Received: 2 September 2023 | Revised: 21 December 2023 | Accepted: 19 February 2024

DOI: 10.1111/ejn.16303

RESEARCH REPORT

EJN European Journal of Neuroscience FENS WILEY

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

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Funding information
Wellcome Trust, Grant/Award Numbers: 108891/B/15/Z, 103722/Z14/Z

Edited by: Gal Richter-Levin

Abstract

In a changing environment, animals must process spatial signals in a flexible manner. The rat hippocampal formation projects directly upon the retrosplenial cortex, with most inputs arising from the dorsal subiculum and terminating in the granular retrosplenial cortex (area 29). The present study examined whether these same projections are required for spatial working memory and what happens when available spatial cues are altered. Consequently, injections of iDREADDs were made into the dorsal subiculum of rats. In a separate control group, GFP-expressing adeno-associated virus was injected into the dorsal subiculum. Both groups received intracerebral infusions within the retrosplenial cortex of clozapine, which in the iDREADDs rats should selectively disrupt the subiculum to retrosplenial projections. When tested on reinforced T-maze alternation, disruption of the subiculum to retrosplenial projections had no evident effect on the performance of those alternation trials when all spatial-cue types remained present and unchanged. However, the same iDREADDs manipulation impaired performance on all three alternation conditions when there was a conflict or selective removal of spatial cues. These findings reveal how the direct projections from the dorsal subiculum to the retrosplenial cortex support the flexible integration of different spatial cue types, helping the animal to adopt the spatial strategy that best meets current environmental demands.

KEYWORDS
anatomy, Chemogenetics, dorsal subiculum, Retrosplenial cortex, spatial memory, working memory

Abbreviations: AD, anterodorsal nucleus; AM, anteromedial nucleus; ANOVA, analysis of variance; AV, anteroventral nucleus; CA1, Cornu ammonis 1; Cg1, anterior cingulate cortex area 1; Cg2, anterior cingulate cortex area 2; DS, dorsal subiculum; GFP, green fluorescent protein; iDREADDs, Inhibitory designer receptors exclusively activated by designer drugs; M2, secondary motor area; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex; V2, secondary visual area.

1 | INTRODUCTION

Flexible spatial learning and navigation are essential for humans and animals alike. These complex, multisensory processes require the integration of changing 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 starting 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 importance as a route for the hippocampus proper to engage distal sites (Aggleton & Christiansen, 2015; Kitanishi et al., 2021; Lever et al., 2009; O'Mara, 2005; Witter, 2006; Yamawaki, Corcoran, et al., 2019; Yamawaki, Li, et al., 2019). These distal sites include the retrosplenial cortex (Vann et al., 2009).

Neurotoxic lesions of the subiculum impair location learning in the Morris Water Maze, with more persistent deficits being found for matching-to-place, a test of working memory (Morris et al., 1990). A combination of anatomical and electrophysiological gradients points to functional changes along the longitudinal (dorsal-ventral) axis of the hippocampal formation (Strange et al., 2014). Such evidence suggests that the dorsal subiculum is 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). Support for this conclusion includes how permanent lesions of the dorsal subiculum are sufficient to impair T-maze alternation (Potvin et al., 2007), as well as object-location memory (Potvin et al., 2010) and the ability to distinguish adjacent-arm trials in the radial-arm maze, the latter deficit pointing to a role in pattern-separation (Potvin et al., 2009).

There are dense hippocampal projections to the retrosplenial cortex that in rodents preferentially target the granular subdivision (area 29). These projections principally arise from the dorsal subiculum (Kinnavane et al., 2018; Sugar et al., 2011; van Groen & Wyss, 1992). Like the subiculum, retrosplenial cortex is repeatedly implicated in spatial memory and navigation (Harker & Whishaw, 2004; Nelson et al., 2015; Nelson et al., 2018; 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). These parallel findings raise the question of when and why subiculum–retrosplenial connections are most critical for spatial processing.

One clue comes from the finding that retrosplenial cortex lesions are often most disruptive on those spatial tasks 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). Unlike the subiculum, the retrosplenial cortex is also closely connected with motor, sensory and visual cortices (Miyashita & Rockland, 2007; Sugar et al., 2011; Yamawaki et al., 2016), leaving it particularly well placed to integrate information between different sensory modalities to help navigation (Byrne et al., 2007; Mizumori et al., 2000; Powell et al., 2001).

The present study examined whether the direct subiculum–retrosplenial projections are required for flexible spatial processing. The few previous studies that have targeted these connections have helped to reveal the importance of subiculum and CA1 inputs to the retrosplenial cortex in the mouse for contextual fear conditioning when cue conditions remain stable (Yamawaki, Corcoran, et al., 2019; Yamawaki, Li, et al., 2019). To examine more flexible forms of spatial learning, rats were trained on a T-maze alternation task, followed by four variants with different demands. This test of working memory benefits from how the same animals can be tested on successive variants that tax different strategies. 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).

2 | MATERIALS AND METHODS

2.1 | 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 the retrosplenial cortex. For some sessions clozapine was infused, in other sessions it was saline. Animals were then tested on reinforced alternation in a T-maze, using five successive

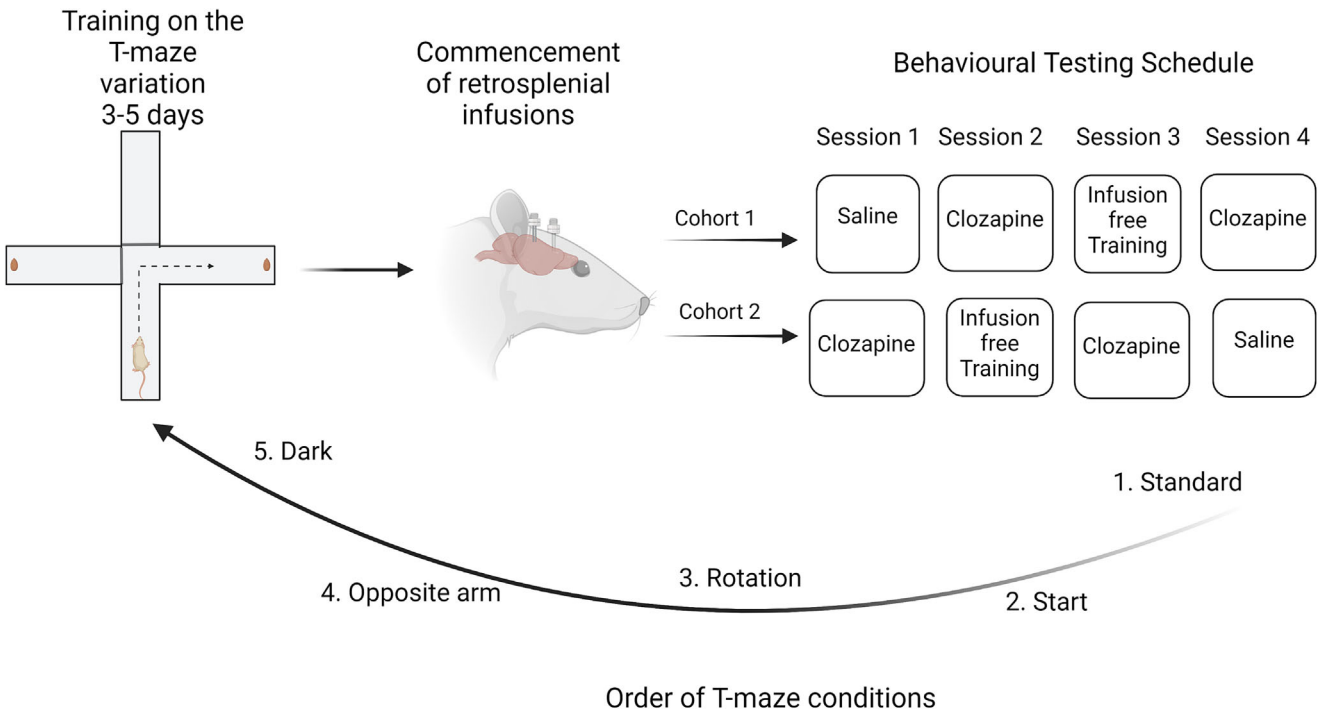

FIGURE 1 **Schematic illustration of the behavioural training and testing schedule post-surgery.** Animals received a sequence of alternation sessions that involved preceding intracerebral infusions of either clozapine or saline. There was also an additional infusion-free session. After the four sessions, rats moved on to 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.

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).

2.2 | 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 eight iDREADDs and four GFP-control animals. The second cohort had 12 iDREADDs and 12 GFP-control animals, giving a total of 20 in the iDREADDs group and 16 GFP-controls. At the time of surgery, all rats weighed between 236 and 360 g. They were housed in pairs in a temperature-controlled room, under a 12 h 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.

2.3 | 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 (0.06 ml) was administered subcutaneously for analgesic purposes, and lidocaine (0.1 ml of 20 mg/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: 2.6 × 10^13GC/ml, lot:v102676; Addgene, MA, USA) or pAAV-CaMKIIa-GFP (AAV5) (titer: 4.3 × 10^12GC/ml, lot: v5894, Addgene, MA, USA) (GFP-control) virus was injected bilaterally into the dorsal subiculum.

In both cohorts, 0.6 µl of the viral construct was injected into the anterior subiculum injection site and 0.4 µl into the more posterior site. The injection

coordinates, with respect to bregma, were as follows: *Anterior*. Cohort 1: AP: −5.9 mm, ML: ±2.9 mm, DV: −2.6 mm; Cohort 2: AP: −5.9 mm, ML: ±2.7 mm, DV: −2.4 mm; *Posterior*. Cohort 1: AP: −6.2 mm, ML: ± 3.2 mm, DV: −2.5 mm; Cohort 2: AP: −6.2 mm, ML: ± 3.0 mm, DV: −2.3 mm), respectively. The very slight changes in coordinates reflected the individual preferences of two researchers, based on pilot experiments. All injections were made vertically using a 10 μl 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 (1.5 mm length × 1.2 mm 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: −1.5 mm), the other cannula pair (1.7 mm length × 1.4 mm separation; 26-gauge, Plastic One, Virginia, USA) was implanted into the posterior retrosplenial cortex (AP: −6.0 mm, ML: ± 0.7 mm, DV: −1.7 mm). 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 5 ml 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.

2.4 | Apparatus for behaviour

Behavioural testing was conducted in an elevated (94 cm), modifiable cross-maze with clear Perspex walls and wooden floor. Each arm was 70 cm long and 10 cm wide, with 17 cm 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 (280 cm × 280 cm × 20 cm) 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 23-26 lx.

2.5 | 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 were 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).

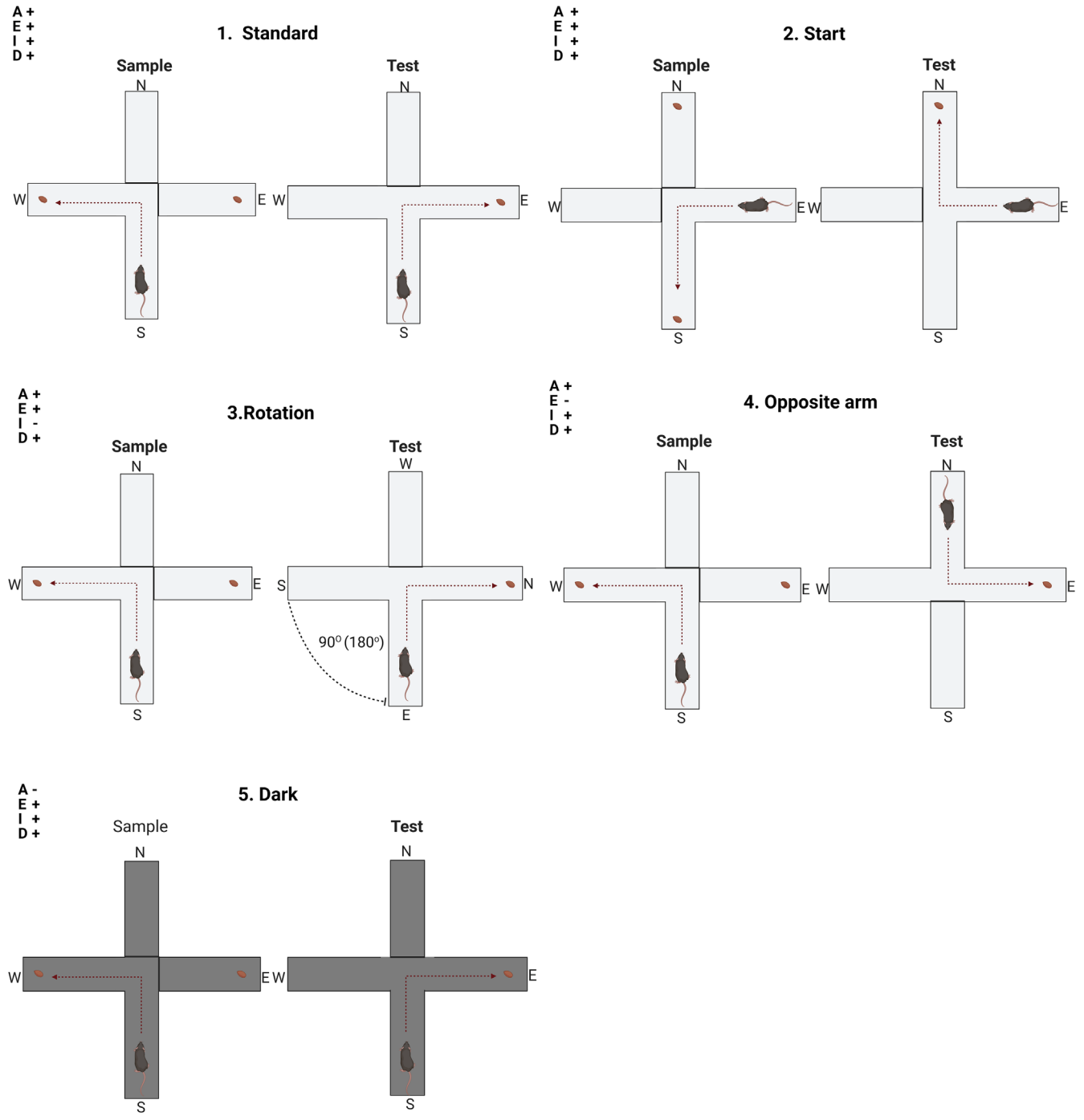

FIGURE 2 **Illustration of the various T-maze conditions.** The figure shows examples of a single trial (sample and test run) for each behavioural condition as follows: 1. Standard T-maze; 2. Start T-maze (with randomized start positions); 3. Rotation T-maze (with either 90° or 180° maze rotation in either direction); 4. Opposite arm T-maze (sample from south, test from north); and 5. 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.

2.6 | Experimental conditions (all with eight 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 was 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 10–15 s. Each rat completed all eight trials prior to running the next animal.

1. *Standard T-maze (all spatial cue types available) (*Figure 2.*1)* – This condition was the same as that used in pre-training. The two phases of each trial started at the same position. In the Standard test, the rats have access to allocentric (distal visual room cues), egocentric (interoceptive cues), directional (turning around a polar coordinate) and intra-maze (e.g., odour trails) cues, all of which might support successful alternation and all of which remained constant.
2. *Start T-maze (flexible learning, all cue types available) (*Figure 2.*2)* – 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 2.*3)* – 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 (allocentric), directional and egocentric cues remained viable, while intra-maze cues were nullified.
4. *Opposite arm T-maze (disrupted egocentric cues) (*Figure 2.*4)* – 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. This meant that a reliance on egocentric cues would consistently cause errors. 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 2.*5)* – The standard T-maze protocol was repeated but now in the dark. In this way, allocentric visual cues were removed. The maze was baited, and barriers were put in place before each trial in dim illumination provided by a 10 W red light facing away from the maze. Then, the light was turned off (∼0.2 lx) and the rat was 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.

2.6.1 | 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 1 μl of sterile saline or 1 μl of clozapine (1 mg/ml) was infused over 1 minute using an infusion pump (11 Plus, Harvard Apparatus, UK). The doses for the local infusions of clozapine were determined from previous studies using the same combinations of drugs but with different cortical targets (Bubb et al., 2021; Nelson et al., 2020). The injector was held in place for a further minute and the dummy cannula was 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 the completion of the experiment, animals were transcranially perfused. All animals received a

lethal dose of sodium phenobarbital (2 ml/kg, Euthatal, Marial Animal Health, UK) administered by intraperitoneal injection. Once completely unresponsive, the animals were transcardially perfused with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde in 0.1 M 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 24 h. A freezing microtome (8000 Sledge Microtome, Bright Instruments) was used to cut the brain into 40 μm 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 -20°C 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 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 5, 6).

Statistical analyses

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

All data were screened for outliers, the assumptions of normality, homogeneity of variances and covariances using SPSS Statistics 27(SPSS Inc., Chicago, Ill., USA). A single outlier score (37.5%) was found for just one animal 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 and 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, 2018). 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.

3 | RESULTS

3.1 | Histological findings

Two criteria were required for inclusion in the experimental analyses. First, the dorsal subiculum virus injections had to result in appreciable bilateral labels within the granular retrosplenial cortex (Figures 5, 6). Second, the infusion placements had to involve the retrosplenial cortex (Figure 3). These decisions were made blind to the pattern of results. Across both cohorts, a total of six iDREADDs and eight GFP-control animals were excluded due to lack of viral expression (unilateral or bilateral) in the retrosplenial cortex (n = 6), off-target cannula placement (n = 2) or both (n = 6). Consequently, the behavioural analyses were derived from

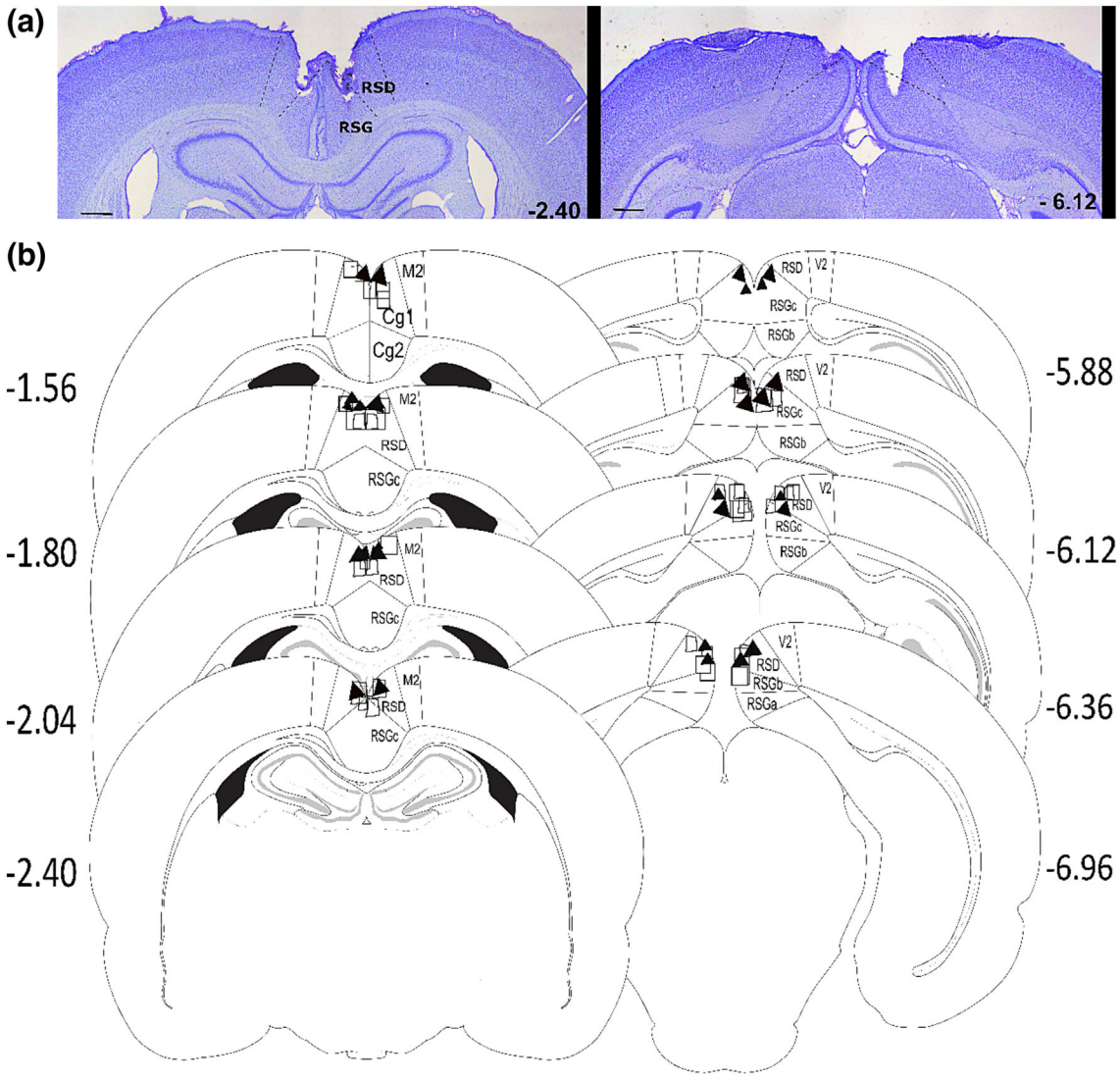

FIGURE 3 **Schematic representation of retrosplenial cannula placement for each experimental animal.** Panel A shows coronal sections (cresyl violet) with cannulation sites in the anterior (left) and posterior (right) retrosplenial cortex. Panel B (below) is a schematic representation of cannula placements 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 a considerable overlap of cannula placements. The numbers represent the approximate distance from bregma in mm. All scale bars are 150 μm. 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.

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 the retrosplenial cortex. In some cases, the virus injection spreads into the dentate gyrus, which does not directly innervate the retrosplenial cortex.

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

A series of independent *t*-tests considered whether there might be pre-surgery (Standard condition only) or post-operative training performance differences between the

iDREADDs and GFP-control rats on the five T-maze task conditions prior to any drug infusions. The two groups did not differ significantly on the pre-surgery training nor on the baseline training prior to the 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 $t_s < 1.01$, $p_s > 0.29$.

3.3 | Performance on test conditions

In a preliminary analysis 'Cohort' was included as a second between-subject factor to establish if there were any differences between the two cohorts. The only main effect of the 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 the subject) while Group refers to iDREADDs or GFP infusions (between subject).

3.3.1 | Standard T-maze

There was a significant main effect of Drug: $F_{1,20} = 7.01$, $p = 0.015$, $\eta_p^2 = 0.25$, but not a main effect of Group or Drug × Group interaction: $F_s < 0.85$, $p_s > 0.37$, $\eta_{ps}^2 < 0.04$ (Figure 4.1). 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.

3.3.2 | Start T-maze

There was a main effect of Drug: $F_{1,20} = 5.76$, $p = 0.03$, $\eta_p^2 = 0.22$, but no main effect of Group or Drug × Group interaction: $F_s < 3.48$, $p_s > 0.08$, $\eta_{ps}^2 < 0.15$ (Figure 4.2). This pattern of results corresponded to that seen for the Standard condition.

3.3.3 | Rotation T-maze

There was a significant main effect of Drug: $F_{1,20} = 12.02$, $p = 0.002$, $\eta_p^2 = 0.37$ but also a Drug × Group interaction: $F_{1,20} = 6.8$, $p = 0.016$, $\eta_p^2 = 0.25$. Simple main effects analyses revealed a significant decline in performance following clozapine infusions within the iDREADDs group that was not seen in the GFP control group: $F_{1,13} = 25.36$, $p < 0.0001$ (Figure 4.3). All other tests were non-significant: $F_s < 2.75$, $p_s > 0.11$.

3.3.4 | Opposite arm T-maze

As in the Rotation condition, there was a significant main effect of Drug: $F_{1,20} = 7.7$, $p = 0.01$, $\eta_p^2 = 0.278$ and a Drug × Group interaction: $F_{1,20} = 4.55$, $p = 0.045$, $\eta_p^2 = 0.18$. Again, there was a 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 4.4). All other tests were non-significant: $F_s < 0.89$, $p_s > 0.35$.

3.3.5 | Dark T-maze

The behavioural analyses revealed a significant Drug × Group interaction: $F_{1,20} = 7.8$, $p = 0.011$, $\eta_p^2 = 0.28$, however, there was no main effect of Drug or Group: $F_s < 0.036$, $p_s > 0.85$. Follow-up simple main effects analyses showed again that the 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 4.5). 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.

4 | DISCUSSION

Although the potential significance of the direct hippocampal projections to the retrosplenial cortex has long been appreciated (Sutherland & Hoesing, 1993; Vann et al., 2009), their importance for spatial memory has only been tested with context fear conditioning (Yamawaki, Corcoran, et al., 2019; Yamawaki, Li, et al., 2019). By combining iDREADDs injections into the dorsal subiculum with clozapine infusions into the retrosplenial cortex, the present study sought to disrupt the direct projections from the dorsal subiculum to the granular retrosplenial cortex. The behavioural analysis involved five variations of reinforced T-maze alternation, a working memory task. The iDREADDs manipulation

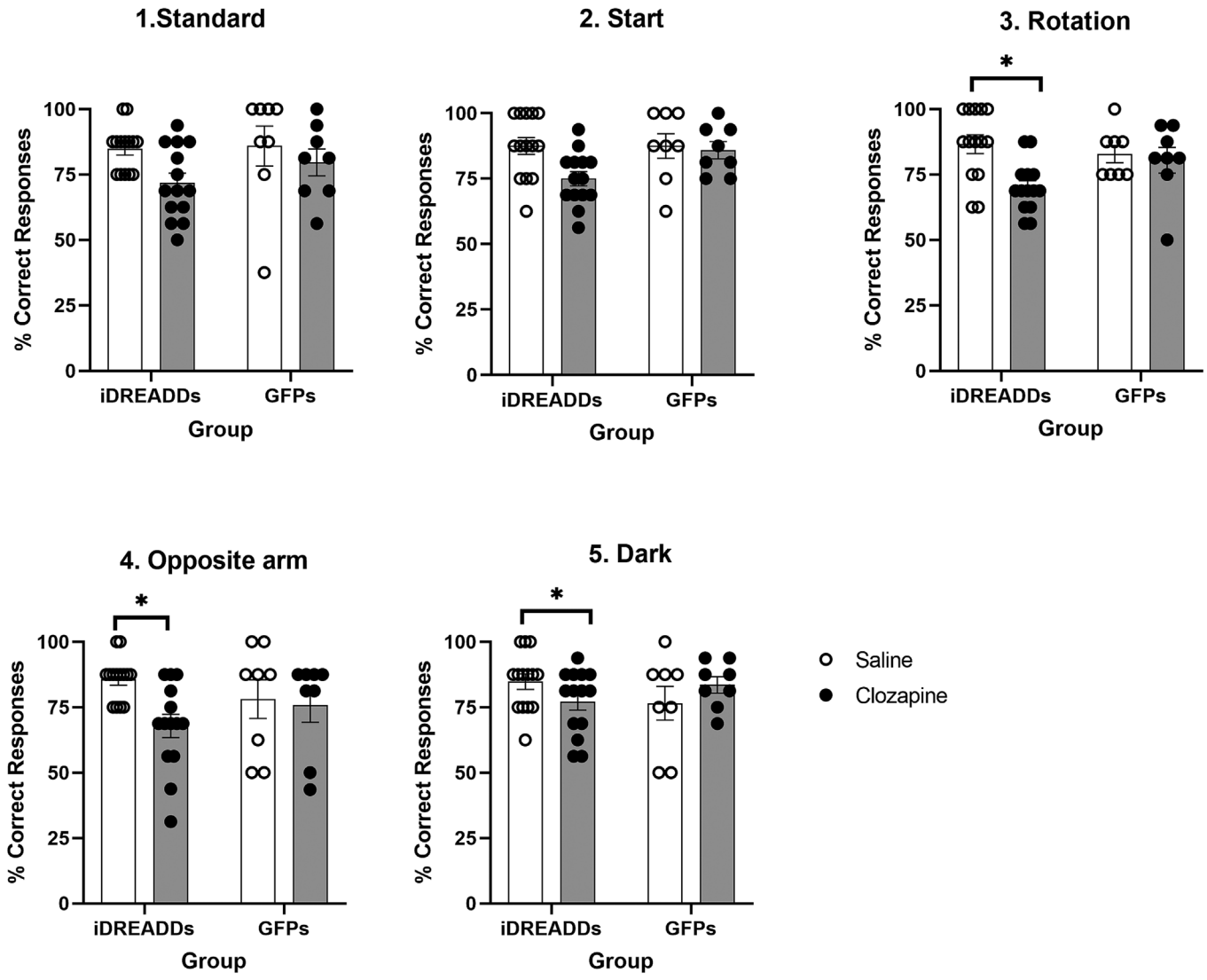

FIGURE 4 Bar graphs depicting the mean and each animal's individual percentage of correct alternation responses for both the iDREADDs and GFP-control groups. From top left to bottom right: 1) standard T-maze; 2) start T-maze; 3) rotation T-maze; 4) opposite arm T-maze; 5) dark T-maze. Despite the within-group 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.

only consistently impaired T-maze alternation on the three test conditions that involved changes to those cues available to solve the spatial working memory task.

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).

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 effects 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. These same two null results are informative as they lessen the likelihood that any subsequent deficits were a result of nonspatial effects, such as a decrease in motivation or the emergence of motor biases.

However, iDREADDs activation more clearly impaired spatial working memory on the Rotation, Opposite arm and Dark alternation conditions. This pattern of deficits does not simply reflect task difficulty, as scores during the intervening infusion-free days, during the iDREADDs/saline condition and by the GFP-control

group (Figure 4) all remained extremely similar across all five conditions. While clozapine infusions into the retrosplenial cortex may have modest effects, it was when the clozapine was able to interact with the transported iDREADDs that more consistent deficits emerged in these three conditions (Figure 4). This pattern highlights how clozapine alone was not responsible for the pattern of effects. A further implication is that the clozapine/iDREADDs interaction disrupted more than one type of task strategy, given the varying demands of the final three conditions (Figure 2). This pattern of results points to the consistent emergence of deficits whenever cue types are changed and restricted.

The temporal pattern of results (the last three conditions impaired) showed that the chemogenetic effects did not disappear over time and training. An opposite concern is that post-operative testing may have resumed too soon so the virus was not fully transported to its target. That possibility is, however, seen as most unlikely as pilot studies repeatedly showed that by two weeks post-surgery there is very extensive transport to the granular retrosplenial cortex. In the present study, the first infusions were a minimum of three weeks post-surgery. By counterbalancing the sequence of the five behavioural conditions it might have been possible to address this issue. In practice, that was not attempted. The problem is that each behavioural condition required different amounts of pretraining 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 have been further exacerbated by the different transfer effects from each specific condition to the next condition.

While the present study lacks direct evidence as to the extent of retrosplenial cortex neuronal disruption associated with clozapine infusions, other studies using comparable methodologies have demonstrated their effectiveness (Bubb et al., 2021; Yamawaki, Li, et al., 2019). That the iDREADDS/clozapine combination disrupted neural processing can also be inferred from the performance disruptions. Consistent with this assumption is how the pattern of behavioural deficits in the iDREADDS rats has obvious similarities with the effects of conventional lesions in the two target sites (Pothuizen et al., 2010; Potvin et al., 2007, 2010). There remains a concern as to whether the clozapine infusions reached sites beyond the 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 the retrosplenial cortex (Nelson et al., 2015; Yamawaki, Li, et al., 2019).

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) or 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; Hunt 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 special 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 considerably more selective, targeting just one set of granular retrosplenial inputs (Figures 5, 6). Together, these findings underline the significance of the hippocampal (subiculum) efferents to granular retrosplenial cortex when spatial cue usage is restricted, so that greater flexibility is required.

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; de 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

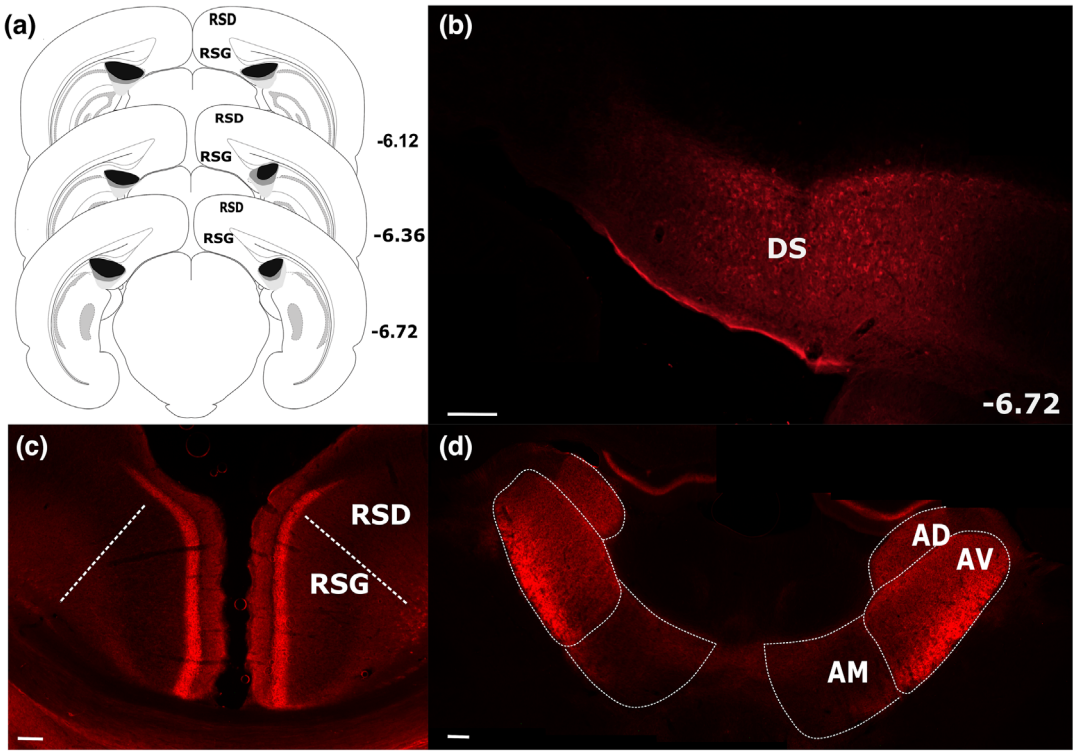

FIGURE 5 **Virus expression in the iDREADDs group.** Panel A shows the smallest (black) and largest (light grey) injection sites across the dorsal subiculum. Numbers refer to the distance from bregma in mm. Panel B shows an example of iDREADDs expression in the dorsal subiculum. Panel C shows the robust 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 150 μm. AD, anterodorsal nucleus; AM, anteromedial nucleus; AV, anteroventral nucleus, DS, dorsal subiculum, RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex.

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 the granular retrosplenial cortex (Yamawaki, Li, et al., 2019). 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 the retrosplenial cortex were silenced, again in a contextual fear conditioning paradigm, and their actions contrasted with those of the anterior thalamic inputs to the retrosplenial cortex (Yamawaki, Corcoran, et al., 2019). While both pathways are involved in the acquisition of contextual fear memory, they act in opposing ways. The inhibitory CA1 projections are normally suppressed, while the excitatory anterior thalamic projections normally enhance the acquisition of context memories (Yamawaki, Corcoran, et al., 2019).

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 the 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 the 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, Corcoran, et al., 2019). In addition, a recent study found that some granular retrosplenial neurons in layer V project directly to

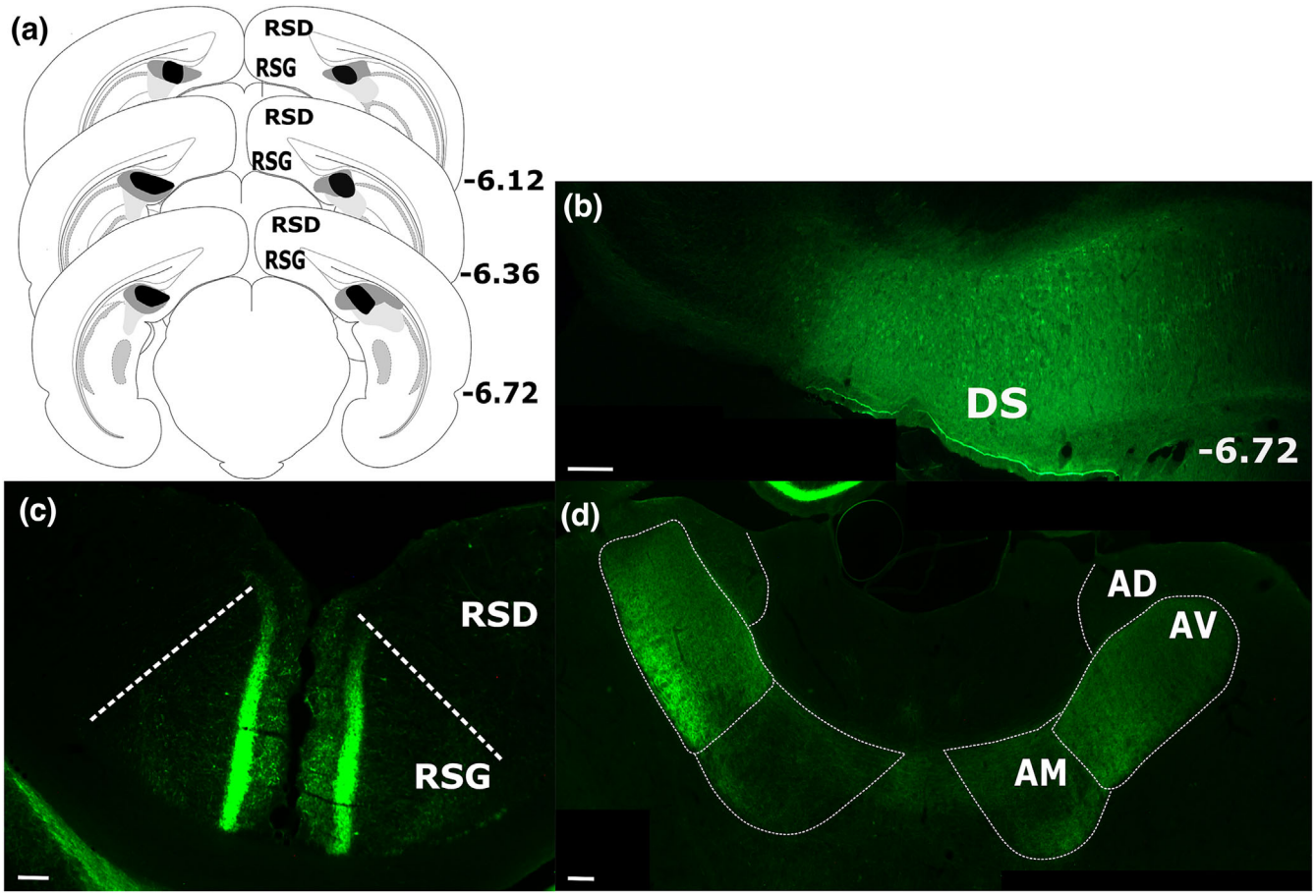

FIGURE 6 **Virus expression in the GFP-control group.** Panel A shows the smallest (black) and largest (light grey) injection sites across the dorsal subiculum. Numbers refer to the distance from bregma in mm. Panel B shows an example of iDREADDs expression in the dorsal subiculum. Panel C shows the robust expression of the 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 150 μm. AD, anterodorsal nucleus; AM, anteromedial nucleus; AV, anteroventral nucleus, DS, dorsal subiculum, RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex.

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).

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 & 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 the 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).

Finally, the finding that the widespread disruption of multiple subiculum efferents has very similar behavioural 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 the retrosplenial cortex enables 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. Within this triangle, the retrosplenial cortex stands out for its additional parietal and occipital connections. In this way, hippocampal-retrosplenial connections help the animal to navigate optimally in a changing environment.

AUTHOR CONTRIBUTIONS

S.Y. surgeries, data collection, histological analyses, data analyses, and manuscript preparation; B.E.F. surgeries and data collection, E.A. histology, A.J.D.N. additional surgeries/cannulations; J.P.A. funding, experimental design, manuscript preparation.

ACKNOWLEDGEMENTS

The study was supported by the Wellcome Trust (Grants: 108891/B/15/Z and 103722/Z14/Z). Illustrations were created with Biorender.com.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ejn.16303.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from https://doi.org/10.6084/m9.figshare.24891234.

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How to cite this article: Yanakieva, S., Frost, B. E., Amin, E., Nelson, A. J. D., & Aggleton, J. P. (2024). Disrupting direct inputs from the dorsal subiculum to the granular retrosplenial cortex impairs flexible spatial memory in the rat. *European Journal of Neuroscience*, 1–17. https://doi.org/10.1111/ejn.16303