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

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Abstract

The dorsal subiculum is the primary source of hippocampal projections to the rat retrosplenial cortex. Although, both regions are implicated in spatial memory and navigation, the significance of their direct interconnections remains poorly understood. The present study selectively disrupted dorsal subiculum projections to retrosplenial cortex with inhibitory designer-receptors exclusively activated by designer drugs (iDREADDs), activated locally by clozapine. iDREADDs were injected in the dorsal subiculum in adult male rats (N=14), where they were transported anterogradely to granular retrosplenial cortex. In a separate control group, GFP expressing adeno-associated virus was injected into the dorsal subiculum (N=8). Both groups received behavioural sessions preceded either by intracerebral infusions of clozapine or saline within retrosplenial cortex. Behavioural testing involved reinforced T-maze alternation, with five test variations that differentially taxed intra-maze, extra-maze, and egocentric strategies. Disruption of the subiculum to retrosplenial projections impaired spatial working memory whenever the test variant created a conflict between cue-types, associated with a switch between different strategies. These findings suggest that the direct projections from the dorsal subiculum to the granular retrosplenial cortex help to maintain the flexible integration of different spatial cue-types.

Introduction

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

Both neurotoxic lesions of the hippocampus proper and lesions of the subiculum impair location learning in the Morris Water Maze, suggesting that together these hippocampal regions are necessary for successful allocentric (world-centred) spatial learning (Morris et al., 1990). Furthermore, the hippocampal formation is not uniform as is shows graded anatomical and electrophysiological changes along its various axes. One reflection is how the dorsal and ventral subiculum appear to be functionally distinct in rodents. Based on a variety of evidence it appears that the dorsal subiculum is the more critical for solving spatial memory tasks (Bannerman et al., 2004; Burzynska et al., 2020; Moser & Moser, 1998; O'Mara, 2005; O'Mara et al., 2009; Strange et al., 2014; Witter et al., 1990). Consistent with this view, permanent lesions of the dorsal subiculum are sufficient to impair T-maze alternation (Potvin et al., 2007), a measure of spatial working memory. The pattern of deficits suggested that the dorsal subiculum is essential for processing idiothetic

cues for navigation (Potvin et al., 2007). In addition, dorsal subiculum lesions impaired both object-location memory (Potvin et al., 2010) and the ability to distinguish adjacent-arm trials in the radial-arm maze, pointing to a role in pattern-separation (Potvin et al., 2009). These same lesion studies also indicated that the dorsal hippocampus proper and the dorsal subiculum can contribute differently to spatial memory (Potvin et al., 2007, 2009, 2010).

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

The effects of retrosplenial cortex lesions on spatial tasks appear to be most pronounced when rats must rely on flexible cue integration, such as when intra-maze and extra-maze cues are opposed (Pothuizen et al., 2008, 2010; Vann & Aggleton, 2004; Vann et al., 2003) or when required to choose between competing relevant and irrelevant spatial information (Wesierska et al., 2009). Like the dorsal subiculum, retrosplenial lesion deficits can also emerge when visual stimuli are removed from spatial tasks (Cooper & Mizumori, 2001; Elduayen & Save, 2014). Given the interconnectivity of retrosplenial cortex with motor, sensory, and visual cortices (Miyashita & Rockland, 2007; Sugar et al., 2011; Yamawaki et al., 2016) this cortical area is well placed to integrate information between different sensory modalities to help navigation (Byrne et al., 2007; Mizumori et al., 2000; Powell et al., 2020). However, it is unclear whether direct hippocampal – retrosplenial connections are required for this process.

While much is known about the effects of permanent retrosplenial lesions on learning and memory, far less is known when just the hippocampal inputs to this area are disrupted. These more targeted studies have, so far, been confined to showing the importance of mouse subiculum and CA1 inputs to retrosplenial cortex for contextual fear conditioning (Yamawaki et al., 2019a,b). The present study sought to examine more flexible forms of spatial learning involving working memory. Consequently, rats were trained on a T-maze alternation task, followed by multiple cue conditions. To disrupt the direct projections from the dorsal subiculum to retrosplenial cortex, inhibitory designer-receptor exclusively activated by designer drugs (iDREADDs) injections in the dorsal subiculum were combined with intracerebral infusions of a ligand (clozapine) at the target site (retrosplenial cortex) to inactivate those projections locally (Gomez et al., 2017; Manvich et al., 2018; Roth, 2016, 2017).

Materials and Methods

Experimental Design

Either iDREADDs or a green fluorescent protein (GFP) expressing adeno-associated virus (control) was injected into the dorsal subiculum in two separate groups of rats. Shortly before each behavioural test, both groups received intracerebral infusions targeted at retrosplenial cortex. For some sessions clozapine was infused, on other sessions it was saline. Animals were then tested on reinforced alternation in a T-maze, using five successive variants that differently taxed the use of available cues. The experiment was repeated with two separate cohorts of rats, both treated in the same ways (Figure 1).

Animals

Two cohorts, respectively of 12 and 24 adult Lister Hooded male rats (Envigo, UK), were trained prior to surgery on reinforced T-maze alternation. The first cohort had 8 iDREADDs and 4 GFP-control animals. The second cohort had 12 iDREADDs and 12 GFP-control animals, giving totals of 20 in the iDREADDs group and 16 GFP- controls. At the time of surgery all rats weighed between 236-360g. They were housed in pairs in a temperature-controlled room, under a 12h light/dark cycle. For all behavioural experiments,

water was available *ad libitum* . The rats were put on a food-restricted diet whereby they were still able to gain weight. None of the rats weighed less than 85% of their free-feeding weight.

All animals were randomly assigned to one of the virus conditions and underwent the same surgical and behavioural procedures. The experimenter was not, however, blind to the group membership of the animals. All experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the local Cardiff University Ethics Committee.

Surgery

Prior to surgery, all rats were anaesthetised with an isoflurane-oxygen mixture (5% induction, 1.5-2.5% maintenance). Then, each rat was placed in a stereotaxic frame (David Kopf Instruments, CA, USA), so that the skull was flat, with respect to the horizontal plane. Chloramphenicol 0.5% eye-gel was applied, meloxicam (0.06ml) was administered subcutaneously for analgesic purposes, and lidocaine (0.1ml of 20mg/ml solution) was applied topically to the incision site. Next, a bilateral craniotomy was performed above the dorsal subiculum, and either pAAV-CaMKIIa- hM4D(Gi)-mCherry (AAV5) (iDREADD)(Titer: 2.6x10ˆ13GC/ml, lot:v102676; Addgene, MA, USA) or pAAV-CaMKIIa-GFP (AAV5)(Titer: 4.3x 10ˆ12GC/ml, lot: v5894, Addgene, MA, USA)(GFP-control) virus was injected bilaterally into the dorsal subiculum.

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

During the same surgeries, pairs of cannulas were implanted into the left and right retrosplenial cortex. One cannula pair (1.5mm length x 1.2mm separation, 26-gauge, PlasticsOne, Virginia, USA) was implanted into the anterior portion of the retrosplenial cortex (from bregma; AP: -2.5 mm, ML: ± 0.6 mm, DV: -1.5mm), the other cannula pair (1.7mm length x 1.4mm separation; 26-gauge, Plastic One, Virginia, USA) was implanted into the posterior retrosplenial cortex (AP: -6.0mm, ML: ± 0.7mm, DV: -1.7mm). The implantation coordinates for both cohorts remained the same. The cannulas were held in place with bone cement (Zimmer Biomet, Swindon, UK) and anchored to the skull with four screws (Precision Technology Supplies, Uckfield, UK). Dummy cannulas were inserted into the guide cannulas to prevent blocking and were secured in place with aluminium dust caps. The analgesic Marcaine Polyamps (AstraZeneca, UK) and antibiotic powder (Clindamycin, Pfizer, UK) were applied to the surgical site. All animals were subcutaneously administered 5ml glucose-saline solution for fluid replacement, prior to placing them in a recovery chamber. Once the animals regained consciousness, they were returned to their home cage and closely monitored.

Apparatus for Behaviour

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

Behavioural Training Prior to Infusions

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

At least seven days post-surgery, and after signs of full recovery, the animals were retrained on the Standard T-maze task for 6 to 10 days, until they reached at least 87.5% on two consecutive sessions. The infusion trials for Standard T-maze then followed, commencing at least three weeks after surgery.

The rats were then trained on the next T-maze condition for 3 to 5 days, followed by the infusion trials for that condition. An additional, infusion-free training day was provided if there had been a gap of more than two days between infusion sessions. An infusion-free training session was also given on the day between the two clozapine infusions, to help performance return to baseline (Figure 1). This testing regime was repeated for the remaining behavioural conditions (Figures 1, 2). The order of clozapine, saline, and injection-free sessions for the various conditions was balanced between the two cohorts (Figure 1).

Experimental Conditions (all with 8 trials per session)

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

To begin the sample run, the rat was released from the start position and ran to the junction of the T-maze, where it turned into the pre-selected arm and ate the reward. The rat was then immediately picked up and the barrier at the choice point removed. Then, the animal was carried to the start position and allowed to begin the test run. After running down the stem of the maze, the rat could choose between the left and right arms. The animal received a food reward only if it alternated, i.e., selected the arm located opposite from the baited sample arm. A test run was considered correct when the animal's back feet crossed markings at the base of each side arm. The animal was picked up and held until, the T-maze was reset, and the next trial commenced after 10-15s. Each rat completed all 8 trials prior to running the next animal.

1. Standard T-maze (all spatial cue types available) (Figure 2A) – This condition was the same as that used in pre-training. The two phases of each trial started at the same position.

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

3. *Rotation T-maze (disrupted intra-maze cues) (Figure 2C)* – The maze was rotated between the sample and the test run, by either 90°or 180° degrees with every trial. The arm on the test run, the degree of rotation and the direction of the rotation were all pseudorandomized so that the same manipulation was not repeated more than two consecutive times. The location of the start position was consistent for all trials, so that extra-maze and egocentric cues remained viable, while intra-maze cues were nullified.

4. *Opposite arm T-maze (disrupted egocentric cues) (Figure 2D)* –The start position of the animal was

rotated 180° between the sample and the test runs. Therefore, each sample run began at the same start arm and each test run began in the arm directly opposite. Critically, the correct arm on the test run remained in the opposite room location to the position of the baited arm in the sample run. No test arm was used as the correct arm on more than two consecutive trials. For all trials, the start position remained in the South (Figure 2D).

5. *Dark T-maze (visual cues removed) (Figure 2E)* – The standard T-maze protocol was repeated but now in the dark. The maze was baited, and barriers put in place before each trial in dim illumination provided by a 10W red light facing away from the maze. Then, the light was turned off (˜0.2lx) and the rat placed in the start position. Once each trial was completed, the rat was picked up and held while the maze was reset. Only Cohort 2 received the additional infusion-free session.

iDREADDs activation

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

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

Perfusions:

Following completion of the experiment, animals were transcranially perfused. All animals received a lethal dose of sodium phenobarbital (2ml/kg, Euthatal, Marial Animal Health, UK) administered by intraperitoneal injection. Once completely unresponsive, the animals were transcardially perfused with 0.1M phosphate-buffered saline (PBS) and 4% paraformaldehyde in 0.1M PBS (PFA). The brains were further post-fixed in PFA for at least 2 hours, and then placed in 25% sucrose solution for a minimum of 24h. A freezing microtome (8000 Sledge Microtome, Bright Instruments) was used to cut the brain in 40µm coronal sections, saved as four simultaneous series. The sections were stored in cryoprotectant (30% sucrose, 1% polyvinyl pyrrolidone, 30% ethylene glycol in PBS) in a freezer at -200C until further processing.

Histology:

One series was mounted onto gelatin-coated slides before being stained for Nissl using cresyl violet. The sections were then dehydrated through increasing concentrations of alcohol (70%; 90%; 100%; 100%) and washed in xylene. Then, the slides were cover-slipped with DPX (Sigma Aldrich, Gillingham, UK) mounting medium. To enhance the fluorescence signal of the mCherry (iDREADDs group) or GFP (control group), additional series were washed three times in PBS and then blocked with 5% normal goat serum (NGS) (Invitrogen, Inchinnan, UK) in Phosphate Buffered Saline with Tritonx-1000 (PBST) for two hours. Both series were then transferred in either a solution of rabbit polyclonal anti-mCherry (Abcam, Cambridge, UK) or chicken polyclonal anti-GFP (Abcam, Cambridge, UK) antibody at a dilution of 1:1000 in PBST with 1% NGS and incubated for 24 hours at room temperature. The sections were then washed three times and transferred to a secondary antibody of either goat-anti-rabbit (Dylight Alexa flour 594, Vector Laboratories, Peterborough, UK) or Alexa Fluor 488 goat-anti-chicken (Invitrogen, Inchinnan, UK) at a dilution of 1:200 in PBST for two hours. The sections were then washed in PBS and mounted onto gelatin-coated slides and cover-slipped using Fluromount (Sigma-Aldrich, Germany).

Image Acquisition and Viral Expression Analysis:

For each animal, cannula placement and viral expression were analysed using a bright field and fluorescent microscope Leica DM5000B, equipped with a DFC310 FX camera. The viral expression was assessed at the injection site, as well as at dorsal subiculum efferent targets. These targets included layers 2 and upper 3 of the retrosplenial cortex, along with the anteroventral and anteromedial thalamic nuclei (Figures 4, 5).

Statistical analyses:

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

All data were screened for outliers, the assumptions of normality, homogeneity of variances and covariances using SPSS Statistics 27(SPSS Inc., Chicago, Ill., USA). A single outlier score (37.5%) was found for just one animal for a single session (GFP group, Standard condition, saline), and so this case remained in the analyses. Levene's test based on medians assessed the homogeneity of variance, showed that the assumption was violated on the opposite-arm saline condition ($p = 0.044$) (Brown & Forsythe, 1974). No violations to the assumption of homogeneity of covariance were found (all $p_s > 0.024$) (see Tabachnick & Fidell, 2013). Where there was a statistically significant interaction term, simple main effect analyses were conducted using pooled error terms in JASP 14.1 (JASP Team, 2022).

Multiple independent t-tests helped to compare control and baseline scores, i.e., the pre-surgery training scores, post-surgery training scores for each alternation condition prior to any infusions, as well as for the infusion-free day scores between the clozapine infusions. These analyses, applicable to Conditions 1-4, were to establish if the performance of the iDREADDs and GFP-controls was statistically comparable, prior to and between iDREADDs activations.

ResultsHistological findings:

Two criteria were required for inclusion in the experimental analyses. First, the dorsal subiculum virus injections had to result in appreciable bilateral label within granular retrosplenial cortex (Figures 4, 5). Second, the infusion placements had to involve retrosplenial cortex (Figure 3). Across both cohorts, a total of 6 iDREADDs and 8 GFP-control animals were excluded due to lack of viral expression (unilateral or bilateral) in retrosplenial cortex (n=6), off-target cannula placement (n=2) or both (n=6). Consequently, the behavioural analyses derive from 14 iDREADDs and 8 GFP-control animals. In four of these animals (n=3 iDREADDs; n=1 GFP) spread from the anterior infusion cannulas may have reached the midcingulate cortex (Vogt & Paxinos, 2012) as well as retrosplenial cortex. In some cases, the virus injection spread into the dentate gyrus, which does not directly innervate the retrosplenial cortex.

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

A series of independent t -tests considered whether there might be pre-surgery (Standard condition only) or post-operative training performance differences between the iDREADDs and GFP-control rats on the five T-maze task conditions prior to any infusions. The two groups did not differ significantly on the pre-surgery training nor on the baseline training prior to commencement of infusion trials for the five test conditions: all $t_s < 1.86$, $p_s > 0.078$. A further set of t -tests took the scores from the non-infusion sessions that were interleaved between the saline and clozapine sessions (Figure 1), for all but the Dark condition. Again, there were no performance differences between the iDREADDs and GFP-control animals on the infusion-free days for each of the four conditions: all $t_s < 1.01$, $p_s > 0.29$.

Performance on test conditions

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

($p<0.05$), and no other main effects or interactions involving this factor were observed (all $F_s < 4.00$; all $p_s > 0.06$). Therefore, the cohort data were pooled and analysed together for each separate condition, though the Dark condition data received extra scrutiny.

In the following analyses, 'Drug' refers to saline or clozapine (within subject) while Group refers to iDREADDs or GFP infusions (between subject).

Standard T-maze: There was a significant main effect of Drug: $F_{1,20} = 7.01$, $p = 0.015$, $\eta_\pi^2 = 0.25$, but not a main effect of Group or Drug × Group interaction: $F_s < 0.85$, $p_s > 0.37$, $\eta_{\pi S}^2 < 0.04$ (Figure 6). This set of results showed that clozapine did not exert a greater effect in the active viral group when compared with the control viral group.

Start T-maze: There was a main effect of Drug: $F_{1,20} = 5.76$, $p = 0.03$, $\eta_\pi^2 = 0.22$, but no main effect of Group or Drug × Group interaction: $F_s < 3.48$, $p_s > 0.08$, $\eta_{\pi S}^2 < 0.15$ (Figure 6). This pattern of results corresponded to that seen for the Standard condition.

Rotation T-maze: There was a significant main effect of Drug: $F_{1,20} = 12.02$, $p = 0.002$, $\eta_\pi^2 = 0.37$ but also a Drug × Group interaction: $F_{1,20} = 6.8$, $p = 0.016$, $\eta_\pi^2 = 0.25$. Simple main effects analyses revealed a significant decline in performance following clozapine infusions within the iDREADDs group that was not seen in the GFP control group: $F_{1,13} = 25.36$, $p < 0.0001$ (Figure 6). All other tests were non-significant: $F_s < 2.75$, $p_s > 0.11$.

Opposite arm T-maze: As in the Rotation condition, there was a significant main effect of Drug: $F_{1,20} = 7.7$, $p = 0.01$, $\eta_\pi^2 = 0.278$ and a Drug × Group interaction: $F_{1,20} = 4.55$, $p = 0.045$, $\eta_\pi^2 = 0.18$. Again, there was decline in performance following clozapine infusions in the iDREADDs group that was not seen in the GFP control group: $F_{1,13} = 16.6$, $p < 0.001$ (Figure 6). All other tests were non-significant: $F_s < 0.89$, $p_s > 0.35$.

Dark T-maze: The behavioural analyses revealed a significant Drug × Group interaction: $F_{1,20} = 7.8$, $p = 0.011$, $\eta_\pi^2 = 0.28$, however, there was no main effect of Drug or Group: $F_s < 0.036$, $p_s > 0.85$. Follow-up simple main effects analyses showed that again iDREADDs animals' performance declined following the clozapine infusions relative to saline: $F_{1,13} = 5.8$, $p = 0.025$. All other tests were non-significant: $F_s < 2.84$, $p_s > 0.10$ (Figure 6). For this one condition, there was a significant main effect of Cohort ($F_{1,18} = 10.6$, $p = 0.004$). Overall, Cohort 1 had lower scores, possibly reflecting the absence of an additional saline trial. Nevertheless, the key intervention comparisons were within-subject (saline vs clozapine), being effective across both Cohorts.

Discussion

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

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

There was no apparent effect of retrosplenial disruption on the Standard or Start T-maze conditions, i.e., when all spatial strategies were available. The null result on the Start condition showed that iDREADDs activation did not affect the ability of the rats to adjust to changes in start position across the different trials. However, iDREADDs activation impaired spatial working memory on the Rotation, Opposite arm, and Dark alternation conditions. This pattern of deficits does not simply reflect task difficulty, as performance during the intervening infusion-free days, during the iDREADDs/saline condition, and by the GFP-control group (Figure 6) all remained extremely similar across all five conditions. The implication is that the clozapine infusions disrupted more than one type of task strategy, given the varying demands of the final three conditions (Figure 2). At the same time, a blanket disruption would most likely have also impaired the Standard and Start condition. This pattern of results points to the emergence of deficits when cue types are changed and restricted.

The temporal pattern of results (last three conditions impaired) showed that the chemogenetic effects did not disappear over time and training. This same temporal pattern does, however, raise the possible concern that post-operative testing may have resumed too soon, so that the virus was not fully transported. That possibility is, however, seen as most unlikely as pilot studies repeatedly show that by two weeks post-surgery there is extensive transport to granular retrosplenial cortex. In the present study, the first infusions were a minimum of three weeks post-surgery. In theory, by counterbalancing the sequence of the five behavioural conditions it would have been possible to address this issue. This was not, however, attempted. Each behavioural condition required different amounts of pre-training to establish appropriate performance levels prior to each set of drug infusions. This variation would have placed testing and testing intervals out of synchrony. The increase in individual variability would be exacerbated by the different transfer effects from each specific condition to the next condition.

While the present study lacks direct evidence as to how the clozapine infusions disrupted retrosplenial activity, other studies using comparable methodologies have demonstrated their effectiveness (Bubb et al., 2021; Yamawaki et al., 2019b). That the iDREADDS/clozapine combination disrupted neural processing can also be indirectly inferred from the performance disruptions seen on the last three conditions. Consistent with this assumption is how the pattern of behavioural deficits in the iDREADDS rats had obvious similarities with the effects of conventional lesions in the two target sites (Pothuizen et al., 2010; Potvin et al., 2007, 2010). A further potential concern is whether the clozapine infusions reached sites beyond retrosplenial cortex. While possible, any such site would also need to receive direct dorsal subiculum inputs to have any functional impact, so the likelihood is low. Furthermore, related cannula studies have concluded that infusions are well retained by retrosplenial cortex (Nelson et al., 2015; Yamawaki et al., 2019b).

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

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

Of especial relevance are those few studies that have made permanent lesions targeting just the granular retrosplenial cortex. Such lesions again appear to leave standard T-maze alternation intact but impair

performance when intra-maze cues are removed by switching to adjacent, parallel mazes (Pothuizen et al., 2010). This profile closely resembles the current findings, even though the present iDREADDs manipulation was even more selective, targeting just one set of granular retrosplenial inputs (Figures 4, 5). Together, these findings underline the significance of the hippocampal (subiculum) efferents to granular retrosplenial cortex when spatial cue usage is restricted.

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

An especially relevant study used chemogenetic methods similar to those in the present study to target hippocampal-retrosplenial projections during contextual fear conditioning. That study showed how the glutamatergic (vGlut1+ and vGlut2+) subiculum projections can differentially regulate the cellular functions of granular retrosplenial cortex (Yamawaki et al., 2019b). That same study also indicated that a major role of the vGlut1+ projections was in processing recent context memories, whilst the vGlut2+ projections assisted with the long-term retrosplenial storage of fear-inducing context memory (see also Czajkowski et al., 2014; De Sousa et al., 2019; Milczarek et al., 2018). In a related study, the sparse inhibitory CA1 projections to retrosplenial cortex were silenced, again in a contextual fear conditioning paradigm, and their actions contrasted with those of the anterior thalamic inputs to retrosplenial cortex (Yamawaki et al., 2019a). While both pathways are involved in the acquisition of contextual fear memory, they act in opposing ways. The inhibitory CA1 projections normally supressed, while the excitatory anterior thalamic projections normally enhanced the acquisition of context memories (Yamawaki et al., 2019a).

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

Finally, clear parallels exist between the present results and those of a previous experiment that also placed iDREADDs in the dorsal subiculum to examine spatial working memory (Nelson et al., 2020). Systemic activation of the iDREADDs did not influence Standard T-maze alternation, but impaired the same Rotation condition (Nelson et al., 2020), consistent with the present study. This same pattern of deficits (Standard - intact; Rotation - impaired) was then seen when just the subiculum projections to the anterior thalamic nuclei were disrupted (Nelson et al., 2020). These parallel effects with the present study again highlight the close anatomical (Bubb et al., 2017; Horikawa et al., 1988; Sripanidkulchai & Wyss, 1986) and functional (Aggleton & O'Mara, 2022; Kinnavane et al., 2019; Pothuizen et al., 2009; Sutherland & Rodriguez, 1989; Sutherland & Hoesing, 1993) relationships between the hippocampal formation, anterior thalamic nuclei, and retrosplenial cortex. Their common actions may reflect the way that many dorsal subiculum neurons collaterise to reach both granular retrosplenial cortex and the mammillary bodies (Kinnavane et al., 2018), the latter site relaying monosynaptically to the anterior thalamic nuclei (Umaba et al., 2021). Furthermore, the

finding that the widespread disruption of multiple subiculum efferents has very similar effects to targeting just those reaching the anterior thalamic nuclei (Nelson et al., 2020) or reaching the retrosplenial cortex (present study) underlines the functional primacy of these particular interactions. Together, these results accord with the influential idea that retrosplenial cortex facilitates the ability to switch between spatial strategies (Byrne et al., 2007; Vann et al., 2009) and that this function is facilitated by direct inputs from the dorsal subiculum, along with anterior thalamic interactions.

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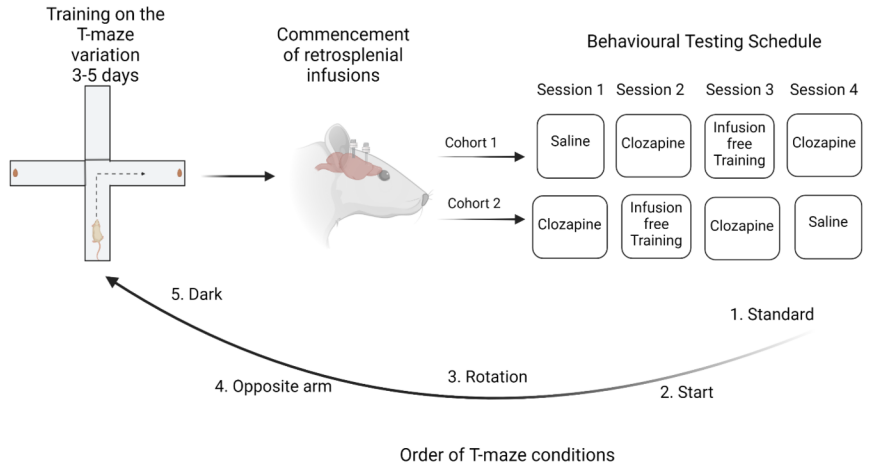

Figure 1. Schematic illustration of the behavioural training and testing schedule post-surgery. Animals received a sequence of alternation sessions that involved preceding intracerebral infusions of either clozapine or saline. There was also an additional infusion-free session. After the four sessions, rats moved onto the next T-maze condition, starting with training sessions followed by infusion sessions. The order of infusions was counterbalanced across the two cohorts as indicated.

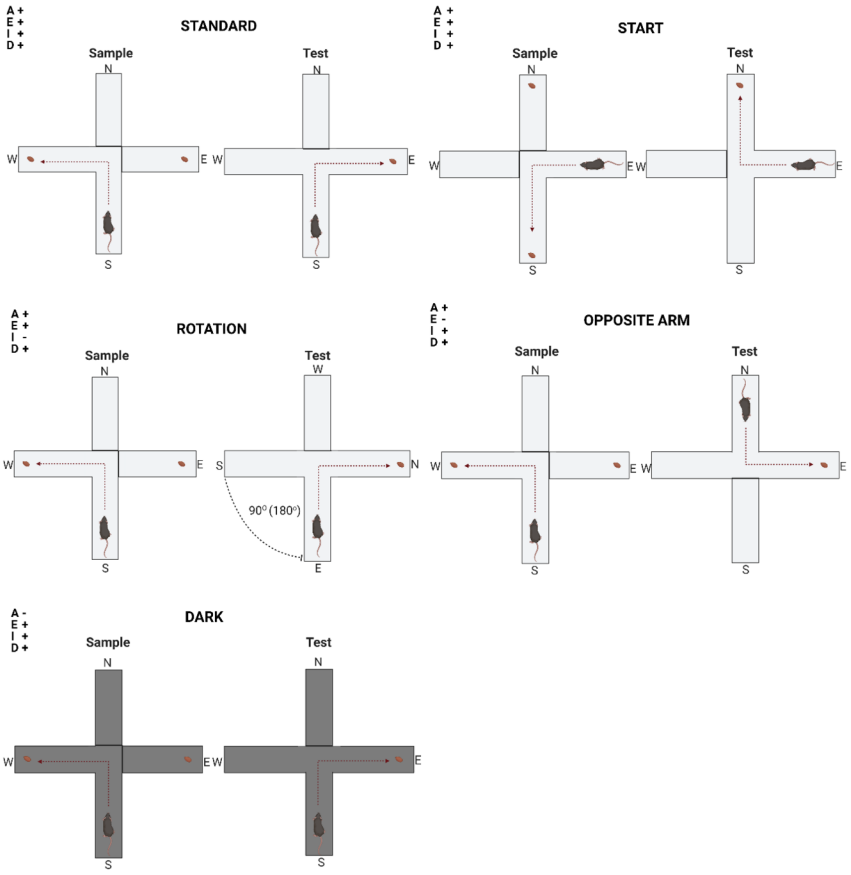

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Figure 2. Illustration of the various T-maze conditions. The figure shows examples of a single trial (sample and test run) for each behavioural condition as follows: Standard T-maze; Start T-maze (with randomized start positions); Rotation T-maze (with either 90° or 180° maze rotation in either direction); Opposite arm T-maze (sample from South, test from North); and Dark T-maze. Abbreviations: A, allocentric cues; E, egocentric cues; I, intra-maze cues; D, directional cues; +, cue is available to solve the maze; -, the cue does not solve the maze.

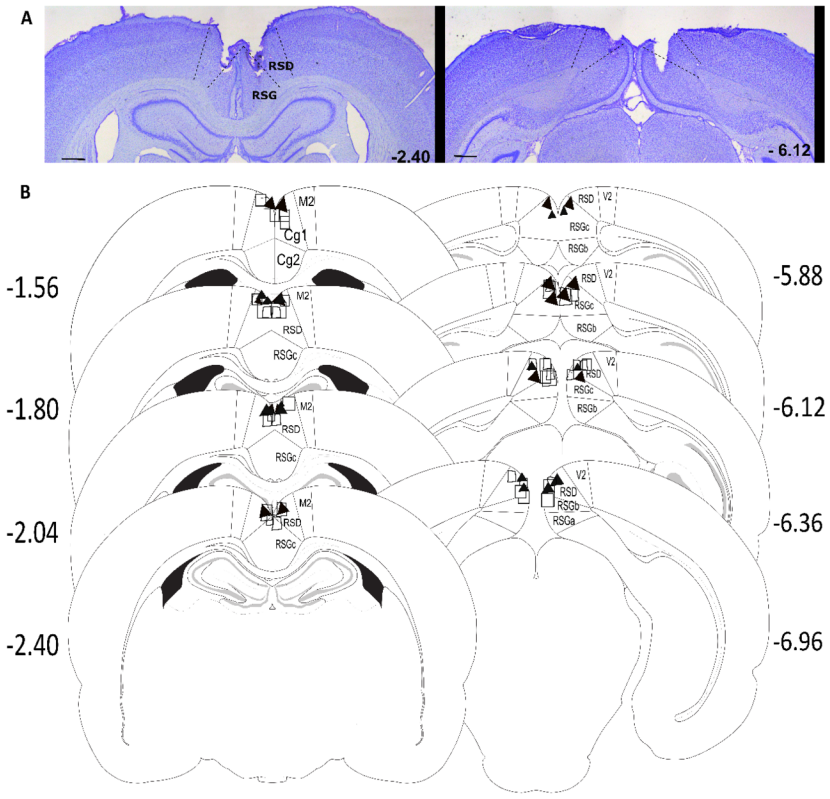


Figure 3. Schematic representation of retrosplenial cannula placement for each experimental animal. Panel A shows coronal sections (cresyl violet) with cannulation sites in the anterior (left) and posterior (right) retrosplenial cortex. Panel B (below) is a schematic representation of cannula placements adapted from the Paxinos and Watson rat atlas (2004) for each animal in both the anterior (left) and posterior (right) portions of the retrosplenial cortex. Squares denote iDREADDs animals and triangles GFP-controls. In three iDREADDs and 1 GFP-control animal, the cannulas also affected the most posterior portions of the cingulate cortex. The same implantation coordinates were used for all animals, producing considerable overlap of cannula placements. The numbers represent the approximate distance from bregma in mm. All scale bars are 150μm. Abbreviations: Cg1/2, anterior cingulate cortex; M2, secondary motor cortex, RSD, dysgranular retrosplenial cortex; RSGc, granular retrosplenial cortex, area c; RSGb, granular retrosplenial cortex, area b; RSGa, granular retrosplenial cortex, area a; V2, secondary visual area.

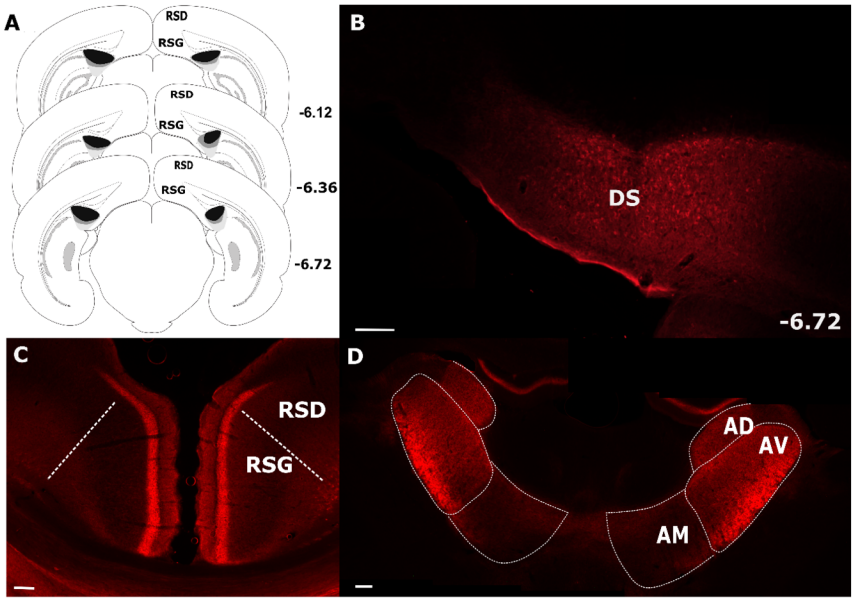


Figure 4. Virus expression in the iDREADDs group. Panel A shows the smallest (black) and largest (light grey) injection sites across the dorsal subiculum. Numbers refer to the distance from bregma in mm. Panel B shows an example of iDREADDs expression in the dorsal subiculum. Panel C shows the robust expression of transported iDREADDs in layers I, II, and upper III of the granular retrosplenial cortex. Panel D shows anterograde transport from the dorsal subiculum to the anterior thalamic nuclei. All scale bars are 150µm. AD, anterodorsal nucleus; AM, anteromedial nucleus; AV, anteroventral nucleus, DS, dorsal subiculum, RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex.

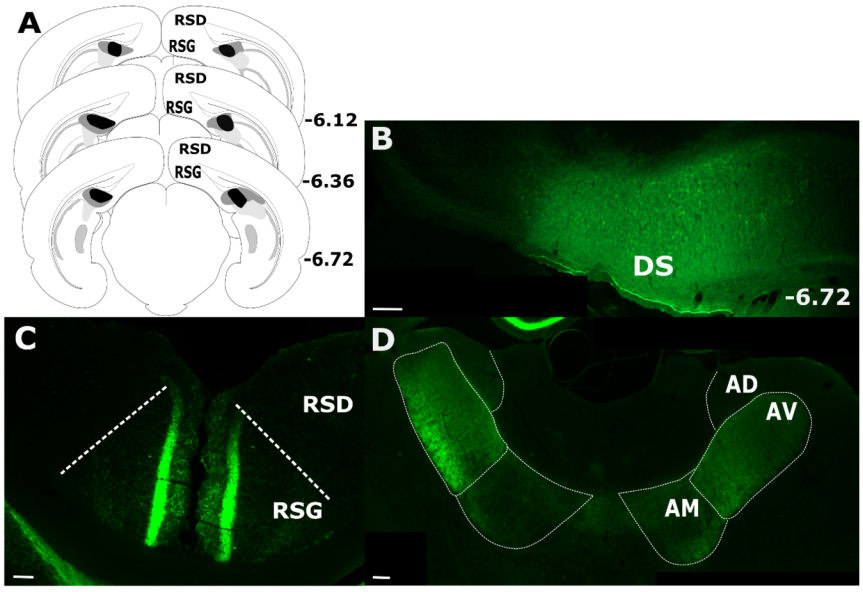


Figure 5. Virus expression in the GFP-control group. Panel A shows the smallest (black) and largest (light grey) injection sites across the dorsal subiculum. Numbers refer to the distance from bregma

in mm. Panel B shows an example of iDREADDs expression in the dorsal subiculum. Panel C shows the robust expression of transported virus in layers I, II and upper III of the granular retrosplenial cortex. Panel D shows anterograde transport from the dorsal subiculum to the anterior thalamic nuclei. All scale bars are 150µm. AD, anterodorsal nucleus; AM, anteromedial nucleus; AV, anteroventral nucleus, DS, dorsal subiculum, RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex.

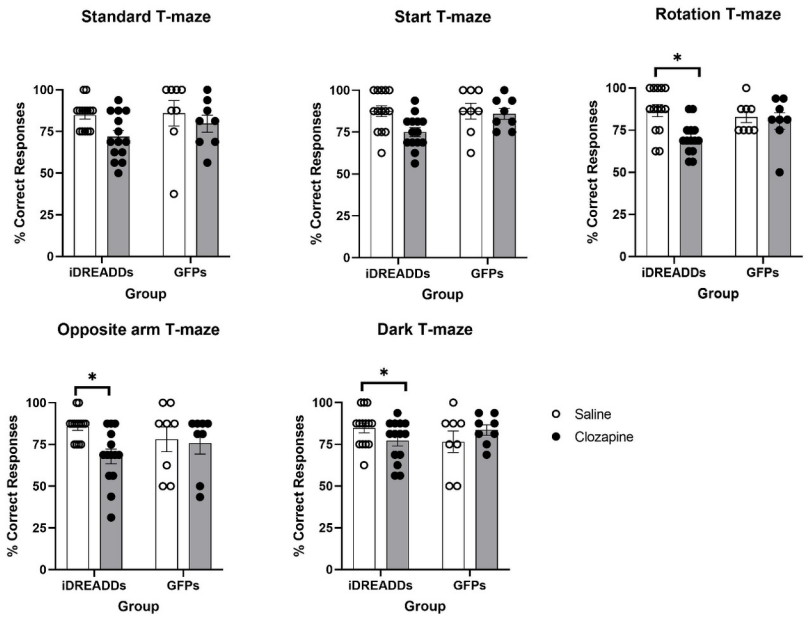
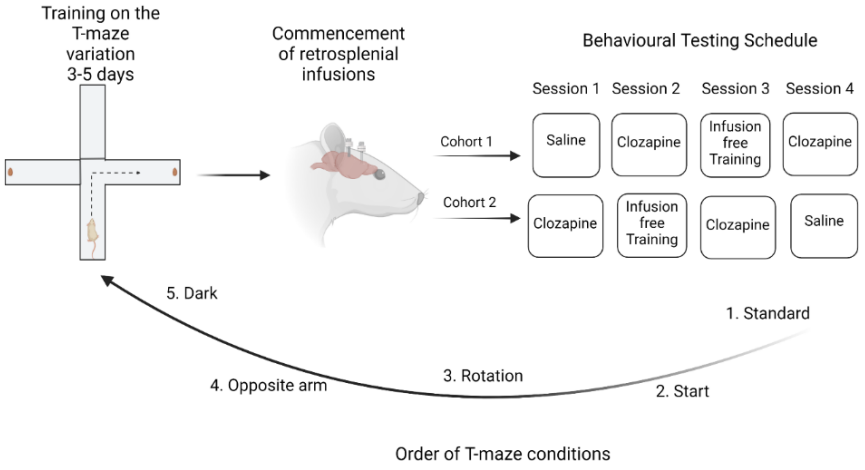


Figure 6. Bar graphs depicting the mean and each animal's individual percentage of correct alternation responses for both the iDREADDs and GFP-control groups. From top left to bottom right: 1) Standard T-maze; 2) Start T-maze; 3) Rotation T-maze; 4) Opposite arm T-maze; 5) Dark T-maze. Despite the within-group differences restricted to the iDREADDs group, there were no between-group differences for the iDREADDs group and the GFP-controls. Error bars indicate SEM.

* Denotes within-group statistically significant differences; the saline condition is presented in white and the clozapine condition in grey.

Graphical abstract



Graphical abstract text

The dorsal subiculum is the primary source of hippocampal projections to the rat retrosplenial cortex. Disruption of the subiculum to retrosplenial projections impaired spatial working memory whenever the test variant created a conflict between cue-types, associated with a switch between different strategies. These findings suggest that the direct projections from the dorsal subiculum to the granular retrosplenial cortex help to maintain the flexible integration of different spatial cue-types.