Cingulate Cortex-Anterior Thalamic Connectivity: Anatomy and Function

This thesis is submitted to Cardiff University for the Degree of Doctor of Philosophy by

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Summary

The cingulum bundle is a highly complex fibre pathway that is implicated in a wide array of functions, yet little is known about its constituent connections and their differential contributions to cognition. This thesis investigated the dense interconnections between the cingulate cortices and the anterior thalamic nuclei, many of which join the cingulum. Initially, contemporary viral-based tract tracing techniques in the rat provided an anatomical reappraisal of this major component of the tract. This investigation revealed that many fibres between the anterior cingulate cortex and the anteromedial thalamic nucleus are present in the anterior cingulum, a subsection typically associated with executive function. Connections between the retrosplenial cortex and the anteroventral thalamic nucleus, meanwhile, primarily occupy the posterior cingulum, a subsection linked to memory.

Next, this thesis investigated the role of anterior cingulate-anterior thalamic interconnectivity in attention. Existing evidence implicates both regions in intradimensional set-shifting, where discriminations are most effectively solved by responding to a stimulus dimension that previously predicted reward. A series of DREADDs manipulations confirmed that the anterior cingulate cortex supports this attentional function in rats, and novel evidence indicated that projections to the anterior thalamic nuclei critically contribute to this capacity. This thesis further found that in the absence of normal anterior cingulate function, inappropriate attention appears to be directed to unreliable reward predictors, facilitating performance when contingencies change (extradimensional shift). These findings are best explained by dual-process theories of attention where competing learning parameters, with distinct neural underpinnings, mediate the allocation of attentional resources. One process directs attention to reliable predictors of outcomes (reliant on the anterior cingulate cortex and its actions on the anterior thalamic nuclei), while another biases attention towards unreliable predictors of outcomes.

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Statement

This is a statement to acknowledge that some of the content in Chapter 1, General Introduction, is adapted from the following published paper:

Bubb, E. J., Metzler-Baddeley, C., & Aggleton, J. P. (2018). The cingulum bundle: anatomy, function, and dysfunction. *Neuroscience & Biobehavioral Reviews, 92*, 104-127.

This relates to sections 1.1, 1.2, 1.3 and 1.4. Reuse of authored material as part of a thesis is permitted by the publishers, Elsevier (https://www.elsevier.com/about/policies/copyright/permissions).

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1 General Introduction

1.1 Overview

The research described in this thesis investigates the cingulum bundle, a medial white matter pathway that provides connectivity to an array of subcortical and cortical brain structures. There is a natural tendency to portray the cingulum as an inert pathway, i.e., its role is just to ensure the transmission of information. Our concept of white matter is, however, changing rapidly. With the advent of diffusion MRI methods to study white matter *in vivo*, widespread, dynamic cingulum contributions to function and dysfunction are being revealed.

The cingulum is, however, highly anatomically complex. Understanding its contribution to cognition must therefore begin with a deconstruction of the many subgroups of connections that together constitute the tract. A particularly substantial subset of cingulum fibres, constant across species, connect the anterior thalamic nuclei and the cingulate cortices. The first aim of this thesis is to provide a reappraisal of these principal connections, using contemporary tract tracing techniques in the rat to examine fibre properties, such as directionality, that cannot be visualised using human neuroimaging. Subsequently, this thesis will examine the contribution of fibres connecting the anterior cingulate cortex and the anterior thalamic nuclei to cognition. Chemogenetic methods are employed to alter brain activity in these circuits *in vivo* in the rat, and the resultant effects of these manipulations on behaviour are measured.

1.2 A brief history of the cingulum bundle

The cingulum bundle is one of the most prominent fibres tracts in the brain. Located within the medial surface of the cortex, it spans the dorsal surface of the corpus callosum to form a near-complete ring from the orbital frontal cortices to the temporal pole (Figure 1.1). Whilst it appears to be Reil (1809) who first appreciated the full extent of the tract, the name ‘cingulum’ is credited to Burdach (1822). While alternative terms have appeared (Swanson, 2015), the name cingulum bundle persists.

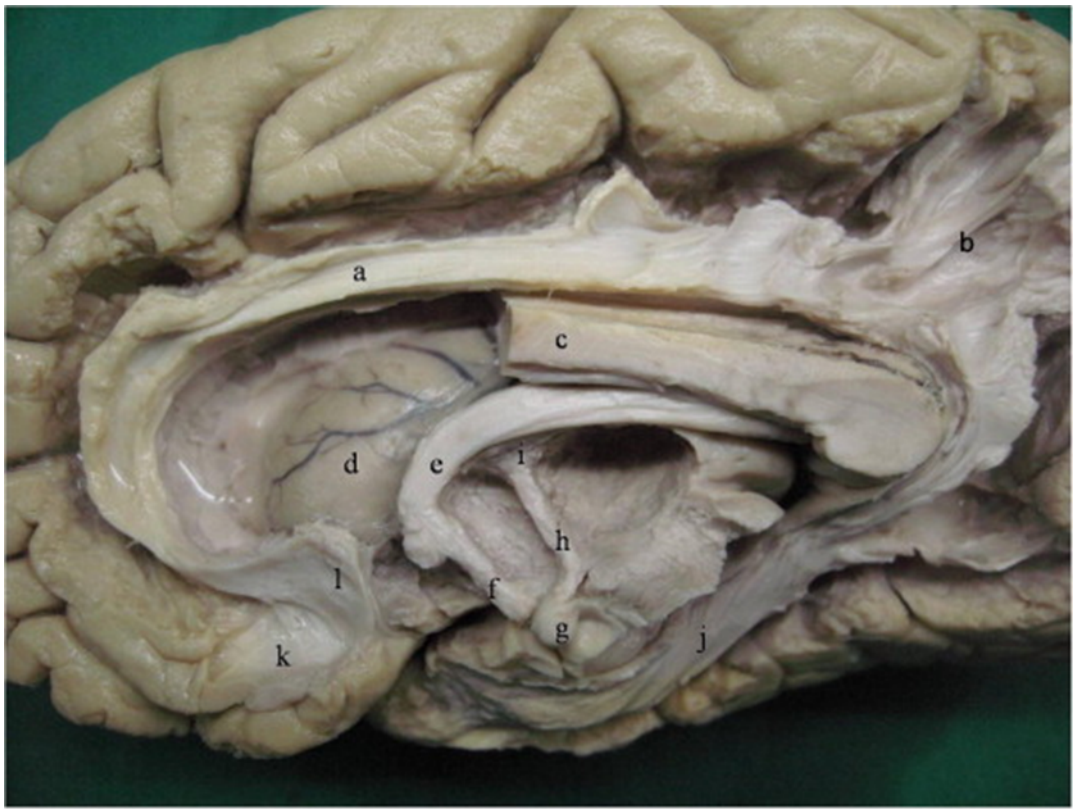

Figure 1.1 Medial aspect of the human brain after partial dissection showing the cingulum

(a) cingulum; (b) cingulum fibres entering parietal cortex (c) corpus callosum (anterior half has been removed); (d) head of caudate nucleus; (e) body of the fornix; (f) columns of the fornix; (g) mammillary body; (h) mammillothalamic tract; (i) anterior nucleus of the thalamus; (j) parahippocampal radiation of the cingulum; (k) paraolfactory gyrus; (l) paraterminal gyrus. (From Shah et al., 2012, with permission).

The cingulum's proximity to the "grand lobe limbique" of Broca (1878) immediately pointed to their close relationship. This cortical relationship was clarified by Beevor (1891), who realised that fibres continuously join and leave the cingulum and emphasised its affinity with the cingulate gyrus. Interest in the cingulum was heightened by Papez (1937), who incorporated the bundle in his influential model of emotion (Figure 1.2). Subsequently, the cingulum was seen as a core part of the limbic system (Dalgleish, 2004; Maclean, 1949). One consequence was that the tract became a target for psychosurgical procedures (section 1.4.3.1). More recently, MRI-based evidence of cingulum dysfunctions in a wide range of neurological and psychiatric disorders (section 1.4.3.2) has highlighted the clinical significance of this fibre bundle.

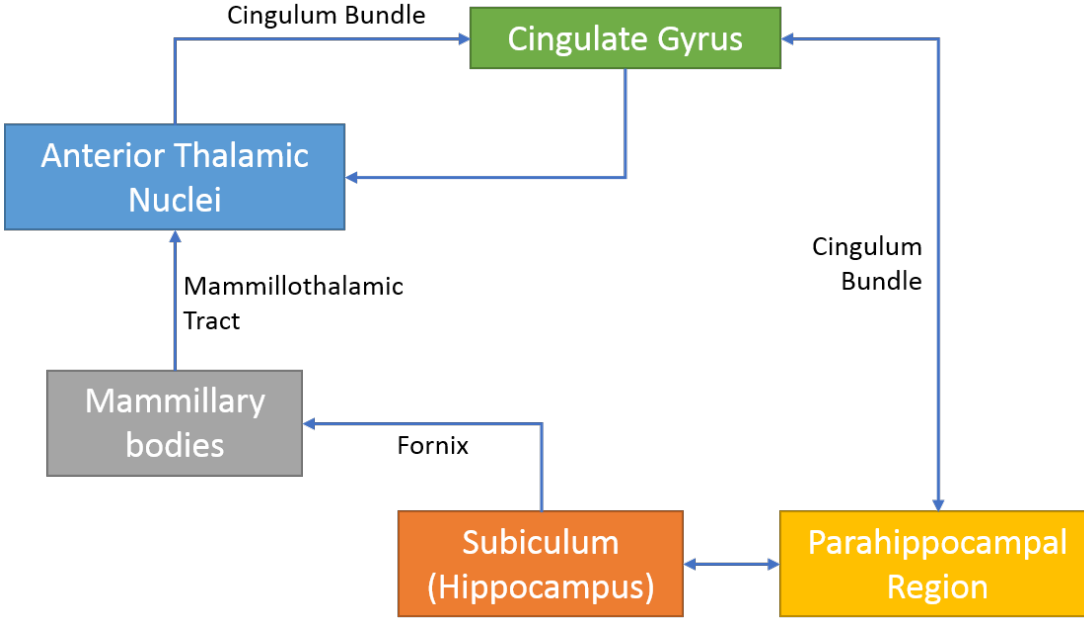

Figure 1.2 Schematic diagram of Papez circuit

Diagram illustrates the central position of the cingulum bundle in Papez circuit (Papez, 1937).

1.3 Structure and connections of the cingulum bundle

It has long been known that the cingulum is not a unitary pathway. It is highly complex, containing many different fibres of different lengths. There are many short cortico-cortical association fibres ('U-fibres'), interlinking neighbouring parts of the frontal, parietal, and temporal lobes, as well as longer association fibres that provide distal connectivity between these regions (Schmahmann & Pandya, 2006). In addition, other cingulum fibres radiate across the tract to reach cortical and subcortical sites (Yakovlev & Locke, 1961). Consequently, few, if any, connections extend the entire length of the tract (Heilbronner & Haber, 2014).

This section provides a detailed anatomical account of the current research on the connections that comprise the cingulum bundle. Findings from the rat, the monkey, and the human, are discussed separately before a cross-species comparison is provided. The principal findings are from animal experiments, where axonal tracers have helped to visualise projections down to the level of single neurons.

1.3.1 Connections in the rat

Our current knowledge of the rat cingulum bundle originates from studies conducted almost fifty years ago. Using lesion degeneration methods, Domesick (1970) described the many anterior thalamic-cingulate projections within the bundle. She described how projections from the anterior thalamus stream forward to form fascicles in the dorsomedial aspect of the caudoputamen. Some fibres turn dorsally before reaching the level of the genu to skirt the lateral ventricle, cross through the corpus callosum, and join the external medullary stratum of the cingulum (Figure 1.3, Domesick, 1970). Other degenerating fibres continue rostrally to the anterior limit of the dorsomedial caudoputamen (some in the internal capsule), then turn medial and dorsal to join the cingulum bundle around the genu of the corpus callosum (Figure 1.4). Together, these efferents form a basket of thalamo-cingulate fibres, with inputs joining the cingulum at different rostro-caudal levels. Posterior to the splenium, the degenerating thalamic fibres in the cingulum divide to form separate fascicles in caudal retrosplenial and parahippocampal regions.

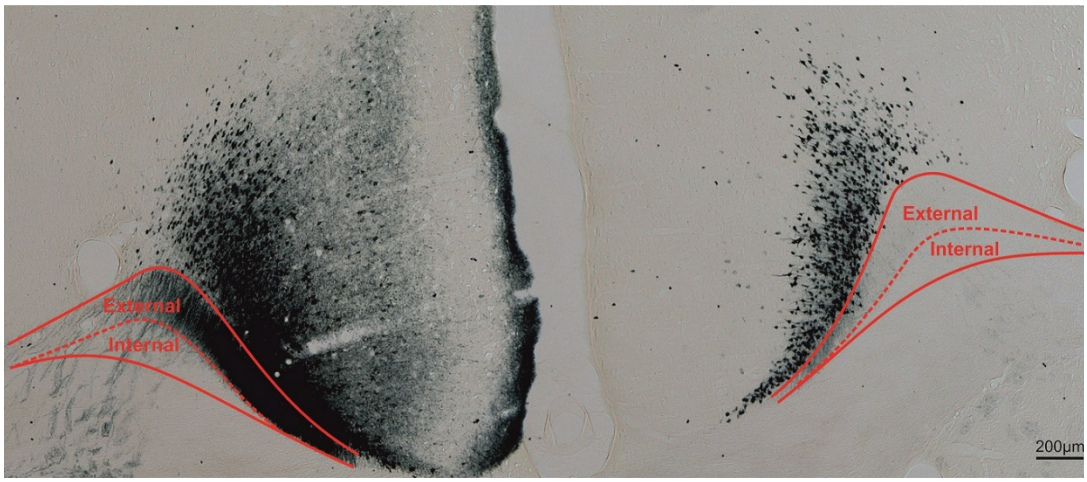

Figure 1.3 Coronal section of a rat brain showing labelled cingulum fibres following an anterior thalamic injection

Image is from a rat brain with an anterior thalamic injection of wheat germ agglutin (WGA) in left hemisphere. Labelled anterior thalamic fibres join the external medullary stratum (Domesick, 1970) of the medial cingulum bundle to reach the cingulate cortex. The lack of corresponding fibres in the right hemisphere is because the thalamo-cortical projections remain ipsilateral, although the reciprocal cortico-thalamic projections are bilateral (Mathiasen, Dillingham, Kinnavane, Powell, & Aggleton, 2017). For methods, see Amin et al., 2010. Scale bar = 200 µm.

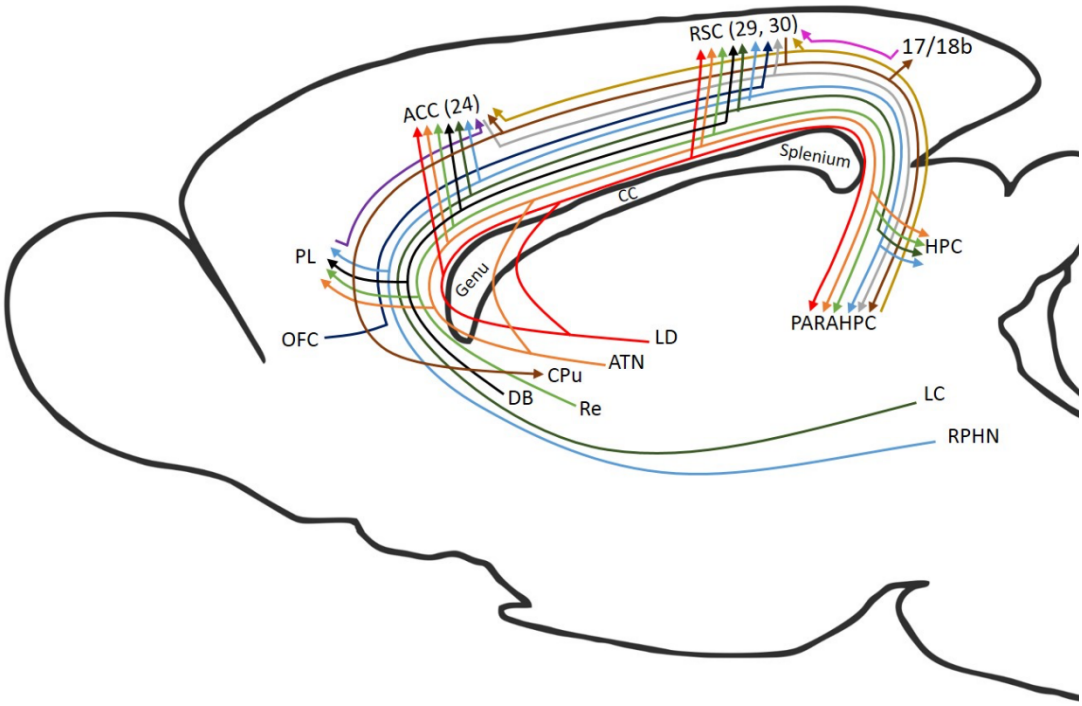

Figure 1.4 Schematic of the rat brain showing connections that provide sagittal fibres to the cingulum bundle

Note cingulate projections that cross through the bundle, e.g., to the anterior thalamic nuclei, are not depicted. The colours help distinguish the multiple pathways. Abbreviations: ACC, anterior cingulate cortex; ATN, anterior thalamic nuclei; CC, corpus callosum; DB, diagonal band; HPC, hippocampus, including subiculum; LC, locus coeruleus; LD laterodorsal thalamic nucleus; OFC, orbital frontal cortex; PARAHPC, parahippocampal region; PL, prelimbic cortex; RPHN, raphe nucleus; RSC, retrosplenial cortex. Note, that offshoots of lines do not represent collaterals.

Domesick (1969) also described how the dense, reciprocal corticothalamic projections typically take a different route with respect to the cingulum. Rather than becoming enclosed in the sagittal course of the cingulum, these fibres were traced passing directly through it and the underlying callosal strata from their point of origin. Fibres originating in the anterior cingulate cortex then form fascicles crossing the caudoputamen in a caudal and ventral direction. These fascicles are not constrained to the dorsomedial aspect of the caudoputamen, as are those of thalamic origin, and traverse the internal capsule to enter the thalamus from its lateral side. Fibres from the retrosplenial cortex follow the same route, with those from the more posterior regions travelling rostralward in the internal stratum of the cingulum to pass into the caudoputamen at thalamic levels.

No subsequent study has focused specifically on the trajectory of thalamic-cingulate fibres. However, studies that confined axonal tracers in different thalamic nuclei tend to support the conclusions made by Domesick (1970). Fibres from the anterodorsal thalamic nucleus have been referred to as following the route described by Domesick (1970) to terminate in granular retrosplenial (area 29) cortex and parahippocampal areas, including presubiculum, and postsubiculum, with lighter terminations reaching the entorhinal cortex and subiculum (Van Groen & Wyss, 1990a, 1990c, 1995). Meanwhile, projections from the anteroventral thalamic nucleus have been described following essentially the same route as anterodorsal efferents before terminating in the anterior cingulate cortex, as well as those other areas innervated by the anterodorsal nucleus (Shibata, 1993a, 1993b; Van Groen & Wyss, 1995).

Similarly, some fibres from the anteromedial thalamic nucleus appear to take the route described by Domesick (1970), streaming forward through the caudoputamen and wrapping around the genu of the corpus callosum, before turning rostral to reach medial frontal areas (Shibata, 1993b; Van Groen, Kadish, & Wyss, 1999). Other anteromedial fibres turn caudally in the cingulum to terminate in the posterior part of the anterior cingulate cortex and retrosplenial cortex (areas 29 and 30), with additional fibres descending behind the splenium to innervate parahippocampal areas such as presubiculum. Some lighter terminations appear to reach entorhinal and perirhinal areas (Shibata, 1993a; Van Groen et al., 1999).

Midline thalamic fibres from the interanteromedial nucleus take a more rostral route, entering the dorsal internal capsule, passing the rostral limit of the corpus callosum in the cingulum to terminate in frontal and orbital areas (Van Groen et al., 1999). Other interanteromedial fibres turn dorsal and then caudal in the cingulum to reach the anterior cingulate cortex, dysgranular retrosplenial cortex (area 30), the subiculum, and perirhinal cortex (Van Groen et al., 1999). Meanwhile, nucleus reuniens efferents also join the rostral cingulum bundle to innervate prelimbic, anterior cingulate, and retrosplenial cortices (Herkenham, 1978; Wouterlood, Saldana, & Witter, 1990). Other nucleus reuniens projections continue caudally around the splenium, where they enter the angular bundle and disperse within hippocampal and parahippocampal regions. These fibres innervate the subiculum,

CA1, presubiculum, parasubiculum, the medial entorhinal and perirhinal cortices, although the cingulum is not the only route (Wouterlood et al., 1990).

Fibres from the laterodorsal thalamic nucleus also head rostral before turning dorsal to enter the cingulum (Figure 1.4), where a small proportion of efferents terminate in anterior cingulate areas (Van Groen & Wyss, 1992). Most laterodorsal fibres continue caudally to terminate in the retrosplenial cortex (areas 29 and 30) and parahippocampal areas including the presubiculum, parasubiculum, and postsubiculum, with lighter inputs reaching entorhinal cortex (Van Groen & Wyss, 1990a, 1990b, 1992). Meanwhile, projections from the mediodorsal thalamic nucleus to the anterior cingulate cortex enter and cross the cingulum around the level of the genu (Leonard, 1969), but do not contribute to the bundle for any length.

Subsequent studies also found support for the more direct route described by Domesick (1969) for retrosplenial, anterior cingulate and prelimbic projections to the anterior thalamic nuclei (Shibata, 1998; Shibata & Naito, 2005; Van Groen & Wyss, 1990b, 1992). That is, fibres pass around the lateral ventricle, briefly joining the internal capsule, before cutting across the thalamus to reach the anterior thalamic nuclei. However, no studies provide a detailed description of the route taken by these cortical-thalamic efferents. Consequently, the anteroposterior level at which these fibres cut down through the white matter, and whether they join the cingulum for any length, is not known. Meanwhile, the dense hippocampal and parahippocampal inputs to the anterior thalamic nuclei rely on the fornix and internal capsule (Dillingham, Erichsen, O'Mara, Aggleton, & Vann, 2015; Meibach & Siegel, 1977), rather than the cingulum.

Both the anterior cingulate (area 24) and retrosplenial (areas 29, 30) cortices have dense intrinsic connections, some of which join the cingulum while others take a direct route within the cortex, i.e., dorsal to the tract (Jones, Groenewegen, & Witter, 2005; Van Groen & Wyss, 1992, 2003). Likewise, some projections from orbital areas to retrosplenial cortex involve the cingulum (Beckstead, 1979; Shibata & Naito, 2008). The cingulum is also the principal route for anterior cingulate and retrosplenial projections (as well as the lighter pregenual cortical projections) to the

parahippocampal region, including inputs to the entorhinal cortex, postrhinal cortex, parasubiculum, and presubiculum (Jones & Witter, 2007). In addition, prelimbic projections to the anterior cingulate cortex briefly join the cingulum (Beckstead, 1979).

Retrosplenial cortex has dense interconnections with the adjacent anterior cingulate and secondary motor areas, though it only has weak projections to prelimbic cortex. Some of these connections join the cingulum (Shibata, Kondo, & Naito, 2004; Van Groen & Wyss, 1990b, 1992, 2003). Meanwhile, rostral projections from the dysgranular retrosplenial cortex (area 30) to the caudoputamen also join the cingulum (Van Groen & Wyss, 1992). Other cingulum fibres include the reciprocal connections between dysgranular retrosplenial cortex (area 30) and visual areas 17 and 18b (Van Groen & Wyss, 1992). Similarly, both retrosplenial areas (29 and 30) have reciprocal connections with the postsubiculum, some joining the cingulum while others take a direct cortico-cortical route (Van Groen & Wyss, 1990c, 2003).

In addition to the thalamus, other subcortical sites contribute to the cingulum. Cholinergic fibres from the diagonal band extend along the cingulum bundle to innervate cingulate and retrosplenial areas, with lighter inputs to frontal areas (Woolf, Hernit, & Butcher, 1986). Noradrenergic fibres from locus coeruleus pass through the anterior thalamus to reach the cingulum, with some fibres terminating in the cingulate cortices and others extending to the hippocampus, including the subiculum (Jones & Moore, 1977; Pasquier & Reinoso-Suarez, 1978; Segal, Pickel, & Bloom, 1973). Additional pontine projections, e.g., from nucleus incertus, course rostrally through the septal region to turn caudally in the cingulum bundle and terminate along the rostrocaudal extent of the cingulate and secondary motor cortices (Goto, Swanson, & Canteras, 2001). Median raphe efferents wrap dorsally around the genu of the corpus callosum (Azmitia & Segal, 1978), joining the cingulum to terminate lightly in frontal cortex, throughout the cingulate cortex, and the entorhinal cortex and dentate gyrus (Pasquier & Reinoso-Suarez, 1978). Finally, some projections reaching the cingulate cortex from the claustrum, lateral hypothalamic area, and amygdala may potentially use the cingulum, though this route does not

seem specified (Berk & Finkelstein, 1982; Krettek & Price, 1977; Van Groen & Wyss, 2003).

Figure 1.4 depicts the main connections that join the sagittal course of the cingulum, rather than principally cross through the bundle. As a consequence of distinguishing the many cortical sites involved, it might appear from Figure 1.4 that the bundle is dominated by cortico-cortical connections. In fact, in the rat, the part of the bundle above the corpus callosum largely consists of thalamic connections with the cingulate cortices, as initially described by Domesick (1969, 1970). In contrast, many of the cortico-cortical connections are not only light, but are also have links directly through the cortex, i.e., not all interconnections use the bundle. Overall, the rat cingulum bundle principally provides subcortical connectivity to cortical regions close to the midline, i.e., medial frontal, cingulate and parahippocampal cortices, as well as interlinking these same cortical areas.

1.3.2 Connections in the nonhuman primate

The primary analysis of the course and composition of the cingulum bundle in the non-human primate was conducted by Mufson and Pandya (1984) using autoradiographic tracer techniques in the rhesus monkey. As in the rat, Mufson and Pandya (1984) found that a large subset of cingulum fibres are thalamo-cortical projections that arise from the anterior and laterodorsal thalamic nuclei. Many anterior thalamic projections travel rostrally below the caudate nucleus to the anterior limb of the internal capsule, where they turn dorsal to join the cingulum close to the level of the genu. Some anterior thalamic fibres, however, stream directly lateral across the dorsal thalamus, around the lateral ventricle, to enter the cingulum from its lateral side (Figure 1.5). The anterior thalamic projections joining the cingulum bundle then terminate in anterior cingulate (areas 24 and 25), posterior cingulate (area 23) and retrosplenial cortices (areas 29 and 30) (Mufson & Pandya, 1984). While some of the cingulate/retrosplenial projections from the laterodorsal thalamic nucleus travel forward to join the cingulum bundle, the majority favour the more direct route around the caudate nucleus and lateral ventricle (Mufson & Pandya, 1984).

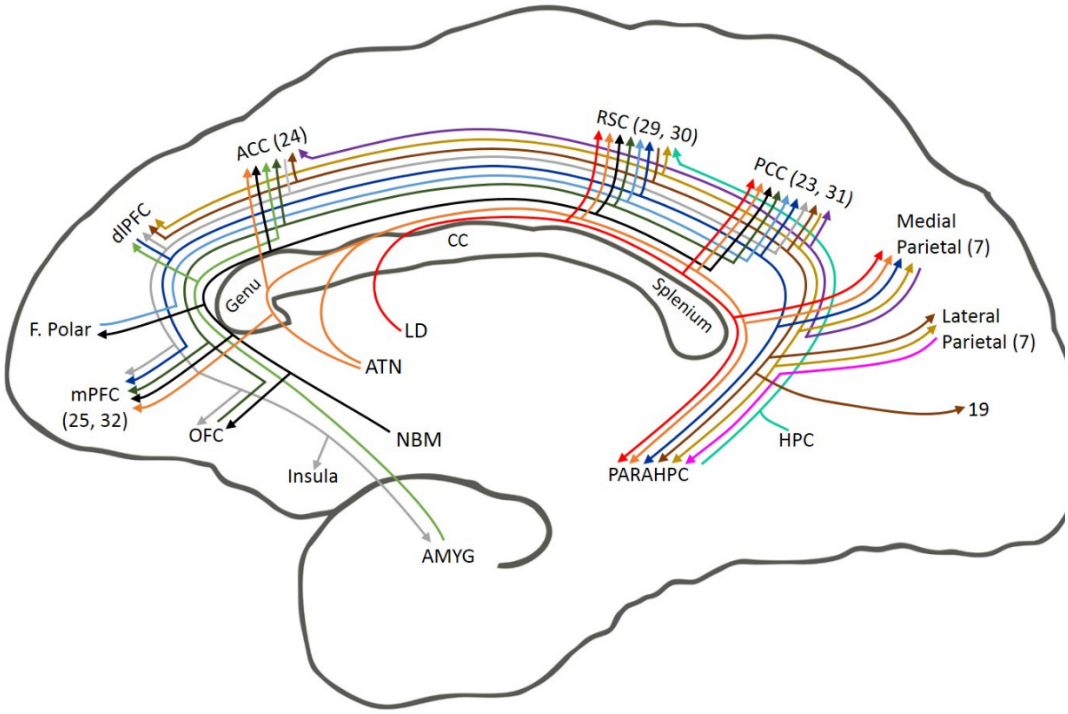

Figure 1.5 Schematic of the monkey brain showing connections that provide sagittal fibres to the cingulum bundle

Note cingulate projections that cross through the bundle, e.g., to the anterior thalamic nuclei, are not depicted. The colours help distinguish the multiple pathways. While it is most likely that additional subcortical projections join the cingulum (see Figure 1.4), explicit descriptions are often lacking. Abbreviations: ACC, anterior cingulate cortex; ATN, anterior thalamic nuclei; CC, corpus callosum; HPC, hippocampus, including subiculum; LC, locus coeruleus; LD laterodorsal thalamic nucleus; NBM, nucleus basalis of Meynert; PARAHPC, parahippocampal region; PL, prelimbic cortex; RSC, retrosplenial cortex. Note, that offshoots of lines do not represent collaterals.

The anteromedial thalamic nucleus is closely connected with the anterior cingulate region (area 24, but also areas 25 and 32) and provides some inputs into posterior cingulate cortex (area 23) (Shibata & Yukie, 2003). Meanwhile, the anteroventral nucleus innervates posterior cingulate (area 23) and retrosplenial cortices (areas 29 and 30), with additional inputs to granular retrosplenial cortex (area 29) arising from the anterodorsal nuceli (Shibata & Yukie, 2003). The laterodorsal nucleus projects to posterior cingulate (area 23) and retrosplenial (area 30) cortices (Shibata & Yukie, 2003), with some fibres also extending to more lateral parietal areas (Morris, Petrides, & Pandya, 1999). It should, however, be noted that these studies did not describe the route of these projections.

One exception is a study conducted by Heilbronner and Haber (2014), where the route of efferents from injections encompassing the anteroventral and laterodorsal nuclei are described. Fibres project around the caudate nucleus and through the internal capsule to join the cingulum as rostral as the anterior commissure and as caudal as the splenium. Therefore, fibres leaving the thalamus rostrally did not travel as far forward as described previously (i.e. they do not extend as far forward as the genu, Mufson & Pandya, 1984). Moreover, a subset of fibres leaving the thalamus caudally were identified (Heilbronner & Haber, 2014). Such fibres, joining the cingulum at anteroposterior levels caudal to the injection site, had not been described previously (Mufson & Pandya, 1984). Finally, Heilbronner and Haber (2014) found additional efferents from the anteroventral and laterodorsal nuclei that reached medial parietal area 7m, parasubiculum, presubiculum, and other parahippocampal cortices.

The second principal group of fibres described by Mufson and Pandya (1984) comprise connections leaving anterior cingulate (area 24) and posterior cingulate (area 23) cortices. As in the rat, return projections from the anterior cingulate cortex (area 24) to the anterior thalamic and laterodorsal thalamic nuclei do not appear to join the cingulum for any length. Instead, fibres cut through the tract, joining the anterior limb of the internal capsule, before entering the thalamus. Other anterior cingulate (area 24) fibres also cross the bundle to reach the caudate nucleus, putamen (Baleydier & Mauguiere, 1980), and brainstem (Mufson & Pandya, 1984). Others join the cingulum and travel forward to terminate in dorsolateral, medial, and orbital prefrontal areas. A further subset of anterior cingulate cortex (area 24) fibres pass through the lateral cingulum to reach the amygdala, perirhinal cortex, insula and superior temporal cortex (Mufson & Pandya, 1984). Rostral area 24 also appears to project to more posterior area 24, as well as posterior cingulate cortex (area 23), via the cingulum bundle (Pandya, Van Hoesen, & Mesulam, 1981; Vogt & Pandya, 1987).

The return projections from monkey posterior cingulate cortex (area 23) to the anterior thalamic and laterodorsal thalamic nuclei further parallel the direct route seen in the rat. Fibres cross through the cingulum, rather than joining it for any

length, and travel ventrally in the internal capsule to reach the anterior, laterodorsal and mediodorsal thalamic nuclei (Mufson & Pandya, 1984). Other posterior cingulate cortex (area 23) projections that cross the bundle terminate in the caudate nucleus and the brainstem (Mufson & Pandya, 1984; Vilensky & Van Hoesen, 1981).

Cortical projections from posterior cingulate cortex (area 23) that join the cingulum include fibres to lateral parietal sites and parahippocampal areas (Kobayashi & Amaral, 2007; Mufson & Pandya, 1984). In return, parahippocampal areas project to posterior cingulate cortex (area 23) (Baleydier & Mauguiere, 1980; Vogt & Pandya, 1987), presumably via the cingulum. In addition, restricted rostral projections from posterior cingulate cortex (area 23) reach dorsolateral prefrontal cortex (areas 9, 46) via the cingulum (Kobayashi & Amaral, 2007; Mufson & Pandya, 1984).

Although not described by Mufson and Pandya (1984), another group of fibres that join the cingulum in the monkey are efferents from retrosplenial cortex (areas 29, 30). Rostrally directed retrosplenial efferents in the cingulum reach the anterior part of posterior cingulate cortex (area 23), caudal anterior cingulate cortex (area 24), as well as the dorsolateral prefrontal cortex, with light inputs to other frontal regions (Kobayashi & Amaral, 2007; Morris, Pandya, & Petrides, 1999; Morris, Petrides, et al., 1999). Caudally directed retrosplenial efferents that join the cingulum terminate in visual (area 19) and parahippocampal areas (Kobayashi & Amaral, 2007; Morris, Petrides, et al., 1999), with return parahippocampal projections to retrosplenial cortex involving the cingulum (Bubb, Kinnavane, & Aggleton, 2017).

The final group of cingulum fibres described by Mufson and Pandya (1984) consists of projections from both anterior frontal and posterior parietal regions. Dorsolateral prefrontal cortical areas project via the cingulum bundle to posterior cingulate (areas 23, 31) and retrosplenial (areas 29 and 30) cortices, as well as to medial parietal and parahippocampal areas (Goldman-Rakic, Selemon, & Schwartz, 1984; Morris, Petrides, et al., 1999; Selemon & Goldman-Rakic, 1988). In addition, the frontal pole (area 10) reaches targets along the cingulate and retrosplenial cortices via the cingulum (Heilbronner & Haber, 2014). Meanwhile, efferents from medial parietal

areas join the cingulum to terminate in anterior cingulate (area 24), posterior cingulate (area 23) (Mufson & Pandya, 1984) and dorsal lateral prefrontal cortices (Petrides & Pandya, 1984). Finally, projections from lateral parietal cortex reach parahippocampal via the cingulum (Seltzer & Pandya, 1984).

Heilbronner and Haber (2014) also described basolateral amygdala projections that join the cingulum to reach the anterior cingulate cortex. Finally, many cholinergic fibres from the nucleus basalis of Meynert in the basal forebrain join the cingulum to run above the corpus callosum and innervate the length of the cingulate gyrus, as well as superior frontal cortices (Kitt, Mitchell, DeLong, Wainer, & Price, 1987).

Figure 1.5 depicts the connections that join the sagittal course of the cingulum, rather than principally cross through the bundle. As in the rat, the cingulum connections include noradrenergic, serotonergic, and cholinergic fibres. Again, as in the rat, the part of the bundle above the corpus callosum has many thalamic connections with the cingulate cortices. However, in comparison to the rat it contains more cortico-cortical connections, including inputs from almost all prefrontal cortical areas (Heilbronner & Haber, 2014). Nonetheless, it is clear from Figure 1.5 that no single site dominates the tract, as its component connections shift along the length of the bundle.

1.3.3 Connections in the human

Unlike in animal experiments, it is not currently possible to visualise projections down to the level of the single neuron in humans. Therefore, initial anatomical findings regarding the human cingulum bundle came from microdissections and reconstructions based on cellular and white matter stains. More recently, major advances have come from non-invasive diffusion weighted magnetic resonance imaging (dMRI), which exploits the motion of water protons in brain tissue for *in vivo* reconstruction, visualisation and quantification. Diffusion tensor imaging (DTI) (Basser, Mattiello, & LeBihan, 1994) has been the most influential dMRI method for studying white matter microstructure as it uses measurements of the restricted diffusion of water molecules to produce images of fibre bundles. These white matter

bundles can be selected and followed through the brain in a process known as tractography (Basser, Pajevic, Pierpaoli, Duda, & Aldroubi, 2000).

Tractography has allowed researchers to reconstruct the cingulum bundle, advancing knowledge of its structural properties. Whilst initial DTI tractography studies portrayed the cingulum as a unitary bundle (e.g., Catani et al., 2002), more recent reconstructions increasingly distinguished between subdivisions of the tract that were found to have different anatomical properties. One example is differentiation of the 'dorsal' and 'ventral' cingulum, i.e., above or below the splenium (e.g., Budisavljevic et al., 2015). Other researchers have divided the tract three-ways, e.g., into its 'subgenual', 'retrosplenial' (supracallosal), and 'parahippocampal' (ventral) components (Jones, Christiansen, Chapman, & Aggleton, 2013). These three subdivisions were found to exhibit distinct fractional anisotropy (FA) measures, even in areas where they overlapped, and occupied different medial-lateral positions within the bundle. These differences, which suggest qualitative changes along the length of the tract, add support to similar tract subdivisions used in other dMRI studies (e.g., Concha et al., 2005; Lin et al., 2014).

Nonetheless, there is much heterogeneity in cingulum reconstructions across different studies. It is also pertinent that there is an inability to determine the direction of white matter (afferent or efferent), a limitation that is highly problematic for studying the cingulum bundle. This, along with the problem of disentangling complex fibre architecture (Jeurissen, Descoteaux, Mori, & Leemans, 2019), limits the extent to which the specific connections that constitute the human cingulum bundle can be determined.

1.3.4 Cross-species comparison and outlook for investigation

Although many of the details of the human cingulum bundle remain to be specified, it is possible to make comparisons across the three highlighted species. It is clear that the set of subcortical – cortical connections, which appear to dominate the rat cingulum bundle, are also present in the monkey. Given the strong homologies between the cytoarchitecture of the rodent and primate (including human) cingulate

cortex, including its major subdivisions (Vogt & Paxinos, 2014), it seems highly likely that these connections will also be present in humans.

In particular, there is strong evidence that connections between the anterior thalamic nuclei and the cingulate cortices form a major component of both the rat (Figure 1.4) and the monkey (Figure 1.5) cingulum bundle. The relationship between these regions is complex and there are clear differences in connectivity between each of the individual anterior thalamic nuclei, and each respective region of the cingulate cortex. For instance, the anteromedial nucleus is closely associated with the anterior cingulate cortex, whereas the anterodorsal and anteroventral nuclei share more connections with the retrosplenial cortex, in both the rat and the monkey.

There has been a wealth of research focusing on the origins and terminations of these connections, which has been reviewed here and, in more detail, elsewhere (Bubb et al., 2017). On the other hand, the trajectories of these fibres, with respect to the cingulum bundle, are less well understood. The last studies to focus on the routes taken by anterior thalamic-cingulate connections were conducted in 1970 (Domesick, 1969, 1970) in the rat, and in 1984 (Mufson and Pandya, 1984) in the monkey. Owing to methodological limitations, both studies left several details to be clarified.

Chapter 3 outlines and addresses the outstanding questions concerning the interconnectivity of the anterior thalamic nuclei and the cingulate cortices. The relative stability of this subset of connections across species makes them a good candidate for investigation in the rat, where recent anatomical tract tracing techniques involving anterogradely transported viruses (Osten & Margrie, 2013) allow the connections to be described with a level of precision that has not been achieved previously.

Meanwhile, there is an obvious increase in cortico-cortical connectivity within the primate cingulum bundle, making it more difficult to draw conclusions on this aspect of the cingulum bundle across species. Rather than just involving cingulate, medial frontal and parahippocampal areas, there is a marked extension as the tract contains

some fibres from across almost all parts of the prefrontal cortex (Heilbronner & Haber, 2014) as well as reaching more dorsal and lateral parts of parietal cortex. In addition to an apparent increase in parietal – frontal connectivity within the bundle from rat to non-human primate, DTI reconstructions suggest a further increase in parietal – temporal connections within the human parahippocampal cingulum bundle. This may reflect further cross-species differences in cortico-cortical cingulum connections, between different primates.

1.4 Functions of the cingulum bundle

By virtue of the core set of connections found across species, the cingulum has been placed within the limbic system. Therefore, attempts to understand the functions of its connections have often focussed on emotion and memory (Aggleton, Neave, Nagle, & Sahgal, 1995; Bubb, Metzler-Baddeley, & Aggleton, 2018; Ennaceur, Neave, & Aggleton, 1997). Meanwhile, the greater emphasis on frontal connections in the primate cingulum bundle has led researchers to consider its potential contributions to cognitive control, attention, pain, motor mechanisms, and reward signalling (Beckmann, Johansen-Berg, & Rushworth, 2009).

Given the many different connections that comprise the cingulum, outlined in the previous section (1.3), it is perhaps in some ways unsurprising that it is associated with such a diverse array of functions. Consequently, it is becoming increasingly apparent that different parts of the tract are involved in different aspects of cognition, reflecting changing composite connections.

This section provides a detailed account of the current research on the functions of the cingulum bundle. As in the previous section (1.3), findings from the rat, the monkey, and the human are discussed separately. A cross-species comparison is then provided, highlighting where evidence converges to implicate different subsections of the tract in specific functions. The principal findings in this area come from human research, where the results of psychosurgeries targeting the bundle, alongside recent advances in neuroimaging, have led to a surge of empirical data regarding the functions of the cingulum.

1.4.1 Functional analyses in the rat

In the rat, studies of lesions targeted at the cingulum bundle have been predominantly limited to examining pain perception and spatial memory. The former follows from the introduction of anterior cingulotomies for intractable pain in humans (section 1.4.3.1), the latter from the many hippocampal and parahippocampal connections within the tract (section 1.3). A limitation of all of these studies is that they are not accompanied by experiments that identify the extent to which the various brain regions that contribute fibres to the tract are disconnected by the various interventions.

Initial research into the anterior part of the rat cingulum bundle showed that its blockade can cause analgesia (Vaccarino & Melzack, 1989, 1992). Similarly, related studies described how cingulum bundle anaesthesia delays the onset of self-mutilation, a behaviour that is thought to be an index of pain, following peripheral neurectomy (Magnusson & Vaccarino, 1996; Vaccarino & Melzack, 1991). In contrast, stimulation of the cingulum can precipitate self-mutilation (Pellicer, López-Avila, & Torres-López, 1999). Meanwhile, the finding that electrical stimulation of the cingulum bundle reduces pain in the formalin test (Fuchs, Balinsky, & Melzack, 1996) was interpreted as a disruption of patterned activity that would normally signal pain.

The contribution of the cingulum bundle to pain perception has been interpreted in different ways. Melzack (2005) regarded the cingulum as part of a widely distributed ‘neuromatrix’ of structures, which together provides pain perception. Alternatively, Vogt (2005) argued for a ‘dual pain system’, involving both affective-motivational and sensory-discriminative components. The anterior cingulate cortex is proposed to be involved in both systems, with the latter reflecting specific nociceptive information that may come from midline and intralaminar thalamic nuclei (Vogt, 2005), helping to explain the significance of the cingulum. (See also section 1.5.1 for a discussion of the involvement of the anterior cingulate cortex in pain perception).

The second topic of investigation in the rat, spatial memory and navigation, arises from the close links between the cingulum bundle and brain sites known to

contribute to spatial processes (see section 1.3). Accordingly, Table 1 compares the behavioural effects of cingulum bundle lesions with damage to associated areas (anterior cingulate cortex, retrosplenial cortex, anterior thalamic nuclei) on memory tasks, particularly those taxing spatial functioning. It is noteworthy that such studies differ in their location and number of cingulum lesions, so cannot be used to infer the function of subregions of the tract per se. The fornix is included in Table 1 as it contains the connections from the hippocampus to the anterior thalamic nuclei, forming another key white matter tract in Papez circuit (section 1.2, Figure 1.2). All of the tasks in Table 1 are highly sensitive to hippocampal lesions (Jarrard, 1993; Morris, Garrud, Rawlins, & O'Keefe, 1982; E. C. Warburton, Baird, Morgan, Muir, & Aggleton, 2001). The terms 'reference' and 'working' memory refer, respectively, to when a fixed piece of information is learnt, or when the information changes across trials/sessions. All cortical lesions were made by cytotoxins, to avoid cingulum bundle damage.

Table 1. Cingulum bundle lesion effects in rats on spatial memory tasks

Task	Cingulum Bundle study	Cingulum Bundle	Retrosplenial Cortex	Anterior Thalamic Nuclei	Fornix	Anterior Cingulate
Water-maze reference acquisition	Warburton et al. 1998 (2)	X	X[1] X[6]	XXX[2]	XX* XX[2] XXX[12]	X*
	Harker and Whishaw 2004 (1)	X	X*			
Water-maze working	Harker and Whishaw 2004 (1)	X	X* X[1]	XXX[15]	XXX[14]	
T-maze alternation acquisition	Aggleton et al. 1995 (3)	XXX	√* (ant +post cingulate) X[8] √[10]	XXX[3] XXX[7]	XXX* XXX[7]	√[10] X[13] marginal
	Neave et al. 1996 (2,1)	X CB2 X CB1				
	Neave et al. 1997 (2,1)	XX CB2 √ CB1			XXX*	

	Warburton et al. 1998 (2)	X			XXX*	
T-maze alternation delays	Aggleton et al. 1995 (3)	XX	√* (ant +post cingulate) √[10]	XX[3] XXX[4]	XXX[3,4]	√[10]
	Neave et al. 1996 (2,1)	√ CB2 √ CB1 (X when groups combined)				
Cross-maze alternation	Neave et al. 1997 (2,1)	√ CB2 √ CB1		XXX[4]	XXX*	
Delayed nonmatch to position in operant box	Aggleton et al. 1995 (3)	√	√* (ant +post cingulate)	XX[5]	XX*	√[10]
	Neave et al. 1996 (2,1)	√ CB2 √ CB1				
Lever discrimination and reversals	Aggleton et al. 1995 (3)	√	√* (ant +post cingulate)		XX*	√[10]
Radial arm maze (working)	Neave et al. 1997 (2,1)	XX CB2 √ CB1	XX[6]	XXX[9]	XXX*	√[11]
Object recognition	Ennaceur et al. 1997 (3)	√	√* √[6]	√[2]	√*	√*
Object location recognition	Ennaceur et al. 1997 (3)	√	XX* (ant +post cingulate)		XX*	

Table provides a comparison of cingulum bundle lesion effects in rats with other, related brain sites. Symbols: * results from same study as cingulum bundle (CB) lesion; √, no lesion effect; X, mild/borderline effect; XX, clear deficit; XXX, severe deficit. Numbers in parenthesis show the number of cingulum lesions in each hemisphere. In two studies (Neave et al., 1996, 1997) there were two groups with cingulum bundle lesions, which differed in the number of lesion placements per hemisphere (one, CB1 or two, CB2). The superscript numbers refer to appropriate comparison studies: 1. Vann et al., 2003: 2. Warburton and Aggleton, 1999: 3. Aggleton et al., 1995: 4. Warburton et al., 1997: 5. Aggleton et al., 1991: 6. Vann and Aggleton, 2002: 7. Warburton et al., 1999: 8. Nelson et al., 2015: 9. Aggleton et al., 1996: 10. Neave et al., 1994: 11. Ragozzino et al., 1998: 12. Sutherland and Rodriguez, 1989: 13. Sanchez-Santed et al., 1997: 14. Cassel et al., 1998: 15. Perry et al., 2018.

Several conclusions emerge from Table 1. The first is that cingulum bundle lesions most consistently affect spatial tasks involving allocentric cues, i.e., when the

relationships between distal cues specify location. Nevertheless, despite the dense contributions to the bundle from the anterior thalamic projections to the cingulate cortices, cingulum bundle lesions are far less disruptive than anterior thalamic lesions. This difference reveals the relative importance of those anterior thalamic connections that avoid the cingulum bundle, e.g., its inputs from the hippocampal region, the mammillary bodies, and frontal cortices, while also signifying how these thalamic nuclei are a key point of convergence for spatial processing (Bubb et al., 2017).

Table 1 also highlights the close correspondence between the effects of retrosplenial cortex lesions and cingulum bundle lesions on spatial memory (Harker & Whishaw, 2004). These cingulum effects become more robust as more lesions are placed along the tract, presumably reflecting the additive effects of more anterior cingulate and retrosplenial cortex disconnections (see Vann et al., 2003). Harker and Whishaw (2002) found that the reference and working memory deficits of retrosplenial lesions were not exacerbated by additional cingulum bundle damage, indicating that retrosplenial disconnection largely accounts for the effects of cingulum bundle lesions on spatial tasks. Table 1 further demonstrates how fornix lesions produce more severe spatial memory deficits than cingulum bundle lesions. This is notable as both tracts are serially linked within 'Papez circuit' (section 1.2, Figure 1.2), which is assumed to be vital for memory (Aggleton & Brown, 1999; Rolls, 2015).

Overall, it is clear that functional analyses of the rat cingulum bundle are lacking. Interventions targeting the anterior portion of the tract indicate a potential role in pain processing. However, human research further implicates the anterior cingulum in many aspects of executive function and emotion (see section 1.4.3), both of which are yet to explored in the rat. Meanwhile, it is somewhat surprising that cingulum bundle lesions often have such mild effects on spatial learning, given the significance of this tract for both anterior thalamic and parahippocampal fibres (section 1.3). This suggests there may be a missing piece to the puzzle, while also emphasising the importance of other pathways, such as the fornix.

1.4.2 Functional analyses in the nonhuman primate

Selective cingulum bundle lesions have not been investigated in nonhuman primates. For this reason, experiments studying the effects of surgical lesions in adjacent cingulate areas, that contribute many fibres to the tract, are the most relevant body of research. Inspired by Papez' model (1937; section 1.2, Figure 1.2), many initial studies of the monkey cingulate gyrus focussed on emotion. However, the effects of cingulate gyrus lesions on social and affective behaviour appear inconsistent. Cingulate resections of both anterior cingulate and posterior cingulate cortices (which also perturbed the underlying white matter of the cingulum bundle) have been found to have little effect on social and affective behaviours in some studies (Franzen & Myers, 1973; Kimble, Bagshaw, & Pribram, 1965; Myers, Swett, & Miller, 1973; Pribram & Fulton, 1954). Others, however, have found evidence of reduced social and emotional responsiveness following anterior cingulate lesions (Anand, Dua, & Chhina, 1957; Glees, Cole, Whitty, & Cairns, 1950; Hadland, Rushworth, Gaffan, & Passingham, 2003; Rudebeck, Buckley, Walton, & Rushworth, 2006).

Evidence for disruption of memory following cingulate lesions is also mixed. The majority of studies of extensive cingulate resections, encompassing both anterior and posterior/retrosplenial areas and involved the cingulum, have found little apparent effect on spatial (Murray, Davidson, Gaffan, Olton, & Suomi, 1989; Pribram & Fulton, 1954) and episodic (Parker & Gaffan, 1997) memory tasks that are sensitive to lesions of the hippocampus (Gaffan, 1994; Murray et al., 1989). One exception is a study that found extensive anterior cingulate removal to cause mild deficits on spatial reversal learning, delayed response, and object recognition memory (Meunier, Bachevalier, & Mishkin, 1997). Another exception is a study that found that although posterior cingulate/retrosplenial lesions spared the acquisition of scene discriminations, they did disrupt the retention of these discriminations (Buckley & Mitchell, 2016).

Anterior cingulate lesions centred around the level of the genu have, however, been found to impair performance on a conditional task in which different actions were linked with different rewards (Hadland et al., 2003). This, and other related findings

(Kennerley, Walton, Behrens, Buckley, & Rushworth, 2006; Rushworth, Walton, Kennerley, & Bannerman, 2004), has led to the proposal that the macaque anterior cingulate cortex helps in monitoring and reacting to particular forms of conflict. Related evidence from monkeys performing a Wisconsin Card Sorting Test (WCST) again points to a role for the anterior cingulate cortex in representing error likelihoods and adjusting behavioural patterns following an error (Buckley et al., 2009; Kuwabara, Mansouri, Buckley, & Tanaka, 2014). The proposal that the anterior cingulate cortex, and the underlying cingulum, is involved in cognitive control, conflict, and error monitoring is discussed in section 1.5.1. In contrast, lesions to the posterior cingulate/retrosplenial cortices have been found to have no effect on WCST performance (Mansouri, Buckley, Mahboubi, & Tanaka, 2015).

Overall, the effect of cingulate resections in monkeys seem to be remarkably slight, echoing that of cingulum bundle lesions in rats. Because studies have not targeted the cingulum itself, but often included it within larger lesions, some of the most telling findings are null results. Despite the presumed disconnections caused by the various surgeries, social, emotional and memory functioning appear mostly preserved. It follows that damage confined to the bundle might be even less disruptive. There is more evidence to support deficits in executive function as a result of anterior cingulate lesions that also disrupt the anterior cingulum bundle, a matter which is explored further in section 1.5.1.

1.4.3 Functional analyses in the human

1.4.3.1 Cingulotomy

The 1930s saw the introduction of prefrontal lobotomy for psychiatric disorders (Moniz & de Almeida Lima, 1935). As a key structure in Papez's (1937) circuit for emotion, a subset of later, more targeted surgeries lesioned the white matter of the anterior cingulum bundle (Turner, 1973). Consequently, an unusual body of empirical data exists; from which analysis of the effects of such surgeries on clinical symptoms, and on cognitive measures, can provide insight into the function of the cingulum bundle.

With the advent of computerised tomography and subsequent MR imaging it was confirmed that although anterior cingulotomies principally compromise the cingulum (Steele, Christmas, Eljamel, & Matthews, 2008), they also cause cortical damage, principally in anterior cingulate cortex (area 24) (Spangler et al., 1996; Steele et al., 2008). Further, many of the early clinical reports lacked formal post-operative assessments, appropriate controls and failed to test blind to surgical treatment. For these reasons, this section focuses on the more rigorous, recent research, highlighting those that use formal cognitive measures and those that benefitted from better visualisation of the surgery. Using the latter information, Heilbronner and Haber (2014) concluded that typical cingulotomies compromise a great many fibres in the supracallosal cingulum bundle, and consequently disrupt a wide variety of cortical and subcortical connections, including projections from the amygdala and anterior thalamic nuclei.

Anterior cingulotomies were first conducted to treat schizophrenia (Whitty et al., 1952), but were generally abandoned for psychotic patients due to no lasting benefits being observed (Ballantine, Cassidy, Flanagan, & Marino, 1967). There was greater success, however, in patients with obsessional and anxious characteristics (Whitty, Duffield, Tow, & Cairns, 1952), prompting a switch to these conditions. In an analysis of 198 patients with bilateral cingulum bundle lesions (Ballantine, Bouckoms, Thomas, & Giriunas, 1987) more than half of those treated for obsessive compulsive disorders (OCD) (Jung et al., 2006), anxiety disorders and affective disorders, including depression (Ballantine et al., 1987; Shields et al., 2008) seemingly had lasting improvements. These results are supported by other analyses (Corkin, 1980; Feldman, Alterman, & Goodrich, 2001; Spangler et al., 1996; Steele et al., 2008), including those using standardised assessments (Shields et al., 2008).

While the apparent ability of anterior cingulotomy to provide some level of relief for such a range of psychiatric disorders is striking (Feldman et al., 2001; Linden, 2014), the nature of the improvements reveals similarities across patient groups. Those treated for anxiety, OCD and bipolar depression are described as exhibiting less anxiety, depression, hostility and obsessional thinking post-cingulotomy (Brown & Lighthill, 1968). These improvements seem to manifest in a reduction of attention to

negative thoughts, anxieties, and tension (Cohen et al., 2001), which still occurred post-cingulotomy, but ‘no longer bothered’ patients (Whitty et al., 1952). Related studies have described shallower positive affect after surgery, while motivation is depressed, but not to a clinical degree (Tow & Whitty, 1953; Whitty et al., 1952; Wilson & Chang, 1974).

In addition to treatment for psychiatric disorders, neurosurgeons also targeted the anterior cingulum for chronic pain, with appreciable relief reported in more than half of patients (Pouratian, 2016; Rawlings, Rossitch Jr, & Nashold Jr, 1992). In parallel to the nature of the improvements seen in psychiatric populations, chronic pain patients continued to experience pain but perceived it as less distressing or worrying (Corkin & Hebben, 1981; Foltz & White, 1962). Using standardised assessments in a group of 18 patients, Cohen et al. (2001) found improvements in tension and anger scales a year after undergoing cingulotomy for chronic pain. Meanwhile, although no alterations were observed in measures of self-perceived energy and emotional vibrancy, behavioural passivity and apathy were frequently reported by families of patients (Cohen et al., 2001).

The observation of apathetic characteristics following anterior cingulotomy is particularly interesting because limbic-frontal-subcortical circuits are frequently implicated in the pathophysiology of clinical apathy (Kos, van Tol, Marsman, Knegtering, & Aleman, 2016). Neuroimaging methods consistently reveal robust changes in the anterior cingulate cortex, orbital frontal cortex, and medial thalamus (Le Heron, Apps, & Husain, 2018) in this patient group. Furthermore, apathy is a well-recognized feature of strokes affecting similar regions of the medial frontal cortex (Kang & Kim, 2008; Le Heron et al., 2018) and the medial and anterior thalamus (Carrera & Bogousslavsky, 2006; Krause et al., 2012; Serra et al., 2013). Consequently, the interconnections between these structures take prominence in circuit level explanations of apathy (Kos et al., 2016; Le Heron et al., 2018) and the likely disruption of these fibres in anterior cingulotomy may explain the prevalence of this characteristic in these patients.

Overall, surprisingly few lasting cognitive disturbances appear to result from anterior cingulotomies. Confusion, disorientation, and memory loss can occur transiently (Dougherty et al., 2003; Pouratian, 2016), but after a short postoperative period cognition (Cohen, Kaplan, Moser, Jenkins, & Wilkinson, 1999; Cohen, Kaplan, Zuffante, et al., 1999; Corkin, 1980; Tow & Whitty, 1953; Turner, 1973) and IQ (Ballantine et al., 1967; Brown & Lighthill, 1968; Cohen, Kaplan, Moser, et al., 1999; Cohen, Kaplan, Zuffante, et al., 1999; Fedio & Ommaya, 1970; Kim et al., 2003; Steele et al., 2008) appear largely preserved. There is also a consistent lack of deficits on formal assessments of various types of memory, such as the Wechsler Memory Scale (Cohen, Kaplan, Zuffante, et al., 1999; Corkin, 1980; Fedio & Ommaya, 1970) recall of the Rey-Taylor complex (Corkin, 1980; Jung et al., 2006), delayed alternation (Corkin, 1980), the Hopkins Verbal Learning Test (Jung et al., 2006; Kim et al., 2003) and digit span (Steele et al., 2008). In fact, there have been occasional reports of improvements on some memory scales, including in paired-associate learning and tests of spatial working memory (Steele et al., 2008).

Assessments of executive function have returned more mixed results. In an extensive study of cingulotomy for pain or depression, Corkin (1980) found no deficits on multiple tests of frontal-lobe function including fluency tests, maze tracing and the WCST for 34 patients tested both before and over one year after surgery. In contrast, a smaller group of cingulotomy patients (n=14) treated for OCD showed impaired WCST performance on several measures (Kim et al., 2003). Meanwhile, a study of eight patients surgically treated for depression revealed unaltered performance on tests of executive function, including block design and verbal fluency, and on the trail making test, which taxes visual attention and task switching (Steele et al., 2008). On the other hand, borderline deficits on trail making have been reported in a larger cohort of 18 cases (Cohen, Kaplan, Moser, et al., 1999). Frontal-type deficits have also been seen in a Stroop Interference task, with borderline deficits on a Go/No-Go executive task (Cohen, Kaplan, Moser, et al., 1999). Post-surgical deficits were also found in older patients on a test of visual perception (Thurstone's Hidden Figures) that taxes executive function (Corkin, 1980).

The most consistent difficulties following anterior cingulotomy appear to be in tasks that require focused and sustained attention (Cohen, Kaplan, Moser, et al., 1999; Janer & Pardo, 1991), as well as those that are associated with self-initiated response generation and persistence (Cohen, Kaplan, Zuffante, et al., 1999). This points to a role for the anterior cingulum/anterior cingulate cortex in the initiation and maintenance of behaviour (see also section 1.5.1). Meanwhile, the observation that not all frontal-executive functions are negatively affected by cingulotomy points to the contribution of other pathways.

The cingulotomies described thus far targeted the white matter under the anterior cingulate cortex, with lesions around the level of the genu typically associated with better outcomes than those further caudal, at mid cingulate levels (Corkin, 1980; Richter et al., 2004; Steele et al., 2008). Posterior cingulotomies have rarely been performed. One exception was an attempt to treat chronic aggression in five patients with cases of extreme paranoia or personality disorder (Turner, 1973). The procedure involved the posterior cingulate gyri above the splenium. This presumably disrupted cingulum fibres, but the extent of damage is not clear. Post-operatively, reductions in patient aggression were reported and, surprisingly, memory appeared to be unaffected. There were, however, no formal assessment in this study (Turner, 1973).

Overall, many of the descriptions of cingulotomy appear consistent with the anterior cingulate and adjacent cingulum bundle having a role in the integration of visceral, and affective processes (Dalgleish, 2004), e.g., causing less attention to negative states. Related evidence implicates the anterior cingulate cortex in the maintenance and cortical integration of information from limbic structures, which includes conflict between the current status and perceived indicators of change (Mansouri, Egner, & Buckley, 2017; Rushworth et al., 2004). A related role for the cingulum would be in amplifying or attenuating attention to pain signals (Cohen, Kaplan, Moser, et al., 1999). The discovery of selective deficits in attention and cognitive control also points to a contribution to executive tasks (Cohen, Kaplan, Moser, et al., 1999; Cohen, Kaplan, Zuffante, et al., 1999; Janer & Pardo, 1991). The role of anterior cingulate cortex, and underlying anterior cingulum, in these aspects of cognition is considered further in section 1.5.1.

1.4.3.2 Diffusion tensor imaging (DTI)

Rather than studying intentional cingulum bundle damage (cingulotomies), brain imaging allows the non-invasive investigation of cingulum function by means of correlational analysis. Diffusion tensor imaging (DTI) indices of white matter integrity can be compared between groups (i.e. between clinical groups and controls), can be correlated with clinical symptoms, or can be correlated with performance on cognitive measures. For an overview of the use of diffusion tensor imaging (DTI) to study the microstructure of white matter, see section 1.3.3.

1.4.3.2.1 Psychiatric conditions and the cingulum bundle

There is repeated evidence of cingulum change in a number of psychiatric conditions, including schizophrenia, attention deficit hyperactivity disorder (ADHD), depression, post-traumatic stress disorder (PTSD), obsessive compulsive disorder (OCD), and autism spectrum disorder (ASD). Table 2 highlights the status of specific subdivisions of the bundle in these various psychiatric conditions, alongside correlations with psychometric data.

It is important to remember that such DTI analyses are only correlative and not causal in nature. Indeed, the growing realisation that experience can alter white matter microstructure (McKenzie et al., 2014; Metzler-Baddeley et al., 2017; Zatorre, Fields, & Johansen-Berg, 2012) highlights the problems of separating cause from effect. It must also be remembered that in none of the clinical conditions described is pathology restricted to the cingulum bundle, rather, it is often combined with widespread white matter changes in other frontal pathways.

Table 2. Diffusion MRI studies reporting cingulum bundle changes in psychiatric conditions

Clinical group	Cingulum subsection	Structural change	Supporting research	Neuropsychological correlations	Meta-analysis conclusions
Schizophrenia	Dorsal	FA -	Takei et al., 2009; Kubicki et al., 2003	Lower FA in the left dorsal cingulum correlated with poorer performance on the Wisconsin Card Sorting Test (Kubicki et al., 2003).	Moderate to high quality evidence of a reduction in white matter density and FA in the cingulum in schizophrenia

	Dorsal	MD +	Takei et al., 2009	Higher MD in dorsal cingulum correlated with a longer reaction time on the Stroop Test.	(Shepherd, Laurens, Matheson, Carr, & Green, 2012).
	Dorsal, pregenual anterior	FA -	Takei et al., 2009		
	Dorsal, anterior (RH)	FA -	Sun et al., 2003; Wang et al., 2004; Fujiwara et al., 2007a ; Fujiwara et al., 2007b; Hao et al., 2006 ; Whitford et al., 2014	Lower FA in the right dorsal anterior cingulum correlated with patient scores of hallucinations and delusions (Whitford et al., 2014).	
	Dorsal, anterior (LH)	FA -	Sun et al., 2003; Wang et al., 2004; Fujiwara et al., 2007a; Fujiwara et al., 2007b; Mitelman et al., 2007		
	Dorsal, posterior (RH)	FA -	Fujiwara et al., 2007a		
	Dorsal, posterior (LH)	FA -	Fujiwara et al., 2007a, Mitelman et al., 2007		
	Ventral (RH)	FA -	Whitford et al., 2014	Lower FA in the right ventral cingulum correlated with patient scores of affective flattening and anhedonia/associability	
ADHD	Dorsal, anterior (RH)	FA -	Makris et al., 2008; Konrad et al., 2010		Evidence exists of disturbed white matter integrity in the cingulum in ADHD, but it is not one of the structures most reliably reported to be affected (van Ewijk, Heslenfeld, Zwiers, Buitelaar, &

					Oosterlaan, 2012).
	Dorsal, posterior	FA +	Svatkova et al., 2016		
Depression (bipolar)	Dorsal (RH)	MD +	Benedetti et al., 2011		Evidence of disturbed white matter integrity in the cingulum is mixed in depressive clinical populations. Stronger evidence exists of microstructure alteration in 'at risk' groups (Bracht et al., 2015).
	Dorsal (RH)	RD +	Benedetti et al., 2011		
	Dorsal, anterior	FA -	Wang et al., 2008		
	Dorsal, posterior (LH)	FA -	Wise et al., 2016		
Depression (MDD)	Dorsal	FA -	de Diego-Adelino et al., 2014		
	Dorsal, subgenual anterior	FA -	Cullen et al., 2010		
PTSD	Dorsal	FA +	Kennis et al., 2015	Greater FA in the dorsal cingulum correlated with symptom severity and persistence (Kennis et al., 2015; Kennis et al., 2017).	A small meta-analysis concluded there is preliminary evidence of group differences in cingulum integrity in PTSD. Evidence indicates increases and decreases in FA in different sections of the cingulum (Daniels et al., 2013).
	Dorsal (LH)	FA -	Kim et al., 2006; Sanjuan et al., 2013		
	Dorsal (RH)	FA -	Sanjuan et al., 2013		
	Dorsal, anterior	FA -	Zhang et al., 2011		
OCD	Dorsal (LH)	FA +	Cannistraro et al., 2007; Gruner et al., 2012	Greater FA in the left dorsal cingulum correlated with better performance in response inhibition and cognitive control measures; the Stroop Test and the Trails Making Test (Gruner et al., 2012).	1. There is robust evidence of increased white matter volume and decreased FA in anterior midline tracts (including the cingulum) in OCD (Radua et al., 2014). 2. There is evidence FA is typically
	Dorsal (RH)	FA -	Cannistraro et al., 2007		

	Dorsal, anterior (LH)	GFA -	Chiu et al., 2011	GFA in left anterior cingulum correlated with higher scores in measures of obsession.	reduced in the cingulum in adults and increased in paediatric and adolescent samples (Koch, Reeß, Rus, Zimmer, & Zaudig, 2014).
	Dorsal (RH)	MD -	Lochner et al., 2012	MD in the right body of the dorsal cingulum negatively correlated with scores in measures of anxiety and depression.	
	Dorsal, anterior (LH)	MD -	Lochner et al., 2012	MD in the left anterior cingulum correlated with scores on an obsessive compulsive scale.	
	Ventral (LH)	FA -	Fan et al., 2016		
ASD	Dorsal	FA -	Ikuta et al., 2014; Shukla et al., 2011	Reduced FA in the cingulum correlated with poorer behavioural regulation scores (Ikuta et al., 2014).	1. There is evidence of cingulum microstructure changes in autism, most consistently reduced FA and/or increased MD in the anterior cingulum (Travers et al., 2012). 2. Combining datasets from five studies found no evidence to support a significant difference in cingulum FA between autistic subjects and typically developing controls (Aoki, Abe, Nippashi, & Yamasue, 2013).
	Dorsal	MD +	Shukla et al., 2011		
	Dorsal	RD +	Shukla et al., 2011		
	Dorsal, anterior	FA -	Jou et al., 2011		

Examples of diffusion MRI studies that have reported cingulum bundle changes in schizophrenia, attention deficit hyperactivity disorder (ADHD), depression (including major depressive disorder, MDD), post-traumatic stress disorder (PTSD), obsessive compulsive disorder (OCD), and autism spectrum disorder (ASD). The columns show which portion of the cingulum appeared abnormal and

provide neuropsychological correlations. Relevant meta-analyses are in the right column. Other abbreviations: FA, fractional anisotropy; GFA, global FA; MD, mean diffusivity; RD, radial diffusivity; +, increase; -, decrease. Reductions in FA and increases in diffusivity are usually seen as evidence of a loss of white matter integrity.

Several interesting conclusions emerge from Table 2. The commonest reported cingulum changes are reduced fractional anisotropy (FA) and increased diffusivity (mean diffusivity, MD, and radial diffusivity, RD), both of which are thought to reflect a disruption of white matter integrity. However, the opposite is sometimes found (increased FA and decreased MD/RD), and there are instances where different parts of the cingulum have been shown to exhibit opposite changes in diffusivity, such as in PTSD (Daniels et al., 2013) and OCD (Cannistraro et al., 2007). Furthermore, microstructure changes frequently appear to be restricted to subsections of the cingulum, rather than affecting the entire tract. Taken together, these observations suggest that psychiatric conditions are underpinned by cingulum changes that differ along the length of the tract; pointing to the need for further investigation into how subgroups of cingulum connections map onto symptomatology.

It is the dorsal cingulum, above the corpus callosum, that appears to be most robustly affected in each of the psychiatric conditions where DTI changes most consistently appear (Table 2, schizophrenia, ADHD, depression, PTSD, OCD, and ASD). Unfortunately, many studies do not differentiate between subdivisions of the dorsal cingulum. Of those that do, changes to the anterior portion are most common, but changes that extend to the posterior dorsal cingulum in schizophrenia, ADHD, bipolar depression have also been found in some studies. This lack of consistency across studies may relate to the need to separate different subtypes within disorders, which are associated with differing profiles of cognition (Svatkova et al., 2016). In discerning how white matter changes relate to function, it is those studies that correlate microstructure changes with symptoms and measures of cognition that are of particular interest (Table 2).

1.4.3.2.2 Cingulum DTI indices and cognition

There is accumulating evidence that the cingulum bundle, notably its dorsal/anterior portion, mediates performance in ‘frontal’ tests of cognitive control and executive

function. For example, a recent study of 202 cognitively normal, healthy adults (Bettcher et al., 2016) found that FA differences in the dorsal cingulum correlated independently with all executive functions measured (shifting/inhibition, updating/working memory and processing speed). In contrast, prefrontal cortex grey matter volume did not independently predict executive performance. These results are consistent with a battery of previous reports of correlations between FA metrics in the dorsal/anterior cingulum and executive functions, attention and working memory (Charlton, Barrick, Lawes, Markus, & Morris, 2010; Chiang, Chen, Shang, Tseng, & Gau, 2016; Kantarci et al., 2011; Metzler-Baddeley et al., 2012; Takahashi et al., 2010).

Further evidence of an association between the dorsal cingulum and executive function comes from clinical groups (Table 2). In schizophrenia, associations have been found between DTI indices of the dorsal cingulum and performance on the Wisconsin card sorting task (Kubicki et al., 2003) and the stroop test (Takei et al., 2009). Meanwhile, in autistic spectrum disorder correlations were found for behavioural regulation scores (Ikuta et al., 2014), consistent with executive dysfunction, though other studies have failed to find clear cingulum correlations (Rane et al., 2015). In attention deficit hyperactivity disorder (ADHD), studies reveal that clinical symptoms and executive dysfunction correlate most with the status of fronto-striatal tracts (Angriman, Beggiato, & Cortese, 2014). Nevertheless, Chiang et al. (2016) reported lower cingulum FA in ADHD that correlated with inattention, alongside the loss of cingulum correlations with executive functions seen in controls.

Meanwhile, FA in the posterior dorsal cingulum has been found to contribute to attention/executive, language and visuo-spatial function in a group of 220 cognitive healthy older adults (Kantarci et al., 2011). Another study (Metzler-Baddeley et al., 2012) found individual FA differences in the anterior and posterior cingulum portion, but not the middle or the parahippocampal portion, to correlate with executive function tasks (category fluency and stroop test). It is apparent that while this relationship with executive function echoes that reported in some studies of cingulotomy (Cohen, Kaplan, Moser, et al., 1999; Cohen, Kaplan, Zuffante, et al.,

1999), the DTI literature suggests that tract involvement is not limited to the anterior portion, and contributes to a wider range of attributes and tests.

Evidence concerning the association of the cingulum bundle with memory functions (other than working memory) is more complex. While some studies have found correlations between episodic memory and the parahippocampal cingulum (Ezzati, Katz, Lipton, Zimmerman, & Lipton, 2016), correlations between white matter microstructure and episodic memory in healthy populations are much more reliably found for the fornix than the cingulum (Douet & Chang, 2015; Rudebeck et al., 2009). However, in contrast to the evidence from healthy populations, correlations between cingulum microstructure and memory are more frequently described within some clinical groups, such as older adults with Mild Cognitive Impairment (Yu, Lam, & Lee, 2017), and Alzheimer's disease (Kantarci et al., 2011).

Metzler-Baddeley et al. (2012) found that while healthy controls showed correlations between performance in episodic memory tasks and FA in the fornix, but not the parahippocampal cingulum, patients with Mild Cognitive Impairment showed correlations between memory and microstructure in both pathways (Metzler-Baddeley et al., 2012). A follow-up study (Ray et al., 2015) then demonstrated that the shift from correlations between memory and the fornix to correlations between memory and the parahippocampal cingulum was largest for MCI patients with better memory performance; suggesting that in the presence of fornix impairments, episodic memory can be supported by the parahippocampal cingulum.

Overall, DTI data reveal overlapping patterns of fronto–cortical and fronto-limbic changes across a variety of disorders, with cingulum alterations a frequent component (Table 2). The dorsal/anterior cingulum appears to be most affected in psychiatric conditions characterised by changes to emotional and/or executive function, while alterations in more posterior parts of the cingulum tend to be associated with conditions associated with memory dysfunction. Supported also by correlations observed between cingulum DTI indices and cognition in healthy populations, there appears to be a shift in function from genual parts of the cingulum (executive function/emotion) to parahippocampal parts (memory). This transition

seems to be gradual in nature and the latter association appears more robust in some clinical populations.

1.4.4 Summary and outlook for investigation

Table 3 brings together findings from multiple studies of cingulum bundle activity or dysregulation in order to highlight any recurring sets of functions. From the data discussed in this section, those functions most consistently linked to different parts of the cingulum bundle are emotion (including social interactions), motivation, executive functions (including aspects of attention), pain, and memory. Consistent with how the core connections within the cingulum bundle are retained across species, it is notable that these functional categories are supported by research in the rat, the monkey and the human.

Table 3. Major functions ascribed to various parts of the cingulum bundle

Function	Principal connections	Suggested subsection	Evidence
Emotion (note link with pain as well as aspects of empathy)	Amygdala, medial and orbital prefrontal cortices, anterior cingulate cortex	Subgenual, anterior cingulate	**R** Anterior cingulate cortex lesions disrupt social responsiveness **M** Lesions involving CB cause subtle social deficits **H** Anterior cingulotomy is partially effective in treating affective disorders **H** Anterior cingulotomy is sometimes associated with decreased anxiety, depression, and hostility across clinical groups **H** Affective disorders are associated with dMRI changes in white matter tracts, including the CB **H** Emotion and reward related fMRI activity in subgenual and anterior cingulate cortex as well as amygdala.
Motivation	Anterior cingulate cortex, medial and anterior thalamus, medial and orbital frontal cortices	Anterior cingulate, subgenual	**R** Anterior cingulate lesions affect response cost judgements **H** Apathy is sometimes associated with anterior cingulotomy **H** Importance of orbital and medial frontal areas for hedonics **H** Reward related fMRI activations in ventromedial frontal and anterior cingulate areas

Executive function (including attention)	Dorsolateral and anterior cingulate cortices, Medial, anterior and midline thalamus, ascending cholinergic fibres	Anterior cingulate, subgenual	**R** **M** **H** **H** **H**	Anterior cingulate lesions disrupt attentional tasks dependent on cholinergic inputs Rostral cingulate lesions involving the CB can disrupt some executive functions Anterior cingulotomy is associated with deficits in high level processing and selection dMRI correlations between anterior/dorsal cingulum and tests of cognitive control and executive function fMRI studies of control tasks
Pain	Midline and intralaminar thalamic nuclei, anterior cingulate cortex	Anterior cingulate, mid-cingulate	**R** **H** **H**	Blockade of CB leads to analgesia and delayed self-mutilation, whereas stimulation precipitates self-mutilation Anterior cingulotomy is partially effective in treating chronic pain Supracallosal cingulate fMRI activity in pain
Memory (including spatial processing)	Hippocampus, anterior thalamic nuclei, retrosplenial and parahippocampal cortices	Parahippocampal, retrosplenial	**R** **M** **H** **H** **H**	CB lesions can disrupt performance on spatial tasks involving allocentric cues Mild, inconsistent memory effects after supracallosal lesions that invade CB Anterior cingulotomy is associated with borderline deficits on some memory measures Link from dMRI between parahippocampal bundle and memory performance Memory loss and topographic amnesia is associated with retrosplenial cortex damage

Column 2 indicates those cortical and subcortical connections most linked with the relevant function. Column 3 refers to that those subdivisions of the cingulum bundle (CB) particularly associated with that class of function. Column 4 gives examples of the relevant evidence from studies of rats (R), nonhuman primates (M), and humans (H). Note that at present there is a lack of evidence concerning selective cingulum bundle disruption in nonhuman primates.

Given the complex nature of the tract and its patterns of connectivity, it should perhaps be no surprise that these different attributes often interact with each other, blurring their distinctions. Memory can be somewhat distinguished from the other functions as it appears to rely upon the posterior part of the cingulum, principally driven by the retrosplenial and parahippocampal cortices (Table 3). The remaining functions appear to rely, at least in part, upon the anterior cingulum. Importantly, the

common denominator of all of these functions appears to be the involvement of the anterior cingulate cortex.

This raises questions regarding the extent to which aspects of emotion, pain, motivation and executive processing overlap; and whether these functions can be dissociated on the basis of further subgroups of fibres reflecting anterior cingulate cortex connections to different structures. To this end, Table 3 speculates which principal cingulum connections are most likely to underlie each function. However, the evidence for these suggestions is primarily indirect, as selective disconnections of structures have very rarely been performed.

As highlighted in section 1.3, connections between the anterior thalamic nuclei and the anterior cingulate cortex form a major component of the cingulum. This subgroup of connections is robust across species and will be the subject of anatomical investigation in this thesis (Chapter 3). Superficially, these regions appear to have little overlap in terms of function. While the anterior cingulate cortex has been linked to a variety of cognitive functions (including emotion, motivation, executive function, pain and conflict, see section 1.5.1, (Beckmann et al., 2009), investigation of the anterior thalamic nuclei has typically been limited to spatial navigation (see section 1.6). What, therefore, is the function of such dense interconnections between these regions? This question will form the focus of the functional investigation of this thesis (Chapters 4, 5 and 6).

1.5 Functions of major contributory regions to the cingulum bundle

In order to investigate the function of their connectivity, this section will first consider the functions of the anterior cingulate cortex and the anterior thalamic nuclei independently. Particular attention will be paid to those aspects of cognition that have also been associated with the anterior cingulum and may therefore be supported by the anterior cingulate – anterior thalamic interconnections provided by the bundle.

1.5.1 Anterior cingulate cortex

1.5.1.1 Executive function and cognitive control

In recent decades there has been a rapid expansion of empirical data relating to the anterior cingulate cortex, and the region has subsequently been associated with many different functions, including emotion, reward, pain, motor, conflict and error processing (Beckmann et al., 2009). Amongst the most widely agreed upon observations is the involvement of the dorsal anterior cingulate cortex in executive function (Shenhav, Cohen, & Botvinick, 2016). Broadly defined, executive function comprises 'higher level' cognitive processes that regulate 'lower level' processes, in order to effortfully guide behaviour towards a goal (Alvarez & Emory, 2006; Banich, 2009).

The dorsal anterior cingulate cortex appears to be important for cognitive control. That is, the ability to adapt behaviour in accord with an internally held goal. This ability is especially important when competing responses, such as those invoked by distraction or habit, must be overcome (Shenhav, Botvinick, & Cohen, 2013). Much of the evidence for a role of the dorsal anterior cingulate cortex in cognitive control comes from human neuroimaging, where multiple meta-analyses have confirmed its activation in tasks that demand control (Nee, Wager, & Jonides, 2007; Niendam et al., 2012; Ridderinkhof, Ullsperger, Crone, & Nieuwenhuis, 2004; Shackman et al., 2011). Importantly, evidence also directly implicates the cingulum bundle, with low white matter integrity in the anterior portion associated with poor performance on control demanding tasks (Metzler-Baddeley et al., 2012)

In particular, activity in the dorsal anterior cingulate cortex has been consistently associated with cognitively challenging situations where there is conflict (Botvinick, Cohen, & Carter, 2004; Carter & van Veen, 2007; Gasquoine, 2013), errors (Gasquoine, 2013; Holroyd & Coles, 2002) and the need for task switching (Nee, Kastner, & Brown, 2011). This has led several researchers to propose that the dorsal anterior cingulate cortex serves a monitoring function, detecting challenges that require an increase in cognitive control (Botvinick, 2007; Carter & van Veen, 2007; Shenhav et al., 2013; Shenhav et al., 2016).

In fact, anterior cingulate cortex activation in situations requiring override of prepotent responses has become one of the most widely replicated findings in cognitive neuroscience (Botvinick, 2007), such as increased anterior cingulate cortex activation on incongruent as opposed to congruent trials in the Stroop task (see Barch et al., 2001, for a review). Supporting the view that this detection leads to reactive adjustments in behaviour, Kerns et al. (2004) found anterior cingulate cortex activity on trials in the Stroop task to be correlated with the strength of top-down control on succeeding trials. Further evidence comes from animal studies, where glutamatergic activation of the anterior cingulate cortex has been seen to produce an aversive learning signal, leading to subsequent avoidance behaviour in the rat (Johansen & Fields, 2004).

One particularly striking study used human single cell recording, along with neuroimaging pre and post anterior cingulotomy, to demonstrate that the dorsal anterior cingulate cortex mediates ongoing behavioural adaptation. Using a Stroop-like task, Sheth et al. (2012) demonstrated that level of cognitive interference correlated with dorsal anterior cingulate cortex activity, recorded from single units and from fMRI regional signal. Moreover, current dorsal anterior cingulate cortex activity was modulated by dorsal anterior cingulate cortex on the previous trial, such that behavioural responses were accelerated when responding to trials of similar difficulty and were slowed when responding to trials of differing difficulty. This conflict adaptation was abolished by anterior cingulotomy. Taken together, these findings suggest that the dorsal anterior cingulate cortex keeps track of expected cognitive demand to optimise behavioural responding (Sheth et al., 2012; Shenhav et al., 2013, 2016).

1.5.1.2 Reinforcement-based decision making, action selection and motivation

A closely related body of research, based mainly on nonhuman animals, links the dorsal anterior cingulate cortex with reinforcement-based decision making (Rushworth, Behrens, Rudebeck, & Walton, 2007; Shenhav et al., 2016). While the orbitofrontal cortex has most typically been associated with reinforcement learning

(Rushworth et al., 2007), more recent evidence has also implicated the anterior cingulate cortex. Both regions respond to reinforcement in both single-unit recordings (Amiez, Joseph, & Procyk, 2005a; Roesch & Olson, 2004) and neuroimaging experiments (Cox, Andrade, & Johnsrude, 2005; Knutson, Taylor, Kaufman, Peterson, & Glover, 2005), while lesions impact on those behaviours that are normally learned through reinforcement (Amiez, Joseph, & Procyk, 2005b; Izquierdo, Suda, & Murray, 2004). Rushworth et al. (2007) have proposed that the two regions contribute differentially to reinforcement, with the orbitofrontal cortex encoding the reinforcement value of a *stimulus* and the anterior cingulate cortex encoding the reinforcement value of an *action.*

Both regions share connections with reinforcement related structures such as the striatum and the amygdala, whereas the orbitofrontal cortex shares more connectivity with areas that encode the properties of stimuli, such as visual areas (Rushworth et al., 2007). Consistent with this observation, anterior cingulate cortex lesions do not impair performance on visual stimulus discrimination tasks that are sensitive to orbitofrontal lesions in the rat (Chudasama & Robbins, 2003) and the monkey (Rudebeck et al., 2006), respectively. On the other hand, the anterior cingulate cortex has more interconnections with motor areas (Miyachi et al., 2005) and regions involved in spatial processing, such as the anterior thalamus (see section 1.3). Indeed, several studies have demonstrated single unit responses in the macaque anterior cingulate cortex to both spatial and non-spatial aspects of reward (Matsumoto, Suzuki, & Tanaka, 2003; Shima & Tanji, 1998).

Kennerley et al. (2006) provided more direct evidence for the central role of the anterior cingulate cortex in action selection using a reward-learning behavioural paradigm in the macaque, where a pull or a turn of the same lever was associated with a food reward. The action an animal typically chooses on a given trial is determined by the reward history that has been associated with that action over time. However, macaques with lesions to the anterior cingulate cortex based their choice solely on the outcome of the most recent action, indicating that the anterior cingulate cortex plays a role in incorporating reward history to determine action selection. Distinguishing this result from a conflict-monitoring or error related function,

Kennerley et al. (2006) reported that animals with anterior cingulate cortex lesions did not differ from controls on single trials that followed an error. Rather, their deficit appeared to be a result of the failure to accrue positive reinforcement over time.

Closely related evidence indicates that the anterior cingulate cortex may also be important for the generation of exploratory actions, potentially through its close links with the motor system (Rushworth et al., 2007). That is, it may encode the value of actions themselves, rather than solely linking outcome/reward history with action choice. In the macaque, single unit recordings reveal anterior cingulate activity both during exploration of the reward environment and during monitoring of the outcome of those actions (Hayden & Platt, 2010). Such anterior cingulate activity is further consistent with the literature connecting the region with effort-based decision making and motivation. Anterior cingulate ablation diminishes the natural willingness of rats to climb over a barrier in order to receive a larger reward (Salamone, Cousins, & Bucher, 1994). Meanwhile, the same rats show no difference in the length of time they are willing to wait for reward, indicating that the deficit is specific to choices that require effortful action. Similarly, anterior cingulate lesions limit the amount of responses a macaque will make in order to receive a reward (Kennerley et al., 2006).

Taken together, these results suggest that the anterior cingulate is involved in evaluating the cost (effort) of an action itself, as well as the association of that action with reward (Rushworth et al., 2007; Rushworth et al., 2004). In this way, the anterior cingulate appears to mediate the relationship between previous action-reinforcements and real-time choices. One interesting possibility is that the anterior cingulate sets the length of reinforcement history that influences the current action evaluation (Rushworth et al., 2007).

1.5.1.3 Emotion and pain

Closely related to an evaluative function of the anterior cingulate cortex is its well-established link to emotion, where it appears to play a role in the appraisal of emotional stimuli and the formation of responses (Etkin, Egner, & Kalisch, 2011). A

long-held view postulates that the dorsal anterior cingulate cortex is involved in 'cognitive' functions, whereas ventral regions are involved in emotion (Bush, Luu, & Posner, 2000). However, recent reassessments have found that the empirical data does not fully support this distinction (Etkin et al., 2011). Several meta-analyses of human neuroimaging confirm activation of the dorsal anterior cingulate cortex, in conjunction with other medial prefrontal cortex (mPFC) regions, in the appraisal of stimuli signalling threat and in the subsequent expression of fear (Etkin et al., 2011; Levenson, 2003; Mechias, Etkin, & Kalisch, 2010). Similarly, engagement of the dorsal (as well as ventral) anterior cingulate cortex has been associated with the experience of perception of pain (Lamm, Decety, & Singer, 2011) and with sensitivity to a range of emotions, including disgust (Mataix-Cols et al., 2008) and rejection (Eisenberger, Lieberman, & Williams, 2003).

Instead, it has been suggested that the dorsal and ventral regions of the anterior cingulate cortex represent an anatomical and functional continuum, blending features of cognitive and emotional processing, as opposed to a strict dichotomy (Gasquoine, 2013; Mohanty et al., 2007). For example, studies using emotional analogues of the Stroop task find dorsal anterior cingulate activation when emotional facial expressions (e.g. fearful) are presented with incongruent word labels (e.g. happy) (Etkin, Egner, Peraza, Kandel, & Hirsch, 2006). Accompanied by a slowing of reaction times on incongruent trials, this activity is thought to reflect the detection of emotional conflict (Etkin et al., 2011; Etkin et al., 2006). Under a cognitive control framework of anterior cingulate function, emotional conflict can be interpreted as a challenging cognitive state that requires additional cognitive control, or attentional resources, in order to modify subsequent behavioural responses (Gasquoine, 2013). Interestingly, the association of pain with the anterior cingulate cortex (see section 1.4.3.1, regarding the treatment of chronic pain by anterior cingulotomy), could be explained under this same framework.

1.5.1.4 Behavioural flexibility and attentional set-shifting

Appropriate cognitive control allows for behavioural flexibility, enabling the updating of responding to effectively navigate a world with changing environmental contingencies (Bissonette, Powell, & Roesch, 2013; Brown & Tait, 2015). A

common method for assessing this capability in animals is the use of an attentional set-shifting paradigm. An analogue of the Wisconsin card sorting task used in humans, these tasks require an animal to learn abstract rules to receive a reward and challenge the ability to shift between available rules (Bissonette et al., 2013; Tait, Bowman, Neuwirth, & Brown, 2018). These tasks are particularly interesting as they involve conflict (competition between behavioural responses), error detection, and reinforcement-based decision making; all of which have been associated with the anterior cingulate cortex, as described in the preceding sections.

In the rat, digging tasks are commonly used (Birrell & Brown, 2000) and tax two key elements of set-shifting. The first is intradimensional set-shifting where, after initial rule acquisition, an animal must continue to attend to new exemplars in a stimulus dimension that is a reliable reward predictor, e.g. odour, while ignoring an irrelevant stimulus dimension, e.g. digging media. The second is extradimensional set-shifting, where an animal must respond to the previously irrelevant stimulus dimension (e.g. digging media) that becomes predictive of reward. Perhaps importantly, this version of set-shifting requires the animal to approach and dig in pots, which contain the stimuli, to obtain the reward. It thus requires *action* selection (some of which is exploratory), rather than just *stimulus* selection, which may be important for engaging the anterior cingulate cortex (Kennerley et al., 2006; Rushworth et al., 2007).

Attentional set-shifting tasks have been used to dissociate between aspects of performance underpinned by the anterior cingulate cortex and other mPFC areas (typically prelimbic, but also infralimbic cortex; Birrell & Brown, 2000). Lesions to either region do not impair the initial learning of rules during set-shifting (Birrell & Brown, 2000; Bissonette et al., 2013; Dias, Robbins, & Roberts, 1996a; Ng, Noblejas, Rodefer, Smith, & Poremba, 2007) suggesting that neither are critical for rule formation *per se*. However, Ng et al. (2007) demonstrated that anterior cingulate lesions impair the ability to shift between rules within the same perceptual dimension (intradimensional set-shifting). On the other hand, mPFC lesions impair performance when shifting between rules relating to different perceptual dimensions (extradimensional shifts) (Birrell & Brown, 2000; Ng et al., 2007).

It appears, therefore, that the anterior cingulate cortex and mPFC play different roles in attentional set-shifting. These roles can be further dissociated from that of the orbitofrontal cortex, which appears to primarily mediate reversal learning (Bissonette et al., 2013; Chase, Tait, & Brown, 2012). That is, where the same stimuli from the previous discrimination are presented, but reward contingencies are reversed so that the previously unrewarded stimulus (from the same dimension) is rewarded. This is thought to reflect a role of the orbitofrontal cortex in reward expectancy signalling, and has been reviewed extensively elsewhere (Schoenbaum, Roesch, Stalnaker, & Takahashi, 2009; Schoenbaum, Takahashi, Liu, & McDannald, 2011). It should be noted that there is evidence that the orbitofrontal cortex also contributes to intradimensional set-shifting, similarly attributed to its role in identifying relevant stimuli following unexpected outcomes (Chase et al., 2012).

The mechanisms underpinning the differential contributions of the anterior cingulate cortex and mPFC are less well understood. The observation that the anterior cingulate cortex is critical for intradimensional, but not extradimensional, shifts might reflect a number of functions. For example, it could reflect engagement in generalising a rule within a dimension, resolving response conflict, detecting errors, or directing attention away from irrelevant stimuli (Bissonette et al., 2013). Within a cognitive control framework (Shenhav et al., 2013; Shenhav et al., 2016) of anterior cingulate function, it may contribute to intradimensional shifts by using reward history to signal reward prediction errors (Bissonette et al., 2013), which, in turn, allows attention to be directed to the most relevant predictors of reward on subsequent trials.

In this way, Bissonnette (2013) has suggested that the anterior cingulate cortex may function to provide recent reward history and information about current behavioural choices. This would allow attention to be directed towards those stimuli that are most relevant to reward, and away from those stimuli that are irrelevant to reward. In the absence of this information from the anterior cingulate cortex, attention may not be appropriately focused within the relevant dimension, explaining the impact of lesions on intradimensional shifts, but not extradimensional shifts (Ng et al., 2007).

The mPFC may, in turn, use this information to switch between rules and hold online that which is currently most rewarding (Bissonette et al., 2013). In the absence of mPFC function, adherence to previously learned rules may be inappropriately maintained, leading to so-called 'stuck in set' behaviour and a resultant deficit in extradimensional set-shifting (Birrell & Brown, 2000; Bissonette et al., 2013).

1.5.1.5 Overview

Debates about the function of the anterior cingulate cortex have persisted for decades. Convincing evidence links this region to executive function, conflict, error monitoring, reward-based decision making, emotion, motivation, and action selection. Progress has been made in recent years towards unifying theories of anterior cingulate function; where the central tenant is its involvement in performing a cost/benefit analysis (that includes the reinforcement history and the cost of performing an action itself) that guides reactive adjustments of behaviours (Gasquoine, 2013; Shenhav et al., 2013; Shenhav et al., 2016; Sheth et al., 2012).

Nonetheless, this functionality is likely to be distributed differentially across subpopulations of anterior cingulate neurons which, in turn, interact with different brain structures (Shenhav et al., 2016). Accordingly, the challenge now is to devise experiments to further parse apart the component contributions of subpopulations of the anterior cingulate cortex to cognitive control and behavioural flexibility. A promising starting point would be to further utilise attentional set-shifting tasks, where different types of shift rely differentially on aspects of the functions consistently associated with the anterior cingulate cortex.

1.5.2 Anterior thalamic nuclei

The anterior thalamic nuclei are closely connected with the hippocampus (Bubb et al., 2017; Irle & Markowitsch, 1982), and have been consistently associated with spatial navigation (Aggleton & Nelson, 2015; O'Mara, 2013). In the rat, lesions to the anterior thalamic nuclei impair many aspects of spatial learning, and are particularly disruptive when distal (allocentric) cues specify location (Aggleton & Nelson, 2015; Wolff, Alcaraz, Marchand, & Coutureau, 2015). However, the anterior thalamic nuclei are also densely interconnected with the prefrontal cortex

(Mathiasen, Dillingham, Kinnavane, Powell, & Aggleton, 2017; Shibata, 1993b; Shibata & Naito, 2005), suggesting that their function might not be limited to the spatial domain (Kinnavane, Amin, Aggleton, & Nelson, 2019).

Initial evidence for a role of the anterior thalamic nuclei in non-spatial functions comes from clinical literature, where damage to the anterior thalamus has been associated with executive dysfunction (Ghika-Schmid & Bogousslavsky, 2000; Little et al., 2010). In cases of localised anterior thalamic infarction (Ghika-Schmid & Bogousslavsky, 2000), patients are described as displaying perseverative behaviour when engaging in executive tasks, as well as being distractible and sensitive to interference. Interestingly, apathy is also a significant and persistent feature in this patient group (Ghika-Schmid & Bogousslavsky, 2000). This parallels the effects of cingulotomy (see section 1.4.3.1), potentially implicating anterior thalamic connections within the cingulum in motivated behaviour. However, it is important to note the potential confound of damaged fibres of passage, for example to frontal areas, in stroke patients.

In traumatic brain injury, integrity of both the anterior thalamic nuclei and their fibres have been found to be correlated with executive function (Little et al., 2010). The same study found an absence of correlations between frontal cortical regions and executive measures, leading the authors to conclude that reduced integrity of anterior thalamic-cortical pathways underlies the executive dysfunction observed in this patient group (Little et al., 2010). Meanwhile, a DTI study in healthy bilingual adults demonstrated that the integrity of anterior thalamic fibres predicts performance in the Stroop test (Mamiya, Richards, & Kuhl, 2018), suggesting a role for this pathway in directing attention in the presence of conflicting cues.

However, evidence is sparse for a role of the anterior thalamic nuclei in non-spatial functions in animals. This is largely because lesion studies have primarily used tasks known to involve the hippocampus (Wolff et al., 2015), motivated by hippocampal-anterior thalamic connectivity, and have therefore focused on memory functions. Of those that have addressed aspects of executive function, most studies have returned null results. Lesions to the anterior thalamus have no apparent effect on simple

discriminations or reversals (Chudasama, Bussey, & Muir, 2001; Wright, Vann, Aggleton, & Nelson, 2015), nor do they impair visual discriminations in a water maze (Moreau et al., 2013).

One exception is a study conducted by Wright et al. (2015), where rats with lesions of the anterior thalamic nuclei displayed a striking deficit in attentional set-shifting. This study used a task involving both intradimensional set-shifting, where animals must respond to new exemplars of the same stimulus dimension, and extradimensional set-shifting, where animals must switch responding to exemplars of a new, previously irrelevant stimulus dimension (as explained in section 1.5.1.4). Anterior thalamic lesions were found to impair intradimensional set-shifting but, paradoxically, to facilitate extradimensional set-shifting (Wright et al., 2015). Subsequent testing revealed that extradimensional shifting was facilitated when the newly relevant stimulus dimension had been previously established as an unreliable reward predictor. This led the authors to suggest a role for the anterior thalamic nuclei in directing attention to task relevant stimuli (Wright et al., 2015), whereby in the absence of anterior thalamic function unreliable reward predictors receive undue attention. This manifests as an advantage when contingencies change, and a previously unreliable stimulus dimension becomes predictive of reward.

Taken together, the effects of anterior thalamic lesions on discrimination learning appear to parallel those of anterior cingulate cortex lesions. Damage to anterior cingulate cortex similarly spares initial acquisition of simple discriminations and reversals, while impairing intradimensional set-shifting (Chudasama & Robbins, 2003; Ng et al., 2007) (see section 1.5.1.4). On the other hand, the effects of lesions of the anterior thalamic nuclei appear to be dissociated from the effects of lesions to other prefrontal cortical areas. Particularly stark is the difference between anterior thalamic and prelimbic lesions in extradimensional set-shifting; with the former facilitating (Wright et al., 2015) and the latter impairing (Birrell & Brown, 2000) these discriminations. Meanwhile, the observations that anterior thalamic lesions do not impair reversal learning or visual discriminations (Chudasama & Robbins, 2003; Wright et al., 2015) stand in contrast to the effects of orbitofrontal lesions (Bissonette et al., 2013; Chudasama & Robbins, 2003).

1.6 Rationale for the following experiments

As has been established in the preceding sections, the cingulum bundle is a highly complex fibre pathway, both anatomically and functionally. DTI indices reveal an array of correlations with functions, and with clinical conditions, that are helping to redefine the importance of the cingulum in cognition. It is becoming increasingly apparent that there is shifting functionality along the length of the tract, reflecting a changing composition of connections. Consequently, there has been a move to divide the cingulum into subdivisions, yet human neuroimaging methods are critically limited by their inability to isolate specific connections that comprise the tract. To address this challenge, this thesis uses contemporary viral-based techniques that allow the structure and function of the cingulum to be investigated with high precision in the rat brain.

Focusing on the fibres that connect the cingulate cortices and the anterior thalamic nuclei, which are retained across species, Chapter 3 uses anterogradely transported viral tract tracing to examine the anatomical course of this substantial subset of cingulum connections. This will provide detailed information on which such connections are present in different parts of the cingulum bundle. Next, Chapters 4, 5, and 6 investigate the function of the fibres that connect the anterior cingulate cortex and the anterior thalamic nuclei. As described in section 1.5, lesions to both regions have been independently found to impair intradimensional, but not extradimensional, set-shifting in rats (Ng et al., 2007; Wright et al., 2015). Given their dense interconnectivity, elucidated in Chapter 3, it seems that both regions may be involved in a functional circuit underpinning aspects of behavioural flexibility that are dissociable from the functions of other prefrontal areas.

To investigate this hypothesis the experiments in this thesis initially examined the effects of effects of downregulating (Chapter 4), and upregulating (Chapter 5), the activity of anterior cingulate cortex on attentional set-shifting. This is achieved using inhibitory (Chapter 4), and excitatory (Chapter 5) Designer Receptors Exclusively Activated by Designer Drugs (DREADDs, see Chapter 2.3). Based on the observation that the anterior cingulate cortex and the anterior thalamic nuclei seem to perform related functions in attentional set-shifting, Chapter 6 uses inhibitory

DREADDs and associated methods (see Chapter 2.3) to selectively inhibit anterior cingulate cortex efferents to the anterior thalamic nuclei. The aim of this final experiment is to establish whether this interconnectivity, involving cingulum fibres, is responsible for the seemingly shared contributions of these two regions to behavioural flexibility.

2 General Methods

2.1 Overview

The general protocol for experiments described in this thesis will be described here. Where specificities differ between experiments, these will be described in the respective chapters. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and associated guidelines and were approved by the local ethical review committees at Cardiff University.

2.2 Anatomical borders and nomenclature

The rat brain atlas of Paxinos and Watson (2005) was used to identify surgical coordinates (mm from Bregma) and to identify brain areas in virus and c-*fos* expression analysis. This atlas was also used for nomenclature (Paxinos & Watson, 2005), where Cg1 refers to the dorsal subdivision and Cg2 refers to the ventral subdivision of the anterior cingulate cortex. One exception is the retrosplenial cortex, where the borders and nomenclature described by Van Groen and Wyss (1990, 1992, 2003) are used; that is dysgranular (Rdg), granular a (Rga) and granular b (Rgb) subdivisions. In this instance, the brain atlas of Swanson (2004) was used to identify brain areas.

2.3 Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)

2.3.1 A brief introduction to DREADD technology

The experiments described in Chapters 4, 5 and 6 of this thesis used designer receptors exclusively activated by designer drugs (DREADDs) (Alexander et al., 2009; Armbruster, Li, Pausch, Herlitze, & Roth, 2007; Roth, 2016) to alter the activity of anterior cingulate cortex neurons. A chemogenetic technology, DREADDs are genetically modified endogenous muscarinic g-coupled protein receptors (GCPRs) with binding sites engineered to interact with previously unrecognised ligands (Armbruster et al., 2007). GCPRs are expressed in the cell body of neurons and when a ligand binds the extracellular receptor, the associated

intracellular g protein is mobilised. This regulates the activity of other proteins in the cell membrane, which, in turn, modifies the excitability of the neuron (Figure 2.1) (Roth, 2016; Samama, Cotecchia, Costa, & Lefkowitz, 1993).

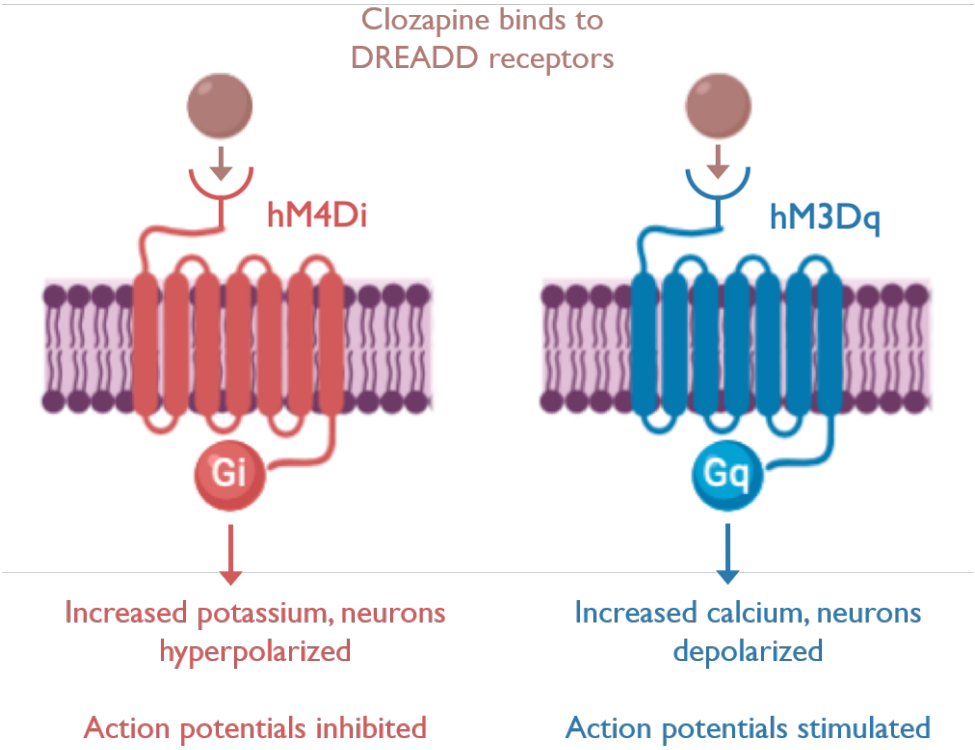

Figure 2.1. Schematic diagram of the ligand clozapine binding to DREADD receptors to influence neuronal activity

Different DREADDs have been developed to decrease (Armbruster et al., 2007) or increase (Alexander et al., 2009) neuronal activity, with the mechanism of action depending upon the associated g protein. Chapters 4 and 6 use a modified version of the human M4 muscarinic receptor coupled to a Gi protein (hM4Di) to decrease the activity of neurons in the anterior cingulate cortex. When these receptors are bound by a ligand, Gi proteins increase the activity of inward rectifying potassium channels (Armbruster et al., 2007; Roth, 2016), hyperpolarising neurons and inhibiting action potentials (Figure 2.1). Chapter 5 uses a modified human M3 muscarinic receptor coupled to a Gq protein (hM3Dq) to increase the activity of neurons in the anterior cingulate cortex. When a ligand binds to the receptor, Gq proteins trigger the release of intracellular calcium (Alexander et al., 2009; Conklin et al., 2008), depolarising neurons and stimulating neuronal firing (Figure 2.1).

DREADDs are packaged into viral vectors, which are introduced to the brain by intracranial injections for neuronal transfection (Roth, 2016). The adeno-associated viral vector serotype 5 (AAV5), used in this experiment, is relatively non-toxic and achieves long term (months to year) expression (Campbell & Marchant, 2018; Morsy et al., 1998). A promoter is also included in the viral construct, helping to determine which cell types express the DREADD (Campbell & Marchant, 2018). This study used calmodulin-dependent protein kinase II (CAMKII), which predominantly targets excitatory glutamatergic neurons (Campbell & Marchant, 2018; Smith, Bucci, Luikart, & Mahler, 2016). Finally, fluorescent reporter molecules are added to allow visualisation of DREADD expression in the brain. All experiments in this thesis used DREADDs tagged with mCherry, a fluorophore that is visible under fluorescent microscopes and can be enhanced using antibodies (Smith et al., 2016). Following intracranial injection of the virus, robust DREADD expression is typically observed in neurons within 2-3 weeks (Smith et al., 2016).

2.3.2 Clozapine as a ligand to activate DREADDs

Animals expressing DREADDs in a target brain area can be administered a systemic injection of a ligand that binds to, and activates, the receptors. The synthetic ligand Clozapine-N-Oxide (CNO) has most readily been used by neuroscientists (Rogan & Roth, 2011; Roth, 2016; Smith et al., 2016), based on the assumption that it is otherwise pharmacologically inert. However, recent research has revealed that CNO is reverse metabolised in vivo into its parent compound clozapine, an antipsychotic that also acts on endogenous receptors and produces physiological and behavioural effects (Gomez et al., 2017).

Further evidence indicates that CNO does not in fact cross the blood brain barrier (Gomez et al., 2017). Rather, the activation of DREADDs by systemic injection of CNO is mediated by its metabolised compound clozapine, which readily crosses the blood brain barrier (Campbell & Marchant, 2018; Gomez et al., 2017). These observations have led to the suggestion that subthreshold doses of clozapine may be a more suitable ligand than CNO (Gomez et al., 2017). One key advantage of using clozapine is that it bypasses the large amount of between-subjects variability in time taken to metabolise CNO into clozapine (Manvich et al., 2018), thus better

controlling for variable onset of off target effects. For these reasons, clozapine was used as the ligand in all DREADD experiments in this thesis.

Clozapine interacts with serotonergic and dopaminergic receptors (Meltzer, 1994), meaning that injections of clozapine (or CNO, reverse metabolised into clozapine) may result in confounding physiological and behavioural effects that are the result of activation of endogenous, rather than DREADD, receptors (Campbell & Marchant, 2018; Gomez et al., 2017). However, because clozapine has a much higher affinity for DREADD receptors than for endogenous receptors (Campbell & Marchant, 2018), effective doses that minimise off-target effects can be found. Unfortunately, given that the majority of studies to date have used CNO as a DREADD ligand, a literature regarding optimal dose ranges of clozapine is lacking. DREADDs have a much higher affinity to clozapine than to CNO (Armbruster et al., 2007; Gomez et al., 2017), indicating that dosages of the former need be orders of magnitude lower than the latter. Furthermore, far greater doses of a ligand are required to activate hM4Di inhibitory DREADDs than hM3Dq excitatory DREADDs (Farrell & Roth, 2013; Mahler et al., 2014; Yau & McNally, 2015), reflected by the higher dosages in Chapters 4 and 6, than in Chapter 5. For further details of the clozapine dosages used in each experiment, see methods in Chapters 4, 5 and 6, respectively.

The ligand is typically administered by intraperitoneal (i.p.) injection, which is the method used in Chapters 4 and 5. Following injection, electrophysiological data indicate a response onset of approximately 5-12 minutes, after which robust changes in neuronal firing are observed (Alexander et al., 2009; Chang, Todd, Bucci, & Smith, 2015; Guettier et al., 2009). The time it takes for firing to return to baseline is less clear, with reports from ranging from 70 minutes (Chang et al., 2015) to 9 hours (Alexander et al., 2009; Guettier et al., 2009). Again, a caveat is that these studies tested CNO, not clozapine. Furthermore, the temporal kinetics of CNO/clozapine appear to be dose dependent (Pati et al., 2019) but, while it can be intuited that response offset will be delayed as dosage is increased, formal investigation in this area is lacking.

2.3.3 Advantages of DREADDs

The advent of DREADDs brought key advantages over traditional loss or gain of function methods for behavioural neuroscience research (Roth, 2016; Smith et al., 2016). One such advantage is the transient nature of the intervention, which circumvents the potential confound of compensatory changes in other brains regions that occur following permanent lesions (Smith et al., 2016). Another is that inhibitory DREADDs dampen activity rather than eliminate it (e.g lesions), and excitatory DREADDs stimulate endogenous cell firing rather than simply causing action potentials (e.g. electrical stimulation)(Smith et al., 2016). This is generally considered to produce more naturalistic down and up regulation of activity, with more physiological relevance to function and dysfunction (Lee, Giguere, & Roth, 2014; Smith et al., 2016).

Of particular significance to the experiments in this thesis (in addition to influencing cell body signalling) DREADDs can be trafficked down axons to their terminals, where they influence the release of neurotransmitters (Mahler et al., 2014; Stachniak, Ghosh, & Sternson, 2014). Therefore, it is possible to locally infuse a ligand directly into a target region, thus selectively manipulating the terminals of DREADD expressing neurons that project there. Several studies have achieved this using implanted intracranial cannulas above the projection target region (Lichtenberg et al., 2017; Mahler et al., 2014; McGlinchey & Aston-Jones, 2018; Stachniak et al., 2014). The final experiment (Chapter 6) uses this method, with inhibitory DREADDs (hM4Di) in the anterior cingulate cortex and cannulae implanted above the anterior thalamic nuclei to deliver clozapine, to selectively inhibit the terminals of anterior cingulate neurons that project to the anterior thalamic nuclei.

2.3.4 Non-DREADD expressing control groups

Well-designed control groups are essential in DREADD experiments, in order to minimise the risk of off-target effects of the either virus or the ligand (Roth, 2016; Smith et al., 2016). In this thesis, each DREADD experiment (Chapters 4, 5 and 6) includes a control group of animals receiving comparative intracranial delivery of a non-DREADD expressing virus. The same viral vector (AAV5) and promoter

(CAMKII) were used, tagged with green fluorescent protein (eGFP) for visualisation of expression.

In an ideal design, both of these groups (DREADD and non-DREADD expressing control) would be combined with both the ligand (clozapine) and the vehicle (saline) (Smith et al., 2016). Whilst this can be implemented with relative ease within-subjects, the version of the attentional set-shifting task used in this thesis is run between-subjects, to avoid carry-over effects of previously learnt discriminations that can occur when retesting animals (Chase et al., 2012; Tait, Chase, & Brown, 2014). Consequently, including a saline control group would require a doubling of the number of animals in each experiment.

Therefore, the experiments in this thesis included two conditions, with DREADD and non-DREADD expressing control groups receiving identical administration of clozapine in each experiment. This controls for any off-target effects of the drug between groups and is considered to be a prudent use of subjects (MacLaren et al., 2016; Smith et al., 2016). Whilst it does not control for potential effects of the drug in the global sense (MacLaren et al., 2016), the behavioural profile of normal animals undertaking the version of the attentional set-shifting task used in these experiments is well characterised (Chase et al., 2012; Wright et al., 2015). Therefore, deviations in the behaviour of non-DREADD expressing control animals from this profile (indicating effects of clozapine administration) should be recognisable without the need for a saline control.

2.3.5 Investigating the influence of DREADDs using c-*fos*

As has been established in the preceding sections, DREADDs are an emerging technology and thus the mechanistic action of DREADDs at the cellular and circuit level is not yet fully understood. It is, therefore, highly valuable to measure how DREADDs impact the brain in experiments, in order to more accurately interpret a given behavioural result (Smith et al., 2016). Consequently, as described in section 2.7, each DREADD experiment in this thesis was followed by investigation into the immediate early gene c-*fos*, an indirect marker of neuronal activity (Dragunow & Faull, 1989; Zhu, Brown, McCabe, & Aggleton, 1995). Following administration of

clozapine, animals were culled, and their brain tissue immunohistochemically stained for the marker. This provides a measure of the impact of DREADDs on activity in brain regions of interest.

2.4 Animals

The subjects in all experiments were male, Lister Hooded rats (Envigo, Bicester, UK). They were housed in groups of two or three under a 12-hour light/12-hour dark cycle. During behavioural testing all animals were food restricted to maintain at least 85% of their free-feeding body weight, while water was available *ad libitum*. All animals were habituated to handling but remained otherwise naïve prior to the start of the experiments.

2.5 Surgery

2.5.1 Anaesthesia, analgesia and surgical site preparation

Animals in Chapters 4, 5 and 6 weighed between 290-350g and were approximately three months old at the start of surgeries (see methods section in Chapter 3.2.1 for respective weights and ages). All animals in all chapters had anaesthesia induced using a mixture of oxygen and 5% isoflurane and, once unresponsive, were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The isoflurane level was lowered to 1.5-2.5% to maintain anaesthesia for the duration of the surgery. Animals were administered a subcutaneous injection of the analgesic Metacam (0.06ml, Buehringer Ingelheim Lid, Bracknell, UK) and a sagittal incision was made allowing the scalp to be retracted to expose the skull. The analgesic lidocaine (0.1ml, Xylocaine, AstraZeneca, Luton, UK) was applied topically to the surgical site.

2.5.2 Intracranial virus injections

Animals from the experiments in Chapters 4 and 5 underwent the same surgical procedure, except for the DREADDs injected in the experimental groups (see sections 4.2.1.2 and 5.2.1.2 for details). The data from some of these same animals were also used in Chapter 3 (see section 3.2.2 for details).

For these surgeries, the incisor bar of the stereotaxic frame was set so that the skull was flat relative to the horizontal plane. A 10µl Hamilton syringe (Bonaduz, Switzerland) was attached to a moveable arm mounted to the stereotaxic frame and anteroposterior (AP) coordinates were taken from Bregma. A craniotomy was made above the injection sites, allowing mediolateral (ML) coordinates to be taken from the sagittal sinus and dorsoventral (DV) coordinates to be taken from the dura. Animals received three injections of the virus (DREADD expressing or control virus, see sections 4.2.1.2 and 5.2.1.2 for details) in the anterior cingulate cortex in each hemisphere as follows: 0.35 µl at AP: +1.9, ML: +/-0.8, DV: -1.2, 0.7µl at AP: +1, ML: +/- 0.8, DV: -1.6 and 0.7µl at AP: +0.1, ML: +/- 0.8, DV: -1.6. The dura was pierced above each injection site and the needle lowered into place. The virus injections were controlled by a microprocessor (World Precision Instruments, Hitchin, UK) set to a flow rate of 0.1 µl/min, and the needle left *in situ* for a further 5 minutes to allow for virus diffusion.

Some animals in Chapter 3 and all animals in Chapter 6 underwent other surgical procedures, see section 3.2.2 and section 6.2.1.2 for details.

2.5.3 Surgical site closure and post-operative care

For all animals in Chapters 3, 4 and 5, the surgical site was closed using sutures and the analgesic bupivacaine (Pfizer, Walton Oaks, UK) was injected between the suture sites. A topical antibiotic powder Clindamycin (Pfizer, Walton Oaks, UK) was then applied to the site. Animals were administered a subcutaneous injection of glucose-saline (5ml) for fluid replacement before being placed in a recovery chamber until they regained consciousness. Animals were monitored carefully postoperatively with food available *ad libitum* until they had fully recovered, with behavioural pre-training commencing approximately two weeks post-surgery.

2.6 Attentional set-shifting task protocol

All animals in Chapters 4, 5 and 6 completed the same standard attentional set-shifting task as described below. In each chapter, this task returned results that warranted further investigation. Consequently, different follow-up experiments were devised and are described in the methods section of each respective chapter.

2.6.1 Apparatus

Training and testing were performed in a black Perspex box which measured 69.5cm long, 40.5cm wide and 18.6cm tall. One end of the testing arena comprised two individual chambers encompassing approximately a quarter of the overall area of the box (Figure 2.2). These two chambers were separated from the remaining open space by black Perspex panels that could be removed by the experimenter to allow access. Each of the three compartments had a hinged, transparent Perspex lid. In each of the two smaller compartments was a circular glass pot (75mm diameter, 45mm height) that contained the digging media. Against the opposite wall, in the larger compartment, there was an identical glass pot containing water.

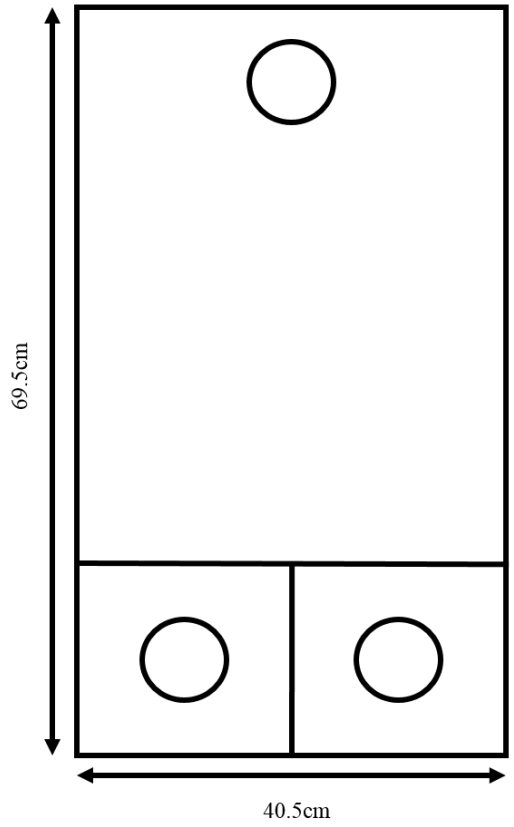

Figure 2.2. Schematic diagram of the test apparatus used to run the attentional set-shifting task

Approximately one quarter of the space is divided into two smaller chambers, separated from the remaining open space by removable Perspex panels. Each small chamber contains a glass pot containing the digging medium and the remaining open space contains an identical glass pot filled with water.

2.6.2 Pre-training

Animals underwent 3 days of pre-training before testing commenced. On the first day of pre-training animals had access to all three chambers with no glass pots present. Animals were placed in the arena for 10 minutes to habituate to the apparatus. On the second day of pre-training, all three glass pots were in place, with the two glass pots in the smaller chambers filled with bedding sawdust. Panels were removed providing access to alternating chambers across trials to prevent the formation of a side bias. On the first trial, half a Cheerio (Nestle, Glasgow, UK) was placed on top of the sawdust and it was progressively buried in subsequent trials to teach animals to dig for the food reward. This was typically completed within 10 trials.

On the third day of pre-training, the day before testing took place, animals were pre-exposed to the test stimuli (Table 4). To avoid exposure to the combinations of stimuli used during the test, each odour was presented with bedding sawdust and each digging media was presented without odour. Animals retrieved half a buried cheerio from each pot of sawdust laced with odour and each pot of odourless digging media. The reward was retrieved from each pot twice, once in each chamber, to prevent formation of a side bias. The purpose of the pre-exposure stage was to prevent any potential refusals to dig in the test stimuli on the test day.

2.6.3 Behavioural testing

2.6.3.1 Clozapine administration

Behavioural testing began three weeks post-surgery, allowing sufficient time for robust DREADD expression in neurons (see section 2.3.1) (Smith et al., 2016). All animals received clozapine as a ligand to activate the DREADDs (see 2.3.2)

Animals in Chapters 4 and 5 were administered an intraperitoneal (I.P.) injection of clozapine (HelloBio, Bristol, UK) fully dissolved in saline at a dilution of 2mg/ml (for dosages see sections 4.2.1.3.2 and 5.2.1.3.2). They were then returned to a holding cage for 20 minutes before testing began. This interval was chosen to allow sufficient time for the DREADDs receptors to be activated by the ligand and produce

any associated behavioural effects (see section 2.3.2) (Smith et al., 2016) in the experimental groups. Delivery of clozapine differed in Chapter 6, where it is was infused directly in the anterior thalamic nuclei intracranially. See section 6.2.1.3.1 for details.

2.6.3.2 Standard attentional set-shifting task

On the test day, animals in Chapters 4, 5 and 6 underwent the same behavioural protocol. The glass pots in the two smaller compartments of the arena were filled with different stimuli pairs (Table 4). Only one pot contained the buried food reward (half a Cheerio, Nestle, Glasgow, UK) and animals encountered a series of discriminations requiring them to respond to the correct stimulus in order to retrieve the cheerio. At the beginning of each trial the dividing panels were removed allowing the animal access to the two smaller compartments. The compartment of the correct pot was pseudorandomly allocated in each trial. If the animal dug in the correct pot, defined as breaking the surface of the digging media with paws or nose, it could retrieve the reward. For the first four trials of each discrimination, the animal was allowed access to the correct compartment to retrieve the reward following an initial dig in the incorrect pot. Thereafter, if the animal dug in the incorrect pot, access to the correct compartment was blocked. The inter-trial interval lasted approximately five seconds during which time the pots were rebaited. Once the animal had acquired a discrimination, quantified by six consecutive correct digs, it moved on to the next discrimination. The discrimination stages proceeded as follows:

1. A simple discrimination (SD) between two sawdusts with different odours or between two (unscented) digging mediums with different textures
2. A compound discrimination (CD), where the same odour or media as the previous trial is rewarded but is presented with irrelevant stimuli from the other dimension
3. Four intradimensional shifts (ID), where different compound stimuli are presented with the previously rewarded dimension remaining relevant
4. An extradimensional shift (ED), where different compound stimuli are presented and the previously unrewarded dimension becomes relevant

5. A reversal (REV), where the same compound stimuli as the previous trial are presented with the previously incorrect stimulus (from the same dimension) being rewarded.

Therefore, for the first six discriminations exemplars from the same stimulus dimension (odour or digging media) were rewarded. This is thought to encourage the formation of an attentional set (Chase et al., 2012; Tait et al., 2014) whereby an animal learns to solve a discrimination by attending to one dimension only (odour or digging media) while ignoring the irrelevant dimension. Successive improvement across these first six discriminations is thought to signify successful set-formation. (Brown & Tait, 2015; Chase et al., 2012; Tait et al., 2018). The stimulus dimension relevant to solving these trials was counterbalanced across animals, as was the order in which the various stimuli were presented, as far as possible.

When challenged with an ED shift, the animal must attend to the dimension previously established as irrelevant to reward. Animals therefore typically exhibit a shift cost (Birrell & Brown, 2000; Chase et al., 2012; Tait et al., 2014; Wright et al., 2015), taking more trials to solve the ED than the preceding ID. The final stage was a reversal, which does not require attention to be reoriented to a different dimension. Rather, the stimuli remain the same as the preceding trial, but the previously non-reinforced stimulus is reinforced, i.e. the reward contingencies are reversed.

Table 4. Depiction of a possible order of stimulus pairings in the attentional set-shifting task

Discrimination	Rewarded dimension	Rewarded Stimuli	Unrewarded Stimuli
SD	Media	**Coarse tea**	Fine tea
CD	Media	**Coarse tea** + cinnamon	Fine tea + ginger
		Coarse tea + ginger	Fine tea + cinnamon
ID1	Media	**Coarse cork** + tarragon	Fine cork + fenugreek
		Coarse cork + fenugreek	Fine cork + tarragon
ID2	Media	**Wood shavings** + marjoram	Wood chip + sage
		Wood shavings + sage	Wood chip + marjoram
ID3	Media	**Short cigarette filters** + cumin	Long cigarette filters + dill
		Short cigarette filters + dill	Long cigarette filters + cumin
ID4	Media	**Beanbag filler** + mint	Polystyrene + turmeric

		Beanbag filler + turmeric	Polystyrene + mint
ED	Odour	Confetti + **cloves**	Oregano + shredded paper
		Shredded paper + **cloves**	Oregano + confetti
REV	Odour	Confetti + **oregano**	Cloves + shredded paper
		Shredded paper + **oregano**	Cloves + confetti

Stimuli from the relevant dimension, which signify reward location, are in bold. In this example, digging media is the first dimension relevant to the location of the buried food reward. From the ED stage onwards, odour is the relevant dimension. Stimuli are always paired as shown, but the discrimination in which animals encounter them is counterbalanced. The first dimension to be rewarded is also counterbalanced across animals.

2.6.3.3 Follow-up experiments

The results of the attentional set-shifting task warranted further investigation in Chapters 4, 5 and 6. Therefore, approximately two weeks after completion of the main behavioural testing stage, follow-up experiments were conducted where animals were challenged to a further series of discriminations. These differed across chapters and are described in each respective methods section.

2.6.4 Analysis of behaviour

Behavioural testing was carried out by an experimenter who scored the number of trials taken to meet criterion (six consecutive correct digs) and total number of errors for each discrimination. The time taken for each animal to complete the task was also recorded.

2.7 Investigation of c-*fos* through novel environment exposure

Each chapter (4, 5 and 6) included an investigation of group differences in the expression of the immediate early gene c-*fos,* an indirect marker of neuronal activity (see also 2.3.5). Exposure to novelty has been shown to induce expression of c-*fos* in the anterior cingulate cortex (Vann, Brown, & Aggleton, 2000; Wirtshafter, 2005; Zhu et al., 1995). In order to investigate the influence of DREADDs on such activity, approximately one week after the testing phase, animals underwent exposure to two novel environments to allow for subsequent quantification of regional activity differences between groups.

Animals were administered clozapine (HelloBio, Bristol, UK, for method of delivery and dosages see methods sections in Chapters 4, 5 and 6) and were placed in a cage in a dark holding room for 20 minutes. The purpose of this interval was to allow sufficient time for DREADD receptor activation by the ligand (Smith et al., 2016) and to habituate animals to the dark room. Animals were then taken to a testing room where they were placed in two novel environments, each for a period of 15 minutes. The first was a large square open field arena measuring 100cm long, 100cm wide and 45cm tall which was filled with bedding sawdust and six novels objects (two drinks cans, two triangular bottles and two cylindrical bottles). The second was a bow tie maze (Albasser et al., 2010) which measured 120cm long, 50cm wide and 50cm tall and was filled with bedding sawdust and food rewards (Cheerios, Nestle, Glasgow, UK). Animals were returned to the dark holding room for 90 minutes, an interval considered to be within the optimal timeframe for neuronal Fos expression after an induction event (Bisler et al., 2002), before perfusion.

2.8 Histology

2.8.1 Perfusion

Animals were administered an I.P. injection of a lethal dose of sodium pentobarbital (2ml/kg, Euthatal, Marial Animal Health, Harlow, Essex, UK) and transcardially perfused with 0.1M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1M PBS (PFA). Brains were removed, postfixed in PFA for 2 hours and then placed in 25% sucrose solution for 24 hours at room temperature on a stirring plate.

2.8.2 Sectioning

Brains were cut into 40 μm coronal sections using a freezing microtome (8000 sledge microtome, Bright Instruments, Luton, UK) and a series of 1 in 4 sections was collected in PBS for fluorescence analysis. The remaining three series were collected in cyroprotectant (30% sucrose, 1% polyvinyl pyrrolidone and 30% ethylene glycol in PBS) and stored in a freezer at -20^{0}C until further processing. In Chapter 6, an additional series was collected for cresyl staining (as described in 6.2.1.4).

2.8.3 Immunohistochemistry for DREADDs

For animals with DREADD injections in Chapters 4, 5 and 6, immunohistochemistry was carried out on the tissue to enhance the fluorescence signal of mCherry as follows. The first series of sections was transferred from PBS into a blocking solution of 5% normal goat serum (NGS) in Phosphate Buffered Saline with Tritonx-1000 (PBST) and incubated for 1 hour. The sections were then transferred into the primary antibody solution of rabbit-anti-mCherry (Abcam, Cambridge, UK) at a dilution of 1:1000 in PBST with 1% NGS and incubated for 24 hours. Sections were washed four times in PBST and transferred to a secondary antibody solution of goat-anti-rabbit (Dylight Alexa flour 594, Vector Laboratories, Peterborough, UK) at a dilution of 1:200 at PBST. From this point onwards the sections were kept in the dark.

Sections were incubated for 1 hour before being washed three times in PBS. For animals with eGFP injections, enhancement of the fluorescence signal was not necessary and therefore no immunohistochemistry was performed on the tissue. Sections were mounted onto gelatine subbed glass slides and were allowed to dry overnight before being immersed in xylene and coverslipped using DPX (Thermo Fisher Scientific, Loughborough, UK). All incubations were on a stirring plate at room temperature and all washes were for 10 minutes.

2.8.4 Immunohistochemistry for c-*fos*

For all animals in Chapters 4, 5 and 6, the second series from each brain was removed from cryoprotectant before being immunohistochemically stained for Fos protein. Sections were washed four times in PBS, once in a peroxidase block (0.3% hydrogen peroxidase in PBST) and four times in PBST. The sections were then transferred to a blocking solution of 3% NGS in PBST and incubated for 1 hour. The sections were then transferred to a primary antibody solution of rabbit-anti-c-*fos* (Millipore, Watford, UK) at a dilution of 1:5000 in PBST and incubated for 10 minutes, followed by 48 hours at 4^{0}C in a refrigerator. Sections were washed four times in PBST and transferred to a secondary antibody solution of goat-anti-rabbit (Vector Laboratories, Peterborough, UK) at a dilution of 1:200 in 1.5% NGS in

PBST. Sections were incubated for 2 hours before being washed four times in PBST and transferred to an avidin/biotinylated enzyme complex (Vectastain ABC HRP kit, Vector Laboratories, Peterborough, UK) in PBST for 1 hour. Sections were washed four times in PBST and twice in a Tris buffer (0.6% trisma base in distilled water). Sections were then immersed in a DAB solution (DAB peroxidase HRP substrate kit, Vector Laboratories, Peterborough, UK) for 1-2 minutes before the reaction was stopped with cold PBS. The sections were mounted onto gelatin-subbed glass slides and allowed to dry overnight before being immersed in xylene and coverslipped using DPX. All incubations were on a stirring plate at room temperature and all washes were for 10 minutes.

2.9 Image capture and virus expression analysis

For all animals in Chapters 4, 5 and 6, virus expression was analysed using a fluorescent Leica DM5000B microscope with a Leica DFC310 FX camera. Images were collected from the anterior cingulate cortex in each case to document the cellular virus expression at the injection sites. Additional images were collected from anterior cingulate cortex projection regions to document the transport of the virus through axons to their terminals. In Chapter 6, additional images were collected from cresyl stained sections to verify cannula placement (as described in section 6.2.1.6).

2.10 Image capture and fos expression analysis

For all animals in Chapters 4, 5 and 6, Fos-positive cells were analysed using a DMRB microscope, an Olympus DP73 camera and cellSens Dimension software (version 1.8.1, Olympus Corporation). For each region of interest, images were taken from consecutive sections (each 120μm apart) from both hemispheres of the brain. A 5x objective lens was used to take multiple images which were combined to create images encompassing each area of interest on each section.

For each hemisphere in each case, eight images were generated for the anterior cingulate cortex, four were generated for both prelimbic cortex and the anterior thalamic nuclei, and three were generated for secondary somatosensory cortex. Prelimbic cortex was included to identify whether the DREADDs influenced activity

in neighbouring medial prefrontal areas other than the target injection region (anterior cingulate cortex). The anterior thalamic nuclei were included as a major target of efferent projections of the anterior cingulate cortex, to identify whether the DREADDs influenced activity downstream. Secondary somatosensory cortex was included as a control region, which neither neighbours nor has known interconnectivity with anterior cingulate cortex.

The numbers of Fos-positive neurons, defined as neurons with a diameter of 4-20μm, sphericity of 0.1-1.0 and stained above a grayscale threshold set 60 units below the peak grey value, were counted in each image (cellSens Dimension software, version 1.8.1, Olympus Corporation). For each case, a mean Fos count was generated for each region of interest: dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL), anteromedial (AM) and anteroventral (AV) thalamic nuclei. This was achieved by averaging the number of Fos-positive cells in the images of that region.

2.11 Statistical analysis

2.11.1 Behavioural data

In Chapters 4, 5 and 6, a series of analysis of variance (ANOVA) and t-tests were conducted on mean trials required to reach criterion at each stage of the attentional set-shifting task. Although errors to criterion were also recorded for each rat at each stage, the two measures are normally highly correlated (Birrell & Brown, 2000). Therefore, provided analysis of each measure produced the same pattern of results in each experiment, only trials to criterion are reported.

All analyses were conducted using JASP computer software (version 0.11.1, Amsterdam, The Netherlands)(Team, 2019). Data were initially checked for normality using the Shapiro-Wilk test. Levene's test for homogeneity of variance was checked for between subject variables and any violations of this assumption are discussed in the respective Chapters. Mauchly's test for sphericity was checked for within-subjects variables and where violated, Greenhouse-Geisser corrections were applied to the degrees of freedom. The alpha level was set at $p<.05$ throughout.

2.11.1.1 Standard attentional set-shifting task

In Chapter 6, a preliminary ANOVA was run to check for any differences between the two cohorts of animals included in the study (see section 6.2.2). In Chapters 4, 5, and 6, an ANOVA was run to check for any effects of rewarded dimension (whether rats required to attend to odour or digging media to solve the first discriminations differed), with stage (eight levels) as a within-subjects factor, and first dimension (two levels) and group (two levels) as between-subjects factors. Provided no main effect of rewarded dimension and no interactions between this factor and group were found, data were pooled across dimensions for all subsequent analyses.

Next, a two-way ANOVA was run with stage (eight levels) as a within-subjects factor and group (two levels) as a between-subjects factor. Where interactions were found between stage and group, simple main effects analyses were conducted by ANOVA on the relevant factors and the pooled error term was applied to between-subjects effects (Howell, 2009). Additionally, paired sample t-tests were conducted on the difference between trials to criterion to complete ID1 and ID4 for each group in each experiment. This was to determine whether ID4 was completed in fewer trials than ID1, an indicator of attentional set-formation.

Further analyses were run on shift costs, the difference between the mean number of trials taken to solve the four ID stages and the number of trials taken to solve the ED stage (Wright et al., 2015). Firstly, one-sample t-tests were conducted for each group to determine whether they displayed a shift cost or benefit (significant difference from zero). Secondly, independent samples t-tests were used to determine whether there was a significant difference in shift cost (/benefit) between the groups in each experiment.

Finally, independent samples t-tests were conducted on times taken to complete the task. The first t-test determined whether there was a difference in mean total time for each group to complete all of the discriminations. A further t-test determined whether there was a difference in time taken per trial (total time/total number of trials) between the groups, thus providing an indication of whether the groups completed trials at different rates.

2.11.1.2 Follow-up attentional set-shifting task

In Chapters 4, 5 and 6, animals also completed follow-up attentional set-shifting tasks, which had four stages in Chapters 4 and 6 and six stages in Chapter 5. First, an ANOVA was run to check for any effects of rewarded chamber, whether rats were required to dig in the left or right chamber to solve the spatial extradimensional discrimination, on performance. This included stage (four or six levels, depending on task) as a within-subjects factor, and first chamber (two levels) and group (two levels) as between-subjects factors. Provided no main effect of rewarded chamber and no interactions between this factor and group were found, data were pooled across dimensions for all subsequent analyses.

Next, a two-way ANOVA was run with stage (four or six levels, depending on task) as a within-subjects factor and group (two levels) as a between-subjects factor. Any interactions between stage and group were investigated with simple main effects analyses restricted to the relevant factors and the pooled error term was applied to between-subjects effects (Howell, 2009).

Further analyses were run on shift costs. In the follow-up task in Chapter 5, the shift cost was calculated by taking the difference between the mean number of trials taken to solve the two ID stages and the number of trials taken to solve the spatial ED stage (Wright et al., 2015). As there was only one ID stage in the follow-up tasks for Chapter 4 and 6, the shift cost was calculated as the difference in trials taken to complete that ID stage and the ED stage. Initially, one-sample t-tests were carried out for each group to determine whether they displayed a shift cost or benefit (significant difference from zero). Next, independent samples t-tests were used to determine whether there was a significant difference in shift cost (/benefit) between the groups.

2.11.2 Fos-positive cell counts

In Chapter 4, 5 and 6 a series of ANOVA were conducted on the mean Fos-positive cell counts (see section 2.10) for each brain region of interest. Analyses were conducted using JASP computer software (version 0.11.1, Amsterdam, The

Netherlands)(Team, 2019) and assumptions were checked and corrected as described in section 2.11.1.

First, a two-way ANOVA was run on mean Fos-positive cell counts in the cortical regions of interest, with region (three levels, Cg1, Cg2, PrL) as a within-subjects factor and group (two levels) as a between-subjects factor. A one-way ANOVA was then conducted on Fos-positive cell counts in the control cortical region, secondary somatosensory cortex (S2), with group (two levels) as a between-subjects factor. Next, a second two-way ANOVA was run on cell counts in the anterior thalamic nuclei with region (two levels, AM and AV) as a within-subjects factor and group (two levels) as a between-subjects factor. Where interactions were found between region and group, simple main effects analyses were conducted by ANOVA on the relevant factors and the pooled error term was applied to between-subjects effects (Howell, 2009).

Finally, Pearson correlation analysis was conducted on Fos-positive cell counts for each group, producing a measure of covariation of activity between different brain regions. Bonferroni corrections were applied to the alpha level to adjust for multiple comparisons.

3 Mapping Fibre Pathways Between the Anterior Thalamic Nuclei and the Cingulate Cortex

3.1 Introduction

The cingulum bundle is one of the most prominent white matter tracts in the mammalian brain, coursing dorsal to the corpus callosum, it spans the length of the medial cortex from the orbital frontal cortices to near the temporal pole. A highly complex pathway, there is a growing realisation that functionality shifts along the length of the tract, reflecting changing underlying connections. Whilst the field of neuroimaging is moving to split the tract into subdivisions, each associated with different functions and dysfunctions (see section 1.4.3.2), it is impeded by its inability to isolate connections within the tract.

The current study investigated the substantial subset of cingulum fibres that connect the anterior thalamic nuclei with the cingulate cortex. Current knowledge of these connections originates from research by Domesick (1969, 1970), where degenerating fibres were traced from lesions in the rat anterior thalamus (1970) and cingulate cortex (1969), respectively (see section 1.3.1). However, the lesion degeneration method used in Domesick's (1969; 1970) pioneering research has limitations. Firstly, it is not possible to distinguish fibres degenerating from the lesion site itself from damaged fibres that pass through the lesion site but originate elsewhere. It is also not possible to differentiate between the efferents of individual anterior thalamic nuclei, and other adjacent nuclei, due to the large sizes of the lesions involved (Domesick, 1970). In the case of reciprocally connected sites, there is the further challenge of distinguishing efferent and afferent fibres (Brodal, 1981). The method also lacks sensitivity, leaving the possibility of false negatives, while fibres that follow alternate routes may not have been described (Brodal, 1981).

Although subsequent studies using axonal tracers located in the anterior thalamus (Shibata, 1993b; Van Groen et al., 1999; Van Groen & Wyss, 1995) and cingulate cortex (Shibata & Naito, 2005) have generally supported the conclusions of Domesick (1969, 1970), no study since has focused specifically on describing these

pathways. Consequently, several details remain to be clarified. One such detail is whether all anterior thalamic nuclei projections take the rostralward trajectory described by Domesick (1970) before interfacing the cingulum. There is evidence that a subset of anterior thalamic projections in nonhuman primates take a more direct route to the cortex (Mufson & Pandya, 1984; Weininger et al., 2019), leaving the thalamus laterally to follow the same route as the return projections (Domesick, 1969). Whether such a route exists in the rat has not been determined. Furthermore, how fibres diverge from the different anterior thalamic nuclei, and whether they interface the cingulum at different points, remains unclear. Uncovering these details will help to establish the presence of specific connections along the length of the tract.

The current study re-examined the routes of anterior thalamic-cingulate-retrosplenial connections, taking advantage of anterogradely transported viruses (Osten & Margrie, 2013). Discrete injections of green fluorescent protein-tagged adeno-associated virus (AAV-eGFP) targeted the anteromedial (AM), anteroventral (AV) and anterodorsal (AD) thalamic nuclei. Meanwhile, complementary anterogradely transported virus injections targeting the anterior cingulate and retrosplenial cortices helped to visualise the return projections to the anterior thalamic nuclei. Anatomical tract tracer, biotinylated dextran amine (BDA) and conjugated cholera toxin subunit B (CTB), injections into the cingulum bundle helped to corroborate the presence of fibres from each of the target regions travelling in different parts of the cingulum. A further subset of injections of the anterogradely and retrogradely transported Equine Infectious Anaemia Virus (EIAV) targeted the anterior thalamic nuclei and the cingulum bundle but were cut sagittally (rather than coronally) to allow visualisation of fibres on this anatomical plane.

3.2 Methods

3.2.1 Animals

Subjects were 35 male, Lister Hooded rats (Envigo, Bicester, UK) housed as described in section 2.4.

3.2.2 Surgery

3.2.2.1 Anaesthesia, analgesia and surgical site preparation

All animals in this Chapter were anaesthetised, administered analgesics and the surgical site was prepared as described in section 2.5.1

3.2.2.2 Intracranial virus injections in anterior thalamic nuclei

A total of 15 animals received injections of the anterogradely transported virus AAV5-CaMKIIa-EeGFP (titre: 4.3x10^12 GC/ml, Addgene, Watertown, MA, USA) targeting the anterior thalamic nuclei. Of these animals, 11 received the injections for the purpose of this anatomical study and injection sites from five of these animals were selected for further analysis. A further four animals received the injections as part of an unrelated behavioural experiment (not included in this thesis) and contributed viral tracing data to this study.

A further one animal received bilateral injections of a lentiviral vector based on the equine infectious anaemia virus (EIAV, Invitrogen, Renrewshire, UK), which is transported both anterograde and retrograde (Mazarakis et al., 2001), into the anterior thalamic nuclei. This brain was cut on the sagittal plane, so that fibres could be visualised joining the cingulum along its length (the majority of brains from the dataset were sectioned on the coronal plane). All animals weighed between 290-420g and were approximately three to six months old at the start of surgeries.

For these surgeries, the incisor bar was set so that the skull was at +5mm relative to the horizontal plane. A 10µl Hamilton syringe (Bonaduz, Switzerland) was attached to a moveable arm mounted to the stereotaxic frame and anteroposterior (AP) and dorsoventral (DV) coordinates were taken from Bregma. A craniotomy was made above the injection sites, allowing mediolateral (ML) coordinates to be taken from the sagittal sinus. Injection site co-ordinates and virus infusion volumes are given in Table 5. The dura was pierced above each injection site and the needle lowered into place. The virus injections were controlled by a microprocessor (World Precision Instruments, Hitchin, UK) set to a flow rate of 0.1 µl/min, with the needle left in situ for a further five minutes to allow for virus diffusion.

3.2.2.3 Intracranial virus injections in anterior cingulate cortex

Viral tracing data from two control animals from the inhibitory DREADDs attentional set-shifting task (ASST) experiment (Chapter 4) and four control animals from the excitatory DREADDs ASST experiment (Chapter 5) contributed to this study. These animals received injections of the anterogradely transported virus AAV5-CaMKIIa-EeGFP (titre: 4.3x10^12 GC/ml, Addgene, Watertown, MA, USA) into the anterior cingulate cortex, as described in section 2.5.2.

A further single animal received unilateral injections of AAV5-CaMKIIa-EeGFP (titre: 4.3x10^12 GC/ml, Addgene, Watertown, MA, USA) into the anterior cingulate cortex, for the purpose of comparing fibres projecting to the ipsilateral and contralateral anterior thalamus. Injections were administered as described in section 2.5.2, with coordinates and volumes as displayed in Table 5.

3.2.2.4 Intracranial virus injections in retrosplenial cortex

Two animals received unilateral injections of AAV5-CaMKIIa-EeGFP (titre: 4.3x10^12 GC/ml, Addgene, Watertown, MA, USA) into the retrosplenial cortex. Animals weighed between 360-420g and were approximately six months old at the start of surgeries.

For these surgeries, the incisor bar was set so that the skull was at +5mm relative to the horizontal plane. A 10µl Hamilton syringe (Bonaduz, Switzerland) was attached to a moveable arm mounted to the stereotaxic frame and anteroposterior (AP) coordinates were taken from Bregma. A craniotomy was made above the injection sites, allowing mediolateral (ML) coordinates to be taken from the sagittal sinus and dorsoventral (DV) coordinates to be taken from dura. The dura was pierced above each injection site and the needle lowered into place. The virus injections were controlled by a microprocessor (World Precision Instruments, Hitchin, UK) set to a flow rate of 0.1 µl/min, with the needle left *in situ* for a further five minutes to allow for virus diffusion. Injection site co-ordinates and virus infusion volumes are given in Table 5.

3.2.2.5 Intracranial virus injections in cingulum bundle

Two animals received bilateral injections of equine infectious anaemia virus (EIAV, Invitrogen, Renrewshire, UK), which is transported both anterograde and retrograde (Mazarakis et al., 2001), into the cingulum bundle. This was for visualisation of fibres on the sagittal plane, so that fibres could be traced joining the cingulum along its length. These animals weighed between 330-360g and were approximately four months old at the start of surgeries.

For these surgeries, the incisor bar of the stereotaxic frame was set so that the skull was flat relative to the horizontal plane. A 10µl Hamilton syringe (Bonaduz, Switzerland) was attached to a moveable arm mounted to the stereotaxic frame and anteroposterior (AP) coordinates were taken from Bregma. A craniotomy was made above the injection sites, allowing mediolateral (ML) coordinates to be taken from the sagittal sinus and dorsoventral (DV) coordinates to be taken from dura. The dura was pierced above each injection site and the needle lowered into place. The virus injections were controlled by a microprocessor (World Precision Instruments, Hitchin, UK) set to a flow rate of 0.1 µl/min, with the needle left *in situ* for a further five minutes to allow for virus diffusion. Injection site co-ordinates and virus infusion volumes are given in Table 5.

3.2.2.6 Tracer injections in cingulum bundle

Seven animals received injections of neuroanatomical tracers directly into the cingulum bundle. Tracers used were biotinylated dextran amine (BDA, 3kD, Life Technologies Ltd, Paisley, UK) and conjugated cholera toxin subunit B (CTB, Invitrogen, Carlsbad, CA, USA). BDA was made up at 10% in sterile, distilled water (pH 7.4) and CTB was made up at 1% in 0.1M phosphate-buffered saline (PBS). Both tracers are transported both anterograde and retrograde, with a stronger anterograde component for BDA (Veenman, Reiner, & Honig, 1992) and a stronger retrograde component for CTB (Dederen, Gribnau, & Curfs, 1994). All animals weighed between 290-390g and were approximately three to six months old at the start of surgeries.

For these surgeries, the incisor bar of the stereotaxic frame was set so that the skull was flat relative to the horizontal plane. Anteroposterior (AP) coordinates were taken from Bregma. A craniotomy was made above the injection sites, allowing mediolateral (ML) coordinates to be taken from the sagittal sinus and dorsoventral (DV) coordinates to be taken from dura. Injections were made iontophoretically using a glass micropipette (18–22-mm tip diameter), using an alternating current (6s on/off) of 6μA for 10 minutes for each injection. Four animals received injections into the anterior cingulum bundle, below the anterior cingulate cortex, and three animals received bilateral injections into the posterior cingulum bundle, below the retrosplenial cortex (Table 5).

3.2.2.7 Surgical site closure and post-operative care

All animals in this chapter had surgical sites closed and received post-operative care as described in section 2.5.3

3.2.2.8 Summary of cases

Table 5 provides information about the individual cases analysed in this chapter. Cases are detailed with a reference number, indication of type of virus/tracer, volume and injection site coordinates. Finally, the injection site (after *post-hoc* histological analysis) of each case is listed.

Table 5. Anterograde and retrograde virus and tracer injections included in anatomical analysis

Case	**Virus / Tracer**	**Volume and coordinates**	**Injection site** (histologically confirmed)
Coronal cases			
Anterogradely transported virus injections in anterior thalamic nuclei			
215#1	eGFP	• 0.4 µl @ AP:-0.2, ML:+/-:0.8, DV:-6.7	AM (CM, MD)
215#12	eGFP	• 0.4 µl @ AP:-0.2, ML:+/-:0.8, DV:-6.7	AM (Re)
218#1R	eGFP	• 0.5 µl @ AP:-0.2, ML:+/-1.5, DV:-6.4	AM (AV, AD)

211#3	eGFP	• 0.4 µl @ AP:-0.1, ML:+/-0.8, DV:-6.8 • 0.6 µl @ AP:-0.2, ML:+/-1.5, DV:-6.2	AM (AV, AD, CM, MD)
211#5	eGFP	• 0.4 µl @ AP:-0.1, ML:+/-0.8, DV:-6.8 • 0.6 µl @ AP:-0.2, ML:+/-1.5, DV:-6.2	AM (AV, AD, CM)
211#21	eGFP	• 0.4 µl @ AP:-0.1, ML:+/-0.8, DV:-6.8 • 0.6 µl @ AP:-0.2, ML:+/-1.5, DV:-6.2	AM (AV, VA, CM, MD)
218#1L	eGFP	• 0.5 µl @ AP:-0.2, ML:+/-1.5, DV:-6.4	AV (AM, VA, LD)
218#3	eGFP	• 0.3 µl @ AP:-0.2, ML: +/-0.8, DV: -6.9	AV/AM (VA, CM)
211#13R	eGFP	• 0.4 µl @ AP:-0.1, ML:+/-0.8, DV:-6.8 • 0.6 µl @ AP:-0.2, ML:+/-1.5, DV:-6.2	AV (AM, VA, CM)
211#14	eGFP	• 0.4 µl @ AP:-0.1, ML:+/-0.8, DV:-6.8 • 0.6 µl @ AP:-0.2, ML:+/-1.5, DV:-6.2	AV (AM, VA, CM)
229#2L	eGFP	• 0.35 µl @ AP:-0.2, ML:+/-1.4, DV:-6.2	AV/AD (AM)
229#2R	eGFP	• 0.35 µl @ AP:-0.2, ML:+/-1.4, DV:-6.2	AV/AD (AM)
211#13L	eGFP	• 0.4 µl @ AP:-0.1, ML:+/-0.8, DV:-6.8 • 0.6 µl @ AP:-0.2, ML:+/-1.5, DV:-6.2	AV/AD/AM (VA, CM, MD, LD)
Anterogradely transported virus injections in anterior cingulate cortex			
224#1	eGFP	All bilateral: • 0.35 µl @ AP:+1.9, ML:+/-sinus, DV:-1.1	ACC, including pregenual
224#2	eGFP		ACC, including pregenual

219#2	eGFP	• 0.7 µl @ AP:+1.0, ML:+/-sinus, DV:-1.6	ACC, including pregenual
219#18	eGFP	• 0.7 µl @ AP:+0.1, ML:+/-sinus, DV:-1.6	ACC, postgenual
224#20	eGFP		ACC, postgenual
224#21	eGFP		ACC, postgenual
215#31	eGFP	• 0.4 µl @ AP:+0.6, ML:+0.7, DV:-1.5 • 0.4 µl @ AP:-0.4, ML:+0.6, DV:-1.7	ACC, postgenual
Anterogradely transported virus injections in retrosplenial cortex			
224#29	eGFP	• 0.6µl @ AP:-2.0, ML: sinus, DV:-1.6 • 0.6µl @ AP:-4.0, ML: sinus, DV:-1.6	Rgb
224#30	eGFP	• 0.6µl @ AP:-2.0, ML: sinus, DV:-1.6 • 0.6µl @ AP:-4.0, ML: sinus, DV:-1.6	Rgb
Anterogradely and retrogradely transported tracer injections in cingulum bundle			
205#5R	CTB	All:	Cingulum, anterior
205#6	CTB	• 0.1 µl @ AP:+0.6, ML:+/-1.4, DV:-2.1	Cingulum, anterior
209#16	CTB		Cingulum, anterior
209#17	CTB		Cingulum, anterior
205#5L	BDA		Cingulum, anterior
205#2R	CTB	All:	Cingulum, posterior
205#2L	BDA	• 0.1 µl @ AP:-2.4, ML:+/-1.1, DV:-2.0	Cingulum, posterior
205#3	BDA		Cingulum, posterior
209#19	BDA		Cingulum, posterior
Sagittal cases			
Anterogradely and retrogradely transported virus injections in anterior thalamic nuclei			
213#2LH	EIAV	All:	AV/AD/LD
213#2RH	EIAV	0.6 µl @ AP:-0.2, ML:+/-1.5, DV:-6.4	AV/AD/LD
Anterogradely and retrogradely transported virus injections in cingulum bundle			
209#5LH	EIAV	All:	Cingulum, anterior (ACC, M2)
209#5RH	EIAV	0.6 µl @ AP:+0.6, ML:+/-1.4, DV:-2.1	Cingulum, anterior (ACC, M2)

213#3LH	EIAV		Cingulum, anterior (ACC, M2)
213#3RH	EIAV		Cingulum, anterior (ACC, M2)

Cases are ordered by confirmed site of injection. Unilateral injections are described unless otherwise stated. In some cases, multiple injections targeted the same structure to ensure sufficient spread across the target region. Where there are meaningfully different injection sites in the same animal, they are treated as separate cases. These case numbers contain an L, indicating left hemisphere injection in that animal, or an R, indicating right hemisphere injection in that animal. ML coordinates of sinus are injections made as close to the sagittal sinus as possible. Sites in parenthesis indicate weak involvement at the injection site. Sites included: anteromedial (AM), anteroventral (AV), anterodorsal (AD), central medial (CM), mediodorsal (MD), reuniens (Re), ventral anterior (VA) and laterodorsal (LD) thalamic nuclei, anterior cingulate cortex (ACC), granular retrosplenial cortex b (Rgb) and secondary motor cortex (M2).

3.2.3 Perfusion

Animals with AAV5-CaMKIIa-EeGFP into the anterior cingulate from the control groups of behavioural experiments were perfused following completion of their testing, approximately five weeks post-operatively. For all other animals with injections of AAV5-CaMKIIa-EeGFP there was a post-operative survival time of approximately three weeks to allow for optimal virus expression (Smith et al., 2016). Animals with injections of BDA and CTB had a post-operative survival time of approximately five to nine days. All animals were given a lethal dose of sodium pentobarbital, transcardially perfused and brains were removed as described in section 2.8.1

3.2.4 Sectioning and histology

Brains were cut into 40 μm sections using a freezing microtome (8000 sledge microtome, Bright Instruments, Luton, UK). Sections were cut on the coronal plane, apart from cases with EIAV virus injections that were cut on the sagittal plane (Table 5).

3.2.4.1 Immunohistochemistry for virus injections

For cases with eGFP virus injections, enhancement of the fluorescence signal was not necessary and, therefore, no immunohistochemistry was performed on the tissue. Each brain was cut into four series, one of which was collected and mounted onto gelatine subbed slides before being immersed in xylene and coverslipped using DPX

(Thermo Fisher Scientific, Loughborough, UK), for fluorescence analysis. The remaining three series were collected in cyroprotectant (30% sucrose, 1% polyvinyl pyrrolidone and 30% ethylene glycol in PBS) and stored in a freezer at -20^{0}C until further processing.

For EIAV injection cases, brains were cut into two series. One series was mounted directly onto gelatine subbed slides, allowed to dry overnight and then stained with cresyl violet, a Nissl stain. Sections were hydrated by two-minute washes in decreasing concentrations of alcohol, followed by distilled water. Sections were then placed in cresyl violet stain for five minutes, followed by distilled water for 30 seconds. The sections were then dehydrated by two-minute washes in increasing concentrations of alcohol, followed by xylene, before being coverslipped using DPX (Thermo Fisher Scientific, Loughborough, UK).

The second series was stained using X-gal. Sections were mounted onto gelatine subbed slides, covered in X-gal solution (50% X-gal, 1.25% dimethyl sulfoxide, 5% 50mM potassium ferricyanide, 5% potassium ferrocyanide, 1% octylphenoxypolyethoxyethanol (1% concentration), 0.5% sodium deoxycholate (1% concentration), 2% magnesium chloride, in PBS) and incubated on a heated bar slide holder in a water bath at 37°C for five hours. The X-gal solution was then removed, and sections were washed three times for by applying PBS to the slides. Slides were left to dry overnight before being immersed in distilled water for 90 seconds, counterstained in eosin for 60 seconds, and immersed in distilled water for a further 60 seconds. Slides were allowed to dry overnight before being coverslipped using DPX.

3.2.4.2 Immunohistochemistry for tracer injections

In cases with BDA or CTB injections, brains were cut into four series. One series was mounted directly onto gelatine subbed slides, allowed to dry overnight and then stained with cresyl violet, as described above (Immunohistochemistry for virus injections3.2.4.1). Another series was collected in 0.1M PBS for immunohistochemistry, and the remaining two series were collected in

cyroprotectant (30% sucrose, 1% polyvinyl pyrrolidone and 30% ethylene glycol in PBS) and stored in a freezer at -20^{0}C.

For BDA immunohistochemistry, sections were first washed three times in tris-buffered saline (TBS). They were then incubated on a stirring plate at room temperature for two hours with fluorophore (A488) conjugated streptavidin (Thermofisher, UK); at a dilution of 1:200 in TBS with 1% NGS and 0.2% Triton X-100. Sections were then washed three times in TBS, twice in trizma non-saline (TNS), and mounted onto gelatine-subbed slides, immersed in xylene and coverslipped using DPX. All washes were for 10 minutes.

For CTB immunohistochemistry, sections were first washed three times in PBS followed by once in Phosphate Buffered Saline with Triton X-100 (PBST). Sections were then transferred into a blocking solution of 5% normal goat serum (NGS) in PBST and incubated for 90 minutes. Sections were then moved into the primary antibody solution of rabbit-anti-cholera toxin (Sigma Aldrich, Gillingham, UK) at a dilution of 1:10,000 in PBST with 1% NGS, and incubated for 24 hours. Sections were then washed four times in PBST and moved to a secondary antibody solution of goat-anti-rabbit (Dylight Alexa flour 594, Vector Laboratories, Peterborough, UK) at a dilution of 1:200 in PBST with 1% NGS. From this point onwards the sections were covered from light. Sections were incubated for two hours and then placed in a refrigerator (4ºC) overnight. Sections were washed four times in PBST before being mounted onto gelatine-subbed slides, immersed in xylene and coverslipped using DPX. All incubations were on a stirring plate at room temperature and all washes were for 10 minutes unless otherwise stated.

3.2.5 Image capture and analysis

Injection sites and virus/tracer transport were analysed using a fluorescent Leica DM5000B microscope with a Leica DFC310 FX camera. Images were collected from the injection site from each case as well as images of regions where anterograde, retrograde or fibre labelling was observed.

3.3 Results

3.3.1 Efferent projections from anterior thalamic nuclei to cingulate cortex

3.3.1.1 Anteromedial thalamic nuclei

In three cases, anterogradely transported eGFP virus injections were centred in the anteromedial (AM) nucleus of the thalamus, with no apparent (Table 5, 215#1 & 215#12, Figure 3.1a), or very limited (Table 5, 218#1R), involvement of the other anterior thalamic nuclei. In all three cases, fibres left the thalamus by the anterior thalamic peduncle and entered the anterior limb of the internal capsule. Travelling rostralward and dorsalward towards the corpus callosum, discrete fibre fascicles crossed through the medial aspect of the caudoputamen (Figure 3.1c). From the level of the anterior commissure forward, some fibres pierced through the body of the corpus callosum along its anteroposterior axis to join the cingulum from its lateral side. Many fibre fascicles extended beyond the anterior limit of the caudoputamen, turning dorsalward and caudalward to wrap around the genu of the corpus callosum to join the cingulum (Figure 3.1d). These fibres aggregated in the medial aspect of the external medullary stratum of the cingulum (Figure 3.1b), following its sagittal course caudalward. Heavy terminal labelling was observed in layers 1 and 4-6 of the dorsal (Cg1) and ventral (Cg2) anterior cingulate cortex (Figure 3.1b), and a light projection appeared to reach the retrosplenial cortex, primarily terminating in layer 1 of granular cortex B (Rgb).

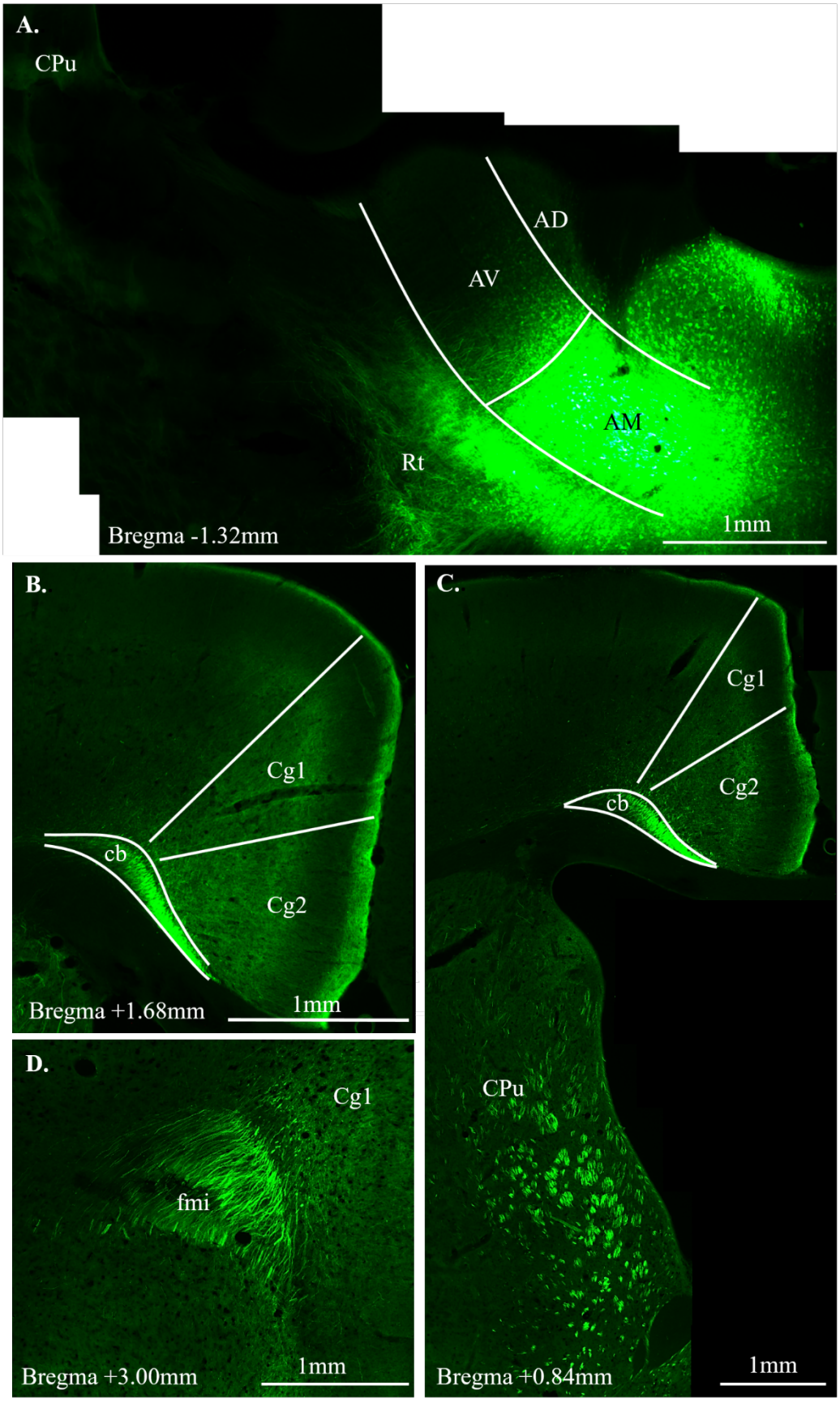
A.
CPu
AD
AV
AM
Rt
Bregma -1.32mm
1mm
B.
Cg1
cb
Cg2
Bregma +1.68mm
1mm
C.
Cg1
Cg2
cb
CPu
Bregma +0.84mm
1mm
D.
Cg1
fmi
Bregma +3.00mm
1mm

Figure 3.1. Photomicrographs showing the trajectory of fibres from the anteromedial (AM) thalamic nuclei to the cingulate cortex.

Images are taken from a eGFP virus injection, case 215#1. Scale bars show approximately 1 millimetre. Coordinates show approximate anteroposterior level in millimetres from Bregma (A.) Injection site centred in AM. Absence of fibres in caudoputamen. (B.) Terminal label in anterior cingulate cortex (Cg1 and Cg2). (C.) Fibre bundles travelling rostralward through the medial caudoputamen (CPu) and fibres travelling caudalward in the medial cingulum bundle (cb). (D.) Fibres wrapping around the genu of the corpus callosum in the forceps minor (fmi). Terminal label in anterior cingulate cortex (Cg1). Other sites included: anteroventral (AV), anterodorsal (AD) and reticular (Rt) thalamic nuclei.

The same fibre pathway was also evident in the three cases with anterogradely transported virus injections that were centred in AM (Table 5, 211#3, 211#5 and 211#11), but had some involvement of anteroventral (AV) and/or anterodorsal (AD) nuclei. Fibres following the trajectory of AV and AD projections, described in the following sections, were also observed in these cases.

In all of the five cases with tracer injections in the cingulum bundle underneath the anterior cingulate cortex (Table 5, 205#5L [BDA], 205#5R, 205#6, 209#16 and 209#17 [CTB]) retrograde label was observed in AM, confirming the presence of projections from this nucleus at this level of the bundle. Conversely, there was no retrograde label in AM following any of the four tracer injections into the cingulum bundle underneath the retrosplenial cortex (Table 5, 205#2R [CTB], 205#2L, 205#3 and 209#19 [BDA]), indicating that almost all projections from AM terminate in cingulate cortex anterior to this level.

3.3.1.2 Anteroventral thalamic nuclei

In six cases, anterogradely transported eGFP virus injections were centred in AV, with varying involvement of other anterior thalamic nuclei (Table 5, 218#1L, 218#3, 211#13, 211#14, 229#2L and 229#2R). Each of these injections produced heavily labelled fibres following the same trajectory as the AM projections described previously (Figure 3.1). Critically, this was observed in cases with limited involvement of AM at the injection site (229#2L, 218#1L, Figure 3.2b); suggesting that AV efferents follow the same route to the cortex. Additionally, all tracer injections in the cingulum bundle underneath the anterior cingulate cortex (Table 5, 205#5L [BDA], 205#5R, 205#6, 209#16 and 209#17 [CTB]) resulted in retrograde

label in AV, confirming the presence of efferents from this nucleus at this level of the fibre pathway.

As well at this rostralward projection, all anterogradely transported virus injections involving AV resulted in labelled fibres following a more direct route to the cortex. Of those fibres leaving the thalamus by the anterior thalamic peduncle, many make a sharp dorsalward turn towards the corpus callosum. As such, fascicles were seen crossing the caudoputamen and into the cingulum under the entire anteroposterior length of the anterior cingulate cortex. A further subset of fibres left the thalamus laterally, to travel dorsalward around the lateral ventricle (Figure 3.2a), crossing directly into the cingulum underneath the retrosplenial cortex. Fibres from these injections occupied both the medial and lateral aspect of the external medullary stratum of the cingulum (Figure 3.2a), and terminal label was observed in layers 1 and 4-6 of the dorsal (Cg1) and ventral (Cg2) anterior cingulate cortex and layers 1 and 4 of retrosplenial granular cortex B (Rgb).

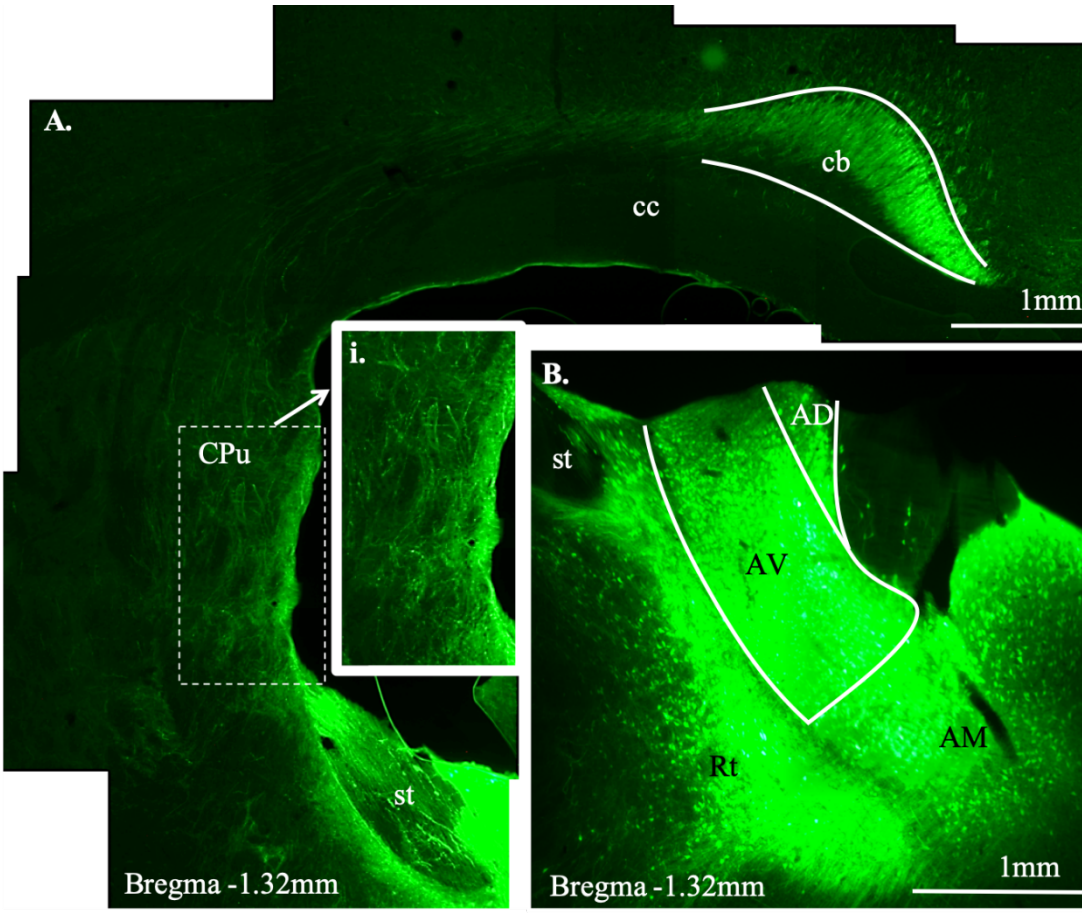

Figure 3.2. Photomicrographs showing the trajectory of a subset of fibres from the anteroventral (AV) thalamic nuclei to the cingulate cortex.

Images are taken from a eGFP virus injection, case 229#2L. Scale bars show approximately 1 millimetre. Coordinates show approximate anteroposterior level in millimetres from Bregma. (A.) Fibres, which have left the anterior thalamus laterally, travelling dorsalward through the caudoputamen (CPu) to join the cingulum bundle (cb). Fibres occupy external medullary stratum of the cingulum. (i.) Inset from (A.) showing fibres crossing caudoputamen at a higher magnification.

There is evidence that projections from AD, described in the following section, and from the laterodorsal (LD) thalamic nucleus, not described here, may follow a similar more direct route to the cortex. It is, therefore, pertinent that injections in the AV nucleus without apparent involvement of either of AD or LD (Table 5, 218#1 & 211#14) produce this pattern of fibre labelling. Meanwhile all tracer injections in the cingulum bundle underneath the retrosplenial cortex (Table 5, 205#2R [CTB], 205#2L, 205#3 and 209#19 [BDA]) resulted in retrograde label in AV, confirming the presence of projections from this nucleus at this level of the bundle.

3.3.1.3 Anterodorsal thalamic nuclei

Three anterogradely transported eGFP virus cases involved AD at the injection site (Table 5, 211#13, 229#2L (Figure 3.2b) and 229#2R), all of which resulted in labelled fibres following both the rostralward and more direct route to the cortex described thus far. Involvement of AV at the injection sites precludes discernment of those fibres originating exclusively from AD in these cases. However, the observation that no retrograde label was observed in AD from any of the five tracer injections in the cingulum bundle underneath the anterior cingulate cortex (Table 5, 205#5L [BDA], 205#5R, 205#6, 209#16 and 209#17 [CTB]) indicates that projections from AD cannot join the cingulum anterior to this level; i.e. fibres from AD do not appear to follow the rostralward route to the cortex described previously.

Further caudal in the cingulum, tracer injections underneath the rostral part of retrosplenial cortex resulted in light (Table 5, 205#2L, 205#3 [BDA]) or no (205#2R [CTB], 209#19 [BDA]) retrograde label in AD. This is consistent with some projections from AD taking the direct route to the cortex described previously, joining the cingulum at levels similar or caudal to the tracer injection site.

3.3.2 Efferent projections from cingulate cortex to anterior thalamus

3.3.2.1 Anterior cingulate cortex

In three cases, bilateral anterogradely transported eGFP virus injections in the anterior cingulate cortex extended to the most rostral limit of this region, encompassing all layers of pregenual Cg1 (Table 5, 224#1 [Figure 3a], 224#2 and 219#2). Projections from this area did not wrap around the genu of the corpus callosum (Figure 3a) but travelled caudally in the internal stratum of the cingulum above the body of the corpus callosum. From here, fibres pierced through the white matter (Figure 3.3b) and aggregated in fascicles travelling caudalward and ventralward in the medial aspect of the caudoputamen (Figure 3.3c). After joining the anterior limb of the internal capsule, fibres turned medial around the stria terminalis (Figure 3.3d) to terminate in AM and dorsomedial AV (AVDM) (Figure 3.3e).

Three additional cases contained bilateral anterogradely transported eGFP virus injections centred in the anterior cingulate cortex over the body of the corpus callosum (Table 5, 219#18, 224#20, 224#21). Injection sites encompassed all layers of Cg1, with some overlap into Cg2. In these cases, labelled fibres passed through the cingulum directly from the injection site, without becoming enclosed in the white matter for any length. From here, fibres followed the same route and terminated in the same anterior thalamic nuclei as projections from pregenual anterior cingulate cortex (Figure 3.3e). A case with unilateral injections in the anterior cingulate cortex (Table 5, 215#31) revealed that projections reached the contralateral thalamus by the same route. Fibres crossed directly through the thalamus to terminate lightly in the same anterior thalamic nuclei in the other hemisphere (Figure 3.3e).

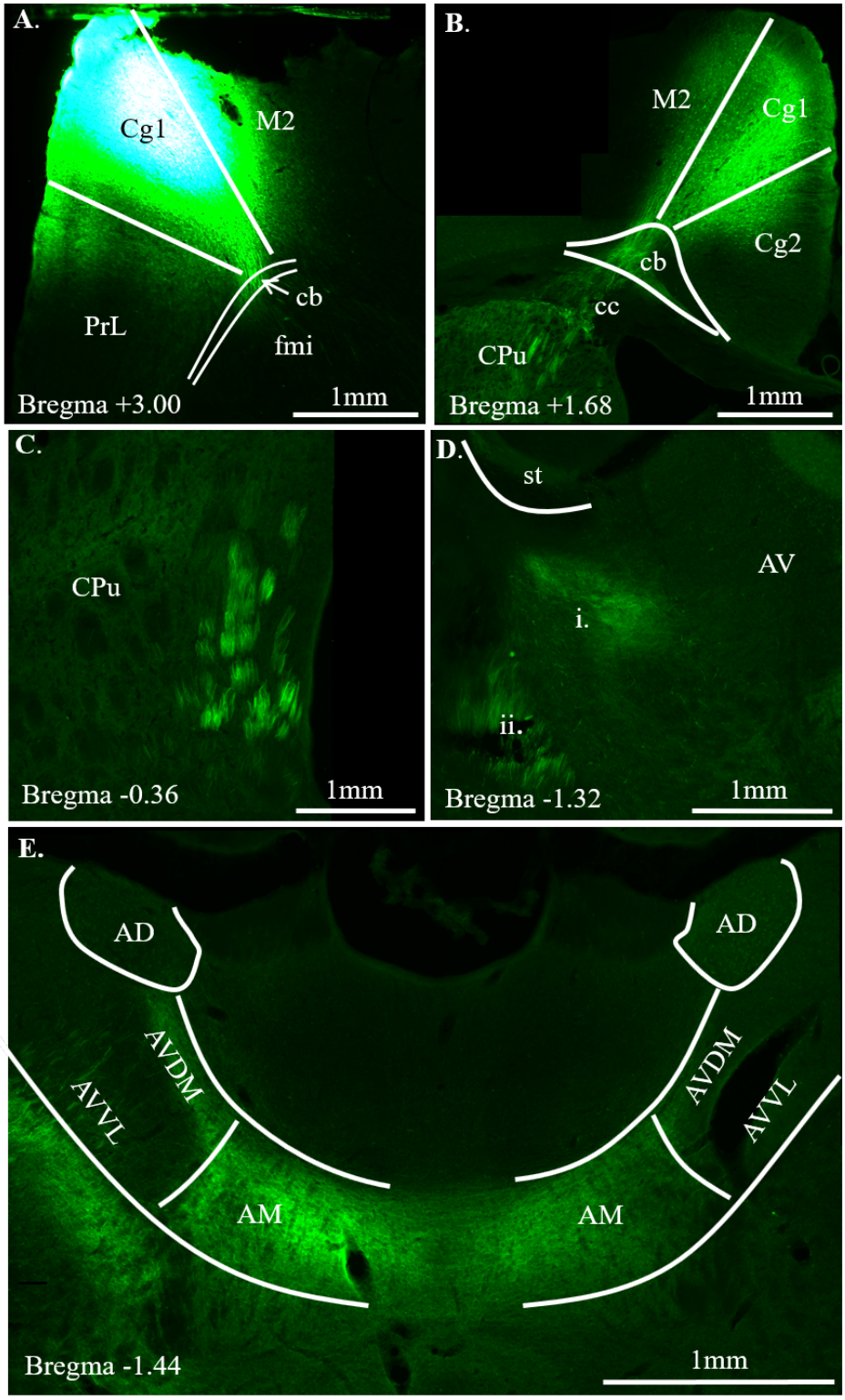

Figure 3.3. Photomicrographs showing the trajectory of fibres from the anterior cingulate cortex to the anterior thalamic nuclei.

Images are taken from eGFP virus injections (A) case 224#1 and (B-E) case 215#31. Scale bars show approximately 1 millimetre. Coordinates show approximate anteroposterior level in millimetres from Bregma. (A.) Injection site centred in pregenual anterior cingulate cortex (Cg1). Fibres entering cingulum bundle (cb) to follow its caudalward course. (B.) Injection site centred in

postgenual dorsal anterior cingulate cortex (Cg1). Fibre crossing cingulum bundle (cb) and corpus callosum (cc) to enter the caudoputamen (CPu). (C.) Fibres travelling caudalward in the medial CPu. (D.) i. Fibres turning laterally to enter the anterior thalamus. ii. Fibres continuing ventrally towards other targets. (E.) Terminal label in the anteromedial (AM) and dorsomedial aspect of the anteroventral thalamic nuclei (AVDM). Fibres crossing midline to terminate in the same nuclei in the other hemisphere, from a unilateral injection. Other sites included: prelimbic cortex (PrL), secondary motor cortex (M2), forceps minor of the corpus callosum (fmi), ventral anterior cingulate cortex (Cg2), stria terminalis (st), anterodorsal thalamic nuclei (AD).

In all five cases with anterograde and retrograde tracer injections into the cingulum bundle underneath the anterior cingulate cortex (Table 5, 205#5L [BDA], 205#5R, 205#6, 209#16 and 209#17 [CTB]), anterograde label was observed in AM and AVDM, but not in AD; supporting the presence of these efferents in the cingulum at this level.

3.3.2.2 Retrosplenial cortex

In two cases, unilateral anterogradely transported eGFP virus injections extended from near the rostral limit of the retrosplenial cortex to just before the level of the splenium (Table 5, 224#29, 224#30). Injection sites were centred in retrosplenial granular cortex, encompassing all layers of area B (Figure 3.4a). Fibres joined the internal stratum of the cingulum and travelled rostralward to the level of the anterior thalamus (Figure 3.4a). From here, fascicles cut down through the white matter and caudoputamen, skirting the lateral ventricle to briefly pass through the internal capsule. Taking a sharp medialward turn around the stria terminalis, fibres enter the thalamus from its lateral side (Figure 3.4c). Terminal label was present in AV and AD (Figure 3.4c), with some fibres seen crossing the midline thalamus to terminate lightly in the same nuclei of the contralateral hemisphere.

A further subset of fibres from these cases entered the cingulum and projected beyond the rostral limit of the anterior thalamus. Cutting down through the white matter underneath the anterior cingulate cortex, these fibres formed fascicles travelling caudalward and ventralward through the medial caudoputamen (Figure 3.4b). It is possible that some of these fibres entered the thalamus from this aspect, but the majority appeared to travel into the posterior limb of the internal capsule (Figure 3.4c). From here, they entered the cerebral peduncle on route to targets in the brain stem.

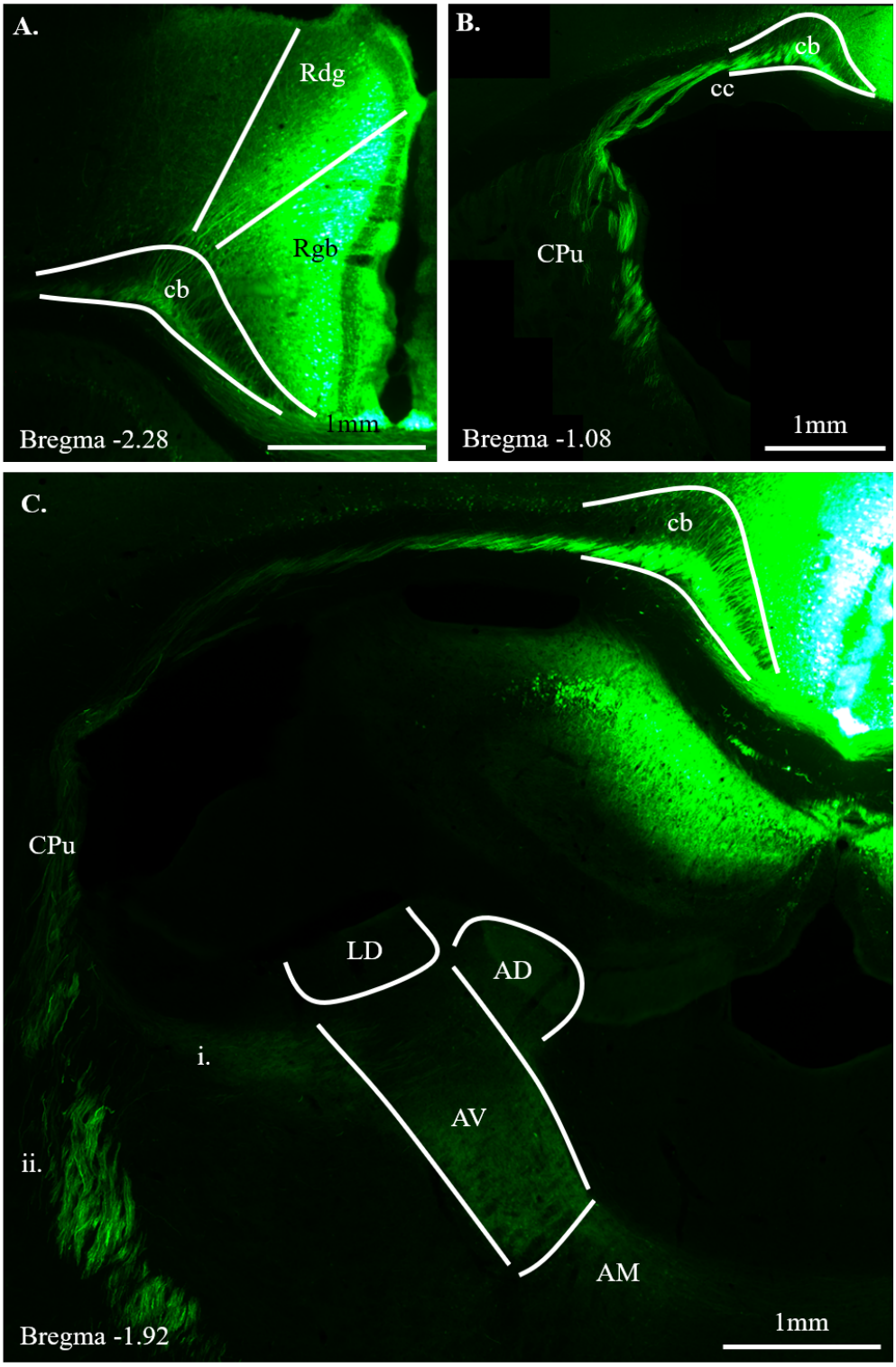

Figure 3.4. Photomicrographs showing the trajectory of fibres from the retrosplenial cortex to the anterior thalamic nuclei.

All images are taken from a eGFP virus injection, case 224#29. Scale bars show approximately 1 millimetre. Coordinates show approximate anteroposterior level in millimetres from Bregma. (A.) Injection site centred in retrosplenial granular cortex B (Rgb). Fibres enter the cingulum bundle (cb) to follow its rostralward course. (B.) Fibres travelling beyond the rostral limit of the anterior

thalamus before crossing the corpus callosum (cc) to enter the caudoputamen (Cpu). (C.) Fibres crossing the caudoputamen at the level of the anterior thalamus. (i) Fibres turning medially to enter the anterior thalamus. Terminal label is observed in the anteroventral (AV) and anterodorsal (AD) nuclei of the thalamus. Note that label in the anteromedial (AM) thalamic nucleus is predominantly from fibres crossing the midline to terminate in AV and AD of the other hemisphere, from a unilateral injection. (ii) Fibres continuing ventrally to reach other targets. Other site included: retrosplenial dysgranular cortex (Rdg).

In all four cases with tracer injections into the cingulum bundle underneath the retrosplenial cortex (Table 5, 205#2R [CTB], 205#2L, 205#3 and 209#19 [BDA]), anterograde label was observed in AV, consistent with efferents from retrosplenial cortex reaching this nucleus via the cingulum at this level. Conversely, only one of these injections (209#19 [BDA]) produced light anterograde label in AD, indicating few efferent fibres from this nucleus in the cingulum at this level.

3.3.2.3 Projections between the anterior thalamic nuclei and the cingulate cortex visualised on the sagittal plane

In two cases EIAV lentivirus injections targeted the anterior thalamus (Table 5, 213#2LH, 213#2RH). Injection sites were centred in the anteroventral (AV) nucleus of the thalamus, with some overlap into laterodorsal (LD), and thalamic reticular nuclei. In a further four cases, EIAV lentivirus injections targeted the cingulum bundle below the anterior cingulate cortex (Table 5, 209#5LH, 209#5RH, 213#3LH, 213#3RH). Injection sites incorporated the cingulum bundle and adjacent anterior cingulate (Cg1) and secondary motor (M2) cortices. These injections were made for the purpose of visualising fibres on the sagittal plane.

Both cases with injections in the anterior thalamic nuclei produced fibre labelling consistent with the anterior thalamic trajectories described in the previous sections. That is, fibres were seen sweeping around the stria terminalis, crossing the caudoputamen in bundles and piercing through the body of the corpus callosum, along its sagittal length. Labelled fibres are also seen travelling in the cingulum Figure 3.5a, b). Similarly, all four cases with injections in the cingulum bundle produced labelled fibres in this same pathway (Figure 3.5c,d).

It is noteworthy that EIAV lentivirus is axonally transported both anterograde and retrograde (Mazarakis et al., 2001) and, therefore, these cases do not distinguish

efferent and afferent fibres between the anterior thalamus and the anterior cingulate cortex. Further, the injections sites were large. This means that fibres from discrete thalamic nuclei, or cortical regions, cannot be delineated based on these data. Nonetheless, the fibres observed in these cases are consistent with those from more targeted injections described in the previous sections of this chapter and provide a sagittal visualisation of these pathways.

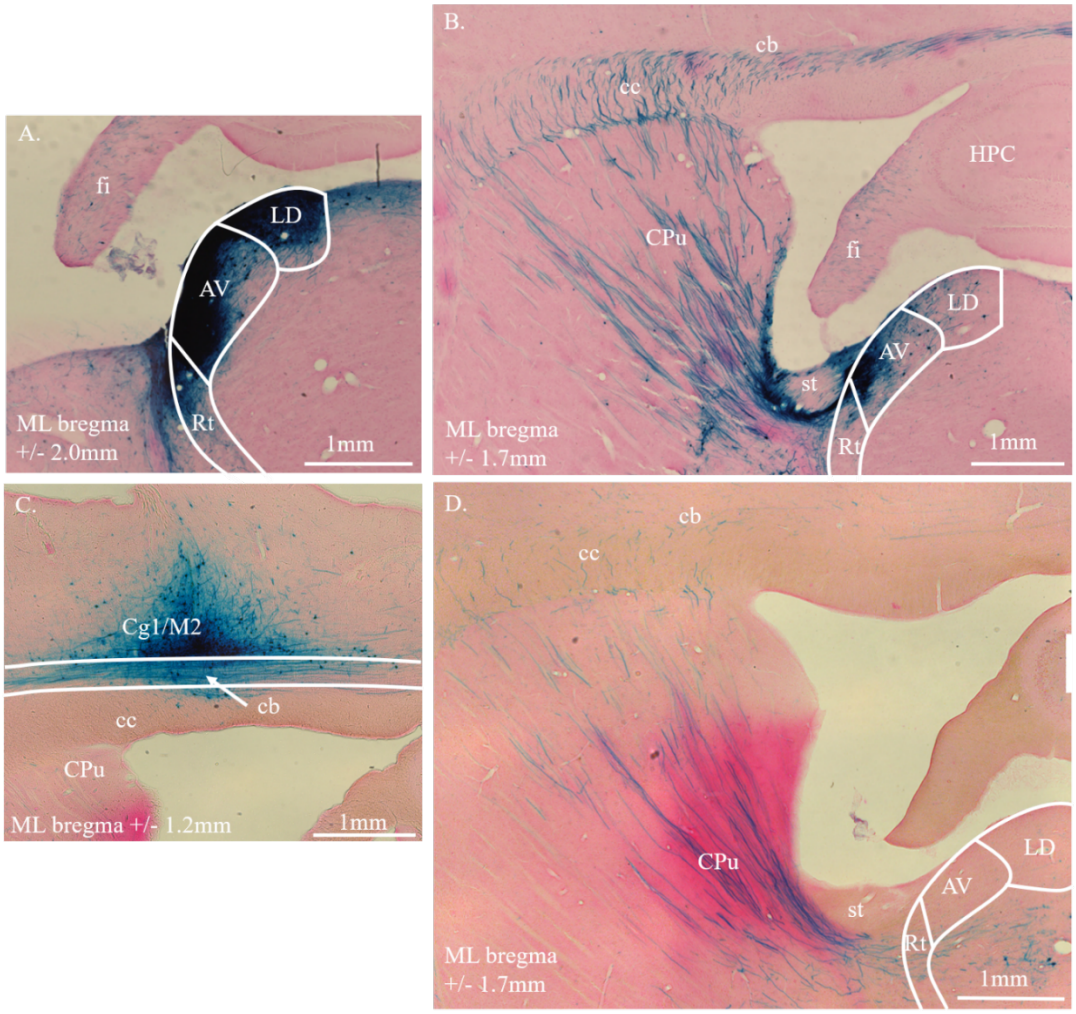

Figure 3.5. Photomicrographs showing the trajectory of fibres between the anterior thalamic nuclei and the cingulate cortex on the sagittal plane.

Images A-B are taken from an EIAV virus injection into the anterior thalamic nuclei, case 213#2. Images C-D are taken from an EIAV virus injection in the cingulum bundle, case 213#5. Scale bars show approximately 1 millimetre. Coordinates show approximate mediolateral level in millimetres from Bregma. (A.) Injection site centred the anteroventral (AV) and laterodorsal (LD) thalamic nuclei (B.) Labelled fibres around the stria terminalis (st), in the caudoputamen (CPu), corpus callosum (cc) and cingulum bundle (cb) (C.) Injection site in the cingulum bundle (cb) and adjacent anterior cingulate cortex (Cg1) and secondary motor cortex (M2) (D.) Labelled fibres around the stria terminalis (st), in the caudoputamen (CPu), corpus callosum (cc) and cingulum bundle (cb). Other sites included: fimbria of the hippocampus (fi), hippocampus (HPC), reticular thalamic nucleus (Rt).

3.4 Discussion

Despite their many interconnections, no study has focused on mapping the fibre pathways between the anterior thalamic nuclei and the cingulate cortex since the seminal work of Domesick (1969, 1970), which left a number of details to be clarified. The current study re-examined the routes of anterior thalamic-cingulate-retrosplenial connections using virus-based anterogradely transported tracers tagged with a fluorescent marker. Injections targeted the anteromedial (AM), anteroventral (AV) and anterodorsal (AD) thalamic nuclei, which allowed efferent fibres from these nuclei to be traced to the anterior cingulate and retrosplenial cortices (Figure 3.6). Corresponding injections targeted these cortical regions, which enabled the mapping of return projections from the cingulate cortex to the anterior thalamic nuclei (Figure 3.7). Anterograde and retrograde tracer injections into the cingulum bundle helped to corroborate the presence of connections from individual anterior thalamic nuclei at different anteroposterior levels of this fibre pathway. A further subset of injections anterogradely and retrogradely transported virus injections targeted the anterior thalamic nuclei and the cingulum bundle and were cut sagittally (rather than coronally), allowing the visualisation of fibres on this anatomical plane.

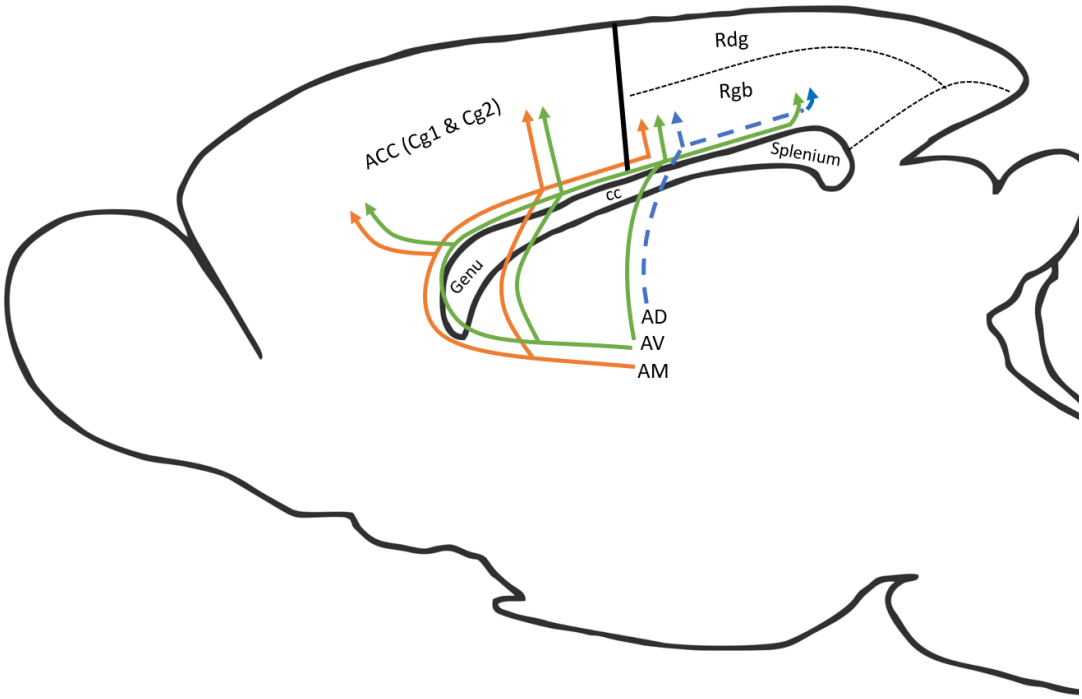

Figure 3.6. Sagittal schematic of the rat brain showing the routes taken by fibres from the anterior thalamic nuclei to the cingulate cortex.

The colours distinguish pathways from different nuclei. Dashed lines represent trajectories that are suggested, but not confirmed, by the data in this study. Lines that run dorsal to the corpus callosum are fibres that become enclosed in the cingulum bundle. All projections to the anterior cingulate cortex terminate in both Cg1 and Cg2. Sites included: anteromedial (AM), anteroventral (AV) and anterodorsal (AD) thalamic nuclei, anterior cingulate cortex (ACC, Cg1 and Cg2), granular retrosplenial cortex b (Rgb) and corpus callosum (cc).

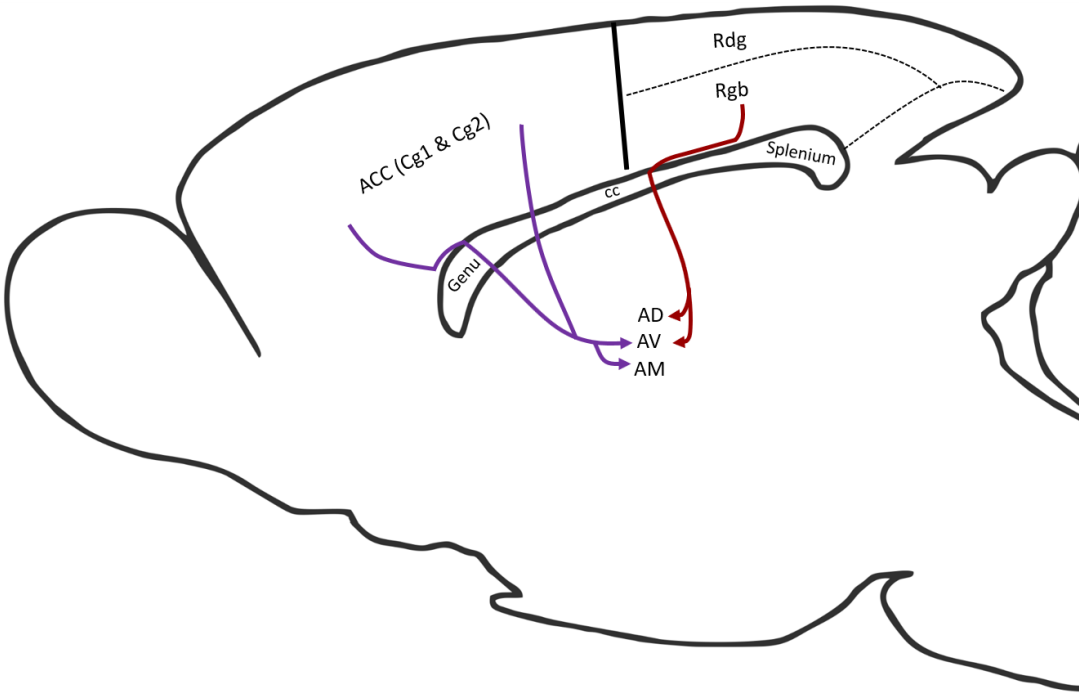

Figure 3.7. Sagittal schematic of the rat brain showing the route taken by fibres from the cingulate cortex to the anterior thalamic nuclei.

The colours distinguish pathways from different cortical regions. Lines that run dorsal to the corpus callosum are fibres that become enclosed in the cingulum bundle. Note that all projections from the anterior cingulate cortex are from both Cg1 and Cg2. Sites included: anterior cingulate cortex (ACC, Cg1 and Cg2), granular retrosplenial cortex b (Rgb), anteromedial (AM), anteroventral (AV) and anterodorsal (AD) thalamic nuclei and corpus callosum (cc).

3.4.1 Anterior thalamic nuclei to cingulate cortex

Anterogradely transported virus injections in AM revealed fibres following the same route to the cortex originally described by Domesick (1970). Fibres leave the thalamus anteriorly, before forming discrete bundles that course rostralward through the medial part of the caudoputamen. These bundles pierce through the body of the corpus callosum from the level of the anterior commissure forward. The longest fibres extend beyond the rostral limit of the caudoputamen, where they travel dorsalward around the genu of the corpus callosum in the forceps minor. Some of these fibres continue rostralward to reach medial prefrontal targets, including the pregenual part of the dorsal anterior cingulate cortex (Cg1). The remaining fibres join the caudalward course of the cingulum bundle, where they aggregate in the medial external medullary stratum of this fibre pathway.

A substantial proportion of efferents from AM targeted the anterior cingulate cortex, principally terminating in layers 1 and 4-6 of the dorsal (Cg1) and ventral (Cg2) anterior cingulate cortex; consistent with previous research (Shibata, 1993b; Van Groen et al., 1999). Only a light projection appeared to reach retrosplenial cortex, terminating in layer 1 of granular cortex B (Rgb). This is a more restricted projection from AM than has been described in previous research, where studies have reported additional termination in granular cortex A (Rga) (Van Groen et al., 1999) and dysgranular cortex (Rdg)(Shibata, 1993b).

The presence, or absence, of retrograde label in AM following tracer injections into different anteroposterior levels of the cingulum bundle sheds further light on the strength of the projection from this nucleus to the retrosplenial cortex. Following tracer injections into the cingulum at the level of the anterior cingulate cortex (at anteroposterior level +0.6mm from Bregma), retrogradely labelled cell bodies were identified in AM. The implication of this is that efferent fibres from AM are present, travelling caudally, in this part of the cingulum bundle. In contrast, tracer injections into the cingulum bundle above retrosplenial cortex (at anteroposterior level -2.4mm from Bregma) resulted in an absence of retrograde label in AM. Consequently, this suggests that most fibres from AM, which travel caudally in the cingulum under the anterior cingulate cortex (anteroposterior level +0.6mm from Bregma), terminate at levels anterior to the retrosplenial level injection site (anteroposterior level -2.4mm from Bregma). Taken together, these findings indicate that there may be a lighter projection from AM to the retrosplenial cortex than described previously, primarily targeting the rostral part of granular cortex B.

Anterogradely transported virus injections centred (or primarily centred) in AM resulted in an absence (or relative absence) of fibres following any other routes to the cingulate cortex. This suggests that projections from this nucleus chiefly follow the pathway to the cingulate cortex originally described by Domesick (1970), a finding that also echoes descriptions of anteromedial thalamic efferents from tracer injection studies (Van Groen et al., 1999). The rostralward trajectory of AM projections may reflect the close affinity of this nucleus with frontal cortical regions (Jones, 2012). Most of the anterior thalamic input to prefrontal, including anterior cingulate, cortex

comes from AM (Shibata, 1993b), as well as from the interanteromedial thalamic nuclei (IAM) (Hoover & Vertes, 2007).

Anterogradely transported virus injections in AV revealed efferent fibres from this nucleus that follow the same route to the cortex as fibres from AM. That is, the trajectory initially described by Domesick (1970). It is important to note that all such cases had some involvement of AM at the injection site. However, if this pathway was exclusively favoured by efferents from AM, limiting the involvement of AM at the injection site should have resulted in a reduction in the number of labelled fibres in this pathway. There was no such reduction, with injections primarily centred in AV produced a comparable amount of labelled fibres in this pathway to injections centred in AM. Furthermore, tracer injections into the cingulum bundle underneath the anterior cingulate cortex (at anteroposterior level +0.6mm from Bregma) resulted in retrogradely labelled cell bodies in AV. This indicates that efferent fibres from AV are present at this anterior level of the fibre pathway. Taken together, these findings indicate that some fibres from AV project rostralward from the thalamus through the caudoputamen before joining the cingulum.

Other fibres from AV appear to favour more direct routes to the cortex. Anterograde tracer injections in AV revealed a subset of fibres that followed essentially the same route as fibres from AM but made a sharper dorsalward turn through the caudoputamen. As such, fibres crossed through the corpus callosum and into the cingulum at more caudal entry points than projections from AM. Another subset of fibres left the thalamus laterally, travelling around the lateral ventricle to enter the cingulum directly under the retrosplenial cortex. Whilst no such route has been described previously in the rat, it has been identified from tracer injections in the monkey. Mufson and Pandya (1984) described fibres from an injection site in AV that left the thalamus laterally, and some posteriorly, before reaching the cingulum bundle and retrosplenial cortex.

Whereas fibres from AM were constrained in the medial aspect of the external medullary stratum of the cingulum, fibres that followed the more direct routes to the cortex from AV were found to occupy the lateral external medullary stratum. It is

noteworthy, however, that the laminar organisation of fibres within the cingulum bundle itself is not comparable across species (Mufson & Pandya, 1984).

As previously stated, all cases with anterogradely transported virus injections in AV also involved other thalamic nuclei at the injection site. Therefore, it is not possible to attribute termination in these case to efferents from this individual nucleus. Consequently, although termination was observed in anterior cingulate cortex (Cg1 and Cg2) from injections centred in AV, this may have resulted from spread of the virus into neighbouring AM. Previous research has reported only a light projection from AV to restricted parts of Cg2 (Shibata, 1993b), and have found that the preponderance of projections from this nucleus were to the retrosplenial cortex (Shibata, 1993b; Van Groen & Wyss, 1995). Consistent with the latter observation, the current study found that injections involving AV resulted in more terminal label in retrosplenial cortex, occupying layers 1 and 4 of Rgb, than injections centred in AM.

All anterogradely transported virus injections involving AD resulted in fibres following both the rostralward route and the more direct routes to the cortex described thus far. Due to the involvement of other anterior thalamic nuclei at the injection site in all these cases, it is not possible to identify the trajectory of fibres originating exclusively from AD. Previous research has found that AD projects to Rga and Rgb (Van Groen & Wyss, 1990b, 1995, 2003), and it has been proposed that fibres from AD follow the rostralward route described previously to their retrosplenial targets (V. Domesick, 1970; Van Groen & Wyss, 1995).

Analysis of retrograde label resulting from tracer injections into different parts of the cingulum bundle, however, appear to contradict this suggestion. If projections from AD follow the rostralward route to the cortex, fibres would pass through cingulum bundle under the anterior cingulate cortex on route to the retrosplenial cortex. However, tracer injections into the cingulum bundle at the level of the anterior cingulate cortex (anteroposterior level +0.6mm from Bregma) did not result in any retrogradely labelled cells in this nucleus. Tracer injection further caudal in the

cingulum, underneath the retrosplenial cortex (anteroposterior level -2.4mm from Bregma), resulted in either a few or no retrogradely labelled cells in AD.

It is interesting that these observations would be consistent with AD projections following the same direct route to the cortex as those from AV. That is, leaving the thalamus laterally to travel around the lateral ventricle. From here, fibres could join the cingulum at levels roughly equivalent to the caudal tracer injection site (anteroposterior level -2.4mm from Bregma) to reach retrosplenial targets. The more caudalward trajectory of fibres emanating from AV, and possibly AD, may reflect the closer affinity of these nuclei with the more caudally situated brain regions such as retrosplenial and parahippocampal cortex (Van Groen & Wyss, 1995); relative to AM.

3.4.2 Cingulate cortex to anterior thalamic nuclei

Anterogradely transported virus injections in the anterior cingulate cortex revealed fibres following a similar route to the anterior thalamic nuclei to that originally described by Domesick (1969). Efferents from the postgenual anterior cingulate cortex do not become enclosed in the cingulum but pierce directly through the white matter from their point of origin. These fibres travel caudally and ventrally through the medial aspect of the caudoputamen, before joining the anterior limb of the internal capsule. Fibres then take a sharp medial turn around the stria terminalis to reach the thalamus. One exception, not described by Domesick (1969), is that some fibres from pregenual dorsal anterior cingulate cortex (Cg1) do join the internal stratum of the cingulum for some length. These efferents travel caudalward in the white matter until they are positioned over the body of the corpus callosum, from which point they follow the formerly described route to the thalamus.

Consistent with previous research (Beckstead, 1979; Shibata & Naito, 2005), many fibres from the anterior cingulate cortex were seen to terminate bilaterally in AM. A light projection targeted AV, terminating in the dorsomedial part of this nucleus (AVDM), which is further consistent with a previous anterograde tracing study (Shibata & Naito, 2005). However, whereas the previous study found the projection to be ipsilateral, terminal label was observed bilaterally in the current study

(Mathiasen et al., 2017). It is noteworthy that this previous study (Shibata & Naito, 2005) found that secondary motor cortex projects bilaterally to AVDM. The contralateral label in the current study may, therefore, be attributable to involvement of neighbouring secondary motor cortex at the injection sites of these cases.

Injections in the present study typically encompassed both dorsal (Cg1) and ventral (Cg2) in anterior cingulate cortex. Therefore, it is not possible to differentiate between the anterior thalamic termination sites of these subregions using the current data set. Previous research, however, has found that discrete injections in each of these areas resulted in comparable distribution of labelled terminals in the anterior thalamic nuclei (Shibata & Naito, 2005).

Anterogradely transported virus injections in the retrosplenial cortex, centred in granular area B (Rgb), revealed fibres following the same route to the anterior thalamic nuclei as described by Domesick (1969). Fibres enter the cingulum and travel rostrally in the internal stratum. At around the level of the anterior thalamus, fibres cut down through the white matter to skirt the lateral ventricle, before briefly joining the internal capsule. Fibres then sweep medially around the stria terminalis to enter the thalamus from its lateral side.

The current study found an additional subset of fibres, not described by Domesick (1969), which course further in the cingulum, to beyond the rostral limit of the anterior thalamus, before cutting down through the white matter. These fibres then turn caudally to cross the caudoputamen before entering the internal capsule. Although it is possible that some fibres enter the thalamus from this aspect, the majority appear to continue into the posterior limb of the internal capsule. From here, fibres can be traced into the cerebral peduncle to reach retrosplenial targets in the pons (Domesick, 1969; Shibata, 1998; Van Groen & Wyss, 2003).

In line with previous research (Shibata, 1998; Van Groen & Wyss, 2003), efferents from rertrosplenial cortex (Rgb) were seen to terminate in AV, with a lighter projection reaching AD. Termination was present in these nuclei in both the ipsilateral and the contralateral hemispheres, as described previously (Mathiasen et

al., 2017). Previous research has further described projections from retrosplenial granular cortex area A (Rga) to AV (Van Groen & Wyss, 2003) and from retrosplenial dysgranular cortex (Rdg) to AM (Shibata, 1998; Van Groen & Wyss, 1992). However, due to the injections in the current study primarily targeting Rgb, the trajectory of efferent fibres from these retrosplenial subdivisions cannot be discerned from the current data set.

3.4.3 Summary and implications

Figure 3.6 provides a sagittal schematic summary of the fibre pathways from the anterior thalamic nuclei to the anterior cingulate and retrosplenial cortices. This study found that all projections from AM and many projections from AV follow the previously depicted route to the cortex (Domesick, 1970). These fibres leave the thalamus anteriorly to travel rostralward through the caudoputamen. Some fibres cross through the body of the corpus callosum, and others wrap around the genu, to join the cingulum. Other projections from AV follow a route to the cortex that has not been described previously in the rat. These fibres leave the thalamus laterally, skirting the lateral ventricle to reach the cingulum more directly. This study found evidence to suggest that efferents from AD may also follow this more direct route to the cortex.

Figure 3.7 provides a sagittal schematic summary of the fibre pathways from the anterior cingulate and retrosplenial cortices to the anterior thalamic nuclei. This study found that fibres from rostral anterior cingulate cortex travel caudally to join the cingulum over the body of the corpus callosum. From here, they join fibres from the postgenual anterior cingulate cortex in crossing through the white matter and the caudoputamen, as described by Domesick (1969). In the thalamus, these fibres terminate in AM and AV. Projections from retrosplenial cortex (Rgb) were also found to follow the classically depicted route (Domesick, 1969). That is, they travel rostralward in the cingulum before crossing through the white matter to skirt the lateral ventricle and enter the thalamus from its lateral side. These fibres terminate in AV and AD of the thalamus.

In animals, these findings have far reaching implications for the interpretation of lesion studies. Firstly, there is the observation that projections from the anterior thalamic nuclei to the cingulate cortices join the cingulum all along the length of the tract, and that projections from the cingulate cortices primarily cross through the cingulum to reach the anterior thalamus, rather than joining its sagittal course. Many these fibres would be left intact when lesioning the cingulum at one anteroposterior, particularly when lesioning the cingulum asymmetrically (to avoid bilateral cortical damage (Neave et al., 1997; Neave et al., 1996). In fact, the distribution of fibres leaving and joining the cingulum would make it very difficult to achieve a complete disconnection of the anterior thalamus and the cingulate cortices by any lesion method, as it appears that even creating multiple lesions along the anteroposterior axis would not damage all fibres.

Meanwhile, this study informs interpretation of the functionality of different parts of the cingulum bundle. For example, the anterior portion of the tract contains many fibres connecting the anteromedial thalamic nuclei to the cingulate cortices and comparatively few serving the anteroventral and anterodorsal thalamic nuclei. Therefore, lesions at this level of the tract would preferentially disrupt anteromedial-cingulate interconnections, without affecting anteroventral/anterodorsal-cingulate interconnectivity. Relatedly, changes to the anterior portion of the cingulum observed in human diffusion tensor imaging (DTI) research (1.4.3.2.11.4.3.2) may reflect differences in anteromedial-cingulate cortex interconnectivity, but is less likely to reflect changes anteroventral/anterodorsal-cingulate interconnectivity. The reverse is true for posterior portions of the tract, with interventions and correlations more closely associated with anteroventral/anterodorsal-cingulate interconnectivity 1.4.3.2.

A further implication relates to the interpretation of lesion studies that may cause unintended disconnection of the anterior thalamus and cingulate cortices. Principally, the extent to which interconnecting fibres between the anterior thalamus and the cingulate cortices disperse across the caudoputamen means that lesions targeting this region will necessarily result in some level of disconnection. Therefore, researchers

interpreting the functional implications of caudoputamen lesions need be aware of the potential confound of disrupting anterior thalamic-cingulate cortex connectivity.

4 DREADD-Mediated Inhibition of Anterior Cingulate Cortex and Attentional Set-Shifting

4.1 Introduction

The anterior cingulate cortex is one of a number of frontal regions with key roles in cognitive control and behavioural flexibility (Shackman et al., 2011; Shenhav et al., 2013; Shenhav et al., 2016). In short, it is repeatedly implicated when behaviour needs to be effortfully guided towards a goal, especially when an action needs to be chosen from a number of competing responses (see also 1.5.1). Such a capacity is vital for an animal to successfully navigate a world full of changing environmental contingencies (Sheth et al., 2012). However, it encompasses many component processes that are, in turn, supported by diverse brain structures and circuits (section 1.5.1.1). Consequently, an ongoing challenge in neuroscience is parsing apart how such structures function, and interact, to support behavioural flexibility.

In rats, Ng et al. (2007) used the attentional set-shifting task to demonstrate that the functionality of the anterior cingulate cortex may be dissociable from that of other medial prefrontal areas. This task requires a subject to orient attention towards relevant, and away from irrelevant, stimuli in order to solve a discrimination and receive a reward (Birrell & Brown, 2000)(see also section 1.5.1.4). Lesions of the anterior cingulate cortex were found to impair intradimensional shifts, but did not impair extradimensional shifts (Ng et al., 2007). This result is striking because damage to other medial prefrontal areas, such as prelimbic cortex (Birrell & Brown, 2000), causes rats to take additional trials to learn extradimensional shifts.

In explanation of their results, Ng et al. (2007) suggested that completing an intradimensional shift requires the irrelevant stimulus dimension to be ignored, whereas an extradimensional shift requires the opposite; attention needs to centre on the stimulus dimension that has previously been established as irrelevant. According to classic theories of attention (Mackintosh, 1965, 1975), the finite nature of attentional resources means that paying more attention to one stimulus dimension involves a lessening of attention paid to another (irrelevant) stimulus dimension. Ng

et al. (2007) argued that rats with anterior cingulate cortex lesions do not demonstrate this weakening salience of irrelevant stimuli, resulting in slower acquisition of discriminations that requires attention to be focused within the relevant dimension (intradimensional shifts). At the same time, less weakening of attention to the irrelevant dimension is not problematic for these animals when the irrelevant dimension becomes predictive of reward (extradimensional shifts).

Across a series of successive intradimensional shifts, normal animals typically display a gradual improvement in performance (Chase et al., 2012). This is thought to signify the formation of an attentional set (Tait et al., 2018; Tait et al., 2014), whereby animals have learnt to orient their attention within the relevant dimension. Whilst Ng et al. (2007) reported that animals with lesions to the anterior cingulate cortex were impaired at intradimensional shift stage, their task design included just one intradimensional shift. The impact of disrupting activity in the anterior cingulate cortex on *attentional set-formation*, therefore, remains unclear. For example, it could either slow, or alternatively abolish, the tendency of an animal to focus attention within the relevant dimension. This is also important when interpreting performance at extradimensional shift stage, where a deficit (or lack thereof) is thought to be an indicator of attentional set-formation (Chase et al., 2012).

The current study aimed to further investigate the impact of disrupting activity in the anterior cingulate cortex on intradimensional set-formation and extradimensional attentional set-shifting in rats, using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs, section 2.3.1) to inhibit the activity of neurons in the anterior cingulate cortex. There are advantages of DREADD technology over traditional lesion methods (see section 2.3.3), that in themselves make this an attractive prospect. Further, the current study used an attentional set-shifting design involving four successive intradimensional shifts, in contrast to the single intradimensional shift used by Ng et al. (2007) aiming to shed light on the role of the anterior cingulate cortex in attentional set-formation.

4.2 Standard attentional set-shifting task (experiment 4a)

4.2.1 Methods

4.2.1.1 Animals

Subjects were 22 male, Lister Hooded rats (Envigo, Bicester, UK) housed as described in section 2.4.

4.2.1.2 Surgery

Animals underwent surgery as described in section 2.5, with 12 animals receiving injections of the inhibitory DREADD AAV5-CaMKIIa-hM4Di-mCherry (titre 4.4x10^12 GC/ml, Addgene, Watertown, MA, USA) and 10 animals receiving injections of a non-DREADD expressing control AAV5-CaMKIIa-EeGFP (titre 4.3x10^12 GC/ml, Addgene, Watertown, MA, USA) into the anterior cingulate cortex.

4.2.1.3 Attentional set-shifting task protocol

Apparatus and pretraining as described in section 2.6.

4.2.1.3.1 Clozapine administration

Three weeks after surgery, animals were administered an intraperitoneal (I.P.) injection of clozapine dihydrochloride (HelloBio, Bristol, UK) fully dissolved in saline at a dilution of 2mg/ml as salt. An injection volume of 2ml/kg was used, resulting in a dosage of 4mg/kg. This dosage was chosen as it is at the higher end dose range found to be effective in our laboratory (unpublished observations); and long-lasting activation of DREADD receptors was desired due to the length of the attentional set-shifting task (1-3 hours).

4.2.1.3.2 Behavioural testing

As described in section 2.6.3.2.

4.2.1.3.3 Analysis of behaviour

As described in section 2.6.4.

4.2.1.4 Histology

Perfusion, sectioning and immunohistochemistry as described in section 2.8

4.2.1.5 Image capture and virus expression analysis

As described in section 2.9.

4.2.1.6 Statistical analysis

As described in section 2.11.1.1

4.2.2 Results

4.2.2.1 Virus expression analysis

Two animals were excluded from the analysis due to a lack of expression of the virus in the anterior cingulate cortex. One animal was from each group such that group numbers were: inhibitory DREADDs, n=11, control virus, n=9. Figure 4.1a illustrates the cases with the smallest and largest spread of the virus in the inhibitory DREADDs group (iDREADD). Comparable expression of the virus was observed in the control group. Figure 4.1b-d depict representative fluorescent expression of mCherry (DREADDs) and eGFP (control virus) in the anterior cingulate cortex in individual rats. Expression of the virus was typically concentrated in the dorsal aspect of the anterior cingulate cortex, Cg1, with some spread into ventral anterior cingulate cortex, Cg2. No cases demonstrated more than limited spread of the virus into neighbouring prelimbic or retrosplenial cortices, but it should be noted that approximately half of all cases exhibited viral expression in the medial aspect of neighbouring secondary motor cortex (Figure 4.1).

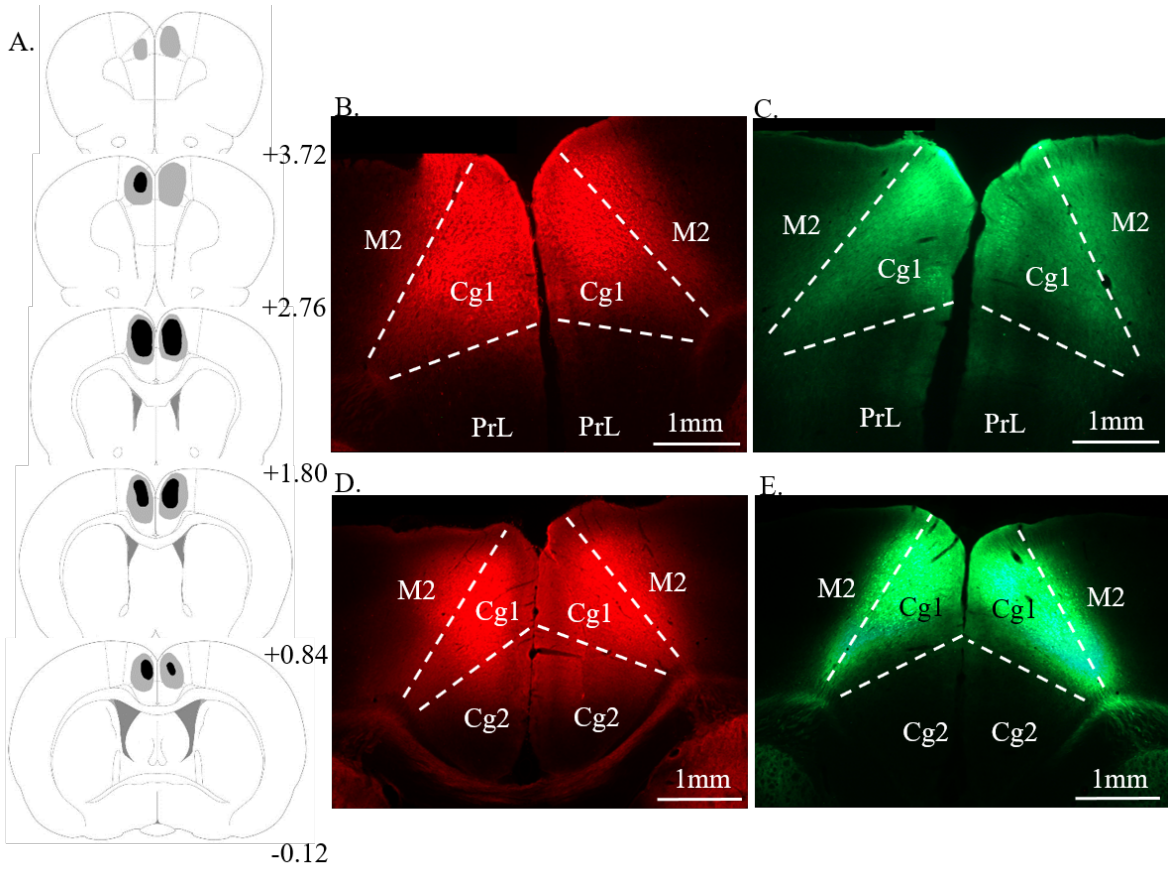

Figure 4.1. Summary of virus expression in the iDREADD and control groups

A. Diagrammatic coronal reconstructions showing the individual cases with the largest (grey) and smallest (black) expression of mCherry in the iDREADD group. Numbers refer to the distance (mm) from Bregma (adapted from Paxinos & Watson, 2005). B-E. Representative examples of mCherry (B & D) and eGFP (C & E) expression in pregenual (B & C) and postgenual (D & E) anterior cingulate cortex. Regions included are dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL) and secondary motor cortex (M2). Scale bars show approximately 1 millimetre.

4.2.2.2 Behavioural testing

As outlined in section 2.11.1.1, a series of ANOVA were conducted on the mean trials required to reach criterion at each stage of the attentional set-shifting task. Although errors to criterion were also recorded for each rat at each stage, the two measures are correlated (Birrell & Brown, 2000) and analysis of each measure produced the same pattern of results across experiments. Therefore, only trials to criterion are reported.

The first analysis revealed that there were no effects of rewarded dimension (whether rats were required to attend to odour or digging media to solve the first

discrimination) on performance and no interactions involving this factor and group ($F<1$). Consequently, the data were pooled across dimensions for all subsequent analyses.

Two-way ANOVA (with stage [eight levels] as a within-subjects factor and group [two levels] as a between-subjects factor) revealed a significant difference in performance between the groups ($F_{(1,18)}=12.39$, $p<.01$, $\eta^2=.41$) and an interaction between group and task stage ($F_{(7,126)}=3.72$, $p=.001$, $\eta^2=.10$). Simple effects analyses revealed that the iDREADD group did not differ from the control group on the simple discrimination (SD), compound discrimination (CD), first intradimensional discrimination (ID1), extradimensional discrimination (ED), or reversal (REV) (maximum $F_{(1,18)}=2.11$, $p=.16$), but took more trials to reach criterion for ID2 ($F_{(1,18)}=6.78$, $p<.05$), ID3 ($F_{(1,18)}=5.42$, $p<.05$) and ID4 ($F_{(1,18)}=9.40$, $p<.01$)(Figure 4.2).

Further, paired samples t-tests were conducted on the difference between trials to criterion for ID1 and ID4 for each group. These analyses found that the control group completed ID4 in fewer trials than ID1 ($t_{(8)}=-2.58$, $p<.05$), indicating attentional set-formation. Meanwhile, there was no difference in trials taken to solve these two stages in the iDREADD group ($t_{10)}=0.11$, $p=.91$)

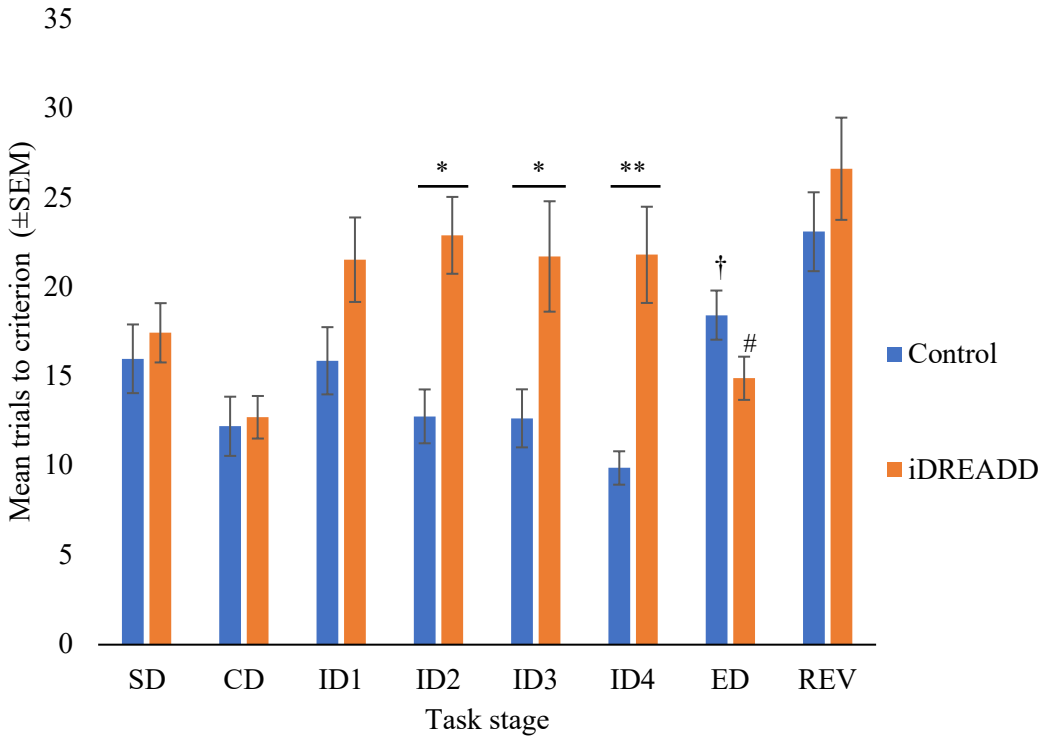

Figure 4.2. Mean (±SEM) trials to criterion on each stage of the attentional set-shifting task.

The group with DREADD-mediated inhibition of anterior cingulate cortex (iDREADD) took significantly more trials to solve several ID stages of the task (than the control group, * p<.05, ** p<.01). The iDREADD group took fewer trials to solve the ED (than the previous ID4, # p<.05), whilst the control group took more trials to solve the ED (than the previous ID4, † p<.001)

As can be seen in Figure 4.2, the control group showed an increase in trials to criterion at ED shift stage. Conversely, the iDREADD group required fewer trials to solve the ED than the preceding intradimensional shift (ID4). ANOVA conducted on ID4 and ED confirmed that there was no main effect of group ($F_{(1,18)}$=4.18, p=.06, η^2=.19) or task stage (F<1), but there was an interaction between group and task stage ($F_{(1,18)}$= 26.21, p<.001, η^2=.31). Simple effects analyses found that while the control group took more trials to solve the ED ($F_{(1,18)}$=37.59, p<.001) than the preceding ID4, the iDREADD group solved it in fewer trials ($F_{(1,18)}$=7.80, p<.05).

One sample t-tests were conducted on shift costs (the difference between the mean trials to criterion from the four ID stages and the ED stage (Wright et al., 2015) and confirmed that the control group showed a shift cost ($t_{(8)}$=3.75, p<.01). Conversely,

the iDREADD group showed a shift benefit ($t_{(10)}$=-3.47, p<.01), taking fewer trials to solve the ED stage. Further, an independent samples t-test revealed that there was a significant difference in shift cost between the groups ($t_{(18)}$=-4.82, p<.001), as displayed in Figure 4.3.

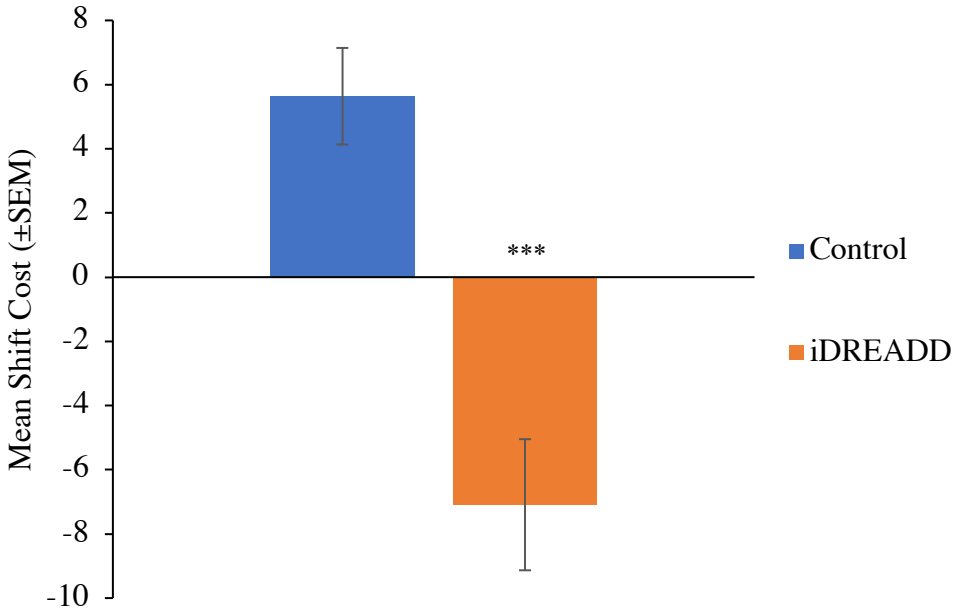

Figure 4.3. Mean shift cost (±SEM), the difference between average trials to criterion for the four ID stages and the ED stage.

There was a significant difference between the groups (***, p<.001). The control group showed a positive shift cost, taking more trials to solve the ED, and the iDREADD group showed a negative shift benefit, taking fewer trials to complete the ED.

Finally, independent samples t-tests were conducted on the mean time taken for the animals to complete the task. These analyses found that the iDREADD group took longer in total than the control group ($t_{(18)}$=3.67, p<.01) to solve all of the discriminations. However, there was no difference in time taken *per trial* between the groups ($t_{(18)}$=0.48, p=0.64). This indicates the iDREADD group took longer to complete the task due to the increased in the number of trials it took them to solve the discriminations, rather than completing trials at a slower rate.

Overall, these results suggest that DREADD-mediated inhibition of anterior cingulate cortex impaired intradimensional set-shifting and the ability to form an attentional set but, paradoxically, improved extradimensional set-shifting.

4.3 Follow-up attentional set-shifting task (experiment 4B)

In the main attentional set-shifting task (experiment 4A, section 4.2), animals with inhibitory DREADD expression in the anterior cingulate cortex (iDREADD) did not appear to require extra trials to solve the extradimensional shift stage. This is in stark contrast with the performance at extradimensional shift stage observed in normal animals, where additional trials are required (Birrell & Brown, 2000). To investigate whether this apparent iDREADD advantage would transfer to perceptual dimensions other than digging media and odour, animals were challenged with a follow-up attentional set-shifting task. Critically, this task included an extradimensional shift involving a perceptual dimension previously experienced as irrelevant to reward in all previous trials; the spatial location of the digging pot.

4.3.1 Methods

Animals, surgeries and clozapine administration as described in section 4.2.1.

4.3.1.1 Behavioural testing

On the test day, the glass pots in the two smaller compartments of the arena were filled with different stimuli pairs (Table 6). Animals were administered an I.P. injection of clozapine (HelloBio, Bristol, UK) as described in section 4.2.1.3.2. The behavioural testing protocol was the same as described in section 2.6.3, except that the experiment took place approximately two weeks after completion of the main behavioural test. The discriminations proceeded as follows:

1. A compound discrimination (CD), where either an odour or a digging media is rewarded but is presented with irrelevant stimuli from the other dimension.
2. An intradimensional shift (ID), where different compound stimuli are presented with the previously rewarded dimension remaining relevant.
3. A spatial extradimensional shift (EDSpatial), where the same compound stimuli are presented but the spatial location of the pot (left or right chamber) is relevant to reward location, i.e. spatial location becomes the new dimension.
4. A spatial reversal (REVSpatial), where the same compound stimuli are presented but the previously incorrect location from the spatial dimension is relevant to reward location.

Therefore, for the first two discriminations, exemplars from one of the stimulus dimensions experienced to be relevant in the main behavioural test (experiment 4A, section 4.2) were rewarded. The rewarded dimension was always the same as that which was most recently rewarded in the main behavioural test for each animal, i.e. in the final two discriminations. This ensured that these discriminations did not require animals to perform an initial extradimensional shift. Animals were then challenged to a new type of extradimensional shift, where the spatial dimension of the pot became relevant to reward. The final stage was a reversal, which helps to test reinforcer guided flexibility. Here, the stimuli remained the same as the preceding trial, but the previously incorrect spatial location became relevant to solving the discrimination.

Table 6. Depiction of a possible order of stimulus pairings in the follow-up attentional set-shifting task (experiment 4B)

Discrimination	Rewarded dimension	Rewarded Stimuli	Unrewarded Stimuli
CD	Odour	**Paprika** + short wire	Coriander + long wire
		Paprika + long wire	Coriander + short wire
ID	Odour	**Lemongrass** + buttons	Nutmeg + beads
		Lemongrass + beads	Nutmeg + buttons
EDSpatial	Spatial location	Lemongrass + buttons **(left chamber)**	Nutmeg + beads (right chamber)
		Lemongrass + beads **(left chamber)**	Nutmeg + buttons (right chamber)
		Nutmeg + beads **(left chamber)**	Lemongrass + buttons (right chamber)
		Nutmeg + buttons **(left chamber)**	Lemongrass + beads (right) chamber)
REVSpatial	Spatial location	Lemongrass + buttons **(right chamber)**	Nutmeg + beads (left chamber)
		Lemongrass + beads **(right) chamber)**	Nutmeg + buttons(left chamber)
		Nutmeg + beads **(right chamber)**	Lemongrass + buttons (right chamber)
		Nutmeg + buttons **(right chamber)**	Lemongrass + beads (right) chamber)

Depiction of one possible order of stimulus pairings in the additional discriminations of the attentional set-shifting task. In this example, odour is the first dimension relevant to the location of

the buried food reward. From the ED stage onwards, spatial location of the pot is the relevant dimension. Stimuli are always paired as shown, but the discrimination in which animals encounter them is counterbalanced between animals. The first dimension to be rewarded is the most recent dimension to be rewarded in the main behavioural test. The first spatial location to be rewarded is counterbalanced across animals.

4.3.1.2 Analysis of behaviour

As described in section 2.6.4.

4.3.1.3 Histology

Perfusion, sectioning and immunohistochemistry as described in section 2.8.

4.3.1.4 Image capture and virus expression analysis

As described in section 2.9.

4.3.1.5 Statistical analysis

As described in section 2.11.1.2.

4.3.1 Results

4.3.1.1 Virus expression analysis

As described in section 4.2.2.1.

4.3.1.2 Behavioural testing

A series of ANOVA were conducted on the mean trials required to reach criterion at each stage of the follow-up attentional set-shifting task, as described in section 2.11.1.2. The first analysis revealed that there were no effects of rewarded chamber (whether the reward was located in the left or the right chamber in the first spatial discrimination) on performance and no interactions involving this factor and group (maximum $F_{(1,16)}$=1.59, p=.23, η^2=.08). Consequently, the data were pooled across dimensions for all subsequent analyses.

Two-way ANOVA (with stage [four levels] as a within-subjects factor and group [two levels] as a between-subjects factor) revealed that there was no significant

difference in performance between the groups ($F<1$) and no interaction between task stage and group ($F_{(2.02, 36.31)}=2.48$, $p=.10$, $\eta^2=.09$), as displayed in Figure 4.4.

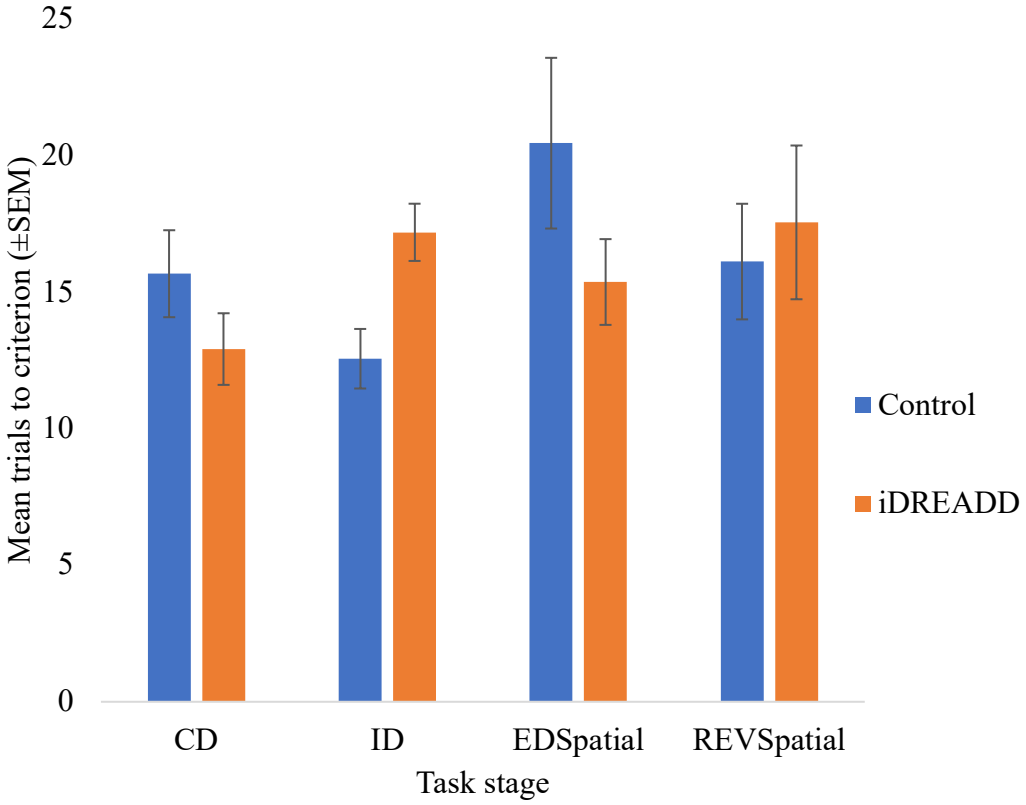

Figure 4.4. Mean (±SEM) trials to criterion on each stage of the follow-up attentional set-shifting task.

No differences were found between the groups and no interaction was found between group and task stage.

One sample t-tests were conducted on shift costs (the difference between the ID stages and ED spatial stage) and revealed that the control group showed a shift cost ($t_{(8)}=2.79$, $p<.05$) and the iDREADD group showed neither a shift cost nor a shift benefit ($t_{(10)}=-1.64$, $p=.13$).

When testing whether there was a significant different in shift cost between the groups, Levene's test was found to be significant ($F_{(1)}=6.41$, $p<.05$). This indicates that the equality of variances assumption for a student's independent samples t-test was violated. Therefore, a Welch's unequal variances t-test (Howell, 2009) was conducted, and revealed that there was a significant difference in shift cost between the groups ($t_{(10.45)=-}3.43$, $p<.01$), as displayed in Figure 4.5.

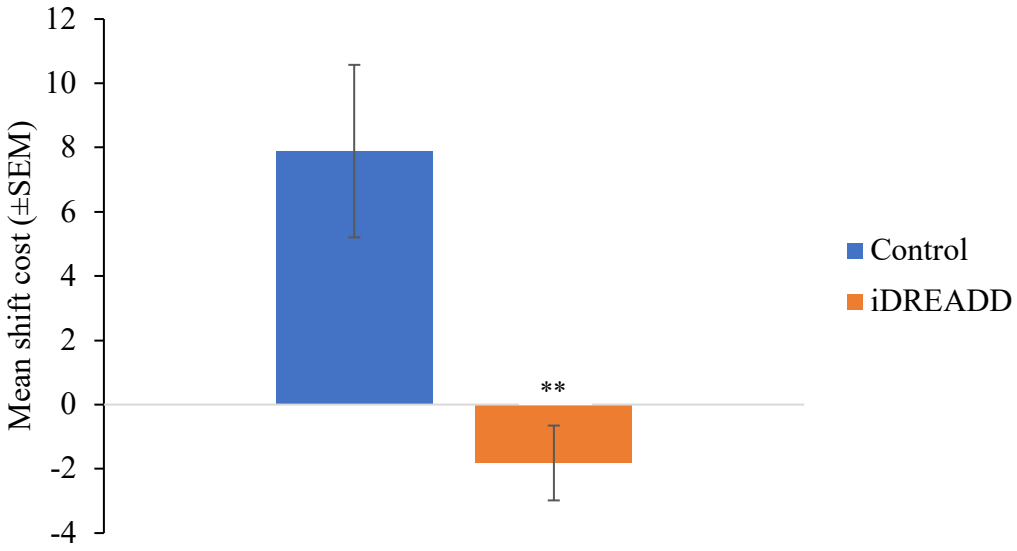

Figure 4.5. Mean shift cost (±SEM), the difference between trials to criterion for the ID stage and the spatial ED stage.

There was a significant difference between the groups (**, p<.01). The control group showed a positive shift cost, taking more trials to solve the spatial ED.

Therefore, animals with DREADD-mediated inhibition of the anterior cingulate cortex did not show an impairment in intradimensional set-shifting tested during the follow-up attentional set-shifting task, perhaps reflecting the fewer number of intradimensional stages. However, their advantage over controls at extradimensional set-shifting transferred to a new perceptual dimension based on the spatial location of the pot.

4.4 Investigation of c-*fos* through novel environment exposure (experiment 4C)

As described in section 2.7, the behavioural experiments were followed by an investigation into the expression of c-*fos,* an indirect marker of neuronal activity. This was conducted to provide an independent measure of the influence of DREADD-mediated anterior cingulate cortex inhibition (iDREADD) on activity in brain regions of interest.

4.4.1 Methods

As described in section 2.7.

4.4.2 Results

4.4.2.1 Fos-positive cell counts

4.4.2.1.1 Analysis of variance

As described in section 2.11.2, a series of ANOVA were conducted on the mean Fos-positive cell counts for the brain regions of interest. Firstly, a two-way ANOVA was conducted on cortical regions of interest, with region (three levels, dorsal anterior cingulate [Cg1], ventral anterior cingulate [Cg2], and prelimbic [PrL] cortices) as a within-subjects factor and group (two levels) as a between-subjects factor. This found a main effect of group ($F_{(1,18)}$=7.53, p<.05, η^2=.30), with higher Fos counts in the iDREADD group than the control group. A region by group interaction ($F_{(2,36)}$=11.16, p<.001, η^2=.05) was also observed (Figure 4.6). Simple effects analyses indicated that Fos counts were significantly higher in the iDREADD group than the control group in Cg1 ($F_{(1,18)}$=6.88, p<.05), but not in Cg2 ($F_{(1,18)}$=2.56, p=.13) or PrL (F<1).

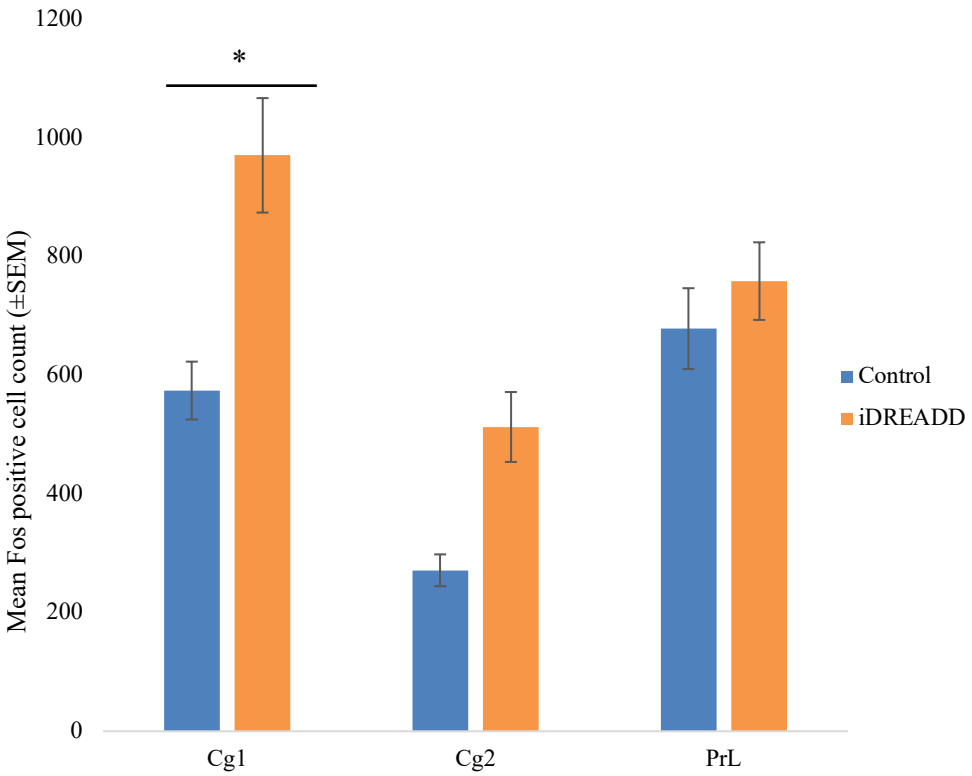

Figure 4.6. Mean (±SEM) Fos-positive cell counts in anterior cingulate (Cg1 and Cg2) and prelimbic (PrL) cortices.

Fos-positive cell counts were higher for the **i**DREADD group than the control group in Cg1 (*p<.05).

Next, a one-way ANOVA was conducted on Fos-positive cell counts in secondary somatosensory cortex (S2), a cortical control region, and found no difference between the groups (F<1). This null result is displayed in Figure 4.7.

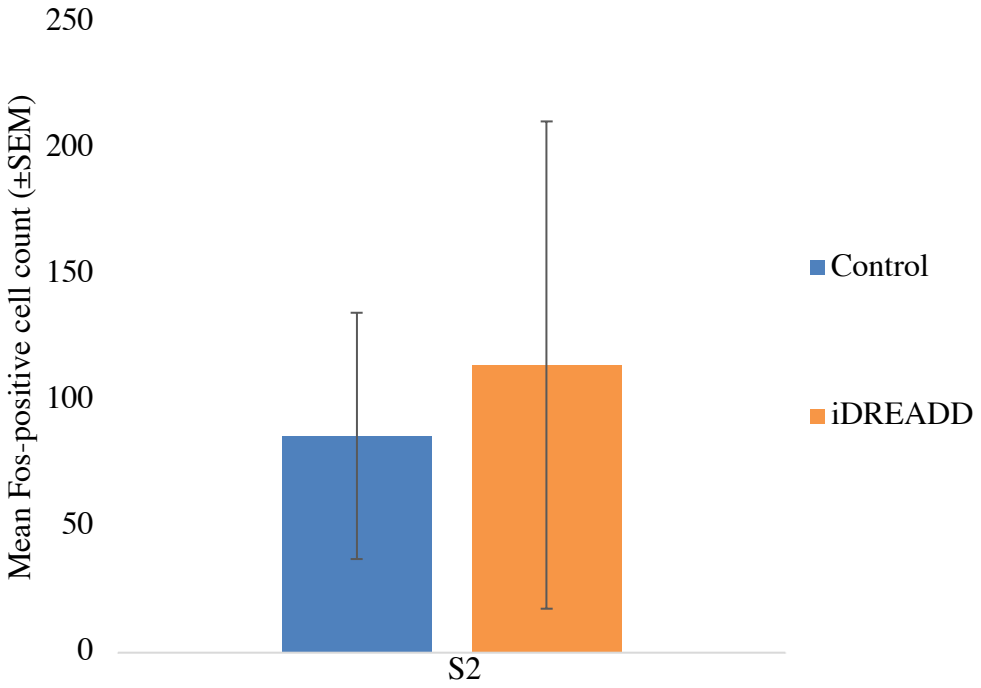

Figure 4.7. Mean (±SEM) Fos-positive cell counts in secondary somatosensory (S2) cortex.

There was no difference between the groups.

Further two-way ANOVA were conducted on Fos-positive cell counts in the anterior thalamic nuclei, with region (two levels, anteromedial nuclei [AM] and anteroventral nuclei [AV]) as a within-subjects factor and group (two levels) as a between-subjects factor. There was a main effect of group ($F_{(1,18)}$=9.73, p<.01, η^2=.35), indicating higher Fos-positive cell counts in the iDREADD group than the control group, as can be seen in Figure 4.8. There was no interaction between region and group (F<1).

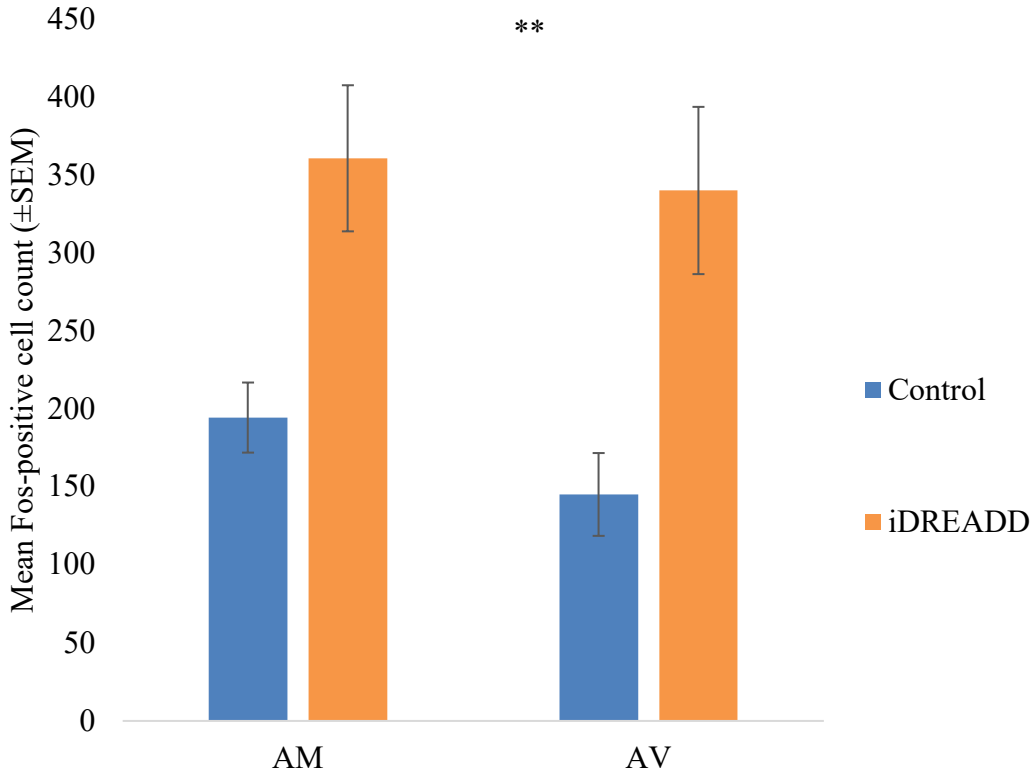

Figure 4.8. Mean (±SEM) Fos-positive cell counts in the anteromedial (AM) and anteroventral (AV) nuclei of the thalamus.

Overall Fos-positive cell counts were higher for the iDREADD group than the control group (**p<.01).

Taken together, these findings indicate that DREADD-mediated inhibition of anterior cingulate cortex increased activity both in the dorsal anterior cingulate cortex (Cg1) and one of its major efferent regions, the anterior thalamic nuclei. This finding, initially counter-intuitive, is discussed further in section 4.5.3. Meanwhile, lack of such differences ventral anterior cingulate (Cg2), prelimbic and secondary somatosensory cortices indicate that this was not a non-specific increase in activity.

4.4.2.1.2 Pearson correlation coefficients

As described in section 2.11.2, Pearson correlation analysis was conducted on Fos-positive cell counts for each group, with Bonferroni corrections for multiple comparisons. As displayed in Table 7, the iDREADD group had strong, positive correlations between many of the regions measured. In particular, both regions of the anterior cingulate cortex (Cg1 and Cg2) correlated both with each other and with

every other region measured, with the sole exception of the somatosensory cortex (S2). Fos-positive cell counts in different anterior thalamic nuclei (AM and AV) correlated with each other, and AV further correlated with S2.

Table 7. Interregional correlation matrix of Fos-positive cell counts in the iDREADD group.

		Cg1	Cg2	PrL	AM	AV
Cg2	**Pearson's r**	0.917***				
	***p*-value**	< .001				
PrL	**Pearson's r**	0.848***	0.841***			
	***p*-value**	< .001	0.001			
AM	**Pearson's r**	0.900***	0.872***	0.724		
	***p*-value**	< .001	< .001	0.012		
AV	**Pearson's r**	0.878***	0.852***	0.550	0.865***	
	***p*-value**	< .001	< .001	0.080	< .001	
S2	**Pearson's r**	0.554	0.395	0.385	0.748	0.865***
	***p*-value**	0.077	0.229	0.243	0.008	< .001

R-values refer to Pearson correlation coefficients, alpha level is adjusted to $p<.003$ (Bonferroni correction).*** $p<.001$. Regions included are dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL), anteromedial thalamic nuclei (AM) anteroventral thalamic nuclei (AV) and secondary somatosensory cortex (S2).

In the control group, the only significant inter-regional correlation following Bonferroni correction was a strong positive relationship between the ventral anterior cingulate (Cg2) and prelimbic (PrL) cortices, as displayed in Table 8.

Table 8. Interregional correlation matrix of Fos-positive cell counts in the control group.

		Cg1	Cg2	PrL	AM	AV
Cg2	**Pearson's r**	0.622				
	***p*-value**	0.074				
PrL	**Pearson's r**	0.563	0.921***			
	***p*-value**	0.115	< .001			
AM	**Pearson's r**	-0.079	-0.040	-0.076		
	***p*-value**	0.839	0.919	0.846		
AV	**Pearson's r**	-0.060	0.013	-0.223	0.831	
	***p*-value**	0.878	0.973	0.564	0.006	
S2	**Pearson's r**	0.475	0.586	0.656	-0.148	-0.285
	***p*-value**	0.197	0.097	0.055	0.704	0.457

R-values refer to Pearson correlation coefficients, alpha level is adjusted to $p<.003$ (Bonferroni correction).*** $p<.001$. Regions included are dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL), anteromedial thalamic nuclei (AM) anteroventral thalamic nuclei (AV) and secondary somatosensory cortex (S2)

Taken together, these results suggest that DREADD-mediated inhibition of the anterior cingulate cortex, counterintuitively, increased interdependent activity between this region and its efferents.

4.5 Discussion

Previous research implicates the anterior cingulate cortex in attentional set-shifting (Ng et al., 2007), a key measure of behavioural flexibility (section 1.5.1.4). However, such processes are multifaceted (Brown & Tait, 2015) and are supported by an array of cortical and subcortical structures (Bissonette et al., 2013; Bissonette & Roesch, 2017), with the unique contributions of the anterior cingulate cortex remaining unclear. To further investigate this matter, the current study used DREADDs (see section 2.3) to inhibit the activity of neurons in the rat anterior cingulate cortex and tested them on two tasks involving intradimensional and extradimensional shifts. This was followed by an investigation into c-*fos*, which provided an independent indication of the impact of inhibitory DREADDs on cellular activity in the brain.

4.5.1 Standard attentional set-shifting task

The first task included a series of four successive intradimensional shifts, designed to test the ability to form an attentional set by focusing attention within a reliably rewarded stimulus dimension. Animals with DREADD-mediated inhibition of the anterior cingulate cortex (iDREADD) were slower than controls to acquire several of the intradimensional shift discriminations (ID2, ID3 and ID4). This supports the results of Ng et al. (2007) who found that rats with lesions of the anterior cingulate cortex were impaired at the single intradimensional shift included in their task. It further extends this observation by demonstrating that performance does not improve across a series of intradimensional shifts. This contrasts with the performance of control animals, who showed the typical reduction in trials to criterion across these shifts, solving the final intradimensional discrimination (ID4) in significantly fewer trials than the first (ID1). This suggests that control animals formed an attentional set, while iDREADD animals did not.

Further evidence that control animals successfully formed an attentional set is that they displayed an extradimensional shift cost, i.e. they required more trials to solve this discrimination than the preceding intradimensional discriminations. Such a deficit is thought to reflect how animals have increased attention to the relevant stimulus dimension, a reliable reward predictor, and decreased their attention to the irrelevant stimulus dimension, an unreliable reward predictor (Chase et al., 2012). When contingencies change, animals must reorient that attention to the stimulus dimension previously experienced as irrelevant, leading to an increase in trials to criterion. Strikingly, iDREADD animals showed the opposite effect. That is, they showed a shift benefit, taking fewer trials to complete the extradimensional shift than the preceding intradimensional shifts. Therefore, while Ng et al.(2007) found that disrupted anterior cingulate functioning did not impair extradimensional set-shifting, the current experiment indicates that, in some circumstances, it might in fact *facilitate* it.

If the anterior cingulate cortex is involved in attending to task relevant stimuli, as suggested by Ng et al. (2007) it is possible that disrupting its activity allows irrelevant stimuli to usurp attentional control. That is to say, there may have been a

relative increase in the salience of task-irrelevant stimuli (Mackintosh, 1975; Pearce & Mackintosh, 2010), equipping iDREADD animals with an advantage when contingencies change and a previously irrelevant stimulus dimension becomes relevant to solving the discrimination (extradimensional shift). It follows that the iDREADD extradimensional shift benefit might be contingent on the extent to which the newly relevant stimulus dimension has been established as an irrelevant/unreliable reward predictor in the preceding trials. Perhaps as Ng et al. (2007) only included one intradimensional shift in their task, there was an insufficient build-up of attention to the task irrelevant stimulus dimension to manifest a shift benefit at the extradimensional stage.

4.5.2 Follow-up attentional set-shifting task

The follow-up attentional set-shifting task investigated whether the iDREADD advantage at extradimensional set-shifting would transfer to a perceptual dimension other than those used in the first task (odour and digging media). This was achieved by including an extradimensional shift in which the spatial location of the digging pot became relevant to solving the discrimination, i.e. whether the pot was in the left or the right chamber of the testing arena.

The task also included an initial complex discrimination and an intradimensional shift, for which there was no difference in performance between the groups. This contrasts with the results of anterior cingulate lesions (Ng et al., 2007), where a deficit was found on a single intradimensional shift. However, it is consistent with results of the main behavioural task (section 4.5.1), where the iDREADD group were impaired at ID2, ID3 and ID4, but did not differ from controls on the complex discrimination or ID1. Together, these results indicate that the iDREADD group were impaired at attentional set-formation, rather than intradimensional set-shifting *per se*. iDREADD animals may not be impaired at the initial stages relative to controls, but as controls improve over a series successive intradimensional shifts and iDREADD animals do not, the disparity in performance between the groups increases.

The control group took more trials to solve the spatial extradimensional shift than the preceding intradimensional shift, presumably reflecting a reorientation of attention to a stimulus dimension previously established as irrelevant to reward. There was a significant difference in shift cost between the groups, signifying that the iDREADD group shifted faster than controls. However, while they did not show a shift cost, neither did they show a shift benefit, as observed in the extradimensional shift in the main attentional set-shifting task (section 4.5.1).

There are several differences between the spatial extradimensional shift and the odour/digging media extradimensional shift from the main task that may be responsible for this minor discrepancy. On the one hand, there is only one intradimensional shift in the follow-up task itself. Looking at this in isolation, one might suggest that the iDREADD group did not display a shift benefit as there was insufficient build-up of interference from the irrelevant stimulus dimensions to manifest as advantageous in an extradimensional shift. In this regard, parallels can be drawn with the findings of Ng et al. (2007) who similarly observed neither a shift cost nor a shift benefit at the extradimensional stage following their single intradimensional shift.

On the other hand, taking the main behavioural task into account, spatial location has been experienced as irrelevant in all discriminations encountered so far. There is mixed evidence regarding carry-over effects from animals completing previous tasks (Chase et al., 2012; Tait et al., 2018), but if iDREADD animals had been attending to spatial location as an irrelevant/unreliable reward predictor in the main task, and carried this information over to the follow-up task, one might predict an extradimensional shift benefit.

Perhaps more importantly, by the spatial extradimensional stage, animals had experience of both original stimulus dimensions being rewarded (digging media and odour). Therefore, there may be competition from switching back to responding to the other stimulus dimension (that was relevant in the first six trials of the main behavioural task, but irrelevant following the first extradimensional shift). It may be

that such competition increased trials to criterion (Dragunow & Faull, 1989) in the iDREADD animals, rendering the lack of a shift benefit unsurprising.

Overall, these results suggest that impaired intradimensional set-shifting in the iDREADD group may be contingent on the number of intradimensional shifts, reflecting a deficit in attentional set-formation. Meanwhile, the follow-up task found further evidence that iDREADD animals were better at extradimensional set-shifting than controls, by demonstrating that their advantage transferred to a new perceptual dimension. They were not, however, faster at the extradimensional stage than the intradimensional stage. This suggests that there were elements of the task design that rendered set-shifting to spatial location different than the extradimensional shift in the main behavioural task.

4.5.3 Investigation of c-*fos* through novel environment exposure

Counterintuitively, the results of the novel environment exposure experiment revealed increases in c-*fos*, a marker of cellular activity, in the iDREADD group relative to controls. There were elevated counts in the dorsal anterior cingulate cortex, where the most robust expression of DREADDs was observed at the injection sites (section 4.2.2.1), and in the anterior thalamic nuclei. Meanwhile, lack of differences between the groups in prelimbic and somatosensory cortices indicate that these increases were specific to DREADD-infected neurons and select efferent projection regions.

Activation of the inhibitory DREADD hM4Di by clozapine is thought to induce hyperpolarisation, suppressing neuronal firing (Rogan & Roth, 2011)(see section 2.3.2). As c-*fos* is a product of activity at the cell body (Dragunow & Faull, 1989; Zhu et al., 1995), inhibitory DREADDs would be expected (if anything) to decrease as a result of neuronal inhibition. Therefore, the cause of the c-*fos* induction observed in the current experiment is unclear. One possibility is that the DREADDs preferentially infected inhibitory gamma-amino butyric acid (GABA) neurons which, although representing a minority of cortical neurons (Rudy et al., 2011), regulate the activity of the whole cortical network (Fino, Packer, & Yuste, 2013). Consequently, hM4Di expression in GABA neurons could disinhibit excitatory

glutamate pyramidal cells, leading to unregulated hyperexcitability and an increase in c-*fos* expression.

Previous research has found that hM4Di expression in the dorsal hippocampus leads to an increase in c-*fos* expression (López et al., 2016) and follow-up experiments revealed that this was a result of decreased GABA receptor function. However, while this study used a neuron-specific virus promoter (hSyn) that does not target any specific neuron subclass, the current study used a promoter thought to target excitatory neurons (CaMKIIα). Therefore, at least theoretically, the increase in c-*fos* in the current experiment should not be due to infection of inhibitory GABA neurons.

Another alternative is that inhibitory DREADDs induced disinhibition through a reciprocal thalamic pathway. Anterior cingulate cortical excitatory neurons innervate the thalamic reticular nucleus, which, in turn, sends inhibitory outputs to the thalamus (Zikopoulos & Barbas, 2006). DREADD-mediated inhibition of these neurons could indirectly reduce the inhibitory activity of the thalamic reticular nucleus, thus disinhibiting thalamocortical circuitry. Such disinhibition could explain not only the increases in c-*fos* in both the anterior cingulate cortex and the anterior thalamic nuclei, but also the increase in covariant activity between these structures.

Overall, the results of the c-*fos* experiment provided *in vivo* verification that inhibitory DREADD hM4Di markedly altered activity in the anterior cingulate cortex and changed network functionality. However, although the behavioural results were consistent with a downregulation of anterior cingulate cortex activity, the underlying synaptology was not. Instead, the results point to unregulated hyperexcitability as the most likely mechanism of action of hM4Di inhibitory DREADDs in the current experiment. This also serves to highlight that the complex relationship between DREADDs, inhibition, and disinhibition in the brain is not yet well understood.

4.5.4 Summary and implications

Overall, the results of these experiments demonstrate a double dissociation between the role of the prelimbic (and infralimbic), and anterior cingulate cortices in attentional set-shifting. A well-established finding is that dysfunction in the former impairs extradimensional set-shifting but spares intradimensional set-shifting (Birrell & Brown, 2000; Tait et al., 2014). The current study demonstrated that DREADD-mediated inhibition of the anterior cingulate cortex improved performance when shifting between rules relating to different perceptual dimensions (extradimensional shifts), while impairing the ability to apply a rule within the same perceptual dimension (intradimensional set-formation). This suggests that the anterior cingulate cortex is involved in focusing attention on task relevant stimulus dimensions. In the absence of its proper functioning, attention appears to be inappropriately directed towards irrelevant, or unreliable, stimulus dimensions.

Meanwhile, the proof-of-principle c-*fos* study returned surprising results. While it verified that the inhibitory DREADD hM4Di significantly changes activity in the anterior cingulate cortex and alters network dynamics, changes were in the opposite direction to what was predicted. Inhibitory DREADDs increased activity in the anterior cingulate and its efferent regions, as well as increasing interdependent activity between them. This suggests that unregulated hyperexcitability might underpin the mechanistic action of inhibitory DREADDs and calls for further investigation of the influence of DREADDs at the cellular level.

5 DREADD-Mediated Excitation of Anterior Cingulate Cortex and Attentional Set-Shifting

5.1 Introduction

Chapter 4 demonstrated that DREADD-mediated inhibition of the anterior cingulate cortex impaired intradimensional set-shifting and attentional set-formation, but, paradoxically, improved extradimensional set-shifting. This indicates that the anterior cingulate cortex may be involved in focusing attention within a relevant, consistently rewarded dimension. To further investigate this, the current study used excitatory Designer Receptors Exclusively Activated by Designer Drugs (DREADDs, section 2.3.1) to increase activity in the anterior cingulate cortex in rats, and tested them on an attentional set-shifting task.

Superficially, given the hypothesis and the supposed finite nature of attentional resources (Mackintosh, 1965, 1975), one might expect excitatory DREADDs in the anterior cingulate cortex to produce the inverse behavioural profile of inhibitory DREADDs. That is, increased attention to the relevant stimulus dimension, facilitating intradimensional set-formation, and decreased attention to the irrelevant dimension, impairing extradimensional set-shifting. However, given that inhibitory DREADDs increased, rather than decreased, cellular activity in the anterior cingulate cortex and its efferent regions (section 4.5.3), it is not clear how excitatory DREADDs will differentially affect network dynamics and the resulting behavioural profile. Therefore, as in Chapter 4, the behavioural experiment was followed by an investigation into c-*fos* to provide an indication of how excitatory DREADDs influenced cellular activity.

5.2 Standard attentional set-shifting task (experiment 5A)

5.2.1 Methods

5.2.1.1 Animals

Subjects were 22 male, Lister Hooded rats (Envigo, Bicester, UK) housed as described in section 2.4.

5.2.1.2 Surgery

Animals underwent surgery as described in section 2.5, with 12 animals receiving injections of the excitatory DREADD AAV5-CaMKIIa- hM3Dq-mCherry (titre 3.1x10^12 GC/ml, Addgene, Watertown, MA, USA) and 10 animals receiving injections of a non-DREADD expressing control AVV5-CaMKIIa-EeGFP (titre 4.3x10^12 GC/ml, Addgene, Watertown, MA, USA).

5.2.1.3 Attentional set-shifting task protocol

Apparatus and pretraining as described in section 2.6.

5.2.1.3.1 Clozapine administration

Animals were administered an intraperitoneal (I.P.) injection of clozapine dihydrochloride (HelloBio, Bristol, UK), fully dissolved in saline at a dilution of 0.01mg/ml as salt. An injection volume of 1ml/kg was used, resulting in a dosage of 0.01mg/kg. Higher dosages (starting at 4mg/kg, as used in section 4.2.1.3) were initially trialled but were found to produce motor effects, such as convulsions, which impaired excitatory DREADD animals' ability to complete the task. The dosage was systematically dropped before reaching 0.01mg/kg, at which no adverse motor effects were observed in most animals.

Clozapine binds with very high affinity to DREADDs, and has been found to produce DREADD-mediated physiological and behavioural effects (Alexander et al., 2009; Boender et al., 2014; Gomez et al., 2017; Gompf, Budygin, Fuller, & Bass, 2015), and to rapidly alter functional connectivity (Zerbi et al., 2019), at ultra-low dosages. Furthermore, lower dosages are required to activate excitatory, as opposed to inhibitory, DREADDs (Farrell & Roth, 2013; Mahler et al., 2014; Yau & McNally, 2015)(see also section 2.3.2). Observed differences in behaviour (section 5.2.2.2)and in c-*fos* expression (section 5.4.2.2) support the conclusion that 0.01mg/kg of clozapine activated DREADD receptors in the current experiment. It should be noted that adverse motor effects continued even at this low dosage in two animals. These animals were excluded from the analysis, resulting in group numbers of eDREADDs, n=10, control virus, n=10.

5.2.1.3.2 Behavioural testing

As described in section 2.6.3.2

5.2.1.3.3 Analysis of behaviour

As described in section 2.6.4.

5.2.1.4 Histology

Perfusion, sectioning and immunohistochemistry as described in section 2.8.

5.2.1.5 Image capture and virus expression analysis

As described in section 2.9.

5.2.1.6 Statistical analysis

As described in section 2.11.1.1.

5.2.2 Results

5.2.2.1 Virus expression analysis

All animals across both groups displayed robust expression of the virus in the anterior cingulate cortex, and therefore no subjects were excluded from the analysis. Figure 5.1a illustrates the cases with the smallest and largest spread of the virus in the excitatory DREADD group (eDREADD). Comparable expression of the virus was observed in the control virus group. Figure 5.1b-d depict representative fluorescent expression of mCherry (eDREADD) and eGFP (control) in the anterior cingulate cortex of each group. Expression of the virus was typically concentrated in the dorsal aspect of the anterior cingulate cortex, Cg1, with some spread into ventral anterior cingulate cortex, Cg2. No cases demonstrated more than limited spread of the virus into neighbouring prelimbic or retrosplenial cortices (see Figure 5.1), but it should be noted that approximately half of all cases exhibited viral expression in the medial aspect of neighbouring secondary motor cortex.

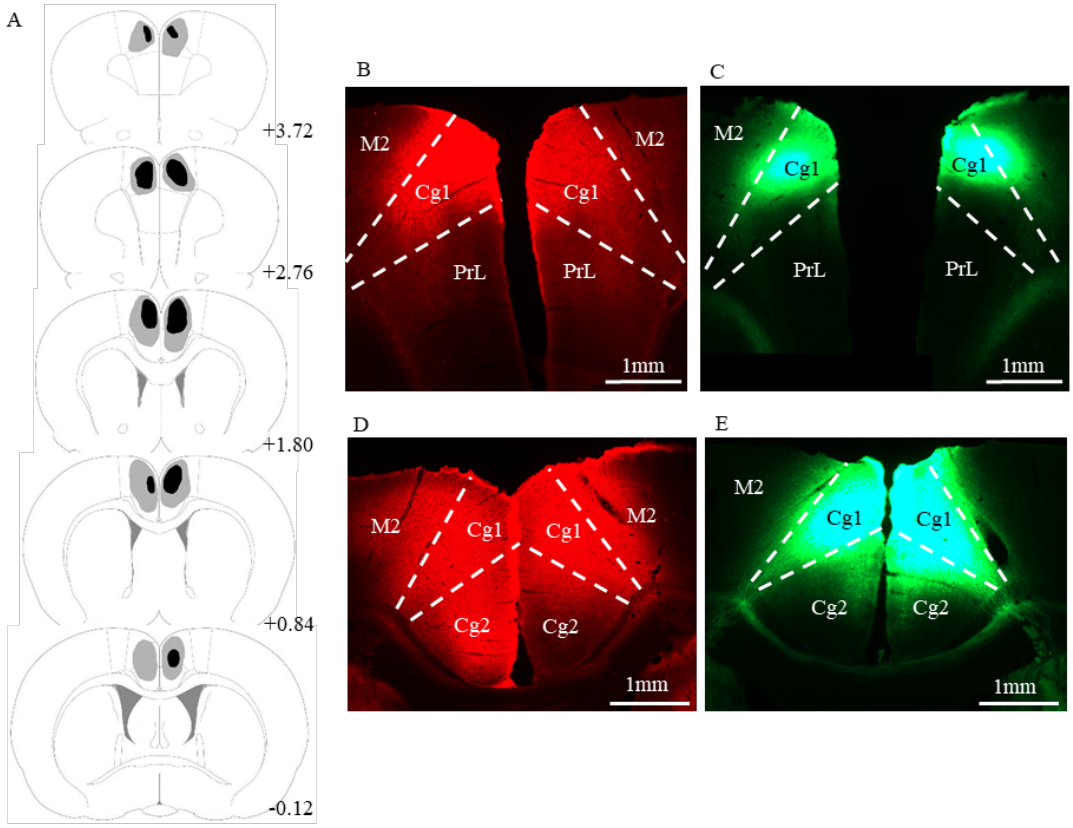

Figure 5.1. Summary of virus expression in the eDREADD and control groups

A. Diagrammatic reconstructions showing the individual cases with the largest (grey) and smallest (black) expression of mCherry in the eDREADD group. Numbers refer to the distance (mm) from Bregma (adapted from Paxinos & Watson, 2005). B-E. Representative examples of mCherry (B & D) and eGFP (C & E) expression in pregenual (B & C) and postgenual (D & E) anterior cingulate cortex.

5.2.2.2 Behavioural testing

Two eDREADD animals displayed adverse motor effects following clozapine injection, as described in section 5.2.1.3.2, impairing their ability to complete the task. These animals were subsequently excluded from all further analysis, resulting in final group numbers eDREADD, N=10, control virus, N=10.

As outlined in General Methods (section 2.11.1.1), a series of ANOVA were conducted on the mean trials required to reach criterion at each stage of the attentional set-shifting task. Errors to criterion were also recorded but as the two measures are correlated (Birrell & Brown, 2000), and analysis of each measure

produced the same pattern of results, only trials to criterion are reported. The first analysis revealed that there were no effects of rewarded dimension (whether rats were required to attend to odour or digging media to solve the discrimination) on performance and no interactions involving this factor and the eDREADDs group ($F<1$). Consequently, the data were pooled across dimensions for all subsequent analyses.

Two-way ANOVA (with stage [eight levels] as a within-subjects factor and group [two levels] as a between-subjects factor) revealed a significant difference in performance between the groups ($F_{(1,18)}=26.87$, $p<.001$, $\eta^2=.60$) and an interaction between group and task stage ($F_{(7,126)}=2.67$, $p<.05$, $\eta^2=.08$, Figure 5.2). Simple effects analyses revealed that the eDREADD group took fewer trials than controls to solve the compound (CD)($F_{(1,18)}=6.01$, $p<.05$), second intradimensional (ID2) ($F_{(1,18)}=4.47$, $p<.05$), extradimensional (ED) ($F_{(1,18)}=19.56$, $p<.001$) and reversal (REV) ($F_{(1,18)}=6.49$, $p<.05$) discriminations. The eDREADD group did not differ from the control group on the simple discrimination (SD) ($F_{1,18)}=1.22$, $p=.28$), ID1 ($F_{(1,18)}=3.38$, $p=.08$), ID3 ($F<1$), or ID4 ($F<1$).

Paired samples t-tests were conducted on the difference between trials to criterion for ID1 and ID4 for each group. These analyses found that the control group completed ID4 in fewer trials than ID1 ($t_{(9)}=-2.61$, $p<.05$), indicating attentional set-formation (Figure 5.2). Meanwhile, there was no difference in trials taken to solve these two stages in the eDREADD group ($t_{6)}=-0.67$, $p=.52$)

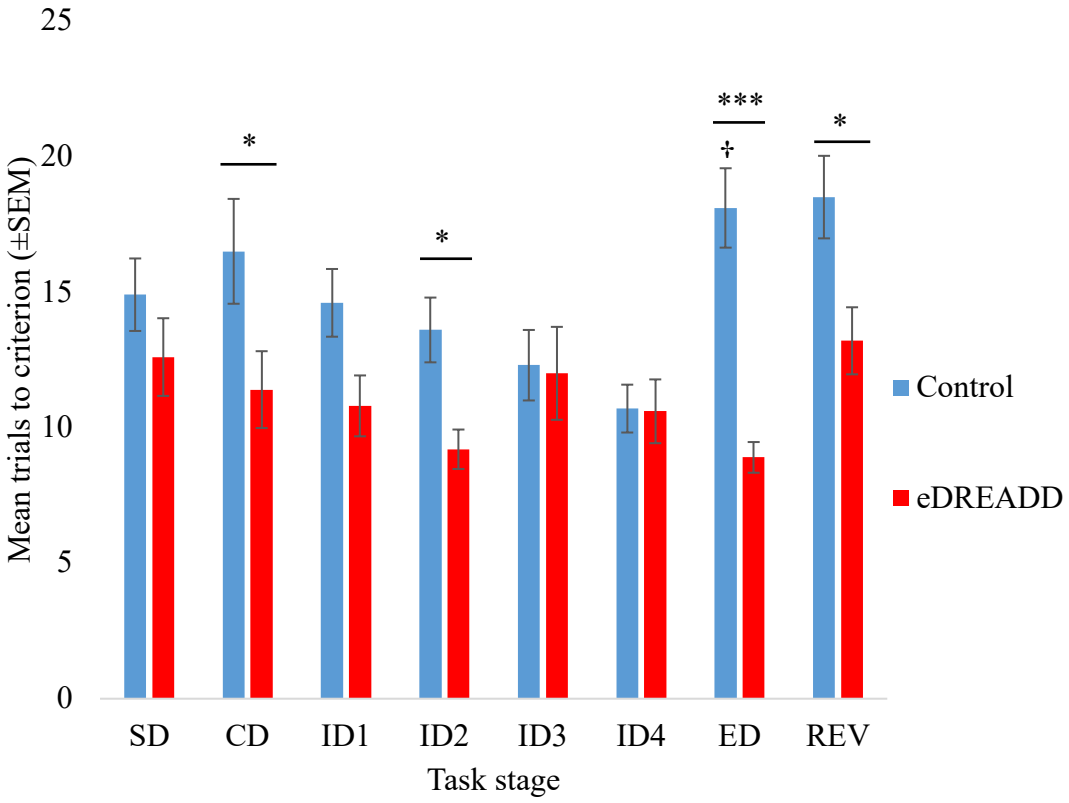

Figure 5.2. Mean (±SEM) trials to criterion on each stage of the attentional set-shifting task.

The group with DREADD mediated excitation of anterior cingulate cortex (eDREADD) took significantly fewer trials to solve several stages of the task (than the control group, * p<.05, ***p<.001). The control group took more trials to solve the ED (than the previous ID4, † p<.001), whilst the eDREADD group did not.

As can be seen in Figure 5.2, the control group showed an increase in trials to criterion at ED shift stage. Conversely, the eDREADD group did not require more trials to solve the ED than the preceding ID4. ANOVA conducted on ID4 and ED revealed that there was a main effect of group ($F_{(1,18)}$=16.7, p<.001, η^2=.48) and task stage ($F_{(1,18)}$=7.98, p<.05, η^2=.09) and an interaction between group and task stage ($F_{(1,18)}$=20.34, p<.001, η^2=.23). Simple main effects analyses confirmed that while the control group took more trials to solve the ED than the preceding ID4 ($F_{(1,18)}$=17.70, p<.01, Figure 5.2), there was no difference between the number of trials taken to solve these two stages for the eDREADD group ($F_{(1,18)}$=2.95, p=.12).

One sample t-tests were conducted on shift costs (the difference between the average trials to criterion from the four ID stages and the ED stage (Wright et al., 2015) and

confirmed that the control group showed a shift cost ($t_{(9)}$=3.91, p<.01) and the eDREADD group showed neither a shift cost nor shift benefit ($t_{(9)}$=-1.77, p=.11). Meanwhile, an independent samples t-test revealed that there was a significant difference in shift cost between the groups ($t_{(18)}$=-4.20. p<.001), as displayed in Figure 5.3.

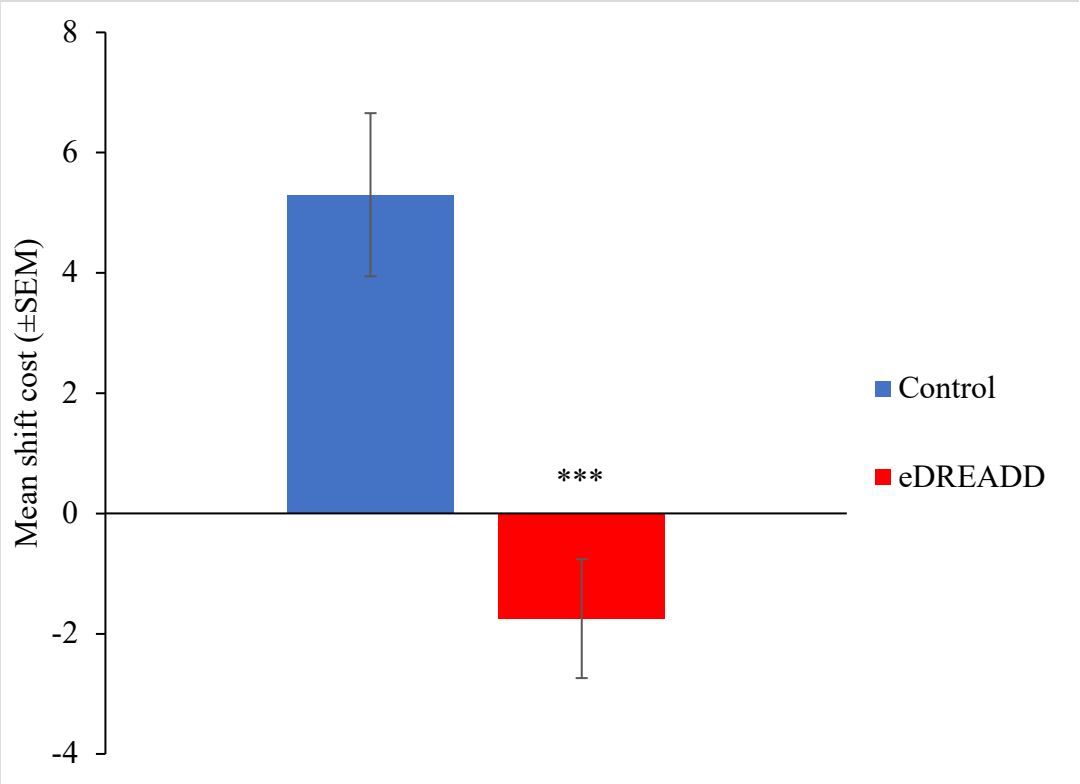

Figure 5.3. Mean shift cost (±SEM), the difference between average trials to criterion for the four ID stages and the ED stage.

There was a significant difference between the groups (***, p<.001). The control group showed a positive shift cost, taking more trials to solve the ED.

Finally, independent samples t-tests were conducted on the mean time taken for the animals to complete the task. These analyses found that the eDREADD group took a shorter time in total than the control group ($t_{(15)}$=-3.74, p<.01) to solve all of the discriminations. However, there was no difference in time taken per trial between the groups ($t_{(15)}$=-0.21, p=0.84). This indicates that the eDREADD group completed the task more quickly due to the reduction in the total number of trials they required to solve all discriminations, rather than due to completing trials at a faster rate.

Overall, these results suggest that DREADD mediated excitation of the anterior cingulate cortex improved aspects of both intradimensional and extradimensional set-shifting.

5.3 Follow-up attentional set-shifting task (experiment 5B)

In experiment 5A, excitatory DREADD expression in the anterior cingulate cortex appeared to produce an improvement at intradimensional (ID) shifts (section 5.2.2.2). However, it is noted that successive improvement across intradimensional shifts, thought to be a hallmark of attentional set-formation (Birrell & Brown, 2000), was not apparent in this group.

This raises the possibility that these animals were employing a strategy other than learning to selectively attend to the relevant dimension to solve discriminations. In reward-based learning, rats may engage in a win-stay, lose-shift strategy (Evenden & Robbins, 1984); where a just-reinforced response is repeated. In the current task, this would manifest as a tendency to return to a pot where a reward had recently been found. This strategy could be sufficient for 'solving' all discriminations encountered in the main behavioural test without necessitating attentional set-formation. Animals need only to register which two of the four pots (or which one of the two in the simple discrimination) has contained a reward previously, without learning about the stimulus dimensions themselves. Notably, this would also predict no difference in how the intradimensional and extradimensional shifts were solved, consistent with the results for this group (section 5.2.2.2).

To investigate this possibility, animals were challenged with a follow-up attentional set-shifting task (experiment 5B), that included a more complex intradimensional shift. Whilst the relevant dimension had two stimuli, as in previous discriminations, the number of stimuli in the irrelevant dimension was increased to four. If eDREADD animals solve discriminations by attending only to the relevant dimension, the introduction of additional stimuli of the irrelevant dimension should not influence their performance. On the other hand, there is evidence that a win-stay, lose-shift strategy is reliant on both working memory capacity and temporal contiguity in the rat (Rawlins, Maxwell, & Sinden, 1988). If an animal is forming

individual stimulus-reward associations for each pot it encounters, the addition of stimuli from the irrelevant dimension will increase working memory load. It will also increase the temporal discontiguity with which rewarded pots were encountered, and a deterioration in task performance might be expected.

eDREADD animals also outperformed controls at extradimensional shifts (see section 5.2.2.2) in this experiment. To investigate whether this would transfer to perceptual dimensions other than digging media and odour, animals were also challenged with an extradimensional shift from a different perceptual dimension (experiment 5B). In this discrimination the spatial location of the pot, experienced as irrelevant in all previous trials, became relevant to reward (see also section 4.3.1.1).

5.3.1 Methods

Animals, surgeries and clozapine administration as described in section 5.2.1

5.3.1.1 Behavioural testing

On the test day, the glass pots in the two smaller compartments of the arena were filled with different stimuli pairs (Table 9). Animals were administered an I.P. injection of clozapine (HelloBio, Bristol, UK), as described in section 5.2.1.3.2. The behavioural testing protocol was the same as described in section 2.6.3, except that the discriminations took place approximately two weeks after completion of the main behavioural test and proceeded as follows:

1. A compound discrimination (CD), where either an odour or a digging media is rewarded but is presented with irrelevant stimuli from the other dimension
2. An intradimensional shift (ID), where different compound stimuli are presented with the previously rewarded dimension remaining relevant
3. A complex intradimensional shift (IDComp), where two stimuli from the relevant dimension are presented, paired with four stimuli from the irrelevant dimension. The previously rewarded dimension remains relevant
4. A complex intradimensional reversal (REVIDComp), where the same complex compound stimuli are presented but the previously incorrect stimuli from the relevant dimension is rewarded

5. A spatial extradimensional shift (EDSpatial), where the same complex compound stimuli are presented but the spatial location of the pot (left or right chamber) is rewarded
6. A spatial reversal (REVSpatial), where the same complex compound stimuli are presented but the previously incorrect spatial location dimension is rewarded

(Note that experiment 5B is the same as experiment 4B [section 4.3], except for the addition of the complex intradimensional shift and complex intradimensional reversal).

Consequently, for the first three discriminations the animal encountered exemplars from one of the stimulus dimensions experienced to be relevant in the main behavioural test. The rewarded dimension remained the same as that which was most recently rewarded in the main behavioural test, such that these discriminations did not represent a type of extradimensional shift for the animals. Animals were then challenged with a reversal, which is an intradimensional shift in the sense that it does not require attention to be reoriented to a different dimension. However, although the stimuli remained the same as the preceding trial the previously incorrect stimuli from the relevant dimension were rewarded. Animals were then challenged with a new type of extradimensional shift, where the spatial dimension of the pot became relevant to reward. The final stage was another reversal, where the previously irrelevant spatial location became relevant to solving the discrimination.

Table 9. Depiction of a possible order of stimulus pairings in the follow-up attentional set-shifting task (experiment 5B).

Discrimination	Rewarded dimension	Rewarded Stimuli	Unrewarded Stimuli
CD	Odour	**Paprika** + short wire	Coriander + long wire
		Paprika + long wire	Coriander + short wire
ID	Odour	**Lemongrass** + buttons	Nutmeg + beads
		Lemongrass + beads	Nutmeg + buttons
IDComp	Odour	**Onion** + coarse cloth	Garlic + fine cloth
		Onion + fine cloth	Garlic + coarse cloth

		Onion + long string	Garlic + short string
		Onion + short string	Garlic + long string
REVIDComp	Odour	**Garlic** + fine cloth	Onion + coarse cloth
		Garlic + coarse cloth	Onion + fine cloth
		Garlic + short string	Onion + long string
		Garlic + long string	Onion + short string
EDSpatial	Spatial location	Garlic + fine cloth **(left chamber)**	Onion + coarse cloth (right chamber)
		Garlic + coarse cloth **(left chamber)**	Onion + fine cloth (right chamber)
		Onion + long string **(left chamber)**	Garlic + short string (right chamber)
		Onion + short string **(left chamber)**	Garlic + long string (right chamber)
		Garlic + short string **(left chamber)**	Onion + long string (right chamber)
		Garlic + long string **(left chamber)**	Onion + short string (right chamber)
		Onion + coarse cloth **(left chamber)**	Garlic + fine cloth (right chamber)
		Onion + fine cloth **(left chamber)**	Garlic + coarse cloth (right chamber)
EDSpatialREV	Spatial location	Garlic + fine cloth **(right chamber)**	Onion + coarse cloth (left chamber)
		Garlic + coarse cloth **(right chamber)**	Onion + fine cloth (left chamber)
		Onion + long string **(right chamber)**	Garlic + short string (left chamber)
		Onion + short string **(right chamber)**	Garlic + long string (left chamber)
		Garlic + short string **(right chamber)**	Onion + long string (left chamber)
		Garlic + long string **(right chamber)**	Onion + short string (left chamber)
		Onion + coarse cloth **(right chamber)**	Garlic + fine cloth (left chamber)
		Onion + fine cloth **(right chamber)**	Garlic + coarse cloth (left chamber)

Depiction of one possible order of stimulus pairings in the additional discriminations of the attentional set-shifting task. In this example, odour is the first dimension relevant to the location of the buried food reward. From the ED stage onwards, spatial location of the pot is the relevant dimension. Stimuli are always paired as shown, but the discrimination in which animals encounter them is counterbalanced. The first dimension to be rewarded is the most recent dimension to be rewarded in the main behavioural test. The first spatial location to be rewarded is also counterbalanced across animals.

5.3.1.2 Analysis of behaviour

As described in section 2.6.4.

5.3.1.3 Histology

Perfusion, sectioning and immunohistochemistry as described section 2.8.

5.3.1.4 Image capture and virus expression analysis

As described in section 2.9.

5.3.1.5 Statistical analysis

As described in section 2.11.1.2.

5.3.2 Results

5.3.2.1 Virus expression analysis

As described in section 5.2.2.1.

5.3.2.2 Behavioural testing

One animal from the eDREADD group displayed adverse motor effects following clozapine injection, as described in section 5.2.1.3.2, impairing its ability to complete the follow-up attentional set-shifting task. This animal was subsequently excluded from this part of the analysis, such that the final group numbers were: eDREADD, n=9, control virus, n=10.

A series of ANOVA were conducted on the mean trials required to reach criterion at each stage of the follow-up attentional set-shifting task. The first analysis revealed that there were no effects of rewarded chamber (whether the reward was located in

the left or the right chamber in the first spatial discrimination) on performance and no interactions involving this factor and group ($F<1$). Consequently, the data were pooled across dimensions for all subsequent analyses.

Two-way ANOVA (with stage [six levels] as a within-subjects factor and group [two levels] as a between-subjects factor) revealed that there was no significant difference in performance between the groups ($F_{(1,17)}=3.08$, $p=.10$, $\eta^2=.15$). There was a main effect of task stage ($F_{(2.76,46.84)}=3.40$, $p<.05$, $\eta^2=.15$) and no interaction between group and task stage ($F<1$), as displayed in Figure 5.4.

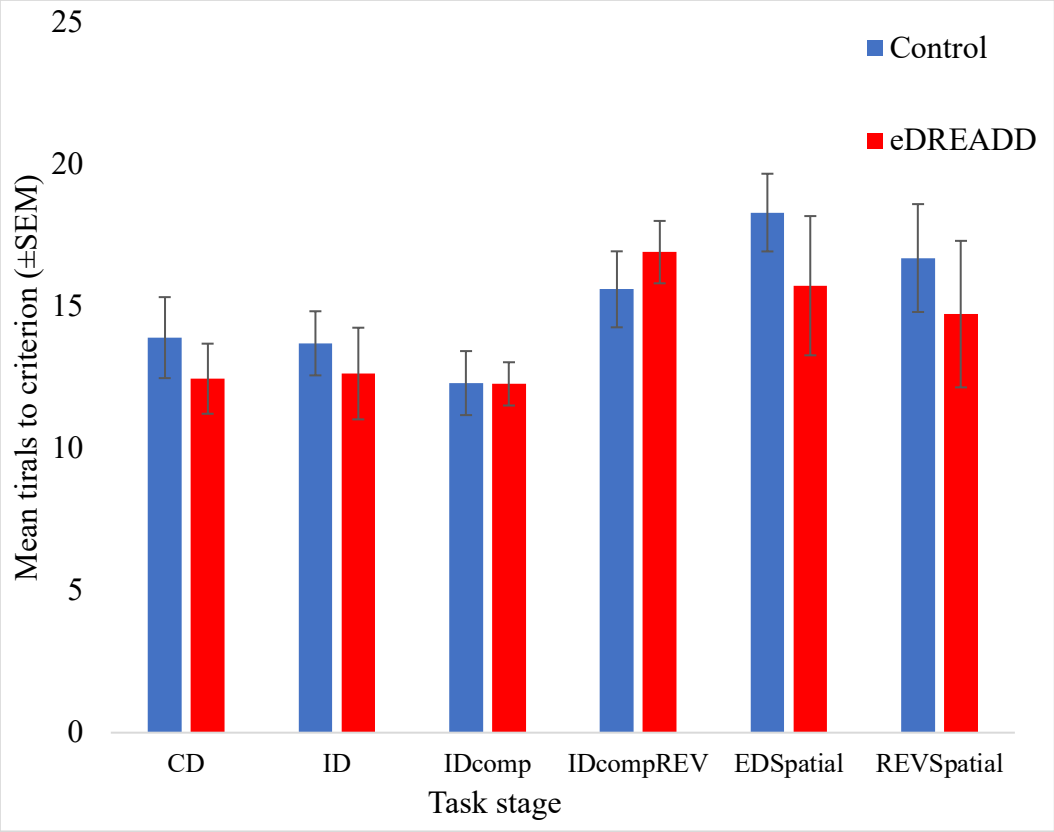

Figure 5.4. Mean (±SEM) trials to criterion on each stage of the follow-up attentional set-shifting task.

No differences were found between the groups and no interaction was found between group and task stage.

One sample t-tests were conducted on shift costs (the difference between the mean trials to criterion from the two ID stages and the ED spatial stage) and revealed that the eDREADD group showed a significant shift cost ($t_{(8)}=3.47$, $p<.01$), taking more trials to complete the ED spatial stage. However, this difference was not significant

for the control group ($t_{(9)}$=1.85, p=.10). An independent samples t-test revealed that there was no significant difference in shift cost between the groups ($t_{(17)}$=-.17, p=.87, Figure 5.5).

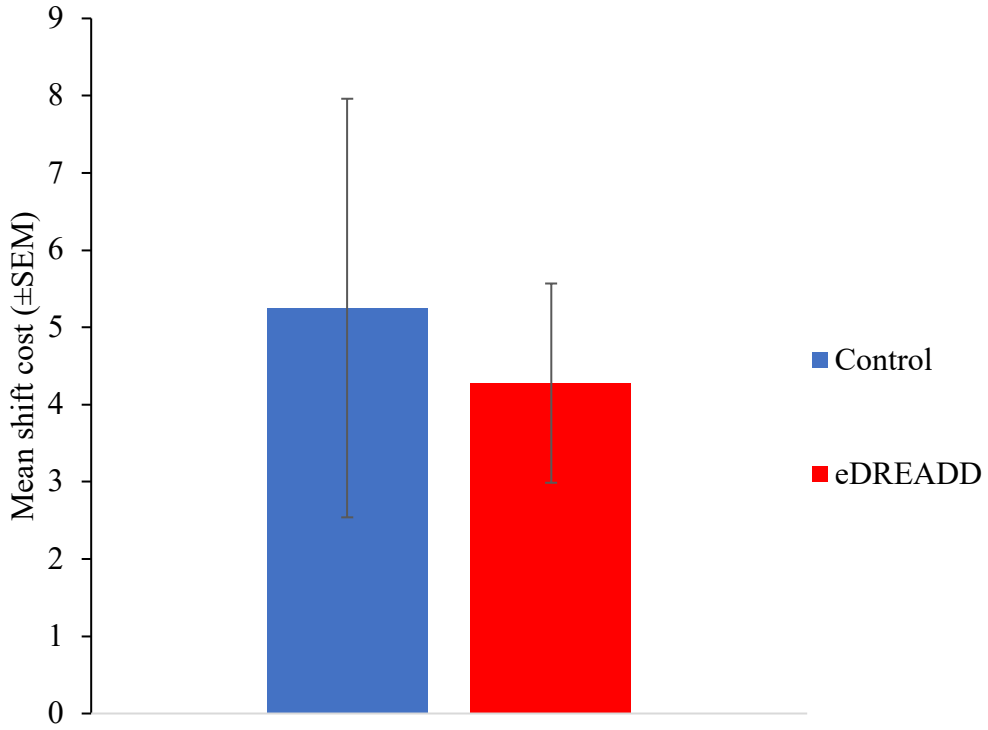

Figure 5.5. Mean shift cost (±SEM), the difference between average trials to criterion for the two ID stages and the ED spatial stage.

There was no significant difference in shift cost between the groups. The eDREADD group showed a positive shift cost, taking more trials to solve the spatial ED.

Therefore, unlike in the main attentional set-shifting task, DREADD mediated excitation of the anterior cingulate cortex did not improve performance on intradimensional or extradimensional set-shifting during the follow-up task.

5.4 Investigation of c-*fos* through novel environment exposure (experiment 5C)

As described in section 2.7, the behavioural experiments were followed by an investigation into the expression of c-*fos,* an indirect marker of neuronal activity. This was conducted to provide an independent measure of the influence of

DREADD-mediated anterior cingulate cortex excitation on activity in brain regions of interest.

5.4.1 Methods

As described in section 2.7.

5.4.2 Results

5.4.2.1 Preliminary analysis

Initial analysis of sections stained for c-*fos* indicated that some cases displayed a widespread increase in Fos-positive cells that was not restricted to the anterior cingulate cortex or its efferent regions. In order to identify cases with non-specific c-*fos* increases, cell counts in the cortical control region, secondary somatosensory cortex (S2), were converted to Z scores and cases with counts +/- two standard deviations from the mean were classified as outliers. Based on this criterion, three cases from the eDREADD group were excluded from the analysis.

5.4.2.2 Fos-positive cell counts

5.4.2.2.1 Analysis of variance

As described in section 2.11.2, a series of ANOVA were run on Fos-positive cell counts. First, a two-way ANOVA was conducted on counts in the cortical regions of interest, with region (three levels, dorsal anterior cingulate [Cg1], ventral anterior cingulate [Cg2] and prelimbic [PrL] cortices) as a within-subjects factor and group (two levels) as a between-subjects factor. There was a significant difference between the groups ($F_{(1,15)}$=7.88, p<.05, η^2=.34), with higher counts observed in the eDREADD group, as can be seen in Figure 5.6. There was no interaction between group and region (F<1).

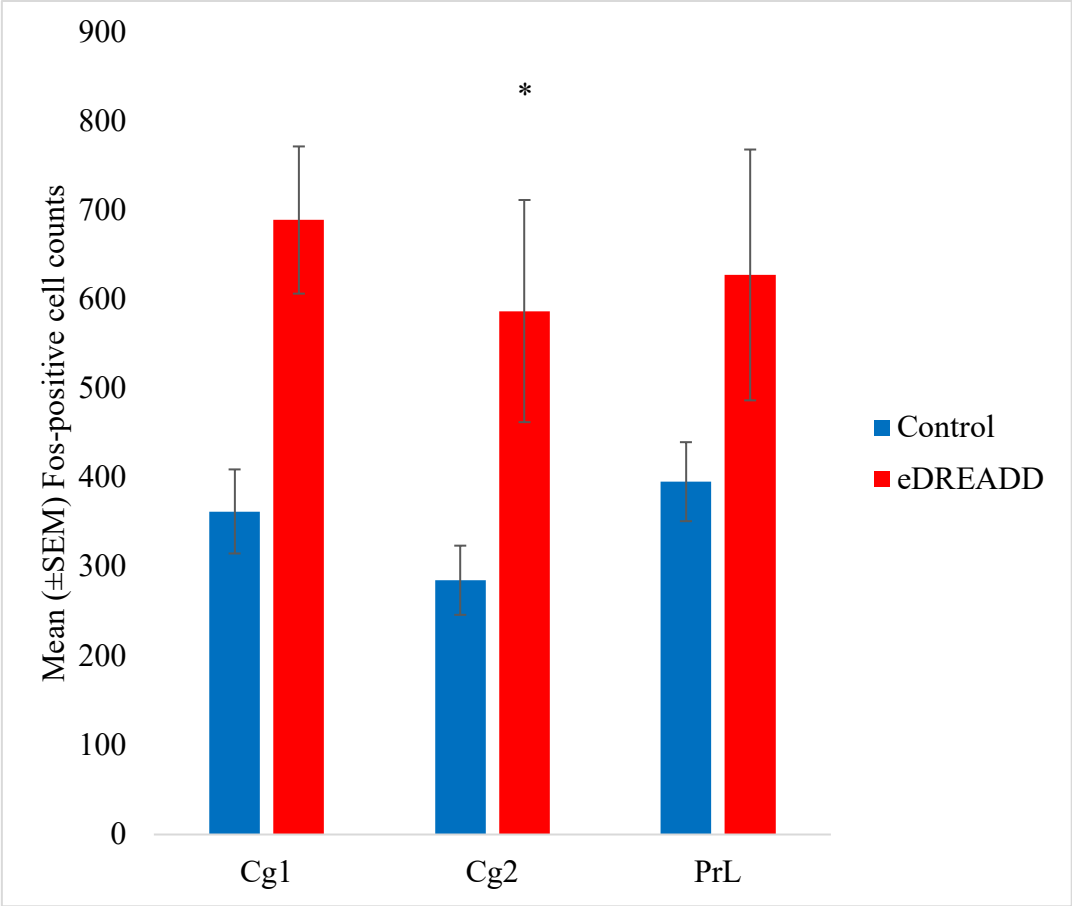

Figure 5.6. Mean (±SEM) Fos-positive cell counts in anterior cingulate (Cg1 and Cg2) and prelimbic (PrL) cortices.

The eDREADD group had a higher number of Fos-positive cells than the control group ($p<.05$). There was no interaction between group and region.

A further one-way ANOVA on counts in secondary somatosensory cortex (S2), a control cortical region, revealed that there was no difference between the groups ($F<1$). This null result is displayed in Figure 5.7.

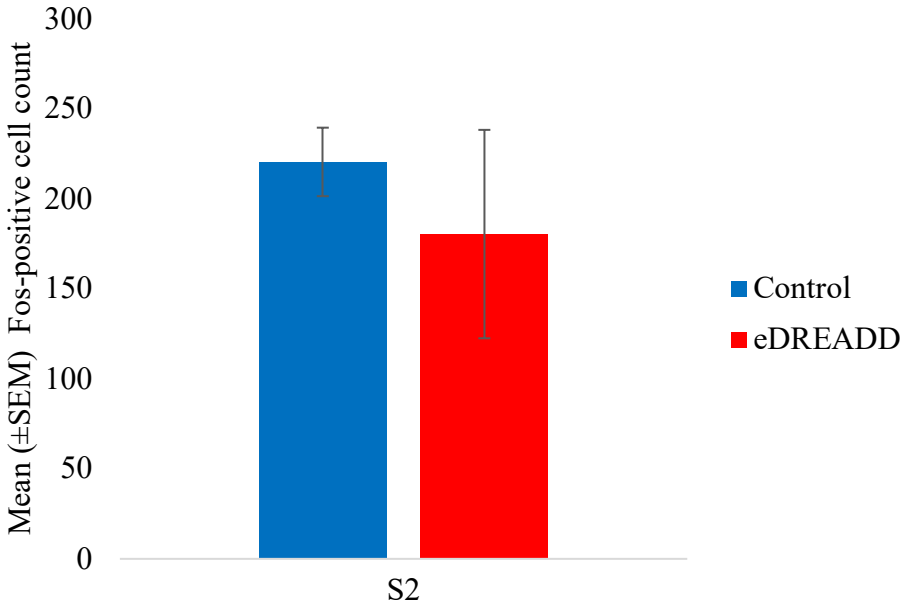

Figure 5.7. Mean (±SEM) Fos-positive cell counts in secondary somatosensory cortex (S2).

There was no difference between the groups.

Finally, a two-way ANOVA was conducted on cell counts in the anterior thalamic nuclei on interest, with region (two levels, anteromedial [AM] and anteroventral [AV] thalamic nuclei) as a within-subjects factor and group (two levels) as a between-subjects factor. This found a main effect of group ($F_{(1,14)}$=6.66, p<.05, η^2=.32), with higher cell counts in the eDREADD group, and an interaction between group and region ($F_{(1,14)}$=22.18, p<.001, η^2=.07). Subsequent simple effects analyses revealed that cell counts were higher in the eDREADD group in AM ($F_{(1,14)}$=8.00, p<.05), but not in AV (F<1), as displayed in Figure 5.8.

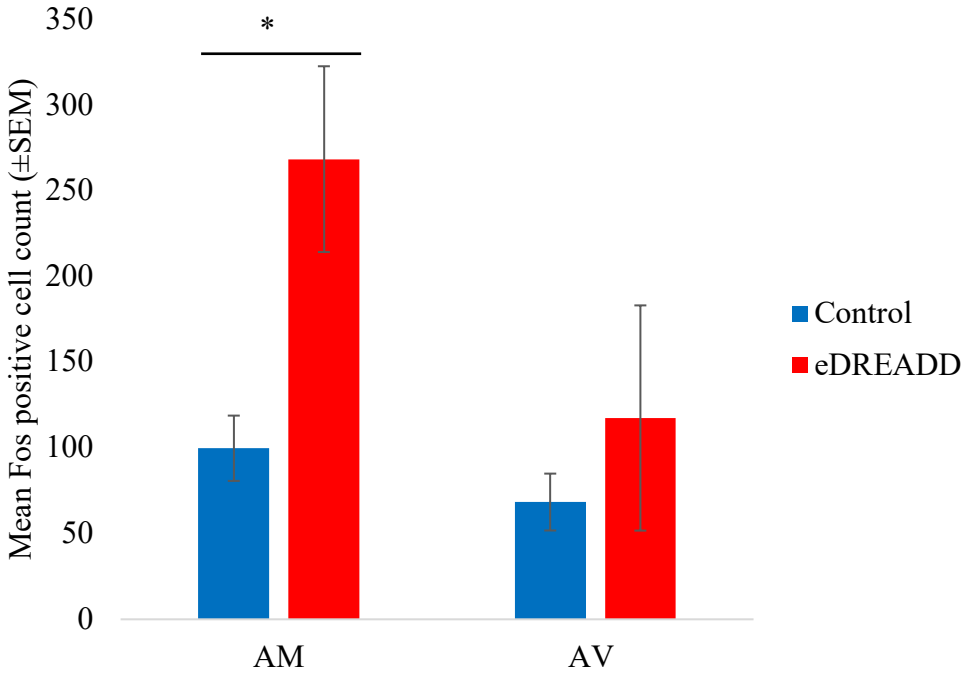

Figure 5.8. Mean (±SEM) Fos-positive cell counts in the anteromedial (AM) and anteroventral (AV) nuclei of the thalamus.

The number of Fos-positive cells in AM was significantly higher in the DREADDs group (*, $p<.05$) than the control group.

Overall, these results suggest that excitatory DREADDs in the anterior cingulate cortex increased activity in the anterior cingulate cortex, prelimbic cortex and the anteromedial thalamic nuclei. Meanwhile, lack of differences between the eDREADD group and the control group in secondary sensory motor cortex and the anteroventral thalamic nuclei indicate that this was not a non-specific increase in activity.

5.4.2.2.2 Pearson correlation coefficients

Pearson correlation analysis was conducted on Fos-positive cell counts for each group with Bonferroni corrections for multiple comparisons (as described in section 2.11.2). In the eDREADD group, there was a strong positive correlation between Fos-positive cell counts in prelimbic cortex (PrL) and ventral anterior cingulate cortex (Cg2), as displayed in Table 10. There were no other significant correlations following Bonferroni corrections.

Table 10. Interregional correlation matrix of Fos-positive cell counts in the eDREADD group.

		Cg1	Cg2	PrL	AM	AV
Cg1	Pearson's r					
	p-value					
Cg2	Pearson's r	0.829				
	p-value	0.021				
PrL	Pearson's r	0.766	0.970			
	p-value	0.045	< .001***			
AM	Pearson's r	0.404	0.432	0.212		
	p-value	0.427	0.393	0.686		
AV	Pearson's r	-0.055	-0.031	-0.257	0.869	
	p-value	0.917	0.954	0.623	0.025	
S2	Pearson's r	-0.059	0.255	0.289	0.190	0.093
	p-value	0.900	0.581	0.530	0.718	0.861

R-values refer to Pearson correlation coefficients, alpha level is adjusted to $p<.003$ (Bonferroni correction).*** $p<.001$. Regions included are dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL), anteromedial thalamic nuclei (AM) anteroventral thalamic nuclei (AV) and secondary somatosensory cortex (S2)

In the control group, strong positive correlations were found between dorsal anterior cingulate cortex (Cg1) and Cg2, and between the anteromedial (AM) and anteroventral thalamic nuclei (AM), as displayed in Table 11.

Table 11. Interregional correlation matrix of Fos-positive cell counts in the control group.

		Cg1	Cg2	PrL	AM	AV
Cg1	Pearson's r					
	p-value					
Cg2	Pearson's r	0.843				
	p-value	0.002**				
PrL	Pearson's r	0.480	0.632			
	p-value	0.160	0.050			
AM	Pearson's r	-0.214	-0.183	0.144		
	p-value	0.553	0.612	0.691		
AV	Pearson's r	-0.249	-0.245	0.203	0.916	
	p-value	0.488	0.495	0.573	< .001***	
S2	Pearson's r	-0.081	-0.118	0.025	0.497	0.483
	p-value	0.825	0.745	0.945	0.143	0.157

R-values refer to Pearson correlation coefficients, alpha level is adjusted to $p<.003$ (Bonferroni correction).**$p<.003$, *** $p<.001$. Regions included are dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL), anteromedial thalamic nuclei (AM) anteroventral thalamic nuclei (AV) and secondary somatosensory cortex (S2)

These findings indicate that DREADD-mediated excitation of the anterior cingulate cortex may have altered the network of interdependent activity between this region and its efferents.

5.5 Discussion

Previous research (Ng et al., 2007) and the results of Chapter 4 (section 4.5) implicate the anterior cingulate cortex in focusing attention on relevant, reliable reward predictors in attentional set-shifting (intradimensional shifts). Chapter 4 further indicated that downregulation of anterior cingulate activity allows unreliable reward predictors to gain attentional control, resulting in improved performance when those predictors became relevant to reward (extradimensional shifts). The current study investigated the impact of upregulating anterior cingulate activity, using excitatory DREADDs (see section 2.3.1), on attentional set-shifting. Rats were

tested on two tasks taxing intradimensional and extradimensional shifts, and an independent investigation into c-*fos* was carried out to provide an indication of the effect of excitatory DREADDs on brain activity.

5.5.1 Standard attentional set-shifting task

In the main attentional set-shifting task, the excitatory DREADDs group (eDREADD) outperformed controls across the entire series of discriminations. This included solving the initial compound discrimination (CD) and the second intradimensional discrimination (ID2) faster than controls, indicating that increasing anterior cingulate activity lead to an increase in attention to reliable reward predictors ([Ng et al., 2007] section 4.5). In fact, the eDREADD group acquired the initial intradimensional shifts so quickly that attentional set-formation (i.e. an improvement from ID1 to ID4, [Chase et al., 2012]) could not be demonstrated statistically. This contrasts with the performance of the control group, where the typical decrease in trials to criterion across these intradimensional stages was observed. As the control group formed an attentional set, their performance became comparable with that of the eDREADD group (on ID3 and ID4).

The eDREADD group also solved the extradimensional shift and its subsequent reversal in fewer trials than the control group. Further, while the control group displayed a shift cost, taking more trials to solve the extradimensional shift than the mean of the preceding four intradimensional stages, the eDREADD group showed neither a shift cost nor shift benefit. These results are somewhat surprising, as one might expect excitation in the anterior cingulate cortex to produce the inverse results of inhibition (see section 4.5). That is, if the eDREADD group are paying more attention to relevant, reliable reward predictors (facilitating intradimensional shifts), this would be at the expense of attending to irrelevant, unreliable reward predictors (thus impairing extradimensional shifts). Indeed, classical attention theories posit attentional resources as finite (Mackintosh, 1965, 1975), such that paying more attention to one stimulus dimension involves a lessening of attention paid to another stimulus dimension.

Taken together, the lack of improvement across intradimensional shifts and the lack of shift cost at extradimensional shift stage suggests that the eDREADD group may not have formed an attentional set. This raises the possibility that animals were using a different strategy, other than selectively attending to the stimulus dimensions, to solve discriminations. One such strategy could be a win-stay, lose-shift approach, where a recently reinforced response is repeated (Evenden & Robbins, 1984), see also section 5.3). This strategy could be sufficient for 'solving' all discriminations as animals need only to register which two of the four pots (or which one of the two in the simple discrimination) contained a reward recently and return to it, without learning about the stimulus *dimensions* themselves. Critically, there would be no difference in how the various shift stages were completed, fitting with the pattern (or lack therefore) of performance in the eDREADD group. This possibility was investigated by challenging animals to a follow-up attentional set-shifting task.

5.5.2 Follow-up attentional set-shifting task

To further investigate the results of the main behavioural task (section 5.5.1), animals were challenged to a follow-up attentional set-shifting task, that included a more complex intradimensional shift and a new type of extradimensional shift. The complex intradimensional shift was designed to test whether eDREADD animals were adopting a win-stay lose-shift strategy to complete discriminations. By increasing the number of stimuli in the irrelevant dimension to four a win-stay lose-shift strategy, contingent on working memory and temporal contiguity, should be more difficult (Rawlins, 1988; see also section 5.3). Meanwhile, the inclusion of a new extradimensional shift based on the spatial location of the digging pot sought to establish whether the eDREADD animals' advantage over controls (section 5.5.1) would transfer to another perceptual dimension.

Analysis found no differences between the groups overall, or at any stage of the follow-up attentional set-shifting task. This null result can be interpreted in several ways. On the one hand, one might postulate that the eDREADD advantage at intradimensional set-shifting in the main behavioural task (section 5.5.1) was contingent on the relative inexperience of the control group. Indeed, the advantage was no longer evident once control animals had formed an attentional set (by ID3).

In the follow-up task, the first rewarded stimulus dimension was the same as that rewarded in the last two stages of the main behavioural task. Given evidence that control animals improve when retested on this version of the attentional set-shifting task (Chase et al., 2012), it seems possible that control animals carried over a partial attentional set, facilitating performance at the initial intradimensional stages of the follow-up task and thus diminishing the eDREADD group advantage.

However, this account does not fully explain why eDREADD animals failed to outperform controls at extradimensional or reversal stages, as they did in the main behavioural task (section 5.5.1). In fact, the eDREADD group displayed a shift cost, taking more trials to solve the spatial extradimensional than the mean of the preceding intradimensional stages, further signifying the loss of extradimensional shift facilitation in the follow-up task. Interestingly, the lack of eDREADD advantage at the spatial extradimensional stage, as well as at its reversal, the complex intradimensional stage and its reversal, is consistent with the hypothesis that eDREADD rats were engaging in a win-stay lose-shift strategy in the main behavioural task. All four of these discriminations had additional distractor stimuli in the irrelevant stimulus dimension, making this strategy a less viable alternative to attentional-set-formation (see section 5.3).

5.5.3 Investigation of c-*fos* through novel environment exposure

The novel environment exposure experiment revealed increases in c-*fos*, a marker of cellular activity, in the eDREADD group. There were higher counts in the dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL) and anteromedial thalamic nuclei (AM) than in the control group. Meanwhile, there were no differences between the groups in secondary somatosensory cortex (S2) or the anteroventral thalamic nuclei (AV), demonstrating that there was not a brain-wide, non-specific increase in activity.

When bound by a ligand, excitatory DREADDs are thought to stimulate neuronal firing by triggering the release of intracellular calcium and depolarising neurons (Alexander et al., 2009; Conklin et al., 2008). The observed increases in c-*fos,* therefore, provide independent evidence that neurons were successfully transfected

by the eDREADDs, resulting in increased activity in the injection target region of anterior cingulate cortex (Cg1 and Cg2). Meanwhile, the anterior cingulate cortex heavily innervates AM (see section 3.4.2), such that the observed c-*fos* difference here indicates that the eDREADDs increased activity in major efferent target regions. However, although these increases are consistent with the proposed mechanistic action of eDREADDs, it is not clear why eDREADDs did not have the opposite effect of inhibitory DREADDs (iDREADDs). The latter manipulation also, paradoxically, increased activity in anterior cingulate and in anterior thalamic nuclei (see section 4.5.3).

Chapter 4 argued that iDREADDs may have preferentially infected GABAergic inhibitory neurons or may have inhibited excitatory pyramidal cells projecting to the inhibitory thalamic reticular nucleus. Either could lead to disinhibition (see section 4.5.3), thus explaining the observed increases in c-*fos.* However, as both inhibitory and excitatory DREADD constructs were injected into the same region with the same promoter (CAMKII) they should, theoretically, have infected the same cell types. Therefore, it is not clear why eDREADDs did not simply reverse the mechanistic action of iDREADDs and lead to decreases in c-*fos*. Again, this highlights how DREADDs do not simply downregulate of upregulate activity, rather, they have a complex influence across a network of interconnected structures.

In this respect, the increase in c-*fos* in prelimbic cortex in the eDREADD group presents an interesting divergence from the activity profile of the iDREADD group. Although prelimbic neighbours the anterior cingulate cortex, there was minimal spread of the virus into this region from the injection sites (section 5.2.2.1). This suggests that the c-*fos* increase is a result of increased activity in efferent projections from the anterior cingulate to prelimbic cortex (Beckstead, 1979). Indeed, Fos-positive cell counts in prelimbic cortex were very strongly correlated with counts in Cg2 (r=.97), with this interregional correlation being the only one to survive Bonferroni correction in the eDREADD group. Furthermore, there was no significant correlation between counts in these two regions in the control group. Taken together, these findings indicate that eDREADDs increased interdependent activity between anterior cingulate and prelimbic cortices.

5.5.4 **Summary and implications**

Overall, the experiments in this chapter found that eDREADDs in the anterior cingulate cortex only partially produce the inverse effects of iDREADDs. Initially, eDREADDs improved aspects of intradimensional set-shifting; the inverse behavioural profile of the iDREADD group. This finding supports the suggestion that the anterior cingulate cortex is involved in attending to reliable reward predictors. However, akin to iDREADDs, eDREADDs also improved extradimensional set-shifting. This unexpected finding reveals that attending to reliable reward predictors need not necessarily be at the expense of attending to unreliable reward predictors.

Furthermore, the results of the c-*fos* study revealed that eDREADDs did not have the opposite effect of iDREADDs (see section 4.5.3) on anterior cingulate activity. Both manipulations increased activity in the anterior cingulate cortex. However, there were differences in the way activity in efferent regions were affected, indicating that DREADDs have a complex influence of network dynamics that is not yet well understood. In the eDREADD group, there was an increase in c-*fos* in prelimbic cortex and strong interdependent activity between this region and the ventral anterior cingulate cortex (Cg2). This is particularly interesting considering the behavioural results because prelimbic has a well-established role in extradimensional set-shifting (Birrell & Brown, 2000; Bissonette et al., 2013). Therefore, it is a good candidate to be involved in a system mediating attention to unreliable reward predictors (Pearce & Mackintosh, 2010). A tentative possibility emerges, that the excitatory DREADDs not only increased attention to reliable reward predictors (anterior cingulate), but also increased attention to unreliable reward predictors (prelimbic), through downstream upregulation of neural activity.

An alternative explanation of the results is that the eDREADD group did not form an attentional set, but instead, were employing a win-stay, lose-shift strategy. That is, they were simply returning to a recently rewarded digging pot, without registering the stimulus dimensions themselves (i.e. that type of digging media/odour reliably predicted reward).

The observation that in the follow-up task, when stimuli were made more complex to make this approach more difficult, the eDREADD advantage over controls disappeared (section 5.5.2), supports this conclusion. In this regard, the results are more in keeping with a hypothesis that the anterior cingulate is involved in exploring better alternative courses of action (Rushworth, Kolling, Sallet, & Mars, 2012). It is possible that although attentional set-formation is the default of normal rats, a win-stay, lose-shift approach was identified and adopted as a more efficient strategy by the eDREADD group.

6 DREADD-Mediated Inhibition of Anterior Cingulate Cortex Efferents to the Anterior Thalamic Nuclei and Attentional Set-Shifting

6.1 Introduction

Chapter 4 revealed that DREADD-mediated inhibition of the anterior cingulate cortex disrupts intradimensional set-formation but facilitates extradimensional set-shifting. This suggests that the anterior cingulate may be involved in a system mediating attention to reliable reward predictors. Meanwhile, Chapter 5 found evidence that excitation of the anterior cingulate facilitates intradimensional set-shifting, partially inverting the behavioural profile of inhibitory DREADDs and further implicating the anterior cingulate in attending to task-relevant stimuli. Strikingly, neurotoxic lesions of the anterior thalamic nuclei produce the same pattern of results as inhibitory DREADDs in the anterior cingulate cortex, impairing intradimensional set-formation but improving extradimensional shifts (Wright et al., 2015).

As characterised in Chapter 3, there is a dense network of fibres connecting the anterior cingulate cortex and the anterior thalamic nuclei. To explore the possibility that these interconnections form part of a system mediating attention to the best predictors of rewards, the current study aimed to disrupt the activity of projections from the anterior cingulate cortex to the anterior thalamic nuclei in the rat. This was achieved by injecting inhibitory DREADDs into the anterior cingulate cortex and infusing clozapine directly into the anterior thalamic nuclei, thus selectively inhibiting the terminals of DREADD expressing neurons that project there (Figure 6.1, see also section 2.3.3). Animals were then challenged to an attentional set-shifting task involving both intradimensional and extradimensional shifts.

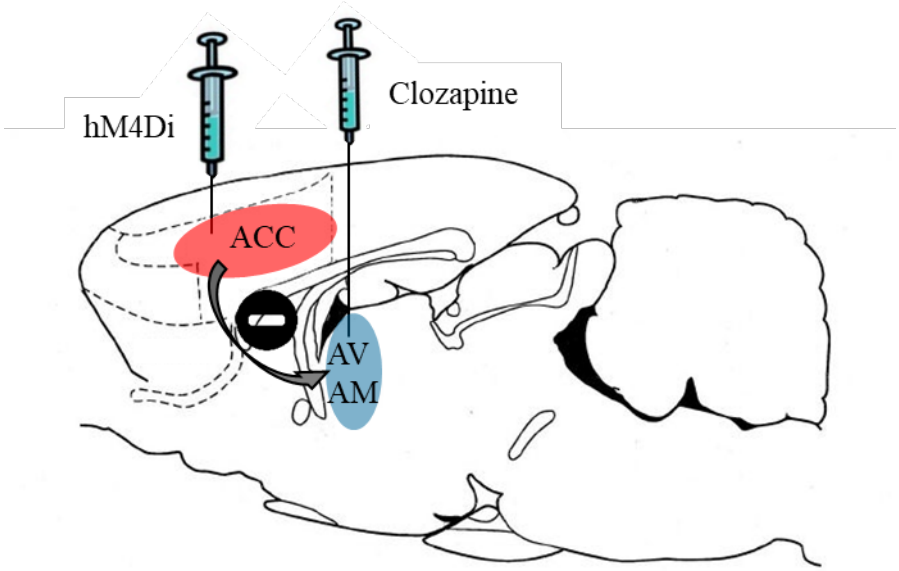

Figure 6.1. Schematic of the rat brain showing DREADD-mediated inhibition of anterior cingulate efferents to the anterior thalamic nuclei

Inhibitory DREADD hM4Di is expressed in the anterior cingulate cortex (ACC). The ligand clozapine is infused directly into the anterior thalamic nuclei via intracranial cannulae. The aim is to downregulate activity at the terminals of DREADD-infected ACC neurons that project to the anteroventral (AV) and anteromedial (AM) thalamic nuclei.

6.2 Standard attentional set-shifting task (experiment 6A)

6.2.1 Methods

This experiment used two separate cohorts of animals, of which the first had 14 animals and the second had eight animals. The experimental design was identical for both cohorts of animals and is described in the following sections.

6.2.1.1 Animals

Subjects were 22 male, Lister Hooded rats (Envigo, Bicester, UK) housed as described in section 2.4.

6.2.1.2 Surgery

Anaesthesia, analgesia and surgical site preparation as described in section 2.5.1.

6.2.1.2.1 Intracranial virus injections and cannula implantation

For these surgeries, the incisor bar of the stereotaxic frame was set so that the skull was at +5mm relative to the horizontal plane. A bilateral guide cannula (26 gauge, 5mm length, 2mm centre to centre width; Plastics One Inc, Roanoke, VA, USA) was attached to a moveable arm mounted to the stereotaxic frame and anteroposterior (AP), mediolateral (ML) and dorsoventral (DV) coordinates were taken from bregma. The cannula was aligned over the implantation site, aimed at the border between the anteromedial and anteroventral thalamic nuclei, at AP: -0.1, ML:+/- 1.0 (mm from bregma), where holes were drilled. The moveable arm with cannula attached was then removed from the stereotaxic frame and replaced with a moveable arm fitted with a 10µl Hamilton syringe (Bonaduz, Switzerland). Anteroposterior (AP) coordinates were taken from Bregma and a craniotomy was made above the injection sites, allowing ML coordinates to be taken from the sagittal sinus and DV coordinates to be taken from the dura.

Animals received three injections of the virus in the anterior cingulate cortex in each hemisphere as follows: 0.35µl at AP: +2.1, ML: +/-0.8, DV: -1.2, 0.65µl at AP: +1.4, ML: +/- 0.8, DV: -1.6 and 0.65µl at AP: +0.7, ML: +/- 0.8, DV: -1.6 (all coordinates are in millimetres). Of these animals, 14 received inhibitory DREADD AAV5-CaMKIIa-hM4Di-mCherry (titre 9.5x10^12 GC/ml, Addgene, Watertown, MA, USA) and eight received injections of a non-DREADD expressing control AAV5-CaMKIIa-EeGFP (titre 4.3x10^12 GC/ml, Addgene, Watertown, MA, USA). Note that injections for this experiment started at a more anterior AP level than in Chapters 4 and 5. This was due to the need to implant a cannula into the anterior thalamic nuclei close to the AP level used for the most posterior injection in Chapters 4 and 5. The dura was pierced above each injection site and the needle lowered into place. The virus injections were controlled by a microprocessor (World Precision Instruments, Hitchin, UK) set to a flow rate of 0.1 µl/min, and the needle left *in situ* for a further five minutes to allow for diffusion of the virus.

The moveable arm fitted with the syringe was removed from the stereotaxic frame and replaced with the moveable arm fitted with the guide cannula. The cannula was lowered into place at AP: -0.1, ML:+/- 1.0, DV:-4.6 (mm from bregma) and was

secured to the skull using four fixing screws (Precision Technology Supplies, East Ginstead, UK) and dental cement (Zimmer Biomet, Winterhur, Switzerland). Removable obturators were inserted into the cannulae to prevent them from blocking.

6.2.1.2.2 Surgical site closure and post-operative care

Loose sutures were placed above and below the dental cement cap. The analgesic bupivacaine (Pfizer, Walton Oaks, UK) and the topical antibiotic powder Clindamycin (Pfizer, Walton Oaks, UK) were applied around the edges of the cap. Animals were administered a subcutaneous injection of glucose-saline (5ml) for fluid replacement and placed in a recovery chamber. Animals were monitored carefully postoperatively with food available *ad libitum* until they had fully recovered, with behavioural pre-training commencing approximately two weeks post-surgery.

6.2.1.3 Attentional set-shifting task protocol

Apparatus and pretraining as described in section 2.6.

6.2.1.3.1 Clozapine administration

The main behavioural test began three weeks post-surgery, allowing sufficient time for robust DREADD expression in neurons (see also section 2.3.1) (Smith et al., 2016). Animals received intracranial infusions of 0.25µl clozapine dihydrochloride (HelloBio, Bristol, UK) fully dissolved in saline at a dilution of 1mg/1000µl as salt. Higher volumes (starting at 1µl of clozapine at a dilution of 1mg/1000µl) were initially trialled but were found to produce motor effects in some DREADD expressing animals that impaired their ability to complete the task. The data from four such DREADD expressing animals was therefore excluded from the analysis, resulting in final group numbers of 10 DREADD expressing animals and eight non-DREADD expressing eGFP control animals. It should be noted that two of these non-DREADD expressing eGFP control animals received the higher volume of 1µl of 1mg/ml clozapine, but this had no apparent effect on their behaviour in the task and therefore their data was included in the analysis.

Clozapine was infused bilaterally over the course of one minute using an injector

(33 gauge, 5.4mm length, 2mm centre to centre width, 2mm projection; Plastics One Inc, Roanoke, VA, USA) inserted into the intracranial cannula, controlled by a microinfusion pump (Harvard Apparatus, Holliston, MA, USA). Injectors were left in place for one minute to allow for clozapine diffusion. Animals were then returned to a holding cage for 15 minutes before testing began. This interval was chosen to allow sufficient time for the DREADDs receptors to be activated by the ligand and produce any associated behavioural effects (see section 2.3.2).

6.2.1.3.2 Behavioural testing

As described in section 2.6.3.2.

6.2.1.3.3 Analysis of behaviour

As described in section 2.6.4.

6.2.1.4 Histology

Perfusion, sectioning and immunohistochemistry were as described section 2.8. However, rather than being collected in cryoprotectant, the second series of sections was mounted directly onto gelatine subbed slides, allowed to dry overnight and then stained with cresyl violet, a Nissl stain. This was to allow for identification of cannula placement. Sections were hydrated by two-minute washes in decreasing concentrations of alcohol, followed by distilled water. Sections were then placed in cresyl violet stain for five minutes, followed by distilled water for 30 seconds. The sections were then dehydrated by two-minute washes in increasing concentrations of alcohol, followed by xylene, before being coverslipped using DPX (Thermo Fisher Scientific, Loughborough, UK).

6.2.1.5 Image capture and virus expression analysis

As described in section 2.9.

6.2.1.6 Image capture and cannula placement analysis

Cannula placement was analysed using the sections stained with cresyl violet and a Leica DM5000B microscope with a Leica DFC310 FX camera. Images were collected

from sections where disrupted cytoarchitecture in the anterior thalamic nuclei was observed, providing an indication of where the guide cannula had been positioned.

6.2.1.7 Image capture and fos expression analysis

As described in section 2.10.

6.2.2 Statistical analysis

As this experiment used two separate cohorts of animals, initial analyses were conducted to check for any effects of cohort on performance. An ANOVA was run with task stage (eight levels) as a within-subjects factor, and cohort (two levels) and group (two levels) as between-subjects factors. Provided no main effect of cohort and no interactions involving this factor and task stage were found, data were pooled across dimensions for all subsequent analyses.

The remaining statistical analyses were carried out as described in section 2.11.

6.2.3 Results

6.2.3.1 Virus expression analysis and cannula placement

There was robust expression of the virus in the anterior cingulate cortex in all animals from both groups. Figure 6.2a illustrates the cases with the smallest and largest injection sites in the inhibitory DREADD anterior cingulate cortex efferents to anterior thalamic nuclei group (iDRAccAtn). Comparable expression of the virus was observed in the control virus group. Figure 6.2b-d show representative fluorescence of mCherry (iDRAccAtn) and eGFP (control virus) in the anterior cingulate cortex of each group. Injection sites were typically located in the dorsal aspect of the anterior cingulate cortex, Cg1, with some spread into ventral anterior cingulate cortex, Cg2. As can be seen from Figure 6.2a, there was limited spread of the virus into neighbouring prelimbic or retrosplenial cortices. Meanwhile, the medial aspect of secondary motor cortex was incorporated at the injection site in approximately half of all cases. Robust virus expression was observed in the anterior thalamic nuclei (Figure 6.2f), indicating that the virus had been trafficked down the axons of neurons in the anterior cingulate cortex to their terminals.

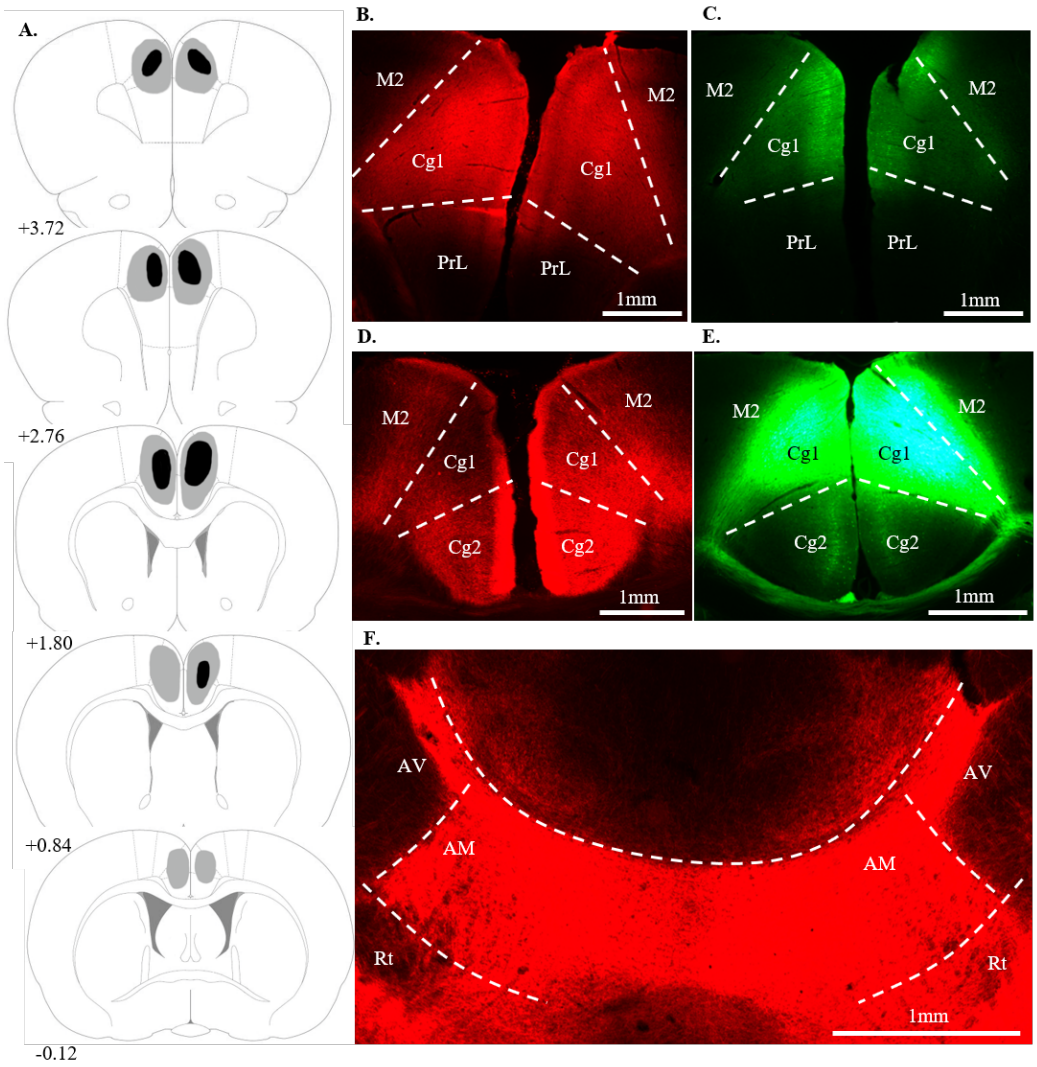

Figure 6.2. Summary of virus expression in the iDRAccAtn and control groups

A. Diagrammatic reconstructions showing the individual cases with the largest (grey) and smallest (black) expression of mCherry in the iDRAccAtn group. Numbers refer to the distance (mm) from Bregma (adapted from Paxinos & Watson, 2005). B-E. Representative examples of mCherry (B & D) and eGFP (C & E) expression in pregenual (B & C) and postgenual (D & E) anterior cingulate cortex. F. Representative example of mCherry expression in the anterior thalamic nuclei. Regions included are dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL), secondary motor cortex (M2), anteromedial thalamic nuclei (AM) anteroventral thalamic nuclei (AV) and reticular thalamic nucleus (Rt). Scale bars show approximately 1 millimetre.

Two animals had cannula tips located outside of the target region of the anterior thalamic nuclei. One was from the iDRAccAtn group and one was from the control virus group, resulting in group numbers of iDRAccAtn, N=9, control virus, N=7. Figure 6.3 illustrates the location of the cannulae tips in the remaining animals,

identified by histological analysis. The injectors had a 2mm projection, resulting in infusion locations approximately 2mm ventral to the tips of the cannulae.

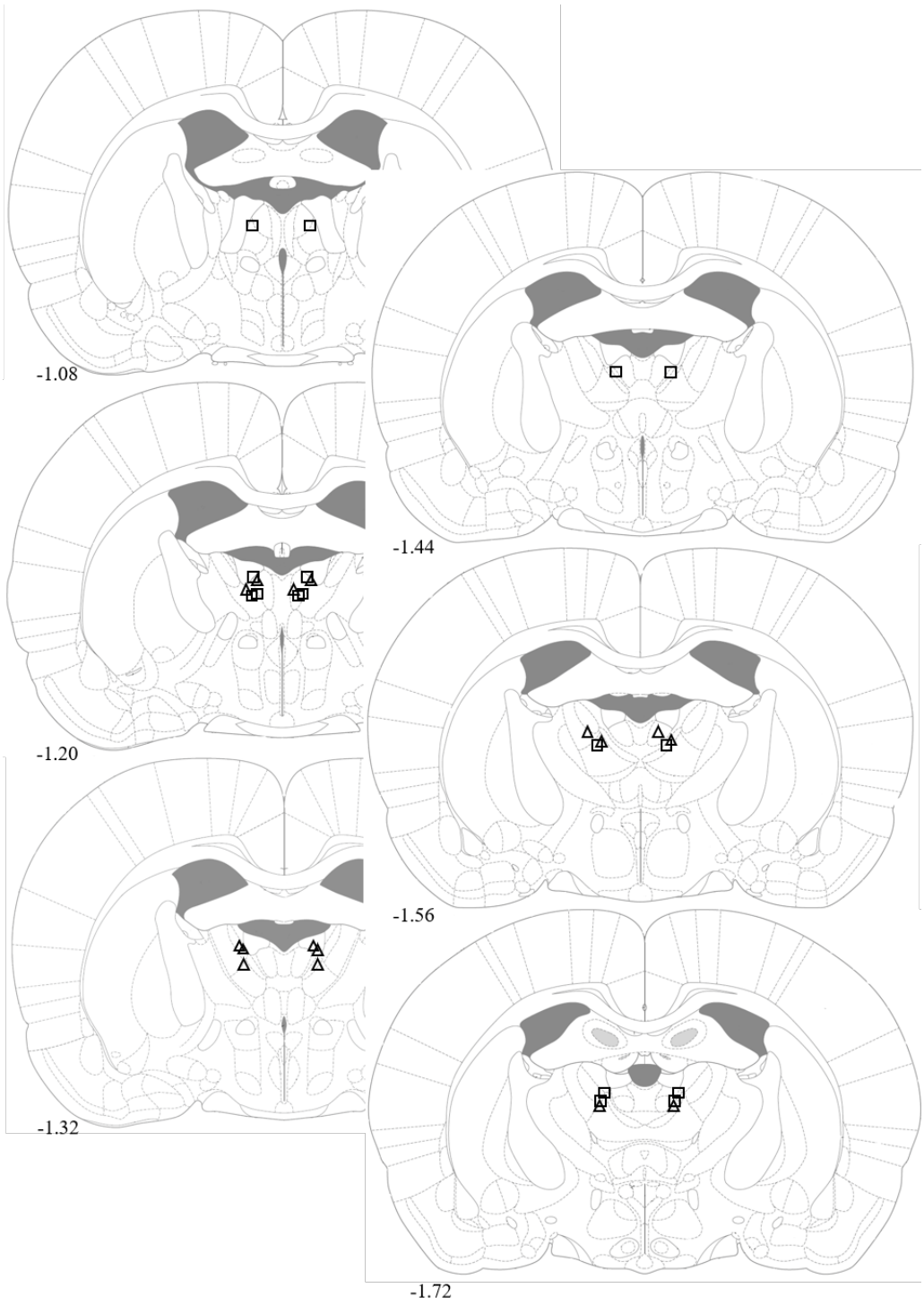

Figure 6.3. Summary of cannulae placement in the iDRAccAtn and control groups

Diagrammatic reconstructions showing the locations of tips of cannulae aimed at the anterior thalamic nuclei. Triangles represent cases from the iDRAccAtn group and rectangle represent cases from the control group. Numbers refer to the distance (mm) from Bregma (adapted from Paxinos & Watson, 2005).

6.2.3.2 Behavioural testing

As outlined in section 2.11.1.1, a series of ANOVA were conducted on the mean trials required to reach criterion at each stage of the attentional set-shifting task. Analyses of errors to criterion were also conducted and produced the same pattern of results. Therefore, only trials to criterion are reported.

As this experiment used two separate cohorts of animals, initial analyses were run to check for any effects of cohort on performance. There was no main effect of cohort ($F_{(1,12)}$=1.36, p=.27, η^2=.06), no interaction between this factor and task stage ($F_{(7,84)}$=1.57, p=.16, η^2=.07), and critically there was no three way interaction between cohort, task stage and group (F<1). Consequently, the data were pooled across cohorts for all subsequent analyses. Further analyses revealed that there were no effects of rewarded dimension (whether rats were required to attend to odour or digging media to solve the discrimination) on performance ($F_{(1,12)}$=2.23, p=.17, η^2=.11) and no interactions involving this factor and group (F<1). Therefore, the data were also pooled across these dimensions.

Two-way ANOVA (with stage [eight levels] as a within-subjects factor and group [two levels] as a between-subjects factor) revealed a significant difference in performance between the groups ($F_{(1,14)}$=5.39, p<.05, η^2=.28) and an interaction between group and task stage ($F_{(7,98)}$=2.93, p<.01, η^2=.13). Simple effects analyses revealed that the iDRAccAtn group did not differ from the control group for SD (F<1), CD ($F_{(1,14)}$=1.85, p=.20), ID3 (F<1), ID4 ($F_{(1,14)}$=1.85, p=.20), ED ($F_{1,14)}$=3.61, p=.08), or REV (F<1). However, they required more trials to reach criterion for ID1 ($F_{(1,14)}$=8.20, p<.05) and ID2 ($F_{(1,14)}$=6.87, p<.05), as displayed in Figure 6.4.

Paired samples t-tests were conducted on the difference between trials to criterion for ID1 and ID4 for each group. These analyses found that no differences between these two trials in the control group ($t_{(6)}$=-1.80, p=.12) or the iDRAccAtn group ($t_{(8)}$=-2.07, p=.07). Therefore, this measure did not find evidence for attentional set-formation in either group.

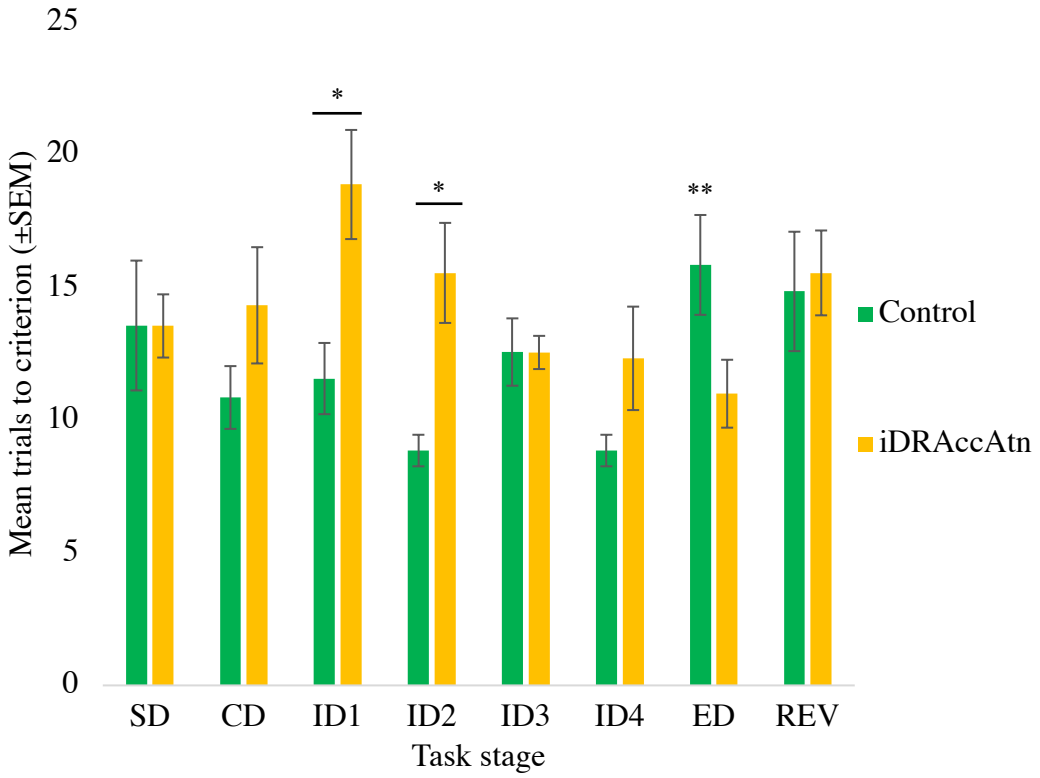

Figure 6.4. Mean (±SEM) trials to criterion on each stage of the attentional set-shifting task.

The iDRAccAtn group took significantly more trials to solve some ID stages of the task (than the control group, * p<.05). The control group took more trials to solve the ED (than the previous ID4, **p<.01), whereas the iDRAccAtn group displayed no difference in trials taken to complete these two stages.

As can be seen in Figure 6.4, the control group showed the expected increase in trials to criterion at the extradimensional (ED) shift stage. An ANOVA conducted on ID4 and ED confirmed that there was no main effect of group ($F<1$) or task stage ($F_{(1,14)}=4.49$, $p=.05$, $\eta^2=.08$), but there was an interaction between group and task stage ($F_{(1,14)}= 9.72$, $p<.01$, $\eta^2=.18$). Simple effects analyses found that while the control group took more trials to solve the ED ($F_{(1,14)}=12.19$, $p<.01$) than the preceding ID4, the iDRAccAtn group showed no difference in the number of trials taken to complete these task stages ($F<1$).

One sample t-tests were conducted on shift costs (the difference between the mean trials to criterion from the four ID stages and the ED stage [Wright, Vann, Aggleton, & Nelson, 2015]). This comparison revealed that while the control group showed a

shift cost ($t_{(6)}$=3.26, p<.05), the iDRAccAtn group showed a shift benefit ($t_{(8)}$=-3.40, p<.01). Further, an independent samples t-test revealed that there was a significant difference in shift cost between the groups ($t_{(14)}$=-4.77, p<.001), as displayed in Figure 6.5.

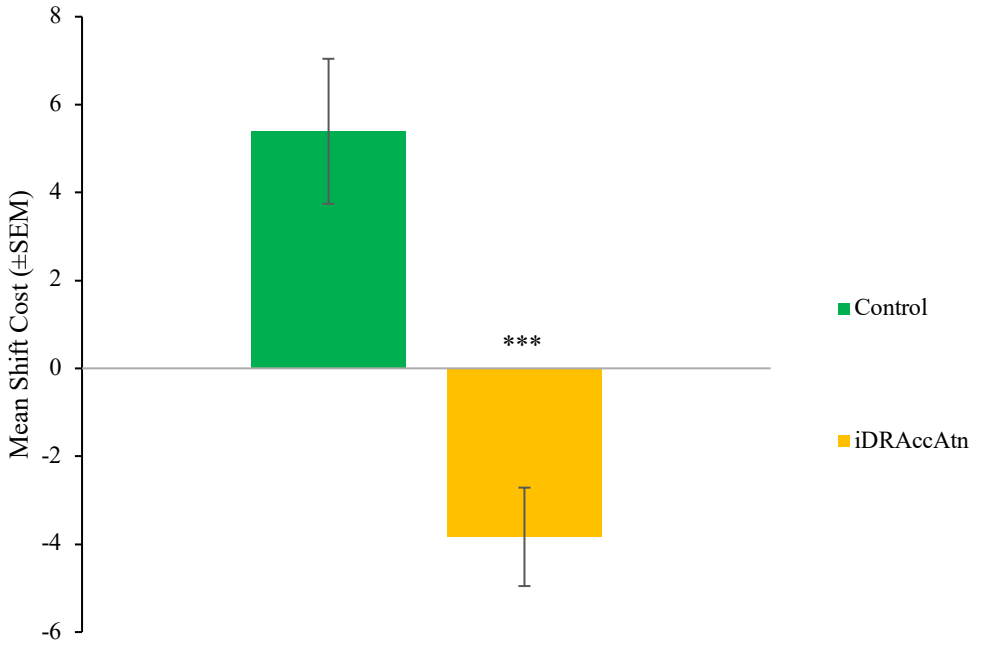

Figure 6.5. Mean shift cost (±SEM), the difference between average trials to criterion for the four ID stages and the ED stage.

There was a significant difference between the groups (***, p<.001). The control group showed a positive shift cost, taking more trials to solve the ED, and the iDRAccAtn group showed a negative shift benefit, taking fewer trials to complete the ED.

Finally, paired samples t-tests were conducted on the mean times taken for each group to complete the task. There were no differences either in total time taken to complete all discriminations ($t_{(14)}$=1.27, p=.22), or in time taken per trial ($t_{(14)}$=1.08, p=.30).

Overall, these results suggest that DREADD mediated inhibition of the anterior cingulate efferents to anterior thalamic nuclei impaired performance on the first two intradimensional shifts. Meanwhile, extradimensional set-shifting was improved relative to mean performance across the four intradimensional shifts, but not relative to the final intradimensional shift (ID4).

6.3 Follow-up attentional set-shifting task (experiment 6B)

Animals with inhibition of anterior cingulate cortex efferents to the anterior thalamic nuclei in experiment 6A did not appear to be impaired at the extradimensional shift stage (section 6.2.3.2). This is the same pattern of results that followed systemic inhibition of anterior cingulate cortex efferents in Chapter 4 (section 4.5.1), and contrasts with additional trials at extradimensional shift stage that were required by control animals.

To investigate whether this apparent advantage would transfer to perceptual dimensions other than digging media and odour, animals were challenged to a follow-up attentional set-shifting task. This was the same task used in Chapter 4, including an extradimensional shift to a perceptual dimension previously experienced as irrelevant to reward in all previous trials; the spatial location of the digging pot.

6.3.1 Methods

Animals and surgeries as described in section 6.2.1. One additional animal from the iDRAccAtn group was included in the follow-up task. This animal successfully completed the main behavioural task, but it did so after receiving a dosage of clozapine that was later found to produce adverse motor effects in other animals (see section 6.2.1.3.1). Because the clozapine dosage was then systematically lowered for all other animals, the data from the higher dosage animal was excluded from the main behavioural task. In the follow-up task however, it received the same dosage of clozapine as all other animals (section 6.2.1.3.1) and it's data was included in the analysis. This resulted in final numbers for the follow-up task of 10 in the iDRAccAtn group and seven in the control group.

Perfusion, sectioning and immunohistochemistry, image capture, virus expression and cannula placement analysis were as described in section 6.2.1. Behavioural testing followed that of Chapter 4, as described in section 4.3.1.1. Statistical analysis was conducted on the behavioural data as described in section 2.11.1.2.

6.3.2 Results

6.3.2.1 Virus expression analysis

As described in section 6.2.3.1.

6.3.2.2 Behavioural testing

A series of ANOVA were conducted on the mean trials required to reach criterion at each stage of the follow-up attentional set-shifting task, where animals were challenged to a different kind of ED shift, based on the spatial location of the pot. The first analysis revealed that there were no effects of rewarded chamber (whether the reward was located in the left or the right chamber in the first spatial discrimination) on performance and no interactions involving this factor and group ($F<1$). Consequently, the data were pooled across dimensions for all subsequent analyses.

Two-way ANOVA (with stage [four levels] as a within-subjects factor and group [two levels] as a between-subjects factor) revealed that there was no significant difference in performance between the groups ($F<1$), but there was an interaction between group and task stage ($F_{(3,45)}=4.19$, $p<.05$, $\eta^2=.15$). Simple effects analyses found that the iDRAccAtn group did not differ from controls on CD ($F<1$), ID ($F_{(1,15)}=2.10$, $p=.16$), or REVspatial ($F_{(1,15)}=4.10$, $p=.06$), but took fewer trials to complete the EDspatial ($F_{(1,15)}=9.60$, $p<.01$); as displayed in Figure 6.6.

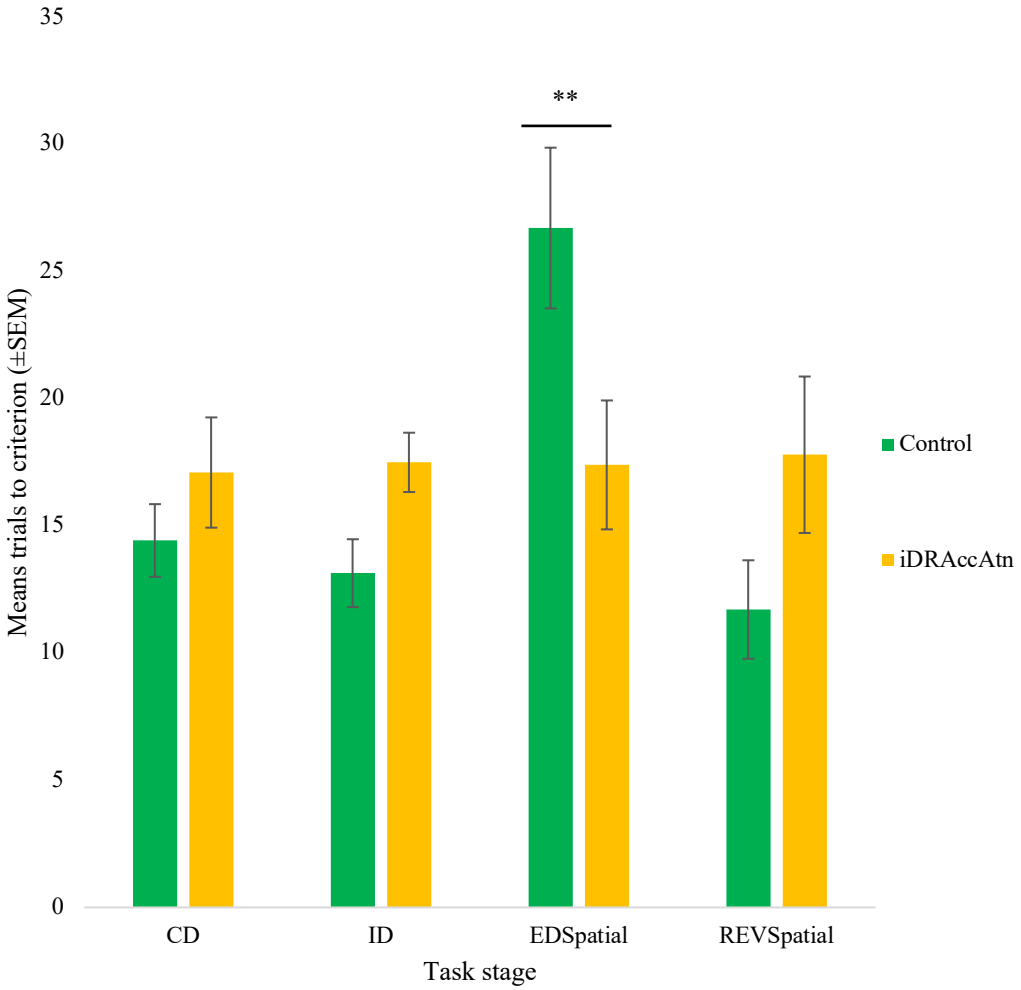

Figure 6.6. Mean (±SEM) trials to criterion on each stage of the follow-up attentional set-shifting task.

The iDRAccAtn group took significantly fewer trials to complete the spatial ED (than the control group, **p<.01)

One sample t-tests were conducted on shift costs (the difference between the ID stage and the ED spatial stage) and confirmed that while the control group showed a shift cost ($t_{(6)}$=5.61, p<.01), the iDRAccAtn group showed neither a shift cost nor a shift benefit ($t_{(9)}$=-.03, p=.97). Further, an independent samples t-test revealed that there was a significant difference in shift cost between the groups ($t_{(15)}$=-3.33, p<.01), as displayed in Figure 6.7.

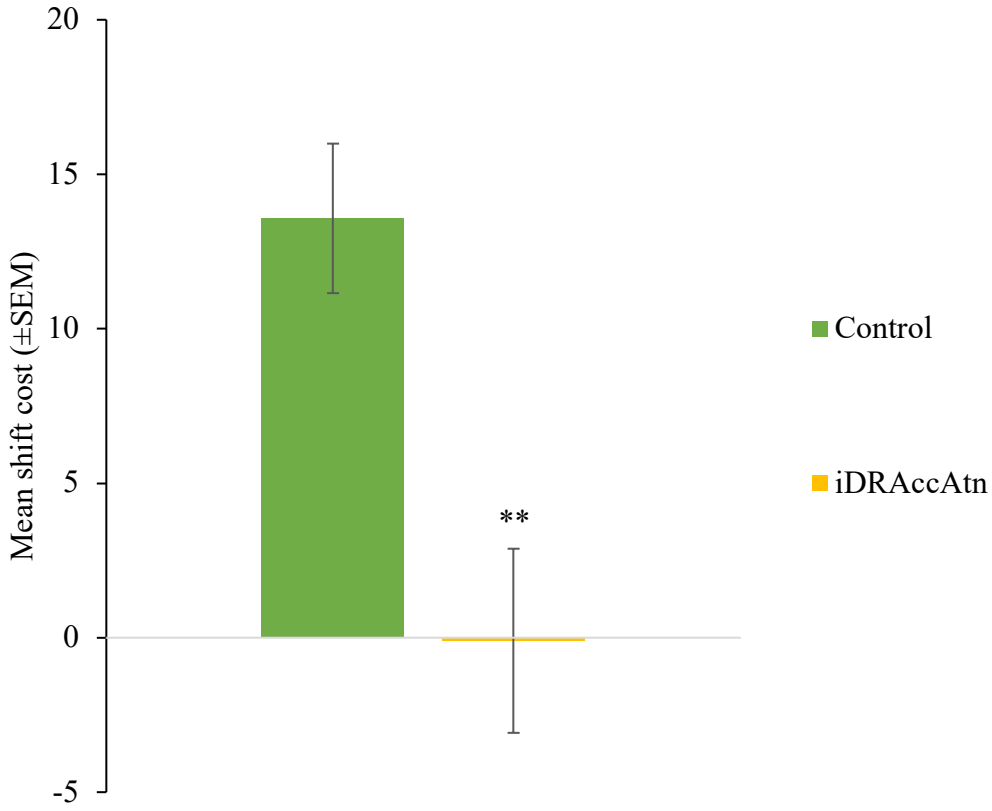

Figure 6.7. Mean shift cost (±SEM), the difference between trials to criterion for the ID stage and the spatial ED stage

There was a significant difference between the groups (**, $p<.01$). The control group showed a positive shift cost, taking more trials to solve the spatial ED.

Therefore, animals with DREADD mediated inhibition of anterior cingulate cortex efferents to the anterior thalamic nuclei did not appear to be impaired at intradimensional set-shifting during the follow-up task. However, their advantage over controls at extradimensional set-shifting persisted when shifting to a new perceptual dimension based on the spatial location of the pot.

6.4 Investigation of c-*fos* through novel environment exposure (experiment 6C)

As described in section 2.7, the behavioural experiments were followed by an investigation into the expression of c-*fos,* an indirect marker of neuronal activity. This was conducted to provide an independent measure of the influence of

DREADD-mediated anterior cingulate cortex inhibition on activity in brain regions of interest.

6.4.1 Methods

As described in section 2.7.

6.4.2 Results

6.4.2.1 Fos-positive cell counts

6.4.2.1.1 Analysis of variance

As outlined in section 2.11.2, a series of ANOVA were run on Fos-positive cell counts. As this experiment used two different cohorts of animals, initial analyses checked for an effect of cohort on performance. There was a main effect of cohort ($F_{(1,11)}$=6.69, p<.05, η^2=.30) and an interaction between this factor and region ($F_{(3,33)}$=5.91, p<.01, η^2=.09). These effects are difficult to interpret, particularly as each cohort had different proportions of animals in iDRAccAtn and control groups, such that group could be a confounding factor. Critically, however, there was no three-way interaction between cohort, region and group (F<1), meaning that the pattern of regional changes in each group did not vary by cohort. Consequently, the data were pooled across cohorts for all subsequent analyses.

First, a two-way ANOVA run on cortical regions of interest with region (three levels, dorsal anterior cingulate [Cg1], ventral anterior cingulate [Cg2] and prelimbic [PrL] cortices) as a within-subjects factor and group (two levels) as a between-subjects factor found no difference in Fos-positive cell counts between the groups ($F_{(1,13)}$=2.50, p=.14, η^2=.16) and no interaction between group and region (F<1). This is displayed in Figure 6.8.

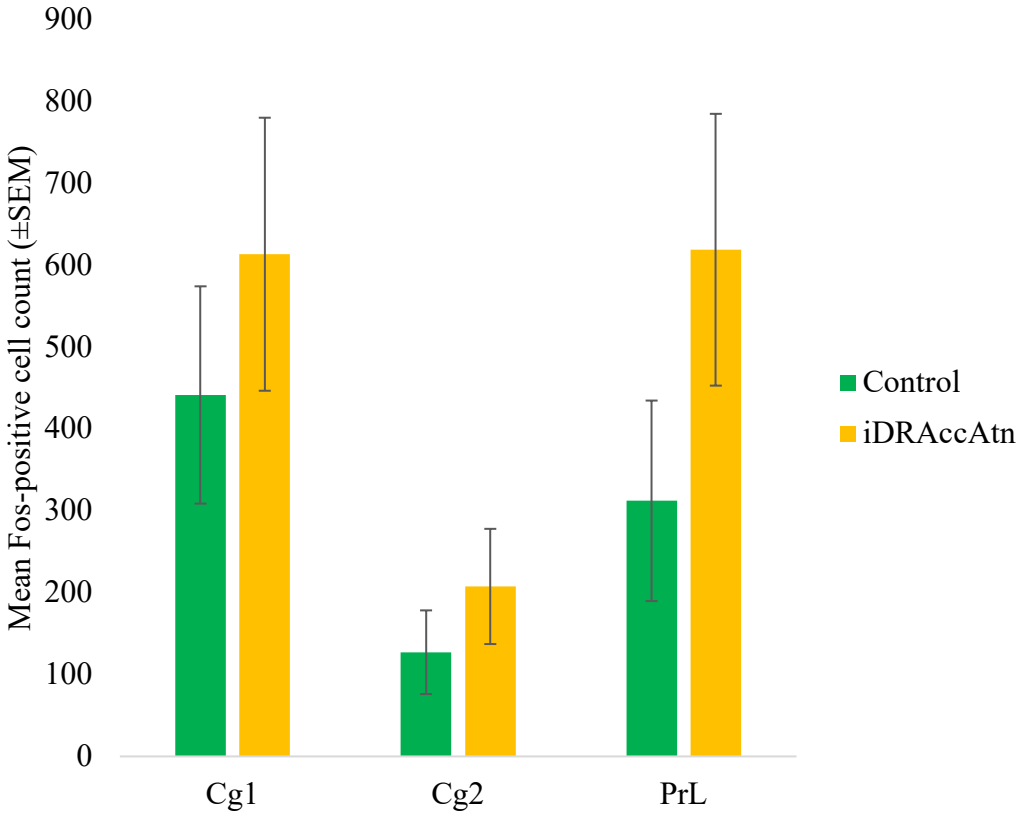

Figure 6.8. Mean (±SEM) Fos-positive cell counts in anterior cingulate (Cg1 and Cg2) and prelimbic (PrL) cortices.

There were no differences between the groups.

Next, a one-way ANOVA revealed no difference in Fos-positive cell counts in the control cortical region, secondary somatosensory cortex (S2), between the groups ($F_{(1,13)}$=3.69, p=.08, η^2=.22). This is displayed in Figure 6.9.

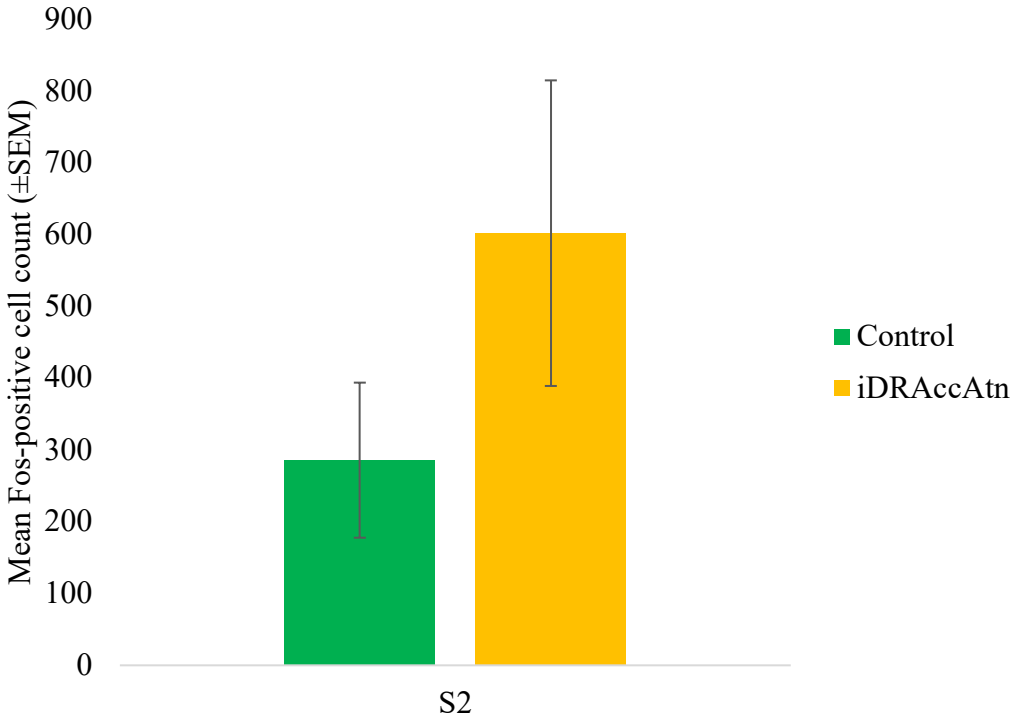

Figure 6.9. Mean (±SEM) Fos-positive cell counts in secondary somatosensory (S2) cortex.

There was no difference between the groups.

Finally, a two-way ANOVA conducted on Fos-positive cell counts in the anterior thalamic nuclei (two levels, anteromedial [AM] and anteroventral [AV]) found no difference between the groups ($F_{(1,3)}$=1.95, p=.26, η^2=.26) and no interaction between region and group ($F_{(1,3)}$=2.18, p=.24, η^2=.24). These data are displayed in Figure 6.10. However, it should be noted that there were only five animals in this analysis (three in iDRAccAtn group, two in the control group), due to damage and poor staining of brain tissue. Therefore, this result lacks both reliability and power.

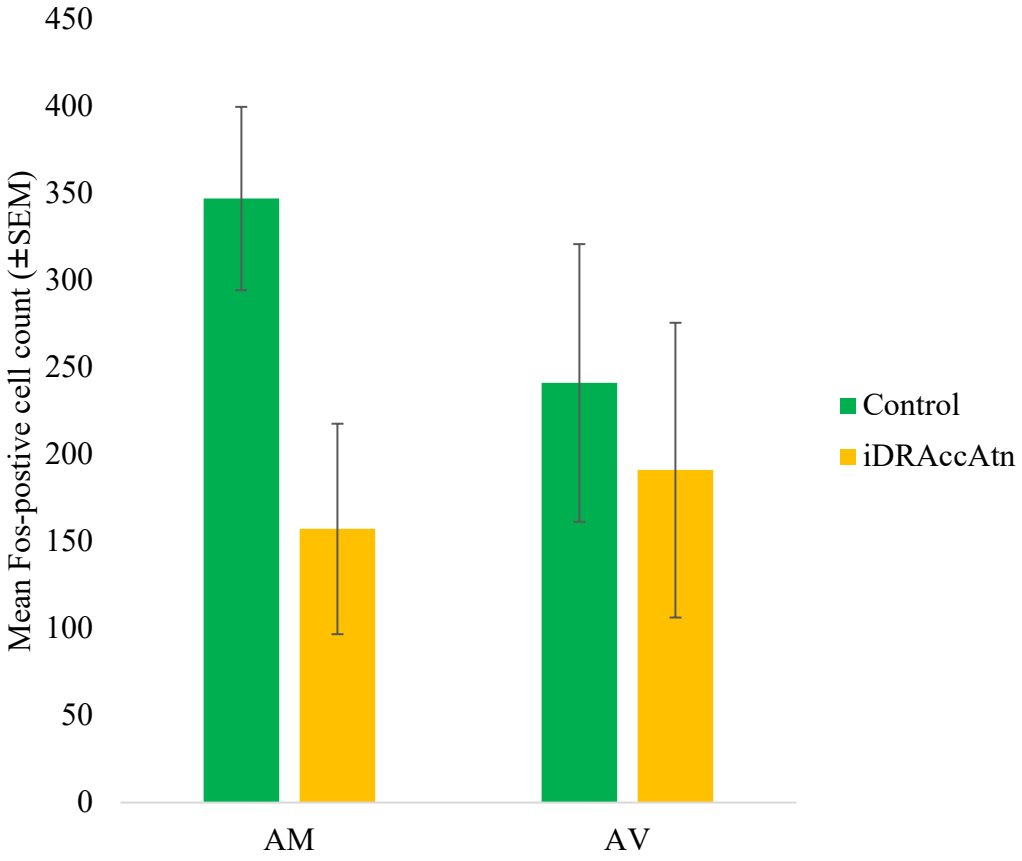

Figure 6.10. Mean (±SEM) Fos-positive cell counts in the anteromedial (AM) and anteroventral (AV) thalamic nuclei

There were no differences between the groups.

Overall, these results indicate that DREADD-mediated inhibition of anterior cingulate cortex efferents to anterior thalamic nuclei did not significantly change activity in any of the regions measured. However, there is a suggestion of increased activity in widespread cortical areas that the current study may have been underpowered to detect.

6.4.2.1.2 Pearson correlation coefficients

Pearson correlation analysis was conducted on Fos-positive cell counts for each group with Bonferroni corrections for multiple comparisons (as described in section 2.11.2). It should be noted that the anterior thalamic nuclei (AM and AV) were not included in this analysis due to insufficient numbers in the control group (N=2, as described above in section 6.4.2.1.1). In the iDRAccAtn group there were strong positive correlations between Fos-positive cell counts in: Cg1 and Cg2, S2 and Cg1,

and S2 and Cg2 (Table 12). No further correlations survived Bonferroni correction (Table 12).

Table 12. Interregional correlation matrix of Fos-positive cell counts in the iDRAccAtn group.

		Cg1	Cg2	PrL
Cg1	Pearson's r			
	p-value			
Cg2	Pearson's r	0.895		
	p-value	0.003**		
PrL	Pearson's r	0.453	0.522	
	p-value	0.259	0.185	
S2	Pearson's r	0.943	0.847	0.527
	p-value	< .001***	0.008**	0.180

R-values refer to Pearson correlation coefficients, alpha level is adjusted to $p<.008$ (Bonferroni correction). ** $p<.01$, *** $p< .001$. Regions included are dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL) and secondary somatosensory cortex (S2)

In the control group, there were no significant correlations between Fos-positive cell counts in any of the regions measured (Table 13).

Table 13. Interregional correlation matrix of Fos-positive cell counts in the control group.

		Cg1	Cg2	PrL
Cg1	Pearson's r	—		
	p-value	—		
Cg2	Pearson's r	0.038		
	p-value	0.935		
PrL	Pearson's r	0.194	0.728	
	p-value	0.677	0.064	
S2	Pearson's r	0.715	-0.070	-0.150
	p-value	0.071	0.881	0.748

R-values refer to Pearson correlation coefficients, alpha level is adjusted to $p<.008$ (Bonferroni correction). ** $p<.01$, *** $p< .001$. Regions included are dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL) and secondary somatosensory cortex (S2)

These findings indicate that DREADD-mediated inhibition of anterior cingulate efferents to anterior thalamic nuclei may have increased the interdependent activity between subdivisions of the anterior cingulate cortex (Cg1 and Cg2), and between these regions and the secondary motor cortex.

6.5 Discussion

The results of Chapters 4 and 5 implicate the anterior cingulate in attentional set-shifting in the rat, particularly when a consistent stimulus class is associated with reward. Previous research has found the anterior thalamic nuclei to be involved in these same processes (Wright et al., 2015), and these nuclei are densely interconnected with the anterior cingulate cortex (Chapter 3). The current study disrupted projections from anterior cingulate to the anterior thalamus by expressing inhibitory DREADDs in the former and infusing the ligand clozapine in the latter (iDRAccAtn). IDRAccAtn rats were then tested on two attentional set-shifting tasks involving intradimensional and extradimensional shifts, followed by an investigation into c-*fos* that provided a measure of iDRAccAtn impact on cellular activity in the brain.

6.5.1 Standard attentional set-shifting task

The first task included a series of four successive intradimensional shifts. The iDRAccAtn group took more trials than the control group to solve the initial two intradimensional discriminations, suggestive of an impaired ability to focus attention on a consistently rewarded stimulus dimension. This echoes the results of Chapter 4, where systemic inhibition of the anterior cingulate cortex (iDREADD group) similarly impaired performance at several of the intradimensional shifts (section 4.5.1).

However, while the iDREADD group showed no improvement across intradimensional shifts, the performance of iDRAccAtn animals was comparable with controls by the third (ID3) intradimensional shift. They did not, however,

statistically complete the final intradimensional discrimination (ID4) in fewer trials than the first (ID1), which is taken to be an indicator of successful attentional set-formation. Together these mixed results suggest that the iDRAccAtn group may have formed a partial attentional set, characterised by impairment of initial attentional set acquisition.

Nonetheless, whereas the control group showed the classic 'shift cost' when challenged to an extradimensional shift, indicating orientation of attention within the relevant stimulus dimension, the iDRAccAtn group showed a shift benefit. That is, they took fewer trials to complete this stage than the mean of the preceding four intradimensional stages. Again, this aligns with the results of the iDREADD group (section 4.5.1) and suggests that iDRAccAtn animals were biased towards poor predictors of reward, facilitating extradimensional shift performance when contingencies changed, and a previously poor predictor became relevant to reward.

6.5.2 Follow-up attentional set-shifting task

As in Chapter 4 (iDREADD group, section 4.5.2), iDRAccAtn animals were challenged to a follow-up attentional set-shifting task to determine whether their extradimensional shift advantage would transfer to a new perceptual dimension, based on the spatial location of the digging pot. The task also included an initial compound discrimination and single intradimensional shift, where there were no differences in performance between the groups. This, again, replicates the performance of iDREADD animals (section 4.5.2).

It was argued in Chapter 4 (section 4.5.2) that the lack of iDREADD impairment at the compound and intradimensional stages of the follow-up task may have reflected the fewer number of intradimensional shifts than in the main behavioural task. The justification was that the iDREADD group were only impaired relative to controls at the later intradimensional stages (ID2, ID3 and ID4) in the main task. This was proposed to reflect a deficit in attentional set-formation (while control animals improve over a series successive intradimensional shifts, iDREADD animals did not, thus the disparity in performance between the groups increased as the intradimensional stages progressed, see section 4.5.2). However, this explanation

does not fit the iDRAccAtn data as these animals were impaired relative to controls at the first intradimensional shift (ID1) of the main behavioural task. Rather, the results indicate that selective inhibition of projections from the anterior cingulate cortex to the anterior thalamic nuclei may not result in complete blocking of intradimensional set-formation.

Nevertheless, mirroring the performance of iDREADD animals once more, the iDRAccAtn advantage over controls at extradimensional set-shifting was replicated in the follow-up task. Whereas the control group showed a shift cost when required to start responding to spatial location to solve the discrimination, the iDRAccAtn group required no more trials. This provides a further indication that iDRAccAtn animals were paying inappropriate attention to stimulus dimensions that were, until now, unreliable predictors of reward.

6.5.3 Investigation of c-*fos* through novel environment exposure

The novel environment exposure experiment revealed that there were no significant differences in c-*fos* counts in any of the regions measured. However, closer inspection of the data reveals a suggestion of higher counts in all cortical regions measured (dorsal anterior cingulate cortex [Cg1], ventral anterior cingulate cortex [Cg2], prelimbic cortex [PrL] and secondary sensory motor cortex [S2]) in the iDRAccAtn group Meanwhile, the number of animals included in counts of the anterior (anteromedial [AM] and anteroventral [AV]) thalamic nuclei was too low to gainfully interpret differences between the groups.

The non-significant cortical increases in the iDRAccAtn group is counterintuitive given the proposed actions of the inhibitory DREADD hM4Di; a reduction in neuronal firing and thus cellular activity (Rogan & Roth, 2011)(see also sections 2.3.1, 4.5.3). However, this observation is consistent with the results of Chapter 4, where systemic activation of inhibitory DREADDs in the anterior cingulate cortex (iDREADD) increased c-*fos* expression in this region and its efferent targets (section 4.4.2.1.1). A tentative suggestion, therefore, is that infusions of clozapine into the anterior thalamic nuclei (iDRAccAtn) may have spread to and inhibited the terminals of DREADD-expressing anterior cingulate neurons projecting to the adjacent

thalamic reticular nucleus. As described in Chapter 4 (section 4.5.3), inhibiting the actions of this inhibitory structure (Zikopoulos & Barbas, 2006) could lead to disinhibition in reciprocal thalamic pathways, leading to cortical c-*fos* increases.

To determine whether iDRAccAtn cortical c-*fos* increases are meaningful, however, future research with a larger number of animals would be required. There are reasons to suspect that the current experiment may have been underpowered to detect real differences in cortical c-*fos* activity. This experiment had fewer subjects (N=15) than the iDREADD experiment (N=20, see section 4.4) and, due to the more selective nature of the iDRAccAtn intervention, the effect sizes of regional differences may be smaller. Furthermore, the use of two separate cohorts likely increased between-subject variability, making differences between groups more difficult to detect. Indeed, the observation of significant differences between the cohorts highlights this potential confound. Meanwhile, due to poor tissue staining and damage, the number of subjects with counts in the anterior thalamic nuclei was too low (N=5) to meaningfully interpret. Accordingly, there is a clear need for further research to determine the effect of iDRAccAtn on activity in this region

The results of the correlation analysis support the notion of real increases in cortical activity in the iDRAccAtn group. There were strong positive correlations between Fos-positive cell counts between subregions of the anterior cingulate cortex, Cg1 and Cg2, and between both of these areas and the secondary somatosensory cortex in this group. Meanwhile, the control group did not exhibit any significant correlations between any of the cortical regions measured. This indicates a potential increase in interdependent regional activity in the iDRAccAtn group that, notably, was also observed in the iDREADD group (section 4.5.3).

However, the existence of strong positive correlations between the anterior cingulate and secondary somatosensory cortex is somewhat unexpected. The latter was counted as a control region, on the basis of an absence of known connectivity between this region and the anterior cingulate cortex. For the same reason, the increase in Fos-positive cell counts in somatosensory cortex in the iDRAccAtn, although not significant, is surprising. These findings speak both to the extent of the

downstream network effects of DREADDs, and to the arbitrariness of determining a region a 'control'; given the currently rudimentary understanding of brain connectivity.

6.5.4 Summary and implications

In striking similarity to the effects of disrupting activity in each region independently, interrupting anterior cingulate efferents to the anterior thalamic nuclei impaired aspects of intradimensional set-shifting and facilitated extradimensional set-shifting. These results provide compelling evidence that normal activity between the rat anterior cingulate cortex and anterior thalamic nuclei is involved in focusing attention on reliable reward predictors. In the absence of proper functioning of these efferents, excessive attention appears to be directed towards unreliable reward predictors. Such a tendency subsequently manifests as advantageous when contingencies change, i.e. in extradimensional shifts. Meanwhile, there was some evidence that the iDRAccAtn group retained some ability to form an attentional set, suggesting that anterior cingulate cortex efferents to anterior thalamic nuclei may have a more selective role in the initial attentional set acquisition.

The proof-of-principle c-*fos* study failed to find significant differences in cellular activity by this measure between the groups. However, there was a hint of widespread cortical increases, not restricted to the anterior cingulate and its known efferent regions, in the iDRAccAtn group. There also appeared to be an increase in interdependent cortical activity in this group. Such increases draw parallels with those observed in the iDREADD group (section 4.5.3) and bolster the suggestion that the inhibitory DREADD hM4Di can, counterintuitively, increase cellular activity. The underlying mechanism of action is unclear, but may involve disinhibition and subsequent unregulated hyperexcitability in reciprocal thalamic pathways (see also section 4.5.3).

7 General Discussion

7.1 Overview

The anterior cingulate cortex and the anterior thalamic nuclei have dense, reciprocal connections, many of which travel in the cingulum bundle. There has been a surge of interest in the cingulum, driven by human neuroimaging literature detailing widespread contributions to function and dysfunction; yet these methods are critically limited by an inability to isolate specific connections in white matter. Accordingly, the first aim of this thesis was to use contemporary viral tract tracing techniques to examine this subgroup of cingulum fibres in the rat. The findings confirmed and extended previous work by demonstrating that connections between the anterior cingulate cortex and the anteromedial thalamic nucleus, but not the anteroventral thalamic nucleus, form a major component of the anterior cingulum bundle.

The next aim of this thesis was to determine the function of these interconnections, a topic that has not been investigated previously. There is existing evidence implicating both the anterior cingulate cortex (Ng et al., 2007) and the anterior thalamic nuclei (Wright et al., 2015) in intradimensional set-shifting, the ability to attend to a stimulus dimension that reliably predicts reward. In this thesis, a series of DREADD manipulations of the anterior cingulate cortex confirmed this region's role in intradimensional set-shifting and provided novel evidence that efferents to the anterior thalamic nuclei critically contribute to this attentional capacity.

Meanwhile, in stark contrast to the effects of damage to other medial prefrontal regions (Birrell & Brown, 2000), manipulations of the anterior cingulate cortex facilitated performance when animals were required to respond to a stimulus dimension that was previously an unreliable predictor of reward. This provides striking evidence for the existence of two competing attentional systems in the brain (Pearce & Mackintosh, 2010). One, involving the anterior cingulate cortex and its connections to the anterior thalamic nuclei, mediates attention to reliable predictors of outcomes (Mackintosh, 1975), while another mediates attention to unreliable, partially reinforced predictors (Pearce & Hall, 1980).

7.2 A complex network of cingulum fibres connects the anterior thalamic nuclei and the cingulate cortex

The cingulum bundle is a highly complex fibre pathway comprising many discrete subpopulations of fibres (see section 1.3). The first aim of this thesis was to provide a contemporary anatomical reappraisal of fibres connecting the anterior thalamic nuclei to the cingulate cortices, known to form a major component of the tract across species (Mufson & Pandya, 1984; Domesick, 1970, section 1.3.4). These details matter because there is growing appreciation, largely driven by human tractography research (section 1.4.3.2.2), that the function of the cingulum changes along its length. A proper understanding of functional subdivisions must be underpinned by knowledge of the changes in regional connectivity such subdivisions provide.

Consistent with previous research, Chapter 3 found that the anteromedial (AM) thalamic nucleus is closely associated with the anterior cingulate cortex (Shibata, 1993b; Van Groen et al., 1999). The experiments in this chapter confirmed that many efferents course forward from this nucleus to wrap around the genu of the corpus callosum, turning caudally to join the dorsal cingulum before terminating in anterior cingulate cortex. Consequently, many fibres in the subgenual and anterior dorsal subdivisions of the cingulum are projections from AM to the anterior cingulate cortex.

This finding has implications for a wealth of human literature. Firstly, anterior cingulotomies for psychiatric illness (section 1.4.3.1) will have disrupted many fibres connecting AM to the anterior cingulate cortex. Such surgeries typically provide relief through a lessening of attention to negative states, often observed alongside selective deficits in attention and cognitive control in executive tasks (Cohen, Kaplan, Moser, et al., 1999; Cohen et al., 2001; section 1.4.3.1). Further, anterior and dorsal cingulum microstructure changes are a consistent feature of a range of psychiatric disorders (such as schizophrenia, ADHD, depression, PTSD, OCD, and ASD, see section 1.4.3.2.1, Table 2) characterised by altered emotional and/or executive function, while anterior cingulum integrity further correlates with these functions in healthy populations (section 1.4.3.2.2). The results from Chapter

3, therefore, highlight a potential contribution of AM-anterior cingulate interconnectivity to such function, and dysfunction, along with other frontal fibres that comprise the cingulum bundle at this level.

Chapter 3 also found support for the view that the anteroventral (AV) thalamic nucleus is more closely associated with the retrosplenial cortex (Shibata, 1993b; Van Groen & Wyss, 1995) than the anterior cingulate cortex, pointing to a potential segregation of information between AM and AV. Indeed, Chapter 3 revealed that only a distinct subdivision of AV, the dorsomedial part (AVDM), receives input from the anterior cingulate cortex. This indicates that there may be further segregated functionality *within* individual anterior thalamic nuclei (Wright, Vann, Erichsen, O'Mara, & Aggleton, 2013). AVDM appears to have a connectivity profile more closely aligned with AM, participating in frontal functional circuits involving the anterior cingulate cortex, described above. The ventral lateral part of AV (AVVL), meanwhile, shares more connectivity the retrosplenial cortex; a functional node of hippocampal-parahippocampal circuitry associated with spatial memory (Bubb et al., 2017).

A related finding from Chapter 3 is that efferents from AV, including those to retrosplenial cortex, do not all travel leave the thalamus anteriorly as described by Domesick (1970). Rather, the majority favour a more direct route to the cortex, meaning that subgenual and anterior dorsal subdivisions of the cingulum contain relatively few fibres from AV. Instead, more AV efferents are present in the caudal dorsal and parahippocampal cingulum. Again, this can help to inform interpretation of human literature, where these caudal cingulum subdivisions are associated with memory function, and dysfunction (section 1.4.3.2). Interestingly, this more direct route of anterior thalamic projections, bypassing the subgenual and anterior cingulum, has been described previously in the monkey (Mufson & Pandya, 1984). Therefore, the results from Chapter 3 depict a stronger homology of anterior thalamic – cingulate connectivity between species than described previously (Domesick, 1970), supporting the cross-species translational value of the research described in this thesis.

Finally, the complexity of the anterior thalamic-cingulate fibre trajectories elucidated in Chapter 3 indicates that attempting to disconnect these structures by conventional lesion methods would be a near-impossible task. While anterior thalamic efferents (particularly those from AV) join the cingulum all along its length to reach the cingulate cortices, the return projections are even more diffuse. Few join the sagittal course of the cingulum at all, instead crossing ventrally through the white matter to reach anterior thalamic targets. This might explain why cingulum bundle lesions often have such slight effects in rats, particularly on tests of spatial memory which likely involve AV connectivity (see section 1.4.1, Table 1). The difficulty of disrupting cingulate-anterior thalamic fibres using lesions further highlights the value of utilising DREADD methodology to manipulate the connectivity between these structures (Chapter 6).

7.3 Manipulations of the anterior cingulate cortex affect attentional set-shifting

7.3.1 Overview

Chapters 4, 5 and 6 used three distinct DREADD methodologies to manipulate activity in the anterior cingulate cortex of rats and tested them on an attentional set-shifting task. Chapter 4 used inhibitory DREADDs (iDREADD) to downregulate anterior cingulate activity systemically. Chapter 6 combined inhibitory DREADDs with local injections of the ligand clozapine in the anterior thalamic nuclei (iDRAccAtn) to selectively disrupt the activity of neurons projecting from the anterior cingulate to the anterior thalamic nuclei. Meanwhile, Chapter 5 used excitatory DREADDs (eDREADD) to systemically upregulate anterior cingulate activity. Table 14 provides a summary of the effects of these manipulations on attentional set-shifting, as revealed by the experiments in these respective chapters.

Table 14. Summary of anterior cingulate DREADD manipulation effects on attentional set-shifting

	iDREADD	iDRAccAtn	eDREADD
Simple discriminations	√	√	√
Intradimensional discriminations	X	X	√+
Attentional set-formation	X	?	?
Extradimensional shifts	√+^{R}	√+^{R}	√+
Reversals	√	√	√+

Table provides an overview of the effects of different anterior cingulate DREADD manipulations on attentional set-shifting: iDREADD, inhibition; iDRAccAtn, inhibition of efferents to anterior thalamic nuclei; eDREADD, excitation. Symbols: √, no effect; X, impairment; √+, enhancement; ^{R}, effect replicated in follow-up experiment; ?, effect unclear based on current evidence. Note the pattern of performance was consistent in each of the control groups (no interaction between control group and task stage).

Initially, it is clear from Table 14 that none of the anterior cingulate manipulations had an effect on the animals' ability to acquire an initial simple discrimination; where one of two stimuli from the same perceptual dimensions signalled reward. This is consistent with the results of lesions of both the anterior cingulate cortex (Ng et al., 2007) and the anterior thalamus (Chudasama et al., 2001; Wright et al., 2015), and indicates that the actions of the anterior cingulate cortex, including its interconnectivity with the anterior thalamic nuclei, are not intrinsically involved in initial discrimination learning.

7.3.2 Intradimensional shifts

All three anterior cingulate manipulations dramatically changed performance at intradimensional (ID) discriminations, where animals must respond to the stimulus dimension (media/odour) that was relevant when solving the previous discrimination. Both the iDREADD and iDRAccAtn groups were slower to acquire several of the ID stages than controls (Table 14). Again, this supports previous observations that lesions to the anterior cingulate cortex (Ng et al., 2007) and anterior thalamic nuclei (Wright et al., 2015) impair intradimensional set-shifting. It further provides novel evidence that the anterior cingulate cortex and the anterior thalamic nuclei interact to provide this shared functionality. Meanwhile, the reversal

of this profile in the eDREADD group (better performance than controls, Table 14) furthers the notion that the anterior cingulate is involved in processes supporting intradimensional set-shifting.

Progressive improvement across intradimensional shifts is thought to signify successful attentional set-formation, whereby animals increasingly orient their attention to the relevant stimulus dimension (Chase et al., 2012; Tait et al., 2018). The role of the anterior cingulate cortex in such a process has not been investigated previously in rodents and, as can be seen in Table 14, evidence from the experiments in this thesis is somewhat mixed. Neither the iDREADD nor the iDRAccAtn group showed statistical evidence of attentional set-formation, implicating both the anterior cingulate and its efferents to the anterior thalamic nuclei in this process. However, the iDRAccAtn group did show an improvement across ID stages, albeit a non-statistical difference. This evidence indicates that anterior cingulate efferents to the anterior thalamic nuclei may be involved in some aspects of initial attentional set acquisition, but the absence of such activity may not completely abolish the ability to form an intradimensional set. Future research with larger groups of animals, and thus more power, would be necessary to determine conclusively whether iDRAccAtn animals retain the ability to form an attentional set.

On the other hand, the eDREADD group acquired the initial intradimensional discriminations so quickly that attentional set-formation (i.e. progressive improvement across successive IDs) could not be demonstrated statistically. There are two possible interpretations of this result. It could represent rapid orientation of attention within the relevant stimulus dimension. Conversely, it could signify the immediacy of an alternate effective strategy, such as win-stay lose-shift (Evenden & Robbins, 1984), underpinned by learning about specific exemplar pairings without attending to stimulus dimensions (see section 5.5.1). Therefore, it is not clear whether this particular finding supports a role of the anterior cingulate cortex in attentional set-formation. Although not conclusive, the observation that the eDREADD advantage over controls was lost in the follow-up task (when animals were challenged to discriminations designed to make specific exemplar learning more challenging, experiment 5B, section 5.3), indicates that eDREADD animals

may have been using such a strategy. Alternatively, during this follow-up task, the performance of the control group may have simply caught up with that of the eDREADD group (see also 5.5.2).

7.3.3 Extradimensional shifts

All three anterior cingulate DREADD manipulations markedly changed performance at extradimensional set-shifting (Table 14). That is, when the animal must solve a discrimination by attending to the stimulus dimension that was irrelevant in the preceding discrimination. Both inhibitory DREADD groups (iDREADD and iDRAccAtn) were facilitated at this type of shift relative to controls, indicating that extradimensional shifting does not require normal activity in anterior cingulate or its efferents to the anterior thalamic nuclei. In fact, disrupting global anterior cingulate activity (iDREADD) appeared to allow the mechanisms underlying extradimensional set-shifting to dominate, allowing this discrimination to be solved in fewer trials than the intradimensional stages.

Akin to the actions of inhibitory DREADDs, excitatory DREADDs (eDREADD) also facilitated extradimensional set-shifting, relative to the performance of control animals. This contrasts with the effect of eDREADDs on intradimensional set-shifting, where the profile of the inhibitory groups (iDREADD and iDRAccAtn) was reversed (Table 14, section 7.3.2). Nonetheless, the same possible explanations of eDREADD facilitation at intradimensional shifts (section 7.3.2) could also apply to their facilitation at extradimensional shifts. The result could be consistent with a role of the anterior cingulate in focusing attention on the relevant dimension. In this respect, the extradimensional facilitation (relative to controls) could be underpinned by a rapid reorientation of attention to the now relevant, but previously irrelevant, stimulus dimension. Alternatively, the eDREADD group could be solving discriminations using another strategy, such as win-stay lose-shift (Evenden & Robbins, 1984, see also section 6.5.1).

7.3.4 Reversals

Finally, as displayed in Table 14, both the iDREADD and iDRAccAtn manipulations did not affect reversals, where animals must respond to the previously incorrect

stimulus from the same dimension as was relevant in the previous discrimination. This is consistent with previous findings that anterior cingulate and anterior thalamic lesions both spare reversal learning (Chudasama, Bussey & Muir, 2001; Ng et al., 2007; Wright et al., 2015).

Somewhat unexpectedly, there was some evidence of reversal facilitation in the eDREADD group, potentially implicating the anterior cingulate cortex in reversal learning. However, there was an overall improvement in this group, evident across all three discrimination types; intradimensional shifts (section 7.3.2), extradimensional shifts (section 7.3.3) and reversals (Table 14). Further, given different baseline levels of performance between the groups, differences in reversal performance are difficult to interpret. These observations, combined with the lack of deficits in both inhibitory groups, suggests that the anterior cingulate is not specifically involved in reversal learning. Instead, there is strong evidence implicating the orbitofrontal cortex in the processes underpinning this type of discrimination (Chudasama & Robbins, 2003; Rushworth et al., 2007; section 1.5.1.4).

7.3.5 Conclusions

Overall, the evidence from these experiments provide strong support for a role of the anterior cingulate cortex and its efferents to the anterior thalamic nuclei in intradimensional set-shifting. Normal anterior cingulate function underpins the ability to form an attentional set, though it is not clear whether its interactions with the anterior thalamic nuclei are essential to this process. Meanwhile, manipulations of the anterior cingulate cortex not only preserve, but facilitate, extradimensional shifts. This result provides a striking double dissociation with the results of lesions of other medial prefrontal areas (mainly prelimbic, but also infralimbic, cortex), which spare intradimensional shifts but impair extradimensional shifts (Birrell & Brown, 2000; Tait et al., 2014).

Meanwhile, it appears that the anterior cingulate does not play a critical role in acquisition of simple discriminations or reversals, an observation that is consistent with previous demonstrations that these processes are independent of

intradimensional and extradimensional set-shifting (Roberts et al., 1994). In turn, this further dissociates the role of the anterior cingulate from that of the orbitofrontal cortex, where lesions result in reversal deficits (Chase et al., 2012), without *necessarily* affecting either intradimensional or extradimensional shifts (Dias, Robbins, & Roberts, 1996b). Together, these results indicate that attentional set-shifting relies upon a number of different processes, with distinct neural underpinnings. The implications of this for theories of attention are discussed in the following section.

7.4 The anterior cingulate cortex, in conjunction with the anterior thalamic nuclei, mediates attention to reliable reward predictors

7.4.1 Implications for theories of attention

As elucidated in the previous section (7.3), the experiments in this thesis found clear evidence that the anterior cingulate cortex, including its efferents to the anterior thalamic nuclei, is involved in intradimensional set-shifting. 'Normal' intradimensional set-shifting, as observed in control rats, is characterised by a successive improvement across intradimensional stages. According to Mackintosh's (1975) classic theory of attention, correlation with reinforcement determines how much attention a stimulus receives. In the case of intradimensional shifts, observations that rats learn a discrimination faster based on the previously relevant dimension reflects a variant of the 'transfer along a continuum' (Lawrence, 1949, 1952) effect. That is, subjects learn to attend to particular features of a stimulus that best predict an outcome (such as odour predicting reward) and transfer those attentional biases to similar stimuli (Pearce & Mackintosh, 2010).

Mackintosh (1975) further denotes that to behave optimally subjects must stop responding to stimuli, and stimulus dimensions, that have a history of irrelevance (Pearce & Mackintosh, 2010). That is, repeated experience of non-association between a stimulus and an outcome should result in 'learned irrelevance' and reduced learning about that stimulus by selective attention. The typical extradimensional shift cost, observed in control animals, supports the existence of

such a process. According to this theory (Mackintosh, 1975; Pearce & Mackintosh, 2010), animals take longer to solve an extradimensional shift because they had previously established the now relevant stimulus dimension as irrelevant and inconsequential to reward and had thus reduced attention to it.

So far then, the role of the anterior cingulate cortex, and its efferents to the anterior thalamic nuclei, in attentional set-shifting appears to be largely consistent with Mackintosh's (1975) theory of attention. Under such a framework, the anterior cingulate would be involved in directing attention towards relevant stimulus dimensions, that reliably predict reward, and/or away from irrelevant stimulus dimensions. It follows that disruption of this process would impair intradimensional shifts and attentional set-formation, resulting in abolition of the intradimensional-extradimensional shift cost; as observed in the iDREADD and iDRAccAtn groups (see section 7.3).

However, there is another classic theory of how attention is allocated. Pearce-Hall (1980) argue that stimuli that uniquely signal reward lose associability because once a stimulus has been established as a reliable predictor of an outcome, learning about that stimulus is 'complete' and no longer warrants attentional resources. Rather, Pearce-Hall (1980) suggest that a better use of attention is to focus on inconsistent, or partially reinforced, stimuli, where a stimulus-outcome association has yet to be established. There is considerable evidence for the existence of such a mechanism (Pearce & Mackintosh, 2010), including demonstrations of new discriminations being learned more quickly when based on a previously partially reinforced stimulus, than when based on one that was consistently rewarded (Haselgrove, Esber, Pearce, & Jones, 2010).

On face value, the mechanisms proposed by Mackintosh (1975) and Pearce-Hall (1980) appear to directly contradict each other. The former suggests that task relevant, reliable predictors of outcome receive more attention, whereas the latter predicts that unreliable, partially reinforced stimuli command attentional control. As aforementioned, the experimental results from this thesis support the role of the anterior cingulate cortex in an attentional mechanism that seems closes aligned with

Mackintosh (1975). However, seemingly paradoxically, the results also support the existence of a mechanism mediating attention to unreliable predictors of outcome, as proposed by Pearce-Hall (1980). This is because the intradimensional-extradimensional shift cost was not just abolished in the iDREADD and iDRAccAtn groups, it was reversed. These animals showed a shift *benefit*, solving the extradimensional shift in fewer trials than the intradimensional discriminations.

It would appear, therefore, that in the absence of normal anterior cingulate function (iDREADD and iDRAccAtn) animals paid more attention to stimulus dimensions that were irrelevant, inconsistent reward predictors during the intradimensional stages. This, in turn, manifests an advantage when contingencies change and a previously inconsistent stimulus dimension predicts reward (extradimensional shift). Consequently, the experiments in this thesis appear to simultaneously support the existence of a mechanism whereby attention is focused on the most reliable predictors of reward (Mackintosh, 1975), underpinned by the activity of the anterior cingulate and its efferents to the anterior thalamic nuclei, and a mechanism where attention is focused on unreliable reward predictors (Pearce & Hall, 1980). This latter mechanism may be supported by activity in medial prefrontal areas, such as prelimbic cortex, given that lesions to this region produce roughly the inverse pattern of results to that of anterior cingulate inhibition described here (Birrell & Brown, 2000; Bissonette et al., 2013).

Indeed, recent reviews have converged upon the conclusion that both attentional theories marshal substantial support, such that the existence of neither mechanism can be discounted (Le Pelley, Haselgrove, & Esber, 2012, Haselgrove et al., 2010). Consequently, several so-called hybrid theories have been suggested (Haselgrove et al., 2010; Le Pelley et al., 2012; Pearce & Mackintosh, 2010) that, whilst differing in their details, all suggest that both attentional mechanisms coexist in the brain. That is, there are two different learning rate parameters that govern the associative strength of a stimulus. The first changes according to the rules of Mackintosh (1975), increasing attention to reliable predictors of outcomes, and the second changes according to the rules of Pearce-Hall (1980), increasing attention to unreliable predictors. Combining these processing under a unifying theory of attention can

explain a breadth of evidence about the way animals learn under different circumstances, that either model alone struggles to account for (Pearce & Mackintosh, 2010).

7.4.2 Implications for theories of anterior cingulate cortex function

As has been established in the preceding sections (sections 7.3, 7.4.17.4), the results of the experiments in this thesis support the involvement of the anterior cingulate cortex, and its efferents to the anterior thalamic nuclei, in mediating attention to reliable reward predictors. Nevertheless, the anterior cingulate cortex has further been implicated in a wide range of candidate functions including reward, motor, executive function, conflict and error processing (Beckmann, 2009; see also section 1.5.1), each supported by a breadth of empirical data. This section aims to establish a place for the current data within this literature, highlighting those areas where the present research draws parallels with existing theory.

As described in section 1.5.1.2, there is a considerable body of research illustrating a role for the anterior cingulate cortex in incorporating reward history to determine action selection (Rushworth et al., 2007; Shenhav et al., 2016). Anterior cingulate lesions result in a failure to accrue positive reinforcement over time (Kennerley et al., 2006), while anterior cingulate neurons respond during the generation of exploratory actions and the monitoring of outcomes of these actions (Hayden & Platt, 2010). Data such as these have led theorists to suggest that the anterior cingulate mediates the relationship between previous action-reinforcements and current behavioural choices (Rushworth et al., 2004; 2007; Quilodran, Rothe, & Procyk, 2008).

A closely related body of evidence implicates the anterior cingulate cortex in cognitive control; the ability to adapt behaviour in line with an internally held goal (Shenhav et al., 2013). Largely supported by data from human neuroimaging, the anterior cingulate cortex has been found to respond during conflict (Botvinick et al., 2004), errors (Holroyd & Coles, 2002), and other control-demanding tasks (Gasquoine, 2013). Taking all this research together, a unified picture of anterior cingulate function starts to emerge. The central tenant is using recent action-outcome

history to drive reactive adjustments in behaviour (Gasquoine, 2013; Shenhav et al., 2013; Shenhav et al., 2016; Sheth et al., 2012).

Such a process is clearly implicated in the attentional set-shifting task. A breakdown in the relationship between recent action-outcomes and current choice behaviour would leave an animal unable to establish those stimulus-dimension choices most associated with reward, and with non-reward, respectively. Therefore, this theory accords with the observed deficits in intradimensional set-shifting in the iDREADD and iDRAccAtn groups. Similarly, if no attentional set was formed, an abolition of the intradimensional-extradimensional shift cost would be predicted (Durlach & Mackintosh, 1986). That is, if animals did not utilise action-outcome history to orient responding to the reliably rewarded stimulus dimension during intradimensional shifts, then there would be no 'shift' necessary when required to respond to a previously unreliably rewarded stimulus dimension (Roberts et al., 1994).

However, iDREADD and iDRAccAtn animals not only showed the loss of a shift cost, they showed a shift benefit; they were able to solve the extradimensional shift in *fewer* trials than the intradimensional discriminations. To fully fit this data therefore, the anterior cingulate need preferentially mediate the relationship between previous action-reinforcements *reliably associated with outcomes* and current behavioural choices. At least, the finding of a shift benefit indicates that the anterior cingulate cannot be fully responsible for updating responding on the basis of partially reinforced action-outcome associations (see also section 7.4).

On the other hand, it is notable that increased integration of recent action-reinforcements into current choice behaviour fully fits the behavioural profile of the eDREADD group. By rapidly updating of internal models of the action-reinforcement environment (Quilodran et al., 2008), it follows that eDREADD animals may have been able to adapt their behaviour more quickly in response to each new type of discrimination. This increased sensitivity to feedback could, therefore, explain why they outperformed controls at intradimensional and extradimensional shifts, and reversals.

Overall, the results of the experiments in this thesis are largely consistent with a cognitive control (Shenhav et al., 2013; Shenhav et al., 2016; Sheth et al., 2012) framework of anterior cingulate function. The anterior cingulate cortex may contribute to intradimensional set-shifting by providing a recent action-outcome history which, in turn, drives responding to reliable predictors of reward on subsequent trials (Bissonette et al., 2013). However, it is not clear why disruption of such processes would bias responding to unreliable reward predictors, as indicated by the extradimensional shift benefit observed in the iDREADD and iDRAccAtn groups. Instead, this result would suggest that integrating the action-outcome history of inconsistent predictors may not rely on the anterior cingulate cortex (see section 7.4).

7.5 The mechanistic action of DREADDs is complex and poorly understood

Following the main behavioural experiments in Chapters 4, 5 and 6, investigations into c-*fos*, a marker of cellular activity (Dragunow & Faull, 1989; Zhu et al., 1995), were conducted to provide an independent *in vivo* measure of the effects of the DREADD manipulations in the brain. A summary of the differences in regional Fos-positive cell counts in each experimental group, relative to controls, is provided in Table 15.

Table 15. Summary of the effects of anterior cingulate DREADD manipulations on Fos-positive cell counts

	iDREADD	iDRAccAtn	eDREADD
Cg1	↑	–[?]	↑
Cg2	–	–[?]	↑
PrL	–	–[?]	↑
S2	–	–[?]	–
AM	↑	–[?]	↑
AV	↑	–[?]	–
Interregional correlations (positive)	Cg1&Cg2, Cg1&PrL, Cg2&PrL[C], Cg1&AM, Cg1&AV, Cg2&AM, Cg2&AV, AM&AV, AV&S2.	Cg1&Cg2, Cg1&S2, Cg2&S2* Note: counts for AM and AV were not included in correlation analysis for this group.	Cg2&PrL.

Table provides an overview of the effects of different anterior cingulate DREADD manipulations on c-*fos* expression, relative to control animals: iDREADD, inhibition; iDRAccAtn, inhibition of efferents to anterior thalamic nuclei; eDREADD, excitation. Symbols: ↑, increase; –, no difference; [?], effect unclear based on current evidence; [c], correlation also present in control group. Regions included are dorsal anterior cingulate cortex (Cg1), ventral anterior cingulate cortex (Cg2), prelimbic cortex (PrL), anteromedial thalamic nuclei (AM), anteroventral thalamic nuclei (AV), and secondary somatosensory cortex (S2). *Note, low N in this group, could be underpowered to detect further correlations.

As can be seen from Table 15, there were increases in c-*fos* expression in the anterior cingulate cortex (Cg1) and the anterior thalamic nuclei (AM and AV), a major efferent target (3.4.2), in the iDREADD group. Given that the inhibitory DREADD hM4Di is thought to suppress neuronal firing (Rogan & Roth, 2011; Armbruster et al., 2007; see section 2.3.2), and that c-*fos* is a product of cell body activity (Dragunow & Faull, 1989; Zhu et al., 1995), such increases are counterintuitive. Complicating the matter further, c-*fos* increases were also observed in the eDREADD group (Table 15). Although this is consistent with the proposed stimulation of neuronal firing by excitatory DREADD hM3Dq (Alexander et al., 2009; Conklin et al., 2008; see section 2.3), it is not clear why eDREADDs did not simply reverse the action of iDREADDs.

Importantly though, while eDREADDs did produce the opposite c-*fos* profile of iDREADDs, neither did they replicate it. As can be seen in Table 15, eDREADDs increased activity in more cortical regions (Cg2 and PrL) than iDREADDs. Meanwhile, the iDREADD increase in AV activity was not seen in the eDREADD group. Perhaps more strikingly, the iDREADD group displayed a multitude of strong, positive correlations between Fos-postive cell counts in the anterior cingulate and its efferent target regions that were not present in controls, or in the eDREADD group (Table 15). Together, these findings indicate that iDREADDs and eDREADDs differentially affected both regional activity, and covariant activity between these regions.

The question of how DREADDs influenced cellular and network activity in these experiments requires further investigation. It was suggested in Chapter 4 that the inhibitory DREADD hM4Di could have preferentially infected GABAergic inhibitory interneurons, resulting in disinhibition of excitatory neurons (see section 4.5.3) and the observed increases in c-*fos* expression. This hypothesis could be tested by immunohistochemically staining brain tissue for parvalbumin, a calcium-binding protein expressed in GABA-ergic interneurons (Carlen et al., 2012). This would allow colocalization of neurons expressing a fluorescent marker for parvalbumin and the fluorescent marker mCherry, tagged to DREADD-infected neurons (see section 2.3.1). From the number of DREADD-infected neurons co-expressing both markers, the proportion that were GABA-ergic interneurons could be estimated.

However, it is important to note that the excitatory DREADD hM3Dq (eDREADD) was injected into the same region with the same promotor (CAMKII) as the inhibitory DREADD hM4Di (iDREADD), so there is no reason why they would have infected different cell types (Campbell & Marchant, 2018; Smith et al., 2016). The iDREADD c-*fos* increases, therefore, appear to be contingent on complex, downstream network effects. One possibility is that iDREADDs inhibited excitatory anterior cingulate efferents to the thalamic reticular nucleus, an inhibitory structure (Zikopoulos & Barbas, 2006), thus disinhibiting thalamocortical circuitry (see section 4.5.3). The observation of increased covariant activity between the cortex and the anterior thalamic nuclei is consistent with this suggestion (Table 15).

Nonetheless, the question of why eDREADDs did not simply produce the opposite action again signifies that a series of interconnected structures are affected; where upregulating or downregulating a single node leads to differential cascading effects of inhibition and excitation. One possibility for future research would be to investigate these network effects formally, using structural equation modelling on c-*fos* counts. This technique applies multiple-equation regression models to quantify the causal relationships between observed variables (Lomax & Schumacker, 2004) and could be used to infer the direction of influence between regions as well as the strength of the relationships (Lomax & Schumacker, 2004). Consequently, one could include counts from the thalamic reticular nucleus, in addition to those other regions measured, to characterise the influences of the various nodes in the network following each DREADD manipulation.

Meanwhile, due to tissue damage and poor staining, the number of subjects included in the Fos-positive cell count analysis in the iDRAccAtn group was very low (see section 6.4.2.1). Therefore, although the observed increases in cortical areas were non-significant, this may be due to lack of power. Indeed, the indication of an increase in covariant regional activity (Table 15), suggests that, akin to the iDREADD group, there may have been unregulated hyperexcitability in thalamo-cortical circuitry in the iDRAccAtn group. In the case of this group, infusions of clozapine directly into the anterior thalamic nuclei may have spread into the neighbouring thalamic reticular nucleus, inhibiting the terminals of anterior cingulate neurons projecting there (Zikopoulos & Barbas, 2006; section 6.5.3).

Overall, the results from the c-*fos* experiments provide a clear indication that DREADDs have a substantial influence not only on the activity of neurons at the injection site, but also on the network dynamics of a range of interconnected structures. However, the mechanistic action of DREADDs is poorly understood, not least bolstered by observation that inhibitory DREADDs can lead to counterintuitive increases in activity. Future research needs to investigate the widespread influence DREADDs have across the brain, acknowledging that they do not simply upregulate or downregulate activity in the target structure. Findings in this area will, in turn,

greatly aid interpretation of behavioural changes resulting from DREADD manipulations.

7.6 Conclusions and future directions

The primary findings in this thesis reveal that the anterior cingulate cortex, in conjunction with the anterior thalamic nuclei, plays a crucial role in focusing attention on stimuli that are reliably associated with rewarding outcomes. Disrupting this function appears to allow unreliable, inconsistent predictors to receive inappropriate attention, which facilitates learning when contingencies change. These findings support dual-process theories of attention (Pearce & Mackintosh, 2010) that denote that learning is optimised through competing learning parameters, one directing attention towards reliable predictors and the other towards unreliable predictors.

This thesis also illustrated how many of the fibres comprising the anterior cingulum are connections between the anterior cingulate cortex and the anterior thalamic nuclei. The behavioural results implicate this subgroup of cingulum fibres in the aforementioned attentional function. This has not been described previously and is consistent with a large literature regarding anterior cingulum abnormalities in disorders characterised by attentional dysfunction; including schizophrenia, attention deficit hyperactivity disorder and obsessive-compulsive disorder. More broadly, by demonstrating that normal function in the anterior cingulate cortex plays a crucial role in the appropriate allocation of attention, the behavioural results converge with evidence that anterior cingulate cortex dysfunction forms a major component of these disorders

The results of this thesis raise many interesting avenues for future research. For example, the connections between the anterior cingulate cortex and the anterior thalamic nuclei are bidirectional (Chapter 3), yet Chapter 6 only disrupted the efferents from the former to the latter. An obvious next step, therefore, would be to reverse the DREADD methodology to disrupt the projections from the anterior thalamic nuclei to the anterior cingulate cortex and to assess the impact on attentional set-shifting. The impact of further DREADD manipulations could also be

tested, such as inhibiting the activity of prelimbic cortex to assess the hypothesis that this region mediates attention to unreliable predictors. Studying the effects of such manipulations on attentional set-shifting would shed further light on how structures function and interact to support cognitive flexibility.

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