Investigating the behavioural significance of the claustrum



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Summary of Contributions

I declare that this thesis is my own work and that attribution has been given where information was derived from other sources. Aside from the exceptions outlined below, I conceptualised the project, performed the experiments, analysed the data, and constructed all figures. While all writing contained herein is my own, the project as a whole has relied on key contributions from others. Specific contributions are described following each chapter. The pronoun 'we' is used throughout the text to indicate myself and the contributors specified for each chapter. In addition to the chapter-specific contributions, my supervisors Adam Packer and Simon Butt provided invaluable guidance throughout and contributed to the design and conceptualization of experiments.

Appendix II contains the co-first authored paper "Human lesions and animal studies link the claustrum to perception, salience, sleep and pain". This paper began as an essay written by M Doody, but the final text was predominantly written by H Atilgan and myself. This paper is the result of a collaboration between all authors. It is included here to supplement the background information provided in Chapter 1.

Table of Contents

Abstract 5
1 Introduction 6
1.1 Location & Identification 7
1.2 Connectivity & Physiology 10
1.3 Activity & Function 14
2 Sensory response properties of the mouse claustrum 19
2.1 Methods 19
2.2 Results 26
2.3 Summary 32
2.4 Acknowledgements & Contributions 33
3 Impact of claustrum silencing on ethologically motivated behaviours 34
3.1 Methods 34
3.2 Results 40
3.3 Summary 47
3.4 Acknowledgements & Contributions 48
4 Impact of claustrum silencing on circadian and locomotor activity 49
4.1 Methods 49
4.2 Results 55
4.3 Summary 63
4.4 Acknowledgements & Contributions 64
5 Impact of claustrum silencing on learned behaviour 65
5.1 Methods 66
5.2 Results 71
5.3 Summary 86
5.4 Acknowledgements & Contributions 87
6 Discussion 88
6.1 In-vivo Axon Imaging 88
6.2 Sensory responsive neurons in the claustrum 91
6.3 CLCX:EDGE Transgenic line 92
6.4 Chronic claustrum silencing with Tetanus Toxin 93
6.5 Behavioural effects of claustrum silencing 95
6.6 Summary 98
7 Acknowledgements 99
8 Works Cited 100
9 Appendix I — Supplementary Figures 119
10 Appendix II — Atilgan, Oliver, Doody et al. 131

Table of Figures

Figure 1-1 7
Figure 2-1 26
Figure 2-2 27
Figure 2-3 28
Figure 2-4 29
Figure 2-5 29
Figure 2-6 30
Figure 2-7 31
Figure 3-1 40
Figure 3-2 40
Figure 3-3 41
Figure 3-4 41
Figure 3-5 42
Figure 3-6 43
Figure 3-7 44
Figure 3-8 44
Figure 3-9 46
Figure 4-1. 55
Figure 4-2. 56
Figure 4-3 57
Figure 4-4 58
Figure 4-5 59
Figure 4-6 60
Figure 4-7 61
Figure 4-8 62
Figure 5-1 71
Figure 5-2 72
Figure 5-3 73
Figure 5-4 74
Figure 5-5 75
Figure 5-6 76
Figure 5-7 78
Figure 5-8 79
Figure 5-9 80
Figure 5-10.. 81
Figure 5-11 81
Figure 5-12. 82
Figure 5-13. 83
Figure 5-14.. 84

Abstract

Sequestered deep beneath the cortex, the claustrum is the most densely connected structure in the brain. This tantalizing anatomy has spawned a wide range of hypotheses regarding the function of the claustrum, but technological and biological constraints have hindered research. In this thesis, I have investigated the activity and behavioural function of the claustrum. I developed a novel preparation for recording claustrum activity using two-photon calcium imaging of claustrum axons in dorsal cortex. I show that claustrum axons display stimulus-locked changes in activity following uni- and multimodal sensory stimuli. While some axons were selective to one sensory modality, most were promiscuously responsive to multiple sensory modalities. I then investigated the behavioural consequences of silencing the claustrum. I detected no effects of claustrum silencing on home-cage activity, circadian behaviour, or a battery of tests assessing a wide range of behavioural domains. Finally, I investigated the impact of claustrum silencing on two behavioural tasks. While I detected no effects on a reversal learning task, I found that claustrum silencing reduced animals' sensitivity to multimodal stimuli in a classical conditioning-like paradigm. Collectively, these results support the hypothesis that the claustrum may be involved in processing multimodal information and highlight the need for more research into the function of the claustrum.

"…for no one can suppose that in each of us, as in a sort of Trojan horse, there are perched a number of unconnected senses, which do not all meet in some one nature, the mind, or whatever we please to call it, of which they are the instruments, and with which through them we perceive objects of sense." (Plato; Theaetetus 184d1–5)

1 Introduction

How do our senses, each a distinct stream of information, come together in united perception? In the passage above, Socrates argues for a conscious soul, capable of receiving the information of the senses and thinking about their meanings. Aristotle later disagreed, separating the integration of the senses from the emergence of thought. Instead, he argued for a 'common sense' that existed above the five conventional senses and unified their reports. While Plato and Aristotle disagreed on how and where, they both recognized the need for a unifier, for an organ of integration which fused separate sensations into unified perception. In their 2005 paper "What is the function of the claustrum?" Francis Crick and Christof Koch asked us to consider if the claustrum might perform this function, writing that the claustrum "… appears to be in an ideal position to integrate the most diverse kinds of information that underlie conscious perception, cognition and action."

The claustrum is a small strip of tissue sequestered deep within the brain. First described in the 18th century by Felix Vicq D'Azyr, its contorted shape and inaccessible location have frustrated generations of neuroscientists[1,2]. A putative claustrum has been identified in all extant mammals — though some controversy exists regarding the presence of a claustrum in monotremes — and recent studies have identified homologous structures in birds and reptiles[3–7]. Evolutionary conservation of the claustrum since the common ancestor of mammals and reptiles suggests a fundamental function. However, that function has eluded generations of researchers. The claustrum is remarkable for its strong reciprocal connectivity with nearly every cortical region, the highest connectivity of any region in the human brain by volume[8–14]. This unique anatomy has spawned a host of functional hypotheses, linking the claustrum to everything from consciousness and sleep to sexual function and aesthetic judgement[6,15–20].

Despite the numerous hypotheses and a recent increase in research, the claustrum's contribution to behaviour remains a mystery.

In this thesis, I will first provide relevant background information to outline current knowledge on the anatomy, connectivity, physiology, and function of the claustrum. I will then explain the methods and results of my experiments on this topic and place these results within the context of the field.

1.1 Location & Identification

To study a brain region, one must first be able to locate it. While this may sound trivial, claustrum research has been plagued by inconsistencies in the nomenclature, labelling, and delineation of the claustrum that reduce the reproducibility and applicability of results among studies and labs. This section will discuss current evidence regarding the anatomical boundaries of the claustrum and techniques for reproducibly delineating the claustrum and its neighbors. This section will focus primarily on the rodent claustrum, but similar difficulties exist in other species.

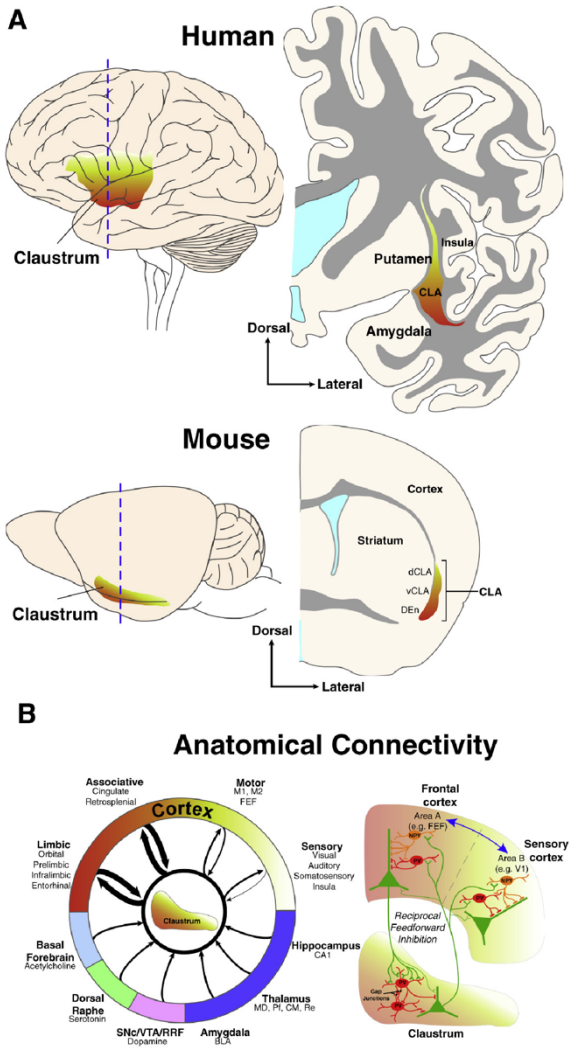


Figure 1-1. (A) Diagram showing the anatomical location of the human (top) and mouse (bottom) claustra. Coronal sections taken at the dashed line are shown on the right. (B) Diagram illustrating the connectivity pattern (left) and cortico-claustro-cortical circuit structure (right) of the claustrum. Adapted from Smith et al. Curr Biol. 2020.[1]

The claustrum is a long, thin, bilateral sheet of grey matter running dorsoventrally through the brain (Figure 1-1A). Lying beneath the insular cortex, the human claustrum is encapsulated between the extreme and external capsules. In animals (including rodents) that lack a robust extreme capsule, the claustrum is contiguous with the cortex. Dorsally and laterally, the rodent claustrum is contiguous with the deep layers of the insular cortex. It is separated from the striatum medially by the external capsule, though some authors suggest that the claustrum is fully surrounded by deep layers of the cortex[21–23].

Ventrally, the claustrum is contiguous with the dorsal endopiriform nucleus (dEN). The lack of clear anatomical borders for the mouse claustrum presents a challenge for claustrum researchers.

Gene expression studies have identified numerous genes that are enriched within the claustrum region. While some genes like Gnb4, Ntng2 and Slc17a6 express broadly across the claustrum, other genes such as Cplx3, Ctgf, Nr2f2, and Npsr1 appear to highlight different subregions within the claustrum[5,24]. Various transgenic mouse lines, including the Cla-Cre[19], Egr2-Cre[25], Gnb4-Cre[13], and VGLUT2-Cre[26] lines have also been used to label the claustrum with varying degrees of strength and specificity. Ongoing work to develop more claustrum-specific transgenic lines may alleviate some of the challenges associated with claustrum research.

In addition to transgenic mouse lines, the claustrum is also commonly labelled using retrograde tracers[10,27–30]. Zingg et al. 2018 showed that the claustrum projects strongly to the retrosplenial cortex (RSP) but regions adjacent to the claustrum do not[10]. Injections of retrograde tracers into the RSP label a small pocket of cells within the claustrum with minimal off target labelling[10,28]. Retrograde tracers injected into other cortical areas are useful for labelling various projection-defined claustrum subpopulations but run the risk of off-target expression.

Immunohistochemical staining for parvalbumin (PV) reveals a dense plexus of PV-labelled fibres within the claustrum[24,28,31,31–35]. The PV labelled plexus corresponds closely to the area labelled by retrograde tracing from the RSP[28]. While PV staining is useful for identifying the broad location of the claustrum, additional debate surrounds the existence of subregions within and beyond the PV/RSP defined region. While some studies have viewed the claustrum as a monolith, others have subdivided it into dorsal and ventral subregions and still other studies have divided the claustrum into a core and shell arrangement[4,13,22,31,32,32,33,36–39]. In rodents, the claustrum core has classically been identified as the PV-rich plexus, while the extent of the shell is less clearly defined. Recent connectivity data shows clear dorsoventral and centre surround gradients in both the inputs and outputs of the claustrum[27,28]. These gradients provide compelling evidence of regional sub-specialization within the claustrum and support the existence of claustral subregions beyond the PV/RSP defined region. However, to delineate the claustrum based on

every different combination of gradients would yield an unwieldy and unhelpful number of subregions.

In addition to the evidence supporting the existence of functional subregions within the claustrum, some have argued that the claustrum itself should be considered a subregion of a larger complex[24,40,41]. Genetic and developmental overlaps between the claustrum and DEn provide compelling evidence that they should be considered separate but related structures[24,40]. While the claustrum and DEn were previously considered to have different developmental origins, recent data suggests that both structures arise from the same region of the pallium[4,7]. However, birth dating studies suggest a gradient in the birth date of claustrum and DEn neurons[23,42]. Connectivity data suggests that while the claustrum and DEn participate in circuits involving different cortical regions, they share similar patterns of connectivity (ex. in the relative strength of ipsi- and contralateral cortico-claustral projections)[40,43–46]. Genetic evidence also supports a link between the claustrum and DEn with numerous genes co-expressed in the claustrum and DEn (ex. Npsr1)[5,13,24,40]. Considering these similarities in genetics, development, and connectivity between the claustrum and dEN, it is important for researchers to consider both regions and to delineate and report the cell population under investigation in each individual experiment.

The discussion above highlights the fact that significant uncertainty persists regarding the internal and external borders of the claustrum. While the region containing the claustrum and DEn is unlikely to be a functionally homogenous monolith, it is not clear exactly how it should be subdivided, whether by genetics, cytostructure, input connectivity, output connectivity, development, or behavioural relevance.

To facilitate transparent reporting and comparisons across labs, Grimstvedt et al. have recently reviewed existing methods of delineating the claustrum complex. The paper presents novel anatomic and genetic data to support a new set of boundaries for the claustrum complex[24]. These delineations are informed by gene expression and connectivity, as well as fiber- and cyto-architecture. Staining for myelin basic protein (MBP) in the mouse reveals a diagonal band of myelinated fibers that extends from the external capsule and runs along the dorsolateral edges of the claustrum. MBP staining also shows an absence of myelinated fibers within the claustrum itself, largely corresponding with the RSP/PV-rich claustrum, and characteristic patterns in the dEN. While the proposed delineations do not capture the full complexity of dorsoventral and

centre-surround gradients present in the claustrum, they can be drawn based on easily replicated immunohistochemical stains[27,28,30]. This simplicity means that the proposed delineation can easily be adopted by the claustrum research community to provide a common reference framework. Presenting claustrum histology with reference to either or both stains will enable greater reproducibility among labs and shed light on the overlap between the cell populations investigated in different studies.

1.2 Connectivity & Physiology

The claustrum is perhaps best known for its remarkable connectivity, which has repeatedly been demonstrated across species and decades[8–13,44,46–70]. Using diffusion tensor imaging (DTI), Torgerson et al. found that the human claustrum had the highest connectivity per unit volume of any brain region[11]. Individual claustrum neurons have also been shown to circle the entire brain[71]. In a major study investigating the morphology of neurons across the mouse brain, claustrum neurons were found to have the highest number of cortical targets of all neurons studied[72]. This section will discuss the input and output connectivity of the claustrum along with its cellular and circuit properties.

The claustrum has repeatedly been shown to receive inputs from nearly every region of the neocortex (Figure 1-1B; left)[10,12,13]. While there is some degree of input from most regions, the strongest projections in the rodent brain come from regions in the anterior dorsal midline such as the orbitofrontal and cingulate cortices[10,13,19]. These inputs are predominantly bilateral with most cortical regions projecting to both ipsi- and contralateral claustrum[13,43–46]. The claustrum receives inputs from sensory areas, albeit weakly, as well as motor and premotor regions[9,13,44–46]. Cortico-claustral inputs arise from various cortical layers, with some variation in output layer by cortical region[45–47,65]. While the precise cortical inputs to claustrum vary across species, the existence of broad cortico-claustral connectivity appears to be a general feature of the claustrum[14].

The claustrum's connections with the cortex are predominantly reciprocal, with the claustrum sending projections to most cortical regions. The strength of these claustro-cortical projections is broadly related to the strength of the corresponding input, meaning that brain regions sending weaker projections to claustrum receive weaker innervation in return[13]. While the claustrum tends to receive input from both ipsi- and contralateral cortex, claustrum output

is predominantly ipsilateral, although there is variation among brain regions[13,43–46]. As with cortico-claustral inputs, claustro-cortical outputs have been shown to vary among species[73]. The terminals of claustro-cortical projections have been shown to be distributed across cortical layers, but with variation in the degree of layer specificity among cortical regions[9,14,47,55,74].

Claustro-cortical outputs also show topographic organization with groups of claustrum neurons projecting to different cortical areas and vice-versa. This topographical organization within the claustrum occurs along both the anteroposterior and dorsoventral axes[12,13,28,30,40,45,49,55–57,75–77]. This organization is both spatial and functional, with anterior areas of cortex connected with anterior areas of claustrum and posterior areas of cortex connected with posterior areas of claustrum[10,13]. The claustrum has been proposed to be organized in a series of partially overlapping functional ensembles where neighboring and functionally related cortical regions tend to receive input from neighboring claustrum neurons[14,40,45,46,64,75]. Nearby claustrum neurons are thought to target related cortical regions, such that closely linked cortical regions tend to receive input from the same group of claustrum cells[45,46]. The observed overlap of cortical inputs to claustrum can be seen as evidence that multisensory* processing could occur in the claustrum, but the existence of segregated functional modules may argue against claustral integration.

When compared to its cortical connectivity, the claustrum has relatively little connectivity outside the neocortex. However, the claustrum has been shown to receive input from various subcortical and allocortical structures[10,70,78]. Notably, the claustrum receives input from the basolateral amygdala, dorsal raphe, hippocampus, and mediodorsal thalamus[10]. Unlike in the cortex, most subcortical projections to the claustrum are not reciprocal. However, there are some differences among studies in the reciprocity of subcortical connections which may be attributable to differences in the techniques used to label the claustrum[10,13].

These subcortical structures provide neuromodulatory input to the claustrum. Serotonergic input comes from the dorsal and median raphe nuclei, with the claustrum expressing the highest concentration of 5HT2A receptors of any region in the mouse brain along with varying levels of other serotonergic receptors[10,19,78,79]. In addition to serotonergic innervation, the claustrum receives dopaminergic input from the ventral tegmental area and the

* Note, in this thesis the terms 'unimodal' and 'multimodal' are used to refer to sensory stimuli, while 'unisensory' and 'multisensory' are used to refer to neural responses to said stimuli. See Stein et al 2010.

substantia nigra. The claustrum has been shown to express both D1 and D2 dopaminergic receptors, and D1 expressing claustrum neurons have been implicated in incentive salience[10,78,80]. Cholinergic inputs to claustrum have been identified from the basal forebrain, with the claustrum expressing both nicotinic and muscarinic receptors[10,19,25,78]. The claustrum also receives noradrenergic innervation from the locus coeruleus, albeit weakly, and contains noradrenergic receptors[10,78]. Intriguingly, the claustrum also contains high concentrations of kappa opioid receptors[81]. Both kappa opioid receptors and 5HT2A receptors have been implicated in delusions, highlighting a potential role for the claustrum in disordered perception[82–84].

Various attempts have been made to categorize the cell types present in the claustrum[27,30,85–88]. The claustrum is known to contain both excitatory projection neurons as well as local interneurons[76,86,89]. Recent evidence has also suggested that there may be a population of projection interneurons that directly innervate cortex[27]. Among inhibitory neurons, the claustrum has been shown to contain PV, neuropeptide Y (NPY), and somatostatin interneurons[86,90]. PV positive interneurons have been shown to be densely interconnected by both chemical and electrical synapses, but less is known about the relative connectivity of other interneuron subtypes[86,89]. Significant variation exists in the spatial distribution of these interneuron subtypes, with PV interneurons located in the claustrum core while NPY and somatostatin interneurons are primarily located in the shell[28]. This suggests that different interneuron subtypes may be involved in regulating different functional modules of excitatory neurons. Further work is needed to understand the relationship between different interneuron subtypes and different groups of projection neurons.

The claustrum is primarily composed of excitatory projection neurons. Several studies have aimed to identify the cell types present in the claustrum based on their electrophysiological and transcriptomic profiles. While the precise cell types identified in these studies are difficult to compare, it is clear that the claustrum contains excitatory neurons which differ in their projection targets, intrinsic electrophysiological properties, and genetic signature[27,30,85–88]. Within the claustrum, the various cell types appear to be organized topographically[30]. Electrophysiologically distinct cell types have also been shown to receive input from different cortical regions[27,85]. Individual excitatory neurons have been shown to receive input from multiple cortical areas, highlighting the possibility that the claustrum may be able to integrate

information from different cortical areas[27]. Further evidence is required to understand the full diversity of cell types within the claustrum and how they relate to different cortical projection targets.

A significant body of work has examined the impact of claustrum activity on downstream cortical targets. Claustrum stimulation has been shown to evoke both excitatory and inhibitory currents in cortical cells[19,26,29,91,92]. While excitatory claustro-cortical projections can target cortical excitatory neurons, projections to cortical interneurons are thought to dominate. This leads to an overall inhibitory effect of claustrum stimulation on the cortex, where claustrum stimulation acts to dampen activity across the cortex[19,27,29]. This inhibitory effect does not appear to be universal, with variation observed across brain regions and cortical layers[26,91]. However, these differences among studies may also be the result of different techniques used to label the claustrum.

Like claustro-cortical projections, cortico-claustral projections are also thought to lead to inhibition. Cortico-claustral inputs target both excitatory and inhibitory cells within the claustrum. Excitation of these inputs has been suggested to lead to feed-forward inhibition, causing a brief excitation of excitatory claustrum neurons followed by interneuron-mediated inhibition[89]. Due to the dense connections, including electrical synapses, observed between claustrum interneurons, cortico-claustral input can lead to widespread inhibition. However, experiments demonstrating cortico-claustral feedforward inhibition have mainly involved PV interneurons. Given the observed differences in the topographic arrangement of interneuron subtypes and cortically projecting ensembles of claustrum neurons, further experiments are required to understand the prevalence of feed-forward inhibition within different populations of excitatory and inhibitory claustrum neurons[14,28,86]. Figure 1 B (right) illustrates the cortico-claustro-cortical circuit structure of the claustrum.

There has been significant debate within the field regarding the existence of intra-claustral communication networks. In their seminal review linking the claustrum to consciousness and multisensory integration, Crick and Koch highlighted that these hypotheses hinged on the existence of intra-claustral information transfer[16]. Not only would this require communication among claustrum neurons, but it would also require communication among projection defined claustrum submodules. While intra-claustral inhibitory communication has been well documented, excitatory to excitatory communication has been more

controversial[10,89,93]. Tracer injections into the claustrum have revealed extensive intra-claustral projections along the rostro-caudal axis[45]. The lack of dorsoventral connectivity was seen as evidence that intra-claustral communication was likely restricted within dorsoventrally distributed functional modules[14,40]. In addition, when performing patch-clamp electrophysiological recordings of excitatory claustrum neurons, Kim et al. found very limited intra-claustral connectivity[89]. However, these recordings were taken from coronal slices which may have severed long range connections. More recently, Shelton et al. used optogenetic stimulation to investigate the possibility of intra-claustral excitatory communication[27]. Patch-clamp recordings revealed high rates of intra-claustral connectivity. Moreover, these experiments found evidence for communication among projection-defined claustral submodules. While further work is required, current evidence suggests the existence of intra-claustral connectivity both within and between functional submodules.

1.3 Activity & Function

Assigning functional significance to a brain region is not easy. Examples of human lesions — whether they resulted from accidental damage, encephalitis, surgical resection, or some other cause — have historically provided a window into the behavioural role of the lesioned area[94,95]. Behavioural changes occurring after the loss of a region provide evidence that the altered behavioural processes might involve the lesioned region. However, compensation across regions and hemispheres, combined with the complexity of many behavioural functions means that the impact of lesions can be difficult to understand[95]. In the case of the claustrum, the issue is further complicated by a dearth of highly specific lesions. Many lesions involving the human claustrum impinge upon the surrounding white-matter tracts as well as adjacent brain regions such as the insula. Atilgan*, Oliver*, Doody* et al. (2022) includes a comprehensive review of all known examples of human claustrum lesions. In consideration of the lesion data, the paper also includes a review and critical reappraisal of major hypotheses regarding the function of the claustrum[96]. This paper is reproduced here in Appendix II and provides additional background on the effects of claustrum lesions and current functional hypotheses.

In addition to acute lesions, associations between a brain region and neurological disorders may provide evidence of its function. Changes in the claustrum have been noted in neurodegenerative conditions including Parkinson's Disease, Alzheimer's Disease, and Dementia

with Lewy Bodies[97–103]. The claustrum has also been linked to Schizophrenia[104–107]. In part due to its connection with Schizophrenia, the claustrum has recently been proposed as a centre for delusions and hallucinations[82]. With its widespread connectivity, the claustrum has also been linked to seizures[96,108–111]. With the possible exception of certain cases of status epilepticus, a key causal role for the claustrum has not been identified in any major neurobiological disorder[112].

Another window into the function of a brain region can be found by recording its activity during normal functioning. If a brain region's activity is correlated with certain behavioural states or processes, this may suggest that the brain region supports these behavioural functions. In the case of the claustrum however, notable challenges make *in vivo* recordings of claustrum activity difficult. The claustrum is too deep in the brain to be visualized using commonly available microscopy techniques. While implanted electrodes and optical devices can reach the claustrum, its elongated shape means that only a small rostro-caudal slice can be recorded. Non-invasive techniques like fMRI have historically struggled to separate claustrum signal from that of nearby brain regions, although recent improvements in field strength and analytical techniques have made claustrum recordings more feasible[113,114]. Despite these challenges, numerous studies have succeeded in recording the activity of claustrum neurons *in vivo*. This section will provide an overview of their findings along with a brief discussion of prominent functional hypotheses. For further discussion of these functional hypotheses, please see Appendix II.

Several pioneering studies of the mid to late 20th century used implanted electrodes for extracellular recordings in the cat claustrum. These studies established the existence of sensory responsive neurons in the claustrum. They identified neurons in the claustrum responsive to somatosensory, auditory, visual, and painful stimuli[48,53,61,115–120]. Visual and somatosensory responsive neurons showed retinotopic and somatotopic organization within the claustrum[48,61,118]. Some studies identified discrete zones responsive to individual sensory modalities while others found heterogeneously distributed responses[48,61,117,118]. Studies which presented combinations of sensory stimuli to investigate the existence of multisensory responses found mixed results. Some authors found little overlap between sensory modalities, while others found that a large majority of neurons displayed multisensory responses[48,115,117,119].

More recent studies have also investigated the sensory response properties of the claustrum in various species. Remedios et al. recorded claustral responses to audio and visual stimuli in primates. While they identified units responsive to both modalities, they found

responses to be primarily unisensory with little evidence of multisensory responses. Instead, they proposed that claustrum neurons were responsive to changes in the auditory environment[75,121]. Other recent studies have assessed the sensory response properties of the claustrum during a behavioural task. Garcia et al. recorded claustrum activity in rabbits during an eyeblink conditioning task and found that few claustrum cells were responsive to unimodal stimuli. Instead, they found cells responsive to the combination of the conditioned and unconditioned stimuli, but only in untrained animals[122]. Chevee et al. recorded from neurons in the anterior claustrum of mice during a multimodal behavioural task. They found relatively few cells that were responsive to sensory stimuli, instead finding that cells were primarily modulated by motor signals[123]. In a preprint investigating sensory responses in mice, Ollerenshaw et al. found only a small percentage of neurons in the anterior claustrum were responsive to passive sensory stimulation[124]. They noted that a slightly higher percentage of cells were responsive to multimodal stimuli, but this remained a small proportion of the overall population. However, they found that many more neurons were active during a visually guided behavioural task, particularly on trials where the mouse executed a motor response[124]. Interestingly, a recent study noted unisensory responses to aversive stimuli in claustrum neurons projecting to anterior cingulate cortex (ACC)[125]. Taken together, these results provide mixed evidence for the existence of sensory responsive cells in the claustrum. While sensory responses appear more common in cats and primates, there is mixed evidence for simple sensory responses in mice. However, this difference may partly be due to different techniques used to label and record from the claustrum.

To avoid the challenges associated with recording claustrum activity directly, some authors have investigated immediate early gene (IEG) expression. IEG expression has been observed following rewarding stimuli (cocaine), as well as aversive stimuli[80,126,127]. Another study observed increased IEG expression in claustrum when mice were exposed to a novel environment[77]. IEG expression was also seen as evidence that claustrum cells might support cortical activity during rapid eye movement (REM) sleep hypersomnia[20]. Other studies have also connected the claustrum to sleep. Notably, Narikiyo et al. linked claustrum activity to the generation of cortical slow waves in mice[19]. Intriguingly, evidence linking the claustrum to sleep has also be found in reptiles and humans[6,128]. Further work is needed to understand this emerging possibility.

Claustrum activity can also be recorded using non-invasive techniques such as PET scanning and MRI. MRI recordings have noted altered claustrum activity linked to psilocybin, which is significant considering evidence linking the claustrum to delusion[82,84,96]. Similar to the IEG studies mentioned above, numerous human imaging studies have seen claustrum activation following aversive stimuli (pain and itch)[129–143], as well as salient non-aversive stimuli (sexual imagery)[15,144,145]. Resting state fMRI imaging in humans and rodents has suggested that the claustrum may be linked to various brain networks including the task positive network, the default mode network, and the salience network[113,114,146,147].

Invasive recording techniques have been used to record claustrum activity in animal models. White et al. noted increased claustrum activity preceding correct trials on a five-choice serial reaction time test compared to a one-choice serial reaction time test, linking claustrum activity with task difficulty[148]. In a recent preprint, Fodoulian et al. found activation of various subgroups of claustrum neurons during an attentional set shifting task[26]. In addition to task-related activation, claustrum recordings have also identified cells encoding spatial information[149].

The diverse results presented above highlight the challenge of designing causal manipulations to investigate the function of the claustrum. Claustrum activity in various subpopulations has been linked with a wide array of behavioural processes, sensory stimuli, and brain states. This is perhaps not surprising given the extensive connectivity of the claustrum with the cortex. Any neural process occurring in the cortex could conceivably be conveyed to the claustrum, potentially giving rise to activity. Despite these obstacles, decades of research into the physiology, connectivity, and activity of the claustrum have coalesced around several leading functional hypotheses. Notably, several recent studies in mice have implicated the claustrum in processes related to cognitive control and executive function. Claustrum manipulations in these studies have led to changes in impulsivity, resilience to distraction, cognitive flexibility, reversal learning, and performance on a complex task requiring focussed attention[25,26,87,123,148,150,151]. While each result is slightly different, all can be placed within the broader framework of executive function[152,153]. This link is also consistent with the strong connectivity observed between the claustrum and frontal cortical structures in mice. This trend is not universal, with other claustrum manipulations supporting a role for the claustrum in sleep, anxiety, and other diverse processes[6,19,122,125,126] .

In short, the function of the claustrum remains a mystery. While a plethora of functional hypotheses have been proposed, no hypothesis has garnered sufficient evidence to overtake the field. As discussed above, the inaccessible location of the claustrum complicates any attempt to record or manipulate its activity. With improvements in the techniques available for interrogating and manipulating the activity of the claustrum an increasing number of functional studies have been published in recent years. However, the diversity of the methods employed to label, record, and manipulate the claustrum (ex. different transgenic lines, retrograde tracing from various cortical regions) combined with inconsistent reporting of the precise population under examination reduce the comparability of studies. More work is required to understand this enigmatic region, but with the rapid advance in knowledge and methodology researchers may soon be able to uncover the function of the claustrum.

The experiments described in this thesis sought to help elucidate the behavioural relevance of the mouse claustrum. In Chapter Two, I aimed to record the activity of the claustrum. I first developed a novel technique to record from the claustrum, and later used this technique to investigate the existence of sensory responsive neurons in the claustrum. In Chapter Three, I investigated the effects of chronic claustrum silencing on ethologically motivated behaviours. After silencing the claustrum in a novel transgenic mouse line, I measured the effects of silencing on a battery of behavioural tests. In Chapter Four, I investigated the effects of chronic claustrum silencing on home-cage behaviour. I measured the impact of chronic claustrum silencing on home-cage activity levels as well as metrics of circadian behaviour in both transgenic and wildtype mice. Finally, in Chapter Five I investigated the impact of chronic claustrum silencing on learned behaviours. I trained mice with chronic claustrum silencing on a classical conditioning-like task and a reversal learning task to measure the impact of silencing on animals' performance. The results of these experiments are described in the chapters below and discussed in context of the relevant literature in Chapter Six.

2 Sensory response properties of the mouse claustrum

Of the numerous hypotheses that have been proposed for the function of the claustrum, Crick and Koch's proposal that the claustrum might act to integrate different modalities of sensory stimuli has perhaps generated the most excitement. This proposal was consistent with the connectivity of the claustrum and supported by early studies in cats that identified claustrum neurons responsive to various modalities of sensory stimuli[48,118]. Some early studies also reported populations of neurons responsive to multiple sensory modalities[117]. However, an impactful study from Remedios et al. in 2010 found that while the primate claustrum contained populations of sensory responsive neurons, they were not responsive to multimodal stimuli[75]. While supporting the idea that the claustrum is a 'multisensory' structure, this argued against the idea that the claustrum could be a site of integration. More recent work in mice has continued to argue against multisensory integration in the claustrum, with studies finding few sensory responsive neurons in the claustrum[123,124].

In this chapter, we sought to extend Remedios et al.'s results in a mouse model. First, we developed a novel preparation to facilitate minimally invasive recordings of the mouse claustrum. Next, we aimed to test whether we could identify neurons responsive to individual modalities of sensory stimuli. Finally, we investigated whether sensory responsive neurons were exclusive to individual modalities or displayed multisensory responses.

2.1 Methods

10 mice (2 male and 8 female) received intracranial injections of virus and were implanted with cranial windows. Two-photon calcium imaging was then used to record the sensory-evoked activity of claustrum axons in dorsal cortex. A first cohort of mice was presented with only unimodal sensory stimuli, and a second cohort was presented with both uni- and multimodal stimuli.

2.1.1 Animals

All experiments were carried out on wild type C57Bl/6 mice. Littermate groups of 2–5 were housed under standard conditions with wood chip bedding and ad libitum access to food and

water. All experiments were subject to ethical approval and conducted in accordance with local laws and regulations.

2.1.2 Injection Surgery

Intracranial injections were performed in accordance with the method outlined below. All mice were injected with 80nl of retro-Cre virus (AAV.hSyn.Cre.WPRE.hGH, Addgene) in both the RSP (bregma lambda coordinates: -3.0 AP, 0.5 ML, -1 DV) and ACC (bregma lambda coordinates: 1.34 AP, 0.3 ML, -1.25 DV). In addition, mice were injected with 250nl of Cre-dependent GCaMP7b virus (AAV-syn-FLEX-jGCaMP7b-WPRE, Addgene) in the claustrum (bregma lambda coordinates: 1.0 AP, 3.4 ML, -2.7 DV).

All recovery surgical procedures were carried out under sterile conditions. Anesthesia was induced with 5% isoflurane and maintained with ~1.5%. Once mice were anesthetized, the incision area was shaved, and mice were placed on a stereotaxic frame with an integrated heater to maintain body temperature (Stoelting). Mice received an intraperitoneal injection of 5 mg/kg meloxicam and 0.1 mg/kg buprenorphine. The incision area was prepared with chlorhexidine gluconate and isopropyl alcohol (ChloraPrep) along with an injection of local anesthetic (bupivacaine) under the scalp. A single incision was made, and skin retracted to expose the skull. The surface of the skull between bregma and lambda was levelled relative to the stereotaxic frame.

Small craniotomies were made using a dental drill (500um tip) at the indicated bregma lambda coordinates. Dorsal-ventral coordinates were measured from the surface of the dura. Borosilicate glass injection pipettes were backfilled with oil and loaded with the appropriate virus. Injection pipettes were then lowered slowly into the brain and allowed to rest for two minutes prior to injection. Injections were performed at ~50nl/minute using a manually controlled hydraulic microinjector (Narishige). Pipettes were allowed to rest for 10 minutes after each injection and retracted slowly.

After all injections were completed, incisions were sutured (Vicryl) and sealed with Vetbond (3M). Mice were then transferred to a fresh cage. The recovery cages were heated until mice regained normal locomotion and included sterile bedding and excess palatable food (mash, forage mix). In addition to normal monitoring from veterinary staff, mice received twice daily welfare checks and weighings for two days following surgery and daily welfare checks and

weighings for an additional five days. Mice were provided with analgesic jelly (meloxicam) daily during recovery. All mice continued to be group-housed following surgery except where specified.

2.1.3 Cranial Window Implantation

Cranial window implantation surgeries were carried out using the same anesthetic, analgesic, and sterilization procedures described above for intracranial injection surgeries. After mice were anesthetized and the scalp prepared, a section of scalp was resected. The skull was then cleaned to remove the periosteum and lightly scored using a bone scraper for better adhesion. An aluminum headplate was secured in place using dental cement (Sun Medical). A dental drill was used to make a 4mm diameter circular craniotomy centred on bregma. The imaging well was filled with sterile saline and the craniotomy was left to soak for 5 minutes before the bone chip was lifted from the craniotomy. The craniotomy was then flushed with saline to clear any bleeding. The dura was removed from the surface of the brain using fine forceps. Finally, a cranial window composed of a 4mm glass coverslip glued to a 5mm glass coverslip was secured into the craniotomy with Vetbond and dental cement. Recovery and post-op monitoring was the same as for intracranial injections above.

2.1.4 *In Vivo* Calcium Imaging

All imaging was conducted on a Bruker Ultima 2P+ two photon microscope using a Coherent Chameleon femtosecond laser. The microscope was housed in an enclosed hood to minimize external sound and light. The Nikon 16x 0.8NA water immersion lens was further shielded from external light using a custom 3D printed cone connected to a flexible rubber sleeve. The laser was tuned to a wavelength of 920nm with ~50mW of power on sample. Videos were recorded using PrairieView software at 30 frames per second with 512x512 pixel resolution and 4x optical zoom.

After surgery, mice were given at least 3 weeks for viral expression. Mice were acclimated to head-fixation over three days with increasingly long periods of head-fixation under the microscope. Next, axonal GCaMP7b expression levels were assessed by eye. Animals without detectable GCaMP7b were excluded from further experiments. Mice with expression moved on to imaging and sensory stimulation.

At the start of each imaging session, a mouse was head fixed under the microscope inside the imaging hood. White noise at ~60dB SPL was played using a loudspeaker (Philips A2.310/05) throughout the session to help mask unwanted sound. The surface of the cranial window was first leveled relative to the imaging plane using a tip-tilt stage (Thorlabs). Next, the coordinates of the microscope field of view were zeroed at the centre of the cranial window to allow for comparison across imaging sessions. Individual imaging fields of view were selected manually based on visible axon expression. Axon activity was then recorded for the duration of the sensory stimulation protocol. Once all sensory stimuli had been delivered, a new field of view was selected, and the stimulation repeated. The approximate X-Y-Z coordinates of each field of view were recorded to reduce the likelihood of recording from the same axons multiple times. However, due to the length and branched shape of individual axons, it is possible that the same axons may have been visible in multiple fields of view. Imaging and field of view selection was done in the same manner for both the uni- and multi-modal imaging cohorts.

2.1.5 Sensory Stimulation

Calcium activity was continuously recorded for each field of view while mice were presented with a series of sensory stimuli. Stimuli lasted 500ms and consisted of a complex tone, a flash of white light, and a paddle moving through the whiskers. The complex tone was amplitude and frequency modulated with a carrier frequency of 5kHz. The white light was generated by an LED (Thorlabs) emitting ~5.5 mW ~20cm from the mouse. Finally, the whisker stimulus consisted of a paddle attached to a piezoelectric whisker stimulator (Physik Instrumente) moving within the whiskers at 20Hz. Stimulus presentation was controlled by a computer running PackIO software to send and receive voltage signals through a National Instruments data acquisition card. Voltage traces were generated using custom MATLAB (Mathworks) code.

For the unimodal cohort, mice were presented with a series of 60 sensory stimuli separated by randomly selected 8–11 second inter-trial intervals. These stimuli consisted of either sound, light, or whisker stimulation alone, or a blank condition in which no stimulus was presented. Trial order was randomly generated each day. The multimodal stimulation protocol was identical to the unimodal protocol but included an additional 60 randomly interleaved trials consisting of either: whisker and sound; whisker and light; sound and light; or sound, light and whisker stimuli.

2.1.6 Histology

Histological validation of claustrum-specific viral expression was performed according to the general antibody staining protocol described below. To cover the full extent of the claustrum, 12 coronal sections spaced 250um apart were selected such that the third section was located at the anterior union of the corpus callosum. Slices were then stained with antibodies against PV (SWANT; 1:500; RRID: AB_2631173) to facilitate identification of the claustrum, and GFP (AVES; 1:2500; RRID: AB_10000240) to clearly label all GCaMP7b positive cells. All secondary antibodies were used at a concentration of 1:500.

After all *in vivo* experiments were completed, mice were deeply anesthetized with 5% isoflurane before receiving an intraperitoneal overdose of pentobarbital. Mice were transcardially perfused with 0.01M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were then extracted and left to fix overnight in PFA at 4°C. Brains were then stored in PBS with 0.0005% sodium azide at 4°C. Brains were sliced into 50um coronal sections using a vibratome (Leica VT1000S). Slices were then stored in sterile 24 well plates with PBS and sodium azide.

For antibody staining, slices were first rinsed 3 x 5 minutes in PBS. They were then blocked for 2 hours at room temperature in PBS with 0.5% Triton X-100 and 5% normal donkey or goat serum as dictated by the secondary antibodies used. Slices were then incubated overnight at 4°C with primary antibodies diluted in blocking solution. Slices were then rinsed 3 x 5 minutes in PBS and incubated for 2 hours at room temperature with secondary antibodies diluted in blocking solution. Finally, slices were rinsed 3 x 5 minutes in PBS and incubated for a further 30 mins with DAPI (1:1000) if needed. Slices were then mounted on slides and cover-slipped using fluoromount mounting medium. Slides were allowed to dry overnight and sealed with nail polish before imaging. Images of post-hoc histology from calcium imaging animals were taken using a Zeiss LSM710 confocal microscope at 10x magnification and a Leica epifluorescence microscope at 1.6x magnification.

2.1.7 Data Analysis & Statistics

Data was analyzed using Python 3.9.13 and MATLAB 2022b using built-in and open-source packages including Suite2P and GRAMM[154,155]. Code and data are available upon request. Figures were generated using Python and MATLAB and formatted using Adobe Illustrator.

Calcium Signal Preprocessing & Region of Interest (ROI) Selection

Calcium imaging videos were first motion corrected with Suite2P. After motion artifacts had been removed, axonal regions of interest were selected. In the unimodal cohort, axonal ROIs were selected manually using ImageJ. In the multimodal cohort, axonal ROIs were selected automatically using Suite2P and curated manually. Axons were selected based on both their morphology and activity traces.

After regions of interest had been selected, calcium activity was extracted and converted to $\Delta F/F$ using the formula $\Delta F/F = (F - \bar{F})/\bar{F}$ where $\bar{F}$ represents the mean fluorescence across the entire session. Fluorescence traces from the multimodal cohort were first corrected based on neuropil fluorescence using the formula $F = F - 0.7F_{Neu}$ where F_{Neu} represents the fluorescence of the area immediately surrounding the axonal ROI.

Identification of Responsive Axons

After preprocessing and ROI selection, fluorescence traces were then analyzed to identify axon segments displaying significant stimulus-locked activity. Axonal fluorescence traces were first converted to stimulus triggered averages for each trial type by averaging together the axon's activity from 2 seconds before to 6 seconds after each stimulus presentation. Significant responses were then identified by comparing the activity in the 1 second before and after stimulus onset using a Mann-Whitney test corrected for multiple comparisons using a Benjamini-Hochberg false discovery rate analysis with an alpha of 1%.

Identification of Uni- and Multisensory Responses

Axons were classified as either uni- or multisensory based on the trial types to which they responded. Unisensory axons were those which responded to only one modality and whose response was not modulated by other modalities (ex. an axon which responded to all trial types

that included the sound stimulus and none which did not include the sound stimulus). Multisensory axons were those which either a) responded to multiple sensory modalities (including those which only responded to multimodal trial types) or b) whose response to a unimodal trial type was modulated by the addition of other modalities (ex. an axon which responded to the unimodal sound stimulus but did not respond to multimodal trial types that included the sound stimulus).

Understanding the Diversity of Axonal Responses

To better understand the variability of axonal responses we examined the response to each individual stimulus presentation. To do this, we calculated the area under the $\Delta F/F$ curve for each axon on each stimulus presentation. This value was then corrected by subtracting the area under the $\Delta F/F$ curve in the second preceding stimulus onset to give us the change in area under the curve (ΔAUC). To examine the reliability of axonal responses, we calculated the probability of a stimulus-locked change in $\Delta F/F$ for each axon. To do this, we defined an arbitrary threshold of 1 standard deviation above the mean ΔAUC. We could then calculate the probability of each axon displaying stimulus-locked activity as $p_{activity} = \frac{\#\ stimulus\ presentations\ evoking\ change\ of\ 1\ STD}{total\ \#\ stimulus\ presentations}$.

2.2 Results

Two cohorts of mice were implanted with cranial windows for *in vivo* calcium imaging of claustrum axons in dorsal cortex. Mice were presented with a series of uni- and multimodal sensory stimuli. Analysis revealed stimulus-locked responses to both uni- and multimodal sensory stimuli. Axons were responsive to all stimulus types and a population of axons were found to be exclusively responsive to multimodal stimuli.

2.2.1 Labelling the Claustrum

Specifically and reliably labelling the claustrum remains a challenge for the field. To gain access to the claustrum, we explored a dual viral retrograde approach based on the work of Zingg et al. (2018)[10]. Briefly, we injected Cre-dependent GCaMP7b into the claustrum and retro-Cre into the RSP and ACC (Figure 2-1 A). This approach yielded strong expression in claustrum with limited off-target expression in nearby cortical areas (Figure 2-1 B C).

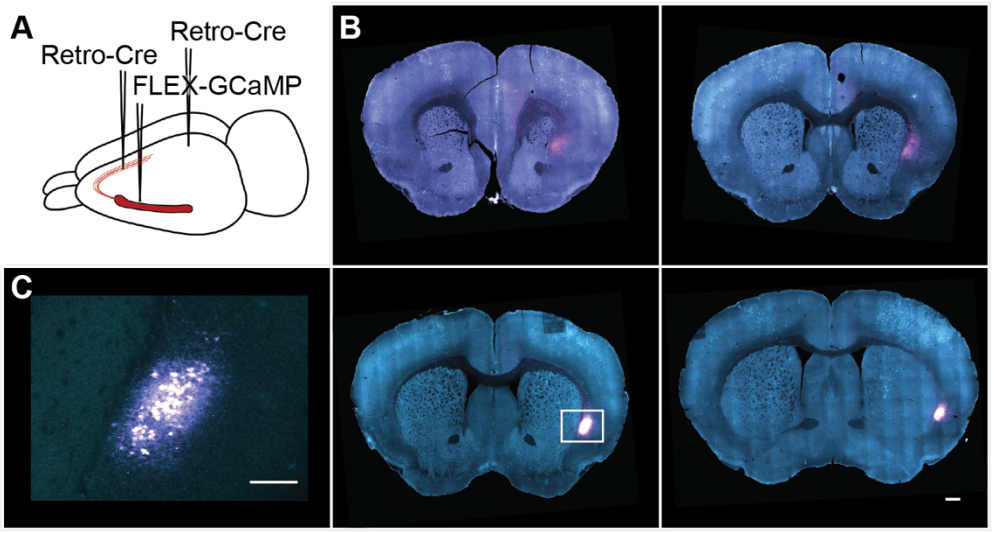


Figure 2-1. (A) Schematic showing the dual retrograde viral strategy for labelling the claustrum. Representative low-magnification (B; scale bar is 500um) and high-magnification (C; scale bar is 250um) sections showing claustrum labelling resulting from dual ACC RSP retro-Cre injections. Panel C is oriented with dorsal at the top of the frame and lateral on the right.

2.2.2 Visualizing Claustrum Axons

The inaccessible location and elongated shape of the claustrum make *in vivo* recordings of claustrum activity challenging. To circumvent some of these challenges, we chose to record the activity of claustrum axons in dorsal cortex as a proxy for the activity of cells within the claustrum. This strategy potentially allowed us to record from cells throughout the anterior-

posterior extent of the claustrum without causing significant damage to the overlying cortex. Briefly, we first unilaterally injected retro-Cre into the ACC and RSC and GCaMP7b into the claustrum before implanting a cranial window centred on bregma (Figure 2-2 A). After allowing >3 weeks for viral expression, we were then able to visualize GCaMP7b labelled axons throughout the hemisphere ipsilateral to the injection. Cranial windows remained stable over time allowing us to perform long term imaging (Figure 2-2).

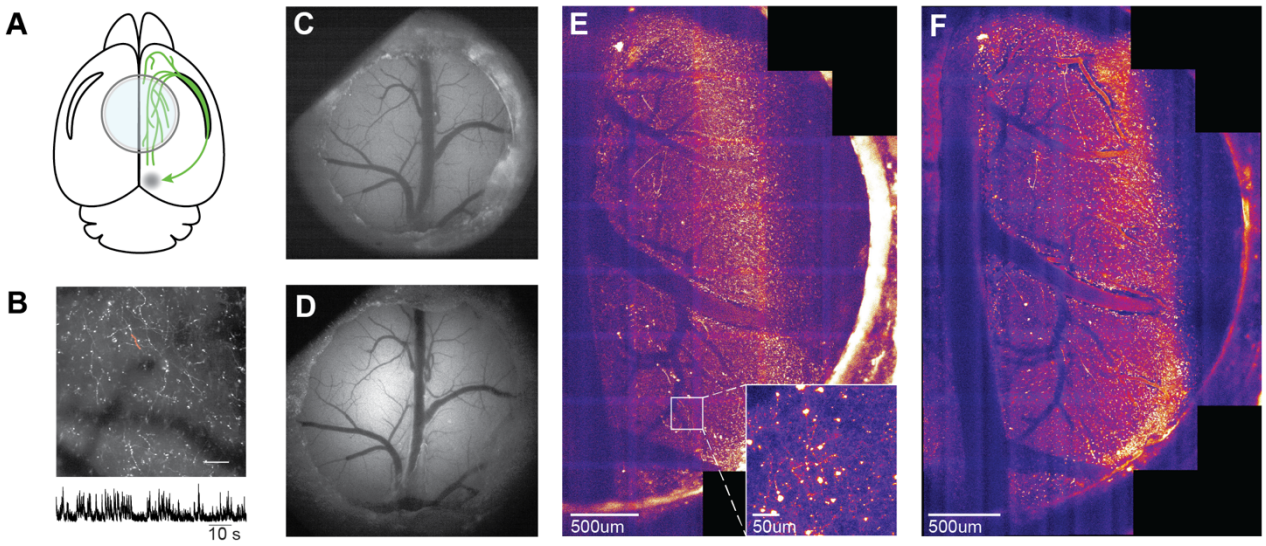


Figure 2-2. (A) Schematic showing the strategy for recording from claustrum axons in cortex. (B) Representative field of view containing GCaMP7b labelled axons with a calcium trace extracted from highlighted axon segment. Brightfield images showing the extent of the cranial window ~2 weeks (C) and ~ 10 weeks (D) after implantation. Two-photon images showing GCaMP7b labelled axons in a single plane of the cranial window ~2 weeks (E) and ~ 10 weeks (F) after implantation.

2.2.3 Claustrum Activity Following Unimodal Sensory Stimulation

To better understand the sensory response properties of the claustrum, we first presented mice with a random series of unimodal sensory stimuli while recording the activity of claustrum axons in dorsal cortex. Specifically, the sensory stimuli consisted of a flash of white light, a complex tone, and a paddle oscillating within the whiskers. In addition, we included a fourth trial type in which no stimuli were presented. Figure 2-3 A shows a schematic representation of the imaging set-up and trial structure.

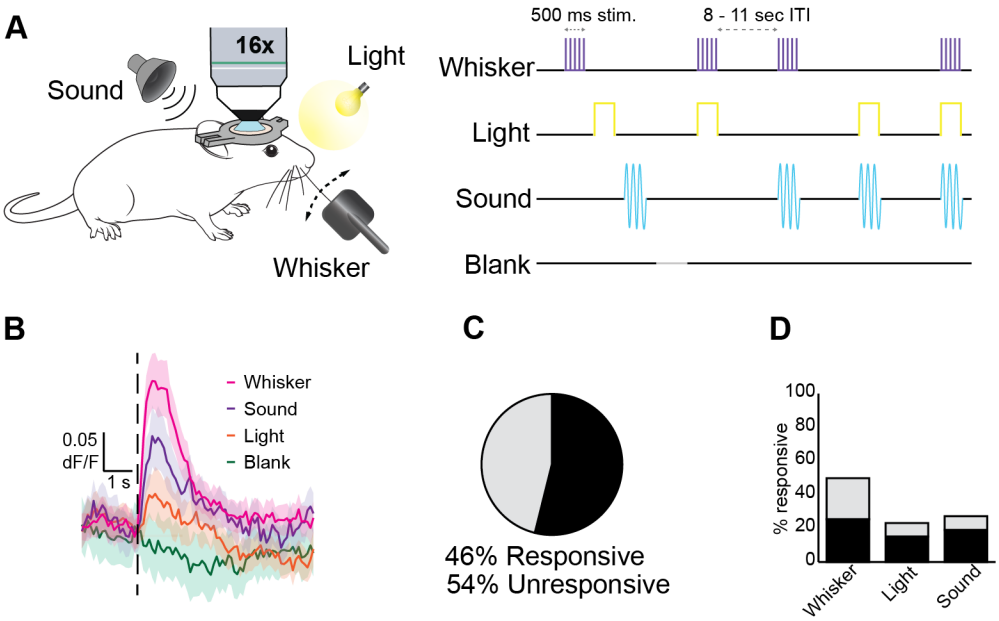


Figure 2-3. (A) Schematic representation of stimulation protocol including both uni- and multimodal trials. (B) Calcium traces showing the average activity of responsive claustrum axons from 2 seconds before until 6 seconds after stimulus onset (dashed line). Shaded outlines indicate standard error of the mean. Traces are derived from axons found to be responsive to that trial type, regardless of whether they were also found to be responsive to other trial types. (C) Percentage of all axons responsive to sensory stimuli. (D) Percentage of responsive axons that responded to each stimulus with light grey bars showing those which are uniquely responsive to that modality.

We observed significant stimulus-locked activity in claustrum axons to all modalities of sensory stimulation (n = 4 mice, 1342 axons, 117 recordings). Axons were classified as stimulus responsive by comparing their activity in the second before and after stimulus onset (Mann-Whitney Test). When averaged across all stimulus presentations, 46% of all axons were found to be responsive to one or more unimodal sensory stimuli. Of these, the greatest proportion were responsive to tactile stimuli, followed by auditory and visual stimuli. While some axons were exclusively responsive to one sensory modality, most axons were promiscuously responsive to multiple different types of unimodal sensory stimuli (Figure 2-3 D).

2.2.4 Claustrum Activity Following Uni- and Multimodal Sensory Stimulation

Based on these results, we chose to expand this experiment to include both unimodal and multimodal sensory stimuli. The sensory stimuli and stimulation protocol were similar to those described above, but the range of trial types was expanded to include auditory, visual, and tactile stimulation presented both alone and in combination. As in the unimodal only cohort, when averaged across all stimulus presentations we found stimulus-locked axonal activity following

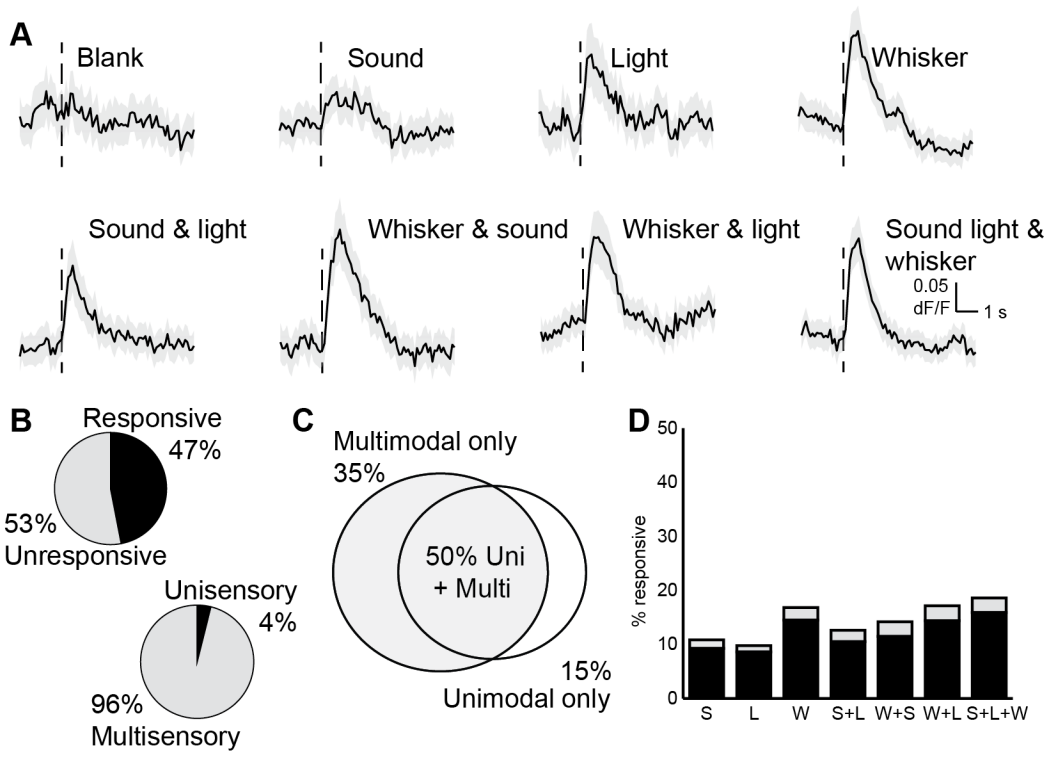


Figure 2-4. (A) Calcium traces showing the average activity of responsive claustrum axons from 2 seconds before until 6 seconds after stimulus onset (dashed line). Shaded outlines indicate standard error of the mean. Traces are derived from axons found to be responsive to that trial type, regardless of whether they were also found to be responsive to other trial types. (B) Percentage of all axons responsive to one or more trial types (top) and displaying a unisensory response pattern (bottom). (C) Percentage of responsive axons exclusively modulated by multimodal stimulus types (left), unimodal stimulus types (right) or modulated by both unimodal and multimodal stimulus types (centre). (D) Percentage of responsive axons that responded to each trial-type. Light grey bars show the proportion of axons uniquely responsive to that trial-type.

all unimodal and multimodal trial types (Figure 2-4; n = 4 mice, 1364 axons, 78 recordings). As before, axons were classified as responsive to a given trial type by comparing their activity in the second before and after stimulus onset (Mann-Whitney Test) and correcting for multiple comparisons (Benjamini-Hochberg). Overall, 47% of axons were found to be responsive to one or more unimodal or multimodal trial type (Figure 2-4 B top). Each axon was then classified as either uni- or multisensory based on the modalities present in the trial types to which they responded (Figure 2-4 B bottom). Only 4% of axons were found to display unisensory response patterns, while 96% displayed multisensory response patterns.

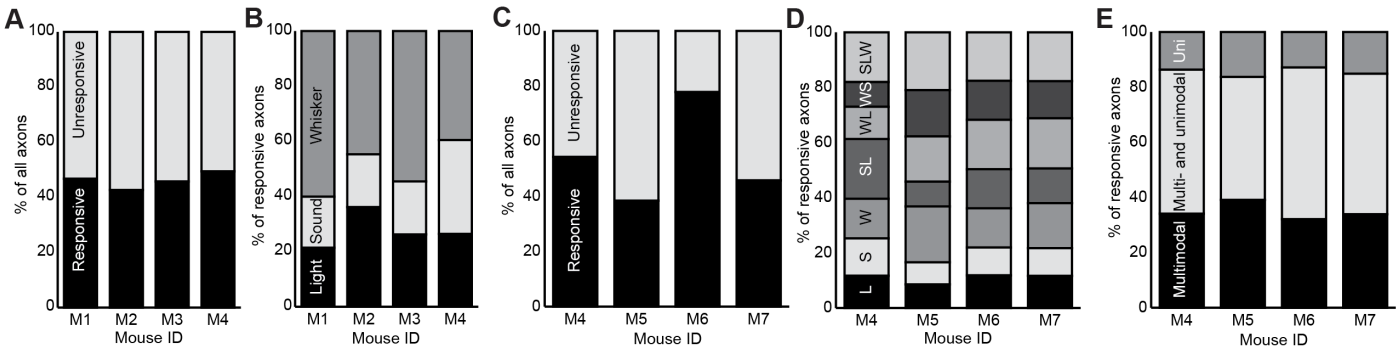


Figure 2-5. For mice in the unimodal cohort, graphs show the percentage of axons responsive to one or more trial types (A), and the percentage of axons responsive to each trial type (B). For mice in the multimodal cohort, graphs show the percentage of axons responsive to one or more trial types (C), and the percentage of axons responsive to each trial type (D). (E) Percentage of axons found to be exclusively responsive to unimodal trial types (top), multimodal trial types (bottom), or found to be responsive to both uni- and multimodal trial types (middle). Note, individual axons may be included multiple times in B and D for each trial type.

We then examined the trial types to which axons responded regardless of the modalities present in those trial types. While a small proportion of axons were found to be uniquely responsive to each unimodal or multimodal trial type, most axons were found to be promiscuously responsive to multiple uni- or multimodal trial types. Among stimulus responsive axons, 50% were found to be responsive to both unimodal and multimodal trial types. Interestingly, 35% of all stimulus-responsive axons were exclusively responsive to multimodal trial types while just 15% were exclusively responsive to unimodal trial types. These metrics are shown for individual mice in Figure 2-5.

2.2.5 Trial-wise responses

To understand the diversity of responses to individual stimulus presentations, we calculated the change in area under the $\Delta F/F$ curve (ΔAUC) before and after stimulus onset (Figure 2-6). Linear regression found no meaningful relationship between the probability of a stimulus-locked response and the imaging session (y = -0.001x + 0.26), suggesting that response probability did not change over time. However, the probability of stimulus-locked activity did vary across stimulus types (Kruskal Wallis; $p < 0.001$). Moreover, the size of the stimulus-locked ΔAUC was also found to vary across trial types (Kruskal Wallis; $p < 0.001$).

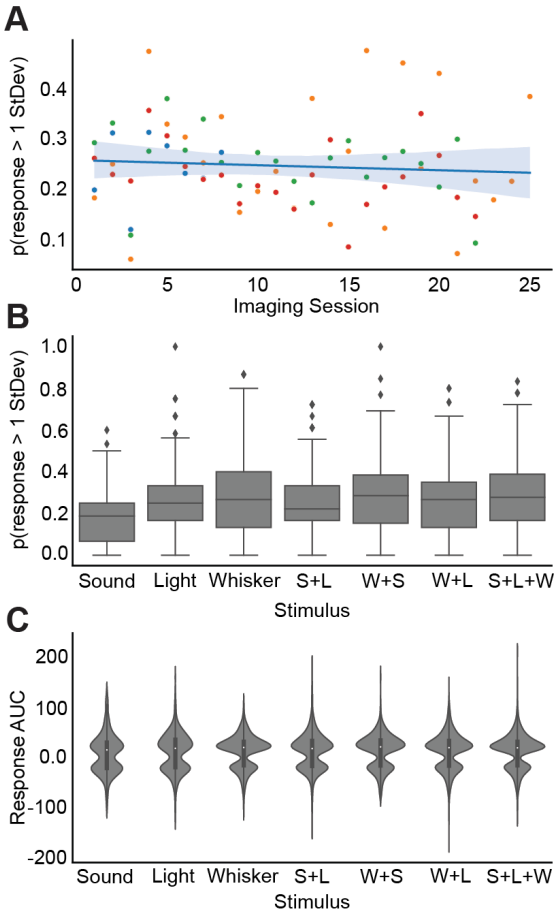


Figure 2-6. (A) Dots show the proportion of stimulus presentations evoking a change in AUC greater than 1 standard deviation in each imaging session for each mouse. Colors denote individual mice. Linear regression line plotted in blue with shaded 95% confidence intervals. (B) Boxes show the distribution of data points for each stimulus type. Data points show the percentage of stimulus presentations evoking a change in AUC greater than 1 standard deviation for each axon responsive to that stimulus type. (C) Violins show the distribution of data points for each stimulus type. Data points show the average change in AUC for each axon responsive to that stimulus type.

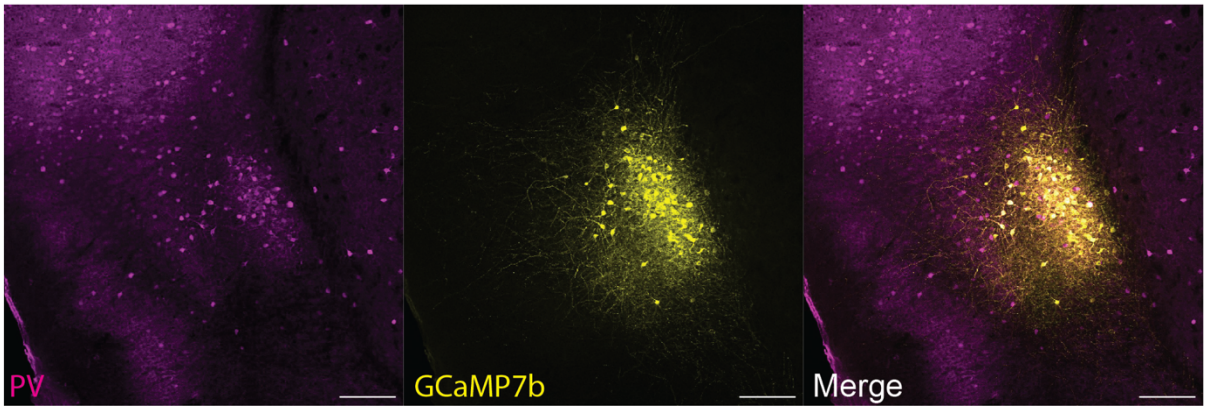


Figure 2-7. Immunofluorescent staining showing parvalbumin (PV, magenta, left), GCaMP7b (yellow, centre), and overlay (right). Scale bar is 200um. Panels oriented with dorsal at the top of the frame and lateral on the left.

2.2.6 Histology

Post-hoc histology was used to confirm unilateral claustrum-specific expression of GCaMP7b in all mice. Representative sections were stained for GFP, to visualize GCaMP7b, and PV to identify the location of the claustrum (Figure 2-7). Staining identified expression in the claustrum of all animals. However, two animals were found to have strong off-target GCaMP7b expression and were excluded from all analyses. Histology could not be recovered from one animal which was excluded from all analyses. Histology from all experimental animals is included in Supplementary Figure S2-1

2.3 Summary

In this chapter we aimed to better understand the sensory response properties of the claustrum by extending the results of Remedios et al. from the primate to the mouse. We first developed a novel technique for recording from the mouse claustrum by using two-photon calcium imaging to record the activity of claustrum axons in the dorsal cortex. Using this technique, we found that nearly half of all recorded axons displayed stimulus-locked activity following one or more modalities of sensory stimuli. In contrast to the dominant unisensory responses reported by Remedios et al., we found unisensory response patterns to be relatively rare[75]. Instead, we observed that most responsive axons displayed multisensory response patterns. However, trial-wise analysis of the data showed that stimulus responsive axons did not respond consistently across stimulus presentations.

These results suggest that claustrum neurons may respond inconsistently to multimodal sensory stimuli, but further evidence is required. While we observed stimulus-locked activity, we cannot conclude that this activity is conveying sensory information. The sensory stimulation may be salient, aversive, instructive, or provoking motor movement, any of which might lead to activity in the claustrum. Moreover, the non-linear relationship between neuronal activity and calcium-evoked fluorescence complicated any analysis of multisensory integration[156,157].

The results presented here represent a step towards understanding the activity of the claustrum. Future experiments including more complex sensory stimuli of differing intensities along with behavioural monitoring will help to conclusively determine the sensory response properties of the mouse claustrum.

2.4 Acknowledgements & Contributions

The experiments in this chapter benefited from contributions by Dr Huriye Atilgan (HA), Dr Robert Lees Fodder (RLF), Dr Andrew Shelton (AS), Dr Anna Hoerder-Suabedissen (AHS), Hamish Forrest (HF), and Niall Conway (NC). HA provided guidance for how to analyze the calcium imaging data. RLF provided guidance for how to build the sensory stimulation set-up and how to analyze the calcium imaging data. AS provided input on the design of some figures in this chapter and drew the schematics in figures 2-2 A and 2-3 A. AHS provided guidance for the histological experiments. HF designed the original light attenuating system that was adapted for use during two-photon imaging. As part of a Master's rotation and under close supervision, NC assisted with data collection and completed the manual ROI selection for the unimodal-only sensory stimulation data set.

3 Impact of claustrum silencing on ethologically motivated behaviours

The claustrum is reciprocally connected to nearly every cortical region. With this exceptionally widespread connectivity, the claustrum could theoretically play a role in a dizzying number of behavioural processes. Functional studies have linked the claustrum to such diverse processes as spatial navigation, attention, motor movement, anxiety, pain, and sleep. The breadth of possible functions underscores the challenge facing researchers trying to understand the function of the claustrum. With so many possible avenues to explore, it is difficult to know which is most likely to be informative. This issue is further compounded by disagreements about how to identify the claustrum, and whether to include it as part of a broader claustrum complex.

In this chapter we took a bottom-up approach to investigating the function of the claustrum. We used a simple manipulation, chronic neural silencing, combined with a novel transgenic line for labelling the claustrum complex. We then compared mice with active and sham claustrum complex silencing on a battery of ethologically motivated behavioural tests selected to cover a wide range of behavioural domains. These experiments will help us to understand the utility of a novel transgenic line and lay the foundation for more complex behavioural experiments to come.

3.1 Methods

24 male transgenic mice received intracranial injections of virus to silence claustrum complex output (TetTox) or PBS as a control (sham). The behavioural phenotype of claustrum silencing was then assessed on a battery of ethologically motivated behavioural tests before sacrifice and staining.

3.1.1 Animals

The 'claustrum complex — enhancer driven gene expression' (CLCX:EDGE) line was used to provide access to a subpopulation of claustrum complex neurons through genetically restricted expression of tetracycline transactivator (tTA) protein. The transgenic line was first described by Blankvoort et al. (2018) and generously provided by the Kentros Lab (Norwegian University of Science and Technology, NTNU).

Groups of 2–4 littermates were housed under standard conditions with wood chip bedding and ad libitum access to food and water. All cages included mice from both the sham and active claustrum silencing groups. All experiments were subject to ethical approval and conducted in accordance with local laws and regulations.

3.1.2 Surgery

Intracranial injections were performed in accordance with the method outlined in section 2.1.2. Silenced mice received injections of 500nl TreTight-TetTox virus (AAV2/1-TRE-TeTxLC, NTNU Viral Vector Core) in claustrum while sham animals received equivalent volumes of sterile PBS. The custom virus was prepared based on a FLEX-TetTox virus (ssAAV-DJ/2-hEF1a-dlox-FLAG_TeTxLC(rev)-dlox-WPRE-hGHp(A), ETH Zurich v63) but adapted for use in the CLCX:EDGE transgenic line. The TetTox construct included a FLAG tag to allow immunohistochemical identification.

3.1.3 Behavioural Testing

Figure 3-1 provides a schematic outline of the experimental structure. Briefly, animals aged p32–p55, with all but three animals aged p50–55, received intracranial injections of either PBS (sham; n = 12) or TreTight tetanus toxin light chain virus (TetTox; n = 12). Animals were then given four weeks for recovery and viral expression. Next, behavioural phenotype was assessed on a battery of ethologically motivated behavioural tests. Finally, mice were sacrificed, and brains were extracted for post-hoc histology.

Tests were selected to cover a wide range of behavioural domains without the need for extensive behavioural training or conditioning. Details of each test are described below in the order in which they were performed. Test order was selected to minimize the effects of previous testing and all tests were separated by a minimum of 3 days. Mice were given >30 minutes to acclimatize to the room prior to each test. Mice who had already been tested were not returned to their home-cages until all cage-mates had been tested. Mice were not able to see the experimenter during testing. All apparatuses were cleaned thoroughly with Anistel between animals. The experimenter was blind to treatment groups during all testing.

Locomotor Activity

Basic locomotor activity was assessed using beam-break boxes (San Diego Instruments). Mice were placed individually into 48 x 26cm cages with woodchip bedding and allowed to explore freely for 60 minutes. Cages were surrounded by an 8 x 4 array of infrared beam-break detectors to record x y motion. A second tier with 8 beam-break detectors was used to record rearing behaviour.

Rotarod

Motor coordination was assessed using a rotarod test (Med Associates). Mice were placed on to a rotating cylinder above a pressure plate. Mice from each cage were tested at the same time but were physically and visually separated by plastic dividers. The cylinder accelerated from 5 to 50 rotations per minute over the course of five minutes. Tests were deemed to have ended when a mouse fell onto the pressure plate or completed two passive rotations around the cylinder. Each mouse was tested four times.

Three-Chamber Social Interaction

Animals' innate preference for social interaction and social novelty was tested using a three-chamber social interaction time test. Testing occurred in a 63 x 30cm arena divided into three evenly sized chambers. The test involved three trials in the apparatus on the same day. Mice were able to move freely between chambers and were always placed into the central chamber at the start of each trial. First, in the habituation trial, mice were allowed to explore freely for five minutes. During this trial, each side chamber contained an empty wire cage. Next, in the sociability and social novelty trials, mice were allowed to explore freely for 10 minutes. In the sociability trial, one wire cage contained a novel mouse while the other contained a novel object (a small metal ring on a stand). In the social novelty trial, the novel object was replaced with a second novel mouse.

Y-Maze

A y-maze test was used to assess novelty seeking and short-term spatial memory. The test apparatus consisted of three 29 x 9cm arms joined together at 120° angles from one another. The first stage of the test involved five habituation trials during which the mouse was able to explore two of the arms (the Start and Other arm) with the third arm blocked off (the Novel

arm). At the start of each trial mice were placed at the end of the Start arm and allowed to explore the Start and Other arms for two minutes before being removed from the apparatus for a 1-minute inter-trial interval. In the second stage of the test, the mouse was allowed to explore all three arms for five minutes. Visual cues outside of the maze were kept consistent between trials.

Puzzle Box

The puzzle box test was used to assess general cognitive function. The test apparatus consisted of an open 58 x 28 cm start chamber connected to a covered 15 x 28cm goal chamber by an interchangeable door or underpass. The start chamber was brightly lit, while the goal chamber was dark and contained bedding and a cardboard tube. Mice performed 11 trials over 4 days. They were naturally motivated to move from the start chamber to the goal chamber but had to overcome obstacles of varying complexity (Table 2-1). In the first trial on the first day, both the door and underpass were left open. In the second and third trials on the first day, the door was removed but the underpass was left open. In the first trial on the second day, the underpass was left open. In the second and third trials on the second day, the underpass was filled with woodchip bedding. In the first trial on the third day, the underpass was filled with woodchip bedding. In the second and third trials on the third day, the underpass was blocked by a T-shaped cardboard barrier. In the first trial on the fourth day, the underpass was blocked by a T-shaped cardboard barrier. Finally, in the second trial on the fourth day, the entry to the goal box was left unobstructed to assess motivation.

Table 2-1. Obstacle to entering the goal box

	Day 1	Day 2	Day 3	Day 4
Trial 1	Open door	Underpass	Woodchip bedding	Cardboard barrier
Trial 2	Underpass	Woodchip bedding	Cardboard barrier	Open door
Trial 3	Underpass	Woodchip bedding	Cardboard barrier	

Elevated Plus Maze

Anxiety-like behaviour was assessed using an elevated plus maze. The apparatus consisted of four 34 x 7cm arms joined together at a central square. Two opposing arms were enclosed by

20cm walls while the other arms were left exposed. The apparatus was raised 50cm above the ground. Mice were placed at the end of one of the enclosed arms and allowed to explore freely for five minutes.

Prepulse Inhibition (PPI)

Sensorimotor gating was assessed with a PPI test (SR-Lab Startle Response System, San Diego Instruments). Mice were placed individually into dimly illuminated sound attenuating chambers. Inside the chambers, mice were confined to a 13cm long tube with a 4cm internal diameter. The mouse enclosure was attached to an accelerometer allowing the system to record the mouse's startle response. The accelerometers and speakers were calibrated before testing to ensure comparable testing among mice.

Mice were presented with a sequence of trials drawn from three trial types: startle alone (S); prepulse alone (P); or prepulse and startle trials (PPI). S trials included a 50ms stimulus at 120dB SPL. P trials included a 20ms stimulus at one of five intensities ranging from 65–81dB SPL. PPI trials consisted of a P trial 50–100ms before an S trial. Testing began with a 5-minute acclimation period during which five S trials were presented to acclimate mice to the startle stimulus. Next, the testing protocol included 10 P and PPI trials for each sound intensity along with 10 S trials. Finally, five additional S trials were presented. The PPI ratio was calculated as $PPI_{ratio} = 1 - \frac{m_p}{m_b}$ where m_b represents the baseline response to the startle pulse alone and m_p represents the attenuated response to the prepulse and startle together.

3.1.4 Histology

Histological validation of claustrum-specific viral expression was performed according to the general antibody staining protocol in section 2.1.6. To cover the full extent of the claustrum, 6 coronal sections spaced 500um apart were selected such that the second section was located at the anterior union of the corpus callosum. Slices were then stained with antibodies against MBP (Abcam; 1:500; RRID: AB_305869) to facilitate identification of the claustrum and FLAG (Cell Signalling Technology; 1:500; RRID: AB_2572291) to clearly label all TetTox positive cells. All secondary antibodies were used at a concentration of 1:500. Imaging was performed using a Zeiss Axioscan Z1 automated slide scanner with a 10x objective and a Leica epifluoresence

microscope at 4x magnification. Lines to delineate the approximate boundaries of the claustrum complex were drawn based on MBP expression.

3.1.5 Data Analysis & Statistics

Video recordings of the social interaction, y-maze, puzzle box, and elevated plus maze tests were analyzed by a blinded observer using Anymaze 7.3 (Stoelting) to extract the mouse's location and movement within the behavioural apparatus. The mouse was deemed to be inside a given region when 85% of its body crossed into the zone. For the puzzle box, time to enter the goal box was measured manually. One mouse was excluded from each of the PPI and three-chamber social interaction tests due to hardware errors. Data was analyzed and plotted using Excel (Microsoft) and Prism 9 (GraphPad). Plots were formatted using Adobe Illustrator.

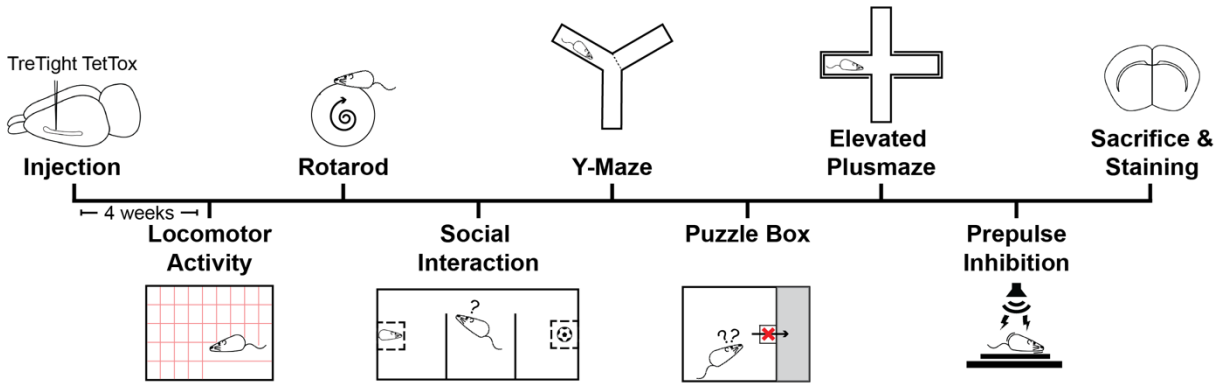


Figure 3-1. Experimental Protocol. Schematic representation showing the timeline of experiments.

3.2 Results

A cohort of mice with sham and active claustrum complex silencing were tested on a battery of seven ethologically motivated behavioural tests (Figure 3-1). Testing did not reveal any significant behavioural differences between the sham and active silencing groups. Both groups exhibited the expected behaviour on all tests. Results are displayed in the order in which testing occurred.

3.2.1 Locomotor Activity

Mice were placed individually into fresh cages surrounded by infrared motion detectors and allowed to explore for one hour to assess their baseline locomotor activity. Unpaired two-sample Welch's t-tests detected no significant differences in locomotion (Figure 3-2 A; $t(21) = 0.13$, $p = 0.89$) or rearing behaviour (Figure 3-2 B; $t(21) = 0.92$, $p = 0.37$). These results do not provide evidence for a difference in baseline locomotor activity between sham and TetTox animals.

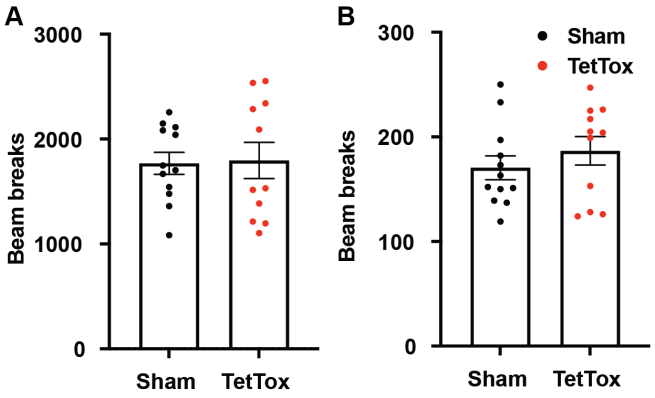


Figure 3-2. Locomotor activity. Graphs showing the number of x-y beam breaks recorded (A) and the number of rearing beam breaks (B) recorded in the locomotor activity test. Data from sham mice (n=12) are plotted in black and TetTox mice (n=11) are plotted in red. Error bars show the standard error of the mean.

3.2.2 Rotarod

Motor coordination was assessed over four trials on a rotarod test (Figure 3-3). Mice were placed onto a gradually accelerating rotating cylinder. A trial was deemed to have ended if a mouse fell from the cylinder or completed two passive rotations. A two-way ANOVA detected a significant effect of trial ($F(2.04, 42.84) = 4.33$, $p = 0.02$), but no significant interaction or effect of group on the latency to fall/end of trial. These results suggest performance may have changed with experience of the test for both groups, but do not provide evidence of an effect of claustrum complex silencing on motor coordination.

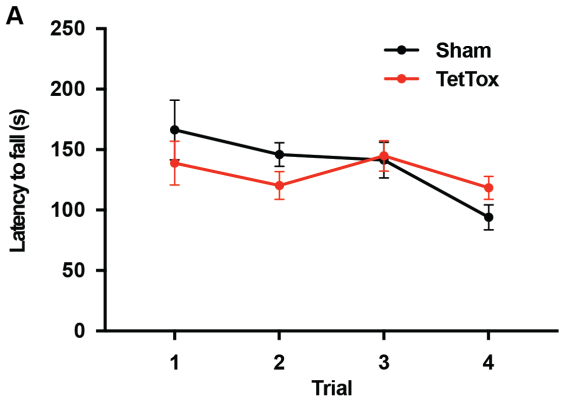


Figure 3-3. Rotarod. Graph showing the time to fall (s) for four repetitions of the rotarod test. Data from sham mice (n=12) are plotted in black and TetTox mice (n=11) are plotted in red. Error bars show the standard error of the mean.

3.2.3 Three-Chamber Social Interaction

Both group's innate preferences for social interaction and social novelty were assessed using the three-chamber social interaction time test. The test included two stages preceded by a habituation trial. In the first stage, mice could choose to explore either a novel mouse or a novel object. In the second stage, mice could choose to explore a second novel mouse or the now familiar mouse from the previous stage.

In the first stage of the test, a two-way ANOVA detected a significant effect of chamber, suggesting mice spent significantly more time exploring the novel mouse compared to the novel object (Figure 3-4 A; $F(1, 21) = 109.2$, $p<0.0001$). In the second stage, a two-way ANOVA again detected the expected effect of chamber, suggesting that mice spent more time

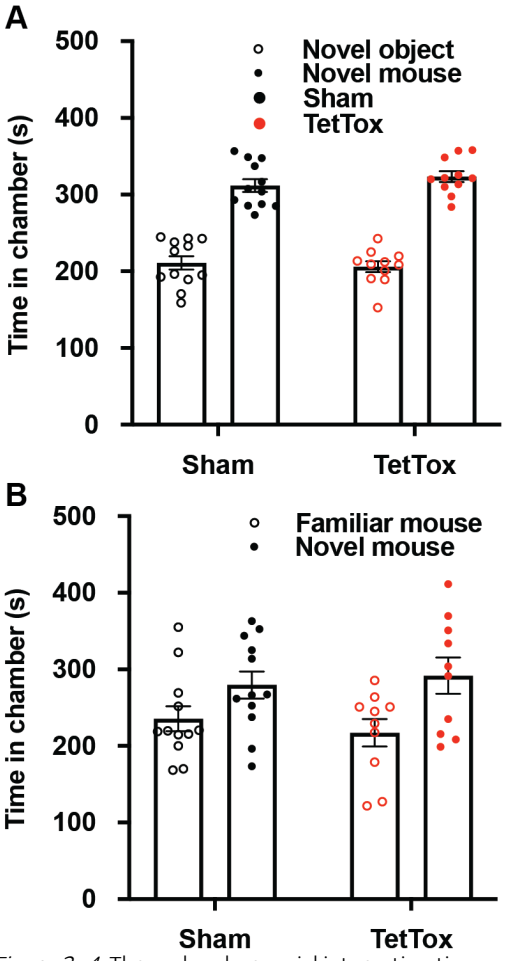


Figure 3-4. Three chamber social interaction time test. Graphs showing the time spent in the chamber with the novel mouse in the sociability (A) and social novelty (B) phases of the test. Data from sham mice (n=12) are plotted in black and TetTox mice (n=10) are plotted in red. Error bars show the standard error of the mean.

exploring the novel mouse compared to the familiar mouse (Figure 3-4 B; F (1, 20) = 5.370, p = 0.03). No significant interaction or effect of treatment was detected in either stage of the test. These results do not provide evidence for an effect of claustrum complex silencing on social behaviour.

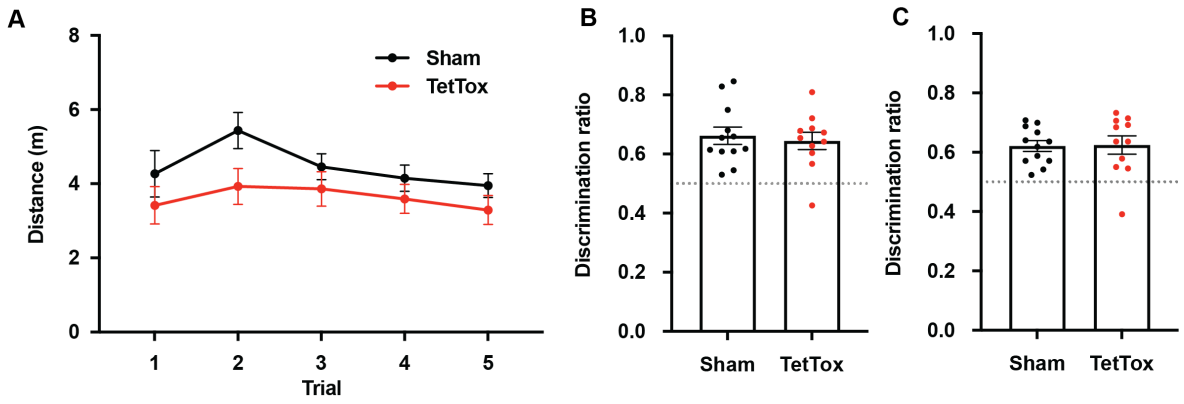


Figure 3-5. Y-Maze. Graphs showing the average distance travelled during the five habituation trials of the Y-maze test (A), and the discrimination ratio for time spent (B) and entries into (C) the novel and other arms. Data from sham mice (n=12) are plotted in black and TetTox mice (n=11) are plotted in red. Error bars show the standard error of the mean.

3.2.4 Y-Maze

In the Y-maze test, mice were first given five trials to habituate to two arms of a Y-shaped maze before a probe trial in which all three arms were accessible. A two-way ANOVA detected a significant effect of trial on distance travelled in the habituation trials but no interaction or effect of group (Figure 3-5 A; F (2.42, 50.91) = 3.55, p = 0.03). Figures 3-5 B and C respectively show the ratio of time spent or entries into the novel arm compared to time spent or entries into both the novel and other arm together during the probe trial. One-sample t-tests showed that both sham (t (11) = 5.6, p < 0.001) and TetTox (t (10) = 4.98, p < 0.001) mice spent significantly more time in the novel arm than would be expected by chance. Similarly, a one-sample t-test showed that both sham (t (11) = 6.78, p < 0.001) and TetTox (t (10) = 4.06, p = 0.002) mice had significantly more entries into the novel arm than would be expected by chance. Finally, two-sample t-tests failed to detect any differences between groups. These

results suggest that both groups displayed the expected preference for exploring a novel environment and provide no evidence of an effect of claustrum complex silencing.

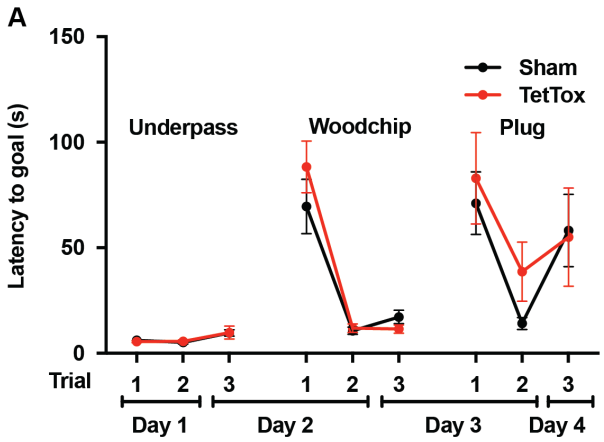


Figure 3-6. Puzzle box. Graphs showing the latency to the goal box on each trial of the puzzle box test (A), and the latency to the goal box on unobstructed trials before and after the test (B). Data from sham mice (n=12) are plotted in black and TetTox mice (n=11) are plotted in red. Error bars show the standard error of the mean.

3.2.5 Puzzle Box

In the puzzle box test, mice overcame obstacles of increasing complexity to move from an aversive chamber into a goal chamber. The test occurred over 4 days, with each obstacle presented three times over two days. Each obstacle was presented twice on its first day to assess learning and short-term memory, and then once on the next day to assess long term memory (Figure 3-6 A).

On the underpass trials, a two-way ANOVA detected a significant effect of trial ($F(1.15, 24.19) = 5.18$, $p = 0.03$), but no interaction or effect of group. The same pattern of results was found on both woodchip trials ($F(1.1, 23.17) = 52.16$, $p < 0.001$) and plug trials ($F(1.75, 36.66) = 7.35$, $p = 0.003$). These results suggest that both groups' performance changed following experience of the task.

To assess motivation, the test trials were bookended by two trials where the entry to the goal chamber was unobstructed (Figure 3-6 B). A two-way ANOVA detected a significant effect of trial ($F(1, 21) = 12.48$, $p = 0.002$), but no significant interaction or effect of group. Importantly, the unobstructed latency to enter the goal box did not increase over the course of testing. This suggests that mice remained motivated to reach the goal chamber at the end of the test. Supplementary Figure 3-1 includes survival plots for each test trial showing the percentage of animals who had yet to reach the goal box at each latency. Collectively, these results do not provide evidence for an effect of claustrum complex silencing on general cognitive performance.



Figure 3-7. Anxiety-like behaviour. Graph showing the time spent in the open (open circles) and closed arms (filled circles) of the elevated plus maze (A). Data from sham mice (n=12) are plotted in black and TetTox mice (n=11) are plotted in red. Error bars show the standard error of the mean.

3.2.6 Elevated Plus Maze

In the elevated plus maze, the approach-avoidance conflict between the animals' innate desire to explore and their aversion to exposed areas was used to assess anxiety levels. A two-way ANOVA detected a significant effect of arm (Figure 3-7; $F(1, 21) = 203.8$, $p < 0.001$), but failed to detect any interaction or effect of group on time spent in the open and closed arms. These results do not provide evidence of a difference in anxiety-like behaviour between groups.

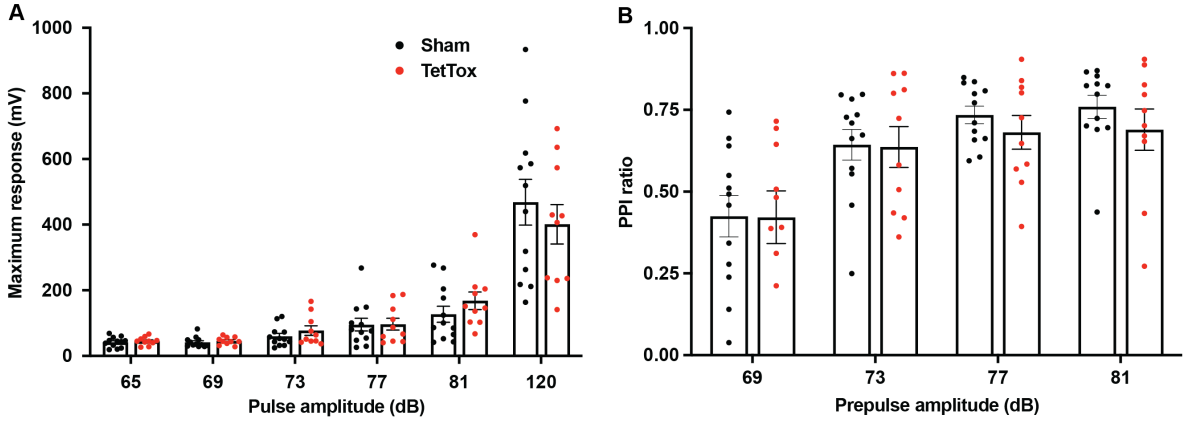


Figure 3-8. Prepulse inhibition. Graphs showing the maximum startle response (A) and PPI ratio (B) recorded on the prepulse inhibition test. Data from sham mice (n=12) are plotted in black and TetTox mice (n=10) are plotted in red. Error bars show the standard error of the mean.

3.2.7 Prepulse Inhibition

The PPI test measured differences in startle responses to quiet and loud sounds presented alone or together. A two-way ANOVA detected an expected effect of pulse amplitude on startle response ($F(1.41, 28.16) = 51.73$, $p < 0.001$), but failed to detect any interaction or effect of group (Figure 3-8 A). This suggests that both groups have similar startle responses to both the prepulse and startle sounds.

The PPI ratio shown in Figure 3-8 B represents the change in response following a 120dB SPL startle pulse if that pulse is preceded by a quieter prepulse — values above 0 indicate a suppression of the startle response by the prepulse. A two-way ANOVA detected a significant effect of prepulse amplitude ($F(3, 60) = 31.11$, $p < 0.001$) but failed to detect a significant interaction or effect of group on the PPI ratio. One sample t-tests were used to assess whether the PPI ratio was different from 0 — the expected value if the prepulse had no effect on the response to the startle pulse. All prepulses elicited a significant reduction in startle response for both groups (sham 69dB SPL: $t(11) = 6.72$, $p < 0.001$; TetTox 69dB SPL: $t(9) = 5.24$, $p < 0.001$; sham 73dB SPL: $t(11) = 13.72$, $p < 0.001$; TetTox 73dB SPL: $t(9) = 10.22$, $p < 0.001$; sham 77dB SPL: $t(11) = 24.46$, $p < 0.001$; TetTox 77dB SPL: $t(9) = 13.21$, $p < 0.001$; sham 81dB SPL: $t(11) = 21.52$, $p < 0.001$; TetTox 81dB SPL: $t(9) = 10.90$, $p < 0.001$). This suggests that both groups displayed a reduction in startle response when the startle tone is preceded by a quieter tone. Collectively, these results do not provide evidence of a difference between groups in the PPI response.

3.2.8 Histology

Post-hoc histology was used to confirm bilateral claustrum complex specific expression of TetTox in all animals with active silencing. Representative sections from all TetTox mice were stained for FLAG to visualize TetTox expression and MBP to delineate the claustrum complex (Figure 3-9). The staining showed bilateral TetTox expression in the claustrum complex of all but one animal. The animal with unilateral expression was excluded from all analyses. Brain sections from two sham animals were also stained and revealed no TetTox expression. Expression varied between mice in both the distribution of TetTox within the claustrum complex and the dorsal-ventral extent of TetTox expression. Within the claustrum complex, TetTox expression was largely confined to the medial DEn and the ventral claustrum. Histology from all experimental animals can be found in Supplemental Figure S3-2.

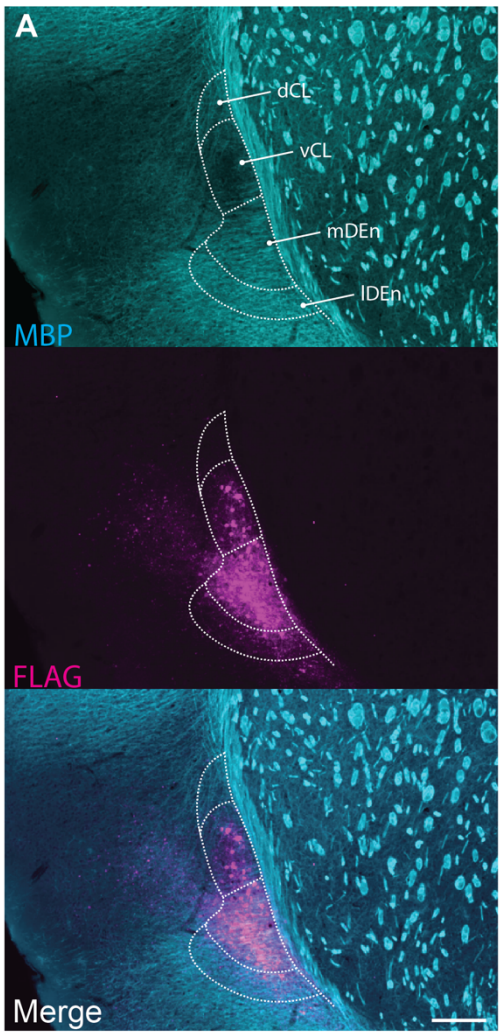


Figure 3-9. Histology. Immunofluorescent staining of TetTox injected brain tissue from CLCX:EDGE mice (A) showing myelin basic protein (MBP, cyan, top), FLAG (magenta, centre), and overlay (bottom). Scale bar is 200um. Panels oriented with dorsal at the top of the frame and lateral on the left. Abbreviations: dorsal claustrum (dCL), ventral claustrum (vCL), medial DEn (mDEn), lateral DEn (lDEn)

3.3 Summary

This chapter began with two aims: first, to characterize the effects of chronic claustrum complex silencing on basic behaviours, and second, to assess the usefulness of a novel claustrum complex-specific transgenic mouse line. A cohort of mice received bilateral intracranial injections for active (TetTox) or sham (PBS) claustrum complex silencing. After allowing for viral expression, the mice were assessed on a battery of common ethologically motivated behavioural tests. Across all tests, no significant differences were identified between mice receiving active or sham silencing. Moreover, post-hoc histology revealed that TetTox expression was predominantly confined to the medial DEn with limited expression in the ventral claustrum. This expression pattern suggests that this transgenic line is of limited use for studying the claustrum in isolation. While these results do not provide evidence for any specific hypothesis for the function of the claustrum, they provide a strong foundation for future experiments.

3.4 Acknowledgements & Contributions

The experiments in this chapter benefited from contributions by Prof David Bannerman (DB), Dr Andrew Shelton (AS), and Theodore Heath-Coleman (THC). DB provided guidance in designing the experiments. AS performed electrophysiological experiments to validate the efficacy of the TetTox virus (described in section 6.4). As part of an undergraduate research project and under close supervision, THC analyzed the data from the behavioural tests and assisted with performing immunohistochemical stains. Imaging of post-hoc histology was completed with guidance from Dr Cyril Lai at the Wolfson Imaging Centre in the MRC Weatherall Institute of Molecular Medicine. Except for the puzzle box, equipment used for behavioural testing was provided by the Behavioural Neurosciences Unit within the Biomedical Services Department with technical support provided by Dr Katie Hewitt. The CLCX:EDGE line was provided by Prof Clifford Kentros at the Norwegian University of Science and Technology.

4 Impact of claustrum silencing on circadian and locomotor activity

Several recent studies have raised the prospect that the claustrum may be involved in sleep. Studies in reptiles, humans, and mice have linked the claustrum with various sleep-related phenomena, but researchers have only recently begun to examine this possibility[6,19,128].

In this chapter we extend the results of the previous chapter into new behavioural domains. Here, we used a recently developed system for 24/7 home-cage activity tracking to measure the impact of claustrum and claustrum complex silencing. We compared the activity and circadian behaviour of three groups of mice: wild type mice with sham silencing, wild type mice with claustrum silencing, and transgenic mice with claustrum complex silencing. To accurately track the behaviour of individual mice, all mice were singly housed for the duration of the study. Chronic social isolation has previously been shown to induce anxiety-like behaviour[158,159]. Given the emerging link between the claustrum and anxiety, we assessed all groups for anxiety-like behaviour using a battery of ethologically motivated anxiety tests[126,127].

4.1 Methods

18 male mice received intracranial injections of virus to silence claustrum/claustrum complex output or PBS for sham silencing. Mice were housed in individual activity-tracking cages which allowed assessment of circadian and locomotor behaviour before sacrifice and staining.

4.1.1 Animals

Both wildtype C57Bl/6 and CLCX:EDGE transgenic mice were used in this experiment. For more information on the CLCX:EDGE transgenic, see section 3.1.1. Animals were single housed throughout the experiment with wood chip bedding and ad libitum access to food and water. All experiments were subject to ethical approval and conducted in accordance with local laws and regulations.

4.1.2 Surgery

Intracranial injections were performed in accordance with the method outlined in section 2.1.2. Silenced wild type mice received bilateral injections of 80nl retro-Cre virus (ssAAV-retro/2-

hSyn1-mCherry_iCre-WPRE-hGHp(A), ETH Zurich v230) in RSP and 500nl FLEX-TetTox virus (ssAAV-DJ/2-hEF1a-dlox-FLAG_TeTxLC(rev)-dlox-WPRE-hGHp(A), ETH Zurich v63) in claustrum. Sham wild type mice received equivalent volumes of sterile PBS. Transgenic silenced mice received bilateral injections of 500nl TreTight-TetTox virus (AAV2/1-TRE-TeTxLC, NTNU Viral Vector Core) in claustrum. The TetTox construct included a FLAG tag to allow immunohistochemical identification.

4.1.3 Behavioural Testing

Three groups of mice were included in this study: a wildtype claustrum silencing group (n = 6), a wildtype sham silencing group (n = 6), and a transgenic claustrum complex silencing group (n = 6). Animals aged p41–p53 were single housed in activity tracking cages. After a two-week acclimation period, animals were returned to standard cages for 5–8 days to receive intracranial injections. Mice were then returned to the activity tracking cages for further six weeks of monitoring. Finally, mice were tested on a series of ethologically motivated tests for anxiety-like behaviour (elevated plus maze, aversive open field, and light-dark box).

Activity Tracking Cages

During the study, mice were housed in Digital Ventilated Cages (DVC; Techniplast). DVCs resembled standard individually ventilated cages, but cage racks included capacitive sensors which permitted continuous, passive, and non-disruptive behavioural monitoring. The sensor consisted of 12 capacitive plates arrayed underneath each cage. Readouts from each of the 12 sensors were recorded every 250ms and enabled tracking of both general locomotor activity as well as distance travelled. Further details about the DVC system and data analysis can be found in section 4.1.5 below as well as in Pernold et al. (2019) and Iannello (2019)[160,161].

Elevated Plus Maze

The elevated plus maze test was conducted according to the protocol described in section 3.1.3.

Aversive Open Field

The apparatus consisted of a brightly illuminated 60cm diameter circular arena. Mice were placed into the arena and allowed to explore for 10 minutes. The apparatus was divided into

three zones: centre, intermediate, and outer. The centre zone was defined as a circle in the middle of the arena with a 5cm radius. The outer zone was defined as a 5cm band running around the extreme outside of the arena. Finally, the intermediate zone consisted of the area between the outer and centre zones. The mouse's location within the arena was tracked throughout the test to measure its preference for the centre, intermediate, and outer zones.

Light Dark Box

The apparatus consisted of a 27 x 27cm box split into two chambers connected through a small doorway. One chamber was open and brightly lit while the other was covered and dark. Mice were placed initially into the dark box and allowed to explore for 5 minutes. The time spent in the light box was recorded to determine animals' preference for the light or dark areas.

4.1.4 Histology

Histological validation of claustrum-specific viral expression was performed according to the general antibody staining protocol in section 2.1.6. To cover the full extent of the claustrum, 6 coronal sections spaced 500um apart were selected such that the second section was located at the anterior union of the corpus callosum. Slices were then stained with antibodies against MBP (Abcam; 1:500; RRID: AB_305869) to facilitate identification of the claustrum and FLAG (Cell Signalling Technology; 1:500; AB_2572291) to label all TetTox positive cells. All secondary antibodies were used at a concentration of 1:500. Imaging was performed using a Zeiss Axioscan Z1 automated slide scanner with a 10x objective and a Leica epifluoresence microscope at 4x magnification. Lines to delineate the approximate boundaries of the claustrum complex were drawn based on MBP expression.

4.1.5 Data Analysis & Statistics

Data was then analyzed using Python 3.9.13 and MATLAB 2022b using built-in and open-source packages. Plots were produced using Python, MATLAB, and Prism 9 (GraphPad) and plots were formatted using Adobe Illustrator. Statistical tests were performed using Python and Prism 9.

The home-cage monitoring study was divided into baseline and expression periods. To allow mice to acclimatize to their new environment, data for the baseline period was collected

starting one week after mice were placed in the activity monitoring cages until they were removed for surgery. To balance the baseline period and allow adequate time for viral expression, data for the expression period was collected for one week starting three weeks after the last mice in each group received their intracranial injections.

Home-Cage Locomotor Activity Tracking

Activity tracking data from the DVC system was preprocessed using the DVC Analytics platform (Techniplast) to extract locomotor activity data. Briefly, readouts from the capacitive plates are affected by the local electromagnetic environment. The presence of water-rich bodies, such as a mouse, near the sensor alters its readout. Alterations to the capacitive readings across the 12 sensors can therefore be used to identify the location of a mouse within the cage. By tracking the change in the location of the mouse across readings, the system can track the distance travelled by the mouse within a given time window.

While the distance travelled metric reveals an important aspect of home-cage behaviour, it is insufficient to distinguish between a truly immobile mouse (ex. during sleep) and a mouse which is stationary but active (ex. grooming, nest building). To provide a more detailed picture of activity, the DVC platform can also calculate an activity index. Activity is detected by computing the change in electrode capacitance between adjacent readings. An electrode is considered activated if the difference between the readings is greater than a threshold chosen by the software to separate activity from noise. This measurement is therefore insensitive to long term changes in capacitance (ex. due to the moisture level of the bedding) while providing a short-term readout of local activity. The density of activation across the electrode array over time can then be used as a general indicator of the animals' activity level. The so-called Animal Activity Index calculated by the DVC analytics platform is expressed in arbitrary units. Both the Animal Activity Index and distance travelled have been validated against video tracking[161].

Circadian Parameters

Python, the pyActigraphy package, and the DVC analytics platform were used to compute circadian metrics from the DVC data[162]. While the pyActigraphy toolbox was written to analyze data collected using devices such as smart watches, it was adapted here to import data produced by the DVC analytics platform. The pyActigraphy toolbox was then used to analyze

animals' mean inter-daily stability (ISm) and mean intra-daily variability (IVm). Inter-daily stability measures the similarity of activity patterns across days. A value of 1 would indicate identical activity patterns across days while a value of 0 would indicate no relationship between daily patterns. Intra-daily variability measures the consolidation of periods of rest and activity. Animals with fragmented activity patterns exhibiting frequent transitions from rest to activity would have high intra-daily variability, where animals with consolidated bouts of activity and inactivity would have low intra-daily variability. Classically, both metrics have been calculated using a one-hour interval for analysis. However, more contemporary actigraphy systems such as the DVCs sample activity at a much higher frequency. The ISm and IVm metrics take advantage of this increased temporal resolution by averaging together values calculated using a range of time intervals, thereby providing a more sensitive readout of circadian rhythmicity. Further details on these metrics can be found in [162–164].

The DVC data was further analyzed using custom python code to calculate the relative amplitude (RA) of animals' daily activity. RA provides a readout of the difference in activity level between active and inactive periods of the circadian day. Disrupted circadian patterns and less consolidated activity patterns can result in reduced RA. RA was calculated using the following formula $RA = \frac{AI_D - AI_L}{AI_D + AI_L}$ where AI_D refers to animals' mean activity index during their dark cycle — when activity is highest — and AI_L refers to their mean activity in the light cycle — when activity is lowest. This metric was calculated using the Animal Activity Index.

Finally, the DVC analytics platform was used to calculate the regularity disruption index (RDI) for all mice at baseline and after claustrum silencing. RDI is a novel biomarker based on sample entropy. It was developed for the DVC system to quantify irregularities in activity patterns. A highly regular pattern of activity would yield a RDI near zero, while highly disrupted activity would yield a high RDI. Notably, RDI values reflect the regularity of activity regardless of its magnitude. RDI was calculated separately for the light and dark cycle to delineate activity patterns in different behavioural states.

Activity Bouts

Actigraphy data can be used to calculate the number and duration of periods of rest and activity. Activity bouts are usually defined as periods of time when activity never falls below a specified

threshold. However, there is variation between studies in the required length and threshold. The parameters of this analysis were selected to maximize the number of bouts detected. Bouts were detected using MATLAB code to identify periods where the Animal Locomotor Index did not fall below a threshold of 0.2 arbitrary units. Bouts had a minimum duration of 1 minute with a minimum inter-bout interval of 1 minute.

Anxiety Tests

Video recordings of the elevated plus maze and aversive open field tests were analyzed by a blinded observer using Anymaze 7.3 (Stoelting) to extract the mouse's location and movement within the behavioural apparatus. The mouse was deemed to be inside a given region when 85% of its body crossed into the zone. Video recordings of the light dark box test were analyzed manually by a blinded observer to record time spent in the light zone of the apparatus.

4.2 Results

Passive home-cage monitoring was used to record the activity of sham and active claustrum-silenced mice. Three groups of mice were used in this study: wildtype C57 mice with sham silencing (sham); wildtype C57 with active silencing of RSP-projecting claustrum cells (CRE-TetTox); and CLCX:EDGE transgenic mice with active silencing of genetically restricted claustrum complex cells (CLCX:EDGE-TetTox). Analysis revealed no meaningful effects of claustrum silencing on home-cage behaviour or circadian parameters. A set of three behavioural tests were also used to examine the interaction between chronic social-isolation and claustrum silencing. No effect of claustrum silencing on anxiety-like behaviour was detected.

4.2.1 Locomotor Activity

To measure animals' activity level during the pre- and post-viral injection periods, the daily distance travelled was recorded using home-cage activity monitoring. The daily distance travelled in the light and dark phase are plotted in Figure 4-1 A and B. Two-way repeated measures ANOVAs were used to compare the effect of treatment between groups and time period (i.e., baseline vs expression) within groups for both the light and dark cycles. No significant effects or interactions of treatment and time period were detected for the dark cycle. For the light cycle, the two-way ANOVA detected a trend towards a significant effect of treatment ($F(2, 15) = 3.25$, $p = 0.07$), but no effect of time period or interaction. After correcting for multiple comparisons, post hoc testing revealed a trend towards a significant difference between CLCX:EDGE-TetTox and sham animals ($T(10) = 2.55$, $p = 0.08$).

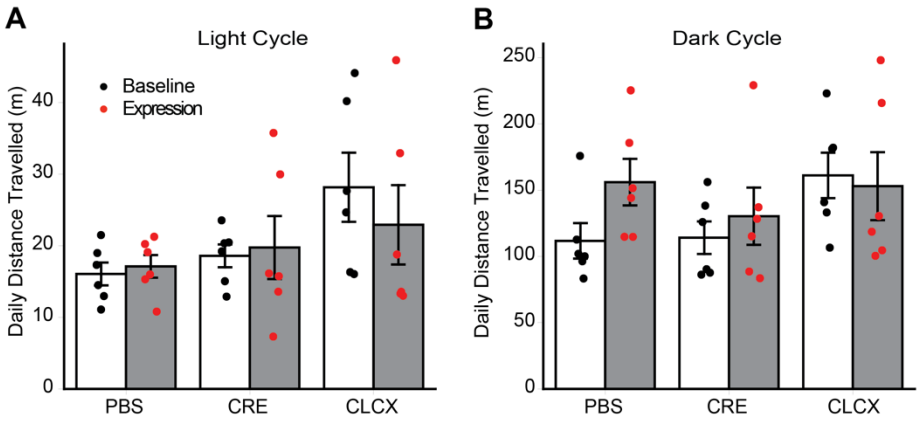


Figure 4-1. Distance Travelled. Graph showing the daily distance travelled by each group in the light phase (A) and dark phase (B). Data from the baseline period is plotted in black with open bars and data from expression period is plotted in red on shaded bars (n = 6 per group). Error bars show the standard error of the mean.

To provide a more detailed picture of the animals' activity levels, the Animal Locomotor Index calculated by the activity tracking cages was compared within the light (Figure 4-2 A) and dark cycles (Figure 4-2 B). Two-way repeated measures ANOVAs were used to compare the effect of treatment between groups and time period within groups for both the light and dark cycles. For the dark cycle, the two-way ANOVA detected a trend towards a significant effect of treatment ($F(2, 15) = 2.73$, $p = 0.097$), and a significant effect of time period ($F(1, 15) = 10.99$, $p = 0.005$), but no interaction. After correcting for multiple comparisons, post-hoc testing detected a significant difference between the Cre-TetTox and CLCX:EDGE-TetTox groups during the baseline period ($T(10) = -3.64$, $p = 0.03$). Similarly, for the light cycle, the two-way ANOVA detected a trend towards a significant effect of treatment ($F(2, 15) = 2.72$, $p = 0.098$) and a significant effect of time period ($F(1, 15) = 8.74$, $p = 0.009$), but no interaction. After correcting for multiple comparisons, post-hoc testing detected a significant difference between the sham and CLCX:EDGE-TetTox groups ($T(10) = 3.5$, $p = 0.03$) during the baseline period, and a trend towards a significant difference between the Cre-TetTox and CLCX:EDGE-TetTox groups ($T(10) = -3.11$, $p = 0.06$) during the baseline period.

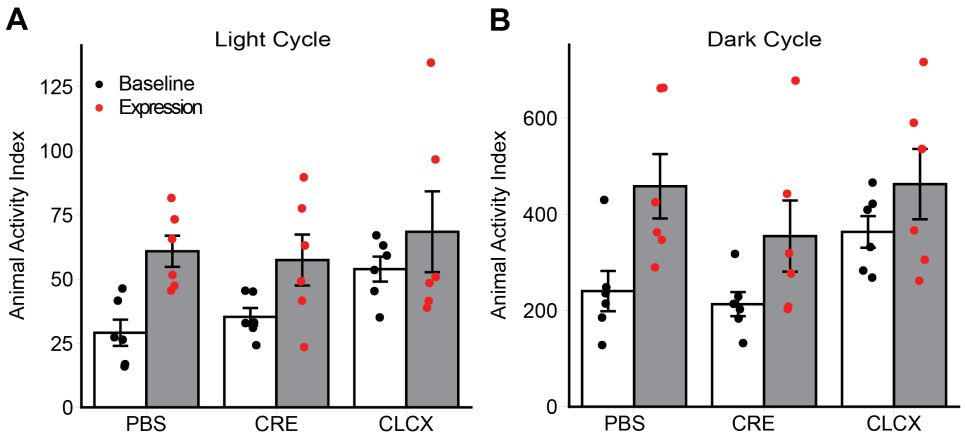


Figure 4-2. Animal Activity Index. Graph showing the activity index for each group in the light phase (A) and dark phase (B). Data from the baseline period is plotted in black with open bars and data from expression period is plotted in red on shaded bars (n = 6 per group). Error bars show the standard error of the mean.

Collectively, this data does not provide compelling evidence that claustrum or claustrum complex silencing affects general locomotor behaviour. Importantly, these results do suggest that differences existed at baseline between the CLCX:EDGE group and the other groups. This baseline difference complicates the analysis of any additional findings.

4.2.2 Activity Patterns

General locomotor behaviour can be analyzed in greater detail by identifying discrete periods of rest and activity. The number, duration, and distribution of these activity bouts can reveal differences in animals' activity levels and patterns.

Time weighted frequency histograms show the distribution of activity bouts by duration for both the light and dark cycles in the baseline and expression periods (Figure 4-3). To analyze these distributions, a baseline-adjusted distribution was calculated for both the light and dark cycles by subtracting the baseline values from the expression values (data not shown). Two-way repeated measures ANOVAs were then run for both the light and dark cycles to compare the effect of bout duration within subjects and treatment group between subjects. A significant effect of duration was detected for both the light ($F(2.27, 34.09) = 4.99$, $p = 0.01$) and dark ($F(1.74, 26.19) = 9.30$, $p = 0.001$) cycles, and a significant interaction between duration and treatment group was detected for the dark cycle ($F(14, 105) = 3.63$, $p < 0.0001$). However, after correcting for multiple comparisons (Sidak), post hoc testing revealed no significant differences.

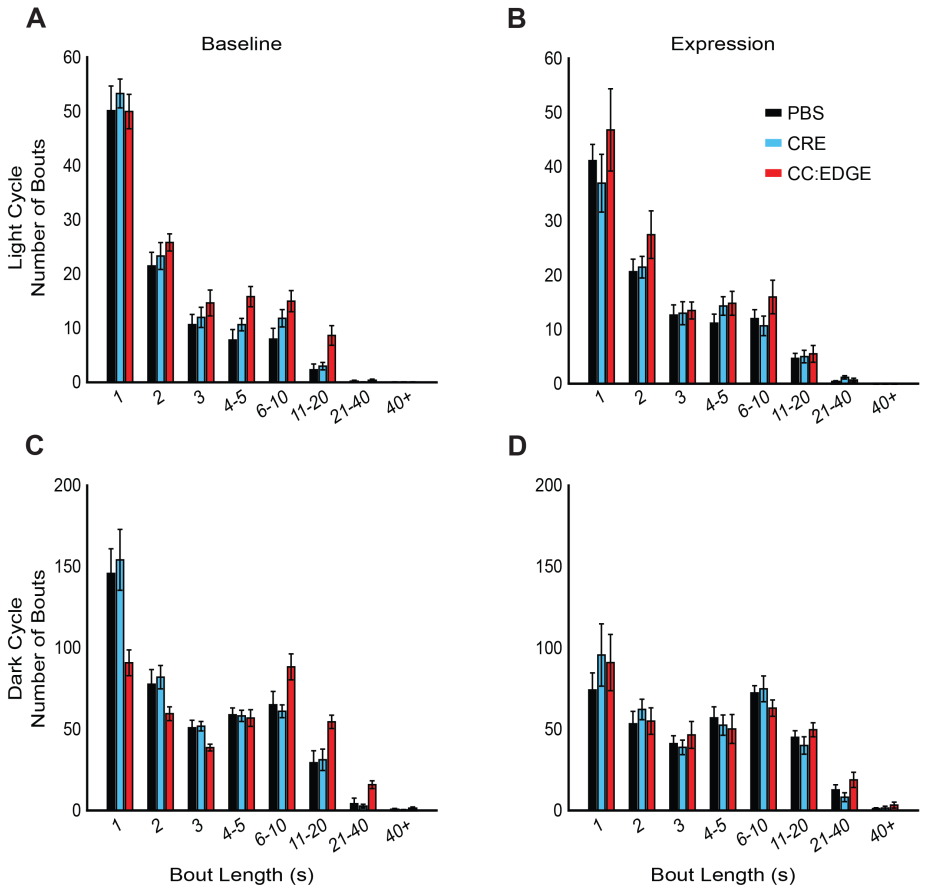


Figure 4-3. Distribution of Activity Bouts. Frequency weighted histograms showing the distribution of activity bouts by duration (s) in the light cycle (top) and dark cycle (bottom) during the baseline (left and expression periods (right). Error bars show the standard error of the mean (n = 6 per group).

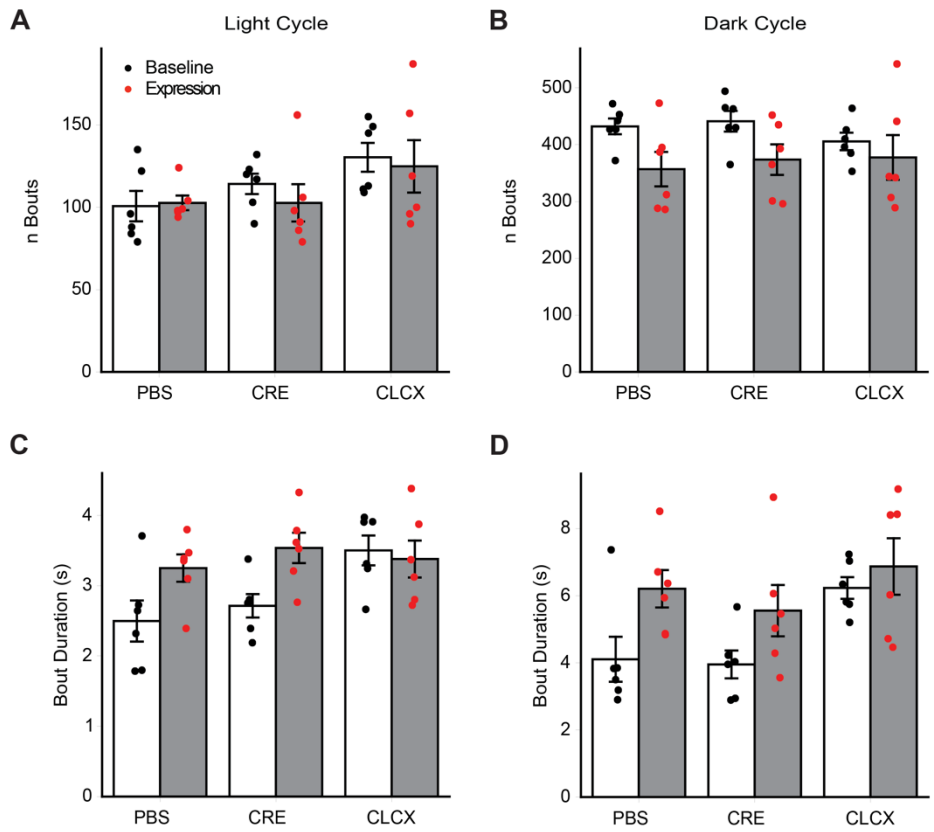


Figure 4-4. Number and duration of activity bouts. Graphs showing the number of activity bouts recorded in the light (A) and dark (B) cycles. Graphs showing the duration of activity bouts recorded in the light (C) and dark (D) cycles. Data from the baseline period is plotted in black with open bars and data from expression period is plotted in red on shaded bars (n = 6 per group). Error bars show the standard error of the mean.

To further understand the activity bouts data, the average number and duration of bouts during the light and dark cycles were plotted for both groups (Figure 4-4). Two-way repeated measures ANOVAs were used to compare the effect of treatment between groups and time period within groups for both the light and dark cycles. For the duration of bouts, a significant effect of time period ($F(1, 15) = 6.27$, $p = 0.02$) was detected for the light cycle, and significant effects of both time period ($F(1, 15) = 7.67$, $p = 0.01$) and group ($F(2, 15) = 4.85$, $p = 0.02$) were detected for the dark cycle. After correcting for multiple comparisons (Sidak), post hoc testing revealed a significant difference between the sham and CLCX:EDGE-TetTox groups at baseline ($p = 0.01$) for the light cycle, and a difference between the Cre-TetTox and CLCX:EDGE-TetTox groups at baseline ($p = 0.04$) for the dark cycle. For the number of bouts, a trend towards a significant effect of group ($F(2, 15) = 3.01$, $p = 0.08$) was detected for the light cycle, and a significant effect of time period was detected for the dark cycle ($F(1, 15) = 6.27$, $p = 0.02$). After correcting for multiple comparisons (Sidak), post hoc testing revealed no significant differences.

To further understand any potential differences in activity, the RDI was calculated for each mouse in both the light and dark cycles (Figure 4-5). The RDI is a novel metric that was recently developed for use with the DVC system. The RDI quantifies irregularities in the activity of mice regardless of the level of activity. Two-way repeated measures ANOVAs were used to compare the effect of treatment between groups and time period within groups. A significant effect of treatment group was detected for both the light ($F(2, 15) = 5.44$, $p = 0.02$) and dark cycles ($F(2, 15) = 7.18$, $p = 0.006$), and a significant interaction between treatment group and time period was detected for the light cycle ($F(2, 15) = 4.83$, $p = 0.02$). After correcting for multiple comparisons (Sidak), post hoc testing revealed a significant difference between the CRE-TetTox and CLCX:EDGE-TetTox ($p = 0.01$) as well as a significant difference between the PBS and CLCX:EDGE-TetTox groups ($p = 0.002$) at baseline in the light cycle. Significant differences were also detected between the CRE-TetTox and CLCX:EDGE-TetTox ($p = 0.005$) as well as between the PBS and CLCX:EDGE-TetTox groups ($p = 0.02$) during the expression period in the dark cycle. Collectively, this data does not provide compelling evidence that claustrum or claustrum complex silencing affects activity patterns. However, these results do suggest that differences exist between the CLCX:EDGE group and the other treatment groups. However, these differences occurred both at baseline and after claustrum silencing, complicating any further analysis.

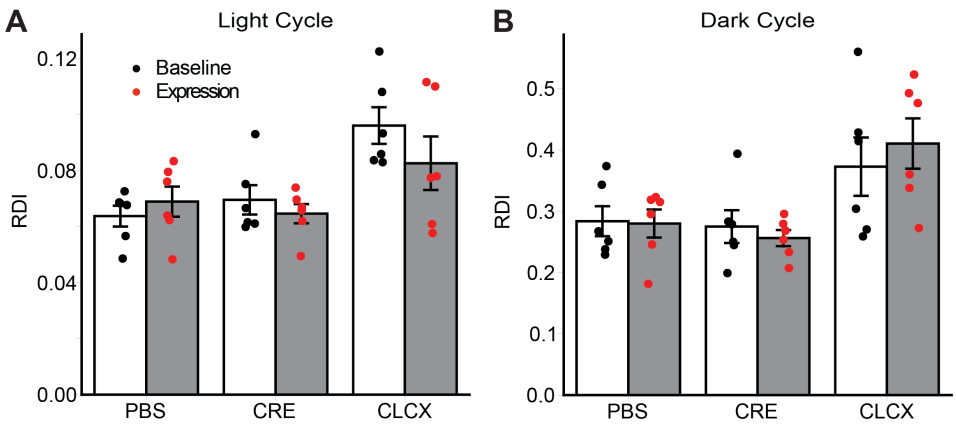


Figure 4-5. Regularity disruption index (RDI). Graphs showing the average RDI from the light (A) and dark (B) cycles. Data from the baseline period is plotted in black with open bars and data from the expression period is plotted in red on shaded bars (n = 6 per group). Error bars show the standard error of the mean.

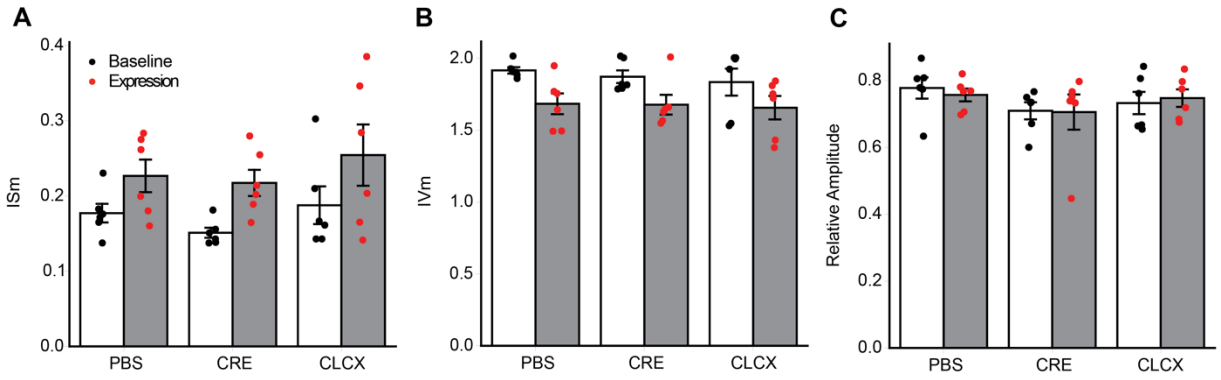


Figure 4-6. Circadian Parameters. Graphs showing the mean inter-daily stability (ISm; A), mean intra-daily variability (IVm; B) and relative amplitude (RA; C). Data from the baseline period is plotted in black with open bars and data from the expression period is plotted in red on shaded bars (n = 6 per group). Error bars show the standard error of the mean.

4.2.3 Circadian Parameters

To examine the effect of claustrum or claustrum complex silencing on circadian rhythms, we calculated common metrics of circadian behaviour. ISm provides a readout of the variability and reproducibility of circadian activity patterns across days (Figure 4-6 A). A two-way repeated measures ANOVA was used to compare the effect of treatment between groups and time period within groups. A significant effect of time period was detected ($F(1, 15) = 12.36$, $p = 0.003$), but no effect of treatment or interaction. After correcting for multiple comparisons (Sidak), post hoc testing revealed no significant differences.

IVm provides a complementary readout of circadian activity, showing the fragmentation of activity patterns within the day (Figure 4-6 B). A two-way repeated measures ANOVA was used to compare the effect of treatment between groups and time period within groups. A significant effect of time period was detected ($F(1, 15) = 15.60$, $p = 0.001$), but no effect of treatment or interaction. After correcting for multiple comparisons (Sidak), post hoc testing revealed no significant differences.

The circadian activity pattern can also be quantified by examining RA. RA measures the difference in activity level between the periods of high and low activity corresponding to the light and dark cycles (Figure 4-6 C). A two-way repeated measures ANOVA was used to compare the effect of treatment between groups and time period within groups. No significant effects or interactions were detected.

Collectively, this data does not provide compelling evidence that claustrum or claustrum complex silencing affects circadian behaviour.

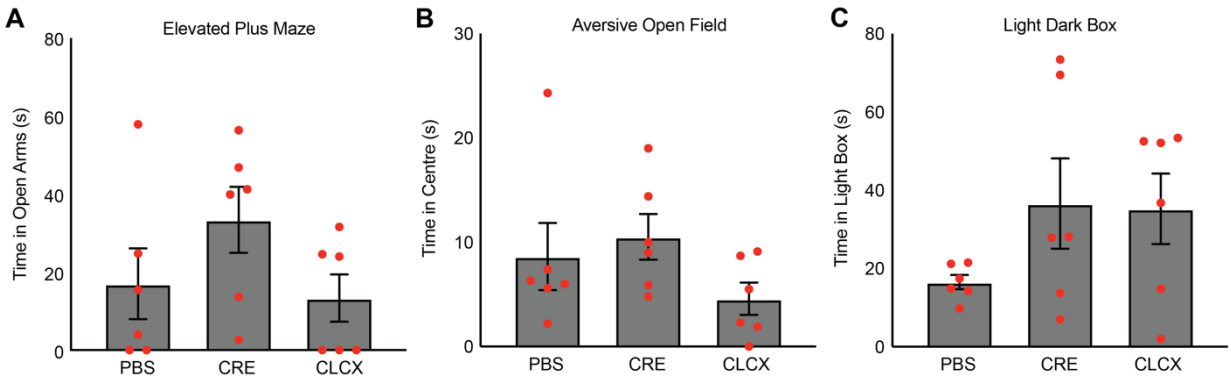


Figure 4-7. Anxiety-like behaviour. Graph showing the time spent in the open arms of the elevated plus maze (A), time spent in the centre zone during the open field test (B), and in the light chamber during the light dark box test (C). Error bars show the standard error of the mean (n = 6 per group).

4.2.4 Anxiety-like Behaviour

For the DVC system to accurately track specific mice, all animals had to be housed individually. While necessary for this experiment, social isolation is known to have numerous behavioural and physiological effects on mice. In particular, social isolation has frequently been shown to induce anxiety-like behaviour.

To quantify changes in anxiety-like behaviour, mice were examined on three behavioural tests: the elevated plus maze, the light dark box, and the aversive open field test. One-way ANOVAs detected no significant differences between groups in the time spent in the open arms of the elevated plus maze (Figure 4-7 A), the centre of the open field (Figure 4-7 B), or the light compartment of light dark box (Figure 4-7 C). Collectively, these results do not provide evidence that claustrum or claustrum complex silencing affects isolation-induced anxiety-like behaviour.

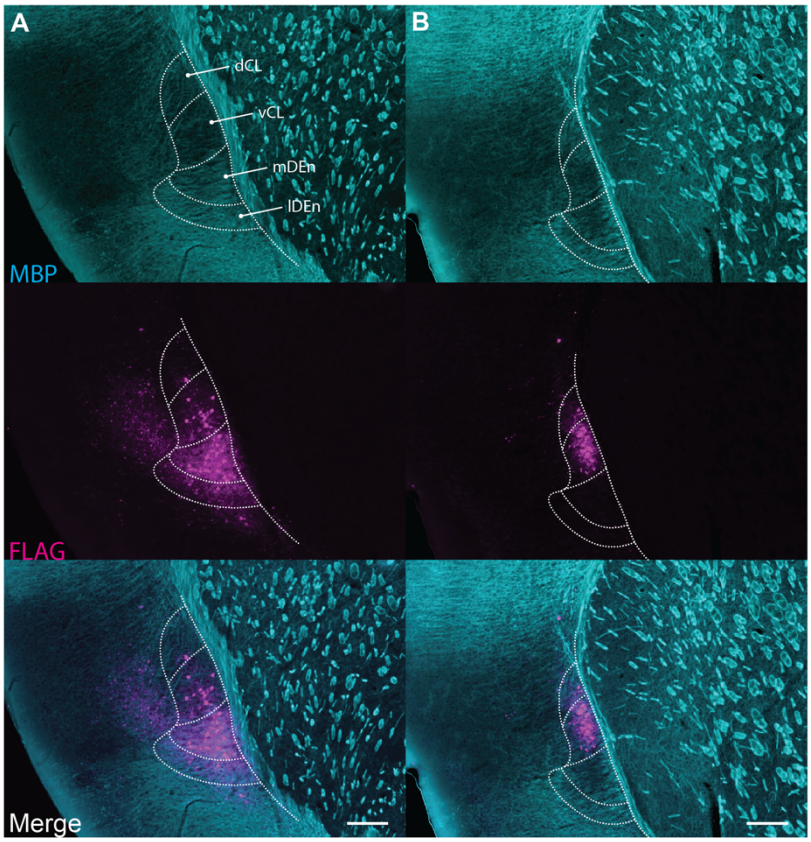


Figure 4-8. Histology. Immunofluorescent staining of TetTox injected brain tissue from CLCX:EDGE (A) and CRE (B) mice showing myelin basic protein (MBP, cyan, top), FLAG (magenta, centre), and overlay (bottom). Scale bars are 200um. Panels oriented with dorsal at the top of the frame and lateral on the left. Abbreviations: dorsal claustrum (dCL), ventral claustrum (vCL), medial DEn (mDEn), lateral DEn (lDEn)

4.2.5 Histology

Post-hoc histology was used to confirm bilateral expression of TetTox in all animals with active silencing. Representative sections from all CLCX:EDGE-TetTox (Figure 4-8 A) and Cre-TetTox (Figure 4-8 B) mice were stained for FLAG to visualize TetTox expression and MBP to delineate the claustrum complex. The staining showed bilateral TetTox expression in the claustrum or claustrum complex of all animals. Expression varied between groups in both the distribution of TetTox within the claustrum complex and the dorsal-ventral extent of TetTox expression. For CLCX:EDGE transgenic animals, TetTox expression was largely confined to the medial DEn and the ventral claustrum. In contrast, in Cre-TetTox animals, TetTox expression was largely confined to the dorsal and ventral claustrum. Histology from all experimental animals can be found in Supplemental Figure S4-1.

4.3 Summary

This chapter aimed to characterize the effect of claustrum and claustrum complex silencing on circadian patterns and home-cage activity. Three groups of mice received bilateral injections for active claustrum silencing (Cre-TetTox), active claustrum complex silencing (CLCX:EDGE-TetTox) and sham silencing (PBS). Mice were single housed in activity tracking cages to allow for 24/7 passive monitoring. While we detected various significant differences between groups, differences were only detected between CLCX:EDGE-TetTox and other groups. These differences do not provide compelling evidence of an effect of claustrum complex silencing as we detected differences between CLCX:EDGE-TetTox and other groups at baseline. This finding complicates the analysis of data from this chapter and highlights the need for further behavioural characterization of the CLCX:EDGE line. These experiments suggest that claustrum silencing does not drastically alter circadian or home-cage activity. Future studies should build upon this result by using electroencephalography to understand the impact of claustrum silencing on sleep-related brain activity.

4.4 Acknowledgements & Contributions

The experiments in this chapter benefited from contributions by Prof Stuart Peirson (SP), Prof Vladyslav Vyazovskiy (VV), and Selma Tir (ST). SP, ST and VV provided guidance in designing the experiments. SP provided access to the DVC system within the Biomedical Services Department. ST provided practical training on the use of the DVC system. SP and VV provided guidance on the analysis of the DVC data. VV provided the base code used to detect activity bouts. Imaging of post-hoc histology was completed with guidance from Dr Cyril Lai at the Wolfson Imaging Centre in the MRC Weatherall Institute of Molecular Medicine. Equipment used for the anxiety tests was provided by the Behavioural Neurosciences Unit within the Biomedical Services Department with technical support provided by Dr Katie Hewitt. The CLCX:EDGE line was provided by Prof Clifford Kentros at the Norwegian University of Science and Technology.

5 Impact of claustrum silencing on learned behaviour

Chapters Three and Four investigated the impact of chronic claustrum silencing on a wide range of behavioural domains. Collectively, these results suggest that claustrum silencing does not impact basic behaviours. This is consistent with recent studies showing that claustrum activity is more important for cognitively demanding tasks[25,113,148].

To build upon these results, we trained mice with active and sham claustrum silencing to complete two complementary behavioural tasks. In the first task, a classical conditioning-like paradigm, water rewards were dispensed from three different nose poke ports corresponding to an auditory stimulus, a visual stimulus, and the two stimuli presented simultaneously. In the second task, a reversal learning paradigm, mice earned water rewards by poking two different nose pokes associated with reversible reward probabilities. These tasks assess common mechanisms of learning, as well as providing insight into diverse behavioural features like impulsivity, perseveration, and motivation. Moreover, these tasks incorporate elements of important hypotheses for the function of the claustrum including multisensory processing and cognitive flexibility.

5.1 Methods

28 mice (14 male and 14 female) received intracranial injections of virus to silence claustrum output or PBS for sham silencing. All mice were then trained on two behavioural tasks before sacrifice and staining.

5.1.1 Animals

All experiments were carried out on wildtype C57Bl/6 mice. Littermate groups of 4–5 were housed under standard conditions with wood chip bedding and ad libitum access to food and water except when required for behavioural experiments. All cages included mice from both the sham and active claustrum silencing groups. All experiments were subject to ethical approval and conducted in accordance with local laws and regulations.

5.1.2 Surgery

Intracranial injections were performed in accordance with the method outlined in section 2.1.2. Silenced mice received injections of 80nl retro-Cre virus (ssAAV-retro/2-hSyn1-mCherry_iCre-WPRE-hGHp(A), ETH Zurich v230) in RSP and 500nl FLEX-TetTox virus (ssAAV-DJ/2-hEF1a-dlox-FLAG_TeTxLC(rev)-dlox-WPRE-hGHp(A), ETH Zurich v63) in claustrum while sham animals received equivalent volumes of sterile PBS. The TetTox construct included a FLAG tag and the retro-Cre included a fluorophore to allow immunohistochemical identification.

5.1.3 Behavioural Testing

Figures 5-1 A provides a schematic outline of the experimental structure. Briefly, animals aged p44–p48 received intracranial injections of either PBS (sham; n= 12) or retro-Cre and Cre-dependent tetanus toxin light chain viruses (TetTox; n= 16). Animals were then given four weeks for recovery and viral expression. Mice were then water restricted and trained on a multimodal conditioning task before a one-week break from water restriction. Next, mice were again water restricted for two days before being trained on a reversal learning task. Finally, mice were sacrificed, and brains were extracted for post-hoc histology.

Water restriction was used to provide task motivation. Mice were weighed twice daily during water restriction (before and after training) to ensure that their weight remained above

85% of baseline. On the first and second days of water regulation, mice were given access to their water bottle for one hour each day. Training commenced on the third day, after which most mice received all their water during the behavioural task. Mice who did not receive sufficient water during their training sessions or whose weight dropped below 85% of baseline were given additional water outside of training to maintain their body weight.

Behavioural Training Setup

Training took place in 12 x 12cm plastic boxes with nine nose-poke ports on the back wall arranged in a diamond shape (see https://github.com/pyControl/hardware/tree/master/Behaviour_box_small). Removeable plastic panels could be attached to the back wall to cover various combinations of nose poke ports. Each port contained an infrared beam to detect nose pokes. Solenoid valves could be used to deliver calibrated water rewards to each port. Each box was housed in a separate sound and light attenuating chamber. A speaker located above the nose poke ports was used to deliver auditory stimuli. Each port could be illuminated individually, and the entire chamber could be illuminated with an LED located above each box. Operant boxes were controlled and programmed using pyControl[165].

Multimodal Conditioning

Water restricted mice were trained to associate uni- and multimodal sensory stimuli with water rewards. Mice were trained in two sessions per day for 11 days with only one session on the last day. Each session included 30 trials and lasted for ~50 minutes. Trials were separated by a random 1–2-minute inter-trial interval. During each trial, mice were presented with either an auditory stimulus (A), a visual stimulus (V), or both stimuli at the same time (AV). Each stimulus was presented for 10 seconds. The auditory stimulus consisted of a stepped sine wave rising from 5 to 15kHz. The frequency was increased in 20 steps, and the steps were cycled at 50Hz, meaning that each step was played for 20ms. The sound level measured inside the behaviour box during stimulus presentation was ~85dB SPL. To investigate the possibility that sound from auditory stimuli might be audible to mice in neighboring boxes, sound measurements were taken in a neighboring box before and during stimulus presentation. No difference in sound level in the neighboring box was detected when the auditory stimulus was presented. The visual stimulus consisted of turning on the houselights in the behaviour box.

As soon as the stimulus began, a reward was made available for collection at one of three nose poke ports arranged in a triangle. Each water reward was 15ul. This was decreased to 12ul for mice whose weight remained at or above 100% of baseline for two consecutive sessions. Mice received a reward following A by poking in the lefthand port, in the centre port following AV, and in the righthand port following V. Note, the reward remained available until the mouse poked in the appropriate port (i.e. they did not have to poke within a specific post-stim time window to receive the reward), but rewards were not cumulative (i.e. they did not receive two rewards if they failed to collect the reward before the next presentation of the same stimulus type).

Reversal Learning

In this task, water-restricted mice learned to flexibly remap probabilistic associations between action and reward. Mice were trained in 2 sessions per day for 10 days. Each session lasted 45 minutes, during which time mice could complete trials to earn rewards.

Training took place in the same boxes as the multimodal conditioning. All poke ports used in the previous paradigm were covered, and an upside-down triangle of poke ports was uncovered. At the start of the training session, the house lights were illuminated and remained illuminated throughout training. Trials were separated by a one second inter-trial interval. At the end of the inter-trial interval, the two top poke ports were illuminated. Mice could initiate a trial by poking either the left- or right-hand poke port. A click was played after poking either port. After making a choice, the top two port lights were turned off and the bottom port was illuminated. Mice could then poke the bottom port for a chance to receive a water reward. Reward volumes were gradually decreased over the course of training to maintain motivation. At the start of the first session, the choice ports were randomly assigned as either the good or bad port. Poking the good port led to an 85% chance of receiving water from the reward port, while poking the bad port led to a 15% chance of receiving a reward. The good and bad side carried over between sessions.

Each mouse's performance was tracked throughout the session with an exponential moving average (tau = 12). If mice surpassed a threshold of >80% correct, they initiated a new block. After an additional 5–10 trials with the same left-right reward probabilities, the good and

bad ports were reversed. The number of trials, number of rewards, and number of blocks were therefore dependent on each mouse's individual performance.

5.1.4 Histology

Histological validation of claustrum-specific viral expression was performed according to the general antibody staining protocol in section 2.1.6. To cover the full extent of the claustrum, 6 coronal sections spaced 500um apart were selected such that the second section was located at the anterior union of the corpus callosum. Slices were then stained with antibodies against MBP (Abcam; 1:500; RRID: AB_305869) and PV (SWANT; 1:500; RRID: AB_2631173) to facilitate identification of the claustrum, and FLAG (Cell Signalling Technology; 1:500; AB_2572291) to clearly label all TetTox positive cells. Further rounds of staining included antibodies against MBP (Abcam; 1:500; RRID: AB_305869), glial fibrillary acidic protein (GFAP; Merck; 1:500; RRID: AB_477035), and NeuN (Merck; 1:100; RRID: AB_2298772). All secondary antibodies were used at a concentration of 1:500. Imaging was performed using a Zeiss Axioscan Z1 automated slide scanner with a 10x objective. Lines to delineate the approximate boundaries of the claustrum complex were drawn based on MBP and PV expression.

GFAP expression was quantified using FIJI (ImageJ). First, ovoid ROIs were drawn surrounding the left and right ventral claustrum of each section. The ROI was drawn by hand using only the MBP image and without reference to the GFAP channel. Next, an ROI for the entire slice was drawn using the default algorithm of the thresholding tool. The mean fluorescence intensity was then recorded for all ROIs. The GFAP fluorescence was then averaged across the two claustrum ROIs on each slice and normalized against the fluorescence of the entire slice.

5.1.5 Data Analysis & Statistics

Data was analyzed using Python 3.9.13 and based on open-source packages. Code for this chapter included important functions from the pyControl library[165]. Statistical analyses were performed using python except for the mixed-models analysis, which was performed using Prism 9 (Graphpad). Statistics were computed by taking the median of the available data for

each mouse and the mean across mice. Code and data are available upon request. Figures were generated using Python and formatted using Adobe Illustrator.

D-Prime

A d-prime (d') was calculated for each stimulus in an effort to compare animals' sensitivity to the different stimuli. To calculate each d', all trials had to be classified according to one of four outcomes: hit, miss, correct rejection, or false alarm. Classically, a hit is defined as a trial in which both the stimulus and response are present, while a miss is a trial where the stimulus is present, but the response is absent. A correct rejection is a trial in which both the stimulus and response are absent, while a false alarm is a trial in which the stimulus is absent, but the response is present. For this calculation, the response was defined as poking the correct port first, and within 10s of stimulus onset. Using the auditory d' as an example, a hit would be a trial where a sound was present, and the mouse poked the auditory port first. A miss would be a trial where the auditory stimulus was present, but the mouse poked a non-auditory port first. An auditory false alarm would be a non-auditory trial (i.e., visual or audiovisual) where the mouse poked the auditory port first. Finally, a correct rejection would be a non-auditory trial (i.e., visual or audiovisual) where the mouse did not poke the auditory port first. In this way, each d' included the outcome for every trial of all stimulus types. The redundancy inherent in these outcomes is essential for assessing how animals behave when each stimulus was present and when it was absent.

Once all trials had been assigned an outcome, they were used to calculate a hit rate and a false alarm rate for each stimulus. These were calculated according to the following formulae: $False\ Alarm\ Rate = \left(\frac{\#\ False\ Alarm}{\#\ False\ Alarm + \#\ Correct\ Rejection}\right)$ and $Hit\ Rate = \left(\frac{\#\ Hit}{\#\ Hit + \#\ Miss}\right)$. The hit and false alarm rates could then be combined according to the formula $d' = Z(Hit\ Rate) - Z(False\ Alarm\ Rate)$ where Z is the normal inverse cumulative distribution function.

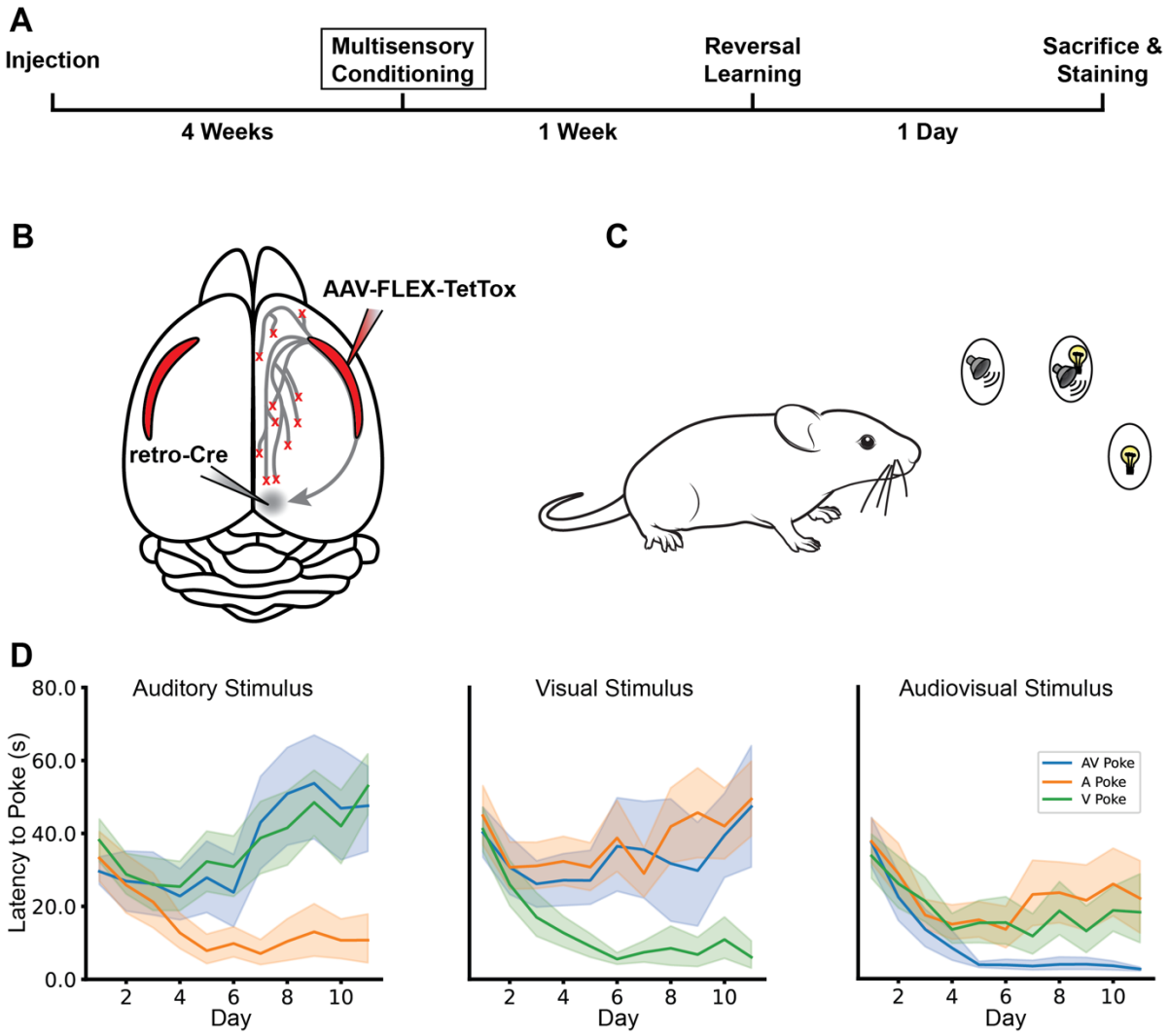


Figure 5-1. Experimental Protocol. Schematic representation showing the timeline of experiments (A), injection strategy for claustrum silencing (B), and behavioural task (C). Mean latency for both groups from stimulus onset until the animal poked the audiovisual port (AV, blue line), audio port (A, orange line), and visual port (V, green line) in response to the indicated stimulus. Latency data is presented across all 11 days of training. Shaded area represents the standard error of the mean.

5.2 Results

A cohort of mice with sham and active claustrum silencing were trained on two behavioural tasks to assess a range of behavioural traits (Figure 5-1 A). First, mice were trained on a classical conditioning-like paradigm where they learned to poke different ports in response to uni- and multimodal stimuli. After a break, mice were then trained to flexibly remap reward associations on a reversal learning task.

5.2.1 Multimodal Sensory Conditioning

A group of 28 mice were trained on two behavioural tasks (Figure 5-1 A). To assess the functional impact of claustrum silencing on complex behaviour, mice either received intracranial injections of FLEX-TetTox & retro-Cre virus (n = 16) or PBS (n = 12; Figure 5-1 B). After allowing four weeks for viral expression, water restricted mice were then trained on a multimodal conditioning task (Figure 5-1 C). In this task, water restricted mice were passively presented with auditory, visual, and combined audiovisual stimuli. At the time that each stimulus was presented, a water reward was made available at a nose-poke port corresponding to one of the three stimuli. Mice could then collect the reward by poking the relevant port. Importantly, the rewards were not contingent on the actions of the mouse. In other words, the reward was made available regardless of when the mouse poked or whether it poked a different port first. In this way, the task resembled a classical conditioning paradigm and allowed an assessment of whether and how the mice learned to link their actions to patterns in their environment. Figure 5-1 D shows the combined latency of both groups to nose-poke following presentation of the auditory, visual, or audiovisual stimulus. These results suggest that the mice learned to associate each stimulus with the corresponding nose poke port.

To better understand the performance of the mice, trials were classified based on their outcome. Trials on which the mouse poked the port corresponding to the presented stimulus first and within 10s of stimulus onset were classified as hits. Trials on which the mouse poked another port first and within 10s of stimulus onset were classified as misses. Finally, trials on which the mouse did not poke any port within

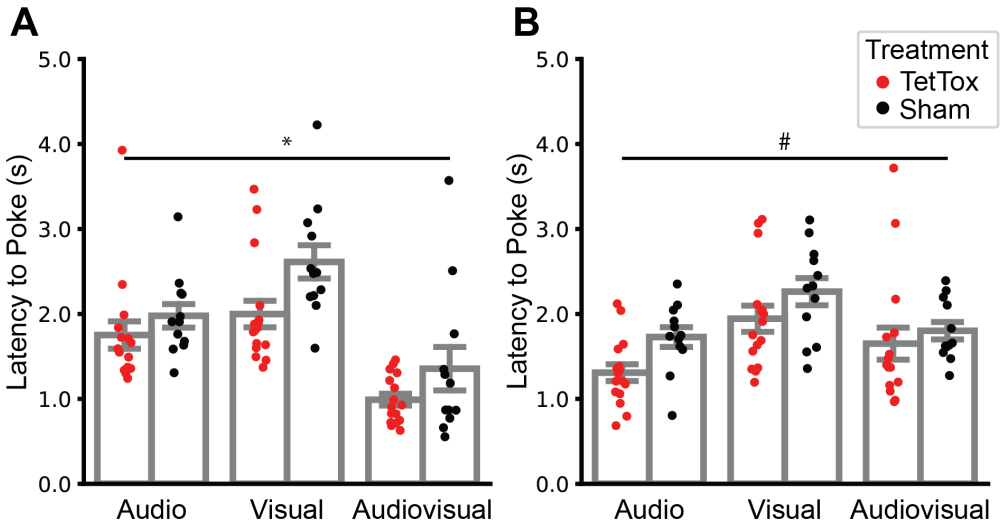


Figure 5-2. Latency to first poke. Graphs show the latency from stimulus onset until the first poke, regardless of which port was poked, on hit (A) and miss (B) trials. X-axis labels indicate the trial type. Plots include data from experienced mice only (training sessions after day 3). Data from sham mice (n=12) are plotted in black and TetTox mice (n=16) are plotted in red. Error bars show the standard error of the mean. Symbols indicate an effect of treatment where $p < 0.1$ (#) or $p < 0.05$ (*).

10s of stimulus onset were classified as omissions. Figure 5-2 shows the latency to the first nose-poke following each stimulus on both hit trials (Figure 5-2 A), where the mouse poked the correct port first, and miss trials (Figure 5-2 B), where the mouse poked a different port first. Two-way repeated measures ANOVAs were used to compare the effect of treatment between groups and stimulus modality within groups for each trial type. Significant effects of both treatment ($F(1, 26) = 6.25$, $p = 0.02$) and stimulus ($F(2, 52) = 29.40$, $p < 0.001$) were detected on hit trials with no significant interaction. After correction for multiple comparisons (Sidak), post-hoc tests detected a trend towards a difference in latency between sham and TetTox mice on visual hit trials ($T(22.7) = 2.45$, $p = 0.07$), but no significant effects. Similarly, a significant effect of stimulus ($F(2, 52) = 13.22$, $p < 0.001$) and a trend towards an effect of treatment ($F(1, 26) = 3.53$, $p = 0.07$) were detected for miss trials with no significant interaction. After correction for multiple comparisons (Sidak), post-hoc tests detected a significant difference in latency between sham and TetTox mice on auditory miss trials ($T(23.6) = 2.75$, $p = 0.03$). Collectively, these results suggest that across trial types and outcomes, claustrum silenced mice displayed shorter latency to poke following stimulus onset.

Based on the trial outcomes discussed above, both groups' relative performance could be compared across trial types. Figure 5-3 shows the percentage of trials classified as a hit for each trial type. The dashed line indicates the performance expected if mice were poking all ports randomly (33%). A two-way repeated measures ANOVA was used to compare the effect of treatment between groups and stimulus modality within groups. The ANOVA detected a significant effect of trial type ($F(2, 52) = 54.0$, $p > 0.001$) but no significant effect of treatment or interaction. These results suggest that mice in both groups have learned to poke the correct port in response to unimodal stimuli but highlight that they performed worse than would be expected by chance on multimodal trials.

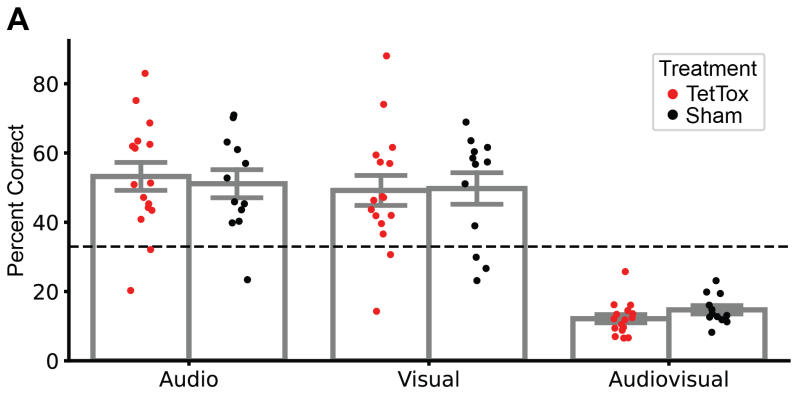


Figure 5-3. Percentage of trials correct. Graphs show the percentage of trials classified as hits for each trial type. Dashed line indicates chance performance of 33%. Plots include data from experienced mice only (training sessions after day 3). Data from sham mice (n=12) are plotted in black and TetTox mice (n=16) are plotted in red. Error bars show the standard error of the mean.

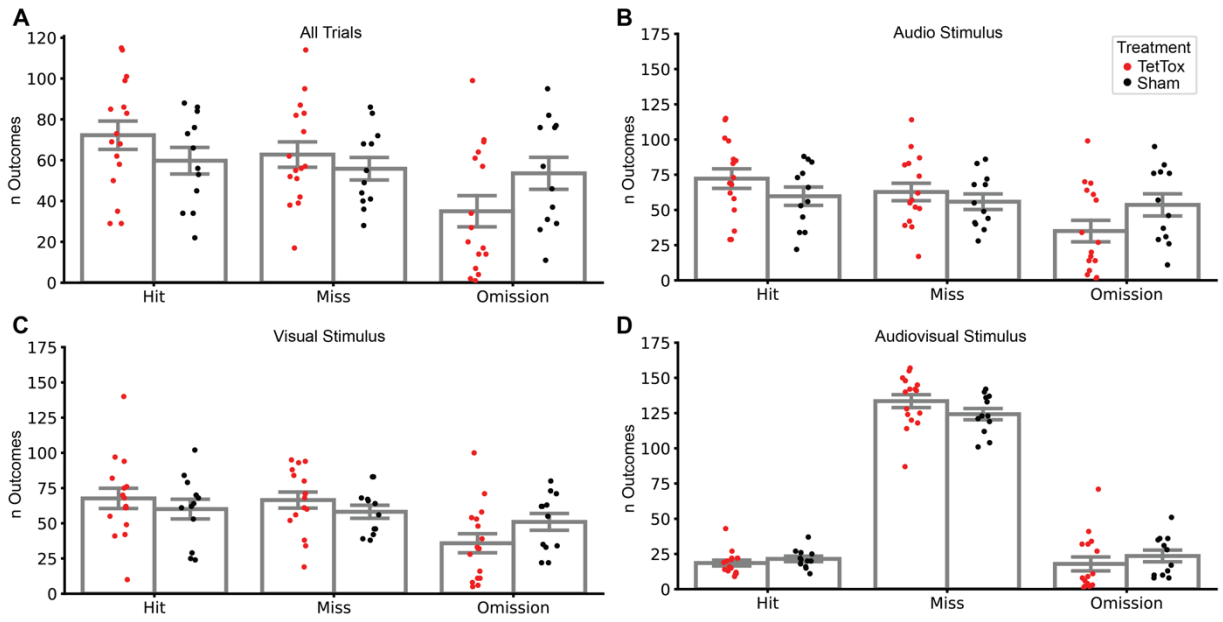


Figure 5-4. Frequency of trial outcomes. Graphs show the number of trials with each outcome across all trial types (A), audio trials only (B), visual trials only (C), and audiovisual trials only (D). Plots include data from experienced mice only (training sessions after day 3). Data from sham mice (n=12) are plotted in black and TetTox mice (n=16) are plotted in red. Error bars show the standard error of the mean.

To understand the factors driving their performance on each trial type, the frequencies of different trial outcomes were compared between groups. Figure 5-4 shows the number of trials with each outcome for each mouse across all trial types and stimulus modalities. This data is also displayed over time within a session in supplemental figures 5-1 – 5-3. While similar patterns of outcome frequency were observed between audio and visual trials, audiovisual trials appeared to be strikingly different, with misses appearing to be far more common than hits or omissions. Two-way repeated measures ANOVAs detected a significant effect of outcome for all trial types combined ($F(2, 52) = 4.77$, $p = 0.01$), auditory trials ($F(2, 52) = 12.38$, $p < 0.001$), visual trials ($F(2, 52) = 11.32$, $p < 0.001$), and audiovisual trials ($F(2, 52) = 88.92$, $p < 0.001$), but no significant effects of treatment or interactions were detected. These results do not provide evidence of a difference in the distribution of outcomes between groups. Moreover, they suggest that the poor performance observed on audiovisual trials in Figure 5-3 was not due to an increase in the number of omissions but could instead be attributed to the high frequency of misses. In other words, mice were still participating in audiovisual trials, but they tended to poke either the audio or visual port immediately before poking the audiovisual port.

From the outcome data in Figure 5-4, it was not clear whether the misses on audiovisual trials were due to mice consistently poking one preferred unimodal port (i.e., the mice learned to associate audiovisual stimuli with either the audio port or the visual port), or if they were instead poking both unimodal ports equally on different audiovisual trials. To investigate this, the number of auditory or visual misses (i.e., the number of times each mouse poked the auditory or visual port first on an audiovisual trial) was plotted for both groups (Figure 5-5 A). A two-way repeated measures ANOVA was used to compare the effect of treatment between groups and stimulus modality within groups. It detected no significant effects or interactions, suggesting that both groups had similar numbers of auditory and visual misses on audiovisual trials. To further quantify this behaviour, each animal's bias was calculated by using the formula $bias = \frac{\#\ auditory\ misses}{total\ \#\ misses} - 0.5$. Based on this formula, animals that exclusively poked the auditory port on audiovisual trials would have a value of 0.5, animals that exclusively poked the visual port would have a value of -0.5, and animals that poked both ports equally would have

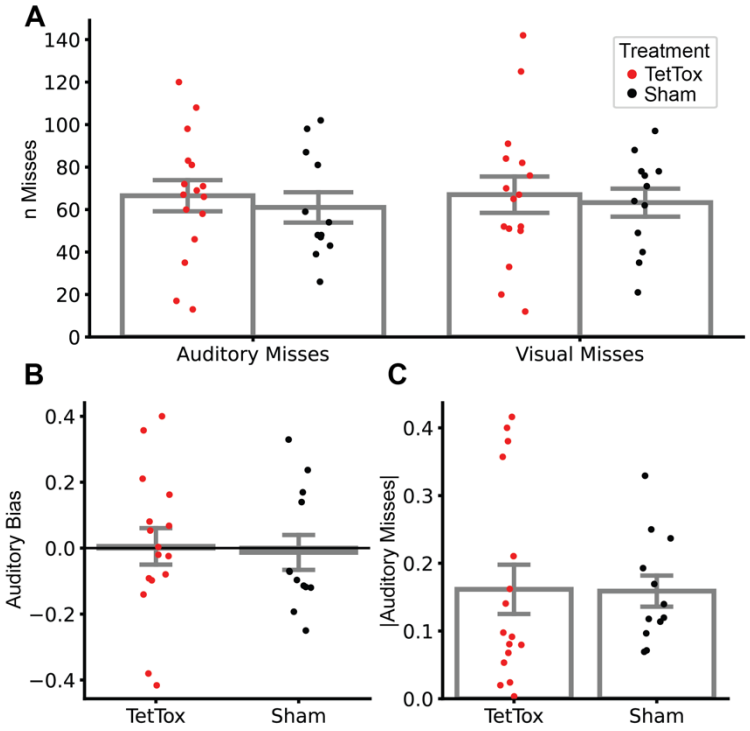


Figure 5-5. Choice bias on audiovisual trials. (A) The number of audiovisual miss trials on which the animal chose the auditory and visual ports. (B) Tendency to choose the auditory port (positive values) or the visual port (negative values) on audiovisual miss trials. (C) Absolute value of the bias in (B) showing the strength of bias regardless of the preferred unimodal port. Plots include data from experienced mice only (training sessions after day 3). Data from sham mice (n=12) are plotted in black and TetTox mice (n=16) are plotted in red. Error bars show the standard error of the mean.

a value of 0. Unpaired Welch's t-tests detected no significant differences between groups in either the bias (Figure 5-5 B) or the absolute value of the bias (Figure 5-5 C). This suggests that both groups displayed similar levels of bias and similar preferences for the two unimodal ports.

Finally, each animal's sensitivity to each stimulus type was quantified using d-prime (d'). Details of the d' calculation can be found in the methods above. Briefly d' was calculated by comparing the hit rate $\left(\frac{\#\ Hit}{\#\ Hit + \#\ Miss}\right)$ and the false alarm rate $\left(\frac{\#\ False\ Alarm}{\#\ False\ Alarm + \#\ Correct\ Rejection}\right)$. A separate d' was calculated for each stimulus type across all trials. Hits and misses occurred on trials where the stimulus was present (i.e., an auditory miss could only occur on an auditory trial), and false alarms and correct rejections occurred on trials where the stimulus was not present (i.e., an auditory false alarm could only occur on a visual or audiovisual trial). In this way, a separate d' was calculated for each stimulus type incorporating all trials across all stimuli. The various possible outcomes were both mutually

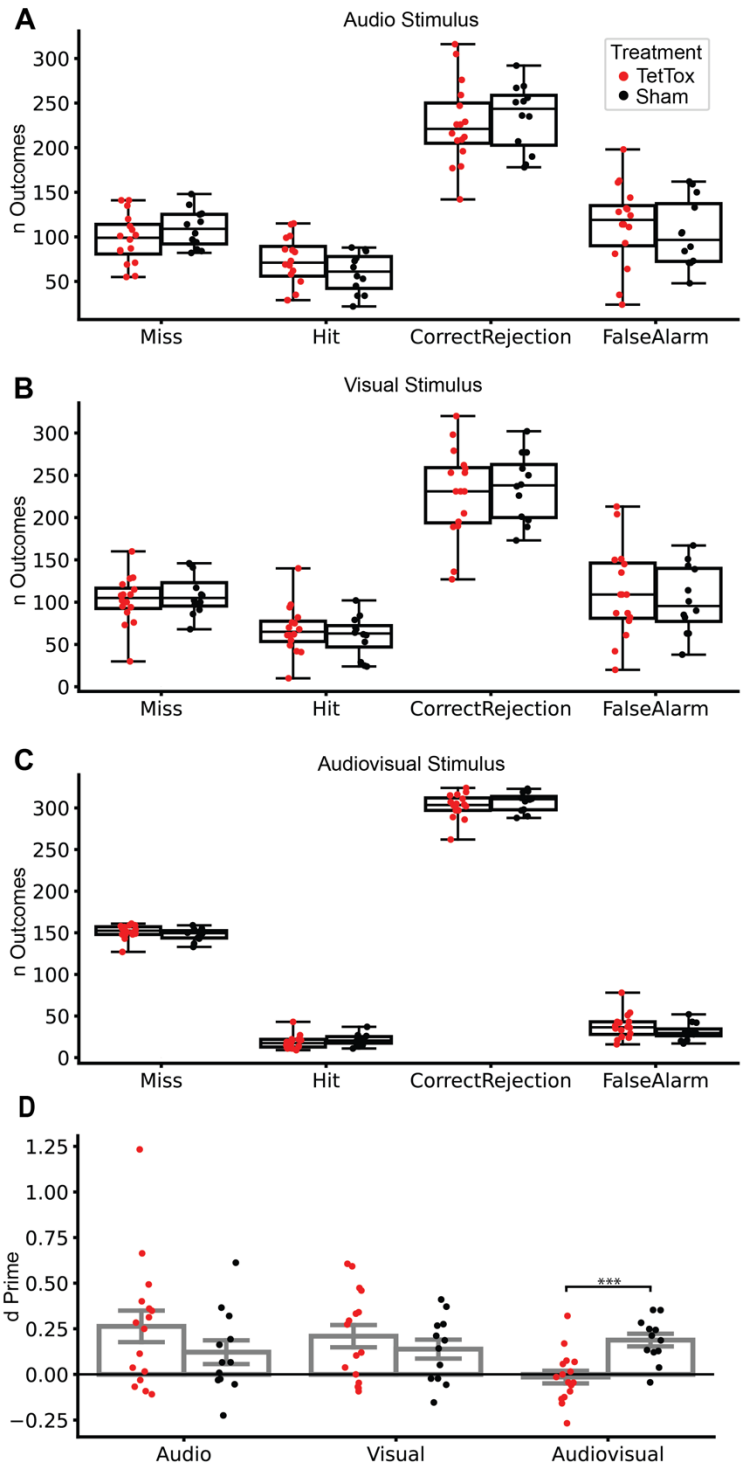


Figure 5-6. D-Prime. Box and whisker plots showing the frequency of different outcomes incorporated into the d' for audio (A), visual (B), and audiovisual stimuli (C). Boxes show the quartiles while the whiskers show the full range of the data. (D) d' values calculated separately for each stimulus type based on the outcomes above. Plots include data from experienced mice only (training sessions after day 3). Data from sham mice (n=12) are plotted in black and TetTox mice (n=16) are plotted in red. Error bars show the standard error of the mean. *** p = 0.001

exclusive and collectively exhaustive, covering all possible trial outcomes with no overlap between outcomes for each d'. The relative frequencies of the outcomes for each stimulus's d' are shown in Figure 5-6 A–C. Two-way repeated measures ANOVAs were used to compare the frequencies of each outcome. A significant effect of outcome was detected for auditory ($F(3, 78) = 85.2$, $p < 0.001$), visual ($F(3, 78) = 72.3$, $p < 0.001$), and audiovisual ($F(3, 78) = 3068.45$, $p < 0.001$) stimuli, but no significant effects of treatment or interactions were identified. Finally, the various outcomes were combined and normalized to calculate d' for each stimulus (Figure 5-6 D). A two-way repeated measures ANOVA was used to compare the effect of treatment between groups and stimulus modality within groups. The ANOVA failed to detect a significant effect of treatment but identified a significant effect of stimulus ($F(2, 52) = 3.29$, $p = 0.04$) and a significant interaction ($F(2, 52) = 5.53$, $p = 0.006$). After correcting for multiple comparisons (Sidak), post hoc testing identified a significant effect of claustrum silencing on d' for audiovisual stimuli alone ($T(25.4) = 4.14$, $p = 0.001$). This suggests that claustrum silencing specifically reduced the animal's sensitivity to multimodal stimuli without impacting their responses to unimodal stimuli.

Taken together, the results presented above demonstrate that animals in both groups were able to adapt their behaviour in response to patterns in their environment. However, the analysis identified two effects of claustrum silencing on the animals' behaviour. First, claustrum silenced animals displayed shorter latencies to poke across various outcomes and sensory modalities. Second, claustrum silenced animals displayed a specific impairment in their sensitivity to multimodal sensory stimuli.

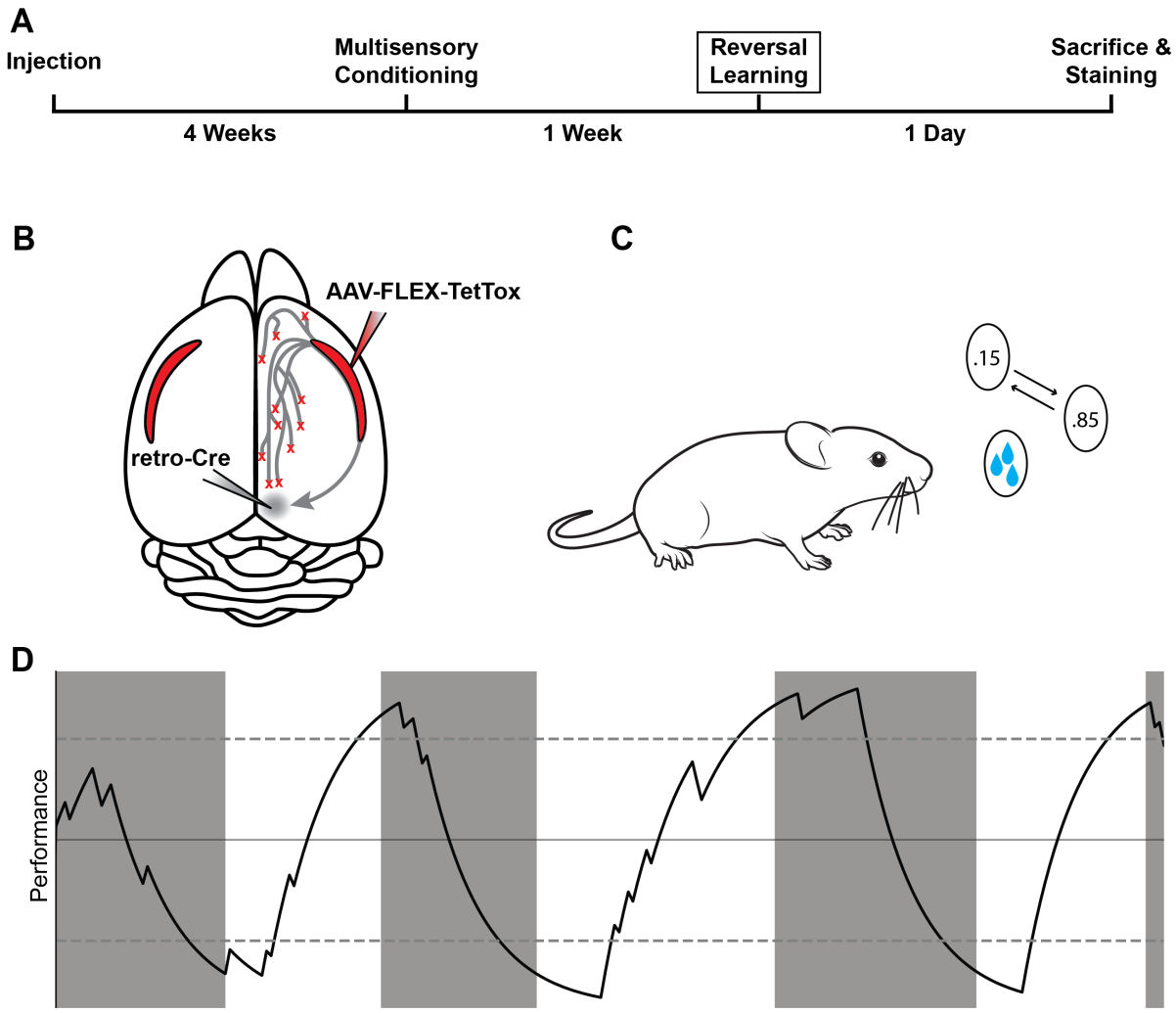


Figure 5-7. Experimental Protocol. Schematic representation showing the timeline of experiments (A), injection strategy for claustrum silencing (B), and behavioural task (C). Performance of an example mouse throughout a single session (D). Black line indicates an exponential rolling average showing the animal's side preference. Dashed lines represent the threshold for triggering a new block. Shaded areas indicate blocks.

5.2.2 Reversal Learning

After completing the multimodal conditioning task, mice were taken off water restriction for ~1 week. Animals were then returned to water restriction and trained on a reversal learning task. Figure 5-7 A, B, and C show schematic representations of the timeline, silencing strategy, and task structure. In this task, mice were trained to alter reward associations in response to a changeable hidden state of the world (i.e., the relative reward probabilities of each port). Mice learned to choose between two nose-poke ports for a chance to collect a water reward at a third port. One choice port led to a reward 85% of the time, while the other led to a reward 15%

of the time. After an individual's performance reached a threshold of 80% correct, the reward probabilities of the two choice ports would be reversed. Reward probabilities were maintained between sessions and each reversal was considered the start of a new block. Unlike in the conditioning task above, an animal's ability to obtain a reward depended entirely on their accuracy and the number of trials they performed. Figure 5-7 D shows an example of the performance of a single mouse during an example session, highlighting the animal's ability to flexibly alter its behaviour in response to the hidden state.

To better understand the animals' performance, various metrics were compared across the training period. Figure 5-8 shows the average number of trials completed (A), rewards earned (B), and blocks completed (C) across the training period. The number of trials and rewards quickly increased to a plateau. The number of blocks completed each day increased steadily throughout the training, suggesting the animals had not yet achieved full mastery of the task. One animal participated minimally in the task, completing only approximately one third as many trials as the others, and was therefore excluded from future analysis (data point not shown). Two-way repeated measures ANOVAs were used to compare the effect of treatment between groups and training day within groups. A significant effect of time was detected on the number of trials ($F(9, 225) = 58.87$, $p < 0.001$), rewards ($F(9, 225) = 57.51$, $p < 0.001$), and blocks completed ($F(9, 225) = 22.67$, $p < 0.001$), but no significant effects of treatment or interactions were detected. Collectively, these results suggest that the animals learned the structure of the task and were able to complete multiple blocks.

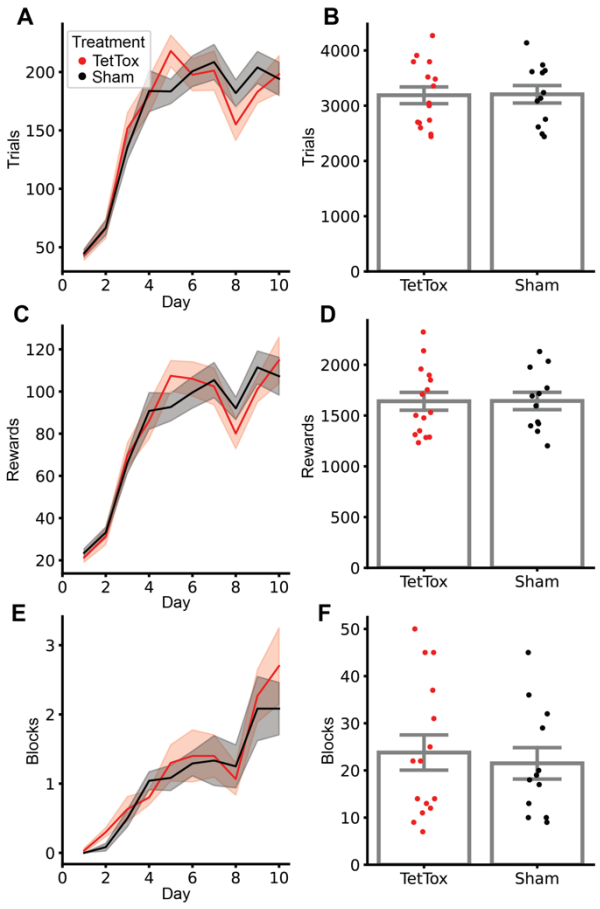


Figure 5-8. Task parameters throughout training. Graphs show the daily and overall mean number of trials completed (A, B), rewards earned (C, D) and blocks completed (E, F). Data from sham mice (n=12) are plotted in black and TetTox mice (n=15) are plotted in red. Error bars show the standard error of the mean.

As in section 5.2.1, the animals' latency to poke was examined across several conditions. Figure 5-9 A and C show the latency from the start of the trial (choice ports illuminated) until the animal poked one of the choice ports. Figure 5-9 B and D show the latency from making a choice (poking a choice port) to poking the reward port. The insets in panels A and B show a zoomed in version of the latencies from day 4 onwards. Two-way repeated measures ANOVAs were used to compare the choice and reward latencies across days. A significant effect of day was detected on both choice ($F(9, 225) = 53.58$, $p < 0.001$) and reward latency ($F(9, 225) = 34.22$, $p < 0.001$), but no effect of treatment or interaction. The latency was further segmented to compare the latencies on trials where the mice chose the high reward probability port (Figure S5-4), the low reward probability port (Figure S5-5), the same port as the previous trial (Figure S5-6), or the opposite port from the previous trial (Figure S5-7). Similar results were obtained for all comparisons. While the mean choice latency of claustrum silenced mice was consistently lower than that of the control group across all measures, this difference never reached the threshold of statistical significance. These results suggest there is no significant difference in the latency to make a choice or collect a reward between groups.

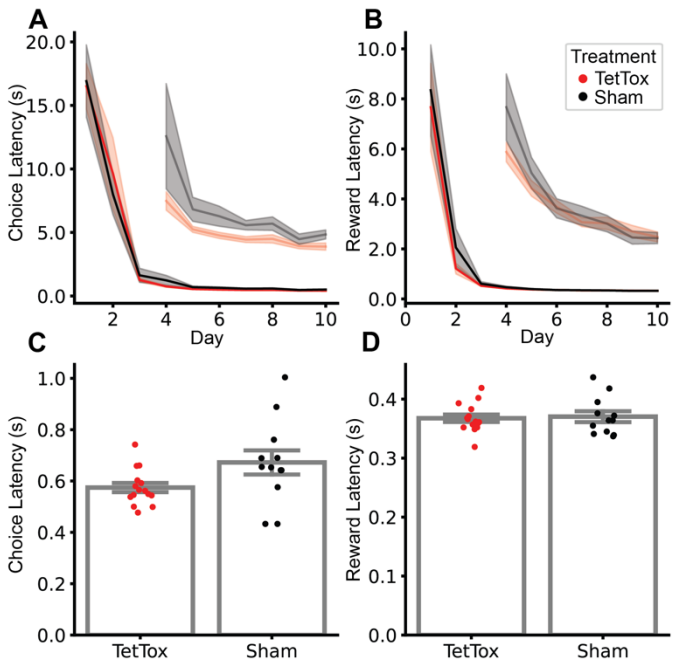


Figure 5-9. Latency to choice and reward. Daily (A) and overall (C) mean latency from trial onset until mouse poked a choice port. Daily (B) and overall (D) mean latency from poking a choice port until poking the reward port. Insets in A and B show a zoomed in version of the latency data from day 4 onwards. Data from sham mice (n=12) are plotted in black and TetTox mice (n=15) are plotted in red. Error bars show the standard error of the mean.

For optimal task performance, animals had to flexibly adjust their behaviour based on unknown reward probabilities. To assess animals' ability to transition to new blocks, the average number of trials separating blocks (i.e., the average number of trials from the start of one block until mice crossed the threshold to initiate a new block) was plotted across time (Figure 5-10 A). A mixed effects model was used instead of a two-way ANOVA to compare the number of trials per block as some mice did not complete a block every day leading to missing values. The mixed effects model detected no significant effect of treatment or day. To further investigate this behaviour, Figure 5-10 C shows the probability of a correct choice (i.e., poking the high reward probability port) aligned to the block transition. Similarly, Figure 5-10 D shows the same data plotted as the probability that the animal will poke the former high reward probability port. A two-way repeated measures ANOVA on the trials following the block transition detected a significant effect of trial ($F(25, 625) = 11.62$, $p < 0.001$), but no significant interaction or effect of treatment. These results suggest that both groups were able to adapt to changed reward probabilities.

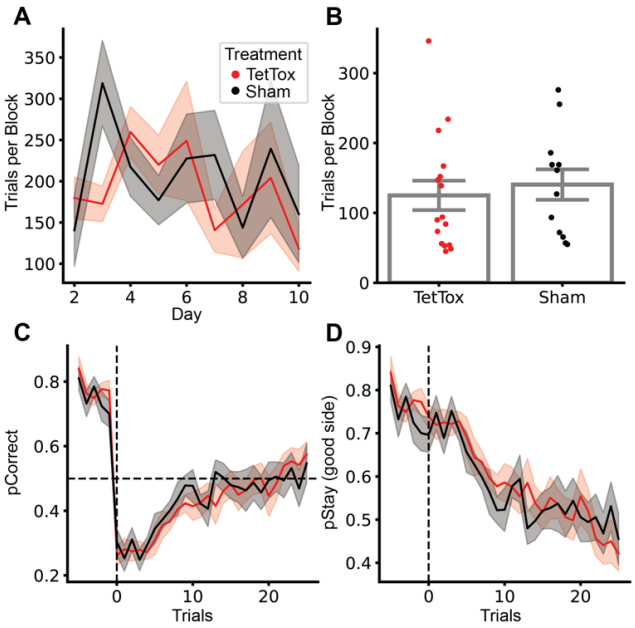


Figure 5-11. Performance on reversals. (A, B) Daily and overall mean length of a block in trials. (C) Likelihood of poking the high reward probability port in the trials before and after the transition to a new block (trial 0). (D) Data from panel C reformatted as the probability of poking the former good side in the trials before and after the transition to a new block. Data from sham mice (n=12) are plotted in black and TetTox mice (n=15) are plotted in red. Error bars show the standard error of the mean.

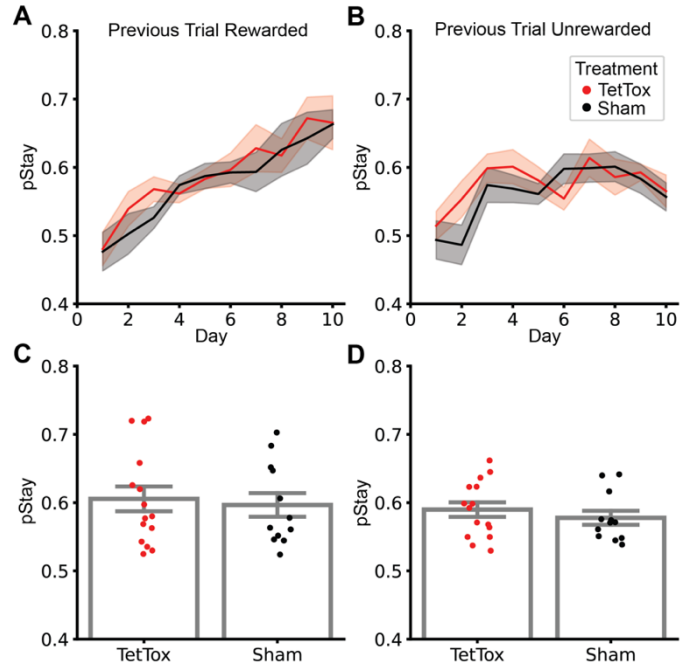


Figure 5-10. Stay vs switch behaviour. Daily and overall mean probability of choosing the same port after a rewarded trial (A, C) or an unrewarded trial (B, D). Data from sham mice (n=12) are plotted in black and TetTox mice (n=15) are plotted in red. Error bars show the standard error of the mean.

To understand the influence of reward on animals' trial-to-trial decisions, trials were classified as stay- or switch-trials depending on whether the animal chose the same port as on the previous trial. Figure 5-11 A and C show the probability of a stay trial given that the animal received a reward on the previous trial while Figure 5-11 B and D show the probability of a stay trial following an unrewarded trial. Two-way repeated measures ANOVAs detected a significant effect of day on both previously rewarded ($F(9, 225) = 12.95$, $p < 0.001$) and unrewarded trials ($F(9, 225) = 4.42$, $p < 0.001$), but no interaction or effect of treatment. These results suggest that animals in both groups learned that it was typically more advantageous to return to the same port following a rewarded trial.

Finally, to further dissect the factors influencing animals' trial-to-trial decisions, a logistic regression was performed. The regression was run individually for each animal and included only data from the final five training sessions (i.e., when the animal was most experienced at the task). The regression aimed to predict the choice on the current trial based on the previous choice (i.e., left or right), the previous outcome (i.e., rewarded or unrewarded), and the interaction of these factors. The loading of each predictor is shown in Figure 5-12. One sample t-tests showed that the previous choice (Sham: $p < 0.001$; TetTox: $p < 0.001$) and interaction coefficients (Sham: $p = 0.05$, TetTox $p = 0.007$) were significantly different from zero, but not the previous outcome coefficient. Two sample t-tests identified no significant differences between groups for any predictor. These results suggest that both groups' trial-to-trial decisions were informed by the choice on the previous trial and the interaction between the previous choice and outcome. Collectively, the results presented above suggest that both groups were able to flexibly remap their associations between action and reward in response to external cues. In contrast to the multimodal conditioning task, no significant differences were observed between groups in any

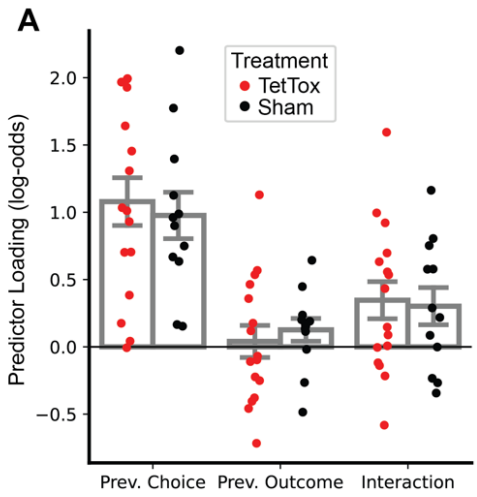


Figure 5-12. Logistic Regression. (A) Bar graph showing the results of a logistic regression using the choice and outcome of the last trial, and the interaction of these factors to predict choice on the current trial. Only data from the final 5 sessions were included in the regression. Data from sham mice (n=12) are plotted in black and TetTox mice (n=15) are plotted in red. Error bars show the standard error of the mean.

metric. This suggests that the behavioural effects of claustrum silencing are complex and highly specific.

5.2.3 Histology

To confirm claustrum silencing, tissue sections from all TetTox mice were assessed for expression of the TetTox virus by staining for the FLAG tag encoded by the virus (Figure 5-13). Histology from all experimental animals can be found in Supplementary Figure S5-8. The stain also included antibodies against MBP and PV (PV) to facilitate identification of the anatomical location of the claustrum. Based on the results presented in section 4.2.5, bilateral expression of the FLAG tag was expected in the claustrum (as identified by the PV plexus and the paucity of MBP staining), along with mCherry from the retro-iCre in both the claustrum and dorsal cortex. However, staining revealed neither FLAG nor mCherry expression in the claustrum (Figure 5-13 B) but strong bilateral mCherry expression in the dorsal cortex (Figure 5-13 C) of all TetTox mice. To confirm that the antibody staining was effective, the same primary antibody, secondary antibody, and blocking solutions used to stain

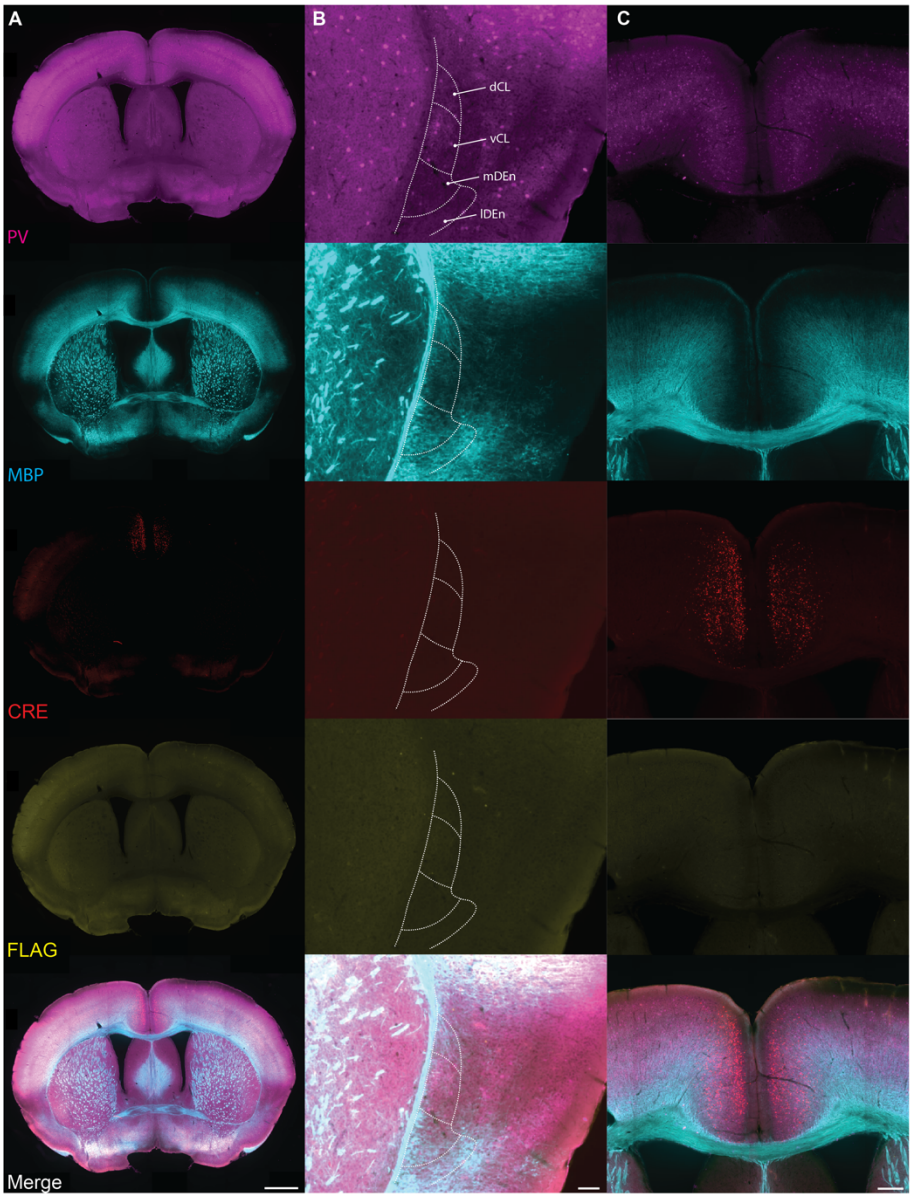


Figure 5-13. TetTox Histology. Immunofluorescent staining of TetTox injected brain tissue showing parvalbumin (PV, magenta, top), myelin basic protein (MBP, cyan, top centre), retro-iCre-mCherry (CRE, red, centre), FLAG (yellow, bottom centre), and overlay (bottom). Staining shows the full slice (A), a zoomed in view of the claustrum (B), and a zoomed in view of the dorsal midline (C). Scale bars are 1mm, 250um and 100um. Panel B is oriented with dorsal at the top of the frame and lateral on the right. Abbreviations: dorsal claustrum (dCL), ventral claustrum (vCL), medial DEn (mDEn), lateral DEn (lDEn)

the TetTox tissue were recovered and used to re-stain tissue with known FLAG expression (see Chapter Four). This second stain revealed the expected FLAG expression, suggesting that the antibody staining was effective (data not shown).

These unexpected and somewhat paradoxical results suggested two possible options: first, that the viral injections had failed to yield TetTox expression, or second, that the combination of viruses employed for this experiment proved cytotoxic, killing dual-infected claustrum cells. To elucidate these possibilities, tissue from TetTox and sham mice was next stained for GFAP, a marker of previous damage or inflammation. This staining revealed strong expression of GFAP in the region of the claustrum for TetTox mice with limited GFAP staining in sham mice (Figure 5-14). Quantification of the fluorescence intensity in the claustrum revealed significantly greater GFAP expression in the claustrum of TetTox injected mice (unpaired Welch's t-test; $p = 0.002$). Finally, additional TetTox tissue was stained for NeuN, a neuron specific marker. However, the age of the tissue at this time prevented effective NeuN staining, thereby precluding neuronal cell counting. While the causes of these unexpected results cannot be determined with complete certainty, the specific lack of mCherry expression in the claustrum, combined with the strong GFAP staining and positive behavioural phenotype, support the hypothesis that the viral injections proved cytotoxic.

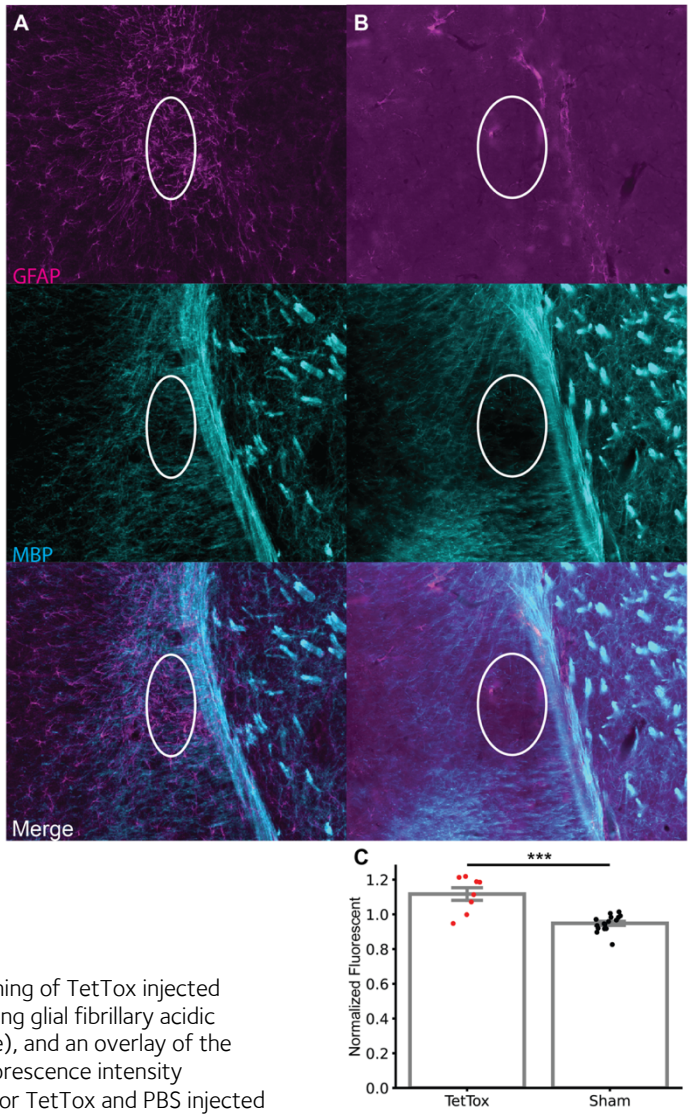


Figure 5-14. GFAP Histology. Immunofluorescent staining of TetTox injected brain tissue (A) and PBS injected brain tissue (B) showing glial fibrillary acidic protein (GFAP; top), myelin basic protein (MBP; middle), and an overlay of the two (bottom). (C) Graph showing claustrum GFAP fluorescence intensity normalized against whole slice fluorescence intensity for TetTox and PBS injected brain tissue Dots show the normalized fluorescence of a coronal section. Panels A and B oriented with dorsal at the top of the frame and lateral on the left.

5.3 Summary

Taken together, the results presented above demonstrate that animals in both groups were able to adapt their behaviour in response to patterns in their environment. In the reversal learning task, animals in both groups were able to flexibly remap their associations between action and reward in response to a hidden state of the world. However, the analysis identified two effects of claustrum silencing on the animals' behaviour on the multimodal conditioning task. First, claustrum silenced animals displayed shorter latencies to poke across various outcomes and sensory modalities. Second, claustrum silenced animals displayed a specific impairment in their sensitivity to multimodal sensory stimuli.

These results, in context of the results from Chapter Two, support a role for the claustrum in multisensory processing. However, further evidence is needed to understand the factors underlying the observed differences. While claustrum silenced mice performed differently on multimodal trials, this does not necessarily mean that claustrum silencing had any impact on their ability to perceive or act upon multimodal stimuli. Future experiments probing the impact of claustrum silencing on different sensory detection tasks may provide further insight into the function of the claustrum.

5.4 Acknowledgements & Contributions

The experiments in this chapter benefited from contributions by Prof Mark Walton (MW), Dr Thomas Akam (TA), and Dr Anna Hoerder-Suabedissen (AHS). TA built the behavioural boxes used in this chapter and wrote the PyControl software used to control them. TA and MW provided guidance in designing the behavioural tasks. TA provided guidance and supervision during data collection and analysis. AHS provided guidance for the histological experiments. The schematics in Figures 5-1 and 5-7 B and C were created using elements originally drawn by Dr Andrew Shelton. Imaging of post-hoc histology was completed with guidance from Dr Cyril Lai at the Wolfson Imaging Centre in the MRC Weatherall Institute of Molecular Medicine.

6 Discussion

In this thesis I have described experiments examining a wide range of topics relevant to the function of the claustrum. Using a novel method for recording the activity of the claustrum, I identified stimulus-locked claustrum activity following uni- and multimodal sensory stimuli. I then examined the effect of claustrum and claustrum complex silencing on a range of basic behaviours, finding no meaningful effects. Finally, I examined the impact of claustrum silencing on a classical conditioning-like task, and a reversal learning task. While claustrum silencing had no impact on the reversal learning task, I found that claustrum silenced mice had reduced latencies to poke and reduced sensitivity to multimodal stimuli. The following sections will place these findings in the context of previous studies and discuss the limitations of these experiments.

6.1 In-vivo Axon Imaging

Chapter Two describes a novel technique for recording claustrum activity *in vivo* using two-photon calcium imaging of claustrum axons in the cortex. This section will discuss some of the advantages and limitations of this technique.

The inaccessible location of the claustrum deep within the brain has presented a challenge to those attempting to record its activity. To circumvent this issue, several studies have employed inserted devices to facilitate direct optical recording or manipulation of the claustrum[19,26,123–126,148]. While effective, these methods have major drawbacks. First, the elongated shape of the claustrum means that implanted devices are restricted to recording activity from only a small section of the claustrum. Given the anteroposterior connectivity gradients present in the claustrum, the anteroposterior position of the implanted device may bias any recordings[13]. Second, the implanted devices can cause widespread damage to the brain. Devices implanted above the claustrum can severely damage the overlying cortex, potentially causing behavioural changes. Moreover, the implanted device may sever connections running to and from the claustrum, potentially altering claustrum activity. This may be especially problematic as the claustrum is located adjacent to a major white matter tract. Finally, some techniques like fibre photometry do not provide single cell resolution, instead providing bulk activity. The axon imaging approach described here may mitigate some of these issues.

Implanting a cranial window to record the activity of axons in dorsal cortex provides single axon resolution with a less invasive surgery. This should preserve the endogenous connectivity and activity of the claustrum and reduce off target behavioural effects caused by cortical damage. In addition, the axons present in the cortex may arise from cells across the dorsoventral and anteroposterior extent of the claustrum.

The axon imaging technique is easily scalable and can be adapted to various experimental requirements. As demonstrated here, the technique is amenable to multimodal stimulation, and could easily be adapted to any head-fixed behavioural task. Moreover, a similar preparation has been used in the lab for manipulating claustrum activity while recording from cortical cells by expressing an opsin in claustrum axons and a calcium indicator in the cortex. This technique could be further extended to allow for simultaneous recording of both claustrum axons and downstream cortical cells using multicolour calcium imaging. In short, the axon imaging technique described here presents an adaptable and scalable system for minimally invasive claustrum recordings at single-axon resolution.

Despite these advantages, there are several major drawbacks to this technique. First, this technique biases the population of claustrum cells recorded. While this technique theoretically allows researchers to record from projection neurons throughout the dorsoventral and anteroposterior extent of the claustrum, the actual distribution of the recorded cells within the claustrum remains unknown. By recording from cortical axons, this technique limits recordings to cells that project out of the claustrum and erases the spatial organization of cells within the claustrum. While the location of the axons recorded within the cortex may provide some hint to their identity, the length and branched character of claustrum axons means that axon segments recorded in one cortical region do not necessarily synapse in that region and may simply be passing through *en route* to their eventual target.

In mice, the claustrum is most densely connected with brain regions in the dorsal midline[10,13,19]. Placing the window over this area should therefore allow the recording of many axons. However, any recorded axon must both originate in a claustrum cell which projects to the ACC or RSP and traverse the upper layers of the cortex visible through the cranial window. The length and branched character of claustrum axons means that a large segment of claustrum cells may meet these criteria, but these axons represent a projection-restricted subset of claustrum cells and may overrepresent certain functional submodules within the claustrum[14,72,146].

In addition to these biological issues, this technique is hampered by several technical constraints. First, it can be difficult to locate axons within the cortex. Using the setup as described in Chapter Two, axons are only visible when active. To avoid recording areas without claustrum axons, recording fields of view were selected based on visible axon expression. While more efficient than randomly sampling the cortex and hoping to record axons, this technique will bias the recorded population towards axons that are either tonically active or responsive to the conditions (i.e., sounds and sensations) present during head-fixed imaging. Future studies may be able to avoid this issue by employing a calcium indicator coupled to a fluorophore[166]. The shape of axons will also bias recordings, as axons parallel to the imaging plane are easier to identify during live imaging. Axons travelling perpendicular to the imaging plane may therefore be underrepresented in this study. Furthermore, despite efforts to avoid recording from the same field of view more than once, the length and branched character of claustrum axons make it impossible to rule out the possibility that multiple independent recordings came from the same claustrum axon.

Experiments using this technique are also relatively low throughput. When using calcium imaging to record from cells, each recorded field of view may include hundreds or thousands of individual cells. In contrast, several fields of view in this study included only a single identifiable claustrum axon. This greatly reduces the range of behavioural paradigms that can be used. The longer the recording that is required at each field of view, the longer it will take to record from a sufficient sample size. Moreover, the behavioural task must be compatible with moving the field of view multiple times within a recording session. Finally, while Suite2P was able to automatically select axonal regions of interest, these selections required extensive manual curation. This introduces further bias and greatly increases the time required for analysis.

Overall, the usefulness of this technique will depend on the biological question being asked. This study only involved a relatively short stimulation paradigm that could be repeated for many fields of view. Expanding this study to include more complex or varied stimuli would greatly increase the time required to collect and analyze the data, reducing the feasibility of such an experiment.

6.2 Sensory responsive neurons in the claustrum

The existence of sensory responsive neurons in the claustrum remains controversial. Numerous experiments have reported sensory responsive neurons in rodents and primates[75,117–119,121]. Within papers that reported the existence of sensory responsive cells, the existence of multisensory responses remains controversial[75]. In contrast to the primate and cat studies, two recent studies in mice have found few sensory responsive cells with one paper reporting only 5% of cells were responsive to passive sensory stimulation[123,124]. The results presented in Chapter Two are strikingly different. First, nearly half of all axons recorded were responsive to one or more sensory modalities. Second, among sensory responsive axons, most displayed multisensory response pattens and many responded exclusively to multimodal trial types. However, several factors may help to explain this difference.

The techniques used to record from the claustrum in Chapter Two were very different from those used in recent studies reporting few sensory responsive cells. Chevee et al. used implanted electrodes to record from S1 projecting cells in the anterior claustrum[123]. Ollerenshaw et al. used an implanted optical device to record from the anterior claustrum in GNB4-Cre transgenic mice[124]. The responses presented in Chapter Two were recorded from ACC or RSP projecting cells located throughout the claustrum. These experiments differed in both the techniques used to label the claustrum, and the spatial location of the cells within the claustrum. Studies reporting sensory responsive cells identified in cats and primates were primarily located in central and posterior regions of the claustrum. The ability to record the activity of axons originating from cells distributed throughout the anteroposterior extent of the claustrum may explain the increased incidence of sensory responsive cells identified in Chapter Two. Moreover, it is possible that the genetically and projection-defined populations recorded in Ollerenshaw et al. and Chevee et al. had little overlap with the projection-defined population of claustrum cells recorded in Chapter Two. In addition, these studies employed different sensory stimuli (i.e., single vs full pad whisker stimuli, white noise vs complex tone). While none of these studies presented a sufficient variety of sensory stimuli to investigate how the properties of the stimulus impacted claustrum responses, it is possible that the stimuli presented in Chapter Two were better able to drive activity in the claustrum. These results highlight the challenges

associated with recording from the claustrum, and the utility of employing multiple different techniques.

It is also interesting to consider what exactly these sensory-locked responses represent. The claustrum does not receive direct sensory input and has relatively little connectivity with primary sensory cortices[10,13]. Importantly, although axons did show significant responses to sensory stimuli on average, these responses were inconsistent following individual stimulus presentations. While the sensory-locked responses might represent the physical features of the stimuli, they might instead represent the behavioural impact of the stimuli upon the mice. A study investigating auditory responses recorded in visual cortex found that these auditory responses encoded little sensory information and were tightly linked to small body movements[167]. Previous work has linked claustrum activity with motor processes, highlighting the possibility that the observed sensory-locked activity did not convey sensory information but was instead related to downstream processes driven by the sensory stimuli[123]. Further investigation is required to understand what behavioural states and stimulus features drive neural activity in the claustrum.

The sensory-locked responses observed in Chapter Two are compatible with various functional hypotheses for the role of the claustrum. Several lines of evidence have linked the claustrum with salience processing[146]. It is conceivable that observed sensory responses are not 'sensory' *per se*, but instead represented the salience of stimuli. To a head-fixed mouse in a light attenuating box with white noise masking background sounds, unpredictable whisker stimulation may be highly salient, driving claustrum activity. Other studies have linked the claustrum to attention and distraction[25,148,168]. Rather than conveying sensory information, sensory-locked claustrum activity may be driven by sensory-evoked shifts in attention. Finally, the claustrum has been linked to multisensory integration. While the evidence presented in Chapter Two does not provide clear evidence that perceptual binding or multisensory integration occur in the claustrum, these results motivate further research.

6.3 CLCX:EDGE Transgenic line

The experiments presented in Chapters Three and Four involved the novel Claustrum Complex Enhancer Driven Gene Expression (CLCX:EDGE) transgenic line. Figure 4-8 highlights the differences between a CLCX:EDGE based labelling strategy and a retro-Cre based labelling

strategy. Based on these results, it appears that CLCX:EDGE based expression is primarily restricted to the medial DEn with limited expression in the ventral claustrum. This is in sharp contrast to the RSP-projecting population located primarily in the ventral claustrum. The lack of overlap observed between these methods of labelling the claustrum reduces the applicability of results obtained in CLCX:EDGE mice to other models. Further, as CLCX:EDGE expression was found primarily in the dEN, this line has limited utility for studying the claustrum alone. However, the CLCX:EDGE line has several important strengths. First, like any transgenic, experiments using the CLCX:EDGE line are less labour intensive than those using projection-defined strategies to label the claustrum. Second, the CLCX:EDGE line provides genetically restricted access to a little-understood population of cells within the dEN. As research on the claustrum advances, it will be important to understand the function of the claustrum in isolation and within the broader claustrum complex. Genetic lines like CLCX:EDGE may be useful for understanding the function of the claustrum complex and its constituent parts.

The experiments presented in Chapters Three and Four are among the first behavioural experiments involving the CLCX:EDGE line. In Chapter Three, two groups of CLCX:EDGE mice were assessed on a battery of common behavioural tests. Both groups displayed expected behaviour on most tests. However, neither group displayed the expected decrease in exploratory behaviour during repeated habituation trials on the Y-maze (Figure 3-5)[169]. In addition, the experiments described in Chapter Four detected several behavioural differences — including increased locomotor activity — between CLCX:EDGE mice and wild type C57 mice at baseline. Further experiments investigating the baseline behavioural phenotype of CLCX:EDGE mice will be required to fully characterize any potential off-target effects of the transgene insertion.

6.4 Chronic claustrum silencing with Tetanus Toxin

Manipulating neural activity can provide causal evidence for the function of a brain region. This can be done both chronically, by killing or silencing cells in the brain region, or acutely using opto- or chemogenetic tools. There are advantages and disadvantages to both chronic and acute lesions[95]. The effects of chronic lesions can be masked by compensation via plasticity and redundancy, but acute lesions can lead to short-lived network responses that obscure real effects. In Chapters Three through Five a virally encoded tetanus toxin light chain construct

(TetTox) was used to chronically silence claustrum neurons. TetTox silences neural output by cleaving synaptobrevin, thereby preventing vesicle release at the synapse[170–172]. In contrast to techniques like diphtheria toxin, which kill infected cells, TetTox mediated silencing has been shown to induce less inflammation in nearby tissue[173]. TetTox has been used in numerous studies and has repeatedly been shown to silence neural activity[122,170–175]. The efficacy of TetTox mediated claustrum silencing was also confirmed electrophysiologically prior to starting behavioural experiments. Briefly, mice received intracranial viral injections in the claustrum of either opsin or opsin mixed with TetTox, along with injections of retro-Cre in the RSC. After allowing time for viral expression, whole-cell patch-clamp electrophysiological recordings were taken from neurons in the RSC. While numerous light-evoked post synaptic potentials were observed in animals that received injections of opsin alone, only one post synaptic potential was recorded in an animal that received injections of opsin mixed with TetTox. These results suggest that TetTox based silencing effectively suppresses claustro-cortical communication.

The virus used in this study also allowed for identification of cells expressing TetTox with post hoc histology. In Chapters Three and Four, staining revealed robust bilateral TetTox expression in all but one animal. However, in Chapter Five staining revealed no TetTox expression. As discussed in section 5.2.3, several lines of evidence suggest that the combination of retro-Cre and TetTox used in Chapter Five caused widespread cell death among co-infected cells. First, cells in the claustrum were expected to co-express TetTox and a fluorophore encoded by the retro-Cre virus. While the fluorophore was present in other areas of the brain, it was specifically absent in the claustrum. This suggests that the retro-Cre virus was injected successfully in all animals. Second, increased GFAP staining was noted in the area surrounding the claustrum in TetTox injected mice. This suggests previous inflammation, possibly resulting from local cell death. Finally, the behavioural experiments in Chapter Five returned a positive result. While the possibility that the viral injections in Chapter Five failed cannot be ruled out, available evidence supports the hypothesis that claustrum silencing was achieved through an unintentional claustrum lesion.

This unintentional claustrum lesion has two major consequences for the analyses presented in this thesis. First, the local inflammation caused by the claustrum lesion may have had off-target behavioural effects on nearby regions[173]. Replicating the behavioural results presented in Chapter Five using a different claustrum silencing strategy would ameliorate this concern.

Second, the occurrence of cell death following TetTox expression precludes quantitative histology. Had no cell death occurred, analyses correlating the strength of a behavioural effect in an individual mouse with the number of cells silenced could provide further support for any putative effect of claustrum silencing. However, if cell death has occurred, any such correlations would be useless as the number of histologically labelled cells would not represent the true extent of claustrum silencing. Despite these limitations, the available evidence suggests that the observed behavioural effects are the result of claustrum silencing.

6.5 Behavioural effects of claustrum silencing

Investigating the consequences of claustrum silencing may shed light on the function of this enigmatic brain region. This thesis has employed two complementary methods for silencing the claustrum using tetanus toxin. First, a transgene-dependent TetTox virus was used to silence the claustrum complex in a novel transgenic line. Second, a Cre-dependent TetTox virus was used to silence the claustrum in mice co-injected with retro-Cre in the RSP. As these two strategies labelled different populations of neurons within the claustrum complex, results should not be generalized between the two. This section will discuss the behavioural effects of these manipulations and their implications for the function of the claustrum.

Many previous studies have investigated the consequences of claustrum silencing. However, most of these studies have employed acute silencing[26,80,123,125,126,148]. Results have been mixed among recent studies employing chronic silencing, with studies reporting diverse effects of claustrum silencing on spatial reversal learning, acquisition of a conditioning task, resilience to distraction, and cocaine-induced locomotor behaviour[25,80,122,150]. This thesis aimed to take a more holistic approach by assessing the impact of silencing on a broad range of behaviours before progressing to more specific behavioural tasks.

The behavioural battery described in Chapter Three suggested that claustrum complex silencing had no significant effects on locomotor behaviour, motor coordination, social interaction, spatial learning, general cognitive function, memory, anxiety, or sensory gating. These results provide the first comprehensive characterisation of the effects of claustrum complex silencing on a wide range of behavioural domains. Specific studies have linked the claustrum to various of these behavioural domains, including motor processes, spatial processing, anxiety, and sensory gating[123,126,149,176]. While the results of Chapter Three do not

support these links, several factors may explain this discrepancy. First, the cell population investigated in the claustrum complex was labelled using a novel transgenic line. Earlier studies linking the claustrum complex to various behavioural processes may have observed effects driven by different cell populations within the claustrum complex. Second, previous studies have suggested that the behavioural effects of the claustrum can only be seen during more complex tasks[25,113,148]. While the behavioural tests used in Chapter Three cover a wide range of behavioural domains, they do not require complex cognitive abilities. Effects of claustrum complex silencing may emerge in more cognitively demanding tasks.

Chapter Four investigated the effects of claustrum and claustrum complex silencing on home-cage behaviour. Several lines of evidence have linked the claustrum to sleep[6,19,20,128]. No effects of claustrum or claustrum complex silencing on home-cage locomotion, activity fragmentation, circadian behaviour, or anxiety were detected. While these experiments do not provide evidence for a role of the claustrum in sleep, more detailed data is required. Had claustrum silencing led to insomnia or deficits in initiating and/or maintaining sleep states, such changes might have led to large effects visible in the home-cage monitoring data. However, more subtle changes would only be detected using electroencephalography. Chapter Four also included an exploratory analysis of the consequences of claustrum silencing on stress-induced anxiety-like behaviour. While no differences were detected in this study, the sample size used in these tests may not have been sufficient given the variability associated with behavioural tests of anxiety.

These results served as a foundation for more complex tests: a multimodal conditioning task and a reversal learning task. No effects of claustrum silencing were detected on the reversal learning task. However, on the multimodal conditioning paradigm, claustrum silencing reduced poke latencies and specifically reduced animals' sensitivity to multimodal stimuli. Previous studies have assessed the effects of both chronic and acute claustrum silencing on reversal learning with mixed results. Grasby et al. found that excitotoxic lesions to anterior claustrum decreased performance on a spatial reversal learning task in rats[177]. Fodoulian et al. found no effect of claustrum inhibition on reversal learning in VGLUT2-Cre transgenic mice but did identify effects on attentional set shifting[26]. These results may suggest a nuanced role for the claustrum in cognitive flexibility that can only be identified under specific behavioural constraints. It should also be noted that the mice tested on the reversal learning task in Chapter

Five did not achieve full mastery of the task. As such, it is possible that this study did not identify deficits occurring at later stages of the reversal learning task.

In addition to the reversal learning task, Chapter Five included a novel classical conditioning-like multimodal behavioural task. The claustrum has frequently been linked to multisensory processing, but recent studies have found little evidence of multisensory activity in the claustrum[16,75,123,124,178]. In contrast to these experiments, analysis of data from the multimodal conditioning task revealed a specific effect of claustrum silencing on multimodal trials. On this task, the mean d' for the TetTox group was approximately zero, meaning that the hit rate was approximately equal to the false alarm rate. In other words, the TetTox group was equally likely to poke the audiovisual port after an audiovisual stimulus as they were after an audio or visual stimulus. While this may provide evidence that the claustrum is involved in multisensory processing, careful examination of the task is warranted. Interestingly, Garcia et al. also noted an effect of TetTox based claustrum silencing on an eye-blink conditioning task in rabbits. The authors reported that bilateral claustrum silencing delayed acquisition of the conditioned response but did not affect animals' ultimate performance. Given this result, it is conceivable that TetTox animals' performance on audiovisual trials might have improved with additional training.

Unlike many sensory detection tasks, the multimodal conditioning task did not force mice to report a choice in order to earn a reward. Rewards were available regardless of the actions of the mouse. In this way, the task did not force mice to learn an association between two stimuli, but instead tested what information from their environment mice chose to use to guide their behaviour. The mice could have received an equal amount of water by poking all three ports randomly once every minute. Instead, the sham group used information from both unimodal and multimodal trials while the TetTox group learned to use information from unimodal but not multimodal trials. While this decreased use of multimodal information could be due to decreased ability of TetTox animals to perceive multimodal stimuli and link them with actions, this is not the only explanation for their behaviour. For example, this result might also be attributed to differences in the complexity of the trials, or animal's motivation to earn rewards. Further experiments are required to understand the factors underlying this difference.

6.6 Summary

Throughout this thesis, I have described a series of experiments investigating the function of the claustrum. I began by investigating the sensory response properties of the mouse claustrum. To do this, I developed a novel technique for recording the activity of the claustrum. With this technique, I identified a population of sensory responsive claustrum cells. Claustrum cells displayed stimulus-locked responses to auditory, visual, and tactile stimuli, as well as combinations of these modalities. Among responsive cells, few were exclusively responsive to any one trial type, and many were responsive to multimodal stimuli. I next described a series of experiments investigating the behavioural consequences of claustrum and claustrum complex silencing. I began by investigating the impact of silencing on basic ethologically motivated behavioural tests, home-cage activity, and circadian patterns. Finding no effects, I then investigated the impact of silencing on a reversal learning task and a multimodal conditioning task. While I found no impact of silencing on the reversal learning task, I found that claustrum silencing led to reduced trial latencies and reduced sensitivity to multimodal stimuli in the conditioning task. This thesis contains many negative results and raises many imponderable questions. Nonetheless, it is a step towards understanding the function of the claustrum.

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I did what was within my range of doing.

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9 Appendix I — Supplementary Figures

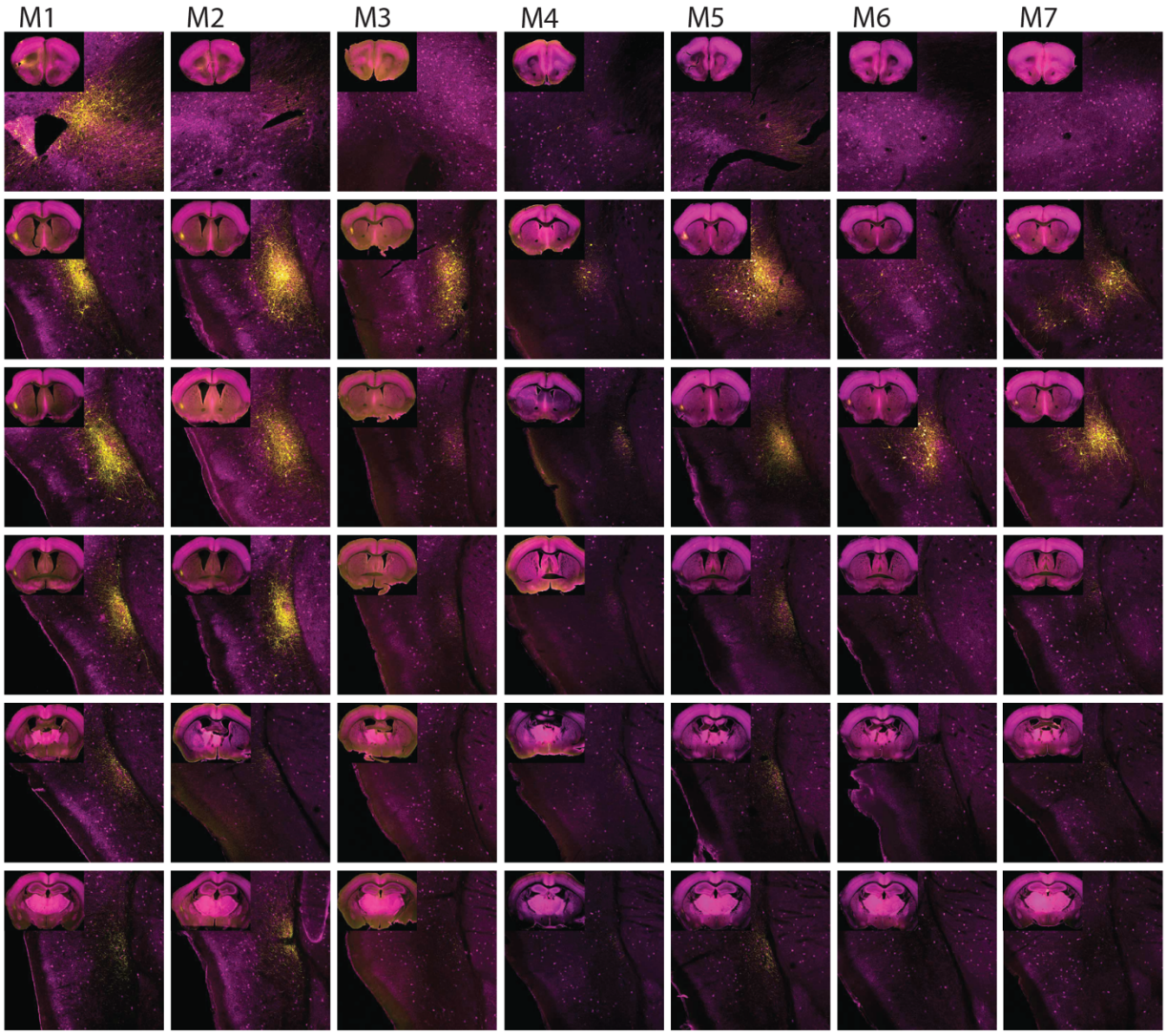


Figure S2-1. Histology. Histological images from all experimental mice Each column corresponds to a mouse. Each row within a subcolumn shows the area surrounding the claustrum right hemisphere of a coronal section. Inset shows full slice image. Sections were spaced 500um apart with the second section located at the union of the corpus callosum. Sections are in order with the most anterior slice in the top row. Parvalbumin is shown in magenta and GFP is shown in yellow.

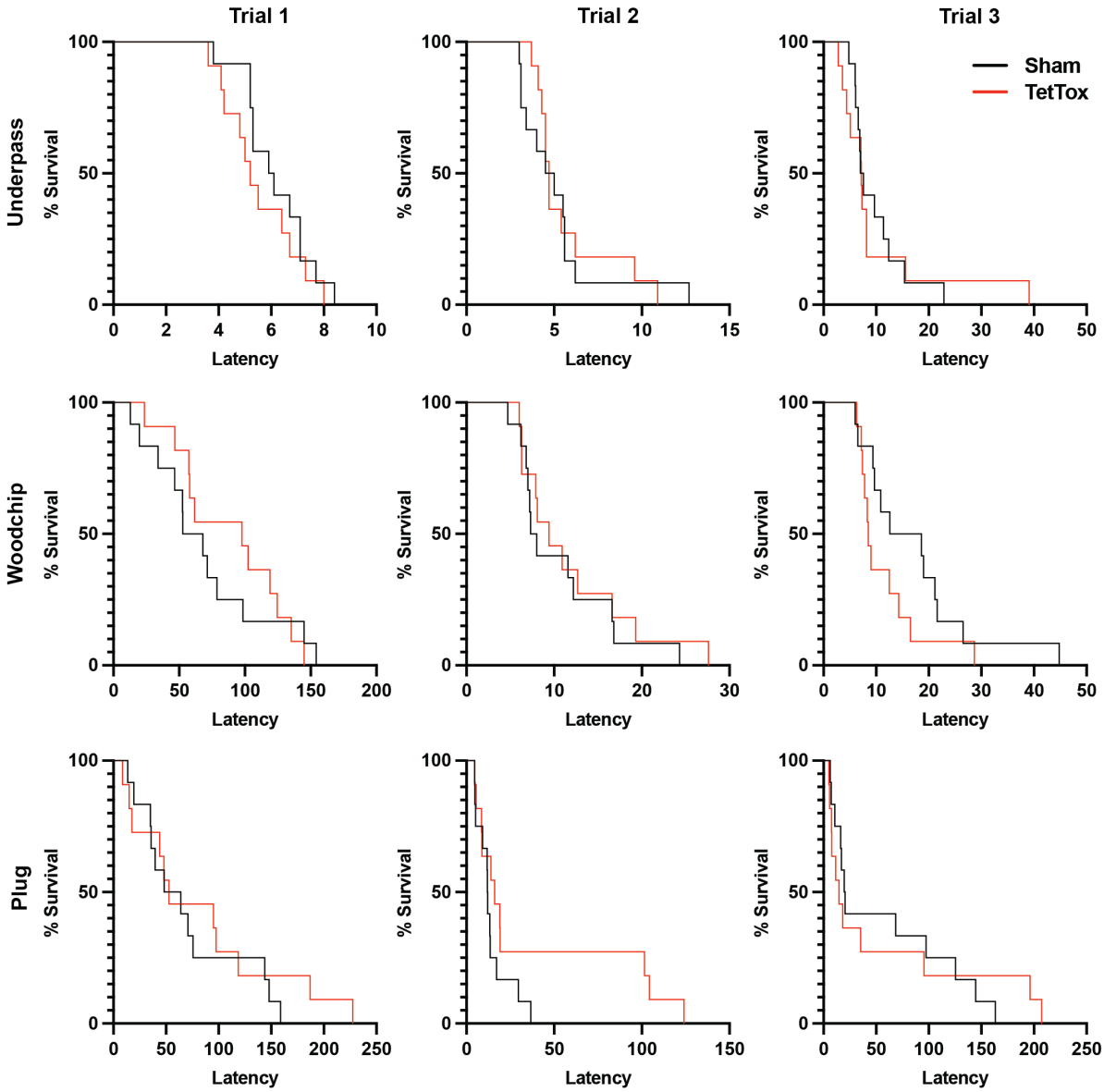


Figure S3-1. Puzzle box. Survival curves showing percentage of animals remaining at each latency on the puzzle box test. Data from sham mice (n=12) are plotted in black and TetTox mice (n=11) are plotted in red. Error bars show the standard error of the mean.

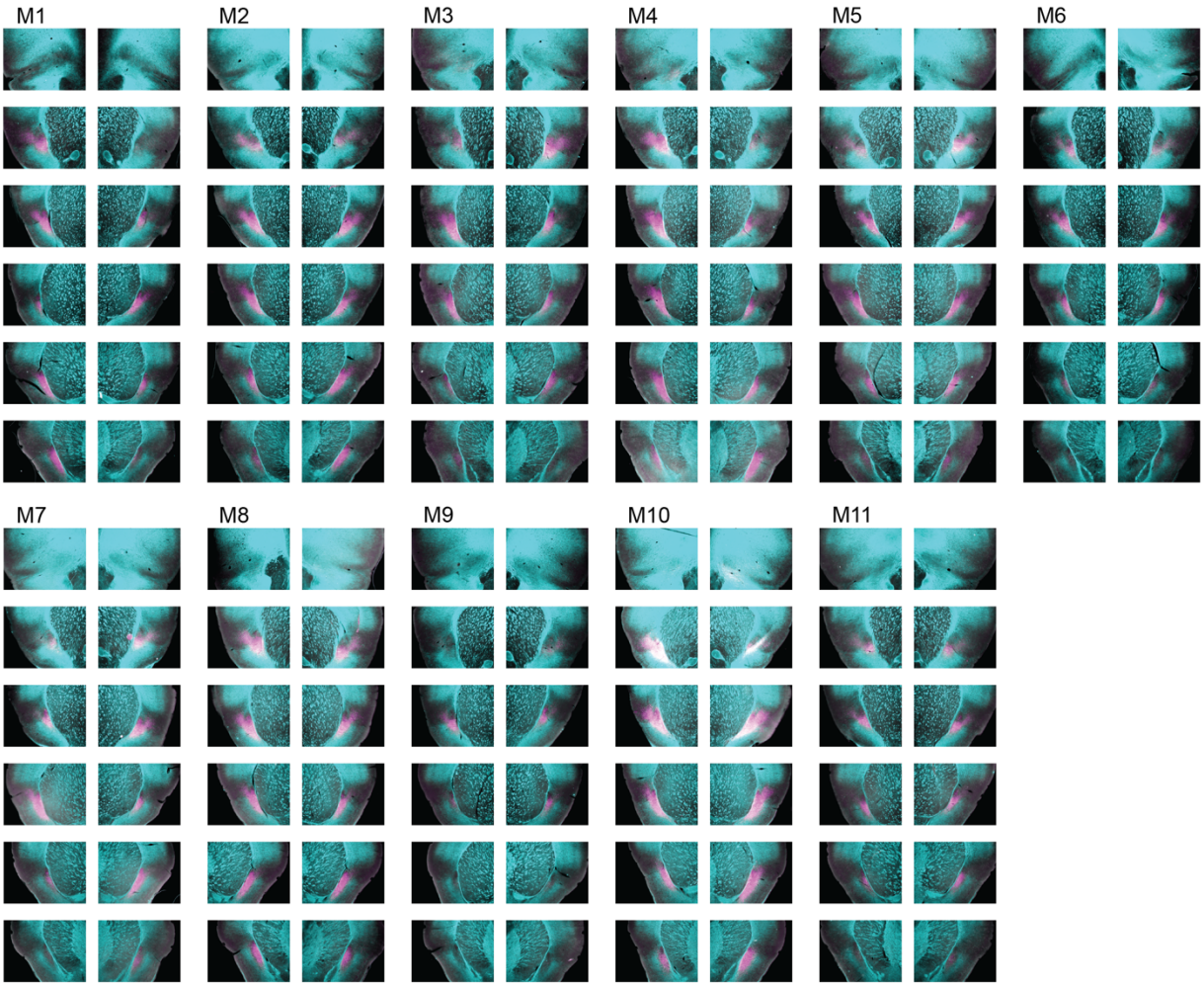


Figure S3-2. Histology. Images display bilateral histology from all experimental animals. Each subcolumn corresponds to a mouse. Each row within a subcolumn shows the area surrounding the claustrum in the left and right hemispheres of a coronal section. Sections were spaced 500um apart with the second section located at the union of the corpus callosum. Sections are in order with the most anterior slice in the top row. Myelin basic protein is shown in cyan and TetTox is shown in Magenta.

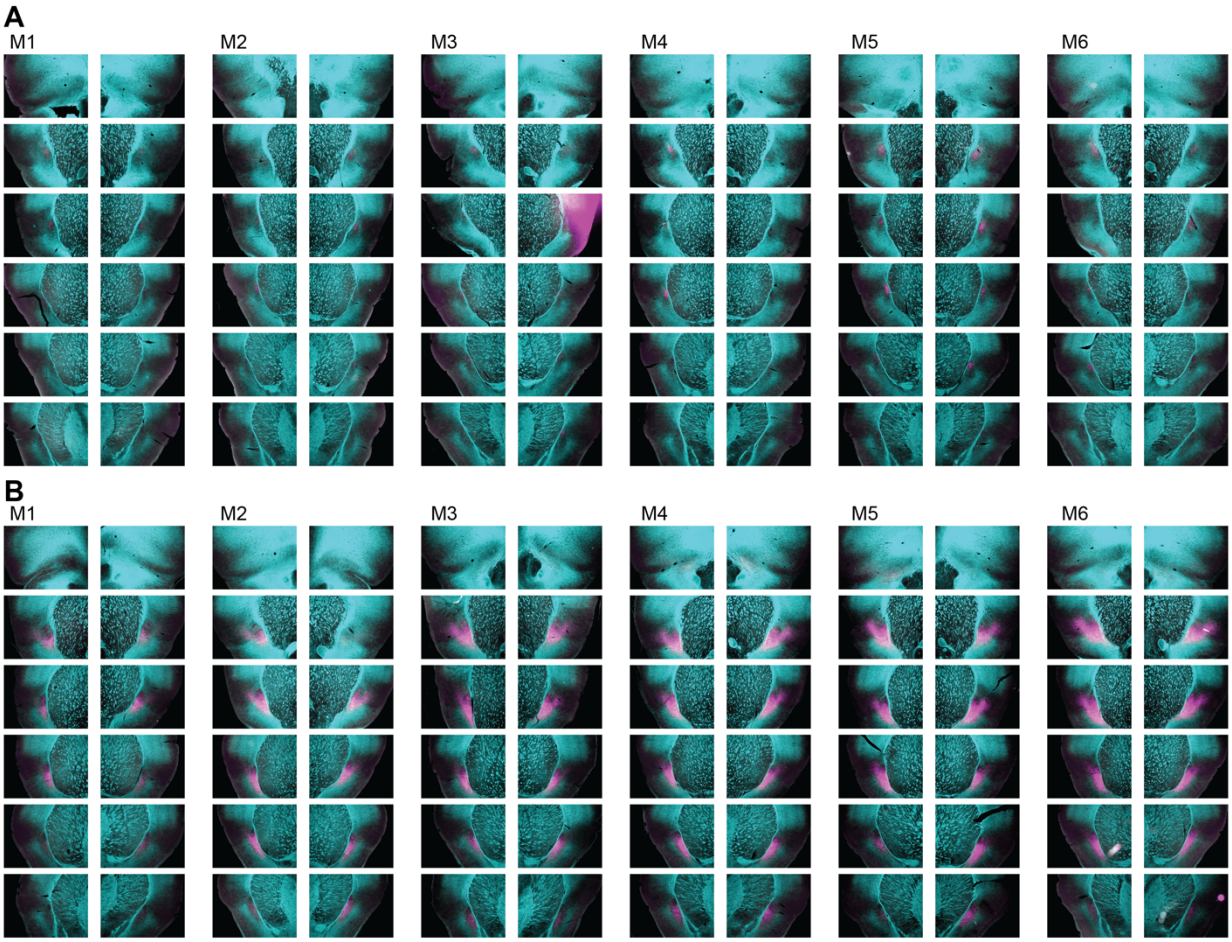


Figure S4-1. Histology. Images display bilateral histology from all experimental animals in the Cre-TetTox (A) and CC-EDGE-TetTox (B) groups. Each subcolumn corresponds to a mouse. Each row within a subcolumn shows the area surrounding the claustrum in the left and right hemispheres of a coronal section. Sections were spaced 500um apart with the second section located at the union of the corpus callosum. Sections are in order with the most anterior slice in the top row. Myelin basic protein is shown in cyan and TetTox is shown in Magenta.

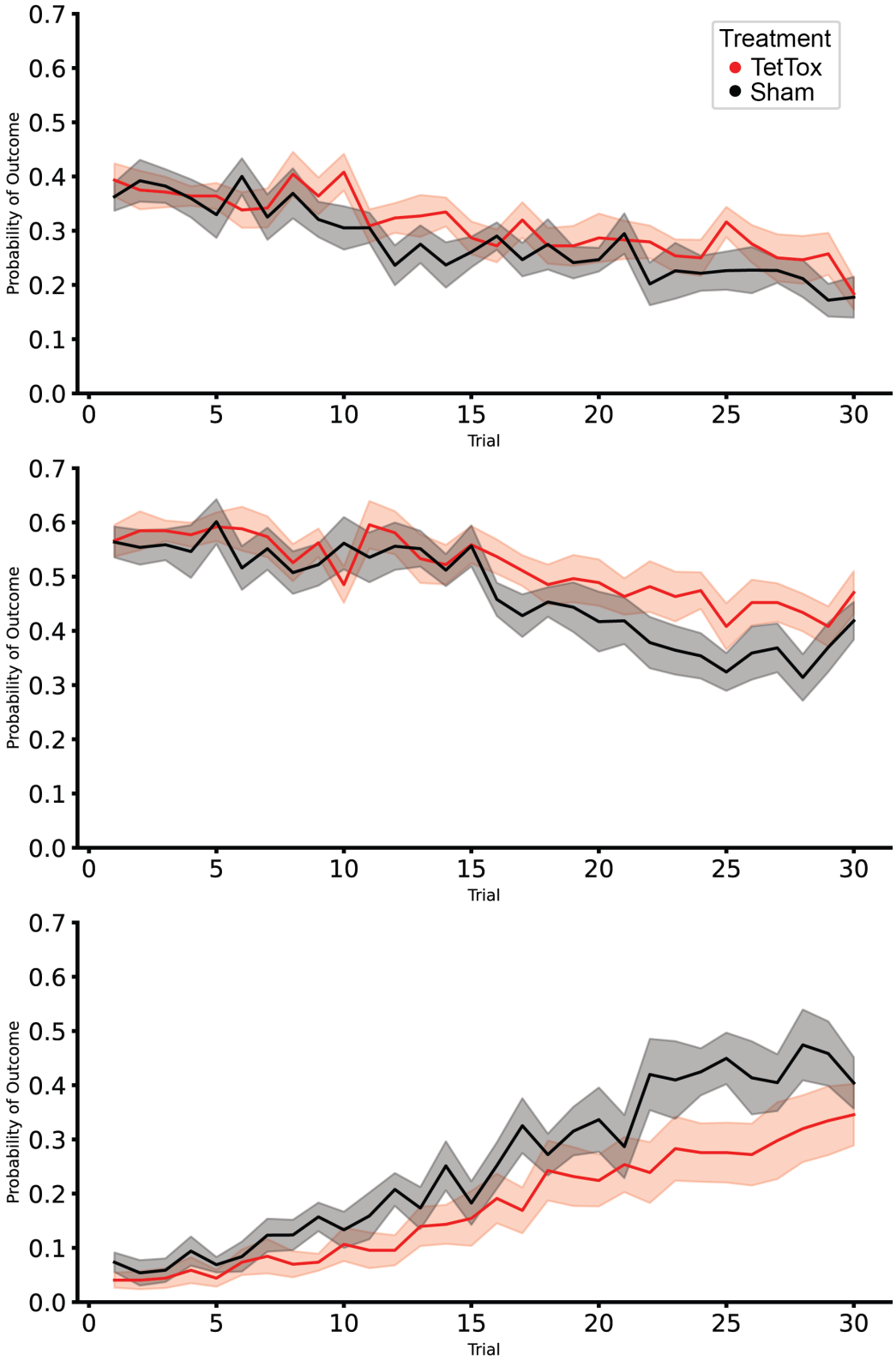


Figure S5-1. Within session probability of outcome. Graphs showing the probability of a hit (top), miss (middle), or omission trial (bottom) averaged across trials within a session. Graphs include data from all trial types. Data from sham mice (n=12) are plotted in black and TetTox mice (n=16) are plotted in red. Error bars show the standard error of the mean.

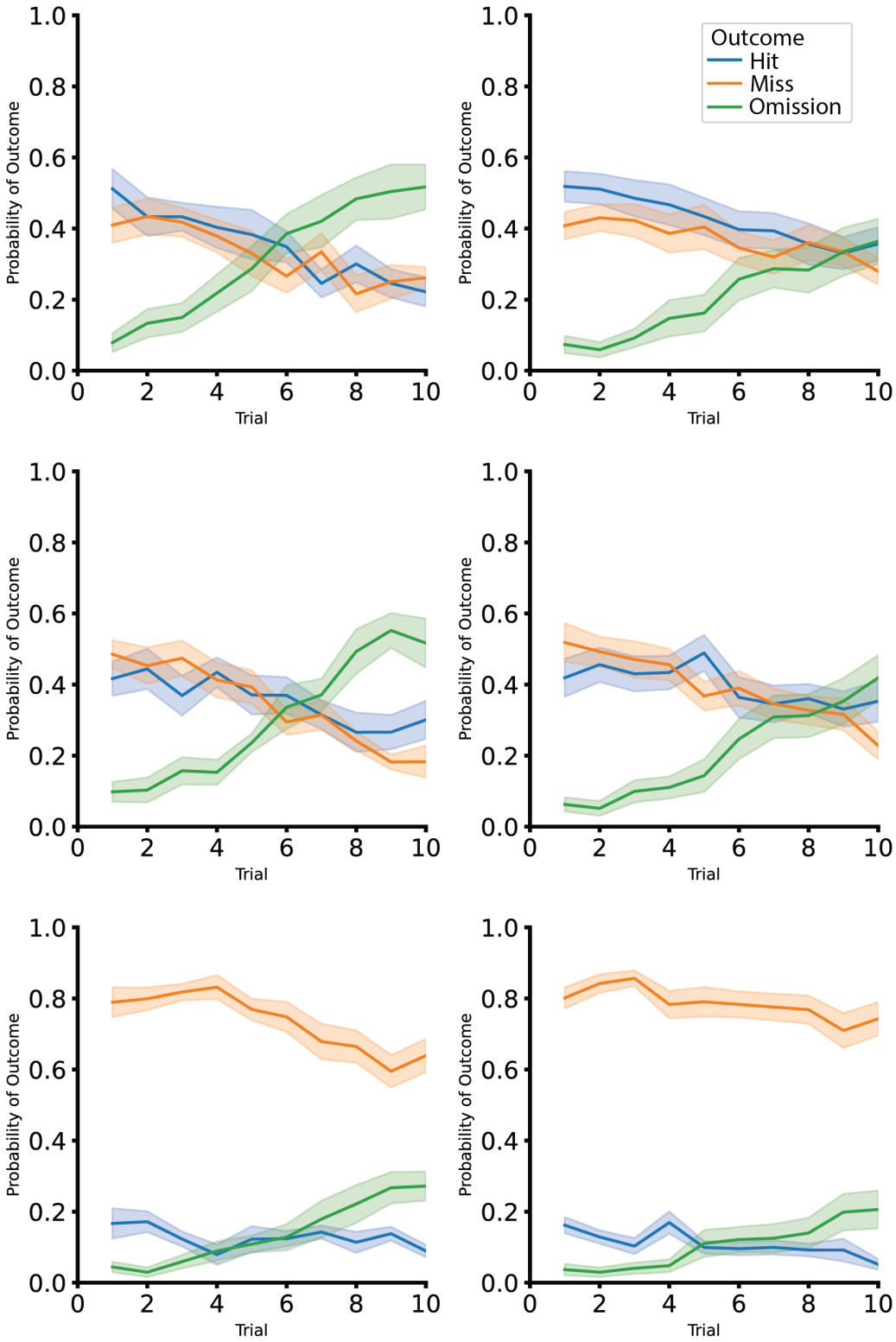


Figure S5-2. Within session probability of outcome by group and trial type. Graphs showing the average probability of a hit (blue), miss (orange), or omission trial (green) within a session for audio (top), visual (middle), and audiovisual trials (bottom) in sham (left; n=12) and TetTox mice (right; n=16). Error bars show the standard error of the mean.

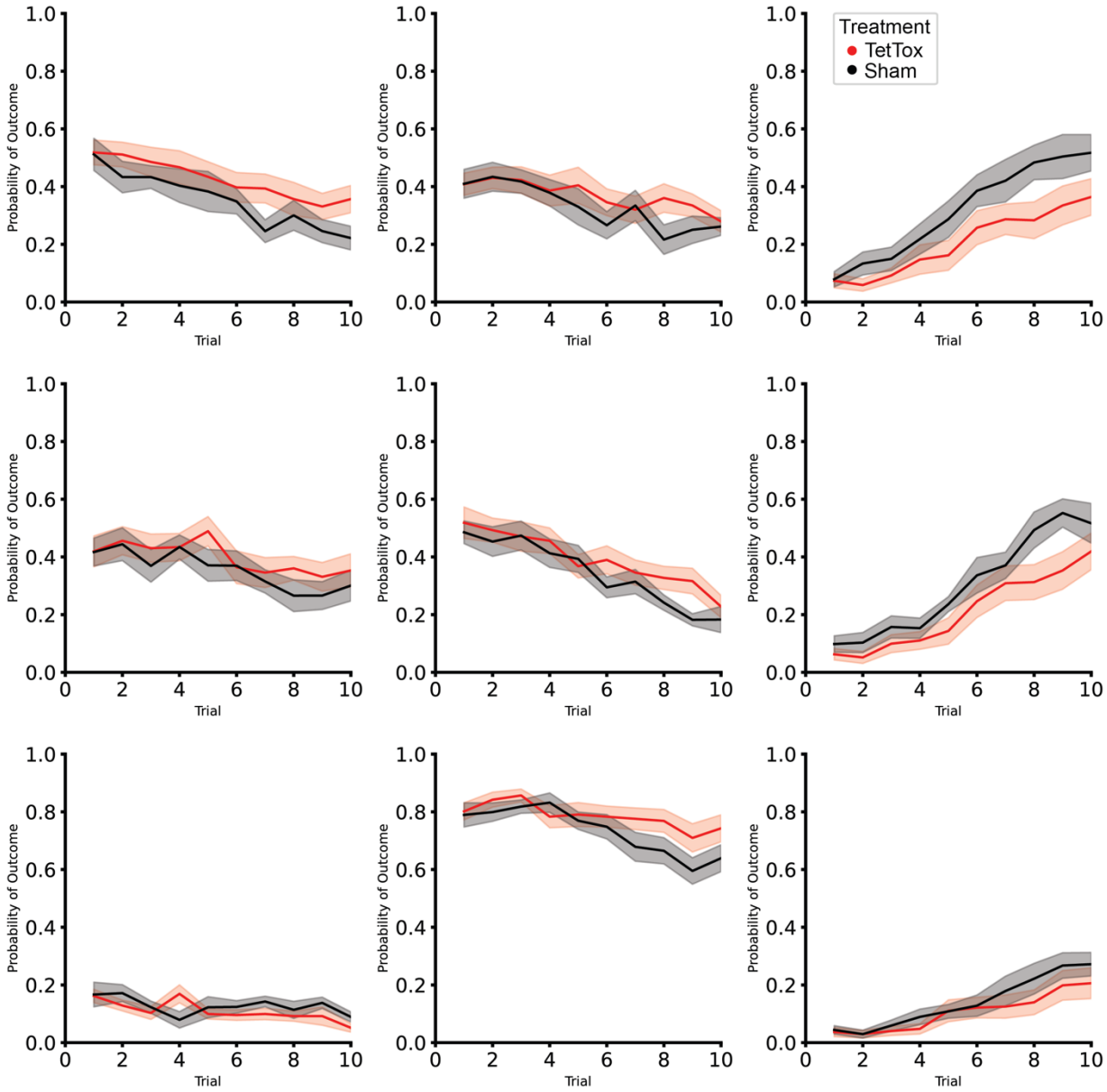


Figure S5-3. Within session probability of outcome by stimulus and trial type. Graphs showing the average probability of a hit (left), miss (centre), or omission trial (right) within a session for audio (top), visual (middle), and audiovisual trials (bottom). Data from sham mice (n=12) are plotted in black and TetTox mice (n=16) are plotted in red. Error bars show the standard error of the mean.

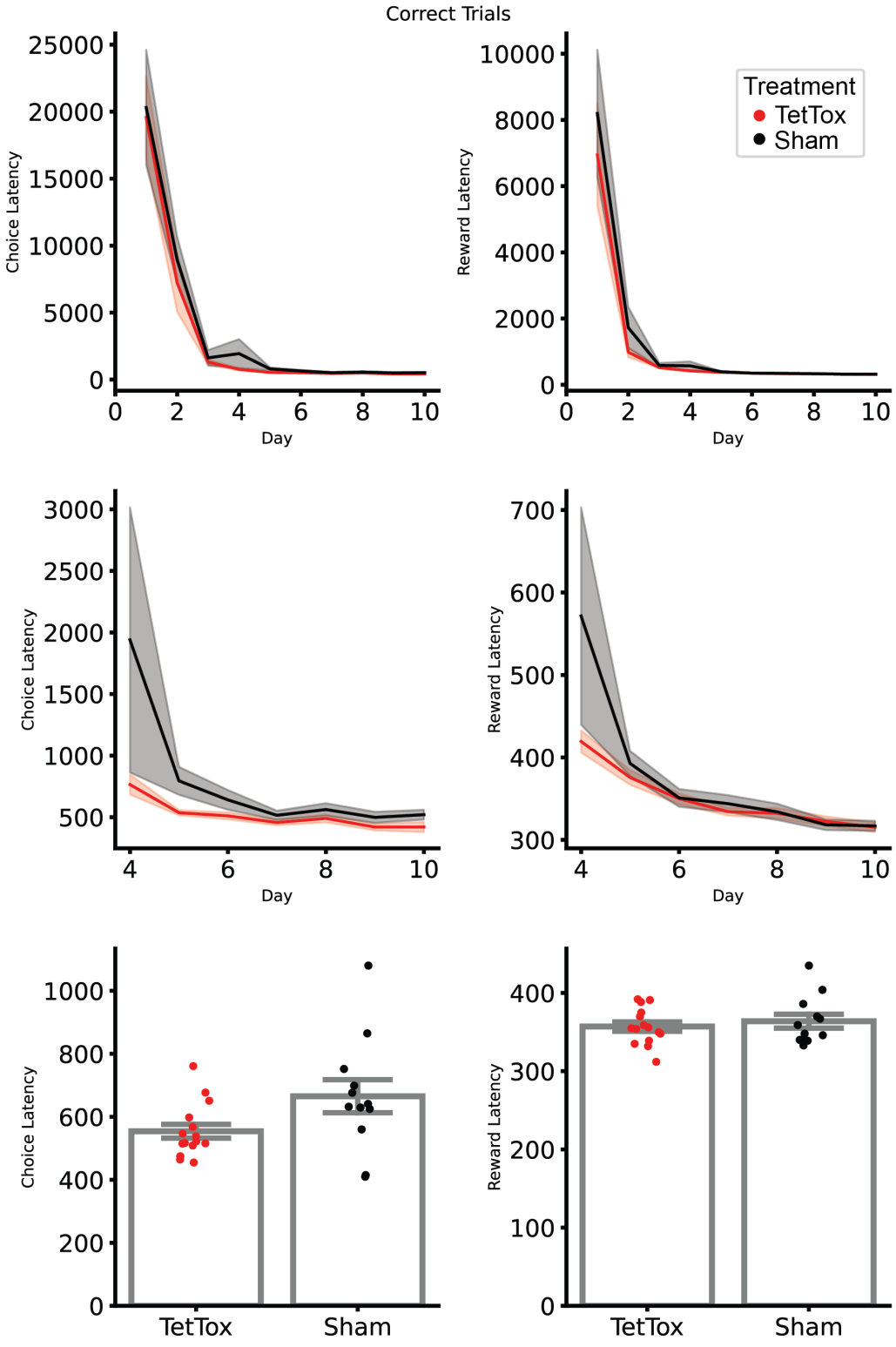


Figure S5-4. Latency on correct trials. Graphs showing the choice (left) and reward latency (right) across all days (top), the last seven days (middle), and on average (bottom) from correct trials only. Data from sham mice (n=12) are plotted in black and TetTox mice (n=15) are plotted in red. Error bars show the standard error of the mean.

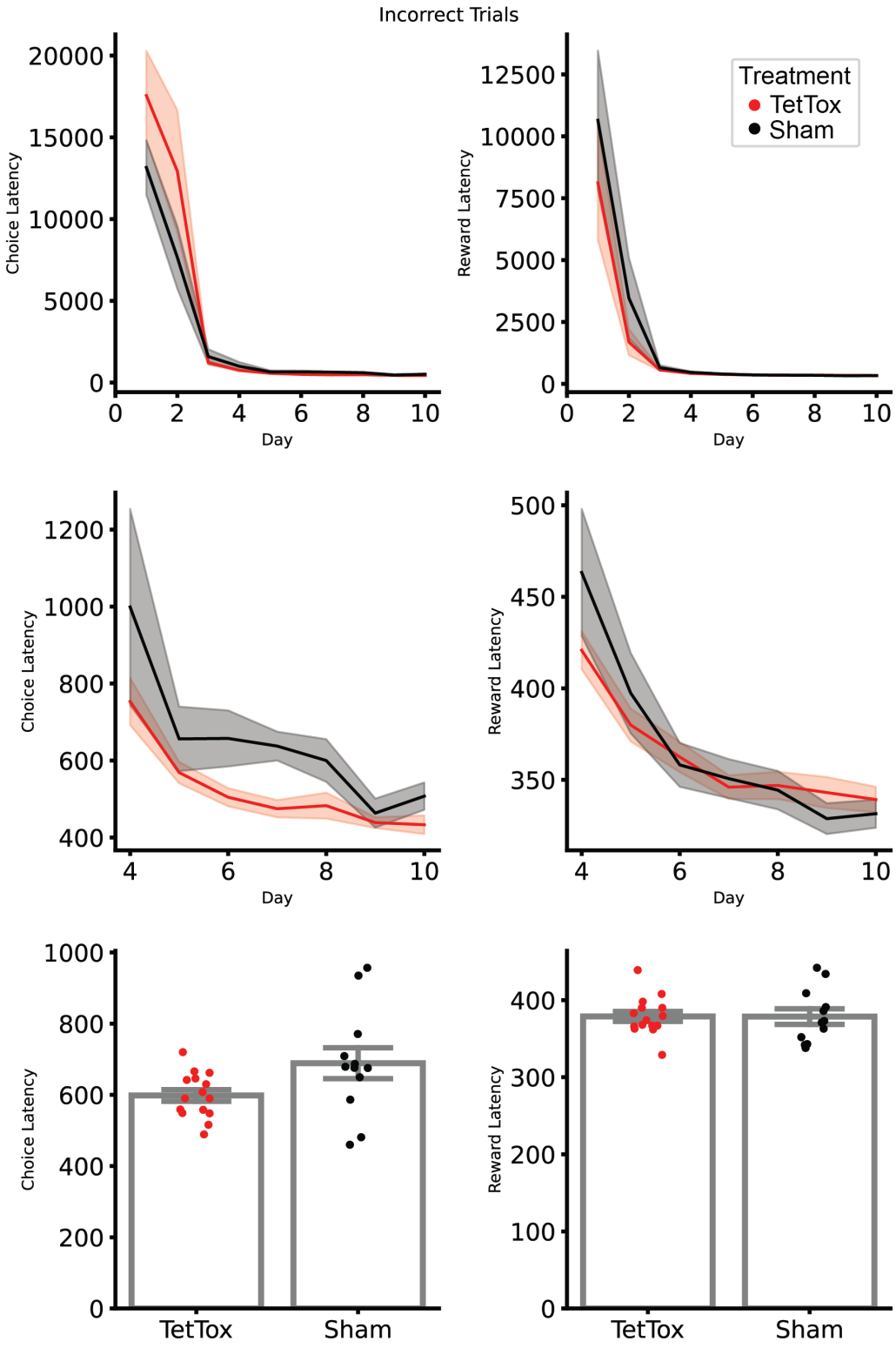


Figure S5-5. Latency on incorrect trials. Graphs showing the choice (left) and reward latency (right) across all days (top), the last seven days (middle), and on average (bottom) from incorrect trials only. Data from sham mice (n=12) are plotted in black and TetTox mice (n=15) are plotted in red. Error bars show the standard error of the mean.

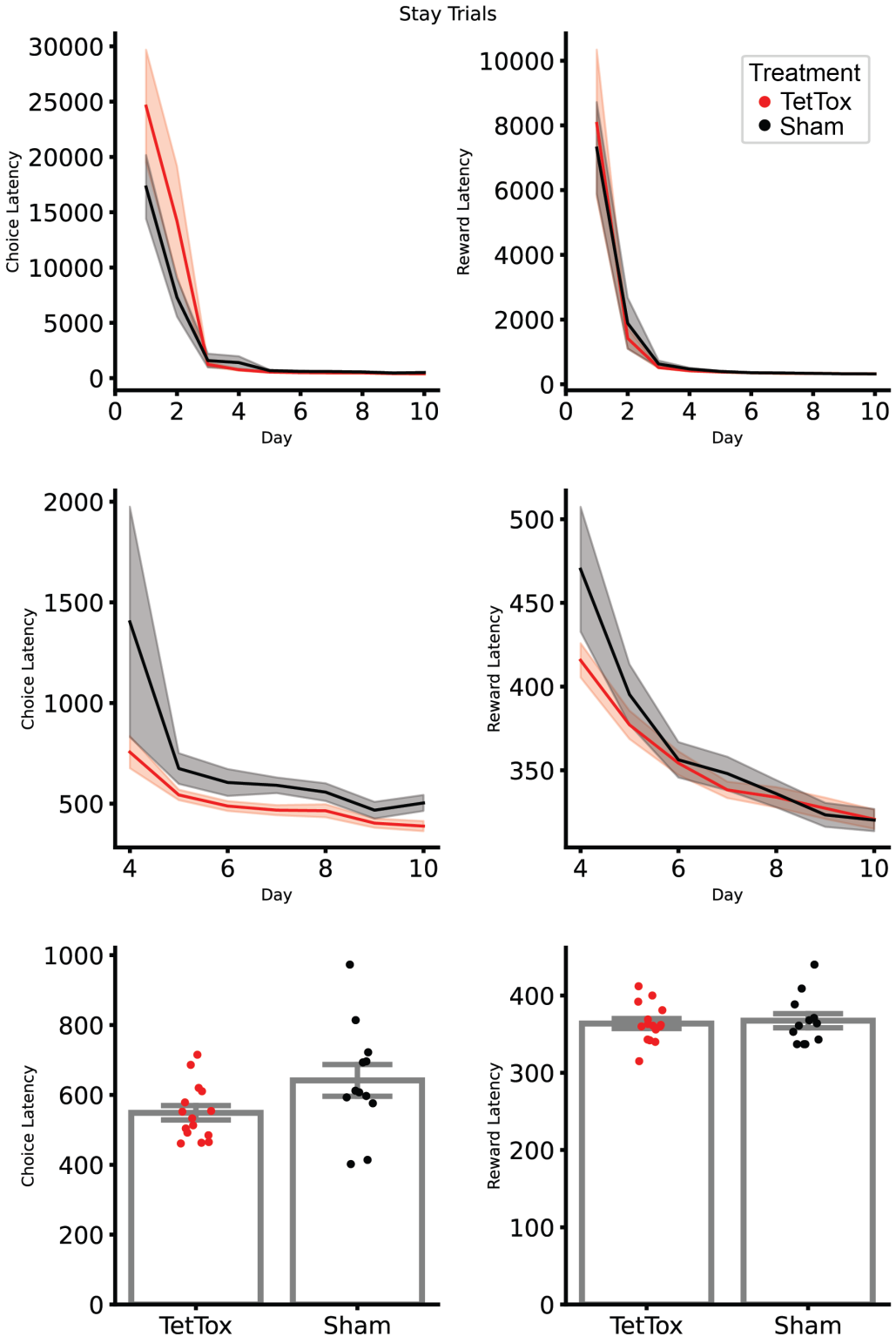


Figure S5-6. Latency on stay trials. Graphs showing the choice (left) and reward latency (right) across all days (top), the last seven days (middle), and on average (bottom) from stay trials only. Data from sham mice (n=12) are plotted in black and TetTox mice (n=15) are plotted in red. Error bars show the standard error of the mean.

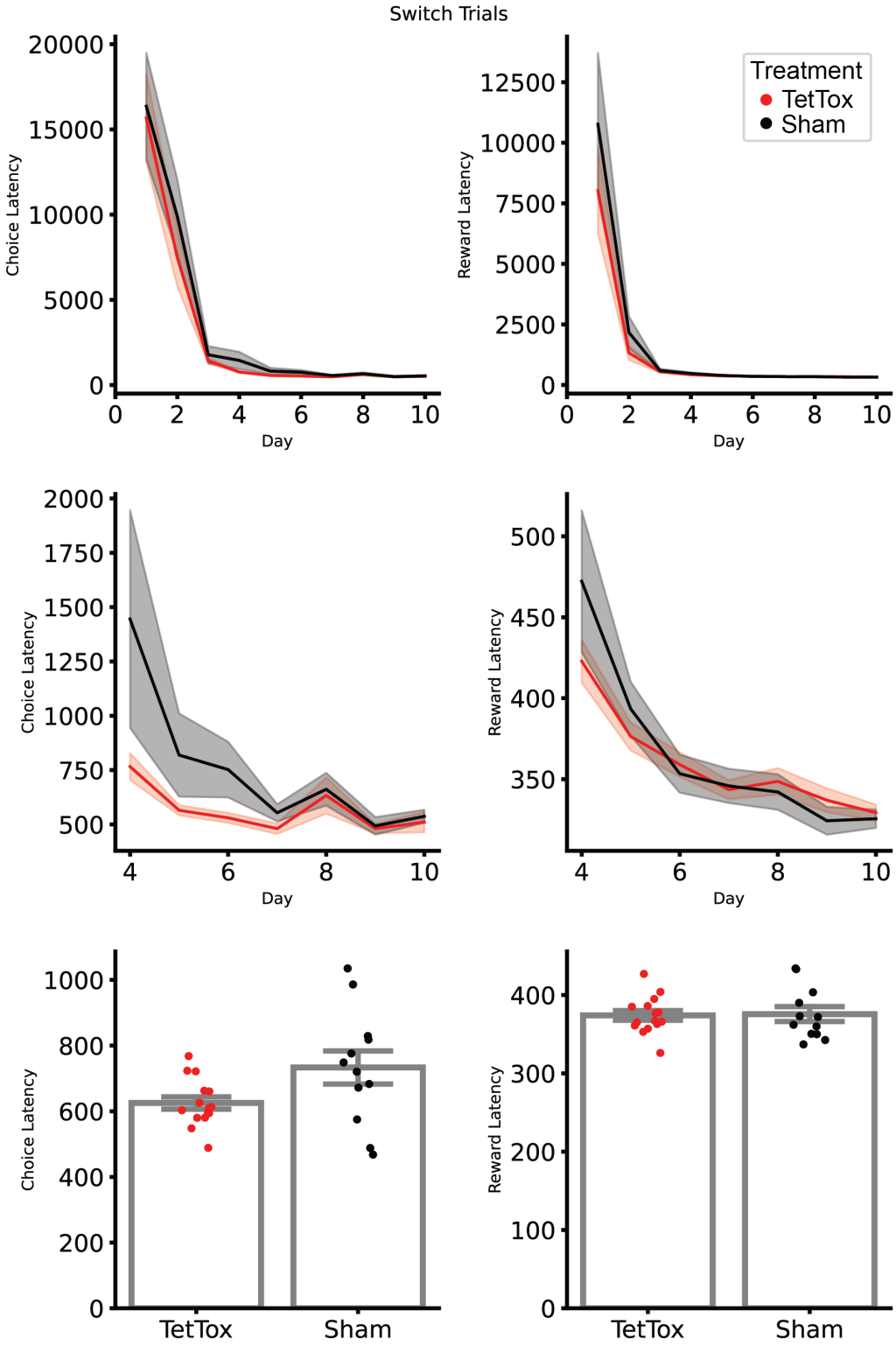


Figure S5-7. Latency on switch trials. Graphs showing the choice (left) and reward latency (right) across all days (top), the last seven days (middle), and on average (bottom) from switch trials only. Data from sham mice (n=12) are plotted in black and TetTox mice (n=15) are plotted in red. Error bars show the standard error of the mean.

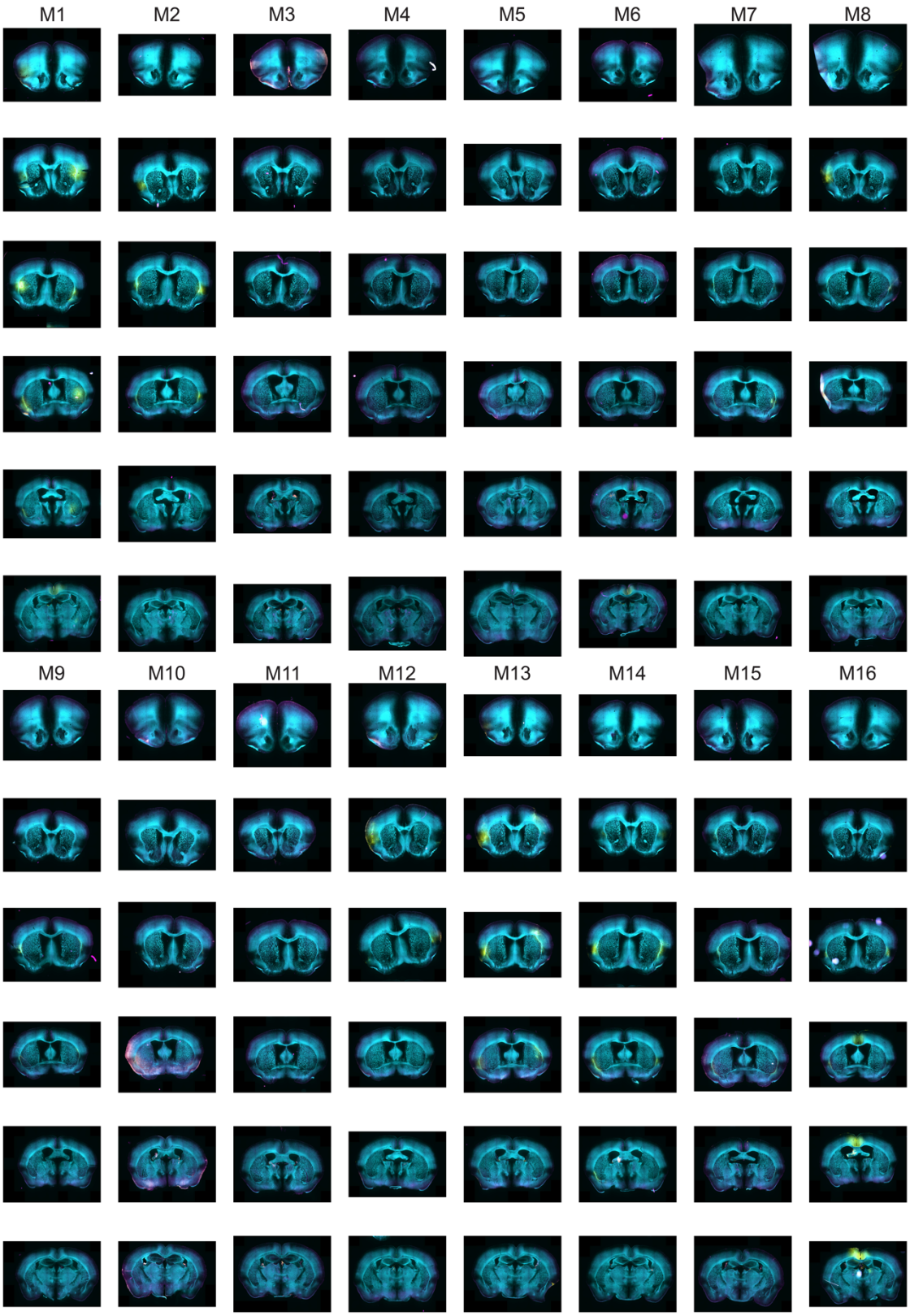


Figure S5-8. Histology. Images display whole slice histology from all experimental animals. Each subcolumn corresponds to a mouse. Coronal sections were spaced 500um apart with the second section located at the union of the corpus callosum. Sections are in order with the most anterior slice in the top row. Myelin basic protein is shown in cyan, retro-iCre-mRuby in magenta, and FLAG in yellow.

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BRAIN
REVIEW ARTICLE

Human lesions and animal studies link the claustrum to perception, salience, sleep and pain

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[†]These authors contributed equally to this work.

The claustrum is the most densely interconnected region in the human brain. Despite the accumulating data from clinical and experimental studies, the functional role of the claustrum remains unknown. Here, we systematically review claustrum lesion studies and discuss their functional implications. Claustral lesions are associated with an array of signs and symptoms, including changes in cognitive, perceptual and motor abilities; electrical activity; mental state; and sleep. The wide range of symptoms observed following claustral lesions do not provide compelling evidence to support prominent current theories of claustrum function such as multisensory integration or salience computation. Conversely, the lesions studies support the hypothesis that the claustrum regulates cortical excitability. We argue that the claustrum is connected to, or part of, multiple brain networks that perform both fundamental and higher cognitive functions. As a multifunctional node in numerous networks, this may explain the manifold effects of claustrum damage on brain and behaviour.

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Keywords: claustrum; lesion; perception; salience; sleep; pain

Introduction

The claustrum is a sheet-like bilateral brain region tucked beneath the insular cortex (Fig. 1). Extensive anatomical work across different species, including humans, monkeys, rodents, reptiles, rabbits, and cats, indicates widespread connectivity between the claustrum and the neocortex,[1–15] and highlights the need for a more thorough investigation of the claustrum's circuitry. Strong reciprocal connectivity with most neocortical areas is perhaps the most noteworthy feature of the claustrum,[2,7,9,10,14–16] which has the highest connectivity in the human brain by regional volume.[17] Recent work has shown cortico-claustral and claustrocortical connections evoke feedforward inhibition in mice,[6,18] similar to previous results in cats.[19–22] While the anatomy, physiology, and putative functions of the claustrum have been reviewed elsewhere,[4,16,23–27] this review seeks to untangle and shed new light on the functional role of the claustrum.

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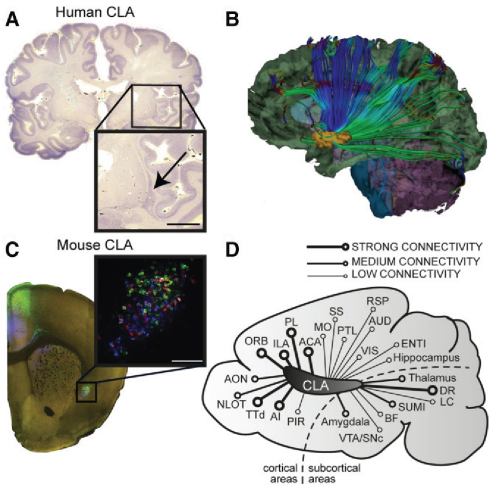


Figure 1 The location and connectivity of the claustrum. (**A**) Nissl stained human coronal brain section with *inset* showing the location of the claustrum. (**B**) White matter tractography image showing outgoing connections from the claustrum reprinted from Torgerson *et al.*[17] with permission from John Wiley and Sons ©2015. (**C**) Mouse coronal brain section with inset showing the claustrum labelled by multicolour retrograde tracers. (**D**) Schematic illustration of a sagittal mouse brain section showing the relative connectivity strength between the claustrum and other brain regions. Connectivity strength was assessed based on the data provided in Zingg *et al.*[15] and Wang *et al.*[12] Most but not all connections are reciprocal (see reviews Mathur[26] and Jackson *et al.*[25]). ACA = anterior cingulate area; AI = anterior insular cortex; Aud = auditory cortex; AON = anterior olfactory nucleus; BF = basal forebrain; CLA = claustrum; DR = dorsal raphe; ENTI = entorhinal cortex; ILA = infralimbic area; LC = locus coeruleus; MO = motor cortex; LOT = the nucleus of the lateral olfactory tract; ORB = orbital area; PL = prelimbic area; PTL = parietal cortex; PIR = piriform area; RSP = retrosplenial cortex; SUMI = supramammillary; SS = somatosensory cortex; TTd = dorsal taenia tecta; VTA/SNc = ventral tegmental area/substantia nigra; VIS = visual cortex.

Understanding the anatomy and connectivity of a brain region can provide important insights into its function. Attempts to find classic structure-function relationships are challenging in highly connected brain regions where many functions are possible due to multiple co-existing pathways. In the case of the claustrum, such widespread connectivity has led to a plethora of hypotheses regarding its function. The claustrum has been implicated in processes including the amplification of cortical oscillations,[28,29] attentional allocation,[2,4,26,30] consciousness,[16,31] cognition,[6] spatial navigation,[32] the transfer of information across sensory modalities,[33] sexual function,[34] and aesthetic judgement.[35]

In addition to investigating its anatomy and connectivity, examining the consequences of damage to a brain region may provide complementary insights into its function. The lesion approach, including both acute and chronic interventions, has been immensely powerful in localizing language, episodic memory, and even aspects of executive function, in both human and animal studies.[36,37] While imperfect, loss-of-function approaches can enable subsequent neural activity recordings to focus on stimuli or behaviours implicated by the lost functions. Moreover, lesion studies can help reveal a region's functional connectivity. However, the claustrum's contorted shape and bilateral location deep in the brain renders this approach difficult, though some studies have attempted to lesion the claustrum in animals.[38–40]

While the claustrum's anatomy renders lesion studies challenging, examples of claustrum lesions exist within the literature. To our knowledge no review has comprehensively assessed clinical studies of human claustrum lesions. In this review, we present a meta-analysis of all studies to date that describe cases of claustrum lesions in human patients. We show that claustrum lesions lead to a wide range of signs and symptoms. Most commonly, patients experienced impairments in different levels of cognitive, perceptual, and motor abilities, as well as seizures and disturbances to mental state and sleep. Using these findings, in conjunction with animal studies, we consolidate evidence concerning the claustrum's role in sensory perception, sleep, pain, and salience, and facilitate a critical re-evaluation of existing hypotheses of the claustrum's function. Our results do not support prominent hypothesized functions such as multisensory integration and salience processing, but may instead provide evidence for a role in regulating cortical excitability. We hypothesize that the claustrum is connected to, or is a part of, many different networks, and propose that the brain-wide connectivity of the claustrum may have provided a useful scaffold onto which many functional roles could subsequently be built. This may explain the apparent role of the claustrum in fundamental functions such as sleep, as well as in more complex functions such as attention and salience. We argue that the claustrum is likely a multifunctional area, or perhaps possesses a global function to regulate neural activity dynamics across the cerebral mantle. Under this schema, damage to the claustrum would disrupt diverse neurological and behavioural processes.

Diverse findings following lesion or stimulation of the human claustrum

Literature search

The potential functions of the claustrum have not been comprehensively analysed by examining human lesion studies because of the relative scarcity of claustral lesions (the only reported work focused specifically on delusional states).[27] To tackle this, we examined claustrum lesion cases by searching the following terms on PubMed and Scopus: 'claustrum AND (lesion OR contusion OR injury OR trauma)' ($n = 103$). Studies were then screened for cases in which reported lesions included the claustrum according to neuroimaging. Thirty-eight individual cases (Supplementary Tables 1 and 2) and 14 cohort studies (Supplementary Table 3) were included in this review. Details of these studies can be found in the Supplementary material.

Specific claustrum lesions

Due to the claustrum's nestled location, vascularization, and morphology, isolated claustrum lesions are rare, and predominantly occur in the spectrum of autoimmune encephalitis. Unfortunately, available clinical imaging techniques lack sufficient resolution to conclusively resolve the claustrum from the external capsule. Studies that reported lesions to the claustrum and external capsule (Fig. 2B), the external capsule only (Fig. 2C), and the claustrum only (Fig. 2D) reveal strikingly similar imaging results. Given this diversity in the reporting of lesions, it is difficult to distinguish truly claustrum specific lesions from those which may impact both the claustrum and adjacent white matter. Disambiguating what caused the lesion can help. For example, autoimmune encephalitis

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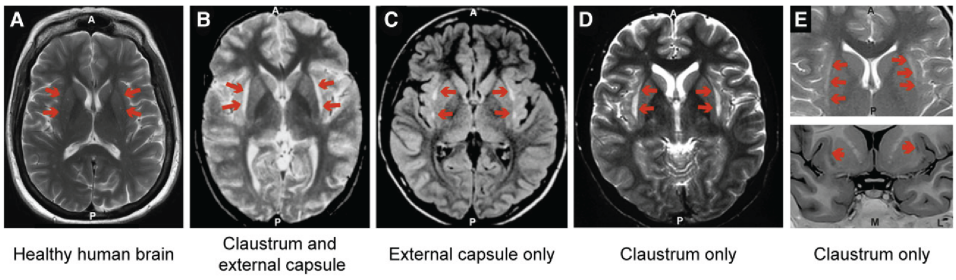


Figure 2 Similar lesions have been described as affecting either the claustrum, the external capsule, or both. (**A**) Representative T_2-weighted image of a healthy human brain. (**B**) T_2-weighted image showing a lesion reported as affecting the claustrum and external capsule. Reprinted from Sperner *et al.*[45] with permission from Springer Nature ©1996. (**C**) Fluid-attenuated inversion recovery (FLAIR) image showing a lesion reported as affecting the external capsule. Reprinted from Mumoli *et al.*[43] with permission from Springer Nature ©2014. (**D**) T_2-weighted image showing a lesion reported as affecting the claustrum. Reprinted from Ishii *et al.*[42] with permission from American Society of Neuroradiology ©2011. (**E**) T_2-weighted image (*top*) and T_1-weighted image (*bottom*) showing a lesion reported as affecting the claustrum. Reprinted from Silva *et al.*[44] with permission from Springer Nature ©2018.

might be caused by anti-neuronal surface antibodies that have a predilection to epitopes on the claustrum, such as voltage-gated potassium channels antibodies and anti-glutamic acid decarboxylase antibody.[41] On the other hand, pathologies that affect white matter such as vasogenic oedema and demyelination might have a predilection for external and extreme capsules. Thus, the diagnosis offers an important clue to claustrum involvement, despite the imaging in these cases being identical. Nonetheless, the lack of isolated claustrum lesions means lesion studies are inherently problematic. It is therefore difficult to attribute a particular functional role to the claustrum based on lesions alone.

Table 1 Lesioned area, symptoms and seizures reported in case studies with claustrum lesions

Hemispheric distribution	Bilateral	22
	Unilateral (Right/Left/Undefined)	16 (9/5/2)
Extent of lesion	Claustrum only	7
	External capsule	14
	Insula	12
	Hippocampus	9
	Other cortices	17
	Other subcortices	22
Signs and symptoms	Cognitive, perceptual and motor abilities	
	Cognitive impairment	19 (50%)
	Motor disturbance	17 (45%)
	Visual disturbances	9 (24%)
	Speech disturbances	8 (21%)
	Auditory disturbances	6 (16%)
	Tremor	5 (13%)
	Paraesthesia	5 (13%)
	Electrical activity disturbance	
	Seizures	18 (47%)
	Non-seizure EEG abnormalities	8 (21%)
	Mental state	
	Loss of consciousness	8 (21%)
	Hallucinations	4 (11%)
	Delusions (Cotard delusion)	5 (2) (13%)
	Sleep disturbances	6 (16%)
Seizure type (patients may fit >1 severities)	1) Partial	7 (18%)
	2) Generalized	15 (39%)
	3) Status epilepticus	13 (34%)
	4) Refractory status epilepticus	10 (26%)

Despite this difficulty, seven case studies have been identified which report relatively exclusive lesions in the claustrum with or without involvement of the external capsule (Supplementary Table 1). Four of these patients presented with status epilepticus and were found to have bilateral lesions affecting the claustrum and external capsule. The four patients also had EEG abnormalities along with a variety of other symptoms including seizures, motor impairment, cognitive impairment, psychotic symptoms, and tremor.[42–45] Another patient with abnormalities in the right claustrum and bilateral external capsule experienced non-epileptiform EEG abnormalities, along with motor and cognitive impairment.[46] A 55-year-old patient with an isolated left claustrum lesion following an ischaemic stroke experienced unilateral paraesthesia of the face, tongue, and head—similar to a lacunar syndrome—along with polymodal gustatory, auditory, vestibular, and visual disturbances.[47] Recently, a patient admitted to hospital with Covid-19 showed bilateral lesions to the claustrum and external capsule. He presented with aggression, cognitive impairment, disorientation, and stupor. Upon follow-up, the claustrum lesions remained despite a complete neuropsychiatric recovery.[48] For further details of these cases, see Supplementary Table 1.

While the underlying cause of the claustrum damage was not always clear, the various authors suspected a range of aetiologies including viral encephalitis, ischaemic stroke, and seizure induced damage. Despite the similar and isolated brain abnormalities, the patients presented with a wide and inconsistent array of signs and symptoms. The effects reported in these diverse cases are difficult to interpret. They do not offer strong support to any single hypothesis of the claustrum's function. Conversely, these results suggest that the claustrum may instead have multiple roles, or an, as yet, unidentified global role that impacts multiple behavioural domains. However, further evidence is required to draw any conclusions.

Non-specific claustrum lesions

Beyond these studies, many others have reported lesions to the claustrum in conjunction with other brain regions (Supplementary

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Table 2). Other areas commonly included the external capsule (14/38) and insula (12/38), between which the claustrum is interposed, and the hippocampus (9/38). In particular, presumed viral and non-viral encephalitis may cause relatively claustrum-specific changes on MRI neuroimaging, sometimes in conjunction with hippocampal damage. Like the studies with isolated claustrum lesions, these less specific lesion studies also reported a wide variety of symptoms (Table 1). The most common signs and symptoms in individual cases were nonspecific cognitive impairment (19/38), seizures (18/38), motor impairment (17/38), visual disturbances (9/38), non-seizure EEG abnormalities (8/38), loss of consciousness (8/38), speech problems (8/38), auditory disturbances (6/38), sleep disturbances (6/38), delusions (5/38), tremor (5/38), and hallucinations (4/38). We separated these observations into the following four categories: disruption in cognitive, perceptual and motor abilities; changes in electrical activity; changes in mental state; and sleep disturbance.

Claustral lesions impair cognitive, perceptual and motor abilities

Patients with claustrum lesions were frequently reported to have perceptual and cognitive impairments. In many cases, the lesions impacted patients' ability to perceive sensory input in various ways. For example, a 12-year-old experienced temporary loss of vision and hearing.[45] Interestingly, another young child experienced permanent bilateral blindness due to damage to the left calcarine cortex and right claustrum, although the most parsimonious explanation for this finding is undetected damage to the right optic radiation.[49] In some cases, lower perceptual sensitivity,[50] and tinnitus[51] were reported. Somatosensory deficits have also been reported.[47,52] Cases presumed to be driven by encephalitis often presented with deficits in episodic memory,[46,53,54] working memory, and reasoning or with disorientation in time.[52] Claustrum damage has even been described as part of neurodegenerative syndromes such as corticobasal degeneration, where it may be accompanied by a disruption in visual attention functions including spatial orientation, optic ataxia,[55] and cognitive decline in Lewy body dementia.[56] Left claustrum damage could lead to aphasia when accompanied by left putamen damage.[57] Despite the varied pathologies, lesion sites, and inconsistent observations across studies, each patient experienced disturbance to some aspect of their cognitive and perceptual abilities. Claustrum lesions led to a wide variety of sensory deficits, suggesting the claustrum may play a functional role in one or more aspects of sensory processing. Notably, no evidence for specific deficits in multisensory integration was reported.

Claustrum lesions result in an increased incidence of electrical disturbances

Patients with claustral lesions often experienced disturbances to the electrical activity of the brain, involving generalized seizures (15/38) and status epilepticus (5 min of constant seizures without any return to consciousness in between; 13/38). Generalized seizures were more common when the claustrum was damaged bilaterally (10/15 studies). Several studies[45,51] reported that patients with claustral lesions developed recurrent generalized seizures, with interictal slowing (3–4 Hz) on EEG. Patients who also had claustrum-associated focal seizures reported experiencing delusions and altered mental state (discussed below), which was coupled with pathological EEG slow-wave activity.[58] Unfortunately, the majority of the case studies reporting generalized seizures do not report EEG recordings. The studies that did include EEG recordings typically reported generalized slow-wave activity present even in the awake state. A few of these also described generalized spikes or sharp waves.[45,59]

Strikingly, several patients with claustrum lesions exhibited a form of severe epilepsy (10/38) known as refractory status epilepticus. Refractory status epilepticus is a subset of these cases that fail to respond to the two frontline pharmacological treatments for seizures (benzodiazepines and one anticonvulsant).[60] Studies examining new-onset refractory status epilepticus reported lesions in the claustrum as a common pathology, concomitant with slow-wave activity, spikes and sharp waves, and periodic lateralized epileptiform discharges in EEG recordings.[61–63] Taken together, these studies suggest that claustrum dysfunction increases global synchronization of the electrical activity of the brain, and potentially leads to seizures.[64] This is consistent with a putative role for the claustrum in regulating cortical excitability.

Claustral lesions disturb mental state

Several studies noted that medicated and unmedicated patients with claustrum lesions experienced altered mental states, which can be defined as general changes in brain function characterized by confusion, disorientation and disordered perceptions.[65] These included non-seizure loss of consciousness, confusion, stupor, delusions, hallucinations, as well as other mild fluctuations in mental state. Loss of consciousness, defined here as an interruption in one's awareness of the self and the environment, was reported in 8 of 38 case studies. Similarly, a study of combat veterans with penetrating traumatic brain injuries reported that patients with lesions to the claustrum showed an increased duration but not frequency of loss of consciousness relative to patients with lesions in other locations.[66] Recent lesion mapping has uncovered a distributed circuit associated with loss of consciousness, which peaks in the bilateral claustrum.[67] These findings suggest that the claustrum may be necessary to support some aspects of maintaining one's level of consciousness. An alternative explanation is that these processes generate 'selfhood', the mental representation of a distinct first-person capable of agency, a role which has been associated with the nearby anterior insula[68] but might be extended to parts of the claustrum.

Altered mental states were reported across many claustrum lesion case studies. While some patients manifested broadly altered mental states including anhedonia, delirium, or confusion,[61,63] some had narrower neuropsychiatric features such as delusions with paranoid or religious content,[58,69] visual hallucinations, and abulia.[70] One patient with claustral dysfunction attributed to COVID-19 exhibited 'delirious behavior'.[48] Even more specific features included delusions of jealousy.[71] The high frequency of visual and auditory hallucinations, confusion, and delusional states has previously been identified and discussed at length by Patru and Reser.[27] They argue persuasively that the claustrum is involved in the process of generating and filtering delusional beliefs (described below).

Intriguingly, claustrum dysfunction may also be linked to Cotard delusion. Patients experiencing Cotard delusion may believe that they are dead, do not exist, are decaying, or have lost internal organs.[72] Intriguingly, two patients described above with claustrum lesions experienced symptoms consistent with Cotard delusion.[69,73] As only a few hundred instances of Cotard delusions are known worldwide, the finding that one confirmed case of Cotard delusion and another patient with highly overlapping symptoms arose from pathology involving the claustrum is highly

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suggestive of an association and perhaps a disconnection with 'self'. Although most reported cases of Cotard delusion do not include claustrum lesions[74–76] the claustrum was the only area of overlap between the two cases described. These examples of altered mental states following claustrum lesions suggest that the claustrum may be required to modulate complex cognitive representations such as beliefs and desires.

Claustral lesions disturb sleep

Sleep disturbances were commonly observed in patients with claustrum lesions, usually with other symptoms present. Some studies reported hypersomnia along with altered mental state[63,70] while others reported restlessness along with motor disturbance.[56] It is unclear whether these sleep disturbances occurred as a direct result of the claustrum lesions themselves, patients' medications, or damage to other brain structures. Additionally, these studies did not assess the polysomnographic features of the sleep disturbances or provide a systematic investigation of sleep. No details were reported about whether patients had difficulty initiating and/or maintaining sleep. Recently, a genome-wide analysis suggested a link between insomnia and a disruption in claustrum-hypothalamus-striatum circuitry,[77] pointing to the role of these areas in sleep initiation. Another mechanism by which circuit-level abnormalities might affect a patient's sleep-wake cycle is through a disturbance in waking resting state functional connectivity in the default mode network.[78] Although evidence points to an important functional role of the claustrum in sleep control, lesion studies are too limited to provide conclusive evidence.

Claustral lesions can result in painful sensations

Multiple case studies included patients who reported painful sensations.[61,79–82] These sensations varied in character and severity. In the 1950s, two separate studies described patients with unilateral lesions to the claustrum, among other regions, who experienced hyperalgesia to hot and/or cold in the contralateral upper limb, together with hyperaesthesia, depression, and suicidal ideation.[80,82] Pain with hypoaesthesia has also been reported.[81] Other cases have described dynamic changes, such as painful cramping beginning in the contralateral hand, with Jacksonian march to the whole of the left side,[79] or discomfort with spreading paraesthesia,[47] suggesting that pain could relate to heightened excitability rather than neural loss.

Despite these positive findings, caution must be exercised as many painful sensations might more parsimoniously be attributed to causes other than claustral lesions (e.g. myalgia to fever in Choi *et al.*[61]). Claustrum stimulation has also induced pain, with a participant reporting painful sensations 'like a knife jabbing' and of 'burning' during claustrum stimulation.[83] This is noteworthy as brain stimulation rarely produces painful sensations, although when it does it has often been reported following stimulation of a region surrounding the posterior insula and claustrum.[84] While these are isolated examples, numerous studies have hinted at a connection between the claustrum and pain. However, this possibility has received comparatively less attention than other brain areas.

The lesion studies were inconsistent in their reporting of the neurological examination, and most reported just whether sensation was intact. The lack of any specific mention of pain may reflect an incomplete report, rather than constituting evidence that pain was unaffected. However, it does seem clear that there is no obvious neuropathic pain syndrome associated specifically with claustrum lesions, perhaps contrasting with the more distinct sensory syndromes associated with insula damage.[85] Future studies should specifically test pain in such focal cases, ideally using quantitative methods.

Human claustrum stimulation

Like lesion studies, brain stimulation studies can provide strong evidence linking a given region to specific functional roles. The usefulness of stimulation studies is, however, limited by the possibility that observed effects result from excitation or inhibition of brain regions downstream of the region being stimulated.[86] This caveat is particularly noteworthy when considering the claustrum given its widespread connectivity throughout the brain. Additionally, due to the small volume of the claustrum, stimulation will almost certainly result in depolarization of neurons in nearby tissue.

A recent study by Bickel & Parvizi[83] obtained claustral stimulation data from five patients with pharmacologically resistant epilepsy, whose implanted electrodes partially overlapped with the claustrum. Four of the five patients experienced changes in somatosensation including 'a strange skin sensation from the face running down to the leg'; 'a sense of pressure in the mouth'; 'sensation around the left knee'; 'hot flashes'; and 'acid-like sensation in the throat'. However, none of them reported changes in their awareness of the situation and their surroundings. In 2014, Koubeissi *et al.*[31] reported that a patient with intractable epilepsy suffered a reversible disruption of consciousness after an electrode near the claustrum was simulated. However, a few caveats apply to this finding. First, the electrode was located in the extreme capsule, near, but not within the claustrum. Second, the patient had previously undergone an ipsilateral temporal lobectomy which may have functional implications. Third, the current applied by Koubeissi *et al.*[31] was greater in both magnitude and duration than in the Bickel and Parvizi[83] study. This may have caused widespread blanket depolarization of claustrocortical neurons, producing cortical inhibition,[6] that subsequently disrupted normal information processes.

The signs and symptoms of claustrum dysfunction caused by claustrum lesions and claustrum stimulation vary based on the pathologies and lesion/stimulation sites. Each patient experienced some aspect of disturbance in cognitive function, perceptual abilities, mental state, electrical activity, or sleep. This great diversity of signs and symptoms suggests a structure with a global role affecting diverse cognitive processes. Remarkably, and in stark contrast to this conclusion, patients with well-delineated surgical removal of unilateral claustra for low-grade cerebral glioma had no observed long-term sensorimotor or cognitive impairment.[87] The functional role of the unilateral claustrum might be efficiently compensated by neural plasticity of the intact claustrum, explaining why these patients did not suffer any permanent cognitive-perceptual impairments. These studies suggest that due to its involvement in multiple functionally compensated dynamic neural networks the claustrum is involved in numerous neural processes including both high-level cognitive processes as well as more fundamental processes. Taken together, these lesion and stimulation studies highlight the need to critically re-evaluate existing theories of the claustrum's function.

Re-evaluating the function of the claustrum

The results described above present the most comprehensive survey of claustrum lesions to date. Paradoxically, the most consistent feature of these claustrum lesion studies is their inconsistency. The

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diverse and overlapping array of clinical pathologies reported in these lesion and stimulation studies suggests that the claustrum may have a far-reaching role. The task of attempting to draw functional conclusions from this rich and varied data is further complicated by limited and inconsistent investigational practices across human studies.

In the sections below we will look more deeply into certain functional hypotheses in light of the lesion studies and propose avenues for future exploration. We will briefly evaluate the claustrum's involvement in neural correlates of sensory perception and salience as two prominent theories of the claustrum's function. We then review the claustrum's involvement in sleep and pain based on recent physiological studies.

Sensory processing

Lesion studies detailing sensory and perceptual abnormalities are suggestive of a functional role for the claustrum in sensory processing. These observations are consistent with anatomical and physiological findings in basic research. Connectivity studies have shown strong claustrocortical projections to sensory cortices and minimal corticoclaustral input from primary sensory areas.[12,15,88] Physiological studies have revealed somatotopic responses, retinotopic responses, and direct responses to auditory stimuli within the claustrum.[89–91] This anatomical connectivity, when considered in conjunction with single neuron recordings, led to the hypothesis that the claustrum is well positioned to play a key role in multisensory integration.[16,28] However, individual claustrum neurons have been shown to respond predominantly to one sensory modality[92] without any preference for the properties of the stimulus (i.e. no differences between vocalizations versus environmental sounds[93]).

These contrary findings might be partially resolved by data showing that claustrum neurons responded to changes in the sensory input[93] and responded to the novelty of stimuli pairs.[94] This hints that the claustrum does not encode the physical properties of the sensory stimuli but may instead shape sensory perception. This is supported by an early animal study showing that a claustrum lesion impacted the way primary visual cortex encoded a visual feature,[95] and by more recent theories that the claustrum may reduce noise in sensory cortices.[2,25]

The link to sensory processing is suggested by several lesion patients experiencing hallucinations and other delusions (as reviewed in-depth by Patru and Reser[27]). In that review, the authors argue that the claustrum is poised to act as a common centre for delusional states. They theorize how the claustrum may be involved in common models of delusion such as the glutamate/dopamine model, K-opioid receptor model, and models based on deficits in attention, sensory-gating, salience processing, and prediction. It is, however, important to note that these claustrum-related mechanisms of delusion may be directly related to the sensory abnormalities described in the lesion section.

As reported above, claustrum lesions can lead to sensory effects in multiple sensory domains, suggesting that the claustrum may be involved in the modulation of sensory processing. While it is possible that the claustrum plays a role in generating sensory perception, physiological evidence more strongly suggests a nuanced role in shaping the transition from raw sensory input to perceived output as reviewed elsewhere in detail.[26]

Salience

Recent studies performed in animals have also suggested a role for the claustrum in salience processing. Salience is the property of a stimulus that makes it noticeable. For example, a sudden loud sound carries perceptual salience, whereas the smell of food has motivational salience for a hungry organism. Put another way, salience can be seen as a process of attributing importance to information, which in turn controls information flow in the brain.

Findings from recent connectivity and physiology studies in animal models support the idea that the claustrum may be part of the 'salience network.'[6,96] The salience network is an operationally-defined group of brain regions including the anterior cingulate cortex, insula, amygdala, hypothalamus, ventral striatum, thalamus, and specific brainstem nuclei.[97–99] Many of these regions are closely connected with the claustrum (Fig. 3A and B). Moreover, the claustrum also receives strong input from regions linked to aversive and rewarding salience through its connections with the amygdala and dopaminergic system, respectively.[100,101] Recent physiological evidence from rodent models also aligns with these connectivity studies. Firstly, claustrum neurons have been reported to respond to salient sensory stimuli.[23,93] Secondly, inhibition of the claustrum decreased performance on a forced-choice task only in the presence of a distractor[2] and regulated the behavioural sensitization to rewarding salient stimuli.[102] Finally, in a classical conditioning paradigm, claustrum responses most closely correlated with the perceptual salience (which was produced by novelty and unexpectedness) of conditioned and unconditioned stimulus associations.[94] Based on these and other studies, researchers have proposed a putative role for the claustrum in salience processing.

While much evidence suggests a link, it remains unclear how the claustrum contributes to salience processing. One hypothesis suggests that the salience network is involved with *switching* between the default mode network and the central executive network.[97,99] This switch allows the brain to deploy the cognitive resources necessary to engage with salient stimuli. Functional MRI studies have suggested that the dorsal anterior insula is linked with this network switch.[97] However, it is possible that this process may also involve the claustrum. Furthermore, given the connectivity between the claustrum and default mode network, and the inhibitory effect of claustrum stimulation on the cortex, the claustrum is well placed to inhibit the default mode network in response to salient stimuli.[6]

The claustrum is suggested to contribute to salience processing by acting as a coincidence detector. Paired recordings within the claustrum have revealed dense electrical and chemical synapses between interneurons and a relative paucity of excitatory-excitatory connections.[18] Stimulation of corticoclaustral projections reliably evokes feed-forward inhibition in claustrocortical projection neurons.[18] This may explain the bimodal activity characteristic of claustrum neuron responses to cortical activation in the presence of feedforward inhibition. As such, strong input within a short temporal window is required to induce suprathreshold responses in the claustrum.[103] By responding to coincident input from sensory, limbic, executive[104] and neuromodulatory areas, this coincidence detection architecture could allow the claustrum to signal the salience of stimuli activating multiple neural circuits simultaneously.[29]

On the other hand, the lesion studies discussed above do not support a role for the claustrum in salience. A common sign for the loss of salience in one's mental representation of the environment is hemispatial neglect.[105] Hemispatial neglect is a common neuropsychological syndrome, in which patients fail to detect stimuli located contralaterally to a focal hemispheric lesion, even in the absence of primary sensory or motor deficits.[106] None of unilateral claustrum lesion studies reported spatial hemispatial neglect.

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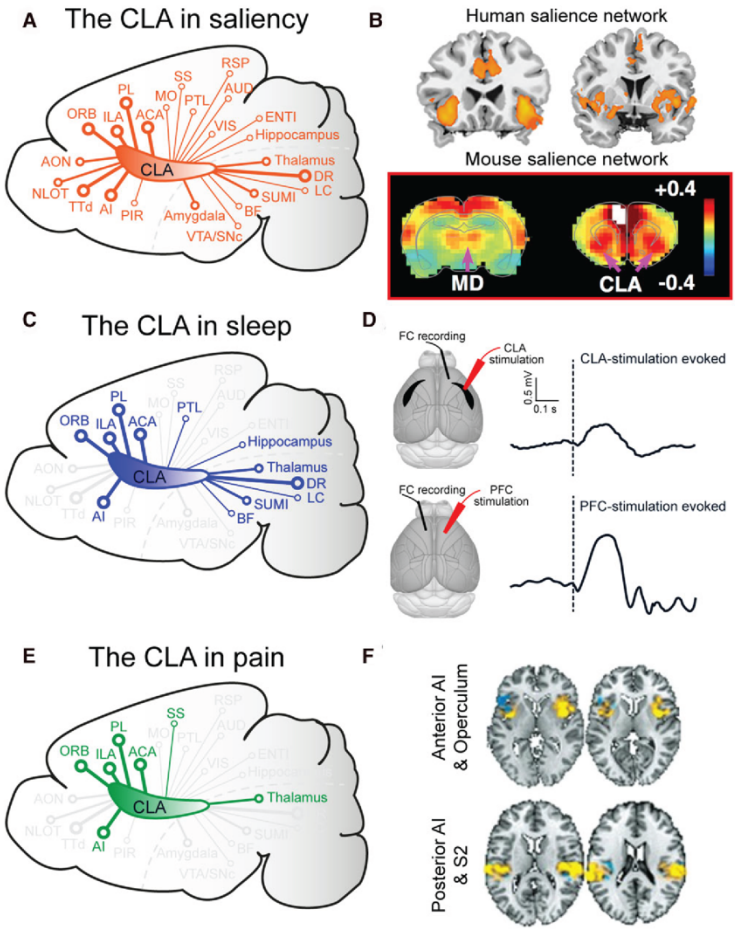


Figure 3 The claustrum is connected with or part of multiple brain networks. (**A**) Schematic illustration of a sagittal mouse brain section highlighting the connections between the claustrum (CLA) and regions important in salience. (**B**) Representative functional MRI images showing the human and rodent salience networks adapted from Seeley *et al.*[98] and Smith *et al.*,[96] respectively (© Society for Neuroscience). (**C**) Schematic illustration of a sagittal mouse brain section highlighting the connections between the claustrum and regions important in sleep. (**D**) Claustrum and prefrontal cortex (PFC) stimulation both evoke slow-wave like activity. Reprinted from Narikiyo *et al.*[7] and Vyazovskiy *et al.*,[122] respectively, with permission from Springer Nature ©2020. (**E**) Schematic illustration of a sagittal mouse brain section highlighting the connections between the claustrum and regions important in pain processing. (**F**) Brain activity to physical pain using multi-study data and machine learning classification tools. Images kindly provided by Tor Wager. FC = frontal cortex; MD = mediodorsal thalamus.

Furthermore, claustrocortical projections do not align well with the salience network based on cortico-striatal-thalamic loops. The strong ipsilateral claustrocortical projections do not match with the strong interhemispheric projections between cortical and subcortical areas posited as crucial due to hypotheses about the salience network.[107] In addition, corticoclaustral projections provide the dominant input to the claustrum (84% of claustrum input originates in the cortex[15]), but salience processing requires substantial non-cortical involvement. While supporting evidence continues to accumulate, questions remain as to whether the claustrum has a role in salience processing.

Sleep

Many of the lesion studies described above include reports of sleep disturbances. While the studies included limited details of these disruptions, recent developments from animal research support the hypothesis that the claustrum may be involved in sleep (for a more general review of sleep, see Brown *et al.*,[108] Liu and Dan,[109] Saper and Fuller[110] and Vyazovskiy and Harris[111]).

The broad anatomical connectivity of the claustrum makes it well placed to play a role in brain-wide processes such as sleep. Sleep is regulated by numerous brain regions, many of which are

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strongly connected to the claustrum, including those that are highlighted in Fig. 3C. Of particular relevance to sleep are the enriched serotonin receptors in the claustrum[112–115] and extensive unidirectional inputs from serotonergic raphe neurons,[7,15] which are involved in sleep-wake control.[116] Additionally, the claustrum's abundant reciprocal connections with the prefrontal cortex, insula, and cingulate cortex[15]—where slow waves can originate[117–119]—make the claustrum a promising candidate for regulating slow waves. These anatomical connections and physiological findings appear to support a role for the claustrum in sleep-related phenomena. Indeed, the claustrum shows increased expression of markers of neural activity following REM sleep hypersomnia, particularly in claustral neurons connected to frontal cortices such as anterior cingulate cortex.[120]

A recent study by Narikiyo *et al.*[7] causally linked the claustrum to slow-wave activity. The authors found that stimulation of the claustrum during slow-wave sleep induced a slow wave-like downstate in the cortex followed by a synchronized transition to an upstate (Fig. 3D). In keeping with this, claustrocortical neurons were more active in slow-wave sleep, firing maximally just before and after the up-state of slow waves. Finally, slow-wave activity was reduced by genetic ablation of the claustrum. While this provides a compelling link between the claustrum and sleep, it is important to note that stimulation of the frontal cortex and thalamus[121,122] as well as peripheral and transcranial magnetic stimulation[123] have both been reported to elicit similar slow-wave activity (Fig. 3D). As such, further research is required to establish a unique role for the claustrum in slow-wave sleep.

Another recent study by Norimoto *et al.*[115] linked the claustrum to other aspects of sleep. The authors identified a reptilian homologue of the claustrum that was important in the generation of sharp-wave ripples. These physiological events are composed of a slow, high amplitude potential (sharp wave) followed by a burst of rapid oscillatory activity (ripple) and are implicated in memory consolidation.[124] Increasing and decreasing serotonin levels in the claustrum led to upregulation and downregulation of spontaneous sharp-wave ripples. They did, however, note that lesions of the claustrum had no effect on the alternation between REM and non-REM sleep. The authors conclude that while the claustrum underlies the generation of sharp-wave ripples during slow-wave sleep, it is not involved in the generation of the sleep rhythm itself.

These studies have carefully dissected several global and specific aspects of sleep and found that while the claustrum was involved with various aspects of sleep in different species, it did not control sleep globally. These results strongly implicate the claustrum as part of a network that subserves some of the neurophysiological correlates of sleep. While the link between the claustrum and sleep remains preliminary, the available evidence from animal studies in combination with the data from human studies presented above might lead to fruitful directions for future research.

Pain

The lesion studies outlined above hint at the intriguing possibility that the claustrum may play a role in pain perception. Pain is a multifaceted sensation, involving both sensory and affective dimensions. Acutely painful sensations activate, in a flexibly accessible manner, a bilateral and widely distributed network of brain regions. These include a core set of regions, such as the thalamus, insula, brainstem, secondary somatosensory, mid- and anterior-cingulate, and prefrontal cortices (Fig. 3E).[125–127] Pain-related activity in the claustrum, which is closely connected with many of these brain regions, may be obscured by activity in the nearby insula, falsely attributed to the claustrum when genuinely within the insula, or, if in both, attributed to one or the other.

Figure 3F illustrates the classification results for physical pain within a region of the posterior insula.[126] It is clear that the extent of activity from the various studies that have contributed to this map might overlap with the adjacent claustrum. Going forward, opportunities with ultra-high field imaging, such as 7 T MRI, with improved signal-to-noise, spatial resolution, and contrast effects are already illustrating for various studies, including pain, the potential it offers for better spatial discrimination between adjacent brain regions or nuclei.[128,129] It will be important for future studies to better delineate the precise roles of the claustrum and divisions of the insula, as well as their interactions, in pain.

Recent advances in rodent and human imaging now also enable further isolation of claustral and insular activity.[130,131] Notably, the insula has been identified as a potential deep brain stimulation target for treatment of pain[132,133]; it is possible that electrodes inserted for this purpose also have contacts within the claustrum or affect it via downstream activation. Despite the challenges of isolating claustrum activity during MRI, several studies of human brain activity during various modalities of experimentally induced pain report increased activity in the claustrum, among many other regions.[134–144]

As well as acute pain, claustrum activity has been reported in a few studies looking at chronic pain.[145,146] Furthermore, studies examining the placebo effect, catastrophizing, and empathy have also reported activity within the claustrum among other regions.[147–152] The claustrum has further been reported as active in studies investigating acupuncture, migraine, oxygen deprivation, and invasive motor cortex stimulation in patients with chronic pain.[153–157] Drawing strong conclusions from these studies is challenging as the authors rarely include any discussion of the claustrum outside of data tables.

In addition to human and animal imaging studies, non-imaging evidence from animal studies also supports a link between the claustrum and pain. The claustrum expresses proteins implicated in pain-related signalling, including kappa, delta, and mu opioid receptors,[158–162] and shows increased neural activity following experimentally-induced pain in various species.[163–167] One early study assessed the correlation between seizure-induced damage to the brain and changes in rats' pain threshold. Intriguingly, of all the structures within the diencephalon and telencephalon, damage to the claustrum explained the largest percentage of variance in the pain threshold.[168] Cautious interpretation is warranted as the pain threshold test in a rat will be confounded with movement and/or motivation deficits.

The claustrum may also play a role in the sensation of itch, a sensation that provokes the desire to scratch. While pain and itch are distinct sensations, the two are closely related.[169–172] Functional neuroimaging studies have noted differences in baseline claustrum activity in patients with chronic pruritus (itching) and healthy volunteers.[173,174] Multiple studies have found increased claustrum activity in response to experimentally induced itch,[174–176] and this activity correlates with itch intensity.[176] The claustrum is also rich in K-opioid receptors which have been linked to itch reduction.[158,177] Moreover, claustrum deactivation is positively correlated with itch-reduction following application of butorphanol, a drug with K-opioid receptor agonist activity.[178]

Although the claustrum is not commonly reported as active in studies of pain and itch, its potential involvement merits further

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investigation. The present lack of discussion, possible reporting bias, and inconsistent activation may be due to the dominance of other structures very near the claustrum that have well defined roles in pain and itch. Despite these challenges, and in light of ultra-high field MRI imaging opportunities and animal imaging techniques, further investigation of a connection between the claustrum and pain is warranted—not least because of its accessibility as a target for deep brain stimulation.

Discussion

Claustrum lesions resulted in numerous symptoms including disturbances in cognitive function, perceptual and motor abilities, mental state, electrical activity, sleep, and pain. Similarly, animal studies have reported roles for the claustrum in numerous functions as outlined above. At first, it seems difficult to reconcile these diverse findings and potential functions. Rather than performing a single function, the claustrum may instead play multiple intersecting roles that underlie the diverse range of disruptions observed following claustrum lesions.

We hypothesise that the widespread connectivity substrate provided by the claustrum may have initially developed for evolutionarily ancient and fundamental functions. Later, it became a useful framework or scaffold upon which more advanced cognitive abilities could be built as they developed over evolutionary timescales. In considering this possibility, it is perhaps helpful to look across the evolutionary history of the claustrum. Putative claustra have been described in all extant mammals, but the claustrum is likely far older. A claustrum homologue was described in birds by Puelles[179] and more recently a reptilian claustrum was described by Norimoto *et al.*[115] By comparing more distant branches of the evolutionary tree, we may perhaps extrapolate backwards to the original function of the claustrum. Sleep, one of the most ancient neural processes, has been linked to the claustrum in species separated by hundreds of millions of years of evolution. Norimoto *et al.*[115] found that the reptilian homologue of the claustrum was involved in the generation of sharp-wave ripples during slow-wave sleep. Similarly, Narikiyo *et al.*[7] proposed that the claustrum was involved with the generation of slow-wave sleep in mice, and Jansen *et al.*[77] linked the claustrum to insomnia in humans. While these studies connecting the claustrum with sleep are preliminary (see above), it is clear that the early claustrum was involved in some facet of sleep.

How can this evolutionarily ancient structure involved in the generation of sleep, one of the most ancient of neural processes, also be linked to more complex cognitive processes like salience or sensory processing? While the extensive connectivity of the claustrum may have evolved to facilitate sleep, it may have gained other uses as the brain increased in complexity. With the expansion of the cortex in mammalian lineages, the scaffold of the claustrum linking these diversifying cortices may have taken on new functions to support new behavioural needs. These new functions, by-products of the claustrum's exceptional connectivity, may have superseded the original function. These functions may even exist as discrete circuits contained within the claustrum complex.

Among the diverse consequences of claustrum lesions, we observed a strikingly high incidence of seizures. Combined with evidence from animal studies linking the claustrum to seizures, these studies are redolent of a role for the claustrum in regulating cortical excitability.[39,40,64] Further research will be required to understand if this increased risk of seizures is specific to the claustrum, or whether similar results would be obtained from any such large disruption of brain connectivity.

Conclusion

Recent work on the claustrum is unravelling the multifaceted nature of its role in the brain. We sought to answer the classic question 'What can't you do without the claustrum?' Unfortunately, lesions to the human claustrum are rarely specific due to its anatomy and bilateral location. Such lesions inevitably impact neighbouring areas, resulting in heterogeneous effects ranging from impaired cognitive, perceptual, and motor abilities, to altered electrical activity in the brain, and profound disturbances to mental states and sleep. These diverse findings suggest a far-reaching function for the claustrum.

While recent anatomical findings exclude the claustrum from some functions such as low-level sensory processing (given the lack of inputs from sensory thalamic nuclei and sparse inputs from primary sensory cortices) and direct generation and/or modification of motor output (given lack of connectivity with striatum), research has not yet yielded a conclusive function for the claustrum. It remains possible that the claustrum underlies many discrete functions and/or possesses a, so far, unidentified global function that ties together these disparate findings. The lesion studies described above do not lend conclusive support to any one theory of the claustrum's function. They do not provide strong evidence for prominent theories such as multisensory integration and salience processing while lending support to other theories such as the regulation of cortical excitability. Further, the lesion studies in the context of current research have led us to hypothesize a role for the claustrum in regulating cortical excitability. While the function—or functions—of the claustrum is not yet known, it is clear that further work is required to understand this enigmatic brain structure.

One possibility is to continue work on the claustrum across species, which has already usefully spanned reptiles, rodents, rabbits, cats, monkeys, and humans. This cross-species approach may be especially important considering that the role of this highly connected brain area could have changed throughout evolution. That the claustrum has been conserved across these diverse lineages over hundreds of millions of years argues for a fundamentally important function.

In neuroscience, we are often tempted to conclude that a brain region or cell type is responsible for a specific function, but this does not always generate understanding. The claustrum is a brain structure where this strategy is particularly unhelpful, urging us to rethink our approach both methodologically and conceptually.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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