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Perirhinal cortex and the recognition of relative familiarity

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ABSTRACT

Spontaneous object recognition (SOR) is a widely used task of recognition memory in rodents which relies on their propensity to explore novel (or relatively novel) objects. Network models typically define perirhinal cortex as a region required for recognition of previously seen objects largely based on findings that lesions or inactivations of this area produce SOR deficits. However, relatively little is understood about the relationship between the activity of cells in the perirhinal cortex that signal novelty and familiarity and the behavioural responses of animals in the SOR task. Previous studies have used objects that are either highly familiar or absolutely novel, but everyday memory is for objects that sit on a spectrum of familiarity which includes objects that have been seen only a few times, or objects that are similar to objects which have been previously experienced. We present two studies that explore cellular activity (through *c-fos* imaging) within perirhinal cortex of rats performing SOR where the familiarity of objects has been manipulated. Despite robust recognition memory performance, we show no significant changes in perirhinal activity related to the level of familiarity of the objects. Reasons for this lack of familiarity-related modulation in perirhinal cortex activity are discussed. The current findings support emerging evidence that perirhinal responses to novelty are complex and that task demands are critical to the involvement of perirhinal cortex in the control of object recognition memory.

1. Introduction

The perirhinal cortex (PRh) has been heavily implicated in the processing of item novelty/familiarity within recognition memory. Its ablation leads to significant item recognition deficits (Aggleton et al., 2010; Barker et al., 2007; Ennaceur et al., 1996; Meunier et al., 1993; Mumby & Pinel, 1994; Nemanic et al., 2004; but see McTighe et al., 2010), while neuroimaging and single unit recordings in animals have repeatedly shown differences in activity within this region for absolutely novel as compared to familiar items (Wan et al., 1999; Xiang & Brown, 1998; Zhu et al., 1995, 1996, Ahn et al., 2019 but see Burke et al. 2012, Deshmukh et al., 2012). In addition, *c-fos* imaging studies have shown greater perirhinal activation after the passive presentation of an absolutely novel compared to a familiar item (Wan, Aggleton & Brown, 1999; Zhu, et al., 1995, 1996). However, no such increased *c-fos* expression is seen in medial temporal lobe regions downstream of the perirhinal cortex such as the lateral entorhinal cortex or the hippocampus (Zhu et al., 1995, 1996). Consistent with these studies, other markers of neural activation have also demonstrated increased perirhinal activation in response to absolutely novel rather than familiar objects including CAMKII (Tinsley et al. 2009) and CREB phosphorylation (Warburton et al. 2005).

More recently, *c-fos* expression in perirhinal cortex has been examined using the gold standard test of item recognition in rats; the spontaneous object recognition task (SOR). This task has been used extremely widely to understand the neural mechanisms supporting animals' ability to distinguish between novel and familiar objects (Ameen-Ali et al. 2015, Sivakumaran et al. 2018). Consistent with previous studies, Albasser et al. (2010a) showed increased perirhinal cortex *c-fos* expression in response to absolutely novel objects in rats actively exploring objects in the SOR task.

These data are largely consistent with the assumption in the animal literature that novelty and familiarity depend upon a single process within perirhinal cortex, where the level of neural response codes for the

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level of familiarity/novelty of an object. However, this assumption has been questioned by both animal and human experiments. In addition to the well described cells that show a decreasing signal in response to multiple presentations of an object (familiarity neurons; Ahn et al, 2019, Fahy et al, 1993; Von Linstow-Roloff et al., 2016), other studies have described a distinct population of cells that showed an enhanced response to the first presentation of a novel object (novelty neurons), providing evidence for two distinct neural mechanisms within the medial temporal lobe to code novelty and familiarity (Xiang & Brown, 1998; Ahn et al, 2019). Consistent with this, structural equation modelling of *c-fos* expression in animals carrying out SOR tasks and human fMRI studies have also shown evidence for anatomically distinct regions signaling familiarity and novelty within the medial temporal lobe (e.g. Daselaar, Fleck & Cabeza, 2006).

These studies showing dissociable novelty and familiarity systems offer a novel perspective on previous rodent SOR data showing perirhinal activation for novelty in this task. Albasser et al. (2010a) used two versions of the SOR task; one employing only absolutely novel and the other very familiar stimulus sets. However, objects encountered in everyday life usually sit on a spectrum of familiarity from things we have encountered on only one or two occasions to things we see everyday. Clearly our memory systems can distinguish things that are highly familiar from things that are only mildly familiar and yet the question of how the perirhinal cortex responds to varying levels of familiarity is still unclear. One way of addressing this issue would be to use stimulus sets with varying levels of familiarity which would allow better understanding of the processes involved in novelty and familiarity detection within the perirhinal cortex. If novelty and familiarity are a single neural process, then a test of SOR in which one item is entirely novel and one is familiar will result in the novelty signal in perirhinal cortex being decreased. Increasing the level of familiarity of the familiar object would lead to a larger reduction in this novelty signal, i.e. the degree to which an object is relatively familiar would be expected to modulate c-fos expression. In contrast, if novelty and familiarity are dissociable mechanisms then the perirhinal activation in response to a novel object will not be mediated by the levels of familiarity of the familiar item. Evidence from Albasser et al. (2010a) shows that these changes in response to novelty may occur differentially within different rostrocaudal perirhinal cortex subregions, implying changes in object novelty do not affect cfos expression uniformly across the perirhinal cortex. Therefore any changes in activity in the current experiments need to be considered at both the level of the perirhinal cortex and at the subregion level to ensure that variations within the perirhinal cortex are not hidden within overall perirhinal activity levels.

Here then, we adapt the SOR task to manipulate the degree of familiarity for the items at test and test these hypotheses using two different experimental approaches. We start in experiment 1 (Fig. 1) by using a standard single trial version of the SOR which mirrors that used in numerous studies of recognition memory in rodents. The task compares multiple groups of animals that have been given different levels of exposure to the object used as the familiar object at test. In all groups the novel item at test is absolutely novel. In experiment 2 (Fig. 2) we use a continual trials approach that has recently been shown to provide significant improvements in statistical power in SOR (Ameen-Ali et al, 2012; 2015; Kinnavane et al, 2015). To maximise the number of test trials and make the design more comparable to previous studies using the continuous trials approach, the familiarity manipulation from experiment 1 (objects experienced in multiple sample sessions) was replaced by an approach in which relative familiarity is manipulated by prior exposure to objects before the testing session. Animals therefore made object recognition judgements with familiar objects (ie making a choice of relative familiarity: both objects have been experienced by the animal, but only one was seen on the sample trial) or novel objects (ie making a choice of absolute novelty: neither object had been seen prior to sample and at test the 'novel' object had never been seen before).

By manipulating relative familiarity of the objects in both

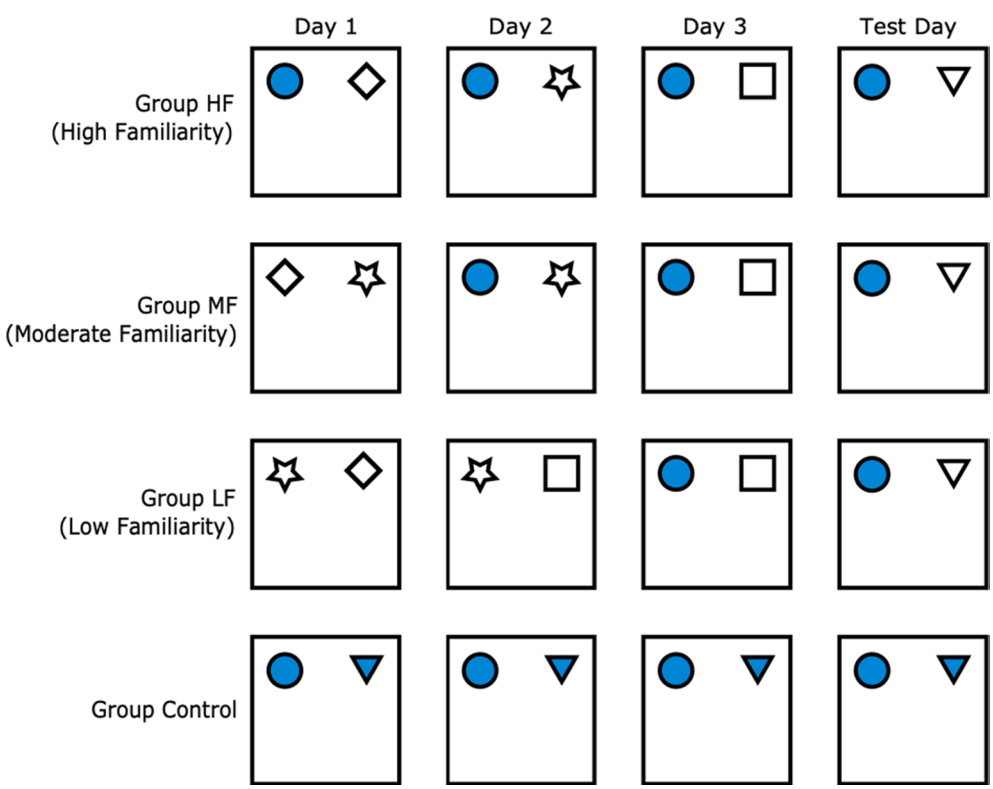

Fig. 1. Schematic of the experimental procedure for Experiment 1 illustrating different levels of relative familiarity of objects at test. Rats in each of the experimental groups were presented with a pair of objects on each day. On the test day, animals experienced the same objects across groups but the level of familiarity for the familiar object (blue circle) differed between groups. For the control group there was no novel object. Repeated objects used on the test day are shown in blue.

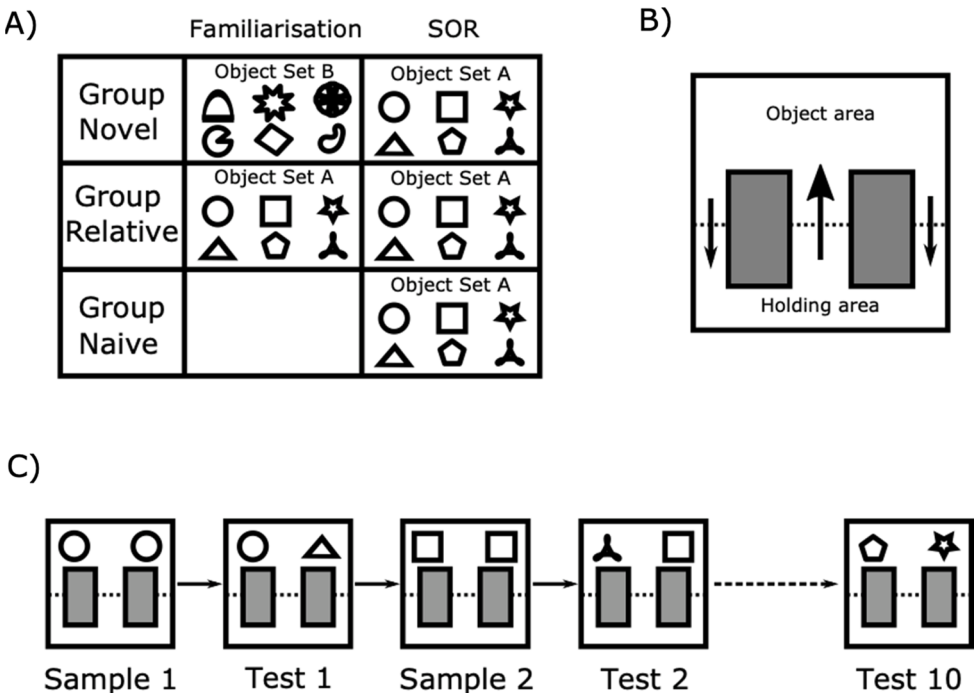

Fig. 2. Schematic of experimental procedure and apparatus for Experiment 2. A) All animals were tested with the same set of objects (Object Set A) in the SOR task. Prior to this, different groups had a different history of exposure to objects. Group Novel had been exposed to a set of distinct, but similar objects (Object Set B), Group Relative had been exposed to the same objects (Object Set A) that were used in the SOR task, Group Naive had no exposure to objects prior to the SOR task. B) The continual trials apparatus for SOR. Animals were tested in this apparatus for multiple trials in a single session. The animal started each trial in the object area and then when a door in the central arm (dotted line) was opened they self-shuttled through to the object area where objects were presented at sample and test. At the end of each exploration period the doors in the side arms (dotted lines) were opened and the animal self-shuttled back to the holding area where it would wait whilst the experimenter changed objects in the object area ready for the next exploration. C) The SOR task. Each trial consisted of one sample and one test, with the test comprising a copy of an object previously seen at sample and one object not seen at sample. Animals completed 10 trials in a single session. Task performance required the animal to make within-session judgements of novelty and familiarity of the objects, however the absolute novelty of the objects was determined by the history of the animal's previous exposure to objects (see A).

experiments we will examine whether the previously reported increased *c-fos* activation to novelty (Albasser et al., 2010a) is modulated by levels of familiarity of the objects at test.

2. Material and Methods:

2.1. Subjects

2.1.1. Experiment 1

Thirty six male naive Lister Hooded rats (Harlan Olac Ltd., UK) weighing on average 350 g at the start of the experiment were used. All animals were housed in pairs on a 12-hour light/dark cycle, with behavioural testing taking place during the light phase. To allow for greater motivation for the rats on the tasks, their food access was controlled such that their weights were maintained at no less than 85% of their free- feeding weight. Rats had ad libitum access to water in their home cages. All procedures were carried out under the Project License numbers 70/8306 and 60/4069, and Personal License number 60/13883. All procedures were approved by the Animal Welfare Ethics Committee of the University of St Andrews, and complied with national (Animal [Scientific Procedures] Act, 1986) and international (European Communities Council Directive of 24 November 1986 [86/609/EEC]) legislation governing the maintenance of laboratory animals and their use in scientific research.

Nine animals were assigned to each of four groups: high familiarity (HF), medium familiarity (MF), low familiarity (LF) and control (Con). Due to health complications, one rat was omitted from testing, such that the final group size for group HF was 8.

2.1.2. Experiment 2

Eighteen male naive Lister hooded rats (Durham University Life Sciences Support Unit in-house breeding colony) weighing on average 300 g at the start of the experiment were used. All animals were housed in pairs on a 12-hr light–dark cycle, with behavioural testing taking place during the light phase. Water was available ad libitum throughout the study, except when in the apparatus. To motivate animals to shuttle continuously in the apparatus, all animals were food deprived to no less than 85% of the free-feeding body weight of aged matched controls throughout testing. All procedures were carried out under the Project License number 40/3388, and Personal License number 70/24277. All procedures were approved by the Animal Welfare Ethics Committee of Durham University, and complied with national (Animal [Scientific Procedures] Act, 1986) and international (European Communities Council Directive of 24 November 1986 [86/609/EEC]) legislation governing the maintenance of laboratory animals and their use in scientific research.

Six animals were assigned to each of three groups: Relative (animals were exposed prior to testing to the same objects used in the SOR task and therefore made relative novelty judgements about familiar objects), Novel (animals had been exposed to objects prior to the SOR task, but within the SOR task itself saw a set of objects not previously experienced and therefore were familiar with objects but made judgements of absolute novelty in the SOR task) and Naive (animals had no experience of any objects prior to testing and therefore in the SOR task made absolute novelty judgements whilst being completely naive to objects). The testing history of the different groups can be seen in Fig. 2

2.2. Apparatus

2.2.1. Experiment 1

All behavioural testing took place in a wooden 67 cm square arena with 40 cm high grey patterned walls and a dark blue floor. Behaviour was monitored live and recorded from via webcam. All objects were 3D easily cleanable household objects and toys of approximately the same size as a rat in one dimension. Objects were made of either plastic or metal, and fixed to the floor using Dual Lock Velcro (3 M2, St. Paul, MN).

2.2.2. Experiment 2

Familiarisation of objects prior to the SOR testing (Groups Relative and Novel) was carried out in a 1 m^2 open field. Each familiarisation phase occurred in two contexts: Context 1- a hatched wire surface on the floor of the apparatus with a coloured circle pattern on the walls; Context 2- a grey smooth surface on the floor of the apparatus with a pink and white striped pattern on the walls.

The SOR task took place in a continual trials apparatus (see Ameen-Ali et al., 2012, for a full description). Briefly, the animals were tested in a square shaped apparatus which comprised of an E-shaped object area abutting an E-shaped holding area. Opaque guillotine doors divided the two areas and could be manually opened and closed to allow the animal to shuttle from one area to the other. During sample and test phases, objects were placed in the top left and top right-hand corners of the object area of the maze approximately 2 cm away from the walls to allow animals to move around the objects and explore them fully. Behaviour in the SOR tasks was recorded from a small camera above the apparatus for scoring off-line.

All objects were 3D easily cleanable household objects and toys of approximately the same size as a rat in one dimension.

2.3. Procedure

2.3.1. Experiment 1

Rats were handled by the experimenter daily for five days prior to any behavioural testing or habituation. During behavioural testing, rats were always brought into the testing room in home-cage groups and placed in a holding cage in the room. They were then tested individually.

2.3.1.1. Habituation. Rats were habituated to the apparatus by being placed into it, facing the back wall, by themselves and allowed to explore the environment for 10 min. This was done on four consecutive days for each rat. All habituation occurred with no objects in the box.

2.3.1.2. Testing. Rats were randomly assigned to one of four groups: High Familiarity (HF), Moderate Familiarity (MF), Low Familiarity (LF) and Control (Con). Testing occurred on four consecutive days and rats were always placed into the box facing the back wall. All rats were presented with two objects on each day (Fig. 1) and given 10 min to explore these objects on sample trials (days 1–3), and 3 min on the test trial (day 4). The difference in the time allowed for exploration was implemented to ensure novel items presented on the test day did not have the time to become familiar, as would be expected within a 10-minute trial.

On sample 1 (day 1) both of the items presented were new to the rat. On subsequent days (including the Test day) one object was familiar, having been seen on the previous day, and one was new (Fig. 1). The test day consisted of two objects, a familiar one seen on the previous day and a new object. For rats in group HF, the familiar object at test had been presented on all three previous days. For rats in group MF, the familiar object at test had been presented on the two previous days. For rats in group LF the familiar object at test had been presented only once, on the day prior to the test day. This ensured that all rats in these conditions had the same number of exposures to objects, and also the same expectation that each day a novel and familiar item would be presented. Rats in group Con were presented with the same two objects on all days.

To control for any object-place confounds, for each rat a given object (e.g. object A) was always presented in the same location (left/right) of the testing box. The novel/familiar status of objects, along with the location of presentation (left/right) of familiar objects was counter-balanced between rats. The same two objects (e.g. A and Z) were presented on the test day for all groups, with half of the rats experiencing one object (e.g. A) as familiar while the other half experienced it as novel.

2.3.2. Experiment 2

Twenty objects were placed randomly around the apparatus at the start of each familiarisation phase with the same objects being used each time.

2.3.2.1. Habituation. All animals were initially given two sessions of handling by the experimenter and two sessions of habituation to the testing room during which they remained in their home cage with their cage mates for a period of 10 min per session in order to acclimatise to the experimental room. Low level diffuse light from a 20 W bulb within a desk lamp was used to dimly light the room without producing shadows on the apparatus. Constant white noise was played to mask any noises from outside the room. These conditions were constant for all habituation, familiarisation, and testing sessions.

2.3.2.2. Pretraining to use the continual trials apparatus. Pretraining involved the completion of five phases over five days aimed at habituating the animals to the continual trials procedure for SOR.

2.3.2.3. Phase 1. Rats freely explored the apparatus in pairs (cage mates) for a period of 30 min. All doors of the apparatus were open to encourage animals to explore the entire environment.

2.3.2.4. Phase 2. Rats freely explored the apparatus individually for 20 min. All doors of the apparatus were open to encourage animals to explore the entire environment.

2.3.2.5. Phase 3. Rats explored the apparatus individually, as for Phase 2, but for only 10 min.

2.3.2.6. Phase 4. Animals were placed individually into the apparatus and trained to shuttle between the two compartments; the holding area and the object area. This phase consisted of three sessions and involved placing dustless precision pellets (20 mg, Purified Diet; BioServ, Frenchtown, New Jersey, USA) on the floor of the apparatus, and using the doors to control the animals' movement between the areas. Each time after the animal had shuttled from one area to the other, the food was replenished.

2.3.2.7. Phase 5. Animals were placed individually into the apparatus in the holding area and shuttled into the object area. Here they were exposed for 3 min to two objects which each concealed two food pellets. After these 3 min, the doors on the outer arms of the apparatus were opened and the animals shuttled through to the holding area which also had two food pellets placed on the floor. Whilst the animal was in the holding area (with apparatus doors closed) the objects in the object area were changed, the central door opened, and the animals shuttled back into the object area. This was repeated for 4 different pairs of objects. No objects used in Phase 5 were used again in the SOR task.

2.3.2.8. Object familiarisation. All animals in Groups Relative and Novel were familiarised to a set of objects prior to SOR testing. The protocol to familiarise animals to objects was adapted from that of Albasser et al. (2010b). Animals received five familiarisation sessions over five days. Each session involved the animal being individually placed within the open field. Within the open field 20 objects were placed randomly within the arena and animals were able to explore these objects. Group Relative was familiarised with Object Set A (the same objects used in the subsequent SOR task), whilst Group Novel were familiarised with Object Set B (a set of objects which would not be used again after this familiarisation period). Group Naive did not experience this object familiarisation stage.

2.3.2.9. Spontaneous object recognition (SOR) testing. On the day immediately following the final object familiarisation session animals

completed SOR within the continual trials apparatus, each completing 10 trials within the single session, as outlined in Ameen-Ali et al (2012). All animals were tested using objects from Object Set A, irrespective of their previous object experience.

At the start of the session, the animal was placed in the holding area with the central door then immediately being opened to allow the animal to move through to the object area. For the sample phase of a trial the animal spent two minutes exploring two identical copies of an object in the object area before the doors on the outer arms of the apparatus were opened and the animal was allowed to shuttle back to the holding area which contained two food pellets. Whilst the animal was in the holding area the experimenter changed the objects in the object area ready for the test. After one minute in the holding area, the central door was opened again to allow the animal back into the object area which now contained a duplicate copy of the object seen at sample (familiar object) and a novel object. The animal would explore these objects for two minutes before the doors on the outer arms of the apparatus were opened for the animal to shuttle back to the holding area. When the animal was in the holding area the experimenter would then prepare objects in the object area for the sample phase of trial 2 and after one minute the central door would open to allow the animal back into the object area. This pattern repeated for the 10 trials (10 samples and 10 tests, each with trial unique objects).

For all samples and tests, both the novel and familiar objects were baited with two food pellets. This encouraged the animals to explore both objects, without differentially rewarding the animal's choice (ie it was a motivation for object exploration but did not reinforce exploration of one object over the other). This was in line with other procedures using continual trials (Albasser et al., 2010b; Ameen-Ali et al, 2012).

Within each trial, for each animal, the novel object referred to the object seen at test but not at sample and the familiar object referred to the object seen at both sample and test. The absolute novelty of the items did not change the nature of their novelty within a trial. Previous exposure to objects either had no impact on the familiarity of the objects in this SOR task (Group Novel) or meant that the animal had to make relative familiarity judgements (Group Relative) where novelty at test represented not having seen the novel item as recently as the familiar item, but being familiar with both through the object familiarisation phase. The location of the novel object was counterbalanced between trials within an animal and between animals within a trial to help counter any bias for exploration of the object on the left or right. Which object from Object set A was novel or familiar for a particular trial was also counterbalanced across animals.

A trial ended if an animal failed to shuttle to the next area of the apparatus after a period of three minutes. This would subsequently cease the testing session and the data for that animal's testing session would not be included in the data analysis.

The testing history of all groups can be seen in Fig. 2.

2.4. Perfusions and histology

An hour after completion of behavioural testing on the test day (day 4), animals were given an overdose of sodium pentobarbitone and then perfused transcardially with 50 ml phosphate-buffer saline, followed by at least 250 ml of 4% paraformaldehyde solution made up with 0.1% phosphate buffer. Brains were removed and cryoprotected in 20% sucrose solution (made up in 0.1% phosphate buffer) until sectioning.

Series of 50 μm coronal sections were cut on a freezing microtome within 6 days of the end of testing, with an equal number of brains from animals in each group being cut on any given day. One in four sections were used for subsequent staining and quantification. The sections were stored in antifreeze in a freezer pending *c-fos* activation immunohistochemistry.

Sections were processed for *c-fos* activation immunohistochemistry as described previously (Ainge, Jenkins, & Winn, 2004; Wilson, Langston, et al., 2013). After being washed in phosphate buffer, sections were placed in blocking solution (20% normal goat serum) for 60 min. These sections were then incubated in anti-*c-fos* primary antibody at a concentration of 1: 8000 (Oncogene Research Products, Calbiochem) overnight. Sections were then removed, washed in phosphate buffer and placed in biotinylated IgG (anti-rabbit, Vectastain Elite ABC kit) in a concentration of 1:200 for 60 min before finally being incubated in avidin–biotin complex (Vectastain Elite ABC kit) at a concentration of 1:50 for a further 60 min. Sections were then reacted with nickel enhanced 3,3-diaminobenzidine tetrahydrochloride (Sigma) before being mounted, dehydrated, and cover slipped with DPX. Negative controls where either primary or secondary antibodies were excluded showed no cellular staining.

2.5. Behavioural analysis

2.5.1. Experiment 1

A behavioural measure of object recognition in the form of an exploration-based Discrimination Ratio (DR) was obtained from the task. The DR is a measure of the exploration allocated to a novel object (T_{novel}) as compared to a familiar one ($T_{familiar}$), as a proportion of total exploration time (T_{total}) to control for intrinsic variability in rats' levels of exploration: $DR=(T_{novel}-T_{familiar})/T_{total}$. Times were scored off-line from recordings of the first three minutes of each session. Animals were scored as exploring an object when the rat had its head oriented towards the object with its nose less than 2 cm away from the object. Moments when the rat was touching the object with another part of the body, or when leaning or rearing against it in order to investigate the area above it were not scored as object exploration. For group Con where there was no novelty at test the DR was calculated based on objects alone, i.e. $DR=(T_{objectA}-T_{objectB})/T_{total}$.

2.5.2. Experiment 2

Scoring of performance on individual trials was as for experiment 1 (see 2.1.5). As continual trial SOR tasks involve multiple trials, the DR was calculated for each trial individually and then an average DR for the 10 trials was calculated for each animal.

2.6. Histological analysis

Regions of interest were identified using a combination of a Digital atlas of the rat hippocampal region (Kjonigsen, Leergaard, Witter & Bjaalie, 2011) and a stereotaxic atlas of the whole rat brain (Paxinos & Watson, 2006). Region boundaries were chosen to parallel those identified by Albasser et al., (2010a). The perirhinal cortex (PRh) was subdivided into three sub regions: rostral (from AP −2.76 to −3.84 relative to bregma), mid (from AP −3.84 to − 4.80 relative to bregma) and caudal (from AP −4.8 to −6.30 relative to bregma). Fig. 3 illustrates the areas of perirhinal cortex sampled.

Fos quantification were as previously described by Wilson et al., (2013) and was carried out blind to the experimental condition. Photographs of the regions of interest were taken at 10x magnification with a consistent light level. Fos expression was quantified bilaterally for a minimum of three (and maximum of four) sections per region of interest. Images were processed using Scion Image software (v4.0.3.2) as follows: Fos expression was identified by taking a mean grayscale for each image and identifying pixels that were 2 standard deviations darker (or more saturated) than the mean. Fos positive neurons were classified as groups of more than 50 and less than 1000 adjacent pixels whose saturation was greater than 2 standard deviations from the mean for that image, and their count recorded. Density of Fos positive neurons was calculated by measuring the area in mm^2 of the region from which Fos positive cell counts within perirhinal cortex were taken and then dividing the number of Fos positive cells by this area. To allow comparisons of activities across different brain regions with differing cell densities, cell counts were normalised by dividing them by the mean count for that area across groups and multiplying by 100.

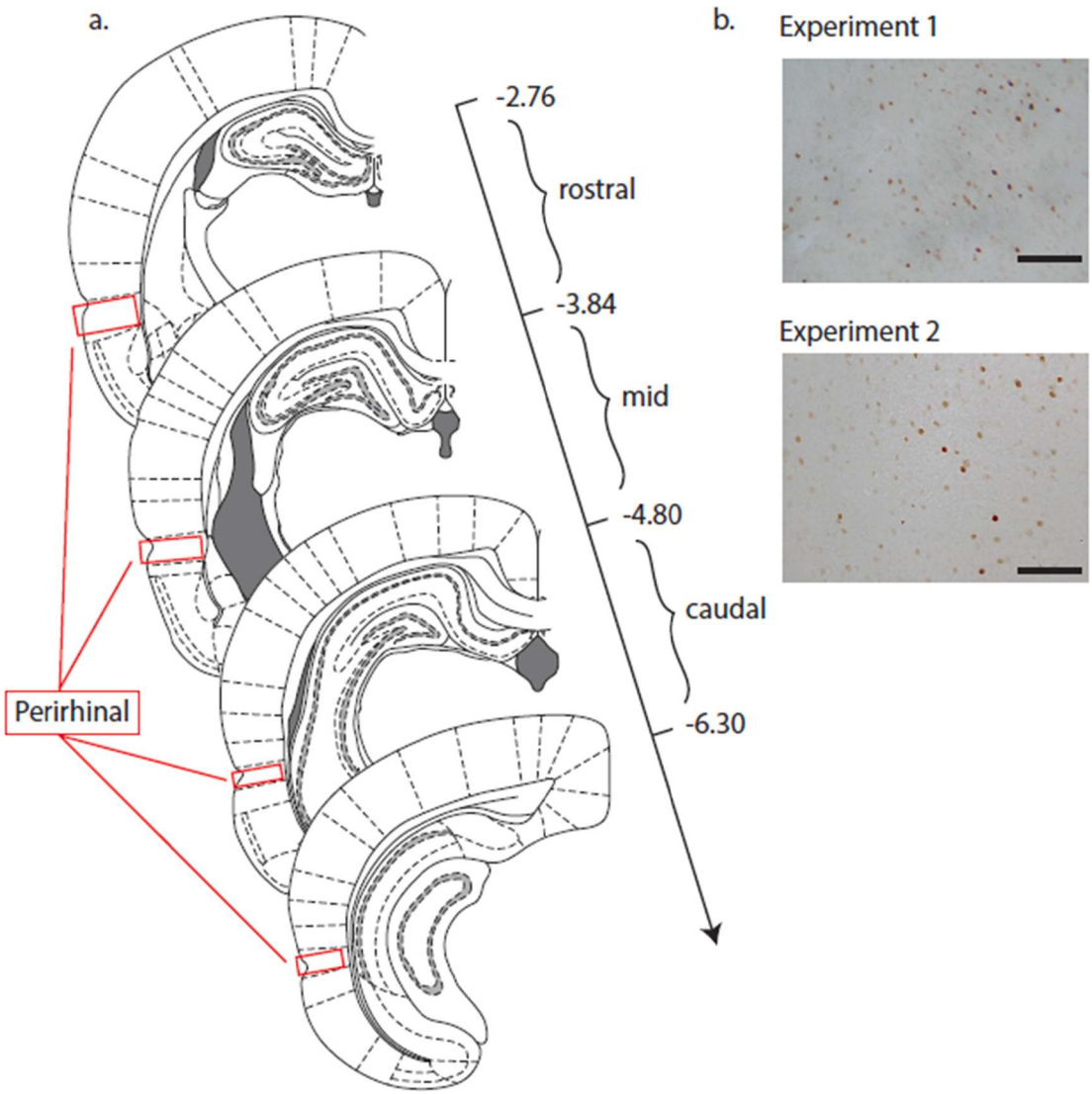

Fig. 3. Perirhinal *c-fos* expression. a. Schematic representation of region of interest sampling. Numbers represent distance from Bregma in mm. Figures adapted from Paxinos & Watson (2006). b. Representative photographs of Fos positive neurons in perirhinal cortex from both experiments.

2.7. Statistical analysis

Preferential exploration of the novel object was compared to chance using 1-sample t-tests, with zero as the value of comparison. Rats discrimination behaviour and total exploration in the test trials were compared across experimental groups using one-way (groups: HF, MF, LF) ANOVA. Where one-way ANOVAs were significant, follow-up Bonferroni corrected pairwise comparisons were carried out. Further analyses of combined Novelty-Naïve vs. Relative groups in experiment 2 used independent samples t-tests. Exploration on Day 1 of training was analysed with mixed-factorial ANOVA with group (HF, MF, LF, control) as between subjects factor and object as within subjects factor to ensure that all groups explored equally when memory status of the object was consistent. SOR performance in training was assessed using a mixed-factorial ANOVA with group (HF, MF, LF) as between subjects factor and day (2,3,4) and object memory status (novel, familiar) as within subjects factors.

Fos expressing cell densities in perirhinal cortex were analysed using a mixed-factorial ANOVA with region (rostral, mid, caudal) as the within subjects factor and group (HF, MF, LF, C) as the between-subjects factor.

Perirhinal Fos expressing cell densities were collapsed across all perirhinal subregions (rostral, mid and caudal) and correlated to DR using Pearsons' correlation to investigate potential relationships between recognition behavior and activity within the perirhinal cortex.

3. Results

3.1. Experiment 1

3.1.1. Behavioural results

Fig. 4a displays the mean DRs for all groups on the test day. The positive DRs for all experimental groups demonstrate preferential exploration of the novel object compared to the familiar object as a proportion of total exploration time. 1-sample t-tests confirmed that this preferential exploration was above chance for all experimental groups: High Familiarity ($t_{(7)} = 4.21$, $p = 0.004$, $d = 1.49$); Moderate Familiarity ($t_{(8)} = 3.97$, $p = 0.004$, $d = 1.32$); Low Familiarity ($t_{(8)} = 4.14$, $p = 0.003$, $d = 1.38$). The control group showed no object preference as depicted by DR not being significantly different from chance ($t_{(8)} = 0.501$, $p = 0.630$, $d = 0.167$). Comparison of the control and experimental groups DRs is not meaningful as the control group only saw familiar objects but rats in the 3 experimental groups showed similar levels of discrimination between novel and familiar objects. This was

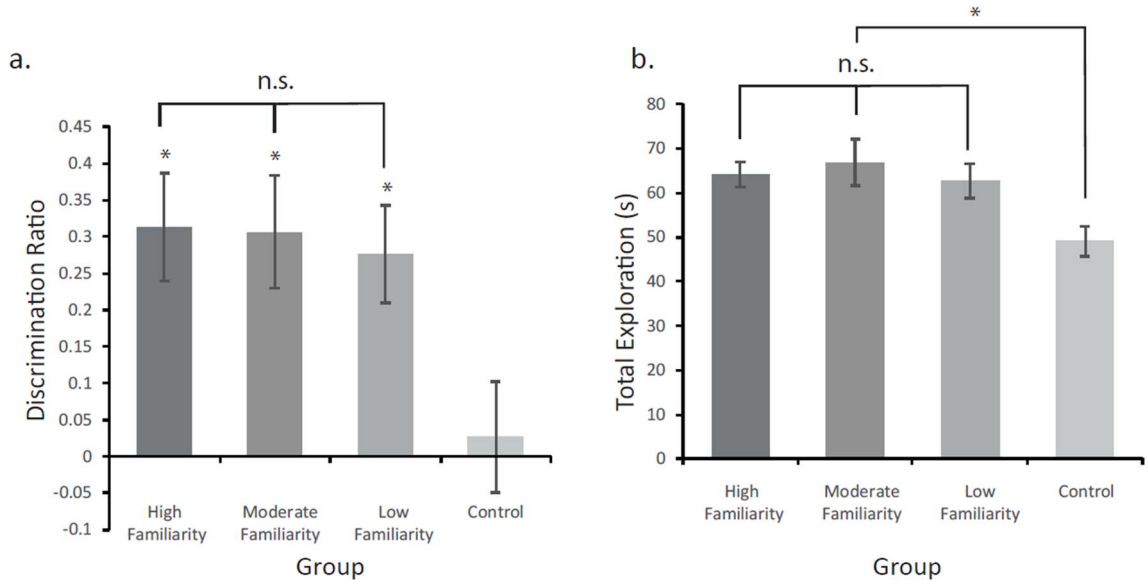

Fig. 4. SOR behaviour on test day in experiment 1. a) Mean discrimination ratios (DRs) for each experimental group. b) Mean total exploration times at test. All error bars show standard error. *p = less than0.05.

confirmed by the non-significant one-way ANOVA ($F_{(2,25)} = 0.075$, $p = 0.928$, $\eta^2 = 0.007$).

Total exploration in the test trials is presented in Fig. 4b. As expected, due to the familiarity of the objects, control animals explored the objects significantly less than the other groups. This was confirmed with a univariate ANOVA showing a significant main effect of group ($F_{(3,31)} = 4.802$, $p = 0.007$, $\eta^2 = 0.317$). Bonferoni corrected posthoc test revealed that the control group explored significantly less than all of the other groups, which did not differ from each other.

It remains possible that initial between group differences in exploration might mask differences between groups in performance. To test whether this was the case, exploration of the objects on day 1 was compared across groups (see Fig. 5a). Animals in all groups explored the objects equally, providing no evidence of side biases, anxiety or object preference between groups. This was confirmed by no significant effects of group or object in a mixed factorial ANOVA.

SOR performance across training days did also not differ across the 3 experimental groups (see Fig. 5b), demonstrating that the exposure of the different groups to different combinations of objects throughout training did not bias overall performance prior to the test day. All groups showed significant preference for novel over familiar objects across days confirmed by a significant main effect of object novelty/familiarity ($F_{(1,23)} = 175.28$, p less than 0.001, $\eta 2 = 0.884$). The groups did not differ in their performance and their performance did not change over days as evidenced by non-significant effects of group and day and a non-significant group × day interaction.

3.1.2. Fos results

Fig. 6a illustrates that there was no difference in the Fos immunoreactivity across groups in any of the perirhinal cortex sub-regions, as confirmed by a mixed-factorial ANOVA revealing no main effect of group, ($F_{(3,31)} = 0.379$, $p = 0.768$, $\eta^2 = 0.035$), and no Group × Subregion interaction ($F_{(6,62)} = 1.021$, $p = 0.419$, $\eta^2 = 0.09$). As the cell densities were normalised for each subregion, a main effect of subregion was not possible. The same pattern of results was observed when the raw cell densities were submitted to the same analysis and these are presented in Table 1.

A Pearsons' correlation confirmed that there was no significant

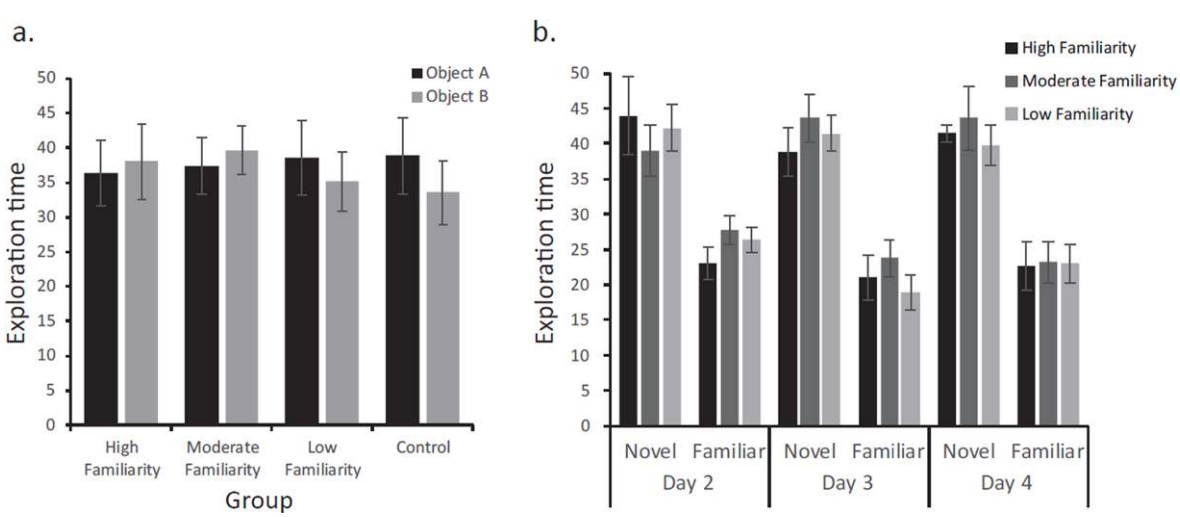

Fig. 5. Object exploration in experiment 1. a. Object exploration on Day 1. Objects A & B are novel for all animals. b. Novel and familiar object exploration across days 2–4.

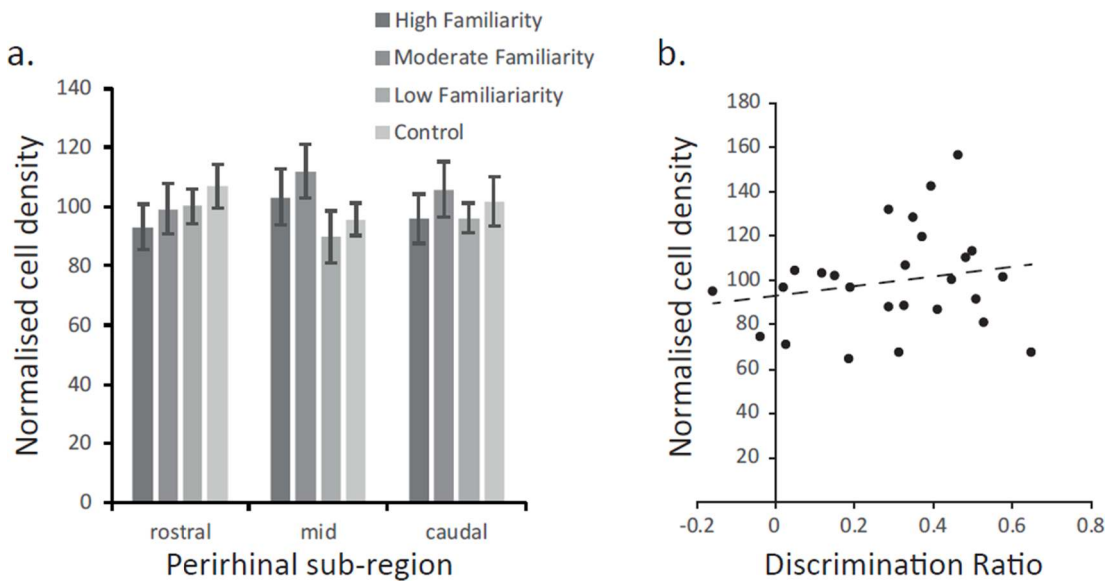

Fig. 6. a. Normalised Fos cell densities in rostral, mid and caudal perirhinal cortex for each group in experiment 1. b. Normalised Fos cell densities correlated with SOR discrimination ratio.

Table 1
Average cell densities (per mm2) in rostral, mid and caudal perirhinal cortex for each group in experiment 1 (mean ± SEM).

Perirhinal region	Rostral	Mid	Caudal
High familiarity	524.90 (±43.42)	472.40 (±43.32)	387.89 (±33.93)
Moderate familiarity	558.96 (±47.73)	512.74 (±41.32)	427.87 (±38.04)
Low familiarity	565.06 (±34.02)	412.15 (±39.78)	388.49 (±20.74)
Control	602.92 (±42.06)	438.77 (±25.59)	411.74 (±33.70)

relationship between DR and perirhinal cortex Fos expressing cell density (Fig. 6b; $r_{(24)} = 0.118$, $p = 0.567$).

3.2. *Experiment 2:*

3.2.1. *Behavioural results*

Fig. 7a displays the mean DRs for all groups. The positive DRs for all experimental groups demonstrate a preferential exploration at test of the novel object compared to the familiar object as a proportion of total exploration time. 1-sample t-tests confirmed that this preferential exploration was above chance for all experimental groups: Novel ($t_{(5)} = 5.751$, $p = 0.002$, $d = 2.35$); Relative ($t_{(5)} = 3.347$, $p = 0.018$, $d = 1.41$); Naive ($t_{(5)} = 5.053$, $p = 0.004$, $d = 2.06$). Animals in all groups showed similar levels of discrimination between novel and familiar objects. This was confirmed by the non-significant one-way ANOVA, $F_{(2,15)} = 0.566$,

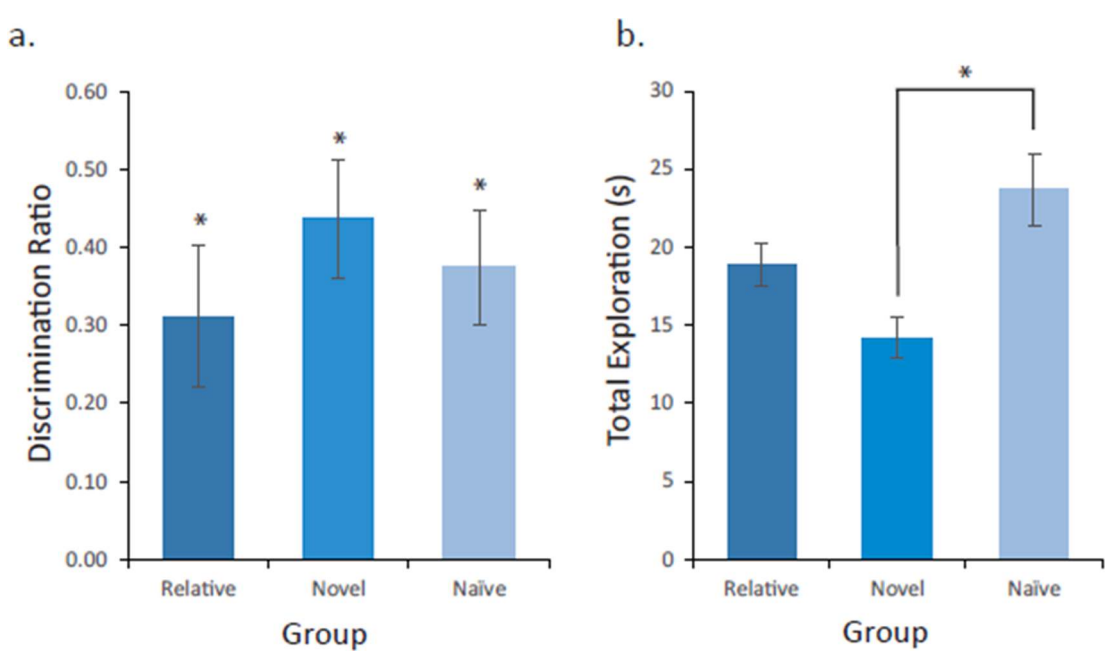

Fig. 7. SOR behaviour in experiment 2. a) Mean discrimination ratios (DRs) for each experimental group. b) Mean total exploration times at test. All error bars show standard error. *p = less than0.05.

$p = 0.579$, $\eta^2 = 0.070$).

Total exploration in the test trials is presented in Fig. 7b. As expected, animals in group Naive that had no prior experience of any objects spent the most time exploring objects at test. This was confirmed with a univariate ANOVA showing a significant main effect of group ($F_{(2,15)} = 7.645$, $p = 0.005$, $\eta^2 = 0.505$). Bonferoni corrected posthoc tests revealed that animals in Group Naive explored significantly more than animals in Group Novel, but there were no other significant differences between groups.

3.2.2. Fos results

Fig. 8a illustrates that there was no difference in the Fos immunoreactivity across groups in any of the perirhinal cortex sub-regions, as confirmed by a mixed-factorial ANOVA revealing no main effect of group, ($F_{(2,15)} = 2.800$, $p = 0.093$, $\eta^2 = 0.272$), and no Group × Subregion interaction ($F_{(4,30)} = 0.518$, $p = 0.723$, $\eta^2 = 0.065$). As the cell densities were normalised for each subregion, a main effect of subregion was not possible. The same pattern of results was observed when the raw cell densities were submitted to the same analysis (see Table 2). Given the trend towards significance in the group effect, combined with the existing literature showing a role for perirhinal cortex in SOR, further analyses aimed to examine whether differences in object familiarity at test mediated perirhinal Fos levels. Previous studies showing mediation of perirhinal activation have used stimuli that had never been experienced before the day of testing, similar to groups Naïve and Novel. To examine whether this is a critical factor Fos levels from these 2 groups were combined and compared to group Relative. Independent samples t-tests revealed no differences between the combined group Novel-Naïve and group Relative.

Next we examined the relationship between DR and perirhinal cortex Fos expressing cell density. Pearsons' correlation confirmed that there was no significant relationship between DR and perirhinal cortex Fos expressing cell density (Fig. 8b; $r_{(16)} = 0.414$, $p = 0.087$) although again a trend towards significance was seen.

4. Discussion

Through two distinct, but related, experiments of SOR in rats we have found little evidence of perirhinal *c-fos* expression being modulated by the relative, rather than absolute, familiarity of stimuli. This is consistent with the behavioural data from both experiments which showed no difference in novelty preference in animals exploring objects that differed in their relative familiarity. These findings contrast with studies that have reported populations of cells within perirhinal cortex which decrease their responding as a function of relative familiarity (Xiang & Brown 1998, Ahn et al. 2019). One interpretation of these data is that the familiarity for the stimuli at test has already reached its highest level and both experiments are demonstrating the maximum threshold of familiarity processing within perirhinal cortex. This would, however, suggest that 3 min of exploration is enough to fully saturate familiarity signaling and while this is possible would mean that either rats are incapable of differentiating the relative familiarity of any stimuli that have been experienced for at least 3 min or that they possess another neural mechanism outside of perirhinal cortex that processes longer term familiarity.

Both experiments have potential limitations for their interpretation. Experiment 1 uses carefully controlled training with objects to calibrate the experience with each object on the test day. However, *c-fos* and behaviour measures in experiment 1 come from a single trial in each animal. This risks both measures being noisy which may mask any true differences between groups. Nonetheless, the behavioural performance of each group is clearly above chance, and the lack of correlation between individual animals' performance and *c-fos* expression suggests that variability in the group measures alone are unlikely to explain the results in their entirety. In contrast, experiment 2 has the benefit of greater number of trials contributing to the behaviour and *c-fos* expression measures, but as a result the prior experience of objects is somewhat less controlled. We can, however, be confident that animals exposed to objects before testing pay attention to those objects as exploration times are highest at test in those animals with no previous exposure to objects. The increased number of trials experienced in

Table 2
Average cell density (per mm^2) in perirhinal cortex in Experiment 2 (mean ± SEM).

Perirhinal region	Rostral	Mid	Caudal
Relative	199.60 (±44.07)	242.60 (±25.33)	279.45 (±37.22)
Novel	262.98 (±46.15)	293.68 (±25.65)	373.80 (±42.68)
Naïve	298.71 (±32.01)	299.27 (±22.19)	329.98 (±21.24)

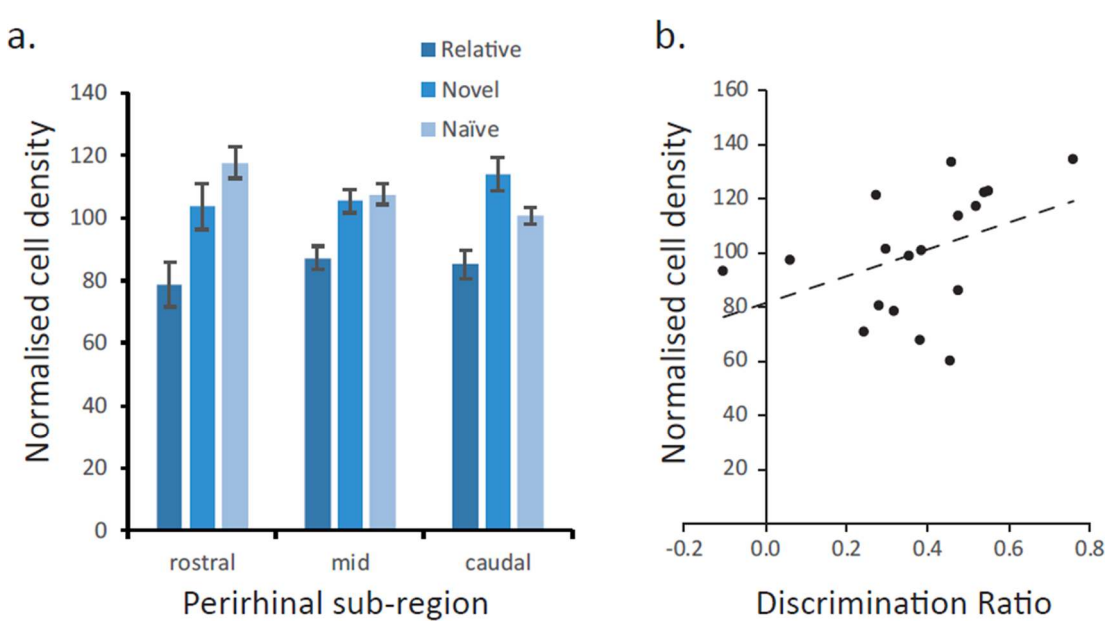

Fig. 8. a. Normalised Fos cell densities in rostral, mid and caudal perirhinal cortex for each group. b. Normalised Fos cell densities correlated with SOR discrimination ratio.

experiment 2 also increases the statistical power derived from small group sizes (Ameen-Ali et al, 2012; 2015; Kinnavane et al, 2015). Therefore whilst group sizes in experiment 2 may appear small (n = 6), behavioural results show robust measures of object recognition in these groups. The lack of interaction between experimental group and *c-fos* expression in perirhinal cortex regions in this experiment shows little sign that this data is impacted by low statistical power.

At the level of the entire perirhinal cortex the statistical results are more marginal (both in terms of mediation of Fos activation by relative novelty, and relationship between *c-fos* activity and performance). Whilst this still stands in marked contrast to the robustness of these group sizes to assess behaviour in the task it does mean we offer some caution to the interpretation of these results as suggesting no involvement of the perirhinal cortex in recognition memory. Rather, we offer an interpretation that both experiments show consistent evidence that the involvement of perirhinal cortex in recognition of objects when their familiarity is on a spectrum is less clear than in many experiments where familiarity is absolute. This was especially clear when discrimination was based on relative familiarity when animals had not been familiarised to stimulus objects (Novel group) while a mild trending effect was observed with animals familiarised with the stimulus objects prior to testing (Relative group) that was tested under experimental conditions close to those implemented in studies relying on absolute familiarity.

Lack of clear evidence of modulation of perirhinal *c-fos* expression by familiarity of objects in SOR tests is consistent with the hypothesis that novelty detection is not a single process mediated by perirhinal cortex. Output from a single novelty/familiarity process would by necessity be mediated by the relative familiarity/novelty of objects at test. Whilst in all conditions in these experiments there is a novel object at test in an SOR trial, the degree of novelty, as judged by a single novelty/familiarity mechanism, would not be constant. In experiment 1 a single novelty/familiarity detection system might be expected to show less Fos expression on the SOR test trial the more familiarised the animal has become to the familiar object (through exposure on previous samples). This was not seen to be the case. In experiment 2 a single novelty/familiarity detection system would be expected to show decreased signals in perirhinal cortex when the object set being used is familiar (compared to a novel object set) even when making 'relative' familiarity judgements within a single test. Again we saw little evidence of such modulation. This lack of modulation would be more consistent with the hypothesis that novelty and familiarity are distinct processes.

Previous studies have reported increased activation in perirhinal cortex to absolute novelty using a variety of tasks and measures of activation (e.g. Zhu et al., 1995; 1996; Albasser et al., 2010a). However, other studies have reported a lack of perirhinal cortex activation to novelty/familiarity (e.g. Kinnavane et al. 2014). Kinnavane et al. (2014) used a continual trials version of the SOR task (as we did in experiment 2) to examine network activity to novelty and familiarity. Interestingly, both Kinnavane et al (2014) and our current data report no differences in levels of Fos expression within perirhinal cortex in groups successfully performing SOR. Differences in perirhinal cortex Fos expression have been seen in SOR in animals with unmatched performance, including failure to discriminate novel objects (e.g. Albasser et al., 2010a). We further extend these findings by showing that relative familiarity of objects does not modulate activation in perirhinal cortex in either traditional single trial or continual trial versions of the SOR paradigm. Consistent with this lack of apparent modulation by relative object familiarity information, Ahn & Lee (2015) reported perirhinal cortex neurons typically responded to complex associations of objects, task choice responses and outcomes, suggesting the nature of perirhinal cortex representations are more complicated than simply representing object familiarity levels.

Previous studies reliably demonstrated greater *c-fos* expression in the perirhinal cortex to novel items by passively presenting 2D and 3D objects to rats while they placed their nose in a nose-poke hole (Zhu, et al., 1995; 1996). Thus, while differential *c-fos* expression was observed, this was in the absence of rats demonstrating memory through a behavioural response to either novel or familiar objects. Indeed, this pattern of perirhinal cortex activity to novel and familiar items appears to be automatic as it is maintained in anesthetized rats (Zhu & Brown, 1995). This leaves open the question of how signals for novelty and familiarity of items in perirhinal cortex are used to support recognition memory. The question is critical given that some studies have argued against the necessity of the perirhinal cortex in novelty recognition (McTighe et al., 2010; Albasser, et al., 2011; Orlate-Sanchez et al., 2015). Indeed, rats with lesions to the perirhinal cortex demonstrate normal levels of heightened exploration for two simultaneously presented novel items compared to two simultaneously presented familiar items (Albasser et al., 2011; Olarte-Sanchez et al., 2015). While displaying this recognition behavior, the same animals were impaired on classical versions of the SOR task in which a novel and a familiar item were presented concurrently (Olarte-Sanchez et al., 2015; Albasser, et al., 2011). Olarte-Sanchez et al. (2015) proposed that a novelty and familiarity signal were still available to the rats, but that these are unable to be bound to the presented objects, such that the animal was unable to identify which of the presented objects was novel and which familiar. Therefore it is possible that the perirhinal cortex may not be necessary to detect the presence of novelty or familiarity, but be essential for binding this to the specific object which is novel or familiar. In the current experiments, the task demands are equal for all groups – each requires the identification and binding of the mnemonic status of two items, potentially explaining the similarity in neural responses between these groups. This would be consistent with studies showing separate novelty and familiarity coding neurons in perirhinal cortex (e.g. Xiang and Brown, 1998; Daselaar, Fleck & Cabeza, 2006) and in turn with the suggestion that novelty and familiarity are distinct processes.

Nonetheless a great many studies show that manipulations of the perirhinal cortex impair SOR (e.g. Aggleton et al, 2010; Barker et al, 2007) and that these impairments are made more severe by increasing the feature overlap of the objects (e.g. Norman & Eacott, 2004). As summarised above our data would be consistent with this deficit being driven by an inability to bind mnemonic status to object identity. When objects are very similar the ability to bind mnemonic status and object identity may be particularly challenging, potentially explaining the more profound SOR deficit in tests using objects with high similarity.

One further issue that could affect interpretation of *c-fos* expression studies in this type of task is how *c-fos* expression maps onto novelty and familiarity responses from perirhinal cortex neurons. Novelty and familiarity signalling in single perirhinal cortex neurons was originally described in monkeys responding to 2D images and these responses changed over a few seconds (Xiang & Brown 1998; Von Linstow-Roloff et al., 2016). *C-fos* expression measures activation levels over minutes to hours and so the question remains as to whether *c-fos* is a sensitive enough tool with which to measure these changes in familiarity responses. A number of observations would suggest that familiarity based changes can be detected over periods of longer than a few seconds. Firstly, studies examining responses of perirhinal cortex neurons to 3D objects in rats have shown clear object related firing from recording sessions lasting up to 15 min (Deshmukh et al. 2012, Burke et al. 2012). These effects are averaged across multiple bouts of object exploration and still reported significant object responsiveness. Ahn et al. (2019) went on to show familiarity based reduction in firing rates, similar to that reported in monkeys, that continue across 40 behavioural trials. This reduction in activity spans a similar period of minutes to hours to that which can be measured using *c-fos* expression.

In summary, the current findings argue against a single mechanism within the perirhinal cortex which signals both familiarity and novelty, which would have been expected to be manifested by modulation of *c-fos* expression in perirhinal cortex by relative familiarity of objects in both experiments. Rather, our data could be consistent with perirhinal cortex being critical for binding signalling of the presence of novelty/

familiarity generated in other parts of the network with object identification. Consistent with this suggestion, studies using structural equation modelling of *c-fos* expression have defined separate networks through lateral entorhinal cortex (LEC) and hippocampus that support novelty and familiarity (e.g. Kinnavane et al 2016; Albasser et al., 2010a; Olarte-Sanchez et al., 2015). In addition, other studies have shown clear evidence of integration of information needed to form episodic memories in other parts of the network including the hippocampus (e.g. Eichenbaum, 2017, Beer et al, 2018, Vandrey et al., in press) and LEC (Wilson et al. 2013, Vandrey et al., 2020).

CRediT authorship contribution statement

Kamar E. Ameen-Ali: Conceptualization, Investigation, Writing - original draft. **Magali H. Sivakumaran:** Conceptualization, Investigation, Writing - original draft. **Madeline J. Eacott:** Conceptualization, Writing - review & editing, Supervision. **Akira R. O'Connor:** Conceptualization, Writing - review & editing, Supervision. **James A. Ainge:** Conceptualization, Investigation, Writing - original draft, Supervision, Project administration, Funding acquisition. **Alexander Easton:** Conceptualization, Writing - original draft, Supervision, Project administration, Funding acquisition, .

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