting claustrum neurons, to obtain whole-cell patch-clamp recordings from these neurons.

**A transgenic mouse line for identification of claustrum in live brain slices**

It was previously reported that YFP-tagged Volvox ChR1 is found in the claustrum in a transgenic mouse line [37]. In living brain slices from these mice, enriched YFP fluorescence could be identified in a region where the claustrum is located, between the insular cortex and ventrolateral striatum (Fig. 1(A)). To determine whether this region is indeed the claustrum, we compared the distribution of YFP to that of PV, a marker of the claustrum core [14,38–41]. For this purpose, immunohistochemistry was performed on brain sections from Thy1-Volvox ChR1 mice (n = 3). Fixed sections were double-labelled to reveal expression of both YFP and PV (Fig. 1(B)). As previously reported [38–42], the anti-PV antibody stained neuropil within the claustrum core. To define the boundary of the claustrum core, the threshold for the PV signal was set at 3 SD above the mean value of background fluorescence, as measured in the surrounding insular cortex region (100 μm away). After the PV signal was thresholded (Fig. 1(B) (3)), the claustrum core was clearly defined and was separated from the medially located striatum and more lateral insular cortex. The YFP fluorescence signal was similarly thresholded to reveal a YFP-positive region in the claustrum, as well as the insular cortex. The YFP-expressing region within the insular cortex was clearly separated from the claustrum core by a YFP-free region that could represent the claustrum shell. The YFP signal within the claustrum closely concurred with the location of PV staining (Fig. 1(B)). Merging of the two thresholded images showed extensive spatial overlap (yellow in Fig. 1(C)), indicating that YFP-channelrhodopsin is indeed enriched in the claustrum. As previously reported [1,3,40], the area of the PV core region decreases from anterior to posterior. The area of the YFP-enriched region similarly decreases from anterior to posterior and overlaps with the PV core region (Fig. 1(D)) (Bregma 0.5, t = 0.07, p > 0.05; Bregma 1.0, t = 0.64, p > 0.05; Bregma 1.5, t = 0.48, p > 0.05, t-test). In summary, we conclude that imaging YFP fluorescence is a reliable means of visualizing the claustrum core in acute brain slices from Thy1-ChR1-YFP transgenic mice.
Location of claustrum cells that project to the ACC

To identify claustrum cells that project to the ACC, fluorescent beads were injected into the ACC of Thy1-Volvox ChR mice. The site of injection can be seen in the bright-field image of Fig. 2(A), where contrast was generated through light absorption by the beads. Using YFP fluorescence to identify the claustrum core, we observed that most retrogradely transported beads were found within the claustrum core (Fig. 2(B)). A small number of labelled cells (8.3%) were found less than 100 µm from the YFP-enriched region, suggesting that these represented neurons reside within the putative claustrum ‘shell’ region [14,40]. We did not observe fluorescent beads in the insular cortex, in agreement with previous findings [12,43]. Fluorescent beads were present in the cell bodies of the projecting neurons (Fig. 2(C)), enabling targeted patch-clamp recordings from their somata. Because of the small number and size of the beads, it is likely that some ACC-projecting neurons did not take up enough beads to be observable. Significantly more labelled neurons were found in the ipsilateral side (86.5%) compared to the contralateral side (13.5%; t = 3.79, p < 0.01, t-test) (Fig. 2(D)). This agrees with previous findings showing an ipsilateral dominance in claustral projections [19]. There was no significant difference in the number of ACC-projecting cells found in the dorsal or ventral halves of the claustrum (t = 0.02, p > 0.05, t-test) (Fig. 2(E)).

Cortical inputs to the claustrum are organized in a topographic manner along the anterior–posterior axis [2,4,19,44]. We therefore next examined differences in the distribution of labelled claustrum neurons along this axis. Labelled cells were found within a 1.5 mm zone anterior from Bregma, corresponding to coronal sections 38–52 in the Allen Institute Mouse Atlas [18]. No labelled neurons were observed more posterior than this (sections 54–69). To determine how ACC-projecting neurons were distributed within this region, sections were subdivided into three 500 µm thick groups: anterior (sections 38–42), middle (sections 43–47) and posterior (sections 48–52). Because of differences in the cross-sectional area of the claustrum along the anterior–posterior axis, we calculated the density of labelled cells by dividing the cell number by the cross-sectional area of the claustrum. Although there were more cells in the anterior and middle portions, there was no significant difference in cell density between the three regions (f = 0.1, p > 0.5, one-way ANOVA) (Fig. 2(F)).
Four claustrum cell types project to the ACC

We performed whole-cell patch-clamp recordings in brain slices from bead-injected Thy1-Volvox ChR mice to characterize the electrophysiological properties of claustral neurons projecting to the ACC. Bead-labelled neurons were depolarized with current pulses (increments of 15 pA per step, 1 s duration) to evoke APs (Fig. 3(A)). Two broad classes of projecting cells could be distinguished according to their AP adaptation patterns: the instantaneous AP frequency of SA cells adapts more than 20 Hz during a 1 s long depolarization, while MA cells adapt less than 20 Hz (Fig. 3(B)). The distribution of adaptation values was discontinuous, allowing clear distinction between SA- and MA-type cells. The average adaptation for MA2 cells was 8.6 ± 0.8 Hz and was higher for SA cells ($f = 39.91, p < 0.01$, one-way ANOVA) (Fig. 3(C)). Pairwise comparisons showed that adaptation was significantly different between all groups, except for the comparison between SA3 and SA4 cells.

The waveform of the first AP in each train was used to distinguish subtypes of SA cells [36]. SA2 cells are unique in showing doublet spikes that are not found in any other cell type under our conditions (Fig. 3(D)). SA3 cells have large (15.0 ± 0.8 mV) AHPs that follow each AP, while SA4 cells have smaller AHPs (7.7 ± 0.4 mV). AHP amplitude was significantly different between SA2 and SA3, SA3 and SA4, SA2 and MA2 ($f = 19.6, p < 0.01$, one-way ANOVA, post-hoc
analysis using Tukey’s HSD test) and SA3 and MA \((f = 19.6, p < 0.05, \text{one-way ANOVA, post-hoc analysis using Tukey's HSD})\) (Fig. 3(E)). In summary, AP waveform properties clearly distinguish each subtype of SA cell.

In addition to these features, which establish the identity of each cell type, two other electrophysiological properties were significantly different between the SA2 and SA3 cell types. The AP threshold for SA2 and SA3 cells (Fig. 3(F)) was significantly different \((f = 3.25, p < 0.01, \text{post-hoc analysis using Tukey's HSD})\), while the AP thresholds of the other cell types did not show

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**Fig. 3.** Anterior cingulate cortex (ACC)-projecting claustrum cells are composed of four electrophysiological cell types. (A) Examples of SA2, SA3, SA4 and MA2 ACC-projecting claustrum cell types, classified according to their action potential (AP) discharge in response to a depolarizing current injection. (B) Averaged plots of instantaneous frequency–time for the four cell types in response to depolarizing current injections. Neurons were determined to be strongly adapting if the change in frequency was more than 20 Hz, 200 ms after stimulus onset. (C) Difference in adaptation frequency between ACC-projecting claustrum cell types. Significant differences in firing frequency adaptation were observed between all cell types except SA3 and SA4. (D) Representative traces of the first AP in SA2, SA3 and SA4 cell types. SA2, SA3 and SA4 cells are classified by the differences in their first AP. SA2 cells have doublet spikes, SA3 cells have single spikes with deep after-hyperpolarizing potential (AHP), while SA4 have single spikes with shallow AHP. The AP threshold is marked by a grey dashed line. (E) AHP amplitudes of ACC-projecting claustrum cell types. Significant differences in AHP amplitude were observed between SA2 and SA3, SA3 and SA4, SA3 and MA2, and SA2 and MA2 cell types. (F) AP threshold values of ACC-projecting claustrum cell types. A significant difference in discharge threshold was observed between SA2 and SA3. (G) Maximal AP rising rates of ACC-projecting claustrum cell types. A significant difference was observed between SA2 and SA3 cell types. *\(p < 0.05\), **\(p < 0.01\).
significant differences. The AP rate of rise was significantly different between SA2 and SA3 cells (t = 2.89, p < 0.01, one-way ANOVA, post-hoc analysis using Tukey's HSD test (Fig. 3G)), while there were no significant differences observed between rate of rise for other cell types. ACC-projecting neurons were represented by four of the seven claustrum electrophysiological cell types: SA2, SA3, SA4 and MA2. A comparison of the electrophysiological properties of these cell types is provided in Table 1.

Because intrinsic neuronal properties change with age [44], we determined whether the parameters used for cell classification changed during development. For this purpose, our data were divided into two age groups: 5–6-week-old and 7–11-week-old mice. Detailed comparisons of SA3, SA4 and MA2 cell types at 5–6 weeks and 7–11 weeks are presented in Supplementary Tables 1–3. Neither AP frequency adaptation nor AHP properties were different between 5–6-week-old mice and 7–11-week-old mice, indicating that the classification system is sufficiently robust to classify cells by adaptation within the age range of 5–11 weeks. Although spike amplitude in MA2 cells and spike half-width in SA3 cells differed between 5–6-week-old and 7–11-week-old animals, these two properties were not used in our classification scheme and, therefore, did not affect cell classification.

**Spatial distribution of ACC-projecting claustrum cells**

ACC-projecting claustral neurons were predominantly ipsilateral (86.5%), with only sparse labelling in the contralateral claustrum. Therefore, most neuron recordings (approximately 90%, 104 of 115 neurons) were obtained from the ipsilateral claustrum (Fig. 4A). The lower number of neurons projecting from the contralateral claustrum agrees with previous observations [15,45] that projections from the claustrum to the cortex are mainly ipsilateral.

In total, 104 recordings were obtained from claustrum neurons ipsilateral to the bead-injected ACC (n = 22 mice). Of the ipsilateral cells, 36 were located in the anterior, 49 were located in the middle and 19 were located in the posterior sections (Fig. 4B). Because recordings were made from only a few labelled cells in the contralateral claustrum (n = 5 mice, 11 cells in total), no firm comparison can be made between neurons found in the ipsilateral and contralateral claustrum. Nonetheless, we note that while there were four cell types projecting to the ACC from the ipsilateral claustrum, only two cell types were observed to project from the contralateral claustrum (Fig. 4A). No MA2 cells were observed in the contralateral claustrum, even though MA2 cells represented the highest proportion of ACC-projecting cells in the ipsilateral claustrum.

The properties of ACC-projecting neurons also varied along the anterior–posterior axis. The representation of different subtypes of projection neurons was significantly different for anterior, medial and posterior brain slices (p < 0.01, chi-squared test) (Fig. 4C). The prevalent cell types at each position were: SA4 for anterior (44%), MA2 for medial (77%) and SA4 for posterior (63%). The cell-type distribution at all three positions was significantly different from the prediction based on the total population of neurons examined, suggesting a non-random spatial distribution of specific cell types along the anterior–posterior axis.

To examine this bias from a different perspective, the data were sorted according to cell types (Fig. 4D). When examined in this way, the distribution of SA2 and SA3 cell types again was not significantly different along the anterior–posterior axis. However, the distribution of SA4 and MA2 cell types was significantly different along this axis (p < 0.01, chi-squared test) (Fig. 4D). We therefore conclude that ACC-projecting claustrum cells display a non-homogeneous spatial distribution, with different cell types represented in different proportions for anterior, medial and posterior claustrum.

### Table 1. Electrophysiological properties of anterior cingulate cortex (ACC)-projecting claustrum cells

|                | SA2         | SA3         | SA4         | MA2         |
|----------------|-------------|-------------|-------------|-------------|
| **Adaptation properties** |             |             |             |             |
| Amount of adaptation (Hz) | 37.4 ± 10.4 | 20.9 ± 1.9  | 20.5 ± 1.9  | 8.6 ± 0.8   |
| Time constant, tau (ms) | 78.1 ± 8.1  | 45.7 ± 13.4 | 58.8 ± 6.6  | 61.6 ± 6.6  |
| RMP (mV)        | −72 ± 3     | −76 ± 3     | −73 ± 2     | −75 ± 1     |
| Input resistance (MO) | 409 ± 81    | 486 ± 35    | 510 ± 21    | 461 ± 21    |
| **AP properties** |             |             |             |             |
| Max firing rate (Hz) | 203 ± 2     | 27.9 ± 6.4  | 27.9 ± 2.3  | 27.6 ± 2.1  |
| AP amplitude (mV) | 85 ± 2.3    | 72.5 ± 2.4  | 74.9 ± 1.8  | 77.7 ± 1.1  |
| Max rate of rise (V/s) | 140.6 ± 5.9 | 96.4 ± 5.8  | 108.0 ± 4.8 | 113.5 ± 3.9 |
| Max rate of fall (V/s) | −29.3 ± 5.1 | −29.5 ± 1.7 | −31.3 ± 1.3 | −32.2 ± 1.1 |
| dV/dt         | −4.2 ± 0.4  | −3.4 ± 0.2  | −3.5 ± 0.1  | −3.6 ± 0.1  |
| AP width at half amplitude (ms) | 2.7 ± 0.9   | 2.6 ± 1.2   | 2.6 ± 0.2   | 2.6 ± 0.1   |
| AHP amplitude (mV) | 4.6 ± 1.4   | 15.0 ± 0.8  | 7.7 ± 0.4   | 10.5 ± 0.6  |
| Fraction of labelled cells (n) | 3.7% (4)   | 14.4% (15)  | 34.6% (36)  | 47.1% (46)  |

Data are shown as mean ± SEM. SA = strongly adapting cell types; MA = moderately adapting cell types; AP = action potential; RMP = resting membrane potential; AHP = after-hyperpolarization.
**ACC-projecting cells in the anterior claustrum display sexual dimorphism**

Because both male ($n = 10$) and female ($n = 12$) mice were used in the recordings described above, we also sorted our data by sex to determine whether there were any sex-based contributions to the distribution of projection neuron types. There was no significant difference in the number of SA2 and MA2 cells between males and females. Remarkably, there were significant differences in the proportion of SA3 and SA4 cells ($p < 0.01$, chi-squared test) (Fig. 5(A)), with SA3 cell type found exclusively in the anterior claustrum of males (50% of recordings in male anterior claustrum) and SA4 cell type found in the anterior claustrum of females (76% of recordings in female anterior claustrum).

These differences were due to heterogeneity in the anterior–posterior axis: SA3 cells were abundant in the anterior claustrum of the male, to the exclusion of SA4 cells, while the opposite was seen in female mice ($p < 0.01$, chi-squared test) (Fig. 5(B)). In contrast, there were no sex-related differences in cell types in the middle ($p > 0.01$, chi-squared test) (Fig. 5(C)) and posterior ($p > 0.01$, chi-squared test) (Fig. 5(D)) regions of the claustrum. This difference in the representation of SA3 and SA4 cells in the anterior claustrum was sufficient to explain the difference in cell-type distribution between male and female mice (Fig. 5(A)). In summary, we observed a surprising sexual dimorphism in the physiological properties of ACC-projecting neurons in the anterior claustrum.

**Discussion**

The ACC is one of the main projection targets of the claustrum. In this study, we characterized the population of claustral neurons that project to the ACC in terms of their electrophysiological properties and topographic distribution within the claustrum. We characterized four classes of claustrum-ACC neurons based on their AP discharge properties. We found a non-random topographic organization of the different cell types along the anterior–posterior axis of the claustrum as well as surprising differences in the cell-type distribution between male and female mice.

**Defining the boundaries of the claustrum in the Volvox transgenic line**

The boundary of the claustrum has been a subject of debate for the past decade [14,15,38–40]. The rodent claustrum is separated from the caudoputamen by an external capsule but has no anatomical marker to

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Fig. 4. Spatial distribution of anterior cingulate cortex (ACC)-projecting claustrum cells.

(A) Retrogradely labelled claustrum cells in the ipsilateral side were identified to be of the SA2, SA3, SA4 or MA2 cell type. MA2 cell types were most prominent, followed by SA4, SA3 and SA2 cell types. Data were obtained from 104 cells ($n = 22$ animals). Retrogradely labelled cells in the contralateral claustrum were fewer ($n = 11$ cells in five animals) and belonged to either SA3 or SA4 cell types. (B) Total number of recorded cells divided according to their prevalence along the anterior–posterior axis. Recorded cells were divided into three topological locations within the claustrum: anterior (slice numbers 38–42), middle (slice numbers 43–47) and posterior (slice numbers 48–52). Each location was 500 μm thick and corresponded to the Allen Institute reference mouse brain atlas ($n = 22$, number of cells = 115). (C) Distributions of ACC-projecting cell types in anterior, middle and posterior claustrum locations differed from the distribution of the pooled data ($p < 0.01$, $n = 22$). (D) Distributions of ACC-projecting cells from different locations on the anterior–posterior axis according to cell type. The spatial distribution of SA4 and MA2 cell types differed from the overall distribution as presented in the pooled data ($p < 0.01$, $n = 22$).
separate it from the insular cortex. In contrast, in addition to an external capsule, the primate claustrum is separated from the insular cortex by an extreme capsule. As such, rodent experiments have required post-hoc PV immunostaining to locate the claustrum. While PV immunostaining is highly selective for the claustrum core, this staining is mostly composed of neuropil and fluoresces weakly, making it difficult to accurately identify the claustrum and its boundaries.

To facilitate our experiments, we developed a method to unambiguously identify the boundaries of the claustrum core region in living brain slices. Our approach was based on the use of brain slices from a transgenic mouse line that has high YFP expression in the claustrum \[37\]. The YFP signal highly overlapped with the PV-rich neuropil of the claustrum core (Fig. 1(C,D)), indicating that the YFP signal can be used to identify the claustrum core in live slices. This eliminates the need for post-hoc PV immunostaining to locate the claustrum.

### Location of ACC-projecting neurons

Central to the hypothesis that the claustrum links the insular cortex and ACC is the prediction that the ACC and claustrum are highly connected, while direct connections between the ACC and insular cortex are sparse. Retrograde tracing provides a means to test this hypothesis: if the ACC and insular cortex were directly connected, then neurons in the insular cortex region would be labelled. Conversely, if most labelled cells were found in the claustrum, then this would argue that the connection between the insular cortex and ACC is indirect. Using retrograde labelling via fluorescent beads, we found that most retrogradely labelled neurons were within the YFP-enriched claustrum core, with some neurons in the surrounding shell region but none in the insular cortex. Similar results have also been observed using retrograde labelling with cholera toxin B \[15\]. These results support the conclusion that the claustrum projects strongly to the ACC, while the ACC and insular cortex are not directly connected \[31\].

Among the labelled claustrum neurons, 87% were located ipsilateral to the injected ACC (Fig. 2(D)). This agrees with previous anatomical evidence that most of the targets of the claustrum are ipsilateral \[14–16\]. Our data also agree with previous results showing that neurons projecting to the ACC are found throughout the dorsal–ventral axis of the claustrum, with no discernible difference between the dorsal and ventral regions \[13\]. Our findings, as well as those of others \[15,17\], differ from those of Smith and Alloway \[19\] showing that claustrum projections to the ACC are found mostly in the most ventral part of the claustrum in rats. The reason for this discrepancy may be found in the anatomical definition of the claustrum. The definition of the claustrum used in Smith and Alloway was broader and based on anatomical landmarks from the Wilson & Paxinos Atlas. The

![Anterior cingulate cortex (ACC)-projecting claustrum cells display sexual dimorphism in the spatial distribution of the different cell types.](image)

(A) The numbers of SA3 and SA4 ACC-projecting claustrum cells are different between male and female mice (p < 0.01, n = 22, 12 female). (B) ACC-projecting SA3 cells were found only in male anterior claustrum, whereas SA4 cells were found only in the female anterior claustrum (p < 0.01). (C, D) ACC-projecting claustrum cells at the middle and posterior locations, respectively, did not differ for any cell type.
most dorsal part of the claustrum, as defined in Smith and Alloway [19], would not be considered part of the PV-stained claustrum core [40]. When using the PV approach to identify the claustrum core, our data agree with previous literature [15] showing that projections from the claustrum to ACC originate from the entire claustrum core.

Properties of claustrum projection neurons

Our patch-clamp recordings allowed us to characterize the intrinsic electrical properties of claustrum neurons that project to the ACC. Out of the seven types of claustrum neurons defined in our laboratory [36], only four types were observed to project to the ACC: SA2, SA3, SA4 and MA2. This is not surprising since the cells recorded in this study constitute a specific subset of claustrum cells, which does not include interneurons and projection neurons that do not target ACC. It is possible that the differences in the intrinsic properties of each type of projection neuron confer differences in the information they transmit to the ACC or the type of inputs they receive. Thus, it will be important to determine the properties of synaptic transmission between each type of projection neuron and their postsynaptic targets within the ACC.

These different cell types were not uniformly distributed within the claustrum, with some types differentially represented across the anterior–posterior axis. Although neurons within different parts of the claustrum are known to project to different cortical regions [2,15], our results are the first to show that cells projecting to the same cortical region have electrical properties that depend on where they are found within the claustrum. This suggests that the different cell types and the different claustrum regions where they are found could have distinct functional roles in regulating ACC activity. Claustrum cells projecting to the ACC are found across the anterior–posterior axis of the claustrum. The ACC-projecting region of the claustrum overlaps with regions that project to other cortical areas [4,6,14,15]. ACC-projecting cell types were found across this anterior–posterior axis. This raises the possibility that the same ACC-projecting claustrum cell could either project to another cortical region or interact with claustrum cells projecting to other cortical regions. It is also possible that the topographic organization of ACC-projecting cells in the claustrum reflects a finer spatial distribution of recipient cells within the ACC.

Sexual dimorphism of claustrum projection neurons

We found differences in the representation of SA3 and SA4 projection neurons in the anterior claustrum of males and females, indicating sexual dimorphism in the distribution of cell types within the claustrum. Sexual dimorphism is a key feature of brain organization: structural differences have been found for the dendritic spines of ACC cells [47,48], differences have been observed in the physiological properties of deep cerebellar nuclei cells [49], functional differences have been shown in circuit activity of oxytocin interneurons [46], differences in circuit responses to social environment have been seen [50] and there are sex-related differences in insular activity during sexual arousal [51–57]. However, to the best of our knowledge, there has been no previous report of sexual dimorphism in the claustrum. Sexual dimorphism among claustrum cells projecting to the ACC could yield sex-related differences in circuit activity and behaviour.

The mechanisms responsible for these sex-related differences in claustrum neuron properties are unclear. One possibility is estrogen receptor-β, which is involved in sexual differentiation during development [58,59]. Estrogen has been shown to regulate potassium channels [60] and it is possible that this action of estrogen could be involved in sexual dimorphism in the anterior claustrum: the two types of sexually dimorphic claustrum neurons – SA3 and SA4 cells – differ in the amplitude of their AP AHPs, which are determined by potassium channel properties. In summary, estrogen receptor-β and its ligand, estradiol, could cause sex-dependent differences in neuronal intrinsic properties. As estrogen levels fluctuate during the estrous cycle, it is also possible that the intrinsic properties of claustrum neurons differ at different stages of the estrous cycle.

Implications for the role of claustrum in salience and attention

The connection between ACC and claustrum has been implicated in both attention [3,4] and saliency [23,61]. Our data show that there are different cell types that project from the claustrum to the cingulate cortex. Four different types of ACC-projecting cells were found in different ratios across topological sections, raising the question of what role each cell type or topological region may perform in attention and/or saliency. Studying the specific role of various ACC-projecting claustrum cell types may provide insights into the mechanism of claustrum modulation in attention or salience.

Central to the SN theory is that the ACC and insular cortex are structurally and functionally connected to each other. However, the spatial resolution of previous fMRI studies in humans and rodents was too low to separate the claustrum from the adjacent insular cortex and, therefore, these studies considered them as a single entity [20–23,30]. Our work shows that the rodent ACC receives projections from the claustrum, rather than the insular cortex. The absence of insular cortex cells
projecting directly to the ACC and the dense connectivity from the claustrum to the ACC provide further support for the hypothesis that the claustrum is part of the SN [3,62]. This anatomical observation raises the possibility that the claustrum links the insula and the ACC. Further work on the local connectivity within the claustrum is required to test this hypothesis, in particular to determine whether inputs from the insular cortex are able to shape the activity of ACC-projecting claustrum cells.

**Summary**

This study characterized the physiological properties of claustrum neurons that project to the ACC. Our experiments were enabled by transgenic mice that allow identification of the claustrum core in living brain slices. We observed that a large number of ACC-projecting neurons arise from the claustrum core. Our work supports and extends the concept of topological specificity by showing that it is not just different regions of the claustrum that send projections to different cortices but there is a second layer of topological specificity in which different cell types that project to the same targets are organized in different parts of the claustrum. Our results also show sexual dimorphism in claustrum cells that project to the ACC. We found that neurons from the claustrum, but not the insular cortex, project to the ACC, which suggests that the claustrum could serve as a link between the insular cortex and the ACC.

**Acknowledgements**

We thank P. Teo, J. Tan, K. Chung, S. Kay and Y. Johansson for technical assistance, Y. Tang for sharing unpublished results, and M. Graf for advice on immunohistochemistry and helpful discussions. This work was supported by a research grant [MOE2015-T2-2-095] from the Singapore Ministry of Education and by a Wallenberg Fellowship from the Knut & Alice Wallenberg Foundation.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was supported by the Knut och Alice Wallenbergs Stiftelse; and Ministry of Education, Singapore [MOE2015-T2-2-095].

**Author contributions**

Z.C. was responsible for experimental work and data analysis. All authors were responsible for experimental design and writing the manuscript.

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