Remote memory of drug experiences coexists with cognitive decline and abnormal adult neurogenesis in an animal model of cocaine-altered cognition

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Abstract

Cocaine addiction is a chronic disorder in which the person loses control over drug use. The past memories of the stimuli associated with the drug are a relevant clinical problem, since they trigger compulsive drug seeking and taking habits. Furthermore, these persistent drug-related memories seemingly coexist with cognitive decline that predicts worse therapeutic output. Here we use a new animal model of cocaine-altered cognition that allowed to observe these events in the same individual and study their relationship. Mice were chronically administered cocaine in a conditioned place preference (CPP) apparatus for 14 days, and control mice received saline. After 28 days of cocaine withdrawal, animals were tested for retrieval of remote drug-associated memory as well as for cognitive performance in a battery of tests, including novel object and place recognition and spatial memory. The cocaine-withdrawn mice showed persistent CPP memory while impaired in the cognitive tasks, displaying deficits in reference memory acquisition and working memory. However, the CPP expression was not associated to the defective cognitive performance, indicating that they were concomitant but independent occurrences. After completion of the experiment, adult hippocampal neurogenesis (AHN) was studied as a relevant neurobiological correlate due to its potential role in both learning and drug addiction. Results suggested a preserved basal AHN in the cocaine-withdrawn mice, but an aberrant learning-induced regulation of these neurons. This paradigm may be useful to investigate maladaptive cognition in drug addiction as well as related therapies.

Keywords: Cocaine addiction, adult hippocampal neurogenesis, cognitive impairment, conditioned place preference, object and place recognition memory, morris water maze

Introduction

Cocaine is currently one of the most widely extended illicit drugs, with an estimate of 18 million of users worldwide[1]. One of the most significant socioeconomic and health burdens induced by cocaine, is the development of a 'drug addiction' disorder that is suffered by almost a 13 per cent of individuals using drugs[1]. Cocaine addiction entails a harmful and uncontrollable pattern of drug use, involving ingrained drug seeking and taking habits that are activated even despite the undesired life consequences and against the person's will[2].

The stimuli associated with the drug, are well-known triggers of compulsive drug use habits[2,3]. By means of Pavlovian associative learning processes, the drug's rewarding effects become paired with concomitant neutral stimuli (e.g. environmental cues in the place of consumption) that acquire a conditioned value; so they automatically evoke the drug-related experience eliciting drug *craving* and relapse in drug use[2,3]. In acute drug exposures, cocaine may even hijack neurobiological mechanisms –neurotransmitters, neurotrophins, hormones,…- in key learning-related brain regions, acting as a cognitive enhancer that potentiates memory formation[4] including the episodic and associative drug-related memories[5]. Consequently, memories for drug-associated stimuli are harmful and long-lasting, and new cognitive therapies aimed to their manipulation (through extinction, forgetting or re-consolidation) are being used in the therapeutic of drug addiction with some preliminary evidence of success[3]. Interestingly, it has been drawn to attention that the strong memories for past cocaine-related stimuli and experiences coexist with drug-induced cognitive decline in chronic cocaine users that may perform under healthy controls in a wide variety of domains -including attention, visuospatial functions, executive processes, working memory and declarative memory-[6,7]. The cognitive status seems a consistent predictor of early treatment dropout and

relapse in cocaine use, thus having implications for the prognosis of the addiction disorder[6,8].

Maladaptive cocaine memories and cognitive decline may converge in common neural substrates as it is the case of the hippocampus[4], a main learning-related brain region. For example, the hippocampal functional activity and connectivity is enhanced in cocaine users that experience cocaine-associated cues, probably to activate memories related to drug use[9] and drug *craving* feelings[10]. In rodents, the drug-induced conditioned place preference (CPP) is one of the main paradigms used to study the acquisition, long-term maintenance and updating of contextual memories associated with drugs[11]. In the CPP, cocaine is administered to the animal in one specific maze compartment decorated with distinctive contextual cues. After several drug-context pairings, the animal is allowed free exploration of the apparatus and it shows a preference to stay in the drug-paired compartment over a neutral one[12]. The expression of such CPP behavior has been interpreted as a rewarding outcome of the substance and also as evidence of associative memory for the drug-related experience (i.e. the animal perceived cocaine as a rewarding stimulus *and* it also learned and remembered which maze contextual cues were associated with the cocaine reward)[11]. Either the lesion or the functional inhibition of the hippocampus –mainly in its dorsal subdivision- prevents the acquisition[13] as well as the long-term retrieval of both recent (i.e. acquired 2 days prior[14]) and remote (i.e. acquired 28 days prior[15]) cocaine-CPP memory. Interestingly, the extent to which CPP relies on the hippocampus may depend on the degree of contextual elements required to remember the reward-paired location[16], suggesting that it is not necessarily a 'cocaine memory' per se that depends upon the hippocampus but rather a memory of the context in which it was received.

Moreover, cognitively-impaired chronic cocaine users may display deficits in hippocampal declarative memory such as in acquiring and recalling new information[6,7,17].

Basic preclinical research has been traditionally more focused on the neurocognitive effects of cocaine on frontal functions[18,19]; but rodents withdrawn from chronic cocaine are also impaired when they are tested on memory paradigms typically linked to the hippocampus –most frequently object and place recognition memory[20-25] but also spatial navigation[26], contextual memory or spatial working memory[21]- . These cocaine-induced hippocampal memory defcits persist even after extended drug withdrawal periods –e.g. 62[21] or 90 days[26]- and are concomitant to both functional and anatomical hippocampal neuroadaptations[4,21]. However, most studies researching hippocampal-dependent memory impairments administered cocaine in the rodents' home cage, and drug-related responses were not researched.

In the present experiment, we extended the number of cocaine-CPP conditioning sessions -typically four according to standard methods[12,27]- to a chronic drug schedule of 14 consecutive cocaine conditioning days -a dosage that would be expected to elicit lasting neurocognitive impairment[21]-. This new protocol will allow to study whether persistent cocaine-associated memories assessed in the CPP paradigm could be remembered by rodents that are also impaired to acquire new hippocampal learnings (**Fig.1a**); and how these cognitive events relate one to the other. Considering the relevance of the hippocampus for the cocaine-induced maladaptive cognition, we will examine adult hippocampal neurogenesis (AHN) as a relevant neurobiological correlate in this model. The adult-born hippocampal neurons have been long involved in the hippocampal cognitive function. While the relationships between neurogenesis and cognition are complex, an increased AHN generally correlates with potentiated learning and memory, and a reduction of these neurons leads to cognitive impairment[28]. Specifically, hippocampal-dependent object and place recognition, spatial navigation and contextual memory tasks have been found impaired in rodents after AHN inhibition[28]. More recently, AHN has been attributed a potential role in cocaine addiction since AHN-reduced rodents engage in more cocaine self-administration[29,30]. Furthermore, AHN

seems involved in cocaine-associated contextual memories as evaluated in the CPP paradigm. Our research group has revealed that a pharmacological reduction of AHN - either before or after cocaine-CPP acquisition- leads to a more persistent CPP behavior[31], but pharmacologically increasing AHN debilitates a previously acquired CPP response[32]. Nevertheless, there is controversy on whether cocaine itself would persistently reduce AHN, which could contribute to the cognitive symptoms during withdrawal[4]. Researching AHN in animal models of cocaine addiction is meaningful, since the recent confirmation of the existence of AHN in the human adult brain, even in the elderly, have highlighted the potential clinical relevance of AHN for disease vulnerability and treatment[33].

Materials and Methods

Ethical guidelines

Procedures followed the European (Directive 2010/63/UE) and Spanish regulations (Real Decreto 53/20130 and Ley 32/2007) for animal research. The experimental protocols were approved by the research ethics committee of the University of Málaga (code: CEUMA 81-2016-A) and Junta de Andalucía (code: 30/03/2017/055).

Animals

Twenty young-adult male C57BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) were used in this study. After acclimation to the animal facility, they were individually housed in standard conditions (temperature: 22°C ± 2°C; 12-h light/dark cycle; lights on at 8:00 AM) with a cotton-like Nestlet® for nesting material (Ancare, New York, USA)

and *ad libitum* access to water and food. Mice started the experiments at 12 weeks of age.

Behavioral testing

CPP acquisition and long-term maintenance

Half of the mice were conditioned to cocaine in the CPP paradigm (COC, n = 10), while the rest were randomly assigned to the 'vehicle' condition (VEH, n = 10). The cocaine-CPP procedure was carried out as in our previously published methods[31,32] (**Supplementary Information**). Exceptionally, the number of cocaine conditioning days was extended for this experiment, so the COC mice received a daily dose of 20 mg/kg of cocaine over 14 days (Days 4-18) confined in the cocaine-paired compartment (**Fig. 1b**; **Supplementary Information**). The COC mice also received a daily saline injection and were confined in the saline-paired compartment for a similar amount of time. The cocaine- and saline-paired sessions were separated by at least 4 h and their daily order ('morning' or 'afternoon') was alternated on consecutive days. The VEH mice were equally exposed to the CPP apparatus but they received saline administrations instead of cocaine (**Supplementary Information**). The initial acquisition of the CPP response was tested 72 h after the last cocaine conditioning session (Test 1 on Day 21); while its long-term maintenance was tested after a retention period of 28 days (Test 2 on Day 46) (**Fig.1b,c**). To measure preference for the cocaine-paired compartment, a 'CPP Score' was calculated: [(seconds spent in the cocaine-paired compartment - seconds spent in the saline-paired compartment)/seconds spent in both compartments] × 100. According to this equation, a preference for the cocaine-paired compartment was indicated by a positive CPP Score significantly greater than zero[4]. The '∇CPP Score' was calculated as the 'CPP Score on Test 1 – CPP Score on Test 2' to measure the magnitude of time-induced decay of the CPP behavior. Importantly, the long-term CPP retention test (Test

2) was carried out before cognitive assessment, in order to avoid any potential influence of the extended behavioral training on the expression of the CPP response.

Behavioral and cognitive assessment

After being evaluated in the second CPP test session, mice were assessed in a battery of behavioral tests for exploratory activity, emotional behavior, and cognitive performance (Days 47-67; **Fig.1b**). Performing this behavioral testing protocol increases AHN in mice, compared with undisturbed control animals (**Supplementary Figure 1**).

The behavioral paradigms included were the elevated plus maze and the open field to assess anxiety like-behavior and locomotor activity (Days 47 and 48), the novel object (Day 49) and the novel place recognition memory (Day 50), the forced swimming test for despair-like behavior (Day 52) and the water maze (Days 53-65) (**Fig.1b; Fig. 2**). Specifically, the water maze included different tasks: habituation to the maze (2 days), visible platform training (2 days), spatial reference memory training with a hidden platform (4 days), probe trials for long-term memory retention at 24 h and 72 h intervals, platform inversion for cognitive flexibility (1 day) and delayed matching-to-place spatial working memory training (4 days) (**Fig.1b; Fig. 3**). Additionally, nest building was scored on Days 20 and 41 (**Fig.1b; Fig. 2a**). All behavioral protocols were performed as detailed in the **Supplementary Information** and in previous publications[21].

Bromodeoxyuridine administration

Bromodeoxyuridine (BrdU, Sigma-Aldrich, Madrid, Spain) was administered after cocaine-CPP conditioning on Days 25-27 (**Fig.1b**) to label the cells that were newly-generated after one week of cocaine withdrawal. Mice received two daily 75 mg/kg intraperitoneal BrdU administrations, separated by 4 h[32].

Assessment of adult hippocampal neurogenesis

Our histological procedures are extensively described elsewhere[31,32] and in the **Supplementary Information**. On day 68 (**Fig.1b**), mice were sacrificed and AHN was quantified in the dorsal hippocampus. The endogenous marker proliferating cell nuclear antigen (PCNA) was used for detection of cells undergoing proliferation; while doublecortin (DCX) labeled young neurons aged up to ~3 weeks old[34]. For the DCX+ neurons, we distinguished two categories according to their morphological features: Type 1: with absent or short dendritic processes (i.e. immature-like morphology); and Type 2: with at least one prominent apical dendrite penetrating the granule cell layer[35] (**Fig.5f**). BrdU was used to detect the labeled cells aged 41-43 days old that survived until the ending of the experiment (**Fig.5a**). To confirm their neuronal phenotype, co-localization of BrdU with the mature neuron marker *neuronal nuclei* (NeuN; that is expressed by neurons from their ~third week of age onwards[34]) was analyzed by immunofluorescence and confocal microscopy (**Supplementary Information**). Cell counting was carried out independently in the suprapyramidal and in the infrapyramidal blades of the DG (SupraDG and InfraDG), considering both the functional and structural asymmetry of AHN in these regions[36].

Statistical analysis

Groups were compared by Student's *t* tests or by analysis of variance (ANOVA with repeated measures) followed by *post-hoc* Fisher's LSD analysis, when appropriate. One-sample *t* tests were used to compare means to a single measure. Correlations were Pearson's. Significance was considered at $p \leq 0.05$. Only significant comparisons are reported.

In addition, we conducted a Principal Component factorial Analysis (PCA) on the cognitive-related measures analyzed in the various tasks, in order to reduce this data to a set of behavioral dimensions (i.e. factors) that underlie cognitive performance (**Supplementary Information**). Data from the whole sample of mice (both VEH and COC, n = 20) was included in the PCA in order to meet sample adequacy criteria (Kaiser-Meyer-Olkin index = 0.528; Bartlett's test for sphericity: $X2 = 72.393$, df = 36, $p < 0.000$) and to calculate a 'factor score' for each animal, that would serve to rank its cognitive abilities in each of the extracted factors. After calculation of the factor scores, they were separated by treatment group in order to perform mean comparisons and relevant correlations.

Results

Chronic cocaine administration induced persistent conditioned memory

Chronic exposure to cocaine in the CPP apparatus induced a significant preference for the cocaine-paired compartment [repeated measures ANOVA on the pre-test session and the two test sessions (treatment x session): 'treatment': $F_{1,18} = 13.405$, $p = 0.002$; 'session': $F_{2,36} = 12.454$, $p < 0.000$; 'treatment x session': $F_{2,36} = 4.795$, $p = 0.014$]. Post-hoc analyses revealed that the COC and the VEH mice were identical during the pre-test session, but they clearly differed in both the recent (Test 1; 72 h retention) and the remote (Test 2; 28 days retention) test sessions where the COC mice showed significant conditioned memory (**Fig. 1c**). Interestingly, the CPP behavior in the COC mice resulted persistent in this experiment, not showing any evident time-induced decay. In this way, the CPP Score in both test sessions (i.e. Test 1 and Test 2; **Fig. 1c**) resulted similar by post-hoc analyses, and the ∇CPP Score (mean = 5.36; SEM = 3.27); was not statistically different from zero. Both treatment groups showed a similar locomotion during the pre-

test and the drug-free test sessions, so no alterations in exploratory activity could affect the expression of the conditioned response (**Fig. 1d**).

Mice conditioned to cocaine were impaired in the acquisition of new learnings during long-term cocaine withdrawal

Motivational, exploratory and emotional behavior

The quality of the nest build was assessed by a 'Nest Score' (as described in the **Supplementary Information**) either at 2 or 23 days of cocaine withdrawal. The COC mice showed deficits when tested 1 h after providing the nesting material, but not after 4 h [repeated measures ANOVA (treatment x hour x day): 'treatment': $F_{1,36}$ = 7.840, p = 0.008; 'hour': $F_{2,72}$ = 161.260, $p < 0.000$; 'day': $F_{1,36}$ = 5.268, p = 0.028; 'treatment x hour': $F_{2,72}$ = 4.986, p = 0.009; post-hoc comparison is shown in **Fig. 2a**]. This suggests that the COC mice were initially less inclined (i.e. 'slower') to perform an intrinsically motivated behavior such as nest building but they were able build a near-perfect nest when they were given enough time (4 h). Therefore, they showed only a mild impairment in this task.

Nonetheless, the COC mice behaved similar to controls regarding the exploratory and anxiety-like behaviors assessed in the elevated plus maze and in the open field tests (**Fig. 2b, d**). Cocaine withdrawal neither affected despair-like responses in the forced swimming test (**Fig. 2c**).

Cognitive assessment

Novel object and place recognition

Regarding the learning-related measures, the cocaine-withdrawn mice showed reduced discrimination of a novel object (t_{18} = -2.184; p = 0.042) and a novel place (t_{18} = -2.117; p = 0.048) from a familiar one at a 24 h retention interval (**Fig. 2e**). Importantly, their total time of object exploration both in the sample and in the test sessions was unaltered compared to the VEH mice [repeated measures ANOVA (treatment x session): 'session': $F_{2,36}$ = 7.620, p = 0.002; **Fig. 2e**], so the memory outcome could not be attributed to changes in the motivation to explore.

Spatial memory in the water maze

The COC and the VEH mice resulted similar in the two habituation sessions in the pool regarding the total distance swan and the exploration of the peripheral region, as well as in the time spent in any of the four imaginary quadrants (**Fig. 3a**).

Subsequently, the COC mice were slower in learning a visible platform task [repeated measures ANOVA (treatment x session): 'treatment': $F_{1,18}$ = 5.392, p = 0.032; session: $F_{7,126}$ = 26.121, p < 0.000; **Fig. 3b**]; and they were also delayed in acquiring a spatial reference memory task with a hidden platform ['treatment': $F_{1,18}$ = 5.144, p = 0.036; 'session': $F_{23,414}$ = 5.900, p < 0.000; **Fig. 3c**]. Nevertheless, if the fourth training day was analyzed separately (**Fig. 3c**), there was no longer a difference between the treatments, suggesting that the COC mice had successfully learned the hidden platform location by the end of the training albeit they slower acquisition.

After spatial memory training, the COC mice showed no alterations in the long-term retention of the spatial memory. In this way, in the first probe trial (24 h retention), both the COC and the VEH mice preferred the target pool quadrant over the opposite one [repeated measures ANOVA (treatment x quadrant): 'quadrant': $F_{1,18}$ = 15.866, p < 0.000; post-hoc analysis is shown in **Fig. 3d**] and they did not differ in parameters of platform

crossings or latency (**Fig. 3d**). The target quadrant was no longer preferred by any group of mice in the second probe trial at 72 h retention ('quadrant' effect: $F_{1,18}$ = 0.032, p = 0.859, non significant); suggesting either inability to recall the platform location at this longer retention interval or a rapid extinction of this memory due to the previous probe trial performed with no platform.

On the platform inversion day, mice learned the new platform location as evidenced by their escape latency across the six daily sessions and by a short-term retention probe trial, in which the new target quadrant was equally preferred by both experimental treatments ('quadrant': $F_{1,18}$ = 14.824, p = 0.001; **Fig. 3e,f**). While the probe trial showed a tendency of the COC mice to be impaired in the latency to find the inverted platform position and in the number of platform crossings, this did not result significant (**Fig. 3f**).

Nevertheless, the cocaine-withdrawn mice were severely impaired in a delayed matching-to-sample working memory task performed over four consecutive days [repeated measures ANOVA on the test sessions (treatment x day): 'treatment': $F_{1,18}$ = 19.583, p < 0.000; 'day': $F_{3,54}$ = 12.511, p < 0.000; **Fig. 3g**].

Importantly, both treatment groups showed a similar swimming velocity (i.e. cm swam per second) during the whole water maze training (**Supplementary Figure 2**).This indicates that the exploratory activity or the motor function did not affect the latency measures. In addition, the fact that the COC mice searched the platform similarly than controls in the probe trials, confirms their motivation to perform the task and to escape from the water.

Factors underlying cognitive behavior and their relationship with CPP maintenance

The PCA reduced the variables to four independent factors that underlay cognitive functioning (**Fig. 4a**). The main factor in terms of variance explained (Factor 1) mostly included measures related to the performance in the visible and hidden platform tasks, behavioral flexibility (inversion day) and spatial working memory. Therefore, this factor was considered to represent short-term memory acquisition and its online manipulation or updating (**Fig. 4a**). Measures of recognition memory for a novel object and place mainly loaded in Factor 2. These functions are different from the spatial abilities tested the water maze and they may entail episodic-like memory components such as 'what' and 'where'. We must note that, because these object memories were only tested at a 24 h retention interval, it is not possible to distinguish an acquisition deficit from one in consolidation or retrieval. Other studies, have reported impaired object recognition memory acquisition by cocaine tested at short retention intervals of few minutes[22,23]. Finally, long-term spatial memory retention at 72 h or 24 h loaded in Factor 3 and Factor 4 respectively (**Fig. 4a**).

A 'factor score' was calculated per animal as a measure of its performance in each of the four cognitive factors. The COC mice showed impairment in the memory functions represented by Factor 1 and Factor 2 (Student's t test: t_{18} = -3.508, p = 0.003; t_{18} = 2.117, p = 0.048; **Fig. 4b**) compared with the VEH animals. However there was no impairment in the factors representing long-term spatial memory retention; which shows a normal consolidation and retrieval of spatial memory in the cocaine-withdrawn mice in despite of delayed acquisition. Correlational analysis in the cocaine-treated mice revealed that their cognitive factor scores were mostly unrelated to the long-term CPP expression (**Fig. 4c**).

Cocaine-withdrawn mice showed abnormal AHN regulation associated to learning

Three days after completion of the behavioral assessment, AHN-related markers were analyzed in the DG (**Fig. 1b; Fig. 5a**). Interestingly, the cocaine treatment did not affect the number of BrdU+ cells -that were generated at one week of cocaine withdrawal and survived until the end of the experiment- (**Fig. 5a,b**) nor their differentiation into mature neurons (**Fig. 5b,c,d**). Furthermore, the COC and the VEH mice displayed a similar proliferative activity in the DG at the conclusion of the experiment, evaluated by the PCNA+ cells (**Fig. 5e**).

Nevertheless, the COC mice showed an abnormally increased number of DCX+ neurons in the SupraDG [repeated measures ANOVA (treatment x DG blade): 'DG blade': $F_{1,18}$ = 4.681, p = 0.044; 'treatment x DG blade': $F_{1,18}$ = 7.361, p = 0.013; post-hoc comparison is shown in **Fig. 5f**] though no difference in the morphological features of these neurons was found. Considering the temporal pattern of DCX expression[34], these young neurons were presumably born at a time period overlapping with the cognitive training (**Fig. 5a**); suggesting that learning induced a transitory increase in the proliferation and/or survival of this specific neuron population in the cocaine-withdrawn mice compared with the control animals. In fact, the density of DCX+ cells in the COC mice was directly correlated with their cognitive performance, specifically with the functions represented by Factor 1 (**Fig. 5g**). None of the AHN-related markers showed a significant correlation with the measures of cocaine-CPP acquisition or long-term maintenance.

Discussion

In the present manuscript, administration of chronic cocaine paired with specific contextual cues (i.e. in the CPP paradigm) revealed that extended cocaine exposure elicited robust CPP behavior, indicating both persistent cocaine reward and drug-associated contextual memory. This remote drug memory coexists with anterograde

reference and working memory deficits in this animal model albeit measures of cognitive performance were mainly unrelated to the CPP response, supporting these phenomena as coincident but independent events.

To the best of our knowledge, this topic has been scarcely addressed in previous literature. A recent work, reported apparently opposite results since an exacerbated cocaine-CPP response was associated with subsequent spatial working memory performance in a radial arm maze, predicting less improvement in the task[37]. But a main difference with the present study is that they focused on the initial cocaine-CPP acquisition rather than on its long-term maintenance, which was not evaluated[37]. In line with our results, animals trained to self-administer cocaine showed memory for remote cocaine-associated contextual cues learned 21-45 days prior; but their working memory or reversal learning performance could not predict the strength of the cue-induced relapse in self-administration[38,39]. It should be noted that the magnitude of the drug 'seeking' or taking responses in the cocaine-CPP or in the self-administration paradigms may be strongly influenced by non-cognitive factors such as stress or drug *craving*[40], which could contribute to explain this dissociation. Nevertheless, despite the present results, it is still possible that testing animals bearing an intrinsic or experimentally-induced vulnerability for the drug (e.g. submitted to a stressful event) may yield particular conditions where both the cognitive performance and the drug seeking/taking responses are modulated at once, and thus result interrelated. Such outcome would agree with clinical evidence suggesting that poorer cognitive performance is a predictor of early relapse in cocaine use[8].

Another relevant aspect is that cognitive impairment in our cocaine-withdrawn mice occurred in absence of affective alterations, though withdrawal from repeated cocaine is often accompanied by a high anxiety state (studies are reviewed in[21,41]). A possible explanation is that a heightened anxiety-like behavior characterizes earlier phases of

cocaine withdrawal but anxiety may become normalized over time, faster than the cognitive status. In this regard, elevated anxiety is often evident in rodents at short cocaine-free periods (24-48 h) but not at longer times after withdrawal, unless anxiety is 'activated' by an aversive or stressful stimulus[41]. Another key variable is the age of the animals at the time of cocaine administration, since chronic cocaine administered in adolescent rodents yields more evident[42] and persistent[43] anxiety-like responses.

In any case, this manuscript shows that chronic cocaine withdrawal impairs the acquisition of new learnings but not the retrieval of remote drug memories. In other words, these two cognitive events should be supported by different neurobiological mechanisms. It is important to emphasize at this point that cocaine administration and withdrawal exert widespread brain neuroplasticity that affects multiple learning systems -that include the hippocampus but also the frontal cortex, the amygdala or the striatum- involved in the acquisition and expression of drug-related responses as well as in cognitive performance[2,4,44]. While the contribution of the extra-hippocampal regions is not considered in this manuscript and it should not be dismissed, the pattern of impaired cognitive performance in the cocaine-withdrawn mice is consistent with a hippocampal malfunction. For example, the hippocampus is required for object and place recognition memory, spatial reference memory or spatial working memory in the water maze[45-48] as well as for nest building[49]. Even the slightly slower acquisition of the visible platform task in the cocaine-withdrawn mice could be influenced by certain procedural rules (i.e. 'how' –not 'where'- to escape from the water) that may involve a hippocampal participation in their learning[50].

Nevertheless, the expression of remote cocaine-CPP memory that may also depend on the hippocampus[15], was preserved in the cognitively-impaired animals. This outcome is not contradictory considering that the neurobiological pathways for reference memory encoding and retrieval are dissociated even at the hippocampal level[51]. This is especially

true for the retrieval of remote old memories that become less supported by the hippocampus as they are progressively stored from the hippocampus to the neocortex[52]. Hippocampal insults are typically characterized by strong (anterograde) amnesia for newly acquired information, but the hippocampal involvement in retrieving remote memories (retrograde amnesia) is less evident and it may depend on the extent of hippocampal damage, the memories' age or its content[52,53]. Accordingly, hippocampal manipulations (e.g. modulation of AHN) may selectively affect recent, but not remote, fear[54] and drug[55] associative memories in animal models. Considering all this, hippocampal dysfunctions preventing reference memory acquisition are compatible with a spared ability to remember older drug-related memories that are persistent in persons abusing cocaine[3]. For its part, hippocampal working memory also engages mechanisms independent of those for reference memory, requiring a close interplay between the hippocampus and the prefrontal cortex[56].

Finally, AHN was studied in this animal model, considering the importance of this process for hippocampal-dependent memory and its potential regulation by cocaine. Specifically, we researched different neuron populations generated at distinct time points during cocaine withdrawal. The number of new neurons (BrdU/NeuN+) born after cocaine administration that survived and reached maturation was normal compared to vehicle-treated mice, supporting that extended cocaine withdrawal is not associated to persistent alterations of basal AHN[4]. However, here we also report that the cocaine-treated mice showed an abnormal increase of the young DCX+ neurons generated up to 3-4 weeks before perfusion, an immature neuron population that is highly susceptible to stimulation by environmental inputs[57]. It is important to note that mice in this study did not remain undisturbed during that time period but they were submitted to an extended behavioral test battery that could stimulate immature hippocampal neurons and proliferative cells (**Supplementary Figure 1**). Therefore, a strong possibility is that the cocaine-treated mice abnormally upregulated their immature DCX+ neurons in response to the learning

experience. In agreement, a previous experiment found that mice withdrawn from chronic cocaine that were left undisturbed displayed normal levels of basal AHN, but behavioral training regulated AHN differently in the cocaine-withdrawn mice. In this case, their AHN and other hippocampal plasticity markers were reduced compared to trained vehicle-treated animals[21]. It is possible for cocaine-withdrawn mice to show either a reduced or an increased learning-induced AHN depending on the task demands, by assuming a 'low efficiency' and 'low capacity' hippocampal network[58]. Under such pathological condition, when learning is moderately successful but excessively effortful, additional AHN may be recruited as a compensatory mechanism; but experimental settings that provide higher task requirements may in turn result in absent or insufficient learning and a diminished recruitment of neurobiological resources[58].

Considering this, a related question is the potential functional role of the increased AHN found in this study in the cocaine-withdrawn mice. While increased AHN is often associated to improved learning[28], this relationship is not always straightforward, since there are also pathological conditions where an aberrantly increased AHN is the cause of cognitive impairment[59]. This latter possibility seems not to hold true for this study since there was a direct relationship of the DCX+ cells in the cocaine-treated mice with their ability for memory acquisition and updating. In other words, AHN was more increased in those cocaine-withdrawn mice that showed better cognitive performance. Nevertheless, this correlational evidence it is still insufficient to elucidate the extent of the causal contribution –if any- of the new neurons recruited by learning. Increased AHN could modulate certain aspects of the cognitive tasks; or it may just be an epiphenomenon of improved learning with not functional implications. While increasing AHN has been shown beneficial to promote forgetting of a previously acquired cocaine-CPP memory[32] it is yet to be investigated whether increasing AHN would improve learning and cognition during withdrawal, which should be tested experimentally by means of AHN-enhancing interventions[29]. Considering the present results, strategies directed to modulate retrieval

of remote cocaine-associated memories may not necessarily alleviate cocaine-induced cognitive impairment since these events seem independent and thus regulated by different neurobiological mechanisms. The animal model reported here will be useful to test the effect of pharmacological or environmental interventions on these two aspects of cocaine-altered cognition simultaneously.

Acknowledgements

experiments and maintenance of the mice, IBIMA's common research support structure of Imaging for the use of the white-light microscope, and the University of Málaga's Research Support Central Services (SCAI) for the confocal microscopy. Authors are grateful to Lourdes Sánchez Salido for her outstanding technical assistance.

Author contributions

Conceived and designed the study (L.J.S., E.C-O.); conducted the behavioral experiments (M.C.M-P., P.S-P., F.A-G., S.G-R.); conducted the histological study (M.C.M-P., S.G-R.); analyzed data (M.C.M-P., E.C-O.); wrote the manuscript (E.C-O.); critically reviewed the manuscript (M.C.M-P., F.R.F., L.J.S., E.C-O.); approved the final version (all authors).

Conflict of interest

The authors declare no competing interests.

References

1. United Nations Office on Drugs and Crime. *World Drug Report 2019*. (United Nations publication, Sales No. E.19.XI.9, 2019).
2. Everitt BJ. Neural and psychological mechanisms underlying compulsive drug seeking habits and drug memories--indications for novel treatments of addiction. *Eur J Neurosci* 2014;40:2163-2182.
3. Torregrossa MM , Taylor JR. Learning to forget: manipulating extinction and reconsolidation processes to treat addiction. *Psychopharmacology (Berl)* 2013;226:659-672.
4. Castilla-Ortega E, Ladron de Guevara-Miranda D, Serrano A, Pavon FJ, Suarez J, Rodriguez de Fonseca F, Santin LJ. The impact of cocaine on adult hippocampal neurogenesis: Potential neurobiological mechanisms and contributions to maladaptive cognition in cocaine addiction disorder. *Biochem Pharmacol* 2017;141:100-117.
5. Muller CP. Episodic memories and their relevance for psychoactive drug use and addiction. *Front Behav Neurosci* 2013;7:34.

6. Sampedro-Piquero P, Ladron de Guevara-Miranda D, Pavon FJ, Serrano A, Suarez J, Rodriguez de Fonseca F, Santin LJ, Castilla-Ortega E. Neuroplastic and cognitive impairment in substance use disorders: a therapeutic potential of cognitive stimulation. *Neurosci Biobehav Rev* 2019;23-48.

7. Vonmoos M, Hulka LM, Preller KH, Minder F, Baumgartner MR, Quednow BB. Cognitive impairment in cocaine users is drug-induced but partially reversible: evidence from a longitudinal study. *Neuropsychopharmacology* 2014;39:2200-2210.

8. Fox HC, Jackson ED, Sinha R. Elevated cortisol and learning and memory deficits in cocaine dependent individuals: relationship to relapse outcomes. *Psychoneuroendocrinology* 2009;34:1198-1207.

9. Ma L, Steinberg JL, Cunningham KA, Bjork JM, Lane SD, Schmitz JM, Burroughs T, Narayana PA, Kosten TR, Bechara A, Moeller FG. Altered anterior cingulate cortex to hippocampus effective connectivity in response to drug cues in men with cocaine use disorder. *Psychiatry Res Neuroimaging* 2018;271:59-66.

10. Fotros A, Casey KF, Larcher K, Verhaeghe JA, Cox SM, Gravel P, Reader AJ, Dagher A, Benkelfat C, Leyton M. Cocaine cue-induced dopamine release in amygdala and hippocampus: a high-resolution PET [(1)(8)F]fallypride study in cocaine dependent participants. *Neuropsychopharmacology* 2013;38:1780-1788.

11. Sorg BA. Reconsolidation of drug memories. *Neurosci Biobehav Rev* 2012;36:1400-1417.

12. Cunningham CL, Gremel CM, Groblewski PA. Drug-induced conditioned place preference and aversion in mice. *Nat Protoc* 2006;1:1662-1670.

13. Meyers RA, Zavala AR, Neisewander JL. Dorsal, but not ventral, hippocampal lesions disrupt cocaine place conditioning. *Neuroreport* 2003;14:2127-2131.

14. Otis JM, Fitzgerald MK, Mueller D. Inhibition of hippocampal beta-adrenergic receptors impairs retrieval but not reconsolidation of cocaine-associated memory and prevents subsequent reinstatement. *Neuropsychopharmacology* 2014;39:303-310.

15. Raybuck JD, Lattal KM. Differential effects of dorsal hippocampal inactivation on expression of recent and remote drug and fear memory. *Neurosci Lett* 2014;569:1-5.

16. White NM, Chai SC, Hamdani S. Learning the morphine conditioned cue preference: cue configuration determines effects of lesions. *Pharmacol Biochem Behav* 2005;81:786-796.

17. Spronk DB, van Wel JH, Ramaekers JG, Verkes RJ. Characterizing the cognitive effects of cocaine: a comprehensive review. *Neurosci Biobehav Rev* 2013;37:1838-1859.

18. Briand LA, Flagel SB, Garcia-Fuster MJ, Watson SJ, Akil H, Sarter M, Robinson TE. Persistent alterations in cognitive function and prefrontal dopamine D2 receptors following extended, but not limited, access to self-administered cocaine. *Neuropsychopharmacology* 2008;33:2969-2980.

19. Calu DJ, Stalnaker TA, Franz TM, Singh T, Shaham Y, Schoenbaum G. Withdrawal from cocaine self-administration produces long-lasting deficits in orbitofrontal-dependent reversal learning in rats. *Learn Mem* 2007;14:325-328.

20. Berardino BG, Fesser EA, Belluscio LM, Gianatiempo O, Pregi N, Canepa ET. Effects of cocaine base paste on anxiety-like behavior and immediate-early gene expression in nucleus accumbens and medial prefrontal cortex of female mice. *Psychopharmacology (Berl)* 2019;

21. Ladron de Guevara-Miranda D, Millon C, Rosell-Valle C, Perez-Fernandez M, Missiroli M, Serrano A, Pavon FJ, Rodriguez de Fonseca F, Martinez-Losa M, Alvarez-Dolado M, Santin LJ, Castilla-Ortega E. Long-lasting memory deficits in mice withdrawn from cocaine are concomitant with neuroadaptations in hippocampal basal activity, GABAergic interneurons and adult neurogenesis. *Dis Model Mech* 2017;10:323-336.

22. Ledesma JC, Aguilar MA, Gimenez-Gomez P, Minarro J, Rodriguez-Arias M. Adolescent but not adult ethanol binge drinking modulates cocaine withdrawal symptoms in mice. *PLoS One* 2017;12:e0172956.

23. Morisot N, Le Moine C, Millan MJ, Contarino A. CRF(2) receptor-deficiency reduces recognition memory deficits and vulnerability to stress induced by cocaine withdrawal. *Int J Neuropsychopharmacol* 2014;17:1969-1979.

24. Fole A, Martin M, Morales L, Del Olmo N. Effects of chronic cocaine treatment during adolescence in Lewis and Fischer-344 rats: Novel location recognition impairment and changes in synaptic plasticity in adulthood. *Neurobiol Learn Mem* 2015;123:179-186.

25. Gong D, Zhao H, Liang Y, Chao R, Chen L, Yang S, Yu P. Differences in cocaine- and morphine-induced cognitive impairments and serum corticosterone between C57BL/6J and BALB/cJ mice. *Pharmacol Biochem Behav* 2019;182:1-6.

26. Mendez IA, Montgomery KS, LaSarge CL, Simon NW, Bizon JL, Setlow B. Long-term effects of prior cocaine exposure on Morris water maze performance. *Neurobiol Learn Mem* 2008;89:185-191.

27. Solinas M, Chauvet C, Thiriet N, El Rawas R, Jaber M. Reversal of cocaine addiction by environmental enrichment. *Proc Natl Acad Sci U S A* 2008;105:17145-17150.

28. Castilla-Ortega E, Pedraza C, Estivill-Torrus G, Santin LJ. When is adult hippocampal neurogenesis necessary for learning? evidence from animal research. *Rev Neurosci* 2011;22:267-283.

29. Castilla-Ortega E , Santin LJ. Adult hippocampal neurogenesis as a target for cocaine addiction: a review of recent developments. *Current Opinion in Pharmacology* 2019;In press. doi: 10.1016/j.coph.2019.10.002.:

30. Deroche-Gamonet V, Revest JM, Fiancette JF, Balado E, Koehl M, Grosjean N, Abrous DN, Piazza PV. Depleting adult dentate gyrus neurogenesis increases cocaine-seeking behavior. *Mol Psychiatry* 2019;24:312-320.

31. Castilla-Ortega E, Blanco E, Serrano A, Ladron de Guevara-Miranda D, Pedraz M, Estivill-Torrus G, Pavon FJ, Rodriguez de Fonseca F, Santin LJ. Pharmacological reduction of adult hippocampal neurogenesis modifies functional brain circuits in mice exposed to a cocaine conditioned place preference paradigm. *Addict Biol* 2016;21:575-588.

32. Ladron de Guevara-Miranda D, Moreno-Fernandez RD, Gil-Rodriguez S, Rosell-Valle C, Estivill-Torrus G, Serrano A, Pavon FJ, Rodriguez de Fonseca F, Santin LJ, Castilla-Ortega E. Lysophosphatidic acid-induced increase in adult hippocampal neurogenesis facilitates the forgetting of cocaine-contextual memory. *Addict Biol* 2019;24:458-470.

33. Moreno-Jimenez EP, Flor-Garcia M, Terreros-Roncal J, Rabano A, Cafini F, Pallas-Bazarra N, Avila J, Llorens-Martin M. Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. *Nat Med* 2019;25:554-560.

34. Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG. Transient expression of doublecortin during adult neurogenesis. *J Comp Neurol* 2003;467:1-10.

35. Plumpe T, Ehninger D, Steiner B, Klempin F, Jessberger S, Brandt M, Romer B, Rodriguez GR, Kronenberg G, Kempermann G. Variability of doublecortin-associated dendrite maturation in adult hippocampal neurogenesis is independent of the regulation of precursor cell proliferation. *BMC Neurosci* 2006;7:77.

36. Jinno S. Topographic differences in adult neurogenesis in the mouse hippocampus: a stereology-based study using endogenous markers. *Hippocampus* 2011;21:467-480.

37. Preston CJ, Brown KA, Wagner JJ. Cocaine conditioning induces persisting changes in ventral hippocampus synaptic transmission, long-term potentiation, and radial arm maze performance in the mouse. *Neuropharmacology* 2019;150:27-37.

38. Bechard AR, LaCrosse A, Namba MD, Jackson B, Knackstedt LA. Impairments in reversal learning following short access to cocaine self-administration. *Drug Alcohol Depend* 2018;192:239-244.

39. Gobin C , Schwendt M. The Effects of Extended-Access Cocaine Self-Administration on Working Memory Performance, Reversal Learning and Incubation of Cocaine-Seeking in Adult Male Rats. *J Addict Prev* 2017;5:

40. Sinha R. Modeling stress and drug craving in the laboratory: implications for addiction treatment development. *Addict Biol* 2009;14:84-98.

41. Erb S. Evaluation of the relationship between anxiety during withdrawal and stress-induced reinstatement of cocaine seeking. *Prog Neuropsychopharmacol Biol Psychiatry* 2010;34:798-807.

42. Valzachi MC, Teodorov E, Marcourakis T, Bailey A, Camarini R. Enhancement of behavioral sensitization, anxiety-like behavior, and hippocampal and frontal cortical CREB levels following cocaine abstinence in mice exposed to cocaine during adolescence. *PLoS One* 2013;8:e78317.

43. Zhu W, Mao Z, Zhu C, Li M, Cao C, Guan Y, Yuan J, Xie G, Guan X. Adolescent exposure to cocaine increases anxiety-like behavior and induces morphologic and neurochemical changes in the hippocampus of adult rats. *Neuroscience* 2016;313:174-183.

44. Sampedro-Piquero P, Santin LJ, Castilla-Ortega E. Aberrant Brain Neuroplasticity and Function in Drug Addiction: A Focus on Learning-Related Brain Regions. in: *Behavioral Neuroscience*. S. Palermo and R. Morese (eds.). London:IntechOpen; 2019. 1-24. Available from: https://www.intechopen.com/online-first/aberrant-brain-neuroplasticity-and-function-in-drug-addiction-a-focus-on-learning-related-brain-regi.

45. Barker GR , Warburton EC. When is the hippocampus involved in recognition memory? *J Neurosci* 2011;31:10721-10731.

46. Broadbent NJ, Squire LR, Clark RE. Spatial memory, recognition memory, and the hippocampus. *Proc Natl Acad Sci U S A* 2004;101:14515-14520.

47. Cohen SJ , Stackman RW, Jr. Assessing rodent hippocampal involvement in the novel object recognition task. A review. *Behav Brain Res* 2015;285:105-117.

48. Morris RG, Schenk F, Tweedie F, Jarrard LE. Ibotenate Lesions of Hippocampus and/or Subiculum: Dissociating Components of Allocentric Spatial Learning. *Eur J Neurosci* 1990;2:1016-1028.

49. Jirkof P. Burrowing and nest building behavior as indicators of well-being in mice. *J Neurosci Methods* 2014;234:139-146.

50. Micheau J, Riedel G, Roloff E, Inglis J, Morris RG. Reversible hippocampal inactivation partially dissociates how and where to search in the water maze. *Behav Neurosci* 2004;118:1022-1032.

51. Eldridge LL, Engel SA, Zeineh MM, Bookheimer SY, Knowlton BJ. A dissociation of encoding and retrieval processes in the human hippocampus. *J Neurosci* 2005;25:3280-3286.

52. Wiltgen BJ, Zhou M, Cai Y, Balaji J, Karlsson MG, Parivash SN, Li W, Silva AJ. The hippocampus plays a selective role in the retrieval of detailed contextual memories. *Curr Biol* 2010;20:1336-1344.

53. Moscovitch M, Nadel L, Winocur G, Gilboa A, Rosenbaum RS. The cognitive neuroscience of remote episodic, semantic and spatial memory. *Curr Opin Neurobiol* 2006;16:179-190.

54. Gao A, Xia F, Guskjolen AJ, Ramsaran AI, Santoro A, Josselyn SA, Frankland PW. Elevation of Hippocampal Neurogenesis Induces a Temporally Graded Pattern of Forgetting of Contextual Fear Memories. *J Neurosci* 2018;38:3190-3198.

55. Rivera PD, Simmons SJ, Reynolds RP, Just AL, Birnbaum SG, Eisch AJ. Image-guided cranial irradiation-induced ablation of dentate gyrus neurogenesis impairs extinction of recent morphine reward memories. *Hippocampus* 2019;29:726-735.

56. Yoon T, Okada J, Jung MW, Kim JJ. Prefrontal cortex and hippocampus subserve different components of working memory in rats. *Learn Mem* 2008;15:97-105.

57. Tashiro A, Makino H, Gage FH. Experience-specific functional modification of the dentate gyrus through adult neurogenesis: a critical period during an immature stage. *J Neurosci* 2007;27:3252-3259.

58. Steffener J , Stern Y. Exploring the neural basis of cognitive reserve in aging. *Biochim Biophys Acta* 2012;1822:467-473.

59. Cuartero MI, de la Parra J, Perez-Ruiz A, Bravo-Ferrer I, Duran-Laforet V, Garcia-Culebras A, Garcia-Segura JM, Dhaliwal J, Frankland PW, Lizasoain I, Moro MA. Abolition of aberrant neurogenesis ameliorates cognitive impairment after stroke in mice. *J Clin Invest* 2019;129:1536-1550.

Figure Legends

Fig. 1: Chronic cocaine induced persistent cocaine-associated memory.

(a) Rationale of the model or cocaine-altered cognition used in this study, with a focus on the hippocampus. The hippocampus is a key brain region for the acquisition and consolidation of declarative memory, including associative and episodic memory. At the first exposures to the drug, acute cocaine acting on a 'healthy' hippocampus activates neurobiological mechanisms that may contribute to the formation of strong and lasting memories for cocaine-related experiences[4]. However, after repeated cocaine exposures, the hippocampus becomes corrupted by drug-induced neuroplastic changes, impeding the acquisition of new hippocampal learnings[4]. Therefore, persistent memories for past drug experiences may coexist with cognitive decline during withdrawal from chronic cocaine. **(b)** Schedule of the experiment. Mice were conditioned to cocaine in a chronic CPP task (COC mice, 14 days) or received saline solution (VEH mice). Subsequently, they were tested for both long-term maintenance of the remote cocaine-CPP memory and for emotional and cognitive behavior in a battery of tests. **(c)** Cocaine-induced CPP task. The COC mice acquired a lasting memory for the contextual stimuli associated with cocaine that persisted after 28 days of drug withdrawal. Accordingly, the CPP Score of the COC mice was significantly greater than zero during both test sessions (one-sample Student's *t* test on Test 1: $t_9 = 5.340$, $p < 0.000$; on Test 2: $t_9 = 4.911$, $p = 0.001$). **(d)** Both groups of mice showed a similar locomotor activity during the drug-free Test sessions in the CPP apparatus.

Results are expressed as mean ± SEM.

Post-hoc difference *vs* the other treatment group: *$p < 0.05$ and **$p < 0.001$

Significant difference vs zero: $$$p < 0.001$

Fig. 2: Cocaine-withdrawn mice showed cognitive deficits in absence of emotional and exploratory alterations. (a) The cocaine-withdrawn mice were slightly impaired in an intrinsically motivated behavior such as nest building, especially at 1 h after the addition of the nesting material. However, cocaine withdrawal did not affect behavior in **(b)** the elevated plus maze, **(c)** the forced swimming and **(d)** the open field tests for emotional and exploratory responses. **(e)** Schedule of the object and place memory task. The cocaine-withdrawn mice showed deficits in both memory domains.

Results are expressed as mean ± SEM.

Difference *vs* the other treatment group by post-hoc analysis (a) or Student's t test (e): *$p < 0.05$

Fig. 3: Reference and working memory assessment in the water maze. (a) Habituation sessions. The time swimming into each pool quadrant shows the two sessions averaged. The figure includes a representation of the circular pool, the peripheral region (blue), the four imaginary quadrants and the starting positions. Both groups were similar in the pool exploration parameters. **(b)** Visible platform training and **(c)** spatial memory training with hidden platform revealed a delayed acquisition in the COC mice. The figure **(c)** shows a representative swimming path of each treatment group on the third training day. **(d)** Both groups were similar on the probe trials for long-term memory retention performed at 24 h and 72 h. **(e)** Training day with inverted platform location and **(f)** probe trial for short-term memory retention at 30 min. **(g)** Delayed matching-to-sample working memory training showed a severe impairment in the COC mice. Each training day included four sample and test pairings, averaged per day in the graph.

Results are expressed as mean ± SEM.

Significant effect for 'treatment' in the repeated measures ANOVA: *$p < 0.05$; **$p < 0.001$

Post-hoc difference *vs* the opposite quadrant: &$p < 0.05$

Figure legend: E: East; ITI: inter-trial interval; N: North; O: Opposite quadrant; S: South; T: Target quadrant; W: West.

Fig. 4: Relationship between cognitive performance during cocaine withdrawal and the persistence of the cocaine-associated memory. (a) The PCA analysis on the whole sample of animals reduced the cognitive-related measures to a set of four different constructs or factors. The variables included in each factor are highlighted in bold. Factors were named after their most representative variables (i.e. those that, in absolute value, loaded higher the factor but lower in the others). Factors cumulatively explained a 82.668 % of the total performance of the animals. **(b)** Comparison of factor scores between the two treatments revealed that the COC mice were impaired in Factor 1 and Factor 2. #Attending to the sign of the variables that loaded in each factor, the factor scores of Factor 2 and Factor 3 were multiplied by '-1' so a positive factor score corresponded to a higher performance, for easier interpretation. **(c)** Cognitive factor scores and measures of long-term CPP maintenance were mainly unrelated according to linear correlations. The only significant correlation (∇ CPP Score and Factor 4) suggested that mice with better long-term memory abilities showed a greater decay of their CPP Score in the Test 2 –maybe they could remember more easily that the drug was no longer administered in the CPP apparatus, as they had experienced this outcome in the Test 1-. However, we believe this correlation should be interpreted with caution as it seemed highly driven by the score of one particular animal.

Results in (b) are expressed as mean ± SEM.

Difference vs the other treatment group by Student's t test: *$p < 0.05$

Figure legend: RM: Reference Memory.

Fig. 5: Altered learning-induced regulation of adult hippocampal neurogenesis in the cocaine-withdrawn mice. (a) Schedule of the experiment (as shown in **Fig. 1b**) displaying the timing of expression of each AHN marker analyzed. **(b)** The cocaine-withdrawn mice did not show alterations in the number of BrdU+ cells nor in their differentiation into mature neurons (i.e. co-expression of BrdU and the mature neuron marker NeuN). Figures **(c, d)** show representative confocal images. Arrows in **(c)** point to positive nuclei for the markers BrdU, NeuN or both (highlighted in orange). White arrows in **(d)** point to nuclei positive for BrdU, some of them also co-label with NeuN. **(e)** Both treatment groups showed similar DG cell proliferative activity at the completion of the experiment, as studied by the PCNA+ cells. **(f)** The percentage of young neurons (DCX+) showing either an immature-like (T1) or a mature-like morphology (T2) was similar in both treatments, but the cocaine-withdrawn mice displayed an increased number of these neurons in the SupraDG. **(g)** The young neurons in the cocaine-withdrawn mice, especially in the SupraDG, were directly correlated with their cognitive performance (factor scores in Factor 1; **Fig. 4**). There was a non-significant tendency for correlation of cognitive performance with the young neurons in the InfraDG ($r = 0.619$, $p = 0.056$). On the contrary, the AHN-related measures were uncorrelated with the measures of cocaine-CPP maintenance (not shown).

Black arrows in **(e, f)** point to positive cells. In (**b, f**), the circular graphs shows percentage data for the total DG; analyses per each DG blade separately did not reveal differences either. Scale in **(e)** is valid for **(f)**.

Results are expressed as mean ± SEM.

Post-hoc difference *vs* the other treatment group: *$p < 0.05$

Figure legend: DG: Dentate gyrus; InfraDG: infrapyramidal blade of the DG; SupraDG: Suprapyramidal blade of the DG; T1: Type-1; T2: Type 2.
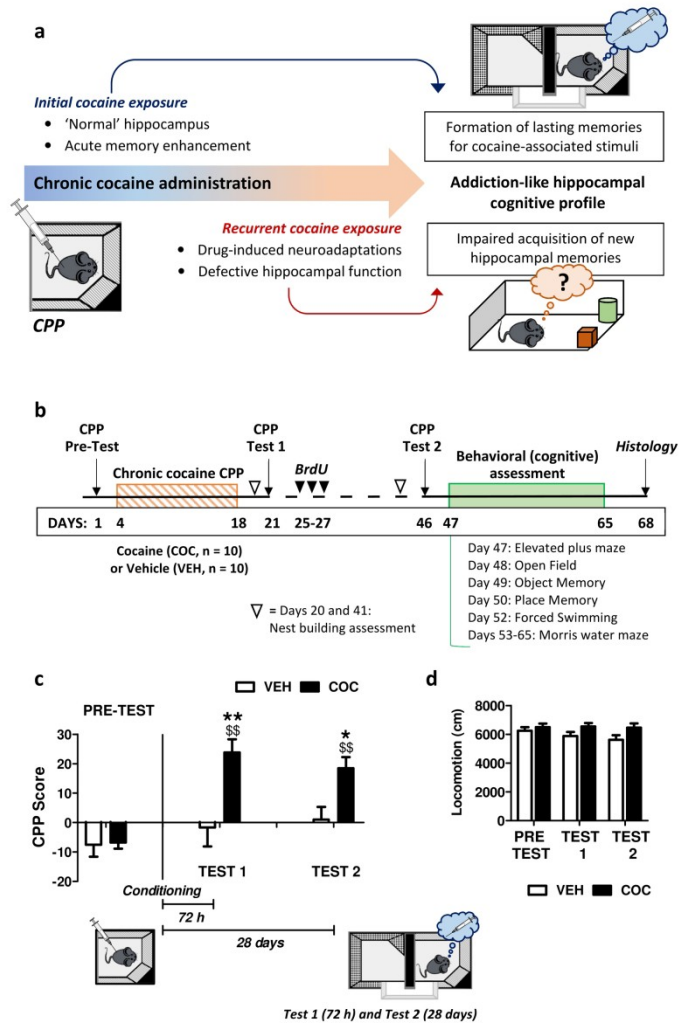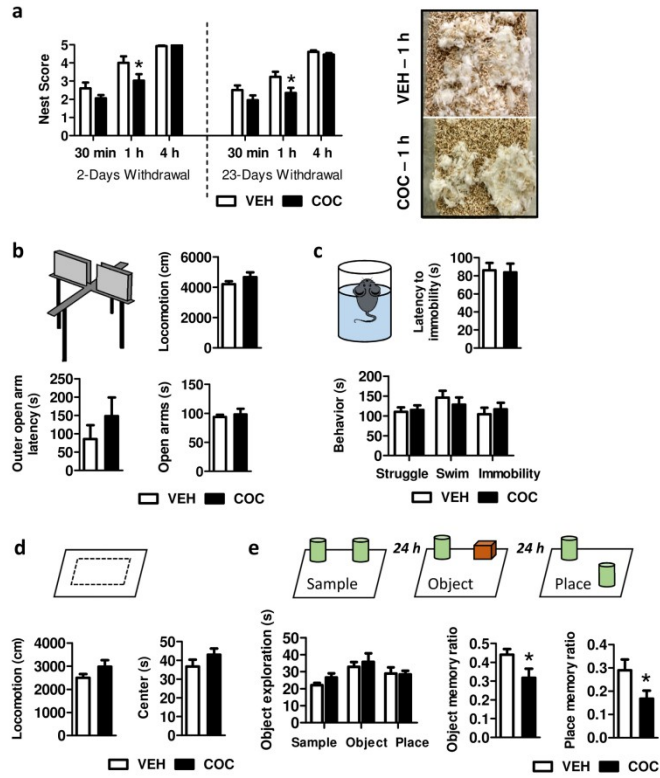
Figure 1

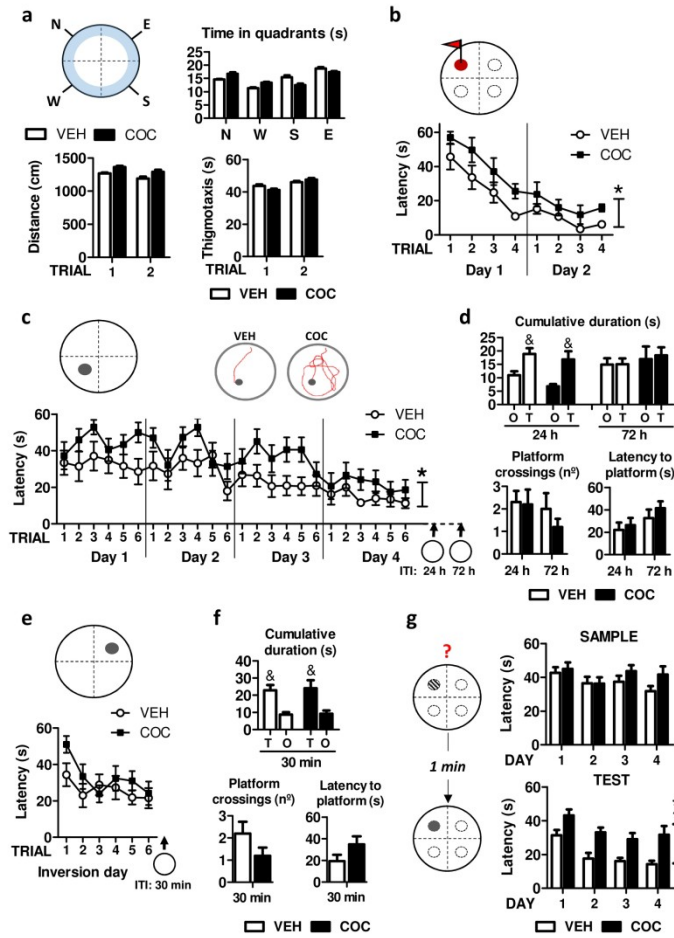209x297mm (300 x 300 DPI)

Figure 2

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Figure 3

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a

Variables	Factor 1 Memory acquisition and manipulation	Factor 2 Object and place memory	Factor 3 72 h spatial memory retention	Factor 4 24 h spatial memory retention
Object Memory ratio	0.335	**-0.586**	0.168	**0.579**
Place memory ratio	0.117	**-0.834**	-0.020	-0.257
Latency to visible platform	**-0.732**	0.062	0.302	0.407
Latency to platform (RM)	**-0.822**	-0.067	-0.003	0.357
Latency to platform (inversion)	**-0.749**	-0.113	**-0.530**	0.054
24 h Probe trial latency (RM)	-0.492	-0.367	0.131	**-0.669**
72 h Probe trial latency (RM)	-0.319	-0.044	**0.916**	-0.074
30 m Probe trial latency (inversion)	**-0.715**	-0.357	-0.222	0.135
Working memory test latency	**-0.860**	0.291	0.041	-0.246
Eigenvalue	3.493	1.410	1.308	1.229
% Explained variance	38.809	15.672	14.532	13.654

b

Factor 1   Factor 2#   Factor 3#   Factor 4

c

FACTOR 1   FACTOR 2#   FACTOR 3#   FACTOR 4
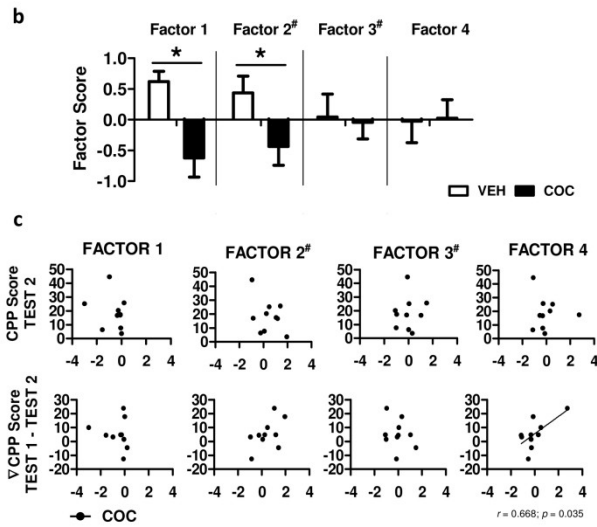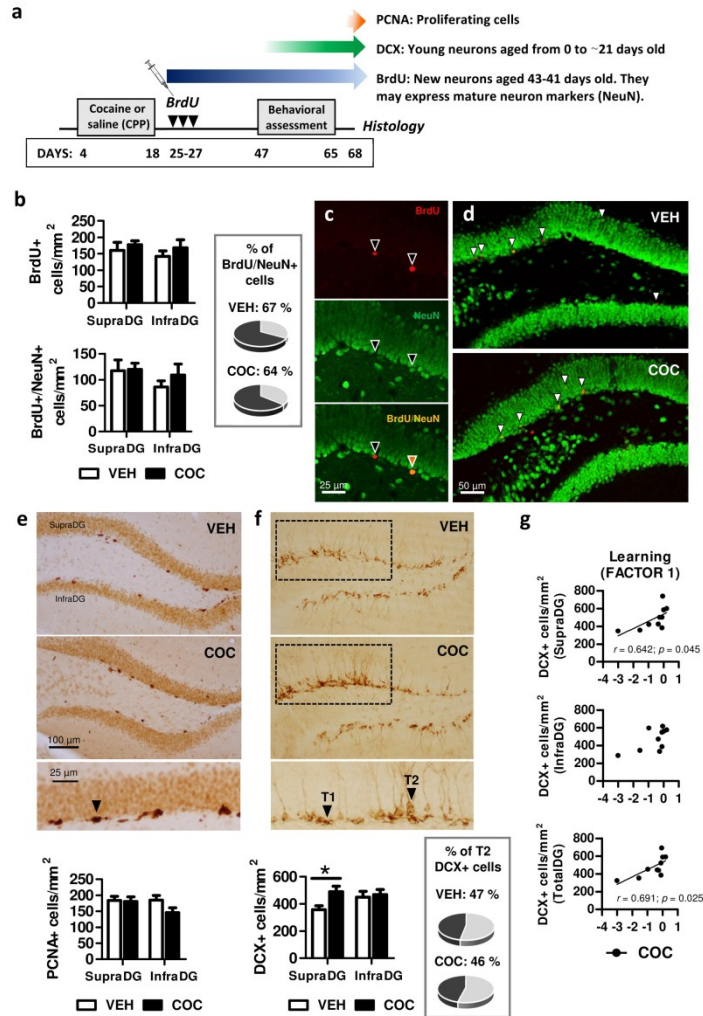
$r = 0.668; p = 0.035$

Figure 4

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Figure 5

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