CHEMOGENETIC SILENCING OF HIPPOCAMPUS AND AMYGDALA REVEALS A DOUBLE DISSOCIATION IN PERIADOLESCENT OBESOGENIC DIET-INDUCED MEMORY ALTERATIONS

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1 ABSTRACT

In addition to numerous metabolic comorbidities, obesity is associated with several adverse neurobiological outcomes, especially learning and memory alterations. Obesity prevalence is rising dramatically in youth and is persisting in adulthood. This is especially worrying since adolescence is a crucial period for the maturation of certain brain regions playing a central role in memory processes such as the hippocampus and the amygdala. We previously showed that periadolescent, but not adult, exposure to obesogenic high-fat diet (HFD) had opposite effects on hippocampus- and amygdala-dependent memory, impairing the former and enhancing the latter. However, the causal role of these two brain regions in periadolescent HFD-induced memory alterations remains unclear. Here, we first showed that periadolescent HFD induced long-term, but not short-term, object recognition memory deficits, specifically when rats were exposed to a novel context. Using chemogenetic approaches to inhibit targeted brain regions, we then demonstrated that recognition memory deficits are dependent on the activity of the ventral hippocampus, but not the basolateral amygdala. On the contrary, the HFD- induced enhancement of conditioned odor aversion specifically requires amygdala activity. Taken together, these findings suggest that HFD consumption throughout adolescence impairs long-term object recognition memory through alterations of ventral hippocampal activity during memory acquisition. Moreover, these results further highlight the bidirectional effects of adolescent HFD on hippocampal and amygdala functions.

2 KEYWORDS (6)

obesity, adolescence, memory, DREADD, hippocampus, amygdala

1 INTRODUCTION

Obesity is one of the most important public health challenges and is linked to the overconsumption of energy-dense food combined with a sedentary lifestyle. In addition to being associated with several peripheral comorbidities including cardiovascular and metabolic disorders (Head, 2015; Malnick & Knobler, 2006; Walls et al., 2012), obesity is also associated with cognitive and neurobiological dysfunctions (Francis & Stevenson, 2013; Wang et al., 2016). Previous studies have demonstrated that obesity is associated with deficits in episodic and spatial memory (for reviews see Francis & Stevenson, 2013; Martin & Davidson, 2014; Sellbom & Gunstad, 2012; Yeomans, 2017) but also with increased emotional, mood and affective disorders (Mansur et al., 2015).

The prevalence of obesity has also risen in young people (Ogden et al., 2016; Sahoo et al., 2015). Recent studies indicate indeed that obese adolescents display blunted performance in geometric/visuospatial problems or relational memory (Khan et al., 2015; Nyaradi et al., 2014; Øverby et al., 2013). Given that childhood and adolescence are crucial periods for cognitive and brain development (Spear, 2000), they represent a window of vulnerability to external insults such as the deleterious impact of various diets (for reviews see Andersen, 2003; Noble & Kanoski, 2016; Reichelt & Rank, 2017). In rodents, we recently showed that high-fat diet (HFD) exposure from weaning to adulthood, defined as periadolescent exposure (see Labouesse et al., 2017) induced complex motivational (Naneix et al., 2017; Tantot et al., 2017) and memory deficits (Boitard et al., 2012, 2014, 2015, 2016; Khazen et al., 2019). Importantly, we found that similar duration of HFD exposure at adulthood does not have any effect on memory (Boitard et al., 2012, 2014, 2015; Khazen et al., 2019) and that periadolescent HFD alters relational and spatial memory but enhances emotional memory (for reviews see Del Olmo & Ruiz-Gayo, 2018; Morin et al., 2017; Murray & Chen, 2019). However, how these memory alterations are supported by specific neurobiological changes is still unclear.

Relational memory and emotional memory are respectively dependent on the hippocampus (Bunsey & Eichenbaum, 1996; Hartley et al., 2014) and the amygdala (LeDoux, 2003; McGaugh, 2004; Paré, 2003). In humans, clinical studies have shown that obese patients present hippocampal (Mestre et al., 2017; Mueller et al., 2012) and amygdala alterations (Connolly et al., 2013; Pasquali et al., 2006; Widya et al., 2011). It is noticeable that both the hippocampus and the amygdala complete their development during adolescence (for review see Casey et al., 2010; McCormick & Mathews, 2010; Saygin et al., 2015; Spear, 2000). Interestingly, overweight/obese children present reduced hippocampal volumes (Bauer et al., 2015) and increased amygdala activation (Boutelle et al., 2015). Similar patterns have been reported in HFD animal models (Abbott et al., 2019; Bose et al., 2009). Therefore, hippocampus and amygdala may then be highly vulnerable to the long-term deleterious

effects of periadolescent HFD. However, the causal role of these two brain areas in periadolescent HFD-related memory changes remain to be demonstrated.

Here we investigated the causal role of ventral hippocampus (vHPC) and the basolateral amygdala (BLA) in memory deficits induced by HFD consumption, from weaning to adulthood (covering adolescence). We previously assessed spatial and relational memory using aversive (Boitard et al., 2014, 2016) or rewarded (Boitard et al., 2012) learning tasks. Here we used different variations of non-aversive, non-rewarded, spontaneous learning tasks using objects, i.e. object recognition memory (ORM). By manipulating the delay between training and test, as well as the arousal levels during training (through habituation or not to the training context), we used different situations that could differentially recruit the hippocampus (for review see Cohen & Stackman, 2015) and the BLA (Maroun & Akirav, 2008; Okuda et al., 2004; Roozendaal et al., 2006), respectively. Regarding hippocampus, if most of the ORM studies have focused on the role of the dorsal hippocampus (dHPC), manipulations of both dHPC and vHPC have stronger effect on ORM performance than similar manipulations restricted to the dHPC suggesting a complementary role of vHPC in ORM (Broadbent et al., 2004; Hales et al., 2015). Here, we first show that periadolescent HFD decreased specifically hippocampal-dependent form of long-term ORM in non-habituated rats (high arousal conditions). Using a chemogenetic DREADD approach (Armbruster et al., 2007; Rogan & Roth, 2011), we then demonstrate that this ORM deficit is abolished by the inhibition of the vHPC, but not the BLA projecting neurons during the acquisition. Interestingly, we additionally observed that the HFD-induced enhancement of aversive odor memory is dependent on BLA, but not vHPC, activity.

2 METHODS

2.1 Animals and diets

Naïve male Wistar rats (Janvier), aged 3 weeks when they arrived, were housed in groups of two to four individuals in polycarbonate cages (48 x 26 x 21 cm) in an acclimatized (22 ± 1°C) housing room maintained under a 12 h light/dark cycle (lights on at 8:00 am, lights off at 8:00 pm). They had *ad libitum* access to food and water from their arrival until euthanasia day. At their arrival, rats were maintained either on a control diet (CD; 2.9 kcal/g; 8% lipids, 19% proteins, 73% carbohydrates; A04, SAFE) or on a high fat diet (HFD; 4.7 kcal/g; 45% lipids, 20% proteins, 35% carbohydrates; D12451, Research Diet). Animals' body weight was recorded weekly. Rats were exposed to CD or HFD for 12 weeks (from weaning to adulthood) before the start of the behavioral experiments (**Figure 1A**). After the completion of the ORM task, rats were housed individually in identical cages (48 x 26 x 21 cm) to

measure individual drinking behavior during all phases of the conditioned odor aversion (COA) procedure.

All procedures were performed in agreement with the French (Directive 2013-118, 1 February 2013) and international (directive 2010-63, 22 September 2010, European Community) legislations and received approval from the local Ethics Committee (5012047-A).

2.2 Viral vector and drugs

An adeno-associated viral vector (AAV) carrying the inhibitory hM4D(Gi) DREADD driven by the CaMKII promoter (to limit expression to projecting neurons) was obtained from University of North Carolina Vector core (Chapel Hill, NC, USA). The vector used was an AAV8-CaMKII-hM4D(Gi)-mCherry (3–4×10^{12} vp/ml).

The exogenous ligand Clozapine-N-Oxyde (CNO; Enzo Life Sciences) was dissolved in 0.9% saline containing 0.5% of dimethyl sulfoxide (DMSO; Sigma) at a final concentration of 1 mg/ml. Saline solution (0.9%) with 0.5% DMSO was used for vehicle injections. Both CNO and vehicle were prepared fresh for every injection day and injected (i.p.) 45 min before behavioral testing.

2.3 Surgery

After 7-8 weeks under CD or HFD, rats were anaesthetized under isoflurane (5% induction; 1–2 % maintenance), injected with the antalgic buprenorphine (Buprecare; 0.05 mg/kg, s.c.) and mounted on a stereotaxic apparatus (David Kopf Instruments). The scalp was shaved, cleaned and locally anaesthetized with and local application of xylocaine. The viral vector was infused using repeated pressure pulses delivered via a glass micropipette connected to a Picospritzer III (Parker, NH, USA). For the vHPC, 1 µl of the AAV was injected over 5 min (200 nl/min) at 2 sites in each hemisphere (i.e. 2 ul per hemisphere). The vHPC coordinates were AP -5.5 mm, ML ±5.5 mm from Bregma, DV -4 and -6 from the skull surface (Paxinos & Watson, 2007). For the BLA, 1 µl of the AAV was injected over 5 min at 1 site in each hemisphere: AP -3.0 mm, ML ±5.5 mm from Bregma, DV -8 mm from the skull surface. The pipette was left in place for 5 additional minutes before being slowly removed. Rats were housed in pairs immediately after surgery and were allowed at least 4 weeks to recover before the start of behavioral testing to allow ample time for virus expression.

2.4 Object recognition memory (ORM)

ORM is a classical procedure to assess non-spatial memory based on the recognition of a familiar object and rodent's natural tendency to explore novel, non-threatening, object. ORM requires a single trial and does not involve any aversive or food reward component (Ennaceur, 2010; Ennaceur & Delacour, 1988).

ORM task was performed in an arena sized 1.0 m x 1.0 m x 0.80 m (W x L X H), between 9:00 am and 1:00 pm. During the acquisition phase, rats were placed in the apparatus for 10 min and the time spent exploring two identical unfamiliar objects (either pairs of glass jars or milk cans; counterbalanced between groups) was recorded. Three or 24 h later, rats were placed back into the same apparatus containing a familiar and a novel object for 5 min at the same location than during the acquisition and the time spent exploring each object was recorded. The position of the familiar and the novel object (left or right) was counterbalanced between animals. Both objects and apparatus were cleaned with 70% of ethanol between each animal. Naïve rats usually prefer exploring the novel object, indicating memory for the familiar one, while a failure of recall is considered as a memory deficit (Cohen & Stackman, 2015; Ennaceur, 2010; Ennaceur & Delacour, 1988). Videos were recorded for each individual rat. Object exploration was analyzed offline in blind conditions using a video tracking software (Videotrack; Viewpoint, France). Object exploration was considered when the rat was at a distance of at least 1.0-1.5 cm and moved its whiskers towards the object. Exploration values were excluded if the animal was not exploring during either the training or the testing phase, and if one object was moved during the test. Exploration is represented as the absolute time exploring each object in seconds. ORM was expressed as the percentage of exploration of the novel object during the testing phase, calculated as following: time spent exploring the novel object / (time spent exploring the novel object + time spent exploring the familiar object) x 100. A value above 50% indicates a higher exploration of the new object over the familiar one. In chemogenetic experiments, rats received either vehicle or CNO (i.p.) 45 min before the acquisition session.

In some experiments, an initial context habituation phase was performed before the acquisition session in order to decrease arousal during training (Maroun & Akirav, 2008; Okuda et al., 2004; Roozendaal et al., 2006). Context habituation consisted of 3 x 5 min daily sessions during which rats were free to explore the arena without objects.

2.5 Conditioned odor aversion (COA)

COA results from the association of an odorized tasteless solution with a visceral malaise. In the present experiment, COA was evaluated using a previously described procedure (see Boitard et al., 2015). Rats were first acclimated to a water-deprivation regimen for 4 days. Access to water was provided in a graded bottle (with 0.5 ml accuracy) placed in the rats' home cage for 15 min each day between 9am and 11am. Baseline water consumption was obtained by averaging the intake of the last 3 days. On the fifth day, rats had access for 15 min to almond- (0.01% benzaldehyde; Sigma Aldrich) or banana-scented (0.01% isopentyl acetate; Sigma Aldrich) water, counterbalanced between rats. The percentage of odorized solution consumption with respect to water baseline was used as a measure of neophobia. Thirty minutes after, rats received an intraperitoneal injection of lithium chloride (LiCl; Sigma Aldrich; 25 mg/kg, 0.075M, 0.75 % of body weight). On days 6 and 7, rats had access to water for 15 min each day to re-establish baseline water intake. Finally, on day 8, long-term memory of the odor aversion was assessed by providing access to the almond- or banana-odorized water for 15 min, immediately followed by 15 min of water. The percentage of odorized water consumption with respect to the initial consumption of the same solution during conditioning was used as a measure of the strength of COA. In chemogenetic experiments, CD and HFD rats received either vehicle or CNO (i.p.) 45 min before the first presentation of scented water on day 5.

2.6 Histology

After the completion of behavioral testing, rats were deeply anaesthetized using a pentobarbital monosodic/lidocaine solution (20 mg/kg) before being transcardially perfused by ice-cold saline (0.9%) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were post-fixed overnight in 4% paraformaldehyde and then switched in 0.1 M phosphate buffer saline (PBS) solution and stocked at -4 ^{o}C before slicing. Serial coronal sections (40 µm) were cut using a vibratome (VT1200S, Leica Microsystems). Free-floating sections were prepared by rinsing in 0.1 M PBS for 20 min (4 x 5 min rinses), blocked for 1 h (PBS 0.1 M, 0.2% Triton-X, 4% normal goat serum) and placed in 1:1000 rabbit anti-RFP (red fluorescent protein; PM005, CliniSciences) at 4° C for 48 h. Sections were then washed in PBS for 20 min (4 x 5 min rinses), incubated in 1:200 AffiniPure rhodamine goat anti-rabbit (11-025-003; Jackson Immunoresearch) diluted in PBS for 2 h at room temperature and counterstained with 1:5000 Hoestch solution (bisBenzimide H 33258, Sigma-Aldrich). Sections were washed for 20 min in PBS (4 x 5 min rinses), mounted, and cover-slipped with Fluoromount-G (SouthernBiotech). Sections were imaged using a Nanozoomer slide scanner and analyzed with the NDP.view 2 freeware (Hamamatsu Photonics, Bordeaux Imaging Center).

2.7 Data analysis

The data never violated the homogeneity of variance measured with Levene's test or normality measured with the Shapiro–Wilk normality test. Weight was analyzed using two-way repeated measures ANOVA, followed by Bonferroni's *post hoc* tests. ORM and COA measures were analyzed using one- or two-way ANOVA followed by Bonferroni's *post hoc* tests (between groups comparisons) or Dunnet's *post hoc* tests (versus the CD-Vehicle group). ORM performance was also compared against 50% (no significant novel object exploration) using one-sample t-test. In case of missing values in the behavioral results from the ORM task (e.g. animal excluded for an absence of exploratory behavior), a Mixed-effect model was used instead for repeated measures analyses. Planned comparisons restricted to the HFD fed groups were done using multiple t-tests with Bonferroni's correction. Statistical analyses were carried out on GraphPad Prism version 7 and SPPS (IBM SPSS Statistics 25). All values were expressed as mean ± standard error of the mean (SEM). The alpha risk of rejection of the null hypothesis was 0.05.

3 RESULTS

3.1 HFD intake significantly induces overweight

Upon arrival, animals were randomly divided to create two groups of similar body weight, and then exposed to either CD or HFD. HFD rats were significantly heavier than CD animals from 6 weeks of diet exposure until the completion of the experiments (2-way repeated measures ANOVA: Diet $F_{1,78}$ = 23.9, $p < 0.001$; Week x Diet $F_{12,546}$ = 11.2, $p < 0.001$; Bonferroni's *post hoc* tests: all $p<0.001$ from week 6; **Supplemental Figure 1**) as previously reported (Boitard et al., 2012, 2014, 2015, 2016).

3.2 HFD intake induces long-term ORM deficits when training takes place in a novel context

Without habituation, CD (n = 11) and HFD-fed (n = 17) rats were placed into a novel arena containing two novel and identical objects (**Figure 1B**). During the acquisition phase, all groups similarly explored the two objects (**Supplemental Figure 2A**; Diet $F_{1,26}$ = 1.4, p =0.2; Diet x Retention Time $F_{1,18}$ = 5.9, p = 0.03). Three or 24 h after training, rats were exposed to a familiar and a novel object. When tested 3 h after the acquisition phase, CD- and HFD-fed rats exhibited a similar higher exploration of the novel object (**Figure 1B**; one-sample t-test versus 50%: CD t_9 = 2.7, p = 0.026; HFD t_{16} = 2.4, p = 0.03). However, when tested 24 h after the acquisition, only CD rats showed a significant preference

for the novel object (one-sample t-test versus 50%: CD $t_7 = 2.4$, $p = 0.047$; HFD $t_{13} = 1.2$, $p = 0.3$). Mixed-effect analysis confirmed a significant Diet x Retention time interaction ($F_{1,18} = 4.9$, $p = 0.04$; Diet $F_{1,26} = 3.9$, $p = 0.06$). More importantly, *post hoc* analyses confirmed a significant difference between the two diet groups when tested at 24 h ($p < 0.05$) but not at 3 h ($p > 0.9$; Bonferroni's *post hoc* tests), indicating that HFD consumption during adolescence impairs long-term, but not short-term, ORM when testing occurred without habituation to the arena.

We then evaluated whether previous habituation to the context would alleviate HFD-induced long-term ORM deficits. For this purpose, another batch of CD (n = 6) and HFD-fed (n = 7) rats was first tested in the ORM task at 24h after training without exposure to the training arena (context A). As previously observed, HFD rats tested without habituation showed a deficit in long-term ORM (one-sample t-test versus 50%: CD, $t_5 = 6.0$, $p = 0.001$; HFD, $t_6 = 1.6$, $p = 0.2$; **Figure 1C**). The same rats were then habituated during 3 days to a new training arena (context B) before being trained and tested for their ORM in this arena. Throughout context exposure, all rats maintained similar levels of locomotor activity (**Supplemental Figure 2B**; two-way repeated measures ANOVA: Diet $F_{1,11} = 0.3$, $p = 0.6$; Day $F_{3,33} = 0.8$, $p = 0.5$; Diet x Day $F_{3,33} = 0.7$, $p = 0.6$) and increased their exploration of the center of the arena (Entries in the center: Day $F_{3,33} = 3.2$, $p = 0.04$; Diet $F_{1,11} = 3.2$, $p = 0.1$; Diet x Day $F_{3,33} = 0.7$, $p = 0.6$; Time in the center: Day $F_{3,33} = 2.5$, $p = 0.07$; Diet $F_{1,11} = 0.4$, $p = 0.6$; Diet x Day $F_{3,33} = 0.3$, $p = 0.8$) indicating efficient habituation. Context habituation decreased the time spent exploring novel objects during training in all rats (**Supplemental Figure 2C**; two-way repeated measures ANOVA: Habituation $F_{1,11} = 5.6$, $p = 0.04$) independently of their diet (Diet $F_{1,11} = 0.5$, $p = 0.5$; Diet x Habituation $F_{1,11} = 1.1$, $p = 0.3$). As a result of habituation, both groups significantly preferred to explore the novel object than the familiar one during the test (one-sample t-test versus 50%: CD, $t_5 = 5.5$, $p = 0.003$; HFD, $t_5 = 4.1$, $p = 0.007$). Two-way repeated measures ANOVA on ORM test with and without habituation revealed that the time exploring the novel object was increased selectively in the HFD group after habituation (**Figure 1C**; Diet $F_{1,11} = 8.8$, $p = 0.01$; Diet x Habituation $F_{1,11} = 5.8$, $p = 0.04$). Indeed, HFD rats tested without habituation presented a lower exploration of the new object compared to the CD group ($p < 0.01$; Bonferroni's *post hoc* test), whereas, after habituation, HFD and CD animals similarly preferred to explore the novel object than the familiar one ($p = 0.6$; Bonferroni's *post hoc* test), demonstrating in this case an intact long-term ORM.

3.3 *Chemogenetic inactivation of the ventral hippocampus, but not the basolateral amygdala, rescues HFD-induced recognition memory impairment*

We then investigated the contribution of the vHPC and the BLA in periadolescent-HFD-induced memory alterations, using a chemogenetic approach involving the targeted expression of the inhibitory DREADD hM4Di (**Figure 2**). After histological analyses, HFD rats presenting bilateral hM4Di-mCherry expression in either the vHPC (n = 15) or in the BLA (n = 13) were kept for the statistical analyses. These rats were further divided depending if they received vehicle (vHPC, n = 8; BLA, n = 8) or CNO injection (HFD-vHPC-CNO, n = 7; HFD-BLA-CNO, n = 5). An additional group of HFD-fed rats did not receive any virus injection to control for the specificity of the CNO effects on behavioral measures (n=26). They were either injected with vehicle (n=11) or CNO (HFD-No DREADD-CNO, n = 15). The HFD-fed groups injected with vehicle (with DREADD, n = 16; without DREADD, n= 11) were pooled to form an HFD group receiving vehicle (HFD-Vehicle, n = 27). Finally, control CD (with or without DREADD) rats which received vehicle injections were pooled to provide a single CD-Vehicle group (n = 21). To summarize, the statistical analyses were performed on the following 5 final groups: CD-Vehicle, n = 21; HFD-Vehicle, n = 27; HFD-No DREADD-CNO, n = 15; HFD-vHPC-CNO, n = 7; HFD-BLA-CNO, n = 5.

Four weeks after surgery, long-term ORM was tested without habituation using the exact same procedure as previously described (**Figure 3A**). All rats received an injection of either CNO or its vehicle 45 min before the acquisition phase. CNO injection did not alter the exploration of the objects during this phase (**Supplemental Figure 3A;** $F_{4,70} = 2.2$, $p = 0.08$). HFD-Vehicle exhibited an absence of long-term ORM when tested 24 h after acquisition (one-sample t-test versus 50%: $t_{26} = 1.9$, $p = 0.07$), whereas CD-vehicle animals showed a higher exploration of the novel object (**Figure 3B**; one-sample t-test versus 50%; CD-Vehicle $t_{20} = 7.8$, $p < 0.001$) as all HFD groups receiving CNO (HFD-No DREADD-CNO $t_{14} = 4.6$, $p < 0.001$; HFD-vHPC-CNO $t_{6} = 6.7$, $p < 0.001$; HFD-BLA-CNO $t_{4} = 4.1$, $p = 0.02$).

Group comparisons confirmed that only the HFD-Vehicle group exhibited a lower ORM performance than CD control rats (one-way ANOVA: $F_{4,70} = 9.0$, $p < 0.001$; Dunnett's *post hoc* tests versus CD-Vehicle: HFD-Vehicle $p < 0.001$, all other groups $p > 0.3$). Further analyses restricted to the HFD groups showed that both HFD-No DREADD-CNO and HFD-vHPC-CNO groups exhibited a higher ORM performance than HFD-Vehicle rats ($p < 0.05$ and $p< 0.001$ respectively; t-test with Bonferroni's correction), suggesting a potential nonspecific CNO effect. However, the ORM performance of the HFD-No DREADD-CNO group remained lower than HFD-vHPC-CNO rats ($p = 0.05$; t-test with Bonferroni's correction) indicating that silencing vHPC improved HFD-induced ORM deficits.

3.4 Chemogenetic inactivation of the basolateral amygdala, but not the ventral hippocampus, prevents HFD-induced aversion memory enhancement

The results of the previous experiment raised an issue regarding the efficiency of our BLA silencing procedure. We therefore investigated in the same rats the impact of chemogenetic silencing of BLA and vHPC projecting neurons on the enhancement of aversion memory induced by periadolescent HFD exposure using a COA procedure (Boitard et al., 2015, 2016; **Figure 4A**; CD-Vehicle, n = 18; HFD-Vehicle, n = 23; HFD-No DREADD-CNO, n = 9; HFD-vHPC-CNO, n = 7; HFD-BLA, n = 5). Neither the diet nor the injection of CNO/vehicle affected the consumption of odorized water during its first presentation, i.e. odor-malaise association, compared to their respective water baseline consumption (**Figure 4B**; one-way ANOVA Group $F_{4,57} = 0.9$, $p = 0.5$; see also **Supplemental Figure 3B**), and all groups presented a low level of neophobia toward the new odorized water (all $p > 0.09$; one-sample t-test versus 100% water baseline). However, the long-term aversion memory was differently impacted by HFD and chemogenetic BLA or vHPC silencing during the odor-malaise pairing (**Figure 4C**; one-way ANOVA Group $F_{4,57} = 3.9$, $p = 0.007$; see also **Supplemental Figure 3C**). As previously observed (Boitard et al., 2015, 2016), there was a stronger aversion memory in HFD-Vehicle group as compared to the control CD group ($p = 0.05$, Dunnett's *post hoc* test). Further analysis restricted to the HFD groups showed that BLA silencing reduced the aversion memory as compared to HFD-Vehicle and HFD-No DREADD-CNO groups ($p = 0.004$ and $p = 0.03$, respectively; Bonferroni's corrected multiple t-tests), whereas vHPC silencing had no effect ($p > 0.9$). These results demonstrate that BLA activity controls HFD-induced aversive memory enhancement.

4 DISCUSSION

Here, we demonstrated that periadolescent HFD consumption (from weaning to adulthood) induced long term memory alterations. Specifically, we showed that HFD-fed rats presented a deficit in long-term, but not short-term, ORM when they are exposed to a novel context. Using chemogenetic silencing with high accuracy in anatomical boundaries, we found that manipulation of the vHPC, but not of the BLA, restored HFD-induced long-term ORM deficit. On the contrary, chemogenetic silencing of the BLA, but not of the vHPC, prevented the increased aversive memory observed in HFD-fed animals.

4.1 Effects of periadolescent high-fat diet on object recognition memory

The effects of HFD on object-related memory has led to contradictory results (for reviews see Abbott et al., 2019; Cordner & Tamashiro, 2015). The present study indicates that 12 weeks of exposure to HFD, starting at weaning, is sufficient to alter rats' object memory but only under certain conditions. Indeed, we observed that periadolescent HFD impaired long-term ORM tested 24h after sampling novel objects in a novel context but had no effect on short-term ORM tested 3 hours after training. These results are consistent with previous studies indicating no effect of HFD on short-term ORM (Beilharz et al., 2014, 2016; Kendig et al., 2019; Kosari et al., 2012; Lavin et al., 2011; McLean et al., 2018; Tran & Westbrook, 2015, 2017, 2018; Tucker et al., 2012), but an impairment of long-term object memory (Ayabe et al., 2018; de Andrade et al., 2017; Mucellini et al., 2019; Wang et al., 2016; Zuloaga et al., 2016). This differential impact of diet suggests a specific effect of periadolescent HFD on memory consolidation processes. Interestingly, we previously reported a similar effect on consolidation of spatial memories (Boitard et al., 2014, 2016) and emotional memories (Boitard et al., 2015), but also of object location memory (Khazen et al., 2019), which suggests that HFD consumption during early life periods could interfere with cellular substrates specifically involved in memory consolidation.

Importantly, we also identified that habituation to the arena prevented HFD-induced long-term ORM deficits. This could explain the absence of HFD-induced memory deficits reported in the literature after habituation and/or repeated tests using ORM or object location memory (Heyward et al., 2012, 2016; Tran & Westbrook, 2017; Tucker et al., 2012). Prior habituation and exploration of the arena is known to reduce the processing of contextual information during memory consolidation (Cohen & Stackman, 2015; Oliveira et al., 2010) and the arousal component of the task (Maroun & Akirav, 2008; Okuda et al., 2004; Roozendaal et al., 2006). Then, the specific diet-induced deficit of long-term ORM reported here may be supported by differential neurobiological substrates involved in multiple memory systems, particularly the hippocampus and the amygdala.

4.2 Effects of chemogenetic manipulation of vHPC and BLA on periadolescent HFD-induced object recognition memory deficits

The hippocampus and the amygdala play a crucial role in long-term ORM (Cohen & Stackman, 2015; Roozendaal et al., 2008) and are profoundly affected by exposure to HFD during the periadolescent period (Del Olmo & Ruiz-Gayo, 2018; Morin et al., 2017; Murray & Chen, 2019; Reichelt, 2016; Reichelt & Rank, 2017). We therefore wondered whether chemogenetic inhibition of projecting,

putative excitatory, neurons in these two brain regions could alleviate the diet effects on object memory.

Recent studies have suggested that CNO may be metabolized *in vivo* to clozapine, an atypical antipsychotic drug, able to interact with DREADD receptors but also to induce non-DREADD related effects (Gomez et al., 2017; Ilg et al., 2018; MacLaren et al., 2016). To rule out this possibility, we included a control group which received CNO at a dose known to induce marginal behavioral effect. Our results showed a slight improvement in long-term ORM in this group which suggests that metabolism of CNO to clozapine might induce some behavioral effects as shown following clozapine administration (Addy et al., 2005; Mutlu et al., 2011). This pattern of results in the control group cannot however account for the complete restoration of ORM deficits following chemogenetic inhibition of vHPC, hence highlighting the central role of this brain region in HFD-induced long-term ORM deficits.

Such results are in agreement with previous research which has demonstrated that long-term ORM, but not short term ORM, relies on the hippocampus (for review see Cohen & Stackman, 2015). Moreover, previous studies have shown that hippocampal manipulations have a greater impact on long-term ORM when performed in an unfamiliar context (Kim et al., 2014; Oliveira et al., 2010), whereas the perirhinal cortex is crucial in both familiar and unfamiliar contexts (Kim et al., 2014). These results suggest that, in a novel context, novel objects may be encoded as part of the context thereby involving the hippocampus, whereas if the novel objects are presented in a familiar environment they are encoded under a process that probably does not involve contextual information processing and therefore does not rely on the hippocampus. We could then hypothesize that HFD-fed animals did not exhibit a memory deficit when they were previously habituated to the arena, as in that case ORM performance is not dependent on a dysfunctional hippocampus.

Habituation to the training context also greatly influences the impact of emotional arousal and consequently the BLA involvement in long-term ORM (Maroun & Akirav, 2008; Roozendaal et al., 2006). However, chemogenetic BLA silencing did not have a greater effect than those of CNO alone on HFD-induced ORM deficit. The amygdala is one of the major target of vHPC projection neurons (Pitkänen et al., 2000), suggesting that the vHPC-to-BLA pathway is not involved in the beneficial effect of silencing vHPC projecting neurons on long-term ORM. The vHPC involvement in memory processes also involves other projections to the nucleus accumbens or the ventromedial prefrontal cortex (Barker et al., 2019; Hsu et al., 2018; Okuyama et al., 2016; Phillips et al., 2019) and future studies are warranted to determine the role of these circuits in HFD-induced memory deficits.

4.3 Effects of chemogenetic manipulation of BLA and vHPC on periadolescent HFD-induced aversive memory enhancement

Few studies have examined the effects of HFD on aversive memory. Aversive cue-based memory is highly dependent on the BLA (LeDoux, 2003; McGaugh, 2004; Paré, 2003). We previously found that periadolescent HFD enhanced long-term, but not short-term, odor aversion memory as well as long-term auditory fear memory (Boitard et al., 2015, 2016). Here we replicate this finding and we provide evidence that chemogenetic silencing of the BLA, but not the vHPC, prevented the increased odor aversion memory observed in the HFD group. It is noticeable that, contrary to the ORM, CNO injection alone (without any DREADDs) did not have any effect by itself on HFD-induced aversive memory enhancement.

It is generally considered that during emotional arousal the activity of the BLA is modulated by glucocorticoids and noradrenaline, and eventually impacts aversive memory via glutamatergic projections to other structures, including the hippocampus (McEwen et al., 2016; McGaugh, 2004). In this context, we previously found that blockade of glucocorticoid receptors in the BLA is able to normalise the enhanced aversive memory of HFD group (Boitard et al., 2015). Taken together, these data suggest that periadolescent HFD consumption increases the activation of BLA through glucocorticoids in response to emotional experience, leading to an enhanced odor aversion memory. Furthermore, a recent study showed that chemogenetic inactivation of the noradrenergic pathway from the locus coeruleus to the BLA abolished aversive memory enhancement, but not ORM impairment, induced by chronic pain (Llorca-Torralba et al., 2019). According to the differential effect of chemogenetic BLA silencing on aversive memory and ORM in HFD-fed rats, a similar impact of periadolescent HFD on the noradrenergic modulation of BLA may also be involved.

In contrast, chemogenetic inactivation of the vHPC did not modify odor aversion memory in HFD-fed animals. Even though the BLA is highly connected to the vHPC (Pitkänen et al., 2000) and that the BLA-to-vHPC pathway plays a central role in emotional processes (Beyeler et al., 2016; Felix-Ortiz et al., 2013; Rei et al., 2015), our results suggest that the HFD-induced enhancement of aversive memories may rather involve projections to the nucleus accumbens (Beyeler et al., 2016; Stuber et al., 2011) or to the ventromedial prefrontal cortex (Burgos-Robles et al., 2017; Felix-Ortiz et al., 2013).

Altogether our results demonstrate in periadolescent HFD-fed rats, that silencing vHPC, but not BLA, improves long-term ORM deficits, while silencing BLA, but not vHPC, prevents COA enhancement. This double dissociation suggests that vHPC and BLA, though related structures, can have distinct and independently-driven functions in HFD-induced memory changes.

4.4 Conclusions

The adolescent brain is highly sensitive and prone to cognitive alterations promoted by diets rich in fat and/or sugar (for reviews see Del Olmo & Ruiz-Gayo, 2018; Morin et al., 2017; Murray & Chen, 2019; Noble & Kanoski, 2016; Reichelt, 2016; Reichelt & Rank, 2017). Our study demonstrates that periadolescent HFD alters long-term memory processes, impairing recognition memory through vHPC-dependent processes while enhancing emotional memory through BLA specific effects. Such bidirectional effect on hippocampal and amygdala memory functions have also been reported in chronic stress and post-traumatic stress disorder (Elzinga & Bremner, 2002; Kaouane et al., 2012; Layton & Krikorian, 2002; Mahan & Ressler, 2012). Interestingly, obesity is linked to a higher prevalence of post-traumatic stress disorder, especially during adolescence (Pagoto et al., 2012; Perkonigg et al., 2009). Future investigation is necessary to evaluate how HFD consumption during adolescence impacts preferentially the medial temporal lobe, and how the potential alterations of specific hippocampal and amygdala circuits may mediate the cognitive impact of juvenile obesity.

5 AUTHORS CONTRIBUTION

C.B.B, G.P.L., E.C. and G.F. acquired funding; F.N., E.C. and G.F. designed research; F.N., I.B., M.S.Z. performed research; F.N., I.B., E.C. and G.F. analysed data; E.C. and G.F. supervised research; F.N., I.B., E.C. and G.F. wrote the manuscript; C.B.B, M.S.Z. and G.P.L. edited and approved the manuscript.

6 ACKNOWLEDGMENTS

The microscopy was completed at the Bordeaux Imaging Center, a service unit of CNRS-INSERM and Bordeaux University and member of the national infrastructure, France BioImaging. We thank María-José Olvera for technical assistance and Yoan Salafranque for the care provided to the animals during experiments.

7 FUNDING AND DISCLOSURE

OBETEEN consortium received financial support by the French National Research Agency (*Agence Nationale de la Recherche: ANR*) by the grant No. ANR-15-CE17-0013 and the Mexican National Council for Science and Technology (*Consejo Nacional de Ciencia y Tecnología: CONACYT*) by the

grant No. 273553. This work was also supported by INRA (to G.F.), CNRS (to E.C.), and French National Research Agency (ANR-14-CE13-0014 GOAL to E.C. and G.F.; ANR-16-CE37-0010 ORUPS to G.F.). F.N. was recipient of a postdoctoral fellowship from ANR OBETEEN (2015–2016), I.B. is the recipient of a PhD fellowship from the French Ministry of Research and Higher Education (2018-2021) and M.S.Z. is the recipient of a PhD fellowship from CONACYT (2017-2019). The authors declare no conflict of interest.

8 ANNEXE 1

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Note: Consortium author list recognizes the significant and irreducible commitment with the conceptualization, management, and realization of this international research project.

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CAPTIONS

Figure 1. Periadolescent HFD exposure altered long-term object recognition memory. (A) Schematic representation of the experimental design. Rats had *ad libitum* access to either CD (grey bars) or HFD (red bars) from weaning to adulthood. All behavioral testing occurred at adulthood after at least 12 weeks of diet. (B) Periadolescent HFD exposure alters long-term (24h testing), but not short-term (3h testing) object recognition memory (ORM). (C) Diet-induced ORM deficit at 24h is abolished when the animals were previously habituated to the arena. Data are represented as mean ± SEM and circles show individual data points. * $p < 0.05$, ** $p < 0.01$ (one sample t-test versus 50%), # $p < 0.05$, ## $p < 0.01$ (Diet effect, two-way ANOVA followed by Bonferroni's *post hoc* tests).

Figure 2. Chemogenetic targeting of the ventral hippocampus or the basolateral amygdala. *Left,* Representative images illustrating the placement of AAV8-CaMKII-hM4Di-mCherry expression in the ventral hippocampus (vHPC, A) and basolateral amygdala (BLA, B). Insets represent magnification of the area of interest (white square). *Right,* Schematics adapted from Paxinos and Watson (2013) showing the largest (light pink) and smallest (dark pink) viral infection for rats included in behavioral experiments.

Figure 3. Chemogenetic inhibition of the ventral hippocampus, but not the basolateral amygdala, restored long-term ORM induced by periadolescent HFD exposure. (A) Schematic representation of the experimental design. Rats had *ad libitum* access to either CD or HFD from weaning to adulthood. DREADD surgery was performed at adulthood (7-8 weeks of diet) and rats recovered 4 weeks before the start of behavioral testing. (B) Long-term ORM performance in HFD-fed rats treated with vehicle (red bars; ± indicating with or without DREADD expression), CNO but without DREADD expression (orange bars), or CNO with DREADD expressed in the vHPC (dark blue) or the BLA (light blue). Expression of the inhibitory DREADD hM4Di is depicted by structure (vHPC or BLA), except for the vehicle group. Data are represented as mean ± SEM and circles show individual data points. ### $p < 0.001$ (one-way ANOVA followed by Dunnett's *post hoc* test vs. CD-Vehicle), * $p \leq 0.05$, *** $p < 0.001$ (planned t-tests with Bonferroni's correction).

Figure 4. Chemogenetic inhibition of the basolateral amygdala, but not the ventral hippocampus, prevented enhanced aversion memory induced by periadolescent HFD exposure. (A) Schematic representation of conditioned odor aversion (COA) protocol and chemogenetic inhibition of vHPC or BLA before COA conditioning. (B) Neither HFD or CNO injection impacted the consumption of odorized water during the conditioning phase (in percentage of water baseline consumption). (C) Long-term COA memory in HFD-fed rats treated with vehicle (red bars; ± indicating with or without DREADD expression), CNO but without DREADD expression (orange bars), or CNO with DREADD expressed in the vHPC (dark blue) or the BLA (light blue). Expression of the inhibitory DREADD hM4Di is depicted by structure (vHPC or BLA), except for the vehicle group. Data are represented as mean ± SEM and circles show individual data points. # $p < 0.05$ (one-way ANOVA followed by Dunnett's *post hoc* test vs. CD-Vehicle), * $p < 0.05$, ** $p < 0.01$ (planned t-tests with Bonferroni's correction).

Supplemental Figure 1. Weight curve of rats exposed to standard or high-fat diet since weaning. Effects of control (CD; n = 38) and periadolescent high-fat diet (HFD, n = 41) on rats' body weight (g). Diet started at weaning (3-weeks old, Week 0) and lasted at least 12 weeks before the start of behavioral testing. *** $p < 0.001$ Diet effect (two-way repeated measures ANOVA).

Supplemental Figure 2. Periadolescent HFD did neither alter object exploration during ORM training nor context habituation. (A) Total object exploration during the acquisition phase of the ORM task for CD and HFD groups being tested at 3 or 24 h without context habituation. (B) Locomotor activity (distance traveled) and exploration of the center of the arena (total entries and time spent) during context habituation sessions. Pictures show representative animal track in the open-field chamber for a CD and a HFD rat. (C) Total object exploration during the acquisition phase of the ORM task for CD and HFD groups being tested without or with context habituation. Data are represented as mean ± SEM and circles show individual data points.

Supplemental Figure 3. Effects of chemogenetic inhibition of vHPC or BLA on object exploration during ORM training and intake during COA acquisition and test.

(A) Total object exploration during the acquisition phase of the ORM task for CD and HFD groups receiving CNO or Vehicle prior to the session. (B) Odorized water consumption during COA acquisition for CD and HFD groups (one-way ANOVA: $F_{4,57} = 3.8$, $p = 0.02$; Dunnett's *post hoc* tests: CD-Veh vs HFD-Veh and HFD-CNO groups, $p < 0.05$). (C) Odorized water consumption during COA testing for CD and HFD groups (one-way ANOVA: $F_{4,57} = 7.1$, $p = 0.0001$; Dunnett's *post hoc* tests: CD-Veh and HFD-vHPC groups vs HFD-Veh, HFD-CNO and HFD-BLA groups, $p < 0.05$). Data are represented as mean ± SEM and circles show individual data points.

Figure

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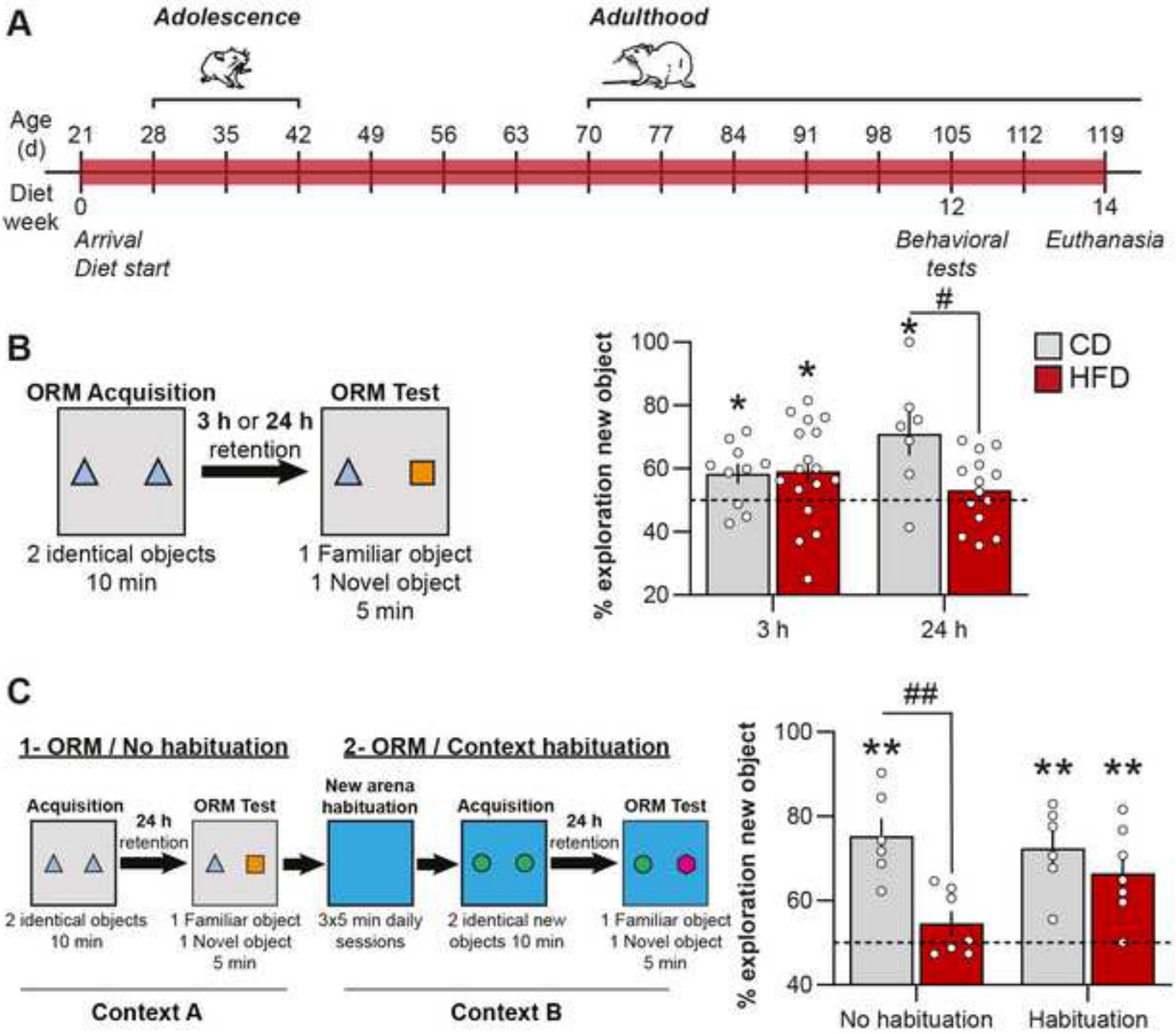

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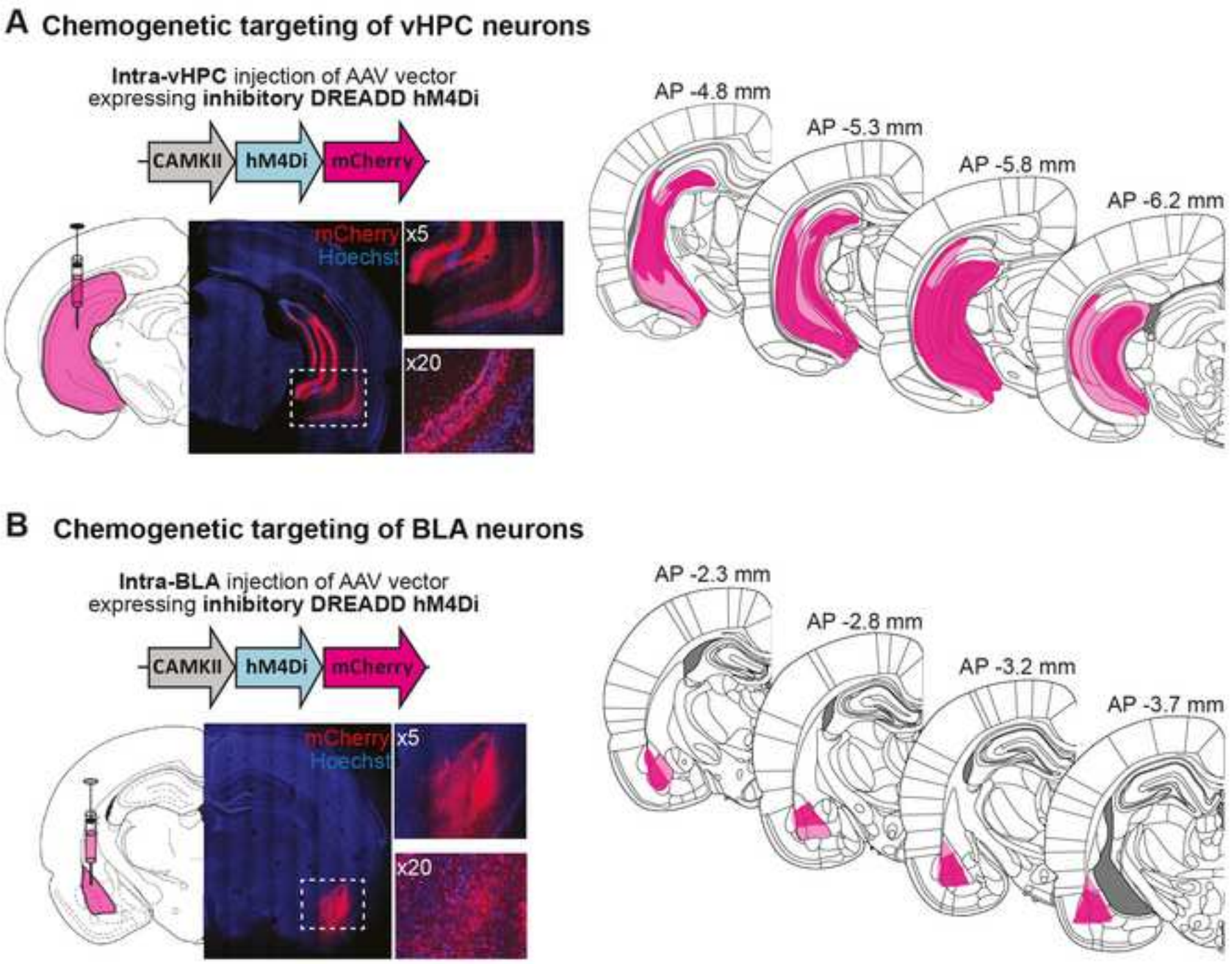

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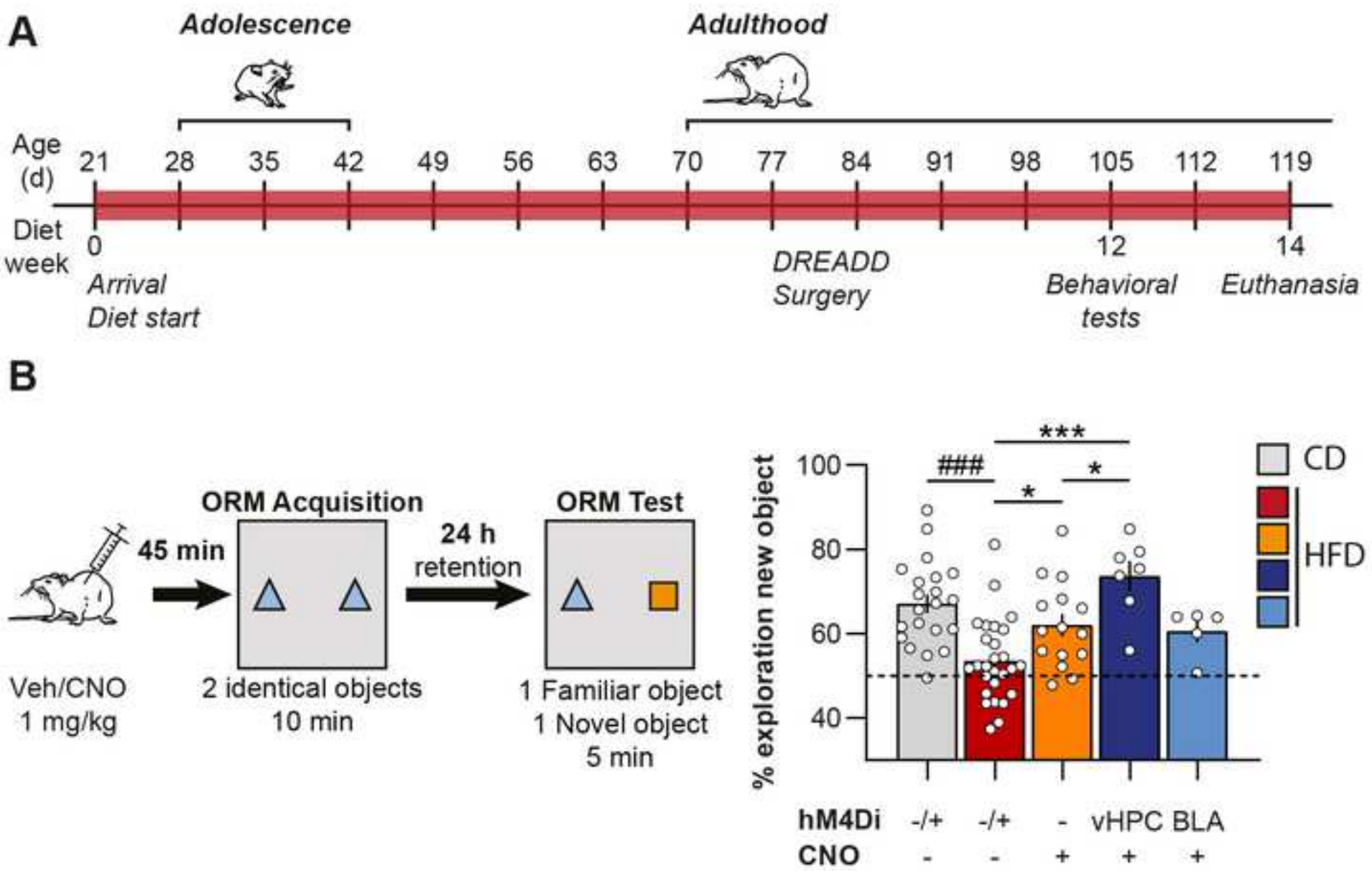

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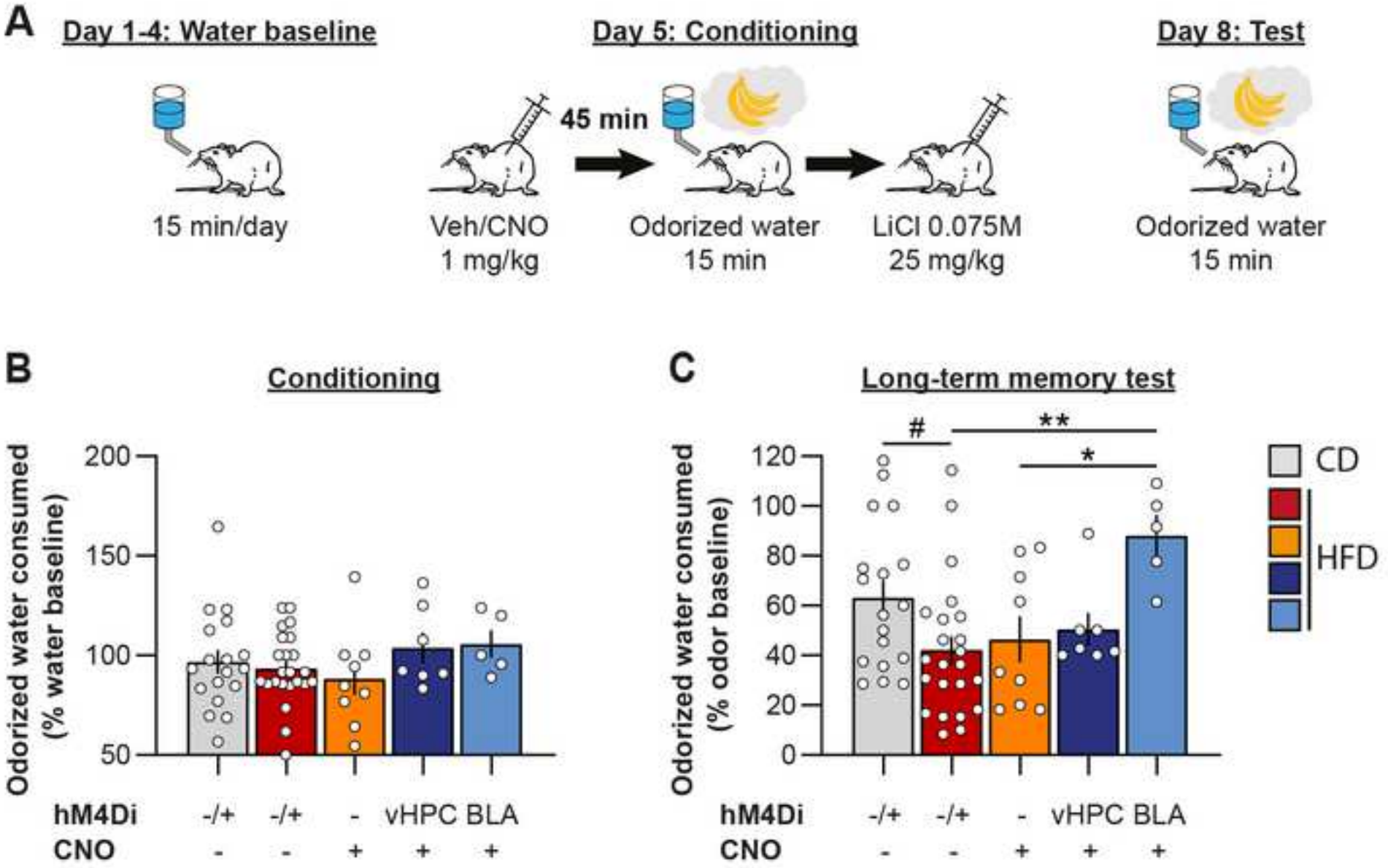

***Credit Author Statement**

AUTHORS INDIVIDUAL CONTRIBUTION

Guillaume Ferreira, Etienne Coutureau, Clémentine Bosch-Bouju and Gustavo Pacheco-Lopez acquired funding;

Fabien Naneix, Etienne Coutureau and Guillaume Ferreira designed research;

Fabien Naneix, Ioannis Bakoyiannis and Marianela Santoyo-Zedillo performed research;

Fabien Naneix, Ioannis Bakoyiannis, Etienne Coutureau and Guillaume Ferreira analyzed data;

Etienne Coutureau and Guillaume Ferreira supervised research;

Fabien Naneix, Ioannis Bakoyiannis, Etienne Coutureau and Guillaume Ferreira wrote the manuscript;

Clémentine Bosch-Bouju, Marianela Santoyo-Zedillo and Gustavo Pacheco-Lopez edited and approved the manuscript.