Delineating The Unique Functional Contribution of the Retrosplenial Cortex in the Hippocampal-Diencephalic-Cingulate Network

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Summary

The research described in this thesis investigates the unique anatomy of the retrosplenial cortex and its functional contributions to spatial working memory in the rat. The retrosplenial cortex, which is composed of Brodmann's areas 29 and 30, has attracted attention due its apparent variable size across species and its strategic anatomical position. Reflecting its anatomical connectivity, the area has been associated with a range of cognitive functions including but not limited to episodic memory, visual processing, and navigation, but yet its exact functions have been hard to define.

In humans, damage to the retrosplenial cortex can result in both anterograde and retrograde amnesia (Valenstein et al., 1987; Maguire, 2001) and can cause an interesting type of topographical disorientation where patients can recognise landmarks but are unable to utilise them to orient themselves and navigate an environment (Maguire, 2001; Vann et al., 2009). Additionally, the retrosplenial cortex is one of the first regions to exhibit pathological changes in Alzheimer's disease and its deterioration can predict mild cognitive impairment (Pangas et al., 2010). Most recently, the area has also been associated with schizophrenia (Bluhm et al., 2009) and states of dissociation (Vesna et al., 2020). Due to its deep anatomical position in the human brain, the majority of intervention research concerning the functions of the retrosplenial cortex comes from animal studies using rodents. Although there is no consensus to its precise function, rodent studies point to multiple roles in spatial cognition including landmark coding, consolidation of spatial knowledge, and particularly, the integration between spatial reference frames.

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Statement

This is a statement to acknowledge that some of the contents of the thesis or the methodology used have been adapted and published or submitted for publication. This applies to the following papers and chapters:

Methodology and findings used in Chapter 1:

Andrianova, L., Yanakieva, S., Margetts-Smith, G., Kohli, S., Brady, E. S., Aggleton, J. P., & Craig, M. T. (2023). No evidence from complementary data sources of a direct glutamatergic projection from the mouse anterior cingulate area to the hippocampal formation. *eLife*, *12*, e77364. https://doi.org/10.7554/eLife.77364.

Aggleton, J. P., Yanakieva, S., Sengpiel, F., & Nelson, A. J. (2021). The separate and combined properties of the granular (area 29) and dysgranular (area 30) retrosplenial cortex. *Neurobiology of learning and memory*, *185*, 107-516. https://doi.org/10.1016/j.nlm.2021.107516.

Chapter 2 (accepted):

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Chapter 3 (Pre-print, under-review):

Yanakieva, S., Frost B., Amin, E., Nelson A., & Aggleton, J.P. (2023). Disrupting the direct inputs from the dorsal subiculum to the granular retrosplenial cortex impairs spatial memory in the rat. *Authorea,* September 05,23. https://doi.org/10.22541/au.169389204.46880548/v.

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List of Abbreviations

(e)GFP: (enhanced) green fluorescent protein;
AD: anterodorsal nucleus;
AM: anteromedial nucleus;
AV: anteroventral nucleus;
Cb: cingulum bundle;
Cg1/2: anterior cingulate cortex;
Cg1: cingulate cortex area 1;
Cg2: cingulate cortex area 2;
Cing/RSP: cingulate/retrosplenial group;
CTB: cholera toxin B;
DLO: dorsolateral orbital cortex;
DMN: default mode network;
DP: dorsal peduncular cortex;
DREADDs: designer receptors exclusively activated by designer drugs;
DS: dorsal subiculum;
FB: fast blue;
Fr3: fasciculus retroflexus 3;
I.p.: intraperitoneal injection;
IAM: interanteromedial nucleus;
IL: infralimbic cortex;
LD: laterodorsal nucleus;
LO: lateral orbital cortex;
M1: primary motor cortex;
M2: secondary motor cortex;
MO: medial orbital cortex;
mPFC/Cing: medial prefrontal/anterior cingulate group;
mPFC/RSP: medial prefrontal/retrosplenial group;
NA: not analysed;
PBS: phosphate-buffered saline;
PFA: paraformaldehyde;
Post: postsubiculum;
PrL: prelimbic cortex;

PT: parataenial nucleus;

RE: nucleus reuniens;

RSD (area 30), dysgranular retrosplenial cortex;

RSG (area 29): granular retrosplenial cortex (a,b,c);

RSGa: granular retrosplenial cortex, a;

RSGb: granular retrosplenial cortex, b;

RSGc: granular retrosplenial cortex, c;

RSP/RSP: retrosplenial/retrosplenial group;

RSP: retrosplenial cortex (granular and dysgranular);

SUB: submedius thalamic nucleus;

TT: tenia tecta;

V1B: primary visual cortex, binocular area;

V1M: primary visual cortex, monocular area;

V2: secondary visual area;

VA: ventral anterior thalamic area;

VISa: anterior visual area;

VISam:anteromedial visual area;

VISl: lateral visual area;

VISli: latero-intermediate visual area;

VISp: primary visual area;

VISpl: posterolateral visual area;

VISpm: posteromedial visual area;

VISpor: postrhinal visual area;

VISrl: rostrolateral visual area.

Chapter 1

1. General Introduction

1.1. A brief overview of the hippocampal-diencephalic-cingulate network in the rat

In 1937, James Papez, brought together anatomical and behavioural studies to formulate a neuroscientific model of emotions. At the centres of the circuit proposed by Papez is a set of connections linking the hippocampus with the hypothalamus, thalamus, and the cingulate cortex (including the retrosplenial cortex), which returned back to the hippocampus, via the parahippocampal region, to form a complete loop (Papez, 1937). As knowledge kept emerging, the circuit's roles have been redefined multiple times, with increasing belief that it may be vital for memory formation (Aggleton & Brown, 1999; Catani et al., 2013; Ranganath & Ritchey, 2012). Since then, the circuit has been given a variety of names, often placing more weight on the importance of the hippocampus due its undoubted role in memory (Bubb et al., 2017).

In 2017, Bubb, et al., introduced the term "hippocampal-diencephalic-cingulate network", which did not place particular functional weighting to just one structure, but instead emphasised the interdependence of these structure on each other. Broadly, the model describes the direct and indirect connections between the hippocampal formation (including the subiculum) to the mammillary bodies, mammillary bodies to the anterior thalamus, anterior thalamus to the cingulate cortex and the connections from the cingulate cortex to the parahippocampal and hippocampal regions (Bubb et

al., 2017). Meanwhile, Aggleton and O'Mara (2022) proposed that there are two separate memory systems: the hippocampal – cortical system and the medial diencephalic – cortical system, the second of which places the anterior thalamic nuclei at its core. Both of these systems project to the parahippocampal, prefrontal, anterior cingulate, and retrosplenial cortices, and although they make different contributions, they interact to jointly to influence cortical areas.

In this thesis, of particular interest are the interconnections between the anterior thalamus and the cingulate cortex (anterior cingulate and posterior cingulate/retrosplenial cortex) as well as the hippocampal formation to the retrosplenial cortex (Figure 1.1). These projections and their functions are characterised and described in detail in the following sections.

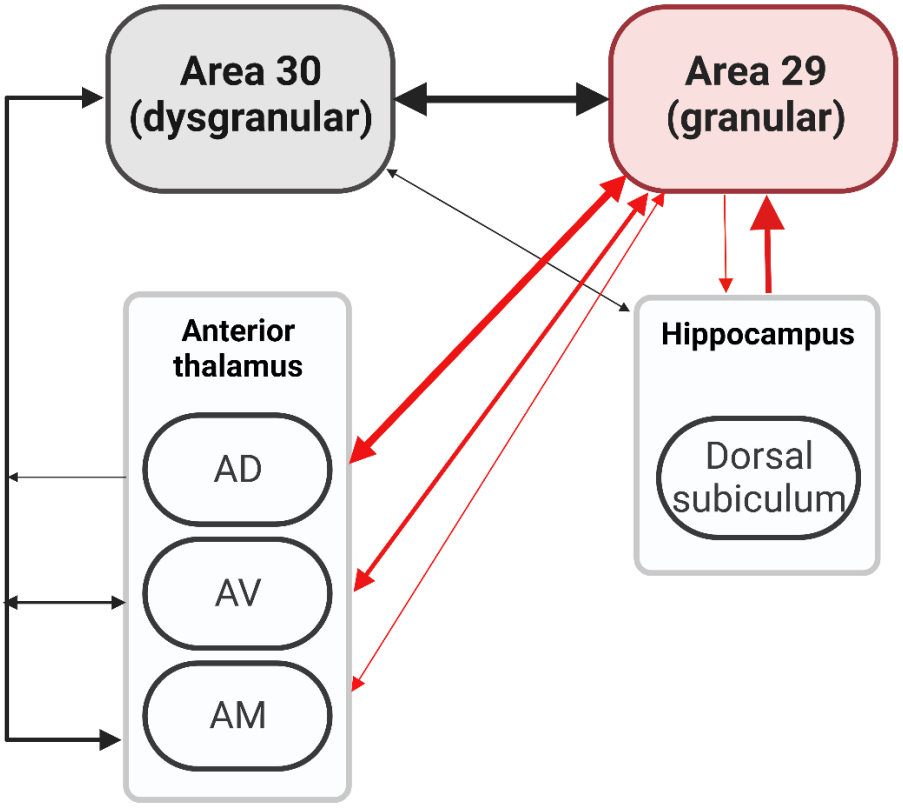


Figure 1.1. Schematic illustration of the connectivity of the retrosplenial cortex with the anterior thalamic nuclei and dorsal subiculum.

The schematic shows the circuits that will be investigated in the thesis. The granular retrosplenial cortex and its projections are illustrated in red and the dysgranular

retrosplenial in black. The arrows show the direction of the projections to and from the subregions, while the thickness indicates their respective densities. Thicker lines indicate denser projections and thinner lines, lighter projections. The figure was created using Biorender.com. Abbreviations: Area 29: granular retrosplenial cortex; Area 30: dysgranular retrosplenial cortex; AD: anterodorsal nucleus; AV: anteroventral nucleus; AM: anteromedial nucleus.

1.2. Anatomy, structure, and nomenclature of the retrosplenial cortex

Retrosplenial cortex gets its name from its anatomical position within the brain. From the Latin word for "behind" – *retro,* and the word *splenion* (from Greek meaning "a bandage"), the region is positioned immediately behind the splenium, the most posterior part of the corpus callosum. The region consists of Brodmann's areas 29 and 30, which together with Brodmann areas 23 and 31, in human and non-human primates form the posterior cingulate cortex. While rats do not have clearly defined areas 23 and 31 (Vann et al., 2009), their retrosplenial cortex is relatively enlarged. Another point of difference concerns the midcingulate area, at the borders of areas 23 and 24. In the human brain this transition areas is relatively extensive while in rodents it appears diminished (Vogt & Paxinos, 2014).Together, areas 29 and 30 extend for more than half of the dorsoventral cortex, making it one of the largest cortical regions in the rodent (Vann et al., 2009) (Figure 1.2). While in primate brains the retrosplenial cortex lies deep within the medial wall of the cerebral cortex, in the rat it is easily accessible through the cranium. Due to its relative accessibility for neural manipulations, much of what is known about the retrosplenial cortex connectivity and function is derived from rodent studies.

On the basis of its cytoarchitecture, the retrosplenial cortex can be divided into subregions. Multiple designations exist for its subdivision (Jones & Witter, 2007), however, the major subregions in the rat's brain consist of area 29 which corresponds to the granular retrosplenial cortex and area 30, and the dysgranular

retrosplenial cortex. The term “granular” refers to the presence of layer IV cells in the region. Compared to the granular retrosplenial cortex, the dysgranular retrosplenial cortex has wider layers II, III and IV (Wyss & Groen, 1992)(Figure 1.2). Some anatomists subdivide the granular area 29 further, with one of the most common denominations being areas 29a, 29b, and 29c (Shibata et al., 2009; Sugar et al., 2011; Vogt & Peters, 1981). Area 29a refers to the most ventral subdivision of the subregion, which differs from the dorsally adjacent area 29b by its homogeneous layers II and III. Area 29b differs from area 29c mostly in layer III, as in area 29c the layer appears to be thinner and its cells are more randomly spaced (Vogt & Peters, 1981; Sugar et al., 2011).

Other anatomists do not distinguish between areas 29a, 29b, and 29c or use different criteria for differentiation (Rose & Woolsey, 1948; Sripanidkulchai & Wyss, 1987; Wyss & Groen, 1992). Variations in the nomenclature of the dysgranular area 30 also exist (Rose, 1927; Jones et al., 2005; Shibata, 1994; Shibata et al., 2009). Due to the considerable variation in nomenclature and particularly the subdivision of the granular area 29, throughout the thesis area 29 will be treated as one, helping to bring together findings from different sources. If distinguishing between its anatomical connections is necessary, areas 29a and 29b will be referred to as the ventral granular area 29 and area 29c will be referred to as dorsal granular area 29 (Figure 1.2).

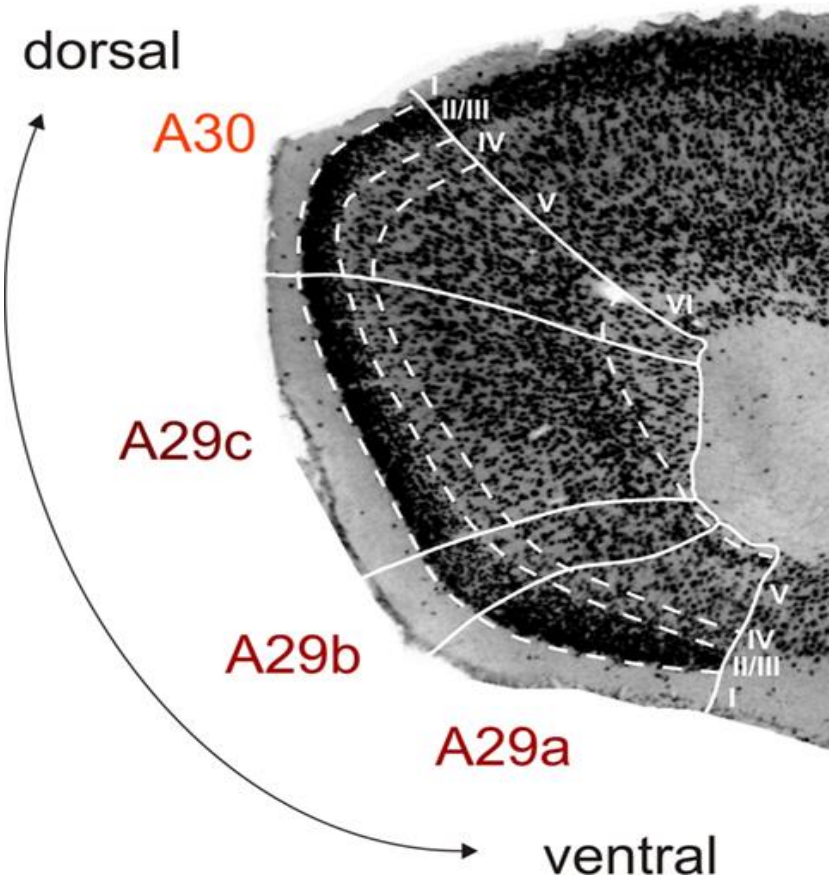


Figure 1.2. Cytoarchitecture and subdivisions of the retrosplenial cortex.

Photomicrograph of a coronal section stained for NeuN, illustrating the cytoarchitecture of the retrosplenial cortex, distinguishing the granular area 29 and its subdivision and the dysgranular area 30. The figure was originally published by Frontiers by Sugar et al. (2011). The figure was used with permission and no changes to it were made.

1.3. Connectivity of the retrosplenial cortex

Despite the apparent size differences of the region across species and the apparent gross anatomical differences, the connectivity of the retrosplenial cortex across species is overwhelmingly similar. In both rats and primates, the majority of retrosplenial connections (up to 78%) originate in other parts of the retrosplenial cortex or posterior cingulate cortex, respectively (Kobayashi & Amaral, 2003).

Furthermore, anatomical studies carried out in rats and non-human primates have noted multiple interconnections between the retrosplenial cortex, the thalamus, hippocampal formation, the parahippocampal region, prefrontal cortex, and the sensory cortices (Aggleton, 2010; Aggleton et al., 2016; Jiang et al., 2018; Kobayashi & Amaral, 2003; Robinson et al., 2014; Sugar et al., 2011) (Figure 1.3).

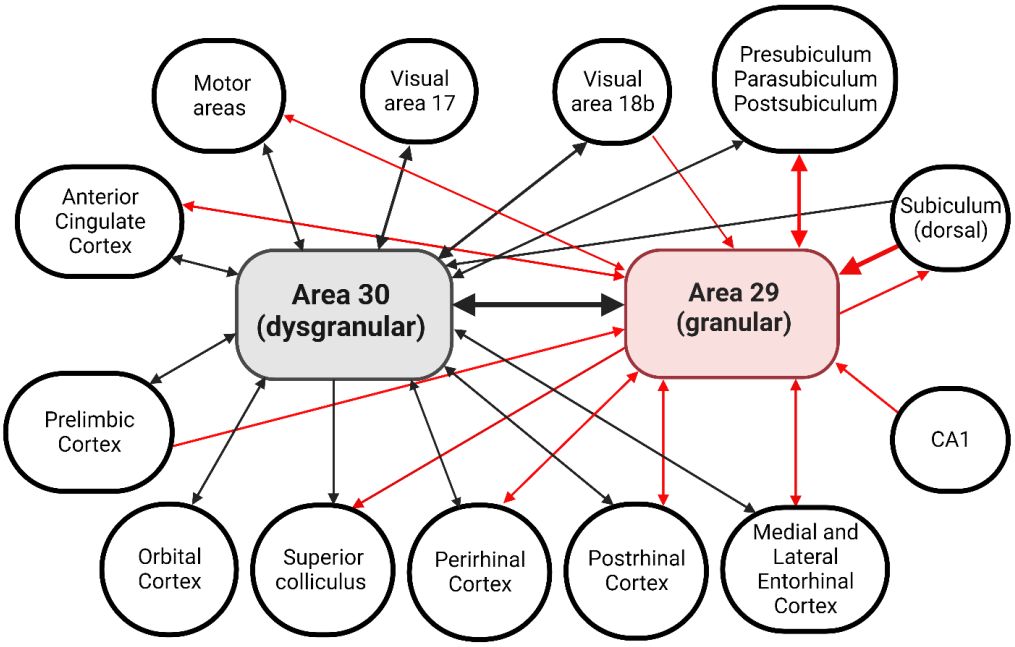


Figure 1.3. Connectivity of the rat retrosplenial cortex (areas 29 and 30).

The figure shows the connectivity of the retrosplenial cortex, emphasising the differences in the connections of areas 29 and 30. The thickness of the arrows indicated the relative strength of the connections. The figure was adapted and published in Aggleton et al. (2021). The figure was created using *Biorender.com.* Abbreviations: Area 29: granular retrosplenial cortex; Area 30: dysgranular retrosplenial cortex; CA1: Cornu Ammonis 1.

1.3.1. Intrinsic connectivity of the retrosplenial cortex in the rat

The ventral parts of the granular area 29 project to the entire rostro-caudal length of the dorsal granular area 29. These projections are also reciprocal so that the entire rostro-caudal length of dorsal granular area also projects to the ventral granular area (Sugar et al., 2011). Projections from the ventral granular area to the dysgranular area 30 tend to originate caudally in layers VI and more rostrally in layers III (Sugar et al., 2011; Van Groen & Wyss, 2003; Wyss & Groen, 1992). On the other hand, projections from the dorsal granular area to the dysgranular area, originate and terminate in all levels of the subregions (Shibata et al., 2009; Sugar et al., 2011). Area 30, projects from its entire rostro-caudal length to the entire dorsal and ventral granular areas 29 (Sugar et al., 2011).

In summary, areas 29 and 30 share many intrinsic connections. These reciprocal intrinsic connections (Figure 1.4) become even more apparent with subsequent division of area 29 and presumably reflect the functional interdependence of the subregions (Shibata et al., 2009; Sugar et al., 2011), making it more challenging to disentangle their respective functions.

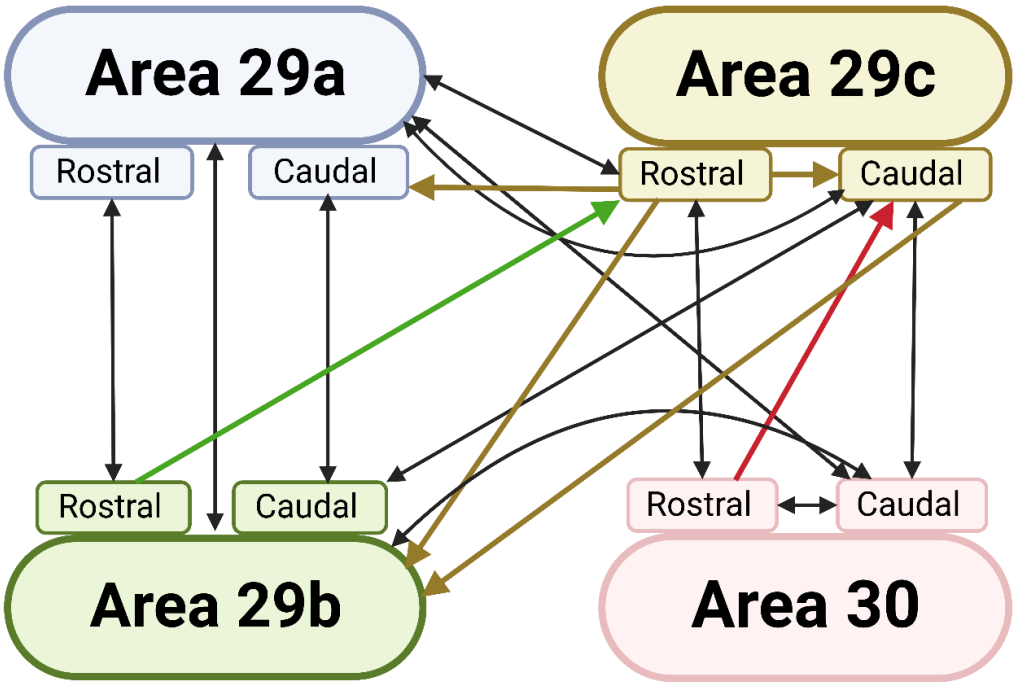


Figure 1.4. Intrinsic connectivity of the rat retrosplenial cortex (areas 29 and 30).

The figure shows the main intrinsic connectivity of the retrosplenial cortex as demonstrated by Shibata et al. (2009). The different subregions of the retrosplenial cortex are depicted in different colours: (1) Area 29a: blue; (2) Area 29b: green; (3) Area 29c: brown; (4) Area 30: red. Double headed arrows indicate reciprocal connections and are presented in black. Non-reciprocal projections are presented with arrows coloured in the representative subregion of origin. The figure was created using *Biorender.com*

1.3.2. Extrinsic connectivity of the retrosplenial cortex in the rat

Consistent with its role in spatial memory and navigation, the retrosplenial cortex is strongly interconnected with the hippocampal formation and the anterior thalamic nuclei. Perhaps the most striking differences between the connectivity of areas 29

and 30, can be observed in their hippocampal and thalamic connections. The majority of hippocampal projections to the retrosplenial cortex originate in the dorsal subiculum and terminate densely in layers II and III of the granular area 29. Direct hippocampal innervations are much sparser in area 30. Additionally, there seems to be small population of CA1 neurons that reach area 29 (Yamawaki, et al., 2019).

Perhaps surprisingly, there is almost a complete lack of direct return projections from the retrosplenial cortex to the hippocampus (Shibata, 1994; Shibata et al., 2009; Sugar et al., 2011). Although some light projections have been observed from layer V to the subiculum (van Groen & Wyss, 1990) and to CA1 (Tsai et al., 2022), it is likely that the retrosplenial cortex largely exerts its influences on the hippocampus through indirect routes via the parahippocampal regions and the anterior thalamus (Prasad & Chudasama, 2013). Both area 29 and 30 project to all parahippocampal regions, including the presubiculum, parasubiculum, postsubiculum, entorhinal, postrhinal and perirhinal cortices (Jones & Witter, 2007), and both have been found to receive reciprocal connections from these areas (Shibata et al., 1994; Sugar et al., 2011) (Figure 1.3).

Some of the differences regarding the thalamic connectivity of areas 29 and 30, may reflect the respective importance of area 30 in sensory processing due to its preferential connectivity to the visual cortical areas, unlike area 29, which is believed to be more critical for spatial navigation. The granular area 29, projects mostly to the anterodorsal nucleus, intermediate and dorsal laterodorsal nucleus, the dorsal parts of the anteroventral nucleus, and nucleus reuniens (van Groen & Wyss, 1990). On the other hand, area 30 has denser projections to the anteromedial nucleus, medial laterodorsal nucleus and the lateroposterior nucleus. Area 30 has very few projections to the anterodorsal nucleus while those to the anteroventral nucleus are also very sparse (van Groen & Wyss, 1990).

Thalamic projections from the anterodorsal and anteroventral nuclei terminate densely in area 29, with much lighter projections from the anteromedial and

laterodorsal nuclei. In contrast, area 30 receives much lighter inputs from the anterodorsal and anteroventral nuclei, and is more densely interconnected with the anteromedial, laterodorsal and lateroposterior nuclei (Sripanidkulchai & Wyss, 1986; van Groen & Wyss, 1990).

Both retrosplenial subregions also have extensive prefrontal connectivity (Monko & Heilbronner, 2021)(Figure 1.3), which may aid more complex cognitive processes such as task-switching and working memory (Alvarez & Emory, 2006). Both areas 29 and 30 are reciprocally connected to the anterior cingulate cortex. Projections from area 29 mostly originate in layer V and terminate in layers I and III, while area 30 projections seem to be topographically organised such that caudal area 30 projects to the mid-rostrocaudal anterior cingulate and rostral area 30 to the most caudal anterior cingulate (Shibata et al., 2004; Wyss & Groen, 1992). The anterior cingulate cortex also returns projections to both area 30 (Fisk & Wyss, 1999) and the entire area 29, again in a mostly topographically organised manner (Shibata et al., 2004). Both subregions are contralaterally and ipsilaterally connected to the prefrontal cortex. Granular area 29 has light to medium projections to all parts of the medial frontal cortex, prelimbic and infralimbic cortices (Condé et al., 1990; Hoover & Vertes, 2007), contrary to the dysgranular area 30 whose corresponding projections are very weak. Area 30 also sends projection to mainly layers I to III of the ventral, medial and lateral orbital cortex (van Groen & Wyss, 1992; Shibata et al., 2004) (Figure 1.3).

The retrosplenial cortex is also connected to the major sensory cortical areas including the motor, auditory and visual cortices. While area 29 has some very light connections with the visual area 18b (van Groen & Wyss, 1990), area 30's connections to the visual areas are much more extensive (Figure 1.3). It receives inputs from both areas 18b and 17, whose projection mostly terminate in layer I. These connections are reciprocal, in addition to limited connections with area 18a (Vogt & Miller, 1983; Wyss & Groen, 1992). The dorsal part of the granular area 29 also has dense projections to the caudal parts of the primary and secondary motor cortices, which originate mostly in layer V and to lesser extend layers II-IV (Reep et

al., 1990; Shibata et al., 2004). The dysgranular area 30 also projects to the motor areas and similarly these projections originate in layer V (Van Groen and Wyss, 1992; Shibata et al., 2004). The retrosplenial cortex also receives auditory inputs (Vogt & Miller, 1983) as is reciprocally connected with the posterior parietal cortex (Reep et al., 1994) (Figure 1.3).

In summary, the gross connectivity of the retrosplenial cortex points out to a role in spatial memory and navigation due to its reciprocal connections to the hippocampal, parahippocampal, and the anterior thalamic regions (Figure 1.3). However, there are extensive connections between the retrosplenial cortex and the prefrontal and sensory cortices, leaving the possibility that the area is also involved in sensory processing and executive function. The two main subregions of the retrosplenial, the granular area 29 and the dysgranular area 30, are distinguished by their connectivity (Figure 1.3), suggesting that although the subregions may be interdependent (Figure 1.4), their main functions may be distinct. Overall, the pattern of their connectivity suggests that area 29 is critical for spatial navigation, while area 30 is needed for sensory processing.

1.3.3. Brief overview of between-species differences in the retrosplenial anatomy and connectivity

As mentioned previously, the size and the gross division of the retrosplenial cortex varies among different species. Brodmann examined, defined, and compared the brain anatomy of various species including guenon and marmoset monkeys, lemurs, flying fox bat, the kinkajou, rabbit, ground squirrel, as well as the hedgehog (as translated by Garey, 2006). He found that while in the monkey species the retrosplenial region was very small, it was considerably developed in the lemur and could be subdivided into three subregions with clearly defined borders (see Garey, 2006, p.151). Although related to the size of the posterior cingulate cortex, the retrosplenial region is even bigger in the kinkajou and especially the rabbit and ground squirrel (see Garey, 2006, p.156). Interestingly, Brodmann described major

differences of the subdivision of the retrosplenial cortex among species with the rabbit having six or seven distinguishable subareas, three or four in the ground squirrel, three in the hedgehog, four in the flying fox, and three in the lemur (see Garey, 2006, p. 197). The retrosplenial cortex displayed some of the most marked differences in its size and architectonic development compared to other brain structures, possibly reflecting the cognitive demands of the various species and its function. More resent research also demonstrated differences in the size of the retrosplenial cortex between the degu and the Wistar rat. The relative size of the retrosplenial cortex to the entire cortex was significantly larger in the degu, as was the estimated volume of area 29 to that of area 30 (Shibata & Kigata, 2022). These differences suggest that the structural features may reflect differences in spatial processing between the species.

Anatomical studies investigating the connectivity of the retrosplenial cortex in non-human primates such as macaque monkey, indicate that its connectivity is largely consistent with that observed in the rat. In both species, the retrosplenial cortex receives dense projections from the hippocampal formation, including the subiculum, presubiculum, parasubiculum and the entorhinal cortex. These hippocampal and subicular projections also appear to primarily terminate in area 29 (Aggleton et al., 2012). The parahippocampal, perirhinal and the prefrontal areas also project to the retrosplenial cortex and the posterior cingulate region (Kobayashi & Amaral, 2003). The prefrontal- retrosplenial connections appear to be reciprocal as are the connections with lateroposterior, laterodorsal, anteroventral nuclei of the thalamus, the medial temporal lobe, parietal cortex, and the sensory areas (Kobayashi & Amaral, 2003; Morris et al., 1999; Rosene & Van Hosen, 1977; Seltzer & Pandya, 2009; Vogt & Pandya, 1987).

In the human and non-human primate brain, retrosplenial cortex is buried deep within the brain and is relatively inaccessible. Consequently, *in vivo* studies of its anatomy and functions are mostly carried out in small rodents such as laboratory rats and mice. Findings are often directly compared as it is presumed that retrosplenial anatomy and function is closely related in these rodents, although, some connectional and functional differences inevitably exist between the rat and

mouse brain (Ellenbroek & Youn, 2016), and possibly between different strains, genders, and ages (Keeley et al., 2015). For example, a series of tracing experiments in Long Evans rats showed that hippocampal-parahippocampal projections to retrosplenial cortex have low densities around birth and develop around week one, reaching adult-like densities (Haugland et al., 2019). However, it is unclear how these change over the lifespan and if densities reduce with age, leaving uncertain their potential significance.

1.3.4. Systematically mapping retrosplenial connections in the mouse brain

Connectivity studies of retrosplenial cortex were, for decades, focussed on the rat brain. Far less information was available for the mouse brain. The move to mouse models to study retrosplenial cortex function increased the value of accurately mapping the connectivity of areas 29 and 30 in the mouse. To help correct this imbalance, I examined the pattern of retrosplenial connections in the adult mouse brain, as reported in the Allen Mouse Brain Connectivity Atlas (2011).

The Allen Mouse Brain Connectivity Atlas (2011) is an open-source comprehensive database of axonal projections (for details see Kuan et al., 2015). Each mouse in the database received an injection into a *source* brain region of enhanced green fluorescent protein (EGFP) expressing adeno-associated virus (AVV), which acts as an anterograde tracer. The axonal projections were then systematically imaged using a TisseCyte 1000 serial two-photon tomography system (Oh et al., 2014) and organised into a searchable atlas. Each case in the atlas contains high resolution images and quantified projection information based on the optical density of label. Detailed histograms of the signal in each structure are presented giving the projection volume (mm^3) and projection density (the fraction of area occupied by a fluorescent signal from the viral construct used relative to the whole structure). The data for the atlas were collected from adult mice in postnatal day P56 ± 2 and details of the algorithms used are provided in Kuan et al. (2015). The Allen Atlas adopts its own division of the retrosplenial cortex, consisting of three areas. The “ventral”

retrosplenial cortex, which corresponds to the granular area 29, and “dorsal” and “agranular” areas, collectively corresponding to the dysgranular area 30.

When selecting the cases for comparison, there were a number of regions of interest, i.e., the source areas containing the tracer injection. A complete list is reported in Appendix A, however, there was not sufficient data for all source areas. The data discussed and presented here are from the following regions of interest: (1) anteroventral nucleus (AV); (2) anteromedial nucleus (AM); (3) anterodorsal nucleus (AD); (4) laterodorsal nucleus (LD); (5) subiculum (SUB); and (6) the following visual areas, anteromedial (VISam), primary (VISp), latero-intermediate (VISli), posteromedial (VISpm), lateral (VISl), postrhinal (VISpor), anterolateral (VISal), posterolateral (VISpl), anterior (VISa), and rostrolateral (VISrl). These regions were selected based on previous anatomical studies.

Each of the above regions of interest was inputted as a potential source and the retrosplenial cortex as a target. The experiments were further filtered for: (1) Mouse line: C57BL/6J; (2) Tracer Type: EGFP; (3) Hemisphere: Either; (4) Min Target Volume: 0.01mm^3. Only the experiments which had an injection volume of 0.02-0.3 mm^3, volume of tracer of min 50% within the region of interest, and at least one end projection of over 0.0005 mm^3 in retrosplenial cortex were retained (see Appendix A for details of the cases included). The volume of the injection was selected as it is within the standard tracer volumes used within existing anatomical studies. The reverse search with the retrosplenial cortex as a filter source structure and each region of interest as a target structure was also performed. The search filters were set as described above.

Interestingly, the analysis showed that the densest thalamic projections to the different layers of area 29 of the retrosplenial cortex, originated in the anteroventral nuclei (0.2- 0.4 mm^3) followed by projections originating in the laterodorsal nucleus (0.05- 0.1 mm^3), and finally the anteromedial nucleus (0.05- 0.1 mm^3). The pattern of distribution was the same for projections terminating within area 30, although the

anteroventral (0.05- 0.3 mm^3) and anteromedial (0.0005- 0.05 mm^3) projections appeared to be of slightly lower densities, while the laterodorsal projections were denser (0.05 - 0.15 mm^3) (see Appendix A, Figure 1). These findings are largely consistent with the thalamic connections observed in rats (Sripanidkulchai & Wyss, 1986; van Groen & Wyss, 1990). Note, the Allen atlas did not seem to contain any studies with injections confined within the anterodorsal nucleus (see Appendix A), and so it cannot be compared.

The return projections from area 29 to the anteroventral and laterodorsal nuclei appeared to have higher densities than its projections to the anterodorsal and anteromedial nucleus (see Appendix A, Figure 2) unlike in rats, where it projects mostly to the anterodorsal nucleus (van Groen & Wyss, 1990). On the other hand, area 30 connectivity is consistent with the rat literature in that projections to the anterodorsal nucleus seem almost non-existent, although its projections to the anteroventral and laterodorsal nuclei are denser than those terminating in the anteromedial nucleus (see Appendix A, Figure 2). As in the rat, the subiculum projections were densest to area 29 (max 0.1 mm^3) and were almost non-existent to area 30 (all < 0.03 mm^3).

Regarding retrosplenial afferents, both area 17 and the extrastriate cortex (except for the latero-intermediate and the anterolateral visual areas where data were not available) had axonal terminations in both areas 29 and 30. Unlike in the rat, these appeared to be comparable in density as there was not a strong preference for area 30. Interestingly, tracers placed in area 29 led to denser signals in the visual areas than tracer injections placed in area 30 (Figure 1.5). These findings suggest that in the mouse there may be greater balance regarding visual processing in areas 29 and 30 (Figure 1.5). Although both rats and mice are nocturnal, these structural differences may reflect the differences in their visual abilities. It must be borne in mind, that when making such comparisons between species and studies, differences in the subdivision of the retrosplenial are an added complication.

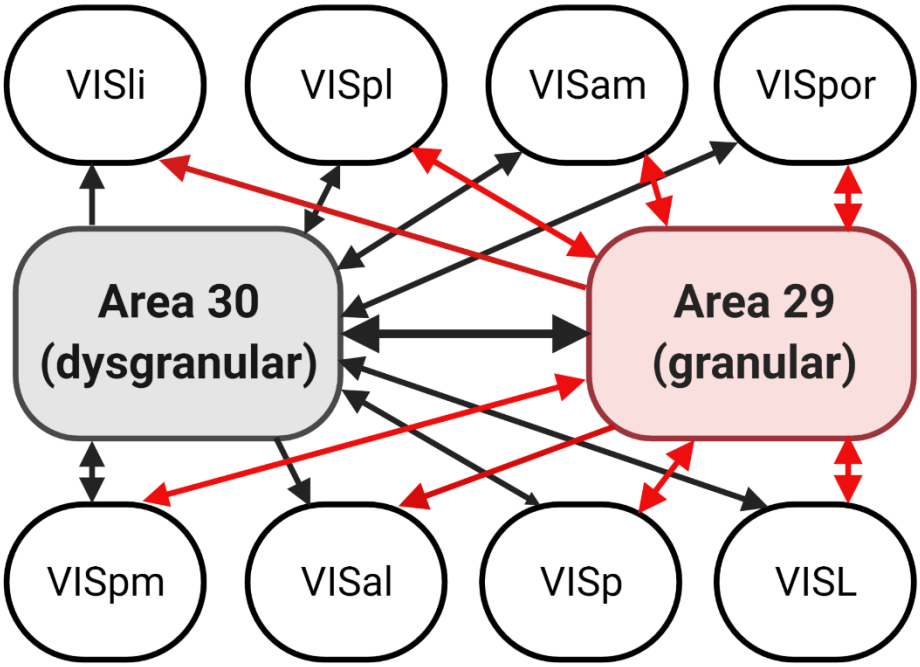


Figure 1.5. Connectivity of areas 29 and 30 with visual cortical areas in the mouse brain according to the Allen Mouse Connectivity Atlas (2011).

A total of 31 cases were used where over 50% of the injection was within the projection source area. The reciprocal connections between area 29 and visual cortical areas appeared denser than the corresponding connections with area 30. Note, no cases with tracer injections in VISli or VISal were found and so their efferents could not be depicted. Abbreviations: VISli: laterointermediate area; VISpl: posterolateral area; VISam: anteromedial area; VISpor: postrhinal area; VISpm: posteromedial visual area; VISal: anterolateral medial area; VISp: Primary visual area (area 17); VISL: lateral visual area. The figure was created with *Biorender.com.*

To summarise, the relative position, size, cytoarchitecture, and connectivity of the retrosplenial cortex seem to vary greatly between different species, and possibly strains, genders, and ages within the species. The various subdivisions given within both the granular area 29 and the dysgranular area 30 add to the complications.

Nevertheless, as outlined, there are core features that bind this area together across species. Systematically mapping both differences and common attributes can help to bring functional studies together and help to bridge the gap in translation to human function.

1.4. Functions of the retrosplenial cortex in humans

1.4.1. Imaging studies of the retrosplenial cortex in humans

In primates and humans, part of the posterior cingulate cortex (areas 29 and 30) is thought to correspond to the retrosplenial cortex in rodents. Due to its position within the brain as discussed above, human research is often limited to the use of non-invasive imaging techniques or case studies of patients who present with lesions and/or tumours involving the retrosplenial cortex. Although such methods are informative, it is generally difficult to distinguish the precise anatomical boundaries of the area. For that reason, imaging studies rarely isolate the retrosplenial cortex strictly to areas 29 and 30, and tend to include the retrosplenial cortex, posterior cingulate cortex, and the medial parietal region (Baumann & Mattingley, 2021; Epstein, 2008; Svoboda et al., 2006), which should be held in mind when interpreting such findings.

A meta-analysis of functional magnetic resonance imaging (fMRI) data in humans showed that that there may be functional subregions across the retrosplenial cortex. The analysis found that the rostral parts of the retrosplenial cortex seem to be associated with episodic memory, while the caudal areas were key in navigation and scene processing (Chrastil et al., 2018). Indeed, these are consistent with rodent studies and supported by both its connectivity and rodent imaging studies (Powell et al., 2020a, also see *section 1.4*). Further examination of the data in that same meta-analysis revealed consistent connectivity differences with the more rostral retrosplenial regions being connected to the default mode network (DMN) and the caudal retrosplenial region to visual areas (Chrastil et al., 2018). Indeed, imaging

research has demonstrated that the retrosplenial cortex is a key region within the DMN. The network consists of the medial prefrontal cortex, posterior cingulate cortex, retrosplenial cortex, and the medial temporal lobe, and is usually active at wakeful rest such as mind-wandering and daydreaming (Horn et al., 2014).

Due to its position and connectivity, the retrosplenial cortex has been proposed to facilitate information transfer between cortical and subcortical regions with the DMN and has been reported to be active in both resting state and during tasks that involve in other DMN regions (Maguire, 2001; Svoboda et al., 2006). One study analysed brain oscillation recordings during autobiographical memory retrieval obtained from patients undergoing invasive electrophysiology. The authors showed retrosplenial theta activity in the 3-4Hz coupled with activity in the medial temporal lobe (Foster et al., 2013). This coupling was not present at other bands (1-20Hz) (Foster et al., 2013) suggesting that indeed the retrosplenial may serve as facilitator of memory formation and navigation within the structures of the DMN through its connectivity with the hippocampus (Lega, 2012; Tesche & Karhu, 2000). In fact, when at rest, and daydreaming, individuals often report remembering past events, planning future events, and recalling personal experiences (Andreasen et al., 1995), which supports the role of the retrosplenial cortex in memory and explains some of the activity observed at “rest”. Interestingly, it has been found that performance on episodic memory tasks is associated with the degree to which the retrosplenial cortex mediates activity in these regions (Kaboodvand et al., 2018). Abnormalities in the DMN and aberrant retrosplenial activity have also been observed in schizophrenia patients. The correlations of activity in the retrosplenial cortex and the superior temporal gyrus (which is associated with hallucinations), were increased in patients with more positive symptoms, while the correlations between retrosplenial activity and the medial temporal lobe were lower (Bluhm et al., 2009).

Retrosplenial dysfunction has been reported in disorders other than schizophrenia, including epilepsy (Archer et al., 2003), and most notably disorders affecting memory such as Korsakoff syndrome (Aupee et al., 2001; Reed et al., 2003), vascular dementia (Martinez-Bisbal et al., 2004), and Alzheimer’s disease (Minoshima et al.,

1997). Positron emission tomography (PET) studies in patients with early stages Alzheimer's disease have demonstrated decreased glucose metabolism in the area compared to controls (Minoshima et al., 1997). Often, these changes can be observed before any cognitive and behavioural symptoms are present (Haxby et al., 1986). Although retrosplenial atrophy has also been observed in patients (Pengas et al., 2010), this need not the driving reason for the hypermetabolism of glucose (Mosconi et al., 2006). Additionally, patients with Mild Cognitive Impairment, which often precedes and predicts the onset of Alzheimer's disease, show metabolic changes in the posterior cingulate area, without evident changes in the hippocampus and the mamillary bodies (Nestor et al., 2003). Single-photon emission computed tomography scans of mild cognitive impairment patients who eventually develop Alzheimer's disease demonstrate that there is a decreased blood flow to the posterior cingulate (Huang et al., 2002; Johnson et al., 1998), likely leading its further deterioration.

Imaging studies of healthy adults also show that the retrosplenial cortex is activated during memory tasks (Gilboa et al., 2004; Wiggs et al., 1998). Most consistently, the posterior cingulate and retrosplenial areas are activated in tasks requiring autobiographical memory (Svoboda et al., 2006). Stronger activation is observed when participants recall more recent autobiographical memories compared to remote ones, although activation is present in both (Gilboa et al., 2004; Piefke et al., 2003; Steinvorth et al., 2006). In addition, the retrosplenial cortex and its adjacent areas also appear to play a more general role in the encoding, consolidation, and retrieval of spatial memories as indicated by positive correlation between functional magnetic resonance imaging (fMRI) activity in the region and performance on a topographic learning task (Wolbers & Büchel, 2005) and sensitivity to overlapping versus nonoverlapping scenes (Park & Chun, 2009; Robertson et al., 2016). Consistently, activation within the retrosplenial cortex is observed when participants are exposed to familiar scenes (Epstein, 2008; O'Craven & Kanwisher, 2000). Together these suggest that the retrosplenial cortex is involved in multiple aspects of spatial processing, with it being particularly important for switching between different spatial frameworks (Figure 1.6).

Although, the retrosplenial cortex seems to be involved in learning new environments (Iaria et al., 2007), activation within the region is stronger when recognizing a location as opposed to when participants are organizing information into spatial categories (Epstein et al., 2007). Interestingly, the retrosplenial cortex preferentially activates when participants observe permanent landmarks rather than those likely to change (Auger et al., 2012). Virtual environment studies confirm that the retrosplenial region may be critical for encoding location of landmarks. When participants are asked to learn the locations of several buildings, and brain activity is measured during retrieval of directional information, the retrosplenial region is more sensitive to the location of the buildings rather than the type of buildings, e.g., coffee shops, gyms (Persichetti & Dilks, 2019; Vass & Epstein, 2013).

In addition, the fMRI signals in the retrosplenial area scale with the size of scenes, with higher levels of activity in response to larger environments (Park et al., 2015). This effect may be unsurprising given that alongside the parahippocampal place area and the occipital place area, the retrosplenial cortex is key for representation of large-scale information in natural scenes (Çukur et al., 2016). The level of activity also correlates with green-space density when individuals view green urban landscape, which in turns correlates with behavioural stress response (Chang et al., 2020). One interpretation about these findings is that the regions are more tuned to larger environments since they may be more relevant for navigation due to the landmarks being present (Baumann & Mattingley, 2021).

Further to the retrosplenial cortex involvement in landmark recognition, evidence from fMRI studies has also demonstrated that the retrosplenial is involved in the encoding of perceived heading direction (Baumann & Mattingley, 2010; Marchette, et al., 2014). The pattern of activation within the retrosplenial cortex may also relate to individuals' ability to navigate. Participants who are better at making judgements about landmarks exhibit more activation (Auger et al., 2012). These individual differences may stem from differences in navigational strategies and the navigational frame that participants use to solve the task. There is a marked increase in

retrosplenial (and hippocampal) activity during navigation when participants use an allocentric frame (Auger & Maguire, 2013; Burgess, 2008).

This conclusion was further supported in a study investigating the neural dynamics of the retrosplenial cortex during an active spatial navigation task. Participants had to navigate to different locations while the positions of landmarks and starting positions were updated. The study showed that theta activity in the retrosplenial cortex increased with changes in heading direction, suggesting that the retrosplenial cortex may be involved in the head-direction computation in humans (Do et al., 2021). Interestingly, that same study found that individuals changed from egocentric to an allocentric frame when they were required to move through space. More specifically, most egocentric navigators switched to an allocentric reference frame during physical navigation, while the allocentric group consistently used their preferred allocentric strategy (Do et al. 2021). This suggests that the retrosplenial cortex may also facilitate switching between cue types and navigation strategies. A more general implication is that tasks which do not involve active physical movement through space may recruit different underlying circuits and mechanisms, as implied by rodent studies highlighting the integration of motor and visual stimuli within the retrosplenial cortex (Powell et al., 2020). A further finding is that individual differences in grey matter volume in the retrosplenial cortex, medial prefrontal cortex and the hippocampus may also correlate with path integration effectiveness (the ability to integrate self-motion cues over time, providing an estimate for total displacement from the starting point) (Chrastil et al., 2017).

Together the literature suggests that in humans, the retrosplenial cortex is involved in episodic memory, spatial memory, and navigation. Unsurprisingly, its dysfunction is associated with disorders affecting memory. The early changes observed in this region in patients with Mild Cognitive Impairment and Alzheimer's disease may be the cause for the topographical disorientation observed in the early development of the disease. Additionally, as a part of the default mode network, retrosplenial cortex may serve as a facilitator of cortical and subcortical information. Although imaging studies are useful in correlating function to cortical activity in humans, these studies

are limited in the insight they can provide. Due to its anatomical position within the human brain, the retrosplenial cortex is inaccessible to techniques such as transcranial electric and magnetic stimulations, and therefore temporary lesions cannot be evoked to experimentally examine loss of function. As a result, loss of function in humans is usually studied in patients who present with retrosplenial lesions.

1.4.2. Retrosplenial lesions in humans

In 1987, Valenstein et al. (1987) described a case of "retrosplenial amnesia" in a patient presenting with a lesion of the splenium and the region of the retrosplenial cortex and cingulum bundle. The patient experienced both anterograde and retrograde amnesia and demonstrated the role of the retrosplenial cortex in memory, beyond its role in the Papez circuit (1937). Later, Gainotti et al. (1989) described a patient who developed amnesia following anaplastic astrocytoma in the retrosplenial cortex region. The patient experienced severe retrograde amnesia for personal events, difficulties learning new verbal information, and poor ability to learn from visual information. This pathology appears consistent with results from imaging studies, as well as the connectivity of the retrosplenial cortex and suggest that the region may be vital for retrieval of autobiographical memories and integration of visual information (Gainotti et al., 1989). Indeed, deficits associated with memory are consistently reported in patients with retrosplenial damage (Bowers, et al., 1988; Maeshima et al., 2013; Masuo et al; 1999; Rudge & Warrington, 1991; Takayama et al., 1991). One frequent issue is that retrosplenial damage is typically accompanied by cingulum bundle disruption, while the ascending fornix may also be at risk. For these reasons, it is often impossible to distinguish the unique contribution of the retrosplenial cortex.

Consistent with the imaging literature, patients with damage to the retrosplenial also present with spatial deficits (Figure 1.6). One of the most fascinating types of spatial deficits caused by retrosplenial dysfunction is a type of topographical disorientation.

These are typically caused by unilateral lesions to the right retrosplenial cortex and cause navigational difficulties (Katayama et al., 1999; Sato et al., 1998; Takahashi et al., 1997; Yasuda et al., 1997). Patients with such damage can recognise landmarks, however, they are unable to use these marks to orient themselves and use them to navigate orient (Maguire, 2001; Osawa et al., 2008). Although, such deficits may improve over time, these deficits are consistent with observations in imaging studies that during navigation, the right hemisphere often shows higher activation than the left (Baumann & Mattingley, 2021).

However, spatial deficits can also be reported following damage to the left hemisphere. For example, a driver who presented with haemorrhage to the left retrosplenial cortex was unable to plan his route home. The driver was able to recognise landmarks on his route but was unable to extract directional information from them (Ino et al., 2007). Another study looked at a patient's ability to take a viewpoint. They were asked to identify the position from which a picture of their house was taken, and while the patient could recognise the landmarks and single viewpoints, the patient was unable to integrate the information and estimate the angle from which the picture was taken. The authors concluded that the inability to identify these viewpoints may be the reason behind the topographical disorientation (Suzuki et al., 1998).

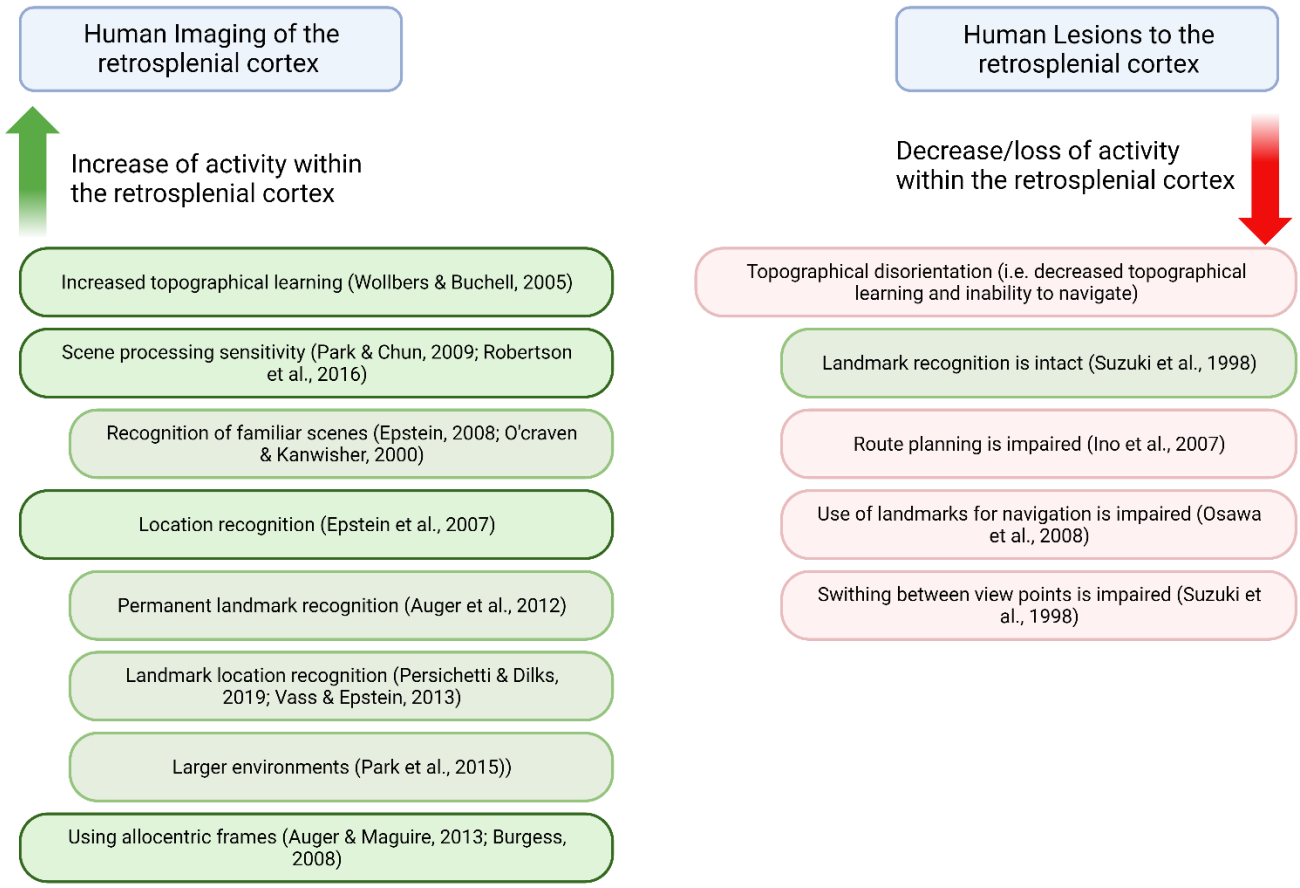


Figure 1.6. Schematic summary of the key human literature implicating the retrosplenial cortex in spatial memory and navigation.

The left-hand side of the schematics shows a summary of the spatial functions associated with increased activity of the retrosplenial cortex in humans. The right-hand side shows intact and impaired functions associated with decreased retrosplenial activity following lesions to the region in humans. Functions that remain intact following damage and which are positively associated with activity are depicted in green, while functions that are impaired are depicted in red. The figure was created using *Biorender.com.*

The functional deficits following retrosplenial cortex lesions in humans are consistent with the imaging literature review in *section 1.4.1 and* point to a clear role of the retrosplenial cortex in episodic memory, as well as navigation and spatial memory (Figure 1.6). Imaging studies suggest that increased retrosplenial activity is associated with various navigational functions, including landmark recognition

(Figure 1.6), although decreases in retrosplenial cortex activity following lesions do not seem to impair the latter *per se*, but rather how the information from the landmarks is used. This points to the possibility that while the retrosplenial cortex may support landmarks recognition in humans, it is not essential for this function. However, as mentioned earlier, imaging studies in humans often fail to specifically isolate the retrosplenial cortex, while lesions can cause varying degrees of impairment based on the lesion location and individual differences. Thus, the unique contributions of the retrosplenial cortex are difficult to examine in humans and as a result, anatomical and functional studies are often carried out using animal models. These are reviewed in *section 1.5* below.

1.5. Functions of the retrosplenial cortex in rodents

1.5.1. Electrophysiology of the retrosplenial cortex in rodents

Further evidence of the retrosplenial cortex involvement in spatial memory and navigation comes from electrophysiology studies. Like the hippocampus and the anterior thalamus, the retrosplenial cortex contains several different cell-types with spatial properties, such as head-direction cells (Chen et al., 1994a,b), angular velocity, and place cells (Cho & Sharp, 2001; Alexander & Nitz,2015; Lozano et al., 2017). Together, these cells modulate spatial representations and activity within the retrosplenial cortex and support navigation by creating cognitive maps (Alexander & Nitz, 2015).

Head-direction cells support navigation as their activity distinguishes the direction which animals are facing. In rodents, approximately 8.5% of the cells in each subregion of the retrosplenial cortex respond to head-direction information and fire when the animal orients in certain position (Chen et al., 1994a,b), which is interesting considering their presumably different functions and likely reflects the intrinsic connectivity of the region (Figure 1.4). Additionally, retrosplenial cortex is densely connected to regions with large populations of head-direction cells such as the

anterior thalamic nuclei (Taube, 1995) dorsal subiculum (Taube et al., 1990) and the entorhinal cortex (Sargolini et al., 2006). Given these inputs and other cortical connections of the retrosplenial cortex, its potential role may be to register and integrate head-direction signals from different sensory sources (Roth et al., 2016).

For instance, head-direction cell activity in the mammillary bodies can influence the anterodorsal nucleus and, in turn, primarily area 29 of the retrosplenial cortex, while visual influences could reach area 30 though the laterodorsal and lateral posterior nuclei of the thalamus (Clark et al., 2010). The head-direction signals these cells receive seem to rely on landmarks (Lozano et al., 2017). Indeed, some head-direction cells alter their directional preferences depending on the position of landmarks within the environment, suggesting that visual information is also incorporated into these cells (Chen et al., 1994a,b). Interestingly, directional sensitivity is also maintained if landmarks are lacking or even in the dark, suggesting that some head-direction cells are responsive to body movement (Chen et al., 1994a,b).

The ability of the retrosplenial cortex to support-navigation by using body-movement may also be supported by a separate class of cells. Widely spread across the retrosplenial cortex are angular head velocity cells that can reliably track the direction and speed of the head of an animal, even in complete darkness, by relying on vestibular cues (Keshavarzi et al., 2021a, 2021b). Interestingly, these cells are more responsive and reliable to perceive self-motion when visual information is added (Keshavarzi et al., 2021a). Large proportions of neurons within the retrosplenial cortex are active when environmental boundaries are positioned at orientation and distance from an animal. Most signals from these neurons seem to be centred in area 30 and are independent from self-motion, while a subpopulation of them is also synchronised with hippocampal theta activity (Alexander et al., 2020). Area 30 also contains the cells that still fire in the dark (Jacob et al., 2017), which is consistent with the proposed role of the subregion in sensory integration (Fischer et al., 2020).

Approximately, 13% of cells in area 30 are visually responsive when mice are sedated compared to 40% when awake (Powell et al., 2020). However, only 6% in the rostral retrosplenial cortex are visually responsive, which reflects the stronger connections between the visual fields and the caudal area 30 (van Groen & Wyss, 1992). These neurons are strongly modulated by locomotion in both the presence and absence of visual stimulation as layer VI head-motion signals within the primary visual areas seem to be conveyed via the retrosplenial cortex (Flossmann & Rochefort, 2021). These findings are consistent with the distinction between traditional head-direction cells (within the anterodorsal nucleus) and sensory head-direction cells that are driven by visual information from the environment (Dudchenko et al., 2019).

Indeed, when the activity of area 30 is selectively recorded, some head-direction cells change their preferred direction when rats walk between two connected compartments, each containing landmarks that were reversed in orientation relative to the other (Jacob et al., 2017). Contrary, cells within the anterodorsal nucleus, for example, maintain their directionality (Dudchenko & Zinyuk, 2005). Although, lesions and inactivation of the retrosplenial cortex can cause changes to head-direction cells in the anterodorsal nucleus (Clark et al., 2010) and to place cells in the hippocampus (Cooper & Mizumori, 2001) by decreasing stability of their firing, spatial deficits are not always observed. The suggestion that there are two different classes of interdependent cells may explain why changes in distal regions do not necessarily affect behaviour (Dudchenko et al., 2019). The interaction and interdependence of the activity within these regions is also evident in studies examining theta wave activity (4-12Hz), which is associated with spatial navigation in rodents (O'Keefe & Reece, 1993; Vanderwolf, 1969; Mizuseki et al., 2009).

Theta wave activity has been recorded in granular area 29, showing similar activation patterns to the theta activity observed in the hippocampus (Leung & Borst, 1987). Studies have shown that theta band oscillations in CA1 within the hippocampus are in coherence with these oscillations recorded within the retrosplenial cortex, even though their amplitudes are different (Young &

McNaughton, 2009). Indeed, Miller et al. (2021) identified two types of context representations within the retrosplenial. One that involved a novel code where neurons fire at a higher rate in preferred context regardless of the spatial location and a second that has context-dependent spatial firing patterns, like those observed in the hippocampus. The activity of some neurons within area 29 is strongly entrained by theta oscillations, showing strong coupling between the activity of the region and the hippocampal formation (Lomi et al., 2021). Additionally, the dorsal subiculum and the anterior thalamus selectively recruit small low-rheobase pyramidal cells within area 29, contrary to regular cells, which are preferentially recruited by the claustrum and anterior cingulate cortex (Brennan et al., 2021).

Within both regions, theta appears to increase in frequency (7-9Hz) when active movements such as walking are executed and decrease (6-8Hz) during movements of the head or postural changes (Leung & Borst, 1987). Interestingly, some of the theta activity in the retrosplenial cortex may be independently generated since septal lesions have been shown to abolish hippocampal theta activity while preserving (and sometimes increasing) theta waves in the retrosplenial cortex (Borst et al., 1987). Although theta activity in the retrosplenial area is observed even in the absence of hippocampal theta rhythms (Young & McNaughton, 2009), it is unclear whether they are generated within the retrosplenial or supported by other regions projecting information to the retrosplenial cortex.

Within both area 29 and area 30 of the retrosplenial cortex there are place cells that are like those found in the hippocampus (Mao et al., 2017). The place field responses within area 30 are critically dependent on the inputs which they receive from the hippocampus (Mao et al., 2018). Both areas also contain border cells (van Wijngaarden et al., 2020). Within the retrosplenial cortex these cells contain multiple firing fields and maintain their properties in the dark and when tactile information is removed (van Wijngaarden et al., 2020). Furthermore, the authors found that the cells did not respond to changes in the global environment implying that they may be egocentric. Additionally, using virtual reality (Mao et al., 2020) and a linear treadmill, the authors imaged mice in a head-fixed frame, thereby removing vestibular self-

motion cues. They found that retrosplenial neurons responded as a function of location in the virtual environment but not on the belt. When the running speed on the belt was varied relative to the speed in the virtual environment, the neurons were unaffected, while when four identical landmarks were added to the virtual environment at fixed positions, a minority of neurons responded repeatedly. Together these suggest that the retrosplenial cortex also encodes internal and external representation of visual space.

The variety of cells that are contained within the retrosplenial broadly supports the conclusion by Alexander and Nitz (2015) that the retrosplenial cortex maps egocentric and allocentric space and is consistent with the idea that the region is crucial for switching between cue types and navigational strategies. Additionally, evidence shows that the retrosplenial cortex is involved in other aspects of navigation such as route planning (Miller et al., 2019) and path integration (Ju & Gaussier, 2020).

1.5.2. Non – spatial functions of the retrosplenial cortex in rodents

Retrosplenial cortex has been associated with a variety of deficits in functions beyond spatial processing and navigation. For instance, evidence shows retrosplenial involvement in a number of learning tasks, including sensory preconditioning (Fournier et al., 2020; Robinson et al., 2011, 2014), retrieval of remotely acquired cued fear (Jiang et al., 2018; Todd et al., 2016), context-preexposure facilitation (Todd et al., 2017), time-based feature discriminations (Todd et al., 2015), discrimination learning (Keene & Bucci, 2008; Robinson et al., 2011), negative pattern learning (Fournier, Todd, et al., 2019), associative object recognition memory (Ennaceur & Aggleton, 1997; Hindley et al., 2014; Vann & Aggleton, 2002), and contextual fear conditioning (Keene & Bucci, 2008). These functions reflect the strategic position of the retrosplenial cortex in the brain and its anatomical connections (see *section 1.3*) with brain regions involved in sensory processing, executive function, memory, and attention. Indeed, spatial, and non-

spatial deficits alike, are underlined by a global failure in memory, learning and attention. It is possible that the various deficits associated with retrosplenial dysfunction are driven by cognitive demands such as decision making and temporal processing that are difficult to measure in rodents.

Dysfunctions in the retrosplenial cortex can be induced by damage to distal sites such as the hippocampus and the anterior thalamic nuclei (Albasser et al., 2007; Amin et al., 2010; Jenkins et al., 2004; Poirier & Aggleton, 2009). Although sometimes changes do not produce observable behavioural effects, the expression of two immediate early genes (c-fos and zif268) in some retrosplenial laminae can be reduced by up to 90% (Albasser et al., 2007; Jenkins et al., 2004). Following lesions to the anterior thalamic nuclei, these decreases are observed in the superficial layers of area 29 (Garden et al., 2009), and interestingly similar effects can be observed following lesions to regions that do not have direct projections to the retrosplenial cortex, such as the mammillothalamic tract and the ventral tegmental nucleus (Vann & Albasser, 2009; Vann, 2013). Meanwhile, optogenetic inhibition of either rostral or caudal retrosplenial cortex results in decreased local cellular activity as indicated by zif268 expression, which is restricted to the targeted region within the retrosplenial cortex (Trask, Ferrara, Jasnow, et al., 2021; Trask, Pullins, et al., 2021). Additionally, retrosplenial lesions have little or no effect on c-fos activation in the hippocampus (Powell et al., 2018), which may be due to the sparse projections to the hippocampus originating in the retrosplenial cortex or potentially reflecting a role of the retrosplenial cortex that is independent of its output to the hippocampus. Indeed, lesions to the retrosplenial cortex produce both retrograde and anterograde context amnesia when rats undergo strong fear conditioning, suggesting that the retrosplenial may have a role in contextual fear conditioning that cannot be compensated by for other regions, such as the hippocampus (Fournier et al., 2019).

Both c-fos and zif26 are believed to be vital for the coordination of neuronal responses following incoming information and are thereby often considered as markers of learning. For instance, infusion of c-fos antisense oligodeoxynucleotides (molecules used to inhibit gene expression) in the hippocampus seemingly abolished

long-term memory (Countryman et al., 2005), reinforcing the likely importance of c-fos for learning. Similarly, long-term maintenance of long-term potentiation is disrupted in mice without the zif268 gene (Jones et al., 2001). Given these genes' importance in learning and memory, it is possible that damage in sites such as the anterior thalamus and the hippocampus may have additional impact given the disruption of c-fos and zif268 activity within the retrosplenial cortex.

When expression of c-fos and zif268 is compared across the retrosplenial subregions following a spatial task, expression of both genes is increased in area 29 regardless of the light conditions, however, increases in area 30 are only observed when the task is performed in the light (Pothuizen et al., 2009). These subregional differences presumably reflect area 30 involvement in visual processing. Using optical methods to stimulate retrosplenial c-fos neurons (primarily in area 30), in an engram-like paradigm, resulted in freezing behaviour independent of hippocampal inactivation (Cowensage et al., 2014). Similarly, further studies using optogenetics targeted at area 30 in contextual fear conditioning showed that activation of the region promoted learning and contextual generalization irrespective of the hippocampus (De Sousa et al., 2019). These findings imply that although the hippocampus is needed to facilitate learning, there may be another network of structures, which includes the retrosplenial cortex and supports these behaviours independently.

Indeed, Pan et al. (2022) showed that impaired retrieval of contextual fear memory occurred in animals with disrupted area 30 but not in animals with disruption of function of area 29, which receives most hippocampal inputs. Interestingly, Pan et al. (2022) also observed that area 30 is more critical for encoding of memories suggesting that different subregions of the retrosplenial may play different roles in both encoding and retrieval of contextual fear memories. This idea is partially supported by a study that used chemogenetic and optogenetic inhibition to silence neurons projecting from layer V of area 29 to CA1 of the hippocampus. Disrupting the activity of the projection selectively impaired retrieval of remote fear conditioning memories (Tsai et al., 2022), suggesting that the temporal characteristics of a

memory may also be relevant when considering subregional contributions. To complicate things further, there may well be functional gradients on the rostro-caudal axis of the retrosplenial cortex, such as that inhibiting activity in the rostral retrosplenial cortex impacts behaviour evoked by auditory stimuli, while inhibition of the caudal retrosplenial cortex selectively impaired memories for context (Trask, Ferrara, Grisales, et al., 2021).

When NMDA receptors are blocked during fear conditioning in the retrosplenial cortex, the ability of the animal to retrieve both recent and remote fear conditioning memories is impaired (Corcoran et al., 2011). Recent and remote memories are believed to rely on different circuits and mechanisms, with the retrosplenial cortex being robustly activated during retrieval of remotely acquired contextual fear memories. When chemogenetics are used to temporarily inactivate the retrosplenial cortex during either retrieval or encoding of delayed auditory fear conditioning, animals' ability to retrieve a remotely conditioned auditory cue is impaired while recently conditioned one is intact (Fournier et al., 2021). Additionally, this same study found that inactivating the retrosplenial during encoding had no impact on freezing behaviour during later retrieval testing for both a remotely and recently conditioned auditory cues, suggesting that the retrosplenial cortex is necessary for retrieval, but not encoding, and is more important for the retrieval of remotely acquired memories (Fournier et al., 2021). The necessity of the retrosplenial cortex for retrieval of remotely acquired cued fear memories also extends to conditioning using visual stimuli (Jiang et al., 2018), supporting the idea that the retrosplenial cortex has a role in sensory integration for forming complex representations (Fournier et al., 2019; 2020). Fournier's et al. (2021) findings partially contradict Pan et al. (2022), which may be due to differences in the experimental paradigms and general difficulties to separate the functions of area 29 from these of area 30 due to their connectivity. In fact, an extensive literature review suggests that the anterior cingulate cortex may be necessary for retrieval of memories that occurred at remote time points, while the role of the retrosplenial cortex is more uniform (Trask et al., 2021b).

The role of the retrosplenial cortex in object recognition has also been studied and the involvement of the region in this process may be critical for its involvement in topographical orientation. In the standard spontaneous object recognition task (Ennaceur & Delacour, 1988), rats are presented with two identical objects and after a certain interval they are allowed to explore a familiar and a different novel object. Typically, rats prefer to spend more time exploring novel objects. Following retrosplenial cortex lesions, animals' performance on the task is unimpaired (Ennauceur et al., 1997; Parron & Save, 2004; Vann & Aggleton, 2002), possibly due to the nonspatial nature of the task. However, deficits are revealed in an object-in-place task when the spatial location of the object becomes relevant. That task, however, varies in its navigational demands, helping to separate spatial location processing from ability to reach a particular location. Typically, four different objects are presented to animals, and these are positioned in four corners of an arena. Before testing the animal, the position of two objects is switched. Healthy rats will usually spend more time exploring the objects that are moved, however, rats with lesions to the retrosplenial cortex do not seem to differentiate between objects (Vann & Aggleton, 2002). Since the task requires rats to create a link between an object and its location, it may reflect the importance of retrosplenial in recognizing and using allocentric landmarks. If one object is moved only, rats still display deficits suggesting that the retrosplenial cortex is necessary for processing the spatial properties of the environment (Ennauceur et al., 1997; Parron & Save, 2004).

Indeed, similarly to findings in fear conditioning paradigms discussed above, retrosplenial cortex seems to be necessary to integrate information across different sensory, spatial, and non-spatial modalities. Hindley et al. (2014) tested rats with lesions to either area 29 or area 30 on cross-modal object recognition. Rats used different sensory modalities when exploring and subsequently recognizing the same test objects. In the task, rats were first presented to the object either in the dark or in the light behind a clear barrier. Then, they tested the animals with either constant combinations of sample and test conditions (light to light, dark to dark), or changed "cross-modal" combinations (light to dark, dark to light). Then the animals were tested on a visual object recognition task without clear barriers, using objects that could not be distinguished in the dark. Hindley et al. (2014) found that that the rats

with lesions to area 30 were selectively impaired on cross-modal recognition when animals switched cues from dark to light, but not when the conditions of sample and test remained constant in either dark or light. Furthermore, rats with lesioned areas 29 and 30 also failed the dark to light cross-modal condition but this impairment was less selective. Hindley et al. (2014) concluded that area 30 has a mediating role in integration of information across multiple cue types, which may apply to both spatial and non-spatial domains. Similar object recognition paradigms have been used to investigate episodic-like memory in rats.

One study, focused on examining “what”, “where”, “when” properties of objects. Animals were tested on four conditions: the identity of an object (what); the location of an object (where); the temporal order in which an object was presented (when); and the integration of the three, which is believed to test episodic-like memories in rats. The authors showed that rats with retrosplenial cortex lesions, preferred novel objects in “what” condition, but not in the temporal and episodic-like memory conditions (Hayashi et al., 2020). Deficits in rats’ temporal discrimination following retrosplenial cortex lesions had been previously demonstrated (Todd et al., 2015) and it is possible that the episodic-like memory deficits exhibited by animals are driven by deficits in temporal order and discrimination. Indeed, in a delayed matching-to-position task where rats are required to remember the location of a lever or to discriminate between two tones, they were able to demonstrate an ability to use self-behaviour (i.e., episodic-like memory). When excitotoxic lesions were made to the retrosplenial cortex, and animals were tested again, this ability disappeared (Sato, 2021), demonstrating that the retrosplenial cortex may play a role in retrospectively accessing episodic-like memories.

Regarding the role of the retrosplenial cortex extending beyond that of the hippocampus, long-term object recognition in rats has been tested in animals with lesions to the retrosplenial cortex and other cortical and subcortical regions known to be involved in the process. Landeta et al. (2021) observed that rats with ipsilateral inactivation of the rostral retrosplenial cortex in a combination with the perirhinal cortex, medial prefrontal, anteromedial thalamic nuclei, and medial entorhinal cortex

were impaired on the task. When inactivation between regions was done in opposite hemispheres, the rats showed deficits in long-term object recognition in the rostral retrosplenial/anterior cingulate group. Interestingly, however, effects were not observed with inhibition of the rostral retrosplenial/dorsal hippocampus, suggesting that the retrosplenial cortex is key to consolidation of object recognition, although there appear to be multiple cortico-cortical and cortico-thalamic pathways (de Landeta et al., 2020, 2021).

1.5.3. Spatial functions of the retrosplenial cortex in rodents

Even though there are some inconsistencies in the research literature about the role of the retrosplenial cortex in spatial memory, some due to differences in surgical methodologies (Vann et al., 2009), it is now generally agreed that the retrosplenial cortex is crucial for normal spatial memory and navigation. In rodents, lesions can either be made physically, using aspiration or electrolysis or by injecting neurotoxins. Physically induced lesions may damage adjacent white matter tracts, including the cingulum, and disconnect projections running though the targeted area. Meanwhile, neurotoxins can spare fibers of passage. More recently, chemogenetic and optogenetic techniques have also been used to cause reversable temporary lesions, which generally reduce rather than eliminate the activity within the targeted region. These two techniques should also spare fibres of passage. Due to these differences, there are some discrepancies in the results of behavioural experiments.

Early neurotoxic lesions involving the retrosplenial cortex showed either no deficits or very mild deficits on tasks that may show severe impairments following traditional lesions to the retrosplenial cortex (Aggleton et al., 1995; Neave et al., 1994; Warburton et al., 1998). In the earlier studies, the most caudal part of the retrosplenial was often spared (Vann et al., 2003), which may have been sufficient to support spatial memory functions, while traditional lesions would have damaged the cingulum bundle, which would in turn result in a complete lesion (Meunier & Destrade, 1997; Whishaw et al., 2001).

Deficits in spatial memory following retrosplenial cortex lesions are evident on standard reference memory (Harker & Whishaw, 2002; Sutherland & Hoesing, 1993; Vann et al., 2003) as well as on working memory (Haker & Whishaw, 2004; Vann et al., 2003) versions of the Morris water maze. While both tasks rely on the use of distal visual cues. In the former, the platform remains constant during the training sessions, while the latter working memory version, the platform remains stationary within the session but moves between different sessions. Animals with retrosplenial lesions take longer than healthy animals to learn the position of the platform and seem to spend less time in the correct location when the platform is removed (Vann et al., 2003). On the other hand, animals with damage to the caudal parts of the retrosplenial cortex are impaired at initial acquisition, but not when the platform is removed, suggesting that they were still able to learn the location of the platform after training (Vann et al., 2003). These are consistent with results observed after lesions to the more rostral parts of the retrosplenial cortex (Haker & Whishaw, 2002; Warburton et al., 1998) suggesting that both regions are reliant on each other and could compensate for damage.

Although deficits in the water maze task might solely reflect the role of the retrosplenial cortex in spatial memory, there may be contributions from the non-spatial demands of the task. For example, when rats are trained on the non-spatial aspects of a task, i.e., to swim freely in a water-maze in the absence of distal visual cues, they show deficits comparable to those seen in lesioned rats without pre-training (Cain et al., 2006). Additionally, rats without pre-training, improve slower with training that those who have been pre-trained suggesting that the retrosplenial cortex may have role in the learning of navigational strategies and their consolidation (Cain et al., 2006), although it appears that damaging the retrosplenial cortex before training does not completely eliminate the ability of the animals to develop these skills (Lukoyanov et al., 2005), possibly reflecting the role of other brain regions in acquisition.

Deficits following retrosplenial cortex damage also emerge on spatial tasks using the radial-arm maze and the T-maze. The radial-arm maze is typically made up of eight arms that are positioned at equal distances from a central platform (Olton & Samuelson, 1976). Arms are baited with a reward which the animal can collect by entering the arm. However, if the animal enters an arm that has already been visited this is considered an error of working memory. The T-maze on the other hand, has two opposing arms that are baited (Dudchenko, 2001). In the standard reinforced version of the T-maze, animals are forced to enter one arm on the information trial. Then the barrier is removed, and animals should visit the alternative arm to gain a reward. Consequently, revisiting the same arm is considered an error of working memory. Rats have a variety of cues and strategies to solve the task, and these can often be manipulated in experimental paradigms (Dudchenko, 2001). Rats with damage to the retrosplenial cortex exhibit deficits on both spatial memory tasks, although these may be mild and may reflect the various navigational strategies available to solve the maze tasks (Cooper et al., 2001; Pothuizen et al., 2008; Vann & Aggleton, 2002, 2004).

More consistent deficits appear when the radial maze rotates between trials, thereby creating conflict between extra-maze and intra-maze cues. Animals with retrosplenial cortex lesions make significantly more errors following the rotation, even if they are not impaired while initially learning the task (Pothuizen et al., 2008; Vann & Aggleton, 2002, 2004; Vann et al., 2003). The impairments also appear to be larger when the maze is rotated than if the rat is rotated (impairing egocentric cues) or if a longer delay between trials is introduced, suggesting that the retrosplenial is needed for switching between the use of intra-maze and extra-maze cues (Vann & Aggleton, 2004). A similar pattern of results is observed when animals' spatial memory is tested on the T-maze. Although the effects of retrosplenial lesions on the alternating T-maze are inconsistent (Aggleton et al., 1995; Markowska et al., 1989; Neave et al., 1994b) they emerge more clearly when available cue types are restricted. Rats with retrosplenial lesions perform worse than shams when allocentric cues are removed, however, their performance remained unimpaired when directional cues were eliminated, leaving only egocentric cues (Pothuizen et al., 2008).

Retrosplenial cortex appears to also be involved in path integration in rodents, which is known to depend on hippocampal formation function (Maaswinkel et al., 1999). Successful path integration requires information derived from multiple domains such as vestibular cues, head-direction, signals from muscles, joint and tendons. Given the connectivity of the retrosplenial cortex with the motor and sensory areas, as well as the variety of cells found within the region discussed in *sections 1.3 and 1.5.1*, it is possible that the retrosplenial cortex aids path integration by combining information from these different sources to aid goal-oriented navigation (Stacho & Manahan-Vaughan, 2022). Retrosplenial lesions made using ablation techniques reduce the ability of rats to navigate back to their starting point both in the dark and light (Whishaw et al., 2001). Inactivation on the other hand seem to impair animals' performance in darkness but not in light (Cooper et al., 2001) suggesting that the retrosplenial is required when animals cannot rely on visual cues, since rats must be able to integrate movement cues with their knowledge about spatial location.

The inconsistencies observed in the literature following retrosplenial cortex lesions may stem from the extent to which the lesion affects the region, as well as the distinct functional contributions of its subregions mentioned in the sections above. For instance, lesions to area 30 impair rats' performance on the rotated radial-arm maze biasing the strategy chosen by rats to solve task from visual to motor (Pothuizen et al., 2010). In addition, lesions to area 29 cause comparable deficits on the same task (Pothuizen et al., 2010), suggesting that the two regions can operate separately, but are needed to optimally solve spatial problems. The functional differences of area 29 and area 30 become apparent in the acquisition phase where animals with lesions to area 29 and complete area 29 and 30 lesions show increases in errors (Pothuizen et al., 2010), contrary to area 30 lesions which do not cause deficits in acquisition (Vann & Aggleton, 2005). Additionally, lesions to area 29 cause deficits on T-maze alternation when intra-maze cues are removed by using pairs of identical, adjacent mazes (Pothuizen et al., 2010). Complete lesions showed smaller deficits than selective area 29 lesions when moving from one to two mazes (Figure 1.7), with the deficits being most prominent when the left arm of one T-maze is next

to the right arm on another T-maze (Figure 1.7B), leading animals to reach the same absolute place. This pattern of results suggested that animals over relied on visual cues due to sparing of area 30 (Pothuizen et al., 2010). Animals' ability to solve the task improves in the dark as visual cues are eliminated, possibly eliminating conflicting information and choice of available strategies. Additionally, c-fos and zif268 activation increases within area 29 during a working memory spatial task regardless of whether in the light or dark, suggesting that this subregion is critical for learning and navigation using both internal and external cues (Pothuizen et al., 2009). In area 30, the expression of c-fos and zif268 increased in the light and decreased in the dark, supporting the role of this subregion in the integration of visual information (Pothuizen et al., 2009).

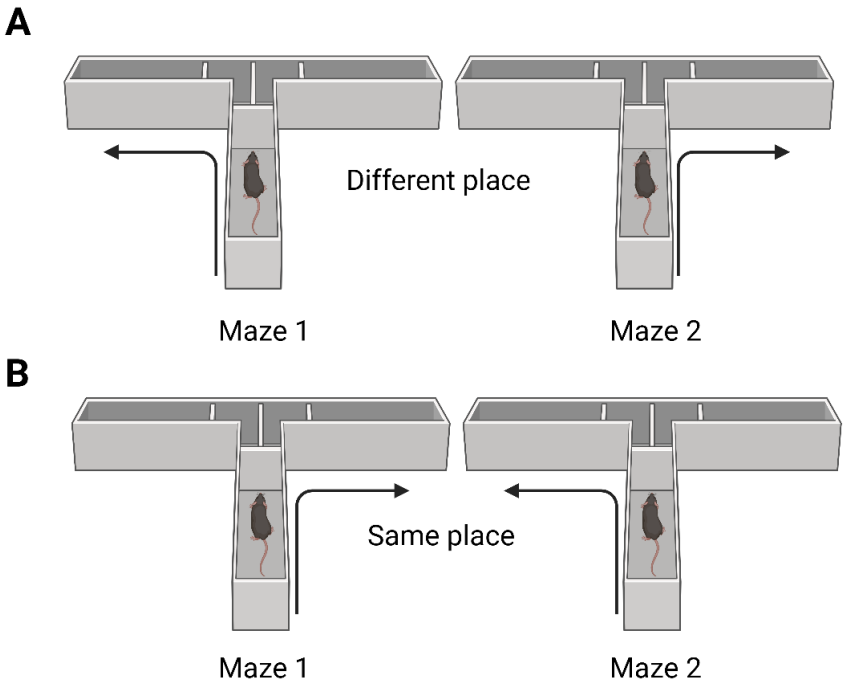


Figure 1.7. Example of test protocol for two parallel T-mazes.

The figure shows the design of a T-maze task using two parallel mazes. While in the standard (one maze) T-maze task, the animal will reach two different absolute locations, in the two-maze version of the task, the correct alternation will lead the animal to the same absolute location on some trials (panel B). This design effectively

eliminates intra-maze cues such as odour, and potentially disrupts directional cues. The figure was created using *Biorender.com.*

1.6. Theories about the current functions of the retrosplenial

The impact of retrosplenial cortex disfunction is still not completely understood, however, it seems to be most clearly observed when animals are required to switch between the type of navigational strategies and cues that they use to solve a spatial problem. For example, deficits are observed when animals have to switch between solving a maze task in the light and then dark (Chen et al., 1994), changing from allocentric to directional cues (Pothuizen et al., 2008) or between extra-maze and intra-maze cues (Pothuizen et al., 2008; Vann & Aggleton, 2004, 2005). Due to this, it is believed that the retrosplenial cortex has a translational role that allows animals to utilise the most appropriate spatial code to solve a spatial problem (Burgess et al., 2001; Byrne et al., 2007). This process is required for successful navigation and requires the ability to estimate and update spatial position based on both self-generated movement and by extracting spatial information from the environment by relying on landmarks, which as discussed in *section 1.4.1,* the retrosplenial cortex is well suited to do. Such a "translational" function would require sensory information from multiple sensory modalities including visual, motor, and olfactory (Sheri J.Y. Mizumori et al., 2000; Wolbers & Büchel, 2005), a property supported by the region's connectivity (Figures 1.3, 1.4).

1.7. Functions of major contributory regions to the retrosplenial cortex

As reviewed in *section 1.3*. and its subsections, the retrosplenial cortex has extensive reciprocal connections with various cortical and subcortical areas. In this thesis, of particular interest are the interactions between the dorsal subicular region

of the hippocampus and the anterior thalamic nuclei. The structure and relative functions of these two regions are discussed in more details in this section.

1.7.1. Structure and connectivity of the subiculum

The subiculum is positioned within the hippocampal formation, distal to the dentate gyrus and the CA fields of the hippocampus proper (Figure 1.8). The subiculum is just one structure from the subicular complex, which in the rat also includes the presubiculum, parasubiculum and postsubiculum (van Groen & Wyss, 1990). In the rat the subiculum can be divided into "ventral" and "dorsal", which corresponds to the most temporal and septal parts of the subiculum, respectively. This distinction is important due to the different connectivity and potential functional separation of these subregions within the subiculum (Aggleton & Christiansen, 2015; O'Mara, 2005).

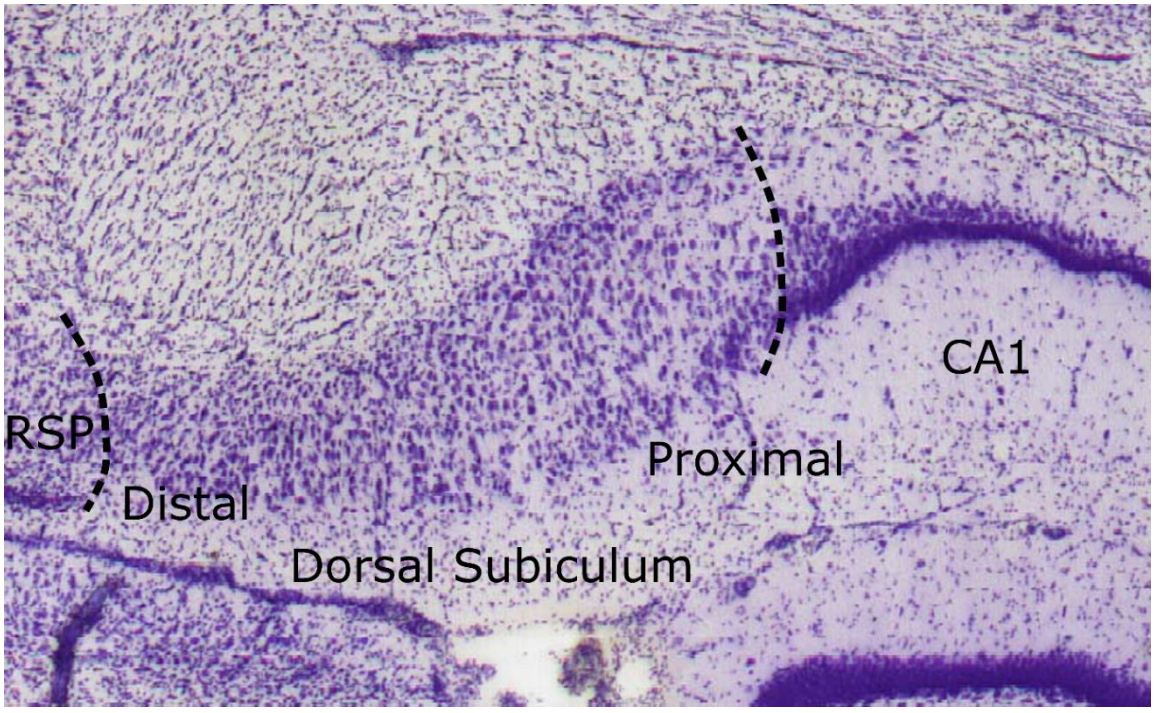


Figure 1.8. Cresyl violet stain of the dorsal subiculum in the rat.

Photomicrograph from a coronal section in the rat showing the dorsal subiculum. The term proximal refers to the area of the region that is closer to the border with CA1 of the hippocampus. The term distal refers to the area of the region that is furthest

away from the hippocampus proper. The photomicrograph was adapted from Rat brain atlas (Paxinos & Watson, 2006).

The subiculum is densely interconnected with other areas of the hippocampus. In the rat, there are also projections from the subiculum, CA1, and CA3 to the septum. The subiculum and CA1 also project to the entorhinal, perirhinal, postrhinal, prefrontal, and retrosplenial cortices (Beerens et al., 2021; Ding, 2013; Haugland et al., 2019; Witter, 2006). As mentioned previously, CA1 projections to the retrosplenial are relatively light and so the dorsal subiculum is the main direct link from the hippocampus to the retrosplenial cortex (Haugland et al., 2019; Sugar et al., 2011). The subiculum and CA1 have been observed to project to some hypothalamic nuclei, various midline nuclei, nucleus accumbens, and the amygdala (Agster & Burwell, 2013; Aggleton & Christiansen, 2015; Witter, 2006). Additionally, the subiculum and postsubiculum are the source of hippocampal projections to the anterior thalamic nuclei and the mammillary bodies (Aggleton, 2010; Aggleton & Brown, 1999; Maguire, 2001; Witter, 2006). Many individual dorsal subicular neurons project to both the mammillary bodies and the retrosplenial cortex (Kinnavane et al., 2018) (Figure 1.8).

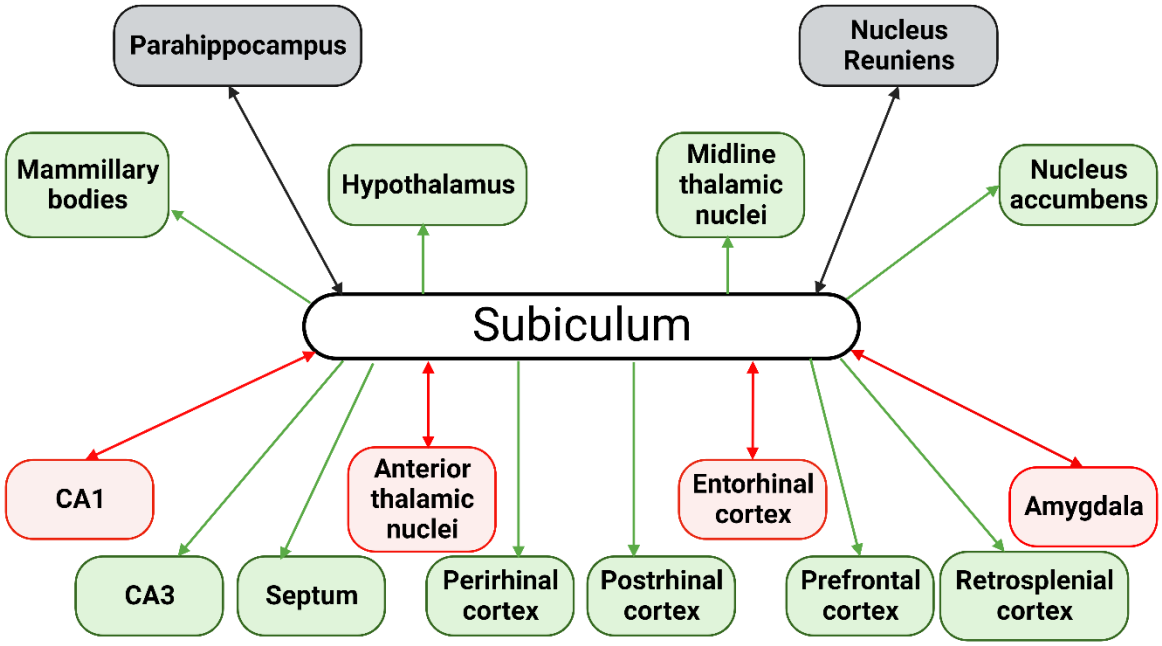


Figure 1.9. Schematic representation of the connectivity of the subiculum.

The subiculum is interconnected with various cortical and subcortical structures. The schematic shows a summary of its reciprocal, afferent and efferent connections. Reciprocal projections are depicted in red, afferent projections in black, and finally efferent projections are shown in green. The figure was created using *Biorender.com.*

Afferent projections to the subiculum come from CA1, the entorhinal cortex, the parahippocampus, and the amygdala (Aggleton et al., 2012; Agster & Burwell, 2013). The anterior thalamus also projects densely onto the subiculum (Shibata, 1993), and the rat nucleus reuniens has extensive projections to the ventral subiculum (Prasad & Chudasama, 2013). The retrosplenial cortex itself, does not project to the subiculum itself, but its projections extend to the presubiculum and postsubiculum (Van Groen & Wyss, 2003; Wyss & Groen, 1992)(Figure 1.8).

This brief overview of the subicular connectivity demonstrates the complex connectivity of the area which is also topographically organised along its dorsoventral axis in the rat. It is apparent that the subiculum is a critical source of hippocampal efferents to many sites, such as the thalamus, hypothalamus (including mammillary bodies), and nucleus accumbens. Of particular relevance to this thesis are the dorsal subiculum connections to retrosplenial cortex, some of which bifurcate to terminate in both the retrosplenial cortex and mammillary bodies (Aggleton et al., 2005, 2012). Alongside these projections, its additional connectivity to the anterior thalamic nuclei reinforce the notion that the subiculum is often associated with spatial memory (Aggleton & Christiansen, 2015). Although the dorsal subiculum has a role in other memory related processes beyond the spatial domain, such as consolidation (Melo et al., 2020), the experimental work presented in this thesis is concerned with spatial working memory and next, these functions are discussed in more detail (Kitanishi et al., 2021; O'Mara, 2005).

1.7.2. Brief overview of the dorsal subiculum role in spatial memory

The functions of the subiculum have been primarily studied in rodents, often with lesions to investigate the loss of any functions. Neurotoxic lesions of either the subiculum or the hippocampus impair the acquisition of spatial navigation strategies suggesting that both regions are vital for this type of learning (Morris et al., 1990). More recent studies have focused on targeting particular parts of the subiculum and it appears that dorsal subiculum lesions are sufficient to impair rats' performance on T-maze alternation (Potvin et al., 2007) although, unsurprisingly, these effects are more pronounced when the lesions affect the dorsal hippocampus as a whole (Potvin et al., 2007). Lesion induced impairments were also observed on the radial-arm maze task when distal cues are overlapping (Potvin et al., 2009). Dorsal subicular deficits can be clearest when testing is in the dark, implying that the region may be particularly important for the integration of idiothetic cues (Potvin et al., 2007, 2010). Additionally, the dorsal subiculum may be essential for detecting novel spatial locations by integrating place information from CA1 and body-movement (O'Mara, 2005).

The connections between the subiculum, the anterior thalamus, and mammillary bodies are of particular relevance to its role in spatial navigation as tasks of spatial working memory, e.g., T-maze alternation, are also sensitive to lesions in these same areas (Aggleton & Nelson, 2015; Nelson et al., 2020; Vann, 2010). Interestingly, the functional support that the subiculum provides to these regions may differ. When subicular inputs to the mammillary bodies were cut, rats did not show evident deficits on matching-to-place in the water maze (Vann et al., 2011). Meanwhile, interrupting the subicular inputs to the anterior thalamic nuclei using DREADDs was sufficient to disrupt, rats' spatial working memory on the T-maze task (Nelson et al., 2020). Although these contrasting effects may be due to the differences in surgical techniques (i.e., permanent lesions vs temporary lesions) and the limitations associated with these different approaches, a possible explanation relates to how almost every mammillary body neuron projects to the anterior thalamic nuclei. It might, therefore, be anticipated that the effects of anterior thalamic nuclei lesions should be at least as severe as the effects of mammillary body lesions, and sometimes more severe (Aggleton & Christiansen, 2015; Potvin et al., 2007).

Further evidence for the role of the subiculum in spatial memory comes from immediate early gene expression studies and electrophysiology findings. For example, c-fos expression increases in the rat hippocampus following spatial working memory tasks. This increase includes the subiculum (Vann et al., 2000), and this increase seem to be particularly linked to the presence of novel stimuli (Jenkins et al., 2003, 2004; Vann et al., 2000). Conversely, blocking the expression of c-fos in the dorsal hippocampus impairs spatial memory on the radial-arm maze (He et al., 2002).

The subiculum also contains place cells, although they are less numerous and may be functionally different to those place cells found in CA1. The subicular place cells appear to have lower spatial resolution and a higher threshold for remapping place representations (Brotons-Mas et al., 2010; Sharp & Green, 1994). Within the subiculum itself, the cells in the distal subiculum exhibit slightly higher spatial

resolution and greater potential to process spatial information (Kim et al., 2012; Sharp & Green, 1994). Additionally, the subiculum also contains boundary vector cells (Lever et al., 2009). These cells are found in the dorsal subiculum and both within the superficial and deep layers (Lever et al., 2009). Given that the retrosplenial cortex receives its inputs from the dorsal subiculum, which may well include these cells, one of the roles of the dorsal subicular-retrosplenial projections may be to update spatial representations. In turn, the retrosplenial cortex may play crucial role in regulating the activity of the dorsal hippocampus by providing a pathway to and from prefrontal cortex (Aggleton & Christiansen, 2015).

A pair of studies that inform these interactions, selectively targeted hippocampal projections to the retrosplenial cortex, which principally terminate in area 29 (Yamawaki et al., 2019a,b). Chemogenetic inhibition revealed that the glutamatergic projections that the retrosplenial cortex receives from the subiculum can be subdivided inro two different types of supporting roles. The first type is the vGlut1+ projections that are principally involved in processing recent context memories, and the second parallel type, vGlut2+ projections, aid long-lasting storage of fear inducing context memories (Yamawaki et al., 2019b). Additionally, disruption of the inhibitory CA1 projections to the retrosplenial cortex during memory acquisition, seem to result in enhanced contextual fear conditioning (Yamawaki et al., 2019a). Contrary, when the anterior thalamic projections to the retrosplenial cortex were silenced, contextual fear conditioning was impaired (Yamawaki et al., 2019a). The conclusion that can be drawn from these studies is that the hippocampus and the anterior thalamic nuclei work together to support retrosplenial function, where the former suppresses and the latter enhances the expression of context memories (Yamawaki et al., 2019a).

In summary, the dorsal subiculum and the retrosplenial cortex are interconnected components of the hippocampal-diencephalic-cingulate network with significant importance in spatial processing. The regions appear to work together, as a part of a broader network, to process spatial information and aid spatial navigation. However, behavioural studies investigating dysfunction, usually cause impairments in the

entire dorsal subiculum and in some instances the dorsal hippocampus, thereby affecting other efferent and afferent outputs. Thus, the specific functions of the individual dorsal subicular outputs are not well understood.

1.7.3. Structure and connectivity of the anterior thalamic nuclei

The anterior thalamic nuclei are a collection of nuclei that lie in the rostral part of the thalamus. The three major nuclei within the anterior thalamus are the anterodorsal nucleus (AD), anteroventral nucleus (AV) and the anteromedial nucleus (AM) and their appearance and structure is similar across mammalian species (Figure 1.9).

In rats the interanteromedial nucleus (IAM) is sometimes recognised as a part of the anterior thalamus (Shibata & Kato, 1993). Since the interanteromedial nucleus is also often regarded as a midline nucleus, in this thesis, the term *anterior thalamus,* refers to the three main nuclei, and the interanteromedial nucleus is referred to separately. Additionally, some anatomists consider the laterodorsal nucleus to be a part of the anterior thalamus as it shares many similar connections and electrophysiological properties to the three main nuclei (Taube, 2007). A key difference is the lack of projections from the mammillary bodies to the laterodorsal nucleus (Vann et al., 2007) and, therefore, throughout this thesis the laterodorsal nucleus will be referred to separately from the three main thalamic nuclei. It is noteworthy, however, that permanent and temporary lesion studies in rats will often unintentionally affect both the interanteromedial and laterodorsal nuclei and thereby their projections and functions.

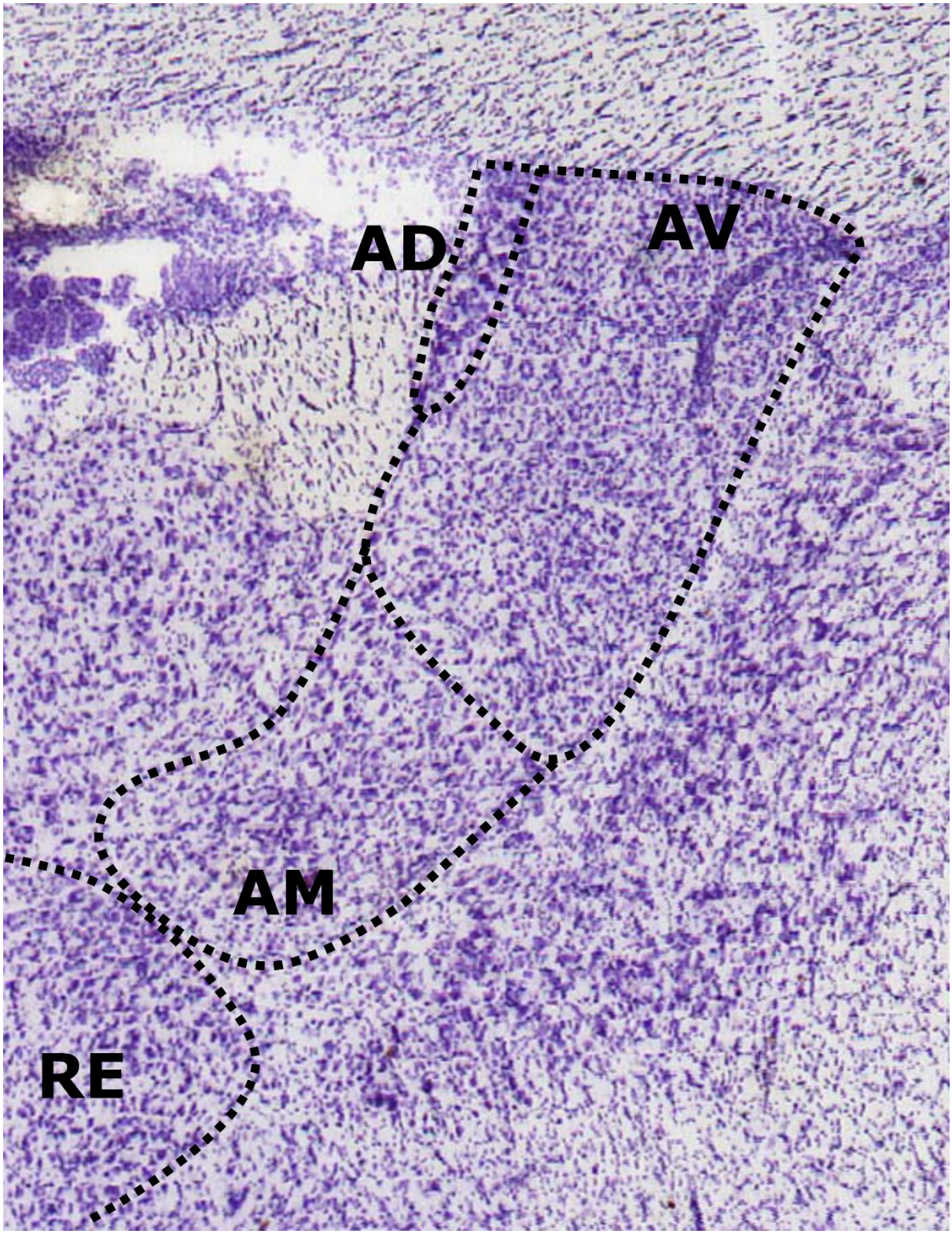


Figure 1.10. Cresyl violet section showing the anterior thalamic nuclei in the rat.

Photomicrograph from a coronal section in the rat showing the three principal nuclei of the anterior thalamus and the midline nucleus reuniens. The photomicrograph was

adapted from Rat brain atlas (Paxinos & Watson, 2006). Abbreviations: AD, anterodorsal; AV, anteroventral; AM, anteromedial; RE, nucleus reuniens.

Although the principal connections of the three thalamic nuclei are often similar, there are some differences in their efferent and afferent connections, as well as topographical differences in their overlapping connections (Phillips et al., 2019). This heterogeneity is sufficient to justify the need to consider the three nuclei separately as there is evidence that they may each make distinct functional contributions (Aggleton et al., 2010). A brief overview of the connectivity and functions of each nucleus is presented before their role is spatial memory is considered together.

1.7.3.1. Anterodorsal nucleus (AD)

The anterodorsal nucleus receives inputs from the lateral mammillary nucleus (Watanabe & Kawana, 1980; Shibata, 1992) as well as the hippocampus (van Groen & Wyss, 1990). The hippocampal inputs to the anterodorsal nucleus originate primarily in the parasubiculum and the postsubiculum, and these appear to be topographically organised (van Groen & Wyss, 1990). There is evidence that the anterodorsal nucleus also receives retinal inputs (Itaya et al., 1981; 1986) and is densely interconnected with the retrosplenial cortex (van Groen & Wyss, 1990; Figure 1.1.; see also section 1.3.2.). The anterodorsal nucleus also has return projections to the deep layers (IV-VI) of the parasubiculum and the more superficial layers (I, III and IV) of the postsubiculum (van Groen & Wyss, 1990). As mentioned previously, the anterodorsal nucleus projects to area 29, where the ventral portion of the nucleus projects to layers I, III and IV of the rostral parts of the retrosplenial cortex, while the more dorsal parts of the anterodorsal nucleus project to the more caudal parts of the retrosplenial cortex (Shibata, 1993). In addition, the more caudal parts of the anterodorsal nucleus also project to the rostral retrosplenial cortex, while the rostral anterodorsal nucleus reaches the caudal part of the granular retrosplenial cortex (Van Groen & Wyss, 2003).

Functionally, due to its connectivity and electrophysiological properties, the anterodorsal nucleus is often associated with spatial memory and navigation. This nucleus is a key element of the "head-direction system" (Taube, 1995), which provides guidance signals that assist navigation (Taube, 2007). The proportion of head-direction cells in this nucleus is much higher than the proportions of cells that rhythmically fire with theta (Albo et al., 2003). Given the electrophysiological properties of the cells within this nucleus and its dense interconnectivity to regions such as the retrosplenial cortex, which are key for spatial memory and navigation, it has been proposed that the primary function of the anterodorsal nucleus is to support navigation (Aggleton et al., 2010).

1.7.3.2. Anteroventral nucleus (AV)

The anteroventral nucleus also receives projections from the mammillary bodies, but from the medial nucleus (Watanabe & Kawana, 1980). Its hippocampal inputs originate in the presubiculum (van Groen & Wyss, 1990), the dorsal subiculum and the postsubiculum (Wright et al., 2010). Area b (see Figure 1.1.) of the granular retrosplenial cortex also projects to the anteroventral nucleus in a topographically organised manner. The rostral portions of the retrosplenial cortex, appear to project to the caudal anteroventral nucleus, while the caudal portion of the retrosplenial cortex projects to the rostral anteroventral nucleus (van Groen & Wyss, 1990, 2003; Wright et al., 2010). The caudal dorsal reticular nucleus and the laterodorsal tegmental nucleus also project to the anteroventral nucleus (Shibata, 1992).

The anteroventral nucleus has return projections to the presubiculum that terminate in layers I and III near the postsubiculum border (van Groen & Wyss, 1990; Shibata, 1992) although projections to the deeper layers IV to VI and from different portions of the anteroventral nucleus have also been identified (Shibata, 1993). The anteroventral nucleus also projects to the anterior cingulate cortex, as well as the retrosplenial cortex. The ventral parts of the anteroventral nucleus project to the rostral granular retrosplenial cortex and the more dorsal parts project to the caudal

granular retrosplenial cortex (Shibata, 1993; van Groen & Wyss, 2003). There are also some projections from the dorsolateral parts of the anteroventral nucleus to the dysgranular retrosplenial cortex (Shibata, 1993).

Functionally, given the overlapping connectivity between the anterodorsal and anteroventral nucleus, which to an extent contrast with the connections of the anteromedial nucleus, it has been suggested that the role of the anteroventral nucleus may be that of a “return-loop system” involving the hippocampus (Aggleton et al., 2010). Contrary to the anterodorsal nucleus, a large proportion of anteroventral cells fire rhythmically with theta (Albo et al., 2003; Lomi et al., 2023), which suggest that the nucleus could convey that information to the hippocampus and the retrosplenial cortex.

1.7.3.3. Anteromedial nucleus (AM)

Like the anteroventral nucleus, the anteromedial nucleus also receives inputs from the medial mammillary bodies, but from different cell populations (Seki & Zyo, 1984; Watanabe & Kawana, 1980). It also receives subcortical inputs from the reticular nucleus (Shibata, 1992). Unlike the anterodorsal and anteroventral nuclei, the anteromedial nucleus receives dense prefrontal inputs from the prelimbic, medial orbital and anterior cingulate, and the secondary motor cortices (Shibata & Naito, 2005). Concerning, its hippocampal inputs, evidence suggests that the anteromedial nucleus receives inputs from the presubiculum and the postsubiculum, as well as the subiculum (Seki & Zyo, 1984). Retrograde tracer studies have also found projections originating in the dorsal subiculum near CA1 (i.e., the proximal subiculum), the ventral subiculum, and the entorhinal cortex (Wright et al., 2010).

The anteromedial nucleus has return projections to the medial orbital cortex, entorhinal cortex, perirhinal cortex, the subiculum, visual areas 18b, the amygdala, anterior cingulate and the retrosplenial cortex (Shibata, 1993; van Groen & Wyss, 1999). The projections appear to be topographically organised and often originate in

different neurons (Shibata, 1993). With regards to the retrosplenial cortex, the rostral anteromedial nucleus projects to the caudal retrosplenial, with projections terminating in layers I, V and VI, while the caudal anteromedial nucleus projects to layers I and V of the rostral areas 29 and 30 (van Groen & Wyss, 1992; Shibata, 1993). Direct projections from the anteromedial nucleus to the hippocampus have also been documented (Wyss et al., 1979). Of all the three anterior thalamic nuclei, the anteromedial nucleus has a distinct connectivity pattern with its extensive prefrontal links. Due to these, it has been suggested that the role of the anteromedial nucleus is to serve as a "feed-forward" system (Aggleton et al., 2010). When compared with the anteroventral nucleus, a far smaller proportion of cells within this nucleus fires rhythmically with theta (Albo et al., 2003), and it has been speculated that primary function of this nucleus is to convey hippocampal-diencephalic-cingulate information to rostral cortices, supporting cognitive flexibility and executive function.

In summary, the three main nuclei of the anterior thalamus appear to differ in both their connectivity and function. It is clear however, that these distinct patterns of connectivity are often complementary, such that their functions support aligned, complex behaviours. Together, the three nuclei are perfectly suited to receive and integrate information either directly through the hippocampus (van Groen & Wyss, 1990; Wright et al., 2010) or indirectly through the mammillary bodies and/or the retrosplenial cortex (Watanabe & Kawana, 1980; Seki & Zyo, 1984; van Groen & Wyss, 2003; Wright et al., 2010). In turn, the connectivity of the nuclei allows for information to either reach the hippocampus directly, or indirectly through the retrosplenial cortex (Wyss et al., 1979; van Groen & Wyss, 1990, 2003; Shibata, 1993). These multiple connections often originate in different neuronal populations, providing various pathways and possibly parallel circuits, which may be involved in learning and memory (Vann et al., 2007; Wright et al., 2010).

1.7.4. Brief overview of the role of the anterior thalamic nuclei in spatial memory

Collectively, the three main nuclei of the anterior thalamus are considered a central node of the hippocampal-diencephalic-cingulate network. The functions of the anterior thalamic nuclei have been extensively and rigorously investigated and the region has been implicated in numerous cognitive processes and diseases in both animal models and humans. In the clinical literature, damage to the anterior thalamic nuclei is associated with severe cognitive dysfunction, where patients display deficits in cognitive skills such as memory, attention, and mental flexibility (Ghika-Schmid & Bogousslavsky, 2000; Mamiya et al., 2018). In the animal literature, rats with lesions to the anterior thalamic nuclei exhibit deficits in set-shifting (Wright et al., 2015), temporal order judgements (Wolff et al., 2006), recency judgements (Dumont & Aggleton, 2013), contextual memory (Dupire et al., 2013; Dumont & Aggleton, 2012; Haddon & Killcross, 2006, 2007), and attention (Chudasama & Muir, 2001; Wright et al., 2015) among others [see Nelson (2021) for a review]. Many of these functions are not yet well understood, partly because research efforts have often focused on their role in spatial processing.

Lesions to the anterior thalamic nuclei, repeatedly and consistently impair rats' performance on T-maze alternation (Aggleton et al., 1995; 1996; 2009;; Warburton et al., 1997; 2001). Another task that is often used to test spatial memory in rats is the radial-arm maze. Although these two tasks are similar, the choice in distinguishing the correct arm in the radial-arm maze should be harder than the choices in the T-maze, although there is arguably less proactive interference in the radial-maze as rats usually complete just one complete session, unlike the T-maze where rats may complete 8-12 trials in a relatively short time span.

Unsurprisingly, lesions to the anterior thalamic nuclei cause impairments to rats' performance on the Morris water maze, radial-arm maze, as well as on T-maze alternation (Aggleton et al., 2009; Alexinsky, 2001; Mitchell & Dalrymple-Alford,

2005; Warburton et al., 1997; 1998; 1999; 2000; 2001). These deficits are also present, when particular projections are targeted (e.g., dorsal subicular inputs to the anterior thalamus or anterior thalamic projections to the dorsal subiculum), even when the rest of the region and their inputs/outputs remain intact (Nelson et al., 2020).

Rats with anterior thalamic lesions are also impaired on tasks testing spatial reference memory, such navigating to the same location. For example, rats with damaged anterior thalamic nuclei exhibit higher latencies to locate a hidden platform in the Morris water maze compared to controls (Sutherland & Rodriguez, 1989; Van Groen et al., 2002; Warburton et al., 1997; Warburton & Aggleton, 1998). Additionally, Aggleton et al. (2009) showed that rats with anterior thalamic lesions were struggle to learn the geometrical properties of a rectangular maze. Together these findings suggest that the anterior thalamic nuclei are involved in various domains of spatial navigation.

In fact, the nature of the spatial deficits following anterior thalamic damage can be further qualified on the basis of the cues that rats are using to solve spatial tasks. For example, lesions to the anterior thalamus do not impair egocentric spatial learning (Aggleton et al., 1996; Warburton et al., 1997, 1998; Mitchell & Dalrymple-Alford, 2005; Wolff et al., 2006), contrary to allocentric learning. Allocentric learning requires rats to use distal cues around the room to orient themselves, unlike egocentric cues that are determined relative to the rats' body movement. This distinction becomes apparent in a recent study, using temporary lesions. Deficits were seen on T-maze alternation following inactivation of the anterior thalamic nuclei, but only when the maze was rotated 90 or 180 degrees, placing intra-maze and extra-maze cues in conflict (Nelson et al., 2020). Thats same study did not find deficits following anterior thalamic inhibition on the standard alternating T-maze when all cues were available to solve the task.

In the rotation version, it is likely that the animals relied on the spatial disposition of distal cues (allocentric) rather than on body turn response (egocentric). This is because in this version of the task, where trials are discrete (i.e., animals are picked up and carried back to the starting location), self-motion cues are compromised (Baird et al., 2004; Futter & Aggleton, 2006). However, the possibility that animals still utilise egocentric and directional information (Douglas, 1996; Dudchenko, 2001; Futter & Aggleton, 2006) cannot be completely eliminated. It is likely that animal's intact ability to solve the standard alternating T-maze reflects their flexible reliance on intra-maze cues and any remaining spatial information that animals can access.

Although the study by Nelson et al. (2020) observed slightly decreased performance on the standard alternating T-maze task following anterior thalamic inhibition, the effects were not significant and were less pronounced than the effects found in surgical-disconnection studies (Warburton & Aggleton, 1998)(Warburton et al., 1998, 2000, 2001). These discrepancies may reflect the differences in which the techniques affect underlying functions, for example, how chemogenetics attenuate rather than eliminates neuronal activity. It is also worth noting that not all studies have observed spatial deficits after anterior thalamic lesions (Greene & Naranjo, 1986; Beracochea et al., 1989; Beracochea & Jaffard, 1991).

Among these examples of spared performance, Greene and Naranjo (1989) used a self-return T-maze where animals could leave the choice arms through a one-way door and return to the start arm, allowing the animals to draw on egocentric cues. Although, anterior thalamic lesions do not usually produce observable effects on tasks using egocentric strategies (Aggleton et al., 1996; Warburton et al., 1997; Mitchell & Dalrymple-Alford, 2005; Wolff et al., 2006) it is additionally interesting that the lesions were often mostly confined to either the anteroventral or anteromedial nucleus, while the anterodorsal nucleus was largely spared.

Indeed, studies that have investigated the effects of selective lesions within the anterior thalamic nuclei, demonstrate that the most severe memory deficits usually

emerge when all three main nuclei are damaged (Aggleton et al., 1996; Byatt & Dalrymple-Alford, 1996; van Groen et al., 2002) and the deficits tend to correlate with the amount of damage to the nuclei (Warburton et al., 1998, 1999). Interestingly, although less severe, damage to the anterodorsal and anteroventral nucleus were also sufficient to cause deficits, when the anteromedial nucleus was intact (van Groen et al., 2002). These outcomes point to different functional specialisation between the nuclei that have complementary spatial functions.

In summary, this brief overview of anterior thalamus connectivity and function suggests that the three main nuclei are critical for various cognitive functions. They are particularly important for spatial processing and navigation, presumably reflecting their connectivity to other regions key for successful spatial navigation such as the mammillary bodies, the hippocampus, and the retrosplenial cortex (see Aggleton & Nelson, 2015b for a review). This is reflected by the sensitivity of tasks, such as the T-maze, to anterior thalamic damage. The intrinsic and extrinsic connectivity of the anterior thalamic nuclei makes it difficult to disentangle its specific function in the spatial domain and may be the reason why permanent lesion studies might overestimate deficits caused by its damage. Given how the nuclei have complementary and additive contribution to spatial processing, it is of particular interest to understand both their unique anatomy and the separate behavioural significance of their afferent and efferent connections.

1.8. Chemogenetic approaches for in-vivo neural manipulation

Chemogenetics is a technique that uses chemically engineered molecules and ligands that can aid our understanding of the relationship between brain activity and behaviour. There are various types of molecules that can be engineered for use in chemogenetics, with the most common being G-protein coupled receptors, which were used in this thesis. Chemogenetics were used in chapters 3 and 4 to disrupt neural activity in the dorsal subiculum and anterior thalamus, respectively.

1.8.1. Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)

Designer receptors exclusively activated by designer drugs (DREADDs) are a chemogenetic technology that has a genetically modified endogenous muscarinic g-coupled protein receptors (GPCRs) with binding sites that are engineered to be activated by synthetic small molecules (designer drugs) but not by their endogenous ligand (Armbruster et al., 2007; Roth, 2016). Therefore, DREADDs can be expressed in specific neuronal populations *in vivo,* and consequently temporarily manipulate neural signalling by exciting or inhibiting cell activity (Figure 1.10). This property makes them a valuable tool in the identification of circuitry and cellular signals that govern behaviour (Roth, 2016).

Since GPCRs are expressed in the body of the neuronal cells, when ligands bind the extracellular receptor, the corresponding intracellular g-protein is mobilized. In turn, this regulates the activity of the other proteins in the cell membrane, determining the excitability of the neurons (Figure 1.10) (Roth, 2016). Currently, there are DREADDs that can increase (excitatory) (Alexander et al., 2009) or decrease (inhibitory) (Armbruster et al., 2007) neuronal activity. The two most commonly used types of DREADDs are derived from the muscarinic M_4 receptor coupled to a G_i protein (hM4Di) and the M_3 muscarinic receptor coupled to a G_q protein (hM3Dq). The hM4DI DREADD silences neuronal activity by inhibiting adenylate cyclase and downstream cAMP production, while the hM3Dq DREADD can increase neuronal signalling by stimulating phospholipase C, which releases calcium stores in the cell (Roth, 2016; Dobrzanski & Kossut, 2017)(Figure 1.10).

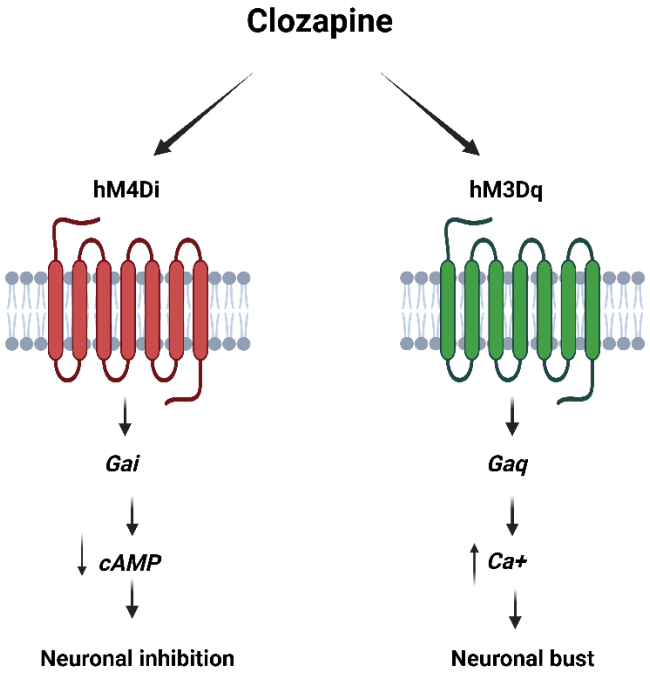


Figure 1.11. Schematic representation of the mechanism of inhibitory and excitatory DREADDs.

When the ligand is introduced and binds to the DREADDs receptor, the associated protein is mobilized, which in turn changes the neurochemistry to cause neuronal inhibition or activation. The figure was created using *Biordendr.com.*

To transport, DREADDs are typically packaged into adeno-associated viral vector serotype 5 (AAV5), which is relatively non-toxic and can be introduced directly to the brain through intracranial injection (Roth, 2016). Their effectiveness appears to be long-term, lasting for months (Campbell & Marchant, 2018; Morsy et al., 1998). The viral construct contains a promoter that helps to determine what cell types express the DREADD. The experiments described in chapters three and four, used a viral construct containing calmodulin-dependent protein kinase II (CAMKII) promoter, which predominately targets excitatory glutamatergic neurons (Campbell & Marchant, 2018; Smith et al., 2016) and transport anterogradely. The anterograde transport allows the virus to propagate in a forward manner from the injection sites (e.g., dorsal subiculum in Chapter 3 and the anterior thalamic nuclei in Chapter 4) to

the projection sites (e.g., the retrosplenial cortex). Additionally, fluorescent reporter molecules are added to the viral constructs to aid visualization of DREADDs expression in the brain. Following, the intercranial injections of the virus, robust expression of the DREADD is usually observed in neurons and projection sites within 2-3 weeks (Roth, 2016; Smith et al., 2016). The experiments in chapters 3 and 4 allowed at least three weeks for the virus to express, prior to commencing behavioural testing.

1.8.2. Clozapine as a ligand to activate DREADDs

To achieve excitation or inhibition of neural activity in animals expressing DREADDs in a targeted brain region, it is necessary to administer a ligand that binds to, and activates, the DREADD expressing receptors. Commonly, Clozapine-N-Oxide (CNO) is used by neuroscientists (Roth, 2016; Smith et al., 2016), presumably because it is otherwise pharmacologically inert. However, concerns have been raised over the use of CNO as a ligand. For instance, higher doses of CNO administered systematically exhibit off-target effects that affect behaviours in mice and rats (MacLaren et al., 2016; Gomez et al., 2017). Additionally, it appears that CNO may not cross the blood-brain-barrier and is reverse metabolized into clozapine (Gomez et al., 2017). Clozapine is an atypical antipsychotic drug that has numerous endogenous targets including serotoninergic, muscarinic, and dopaminergic receptors (Meltzer, 1994), however, directly using clozapine in subthreshold doses may bypass between-subject variability in metabolism, potentially reducing off target effects and individual variability (Manvich et al., 2018). Despite these concerns, clozapine appears to have much higher affinity for DREADDs receptors than for endogenous receptors (Campbell & Marchant, 2018; Gomez et al., 2017).

Interestingly, DREADDS appear to have higher affinity to clozapine than to CNO (Armbruster et al., 2007; Gomez et al., 2017) and usually much lower doses of clozapine are sufficient to successfully activate DREADDs, which should potentially reduce off-target effects. Additionally, different doses of ligand may be needed to activate inhibitory and excitatory DREADDS, with the former requiring smaller doses

(Farrell & Roth, 2013; Mahler et al., 2014; Yau & McNally, 2015). Furthermore, higher doses of clozapine may be needed than when it is administered systemically through intraperitoneal (i.p.) injection than when the ligand is locally delivered through cannulations, although research on the minimum effective dosage is missing. So far, effective doses of CNO for local infusions seem to be in the range of 0.1-3mg/kg (Roth, 2016; Stachniak et al., 2014), while a study using clozapine reported a dosage of 1mg/kg (Nelson et al., 2020). The use of clozapine in rat studies is relatively limited and dosages reported vary considerably from 0.05mg/kg to 4mg/kg (Ilg et al., 2018; Jendryka et al., 2019; Nelson et al., 2020), demonstrating the uncertainty around optimal doses of clozapine. Importantly, care should be taken when using clozapine and animals should be monitored for its potential side effects such as sedation, which would affect mobility (Ilg et al., 2018; Roth, 2016).

Following administration of the ligand, electrophysiological data indicate onset of approximately 5 -12 minutes, when changes in neural activity are observed (Alexander et al., 2009; Chang et al., 2015). The time it takes for the neural firing to return to baseline is less clear, however, as effects appear to last anywhere between 70 minutes to 9 hours (Alezander et al., 2009; Chang et al., 2015). The caveat is that these studies tested i.p. injections of CNO. Not only do the temporal kinetics of CNO and clozapine appear to be dose dependent (Pati et al., 2019), but also it is likely that the method of delivery (i.p. injection vs local infusion) will also have an impact. Although formal direct comparisons and consensus seem to be lacking, previous research allowed at least 15 minutes post local infusions and 30 minutes post i.p. injections before commencing any behavioural testing (Bubb et al., 2021; Nelson et al., 2020). These same delays produced demonstrable effects on targeted behaviours, consistent with DREADDs activation (Bubb et al., 2021; Nelson et al., 2020). Therefore, chapters 3 and 4, followed the same time intervals for local infusions and i.p. injections, respectively. However, given the uncertainties around the dosage and off-target behavioural effects that DREADDs may have, it is essential to adopt proper controls in behavioural experiments (Goutaudier et al., 2019; Roth, 2016).

1.8.3. Non-DREADD expressing controls

To minimize the risk of off-target effects of either the virus or the ligand it is important to adopt strict control measures in DREADDs experiments (Goutaudier et al., 2019; Roth, 2016; Smith et al., 2016). In the thesis where DREADDs were used in the behavioural experiments, the experiment included a control group of animals receiving comparable intracranial injections of non-DREADD expressing virus. The same viral vector (AVV5) and promoter (CAMKII) as described in *section 1.9.1.* were used. The control-expressing virus was tagged with green fluorescent protein (GFP) to aid visualization of the expression. To provide further control for off-target behavioural effects of the drug, both groups (DREADDs and non-DREADD expressing controls) received i.p. injections or infusions of both the ligand (clozapine) and the vehicle (sterile saline) (Smith et at., 2016). The specific number of testing sessions and drug administrations, and doses are described in the methods section of the respective chapters.

1.8.4. Advantages of DREADDs

The advancement of the DREADDs technology led to key advantages over traditional loss or gain of function approaches traditionally used in behavioural neuroscience (Roth, 2016). The main advantage of DREADDs is their transient nature. Not only, do they circumvent potential compensatory changes that may occur in other regions following permanent lesions (Smith et al., 2016), but they also allow for multiple behavioural manipulations and interventions over prolonged periods of time, thereby reducing the number of animals needed. Additionally, inhibitory DREADDs (iDREADDs) suppress the activity of neurons without completely eliminating it (as opposed to conventional lesions), while excitatory DREADDs stimulate endogenous cell firing rather than just stimulating action potentials (e.g. electrical stimulation) (Roth, 2016; Smith et al., 2019). These properties are thought

to produce a more natural up and down regulation of neural activity since some neurons may stay intact (Roth et al., 2016).

Importantly, DREADDs can be tracked down to their axon terminals, where they influence neurotransmission (Mahler et al., 2014; Stachniak et a., 2014). This property makes it possible to selectively manipulate the terminals of DREADD expressing neurons that project to the region of interest. To achieve this, an intracranial cannula is implanted above the projection termination target site to allow for the local infusion of clozapine (Figure 1.11) (Lichtenberg et al., 2017; Mahler et al., 2014). Chapters 3 and 4 use this method with inhibitory DREADDs (hM4Di) in the dorsal subiculum and the anterior thalamic nuclei, respectively, with cannulas implanted above the rostral and caudal retrosplenial cortices.

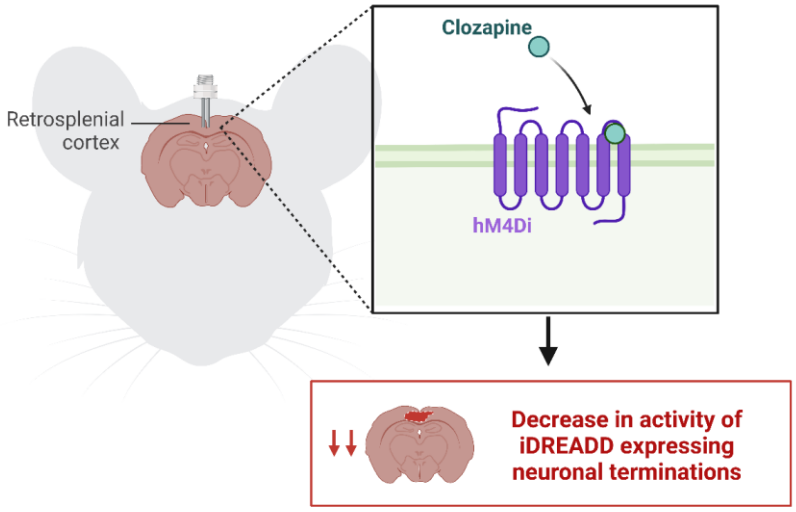


Figure 1.12. Schematic representation of cannulation for local cortical infusion.

The schematic shows permanent bilateral guide canula implanted through the skull into the retrosplenial cortex. The position of the cannula allows for local targeted

delivery of clozapine, which in turn would decrease the activity of iDREADD expressing neuronal terminations. The figure was created using *Biorender.com.*

1.9. Rationale for the experiments

As reviewed in the preceding sections, the retrosplenial cortex is a key structure for mnemonic processes. Human and animal studies consistently support its role in spatial memory and navigation. Despite its strategic location, little is known about the contributions of the retrosplenial cortex to other areas of the hippocampal-diencephalic-cingulate network as these have been difficult to disentangle. As already mentioned, its anatomical location in the rodent brain is more accessible in the rodent than the primate brain, adding to its attractiveness as an experimental target.

The key objectives of this thesis were to perform a detailed investigation of the circuitry of the retrosplenial cortex and then experimentally manipulate and compare some of these brain circuits associated with the retrosplenial cortex. These objectives are achieved by using contemporary viral-based techniques for retrograde tracing and neuronal manipulation. Both of these allow for high precision anatomical investigation and *in-vivo* behavioural manipulation, respectively.

Focusing on the interactions between the anterior thalamic nuclei, medial prefrontal cortex, anterior cingulate cortex and the retrosplenial cortex, Chapter 2 uses double-retrograde tracing technique to examine the complex anatomical interaction of these regions. This will provide information on how the anterior thalamic projections extend and compare across a number of frontal regions and the retrosplenial cortex. Next chapters 3 and 4 directly investigate the functions of the dorsal subicular and anterior thalamic connections with the retrosplenial cortex in spatial working memory, utilising DREADDs technology. Once DREADDs are activated, their effects are only temporary allowing for extended behavioural testing during ‘on’ and ‘off’ periods,

while various behavioural manipulations may be conducted. To take advantage of this methodology, novel variations of the reinforced alternating T-maze were used to manipulate the availability of cues and navigational strategies. Understanding the precise role of these circuits in spatial working memory may aid the development of treatments for disorders affected by their dysfunction.

Chapter 2

2. Collateral rostral thalamic projections to prelimbic, infralimbic, anterior cingulate and retrosplenial cortices in the rat brain

2.1. Introduction

The function of any given cortical area is, in part, determined by its thalamic inputs (Jones, 2002; Sherman, 2007). Numerous tracer studies have identified those rat thalamic nuclei that project to frontal and cingulate regions such as the orbital, prelimbic, anterior cingulate, and retrosplenial areas. These studies reveal that a range of nuclei, which include the anterior thalamic nuclei, the laterodorsal nucleus, and nucleus reuniens project to more than one of these cortical areas (Condé et al., 1990; Hoover & Vertes, 2007; Krettek & Price, 1977; Shibata, 1993; Wyass & Van Groen, 1992). Far less is known, however, about whether these diverse cortical inputs arise from segregated populations of thalamic neurons or whether they reflect collateral projections that enable individual neurons to influence different cortical areas simultaneously.

An initial investigation placed different retrograde tracers into the anterior cingulate and retrosplenial cortices (Horikawa et al., 1988). Between 8-14% of the labelled efferents in the three principal anterior thalamic nuclei (anterodorsal, anteromedial, and anteroventral) projected to both the caudal anterior cingulate cortex and nearby rostral retrosplenial cortex (Horikawa et al., 1988). Lower percentages were seen as the cortical injection sites were increasingly separated in distance. Meanwhile, no laterodorsal nucleus neurons were observed projecting to both the anterior cingulate and retrosplenial cortices (Horikawa et al., 1988). No other thalamic nuclei were

included in that study. A related investigation (Condé et al., 1990), which looked for inputs to the anterior cingulate cortex and other medial prefrontal areas, described how midline thalamic sites, such as nucleus reuniens contain modest numbers of bifurcating neurons. A more recent study (Pei et al., 2021) extended the termination areas under investigation by including the hippocampal formation but just focussed on projections from the anteromedial nucleus and nucleus reuniens. That study reported appreciably higher proportions of double-labelled neurons that collaterise to terminate in multiple areas. For example, the proportion of anteromedial nucleus and nucleus reuniens labelled projections that innervated both the medial prefrontal cortex and dorsal subiculum was over 30% for both nuclei (Pei et al., 2021). Meanwhile over 19% of anteromedial nucleus and nucleus reuniens labelled efferents reached both the medial prefrontal cortex and caudal retrosplenial cortex (Pei et al., 2021). Together these studies confirm that such collaterals exist but leave unreported various medial cortical terminal combinations alongside gaps in the rostral thalamic nuclei under investigation.

A closely related question is whether individual rostral thalamic neurons terminate in separate parts of the *same* cortical area. To address this question, pairs of retrograde tracers were injected, one within the rostral retrosplenial cortex, the other within the caudal retrosplenial cortex (Sripanidkulchai & Wyss, 1986). No double-labelled cells were observed in the anterior thalamic nuclei or the laterodorsal nucleus despite considerable numbers of single-labelled cells (Sripanidkulchai & Wyss, 1986).

However, a very different outcome was described in a later study that also placed pairs of retrograde tracers in separate rostral and caudal retrosplenial locations (Horikawa et al., 1988). That study reported double-labelled cells in all three anterior thalamic nuclei (anterodorsal, anteromedial, anteroventral), although extremely few were observed in the laterodorsal nucleus (Horikawa et al., 1988). These double-labelled cells were most numerous in the anterodorsal nucleus (up to 22% of labelled cells). These two conflicting sets of results (Horikawa et al., 1988; Sripanidkulai & Wyss, 1986) leave uncertain whether individual anterior thalamic projections collaterise across retrosplenial cortex. This issue should be resolved given the

significance of these thalamo-cortical projections for learning and memory (Vann et al., 2009; Yamawaki et al., 2019a).

In view of these uncertainties and the many gaps in our current knowledge, the present study further examined whether rostral thalamic neurons collaterise to reach separate medial cortical areas and how these compared to the populations of neurons reaching the retrosplenial cortex. Most authorities place the infralimbic, prelimbic, anterior cingulate, and medial agranular cortices within the 'medial prefrontal cortex' (Ongur & Price, 2000; Hoover & Vertes, 2007). Given the need to separate anterior cingulate cortex tracer injections from those in other medial prefrontal areas, the group designation 'medial prefrontal' was used for medial cortical areas ventral to the cingulate area. Consequently, different retrograde tracers were placed in four pairs of cortical areas: medial prefrontal/anterior cingulate (mPFC/Cing), anterior cingulate/retrosplenial (Cing/RSP), medial prefrontal/retrosplenial (mPFC/RSP), and retrosplenial/retrosplenial (RSP/RSP). The single and double retrogradely labelled cell populations were counted within the three principal anterior thalamic nuclei, the interanteromedial nucleus, and the laterodorsal nucleus, thereby adding to those nuclei included in previous investigations (Condé et al., 1990; Horikawa et al., 1988; Pei et al., 2021). These same thalamic nuclei are of related interest given their various roles in spatial learning and cognition (Aggleton et al., 2010; Cassel et al., 2021; Griffin, 2021; Mathiasen et al., 2021; Van der Werf et al., 2002; Van Groen et al., 2002) and interconnectivity with the retrosplenial cortex.

2.2. Materials and Methods

2.2.1. Statement of Contributions

The work presented in this chapter contains data collected from colleagues. As stated in the acknowledgement section of the thesis, Mathias Mathiasen completed the iontophoretic injections and the majority of the surgeries. Eman Amin assisted with the histological processing of the tissue and completed the

blind cell-count verification. Anthony Hayes acquired the confocal images. I performed additional surgeries, acquired images, completed all cell counts and analysed the data. The chapter has been peer-reviewed and published, containing additional data on nucleus reuniens.

2.2.2. Animals

Thirty-three Lister Hooded male rats (Envigo, UK) underwent surgery. Nine animals were excluded due to lack of spread of the tracer or injections beyond the target area. The data reported are from the remaining 24 animals. The rats were allocated in the four experimental groups as follows: (1) mPFC/Cing, n = 7; (2) Cing/RSP, n=5; (3) mPFC/RSP, n = 5; (4) RSP/RSP, n = 7. At the time of the surgery the animals weighed between 284g and 663g (*M* = 348.8g). Prior to surgery, all animals were housed in pairs, in a temperature-controlled room, under 12h light/dark cycle. Food and water were available *ad libitum.* All animals were randomly assigned to each group and underwent the same surgical procedures. All animal procedures were carried out in accordance with U.K. Animals (Scientific Procedures) Act 1986 and were approved by the local Ethics Committee at Cardiff University. Six of the cases were also included in an analysis of the topography of anteroventral nucleus projections to retrosplenial cortex (Lomi et al., 2021).

2.2.3. Surgeries and Tracer Infusions

All surgeries took place under isoflurane-oxygen mixture anaesthesia (5% induction, 1.5-2.5% maintenance). Each rat was placed in a stereotaxic frame (David Kopf Instruments, CA, USA), so that the skull was flat. Chloramphenicol 0.5% eye-gel was applied, meloxicam (0.06ml) was administered subcutaneously for analgesic purposes, and lidocaine (0.1ml of 20mg/ml solution) was applied topically to the incision site. Craniotomies were made over the right hemisphere in 21 animals and in three animals (from the Cing/RSP group) over the left hemisphere. The anterior

cingulate injections deliberately targeted the more rostral parts of this area to avoid the transitional, midcingulate area 24' (Vogt & Paxinos, 2014).

Combinations of two retrograde tracer injections at different cortical locations were used. The tracers were fast blue (FB, Sigma-Aldrich, Gillingham, UK; 3% solution in PBS) and non-conjugated cholera-toxin b (CTB, List Biological Laboratories Inc, CA; 1% solution in 0.05M tris). These two tracers exhibit different patterns when filling neuronal cell bodies (Köbbert et al., 2000). All FB injections were made mechanically and CTB injections were made either mechanically (22 cases) or iontophoretically (2 cases). All mechanical injections used a 1.0 µl Hamilton Syringe (Hamilton, Bonaduz, Switzerland). A dedicated syringe was used for FB and another for CTB. The tracers were infused at a flow rate of 20ηl/min for 3-5 minutes and the needle was left *in situ* for further 5 minutes before retraction. Iontophoretic injections were infused by a 6s on/off pulse with 2µA, 6µA, 7µA current for approximately 5 min each setting (total 15 min). The volume of the FB injections was 25-150ηl (*M*=73.75 ηl) and for CTB injections was 60-180ηl (*M*=78.33 ηl).

At the end of the surgeries, the analgesic lidocaine, and an antibiotic powder (Clindamycin, Pfizer, UK) were applied to the surgical site. All animals were subcutaneously administered 5ml glucose-saline solution for fluid replacement, prior to placing them in a recovery chamber. When conscious, the animals were returned to their home cage and closely monitored until they were sacrificed.

2.2.4. Perfusions

After a survival time of 6-8 days, the animals received a lethal injection of sodium phenobarbital (2ml/kg, Euthatal, Marial Animal Health, UK) administered intraperitoneally. This period was selected since effective retrograde transport is usually achieved after 2 to 7 days (Saleeba et al., 2019). Then, the animals were transcardially perfused with 0.1M phosphate-buffered saline (PBS) and 4% paraformaldehyde in 0.1M PBS (PFA). The brains were further post-fixed in PFA for

at least 2 hours, and then placed in 25% sucrose solution for minimum of 24h. The tissue was cut into 50 µm coronal sections in 4 series (i.e., 1 in 4), using a freezing microtome (8000 Sledge Microtome, Bright Instruments). The tissue was stored in cryoprotectant (30% sucrose, 1% polyvinyl pyrrolidone, 30% ethylene glycol in PBS) in a freezer at -20ºC.

2.2.5. Histology

For CTB staining, the sections were washed for 3 x 10 min in a 0.1 M PBS, followed by 3 x 10 min washes in PBST (0.2% Triton X-100 in 0.1M PBS). Sections were then incubated with the primary antibody rabbit-anti-CTB overnight (1:3000) (Sigma-Aldrich, UK) for 16-24 hours at room temperature. The sections were then washed three times for 10 min with PBST and transferred to a secondary antibody of goat-anti-rabbit (Dylight Alexa flour 594, Vector Laboratories, Peterborough, UK) for 2 hours on a stirrer. Finally, the sections were washed with PBS and mounted onto gelatine-coated slides and cover-slipped using Fluromount (Sigma-Aldrich, Germany) or DPX (Thermo Fisher, Waltham, MA) mounting medium.

Where necessary to confirm the boundaries of the regions of interest, an additional series was mounted onto gelatine-coated slides and Nissl-stained using cresyl violet. The sections were then dehydrated through increasing concentrations of alcohol (70%; 90%; 100%; 100%) and washed in xylene. Then, the slides were cover-slipped with DPX (Thermo Fisher, Waltham, MA) mounting medium.

2.2.6. Image Acquisition

Sections were viewed in the dark using a Leica DM5000B fluorescent microscope. Images of the regions of interest and the injection sites were acquired using Leica DFC310FX digital camera in the Leica Application Suite. An A4 DAPI filter was used to view the FB label and N21 filter for the CTB label. The images were acquired at

magnifications of 5x and/or 10x for single channels and a combined overlay of both channels. Photomicrographs acquired for illustration purposes were occasionally adjusted for contrast, brightness, and intensity.

2.2.7. Cell counts

While stereological methods are essential to derive absolute cell counts (Coggeshall & Lekan, 1996), the present study sought to compare the relative numbers of double-labelled cell profiles within the regions of interests, between the four sets of pairings. For this purpose, non-stereological methods are appropriate when certain conditions are met (e.g., random tissue sampling is used and there are no systematic changes in the volume or packaging of neurons across different regions) (Coggeshall & Lekan, 1996).

In the present study we targeted the entire nucleus, counteracting the need for random tissue sampling within a given region of interest. Although the volume and packaging of cells undoubtedly vary between different nuclei, the focus here centres on the proportions of double-labelled profiles within and between the regions of interest, rather than the absolute number of profiles. This same focus meant that we did not attempt to estimate total neuronal numbers within a given nuclei, e.g., after Nissl staining.

With these constraints in mind, profiles of cells were counted manually using Image J 1.53 (Rasband, 2011, NIH, USA). Single-labelled FB and CTB cells were counted across each region of interest within the three major anterior thalamic nuclei (the anterodorsal, anteroventral, and anteromedial thalamic nuclei) as well as the interanteromedial nucleus and nucleus reuniens. Additionally, cell counts were made in the laterodorsal nucleus for the Cing/RSP and RSP/RSP pairs. Laterodorsal cell counts were not made for the mPFC injection combinations as that nucleus does not project to the infralimbic, prelimbic, or rostral anterior cingulate cortices (Condé et al., 1990; Hoover & Vertes, 2007).

The fluorescent labelled cells within the entire regions of interest were counted separately for each tracer. The boundaries of the regions were guided by the Paxinos and Watson rat atlas (5th edition, 2004). A minimum of two sections per region were counted for each case (mean = 3). 'Double-labelled neurons' were defined as those showing evident blue FB label at the centre of the cell body surrounded by red (CTB) ring-like label. Where it was unclear whether a neuron was double-labelled, Corel Draw 2019 (Corel Corporation, USA) was used to create an overlay between the two separate channels and the opacity option was used to gradually transition between the single channel images, confirming the identical location and shape of the double-labelled neurons. Next, double-labelled cells were also counted in a subset of cases (at least one case per group) by a second researcher, blind to the original counts, to confirm inter-rater reliability ($r = 0.935$, $p < 0.01$). The experimenters were not blinded to the group membership of the animals or the purpose of the experiment.

To further validate the double-labelled counts, we used a Zeiss LSM880 Airyscan confocal microscope to acquire higher magnification images (20x and 40x magnification) from a minimum of two sections of each of the principal thalamic nuclei. Data were obtained from a subset of cases (225#8, 232#16, 604#5) that represented different cortical combinations, each with relatively high proportions of double-labelled cells (confocal images were not acquired for a mPFC/RSP pair as double-labelling was almost absent). Using a maximum intensity image projection, the same counting procedure and proportion estimation methods were used as described above. Where it was unclear if a cell was double-labelled, a

z-stack of images of the corresponding region was inspected.

2.2.8. Analysis of double-labelled cell counts

To provide a single measure for each thalamic nucleus we first calculated the total number of labelled cells for each case (cell numbers from both individual tracers

added to the number of double-labelled cells for that same region). This total was then used as a denominator to determine the percentage of double-labelled cells with respect to all labelled cells (i.e., within a region of interest, the number of double-labelled cells, was divided by the sum of just FB, just CTB, and all double-labelled cells). This measure of collaterisation is the same as that used in previous studies (Horikawa et al., 1988; Conde et al., 1990). To avoid violations to the assumptions of parametric tests, a series of non-parametric Friedman tests helped to compare the proportions of double-labelled cells between nuclei, within each injection pairing.

For a more complete picture we also calculated the percentage of double-labelled cells in each thalamic nucleus with respect to the number of single-labelled cells from each of the two injections in that same case, i.e., the two separate counts of single-labelled cells in each case (Table 1). Because there is only one number of double-labelled cells in an individual animal, the proportion of double-labelled cells will always be highest for the tracer injection resulting in the smaller number of single-labelled cells (Table 1).

Table 2.1. Mean percentages of double-labelled cells in each thalamic nucleus relative to the numbers of single-labelled cells (either FB or CTB) resulting from each separate cortical injection.

The table shows, from left to right, the proportions of double-labelled cells with respect to the single-labelled cell counts for each injection site within the mPFC/Cing, Cing/RSP, mPFC/RSP, and RSP/ RSP pairings. Since the number of double-labelled cells is the same for both injection sites, the percentage of double-labelled cells will always be highest with respect to the cortical area associated with the smaller numbers of single-labelled cells. The numbers shown for each thalamic nucleus are the mean percentages of double-labelled cells for all cases within that pairing. The numbers in brackets are the minimum and maximum percentages of double-labelled cells observed within that group of cases (to nearest whole integer).

Abbreviations: AD, anterodorsal; AV, anteroventral; AM, anteromedial; Cing, anterior cingulate corte; IAM, interanteromedial; LD, laterodorsal; mPFC, medial prefrontal cortex; RSP, retrosplenial cortex.

	mPFC/Cing N = 7		**Cing/RSP N = 5**		**mPFC/RSP N = 5**		**RSP/RSP N = 7**	
	% of mPFC cells	**% of Cing cells**	**% of Cing cells**	**% of RSP cells**	**% of mPFC cells**	**% of RSP cells**	**% of rostral RSP cells**	**% of caudal RSP cells**
AD	0	0	0	0	0	0	17 (1-46)	13.3 (1-27)
AV	0	0	0	0	1.4 (0 – 7)	1 (0-5)	6.7 (0.7-23)	10.4 (1-38)
AM	3.3 (0-8)	3.1 (0-17)	7.9 (0-19)	6 (0-19)	0.1 (0-0.1)	0.5 (0-2)	5.7 (0-17)	9.2 (0-46)
IAM	12.2 (0-47)	5.8 (0-24)	3.3 (0-11)	12.4 (0-45)	0.12 (0-1)	4 (0-20)	2.2 (0-12)	6.6 (0-39)
LD	NA	NA	1.4 (0-4)	0.7 (0-2)	NA	NA	6.1 (0-13)	2.45 (0-5)

2.4. Results

Although cell counts are provided for all cases, the focus is on those cases giving the highest numbers and proportions of double-labelled cells for a given combination of cortical injections. This focus reflects how the technique will inevitably underestimate the full extent of bifurcating neurons as the tracer injections are discrete, i.e., they do not completely fill a given cortical area. Care was also taken to ensure that the injection sites in an individual cases did not overlap. A further concern was the possibility of direct tracer uptake by the cingulum bundle following cingulate and retrosplenial injections. For this reason, we compared the observed distribution of retrograde label with past, published topographies (Perry et al., 2021;

Shibata, 1993; Sripanidkulchai & Wyss, 1986). In no case did we observed patterns of retrograde label indicative of direct cingulum uptake (Bubb et al., 2020).

2.4.1. Collateral projections to both medial prefrontal and anterior cingulate cortices (mPFC/Cing)

In seven cases (Figures 2.1, 2.2, 2.3) a retrograde tracer was infused into different portions within the medial prefrontal cortex, combined with a second retrograde tracer into the anterior cingulate cortex. The medial prefrontal cortex injections variously included the ventral prelimbic cortex, dorsal prelimbic cortex, infralimbic cortex, dorsal peduncular cortex, medial orbital cortex, and tenia tecta. In one case (223#27) the more dorsal injection involved both the anterior cingulate and prelimbic cortices. Consequently, the two tracers included adjacent portions of prelimbic cortex, but at different heights. Given the potential for tracer overlap it is notable that the double-labelled cell counts in this case were representative of the group.

Across the mPFC/Cing tracer pairings, the following percentages of double-labelled cells represent the maximum found in the anteromedial nucleus (max ~6%), and the interanteromedial nucleus (max ~11%) (see Figure 2.4). The anteromedial single-labelled cells following tracer injections within the anterior cingulate cortex were often concentrated in the most medial and ventral parts of the nucleus, i.e., close to interanteromedial nucleus.

In those cases where double-labelled cells were observed they were mostly scattered across the interanteromedial nucleus, as well as located in the medial and ventral parts of the anteromedial nucleus. In contrast, only one of the seven cases contained any single-labelled neurons in either the anterodorsal or anteroventral nuclei following a tracer injection within the anterior cingulate cortex. Consequently, double-labelled cells were not observed in either of these two anterior thalamic nuclei.

When considered against the single-labelled cells projecting to the medial prefrontal cortex, the highest proportion of bifurcating neurons was found within the interanteromedial nucleus. Likewise, the interanteromedial nucleus contained the highest proportion of double-labelled cells when compared with those projecting to the anterior cingulate cortex (Table 2.1). For both measures, the anteromedial nucleus contained the lowest proportions of those nuclei with label from both injection sites (Table 2.1), after excluding the anteroventral and anterodorsal nuclei which did not innervate these sites.

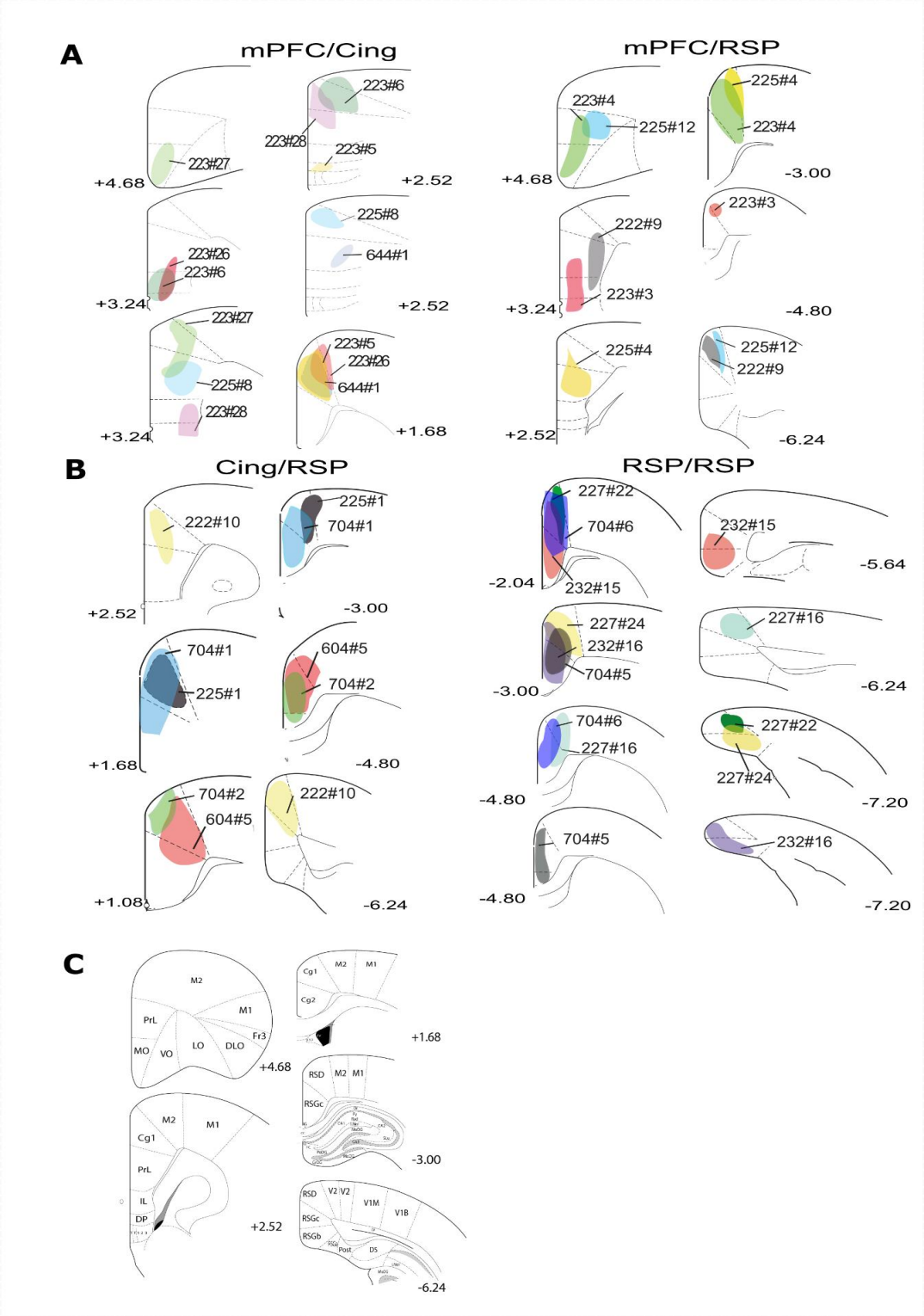


Figure 2.1. Schematic representation of the core of each injection site for the individual animals in each of the four groupings.

Panel A shows those cases with a medial prefrontal cortex (mPFc) injection combined with either anterior cingulate (Cing) or retrosplenial cortex (RSP) injections. Panel B shows the injection sites for the Cing/RSP pairings, as well as the

RSR/RSP pairings. Panel C shows annotated coronal sections that help to locate the various injection sites (sections based on Paxinos & Watson, 2004). A given animal has the same colour and case number within each of the four injection pairings. Other numbers refer to the location of the centre of the injections on the anterior-posterior axis (from bregma in mm). Abbreviations: Cg1: cingulate cortex area 1; Cg2: cingulate cortex area 2; DLO: dorsolateral orbital cortex; DP: dorsal peduncular; Fr3: fasciculus retroflexus; LO: lateral orbital cortex; M1: primary motor cortex; M2: secondary motor cortex; MO: medial orbital cortex; Post: postsubiculum; PrL: prelimbic cortex; RSD (area 30), dysgranular retrosplenial cortex; RSGa: granular retrosplenial cortex, area a; RSGb: granular retrosplenial cortex, area b; RSGc: granular retrosplenial cortex, area c; V1B: primary visual cortex, binocular area; V1M: primary visual cortex, monocular area; V2: secondary visual area; VA: ventral anterior thalamic nucleus.

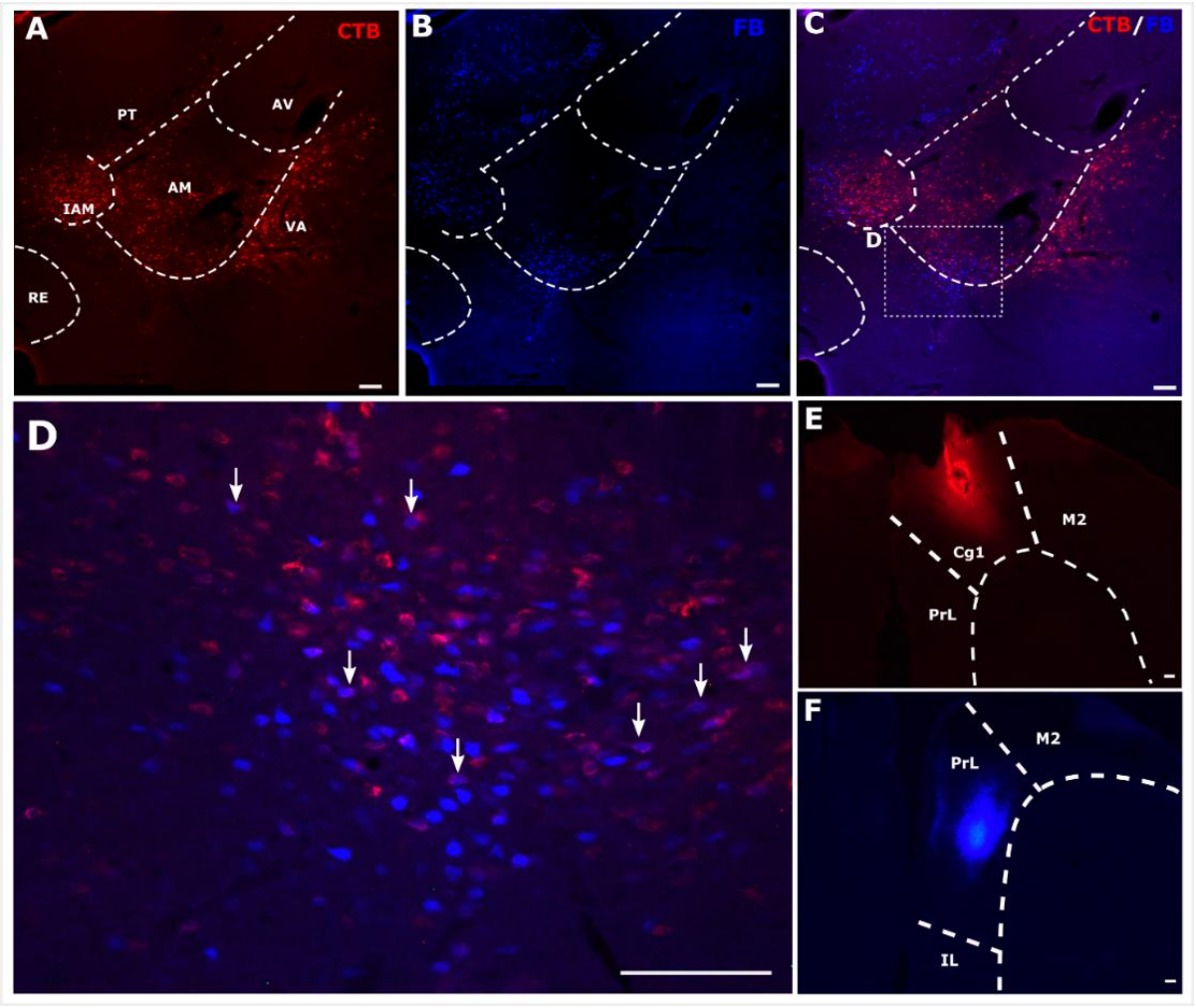


Figure 2.2. Example of combined medial prefrontal (FB) and anterior cingulate (CTB) tracer injections (case 225#8).

Coronal sections (approx. AP: - 1.9mm) containing the various thalamic nuclei under investigation. The upper panels (A, B, C) show label from the CTB (A) and FB (B) injections, as well as the overlay of the two channels (C). Panels E and F depict the CTB and FB injection sites, respectively. The enclosed area in panel C shows that part of the section magnified in panel D. The arrows in panel D point to examples of double-labelled cells, which show a blue centre (FB label) surrounded by red halo (CTB label). All scale bars are 150µm. Abbreviations: AV, anteroventral; AM, anteromedial; Cing1, cingulate cortex area 1; IAM, interanteromedial; IL, infralimbic cortex; LD, laterodorsal; M2, secondary motor cortex; mPFC, medial prefrontal cortex; PrL, prelimbic cortex; PT, parataenial nucleus; RE, nucleus reuniens; RSP, retrosplenial cortex; VA, ventral anterior thalamic nucleus.

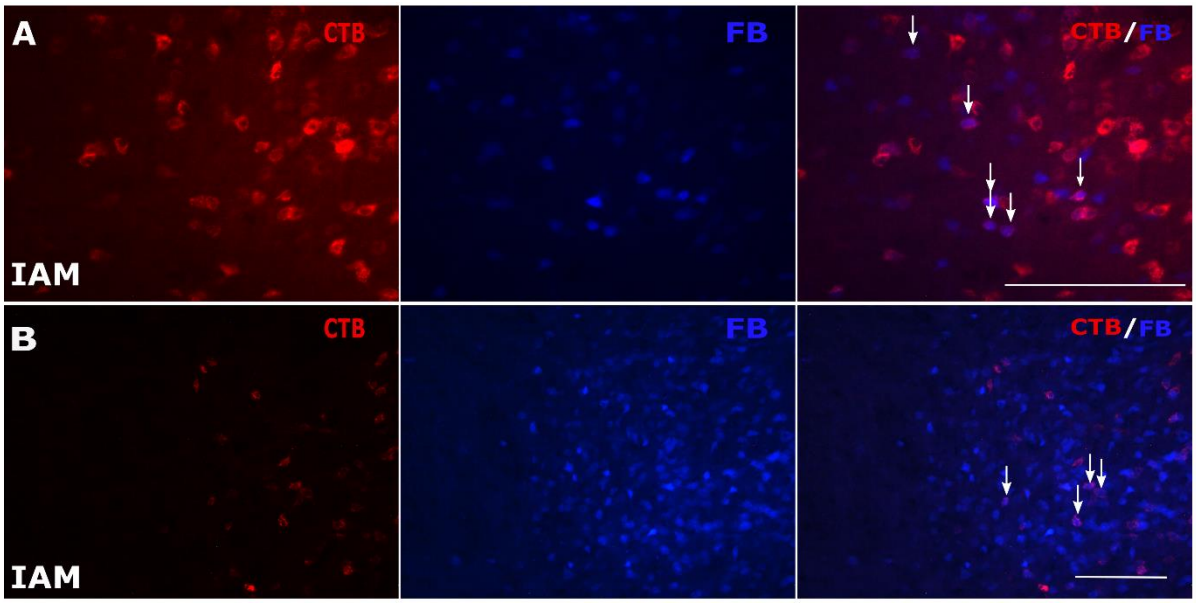


Figure 2.3. Labelling in the interanteromedial nucleus.

Panels in row A show a portion of the interoanteromedial nucleus (case 225#8) in a mPFC/Cing pair. Panels in row B, show a portion of the interanteromedial nucleus (case 604#5) in a Cing/RSP pair. The approximate AP axis is -2.16mm. Both panels show individual CTB and FB channels, with the overlays of the two pointing to double-labelled cells. All scale bars are 150µm. Abbreviations: IAM, interanteromedial nucleus.

2.4.2. Collateral projections to both the anterior cingulate and retrosplenial cortices (Cing/RSP)

In five cases (Figures 2.1, 2.3, 2.5), FB was injected into the anterior cingulate cortex and CTB in different portions of the retrosplenial cortex (AP: -3mm to -5mm). Like the mPFC/Cing pairs, albeit in slightly lower proportions, double-labelled cells were observed within the anteromedial nucleus (max ~5%), and the interanteromedial nucleus (max ~10). Again, double-labelled cells were not observed in the anteroventral or anterodorsal nuclei, but very occasional double-labelled cells were seen in the laterodorsal nucleus (max ~1.4%) (Figure 2.4).

The cell label following the injections in the anterior cingulate cortex was once again distributed mostly in medial and ventral portions of the anteromedial nucleus and in the interanteromedial nucleus. Following the tracer injections to retrosplenial cortex, single labelled cells were distributed across all three anterior thalamic nuclei. In cases with injections in the more anterior retrosplenial portions, there was very dense labelling within the anterodorsal nucleus. Single-labelled cells were also scattered across the anteromedial and anteroventral nuclei. Within the anteroventral nucleus the single-labelled cells were often densest close to the border with the anteromedial nucleus. Meanwhile, following the anterior cingulate injections there was a dense clustering of single-labelled cells in the medial and most ventral portions of the anteromedial nucleus, where most of the double-labelled cells within the nucleus were also found.

Overall, relative to the number of cells projecting to the retrosplenial cortex, the proportion of bifurcating neurons was the highest for the interanteromedial nucleus and lowest for the anteroventral and anterodorsal nuclei, which appeared to contain no double-labelled cells (Table 2.1). The laterodorsal nucleus had very low numbers of single-labelled cells following the anterior cingulate injections, which were concentrated in the most ventral parts of the nucleus. In contrast, the retrosplenial injections resulted in single-labelled cells in the dorsal parts of the laterodorsal nucleus. Consequently, there was very little overlap between the two areas of label, resulting in the very small proportion of double-label around the mid-depth of the nucleus. This very sparse double-labelling in the laterodorsal nucleus was seen in those cases where a tracer was placed in the more caudal portions in the anterior cingulate cortex. Meanwhile, relative to the number of cells projecting to the anterior cingulate cortex, the anteromedial thalamic nucleus contained the highest proportion of double-labelled cells (Table 2.1).

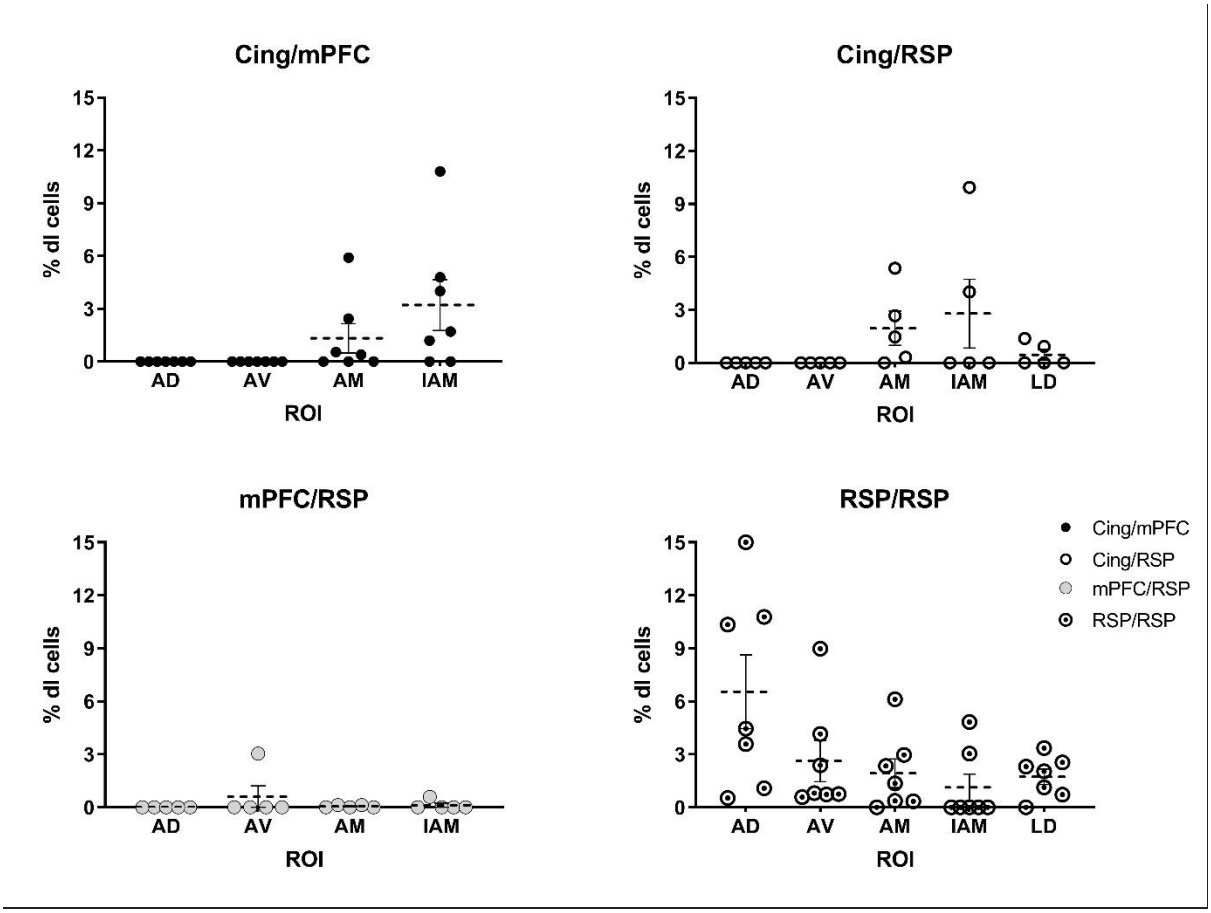


Figure 2.4. Proportion of double-labelled cells relative to all single-labelled cells (FB and CTB cell counts combined), expressed as percentages in the regions of interest (ROI) for each rat.

The different symbols (black circles, white circles, grey circles, white circles with dot) indicate individual animals in the mPFC /Cing (n=7), Cing/RSP (n= 5), mPFC/RSP (n=5), and RSP/RSP (n=7) groups. The dashed horizontal lines signify the mean percentages while the vertical lines show the standard errors for each thalamic nucleus. Abbreviations: AD, anterodorsal; AV, anteroventral; AM, anteromedial; IAM, interanteromedial; LD, laterodorsal.

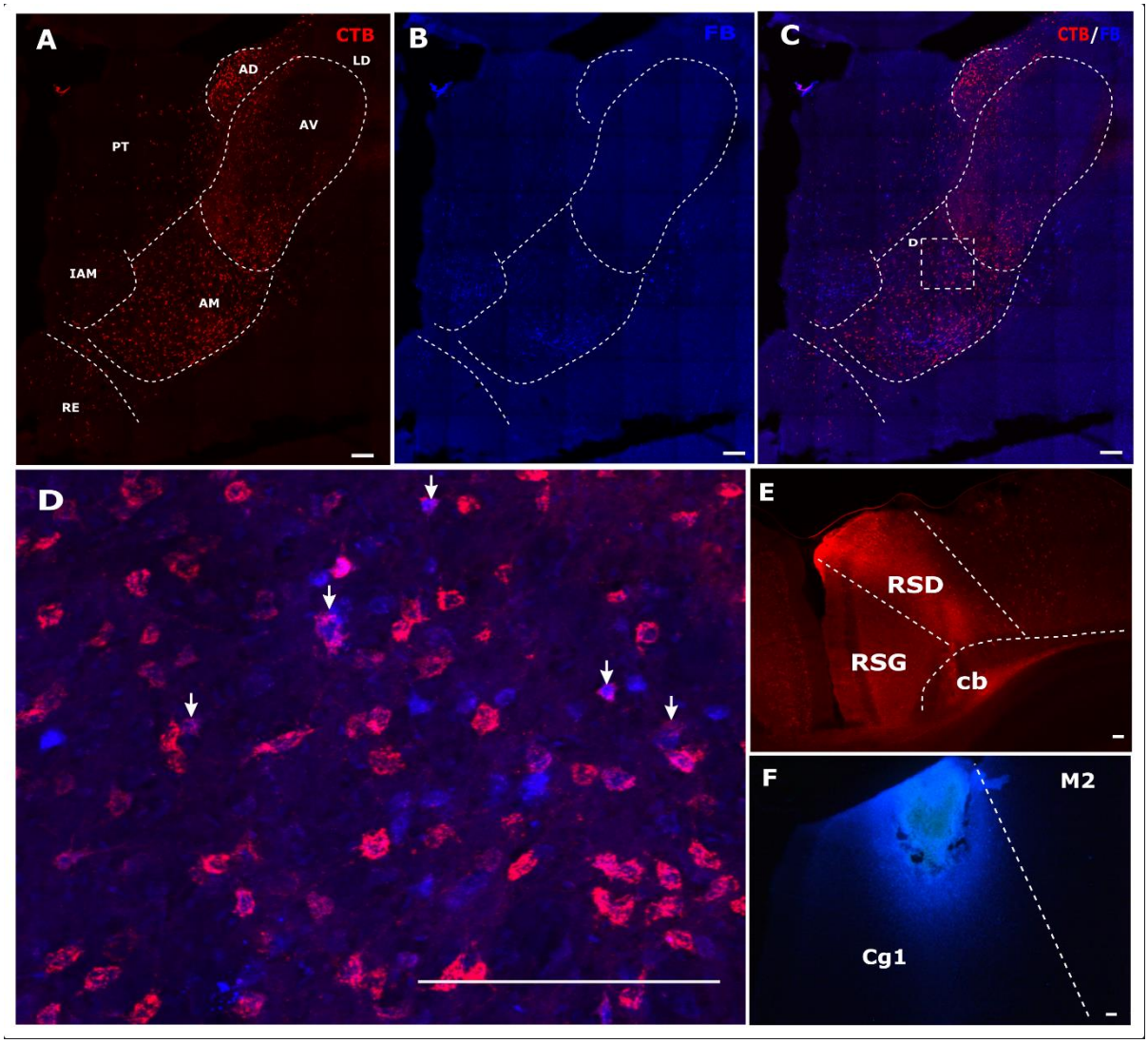


Figure 2.5. Example of combined retrosplenial (CTB) and anterior cingulate (FB) tracer injections (case 604#5).

Coronal sections showing the target thalamic nuclei (approx. AP: - 2.16mm). Panels A and B show individual channel labels and panel C shows the overlay of the two. Panels E and F show, respectively, the injections in the retrosplenial cortex and the anterior cingulate cortex. The enclosed area in panel C is magnified in panel D. The arrows point to examples of double-labelled cells, which show a blue centre (FB label) surrounded by a red halo (CTB label) within the AM. All scale bars are 150µm. Abbreviations: AD, anterodorsal; AV, anteroventral; AM, anteromedial; Cing1, cingulate cortex area 1; IAM, interanteromedial; LD, laterodorsal; M2, secondary motor cortex; PT, parataenial nucleus; RE, nucleus reuniens; RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex.

2.4.3. Collateral projections to both the medial prefrontal and the retrosplenial cortices (mPFC/RSP)

In five cases (Figures 2.1, 2.4, 2.6) FB was injected into different portions of the medial prefrontal cortex (prelimbic, medial orbital, infralimbic) and CTB into different portions of the retrosplenial cortex (AP: -3.3mm to -5.3mm from bregma).

Following these pairs of injections, very low overall proportions of double-labelled cells were seen in the anteroventral (max ~3%), anteromedial (max ~ 0.13%), and interanteromedial (max ~ 0.6%) nuclei, with no such cells being observed in some cases (Figure 2.6). The proportion of bifurcating neurons relative to single-labelled cells was highest in the interanteromedial nucleus, with respect to cells projecting to the retrosplenial cortex, and the lowest within the anteromedial nucleus with respect to single-labelled cells projecting to the mPFC (Table 2.1).

Following tracer injections in the retrosplenial cortex there was a dense clustering of single-labelled cells in the lateral portions of the anteroventral nucleus, with the rest of the label scattered across other portions of the anteroventral, anterodorsal, and the anteromedial nuclei. At the same time, the single-labelled cells following tracer injections in the mPFC were mostly in the anteromedial nucleus and interanteromedial nucleus.

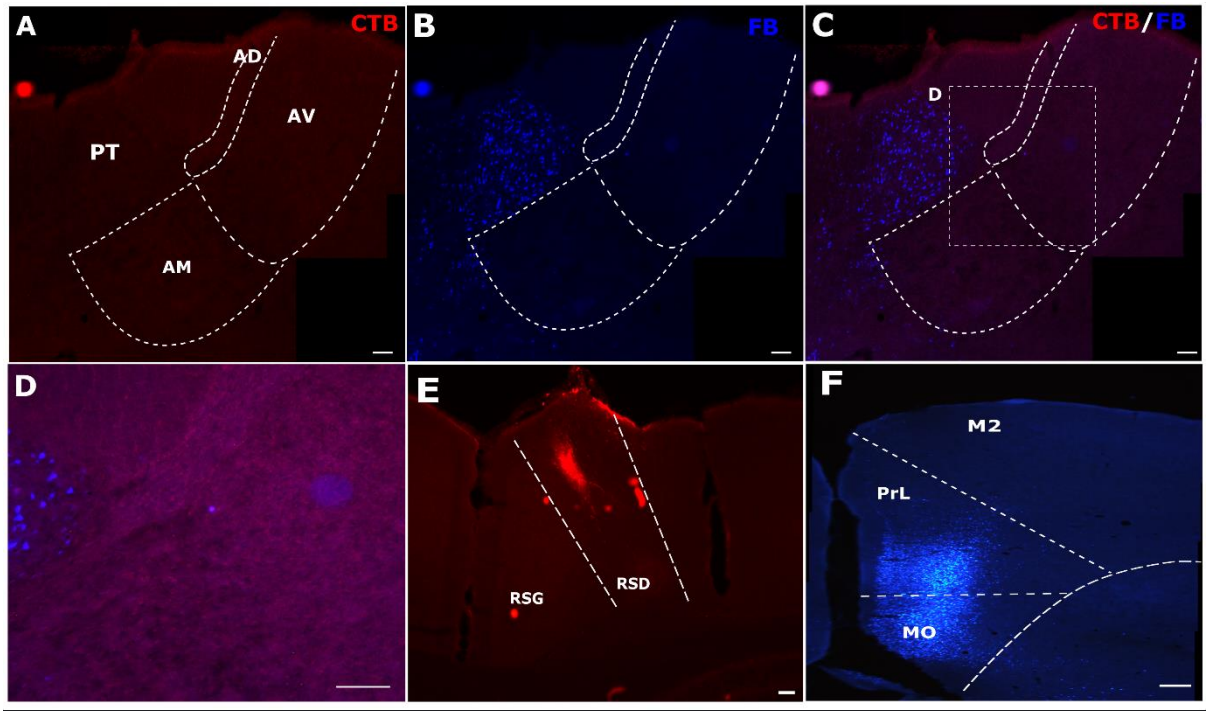


Figure 2.6. Example of combined medial prefrontal (mPFC) and retrosplenial cortex (RSP injection pairing (case 223#3).

Panels A and B show individual channels with CTB and FB cells following injections centred in the dysgranular retrosplenial cortex (E) and prelimbic /medial orbital cortices (F) (approx. AP: - 1.56mm). Panel C, which shows the overlay of the two channels, and panel D shows the lack of double-labelled cells. All scale bars are 150µm. Abbreviations: AD, anterodorsal; AV, anteroventral; AM, anteromedial; M2, secondary motor cortex; MO medial orbital cortex; PrL, prelimbic cortex; PT, parataenial nucleus; RE, nucleus reuniens; RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex.

2.4.4. Collateral projections to different portions of retrosplenial cortex (RSP/RSP).

In seven cases the tracers FB and the CTB were infused in different portions of retrosplenial cortex. The injections were separated along the anterior-posterior axis (AP: -3mm to -6.8mm from bregma) with varying involvement of dysgranular and granular portions. In two cases (704#5 and 704#6) both injections were rostral to the

splenium, i.e., the rostral half of the overall area, but the tracer spread did not overlap (Figure 2.1).

Unlike the previous injection pairings, the highest proportion of double-labelled cells relative to the sum of all single-labelled cells was in the anterodorsal nucleus (max ~15%) (Figures 2.1, 2.4, 2.7). The proportion of double-labelled cells within this nucleus appeared higher when the tracer was positioned in the more anterior portions of the retrosplenial cortex. The single labelling observed was often very dense and uniformly distributed across the anterodorsal nucleus regardless of the positioning of the tracers.

Also, unlike the other injection pairings, there was double labelling in the anteroventral nucleus (max ~9%), although it was slightly less frequent than that in the anterodorsal nucleus (Figure 2.4, Table 1). Furthermore, the single-labelled cells in the anteroventral nucleus showed a clear topography, as label was concentrated in a plexus along the ventrolateral border, in the rostral portions of the nucleus, but positioned closer to the anteromedial nucleus at more caudal levels.

The single-labelled cells from both the anterior and posterior retrosplenial injections were scattered and more uniformly distributed within the anteromedial nucleus, which may explain the lower proportion of double-labelled cells (max ~6%; Figure 2.4), although this is comparable to the proportions in the Cing/mPFC and Cing/RSP pairings. Notably lower was the double-labelling in the interanteromedial nucleus (max ~5%) (Table 2.1).

Unlike the Cing/RSP pairing, there were double-labelled cells in the laterodorsal nucleus. While the numbers of such cells remained low (max~3%; Figure 2.8), double-labelled cells were seen in most cases. The projections from this nucleus were topographically organised as more posterior retrosplenial injections led to label in the most dorsal part of the laterodorsal nucleus while the more anterior

retrosplenial injections led to label in its most ventral parts. This topography helped to separate the populations of labelled cells so that double-labelled cells occurred at the point where these two single-labelled populations of cells met.

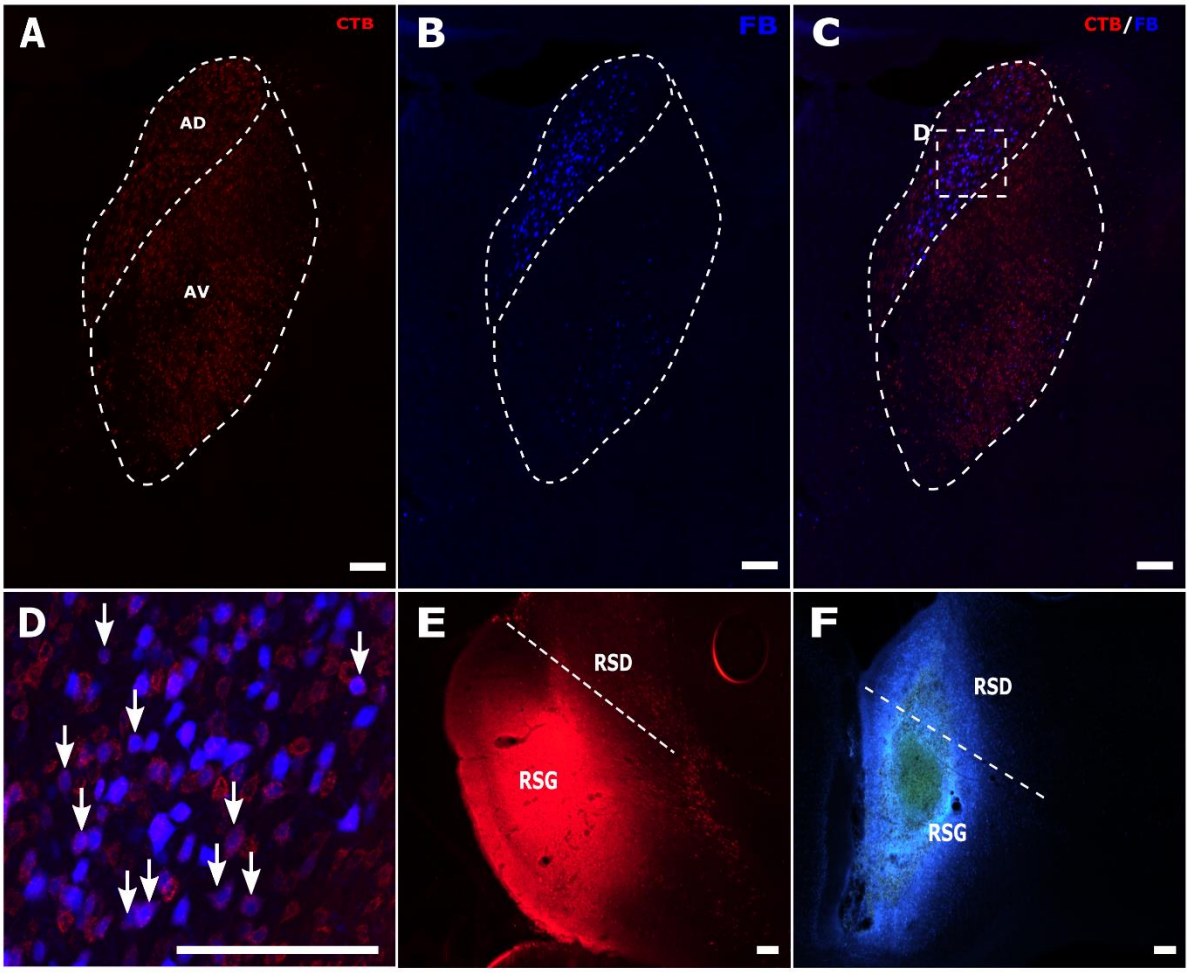


Figure 2.7. Example of combined retrosplenial cortex with retrosplenial cortex (RSP/RSP) injection pairing with double-labelling in the anterodorsal nucleus (case 232#16).

Panels A and B show individual channels for the CTB cells (A) that terminate in more caudal retrosplenial cortex and the FB cells (B) that terminate in the rostral retrosplenial cortex arising from within the anterodorsal and anteroventral nuclei. Panel C shows the overlay of both channels (approx. AP: - 1.72mm). Panel E shows the CTB injection site (caudal granular retrosplenial cortex). Panel F shows the FB injection site (rostral granular with some dysgranular retrosplenial cortex). Panel D is a magnified image of the boxed region in panel C, showing the appearance of double-labelled cells within the anterodorsal nucleus. Arrows point to some of the

double-labelled cells [blue centre (FB) surrounded by red halo (CTB)]. All scale bars are 150 µm. Abbreviations: AD, anterodorsal; AV: anteroventral; RSD: dysgranular retrosplenial cortex; RSG: granular retrosplenial cortex.

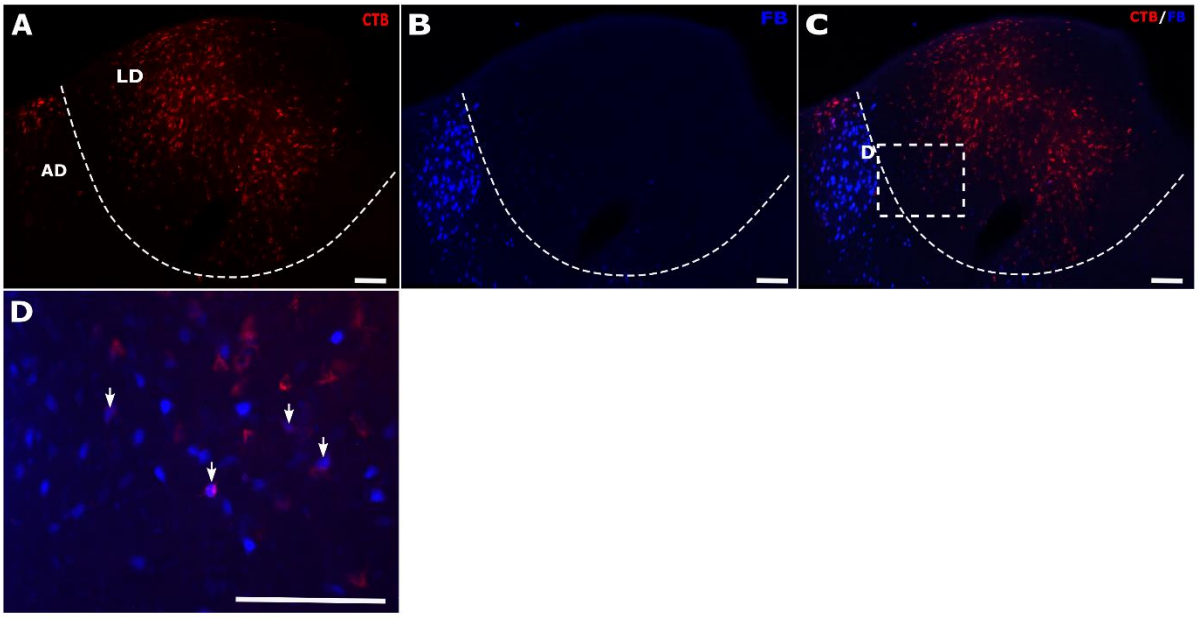


Figure 2.8. Example of laterodorsal nucleus labelling in a RSP/RSP pair (case 232#16).

Panel A shows CTB labelling following an injection in the caudal retrosplenial cortex (approx. AP: -2.28mm). Panel B left shows light FB labelling in the laterodorsal nucleus (alongside dense anterodorsal label) following an injection in the rostral retrosplenial cortex. Panel C shows the overlay of two. The enclosed square is magnified in panel D where arrows point the double-labelled cells within the laterodorsal nucleus. All scale bars are 150 µm. Abbreviations: AD, anterodorsal nucleus; LD, laterodorsal nucleus.

2.4.5. Quantitative Analyses

Friedman tests were conducted within each pair of injections to compare the distributions of the proportion of double-labelled cells between the nuclei of interest. The overall Friedman tests revealed statistically significant differences between the distributions of double-labelled cells in two of the groups: (1) mPFC/Cing: $\chi 2(4) =$

14.41, p = .006. Initial *post-hoc* comparisons revealed statistically significant differences between the anterodorsal and interanteromedial nuclei (p = 0.011) and the anteroventral and interanteromedial nuclei (p = 0.011) double-labelled proportion distributions, however these comparisons did not survive the Bonferonni multiple comparison adjustments (p_s = 0. 11); (2) RSP/RSP: $\chi2(5) = 18.68$, p = .002. Initial *post-hoc* analyses revealed statistically significant differences between the interanteromedial and anteroventral nuclei (p = 0.018), interanteromedial and anterodorsal nuclei (p < 0.001), laterodorsal nucleus and anterodorsal nucleus (p = 0.022), and the anteromedial and anterodorsal nuclei (p = 0.038). Of these, the differences in the distributions between the interanteromedial and anterodorsal nuclei (p = 0.007) survived the Bonferroni multiple comparison correction. There was no overall model effect in the mPFC/RSP pair, $\chi2(4) = 2$, p = 0.74 or in the Cing/RSP pair, $\chi2(5) = 8.5$, p = 0.13.

2.4.6. Further appraisal of double-labelled cell counts

Tissue from three selected cases, each with relatively high counts of double-labelled cells were investigated using confocal microscopy. These additional analyses informed several issues. Despite the thickness of the sections (50 µm), there was no evidence of false-positive counting in the main study as, overall, the labelled cell counts from the confocal images were often higher. Nevertheless, the rank order of proportions of double-labelled cells across different thalamic nuclei remained little changed. In one case (225#8; mPFC/Cing) the fluorescent data and confocal data were closely matched across all nuclei. In a second case (232#16; RSP/RSP) there were very small increases in the proportions of double-labelled cells, with the exception of AD where this increase was greater (rising from 13.7 to 21.8%). However, the Cing/RSP case (604#5) gave higher proportions of double-labelled cells for almost all nuclei (most notably for IAM rising to 21.3% and AM to 11.4%). These additional analyses indicate that the fluorescent cell counts reflected the rank order of collateral projections across nuclei, but the method can underestimate labelled-cell numbers, including the upper limit of double-labelled cells (Figure 2.9).

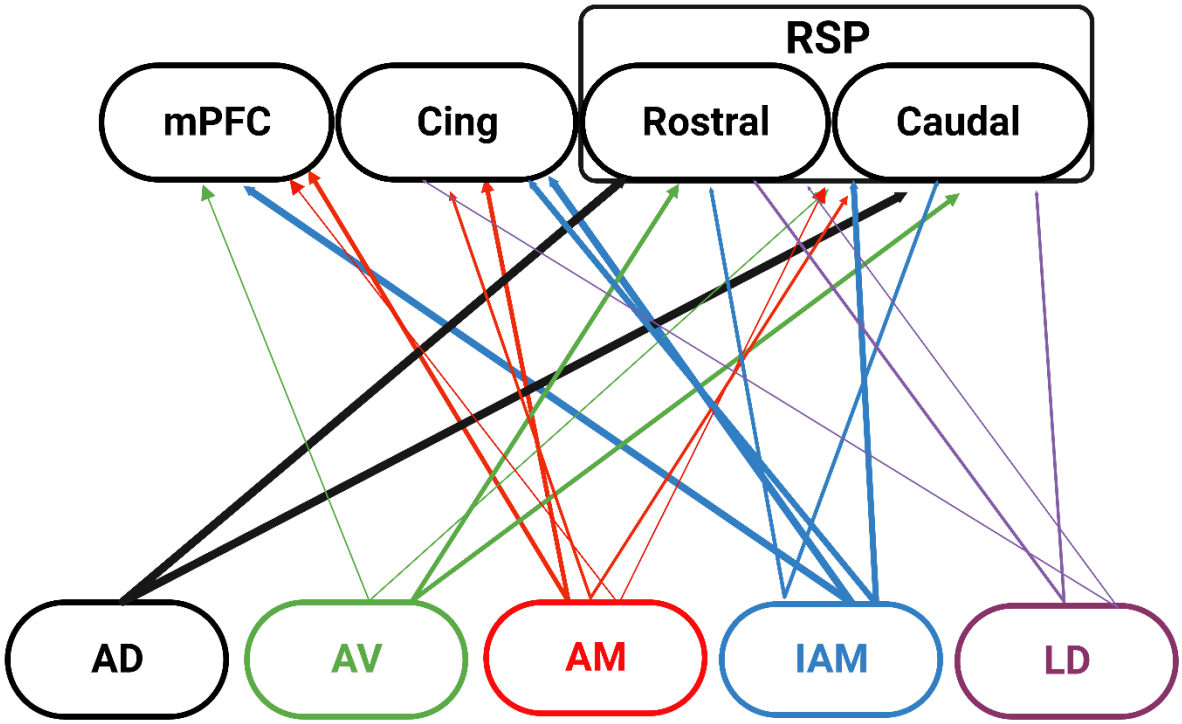


Figure 2.9. Schematic representation of the bifurcating anterior thalamic connectivity.

The schematic presents the projections from each nucleus to each of the investigated cortical regions. The thickness of the lines represents the proportions of double-labelled cells observed, with thicker lines representing larger proportions and thinner lines smaller proportions. The projections from different nuclei are presented in different colours: (1) AD: black; (2) AV: green; (3) AM: red; (4) IAM: blue; and (5) LD: purple. Abbreviations: AD, anterodorsal; AV, anteroventral; AM, anteromedial; Cing, anterior cingulate cortex; IAM, interanteromedial; LD, laterodorsal; mPFC, medial prefrontal cortex; RSP, retrosplenial cortex.

2.5. Discussion

To help understand how rostral thalamic nuclei influence medial cortical areas, pairs of tracers were injected into specific areas within the medial prefrontal and retrosplenial cortices. Retrograde label was then examined in the three principal

anterior thalamic nuclei, the interanteromedial nucleus, and the laterodorsal nucleus. In addition to confirming previously established thalamo-cortical projections (Conde et al., 1990; Horikawa et al., 1988; Shibata, 1993; Vertes, 2004), two patterns of bifurcating projections were observed. Neurons innervating both medial prefrontal/anterior cingulate cortices and anterior cingulate/retrosplenial cortices were most evident at or close to the thalamic midline. In contrast, neurons that collaterise to reach different parts of retrosplenial cortex were most frequent in the anterodorsal thalamic nucleus, although all selected thalamic nuclei contained some bifurcating neurons that simultaneously reach different parts of retrosplenial cortex. A third category was represented by a lack of collateralisation, e.g., very few thalamic neurons were observed that project to both medial prefrontal and retrosplenial cortices.

One task was to resolve whether neurons from the anterior thalamic nuclei collaterise to reach different parts of retrosplenial cortex. Our results closely follow one study (Horikawa et al., 1988) by finding a modest minority of such anterior thalamic cells, but contrast with another (Sripanidkulai & Wyss, 1986) that reported no such collaterals.

In view of these differences, we should consider the possibility of false positives in our study (and in that of Horikawa et al., 1988). Potential causes would include an overlap between pairs of adjacent cortical injections or direct tracer uptake by the cingulum bundle (Bubb et al., 2020; Domesick, 1970). The former explanation (direct spread) appears unlikely given the wide spacing of the retrosplenial injections in this and the previous study (Horikawa et al., 1988). To test for the second explanation (direct cingulum body uptake) both the injections site and the topography of retrogradely labelled cells within the anterior thalamic nuclei were examined to see if they matched previous descriptions for that part of the retrosplenial cortex (Bubb et al., 2020; Perry et al., 2021; Shibata, 1993; Sripanidkulchai & Wyss, 1986) or whether the labelled cells were distributed broadly across the nuclei, i.e., more consistent with cingulum uptake. By these measures there was no evidence of direct cingulum bundle uptake. Therefore, there is a modest population of anterior thalamic neurons with collaterals that reach widely separated parts of retrosplenial cortex (see also Horikawa et al., 1988). This conclusion can be extended as an

earlier report noted that ~10% of labelled anteromedial nucleus neurons project to both the rostral and caudal portions of anterior cingulate cortex (Horikawa et al., 1988).

Of the anterior thalamic nuclei, the anterodorsal nucleus typically contained the highest proportion of retrosplenial/retrosplenial collaterals, often in modest numbers, although confocal microscopy indicated that this proportion might reach 22%. A very similar preponderance in the anterodorsal nucleus (reaching 21%) was previously reported (Horikawa et al., 1988). Together, these findings (Figure 2.4) reinforce other differences qualitative between the three major anterior thalamic nuclei (Aggleton et al., 2010; Byatt & Dalrymple-Alford, 1996; Safari et al., 2020).

The anterodorsal nucleus is a key element of the head-direction system (Taube, 1995), providing compass-like signals and assisting navigation (Taube, 2007). This nucleus is heavily interconnected across the retrosplenial cortex, a cortical region also importantly involved in spatial memory and navigation (Cooper & Mizumori, 2001; Hindley et al, 2014; Mitchell et al., 2018; Nelson et al., 2015; Harker & Whishaw, 2004; Vann et al., 2009; Wolbers & Büchel, 2005). One implication of the current findings is that the information provided by the rat's current heading direction can simultaneously influence diverse areas of retrosplenial cortex, reflecting the relevance of this information for on-line navigation.

In contrast, very few bifurcating neurons originated in the laterodorsal nucleus to reach different parts of retrosplenial cortex, consistent with a previous study (Horikawa at al., 1988). This contrast with the anterodorsal nucleus is all the more striking as both thalamic nuclei contain numerous head-direction cells (Mizumori & Williams, 1993; Taube, 2007) and both project to the granular and dysgranular retrosplenial cortices (Shibata, 1993; Sripanidkulchai & Wyss, 1986; van Groen & Wyss, 1990; Wyss & Groen, 1992). But, unlike the anterodorsal nucleus, the laterodorsal nucleus does not receive direct head-direction information from the lateral mammillary bodies (Dillingham et al., 2015). Rather, the laterodorsal nucleus receives a greater array of cortical and subcortical visual inputs than the anterior thalamic nuclei (Bezdudnaya & Keller, 2008). Consequently, it has been argued that the laterodorsal nucleus head-direction neurons have qualitatively different

properties from those in the anterodorsal nucleus (Dudchenko et al., 2019). One of these different properties appears to be the nature of their inputs to retrosplenial cortex.

The findings also align with previous descriptions of collateral projections from the anterior and midline thalamic nuclei that reach both prefrontal and anterior cingulate areas (Condé et al., 1990) as well as those that reach both anterior cingulate and retrosplenial areas (Horikawa et al., 1988). Like the former study (Condé et al., 1990), double-labelled cells in the mPFC/Cing cases were observed within those thalamic nuclei at or adjacent to the midline, i.e., the interoanteromedial nucleus. The present study extended that of Condé et al. (1990) by describing double-labelled cells in the anteromedial nucleus and including a wider combination of prefrontal areas to receive tracer injections.

Meanwhile, Condé et al. (1990) also reported double-labelled cells in the ventromedial thalamic nucleus, rhomboid nucleus, and mediodorsal thalamic nucleus. Remarkably, the many double-labelled cells in select lateral part of the mediodorsal nucleus reached up to 90% of the neurons labelled by one of the tracers (Condé et al., 1990). This striking difference between the properties of the anterior thalamic nuclei (limited bifurcation) and mediodorsal nucleus (considerable bifurcation) highlights how these integral parts of the 'cognitive thalamus' have contrasting roles (Clark & Harvey, 2016; Perry et al., 2021; Sweeney-Reed et al., 2021). While the parallel mediodorsal nucleus efferents are more consistent with a regulatory role across multiple prefrontal functions (Mitchell & Chakraborty, 2013; Pergola et al., 2018), those from the anterior thalamic nuclei imply the conveyance of more specific information, e.g., relating to space (O'Mara & Aggleton, 2019).

The proportions of anterior thalamic neurons reaching both the anterior cingulate and retrosplenial cortices in the present study appeared slightly lower than those in a previous study (Horikawa et al., 1988). That study reported how, within the anterior thalamic nuclei, the anteromedial nucleus contained the highest proportion (~13%) of double-labelled cells projecting to both anterior cingulate and retrosplenial cortices (Horikawa et al., 1988).

In the present study, the anteromedial nucleus again contained the highest proportion from the three principal anterior thalamic nuclei (~ 6% but reaching 11% in the confocal case). A partial explanation is that the counting method tended to provide conservative counts, as indicated by the confocal data. Meanwhile, the adjacent interanteromedial nucleus contained higher proportions (~ 9%). The double-label in the interanteromedial nucleus is informative as Horikawa et al. (1988) did not separate this area from the anteromedial nucleus, partly explaining their higher counts.

In both studies, the anterodorsal and anteroventral nuclei contained no labelled neurons reaching both the rostral anterior cingulate and retrosplenial cortex, while the laterodorsal nucleus contained < 2% of labelled cells. It was the case, however, that injections involving the caudal anterior cingulate cortex and retrosplenial cortex (Horikawa et al., 1988) led to modest numbers of double-labelled cells also being observed in the other anterior thalamic nuclei. This apparent difference from the present study may reflect how those more caudal anterior cingulate injections involved the midcingulate area 24' (Vogt & Paxinos, 2012). This transition area, which had not been distinguished at the time of the earlier study, was deliberately avoided in the present study.

A largely complementary study also investigated collateral cortical projections from the anteromedial nucleus and the adjacent nucleus reuniens (Pei et al., 2021). Much of their focus was on whether cortical collaterals reach the hippocampal formation (Pei et al., 2021). Consequently, that study included medial prefrontal cortex/dorsal subiculum, medial prefrontal cortex/ventral subiculum, caudal retrosplenial/dorsal subiculum, and caudal retrosplenial/ventral subiculum injection pairings. All injection pairings led to double-labelled cells in the two thalamic nuclei, with the highest proportions in the anteromedial nucleus and nucleus reuniens for the medial prefrontal/dorsal subiculum pairing (both >30% of all label), as well as, the anteromedial nucleus (~20%) and nucleus reuniens (26%) for the medial prefrontal cortex/retrosplenial cortex pairing (Pei et al., 2021). Yet the same group pairing gave proportions closer to zero in the present study for the anteromedial nucleus.

Overall, the study by Pei et al. (2021) produced higher proportions of double-labelled thalamic cells than reported in previous analyses of similar target pairings (Condé et al., 1990; Horikawa et al., 1988; Varela et al., 2014), including the present one. One potential explanation for these higher proportional cell counts is that Pei et al. (2021) focused on those cases with more extensive tracer injections within the target areas. (Unless both target areas are filled with tracer, the resulting double-cell counts will always be an underestimate.) One unintended consequence, however, was that the 'medial prefrontal' cortex injections extended into anterior cingulate cortex (Pei at al., 2021), a factor that might increase the proportions of double-labelled cells within the anteromedial nucleus (see anterior cingulate/retrosplenial pairs here and Horikawa et al., 1988).

Furthermore, their cell counts (Pei et al., 2021) just involved a restricted subarea of each target thalamic nucleus. This method will give higher double-cell counts if there is a bias towards zones of label overlap. In contrast, the present study counted cells across each entire nucleus, a method likely to reduce the proportions of double-labelled cells given the topographic origins of many of the cortical projections from within the target nuclei (Lomi et al., 2021; Shibata, 1993; Sripanidkulchai & Wyss, 1986). Even though the counting methods in the present study tended to be conservative (as indicated by parallel confocal analyses), for those studies that took cell counts across the entire nucleus (Horikawa et al., 1988; Condé et al., 1990; Varela et al., 2014) the proportions of double-labelled cells were more comparable to those in the present study.

Consistent with previous studies, the distribution of single-labelled cells highlights how rostral thalamic nuclei and those close to the midline project to a wide array of frontal and cingulate sites, a pattern seen not only in rats (Condé et al., 1990; Van Der Werf et al., 2002; Vertes, 2004), but also in non-human primates (Kievit & Kuypers, 1977)).

One goal of the present study was to examine the properties of efferents from the three main anterior thalamic nuclei, while also creating comparisons to the midline thalamic nucleus reuniens. Both nucleus reuniens and the anterior thalamic nuclei are interconnected with many of the same sites and both are presumed to make

important contributions to cognition (Aggleton et al., 2010; Cassel et al., 2021; Griffin, 2021; Mathiasen et al., 2020, 2021; Pei et al., 2021). For example, both the anteromedial nucleus and nucleus reuniens contain a similar modest proportion of thalamo-cortical neurons that innervate multiple frontal areas (see also Condé et al., 1991). A small minority of bifurcating projections is also found when looking for individual prefrontal neurons that reach both nucleus reuniens and the anterior thalamic nuclei (Mathiasen et al., 2021), though some prefrontal inputs to nucleus reuniens also collaterise to reach medial temporal sites (Schlecht et al., 2022; Varela et al., 2014).

The observed bias to neuronal separation is consistent with the prevailing view that the various anterior thalamic nuclei and nucleus reuniens operate in parallel, complementary ways that reflect subtle topographic and functional differences (Aggleton et al., 2010; Ferraris et al., 2021; Griffin, 2021; Mathiasen et al., 2020, 2021). These same results also highlight the presence of distinct, parallel prefrontal – thalamic – hippocampal pathways, one involving the anterior thalamic, the other involving nuclei nucleus reuniens (Prasad & Chudasama, 2013). The former reaches the dorsal hippocampus, while the latter is more focussed on the ventral hippocampus.

Within the anterior thalamic nuclei, the relative rates of collaterisation strengthen proposals concerning the respective functions of its principal nuclei (Aggleton et al., 2010). The anteromedial (and interanteromedial) nucleus reflecting prefrontal attributes, while the anteroventral and anterodorsal nuclei are more closely linked with retrosplenial and hippocampal formation functions (Yamawaki et al., 2019a,b; Aggleton & O'Mara, 2022). As discussed in *section 1.8.3*, the position and connectivity of the anterodorsal nucleus make it a possible candidate for being a central node of the head-direction system, while the anteroventral nucleus is better suited as "return-loop" node, conveying information between the hippocampus and the retrosplenial cortex. The anteromedial nucleus, on the other hand, which is distinguished by its prefrontal connections, assumes the role of a "feed-forward" node, providing an indirect pathway for the retrosplenial cortex to reach the prefrontal cortices (Aggleton et al., 2010). Understanding the presence and absence of bifurcations from the anterior thalamic neurons to these sites provides valuable

information about how their interactions may support memory and helps to distinguish the individual functional contributions of the nuclei, which may have implications for neurological conditions involving those sites.

Chapter 3

3. Disrupting direct inputs from the dorsal subiculum to the granular retrosplenial cortex impairs spatial memory in the rat

3.1. Introduction

Effective spatial learning and related navigation are essential skills for humans and animals alike. These are, however, complex, multisensory processes that require the integration of external visual cues with internally generated movement-related cues (Johnsen & Rytter, 2021). The mechanisms required to create a coherent representation of the external environment have been intensively investigated, with the hippocampal formation and parahippocampal region often providing the start point (Eichenbaum, 2017; Moser et al., 2008; O'Keefe & Nadel, 1979). Within the hippocampal formation, the subiculum, may make specific contributions given its diverse spatial cells and its significance as a route for the hippocampus proper to influence distal sites (Aggleton & Christiansen, 2015; Lever et al., 2009; O'Mara, 2005; Witter, 2006; Yamawaki, Corcoran, et al., 2019; Yamawaki, Li, et al., 2019).

Both neurotoxic lesions of the hippocampus proper and lesions of the subiculum impair location learning in the Morris Water Maze, suggesting that together these hippocampal regions are necessary for successful allocentric (world-centred) spatial learning (Morris et al., 1990). Furthermore, the hippocampal formation is not uniform as is shows graded anatomical and electrophysiological changes along its axes. One consequence is that the dorsal and ventral subiculum appear to be functionally distinct in rodents. Based on a variety of evidence it seems that the dorsal subiculum is the more critical for solving spatial memory tasks (Bannerman et al., 2004;

Burzynska et al., 2020; Moser & Moser, 1998; O'Mara, 2005; O'Mara et al., 2009; Strange et al., 2014; Witter et al., 1990). Consistent with this view, permanent lesions of the dorsal subiculum are sufficient to impair T-maze alternation (Potvin et al., 2007), a measure of spatial working memory. The pattern of deficits suggested that the dorsal subiculum is essential for processing idiothetic cues for navigation (Potvin et al., 2007, 2010). In addition, dorsal subiculum lesions impaired both object-location memory (Potvin et al., 2010) and the ability to distinguish adjacent-arm trials in the radial-arm maze, pointing to a role in pattern-separation (Potvin et al., 2009, 2010). These same lesion studies also indicated that the dorsal hippocampus proper and the dorsal subiculum can contribute differently to spatial memory (Potvin et al., 2007, 2009, 2010).

There are dense projections from the subiculum to the retrosplenial cortex that in rodents preferentially target the granular subdivision (area 29). These same projections principally arise from the dorsal subiculum (Kinnavane et al., 2018; Sugar et al., 2011; van Groen & Wyss, 1992)(see also *Chapter 1, sections 1.3 and 1.7.1*.). Like the hippocampus, retrosplenial cortex is repeatedly implicated in spatial memory and navigation (Nelson et al., 2018; Nelson et al., 2015; Harker & Whishaw, 2004; Vann et al., 2009; Wolbers & Büchel, 2005) as well as episodic memory (Hayashi et al., 2020; Maguire, 2001; Nestor et al., 2003; Vann et al., 2009). Furthermore, recent studies suggest that these subiculum projections may facilitate the flow of contextual information to retrosplenial cortex, thereby enabling memory formation (Gao et al., 2021; Yamawaki et al., 2019b).

The effects of retrosplenial cortex lesions on spatial tasks appear to be most pronounced when rats must rely on flexible cue integration, such as when intra-maze and extra-maze cues are opposed (Pothuizen et al., 2008, 2010; Vann & Aggleton, 2004; Vann et al., 2003) or when required to choose between competing relevant and irrelevant spatial information (Wesierska et al., 2009). For example, rats with retrosplenial cortex lesions showed alternation impairments when the T-maze was rotated between the sample and test runs (Nelson et al., 2015), thereby bringing intra-maze and extra-maze cues into conflict. Like the dorsal subiculum, retrosplenial lesion deficits can also emerge when visual stimuli are removed (Cooper & Mizumori, 2001; Elduayen & Save, 2014). Given the interconnectivity of

retrosplenial cortex with motor, sensory, and visual cortices (Miyashita & Rockland, 2007; Sugar et al., 2011; Yamawaki et al., 2016) this cortical area is well placed to integrate information between different sensory modalities to successfully navigate an environment (Byrne et al., 2007; Mizumori et al., 2000; Powell et al., 2020). However, it is unclear whether direct hippocampal – retrosplenial connections are required for this process.

While much is known about the effects of permanent retrosplenial lesions on learning and memory, far less is known when the hippocampal inputs to this area are targeted (Yamawaki,et al., 2019a,b). These more targeted studies have, so far, been confined to showing the importance of these retrosplenial inputs for contextual fear conditioning (Yamawaki et al., 2019a,b). The present study sought to examine a more flexible form of spatial learning involving working memory. Consequently, rats were trained on a T-maze alternation task, followed by multiple cue conditions. To disrupt the direct projections from the dorsal subiculum to retrosplenial cortex, inhibitory designer-receptor exclusively activated by designer drugs (iDREADDs) injections in the dorsal subiculum were combined with intracerebral infusions of a ligand (clozapine) at the target site (retrosplenial cortex) to inactivate those projections locally (Gomez et al., 2017; Manvich et al., 2018; Roth, 2016, 2017).

3.2. Materials and Methods

3.2.1. Statement of Contributions

The work presented in this chapter contains data collected from colleagues. Bethany Frost performed the surgeries and collected the data in Cohort 1. Eman Amin assisted with the histological data analysis in Cohort 1. I performed the surgeries and data collection in Cohort 2, as well as the histological analysis, imaging, and statistical analyses. The chapter has been submitted for a peer-review.

3.2.2. Experimental Design

Either iDREADDs or a green fluorescent protein (GFP) expressing adeno-associated virus (control) was injected into the dorsal subiculum in two separate groups of rats. Due to the potential off-target effects of the intervention and of clozapine (*see section 1.8.2*), it was necessary to include a viral control (GFP) group (*see section 1.8.3*). Shortly before each behavioural test, both groups received intracerebral infusions targeted at retrosplenial cortex. For some sessions clozapine was infused, on other sessions it was saline. Animals were then tested on reinforced alternation in a T-maze, using five successive variants that differently taxed the use of available cues. The experiment was repeated with two separate cohorts of rats, both treated in the same ways (Figure 3.1).

3.2.3. Animals

Two cohorts, respectively of 12 and 24 adult Lister Hooded male rats (Envigo, UK), were trained prior to surgery on reinforced T-maze alternation. The first cohort had 8 iDREADDs and 4 GFP-control animals. Given the usual effects observed on T-maze alternation following retrosplenial lesions (*see section 1.5.3*) the first cohort appeared to be underpowered and, therefore, additional data were needed to establish if there were any true effects. The second cohort had 12 iDREADDs and 12 GFP-control animals, giving totals of 20 in the iDREADDs group and 16 GFP- controls. At the time of surgery all rats weighed between 236-360g. For all behavioural experiments, water was available *ad libitum*. The rats were put on a food-restricted diet whereby they were still able to gain weight. None of the rats weighed less than 85% of their free-feeding weight.

All animals were randomly assigned to one of the virus conditions and underwent the same surgical and behavioural procedures. The experimenter was not, however, blind to the group membership of the animals. All animals were housed in pairs, in a temperature-controlled room, under 12h light/dark cycle (*see Chapter 2, section*

2.2.2). All animal procedures were carried out in accordance with U.K. Animals (Scientific Procedures) Act 1986 and were approved by the local Ethics Committee at Cardiff University.

3.2.4. Surgery

All surgeries took place under isoflurane-oxygen mixture anaesthesia (5% induction, 1.5-2.5% maintenance). Each rat was placed in a stereotaxic frame (David Kopf Instruments, CA, USA), so that the skull was flat, with respect to the horizontal plane. Chloramphenicol 0.5% eye-gel was applied, meloxicam (0.06ml) was administered subcutaneously for analgesic purposes, and lidocaine (0.1ml of 20mg/ml solution) was applied topically to the incision site. Next, a bilateral craniotomy was performed above the dorsal subiculum, and either pAAV-CaMKIIa- hM4D(Gi)-mCherry (AAV5) (iDREADD)(Titer: 2.6x10^13GC/ml, lot:v102676; Addgene, MA, USA) or pAAV-CaMKIIa-GFP (AAV5)(titer: 4.3x 10^12GC/ml, lot: v5894, Addgene, MA, USA)(GFP-control) virus was injected bilaterally into the dorsal subiculum.

In both cohorts, 0.6µl of the viral construct was injected in the anterior subiculum injection site and 0.4µl into the more posterior site. The injection coordinates, with respect to bregma were as follows: *Anterior*. Cohort 1: AP: -5.9mm, ML: ±2.9mm, DV: -2.6mm; Cohort 2: AP: -5.9mm, ML: ±2.7mm, DV: -2.4mm; *Posterior*. Cohort 1: AP: -6.2mm, ML: ± 3.2mm, DV: -2.5mm; Cohort 2: AP: -6.2mm, ML: ± 3.0mm, DV: -2.3mm), respectively. The very slight changes in coordinates reflected individual preferences of two researchers across the two cohorts, based on pilot experiments. All injections were made vertically using a 10µl Hamilton Syringe attached to a movable arm. A micro-syringe pump (World Precision Instruments, Florida, USA) controlled the injection, with the flow rate set at 150ηl/min. The injection needle was left *in situ* for further 5 minutes, before retracting it. The order of the iDREADDs and GFP injections was randomized, so that animals were randomly allocated to either group.

During the same surgeries, pairs of cannulas were implanted into the left and right retrosplenial cortex. One cannula pair (1.5mm length x 1.2mm separation, 26-gauge,

PlasticsOne, Virginia, USA) was implanted into the anterior portion of the retrosplenial cortex (from bregma; AP: -2.5 mm, ML: ± 0.6 mm, DV: -1.5mm), the other cannula pair (1.7mm length x 1.4mm separation; 26-gauge, Plastic One, Virginia, USA) was implanted into the posterior retrosplenial cortex (AP: -6.0mm, ML: ± 0.7mm, DV: -1.7mm). The implantation coordinates for both cohorts remained the same. The cannulas were held in place with bone cement (Zimmer Biomet, Swindon, UK) and anchored to the skull with four screws (Precision Technology Supplies, Uckfield, UK). Dummy cannulas were inserted into the guide cannulas to prevent blocking and were secured in place with aluminium dust caps. Testing with clozapine or saline commenced at least three weeks after surgery.

3.2.5. Apparatus for Behaviour

Behavioural testing was conducted in an elevated (94cm), modifiable cross-maze with clear Perspex walls and wooden floor. Each arm was 70cm long and 10cm wide, with 17cm high walls. Inset food wells were positioned at the end of each arm so that the food rewards could not be seen from the choice point. An aluminium barrier was used to block one arm to create a T-shape, while a second transferable barrier was used to temporarily block access to one of the T-maze arms during the sample run. Unless otherwise specified in the experimental condition, the location of the start arm remained constant across experiments. The maze was positioned in the centre of a room (280cm x 280cm x 20cm) with salient visual cues on the walls. All cues remained constant throughout the experiments. For both pre-training and the five experimental conditions the experimenter stood behind the start arm for both the ‘sample’ and ‘test’ runs while the rat completed the trial. The illumination in the room for all conditions, unless otherwise specified, was 23-26lx.

3.2.6. Behavioural Training Prior to Infusions

Prior to surgery, all rats were habituated to the maze for four sessions. During the first habituation session, multigrain hoops (Crownfield, UK) were placed in the food wells in the choice-arms, and the rats were placed in the maze in cage-pairs to

explore the start arm for 5 minutes. Then, they were placed in the choice-arms where they could collect food rewards for a further 5 minutes. During sessions 2 and 3, the above procedure was repeated for each rat individually for 5 minutes. In session 4, the aluminium barrier was introduced at the entrance of one arm and the rats allowed to explore for 5 minutes. The same procedure was repeated but now the barrier blocked the other arm. The food in the wells was continuously replaced. The rats were then run on the 'Standard' T-maze procedure (see below) for 5 to 9 days and the animals for surgery were selected, based on their performance and willingness to run.

Post-surgery, the animals were retrained on the Standard T-maze task for 6 to 10 days, until they reached at least 87.5% (7/8) on two consecutive sessions. The infusion trials for that condition then followed.

The rats were then trained on the next T-maze condition for 3 to 5 days, immediately prior to the infusions for that condition. An additional, infusion-free training day was provided if there had been a gap of more than two days between infusion sessions. An infusion-free training session was also given on the day between the two clozapine infusions, to help performance return to baseline (Figure 3.1). This testing regime was repeated for the remaining behavioural conditions (Figures 3.1 and 3.2).

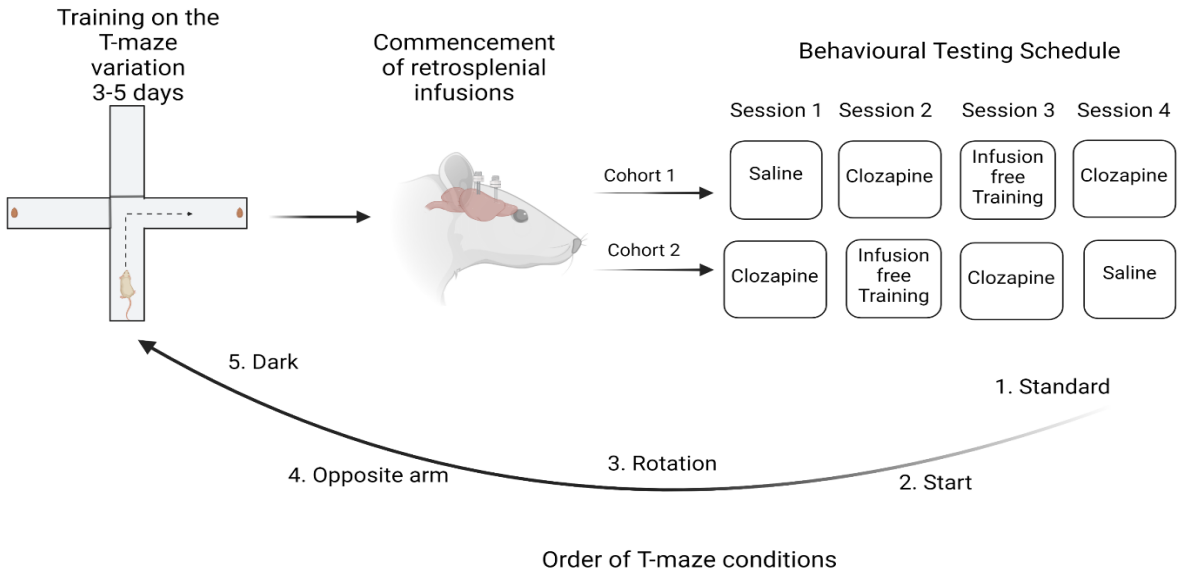


Figure 3.1. Schematic illustration of the behavioural training and testing schedule post-surgery.

Animals were trained on each variation just prior to the commencement of the intracerebral infusions. The order of infusions was counterbalanced across the two cohorts as indicated.

3.2.7. Experimental Conditions (all with 8 trials per session)

Each experimental condition consisted of a forced (i.e., 'sample') run, followed by a free (i.e., 'test') run in the T-shaped maze. The correct choice arm across the block of 8 trials was pseudorandomized so that the same choice arm was not repeated more than twice consecutively. To start each trial both T-maze arms were baited with a quarter of a multigrain hoop before the sample run, but access to one arm was blocked at its base with an aluminium barrier.

To begin the sample run the rat was released from the start position and ran to the junction of the T-maze, where it turned into the pre-selected arm and ate the reward.

The rat was then immediately picked up and the barrier at the choice point removed. Then, the animal was carried to the start position and allowed to begin the test run. After running down the stem of the maze, the rat could choose between the left and right arms. The animal received a food reward only if it alternated, i.e., selected the arm located opposite from the baited sample arm.

A test run was considered correct when the animal's back feet crossed markings at the base of each side arm. The animal was picked up and held until, the T-maze was reset, and the next trial commenced after 10-15s. Each rat completed all 8 trials prior to running the next animal.

1. Standard T-maze *(all spatial cue types available) (Figure 3.2A)* – This condition was the same as that used in pre-training. The two phases of each trial started at the same position.

2. *Start T-maze* *(flexible learning, all cue types available) (Figure 3.2B)* – The start position was changed after each trial between the four arms of the maze. Importantly, the start arm remained consistent for both the sample and test runs. In all other respects, training followed the 'Standard' procedure. Both the selection of the start-arm and the correct test-arm were pseudorandomized, so that no start-arm or test-arm was repeated more than two consecutive times.

3. *Rotation T-maze* *(disrupted intra-maze cues*) *(Figure 3.2C)* – The maze was rotated between the sample and the test run, by either 90°or 180° degrees with every trial. The arm on the test run, the degree of rotation and the direction of the rotation were all pseudorandomized so that the same manipulation was not repeated more than two consecutive times. The location of the start position was consistent for all trials, so that extra-maze and egocentric cues remained viable, while intra-maze cues were nullified.

4. *Opposite arm T-maze* *(disrupted egocentric cues) (Figure 3.2D)* – The start position of the animal was rotated 180° between the sample and the test runs. Therefore, each sample run began at the same start arm and each test run began in the arm directly opposite. Critically, the correct arm on the test run remained in the

opposite room location to the position of the baited arm in the sample run (Figure 3.2D). No test arm was used as the correct arm on more than two consecutive trials. For all trials, the start position remained in the South (Figure 4.2D).

5. *Dark T-maze* *(visual cues removed) (Figure 3.2E)* – The Standard T-maze protocol was repeated but now in the dark. The maze was baited, and barriers put in place before each trial in dim illumination provided by a 10W red light facing away from the maze. Then, the light was turned off (~0.2lx) and the rat placed in the start position. Once the trial was completed, (i.e., both sample and test phase), the rat was picked up and held while the maze was reset.

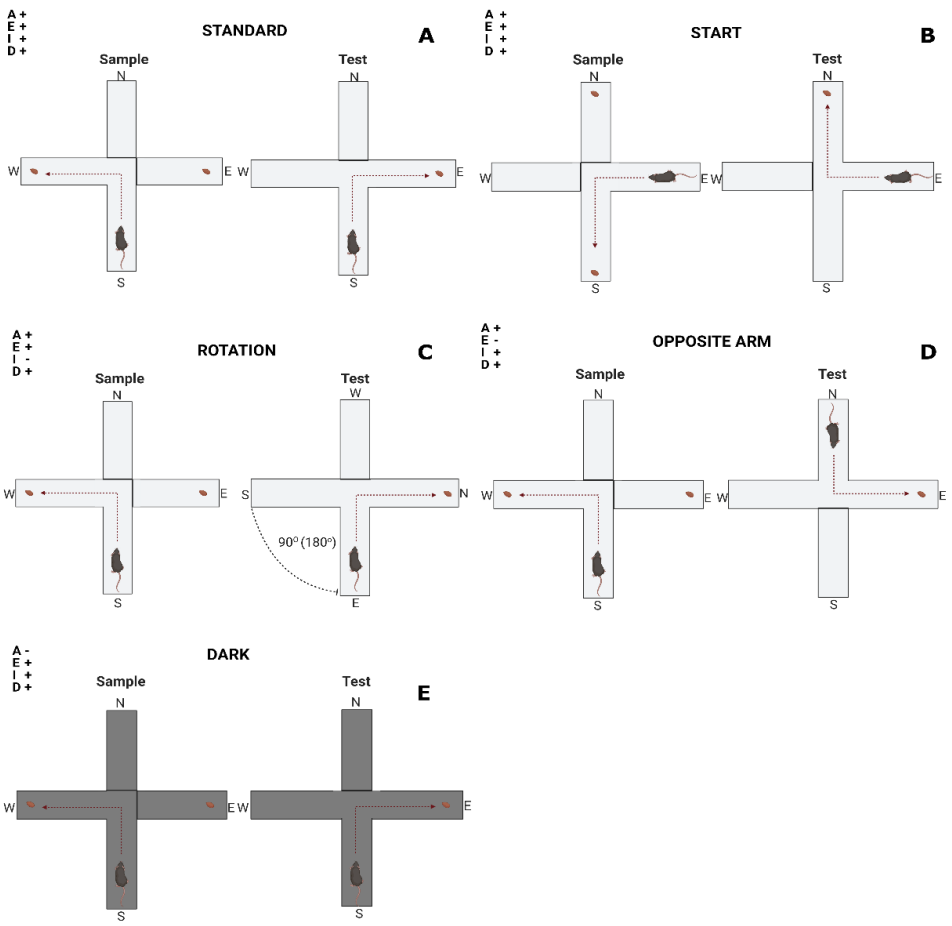


Figure 3.2. Illustration of the T-maze experimental manipulations.

The figure shows examples of two trials on each experimental manipulation as follows: A) Standard T-maze; B) Start T-maze (with randomized start positions); C)

Rotation T-maze (with either 90º or 180º maze rotation in either direction); D) Opposite arm T-maze; and E) Dark T-maze. Abbreviations: A, allocentric cues; E, egocentric cues; I, intra-maze cues; D, directional cues; +, cue is available to solve the maze; -, the cue does not solve the maze.

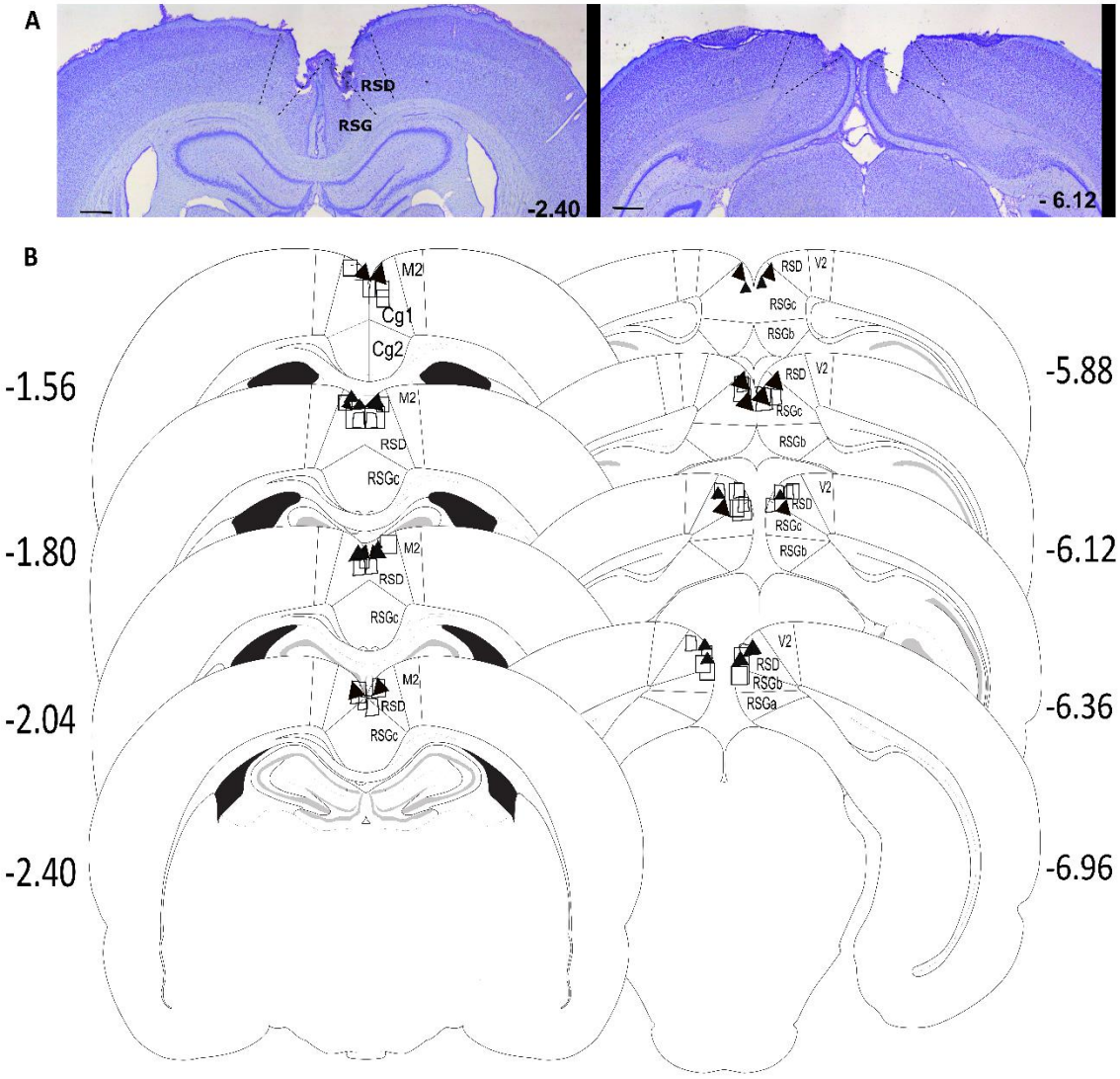


Figure 3.3. Schematic representation of retrosplenial cannula placement for each experimental animal.

Panel A shows coronal sections (cresyl violet) with cannulation sites in the anterior (left) and posterior (right) retrosplenial cortex. Panel B (below) is a schematic representation of cannula placements adapted from the Paxinos and Watson rat atlas (2004) for each animal in both the anterior (left) and posterior (right) portions of

the retrosplenial cortex. Squares denote iDREADDs animals and triangles GFP-controls. In three iDREADDs and 1 GFP-control animal, the cannulas also affected the most posterior portions of the cingulate cortex. The same implantation coordinates were used for all animals, producing considerable overlap of cannula placements. The numbers represent the approximate distance from bregma in mm. All scale bars are 150µm. Abbreviations: Cg1/2, anterior cingulate cortex; M2, secondary motor cortex, RSD, dysgranular retrosplenial cortex; RSGc, granular retrosplenial cortex, area c; RSGb, granular retrosplenial cortex, area b; RSGa, granular retrosplenial cortex, area a; V2, secondary visual area.

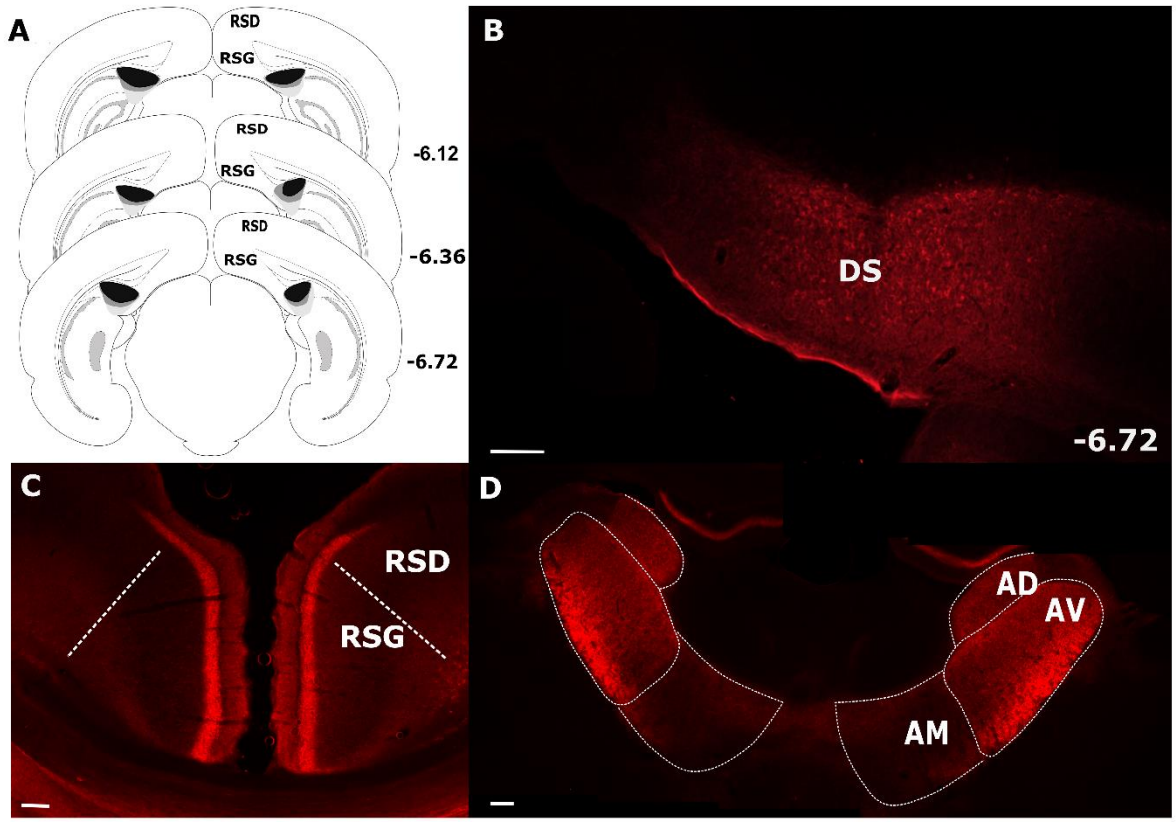


Figure 3.4. Virus expression in the iDREADDs group.

Panel A shows the smallest (black) and largest (light grey) injection sites across the dorsal subiculum. Numbers refer to the distance from bregma in mm. Panel B shows an example of iDREADDs expression in the dorsal subiculum. Panel C shows the robust expression of transported iDREADDs in layers I, II, and upper III of the

granular retrosplenial cortex. Panel D shows anterograde transport from the dorsal subiculum to the anterior thalamic nuclei. All scale bars are 150µm. AD, anterodorsal nucleus; AM, anteromedial nucleus; AV, anteroventral nucleus, DS, dorsal subiculum, RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex.

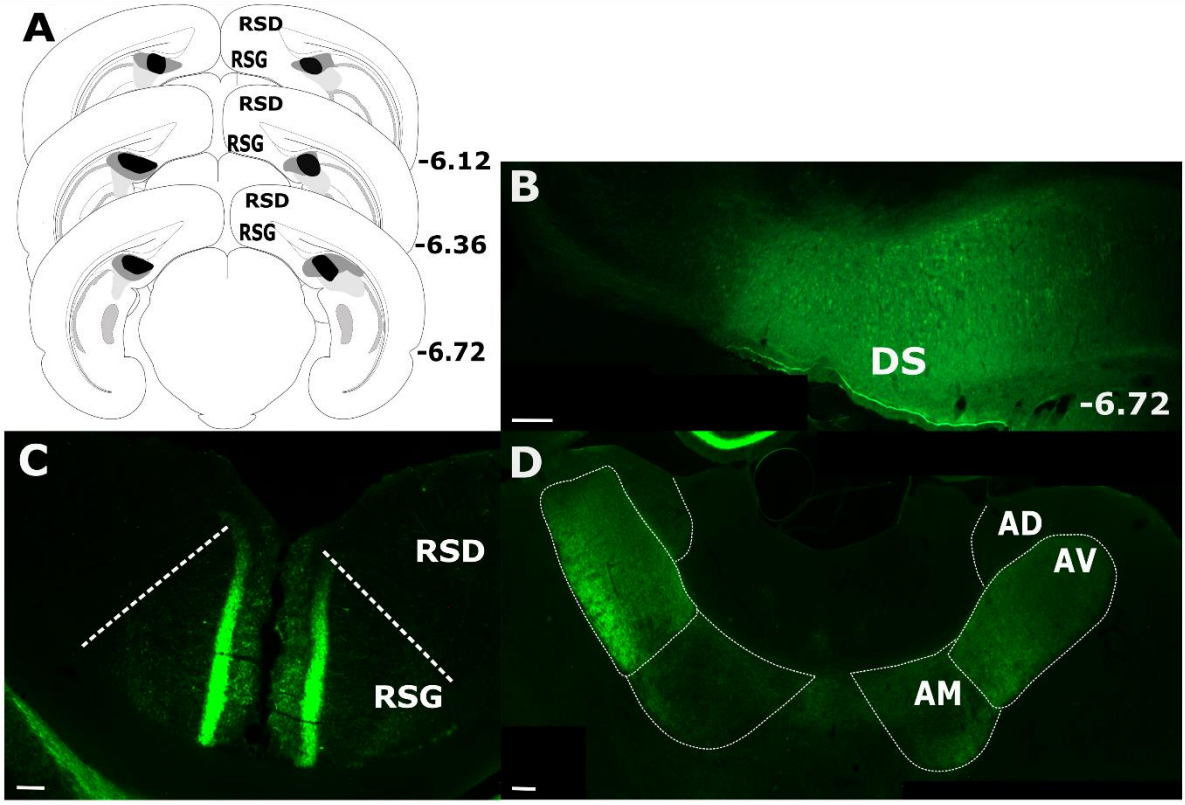


Figure 3.5. Virus expression in the GFP-control group.

Panel A shows the smallest (black) and largest (light grey) injection sites across the dorsal subiculum. Numbers refer to the distance from bregma in mm. Panel B shows an example of iDREADDs expression in the dorsal subiculum. Panel C shows the robust expression of transported virus in layers I, II and upper III of the granular retrosplenial cortex. Panel D shows anterograde transport from the dorsal subiculum to the anterior thalamic nuclei. All scale bars are 150µm. AD, anterodorsal nucleus; AM, anteromedial nucleus; AV, anteroventral nucleus, DS, dorsal subiculum, RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex.

3.2.8. iDREADDs activation

Each behavioural condition was run both after an infusion of clozapine and after an infusion of saline, which served as a within-subject control. Clozapine was infused on two separate occasions per condition, reflecting the potential for greater within-subject variability. The infusion order was counterbalanced between the cohorts (Figure 3.1). There was always an added infusion-free test day between the two clozapine infusions for Conditions 1-4, i.e., apart from the Dark condition.

Animals were first habituated to the infusion procedure, using saline, prior to the commencement of behavioural testing with clozapine. On infusion days, the animals were taken to a separate room in pairs and lightly anaesthetized with an isoflurane-oxygen mixture (5% induction, 1.5-2% maintenance). Double infusion injectors (33-gauge, PlasticsOne, Virginia, USA) were inserted into the guide cannula and either 1µl of sterile saline or 1µl of clozapine (1mg/ml) was infused over 1 minute using an infusion pump (11 Plus, Harvard Apparatus, UK). The injector was held in place for a further minute and the dummy cannula replaced. The infusions lasted no more than 4 minutes per animal, and the animal was returned to its home cage. Animals rested for 15-20 minutes prior to behavioural testing.

3.2.9. Perfusions

Following completion of the experiment, animals were transcranially perfused as described in *Chapter 2, section 2.2.4.* A freezing microtome (8000 Sledge Microtome, Bright Instruments) was used to cut the brain in 40µm coronal sections, saved as four simultaneous series. The sections were stored as described in *Chapter 2, Section 2.2.4.*

3.2.10. Histology

One series was mounted onto gelatin-coated slides before being stained for Nissl using cresyl violet following the procedure described in *Chapter 2, section 2.2.5.*

To enhance the fluorescence signal of the mCherry (iDREADDs group) or GFP (control group), additional series were washed three times in PBS and then blocked with 5% normal goat serum (NGS)(Invitrogen, Inchinnan, UK) in Phosphate Buffered Saline with Tritonx-1000 (PBST) for two hours. Both series were then transferred in either a solution of rabbit polyclonal anti-mCherry (Abcam, Cambridge, UK) or chicken polyclonal anti-GFP (Abcam, Cambridge, UK) antibody at a dilution of 1:1000 in PBST with 1% NGS and incubated for 24 hours at room temperature. The sections were then washed three times and transferred to a secondary antibody of either goat-anti-rabbit (Dylight Alexa flour 594, Vector Laboratories, Peterborough, UK) or Alexa Fluor 488 goat-anti-chicken (Invitrogen, Inchinnan, UK) at a dilution of 1:200 in PBST for two hours. The sections were then washed in PBS and mounted onto gelatin-coated slides and cover-slipped using Fluromount (Sigma-Aldrich, Germany).

3.2.11. Image Acquisition and Viral Expression Analysis

For each animal, cannula placement and viral expression were analysed using a bright field and fluorescent microscope Leica DM5000B, equipped with a DFC310 FX camera. The viral expression was assessed at the injection site, as well as at dorsal subiculum efferent targets. These targets included layers 2 and upper 3 of the retrosplenial cortex, along with the anteroventral and anteromedial thalamic nuclei (Figures 3.3, 3.4, and 3.5).

3.2.12. Quantitative iDREADDs expression analysis

Given clozapine's higher affinity to DREADDs, discussed in *Chapter 1, section 1.8.2,* the level of expression of the iDREADDs affects the inhibition that can be achieved, and thus has implications for the observed behavioural responses of the animals. To quantify the expression of the virus, analysis measuring the fluorescence level within the retrosplenial cortex was conducted on two groups of iDREADDs animals. The first group consisted of six iDREADDs test-animals (not included in the behavioural

experiments in this chapter) who were culled two weeks post-surgery. The second group were the six iDREADDs animals from cohort two. The test animals underwent exactly the same surgical procedure as described in *section 3.2.4.* For both groups, the analyses were performed on the raw (unenhanced) iDREADDs signal. One-in-four series were mounted onto a gelatine-coated slides and were rehydrated in decreasing concentrations of alcohol as described in the Nissl stain procedure in *Chapter 2, section 2.2.5.* Images, typically from five sections spread across the anterior-posterior axis of the retrosplenial cortex were acquired for each case as described in *section 3.2.11.* All included cases had bilateral viral expression within the retrosplenial cortex, and both hemispheres were used to calculate the average iDREADDs signal intensity within the section.

To perform the analyses, ImageJ (version 1.54d) was used. The freehand tool was used to select the region of interest (ROI), which was the dense label in layers II and III, since this was the region used to judge if satisfactory viral expression was achieved within the retrosplenial cortex. The background area was selected within the cortex, immediately next to but outside the ROI. The total cell fluorescence (TCF) was calculated using the following formula: TCF = Integrated density – (Area of selected ROI x Mean fluorescence of background reading). The calculation was performed for each section. Then, the mean total cell fluorescence for each case was calculated by taking the average from the five sections and these were compared between the test animals and cohort two animals using an independent t-test. The analysis did not reveal statistically significant differences in the intensity of viral expression between the test animals (M = 0.623; SD = 0.493) and the iDREADDs animals in cohort two (M = 0.899; SD = 0.613): $t(10) = -0.860$, $p = 0.410$, suggesting that the expression of the iDREADDs virus was sufficient at the start of the experimental infusions. This is important since if the iDREADDs expression increases or decreases over time, it may affect the behavioural responses of animals on different manipulations.

3.2.13. Statistical analyses

The principal behavioural measure was the mean percentage of correct choices made across the blocks of 8 trials, for each experimental condition. The behavioural data were analysed using multiple mixed-model analyses of variance (ANOVAs), with the within-subject factor of drug (saline vs clozapine) and between-subject factor of group (iDREADDs vs GFP-control. Partial eta-squared (η_p^2) is reported as a measure of effect size.

All data were screened for outliers, the assumptions of normality, homogeneity of variances and covariances using SPSS Statistics 27(SPSS Inc., Chicago, Ill., USA). A single outlier score (37.5%) was found for just one animal in one condition (GFP group, Standard condition, saline), and so this case remained in the analyses as removing the case did not make a difference to the statistical output. Levene's test based on medians assessed the homogeneity of variance, showed that the assumption was violated on the opposite-arm saline condition (p = 0.044) (Brown & Forsythe, 1974). No violations to the assumption of homogeneity of covariance were found (all p_s > 0.024) (see Tabachnick & Fidell, 2013). Where there was a statistically significant interaction term, simple main effect analyses were conducted using pooled error terms in JASP 14.1 (JASP Team, 2022).

Multiple independent t-tests helped to compare control and baseline scores, i.e., the pre-surgery training scores, post-surgery training scores for each alternation condition prior to any infusions, as well as for the infusion-free day scores between the clozapine infusions. These analyses were to establish if the performance of the iDREADDs and GFP-controls was statistically comparable, prior to and between iDREADDs activation. Note, as Cohort 1 did not receive behavioural training on the infusion-free day on the final (Dark) condition, this comparison only applies to the first four alternation conditions.

3.3. Results

3.3.1. Histological findings

Two criteria were required for inclusion in the experimental analyses. First, the dorsal subiculum virus injections had to result in appreciable bilateral label within granular retrosplenial cortex (Figures 3.4, and 3.5). Second, the infusion placements had to involve retrosplenial cortex (Figure 3.3). Across both cohorts, a total of 6 iDREADDs and 8 GFP-control animals were excluded due to lack of viral expression (unilateral or bilateral) in retrosplenial cortex (n=6), off-target cannula placement (n=2) or both (n=6). Consequently, the behavioural analyses derive from 14 iDREADDs and 8 GFP-control animals. In four of these animals (n=3 iDREADDs; n=1 GFP) spread from the anterior infusion cannulas may have reached the midcingulate cortex (Vogt & Paxinos, 2014) as well as retrosplenial cortex. In some cases, the virus injection spread into the dentate gyrus, which does not directly innervate the retrosplenial cortex.

3.3.2. Pre-surgery training, post-surgery baseline analyses, and non-infusion sessions

A series of independent *t*-tests considered whether there might be pre-surgery (Standard condition only) or post-operative training performance differences between the iDREADDs and GFP-control rats on the five T-maze task conditions prior to any infusions.

Firstly, the iDREADDs and the GFP-control animals between the two cohort were compared on their performance. The comparisons did not reveal a statistically significant difference on pre-surgery training or any baseline between the GFP animals in cohort one and two: all $t_s < 2.19$, $p_s > 0.071$. In contrast, the iDREADDs animals showed statistically significant differences between the two cohorts on the pre-surgery training, along with the baseline scores for the standard, and the start T-maze: all $t_s > 2.53$, $p_s < 0.026$, but not on the rotation, opposite-arm, or dark T-maze:

all $t_s < -1.863$, $p_s > 0.087$ (Table 2, 3). While these differences may reflect minor, unintended effects it is important to appreciate that the key statistical comparisons involved within-subject data, so mitigating against any cohort effects. Furthermore, when the two cohorts are pooled together to achieve greater power, the iDREADDs group and the GFP group did not differ significantly on the pre-surgery training nor on the baseline training prior to commencement of infusions: all $t_s < 1.86$, $p_s > 0.078$. Additionally, the analyses performed use the pooled error term of the groups, which should account for individual group contributions and increase statistical power.

Further *t*-tests were carried out on the scores from the non-infusion sessions that were interleaved between the saline and clozapine sessions, for all but the Dark condition (Figure 3.1), as those data were absent for Cohort 1. There were no performance differences between the iDREADDs and GFP-control animals on the infusion-free days for each of the four conditions: all $t_s < 1.01$, $p_s > 0.29$ (Figure 3.6).

Table 3.1. Means, Standard Deviations and Standard Errors for GFP-control animals by cohort and T-maze conditions on training (non-drug) scores.

	Cohort	N	M	SD	SE
Pre-surgery training	1	2	84.15	1.20	0.85
	2	6	78.82	10.39	4.24
Baseline Standard T-maze	1	2	89.07	4.43	3.14
	2	6	86.76	6.65	2.71
Baseline Start T-maze	1	2	93.75	8.84	6.25
	2	6	81.95	6.05	2.47
Baseline Rotation T-maze	1	2	75.00	5.94	4.20
	2	6	83.87	8.71	3.55
Baseline Opposite arm T-	1	2	87.50	17.68	12.50
maze	2	6	80.92	7.97	3.25
Baseline Dark T-maze	1	2	75.05	8.84	6.25
	2	6	76.68	8.73	3.56

Table 3.2. Means, Standard Deviations and Standard Errors for iDREADDs animals by cohort and T-maze conditions on training (non-drug) scores.

	Cohort	N	M	SD	SE
Pre-surgery training	1	8	89.38	3.53	1.25
	2	6	81.60	5.36	2.19
Baseline Standard T-maze	1	8	88.47	3.44	1.22
	2	6	78.45	8.68	3.55
Baseline Start T-maze	1	8	92.19	6.47	2.29
	2	6	83.36	6.45	2.63
Baseline Rotation T-maze	1	8	86.97	3.49	1.23
	2	6	82.31	7.30	2.98
Baseline Opposite arm T-maze	1	8	81.25	9.45	3.34
	2	6	83.38	5.08	2.08
Baseline Dark T-maze	1	8	78.14	10.03	3.55
	2	6	87.17	7.25	2.96

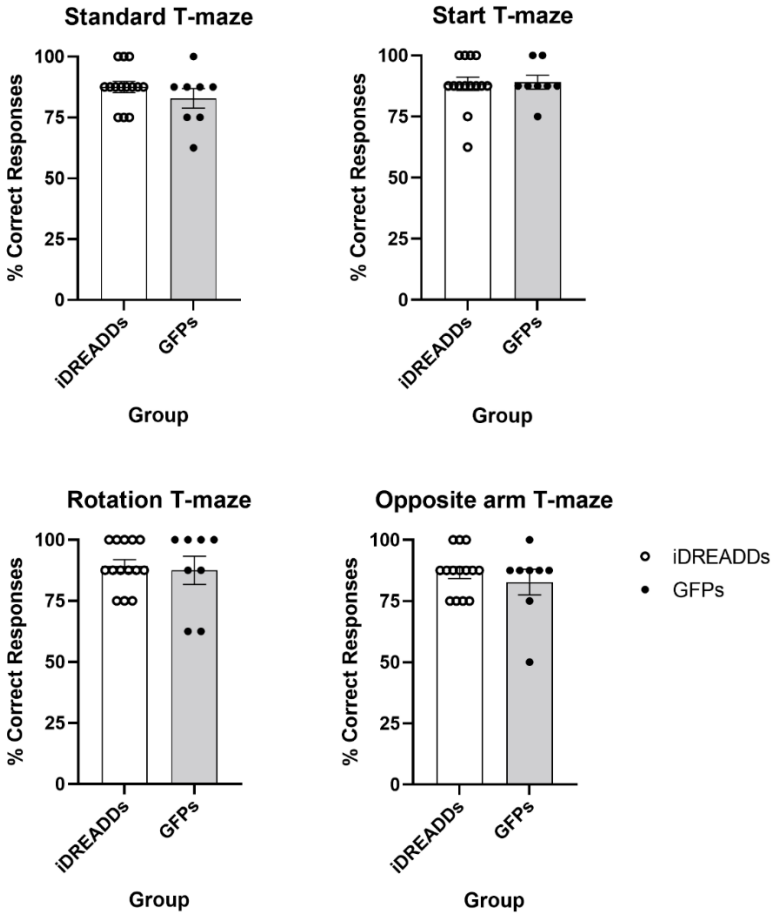


Figure 3.6. Post-surgery performance comparisons across the two groups on the infusion-free days.

Bar graphs depicting the mean and each animal's individual percentage of correct alternation responses for both the iDREADDs and GFP-control groups on the interleaved infusion-free days for the first four conditions. No statistically significant differences were observed between the two groups on the infusion-free day, between the two administrations, suggesting that carry over effects are unlikely between the clozapine administrations. Error bars indicate SEM; iDREADDs animals are presented in white and the GFPs in grey.

3.3.3. Performance on test conditions

In a preliminary analysis 'Cohort' was included as a second between-subject factor to establish if there were any differences regarding the two cohorts. The only main

effect of cohort was for the Dark condition ($p<0.05$), and no other main effects or interactions involving this factor were observed (all $F_s < 4.00$; all $p_s > 0.06$). Therefore, the cohort data were pooled and analysed together for each separate condition, though the Dark condition data were given extra scrutiny. Throughout 'Drug' refers to saline or clozapine (within subject) while Group refers to iDREADDs or GFP infusions (between-subject).

Standard T-maze: There was a significant main effect of Drug: $F_{1,20} = 7.01$, $p = 0.015$, $\eta_p^2 = 0.25$, but not a main effect of Group or Drug × Group interaction: $F_s < 0.85$, $p_s > 0.37$, $\eta_{ps}^2 < 0.04$ (Figures 3.7, 3.8). This set of results showed that clozapine did not have a greater effect in the active viral group when compared with the control viral group.

Start T-maze: There was a main effect of Drug: $F_{1,20} = 5.76$, $p = 0.03$, $\eta_p^2 = 0.22$, but no main effect of Group or Drug × Group interaction: $F_s < 3.48$, $p_s > 0.08$, $\eta_{ps}^2 < 0.15$ (Figures 3.7, 3.8). This pattern of results matched that for the Standard condition.

Rotation T-maze: There was a significant main effect of Drug: $F_{1,20} = 12.02$, $p = 0.002$, $\eta_p^2 = 0.37$ but also a Drug × Group interaction: $F_{1,20} = 6.8$, $p = 0.016$, $\eta_p^2 = 0.25$. Simple main effects analyses revealed a significant decline in performance following clozapine infusions within the iDREADDs group that was not seen in the GFP control group: $F_{1,13} = 25.36$, $p<0.0001$ (Figures 3.7, 3.8). All other tests were non-significant: $F_s < 2.75$, $p_s > 0.11$.

Opposite arm T-maze: As in the Rotation condition, there was a significant main effect of Drug: $F_{1,20} = 7.7$, $p = 0.01$, $\eta_p^2 = 0.278$ and a Drug × Group interaction: $F_{1,20} = 4.55$, $p = 0.045$, $\eta_p^2 = 0.18$. Again, there was decline in performance following clozapine infusions in the iDREADDs group that was not seen in the GFP control group: $F_{1,13} = 16.6$, $p <0.001$ (Figures 3.7, 3.8). All other tests were non-significant: $F_s < 0.89$, $p_s > 0.35$.

Dark Standard T-maze: The behavioural analyses revealed a significant Drug × Group interaction: $F_{1,20} = 7.8$, $p = 0.011$, $\eta_p^2 = 0.28$, however, there was no main effect of Drug or Group: $F_s < 0.036$, $p_s > 0.85$. Follow-up simple main effects

analyses showed that again iDREADDs animals' performance declined following the clozapine infusions relative to saline: $F_{1,13} = 5.8$, $p = 0.025$. All other tests were non-significant: $F_s < 2.84$, $p_s > 0.10$ (Figures 3.7, 3.8). For this one condition only, there was a significant main effect of Cohort ($F_{1,18} = 10.6$, p = 0.004), possibly reflecting the presence of the additional non-infusion training trials in one cohort.

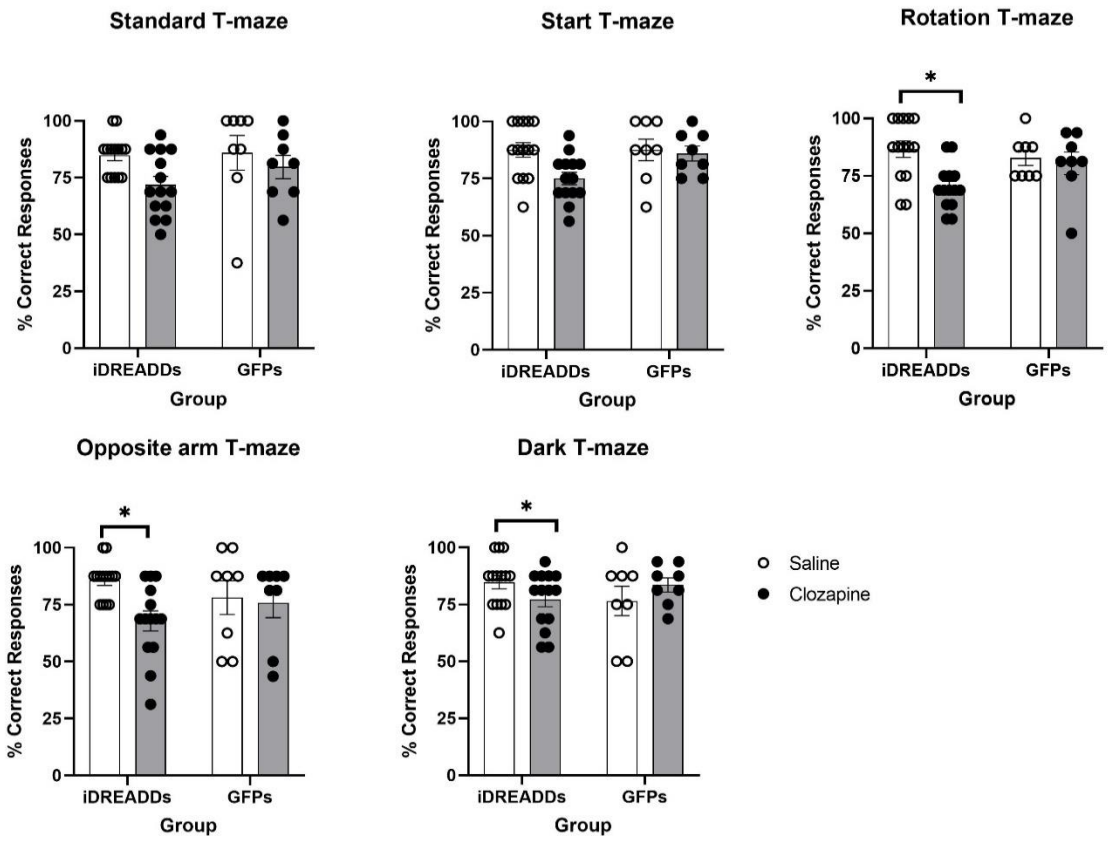


Figure 3.7. Bar graphs depicting the mean and each animal's individual percentage of correct alternation responses for both the iDREADDs and GFP-control groups.

From right to left: 1) Standard T-maze; 2) Start T-maze; 3) Rotation T-maze; 4) Opposite arm T-maze; 5) Dark T-maze. Despite the within-group differences restricted to the iDREADDs group, there were no between-group differences for the iDREADDs group and the GFP controls. Error bars indicate SEM. * Denotes within-group statistically significant differences; the saline condition is presented in white and the clozapine condition in grey.

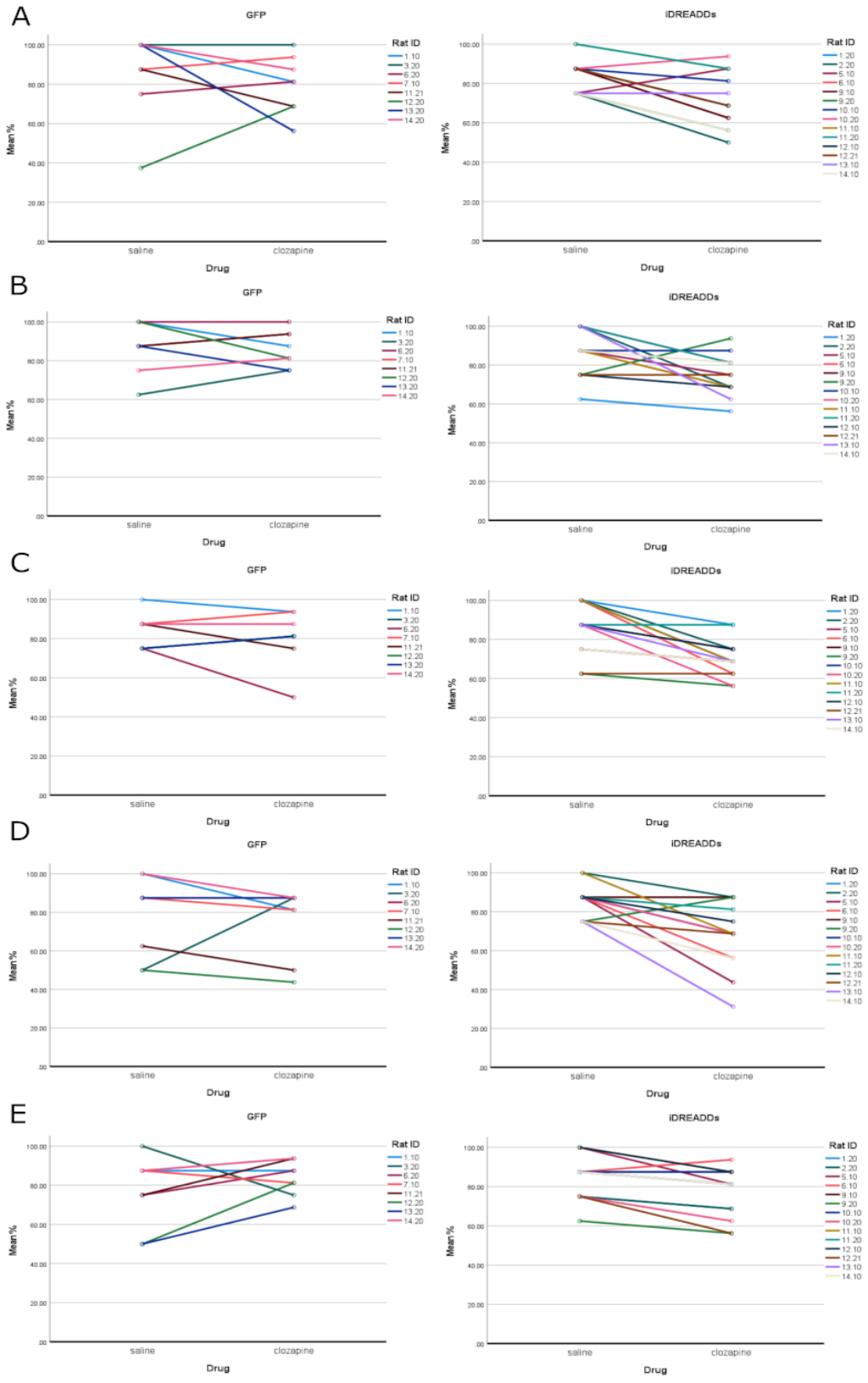


Figure 3.8. Line graph showing individual animals' performance on each T-maze variation by group.

Each line represents different animals' performance following each infusion (saline vs clozapine). The conditions are presented as follows A) Standard T-maze, B) Start T-maze; C) Rotation T-maze, D) Opposite arm T-maze, and E) Dark T-maze. GFP-control animals are presented on the left and the iDREADDs animals are presented on the right. *Note: Some animals' performance overlaps.*

3.4. Discussion

Although the potential significance of the direct hippocampal projections to retrosplenial cortex has long been appreciated (Sutherland & Hoesing, 1993; Vann et al., 2009), their importance for spatial memory has only been tested with classical context conditioning (Yamawaki et al., 2019a,b).The present study investigated the behavioural consequences of disrupting the direct projections from the dorsal subiculum to granular retrosplenial cortex, using five variations of a spatial working memory task, T-maze alternation. By combining iDREADDs injections into the dorsal subiculum with clozapine infusions into retrosplenial cortex, the present study sought to disrupt the direct projections from the dorsal subiculum to granular retrosplenial cortex. This manipulation impaired T-maze alternation on three of the five conditions. No effect of clozapine was seen in the GFP control group.

Despite its apparent simplicity, T-maze alternation remains a complex task (Dudchenko, 2001). In the standard condition animals have access to intra-maze cues, extra-maze (allocentric) cues, along with cues involving proprioception such as egocentric or directional information (Douglas, 1966; Dudchenko, 2001). The latter refers to using a sense of direction to alternate (e.g., east then west), which differs from egocentric strategies (Dudchenko & Davidson, 2002). The various T-maze conditions indicated that disruption of the dorsal subiculum projections to granular retrosplenial cortex impaired performance as soon as specific cue-types were put into conflict or selectively removed.

There was no apparent effect of retrosplenial disruption on the Standard or Start T-maze conditions, i.e., when all spatial strategies remained viable (the Start condition only requires that the animal is not distracted by the changes in start position across

different trials). However, iDREADDs activation impaired spatial working memory on the Rotation, Opposite arm, and Dark alternation conditions. This pattern of deficits does not simply reflect task difficulty, as performance during the intervening infusion-free days (Figure 3.6), during the iDREADDs/saline condition, and by the GFP control group (Figures 3.7, and 3.8) all remained extremely similar across all five conditions. The clear implication is that the iDREADDS infusions could disrupt more than one type of task strategy given the varying demands of the final three conditions (Figure 3.2). At the same time, a blanket disruption would have also impaired the Standard and Start condition. This combination of factors strongly points to the significance of changing and suddenly restricting cue types.

The temporal pattern of results (last three conditions impaired) showed that the chemogenetic effects did not disappear over time and training. This same temporal pattern does, however, raise the potential criticism that post-operative testing may have resumed too soon, so that the virus was not fully transported. This possibility becomes less likely given the results of the viral expression analyses. Comparisons were made between test animals culled two weeks post-surgery and the experimental animals that were finally culled at approximately 12 weeks post-surgery. Although the experimental animals' expression was slightly higher, the same measures were not statistically different. The implication is that the levels of transported virus remained fairly stable across the testing period and all behavioural conditions.

It is true that a counterbalanced sequencing of the five behavioural conditions might have dispelled this possible limitation. In practice, such a design was thought not feasible as each behavioural condition required different amounts of pre-training to establish appropriate performance levels prior to each set of drug infusions. Consequently, a counterbalanced design would increase individual variability, as well as introduce variable transfer effects from condition to condition.

While the present study lacks direct evidence as to how the clozapine infusions disrupted retrosplenial activity, other studies using comparable methodologies have demonstrated their effectiveness (Bubb et al., 2021; Yamawaki, Li, et al., 2019). That the iDREADDS/clozapine combination disrupted neural processing can also be

directly inferred from the contrasts with the GFP/clozapine combination, which did not alter performance levels. Furthermore, the behavioural deficits in the iDREADDs rats had obvious similarities with the effects of conventional lesions in the two target sites (Pothuizen et al., 2010; Potvin et al., 2007, 2010).

A further potential concern is whether the clozapine infusions extended to sites adjacent to retrosplenial cortex. While possible, any such site would also need to receive direct dorsal subiculum inputs to have any impact, so limiting this concern. It is also the case that related cannula studies have concluded that infusions are well retained by retrosplenial cortex (Nelson et al., 2015; Yamawak et al., 2019b).

As already noted, the present results show clear parallels with prior behavioural studies testing either dorsal subiculum or retrosplenial cortex function. Permanent lesions of the dorsal subiculum were found to spare standard T-maze alternation in the light (Potvin et al., 2007). Again, radial-arm maze working memory in the light did not appear affected after dorsal subiculum lesions, although impairments emerged when tested in the dark (Potvin et al., 2007) and when adjacent arms had to be distinguished (Potvin et al., 2009). Other dorsal subiculum lesion deficits include failing to select an object now placed in a novel position (Potvin et al., 2010), indicative of a deficit in location learning.

The present behavioural findings closely resemble those from retrosplenial cortex lesions. Permanent lesions involving both granular and dysgranular retrosplenial cortex can have little or even no apparent effect on standard spatial alternation (Aggleton et al., 1995; Neave et al., 1994), i.e., as in the present study. More reliable spatial working memory deficits are found when, as in the present study, test conditions are changed, such as when intra-maze and extra-maze cues are made incongruent or when strategy switching is required (Nelson et al., 2015; Pothuizen et al., 2008; Vann & Aggleton, 2004; Vann et al., 2003).

Of especial relevance are those few studies that have made permanent lesions targeting just the granular retrosplenial cortex. This is because the virus transported from the dorsal subiculum was heavily concentrated in this division of retrosplenial cortex. Permanent lesions of the granular retrosplenial cortex appear to leave

standard T-maze alternation intact but impair performance when intra-maze cues are removed by switching to adjacent, parallel mazes (Pothuizen et al., 2010). This profile closely resembles the current findings, even though the present iDREADDs manipulation was even more selective, targeting just one set of granular retrosplenial inputs (Figures 3.4, 3.5). Together, these findings help underline the significance of the hippocampal (subiculum) efferents to granular retrosplenial cortex when spatial cue usage is restricted.

Findings from a very different type of behavioural task, contextual fear conditioning, also implicate both the hippocampus (including the dorsal subiculum) and retrosplenial cortex in learning about space (Anagnostaras et al., 2001; Keene & Bucci, 2008; Melo et al., 2020; Miller et al., 2014; Pan et al., 2022; Smith et al., 2012). Meanwhile, immediate-early gene analyses indicate that the two regions have complementary roles in spatial tasks (Czajkowski et al., 2020; Frankland & Bontempi, 2005). In addition, neuronal recordings suggest that the hippocampus may encode and help distinguish contexts, while the retrosplenial cortex may enable behaviourally significant cues to identify the current context (Smith et al., 2012) or help predict future navigational decisions (Miller et al., 2019).

An especially relevant study used chemogenetic methods similar to those in the present study to target hippocampal-retrosplenial projections during contextual fear conditioning. That study showed how the glutamatergic (vGlut1+ and vGlut2+) subiculum projections can differentially regulate the cellular functions of granular retrosplenial cortex (Yamawaki et al., 2019b). That same study also indicated that a major role of the vGlut1+ projections was in processing recent context memories, whilst the vGlut2+ projections assisted with the long-term retrosplenial storage of fear-inducing context memory (Czajkowski et al., 2014; De Sousa et al., 2019; Milczarek et al., 2018; Yamawaki et al., 2019b).

In a related study, the sparse inhibitory CA1 projections to retrosplenial cortex were silenced, again in a contextual fear conditioning paradigm, and their actions contrasted with those of the anterior thalamic inputs to retrosplenial cortex (Yamawaki et al., 2019a). While both pathways are involved in the acquisition of contextual fear memory, they act in opposing ways. The inhibitory CA1 projections

normally supressed, while the excitatory anterior thalamic projections normally enhanced the acquisition of context memories (Yamawaki et al., 2019a).

Further details of retrosplenial-anterior thalamic-hippocampal influences come from an optogenetic study showing how anterior thalamic and dorsal hippocampal projections recruit the same populations of pyramidal cells (layer III) within granular retrosplenial cortex (Brennan et al., 2021). These pyramidal cells are distinct from the cell populations influenced by the claustrum and anterior cingulate cortex (Brennan et al., 2021). Additionally, the timing of late neural spikes in layers II and III by the granular retrosplenial pyramidal neurons appears to be influenced by preceding activation of the subiculum (Gao et al., 2021). Together, these findings emphasise the reliance of the three regions on each other, suggesting that together the subiculum and anterior thalamic nuclei facilitate information processing in the retrosplenial cortex, which is gated by its inputs from CA1 (Aggleton & O'Mara, 2022; Yamawaki et al., 2019a). In addition, a recent study found that some granular retrosplenial neurons in layer V project directly to CA1 of the dorsal hippocampus in mice (Tsai et al., 2022). These projections may help retrieve remotely acquired contextual fear memory, demonstrating a bidirectional interdependence between regions (Tsai et al., 2022).

Finally, clear parallels emerged between the present results and those of a previous experiment that also placed iDREADDs in the dorsal subiculum to examine spatial working memory (Nelson et al., 2020). Systemic activation of the iDREADDs did not influence Standard T-maze alternation, but impaired the same Rotation condition (Nelson et al., 2020), consistent with the present study. This same pattern of deficits (Standard - intact; Rotation - impaired) was then seen when just the subiculum projections to the anterior thalamic nuclei were disrupted (Nelson et al., 2020). These parallel effects with the present study again highlight the close anatomical (Bubb et al., 2017; Horikawa et al., 1988; Sripanidkulchai & Wyss, 1986) and functional (Aggleton & O'Mara, 2022; Kinnavane et al., 2019; Pothuizen et al., 2009; Sutherland & Hoesing, 1993) relationships between the hippocampal formation, anterior thalamic nuclei, and retrosplenial cortex.

Their common actions may reflect the way that many dorsal subiculum neurons collaterise to reach both granular retrosplenial cortex and the mammillary bodies (Kinnavane et al., 2018), the latter site relaying monosynaptically to the anterior thalamic nuclei (Umaba et al., 2021). Furthermore, the finding that the widespread disruption of multiple subiculum efferents has very similar effects to targeting just those reaching the anterior thalamic nuclei (Nelson et al., 2020) or reaching the retrosplenial cortex (present study) underlines the functional primacy of these particular interactions. Together, these results accord with the influential idea that retrosplenial cortex facilitates the ability to switch between spatial strategies (Byrne et al., 2007; Vann et al., 2009) and that this function is facilitated by direct inputs from the dorsal subiculum.

Chapter 4

4. Disrupting direct inputs from the anterior thalamic nuclei to the retrosplenial cortex

4.1. Introduction

The anterior thalamic nuclei form a central node in the hippocampal-cingulate-diencephalic network, having a key function in memory and associated clinical disorders (Aggleton & Shaw, 1996; Aggleton, 2008; Aggleton et al., 2016; Barnett et al., 2021; Carlesimo et al., 2011; Dalrymple-Alford et al., 2015; Harding et al., 2000; Kim et al., 2009; Kopelman, 2015; Liu et al., 2021; Maillard et al., 2021; Sweeney-Reed et al., 2021). These nuclei receive projections from various cortical and subcortical structures including, but not limited to, the mammillary bodies, the subiculum, anterior cingulate cortex, and the retrosplenial cortex (Wright et al., 2010) (*see Chapter 1, section 1.7.3*). Here, of particular interest are the reciprocal connections of the anterior thalamic nuclei with retrosplenial cortex, which likely allow the anterior thalamus to influence hippocampal function and *vice versa* (Vann et al., 2009).

Rodent behavioural studies consistently show that anterior thalamic lesions cause severe spatial deficits that are often of greater severity than those for any other site other than the hippocampal formation (Aggleton et al., 1995,1996, 2009, 2016; Alexinsky, 2001; Mitchell & Dalrymple-Alford, 2005; Warburton et al., 2001), highlighting how the region is essential for spatial memory (see *Chapter 1, section 1.7.4*). Although the evidence is incomplete, it appears that the three main anterior thalamic nuclei may have complementary roles in supporting spatial processes.

The anterodorsal nucleus is a particularly important part of the "head-direction system", receiving inputs from the lateral mammillary nucleus (Taube, 1995; 2007) *(see Chapter 1, section 1.7.3*). Furthermore, as established in *Chapter 2*, there are higher proportions of bifurcating neurons terminating in the retrosplenial cortex, which originate in the anterodorsal nucleus when compared to the anteroventral and anteromedial nuclei. Given the contributions of the retrosplenial cortex and the anterodorsal nucleus for spatial processing, e.g., through their head-direction cells, it is natural to presume a shared role in spatial navigation and memory (Cooper & Mizumori, 2001; Hindley, 2014; Mitchell et al., 2018; Nelson et al., 2015; Harker & Whishaw, 2004; Vann et al., 2009; Wolbers & Büchel, 2005).

Similarly, the anteroventral nucleus receives inputs from the granular retrosplenial cortex, alongside inputs from the subiculum and medial mammillary bodies (Shibata, 1993; Van Groen & Wyss, 2002; Wyss et al., 1979)(*see Chapter 1, section 1.7.3.*). The anteroventral nucleus provides a direct and indirect route to the hippocampus via the retrosplenial cortex, suggesting that this nucleus functions as a relay for information between these regions (Aggleton et al., 2010). Finally, the anteromedial nucleus, receives most of its inputs from the subiculum, the dysgranular retrosplenial cortex, and medial mammillary bodies (Shibata, 1993a; Shibata & Kato, 1993; Van Groen et al., 1999; Wyss et al., 1979)(*see Chapter 1, section 1.7.3*), possibly integrating prefrontal and sensory functions.

The different, complementary properties of the individual anterior thalamic nuclei might together support spatial navigation in multiple ways. Consequently, like the retrosplenial cortex, the anterior thalamic nuclei may contribute to spatial memory by aiding cue switching. For example, the complementary properties of the various nuclei may help to explain why a transient lesion affected T-maze alternation when intra-maze and extra-maze cues were conflicted (Nelson et al., 2020). Nevertheless, most studies have highlighted the importance of the anterior thalamic nuclei for allocentric learning (Aggleton et al., 1996; Warburton et al., 1997; Mitchell & Dalrymple-Alford, 2005; Wolff et al., 2006).

In normal rodents, the preferred firing direction of head-direction cells is determined by visual landmarks (Taube et al., 1990). This is evident from the study of lesions affecting the retrosplenial cortex (Clark et al., 2010), which reduce the effects of landmark position on head-direction cell firing, suggesting a role of the retrosplenial cortex in the coding of visual orientation cues. At the same time, head-direction cells within the anterodorsal nucleus might be more sensitive to proprioceptive cues as disrupting vestibular inputs impairs orientation coding within the anterodorsal nucleus, even when the retrosplenial cortex is intact (Stackman & Taube, 1997). Together these findings, suggest that the integration of anterior thalamic (particularly anterodorsal and anteroventral) and retrosplenial information coding may be necessary for successful navigation and switching between spatial frameworks. This prediction is supported by studies with humans using a novel virtual environment paradigm that provides participants with body-based cues for orientation. When self-motion was introduced, fMRI data showed evidence of head-direction signals in both the retrosplenial cortex and the thalamus, unlike for those tasks that do not provide body-based cues (Shine et al., 2016).

Concerning the specific role of the interactions between the anterior thalamic nuclei and the retrosplenial cortex, the evidence is limited. An early study using crossed unilateral lesions in rats found evidence that anterior thalamic X retrosplenial tissue loss impaired location learning in the Morris Water Maze (Sutherland & Hoesing, 1993). As the two regions are connected reciprocally, this analysis could not separate thalamic efferents from thalamic afferents.

In an electrophysiological study in mice, the interaction of the anterodorsal nucleus and the retrosplenial cortex was looked at during a contextual fear conditioning paradigm. It was found that theta oscillatory coherence between the regions increased with exposure to a novel context and the retrieval of recent memories (Corcoran et al., 2016). Contrary, retrosplenial-anterodorsal theta coherence decreased when mice successfully retrieved remote memories, relative to those mice that failed at retrieval. A similar pattern of results was observed between the retrosplenial cortex and dorsal hippocampus theta coherence (Corcoran et al., 2016) suggesting an interdependent loop that exists between the three structures. Although, further electrophysiological investigations suggest that there may be

distinct underlying circuits, driven by the subregions of the retrosplenial cortex. For example, area 29 contains non-directional cells whose spiking activity is entrained by theta oscillations, suggesting that activity within this subregion is more strongly coupled with the hippocampus (Lomi et al., 2021). Further anatomical examination in that same study found that the dorsomedial anteroventral nucleus has selective projections to area 29, while the ventrolateral anteroventral nucleus targets both areas 29 and 30 (Lomi et al., 2021). Cumulatively, these results suggests that that not only may there be two distinct anterior thalamic – retrosplenial and dorsal hippocampal – retrosplenial pathways, but also potentially distinct, but interacting, retrosplenial subcircuits within these systems.

Additionally, more indirect evidence about the importance of interactions between the anterior thalamus and the retrosplenial cortex comes from gene expression studies. Anterior thalamic lesions appear to cause depletions of immediate-early gene activity within the retrosplenial cortex by largely eliminating c-fos and zif268 expression in layers II and III (Jenkins et al., 2004; Poirier & Aggleton, 2009). Furthermore, lesions to the anterior thalamic nuclei seem to alter the activity of genes associated with metabolism (Poirier et al., 2008) as well as plasticity within the retrosplenial cortex (Garden et al., 2009). Despite the apparent electrophysiological and cellular interactions between the anterior thalamic nuclei and the retrosplenial cortex, the behavioural implications of these interactions are poorly understood. Bearing in mind that both sites are critical for spatial memory, it is valuable to appreciate both their individual and combined contributions.

While the functional interactions between the anterior thalamus and the hippocampal formation have been directly addressed in a series of previous experiments that used iDREADDs (Nelson et al., 2020), the interactions between the anterior thalamus and the retrosplenial cortex have not been examined in this way. Selectively inhibiting the direct projections from the anterior thalamus to the dorsal subiculum and *vice versa*, produced T-maze alternation deficits when intra-maze and extra-maze cues were conflicted, particularly when the inputs from the dorsal subiculum to the anterior thalamic nuclei are silenced (Nelson et al., 2020). Chapter 3 used the same technique to disrupt the inputs from the dorsal subiculum to the retrosplenial cortex and showed similar pattern of results, cumulatively suggesting

that anterior thalami-dorsal subicular-retrosplenial interactions are vital for spatial memory in complex, unstable environments.

The present chapter sought to extend the findings from *Chapter 3* by examining the interactions of the anterior thalamic nuclei with retrosplenial cortex. In this chapter, iDREADDs were first used to systemically inhibit the influences of anterior thalamic nuclei efferents, using i.p. injections of clozapine (*see Chapter 1, section 1.8*). Next, the study sought to target just those projections terminating within the retrosplenial cortex, using methods similar to those in Chapter 3 by infusing clozapine locally into the retrosplenial cortex. Animals were tested on the Standard T-maze task, where all cue types are available, and on the Rotation T-maze, which creates a conflict between intra-maze and extra-maze cues.

Two cohorts of adult rats were trained and tested. The findings from Cohort 1 led to some procedural modifications for Cohort 2, and so they are described separately.

4.2. Materials and Methods

4.2.1. Statement of Contributions

The work presented in this chapter was supported by Eman Amin who assisted with the histological data analysis. I performed the surgeries and data collection, as well as the histological analysis, imaging, and statistical analyses.

4.2.2. Experiment 1 (Cohort 1)

4.2.2.1. Animals

A cohort of 26 adult Lister Hooded male rats (Envigo, UK), were trained prior to surgery on a reinforced T-maze alternation task. Two animals were excluded from

surgery due to their failure to acquire the task. Animals were randomly assigned to groups of 12 iDREADDs animals and 12 GFP-control animals, however, the experimenter was not blind to the group membership of the animals. At the time of surgery all rats weighed between 285g and 355g. Housing conditions and ethical principles with regards to animal care and research were as described in *sections 2.2.1. and 3.2.2.*

4.2.2.2. Surgery

Unless otherwise specified in this section, the animals underwent exactly the same surgical procedures, and were given exactly the same surgical anaesthesia, preoperative and postoperative care as described in *sections 2.2.2 and 3.2.3.* The main difference was that the viral constructs were placed in the anterior thalamic nuclei rather than the dorsal subiculum.

Bilateral craniotomies were performed above the anterior thalamic nuclei and either pAAV-CaMKIIa- hM4D(Gi)-mCherry (AAV5) (iDREADD)(Titer: 2.6x10^13GC/ml, lot:v102676 Addgene, MA, USA) or pAAV-CaMKIIa-GFP (AAV5)(titer: 1.9x10^13GC/ml, lot:v111209, Addgene, MA, USA)(GFP-control) virus was injected into the nuclei. In all animals, 0.5µl of the viral construct was infused in the anterior injection site and 0.6µl into the more posterior site. The injection coordinates, with respect to bregma were as follows: *Anterior*: AP: -0.1mm, ML: ±0.8mm, DV: -6.7mm; *Posterior*: AP: -0.2mm, ML: ± 1.3mm, DV: - 6.0mm.

During the same surgeries, cannulas were implanted into retrosplenial cortex, following craniotomies in both hemispheres. One cannula (1.5mm length x 1.2mm separation, 26-gauge, PlasticsOne, Virginia, USA) was implanted into the anterior portion of the retrosplenial cortex (from bregma; AP: -2.5 mm, ML: ± 0.6 mm, DV: -1.5mm), the other cannula (1.7mm length x 1.6mm separation; 26-gauge, Plastic One, Virginia, USA) was implanted into the posterior retrosplenial cortex (AP: -6.0mm, ML: ± 0.8mm, DV: -1.7mm).

4.2.2.3. Apparatus for Behaviour

The apparatus used for the behavioural testing was as described in *section 3.2.4*.

4.2.2.4. Behavioural Training Prior to Infusions

Prior to surgery, all rats were habituated to the maze for four sessions as described in *section 3.2.6.*

Following habituation to the apparatus, the rats were run on the 'Standard' T-maze procedure as described in *section 3.2.5* for approximately 8 days, consisting of 12 trials a day. The animals for surgery were selected, based on their performance and willingness to run, and thus two animals were excluded pre-surgery due to excessive anxiety and freezing throughout pre-training.

Post-surgery, the animals were retrained on the Standard T-maze task for four sessions, until they reached 83.3% (10/12) correct trials. The infusion trials for the Standard T-maze task then followed. When the experimental testing was concluded, the rats were then trained on the Rotation T-maze task (as described in *section 3.2.6*) for up to four to five sessions, until they reached 83.3% (10/12) correct trials. The infusion sessions for that condition then followed. For all behavioural training and experimental days, the animals were divided and tested in two separate subgroups, consisting of random numbers of iDREADDs and GFP animals, so that there was always a behaviour-free day between the infusion days (Figure 4.1). This

testing regime was repeated for both behavioural conditions.

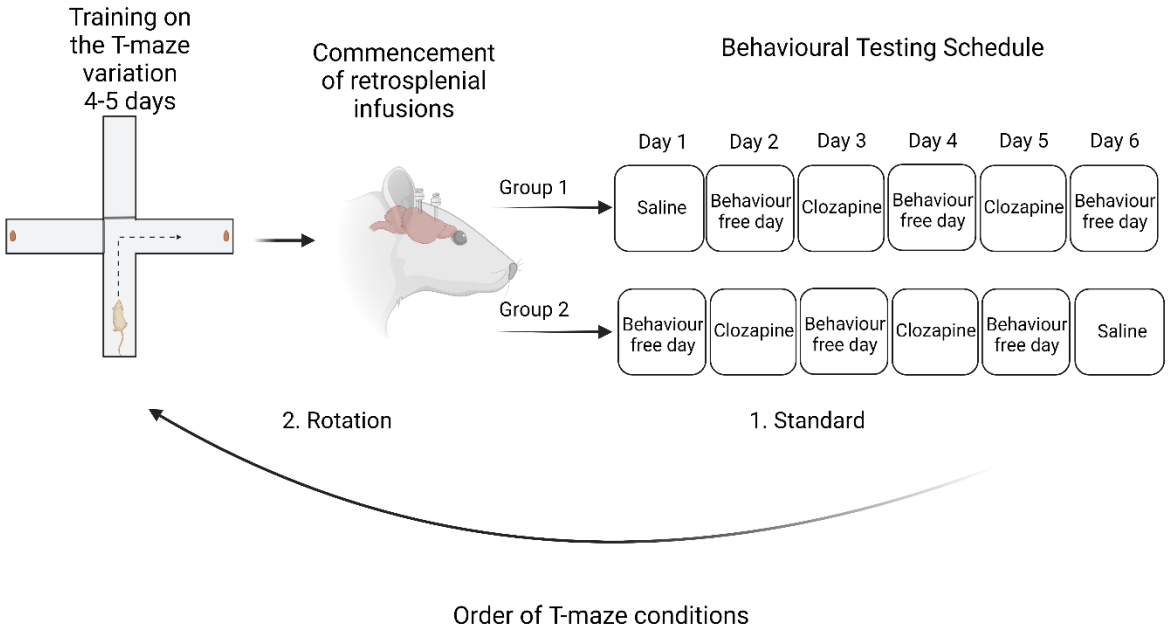


Figure 4.1. Schematic illustration of the behavioural training and testing schedule post-surgery.

Animals received a sequence of alternation sessions that involved preceding intracerebral infusions of either clozapine or saline. There was a behaviour and infusion free day between the experimental days. After all experimental sessions were complete for each group, rats moved onto the next T-maze condition, starting with training sessions followed by infusion sessions. The order of the infusions was counterbalanced across the two groups as indicated.

4.2.2.5. Experimental Conditions (all 12 trials per session)

Both experimental conditions were performed as described in *section 3.2.6 (see also* Figure 4.2A and 4.2B). For this experiment animals completed 12 trials per session

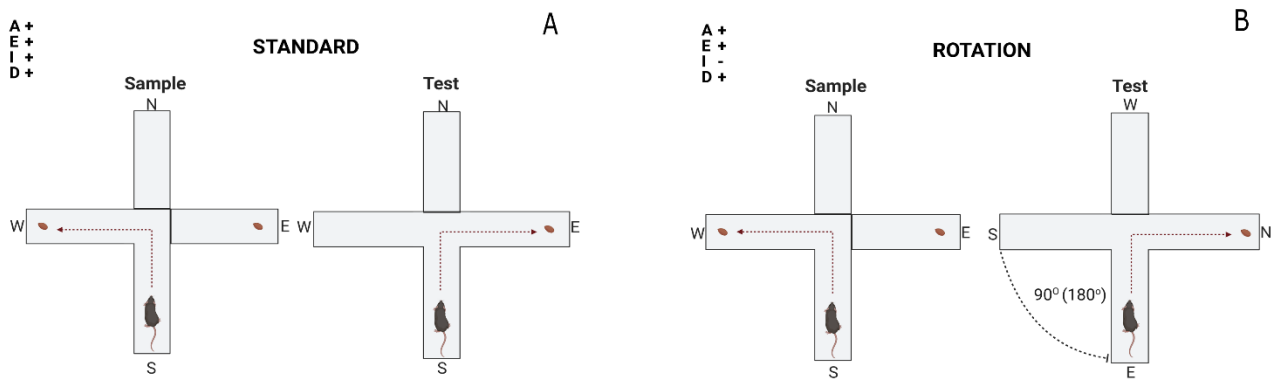


Figure 4.2. Illustration of the T-maze variations.

The figure shows examples of a single trial (sample and test run) for both the standard T-maze and the arm rotation T-maze (with either 90º or 180º maze rotation in either direction. Abbreviations: A, allocentric cues; E, egocentric cues; I, intra-maze cues; D, directional cues; +, cue is available to solve the maze; -, the cue does not solve the maze.

4.2.2.6. iDREADDs activation

The iDREADDs activation procedure was exactly the same as that described in *section 3.2.7 (*Figure 4.1). There was always an infusion and behaviour free day between the infusions.

4.2.3. Experiment 2 (Cohort 2)

4.2.3.1. Experimental

Unlike cohort 1, this experiment adopted two-phased surgical approach. In the first phase of the experiment, either iDREADDs or a green fluorescent protein (GFP) expressing adeno-associated virus (control) was injected into the anterior thalamic in

two separate groups of rats. The animals underwent behavioural testing following a systemic i.p. injections of clozapine or saline. In the second phase of the experiment, a subset of the animals underwent a second surgery where cannulas were implanted into the rostral and caudal retrosplenial cortex. These animals then received localised infusions of either clozapine or saline prior to behavioural testing (Figure 4.3). Unless otherwise specified in the sections below, all animals were housed under the same conditions and underwent the exact same surgical and behavioural procedures as described in *section 4.2.1*

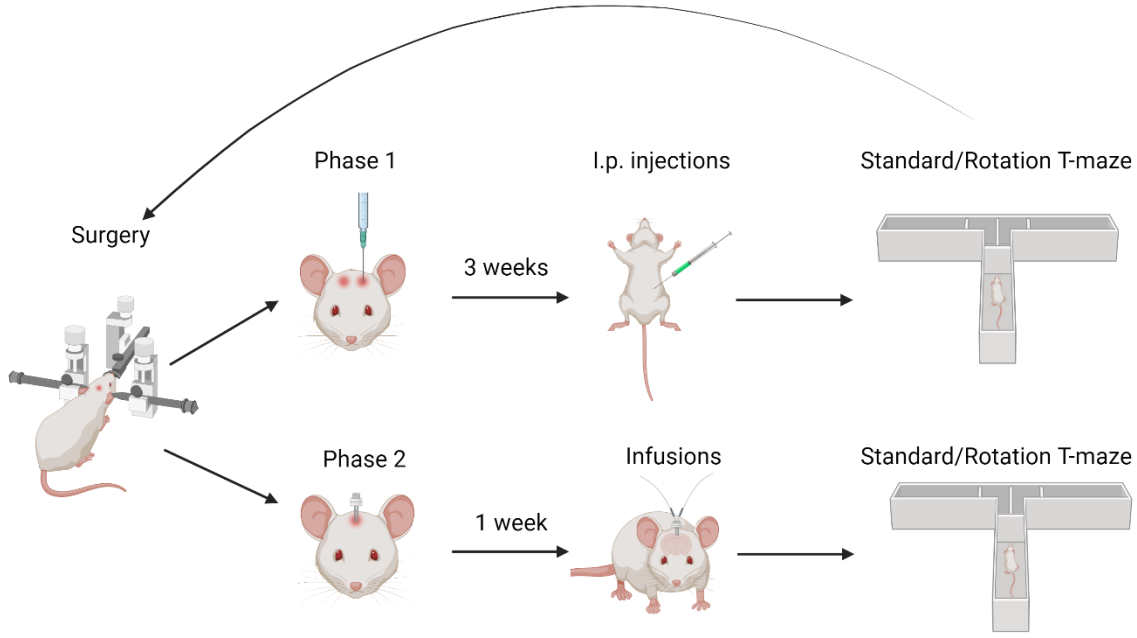


Figure 4.3. Experiment two design schematic.

The schematic shows the two phases of experiment two. The animals in experiment two underwent two separate surgeries. During the first one, all rats received viral infusions of iDREADDs or GFP-control within the anterior thalamus. When the testing for this phase was complete, a second surgery followed. A subset of the animals from Phase 1, then received a second surgery to cannulate the retrosplenial cortex for the local infusions.

4.2.3.2. Animals

For Experiment 2, a second cohort of 26 adult Lister Hooded male rats (Envigo, UK), were trained prior to surgery on a reinforced T-maze alternation task. Two animals were excluded from surgery due to failure to acquire the task. The rest of the animals were randomly assigned to either a group of 12 iDREADDs animals or 12 GFP-control animals. At the time of surgery all rats weighed between 270g and 315g at the time of the first viral infusion surgery, and between 380g and 420g during the cannulation surgery.

4.2.3.3. Surgery

The same surgical procedures and coordinates as described for Experiment 1 (*section 4.2.2.2*) were used to deliver the viral infusions into the anterior thalamic nuclei. The retrosplenial cannulation procedure was completed in a second, separate surgery that took place approximately nine weeks after the first surgery. Due to a delay in the delivery of the cannulas, only the first 14 animals of the cohort underwent this cannulation surgery (4 GFPs and 10 iDREADDs). The cannulas were implanted bilaterally into the retrosplenial cortex. One cannula (1.6mm length x 1.2mm separation, 26-gauge, PlasticsOne, Virginia, USA) was implanted into the anterior portion of the retrosplenial cortex (from bregma; AP: -2.8 mm, ML: ± 0.6 mm, DV: -1.6mm), the other cannula (1.7mm length x 1.6mm separation; 26-gauge, Plastic One, Virginia, USA) was implanted into the posterior retrosplenial cortex (AP: -6.0mm, ML: ± 0.8mm, DV: -1.7mm). All animals recovered for at least 7 days after each surgery took place, prior to commencing behavioural training.

4.2.3.4. Behaviour

Unless otherwise specified the apparatus for behaviour, behavioural training and experimental conditions for Experiment 2 were exactly the same as described for Experiment 1 in this chapter.

After the animals were habituated to the T-maze, they were run on the 'Standard' T-maze procedure for approximately two days prior to surgery to habituate the animals and help them to acquire the task. Two animals were excluded prior to surgery due to heightened freezing behaviour in the maze. In phase one, post viral infusion surgery, the animals were retrained on the Standard T-maze task for eight to twelve days, until they reached at least 83.3% (10/12) correct trials. The systemic activation of the iDREADDs with i.p. administration of clozapine (see *section 4.3.2.5* for details) for that condition then followed. The rats were then trained on the Rotation T-maze condition for one to four days until the majority reached 83.3% (10/12) trials to avoid overtraining, immediately prior to the systemic iDREADDs activation for that condition.

In phase two, post-cannulation surgery, the animals were retrained for up to two days on each condition, just prior the commencement of intracerebral infusions for that condition. The infusion procedure was conducted as described in *section 4.2.2.6*. Finally, the behaviour of the canulated animals was tested once again following a series of i.p. injections of clozapine or saline without any refresher training (Figures 4.4 and 4.5).

4.2.3.5. iDREADDs activation

Each behavioural condition was run both after an i.p. injection or infusion of clozapine and saline, which served as a within-subject control. In phase one, animals received an i.p. injection of 0.6mg/kg of clozapine or saline and the behavioural testing commenced approximately 30 minutes after the administration of the injection. The dose was selected as preliminary data shows that anything above this

range may affect animals' mobility and cognition (see Appendix B). In phase two, animals received intracerebral infusions of clozapine as described in *section 4.2.2.6.* (Figures 4.4 and 4.5).

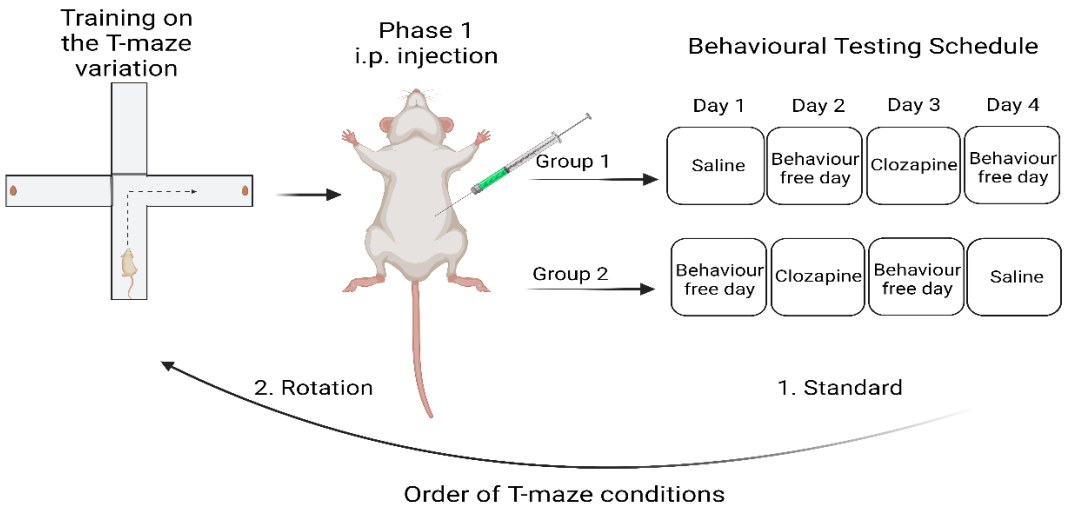


Figure 4.4. Schematic illustration of the behavioural training and testing schedule post-viral infusion surgery for phase 1 of experiment two.

Animals were split into two groups of 12 and received i.p. injections of either clozapine or saline prior to behavioural testing. The injection order was counterbalanced between the groups and all animals had an injection/behaviour free day between each experimental day. After all experimental sessions were complete for each group, rats moved onto the next T-maze variation, starting with training sessions followed by the experimental sessions.

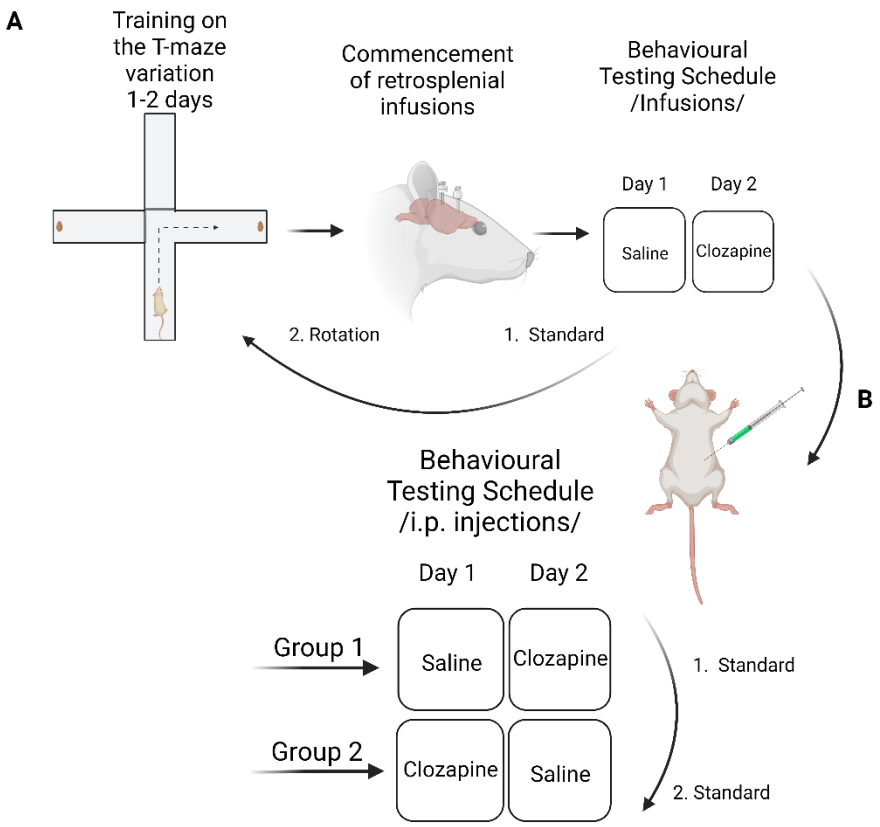


Figure 4.5. Schematic illustration of the behavioural training and testing schedule post-cannulation surgery for phase 2 of experiment two.

In phase two, 14 animals had their retrosplenial cortex cannulated. Panel A shows the testing schedule for the intracerebral infusions, the order of which was counterbalanced between the T-maze variations (i.e., all animals received saline infusion first on the standard T-maze and then all animals received clozapine infusions first on the rotation T-maze). Panel B, shows the schedule for behavioural testing, following repeated i.p. injections in the cannulated animals. The injection order was counterbalanced within the testing day (i.e., group 1 received saline first, and group 2 received clozapine first). Testing was repeated twice for the standard T-maze (injections 2 and 3), to validate the results of the first i.p. injection from phase 1.

4.2.4. Perfusions

Following completion of the experiments, animals from both cohorts were transcardially perfused and their tissue processed as described in *section 3.2.8.*

4.2.5.Histology

All tissue was processed following the histological procedures described in *section 3.2.9.*

4.2.6. Image Acquisition and Viral Expression Analysis

Images were acquired as described in *section 3.2.10,* however, the microscope Leica DM5000B was fitted with a Leica K3M 6.3MP camera and the software used was LAS X Core. The viral expression was assessed at the injection site, as well as in the retrosplenial cortex (Figures 4.6 and 4.7).

4.2.7. Statistical analyses

Unless otherwise specified, the data were screened, analysed, and reported as described in *section 3.2.12.*

In experiment one, there were no extreme outliers or violations to any assumptions (all $p_s > 0.05$). In experiment two, there were no extreme outliers, violations of homogeneity of variances and covariances assumptions (all $p_s > 0.05$), however, the assumption of normality was violated within the GFP group on the standard clozapine injections and within both groups on the saline injection within the rotation variation.

4.3. Results

4.3.1 Experiment 1

As in Chapter 3, this experiment used iDREADDs technology. Here, the viral injections were placed in the anterior thalamic nuclei and the retrosplenial cortex was bilaterally cannulated to facilitate repeated local infusions.

4.3.1.1. Histological findings

Two criteria were required for inclusion in the experimental analyses. First, the anterior thalamic nuclei injections had to result in appreciable bilateral label that had been transported anterogradely to the retrosplenial cortex (Figures 4.6 and 4.7). Second, the cannulation placements had to be within retrosplenial cortex (Figure 4.8). In experiment one, a total of 5 iDREADDs and 5 GFP-control animals were excluded due to lack of viral expression (unilateral or bilateral) in retrosplenial cortex (n=7) or off-target cannula placement (n=3). Consequently, the behavioural analyses derived from 7 iDREADDs and 7 GFP-control animals.

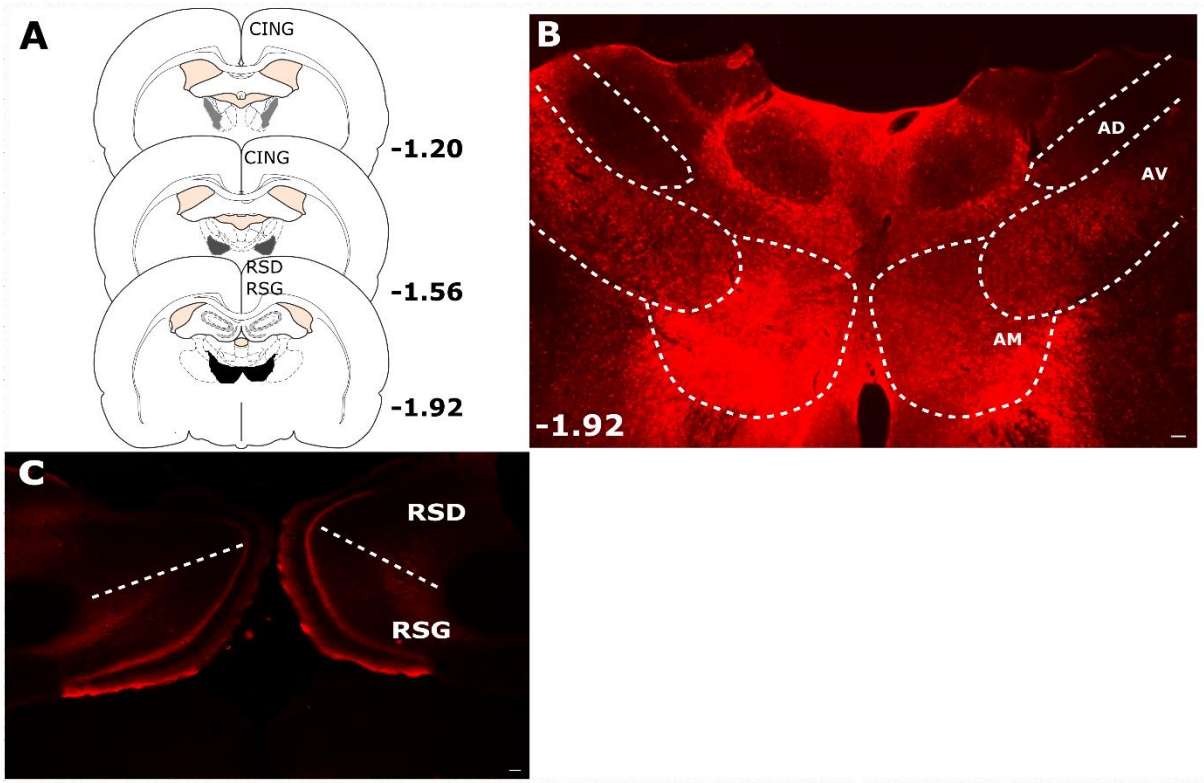


Figure 4.6. Virus expression in the iDREADDs animals.

Panel A of the figure shows the approximate position and spread of the viral expression with light grey signifying lighter expression and black strong viral expression. The viral expression pattern observed within the injection site was lighter in the rostral parts of the anterior thalamus and dispersed within the most ventral parts of the anteroventral nucleus with lighter expression in the anterodorsal nucleus. Moving towards the caudal anterior thalamus, the viral expression appeared strongest in the anteromedial nucleus and around the midline. The pattern of expression was the same for both cohorts of animals. Numbers refer to the distance from bregma in mm. Panel B shows an example of iDREADDs expression in the anterior thalamic nuclei. Panel C shows the robust expression of transported iDREADDs in the retrosplenial cortex, particularly in layers I, II, and III of the granular retrosplenial cortex. All scale bars are 150µm. AD, anterodorsal nucleus; AM, anteromedial nucleus; AV, anteroventral nucleus, CING, anterior cingulate cortex; RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex.

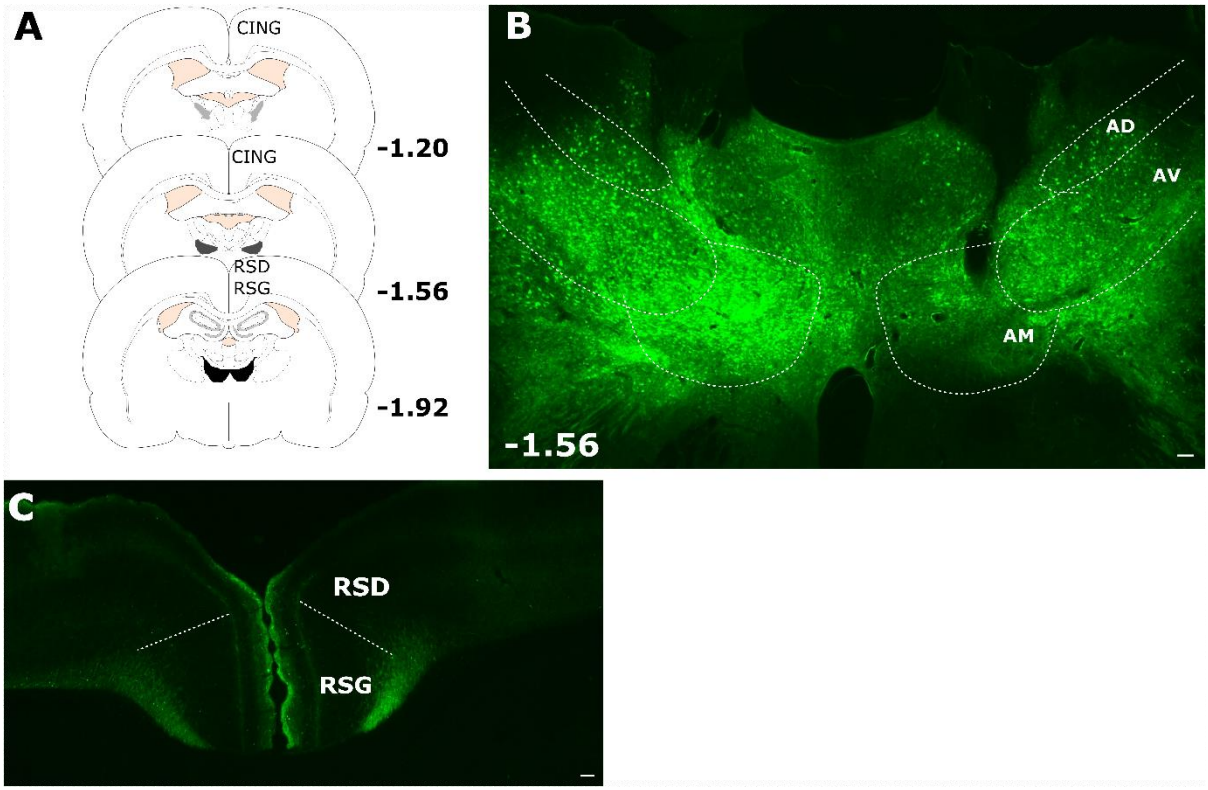


Figure 4.7. Virus expression in the GFP-control animals.

The viral expression observed was similar across both cohorts of animals and followed the pattern of expression observed in the iDREADDs animals. Panel A of the figure shows the approximate position and spread of the viral expression with light grey signifying lighter expression and black strong viral expression. The viral expression was lighter in the most rostral parts of the anterior thalamus (light grey) and mostly within the anteroventral nucleus with lighter expression in the anterodorsal and anteromedial nuclei. Moving towards more caudal parts of the anterior thalamus, the expression was strongest (black) particularly within the anteromedial nucleus and the midline. Numbers refer to the distance from bregma in mm. Panel B shows an example of GFP- control virus expression in the anterior thalamic nuclei. Panel C shows the expression of transported GFP in the retrosplenial cortex, particularly in layers I, II, and III of the granular retrosplenial cortex, as well as the cingulum. All scale bars are 150µm. AD, anterodorsal nucleus; AM, anteromedial nucleus; AV, anteroventral nucleus, CING, anterior cingulate cortex; RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex.

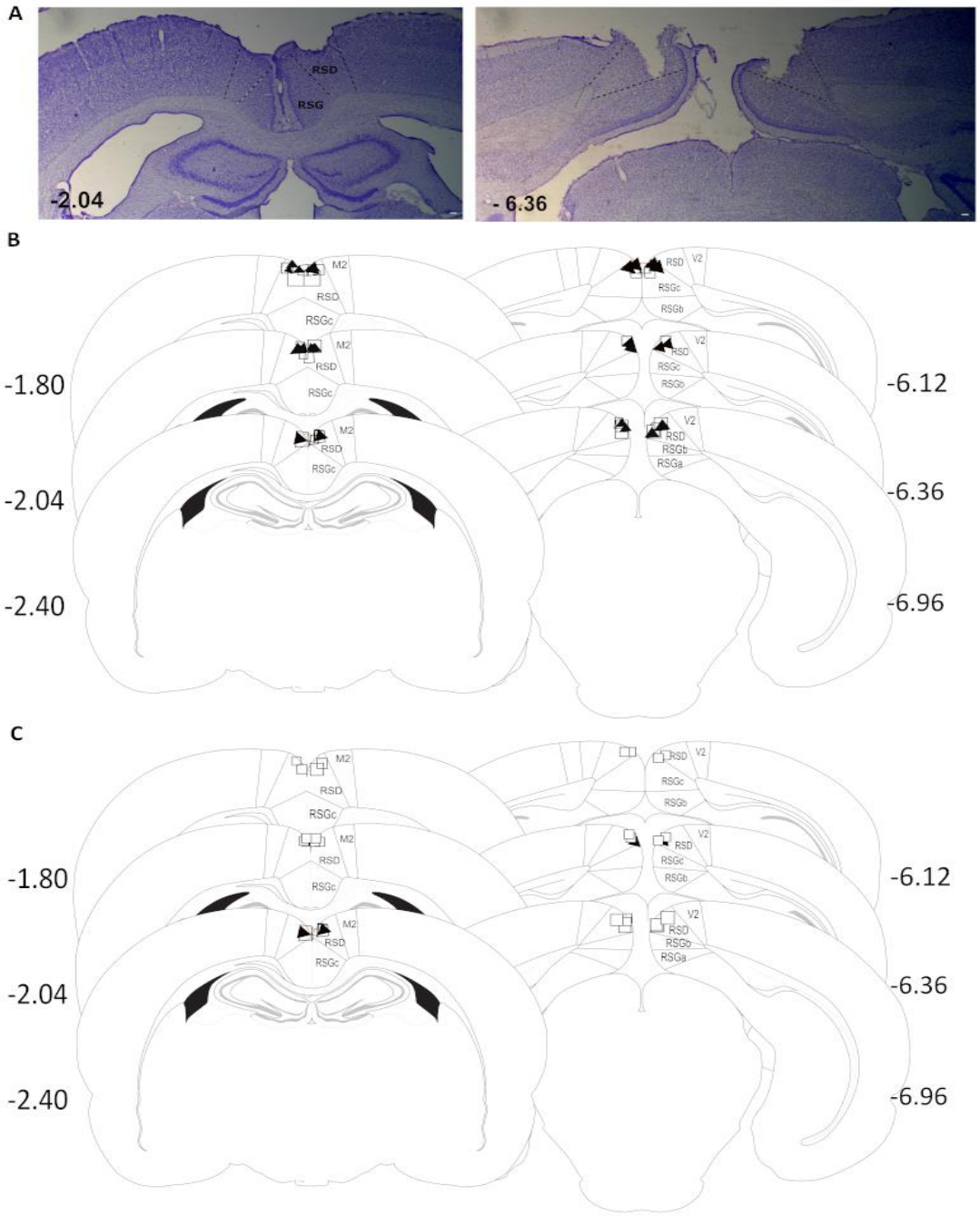


Figure 4.8. Schematic representation of retrosplenial cannula placement for each experimental animal across both cohorts of animals.

Panel A shows coronal sections (cresyl violet) with cannulation sites in the anterior (left) and posterior (right) retrosplenial cortex. Panel B (below) is a schematic representation of cannula placements in Cohort 1, and panel C shows the cannula placements for animals in Cohort 2. The coronal sections in panels B and C were adapted from the Paxinos and Watson rat atlas (2004), with a focus on the anterior

(left) and posterior (right) portions of the retrosplenial cortex. Squares denote cannula placements of iDREADDs animals and triangles GFP-controls. The same implantation coordinates were used for all animals within each Cohort, producing considerable overlap of cannula placements. The numbers represent the approximate distance from bregma in mm. All scale bars are 150µm. Abbreviations: Cg1/2, anterior cingulate cortex; M2, secondary motor cortex, RSD, dysgranular retrosplenial, RSG, granular retrosplenial cortex.

4.3.1.2. Pre-surgery training and post-surgery baseline analyses

A series of independent *t*-tests determined whether there might be pre-surgery or baseline training performance differences between the iDREADDs and GFP control rats on the two variations of the reinforced T-maze task. Animals did not differ significantly on either the pre-surgery training or the baseline training prior to commencement of infusions: all $t_s < -1.029$, $p_s > 0.324$. (Note, an attempt was made to pool the data from experiments one and two and analyse them together. However, initial checks showed that the two cohorts of animals different significantly and the additional analyses were not completed (see Appendix C).

4.3.1.3. Performance on test conditions (Experiment 1- local retrosplenial infusions only)

Standard T-maze: There was no significant main effect of Drug or Group, nor Drug × Group interaction: $F_s < 0.404$, $p_s > 0.54$, $\eta_{ps}^2 < 0.03$ (Figures 4.9 and 4.10).

Rotation T-maze: There was no significant main effect of Drug or Group, nor Drug × Group interaction: $F_s < 2.942$, $p_s > 0.11$, $\eta_{ps}^2 < 0.196$ (Figures 4.9 and 4.10).

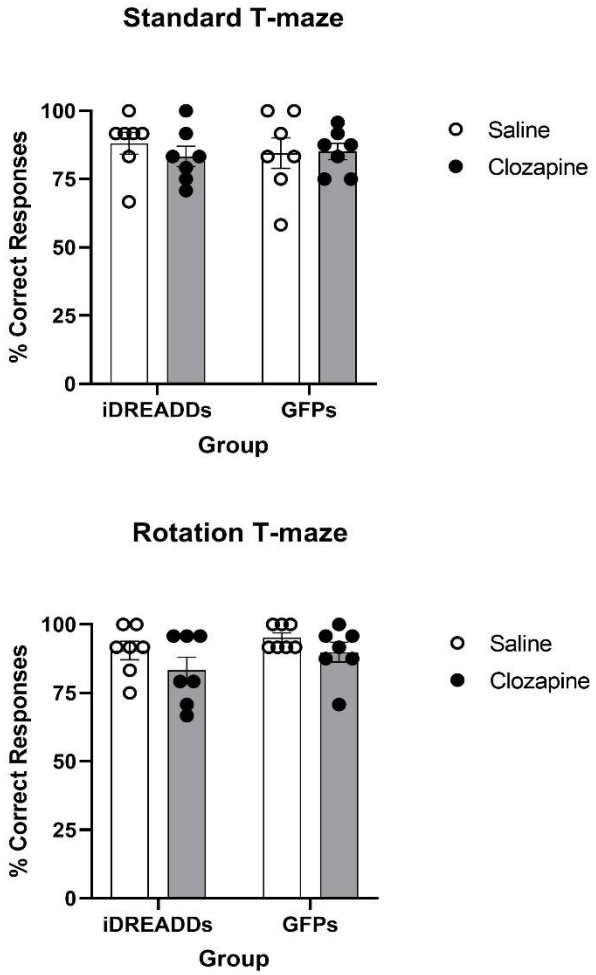
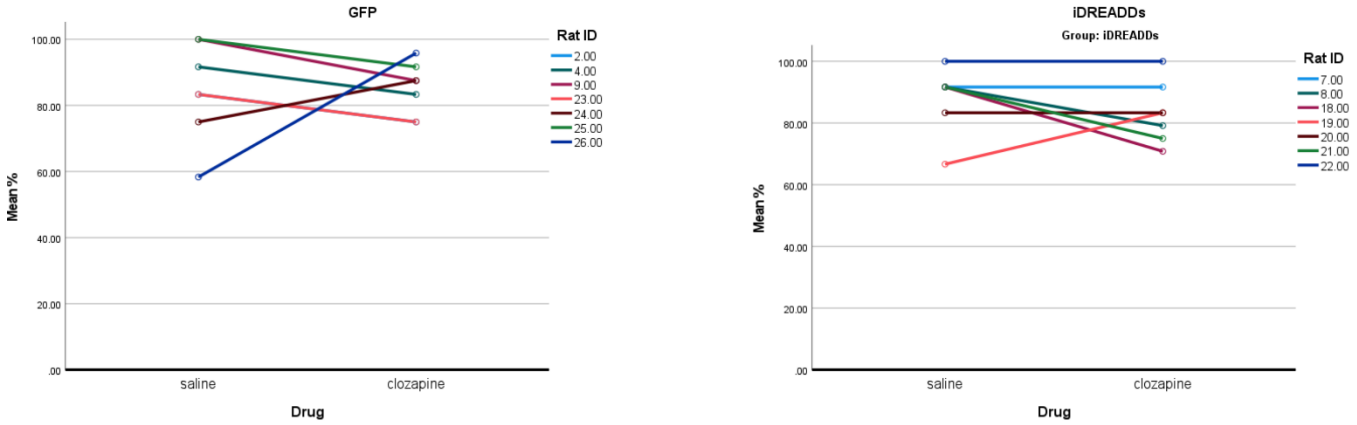


Figure 4.9. Bar graphs depicting the mean and each animal's individual percentage of correct alternation responses for both the iDREADDs and GFP-control groups.

The figure shows only the data for Experiment 1, where the anterior thalamic inputs to the retrosplenial cortex were selectively targeted using intracerebral infusions of clozapine. Top: 1) Standard T-maze and bottom: 2) Rotation T-maze. There were no statistically significant between or within-group group differences in performance. *Error bars indicate SEM; the saline condition is presented in white and the clozapine condition in grey.*

A. STANDARD T-MAZE



B. ROTATION T-MAZE

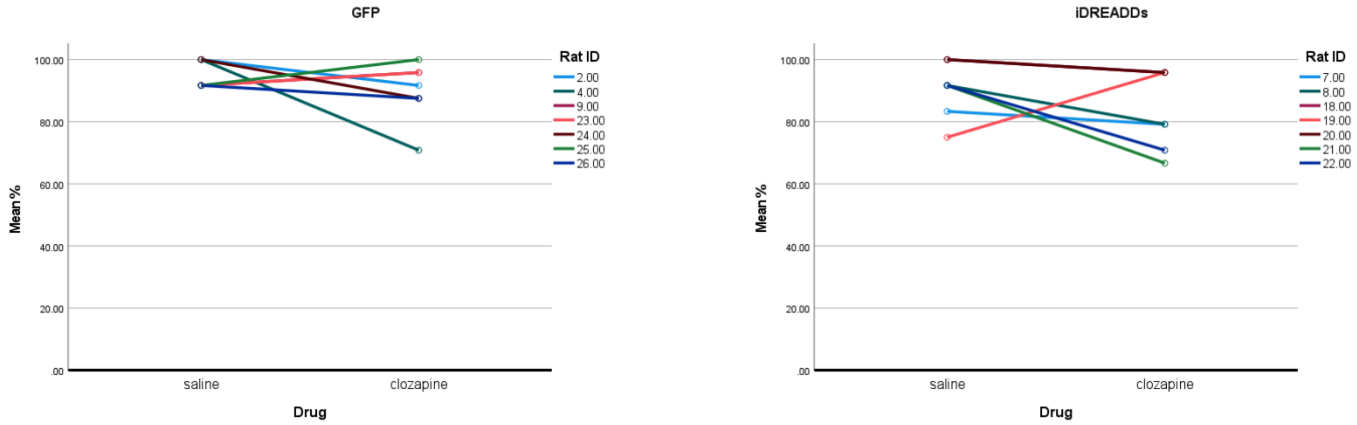


Figure 4.10. Line graph showing individual animals' performance on each T-maze variation by group.

Each line represents individual animals' performance on each drug infusion (saline vs clozapine) in Experiment 1 (retrosplenial infusions only). The top row (A) shows animals' performance on the Standard T-maze and the bottom row (B) shows animals' performance on the Rotation T-maze. GFP-control animals are presented on the left and the iDREADDs animals are presented on the right. *Note: Some animals' performance overlaps.*

4.3.2. Experiment 2 (systemic inhibition of the anterior thalamic nuclei and local inhibition of retrosplenial afferents)

Experiment 2 adopted two-phased approach. In the first phase, animals received iDREADDs injection in the anterior thalamic nuclei. Following recovering they received systemic injection of clozapine to attenuate the activity of all anterior thalamic efferents and to establish the effectiveness of the iDREADDs. In the second phase, a proportion of the animals (iDREADDs only) were cannulated within retrosplenial cortex. This second phase adopted a within-subject design and clozapine was administered locally into the retrosplenial cortex to target only the anterior thalamic projections terminating there.

4.3.2.1. Histological findings

A total of 2 iDREADDs and 6 GFP-control animals were excluded due to lack of viral expression (unilateral or bilateral) in the anterior thalamic nuclei and the retrosplenial cortex (n=8). Consequently, the behavioural analyses derive from 10 iDREADDs and 6 GFP-control animals for the systemic injections in phase 1. Within the 14 cannulated animals, all cannulas were on target, however, the lack of viral expression in some cases led to a final group of 8 iDREADDs and only 1 GFP control in the for the intracerebral infusions in phase 2. Given that only one animal was retained in the GFP group, it was excluded from the statistical analyses (Figure 4.6, 4.7 and 4.8).

4.3.2.2. Pre-surgery training and post-surgery baseline analyses

Animals (iDRREADDs = 10; GFP-controls = 6) did not differ significantly either on the pre-surgery training or on the baseline training prior to commencement of infusions: all $t_s < .490$, $p_s > 0.632$.

4.3.2.3. Performance on test conditions

4.3.2.3.1. Phase 1 – systemic injections (iDREADDs = 10; GFPs = 6)

Standard T-maze: There was a significant main effect of Drug: $F_{1,14} = 5.506$, $p = 0.034$, $\eta_{ps}^2 = 0.28$ and a Drug × Group interaction: $F_{1,14} = 18.1674$, $p = 0.001$, $\eta_{ps}^2 = 0.56$, but not of Group: $F_{1,14} = 0.448$, $p = 0.514$, $\eta_{ps}^2 < 0.03$. Follow-up, simple main effect comparisons revealed significant within-group differences in the iDREADDs group as clozapine was associated with poorer alternation scores: $F_{1,14} = 29.119$, $p = 0.001$ (Figures 4.11 and 4.12). No other comparisons were significant: $F_s < 3.711$, $p_s > 0.074$.

Rotation T-maze: There was not a significant main effect of Drug or Group, nor was there a Drug × Group interaction: $F_s < 1.808$, $p_s > 0.200$, $\eta_{ps}^2 < 0.114$ (Figures 4.11 and 4.12).

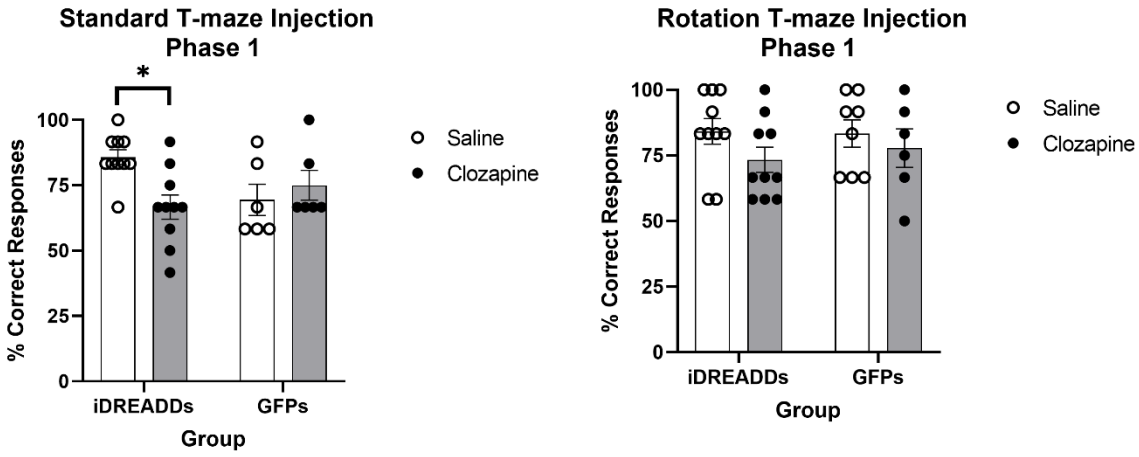


Figure 4.11. Bar graphs depicting the mean and each animal's individual percentage of correct alternation responses for both the iDREADDs and GFP-control groups following i.p. (systemic) injections.

Top: 1) Standard T-maze, and bottom: 2) Rotation T-maze. There was a statistically significant iDREADDs within-group group difference in performance on the Standard T-maze. *Error bars indicate SEM; the saline condition is presented in white and the clozapine condition in grey; "*" denotes statistical significance.*

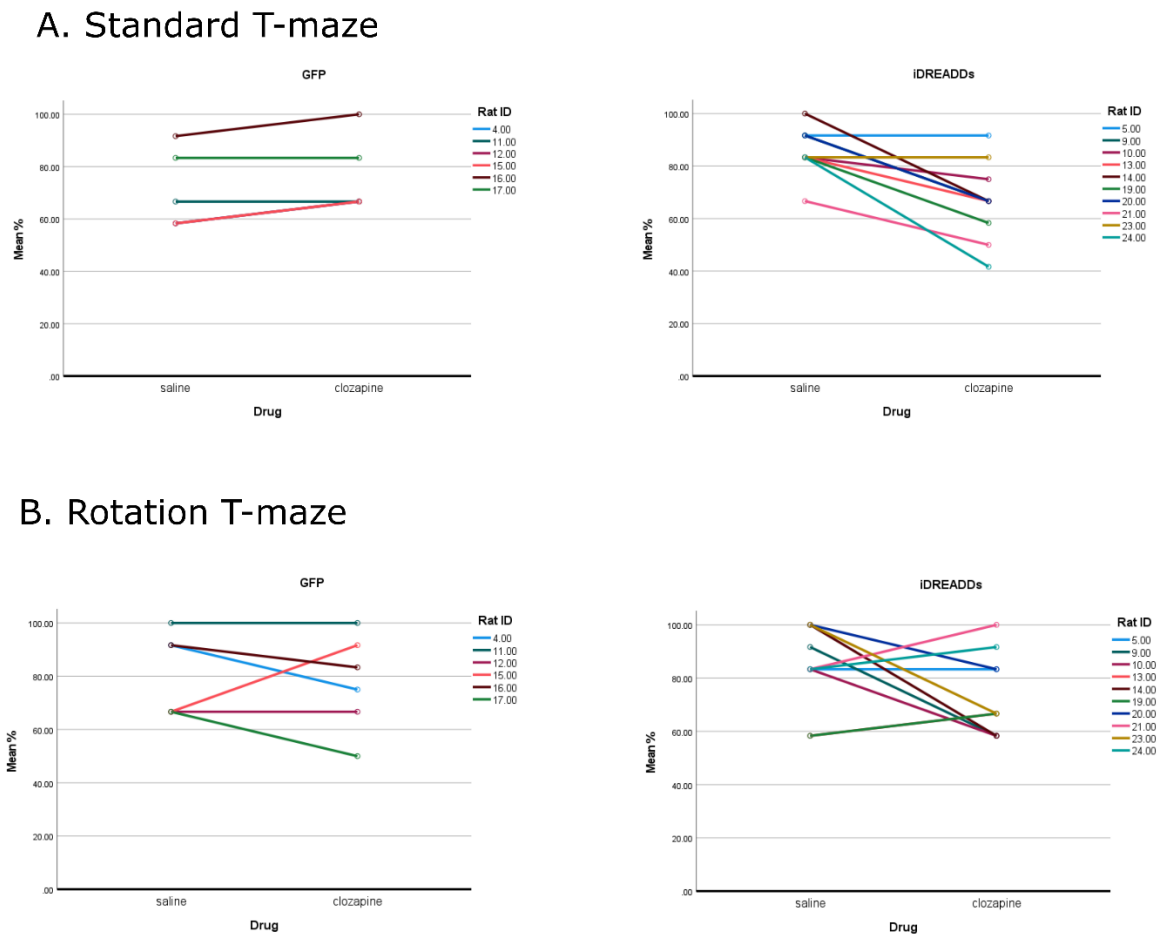


Figure 4.12. Line graph showing individual animals' performance on each T-maze variation by group.

Each line represents individual animals' performance following each i.p. (systemic) injection (saline vs clozapine) in Cohort 2. The top row (A) shows animals performance on the Standard T-maze and the bottom row (B) shows animals performance on the Rotation T-maze. GFP-control animals are presented on the left and the iDREADDs animals are presented on the right. *Note: Some animals' performance overlaps.*

4.3.2.3.2. Phase 2 – systemic injections and infusions of cannulated animals (iDREADDs = 8)

In Phase two, the data from the i.p. systemic injections acquired in Phase one were reanalysed. This time the analysis was carried out to include only the animals who

were cannulated as to provide reference for the effectiveness of the localised retrosplenial cortex infusions. Since, mostly iDREADDs animals were cannulated due to equipment constraints, and only one GFP-animals remained after applying the histology qualifying criteria, the GFP-control animal was excluded. Therefore, all analyses presented from here onwards are within-group analysis for the iDREADDs animals only.

Standard T-maze: The within-group analysis of variance revealed that in the iDREADDs group, animals performed significantly worse following systemic injection of clozapine, relative to saline: $F_{1,8} = 12.30$, $p = 0.008$, $\eta_p^2 = 0.61$. This initial effect diminished, however, with repeated systemic injections: $F_{1,8} < 0.35$, $p_s > 0.57$, $\eta_{ps}^2 < 0.04$. There were no statistically significant differences following the localised infusions, i.e. animals did not perform any worse following clozapine infusions to the terminations of the anterior thalamic projections within the retrosplenial cortex when compared to saline: $F_{1,8} = 0.94$, $p = 0.36$, $\eta_p^2 = 0.105$ (Figures 4.13, 4.14. and 4.15).

Rotation T-maze: The data following the i.p. systemic injections in Phase one were also reanalysed for the Rotation T-maze using within-subject ANOVA. Again, these included only the cannulated iDREADDs animals. The analysis did not find statistically significant effects between the saline and systemic clozapine injections: $F_{1,8} = 2.67$, $p = 0.14$, $\eta_p^2 = 0.25$. Additionally, there was no statistically significant differences following the intracranial infusions: $F_{1,8} = 0.18$, $p = 0.68$, $\eta_p^2 = 0.022$ (Figures 4.13, 4.14, 4.15 and 4.16).

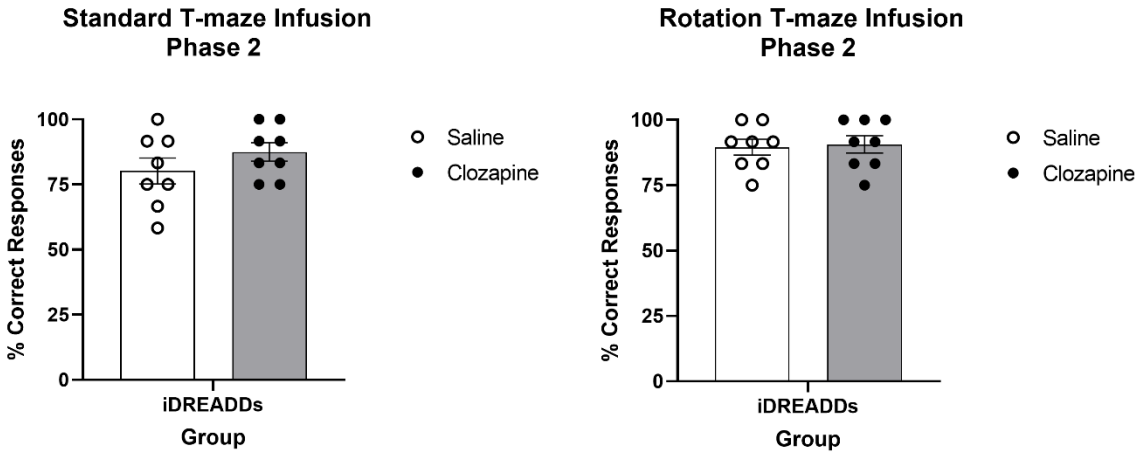


Figure 4.13. Bar graphs depicting the mean and each animal's individual percentage of correct alternation responses for the iDREADDs group following the intracerebral infusions in Phase 2.

Left: 1) Standard T-maze and Right 2) Rotation T-maze. There were no statistically significant differences between the saline and clozapine drug infusion conditions. *Error bars indicate SEM; the saline condition is presented in white and the clozapine condition in grey.*

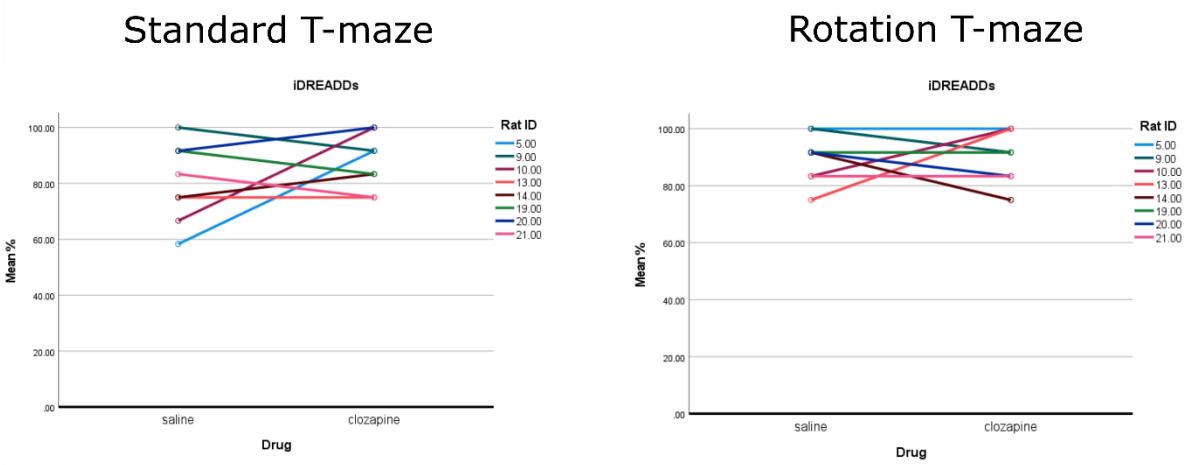


Figure 4.14. Line graph showing individual animals' performance on each T-maze variation (saline vs clozapine, iDREADDs).

Each line represents different animals' performance on each drug infusion (saline vs clozapine) in Cohort 2.

The left side shows animals' performance on the Standard T-maze and the right side shows animals performance on the Rotation T-maze. Both graphs present performance of iDREADDs animals. *Note: Some animals' performance lines may overlap.*

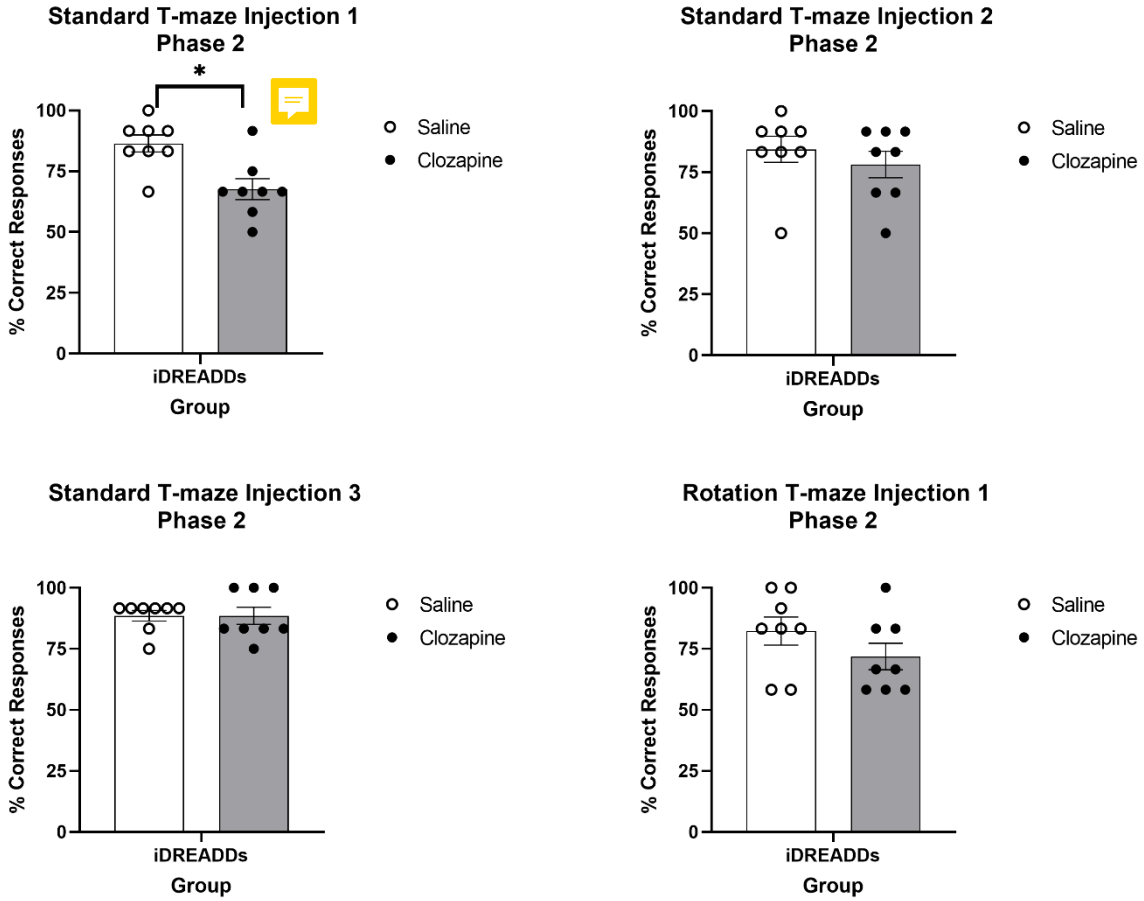


Figure 4.15. Bar graphs depicting the mean and each animal's individual percentage of correct alternation responses for the iDREADDs group following i.p. systemic injections in Phase 2 (cannulated animals only).

The first three panels show the performance of animals on the Standard T-maze, following the first injection (Phase one) and the repeated injections (two and three) in Phase two, where the initial effects diminished. The last panel on the bottom right shows the performance of the cannulated animals on the Rotation T-maze following the i.p. systemic injections in Phase one. Since initial effects were not observed the

testing on the Rotation T-maze was not repeated. *Error bars indicate SEM; the saline condition is presented in white and the clozapine condition in grey.*

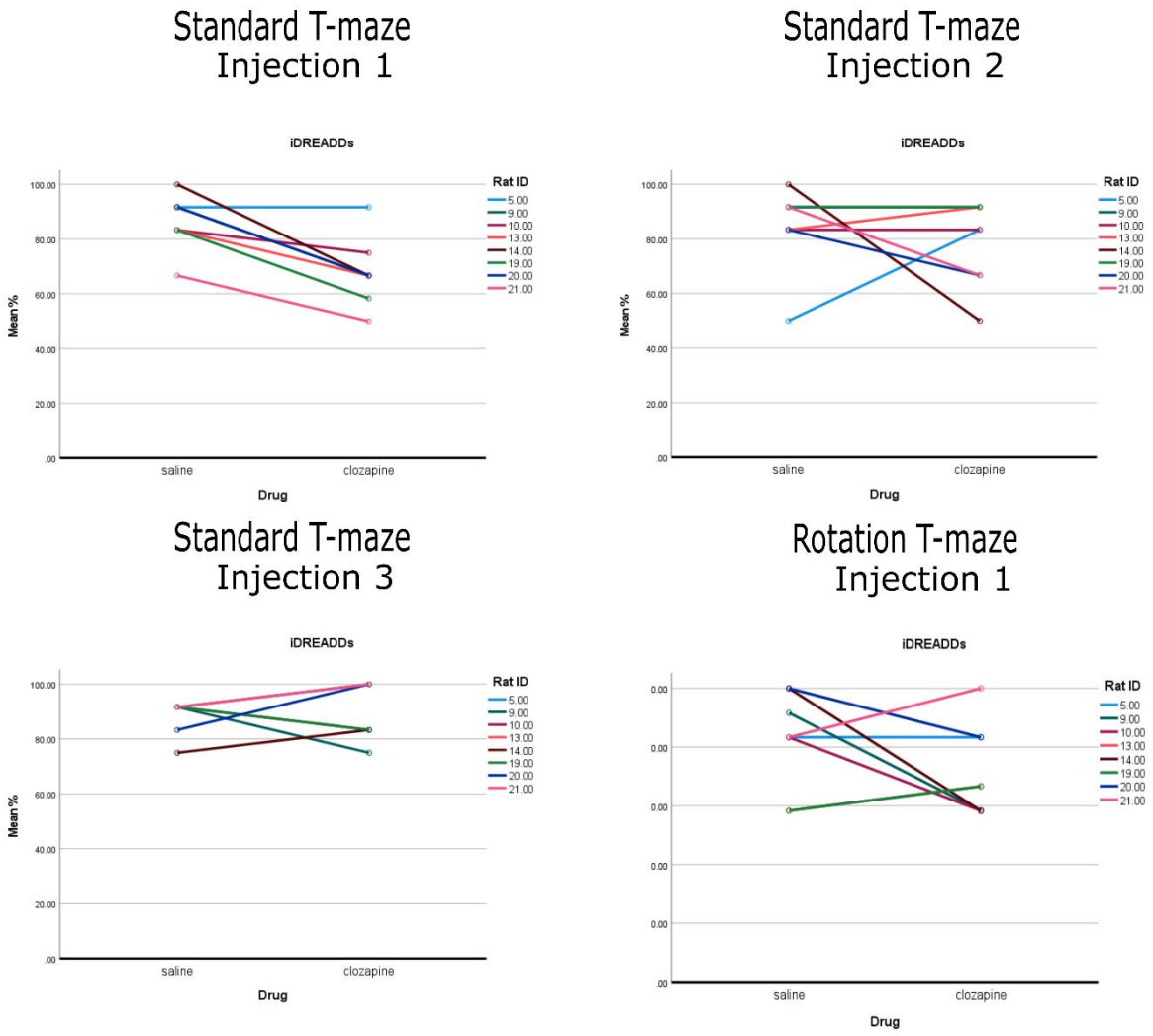


Figure 4.16. Line graph showing individual animals' performance on each T-maze variation (saline vs clozapine, iDREADDs).

Each line represents a different animals' performance following each i.p. systemic injection (saline vs clozapine) in Cohort 2. The first three panels show the performance of animals on the Standard T-maze, following the first injection (Phase one) and the repeated injections (two and three) in Phase two, where the initial effects appear diminished. The last panel on the bottom right shows the performance of the cannulated animals on the Rotation T-maze following the i.p. systemic injections in Phase one. Since initial effects were not observed the testing on the

Rotation T-maze was not repeated. *Note: Some animals' performance line may overlap.*

4.4. Discussion

Although, it has been established that manipulations of the anterior thalamus can produce changes in retrosplenial cortex activity (Garden et al., 2009; Jenkins et al., 2004; Poirier et al., 2008; Poirier & Aggleton, 2009) the potential importance of their direct interactions for spatial working memory has not been studied. In a series of behavioural experiments, I attempted to investigate this interaction by using iDREADDs to disrupt the regions activity both systemically and locally.

The first experiment did not find any effects of the Standard or Rotation T-mazes when the inputs from the anterior thalamus to the retrosplenial cortex were disrupted. Given the nature of the iDREADDs technique, it was unclear whether these results were a true null effect or were due to a technical failure.

To address our uncertainty, a second two-phased experiment was conducted in a separate cohort of animals. In the first phase, systemic clozapine was administered to rats to disrupt the activity of anterior thalamic efferents and to test the effectiveness of the iDREADDs. In this phase, the iDREADDs animals exhibited a spatial deficit in the Standard T-maze condition, but not the subsequent Rotation T-maze. In the second phase of the experiment, a portion of animals underwent local clozapine/saline infusions centred in the retrosplenial cortex in order to selectively disrupt the anterior thalamic projections that terminate there. As with Experiment one, the animals did not display any spatial deficits on either condition. The systemic injection/Standard T-maze condition was then repeated, but the previous deficit appeared to disappear with repeated testing.

The temporal pattern of the results, with an initial deficit following the i.p. injections on the Standard T-maze, may suggest that the chemogenetic effectiveness

disappeared over time, causing a null effect when the anterior thalamic inputs to the retrosplenial cortex were silenced. This is, however, an unlikely explanation given that in Experiment one, the infusions commenced three weeks post the injection of the virus into the anterior thalamus, which as demonstrated in *Chapter 3, section 3.2.12* is sufficient time to achieve effective viral expression and transport. Alternative explanations may be that: (1) the infused clozapine did not disperse enough to cause enough disruption within the retrosplenial cortex and thereby failed to produce observable effects; (2) the position of the injections within the anterior thalamus and consequently the viral expression within the retrosplenial cortex was not optimal, which might also explain why the effects of the systemic clozapine injection did not persist and were not observed in the Rotation condition as in previous research (e.g. Nelson et al., 2020); (3) interrupting these inputs is simply not sufficient to cause behavioural deficits and, therefore, the anterior thalamic-retrosplenial pathway may not be as critical for successful spatial navigation.

In chapter two, it was observed that the anterodorsal and anteroventral nuclei have the highest proportions of bifurcating neurons to the rostral and caudal retrosplenial cortex, contrary to the anteromedial nucleus. Considering that these two nuclei appear to be more critical for spatial memory (Aggleton et al., 2010; Lomi et al., 2023; Taube, 1995), along with evidence that lesions across all three anterior thalamic nuclei produce larger memory deficits than lesions confined to any single nucleus (van Groen et al., 2002), a viral injection with expression largely confined to the anteromedial nucleus (or any individual nucleus) might well show the pattern of results observed here. Although evidence is limited, as discussed in *Chapter 1, section 1.7,* if the three nuclei have different but complementary functions (Aggleton et al., 2010), the anteromedial nucleus may be more sensitive to tasks (such as the Go/No-Go task) and variations of the T-maze (e.g. the start T-maze from *Chapter 3*) that tax attention and cognitive flexibility, given its frontal connections. Consequently, even if the clozapine dispersed as expected, in both the systemic and infusion conditions, one might expect enough intact activity to serve as a compensatory mechanism on the T-maze variations tested. In other words, the complementary actions of the various anterior thalamic nuclei might help to mask the iDREADDs intervention.

A similar explanation would be consistent with the results of an optogenetic study that investigated the interactions of the retrosplenial-anterior thalamic-hippocampal circuit. That study showed that the anterior thalamus and the dorsal subiculum may recruit the same populations of neurons within layer III of the granular retrosplenial cortex (Brennan et al., 2021). Additionally, the neural spikes in layers II and III of the granular retrosplenial cortex appear to be influenced by the activation of the subiculum (Gao et al., 2021) and, therefore, the dorsal subiculum may compensate for the disruption of the direct anterior thalamic inputs to the retrosplenial cortex. In *Chapter 2*, it was suggested that the anterior thalamus and the subiculum together, may facilitate information processing in the retrosplenial cortex (Aggleton & O'Mara, 2022; Yamawaki, Corcoran, et al., 2019). Adding to that, from the results of Experiment one and Phase two of Experiment two presented in this chapter, it appears that the information which the anterior thalamus may contribute to the retrosplenial cortex directly is not vital for spatial working memory as long as the hippocampal inputs are intact.

Although initially the iDREADDs animals showed deficits on the Standard variation of the task following systemic disruption to anterior thalamic activity, the effect attenuated with repeated testing. One possibility is that either the iDREADDs lost their effectiveness over time or animals become more tolerant of the clozapine with repeated systemic administration. Alternatively, since initially the task learning was only just established, it may have been a more sensitive time for disruption of the circuit. Thus, repeated training and testing of the animals would lead to a loss of the observed effects. Given the small group sizes and that the repeat injections were carried out to only a portion of the initial cohort, it may be the case that few animals who were not included in the follow up tests were driving the initial effects (Figures 4.11 and 4.15).

Worryingly, assuming that the initial deficits were genuine (Figure 4.11), it is worth noting that these results seem to contradict findings by Nelson et al. (2020) who used similar methodology. Nelson et al. (2020) placed iDREADDs in the anterior thalamus to examine spatial working memory following both systemic deactivation of the region and then specifically targeting its inputs to the dorsal subiculum (Nelson et al., 2020). Unlike the present results, systemic activation of the iDREADDs did not

influence rats' performance on the Standard T-maze but did impair the maze Rotation condition. Similarly, animals' performance on the Rotation condition was also affected when only the inputs from the anterior thalamus to the hippocampal formation were disrupted (Nelson et al., 2020).

Other than the final level of expression, the position of the viral expression within the anterior thalamus may help explain the differing results. Another potentially important difference between the present experiment and that by Nelson et al. (2020) is the dosage of clozapine used for the i.p. systemic injections. Nelson et al. (2020) used a dosage of 4mg/kg which is more than six times the dose of 0.6mg/kg administered here. The lower dose was deemed appropriate on the basis of unpublished preliminary data showing that doses above this level affect animal's mobility, introducing other potential confounds (see Appendix B).

The appropriate dosage for ligands used to activate DREADDs has long been debated (Roth, 2016; Stachniak et al., 2014) and seems to differ across studies, species, and the constructs used (IIg et al., 2018; Jendryka et al., 2019; Nelson et al., 2020) (also see *Chapter 1, section 1.8.2*) making it harder to compare seemingly similarly designed studies. A further hurdle when interpreting and comparing results from different studies using DREADDs which are systemically activated, lies in the individual differences in drug metabolism and their potential off-target effects (Goutaudier et al., 2019; Roth, 2016). This may be particularly problematic in studies using smaller number of animals, where the attrition rate may be higher due to inadequate viral expression, which can then make a sample more susceptible to ceiling and floor effects. Combining iDREADDs with cannulation and delivering clozapine locally should be one way of avoiding these technical issues.

Both infusion experiments in this chapter and those by Nelson et al. (2020) explored anterior thalamic efferents. In contrast, Nelson et al. (2020) showed robust effects when the inputs from the anterior thalamus to the dorsal subiculum and *vice versa* were silenced. Similarly, when the dorsal subiculum inputs to the retrosplenial cortex are silenced, impairment become apparent (see *Chapter 3*). These effects reflect the complex anatomical and functional relationship between the hippocampal formation, anterior thalamic nuclei and the retrosplenial cortex, which consists of both direct

and indirect interconnections (Aggleton & O'mara, 2022; Bubb et al., 2017; Horikawa et al., 1988; Kinnavane et al., 2019; Pothuizen et al., 2009; Sripanidkulchai & Wyss, 1986; Sutherland & Hoesing, 1993). The same experiments point to a central role for the dorsal subiculum. Although, it can be supposed that the localised infusions showed a null effect due to a failure in the iDREADDs suggested by the lack of effect following systemic inhibition, it is worth noting that not all studies of anterior thalamic lesions have observed spatial deficits (Greene & Naranjo, 1986; Beracochea et al., 1989; Beracochea & Jaffard, 1994) and their presence may depend on the type of cues and strategies available to the animals.

The finding that the disruption of the anterior thalamic afferents to the dorsal subiculum (Nelson et al., 2020) has differing effects to targeting anterior thalamic projections to the retrosplenial cortex (present study) suggests that these inputs may be functionally distinct, reflecting the different functional characteristics of the dorsal subiculum and the retrosplenial cortex. The hippocampus, for example appears to be involved in most if not all aspects of initial acquisition and storing of spatial information (see Bird & Burgess, 2008), and therefore the findings in *Chapter 3*, and those by Nelson et al. (2020) would be consistent with this functions. Contrary, however, given that not all studies of spatial memory have observed deficits following damage to the anterior thalamic nuclei (Greene & Naranjo, 1986; Beracochea et al., 1989; Beracochea & Jaffard, 1994) one may argue that the integrity of anterior thalamic inputs to the retrosplenial cortex are not always essential for spatial working memory (or at least not for the spatial processes measured by the T-maze). One element may be how the extensive pre-training used at every step may have diminished any impact of changing the task conditions between Standard and Rotation.

It is also worth remembering that anterior thalamics function goes beyond that of supporting spatial learning (for a review see Nelson (2021). For instance, behavioural paradigms that required rats to discriminate between multiple objects presented in discrete temporal blocks (between block recency), as well as multiple objects presented at different time points within a single temporal block (within bloc

recency) reveal anterior thalamic lesion impairments for the latter condition (Dumont & Aggleton, 2013). This impairment may reflect the anterior thalamic connectivity with the retrosplenial cortex since animals with retrosplenial lesions, show a similar pattern of impairment on that same task (Powell et al., 2017). Additionally, DREADDs-assisted inhibition of the anterior cingulate cortex terminals within the anteromedial and anteroventral nuclei has confirmed the interaction between the two sites for attentional processes (Bubb et al., 2020), again highlighting non-spatial functions of the anterior thalamic nuclei. Together, these findings point to the conclusion that while the direct dorsal subiculum inputs to the retrosplenial cortex may support animals to attend to relevant information when solving the Rotated T-maze, the anterior thalamic inputs may aid retrosplenial functions that are not captured by this behavioural paradigm.

As discussed in *Chapter 1, section 1.7. 4*, it has been shown that lesions to the anterior thalamic nuclei do not usually impair tasks which can be solved using egocentric strategies (Aggleton et al., 1996; Warburton et al., 1997; Mitchell & Dalrymple-Alford, 2005; Wolff et al., 2006). Both the Standard and Rotation T-mazes can be solved using these strategies and the lack of impairment observed here suggests that, unlike dorsal subicular-retrosplenial interactions, the anterior thalamic – retrosplenial interactions are not critical for the integration of intra-maze cues. Interestingly, Greene and Narajno's (1986) study that described animals with discrete lesions within the anterior thalamic nuclei (anteroventral or anteromedial) used a self-return, continuous T-maze task that encouraged the use of egocentric and intra-maze cues. No deficits were observed. The expression of the iDREADD virus in the experiments described in the current chapter also suggested that these effects may be specific to the position and extent of the lesions, reflecting the cumulative functions of the various individual nuclei.

Finally, it is worth noting that in the discrete trial version of the T-maze used here, animals are picked up after the sample trial. Such interruptions seem to cause animals to lose the use of egocentric working memory (Futter & Aggleton, 2006), contrary to the continuous version of the task which may allow animals to adopt egocentric strategy. Nevertheless, it is only when testing animals on tasks such as

the Opposite arm T-maze condition used in *Chapter 3* that the use of egocentric cues can be removed. Such a condition might have revealed anterior thalamic-retrosplenial cortex deficits. Additionally, it would be interesting to see what the effects of silencing the inputs from the retrosplenial cortex to the anterior thalamus would be, and how these would compare to the results reported here, alongside extending the testing to some of the non-spatial tasks mentioned above.

Chapter 5

5. Discussion

The retrosplenial cortex is one of the largest cortical areas in the rat's brain and a key structure within the hippocampal-diencephalic-cingulate network, yet its exact cognitive functions remain uncertain. The work presented in this thesis sought to examine its unique contribution to spatial working memory by separately examining its role in concert with the dorsal subiculum and the anterior thalamic nuclei. This was achieved by investigating the circuitry of the retrosplenial cortex and then attenuating the activity of just the dorsal subicular or anterior thalamic projections that terminate within retrosplenial cortex.

Chapter 1, section 1.3.4. presented the results of mining publicly available data on the connectivity of the mouse from the Allen Mouse Brain Connectivity Atlas. Data relating to retrosplenial cortex were quantified and used to inform a between species comparison with the known connectivity of the retrosplenial cortex in the rat. *Chapter 2* examined the connectivity of the rat retrosplenial cortex further by placing pairs of retrograde tracers within different portions of the retrosplenial cortex, medial prefrontal cortex, and the anterior cingulate cortex to establish if the anterior thalamic nuclei have joint influences over these sites. *Chapter 3*, used inhibitory DREADDs placed within the dorsal subiculum, combined with localised cannulation of the retrosplenial cortex to inhibit just the dorsal subiculum projections terminating within the retrosplenial cortex. Those animals were tested on several T-maze variations, taxing the use of different spatial cues, and thus making it possible to examine the importance of these inputs for flexible spatial processing. Finally, *Chapter 4* used the same technique and experimental manipulations as *Chapter 3*, but to extend our understanding of the significance of the anterior thalamic inputs to the retrosplenial cortex.

5.1. Summary of findings

A summary of statistical and experimental findings in this thesis can be found in Table 5.1. Many previous studies have investigated the role of the retrosplenial cortex in spatial memory (Aggleton et al., 1995; Dudchenko, 2001; Pothuizen et al., 2009,2009,2010; Vann et al., 2003; Vann & Aggleton, 2002, 2004)(*see Chapter 1, section 1.5.3*). However, a criticism of many of these studies is that they involved varying degrees of tissue damage, often sparing large portions of the retrosplenial cortex (Vann et al., 2009). Depending on the techniques used, there is the added risk of damage to fibres of passage, including those of the cingulum bundle. To complicate matters further, the cytoarchitecture (*see Chapter, section 1.2*) and connectivity (*see Chapter, section 1.3)* of the subregions within the retrosplenial cortex point to functional differences. Only a small number of lesion studies have attempted to separate those functions (e.g., Hindley et al., 2014; Pothuizen et al., 2009, 2010; Vann & Aggleton, 2005) as this has proved technically challenging.

Table 5.1. Summary of Findings.

The table summarises the experimental findings presented in the thesis.

Chapter	Method	Condition	Results
Chapter 1, section 1.3.4.	Data mining of the Allan Mouse Brain Connectivity Atlas	N/A	The connectivity of the mouse retrosplenial cortex is largely consistent with that of the rat. The most striking differences are observed in its connectivity with visual areas, where the mouse area 29 receives denser inputs than the rat's area 29.
Chapter 2, section 2.4.1.	Double-retrograde tracing	mPFC/Cing	Highest proportions of double-labelled cells were observed within the anteromedial nucleus (~6%) and interanteromedial nucleus (~11%) (Figures 2.2., 2.4).

Chapter 2, section 2.4.2.	Double-retrograde tracing	Cing/RSP	The highest proportions of double-labelled cells were observed in the anteromedial nucleus (~5%), interanteromedial nucleus (~10%) and the laterodorsal nucleus (1.4%) (Figures 2.4, 2.5).
Chapter 2, section 2.4.3.	Double-retrograde tracing	mPFC/RSP	Very low proportions of double-labelled cells were observed across the anteroventral nucleus (~3%), anteromedial (~0.13%), and the interanteromedial nucleus (~0.6%)(Figures 2.4, 2.6).
Chapter 2, Section 2.4.4.	Double – retrograde tracing	RSP/RSP	The largest proportions of double-labelled cells were observed in the anterodorsal nucleus (~15%), anteroventral nucleus (~9%), and the anteromedial nucleus (~6%)(Figures 2.4, 2.7).
Chapter 3	Inhibition of dorsal subiculum inputs to the retrosplenial cortex	Standard T-maze (all cue types available)	No deficits were observed between the iDREADDs and GFP-control groups or within the iDREADDs group.
Chapter 3	Inhibition of dorsal subiculum inputs to the retrosplenial cortex	Start T-maze (all cue types available; requires cognitive flexibility)	No deficits were observed between the iDREADDs and GFP- control groups or within the iDREADDs group.
Chapter 3	Inhibition of dorsal subiculum inputs to the	Rotation T-maze (intra-	There was a statistically significant deficit within the iDREADDs group, with animals displaying impaired

	retrosplenial cortex	maze cues are restricted)	performance when these inputs are silenced.
Chapter 3	Inhibition of dorsal subiculum inputs to the retrosplenial cortex	Opposite arm maze (egocentric cues are restricted)	There was a statistically significant deficit within the iDREADDs group, with animals displaying impaired performance when these inputs are silenced.
Chapter 3	Inhibition of dorsal subiculum inputs to the retrosplenial cortex	Dark T-maze (allocentric cues are restricted)	There was a statistically significant deficit within the iDREADDs group, with animals displaying impaired performance when these inputs are silenced.
Chapter 4, Experiment 1, section 4.3.1.	Inhibition of anterior thalamic inputs to the retrosplenial cortex	Standard T-maze (all cue types available)	No deficits were observed between the iDREADDs and GFP- control groups or within the iDREADDs group.
Chapter 4, Experiment 1, section 4.3.1.	Inhibition of anterior thalamic inputs to the retrosplenial cortex	Rotation T-maze (intra-maze cues are restricted)	No deficits were observed between the iDREADDs and GFP- control groups or within the iDREADDs group.
Chapter 4, Experiment 2, section 4.3.2.	Systemic inhibition of the anterior thalamus	Standard T-maze (all cue types are available)	Following the systemic inhibition of the anterior thalamic nuclei, iDREADDs animals showed a significant impairment relative to the saline injections but not to the GFP-control animal. These effects disappeared following repeated systemic injections.
Chapter 4, Experiment 2,	Systemic inhibition of the anterior thalamus	Rotation T-maze (intra-maze cues are restricted)	No deficits were observed between the iDREADDs and GFP- control groups or within the iDREADDs group.

section 4.3.2.			
Chapter 4, Experiment 2, section 4.3.2.	Inhibition of anterior thalamic inputs to the retrosplenial cortex	Standard T-maze (all cue types are available)	No deficits were observed within the iDREADDs group.
Chapter 4, Experiment 2, section 4.3.2.	Inhibition of anterior thalamic inputs to the retrosplenial cortex	Rotation T-maze (intra-maze cues are restricted)	No deficits were observed within the iDREADDs group.

Previous investigations have identified populations of neurons originating in the anterior thalamic nuclei that simultaneously project to retrosplenial cortex and other regions such as the anterior cingulate cortex (Horikawa et al., 1988), and the medial prefrontal cortex (Pei et al., 2021). Additionally, thalamic collateral projections to multiple regions within the retrosplenial cortex have also been observed (Horikawa et al., 1988) although other studies reported contradictory results (Sripanidkulai & Wyss, 1986). Given the significance of thalamo-cortical projections for learning and memory (Vann et al., 2009; Yamawaki et al., 2019a,b) it is important to establish if anterior thalamic projections collaterise across the retrosplenial cortex. To address this gap, *Chapter 2* re-examined the collaterisation of neurons originating from the anterodorsal, anteroventral, anteromedial, interanteromedial, and laterodorsal nuclei (see also Table 5.1, Figure 2.9) to different parts of the rostro-caudal division of the retrosplenial cortex, medial prefrontal, and anterior cingulate cortices.

The analysis showed that neurons innervating the medial prefrontal/ anterior cingulate cortices and anterior cingulate/retrosplenial cortices were more prevalent near the thalamic midline. In contrast, neurons that collaterise to reach different rostro-caudal parts of the retrosplenial cortex were present in all thalamic nuclei, with the highest proportions observed within the anterodorsal nucleus (this is discussed in

more detail in *Chapter 2*). Although these findings concerning dual projections within retrosplenial cortex contradict an earlier study (Sripanidkulai & Wyss, 1986), they are consistent with the findings of Horikawa et al. (1988). Furthermore, as the anterodorsal nucleus provided the highest proportion of bifurcating neurons, this result aligns with its significance as a source of retrosplenial head-direction information (Taube, 1995; 2007). In doing so, it provides one component for the flexible spatial processing provided by retrosplenial cortex.

Like the anterodorsal nucleus, the laterodorsal nucleus contains numerous head-direction cells (Mizumori & Williams, 1993; Taube, 2007), the latter are seemingly more concerned with visual information processing (Dillingham et al., 2015). Moreover, the laterodorsal nucleus does not receive head-direction signals directly from the lateral mammillary bodies Their different connectivity may be reflected by the respective properties of the regions and help explain the differences observed regarding their inputs to the retrosplenial cortex. While the anterodorsal and laterodorsal nuclei functions may be complementary, their inputs to the retrosplenial cortex appear to be widespread and likely support subtly different aspects of direction signalling and topography. To a lesser extent, the anteroventral and anteromedial nuclei also have projections that collateralise across the retrosplenial cortex, as well as some joint projections to the medial prefrontal/retrosplenial cortices and anterior cingulate/retrosplenial cortices. These connectivity patterns are again consistent with the proposed individual functions of the anteroventral and anteromedial nuclei (Aggleton et al., 2010), which emphasise the complementary roles of the three anterior thalamic nuclei when supporting a variety of cognitive functions, including those that relate to the retrosplenial cortex.

Although, *Chapter 2* did not investigate the collaterisation of dorsal subiculum neurons to the retrosplenial cortex, previous research has shown that in both rats and mice almost half of the dorsal subiculum neurons projecting to the retrosplenial cortex simultaneously innervate the mammillary bodies (Kinnavane et al., 2018). Additionally, another study that focused on the projections of the anteromedial nucleus found that about 10% of all identified neurons simultaneously projected to

the caudal retrosplenial cortex and dorsal subiculum (Pei et al., 2021). Together these findings demonstrate the complex circuitry that exists between the mammillary bodies/anterior thalamus, the dorsal subiculum, and the retrosplenial cortex (see Figure 5.1). Given that all of these regions have been repeatedly implicated in spatial memory and navigation (*see Chapter 1, sections 1.5.3, 1.7.2, and 1.7.4*), a key to disentangling their individual contributions is to understand the roles of their separate efferent and afferent projections.

Chapter 3 of the thesis tested the importance of the dorsal subiculum projections to the retrosplenial cortex in spatial working memory. Five novel variations of the reinforced alternating T-maze were used to determine the type of cues that dorsal subicular-retrosplenial inputs utilise to solve the mazes. The results of these experiments showed that disrupting these inputs impaired rats' spatial working memory as soon as specific cue-types were either selectively removed or put into conflict with each other (see Table 5.1). These effects were consistent with previous research using neurotoxic lesions in either of the two regions (Pothuizen et al., 2010; Potvin et al., 2007, 2010) as lesion effects were often most reliable when strategy switching is required or intra-maze and extra-maze cues are incongruent (Nelson et al., 2015; Pothuizen et al., 2008; Vann & Aggleton, 2004; Vann et al., 2003). This pattern of results was also similar to that see in lesion studies that selectively target area 29 of the retrosplenial cortex (Pothuizen et al., 2010), the retrosplenial subdivision that receives the overwhelming majority of dorsal subiculum projections.

The findings in *Chapter 3* indicate that the ability of the retrosplenial cortex to facilitate switching between different cue types and navigational strategies may be supported by the direct inputs of the dorsal subiculum, as a blanket disruption of spatial memory would have impaired animals' performance on all task variations, including when all cue types were available (Table 5.1). The conclusion is supported by how the performance levels were comparable across all five task variations, i.e., the pattern did not merely match task difficulty. It is, however, noteworthy that some of these direct retrosplenial projections simultaneously reach the mammillary bodies (Kinnavane et al., 2018) (Figure 5.1) and, therefore, it cannot be concluded with

certainty that these parallel terminations were not affected by the iDREADDs manipulations within the retrosplenial cortex.

The pattern of the behavioural effects observed in *Chapter 3* has clear similarities to those in a study that placed iDREADDs in the dorsal subiculum (Nelson et al., 2020). In that same study, iDREADDs were also placed in the anterior thalamic nuclei in separate groups of rats. That study, which looked at the effects of inhibiting the direct anterior thalamic to dorsal subiculum inputs and *vice versa*, found that both pathways were necessary for animals to solve the reinforced alternating T-maze when intra-maze and extra-maze cues are conflicted (Nelson et al., 2020). The effect of silencing the dorsal subiculum inputs to the anterior thalamic nuclei appeared somewhat more pronounced, providing further evidence of their role in facilitating strategy switching and cue-type integration. The same study (Nelson et al., 2020) found that silencing the direct anterior thalamic inputs to the dorsal subiculum can also impair these same processes. Given the connectivity of the anterior thalamic nuclei (*see Chapter 1, section 1.7.3*) and the fact that it provides an indirect way for the dorsal subiculum to influence retrosplenial function (and *vice versa*), this raises the question of whether the role of the direct anterior thalamic inputs to the retrosplenial cortex also is to support spatial working memory and cue-integration.

In an attempt to address this issue, *Chapter 4* described how iDREADDs were placed within the anterior thalamic nuclei and combined with cannulations of the retrosplenial cortex. Those experiments investigated the effects of both systemic inhibition (where the activity of the projections from the anterior thalamic nuclei to multiple sites is reduced) and local inhibition (where only the anterior thalamic projections that terminate within the retrosplenial cortex were silenced). The animals were tested on two variations of the reinforced alternating T-maze, one where all cue types are available to solve the maze, and one where intra-maze and extra-maze cues are put in conflict (Table 5.1). While initially systemic inhibition of the anterior thalamic nuclei resulted in significant alternation deficit, the effects diminished with repeated testing. The various possibilities for this inconsistent finding, as well as its implications and study limitations, are discussed in more detail in *Chapter 4*. The

different dosage of clozapine and individual differences in its metabolism, makes it difficult to determine whether the findings in Chapter 4 were a false negative or whether the training regime created sufficient resilience against the procedure.

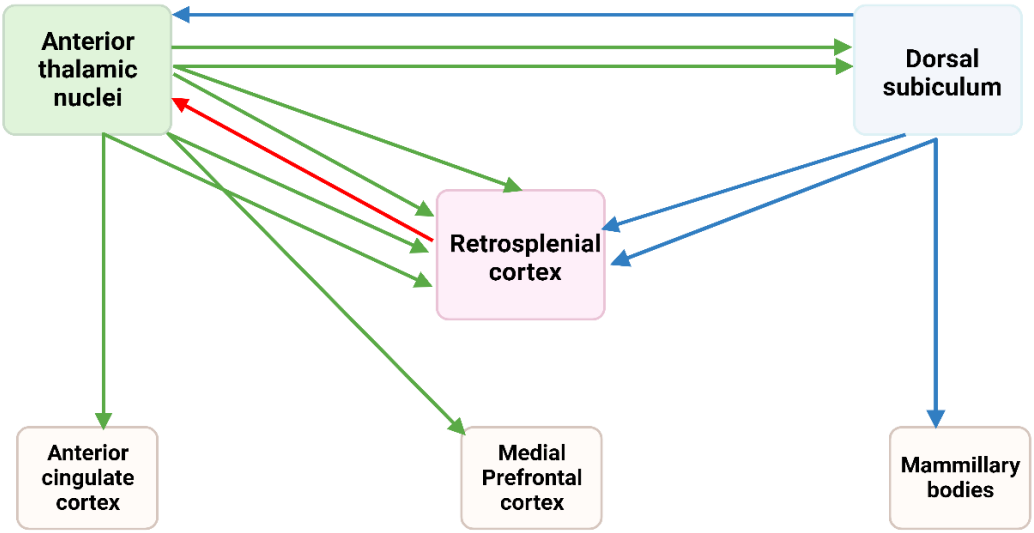


Figure 5.1. Summary of the connectivity between the retrosplenial cortex, dorsal subiculum, and the anterior thalamic nuclei.

Schematic summary of experimental findings described in Chapter 2, including those of Kinnavane et al. (2018) and Pei et al. (2021). The figure shows the direct projections between the retrosplenial cortex-anterior thalamic nuclei and the dorsal subiculum, as well as the bifurcating projections of the dorsal subiculum (to the mammillary bodies) and those from the anterior thalamic nuclei involving the retrosplenial cortex. Retrosplenial cortex projections to the two key regions of interest are presented in red, the projections of the anterior thalamic nuclei are in green, and the projections of the dorsal subiculum in blue.

Although the expression of the iDREADDs virus may not have been sufficient to cause observable effects following systemic inhibition, the expression of the virus within the retrosplenial cortex (*see Chapter 4*) appeared comparable to that

described by Nelson at al. (2020). However, silencing the anterior thalamic projections that terminate within the retrosplenial cortex did not have the anticipated effect on animals' behaviour. While it is possible that this reflects functional segregation within the anterior thalamic nuclei (discussed further in *Chapter 4*), it may be the case that these inputs are not required by the retrosplenial cortex to facilitate cue-integration and switching between navigational strategies once these are established. A major difference between the dorsal subiculum-retrosplenial connectivity and the anterior thalamic-retrosplenial connectivity is that the latter are heavily reciprocal (see *Chapter 1, section 1.7*). As the iDREADDs would not affect the retrosplenial to anterior thalamic projections, this relationship may have dampened any potential effects. In addition, the anterior thalamic nuclei also have indirect pathways to the retrosplenial cortex through the parahippocampal cortex and dorsal subiculum.

Previous research has shown that lesions to the anterior thalamic nuclei do not typically impair egocentric strategies (Aggleton et al., 1996; Green & Naranjo, 1986; Warburton et al., 1997; Mitchell & Dalrymple-Alford, 2005; Wolff et al., 2006) but deficits are apparent when animals rely on allocentric information. Both variations of the T-maze task used in *Chapter 4* allow animals to draw on allocentric information, but this is not the only available strategy (see *Chapter 4*). Additionally, the seemingly different contributions of the anterior thalamic nuclei to cue-type switching within the retrosplenial cortex (Chapter 4) and within the dorsal subiculum (Nelson et al., 2020) possibly reflect the different functions and connectivity of the two sites. It is also worth noting that while the anterodorsal nucleus contains the majority of head-direction cells within the anterior thalamic nuclei and is the main source of single and bifurcating projections (*Chapter 2*) to the retrosplenial cortex, these head-direction cells may not necessarily be needed for successful navigation in the T-maze, where directional choices are highly constrained (Dillingham & Vann, 2019).

In a study closely related to those in *Chapters 3 and 4,* Yamawaki et al. (2019b) targeted populations of neurons that originate in CA1 of the hippocampus, alongside projections originating from the anterior thalamus that terminate within the granular

retrosplenial cortex. Chemogenetically disrupting these projections showed that they have opposing actions on contextual fear conditioning as measured by freezing behaviour. Silencing the CA1 to retrosplenial projections increased freezing behaviour in mice during retrieval while silencing the anterior thalamic projections to the retrosplenial cortex reduced it. Yamawaki et al. (2019b) concluded that while both circuits are engaged in the encoding of the contextual fear memory, they have opposing roles, with the inhibitory CA1 – retrosplenial pathway supressing expression of context memories and potentially modulating the excitatory anterior thalamic-retrosplenial pathway. Although, in Yamawaki et al. (2019b), anterior thalamic injections were targeted at neurons originating in the anteroventral nucleus, meaning that it is possible that their findings reflect functional specialisation within the anterior thalamic nuclei, it would still have been informative to expand the findings of *Chapter 4* to other spatial tasks and non-spatial tasks, such as the Morris Water Maze and object-recognition/recency in order to draw more general conclusions.

Additionally, previous research has identified a small proportion (~10%) of neurons originating in the anteromedial nucleus that project to both the dorsal subiculum and the retrosplenial cortex (Pei et al., 2021). While the proportions of bifurcating neurons that originate in the anterodorsal and anteroventral nuclei and terminate in the dorsal subiculum and retrosplenial cortex has not been investigated, such connections can provide parallel routes by which the anterior thalamic nuclei support retrosplenial function, i.e., indirectly via the dorsal subiculum. Additionally, as established in *Chapter 2* (see Table 5.1), the anterior thalamic nuclei, particularly the anteromedial nucleus (and to a lesser extent the anteroventral nucleus) provides parallel modulation of the medial prefrontal/retrosplenial cortices and anterior cingulate/retrosplenial cortices. Considering the connectivity of the anteromedial nucleus individually (see *Chapter 1*) and the anterior thalamus in general, its reciprocal connections with both the hippocampal formation and the prefrontal cortices (as a part of the Default Mode Network) imply a role of the nucleus in decision making, cognitive flexibility, executive function, and potentially in the facilitation of task and/or cue switching (Chastil et al., 2018; Lega, 2012; Maguire, 2001; Svoboda et al., 2006; Tesche & Karhu, 2000) (see *Chapter 1, section 1.4.1*).

This position as a prefrontal – hippocampal mediator may help to explain why the behavioural tasks adopted in Chapter 4 seemed insensitive, as the retrosplenial cortex could potentially be bypassed. A further implication comes back to the desire to extend the experimental paradigm adopted in Chapter 4 to non-spatial problems such as object-recognition/recency and go-no-go tasks when testing systemic clozapine.

5.2. Implications for retrosplenial cortex function

Beginning with the dense anterior thalamic bifurcating and non-bifurcating inputs to the retrosplenial cortex as described in *Chapter 2*, it remains most likely that these thalamic nuclei are of key importance for retrosplenial function. The experimental findings of Chapters 3 and 4 (see Table 5.1) collectively suggest that while the dorsal subiculum inputs to the retrosplenial cortex are of critical importance for spatial-cue integration and switching between navigational strategies, the anterior thalamic inputs are less so. Instead, as previous research has demonstrated, the anterior thalamus may support the dorsal subiculum in these functions (Nelson et al., 2020) and, therefore, provide its spatial contribution to the retrosplenial cortex, indirectly through the dorsal subiculum pathway. It may also be relevant that despite appearing simple, the T-maze task is complicated, and manipulation of extra-maze and intra-maze cues may not always be sufficient to disrupt rats' performance on the task. Indeed, previous research has shown that although rats may use landmarks to guide their behaviour, animals appear to utilise other sources of information, such as their acquired sense of direction (Dudchenko, 2001).

Thus, it remains possible that the direct anterior thalamic pathway is responsible for aspects of cognition that are not directly measured by the T-maze task or at least not by the variations adopted in *Chapter 4*. For example, initial acquisition of the alternation task was never challenged. Furthermore, the various, parallel pathways that exist between the anterior thalamus and the retrosplenial cortex, as well as the dorsal subiculum and the retrosplenial cortex, imply that while these interactions are

critical for normal functioning, they are able to provide cognitive support when one of the pathways fails. One may speculate that the early metabolic changes observed within the retrosplenial cortex in humans (*Chapter 1, see section 1.4*) reflect more subtle changes in its subcortical connectivity, and thereby functional changes, that cannot be detected until compensatory mechanisms deteriorate, reaching a tipping point from which is impossible to come back.

Topographical disorientation, i.e. getting lost whilst navigating in familiar or unfamiliar environments, is one of the earliest symptoms of Alzheimer's disease (Reisberg, 1982) and often presents subtly before any other symptom appears. This pattern is consistent with the early metabolic changes observed within the retrosplenial cortex (Nestor et al., 2003). It is also clear that spatial navigation is a multifaceted process supported by multiple structures and processes, such as memory, attention, and decision-making. All of these, to at least some extent, require interval timing, that is the ability to execute the correct behaviour and process in the milliseconds to seconds range (see Merchant & Lafuente, 2014 for a review). Within the default mode network, the retrosplenial cortex is perfectly anatomically positioned to support this function (Kaboodvand et al., 2018).

Although Todd et al. (2015) successfully implicated the retrosplenial cortex in temporal discrimination learning in rodents, the lesions also affected the supplementary motor areas, which are known to be some of the structures that govern interval timing in humans (Mita et al., 2009). Additionally, recent evidence shows that the retrosplenial cortex may be critical for guiding decision-making, since its inactivation in mice reduced animals' reliance on rewarded-choice history information, suggesting that the retrosplenial cortex is involved in encoding behaviour-related temporal information (Danskin et al., 2023). However, precisely measuring temporal processing and its relation to decision-making processes in animal models remains challenging, particularly in the context of its application to human behaviour. As such, temporal perceptions and decision-making are often not considered when trying to define the role of the retrosplenial cortex in spatial navigation. Nevertheless, I would speculate that this is one of the reasons why the

retrosplenial cortex appears to be involved in a large variety of functions, spatial and non-spatial alike (see Chapter 1, sections 1.4 and 1.5). In this way it may govern common underlying processes, such as behavioural timing and decision-making, which are challenging to measure in animal models.

5.3. Limitations and future directions

5.3.1. Limitations of retrosplenial cortex tracing studies

As discussed in Chapter 1, the retrosplenial cortex has complex cytoarchitecture and connectivity. Numerous divisions exist based on its anatomy, and there is growing evidence for functional segregation both between its subregions and along its rostro-caudal axis in both rodents (see section 15) and humans (see section 1.4). These functional differences are at least, in part, driven by its cortical and subcortical inputs. As discussed in Chapter 2, contradictions in the literature had existed regarding the collaterised projections that the retrosplenial cortex receives. To an extent these contradictions may be driven partly by different cell-counting and imaging methods. Additionally, the different mechanisms and limitations of the specific tracers used across anatomical studies may also contribute to the contradictory findings.

Chapter 2 used two retrograde tracers: Cholera toxin subunit B (CTB) and Fast Blue (FB). CTB is an organic tracer that was first introduced in 1977 (Stoeckel et al., 1977). Its fluorescent signal strength, rapid transport (2-7days), low toxicity and ease of use makes it suitable for both *in vitro* (Korim et al., 2014) and *in vivo* (Yamashita & Petersen, 2016) studies. Although, it is considered a retrograde tracer, like most conventional tracers, CTB is to some extent bidirectional (Noseda et al., 2010). Fast Blue, on the other hand, is an inorganic tracer that is not dependant on active transport and can be used for identification of projections in fixed tissues postmortem as well as *in vivo* (Saleeba et al., 2019).

Conventional tracers have a number of limitations: (1) they can be taken up by fibres of passage (Dado et al., 1990) which can lead to the erroneous identification of projections; (2) the spread of the tracer around the injection site can result in labelling that is non-specific, and may overemphasise the significance of distant outputs (Saleeba et al., 2019); and (3) tracers such as CTB can travel in both retrograde and anterograde directions in axons, which complicates circuit analysis (Noseda et al., 2010). While as in Chapter 2, care can be taken to avoid uptake by fibres and tracts such as the cingulum bundle, little can be done to control the other limitations. These same limitations may be particularly problematic in regions such as the retrosplenial cortex, which have extensive intrinsic connectivity (see Chapter 1, section 1.3.1).

These limitations may then affect the methodology and technologies used to capture, analyse, and present the findings. As in Chapter 2, data are still mostly presented in terms of numbers of labelled neurons contained within the brain region of interest, averaged across sections and/or estimated by comparing images of sections, yielding highly varying numbers and proportions (e.g. Chapter 2, and Pei et al., 2021). Expressing such large data sets in crude numbers limits the analysis that can be caried out by other researchers and opens the data to future reclassification and criticism (Dempsey et al., 2017; Saleeba et al., 2019). Additionally, the process is very time-consuming and prone to error as it is dependent on correctly and consistently cutting the histological sections, as well as mounting and organising them in the correct order, and orientation. The imaging technique used and the manual and/or automatic cell counting, can yield very different results and require a number of verifications. As it can be seen in Chapter 2, the lack of a preliminary established systematic approach to sectioning and imaging required a number of verifications through confocal microscopy, along with additional counting by a second researcher. Although numerical proportions were sufficient for the purposes of the investigation, i.e., to quantify and contrast the retrosplenial and anterior cingulate thalamic inputs to these of the medial prefrontal cortex, a more systemic

approach to surgical procedures, tissue processing, imaging, and using stereology would have been more informative and extend the longevity of the data set. Such refinements matter as although connectome data cannot by itself explain how the brain works, the connectivity of a region and its cytoarchitecture is one of the way neuroscientists can gain an insight into a brain regions' function.

5.3.2. Limitations of DREADDs as an instrument for neuronal manipulation and behaviour

As discussed in Chapter 4, the results that were observed following the systemic infection of clozapine appear to contradict some previous findings (Nelson et al., 2020) and raise the question of whether the lack of effect on rats' behaviour following the localised inhibition was a genuine effect or simply caused by failure of the iDREADDs to activate, e.g., inhibit transmission. Furthermore, the different dosages of clozapine used between Chapter 4 and Nelson et al. (2020) as well as between Nelson's et al. experiments raises further questions about the effectiveness of iDREADDs as an instrument for neuronal manipulation. As it was discussed in Chapter 1, section 1.8, unlike permanent lesions, iDREADDs do not completely eliminate activity within a region, but rather attenuate it. Consequently, the effect size of the observed behaviour (or lack of) may be proportionate to the level of expression within the targeted site. As discussed in Chapter 4, it is known that lesions of the anterior thalamic nuclei produce the most severe effects when all three nuclei are damaged (Aggleton et al., 1996; Van Groen et al., 2002). Therefore, if iDREADDs did not produce enough attenuation, behavioural effects will be small or not evident at all, reflecting a functional tipping point. Therefore, the ability of iDREADDs to effectively attenuate neuronal function, is in part a consequence of its effective transport within the injection site and its projections.

As discussed in Chapter 1, section 1.8, iDREADDs are packaged into viral vectors that allow them to be transported anterogradely within the brain. The iDREADDs used in Chapters 3 and 4 relied on an adeno-associated virus (AAV) for transport. In recent years AAVs have been developed as alternatives to conventional chemical tracers and, like the conventional tracers discussed in section 5.3.1, they are not free of limitations. Typically, the wild-type virus is modified, removing genes required for viral replication, and replacing them with genetic sequences that encode reporter proteins (e.g. CaMKII), giving static viral tracers similar properties to conventional tracers (Saleeba et al., 2019). The major differences are in the duration of recovery that typically requires 10-20 days, as well as the volumes injected, which are typically larger (Saleeba et al., 2019). Although the AAV stereotype 5, which was used in Chapters 3 and 4, is considered to travel anterogradely when injected in the rodent nervous system, it also exhibits retrograde transport which may vary according to the region that is being targeted (Rothermel et al., 2013; Castle et al., 2016).

The viral titre (which varied across the experiments and groups) also affects the direction of travel as they can become retrograde with higher titres as the immune response limits their efficacy (Howarth et al., 2009). Unsurprisingly, variations in the viral surface properties by human manipulation can also alter their affinity for cellular binding (Kanaan et al., 2017; Seleeba et al., 2019). Additionally, it is widely considered that excitatory principal neurons (which the iDREADDs in Chapter 3 and 4 targeted), but not inhibitory cells express CaMKIIα, resulting in widespread of CaMKIIα promoter-driven protein expression. However, recent research has shown that in addition to pyramidal neurons, GABAergic neurons are also targeted by these viruses in rodents and can be visualised in interneurons, including parvalbumin (PV) expressing cells (Veres et al., 2023). Veres et al. (2023) tested CaMKIIα promoter driven, AAV5 and AAV9 virus stereotypes and found expression in a wide variety of interneurons that together covered around 60% of the whole inhibitory cell population in cortical areas, which challenges the use of CaMKIIα promoter-driven protein expression as a tool to target glutamatergic neurons.

Additionally, although the selectivity of chemogenetic tools is improving (Saleeba et al., 2019), research shows that the level of hM4Di DREADD expression is dependent on the titre and that different titres of viral loads can have different impacts on DREADD-mediated behaviour (Goossens et al., 2021). For instance, when injected in the hippocampus, higher titre DREADDs have higher expression levels than lower titre DREADDs. Interestingly, the different levels of expression observed also produced different effects following clozapine induced activation. The evoked potentials in the higher titre group were inhibited, while the evoked potentials in the low titre group were enhanced (Goossens et al., 2021). While it is difficult to compare titres across different studies, evidence that the same iDREADD can have an opposing action within the same region depending on titres suggests that the magnitude of expression and effects on behaviour will likely vary between structures as well. Further evidence for this comes from a study showing that hM4Di expression in the dorsal hippocampus leads to an increase in c-fos expression instead of a decrease (López et al., 2016), which casts doubt of the effectiveness of using c-fos as a marker to show whether iDREADDS produce the desired effect. A more suitable approach to ascertain that DREADD manipulations are producing the desired effects would be to combine chemogenetic manipulations with electrophysiology. Similarly, to Goossens' et al. (2021) study, that approach will allow researchers to directly confirm that the desired inhibitory effect is achieved. Together, these considerations highlight the complex relationship between DREADDs technology, inhibition, and excitation in the brain. Yet despite their limitations, these technologies keep evolving, opening new pathways for further research (see *section 5.3.3*), which currently cannot be undertaken in humans.

5.3.3. Future directions

Despite the limitations of the tracing experiments in Chapter 2, that were discussed in section 5.3.1, the conclusions were informative in that a quantified proportion of anterior thalamic neurons simultaneously innervate different parts

of the retrosplenial cortex and/or the anterior cingulate cortex. Further research may aim to extend these findings by looking at bifurcating neurons that originate in the dorsal subiculum to reach different parts of the retrosplenial cortex on its rostro-caudal axis. Addressing, some of the methodological limitations by including more animals with injections centred in different medial prefrontal areas and the retrosplenial subregions will shed more light on the topographical organisations and patterns of connectivity of these regions. Furthermore, a more systematic approach to tissue processing and a new generation of automated, standardised approaches to the analysis of tracing data will benefit the generalisation across studies. Developing such methods will simplify analysis, reduce variability, and aid the sharing of data. The Allen Atlas, for instance, has proven to be a useful tool when considering the connectivity of the brain, however, it does not yet provide information on collateral connections.

Concerning the behavioural experiments described in Chapters 3 and 4, despite some limitations, the advances created by DREADDs as a method of neuronal manipulation have made it possible to experimentally examine functional anatomy in a way that is currently not possible in humans. Provided that proper experimental controls (see Chapter 1, section 1.8.3) are adopted to control for some of the limitations discussed in section 5.3.2., the biggest concern in targeting individual projections is the uncertainty around the spread of the clozapine, as well as the surgical complications and limitations that permanent cannulations of rodents can cause. For instance, cannula and repeated infusions can cause damage to the tissue of the cannulated region, limiting the histological analyses that can be carried out and potentially influencing behaviour outputs due to it becoming permanent lesion (although in theory some of these concerns should be mitigated by using cannulated GFP-controls). From an ethical point of view, cannulations can increase the risk of infection in animals and as in Chapter 4 may require an extra surgical procedure. The invention of retro-AAVs (and retrograde DREADDs) makes it possible to remove the requirement of cannulation and use a two-viral approach instead (Campbell & Marchant, 2018). In this type of experiments, a Cre-vector of retrograde type is injected into a brain region that is connected with the region receiving a Cre-dependant vector.

This potentially makes it possible to selectively manipulate projections between regions through a systemic drug injection (Campbell & Marchant, 2018).

Additionally, the development of a newer iDREADD, called k-opioid derived DREADD (KORD), that is activated by using a different ligand, salvinorin B, makes it possible to attenuate the activity of multiple brain regions in the same group of animals providing a direct comparison for behaviour (Roth, 2016; Vardy et al., 2015). For example, injecting hM4Di DREADD in the dorsal subiculum and a KORD DREADD in the anterior thalamic nuclei, combined with traditional cannulation of the retrosplenial cortex, will allow evaluation of both the individual and combined effects of attenuating these inputs. This can be achieved by, for example, first inhibiting just the dorsal subiculum inputs by clozapine infusions, then on a separate occasion inhibiting just the anterior thalamic inputs to the retrosplenial cortex by salvinorin B infusion. Finally, testing animals after infusing both clozapine and salvinorin B, should in theory simultaneously attenuate the contributions of both regions to retrosplenial function, providing a platform for direct statistical comparison of the behavioural impact these manipulations have.

Finally, it would also be interesting to attempt to disentangle the unique contributions of each anterior thalamic nuclei to the functions of the retrosplenial cortex, given speculations about their functional segregation. Most of all, the T-maze task is very functionally specific, testing only one aspect of cognition. Extending the findings contained within this thesis to other spatial tasks, including the Morris Water maze, by adopting the two-viral approach, will allow to extend the findings to other spatial domains. Additionally, attempting to extend the findings to non-spatial tasks, such as object recognition/recency or go-no-go tasks, or attempting to develop new paradigms for testing decision-making, time-perception, and discrimination, will contribute to a better understanding of the roles of the retrosplenial cortex within the cingulate-diencephalic-hippocampal network.

5.4. Conclusions

In conclusion, the results shown in this thesis demonstrate that the retrosplenial cortex connectivity with the anterior thalamic nuclei is complex, providing links between these regions to other cortical structures. Additionally, the experiments demonstrate that the dorsal subiculum inputs to the retrosplenial cortex are of critical importance for spatial working memory when flexibility is required. At the same time, manipulating the corresponding inputs from the anterior thalamus to retrosplenial cortex had little apparent effect, potentially reflecting methodological shortcomings. The current anatomical and behavioural findings highlight the complex anatomical and functional circuitry that exists in the hippocampal-diencephalic-cingulate network and provide insight into the region's unique roles in spatial working memory. It appears that the retrosplenial cortex is vital for changing spatial perspectives, and that it may serve as a facilitating region to engage cortical consolidation. Although, the widespread functional involvement of this same region suggests that some of its main functions may be in a different cognitive domain such as decision-making, something that will need to be assessed further.

Appendices

Appendix A – Allen Atlas supplementary data

Table 1. List of Allen Atlas studies included in the analysis of the connectivity of the retrosplenial cortex in the mouse.

Study ID	Injection site	Injection volume	Tracer	Mouse strain
113784293	Lateral-dorsal nucleus	0.11	EGFP	C57BL/6J
272969333	Lateral-dorsal nucleus	0.03	EGFP	C57BL/6J
272967913	Lateral-dorsal nucleus	0.02	EGFP	C57BL/6J
114427219	Anteroventral nucleus	0.17	EGFP	C57BL/6J
100142569	Anteroventral nucleus	0.16	EGFP	C57BL/6J
146658170	Anteromedial nucleus	0.21	EGFP	C57BL/6J
175374982	Nucleus of reuniens	0.21	EGFP	C57BL/6J
120875111	Paraventricular nucleus	0.13	EGFP	C57BL/6J
266585624	Lateral-posterior nucleus	0.1	EGFP	C57BL/6J
100140949	Ventral RSP	0.18	EGFP	C57BL/6J
112595376	Ventral RSP	0.09	EGFP	C57BL/6J
100148142	Ventral RSP	0.12	EGFP	C57BL/6J
112424813	Dorsal RSP	0.03	EGFP	C57BL/6J
112229103	Agranular RSP	0.04	EGFP	C57BL/6J
152994878	Subiculum	0.12	EGFP	C57BL/6J
640285199	Subiculum	0.03	EGFP	C57BL/6J
146984915	Presubiculum	0.23	EGFP	C57BL/6J
126862385	Primary visual area	0.2	EGFP	C57BL/6J
307296433	Primary visual area	0.29	EGFP	C57BL/6J
272782668	Primary visual area	0.24	EGFP	C57BL/6J
309003780	Primary visual area	0.31	EGFP	C57BL/6J
100141219	Primary visual area	0.2	EGFP	C57BL/6J
174361040	Primary visual area	0.13	EGFP	C57BL/6J
304565427	Primary visual area	0.22	EGFP	C57BL/6J

304586645	Primary visual area	0.21	EGFP	C57BL/6J
100147853	Primary visual area	0.07	EGFP	C57BL/6J
638314843	Primary visual area	0.21	EGFP	C57BL/6J
277714322	Primary visual area	0.05	EGFP	C57BL/6J
277616630	Primary visual area	0.07	EGFP	C57BL/6J
277712166	Primary visual area	0.04	EGFP	C57BL/6J
304762965	Primary visual area	0.08	EGFP	C57BL/6J
277713580	Primary visual area	0.05	EGFP	C57BL/6J
304564721	Primary visual area	0.08	EGFP	C57BL/6J
304585910	Primary visual area	0.09	EGFP	C57BL/6J
146077302	Posteromedial visual area	0.06	EGFP	C57BL/6J
114250546	Lateral visual area	0.13	EGFP	C57BL/6J
116903968	Lateral visual area	0.17	EGFP	C57BL/6J
146858755	Lateral visual area	0.07	EGFP	C57BL/6J
157062358	Postrhinal area	0.07	EGFP	C57BL/6J
272916202	Posterolateral visual area	0.03	EGFP	C57BL/6J
158435116	Ventrolateral orbital area	0.11	EGFP	C57BL/6J
112423392	Ventrolateral orbital area	0.12	EGFP	C57BL/6J
126860974	Medial orbital area	0.28	EGFP	C57BL/6J
112306316	Lateral orbital area	0.32	EGFP	C57BL/6J
180673746	Lateral orbital area	0.1	EGFP	C57BL/6J
170721670	Lateral orbital area	0.23	EGFP	C57BL/6J
157556400	Infralimbic area	0.17	EGFP	C57BL/6J
141603190	Secondary motor area	0.17	EGFP	C57BL/6J
112952510	Secondary motor area	0.29	EGFP	C57BL/6J
100141454	Secondary motor area	0.1	EGFP	C57BL/6J
157710335	Secondary motor area	0.13	EGFP	C57BL/6J
585025284	Secondary motor area	0.17	EGFP	C57BL/6J
141602484	Secondary motor area	0.21	EGFP	C57BL/6J
100141273	Primary motor area	0.24	EGFP	C57BL/6J
100141563	Primary motor area	0.17	EGFP	C57BL/6J
100141780	Primary motor area	0.18	EGFP	C57BL/6J

Figure 1. Projections from the anterior thalamic nuclei to the retrosplenial cortex.

All tracer injections were made in the right hemisphere. The Y-axis refers to the lamina in the ROI, and the X-axis depicts the projection densities in mm^3.The densities of the projections are the mean across all cases. The densest projections are from the anteroventral nucleus to the right ventral RSP (> $0.35mm^3$). Noticeably, the projections from the AM, AV, and LD nuclei have highest densities in the right layers 6a and 6b across all RSP regions. The projections to the left RSP have very low densities (<$0003mm^3$). The granular RSP cortex is termed ventral RSP (RSPv), the dysgranular RSP is subdivided into two areas: dorsal RSP (RSPd) and the most lateral dysgranular RSP (RSPagl) in the Allen Atlas.

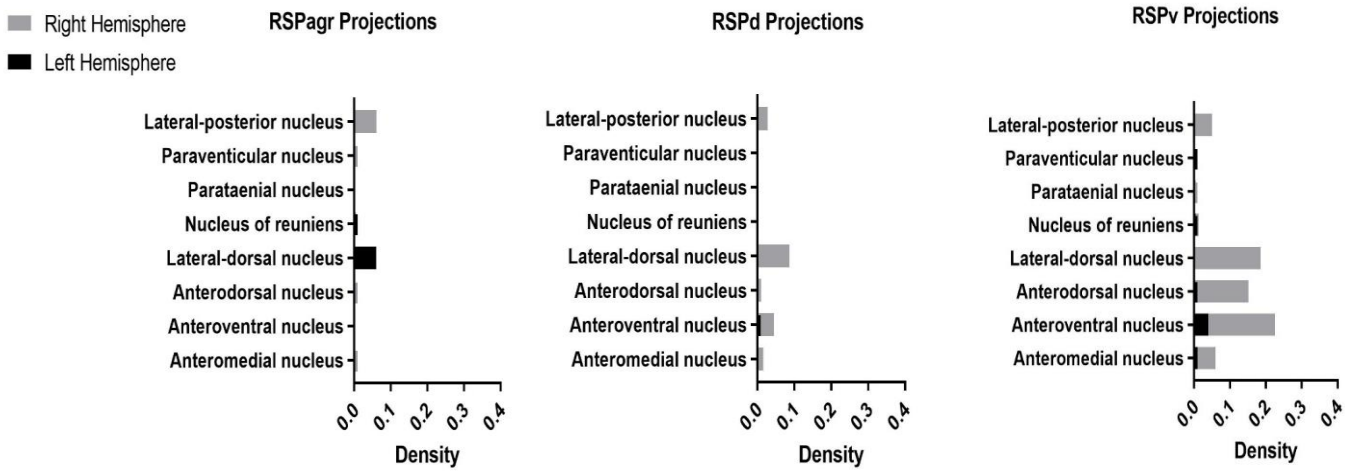
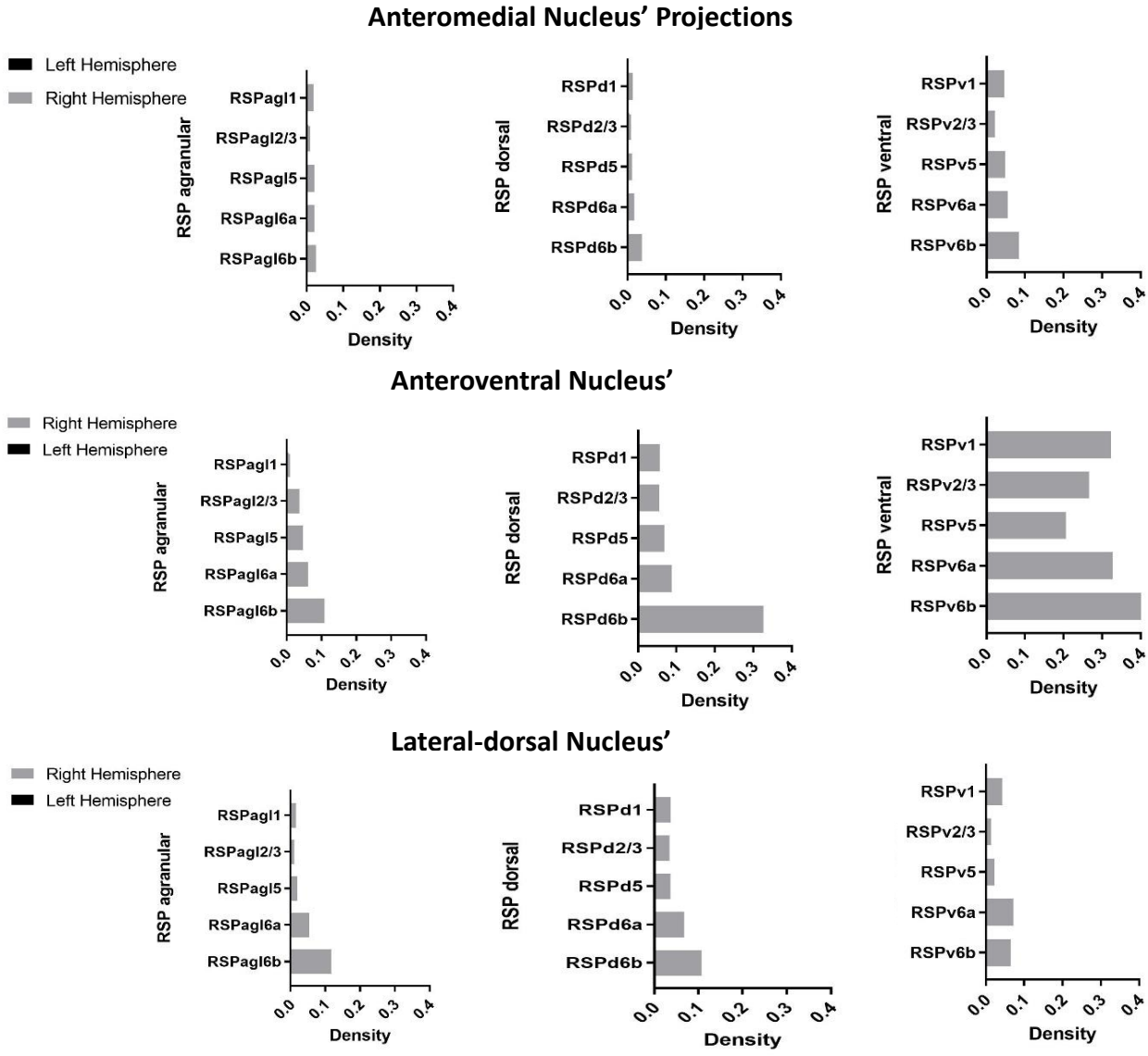


Figure 2. Retrosplenial projections to the thalamic nuclei.

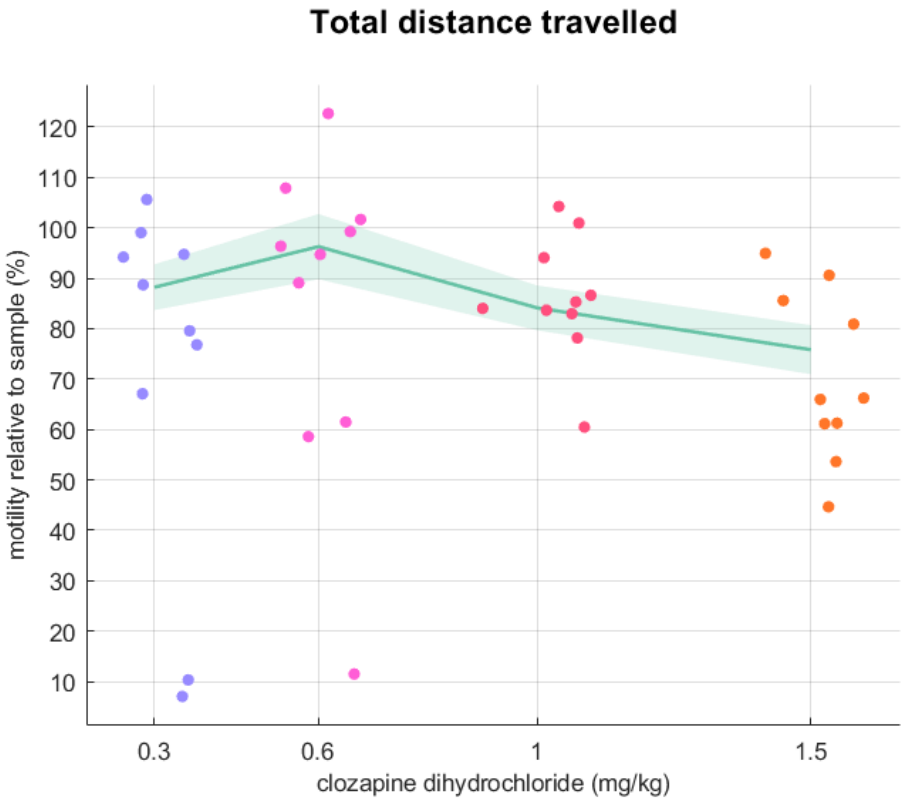
All tracer injections were made in the right hemisphere. The Y-axis refers to the ROI, and the X-axis depicts the projection densities in mm^3.The densities of the projections are the mean across all cases. The agranular RSP (lateral dysgranular) projections are most dense to the LP and the left LD, the dorsal RSP (dysgranular) projections are densest to the LP, LD, AV and AM. Notably, the granular RSP has the densest projections with the entire ATN, particularly the AV nucleus. The granular RSP cortex is termed ventral RSP (RSPv), the dysgranular RSP is subdivided into two areas: dorsal RSP (RSPd) and the most lateral dysgranular RSP (RSPagl) in the Allen Atlas.



Appendix B – Unpublished data testing dose effects of clozapine

The experiment and data collection of these data were carried out by James Perry and Michal Milczarek, who kindly shared their results.

For the experiment, the animals were habituated to a 1m x 1m open field with sawdust on the floor. All animals received three, 10-minute-long habituation sessions. Then, on separate occasions, animals received i.p. injections of different doses of clozapine, and 30 minutes after the injections they were run in the open field for 10 minutes. Their activity levels were measured using ImageJ and expressed as a percentage of sample activity (last habituation session). As it can be seen in the scatterplot below, doses of clozapine of above 0.6 mg/kg produced lower motility scores, and therefore this was the dosage selected as the maximum dose to be administered in the experiments described in Chapter 4.



Appendix C – Additional analyses of data between Cohort 1 and 2 in Chapter 4

All animals' individual data following the intracerebral infusions in Cohorts one and two were plotted as line graphs (Figures 4.10, 4.14). Following visual inspection, animals' performance between the cohorts did not seem to differ. Independent t-tests were carried out to compare the mean percentage performance of the iDREADDs animals between Cohorts one and two during pre-training. Comparisons for GFP-controls were not carried out since in Phase two, there was only one cannulated GFP-control animal. The results are summarised in Table 1 below. The differences described between the cohorts on the Standard T-maze, may be a result of the variable group size and/or the length of training. Therefore, no further attempts were made to analyse data from the two cohorts combined.

Table 1 Results of t-test comparison of training scores between the iDREADDs animals in cohorts one and two.

The table shows the means and standard deviations of iDREADDs animals in cohorts one and two for each training: pre-surgery training on the Standard T-maze, and baseline training scores for both the Standard and Rotation T-mazes.

Condition	Cohort 1 iDREADDs	Cohort 2 iDREADDs	Results
Standard T-maze			
Pre-surgery training	*M* = 85.86 *SD* = 4.76	*M* = 72.90 *S*D = 9.44	$t_s > 3.27$, $p_s < 0.004$
Baseline pre-iDREADDs activation training	*M*=88.39 *SD* = 3.96	*M* = 81.52 *SD* = 3.77	
Rotation T-maze			
Baseline pre-iDREADDs activation training	*M* = 79.46 *SD* = 6.29	*M* = 81.27 *SD* = 7.31	*t*(13) = -0.51, *p* = 0.62

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