THE CLAUSTRUM IS A HIGHWAY NOT A HUB: ORGANIZING PRINCIPLES OF CLAUSTROCORTICAL SYNAPTIC TRANSMISSION

Chia Zhi Qi, Zach

谢智淇

Stockholm 2020
Cover: (Left) A train travelling towards Stockholm’s old town, its tracks laid parallel to the major bridge/highway running through the city. (Right) A plane landing at Singapore’s Changi airport, a global aviation hub. Pictures by Zach Chia.
The claustrum is a highway not a hub: Organizing principles of claustrocortical synaptic transmission

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Chia Zhi Qi, Zach

Principal Supervisor:
Gilad Silberberg
Professor
Karolinska Institutet
Department of Neuroscience

Co-supervisor:
George James Augustine
Irene Tan Liang Kheng Chair Professor
Nanyang Technological University
Lee Kong Chian School of Medicine

Opponent:
Ami Citri
Associate Professor
Hebrew University of Jerusalem
Edmond & Lily Safra Center for Brain Sciences

Examination Board:
Tatiana Deliagina
Professor
Karolinska Institutet
Department of Neuroscience

Balázs Zoltan Gulyás
President's Chair Professor
Nanyang Technological University
Lee Kong Chian School of Medicine

Paolo Medini
Associate Professor
Umeå Universitet
Department of Integrative Medical Biology
To my family.

合抱之木 生于毫末
*The largest trees are born as little saplings;*

九层之台 起于垒土
*A nine-story tower starts with a base of soil;*

千里之行 始于足下
*A journey of a thousand miles begins with a single step.*

Laozi (~6th Century BC)

A.M.D.G.
ABSTRACT
The claustrum (CLA) is a brain nucleus wedged between the cortex and striatum. The behaviors it has been implicated in include consciousness, attention, memory and salience detection; dysfunction of CLA circuits is associated with schizophrenia, epilepsy, parkinsonism and disrupted consciousness.

While previous research has focused on the gross anatomy of the CLA, it is the functional communication of the CLA with other brain regions that generates behavioral output. Understanding CLA functional connectivity will bring us closer to understanding how the CLA is involved in different behaviors and how these dysfunctions can be remedied.

The anterior cingulate cortex-projecting (CLA-ACC) neuron population was used as a model to investigate claustrocortical synaptic transmission. This thesis proposes that the CLA is organized as a highway for connections between brain regions.

Paper I revealed that the CLA is organized as functional modules. Specifically, it showed that CLA-ACC neurons receive multicortical input biased towards frontal & limbic cortices rather than sensory cortices, and that CLA-ACC neurons could be segmented into at least two cortical targeting systems. An insular-claustrum-anterior cingulate cortex circuit, which may be the substrate underpinning the Salience Network, was also identified. These findings support feedforward inhibition as a mechanism of action within the CLA.

Paper II extended the concept of topological selectivity in the CLA to the single-cell level. Topological selectivity was previously known to exist at a population level. Characterization of the intrinsic electrophysiological properties of individual CLA-ACC neurons revealed four types of CLA-ACC populations. These CLA-ACC neurons were distributed heterogeneously with one type predominant in the anterior and posterior CLA and a second type prominent in the middle of the CLA.
**Paper III** identified the cell-type and layer-specific cortical targets of the CLA. It showed that CLA-ACC neurons provide excitatory monosynaptic input to all layers of the ACC and that different neuron populations receive CLA input in a layer-dependent fashion. From these data, Paper III derived a scheme of CLA targets within a cortex.

The findings from this thesis can be summarized using a transportation analogy. Although commonly described as a hub for cortical inputs and outputs, the CLA is likely organized as a collection of highways. A significantly large input should arrive within a small time-window to generate action potentials and enable downstream signal propagation. This is akin to a toll booth with a high toll fee that must be paid-in-full, without delays, before a vehicle can pass through. Projection neurons directed to the same cortical region may have different cell/layer targets. This is comparable to different vehicles on the same highway ending up in different destinations.

The findings in this thesis add to our understanding of CLA functional organization by suggesting that any input received by the CLA must be sufficiently strong in order to overcome FFI and for the signal to be propagated. This implies that only input of ethological relevance is processed. Such a mechanism could underlie CLA action across behaviors.

This thesis is divided into 6 chapters. Chapter 1 is a preamble. Chapter 2 encompasses the state-of-the-art in CLA and describes the gaps in knowledge that this thesis aims to fill. Chapter 3 clarifies the aims of this thesis. Chapter 4 provides an overview of the methods used. Chapter 5 presents and discusses the main results. Chapter 6 explores the main conclusions from this work. Manuscripts and publications are appended after.
LIST OF SCIENTIFIC PAPERS


III. Chia, Z., Augustine, G.J., Silberberg, G. (2020b). Claustrum projections to the ACC are layer and cell-type dependent. *Manuscript in Preparation*. 
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4AP</td>
<td>4-Aminopyridine</td>
</tr>
<tr>
<td>5HT3a</td>
<td>5-hydroxytryptamine receptor type 3a</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ACC</td>
<td>Anterior cingulate cortex</td>
</tr>
<tr>
<td>AHP</td>
<td>Afterhyperpolarization</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>APV</td>
<td>2-Amino-5-phosphopentanoic acid</td>
</tr>
<tr>
<td>CB</td>
<td>Calbindin</td>
</tr>
<tr>
<td>CR</td>
<td>Calretinin</td>
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<tr>
<td>CEN</td>
<td>Central executive network</td>
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<tr>
<td>CLA</td>
<td>Clastrum</td>
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<tr>
<td>CLA-ACC</td>
<td>ACC-projecting clastrum neurons</td>
</tr>
<tr>
<td>ChR</td>
<td>Channelrhodopsin</td>
</tr>
<tr>
<td>CR</td>
<td>Calretinin</td>
</tr>
<tr>
<td>DMN</td>
<td>Default mode network</td>
</tr>
<tr>
<td>DTI</td>
<td>Diffusion tensor imaging</td>
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<tr>
<td>E</td>
<td>Embryonic stage</td>
</tr>
<tr>
<td>EGR2</td>
<td>Early growth response protein 2</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>FFI</td>
<td>Feedforward inhibition</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GNG2</td>
<td>G-protein gamma-2 subunit</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>INS</td>
<td>Insular cortex</td>
</tr>
<tr>
<td>ISH</td>
<td>In-situ hybridization</td>
</tr>
<tr>
<td>MA</td>
<td>Mildly-adapting</td>
</tr>
<tr>
<td>NBQX</td>
<td>2,3-dihydroxy-6-nitro-7-sulfamoyl-benzof[f]quinoxaline</td>
</tr>
<tr>
<td>NCad</td>
<td>N-cadherin</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal stage</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PV</td>
<td>Parvalbumin</td>
</tr>
<tr>
<td>RCad</td>
<td>R-cadherin</td>
</tr>
<tr>
<td>SA</td>
<td>Strongly-adapting</td>
</tr>
<tr>
<td>SN</td>
<td>Salience network</td>
</tr>
<tr>
<td>SOM</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>Tbr1</td>
<td>T-box brain protein 1</td>
</tr>
<tr>
<td>Tbx21</td>
<td>T-box transcription factor 21</td>
</tr>
<tr>
<td>Thy1</td>
<td>Thymus cell antigen 1</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VGlut2</td>
<td>Vesicular glutamate transporter-2</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive Intestinal Polypeptide-expressing neurons</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
1 PREAMBLE

28th July 2004.

The ink had barely dried on the final edits to his manuscript, when Francis Crick breathed his last. A lifetime of scientific breakthroughs had brought him to his last big idea – that the claustrum could be the ‘conductor of consciousness’.

The central thesis of Crick’s last article (2005) was that, “[the claustrum and its neuroanatomy] appears to be in an ideal position to integrate the most diverse kinds of information that underlie conscious perception, cognition and action...”

That seminal paper has since stimulated many new discoveries and opened new avenues for research.

Inspired by Crick and Koch’s question, this thesis seeks to add more illumination on the darkness shrouding the claustrum - the organizing principles behind claustrum functional connectivity. Only by seeing who the claustrum speaks to (and how), will we be able to learn what it says.
2 INTRODUCTION

2.1 CLAUSTRUM IDENTIFICATION

First identified in 1786 by Felix Vicq d’Azyr (chronicled by Edelstein & Denaro, 2004; Parent, 2007) the name claustrum (CLA) both identifies the region and explains why it is difficult to study. The term is derived from Latin and refers to the cloistered part of a monastery open only to the clergy but closed off to the laity.

The brain region CLA is a thin, irregularly shaped sheet of grey matter found on both sides of the brain and hemmed in by the neocortex (specifically the insular, INS) and striatum. It spans the length of, and curves with, the cortex; its thickness varying from anterior to posterior. This anatomy makes lesion studies difficult to accomplish, closing off inquiry into the area for centuries.

Fortunately for modern researchers, this structure has been found throughout the mammalian kingdom (Kowianski et al., 1999). Recent work further suggests that the CLA might predate mammals (Puelles et al., 2016; Tosches et al., 2018). Much of our current understanding has been conducted in model organisms such as non-human primates, cats, rats and mice. The conserved biology across species allows findings in these model organisms to be instructive about the fundamental mechanisms and connectivity of this brain region.

2.1.1 Experimental methods to identify the claustrum

Despite its presence across species, identification of the CLA by eye is not trivial; this is especially so in rodents. Whereas the CLA can be clearly recognized in humans as it is bound by the external and extreme capsules, the CLA in rodents lacks an extreme capsule and therefore requires specific molecular and genetic tools to dissociate it from the adjacent INS. Various studies in mouse and rat models (Asrican et al., 2013; Atlan et al., 2018; Dávila et al., 2005; Graf et al.,
2020; Guirado et al., 2003; Kowiański et al., 2008; Marriott et al., 2020; Medina et al., 2004; Obst-Pernberg et al., 2001; Real et al., 2006, 2003) have shown, among other findings, that 8-12% of CLA neurons are GABAergic and 88-92% are glutamatergic, this data is summarized in Table 1.

Calcium-binding-protein markers have been particularly useful in identifying the CLA (Guirado et al., 2003; Dávila et al., 2005; Hinova-Palova et al., 2014). Researchers identified (Guirado et al., 2003; Real et al., 2003) that a Parvalbumin (PV)-rich-Calretinin (CR)-poor region was surrounded by a PV-poor-CR-rich region. The PV-rich-CR-poor region was defined as the CLA core while the PV-poor-CR-rich region was termed the CLA shell. These different levels of PV-enrichment enable identification of the CLA core (Kim et al., 2016, Fig. 1). The mouse CLA core can also be identified in transgenic mouse lines with molecularly defined populations that preferentially express in the CLA (Thy1 in Asrican et al., 2013; Egr2 in Atlan et al., 2018). A third method uses the connectivity of the CLA and fluorescent reporters from tracer injections (White et al., 2018; Zingg et al., 2018, Fig. 1).

Figure 1 PV-enrichment and tracer methods identify the claustrum. Injection of retrograde AAV virus into the ACC. Retrogradely transported virus was observed in the PV-defined CLA core region with little spread into the surrounding shell/INS region (Adapted with permission from Paper I, Chia et al, 2020a, Current Biology).
2.1.2 Core and shell morphology debate in the clastrum

While the use of molecular markers enabled the visualization of the CLA structure, it also introduced a new question regarding the boundaries of the CLA – does the CLA even have a core-shell arrangement?

The debate emerged when Mathur and colleagues (2009) attempted to redefine the size of the CLA based on what they considered to be a CLA-specific marker, G-protein gamma-2 subunit (Gng2). Gng2 was observed to be solely expressed around the PV-rich region, prompting the argument that the CLA did not have a shell region, and that the CLA comprised the PV-rich region. Later work demonstrated that Gng2 was neither CLA nor neuron specific (Pirone et al., 2012). Connectivity and classification studies further suggested the possible existence of a CLA shell (Atlan et al., 2017; Graf et al., 2020) and that topographic gradients underlie the putative core-shell arrangement (Marriott et al., 2020). It is apparent that this debate will continue for a while (Smith et al., 2019a) before an eventual consensus is reached.

In summary, in-depth interrogation of the structure of the CLA, chemical composition of CLA cells and distribution of GABAergic and glutamatergic neurons has led to the broadly accepted view that a PV-rich region exists in the CLA. Whether the surrounding PV-poor region should be considered the CLA is, however, still the subject of debate. To avoid ambiguity, the PV-rich region is the focus of research in this thesis.

2.2 ANATOMICAL CONNECTIVITY

Advances in anatomical tracing in the 1980s and 90s provided the first evidence of strong connectivity between the CLA and other brain regions. Claustral connectivity first develops around E15/16 (Bayer & Altman, 1991) and the circuitry matures by P28 (Kowianski et al., 1999). This widespread, reciprocal anatomy has been a wellspring for hypotheses regarding CLA function.
Table 1 Neurochemical organisation of the rodent claustrum

<table>
<thead>
<tr>
<th>Marker</th>
<th>Function</th>
<th>Nature</th>
<th>% CLA</th>
<th>Expression</th>
<th>Model</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>CBP involved in calcium signalling</td>
<td></td>
<td></td>
<td>CLA shell. Some cells are GABA-positive</td>
<td>Mouse</td>
<td>IHC</td>
<td>Real et al, 2003 Davila et al, 2005</td>
</tr>
<tr>
<td>CB</td>
<td>CBP involved in development (triggering cell-movement and nervous system outgrowth)</td>
<td></td>
<td></td>
<td>Slight decrease from rostral to caudal CLA. Some cells are GABA-positive</td>
<td>Mouse</td>
<td>IHC</td>
<td>Real et al, 2003 Davila et al, 2005</td>
</tr>
<tr>
<td>PV</td>
<td>CBP implicated in maturation of cortical inhibition circuits and onset of experience dependent activity</td>
<td>Inhibitory</td>
<td>7-12%</td>
<td>Densely spread and coexpressed with GABA-positive cells</td>
<td>Mouse</td>
<td>IHC</td>
<td>Guirado et al, 2003</td>
</tr>
<tr>
<td>NO</td>
<td>Interneural messenger involved in the regulation of metabolic status and dendritic spine growth</td>
<td></td>
<td></td>
<td>In CLA as well as endopiriform nuleus</td>
<td>Rat, Mouse</td>
<td>ICC</td>
<td>Kowianski et al, 2008 Marriott et al, 2020</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide that regulates flow of Ca2+ ions, involved in neuronal fiber growth, protects against excitotoxicity</td>
<td></td>
<td></td>
<td>Sparse, around CLA shell</td>
<td>Rat, Mouse</td>
<td>ICC</td>
<td>Kowianski et al, 2008 Graf et al, 2020 Marriott et al, 2020</td>
</tr>
<tr>
<td>SOM</td>
<td>Neuropeptide that controls growth of neuronal populations and neuronal migration</td>
<td></td>
<td></td>
<td>Sparse, around CLA shell</td>
<td>Rat, Mouse</td>
<td>ICC</td>
<td>Kowianski et al, 2008 Graf et al, 2020</td>
</tr>
<tr>
<td>VIP</td>
<td>Neuropeptide that controls neuronal proliferation, differentiation and confers neuroprotection</td>
<td></td>
<td></td>
<td>CLA shell</td>
<td>Rat, Mouse</td>
<td>ICC</td>
<td>Kowianski et al, 2008 Graf et al, 2020</td>
</tr>
<tr>
<td>Tbr1</td>
<td>Transcription factor protein involved in glutamatergic neuronal development, controls axonal migration and NMDA receptor expression</td>
<td>Excitatory</td>
<td>88-93%</td>
<td>Throughout CLA</td>
<td>Mouse</td>
<td>ISH</td>
<td>Medina et al, 2004</td>
</tr>
<tr>
<td>VGlut2</td>
<td>Vesicular-glutamate transporter for excitatory signals</td>
<td></td>
<td></td>
<td>CLA shell (negative in core)</td>
<td>Mouse</td>
<td>IHC</td>
<td>Real et al, 2006</td>
</tr>
<tr>
<td>Thy1</td>
<td>Neuronal cell surface protein, expressed particularly strongly in the mature axon</td>
<td></td>
<td></td>
<td>Throughout CLA</td>
<td>Mouse</td>
<td>Transgenic Reporter</td>
<td>Asrican et al, 2013</td>
</tr>
<tr>
<td>Egr2</td>
<td>Transcription regulatory factor related to hindbrain development</td>
<td></td>
<td></td>
<td>Throughout CLA, 91% co-express VGlut1</td>
<td>Mouse</td>
<td>ISH</td>
<td>Atlan et al, 2018</td>
</tr>
<tr>
<td>Rcad</td>
<td>Cell-cell adhesion molecule, involved in the formation of neural circuits</td>
<td></td>
<td></td>
<td>Decrease in expression from rostral to caudal CLA</td>
<td>Mouse</td>
<td>ISH, IHC</td>
<td>Obst-Penberg et al, 2001</td>
</tr>
<tr>
<td>Ncad</td>
<td>Cell-cell adhesion molecule, involved in the formation of neural circuits</td>
<td>Both</td>
<td>NA</td>
<td>Moderate expression found throughout CLA</td>
<td>Mouse</td>
<td>ISH</td>
<td>Obst-Penberg et al, 2001</td>
</tr>
</tbody>
</table>

* CBP: Calcium binding protein; ISH: immunohistochemistry; ICC: immunocytochemistry; ISH: in-situ hybridization.
2.2.1 Reciprocal claustrocortical connectivity

Beginning with silver degeneration studies and chemical tracing in model organisms, the CLA has been shown to have vast reciprocal connectivity with almost all cortical regions (Carman et al., 1964; Li et al., 1986; Minciacchi et al., 1985; Sadowski et al., 1997; Sloniewski et al., 1986; Zhang et al., 2001; Wang et al., 2016; White et al., 2016; Atlan et al., 2017; Zingg et al., 2014, 2018). One of its strongest connections is with the anterior cingulate cortex (ACC, Zhang et al., 2001; White et al., 2016; Atlan et al., 2017; Zingg et al., 2018), a frontal cortical region. While not yet systematically investigated, current data suggests that CLA-subcortical connectivity also exists (LeVay & Sherk, 1981a, 1981b; Kenna et al., 2004; Barbier et al., 2017; Pirone et al., 2018; Barbier & Risold, 2019).

These findings have been reproduced in human research. Diffusion tensor imaging (DTI, O’Donnell & Westin, 2011) and functional magnetic resonance imaging (fMRI, Grover et al., 2015) work showed that the CLA is connected with almost all cortical regions, especially with frontal cortices (Fernández-Miranda et al., 2008; Milardi et al., 2015; Torgerson et al., 2015; Torgerson & Van Horn, 2014).

It is thought that cortical projections to the CLA emerge from Layer 6, while CLA projections return to Layer 4 (LeVay & Sherk, 1981a; LeVay, 1986) of the same cortical region. There are, however, different types of cortices (granular, agranular, dysgranular) defined by the presence or absence of a granular layer 4. These different regions have different organizational principles (Nelson et al., 2002; Douglas & Martin, 2004; Beul & Hilgetag, 2015), implying that the input-output layers should be subjected to systematic investigation.

2.2.2 Multicortical connectivity of the claustrum

At a single cell level, a CLA cell can project to more than one cortical region (Minciacchi et al., 1985; Smith et al., 2012). This is, however, highly dependent
on the cortical targets studied (Sloniewski et al., 1986). Afferents to the CLA arrive from both hemispheres while efferent projections from the CLA usually target the ipsilateral hemisphere (Sloniewski et al., 1986; Smith & Alloway, 2010; Smith & Alloway, 2014; Wang et al., 2016; Zingg et al., 2018). These afferent projections to and efferent projections from the CLA tend to have a fixed topographic arrangement (Sloniewski et al., 1986; Sadowski et al., 1997) with discrete regions in the CLA dedicated to certain cortical areas (Olson & Graybiel et al., 1980; Remedios et al., 2010; White et al., 2016; Atlan et al., 2017) Some regions, like the ACC, send projections to and receive projections from the whole span of the CLA (Goll et al., 2015; Atlan et al., 2017).

2.3 SYNAPTIC CONNECTIVITY

While anatomical connectivity hints at the existence of a circuit, it does not:

1) prove the existence of a circuit (tracers may identify running fibers instead of axonal terminals),
2) clarify the intrinsic properties of the neurons,
3) explain how information is propagated or,
4) define what sort of information is propagated.

Electrophysiological approaches are required to address these questions. Single cell electrophysiological methods grew in popularity in the 1990s and enabled a deeper understanding of the physiology of the CLA.

2.3.1 Electrophysiological classification

Research into the electrophysiological properties of claustral cells was initiated by Shibuya and Yamamoto (1998). They performed in-vivo intracellular sharp microelectrode recordings in CLA of anesthetized rats and noted two firing patterns: slow (or mildly) and fast (or strongly) adapting spike-frequencies.
Spike-frequency adaptation is observed during the application of an electrical stimulus step. During this current step protocol, neurons show a decrease in their spike rate after the initial activity. A current step is applied and the response to this artificial stimulus enables neurons to be compared. This feature is controlled by underlying biophysical mechanisms such as the inactivation of depolarizing sodium currents and activation of voltage/spike-dependent slow-hyperpolarizing shunting potassium currents (Benda & Herz, 2003).

By studying phase response curves, Gutkin and colleagues (2005) proposed that strongly adapting neurons behave like coincidence detectors, and mildly adapting neurons with high firing frequencies behave like integrators. Adaptation further influences downstream signal processing hence enabling the postsynaptic neuron to respond to a dynamic environment (Laughlin, 1989; Ermentrout et al., 2001; Fuhrmann et al., 2002).

Recent attempts have built on the work of these early pioneers (Shibuya & Yamamoto, 1998) to incorporate newer tools and techniques for classifying CLA neurons (Kim et al., 2016; White & Mathur, 2018a). The most comprehensive effort thus far has been published by the Augustine Lab (Graf et al., 2020).

2.3.2 Synaptic transmission to the clastrum

A small but growing number of studies have investigated the effect of cortical input on CLA neurons (Clarey & Irvine, 1986; Li et al., 1986; Olson & Graybiel, 1980; Remedios et al., 2010; Spector et al., 1970; White et al., 2018). The earliest published study (Spector et al., 1970) used immobilized cats and presented these animals with four types of stimuli - somatic, click, tone and light flashes. They found that 75% of all responding cells reacted to various sensory stimuli while 25% were specific to a modality. Working on non-human primates, Remedios and collaborators reported that discrete populations of CLA neurons respond to visual and auditory stimuli (Remedios et al., 2010, 2014).
Kim and colleagues (2016) injected Adeno-associated virus tagged Channelrhodopsin-2 (AAV-ChR2) into the auditory, somatosensory, motor and visual cortices of a mouse and utilized ex-vivo whole-cell patch-clamp electrophysiology in CLA slices to show that input from sensory cortices synapsed on both PV and projection neurons in the CLA. This result has been confirmed by another group (White et al., 2018; White & Mathur, 2018) studying ACC inputs to the CLA. Collectively they showed that cortical input synapses on both PV and projection neurons of the CLA.

Kim and colleagues performed paired patch-clamp recordings ex-vivo and compared the difference in response amplitudes between projection neurons and PV interneurons in the CLA. They found that, compared to projection neurons, PV interneurons had faster and larger postsynaptic responses to sensory cortical input. Hence, while both CLA projection and PV cells receive input from the sensory and frontal cortices, the excitatory postsynaptic potential (EPSP) size and response latency of each cell type is different. Further work with different cortical input is required to generalize these observations.

It should be noted that current findings have been mostly limited to comparing projection neurons with PV interneurons. This is in large part due to the ability afforded by PV transgenic mice to identify the CLA core. Separately, the projection neurons were assumed to be a homogenous population in the study discussed above. There is, however, huge diversity of interneurons and projection neurons that needs to be studied in greater detail.

Upon receiving cortical input, the CLA first processes this information locally and subsequently transmits this information to other brain regions (Grillner et al., 2005; Silberberg et al., 2005). This local functional network is often referred to as the “neural microcircuit” and is discussed in the next section.
2.3.3 Claustrum microcircuitry and feedforward inhibition

Kim and colleagues (2016) showed that CLA PV interneurons have both chemical and electrical synapses with other PV interneurons. These PV interneurons were also strongly connected with projection neurons. Projection neurons were sparsely connected with each other, like most cortical circuits. PV interneurons formed robust inhibitory synapses on projection neurons and was the first evidence of feedforward inhibition (FFI) within the CLA.

**Figure 2 Feedforward inhibition.** Excitatory input targets both projection neurons and interneurons in a brain region. The activated interneuron subsequently sends an inhibitory output to the original projection neuron. Abbreviations: PN, projection neuron; IN, interneuron.

FFI is one motif that regulates synaptic transmission within a microcircuit (Fino et al., 2013; Navlakha et al., 2018). FFI occurs when the same glutamatergic input excites both projection neurons and interneurons in the target brain region. The interneuron then sends its output to the same projection neuron to inhibit it (Fig. 2). FFI effectively sharpens neuronal responsiveness to stimuli and shortens the integration time window (Cruikshank et al., 2007; Gabernet et al., 2005; König et al., 1996; Pouille & Scanziani, 2001; Wehr & Zador, 2003). To enable signal propagation, presynaptic excitatory input is needed to cross action potential threshold within a short timeframe. This is defined as coincidence detection which, as of writing, remains a hypothesis to be tested.

After the local circuitry processes cortical input, a population of projection neurons then sends output to their cortical targets.
2.3.4 Synaptic transmission from the claustrum

How does the CLA affect its target cortical population? The answers were first sought in the 1980s by two groups working on cats. Ptito & Lassonde (1981) showed that electrical stimulation of the dorsal CLA of the cat led to a decrease in the spontaneous activity and firing characteristics of visual cortical cells. Tsumoto & Suda (1982) delivered electrical stimulation in the cat CLA and recorded from the striate cortex (primary visual cortex). They found a bimodal response, with excitation followed by induced inhibition in a subset of cells.

Although projection neurons make up the majority of CLA neurons they have also proven the most frustrating to study because of the proximity of the CLA with the INS. To unmistakably study claustrocortical projections, one of two conditions need to be fulfilled: 1) the use of a CLA-specific reporter line, or 2) a cortical region that receives its input mostly from the CLA.

Using the early growth response protein 2 (Egr2) line with strong expression in the CLA, Atlan and colleagues (2018) showed that CLA projections to the auditory cortex helped to modulate an animal’s ability to cope with distractors. What could happen at the circuit level to effectuate this? Narikiyo and colleagues (2020) described the generation of a transgenic line with the T-box transcription factor 21-gene (Tbx21) enriched in the CLA. Activating Tbx21-expressing CLA neurons induced a down state in the cortex followed by prolonged silencing of cortical activity, which was then proceeded by a down-to-up state transition. Jackson and colleagues (2018) proposed the Neuropeptide Y (NPY) interneuron subpopulation (Karagiannis et al., 2009) as the chief modulator of FFI between projections from CLA to prefrontal cortex. Taken together, a potential mode of action emerges:

1. CLA input excites cortical projection and interneurons, causing an initial excitatory burst in a subpopulation of target neurons.
2. This activates a downstream cascade within the cortical column, that is regulated by FFI.
3. The resulting effect is a net inhibition of cortical activity.

Systematic investigation of other neuronal populations is required for a more detailed picture.

2.4 SPECULATED FUNCTIONS OF THE CLAUSTRUM

Hypotheses of CLA function broadly fall into two areas – that of consciousness/awareness (Crick & Koch, 2005) and attention/saliency (Atlan et al., 2018; Goll et al., 2015; Mathur, 2014; Terem et al., 2020). Other more recent hypotheses include spatial organization (Jankowski & O’Mara, 2015; Jankowski et al., 2017), memory retrieval (Kitanishi et al., 2017) and sleep activity (Narikiyo et al., 2020; Norimoto et al., 2020). The hypothesis tested seems to rest on the experimental subject studied – papers published by cognitive neuroscientists and clinicians have focused on the role of the CLA in consciousness whereas animal model-based research has mostly tested hypotheses of saliency and attention.

2.4.1 An integrator of consciousness/awareness

What is consciousness? This thesis uses the definition approached at the Francis Crick Memorial Conference of 2012 – that consciousness is the awareness of one’s body and environment.

The central property of consciousness is the integrated nature of the experience (Crick & Koch, 2005). This requires a continuous stream of communication between distant projection neurons, the contents of which are rapidly integrated across distant cortical and thalamic brain regions.

Crick and Koch argued that the widespread connectivity of the CLA placed it in the right position to be an integrator of multimodal input, serving as the conductor of an orchestra of consciousness. Supporting data for this hypothesis have mostly
come from human studies (Koubeissi et al., 2014; Chau et al., 2015; Kurada et al., 2019). A high profile example showed that perturbation of the CLA caused an epileptic patient to pause midway while performing a task and showing no knowledge of the disruption (Koubeissi et al., 2014). CLA damage was also shown to increase the time taken to regain consciousness (Kurada et al., 2019).

This multimodal integrator model has been challenged by animal researchers (Remedios et al., 2010, 2014; Smith et al, 2012), who argued that the CLA is unimodal and displays little multimodal integration at the level of individual CLA cells. Furthermore, these unimodal cells seem to have a precise, mode-specific topological arrangement that is inconsistent with multimodal integration. Baizer and colleagues (2014) have contended that the structural differences of the CLA and relative decrease in size of the CLA across species argue effectively against a global integration role for this region and by extension the consciousness/awareness hypothesis.

While the CLA may not be a global integrator, arguments that the CLA is therefore, neither involved in consciousness nor an integrator of information are premature:

1. These findings on unimodality were based on studying CLA regions that are known to be discrete and separate, more robustly connected cortices such as CLA-ACC have not been tested for multicortical connectivity.

2. Integration is merely one mechanism by which a cell processes inputs over a longer timespan but is not the only operative mechanism (König et al., 1996).

3. It is synaptic connectivity not morphology of a region that determines function, this is so when circuit connectivity is conserved across species.

2.4.2 A node for attention and saliency

Another group of scientists has focused on the CLA’s role in responding to an external environment. This idea has taken two forms: saliency/attention (Atlan et
Salience refers to how an item (person, object etc.) stands out in contrast relative to its surroundings. It is evolutionarily important to focus on the most outstanding item in an organism’s surroundings. This enables the organism to channel physical and mental resources towards responding to situations that most affect its survival.

The postulated role for the CLA in salience was based on previous experimental work in monkeys (Remedios et al., 2010, 2014), cats (Gabor & Peele, 1964) and rodents (Atlan et al., 2018; Terem et al., 2020).

Goll and colleagues (2015) proposed that the CLA segregates attention between modalities by directing which cortical output will be attended to over others, thereby promoting the preferred modality and reducing the efficacy of less-preferred modalities. The CLA must therefore integrate or process information from various sources, to ensure that attentional strategies are realized. Recent experiments have shown that cortical input to the CLA encodes a top-down preparatory signal on an attentional task (White et al., 2018) and that the inhibition of CLA projections decreases an animal's ability to ignore distractors (Atlan et al., 2018). Another group reported that attentional ability was negatively correlated with CLA activity (Liu et al., 2018).

Terem and colleagues (2020) provided perhaps the most robust argument to date for the CLA’s role in salience. They showed that a subpopulation of CLA neurons were responsible for contextual conditioning and drove incentive salience in rodents, a process that confers a motivational "desire" or "want" in response to a rewarding stimulus. They further showed that a subpopulation of CLA neurons was responsible for sensitization to cocaine.

Based on correlational studies, Patru and Reser (2015) proposed a related idea, that CLA pathology relates to delusions within schizophrenia. They noted that the intensity of delusions in schizophrenia was inversely correlated with the size of the
CLA and INS (Cascella et al, 2011) – the smaller the CLA and INS volume, the more intense the episodes of delusion. The CLA is also highly enriched in k-opioid receptors which are targets of psychoactive drugs (Stiefel et al., 2014).

Consciousness/awareness and salience/attention are not opposing but could be mutually reinforcing (Lamme, 2003). These ideas could be unified by a CLA role in the Salience Network (SN).

### 2.4.3 A CLA role in the salience network

To enable coherent daily function, brain regions activate as a network. Two networks enabling response to interoceptive and exteroceptive stimuli are the default mode network (DMN) and central executive network (CEN) respectively.

DMN to CEN activity is switched on/off through the SN (Seeley et al., 2007; Sridharan, Levitin, & Menon, 2008; Medford & Critchley, 2010; Menon & Uddin, 2010). Central to the function of the SN is communication between the INS and ACC (Seeley et al., 2007; Sridharan, Levitin, & Menon, 2008; Medford & Critchley, 2010; Menon & Uddin, 2010). Physically and emotionally salient stimuli is detected by the INS, which then sends this information to the ACC. As information is passed between INS and ACC, the DMN is deactivated while the CEN is activated. Changes in these networks were observed in diseased states compared to healthy states (White, et al., 2010). Functional connectivity is aberrant in the DMN, CEN and SN after traumatic brain injury (Bonnelle et al., 2012) and in schizophrenia (Manoliu et al., 2014). Recent work has identified a putative SN in mice as well (Sforazzini et al., 2014; Liska et al., 2015; Mechling et al., 2014; Pagani et al., 2016).

While there is functional correlation between the activation of the INS and ACC (Craig, 2002, 2009; Dosenbach et al., 2006, 2007; Harrison et al., 2008; Moisset et al., 2010), no studies have shown unequivocal proof of an anatomical INS-ACC
connection. The original papers used to claim an INS-ACC connection (Mesulam & Mufson, 1982; Mufson & Mesulam, 1982) and attribute von Economo neurons as the main substrate 1) did not dissociate between the CLA and INS, 2) were identified by visual inspection instead of molecular markers and, 3) were only observed in humans and higher order mammals.

Furthermore, the technique used (fMRI) to implicate functional connectivity did not have the spatial resolution then to separate brain regions closely located and connected to each other. The CLA and INS were hence considered as one functional unit. Post-processing analytical methods have recently been developed to dissociate the CLA and INS. These new methods suggest that the correlated activity occurs between the CLA-ACC (Berman et al., 2020; Krimmel et al., 2019; Smith et al., 2019b).

Connectivity between the INS and ACC is only observed when the CLA is also injected with a tracer but not when only the INS is injected (Wang et al., 2016). This suggests that the connection between the INS and ACC might go through the CLA and makes the CLA a potential component of the SN. Another potential interpretation is that, instead of an INS-CLA-ACC circuit, it is the CLA-ACC connection that mediates the SN. However, Occam’s Razor suggests that the former construct should be preferred as it requires less assumptions. More experimental data must be collected to make a more substantive proposal.

The SN effectively requires the generation of awareness to a salient stimulus for attention to be directed, and a response to be delivered, thereby unifying both schools of thought. To move the discussion above from speculation to hypothesis, an INS-CLA-ACC circuit first needs to be discovered.
2.5 RESEARCH PROGRAM

This thesis harnesses a toolbox of anatomical tracers, ex-vivo electrophysiology, optogenetics and pharmacology on the CLA-ACC circuit to provide insights into CLA synaptic transmission and describes organizing principles that could guide future work. Apart from answering questions on synaptic transmission to and from the CLA, this circuit could also be behaviorally relevant via the SN.

*Occam’s Razor is a philosophical approach to problem solving typically attributed to William of Ockham (1287-1347). The approach argues that for any problem, the explanation with the least assumptions required that is consistent with the current available data is more likely to be correct. While not an irrefutable principle of logic, this idea of parsimony enhances falsifiability within the scientific approach. This heuristic is not without its critics and should not be considered an immutable principle.
3 AIMS & RESEARCH FRAMEWORK

The overarching objective of this thesis is:

To elucidate the organising principles of synaptic transmission to and from the CLA using the CLA-ACC pathway in mouse as a model.

Specifically, the thesis has the following aims:

1. To test the hypothesis that CLA-ACC neurons receive input from and target more than one cortical region, & to seek the existence of an INS-CLA-ACC circuit (Paper I).
2. To define the physiological properties of neurons projecting from the CLA to the ACC (Paper II).
3. To investigate the postsynaptic response of ACC neurons to claustral input (Paper III).

The overall research framework is illustrated in Fig. 3.
Figure 3 Summary of research framework.
4 METHODS

The section will briefly recap common considerations across all three papers – CLA identification and animal usage. It will then summarize the main techniques applied in this thesis, ex-vivo patch-clamp electrophysiology and optogenetics. Please refer to the methods section in each paper or manuscript for further details.

4.1 VISUALIZING THE CLAUSTRUM

Acute brain slices were cut coronally in all experiments. The CLA was visualized in three ways: 1) PV-Cre reporter transgenic mice, 2) thymus cell antigen-1 (Thy1) Volvox transgenic mice, 3) retrograde tracers injected to the ACC.

In Papers I and III, a PV-Cre line was crossed with a tdTomato line to create mice expressing tomato in PV-expressing cells. This was used to identify the CLA (Fig. 4).

Figure 4 Parvalbumin expression identifies the CLA core. (A) Illustration of relative position of CLA within a coronal section of the mouse brain. (B, left) Confocal image of a 250 μm thick brain slice from a PV-reporter mouse line. White arrows identify the CLA core, image in box enlarged on the right. (B, right) Enlarge image of PV-reporter defined CLA core (Adapted from Paper III, Chia et al 2020b, manuscript in preparation).

In Paper II, a transgenic mouse line that expresses Thy1 antigen promoter with YFP-tagged Volvox ChR1 was used (Fig. 5). This gene is highly enriched in the CLA.
Further characterization in the same paper showed that the highly enriched CLA region corresponded with the PV-rich CLA core.

**Figure 5.** Thy1-Volvox expression overlaps with PV-rich CLA core. (A) CLA is fluorescently labelled in the Volvox Thy1 line (CLA marked with black arrow). The image shows the identification of CLA in a coronal acute slice under fluorescent microscopy. (B) Immunohistochemistry performed on fixed slices shows the PV neuropil-rich core in the CLA (1) and higher YFP expression in Thy1-Volvox line (2). To identify the CLA, the mean fluorescence of the INS region and 3 SD was used to set the threshold for defining PV (3) and YFP (4) fluorescence. (C) The merged thresholded images show a large overlap between PV and YFP signals. Red represents PV imaging, green represents YFP fluorescence, and yellow represents direct overlap between YFP and PV (Reproduced under Open Access from Paper II, Chia et al, 2017, Claustrum).

**Figure 6 Retrograde tracer injection into ACC enables identification of CLA.** (A) Bright-field image showing the injection site (white arrow) of retrograde beads (250 nl). Injected beads appear as a dark band within the ACC boundaries (dashed white line). (B) Labelled cell bodies (in red) are mostly found in the CLA. CLA was identified as previously described and is marked by the dashed white line. Abbreviations: CP, caudoputamen; CLA, claustrum; INS, Insular cortex. (C) Patch-clamp recordings were obtained from labelled cell bodies. (Reproduced under Open Access from Paper II, Chia et al, 2017, Claustrum).
In Papers I & II, retrograde tracers were injected into the ACC to identify CLA-ACC neurons in non-transgenic mice (Fig. 6).

4.2 ANIMAL USE GUIDELINES
All animal procedures were performed according to the guidelines of the respective local governing bodies, specifically the guidelines of the A*STAR Biological Resource Centre Institutional Animal Care and Use Committee in Singapore (Paper II) and the Stockholm Municipal Committee for Animal Experiments (Papers I, III).

Mice were injected with Lumafluor fluorescent beads (minimum 10 days incubation prior to experiments, Paper I, II) or AAV-ChR2 (minimum 20 days incubation prior to experiments, Paper I and III).

4.3 BRAIN SLICE PREPARATION
Mice were anesthetized with isoflurane and their brains removed in ice-cold cutting solution. Acute brain slices, 250 µm thick, were cut with a vibrotome and then transferred to a water bath (34 – 35°C) for between 30 minutes and 1 hour. These slices were subsequently removed from the water bath and kept at room temperature (22 – 27°C) throughout the length of the experiment. Each experiment lasted no longer than 12 hours from when the brain was sliced. The same brain slice preparation protocol was used across Papers I, II and III.

4.4 EX-VIVO WHOLE-CELL RECORDINGS
Patch pipettes were pulled with Flaming/Brown micropipette puller P-1000 or Narashige PC-10 Puller. Recordings in Paper II were carried out at room temperature
(24 – 27°C) and recordings in Paper I and III were carried out at physiological temperature range (around 35°C). Up to two pipettes were recorded simultaneously in Papers I and III. Recordings were amplified using Multiclamp 700B amplifiers and digitized on ITC-18 (Paper I and III) or Digidata 1440A (Paper II) Data was analyzed in IGOR Pro (Paper I and III) or Clampex (Paper II) software.

4.5 OPTOGENETIC STIMULATION EXPERIMENTS
Optogenetic experiments were performed in Papers I and III. AAV-ChR2 was expressed in various cortical regions or CLA depending on the experimental design. Patch-clamp recordings were made in the CLA or ACC respectively. The axons of these neurons were photostimulated with a 1-watt blue LED (wavelength 465 nm) mounted on the microscope oculars and delivered through the objective lens. Photostimulation was controlled by a LED driver connected to the ITC-18 acquisition board, enabling control over the duration and intensity. The photostimulation diameter through the objective lens was ~400 μm with an illumination intensity of 0.6 – 16 mW/mm² depending on experiment.
5 RESULTS & DISCUSSION

This thesis investigated the organizing principles of synaptic transmission to and from the CLA using the CLA-ACC connection as a model. For comprehensiveness, five aspects of the connection were probed:

1) The sources of and response to cortical input by CLA-ACC cells (Paper I).
2) Other axonal targets of the CLA-ACC neuron population (Paper I).
3) The intrinsic properties of CLA-ACC neurons (Paper II).
4) The cellular and layer targets of CLA-ACC axons at the ACC (Paper III).
5) The postsynaptic response of these target neurons (Paper III).

The key results and findings from these five features are addressed sequentially in the sections that follow. The reader is requested to refer to the results section in each paper or manuscript for further elaboration.

5.1 FUNCTIONAL MODULES IN THE CLAUSRUM (PAPER I)

The question of whether CLA neurons receive multicortical input has been debated for a few decades. This point is important as it underlies various hypotheses behind the function of the CLA.

Paper I (Chia et al., 2020a) tested the hypothesis that CLA-ACC neurons receive synaptic input from multiple cortical regions and showed that the CLA-ACC population received multicortical input biased for frontal and limbic brain regions.

The ACC of mice were injected with retrograde beads to identify CLA-ACC neurons, while anterograde virus expressing ChR2 was injected into various cortices to label neurons projecting to the CLA. The brain regions chosen were frontal and
sensorimotor cortical regions, representing higher-order cognition versus sensory processing respectively. Whole-cell patch-clamp recordings were subsequently made from bead-labelled CLA neurons and synaptic responses were evoked by photostimulation of ChR2-expressing cortical axons (Fig. 7).

Figure 7 CLA-ACC neurons receive robust input from frontal and limbic but not sensorimotor cortices. (A-C) Illustration of the experimental paradigm studying frontal cortical (contralateral ACC), sensorimotor (contralateral motor cortex) and limbic input (insular) to CLA-ACC neurons. Map of injection sites based on the Paxinos and Franklin Mouse Atlas (left) and the corresponding confocal image of 250 μm coronal slice with ChR2 injected into the cortical region (center). Example of responses. Postsynaptic response to photostimulation was abolished with bath application of TTX and recovered upon 4AP application in A and C. The pie chart shows the numbers of responding and non-responding CLA-ACC neurons to axonal projections from the various cortices. Abbreviations as follows: ACC, anterior cingulate cortex; INS, insular; CLA, claustrum; MTR, contralateral motor cortex. (Adapted with permission from Paper I, Chia et al, 2020, Current Biology)

A higher proportion of CLA-ACC neurons responded to frontal cortical input (Paper I, Fig. 1) as compared to sensory cortical input (Paper I, Fig. 2 & 3). This paper
revealed that the disynaptic INS-CLA-ACC circuit did indeed exist (Paper I, Fig. 1). Another finding in this study was that frontal and sensory cortices target different CLA populations. This could explain why previous works focusing on sensory regions were not able to reach a clear conclusion on cortical integration in CLA.

The existence of an INS-CLA-ACC circuit provides support for a CLA connector between the INS and ACC and can stimulate research into the role for the CLA in the SN. This is of medical relevance as both the CLA and the SN have been suggested to play a role in several mental disorders, including schizophrenia and delusions.

The INS-CLA circuit is a poorly studied corticoclastral connection because the proximity of the two structures represents a huge challenge to experimenters. This connection is, however, highly relevant due to its possible role in the SN. To understand how the CLA integrates INS inputs from a cortical region, we injected AAV-ChR2 into the INS of PV-tdTomato transgenic mice (Paper I, Fig. 4), thus enabling photostimulation of INS synaptic terminals in CLA.

Paper I revealed that CLA neurons receive synaptic input from Layer 5 of the ipsilateral INS. Paired whole-cell patch-clamp recordings showed that while both CLA projection neurons and PV interneurons receive INS input, the postsynaptic response of PV interneurons is faster and larger than CLA projection neurons. This supports the proposals of FFI suggested by others (Kim et al., 2016; White et al., 2018). By studying a limbic cortex, Paper I added to the work performed in frontal and sensorimotor regions and showed that the difference in PV-projection neuron postsynaptic responses is generalizable to the CLA regardless of cortical input source.

FFI supports a coincidence detection mechanism (Smythies et al., 2014) of salient or novel information (Remedios et al., 2010; 2014) within the CLA. Stimuli from several cortical regions would have to arrive at the CLA within a short time window to stimulate CLA projection neurons and propagate downstream.
Paper I followed up by asking which other cortices were targeted by CLA-ACC and reported that CLA-ACC neurons co-projected to other limbic or frontal cortical area but very rarely to a sensorimotor cortical area (Paper I, Fig. 5).

The rule that seems to govern CLA projections is that neurons projecting to a frontal cortical region tend to project to other frontal cortical regions rather than sensorimotor cortices. The corollary was true as well, as sensorimotor-projecting CLA neurons sent axons preferentially to other sensorimotor regions rather than frontal/limbic cortices.

In summary, Paper I proposed that there are at least two different targeting systems in the CLA – a higher cognitive system and a sensorimotor system (Fig 8).

**Figure 8 Modular organization of cortico-claustrial interconnectivity.** Cortical inputs from frontal and limbic regions (yellow and orange, respectively) show strong connection probability (solid line arrow) to CLA-ACC neurons while cortical inputs from sensorimotor regions (purple) show either low connection probability (dashed line arrow) or no connection (no arrow). CLA-ACC neurons that receive preferential input from frontal and limbic cortices also preferentially co-project to other frontal cortices (orange). Another population of sensorimotor cortex targeting CLA projection neurons was observed suggesting the existence of a sensorimotor cortex modality (blue). Abbreviations as follows: OFC, orbitofrontal cortex; ACC, anterior cingulate cortex; RSP, retrosplenial cortex; INS, insula; MTR, contralateral motor cortex; SS, somatosensory cortex; VIS visual cortex; AUD, auditory cortex (Reproduced with permission from Paper I, Chia et al, 2020, Current Biology)
While not explored in this thesis, one should not discount the possibility of disparate, system-dependent CLA functions.

5.2 CLA-ACC TOPOLOGICAL HETEROGENEITY (PAPER II)

The state of the art in CLA cell classification by Graf and colleagues (2020) has sought to connect the morphology, biochemistry and electrophysiology of CLA cells into a coherent whole. Paper II (Chia et al., 2017) investigated the intrinsic properties of CLA-ACC neurons and reported that there were 4 different CLA-ACC types with heterogeneous topological distribution (Fig. 9).

CLA-ACC neurons were identified by injecting retrograde fluorescent beads into the ACC. These beads would be retrogradely transported to the sites of ACC input and cell bodies of neurons that project to the ACC could then be observed under fluorescence microscopy. To identify the CLA core, the Thy1-Volvox transgenic mouse line was used. Whole-cell patch clamp recordings were made from these bead-labelled CLA neurons and were used to characterize the intrinsic properties of these neurons (Paper II, Fig. 1-3).

These cell-types were not distributed homogenously but showed topological variation: One population of strongly-adapting (SA) neurons was found in the most anterior and posterior parts of the CLA, while another population of mildly-adapting (MA) neurons was predominant in CLA lateral to the preoptic area (Paper II, Fig. 4).

A surprising sexual dimorphism was observed in the anterior CLA as the male CLA showed a significant bias toward SA3 type cells while in female mice there was a bias for SA4 cells and no SA3 cells (Paper II, Fig. 5). SA3 cells have deep afterhyperpolarization potentials (AHPs), while SA4 cells have shallow AHPs.
Figure 9 Four CLA-ACC cell types display topological heterogeneity. (A) Examples of SA2, SA3, SA4 and MA2 ACC-projecting CLA cell types, classified according to their action potential discharge in response to a depolarizing current injection. (B) Representative traces of the first action potential in SA2, SA3, and SA4 cell types. SA2, SA3 and SA4 cells are classified by the differences in their first action potential. SA2 cells have doublet spikes, SA3 cells have single spikes with deep AHP, while SA4 have single spikes with shallow AHP. The action potential threshold is marked by a grey line. (C) Distributions of ACC-projecting cell types in anterior, middle and posterior CLA 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). (Adapted under Open Access from Paper II, Chia et al, 2017, Claustrum)

It is unclear what mechanisms account for sex-related differences in cell-type distribution. One possibility is estrogen receptor β, which is involved in sexual differentiation during development and de-feminization in the male brain (Kudwa et al., 2005; 2006).

Paper II extended the concept of topological selectivity in the CLA by adding a second layer of topological selectivity – cells with the same target but different intrinsic properties are clustered in different parts of the CLA. How this different distribution affects electrophysiological and behavioral output is ripe for further investigation.
5.3 CELL-TYPE AND LAYER-SPECIFIC CLA-ACC TARGETS (PAPER III)

Where and what do CLA projections target at the cortex? Paper III (Chia et al., 2020b) investigated the ACC targets of CLA input and reported that all layers of the ACC receive CLA input in a cell-type and layer-specific manner.

The CLA of the various transgenic mice was injected with AAV-ChR2. Paired whole-cell patch clamp recordings were subsequently made in the ACC and synaptic responses were evoked by photostimulation of ChR2-expressing CLA axons. Each pair of recordings included a molecularly defined interneuron and a putative pyramidal neuron with the pyramidal neuron serving as a control across the different transgenic mouse lines. [Note: Pyramidal neurons are cortical projection neurons, the term pyramidal is used in Paper III to better align with the convention in the cortical field].

EPSPs were evoked in all layers of the ACC with similar response rates between layers. Pharmacological experiments in a subset of recordings showed that the CLA sent monosynaptic excitatory input to all layers of the ACC (Paper III, Fig. 1).

There are three major cortical interneuron types (Rudy et al., 2011) – Parvalbumin (PV), Somatostatin (SOM) and Serotonin-receptor expressing (5HT) interneurons. PV cells facilitate feedforward inhibition (Pouille & Scanziani, 2001) and control of excitatory and inhibitory balance within the cortex (Haider & McCormick, 2009; Hasenstaub et al., 2005). SOM neurons receive feedforward excitation from pyramidal neurons and suppress the activity other interneurons in the cortex (Urban-Ciecko & Barth, 2016). 5HT neurons receive thalamocortical input and have a larger inhibitory effect on superficial layers of the cortex (Lee et al., 2010).

While all three major interneuron types received CLA input, there was specificity in cell targets and layers (Paper III, Fig. 2-6). PV and 5HT3a interneurons were strongly innervated by CLA input in layers 2/3 while PV interneurons and SOM interneurons were strongly innervated by CLA in layers 5/6.
Figure 10 CLA-ACC neurons target all cortical layers and all major interneuron types. (A) Postsynaptic responses from Layer 1, 2/3 and 5/6 neurons are recorded in Gabazine. The postsynaptic response is subsequently removed on bath application of TTX and recovered with TTX+4AP, this confirms that the input is monosynaptic. The subsequent postsynaptic response is abolished with AMPA and NMDA receptor antagonists, NBQX and APV, confirming that the input is excitatory. (B) Illustration of paired recordings of interneuron (PV, SOM, 5HT3a respectively) and pyramidal neurons. Confocal image of the ACC shows that location of interneurons in the ACC. Example firing pattern of interneuron under current step. Representative postsynaptic responses comparing interneuron (coloured) and pyramidal neuron populations (black trace).

This was also the first work to describe functional CLA inputs into cortical layer 1 cells (Paper III, Fig. S4).
Previous work had described vesicular glutamate transporter-2 (VGlut2) as being expressed in the CLA (Fodoulian et al., 2018; Real et al., 2006) and involved with shifting attention (Mutel et al., 2018). We investigated the cortical targeting pattern of this population. CLA VGlut2-expressing projection neurons were observed to almost exclusively target superficial ACC layers (Paper III, Fig. 7). This suggests that CLA-ACC projections may be mediated by different subpopulations of CLA neurons, with specific targets in ACC. The targeting of different cortical layers draws parallels with thalamocortical circuits and raises questions on how these the thalamocortical and claustrocortical circuits compare and interact with each other.

How do the postsynaptic responses of these different ACC populations differ from each other? Using paired-recordings, this paper reported that pyramidal neurons in both layers 2/3 and 5/6 responded slower than PV neurons. The EPSPs recorded in pyramidal neurons and PV neurons in ACC layer 5/6 differ from each other, with PV cells responding to CLA input with larger EPSPs. Such a difference is not observed between layer 2/3 pyramidal and PV neurons. At the population level, no such difference was found in the EPSP size and response latency of 5HT3a and SOM interneuron populations. The schema in Fig. 11 was developed from the data collected in Paper III.

The results from the three studies are summarised in Fig. 12.
Figure 11 Schema of Claustrum Targets. Illustration showing different claustrum targets in the ACC. Using the CamKIIa promoter, claustrum projections were observed to target all cell types in Layer 1. PV, 5HT3a and NPY neurons in Layer 2/3 as well as PV, SOM and NPY neurons in Layer 5/6. VGlut2-expressing claustrum projection neurons targeted all neurons recorded in Layer 1 and Layer 2/3. A putative claustrum projection neuron population that targets infragranular layers may also exist.
Figure 12 Summary of research answers.
6 CONCLUSIONS & FUTURE DIRECTION

The work completed in this thesis revealed that:

1) CLA-ACC neurons receive multicortical input biased for frontal and limbic cortices rather than sensorimotor input. Among the various circuits found was an INS-CLA-ACC circuit which might be a part of the SN.
2) CLA-ACC neurons also targeted other frontal cortices but were less likely to target sensorimotor cortices.
3) CLA-ACC neurons displayed topological heterogeneity and sexual dimorphism of electrophysiological types in their distribution within the claustrum.
4) Although the CLA provides monosynaptic and excitatory input to all layers of the ACC, the specific targets are organized in a cell-type and layer-dependent manner.
5) The postsynaptic responses of ACC interneuron populations can differ between and within groups.

Three fundamental conclusions can be drawn from this thesis, which hopefully open new vistas for further inquiry.

6.1 A COLLECTION OF HIGHWAYS

Paper I showed that CLA-ACC neurons input was biased for frontal and limbic areas rather than sensorimotor areas. It further showed that these CLA-ACC neurons projected to other frontal areas rather than sensorimotor areas. Collectively, this paper provided evidence that the CLA is organized into at least two functional modules – a higher cognitive module and a putative sensorimotor module. These functional modules are defined by biases towards the input and output cortices.

The term hub is commonly applied when describing CLA connectivity. Rather than a hub-and-spoke projection structure (like an airport) the findings in this thesis suggest that the CLA could be organized more like a point-to-point highway. Highways can merge, split, or run parallel to each other. Connections in the CLA could be organized likewise. How these functional modules interact within the CLA directed by local interneurons is a vital question that will help refine our understanding of CLA function.
A highway is not merely a relay, similarly the CLA likely performs more than a merely passive function. Based on the postsynaptic responses of CLA neurons to cortical input, it was proposed in Paper I that the CLA could be a coincidence detector. This could be likened to a toll—both in a highway (a gatekeeper of sorts) – which requires presynaptic inputs to integrate within a short time window and reach suprathreshold levels, for signal propagation. This is summarized in Fig. 13 below.

![Figure 13 A collection of highways.](image)

**Figure 13 A collection of highways.** The conventionally assumed hub-model on the left, and the highway model proposed by this thesis. The interaction between the functional modules is not known.

Why should this mechanism be biologically relevant? A living organism faces many stimuli at any moment in time. A coincidence detector is ethologically important for an organism to be able to focus on what is particularly relevant, ignore distractors and make an appropriate response. Indeed recent data support this proposed function for the CLA (Atlan et al., 2018).

### 6.2 ONE HIGHWAY, MANY EXITS

A reductionist logic built on Koch’s postulates exists in circuit neuroscience: If activating a subset of neurons in Region A that project to Region B leads to a certain behavioral phenotype, and deactivating Region A subsequently silences that behavioral phenotype, then circuit A-B causes this phenotype. Two assumptions are implicit in this logic: 1) Neurons projecting to
region A target only region B, 2) even if they target other regions (C, D, E etc.) the effect is negligible.

Our results from Paper I call these assumptions into question. Using anatomical methods, we showed that CLA-ACC neurons also project to other regions and are biased for frontal cortices (Fig. 14, middle panel). While Paper III targeted CLA terminals to elucidate organizing principles of ACC in response to the CLA, it did not study what happens to the other regional targets. If the main cortical target (e.g. ACC) is inhibited, do the other cortices get inhibited or excited?

![Diagram](image)

**Figure 14 One highway, many exits each with different destinations.** A CLA projection neuron sends axons to other cortices apart from the cortex of interest (middle panel). Even among projection neurons targeting the same cortex, there exist different populations with specific layer and cell-type targets (right panel). Abbreviation: CLA: Claustrum; CORT: cortex; L: Layer.

**Paper I** showed the existence of an INS-CLA-ACC circuit that could be a part of the SN. The SN switches activity between DMN and CEN by controlling which cortical regions are activated or inhibited. Could the bifurcation of CLA projection neurons be a vital part of cortical switching? Could an FFI mechanism enable this switching to take place?

### 6.3 SAME EXIT, DIFFERENT DESTINATIONS

**Paper I** showed that a single CLA-ACC neuron may send axonal projections to other frontal cortical areas. **Paper II** showed that the intrinsic properties of CLA neurons could be separated into at least four groups and that the spatial distribution of these neurons was topologically heterogeneous. **Paper III** further added to this by showing that there were cell-type and layer-specific targets of the ACC. The same paper also showed that the VGlut-2 subpopulation of
CLA projection neurons sent synapses to superficial layers. Collectively, these data show that at each cortex that the CLA targets, there is a second level of target-heterogeneity (Fig. 14, right panel).

That different molecularly defined projection neuron populations may target different cortical layers is not unique to the CLA. Indeed the striatum (Silberberg & Bolam, 2015) has two types of projection neurons with different pathways identified by different dopaminergic markers. Another example is the thalamus which has two sets of projection neurons that target superficial and deep cortical layers respectively (Sherman, 2012). The observation that claustro cortical projections and thalamocortical projections parallel each other in their cortical layer targets and have opposite effects (CLA inhibits whereas thalamus excites the cortex) raises several questions - How do CLA and thalamus modulate each other? What is the relationship between claustro cortical and thalamocortical inputs at the cortex (e.g. synergistic, antagonistic)? How would this interaction modify behavioral output?
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I end with a poem that has guided me tremendously these few years.

If

If you can keep your head when all about you
Are losing theirs and blaming it on you,
If you can trust yourself when all men doubt you
But make allowance for their doubting too;
If you can wait and not be tired by waiting,
   Or being lied about, don’t deal in lies,
Or being hated, don’t give way to hating,
   And yet don’t look too good, nor talk too wise:

If you can dream—and not make dreams your master;
   If you can think—and not make thoughts your aim;
If you can meet with Triumph and Disaster
   And treat those two impostors just the same;
If you can bear to hear the truth you’ve spoken
   Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to, broken,
   And stoop and build ’em up with worn-out tools:

If you can make one heap of all your winnings
   And risk it on one turn of pitch-and-toss,
And lose, and start again at your beginnings
   And never breathe a word about your loss;
If you can force your heart and nerve and sinew
   To serve your turn long after they are gone,
And so hold on when there is nothing in you
   Except the Will which says to them: ‘Hold on!’

If you can talk with crowds and keep your virtue,
   Or walk with Kings—nor lose the common touch,
If neither foes nor loving friends can hurt you,
   If all men count with you, but none too much;
If you can fill the unforgiving minute
   With sixty seconds’ worth of distance run,
Yours is the Earth and everything that’s in it,
   And—which is more—you’ll be a Man, my son!

Rudyard Kipling (1865 – 1936)
8 REFERENCES


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