Parahippocampal Fos-Like Immunoreactivity After Incidental Association Formation in Rats

Maxwell J. Greene

Advised by Dr. Jane Taylor, Charles B. G. Murphy Professor of Psychiatry, of Psychology and of Neuroscience, and Leah Fleming, PhD Student (Interdepartmental Neuroscience Program)

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Abstract

The crux of sensory preconditioning (and the related phenomenon of representation-mediated aversion) is the formation of associations between neutral environmental stimuli. This process occurs during the preconditioning phase, and involves the encoding and consolidation of associations. Prior work has uncovered the brain regions which may take part in encoding, consolidating, storing, and retrieving an association formed during sensory preconditioning. Still, the exact role played by each neural substrate of such associations, as well as the possible neural circuits formed by these substrates, have not been totally clarified. The aim of this study is to further understanding of how the mammalian brain processes associations between neutral stimuli, with a focus on two parahippocampal structures: the perirhinal and entorhinal cortices. The proposed hypothesis is that the representation of an incidental association will correspond to increased activity in the entorhinal and perirhinal cortices. To test this hypothesis, 44 rats were trained on a representation-mediated taste aversion task, and c-Fos immunohistochemistry was used to visualize brain activity in regions of interest. The results were not significant enough to suggest that rats trained on paired stimuli formed incidental associations. Additionally, no significant main effects or interactions were found on Fos-like immunoreactivity in regions of interest.

Contents

1. Introduction

Associations between neutral environmental stimuli, or "incidental" associations, form when an organism learns that separate sensory events tend to co-occur. Sensory preconditioning (SPC) is a behavioral paradigm (and Pavlovian conditioning phenomenon) used to probe the formation of incidental associations. SPC typically consists of 3 phases. In phase 1, the subject animal is presented with a pair of neutral stimuli, such as a light and a tone. In phase 2, one of the neutral stimuli is repeatedly presented prior to an unconditioned stimulus (US), which may be appetitive or aversive. Finally, in phase 3, the stimulus that was never conditioned with the US is presented. The animal has successfully demonstrated SPC if it responds roughly equally to both of the initial stimuli- the one paired with the US and the one that was never paired with the US (e.g. Brogden 1939, Rescorla & Rizley 1972, Nicholson et al 2000, etc.). This parity in responding is due to an association that formed between the neutral stimuli in phase 1. Presentation of one stimulus may bring up a representation of the other. Researchers have not conclusively and comprehensively determined the brain regions and circuits that support SPC and its constituent subprocesses (Matsumoto et al 2013). Nonetheless, there is some evidence for the involvement of various brain regions in SPC, especially structures in the medial temporal lobe.

SPC (and a related mediated learning paradigm) has the interesting potential to elucidate the cognitive behaviors that underlie psychosis. Specifically, SPC may essentially induce hallucinations via associative learning, and thereby be used to generate animal models of psychotic illnesses like schizophrenia (Powers et al 2017, Dwyer 2017). Therefore, developing a

solid understanding of how SPC works at the neurobiological level will greatly advance knowledge of learning, memory, and the behaviors based on these faculties.

1.1. General computational basis of SPC

After successful SPC, the presentation of one neutral stimulus is able to activate the representation of the other neutral stimulus with which it had been repeatedly paired. It seems quite plausible, then, that the underlying computational mechanism for SPC is Hebbian (e.g. Caporale & Dan 2008). Theoretically, paired presentations of the neutral stimuli may result in a strengthening of the synaptic connections between the neuronal populations which represent each stimulus. As a result, the activation of one population is eventually sufficient to activate the other. Hence, devaluation of one stimulus (by conditioning with an aversive US) also affects the represented value of the other stimulus.

Moustafa et al. (2009) develop a Hebbian model of associative learning between association areas and the hippocampus (between which lie the present study's regions of interest: the rhinal cortices). The model is:

$$w_{ji}(t+1) = w_{ji}(t) + \alpha_h x_i(t) y_j(t)$$

Where w_{ji} is the weight (synaptic strength) between neurons j and i, α_h is the learning rate, x_i and y_j are the activation levels of cortical neuron i and hippocampal neuron j, respectively.

1.2. The perirhinal (PRh) and entorhinal (EC) cortices

Lateral to the hippocampus and towards the ventral part of the rodent brain lie the rhinal cortices (entorhinal, perirhinal, and postrhinal), named for the rhinal sulcus. The rodent (and primate) rhinal cortices play important roles in memory (Burwell 2000).

Area 35 of the rat PRh is devoted to taking in olfactory information, while area 36 receives input in other sensory modalities. PRh receives unimodal and polymodal input from association cortex (Burwell 2000). In turn, EC (primarily the lateral entorhinal area) receives input from PRh, as well as some direct input from association areas. The rhinal cortices channel sensory information to the hippocampus, and this role is explained by the substantial connectivity between the parahippocampal region and association cortex (Burwell 2000). EC sends output to the entire hippocampal formation via the perforant fiber path (Coutureau & Di Scala 2009).

While the superficial layers of EC receive perirhinal and associational input, the deeper layers receive input from the medial prefrontal cortex, retrosplenial cortex, amygdala, medial septal region, and certain thalamic nuclei (Coutureau & Di Scala 2009).

1.3 Perirhinal and entorhinal cortices in representing associations between neutral stimuli

1.3.1. Perirhinal cortex (with basolateral amygdala)

A 2000 study by Nicholson et al. established the importance of PRh for SPC. The authors rooted their hypothesis in knowledge about PRh's connectivity with other structures. PRh receives input from visual and auditory areas, and in turn sends polysensory output to the subiculum and hippocampus, a region known to support key memory processes. Both PRh and

postrhinal cortex project to EC, and this entire parahippocampal area is involved in binding elements of paired stimuli. This feature binding may be important for SPC.

Nicholson and colleagues created bilateral electrolytic lesions of the PRh in their experimental group of rats (2000). Both these experimental rats and a control group were put through a SPC paradigm (in which the neutral stimuli were a light and tone, and in the second phase, one of these stimuli was paired with a periorbital shock). The PRh-lesioned rats did not demonstrate SPC. The experimenters proposed several possible theories about the role of PRh in SPC. The PRh, via EC, may give the hippocampus access to sensory information about the stimuli from phase 1 of SPC. Separately, the PRh's role may be to either encode or store the association between neutral stimuli, or the association between one of the neutral stimuli and the US. Nicholson et al. describe a possible circuit involving PRh and projections from the hippocampus to the cingulate gyrus and retrosplenial cortex, then to the pontine nuclei, and finally to the cerebellum, in order to generate the sensory preconditioned eyeblink response.

In two studies, Holmes et al. elaborate on PRh's contribution to SPC as well as the neurobiological basis for this contribution. In their 2013 study, infusing PRh with muscimol, a $GABA_A$ agonist, or ifenprodil, an *N*-Methyl-D-aspartate (NMDA) antagonist, impaired learning about the association between neutral stimuli. Therefore, NMDA receptor transmission in PRh is necessary for the formation of an association between neutral stimuli in a safe context. In contrast, the basolateral amygdala (BLA; but not PRh) is crucial for forming associations between neutral stimuli in a dangerous context (e.g. a chamber where rats received electric shocks), and for forming an association between a neutral stimuli and a stimulus conditioned to

be a danger signal. PRh is not necessary for direct fear conditioning of a stimulus (i.e., learning the association between a CS and aversive US; Holmes et al. 2013).

The 2018 Holmes et al. study employed a similar paradigm as the 2013 study. Infusions of the AMPA receptor antagonist NBQX or the MEK inhibitor U0126 after presentations of the neutral stimuli revealed that AMPAR transmission and MAPK signalling in PRh is necessary for consolidation of the S1-S2 association. This is the case in a safe context, whereas consolidation of the S1-S2 association in a dangerous context requires the same signalling mechanisms in the BLA. In addition, formation of the association in a safe context followed by learning that the safe context has become dangerous shifts the substrate of the association from PRh to the BLA (Holmes et al. 2018).

The key takeaway from the Holmes et al. studies is that both the PRh and BLA are involved in learning the association between neutral stimuli depending on the context. Shifting the context from safe to dangerous can shift storage of the association from PRh to the BLA, perhaps based on the strong reciprocal glutamatergic connections between the regions. The PRh and BLA are part of different neural pathways, and the extent to which the animal is in a fearful state determines which pathway encodes the S1-S2 association.

Changes in firing rates among perirhinal neurons my underlie PRh-based associative learning. Yanike et al. (2008) recorded perirhinal activity in macaques during a conditioned-motor association task (associative learning that involves matching visuospatial cues to motoric eye responses). They found changes in the firing rates of different perirhinal neuronal subpopulations across the phases of their associative learning paradigm.

1.3.2 Retrosplenial-entorhinal cortical circuit

As noted, EC receives input from the retrosplenial cortex (RSC; Coutoureau & Di Scala 2009). Robinson and colleagues have investigated the role of RSC in SPC. RSC basically sits between medial temporal lobe structures that mediate memory, and sensory areas, making it a good candidate for the substrate of stimulus-stimulus associations (Robinson et al. 2014). In both primates and rodents, projections from RSC to medial EC, postrhinal cortex, and the postsubiculum serve as a conduit for polymodal sensory information (Robinson et al. 2011). Rats with RSC lesions could not form an association between a tone and a light in an SPC task (Robinson et al. 2011). Robinson and the other authors cite various studies in making their claim that the RSC is more "fundamental" to learning relations between sensory stimuli than even the hippocampus. Silencing RSC before the preconditioning phase of SPC (by infusion of clozapine-*N*-oxide to activate hM4Di-mediated inhibition of RSC neurons) prevents the formation of the stimulus-stimulus association (Robinson et al. 2014). RSC is definitely involved in forming associations between neutral stimuli, and the hippocampus is involved in retrieving these associations, but it is still unclear whether RSC is also necessary for retrieval (Robinson et al. 2014).

In their review of entorhinal function, Courtoureau and Di Scala cite one study which found that EC damage does not impair the taste aversion version of SPC. However, the authors also discuss a computational model which suggests that EC conflates/homogenizes simultaneous stimuli, a function that would be consistent with an entorhinal contribution to SPC. (Courtoureau and Di Scala 2009).

In an associative learning task with rats involving odors and locations, a change in coupling between entorhinal and hippocampal oscillations corresponded with learning (Igarashi et al. 2014).

1.4. C-Fos

The present study uses c-Fos immunoreactivity to identify neuronal activity. C-Fos is an immediate early proto-oncogene. Baseline expression of c-Fos in neurons is low. Postsynaptic glutamate binding and depolarization cause an influx of calcium ions via *N*-Methyl-D-aspartate (NMDA) receptors and L-type voltage-sensitive calcium channels. This calcium influx sets off the mitogen-activated protein kinase (MAPK) signalling pathway which ultimately leads to transcription factors binding to the c-Fos promoter. C-Fos mRNA transcripts are translated in the cytoplasm and imported to the nucleus (Chung 2015).

2. Materials and methods

2.1. Subjects

The subjects were 44 naive male Sprague-Dawley rats (Charles River Laboratories), initially weighing approximately 280 to 390 g. Each day of the experiment, it was ensured that the subjects drank at least 10 mL of fluids. All subjects had full access to food for the entirety of the experiment.

2.2. Materials for behavioral component

2.2.1. Housing

When the experiment was not running, the subjects were housed in pairs in 22 cages within a restricted-access facility. During the experiment, the subjects were placed in individual cages where they received fluids, as well as a small amount of food. The experiment took place in two separate rooms to prevent odor cross-contamination.

2.2.2. Stimuli

1 flavor/tastant (T) and 2 odorants (O1 and O2) were used as the neutral stimuli. The tastant was sucrose at 5% concentration (in aqueous solution). The odorants were organic solutions of 0.01% benzaldehyde (almond-like odor) and 0.05% isoamyl acetate (banana-like odor). Henceforth, the almond odor will be designated O1, and the banana odor as O2.

The unconditioned stimulus (US) was 0.3 M lithium chloride in aqueous solution, injected intraperitoneally at a volume of 5 mL/kg body weight to induce illness.

2.3 Materials for immunohistochemistry and requisite preparations

2.3.1. Euthanasia and tissue preparation

An injection of Euthasol (pentobarbital/phenytoin) was used to sacrifice each subject. A 10% formalin solution was used for transcardial perfusion following euthanasia.

Brains were collected and placed in formalin solutions for 1 day, before being transferred to a solution of phosphate-buffered saline (PBS) and sucrose (30%) for cryoprotection. All brains were refrigerated at about 4 C immediately after harvesting.

A cryostat was used to section the brains into 35 μm slices. The slices were placed in wells containing PBS with 0.05% sodium azide.

2.3.2. Immunohistochemistry

The materials used for the visualization of c-Fos expression are described in the procedure.

2.3.3. Data analysis

All statistical analyses were done in R. Fiji was used for cell counting.

2.4 Methods

2.4.1. Design

The design of this study was 2x2 mixed factorial. The basic paradigm consisted of subjects being exposed to paired presentations of either O1 or O2 and T over 3 consecutive days during their 1 h access to water. For 3 additional days, all subjects were exposed to presentations of O2 alone immediately followed by the aversive US (an i.p. injection of LiCl). Wimmer & Shohamy hypothesize that at this devaluation phase, presentation of O2 elicits an internal representation of O1, and a hippocampal-striatal circuit transfers the new value of O2 to O1 (2012). Hence, this is the phase of interest with regard to c-Fos staining. Therefore, at this point half of the subjects were immediately sacrificed, perfused and debrained in preparation for c-Fos immunohistochemical analysis.

The remaining subjects moved on to what is typically the third and final phase of SPC: the test phase. These subjects were given the opportunity to drink either flavored (sucrose) water or unflavored water, and their preference was hence determined. After the test phase, the last surviving subjects were sacrificed, perfused, and debrained.

To reiterate, out of the 44 subjects, 22 were trained with presentations of O1 and T, while another 22 were trained with presentations of O2 and T. Likewise, of the 44 subjects 22 were sacrificed immediately after the conditioning of O2 with the US, while 22 experienced the full SPC paradigm before euthanasia.Therefore, 4 groups of 11 subjects each were established. One group received O1-T pairings and the final phase 3 of the SPC paradigm (group O1-3). The second group received O2-T pairings and the final phase 3 of the SPC paradigm (group O2-3). The third group received O1-T pairings and did not undergo phase 3 (group O1-3'). The fourth group received O2-T pairings and did not undergo phase 3 (group O2-3').

Group	**Phase 1 (Sensory Conditioning**	**Phase 2 (Aversion Training)**	**Phase 3 (Choice Test or Perfusion)**
O1-3	O1→T	O2→US	T vs. water
O2-3	O2→T	O2→US	T vs. water
O1-3'	O1→T	O2→US	Perfused
O2-3'	O2→T	O2→US	Perfused

Fig. 1 The design of the RMTA component of the experiment.

2.4.2. Behavioral procedure

As stated, of the 44 subjects, 22 completed the RMTA task while 22 were sacrificed after the conditioned odor aversion stage. For brevity, the former group will be designated group-COMP (for complete) and the latter group-INCOMP (for incomplete). Group-INCOMP began the experiment a day prior to group-COMP.

Nonetheless, for both groups the first 3 days entailed water restriction. The subjects were transferred from their home cages (where they were housed in pairs and had no water access) to individual cages in two separate rooms. A bottle containing approximately 250 mL of water was placed into each subject's cage, and each subject was allowed 1 h to drink. Subjects who failed to drink at least 10 mL were given additional time until they had consumed the minimum amount.

On day 4, after the above-described 3 days of water restriction, each subject performed a choice test in lieu of normal water access. During the choice test, subjects had the option of drinking either flavored (sucrose) water or unflavored water for 1 h (or more, if needed).

For days 5, 6, and 7, the subjects received odor-taste pairings. 11 rats from group-COMP and 11 rats from group-INCOMP were given access to flavored water paired with O1. Likewise, 11 different rats from each group were given access to flavored water paired with O2.

On day 8, all subjects completed a second choice test, identical to the first.

For days 9, 10, and 11, all subjects were given access to water and simultaneously exposed to O2. Immediately afterwards, each subject received an i.p. injection of LiCl (the US). On day 11, subjects from group-INCOMP were sacrificed using Euthasol, transcardially perfused with saline and formalin, and debrained.

On day 12, group-COMP subjects were given access to unflavored water for 1 h (or more, if needed).

Finally, on day 13, a choice test was administered to group-COMP subjects in which they were given access to flavored and unflavored water. 90 minutes after the choice test, each subject from group-COMP was sacrificed with Euthasol, transcardially perfused with saline and formalin, and debrained.

2.4.3. Immunohistochemical procedure

As described in 2.3.1., collected brains were placed in individual vials containing solutions of PBS and sucrose. After a few days, each brain was mounted on a disk using PolyFreeze support matrix and inserted into the cryostat for sectioning. Each brain was kept in the cryostat at approximately -20 C for 30 minutes prior to slicing.

35 μm slices were collected and placed into wells containing PBS with NaN_3. Ultimately, 2-4 sections containing the regions of interest were selected from 3 group O1-3' rats as well as 3 group O2-3' rats.

During the immunohistochemistry itself, sections from the 6 rats were invariably placed in wells containing 2 mL of the appropriate liquid.

The sections were washed 3 times for 5 minutes in 1xPBS at room temperature. Next, the tissue was placed in a PBS blocking solution (0.3% Triton-X, 3% normal donkey serum) for 2.5 h at room temperature. The sections were incubated at 4 C with 1:1000 rabbit anti-cFos (Synaptic Systems) antibody in a solution of 0.1% Triton-X and 0.1% normal donkey serum. After 24 h, the sections were washed 3 times for 5 minutes in 1xPBS at room temperature. Next,

the tissue was incubated for 2 h with 1:500 donkey anti-rabbit antibody conjugated with Alexa Fluor 555 dye (Invitrogen) at room temperature. After treatment with the secondary antibody, the sections were put onto glass slides and left to dry. The final step entailed placing coverslips over the slides using mounting medium with DAPI (4′,6-diamidino-2-phenylindole; VECTASHIELD) and allowing the slides to dry.

Computer-aided fluorescence microscopy was used to visualize c-Fos expression and capture images of the sections at 10x magnification. Fiji was used to count c-Fos positive cells.

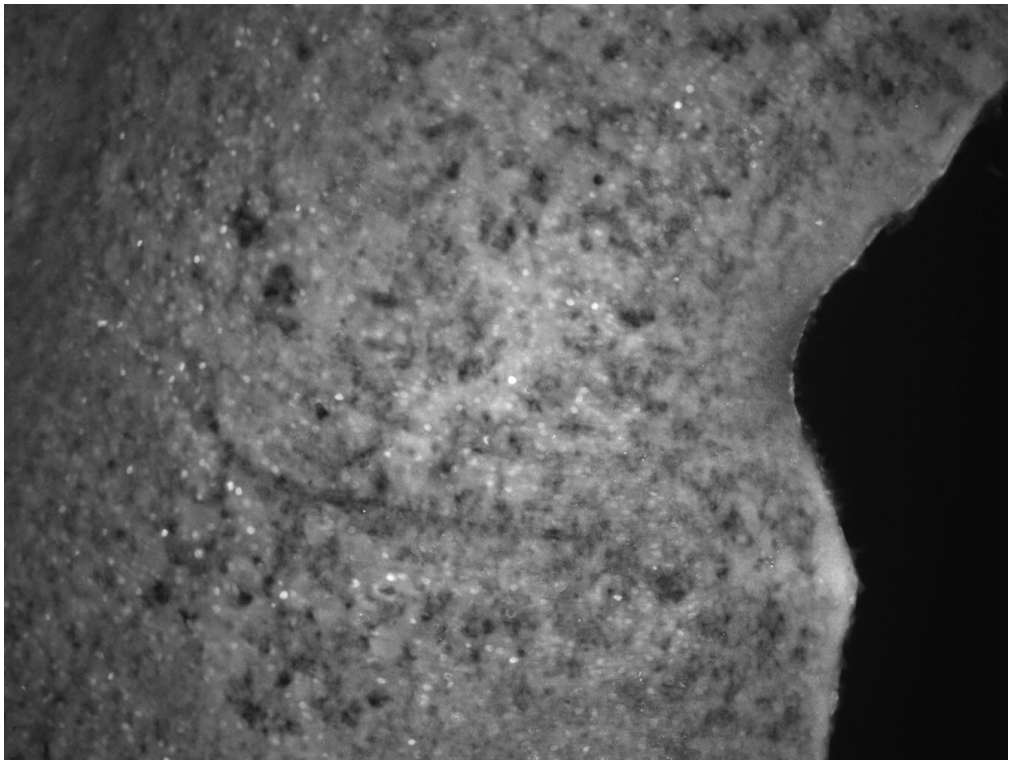

Fig. 2 A slice containing perirhinal cortex at 10x magnification. The bright speckles were counted as c-Fos positive cells based on an appropriate threshold.

3. Results

3.1. Behavioral findings

The dependent variable of interest was defined as the difference between flavored water (water with sucrose) and unflavored water consumption (sucrose - water, in mL). Data were analyzed using a 2x2 mixed analysis of variance (ANOVA) with a within-subjects factor of day (i.e., day of the first choice test or the second choice test) and a between-subject factor of group. The within-subjects variable had only 2 levels, and so no Mauchly's test for sphericity was performed. Levene's test confirmed the homogeneity of variance assumption ($F_{1,20} = 0.7335$, $p = 0.5382$). No significant main effect of group was found ($F_{1,20} = 2.902$, $p = 0.0966$). Neither were a significant main effect of day ($F_{1,20} = 0.154$, $p = 0.6965$) or an interaction between group and day ($F_{1,400} = 0.242$, $p = 0.6255$) found. Perhaps it is notable that the hypothesized main effect of group most closely approaches significance in the hypothesized direction.

The results of the behavioral protocol do not provide strong evidence that the subjects successfully underwent RMTA.

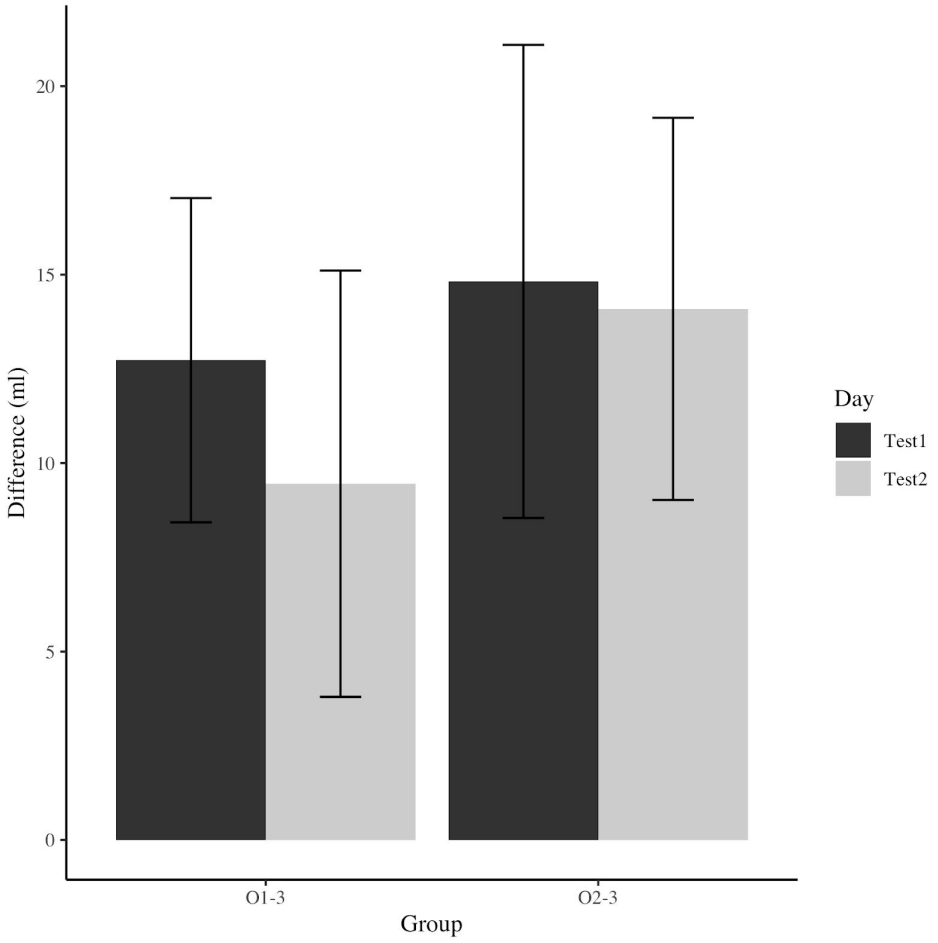

Fig. 3 Bar plot of the mean differences (between flavored and unflavored water consumption) for each group, both on the day of the first (pre-aversion training) choice test and the day of the second (post-aversion training) choice test.

3.2. Immunohistochemical findings

A 2x2 mixed ANOVA was carried out to determine the effects of group (O1-3' vs. O2-3') and region (EC vs. PRh) on c-Fos activity, measured as the number of c-Fos positive cells per area (in pixels^2). Since there were no more than two levels per variable, the sphericity assumption holds. Additionally, Levene's test yielded a non-significant p-value of 0.551 (F=0.7098). No significant main effects of group ($F_{1,20}$=0.343, p=0.561) or region ($F_{1,20}$=0.020,

p=0.887) were found, nor any significant interaction effect ($F_{1,400}$=1.960, p=0.168). The interaction effect comes closest to significance.

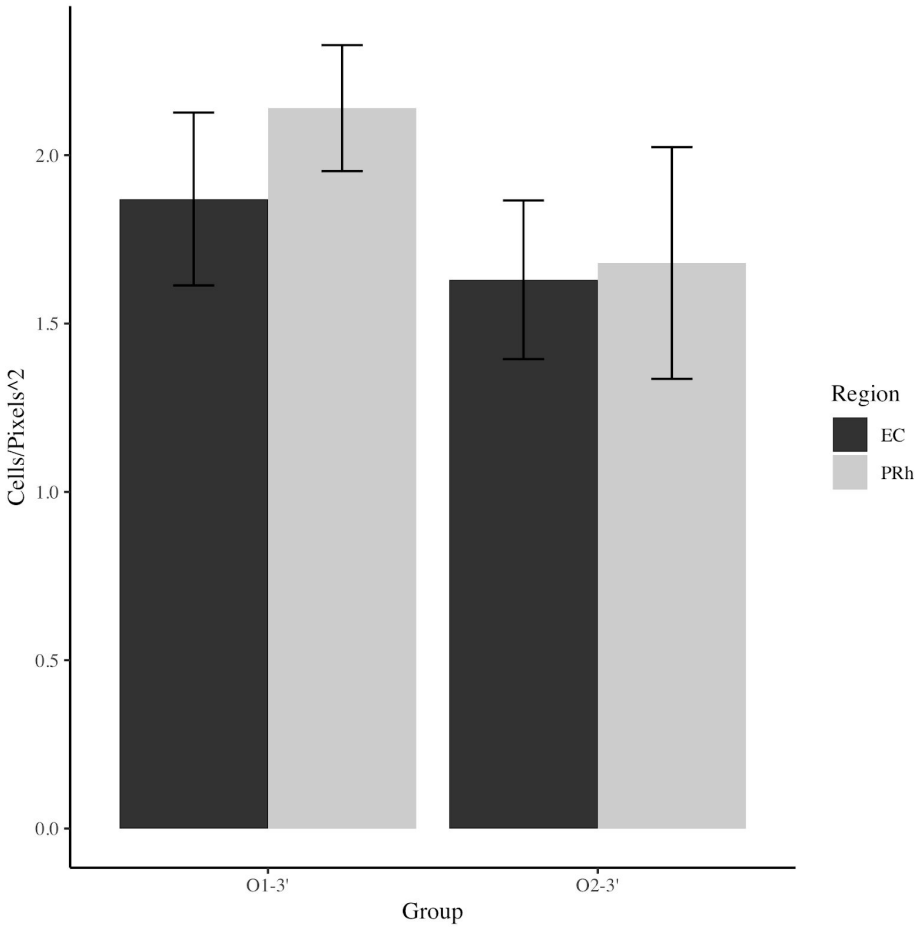

Fig. 4 Barplot of c-Fos activation (c-Fos positive cells per pixel^2) in each region for each group.

4. Discussion

This pair of experiments failed to establish RMTA in the experimental subjects, and failed to show a significant alteration in entorhinal or perirhinal activity following associative learning through RMTA. Because the behavioral component of the study did not yield significant results, it is unsurprising that no significant effects were found in the immunohistochemical

component, as such effects would be dependent on the success of the behavioral training. All in all, the null hypothesis cannot be rejected in favor of the alternative hypothesis that the formation of incidental associations would increase EC and PRh activity (specifically when those associations are recalled in phase 2 of RMTA).

It is plausible that the failure to obtain significant results was due to a lack of statistical power (n=22 for the behavioral experiment, n=6 for the c-Fos experiment). A small sample size increases the likelihood that a large portion of the subjects would never learn the target behavior. Consequently, none of the group O2-3' brains studied may have come from subjects who successfully learned the behavior. If the study was underpowered, it is reassuring that the main effect of group in the behavioral part approaches significance.

Alternatively, the behavioral protocol may not have been optimal for studying RMTA in rats. While this laboratory has previously shown RMTA in Sprague-Dawley rats using similar materials, this prior (unpublished) study had a completely within-subjects design, as opposed to the present study's mixed design.

Clearly, there is a lack of certainty as to whether the subjects acquired the target behavior. This makes it difficult to draw meaningful conclusions from the observed Fos-like immunoreactivity (Fos-LI). It is unexpected that among group O2-3', the only group which could have formed associations between the neutral stimuli, average Fos-LI is depressed in both regions of interest compared with the other group.

While this decrease in entorhinal and perirhinal activity may be due to random variation, it is plausible that it reflects a degree of synaptic depression. Long-term depression (LTD) in the rhinal cortices is thought to play a role in learning and memory (Cho et al. 2000, Kourrich &

Chapman 2003). LTD in PRh may be especially important for recognition memory (Massey & Bashir 2007). NMDA receptor transmission is required for LTD in both EC and PRh, and this receptor activation is also necessary for c-Fos expression (Massey & Bashir 2007, Kourrich & Chapman 2003).

A major limitation of this study was not being able to both examine neural activity during/immediately after the behavior of interest (that is, during O2-US presentations) and distinguish between good and poor learners (since that requires the completion of the RMTA paradigm beyond the O2-US presentations). A way to overcome this limitation would have been to record brain activity *in vivo* during the behavior.

Future studies with greater statistical power should be carried out to establish RMTA in rats and analyze the effect of this behavioral task on parahippocampal activity. Additionally, the mechanism of incidental association representation (that is, synaptic potentiation or depression) should be clarified and other possible neural substrates of incidental associations should be probed. To this end, *in vivo* studies may be preferable to *post mortem* analyses.

Authors' Contributions

MJG conceived the study and LF aided in its design. MJG and LF shared in completing most or all aspects of the behavioral and immunohistochemical components. MJG ran the statistical analyses. HS (Hayde Sanchez) and LF performed injections and perfusions.

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