



## RESEARCH ARTICLE

WILEY

# Unfolding of spatial representation at systems level in infant rats

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## Funding information

China Scholarship Council, Grant/Award Number: 201808080042; Deutsche Forschungsgemeinschaft, Grant/Award Number: DFG In 279/1-1; Hertie Foundation; Spanish Government, Grant/Award Number: PSI2017-83893-R; the European Research Council, Grant/Award Number: ERC AdG 883098

## Abstract

Spatial representations enable navigation from early life on. However, the brain regions essential to form spatial representations, like the hippocampus, are considered functionally immature before weaning. Here, we examined the formation of representations of space in rat pups on postnatal day (PD) 16, using a simple habituation paradigm where the pups were exposed to an arena on three occasions, separated by ~140 min. Whereas on the first two occasions the arena was the same, on the third “test” occasion either proximal cues (Prox group), or distal cues (Dist group), or proximal and distal cues (Prox-Dist group), or no cues (No-change group) were rearranged. Locomotion (distance traveled) was used as behavioral measure of habituation, and c-Fos expression to measure regional brain activity at test. Locomotion generally decreased across the first two occasions. At test, it reached a minimum in the No-change group, indicating familiarity with the spatial conditions. By contrast, the Prox-Dist group displayed a significant increase in locomotion which was less robust in the Prox group and absent in the Dist group, a pattern suggesting that the pups relied more on proximal than distal cues during spatial exploration. c-Fos activity in the No-change group was significantly suppressed in the hippocampus (CA1, CA3, dentate gyrus) but simultaneously enhanced in the prelimbic area (PL) of the medial prefrontal cortex, compared with untreated Home-cage controls, pointing to a possible involvement of the PL in regulating locomotion in familiar spaces. By contrast, in both Prox-Dist and Prox groups c-Fos activity was enhanced in hippocampal CA1 and CA3 regions, suggesting these regions might be particularly involved in regulating exploration of spatial novelty. Our findings show that functional representations of space at a systems level are formed already in pre-weanling rats.

## KEYWORDS

development, distal cues, habituation, hippocampus, medial prefrontal cortex, pre-weanling rats, proximal cues, spatial representation

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## 1 | INTRODUCTION

Animals have the capability to navigate based on spatial maps that integrate information about environmental landmarks and their own movements. The spatial representations can be egocentric, defining spatial elements in relation to the own body, or allocentric, mapping spatial relationships among environmental elements independently of the own body position (Vorhees & Williams, 2014). In adult animals, a network essentially comprising hippocampal place cells, entorhinal grid cells and more distributed head direction cells is central for forming spatial representations, especially when allocentric (Moser et al., 2017).

However, when and how spatial representations emerge during early development is unclear. Recordings of neural activity provided consistent evidence that the different neural components of the spatial network are functioning already early during development even before extensive experience, that is, in pre-weanling and infant rats before PD15 (Ainge & Langston, 2012; Tan et al., 2015, 2017; Wills et al., 2010). Recent findings suggest that, in addition to hippocampal and neighboring areas, other brain regions, specifically the medial prefrontal cortex (mPFC), also contribute to spatial navigation already early in life (Rinaldi et al., 2020). However, although the prefrontal-hippocampal spatial system may be functioning at the level of single neurons in pre-weanling rats, the involved regions are immature and it is unknown whether rats at this age can actually use this system for memory-based navigation.

Behavioral studies in rats using the Morris water maze showed that allocentric spatial navigation emerges after PD20 while the egocentric strategy, tested in the visible platform version, emerges as early as PD17 (Akers & Hamilton, 2007; Rudy et al., 1987). In another test of hippocampus-dependent spatial abilities, the object place recognition task, successful performance emerged only from PD18 on (Contreras et al., 2019; Travaglia et al., 2018; Westbrook et al., 2014), except in one study showing functional spatial representation that, however, were maintained over only a short 10-min interval already at PD16 (Krüger et al., 2012). Likewise, in the object-in-context recognition task which tests the animal's capability to process contextual cues and associates them with objects, rats at PD17 were able to acquire but not to retain these memories longer than 5 min (Ramsaran et al., 2016; Sanders et al., 2020). Thus, it is still an open question whether pre-weanling rats are able to form stable allocentric spatial representations that they use for navigation and last more than a few minutes.

Previous studies show that in tasks, like the object place recognition task, rats before PD18 spend only rather short periods (often less than a second) with exploring an object (Contreras et al., 2019) possibly reflecting an inability to discriminate objects as separate entity, which makes such object-based tasks not suitable for testing spatial capabilities before this age. Therefore, to address the question whether pre-weanling rats explore based on spatial representations, we used here a simple spatial habituation task. Habituation is a basic form of learning which describes the progressive decrease of the

amplitude or frequency of a motor response to repeated sensory stimulation (Domjan, 2002). Our task relied on the pup's capability to explore the environment through locomotion and included two habituation sessions where the rat encountered the same spatial environment, and one test trial with either the same or novel spatial context configurations of proximal and distal cues (Bronstein et al., 1974; Feigley et al., 1972; O'Keefe & Nadel, 1978). Based on evidence that expression of the activity-regulated gene *c-Fos* sensitively differentiates brain areas involved in spatial tasks (Aggleton & Brown, 2005; Guskjolen et al., 2018; Tan et al., 2015; Wan et al., 1999), we used *c-Fos* activity to identify the network of brain regions the rat pup used for exploration during the test session. We focused these analyses on the prefrontal-hippocampal network including neighboring regions such as the perirhinal and entorhinal cortices which altogether are well known to essentially contribute to both episodic and spatial memory formation (Eichenbaum, 2017). Furthermore, we added the parietal and retrosplenial cortices as areas important for regulating spatial navigation (Clark et al., 2018; Wilber et al., 2015), the primary motor cortex as a control region involved in regulating goal-directed locomotion (Gatto & Goulding, 2018), and the primary somatosensory cortex as a control for maturation. We provide evidence that reduced locomotion in a habituated spatial environment involves activation of mPFC regions, whereas increased locomotion in a novel spatial environment involves hippocampal activation.

## 2 | MATERIALS AND METHODS

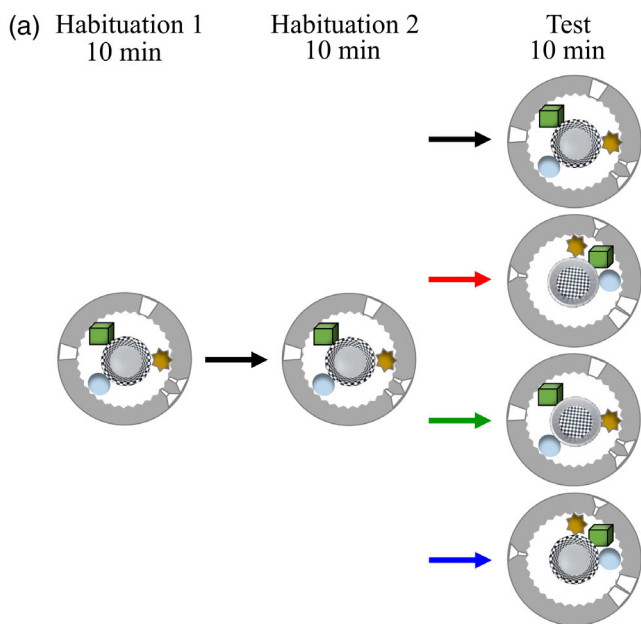
### 2.1 | Animals

A total of 66 male Long-Evans rats were used for the experiments. Animals were taken from 22 litters with each litter including three pups. All pups arrived (from Janvier, Le Genest-Saint-Isle, France) in our facilities on PD8 or PD9 which allowed acclimatization for at least 3 days before any manipulation. The pups were maintained with their dam except during handling and behavioral tasks. All the behavioral tasks were performed on PD16 and all pups had opened their eyes and already started to explore their home cage surroundings on the day of the experiment. The pups were randomly assigned to five groups with the constraint that pre-experimental experience (day of arrival, litter, dam, etc.) was represented in a balanced manner across groups. The five groups named according to the habituation task condition they performed (see below), were the No-change ( $n = 21$ ), Prox-Dist ( $n = 15$ ), Prox ( $n = 12$ ), Dist ( $n = 12$ ); and Home-cage ( $n = 6$ ) groups. (The unequal samples sizes resulted from lab restrictions allowing to perform *c-Fos* analyses only in later experiments). The animal colony was kept at room temperature ( $22 \pm 1^\circ\text{C}$ ) on a controlled 12 h light/12 h dark cycle (lights on at 7:00 h). All experimental procedures were performed in accordance with the European animal protection laws and were approved by the Baden-Württemberg state authority.

## 2.2 | Experimental procedures, design, and task

All experimental procedures were performed between 7:00 and 16:00 h, that is, the light phase. For general habituation of the pups

(from all groups), the respective litter plus their dam was carried in the home cage from the animal facility to the testing room and stayed there (in one of the room corners) together with the dam for 6 h, once every day between PD13 and PD15. During this 6-h room habituation



(b)

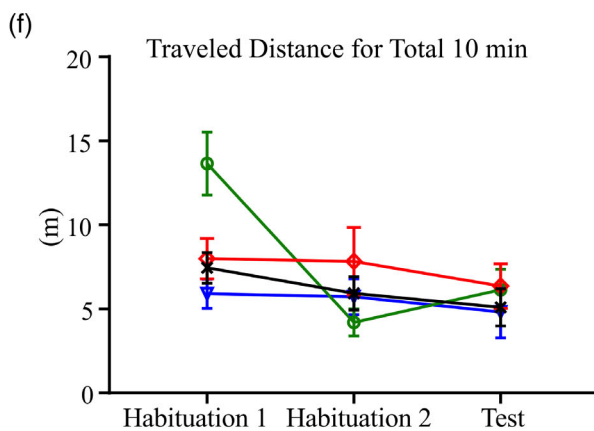
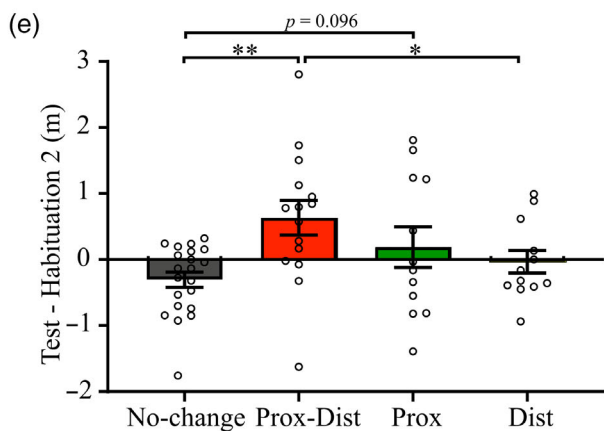
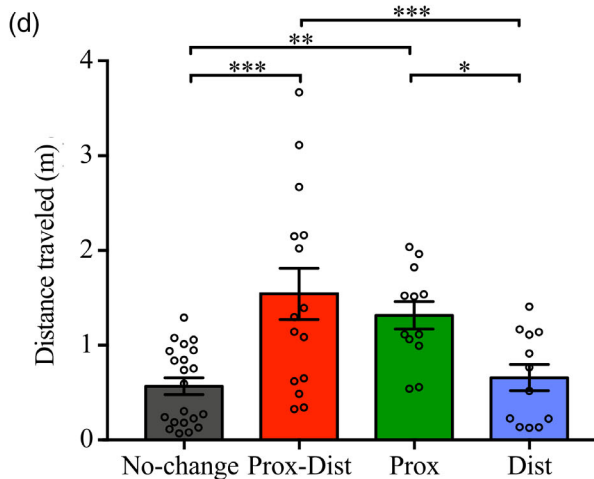
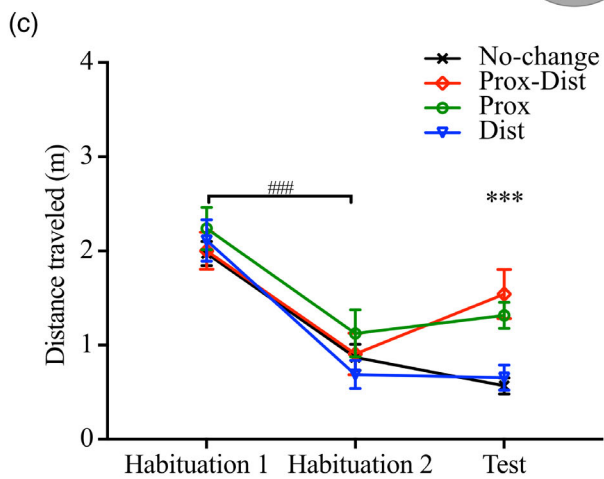


FIGURE 1 Legend on next page.

period, the animals received 5 min of handling twice in order to diminish potential stress.

The spatial habituation task was conducted on PD16. The task consisted of two habituation sessions and one test session which were each separated by ~140 min (Figure 1a). The first two habituation sessions were identical and consisted each of a 10-min period where the pup could freely explore the open field with either the A or B context (counterbalanced within the group). The test session also lasted 10 min and differed depending on the experimental group, that is, the No-change group was exposed to the same arena with the same proximal and distal cue configurations as during the habituation sessions; for the Prox-Dist group novel proximal and distal cue configurations were presented; for the Prox and Dist groups, only the proximal and distal cue configurations, respectively, were changed. The Home-cage control group did not undergo the spatial habituation task but, was kept in the home cage in the testing room during the corresponding intervals, without any further manipulations (e.g., to keep the animal awake). At each session of the task, the animal was introduced in the arena from a different side, in order to facilitate an allocentric spatial representation (Langston & Wood, 2010). During the interval between sessions, the pups were left undisturbed in the home cage with their dam and litter.

### 2.3 | Apparatus and data reduction

The spatial habituation task was always performed in the same experimental room with the pup exposed to a circular open field arena. The arena was placed at the center of the room and surrounded by a circular black curtain, with the south side of the curtain used as entrance for the experimenter (X.S.). The arena had a diameter of 49 cm, a wall of 20 cm height and was made of gray PVC. The upper part of the arena was open, allowing the rat to perceive distal visual cues. For experimentally varying proximal cues, a checkerboard pattern covered either the floor or the arena wall (Figure 1b). The distal cues consisted

of four white curtains, two of them were freely hanging down and two were folded (100 cm × 80 cm and 50 cm × 80 cm). All were attached to the black curtain that surrounded the arena. In addition, 3 different boxes supported by a wooden stick served as distal cues. The distal cues were not more than 200 cm away from the arena. To systematically vary proximal and distal cues between the conditions, the same cues were used but arranged in a different spatial configuration.

The animal's behavior was recorded by a video camera located above the center of the open field. Three fluorescent strip lights were placed on the floor of the room providing indirect light. White noise was presented at a constant intensity during all procedures to mask any disturbing sounds. The open field was cleaned thoroughly between trials with 70% ethanol solution.

The rat's locomotor activity was scored offline using the ANY-Maze tracking software (Stoelting Europe, Dublin, Ireland). Distance traveled was tracked and calculated for each session. Spatial habituation was indicated by a decrease in locomotion (i.e., distance traveled) across two sessions that took place in the same context, and reflected that the animal had formed a spatial memory representation. Correspondingly, an increase in locomotion after introducing a change in proximal and/or distal context cues in the test phase indicated that the animal responded to context novelty based on a memory representation of the habituation sessions. We focused the analyses on the first minute of each session because it is most sensitive to novelty exploration (Winters et al., 2004).

### 2.4 | c-Fos immunocytochemistry

After the test session, the rats were returned to the home cage. Ninety minutes later, they were decapitated, and the brains were removed intact, rapidly frozen in methylbutane (Sigma Aldrich, Taufkirchen, Germany), and stored at  $-40^{\circ}\text{C}$ . The 90-min interval corresponds to the time of peak production of c-Fos protein after an

**FIGURE 1** Experimental design and locomotor behavior. (a) The behavioral experiments included four groups which were all tested on two habituation sessions and one test session, with each session allowing the rat to explore the open field arena for 10 min. Sessions were separated by a ~140 min interval during which the pups were returned to the home cage with their dam. The arenas in the two habituation sessions were identical. On the test session, the No-change group ( $n = 21$ ) was again exposed to the same arena, for the Prox-Dist group ( $n = 15$ ) proximal and distal cues were reconfigured, for the Prox group ( $n = 12$ ) only proximal cues, and for the Dist group ( $n = 12$ ) only distal cues. An additional Home-cage control group ( $n = 6$ ) where the pups remained in their home cage during sessions was used only as control for c-Fos activity and is not shown here. (b) Photo of the arena illustrating the experimental proximal cues (checkerboard pattern covering either the floor or the wall of the arena) and the distal cues (four white curtains and three boxes presented in different spatial configurations). (c) Mean ( $\pm$ SEM) distance traveled for each group during 1st minute of habituation and test sessions (###, \*\*\*  $p < .001$ , ANOVA main effect across habituation session and across groups for the test session, respectively), separately (d) for absolute distance values on the test session and (e) for individual difference values between the test session minus habituation two session (\*  $p < .05$ , \*\*  $p < .01$ , \*\*\*  $p < .001$ , for pairwise comparison). (f) Distance traveled during the entire 10-min interval of first and second habituation and test sessions. Expectedly, given the long (10-min) duration of the intervals, this control analysis did not reflect the habituation-related changes to spatial novelty as sensitively as the analyses focusing on the 1st min of the sessions but still revealed the overall decrease in distance traveled between the first and second habituation session ( $F[1,56] = 14.46$ ,  $p < .001$ ). Statistical controls excluded that the findings on habituation were confounded by the increased locomotion in the first session of the Prox group, inasmuch as locomotion (over 10 min) in this session was uncorrelated with any habituation-related decrease in distance traveled (all  $p > .15$ ). Removing, in an exploratory analysis, the four rats with the longest traveling distance (first min) of this group revealed an even stronger increase in the traveled distance at the test session for the Prox group

event initiation (Bisler et al., 2002; Zangenehpour & Chaudhuri, 2002). We used six pups per group for the immunocytochemistry analyses. Each of these pups was again randomly allocated to the experimental groups, with the constraint that pre-experimental experience was comparable. (Analyses of traveled distance performed in these subsamples of  $n = 6$  rats for each group confirmed the behavioral findings reported for the total groups.) The subsequent procedures were as described previously (Mendez et al., 2015). The brains were cut in coronal serial sections ( $30 \mu\text{m}$ ) at  $-20^\circ\text{C}$  in a cryostat microtome (model HM 505-E, Microm International GmbH, Heidelberg, Germany). The sections were mounted on gelatinized slides, which were post-fixed in buffered 4% paraformaldehyde (0.1 M, pH 7.4) for 30 min and rinsed in phosphate-buffered saline (PBS) (0.01 M, pH 7.4). They were subsequently incubated for 15 min with 3% hydrogen peroxidase in PBS to remove endogenous peroxidase activity, and then washed twice in PBS. After blocking with PBS solution containing 10% Triton X-100 (PBS-T) (Sigma, USA) and 3% bovine serum albumin for 30 min, sections were incubated with a rabbit poly-clonal anti-c-Fos solution (1: 10,000) (Santa Cruz Biotech, sc-52, USA) diluted in PBS-T for 24 h at  $4^\circ\text{C}$  in a humid chamber. Slides were then washed three times with PBS and incubated in a goat anti-rabbit biotinylated IgG secondary antibody (Pierce, USA; diluted 1:200 in incubating solution) for 2 h at room temperature. They were washed three times in PBS and reacted with avidin biotin peroxidase complex (Vectastain ABC Ultrasensitive Elite Kit, Pierce) for 1 h. After two washes in PBS, the reaction was visualized, treating the sections for 3 min in a commercial nickel-cobalt intensified diaminobenzidine kit (Pierce, USA). The reaction was terminated by washing the sections twice in PBS. Slides were then dehydrated through a series of graded alcohols, cleared with xylene, and cover-slipped with Entellan (Merck, USA) for microscopic evaluation. All immunocytochemistry procedures included sections that served as controls where the primary antibody was not added. Slides containing sections of a specific brain region were stained at the same time. Slides were coded so that the experimenters performing the entire analysis were blinded to the conditions of individual subjects.

Regions of interest (ROIs) were defined based on the literature about hippocampal and cortical regions known to be involved in the formation of spatial and episodic memories, and anatomically determined according to the atlas by Khazipov et al. (2015) for PD14 rats. ROIs and their distance (in mm) from bregma were:  $+2.2$  mm for the prelimbic (PL), infralimbic (IL), and cingulate (CG) cortices;  $-2.2$  mm for the hippocampal cornu ammonis 1 (CA1), cornu ammonis 3 (CA3) and dentate gyrus (DG) subfields;  $+1.4$  mm for primary motor (M1) and primary somatosensory (S1) cortices;  $-2.2$  mm for the agranular retrosplenial (RSA) and parietal (PAR) cortices;  $-4.0$  mm for perirhinal (PRH) and entorhinal (ENT) cortices.

The number of c-Fos positive nuclei in ROIs was quantified in two alternate sections  $30 \mu\text{m}$  apart. Quantification was performed by systematically sampling each of the regions selected using superimposed counting frames. Sizes of the counting frames ranged from  $72,000 \mu\text{m}^2$  (RSA) to  $120,000 \mu\text{m}^2$  (ENT and PRH). The total area

sampled by these frames per region in each section was:  $140,000 \mu\text{m}^2$  in PL, IL;  $120,000 \mu\text{m}^2$  in CG;  $144,000 \mu\text{m}^2$  in CA1, CA3;  $80,000 \mu\text{m}^2$  in DG;  $140,000 \mu\text{m}^2$  in M1 and S1 cortices;  $72,000 \mu\text{m}^2$  in RSA;  $144,000 \mu\text{m}^2$  in PAR;  $120,000 \mu\text{m}^2$  in PRH;  $240,000 \mu\text{m}^2$  in ENT. Cell counts were conducted using a microscope (Leica DM6000B, Germany, 20x magnification) and a digital camera (Leica DFC490, Germany) coupled to a computer with software installed (Leica application suite, Germany). c-Fos positive nuclei were defined based on homogenous gray-black stained elements with a well-defined border. Finally, the mean count of two sections was calculated for each subject and region.

## 2.5 | Statistical analyses

Statistical analyses were performed using SPSS software (IBM, Armonk, NY, USA). Analysis of traveling distance was based on a global analysis of variance (ANOVA) with a Group factor (No-change, Prox-Dist, Prox, Dist groups) and a repeated-measures factor Session (first, second habituation sessions, test sessions). The significant Group  $\times$  Session interaction effect from this analysis was followed by one-way sub-ANOVA combined with pairwise LSD post hoc comparisons and paired-samples *t*-tests.

Values of c-Fos activity were likewise first analyzed by a global ANOVA including a Group factor (Home-cage, No-change, Prox-Dist, Prox, Dist groups) and a repeated measures Area factor (PL, IL, CG, CA1, CA3, DG, M1, S1, RSA, PAR, PRH, ENT). The significant Group  $\times$  Area interaction was followed by one-way ANOVA with a Group factor, performed separately for each area, combined with pairwise LSD post-hoc comparisons. For a functional connectivity analysis based on c-Fos activity, Pearson correlation coefficients were calculated between all pairs of brain areas, and the total number of correlations reaching the significance criterion of  $p < .05$  was compared between groups by chi-square test. Connectivity graphs were constructed using both c-Fos quantifications and correlation coefficients. The Igraph package (v1.2.4.2) in R (RStudio, Boston, MA, USA) was used to visualize the networks. Pearson correlations were additionally calculated between c-Fos activity and distance traveled mainly to explore the link between spatial locomotor activity and activity in mPFC and hippocampal areas. Because of their exploratory nature, significance of these coefficients was not corrected for multiple testing.

In cases (of one-way ANOVA, *t*-tests) where normality of the distribution or equal group variances was not assured, we additionally used nonparametric test (Kruskal-Wallis H test, Wilcoxon-signed rank test, respectively), and only when these nonparametric tests confirmed significance, respective results are reported. Normality was tested using Shapiro-Wilk Test that confirmed normal distribution of data in all but one group's locomotion data (No-change group). Re-testing (with the Kruskal-Wallis test) confirmed significance ( $p < .05$ ) for all pairwise comparisons involving this group. Results are presented as means  $\pm$  SEM. A  $p < .05$  level (uncorrected for post-hoc pairwise comparisons) was considered significant.

### 3 | RESULTS

#### 3.1 | Behavior—traveling distance

As expected, distance traveled (in the first min of the exploration interval) uniformly decreased in all groups from the first to the second habituation sessions but, differed between the groups at the test session depending on whether or not novel cue configurations were introduced ( $F[6,112] = 2.557, p = .023$ , for Group  $\times$  Session interaction in the global ANOVA, Figure 1c). Analyses focusing on the first two habituation sessions confirmed the decrease in traveling distance occurring with the repeated exposure of the pups to the same environment, as a most robust phenomenon with no differences between the groups ( $F[1,56] = 92.087, p < .001$ , for Session main effect;  $p = .589$ , and  $p = .766$  for Group main effect and Group  $\times$  Session interaction, respectively;  $p < .003$  for separate pairwise comparison of Sessions in each Group). Yet, the groups showed differential traveling distances in the test session ( $F[3,59] = 8.518, p < .001$ , Figure 1d). With reference to the second habituation session, the No-change group further decreased locomotion ( $p = .015$ ) indicating that the animals confronted to a third presentation of the same spatial configuration continued to habituate. By contrast, the Prox-Dist group showed an increase in locomotion ( $p = .029$ ), indicating that the pups discriminated the change in proximal and distal cue configurations. In the Prox group, traveled distance on average also increased from the second habituation session to the test session, although this was not significant ( $p = .550$ ). Nevertheless, traveled distance at the test session was closely comparable in the Prox-Dist and Prox groups ( $p = .375$ ), and both groups traveled distinctly longer distances than the No-change group ( $p < .001$  and  $p = .003$ , respectively, Figure 1d). By contrast, traveling distance in the Dist group at the test session was similar to that of the No-change group ( $p = .706$ ). Analyses of difference values (traveled distance at test minus second habituation session) revealed basically the same pattern, except that the relative increase in locomotion in the Prox group only approached significance in comparison with the No-change group ( $p = .096$ , Figure 1e, see Figure S1 for an additional analysis on second minus first habituation session difference values).

Control analyses of the time the pups spent in the center of the arena did not provide evidence for any difference in anxiety levels between groups (Figure S2). We also excluded bodyweight as a possible confound of differences in locomotion. Because, unexpectedly, bodyweight at PD16 differed among the groups (mean  $\pm$  SEM, No-change  $33.1 \pm 0.9$  g, Prox-Dist  $37.4 \pm 1.0$  g, Prox  $32.6 \pm 1.0$  g, Dist  $31.3 \pm 1.0$  g,  $F[3,59] = 7.03, p < .01$ ) reflecting greater bodyweight in the Prox-Dist group than in the other groups, we repeated all ANOVA on traveled distance introducing bodyweight as a covariate. These analyses confirmed all Group  $\times$  Session effects of the original analyses, and none of these analyses showed a significant effect for the covariate bodyweight (all  $p > .207$ ). We also did not find any significant correlation between bodyweight and distance traveled during the first min of the Test session ( $r < .209, p > .109$ ) or the time the pup spent in the center area per group ( $r < -.216, p > .098$ ), overall making it unlikely that bodyweight

substantially confounded the observed group differences in spatial locomotion.

In combination, these data provide behavioral evidence that the pups form a representation of the spatial environment during the habituation sessions, that mediates a further down regulation of exploratory locomotion when, at the test session, the pup is exposed to the same environment but that upregulates locomotion when novel proximal cue configurations are introduced. Distal cues as a separate entity do not appear to impact spatial locomotion, but might play a role in combination with proximal cues.

#### 3.2 | Expression of c-Fos

We found group differences in hippocampal and medial prefrontal cortical (mPFC) areas whereas primary motor and primary somatosensory cortices showed little changes ( $F[15.715,98.217] = 15.982, p < .001$  for global ANOVA Group  $\times$  Area interaction, see Figure 2 for pairwise comparisons). Importantly, the No-change group expressed lower c-Fos activity in the hippocampal areas (CA1, CA3 and dentate gyrus,  $p < .001, p = .015, p < .001$ , respectively) but higher c-Fos activity in mPFC areas (PL, IL and CG,  $p < .01, p < .05$ , and  $p < .05$ , respectively) compared with the Home-cage control group. In contrast, in the Prox-Dist, Prox and Dist groups, c-Fos activity was enhanced in hippocampal CA1 and CA3 regions (all  $p < .01$ ). Correlating c-Fos with locomotor activity at the test session revealed that c-Fos expression in the prelimbic cortex was negatively correlated with the distance traveled at test in the No-change group ( $r = -.910, p = .012$ ). However, a positive correlation was found between the c-Fos activity in CA1 and the distance traveled in the Prox-Dist ( $r = .881, p = .020$ ) and Prox groups ( $r = .947, p = .004$ , Figure 2b), pointing toward an opposite functional role for hippocampal and mPFC areas in regulating spatial behavior at this age.

The Prox-Dist group, in mPFC regions displayed low levels of c-Fos activation, comparable to that in the Home-cage control group and distinctly lower than that of the No-change group ( $p < .05$ , for PL, IL, CG). Different from this pattern and similar to the No-change group, the Prox group showed increased c-Fos activity in mPFC regions (PL, IL, CG,  $p < .05$  in comparison with the Home-cage control group), overall suggesting that the mPFC response depends on an intermediate degree of novelty, that is, a high response when only proximal cues are changed but no response when both proximal and distal cues change.

Interestingly, in the Dist group whose behavior during the test session did not differ from that of the No-change group, c-Fos activity was enhanced in the hippocampus (CA1, CA3 and dentate gyrus, all  $p < .01$ ), retrosplenial ( $p < .001$ ), and parietal ( $p < .001$ ) regions compared to the Home-cage control and No-change groups. These increases indicate that the pups neuronally processed the spatial distal cues, although not responding to them at the behavioral level.

We determined connectivity network graphs based on significant (uncorrected for multiple comparisons) Pearson's correlation coefficients, to analyze the functional connectivity within the set of brain structures of interest and their correlation with locomotor behavior

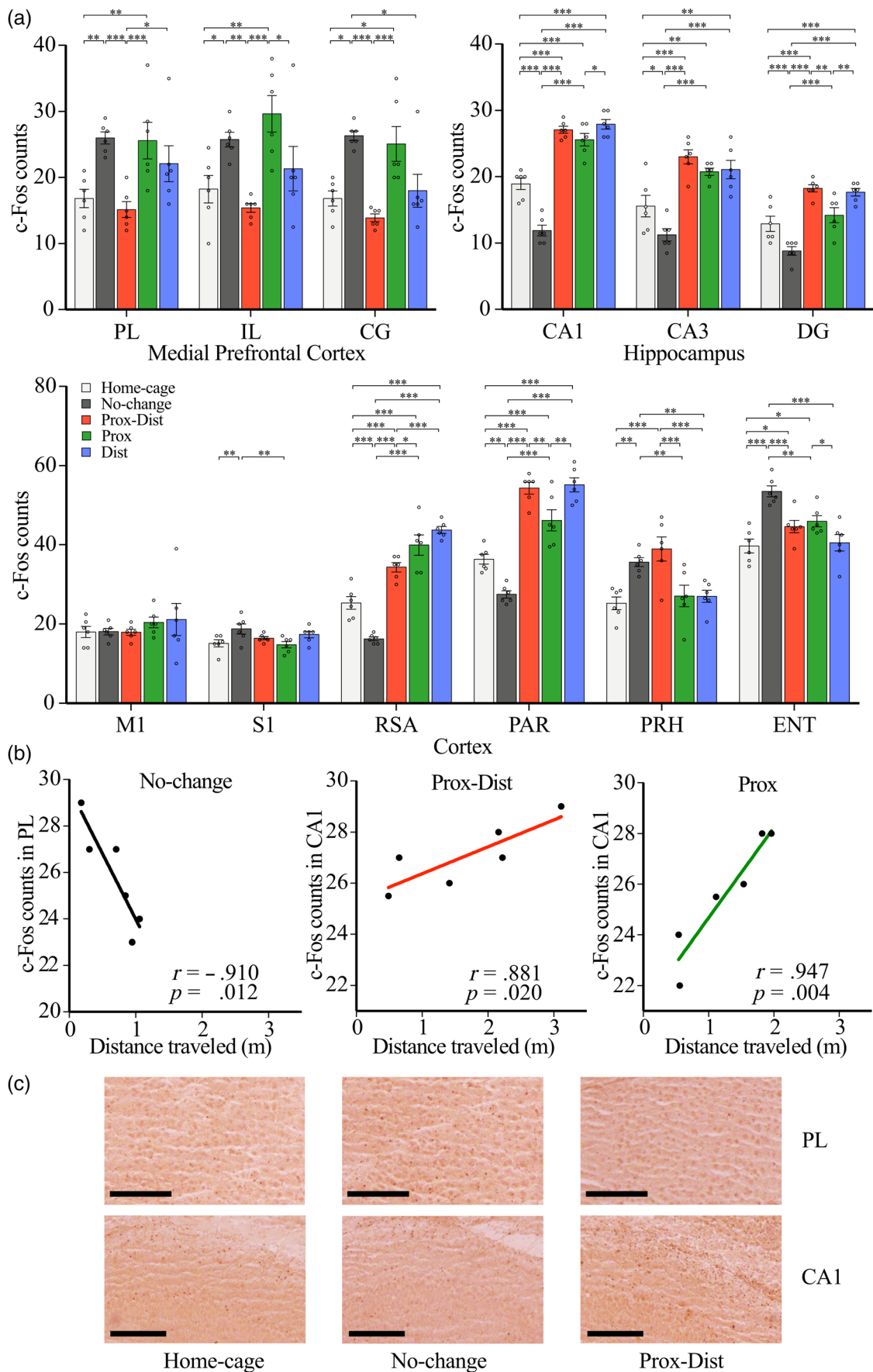
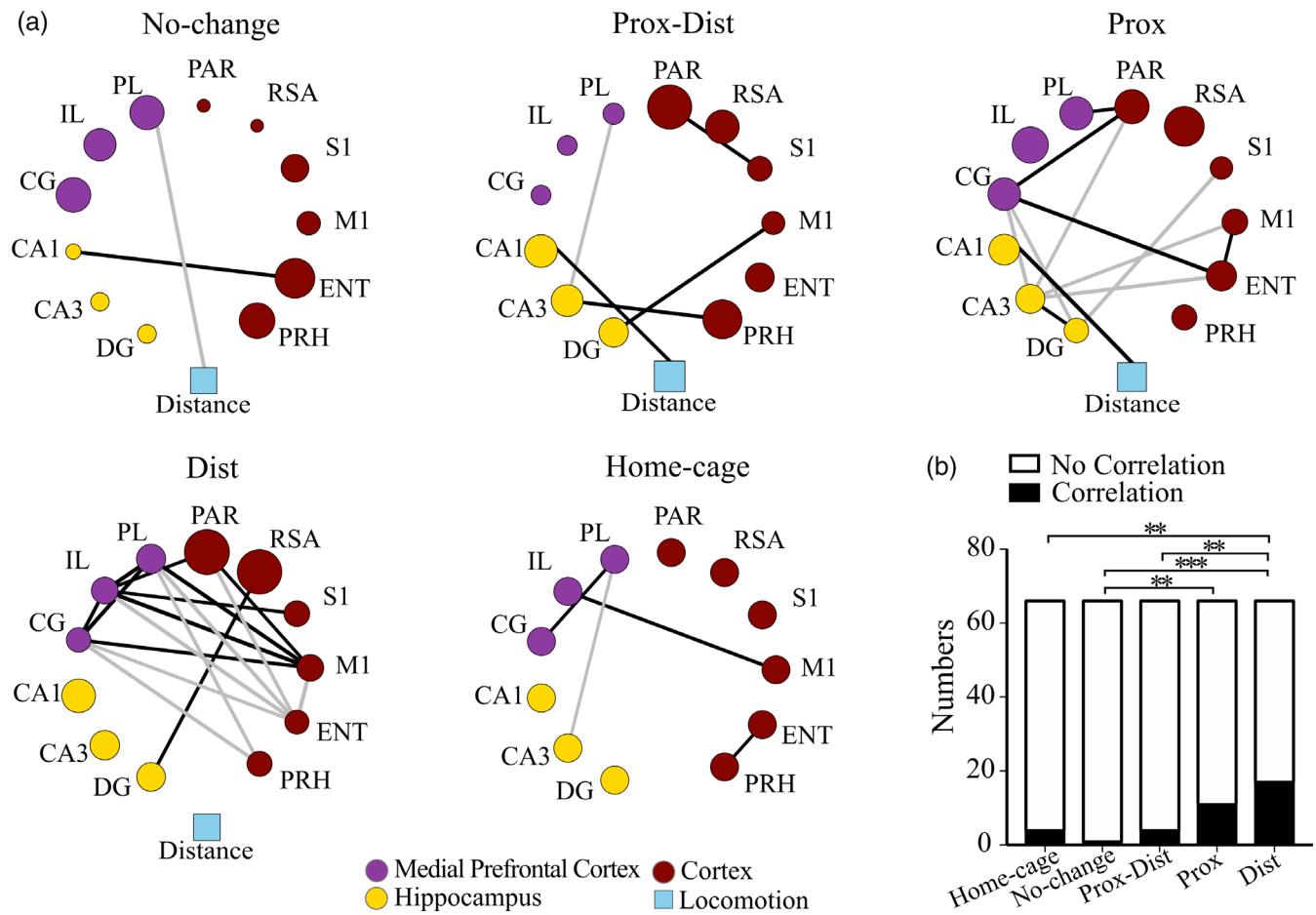


FIGURE 2 Legend on next page.



**FIGURE 3** (a) Network connectivity analysis of c-Fos activity in (top) No-change, Prox-Dist, Prox groups, and (bottom) Dist and Home-cage control groups. Graphs include correlations with distance traveled (distance, blue square) during the test session, and indicate only significant ( $p < .05$ )  $r$  values (positive—black, negative—gray connection lines). Brain regions are color-grouped and node size is proportional to c-Fos activity, with activity of the Home-cage group taken as baseline. (b) Number of significant correlations (black bars) shown as proportion of the total number of possible correlations (i.e.,  $n = 66$ ) between brain regions for the five groups (\*\*  $p < .01$ , \*\*\*  $p < .001$ , for pairwise comparison with  $\chi^2$  test)

during the test session (Figure 3a). The graphs of the No-change, Prox-Dist and Prox groups were hallmarked by the negative correlation of PL cortex and the positive correlation of CA1, respectively, with traveled distance (reported in the previous section). Otherwise, the number of significant interregional correlations in these groups was low and did not differ from that of the Home-cage control group (Figure 3b). The number of interregional correlations was increased in the Prox group, when compared with the No-change group ( $\chi^2 = 9.167$ ,  $p = .002$ ), with the majority of these connections involving the hippocampal CA1, CA3, and DG areas. Interestingly, the Dist group that did not behaviorally respond to the change in distal cue formation, was the group that displayed the strongest increase in the number of interregional correlations in c-Fos activity during the test session ( $\chi^2 > 9.570$ ,  $p < .002$ , for the comparisons with all other

groups except the Prox group; Figure 3b). Unlike in the Prox group, in the Dist group these regional intercorrelations spared hippocampal areas but mainly connected mPFC and parietal cortices, on the one hand, with entorhinal and perirhinal cortices, and on the other hand, with the primary motor cortex. The correlations with entorhinal and perirhinal cortices were negative whereas the correlation with primary motor cortex was positive in direction.

## 4 | DISCUSSION

We tested whether pre-weanling infant rats at PD16 are already able to form persisting (~140 min) spatial representations using a simple spatial habituation paradigm that allowed to assess behavioral

**FIGURE 2** (a) Mean ( $\pm$ SEM) counts of c-Fos positive cells in studied brain regions, top left: Medial prefrontal cortex including prelimbic (PL), infralimbic (IL), and cingulate cortices (CG), and (top right) hippocampal subfields CA1, CA3 and dentate gyrus (DG). Bottom: Primary motor and primary somatosensory cortices (M1 and S1), agranular retrosplenial (RSA), parietal (PAR), perirhinal (PRH), entorhinal (ENT) cortices. (\*  $p < .05$ , \*\*  $p < .01$  and \*\*\*  $p < .001$  for pairwise comparisons between experimental groups). (b) Correlations of locomotion (distance traveled) with c-Fos counts (left) in the PL region of mPFC in the No-change group, and (middle and right) in CA1 in the Prox-Dist and Prox groups. (c) Representative images of c-Fos staining selected for cell count analysis in PL and CA1 (scale bar: 200  $\mu$ m)



(distance traveled during exploration) as well as neuronal (c-Fos activity at the test session) read-outs of spatial memory formation. Results indicate a robust decrease in distance traveled during exploration with the first repetition of exposing the rat pups to the identical arena environment (second Habituation session), followed by a further decrease in locomotion when the rat pup was exposed a third time to the identical environment (test session of the No-change group). By contrast, exposing the rat pup at the third session, that is, the test session to an arena with changed proximal and distal cue configurations invoked a strong increase in distance traveled during exploration. This increase was less clear when only the proximal cue configuration was changed, and absent when only the distal cue configuration was changed. In combination, this pattern indicates that the pups at PD16 indeed form a spatial representation that is used to differentially regulate exploratory behavior in familiar vs. novel spatial environments, and that appeared to integrate proximal cues to a greater extent than distal cues. The picture deriving from locomotor behavior was corroborated by the analysis of regional c-Fos activity levels at the test session. Exposed a third time to the identical spatial environment, rat pups of the No-change group not only traveled the shortest distance during this session but also showed highest c-Fos activity in prelimbic mPFC areas with the c-Fos levels in this region being negatively correlated with locomotor behavior. Although several alternative explanations are possible, this finding is consistent with the notion that inhibition of locomotor activity during spatial habituation is mediated through mPFC regions such as the prelimbic region, participating in the representation of space. We did not find signs of a hippocampal contribution to regulating habituation in the pups of the No-change group. By contrast, the rats which were exposed to changes in the configuration of distal and proximal or only proximal cues at the test session and which responded to these changes with an increase in locomotion, showed enhanced c-Fos activity levels in hippocampal areas, and the increase in c-Fos in CA1 was moreover positively correlated with the distance traveled at the test session. These findings are in line with the view that hippocampal regions particularly contribute to regulating explorative locomotion in response to novel proximal aspects of the spatial environment.

It can be excluded that the differences in locomotor and c-Fos activity between groups were strongly biased by maturational processes, as all experiments took place on the same day (PD16), and there were virtually no differences in locomotion between the groups at the two habituation sessions. Moreover, all groups including the home cage control, showed very comparable c-Fos activity in primary motor cortex, and only minor differences in primary somatosensory cortex. Indeed, locomotion and associated exploratory skills themselves, which were used here as a behavioral indicator of spatial memory, are in essence developed by the end of the second postnatal week (Altman & Sudarshan, 1975). In comparison, the time when response habituation (to repeated stimulation) occurs during development appears to be more variable and depending on the type of stimulation (Bronstein et al., 1974; Einon et al., 1975; Feigley et al., 1972). Against this backdrop, the lack of differences in c-Fos activity in the primary motor cortex between groups that differed in locomotion (i.e., distance traveled) on one side probably reflects that the distance

traveled in our task paradigm does not as sensitively cover principle functions of this cortex in locomotor regulation as other tasks and behaviors (e.g., involving a more specific use of the limbs; Scott, 2003). On the other side, it confirms that our task mainly probed spatial exploration rather than motor regulation.

The consistent decrease in locomotion we found here in all groups across the first two habituation sessions and, in the test session, specifically for the No-change group corroborates the view that habituation is in principle established before PD16. We also can exclude that locomotor responses were confounded by non-spatial aspects of the stimulus conditions, because for manipulating the proximal and distal context of the arena we only changed the spatial configuration of the cues but not the cues themselves. Moreover, to assure the formation of allocentric spatial representations, the pups entered the arena at each session from a different side. Finally, we did not reveal hints that findings on habituation were confounded by general differences in locomotion (across the total 10-min intervals, Figure 1f).

Our findings demonstrating that pups can form persisting spatial representations already at PD16 extends previous studies where this capability emerged later during development. In tasks requiring the discrimination of objects or in the hidden platform version of the Morris water maze task, behavioral hints at the formation of enduring spatial representations (persisting more than 10 min) were revealed in rat pups not before PD17 (Ainge & Langston, 2012; Akers & Hamilton, 2007; Contreras et al., 2019; Ramsaran et al., 2016; Rudy et al., 1987; Sanders et al., 2020; Westbrook et al., 2014). The earlier onset in the formation of spatial memory found here can be explained by the task that did not require discrimination of discrete objects and was performed in stress-free conditions. Whereas tasks like the Morris water maze use a reference object (platform) to specify the kind of spatial memory formed, in the present task paradigm (being not a goal-oriented task) this was achieved by the targeted manipulation of proximal and distal cue configurations. Our finding of an earlier onset of behavioral signs of spatial memory concurs with electrophysiological evidence indicating that the neuronal machinery of place, grid, and head direction cells in hippocampus and adjacent areas is well functioning at PD16 (Tan et al., 2015, 2017; Wills et al., 2010), although these structures as well as prefrontal structures contributing to spatial behavior are by far not fully matured at this age.

The pups increasing locomotion in the test phase when proximal and distal cue configurations changed and, to a lesser extent, when only proximal cues changed but not when only distal configurations changed, suggests that the animal's spatial behavior at this age is preferentially regulated via the integration of proximal landmarks. However, although with designing the arena we aimed at a comparable impact of proximal and distal cues, we cannot exclude that proximal and distal cues differed in salience. Thus, the absent changes in locomotor activity in the Dist group might be related to the specific cues used here, being less salient for the pups. However, an earlier onset for the integration of proximal than distal cues into spatial navigation has been also found in other studies, using other task paradigms such as the Morris water maze task (Akers & Hamilton, 2007; Rudy

et al., 1987) and the object-in-context learning task (Akers et al., 2011; Ramsaran et al., 2016). The pups neglecting distal cues might result from an immature visual acuity with pre-weanling rats not being able to discriminate cues in the distance (Carman et al., 2003; Carman & Mactutus, 2002). However, c-Fos levels in our Dist group provided ample evidence that these rats indeed processed the change in the configuration of distal cues (see below). Hence, rather than not perceiving these cues, it is more likely that the pups at PD16 are just unable to use them for regulating locomotor behavior.

The comparison of c-Fos activity levels between our Prox-Dist and No-change groups can be taken to suggest that both hippocampus and mPFC contribute to the formation of spatial representations, the former mediating increased locomotion to novel cue configurations, the later mediating habituation and suppression of locomotion once the actual environment has been recognized as familiar. As to the hippocampus, our findings in pups concur with a great body of evidence in adult rodents identifying the hippocampus as key structure for navigation and the encoding of novel spatial representations (e.g., Klur et al., 2009; Loureiro et al., 2012; O'Keefe & Nadel, 1978). Similar to our findings in pups at PD16, adult rats showed increased hippocampal c-Fos activity during spatial learning on an object place recognition task as well as upon changes in the spatial configuration of familiar visual stimuli (Jenkins et al., 2004; Mendez et al., 2015; Wan et al., 1999). Moreover, like in our pups, c-Fos activity in CA1 in these adult rats was positively correlated with behavioral indicators of memory (Mendez et al., 2015), although our analyses were exploratory in nature with no statistical correction for multiple testing. In rat pups muscimol-induced inactivation of the hippocampus prevented context fear learning at PD24 (Raineki et al., 2010). Hippocampal c-Fos activity was enhanced in pups at PD20 performing on the Morris water maze and object location task, whereas rats at PD16, the specific age we used here, did not show increased activation in both tasks (Comba et al., 2015). Likewise, hippocampal c-Fos activity was increased during contextual fear learning at PD24 but not at PD21 (Raineki et al., 2010). Against this backdrop, the enhanced hippocampal c-Fos activity in response to novel proximal cue configurations occurring in our rat pups much earlier during development, that is, on PD16, can be well explained by our task paradigm allowing to more sensitively assess at this age the encoding of the specifically spatial aspects of the stimulation. Accordingly, around PD16 place cells in CA1 have been shown to generate new representations upon a novel context and to reactivate familiar representations based on degraded stimuli (Muessig et al., 2016).

Changes in proximal and distal cue configurations also produced distinct changes in c-Fos activity in the retrosplenial, parietal, perirhinal and entorhinal cortices, that is, a network of regions well known to be involved in regulating hippocampus-based spatial navigation (Clark et al., 2018; Wilber et al., 2015). The pattern of changes in c-Fos activity in parietal cortex was on average remarkably similar to that in CA1 supporting the idea that these regions form a circuitry concurrently processing spatial information (Whitlock et al., 2008). Also, like in hippocampal CA1, c-Fos activity in parietal and retrosplenial cortices was at a minimum in the No-change group, consistent

with the view that habituation-related inhibition is not restricted to hippocampal areas but extends to major cortical areas involved in spatial navigation. Curiously, the entorhinal cortex showed an opposite pattern with maximum c-Fos activity in the No-change group. While this as well as the more complex pattern of changes observed in the Prox and Dist groups remain to be clarified in further studies, in combination the observations support the view that spatial exploration already at PD16 represents a network function, relying on a coordinate interaction of multiple hippocampal and cortical regions.

Our findings argue against the view derived from studies of adult rats, that hippocampus-based navigation selectively refers to distal cues (Piterkin et al., 2008; Ramsaran et al., 2016; Rudy et al., 1987). Indeed, the increases in c-Fos activity in hippocampal regions in our Prox group in combination with the positive correlation between CA1 c-Fos levels and locomotor activity in this group, is in line with the assumption that proximal cues alone might be sufficient to regulate hippocampus-based navigation at this age (Rinaldi et al., 2020).

Our data corroborate growing evidence that the formation of spatial representations involves the mPFC already early on in life, although these regions show a rather protracted developmental trajectory (Bitzenhofer et al., 2021; Chini et al., 2020). The increase in c-Fos levels in mPFC regions in the No-change group together with the negative correlation of c-Fos levels in the prelimbic region of the mPFC with locomotor activity is consistent with the view that this region significantly contributes to habituation toward familiar spatial environments, which was established in studies of adult rodents (e.g., Chudasama et al., 2012; Eichenbaum, 2017; Expósito et al., 2020). Interestingly, c-Fos levels in mPFC were not only enhanced in the No-change group but also in the Prox group showing increased locomotor behavior to the novel proximal cue configuration at the test session. This finding might reflect that these areas beyond their involvement in spatial habituation, serve additional functions possibly in the regulation of attention (Birrell & Brown, 2000; Dejean et al., 2016; Hébert et al., 2017).

Notably, c-Fos levels in the Dist group indicated that the pups also processed the changes in the distal cue configuration, although this did not express in behavioral changes. Specifically, the Dist-group upregulating c-Fos activity in CA1 suggests a kind of disconnect, that is, that pups at PD16 are able to encode distal spatial cues but cannot retrieve it for behavioral regulation. This idea is pertinent to findings by Foster and Burman (2010) providing evidence that at PD17 pups could learn about the context in a hippocampus-dependent fear conditioning task, but were able to exhibit fear to this context not before PD23. Those authors argued that the hippocampus being already functional as early as PD17, is not yet well connected to the brain structures regulating respective fear behaviors. In the present study, the pups exposed to changes only in the distal cue configuration, showed maximal c-Fos levels in parietal and retrosplenial areas, that is, interconnected regions well-known to be centrally involved in regulating navigation based on distal cues and allocentric reference frames in the mature brain (Auger et al., 2012; Clark et al., 2018; Mitchell et al., 2018; Vann & Aggleton, 2005). Moreover, the Dist group showed prominently increased overall functional connectivity

between brain regions. Yet, fitting with the findings by Foster and Burman (2010) mentioned above, this increase in functional connectivity appeared to spare hippocampal regions, with this hippocampal disconnect possibly explaining that the processing of distal cue changes was not integrated into the Dist group's locomotor behavior.

We have to caution against any premature conclusions derived from our c-Fos findings as these are basically correlational in nature. Thus, whether or not mPFC regions like the prelimbic cortex are causally involved in regulating spatial navigation and locomotion in rat pups cannot be inferred from the present data. Alternatively, reduced locomotion may produce less c-Fos activity in these regions, or increases in c-Fos could be related to a third factor independently reducing locomotion. Hence, a causal contribution of mPFC regions to spatial navigation needs to be examined in future studies directly manipulating the function of these structures in rat pups at PD16. A further limitation of our study may arise from the lack of a control condition testing the effects of an entirely new spatial environment, in comparison with introducing rather specific changes in the configuration of proximal and distal cues. Although such a control is difficult to integrate in a design requiring all testing to be performed on a single day (PD16), it might have aided the interpretation of our c-Fos data.

In conclusion, our study shows that infant rats at PD16 which is typically the first day they show reliable exploratory locomotor behavior, are able to form persisting spatial representations. Discriminating between decreases in locomotion (habituation) to familiar spatial contexts and increases in locomotion to novel contexts, we provide evidence that these spatial representations are formed at a systems level including not only hippocampal regions regulating behavior to novelty, but also mPFC regions - particularly the prelimbic region - which appear to be centrally involved in locomotor inhibition and habituation to familiar environments and, additionally, in attentional control to conflictual spatial information. This systems view concurs with finding in adult rats, for example, of impaired spatial learning (in the Morris water maze) after lesions to both the prelimbic mPFC as well as to the hippocampus (Wang & Cai, 2008). Coordinate phase-locked oscillatory and spike activity between hippocampal and prefrontal cortical regions emerges already within the first 2 weeks after birth (Brockmann et al., 2011). The present c-Fos data provide additional support for this systems view, in showing that the central structures interfacing the prelimbic mPFC with hippocampal CA1 and CA3 regions, that is, perirhinal and (lateral) entorhinal cortices (Eichenbaum, 2017), displayed significantly enhanced c-Fos activity in both the pups exposed to a highly familiar environment (No-change group) as well as in the pups exposed to clear changes in the spatial context (Prox-Dist group, Figure 2). Collectively, our findings allocating complementary roles to mPFC and hippocampus in mediating responses to familiar and novel spatial contexts, respectively, provide evidence that the mPFC-hippocampal circuit might operate at a systems level during spatial encoding already at PD16. An obvious question arising here is, to what extent such function of the mPFC-hippocampal circuit generalizes already during this early stage of

development to other domains of episodic memory formation (Takehara-Nishiuchi, 2020).

## ACKNOWLEDGMENTS

The authors would like to thank I. Sauter for technical supports and A. Sawangjit for helpful discussions. X.S. gratefully acknowledges funding from the China Scholarship Council (grant no. 201808080042). This research was supported by grants from the Deutsche Forschungsgemeinschaft to M.I. (DFG In 279/1-1), from the European Research Council (ERC AdG 883098 Sleep Balance) to J.B., and from the Spanish Government (PSI2017-83893-R) to M.M. M.I. was supported by the Hertie Foundation (Hertie Network of Excellence in Clinical Neuroscience). Open Access funding enabled and organized by Projekt DEAL.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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**How to cite this article:** Shan, X., Contreras, M. P., Mendez, M., Born, J., & Inostroza, M. (2021). Unfolding of spatial representation at systems level in infant rats. *Hippocampus*, 1–13. <https://doi.org/10.1002/hipo.23392>