Cocaine-conditioned place preference is predicted by previous anxiety-like behavior and is related to an increased number of neurons in the basolateral amygdala

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Abstract

The identification of behavioral traits that could predict an individual's susceptibility to engage in cocaine addiction is relevant for understanding and preventing this disorder, but investigations of cocaine addicts rarely allow us to determinate whether their behavioral attributes are a cause or a consequence of drug use. To study the behaviors that predict cocaine vulnerability, male C57BL/6J mice were examined in a battery of tests (the elevated plus maze, hole-board, novelty preference in the Y-Maze, episodic-like object recognition and forced swimming) prior to training in a cocaine-conditioned place preference (CPP) paradigm to assess the reinforcing value of the drug. In a second study, the anatomical basis of high and low CPP in the mouse brain was investigated by studying the number of neurons (neuronal nuclei-positive) in two addiction-related limbic regions (the medial prefrontal cortex and the basolateral amygdala) and the number of dopaminergic neurons (tyrosine hydroxylase-positive) in the ventral tegmental area by immunohistochemistry and stereology. Correlational analyses revealed that CPP behavior was successfully predicted by anxiety-like measures in the elevated plus maze (i.e., the more anxious mice showed more preference for the cocaine-paired compartment) but not by the other behaviors analyzed. In addition, increased numbers of neurons were found in the basolateral amygdala of the high CPP mice, a key brain center for anxiety and fear responses. The results support the theory that anxiety is a relevant factor for cocaine vulnerability, and the basolateral amygdala is a potential neurobiological substrate where both anxiety and cocaine vulnerability could overlap.

1. INTRODUCTION

Cocaine addiction is a chronic brain disease with an important global burden; it requires complicated treatment and has many health and socioeconomic implications. Cocaine is used by 14 to 21 million people worldwide (prevalence of ~0.4%), most frequently in North and South America, Oceania and Western and Central Europe [1], where 4.2 % of Europeans (15–64 years old) have used cocaine in their lifetime [2]. However, only a subset of cocaine users will become addicted. While some individuals lose control over their cocaine intake and eventually become addicts, others use the drug without developing abusive tendencies or dependence. In fact, it is estimated that only 20.9% of cocaine users become dependent on cocaine at some point in their lives [3].

Key individual differences account for both the acquisition and maintenance of drug-related behaviors. Many biological, socioeconomic and psychological variables modulate the drug's perceived reinforcing value or a person's control over its use, thereby influencing drug addiction vulnerability [3, 4]. Regarding the psychological vulnerability factors, studies have identified several stable, and likely heritable, behavioral characteristics or 'personality traits' that may increase the risk of cocaine addiction. A dimension involving active searching for sensations and new experiences ('sensation seeking' or 'novelty seeking') is often elevated in cocaine addicts compared to controls and is related to the severity of cocaine use [5-7]. Moreover, cocaine addiction usually involves increased impulsivity and willingness to take risks, cognitive deficits and an altered emotional status that frequently corresponds with high anxiety [8, 9], which are expressed as elevated psychiatric comorbidities, such as anxiety and mood disorders [10, 11]. An underlying assumption is that these behavioral attributes reflect the abnormal function of key brain areas that are also part of the addiction circuitry and that respond to drug abuse in a maladaptive way, triggering addiction. In this regard, magnetic resonance studies have associated cocaine addiction with macrostructural abnormalities (i.e., volume alterations) in the limbic brain regions that are involved in motivational, cognitive and emotional behavior, such as the frontal cortices and temporal lobe structures, including the amygdala but usually not the hippocampus [12-19].

Nevertheless, it is difficult for human studies to elucidate whether the maladaptive behavioral traits and the neurobiological hallmarks found in addicts actually predispose individuals to cocaine addiction or if they are a consequence of the neuroadaptations induced by prolonged drug intake. Research on this topic benefits from animal models, as drug administration can be controlled and we can examine their behaviors prior to drug exposure. It is consistently reported that mice or rats that exhibit an increased response to novelty, have elevated impulsivity or high anxiety-like behavior, display increased cocaine-seeking behavior when exposed to self-administration or conditioned place preference paradigms (novelty: [20-23]; impulsivity: [24, 25]; anxiety: [26-29]). However, while rodents' performances in certain tasks could emulate stable behavioral traits similar to those in humans, the meaning of the rodents' behaviors is often

confusing because activity/exploration, emotion and cognition cannot easily be distinguished in animal tests. For example, behaviors that are used as reliable measures for the novelty response (i.e., locomotion or hole exploration) can be influenced by the emotional state experienced by the animal, while the most frequently used anxiety-like measures (i.e., time spent exploring the unprotected zones of a maze) also involve a locomotor/exploratory response (reviewed in [30]). Indeed, this issue may explain some of the complex results on rodents' addiction-vulnerability behaviors in the literature [23, 29, 31]. On the other hand, many studies usually fail to analyze the neurobiological basis of vulnerability in animals.

In this experiment, the mice underwent a battery of tests for a wide range of behavioral domains: novelty-induced exploration and preference for novel contexts, emotion (unconditioned anxiety and despair-like behavior) and hippocampal-dependent memory. Subsequently, the cocaine-seeking behavior was tested in the conditioned place preference (CPP) paradigm, a widely used test to study the reinforcing value of drugs [32]. Correlational approaches were employed to determine whether a behavioral dimension underlying the performance of the mice in the exploratory, emotional or cognitive tests could predict cocaine-seeking behaviors. In addition, animals with high or low place preference were studied for structural differences (the total number of neurons) in the medial prefrontal cortex (mPFC) and the basolateral amygdala (BLA), two key limbic brain areas that may support the link between addiction-vulnerability behavior and cocaine-seeking behavior.

2. MATERIALS AND METHODS

2.1. Animals

The male C57BL/6J mice were acquired from Janvier (Le Genest-St-Isle, France) and arrived at the animal facility when they were 11 weeks of age. After one week of acclimation, the mice were individually housed in standard laboratory cages with nesting material and were handled daily (5 min/day) for five days. The mice were maintained on a 12-h light/dark cycle (lights on at 8:00 a.m.) with water and food provided *ad libitum*.

The experimental procedures were performed in accordance with the European (Directive 2010/63/UE) and Spanish regulations (Real Decreto 53/20130 and Ley 32/2007) for animal research. The protocol was approved by the *Comité Ético de Experimentación Animal* of the University of Málaga (permit number: CEUMA nº 8-2014-A). All surgery (i.e., intravascular perfusion) was performed under sodium pentobarbital anesthesia (200 mg/kg) and all efforts were made to minimize the animal's suffering.

2.2. Experiment I: Behaviors that predict conditioned place preference

Sixteen mice were used for this experiment and began the behavioral protocol at 13 weeks of age. Behavioral testing was performed between 8:00 a.m. and 3:00 p.m. in a noise-isolated room illuminated by 200 lux. The mice were habituated to the room for at least 20 minutes before the assessment began. After each session, the apparatuses were carefully cleaned with a solution of 70 % alcohol to remove the odor cues. The sessions were recorded with a digital camera and analyzed with the Ethovision XT9 software (Noldus, Waninghen, The Netherlands) to determine the spatiotemporal parameters. Stereotypic behaviors were observed by a trained experimenter using the Ethovision's Manual Score module.

2.2.1. Exploratory, emotional and cognitive tests

From Days 1 to 5, the mice were submitted to a behavioral test battery to assess activity/exploration, emotion and cognition, based on previously reported methods [30, 33-37]. Because the mice would progressively become habituated to the behavioral test, the tasks to assess exploration and unconditioned anxiety were performed on the first days, when the testing environment could elicit both novelty and aversion; while a highly stressful task (i.e., the forced swimming test) was administered last to avoid its potential deleterious influence on the subsequent behavioral measures. Thus, the behavioral assessment was performed in the following sequence: the elevated plus maze test (EPM, Day 1) and the hole-board test (HB, Day 2) for exploratory and anxiety-like behavior, the Y-maze test for novelty preference (Day 3), the

episodic-like object recognition memory test (Day 4) and the forced swimming test for despair-like behavior (Day 5). The episodic-like object recognition task provides several memory measures: the 'What-' and 'When-Scores' reveal the animal's ability to discriminate two familiar objects that are presented in a different order (so the older object would be the most explored); while the 'Where-Score' reflects the animal's ability to discriminate the spatial location in which a familiar object was presented (so that a familiar object that was displaced to a familiar but different location would be more explored than a static one) (the calculations for each score are shown in Table 1) [34].

The procedures and the behaviors analyzed for each task (**Tables 1, 2**) are detailed in the Supplementary material.

2.2.2. Cocaine-induced conditioned place preference

Subsequently, the mice were trained in the CPP apparatus on Days 8 to 12 using previously reported methods [38, 39] (Supplementary material). During the conditioning training, one compartment of the maze was paired with cocaine (20 mg/kg in saline, i.p.) and the other with an i.p. injection of saline. A cocaine-paired and a saline-paired session were administered each day, separated by at least four hours, in the following sequence: Day 8: cocaine/saline, Day 9: saline/cocaine, Day 10: cocaine/saline, Day 11: saline/cocaine, and Day 12: cocaine/saline. On day 15, the animals' conditioned place preference was evaluated in a test session in which the mice were administered saline and allowed to explore the entire apparatus for 20 minutes. Place preference was expressed as a CPP-Score **(Table 1)**, such that a preference for the drug-paired compartment over the saline-paired compartment would yield a CPP-Score greater than zero, while a CPP-Score close to zero would indicate an absence of discrimination between the compartments.

2.3. Experiment II: Brain structures associated with conditioned place preference behavior

The animals used for this experiment were selected from the mice included in Experiment I, based on their CPP-Score in the test session. The first group was composed of five mice that exhibited reduced cocaine-seeking behavior (i.e., a CPP-Score below the mean of the sample; LowCPP-mice), and the second group was composed of five mice that exhibited increased cocaine-seeking behavior (i.e., a CPP-Score above the mean of the sample; HighCPP-mice).

2.3.1. Long-term assessment of CPP behavior

After the test session in the CPP apparatus, the LowCPP- and HighCPP-mice underwent 20 days of cocaine withdrawal (i.e., were left undisturbed in their home cage). On day 21, the mice were placed in the CPP apparatus again and were given 12 extinction sessions distributed as one daily session across three weeks, excluding the weekends, to further study the animals' drug-seeking behavior. Each extinction session was performed in a similar manner as the test session reported in Experiment I [38, 39].

2.3.2. Immunohistochemistry and cell quantification

After the last extinction session, the mice were intracardially perfused and histology studies were performed as described in the Supplementary material. The neurons in the mPFC and BLA were immunostained with a neuronal nuclei (NeuN) marker. The total volume of each structure and the density of the positive cells were quantified by stereological methods to estimate the total number of neurons per structure; the right hemisphere was arbitrarily chosen for analysis (Supplementary material). In addition, the dopaminergic neurons expressing tyrosine hydroxylase (TH) were quantified in the ventral tegmental area (VTA) using the same procedures.

2.4. Statistical Analysis

The intra-group or inter-group comparisons were assessed using Student's *t* tests for dependent or independent samples, while comparisons against zero were assessed by one-

sample *t* tests. The extinction data were analyzed by repeated measures ANOVA. In Experiment I, the potential relationships between the variables were tested using Pearson's correlations, and the false discovery rate method ([40] as performed previously [35, 39]) was used to control for correlations driven by chance due to multiple comparisons. A Principal Components Factorial Analysis (PCA) with varimax rotation was performed to reveal the independent dimensions (i.e., factors) underlying the animals' behaviors [30, 41, 42]. The factors were selected using the criterion of an eigenvalue > 1, and the animals' scores in each factor ('Factorial Score': a numerical value indicating each animal's score in the behavioral dimension, represented as a factor) were calculated using the regression method in the SPPS software (SPSS Inc., Chicago, USA). The ability of each factor to predict the cocaine-seeking behavior was tested by Pearson's correlations between the Factorial Score and the CPP-Score.

3. RESULTS

3.1. Experiment I: An 'anxiety' behavioral dimension predicts CPP behavior

The results of the exploratory, emotional and cognitive behavioral assessments of the entire sample of mice are described in **Table 2**. The mice spent more time exploring the closed arms of the EPM than the open arms [*t* test for dependent samples: $t(15) = -15.489$, $P = 0.000$], and preferred the periphery over the central area in the HB test [$t(15) = 12.261$, $P = 0.000$], which was expected due to their natural aversion to open, unprotected spaces. Interestingly, although both the EPM and the HB tasks assessed unconditioned anxiety, anxiety-like and threat-evaluation behaviors, such as grooming and risk-assessment, were increased in the EPM compared to the HB [EPM vs HB: $t(15) = 5.948$, $P = 0.000$ for grooming; $t(15) = 4.142$, $P = 0.001$ for risk-assessment]. The animals preferred the novel arm of the Y-maze over the familiar arm [Novelty-Score vs zero: $t(15) = 3.857$, $P = 0.002$], indicating that the mice demonstrated both novelty preference and a short-term capacity to discriminate among different contexts. In the episodic-like object recognition task, the mice preferred the older objects over the most recent ones [What and When-Scores vs zero: $t(15) = 4.390$, $P = 0.000$ and $t(15) = 4.880$, $P = 0.000$, respectively], which may indicate either memory for the temporal order of object

presentation or recency memory [43]. However, the majority of the mice did not discriminate between the familiar object displaced to a different familiar location and the non-displaced object [Where-Score vs zero: $t(15) = -1.426$, $P = 0.174$]. This is consistent with previous evidence suggesting that the 'Where' memory (i.e., recognizing that a familiar object is displaced in a familiar location that had previously been occupied by a different object) entails a highly complex cognitive process that is usually not present in mice [34, 35, 44]. In the forced swimming test, the mice struggled for the first few minutes after they were placed in the water, and the despair-like behavior (i.e., immobility time) progressively increased over time (from 0.298 ± 0.160 seconds in minute 1 to 28.684 ± 2.426 seconds in minute 5).

A main goal of this experiment was to study the behaviors that could predict the animals' vulnerability to cocaine-seeking. After conditioning in the CPP apparatus, the mice preferred the drug-paired compartment over the saline-paired compartment, as indicated by a CPP-Score higher than zero in the test session [$t(15) = 7.827$, $P = 0.000$, **Table 2**], although there were individual differences. Pearson's correlation between the analyzed behavioral measures and the CPP-Score suggested that the anxiety-like variables in the EPM were the most useful measures to predict the magnitude of CPP behavior (**Table 2**). It is important to note that the significance of these correlations had a low probability of being driven by chance due to multiple comparisons according to the false discovery rate method (< 5 % or < 10 %; **Table 2**).Therefore, a PCA was performed to reveal which behavioral dimensions underlie the EPM behavior. Importantly, the EPM data were adequate to perform the PCA, as indicated by measures for sampling adequacy (Kaiser-Meyer-Olkin: KMO = 0.539; Bartlett's sphericity test: $X^2 = 45.566$, df = 28, $P = 0.019$). The PCA revealed three independent factors that together explained 74.69 % of the total variance (**Fig. 1a**). The most important factor for explaining the EPM behavior (**~**35 % of variance) was 'Anxiolysis' (i.e., reduced anxiety), as it included measures indicating increased open arm exploration and reduced grooming. A second factor (**~**23 % of variance) was named 'Caution' (i.e., reduced risk taking or sensation seeking), as it represented a high frequency of risk assessment behavior and increased latency to the first entry into an open arm. Finally, a third factor (**~**16 % of variance) represented 'Exploration', as it included both locomotion and rearing behavior. Importantly, when the Factorial Scores were

calculated for each factor and tested for correlations with the CPP-Score, the CPP-Score could only be predicted by scores in the 'Anxiolysis' factor (r = -0.762; P = 0.001) (**Fig. 1a, b**), indicating that mice that exhibited increased anxiolysis in the EPM (i.e., the less anxious mice) were less prone to seek cocaine in the CPP apparatus. The 'Anxiolysis' factor scores in the EPM were largely uncorrelated with the behaviors analyzed in the other tasks, including the anxiety-like behavior in the HB (correlations not shown).

3.2. Experiment II: High CPP-expressing mice had an increased number of neurons in the BLA

The LowCPP- and the HighCPP-mice used for this experiment differed in both their CPP-Score in the test session [t(8) = 8.139, P = 0.000] and in the anxiety-like behavioral dimension of the EPM [i.e., the 'Anxiolysis' factor score; t(8) = 2.530, P = 0.035]. Thus, the HighCPP-mice showed an increased CPP-Score and were the most anxious (**Fig. 2a, b**). The CPP extinction sessions confirmed the persistent, long-term differences in the CPP behavior of the LowCPP- and the HighCPP-mice ['group': F(1, 7) = 17.196, P = 0.004; 'day': F(11, 77) = 2.227, P = 0,021; **Fig. 2c**]. Regarding the histological results, there were no structural differences in the brains of the two groups in either the total number of NeuN+ neurons in the mPFC (**Fig. 2d**) or in the number of TH+ neurons in the VTA (**Fig. 2f**). However, the HighCPP-mice displayed an increased number of neurons in the BLA compared to the LowCPP-mice [t(8) = -2.377, P = 0.045, **Fig. 2e**]. The volume and neuronal density measures, as well as the results for each mPFC region, are available in the Supplementary Table.

4. DISCUSSION

The identification of factors that predict susceptibility to cocaine addiction is useful for the development of effective prevention strategies for this disorder. This study highlights the relationship between anxiety and cocaine vulnerability, as the mice that displayed an anxious phenotype (i.e., reduced anxiolysis) in the EPM were more prone to search for cocaine in a CPP paradigm, and, importantly, the mice with a higher CPP also exhibited an increased number of neurons in the BLA, a primary brain center for anxiety and fear responses.

While an individual's behavior is influenced by personality traits, mouse behavior is also driven by different underlying dimensions, such as activity, exploration, risk taking, sensation seeking, emotion and cognition. In this study, a variety of behavioral tests revealed that cocaine-seeking was exclusively predicted by anxiety-like measures in the EPM and not by measures of the novelty response (the exploratory variables in the EPM or the HB), novelty preference, hippocampal-dependent memory or despair-like behavior. In addition, a subsequent PCA analysis confirmed that the predicted anxiety-like measures in the EPM actually represented anxiety. While the animals' EPM performance was also determined by their risk taking and exploratory tendencies, anxiety was independent of these two dimensions and, importantly, was the only dimension associated with cocaine-seeking. On the other hand, it is relevant to note that the anxiety-like measures assessed in the HB did not predict the CPP behavior, in contrast to the EPM. A probable explanation is that the HB task was a less threatening situation than the EPM task for our mice (probably because they were more habituated to the testing procedure the day the HB was performed), due to the reduced frequency of grooming and risk assessment in the HB task, which likely reduced its accuracy for discriminating between anxious and non-anxious animals. Another possibility is that the different anxiety tests in rodents reflect different aspects of their emotionality (reviewed in [45]), such that the obtained conclusions would not necessarily converge between tasks.

Our results agree with studies reporting the usefulness of measuring rodents' unconditioned anxiety for predicting the degree of engagement in addiction-related behavior, such as in cocaine-induced CPP [27, 28] or in cocaine self-administration [26]. However, we did not find that the novelty/exploratory response was a relevant predictor, which differs from other reports (cocaine CPP: [20, 22, 29]; cocaine self-administration: [21, 23, 46]). As an explanation for this apparent controversy, it seems reasonable to consider that several cocaine vulnerable phenotypes (or cocaine vulnerability traits) are likely to exist; therefore, different experimental procedures may reveal one or another of these phenotypes. Interestingly, the studies that observed a relationship between the novelty response and CPP behavior relied on a subthreshold or a low cocaine dose, which likely affected the most susceptible animals (1 mg/kg

[20], 4 mg/kg [22] or 5 mg/kg [29]). However, when higher cocaine doses are used to establish conditioning, a relationship between anxiety and CPP expression is usually reported (10 mg/kg [27], 15 mg/kg [28] or 20 mg/kg in this study). Thus, certain behavioral vulnerability traits may only predict cocaine CPP or self-administration at specific doses (CPP: [22, 27, 29], self-administration [47]) because distinct cocaine dosages may elicit distinct behavioral and neurobiological responses. In this regard, it should also be noted that the theoretical interpretation of the CPP effect could be ambiguous because cocaine dose-dependently triggers the expression of stereotypies and other repetitive behaviors [48] that may be conditioned during the process, causing the animal to remain in the cocaine-paired compartment, regardless of the incentive elicited by the drug [49]. Another important factor is the protocol used to research the cocaine vulnerability behavioral traits. Many variables, such as the specific behavioral task/s used, the testing environment or the animals' previous experiences, may also modulate the elicited novelty and aversion, affecting both the exploratory and anxiety-like measures obtained [50, 51]. Finally, other factors that may explain the discrepancies among experiments are the biological characteristics of the study sample, such as the species used, or the animals' sex, age or their genetic background. For example, exploratory and anxiety-like behavior could greatly diverge among different mouse strains [52], and if rodents are bred for a high or a low expression of a certain behavior (e.g., anxiety [28] or novelty seeking [46]), they may show more extreme performances than a sample of animals that are not bred for any specific purpose.

Considering the different methods and outcomes between studies, the investigation of neurobiological correlates would add relevant information to the characterization of the diverse cocaine vulnerable phenotypes. In recent years, the limbic areas have emerged as key components of the addiction circuit, as they modulate the response of the classic addiction-related regions, such as the accumbens/striatum and the mesolimbic dopamine pathway [53-55]. Interestingly, the limbic system is also a major supporter of emotion and cognition, and it provides a potential neurobiological substrate where the addiction-vulnerability behavioral traits and the addictive behavior could overlap [54, 56]. Therefore, we sought to study whether the cocaine vulnerable mice (expressing both high anxiety-like behavior and increased CPP

behavior) and the non-vulnerable mice differed in the structural organization of the medial prefrontal cortex and the basolateral amygdala, two limbic areas that frequently show macrostructural alterations in cocaine addicts [12, 14-18]. There were no differences in the total number of mPFC neurons in the HighCPP-mice compared to the LowCPP-mice, which also exhibited a similar number of dopamine-releasing neurons in the VTA. However, the BLA of the HighCPP-mice exhibited an increased number of neurons, a structural correlate that may influence both their increased CPP and anxiety-like response. The role of the BLA has emerged to support the acquisition, maintenance, extinction and reinstatement of cocaine-related behaviors in rodents (CPP: [57, 58]; self-administration: [59, 60]). This regulation occurs in part through glucocorticoid hormones, which are secreted by the BLA in response to stress, acting through glucocorticoid receptors located in striatal dopaminoceptive neurons [61]. The BLA also has a widely recognized role in generating anxiety. Animal studies reveal that stimulations of the BLA terminals elicit the expression of unconditioned anxiety responses [62] that are prevented by BLA inactivation [63], and studies in C57BL/6J mice have described the role of BLA glutamatergic transmission for anxiety and stress-responsiveness [64]. In addition, anxiogenic experiences greatly modulate immediate early gene expression in BLA neurons [65], and an increased number of neurons in the BLA (as found in our HighCPP-mice) is a feature of well-known anxiogenic phenotypes, including the Roman low-avoidance rats [66, 67] or rats exposed to prenatal stress [68]. Therefore, as BLA activity is linked to both cocaine-induced CPP and unconditioned anxiety, it is tempting to assume that the increased number of neurons in the BLA of the HighCPP-mice is due to an increased number of excitatory neurons (that normally compose ~85 % of the BLA population [69]). Nevertheless, additional studies aimed at characterizing the different neuronal subpopulations in the BLA are required to establish a conclusion on this hypothesis.

In conclusion, this study shows that, in a sample of male C57BL/6J mice, a behavioral dimension representing unconditioned anxiety is associated with cocaine-seeking behavior (CPP) and structural features in the BLA, which may contribute to both the anxiety response and cocaine vulnerability. Although these results cannot be directly extrapolated to addicts, they support the role of anxiety as a predisposing factor for cocaine addiction [8] and the BLA as one

of its potential neurobiological substrates. Interestingly, a close relationship between anxiety and the BLA morphology and function has already been demonstrated in humans. In healthy individuals, differences in fearfulness or in perceived daily-life stress positively correlate with amygdala volume and gray matter density, particularly in the BLA [70-72]. Anxiety predicts both BLA functional connectivity and its response to certain fearful stimuli [71, 73], and an enlarged amygdala volume is present in anxiogenic pathological conditions, such as generalized anxiety disorder [74, 75]. Nevertheless, this trend seems to be reversed in cocaine addicts, as their amygdala volume is reduced compared to non-drug-users, despite the elevated anxiety characterizing the addicted population [12, 18]. As mentioned above, an unsolved question in studies of cocaine users is whether their characteristics are a cause or a consequence of prolonged drug use. In fact, cocaine itself has anxiogenic effects [76] and may induce multiple brain changes, including some at the macrostructural level, when used at a young age [77]. Therefore, chronic cocaine exposure may explain the reduced amygdala volume in the two available neuroimaging studies (where the addicts ranged from 1 to 27 years of cocaine use [12, 18]), especially considering that one study reported a negative correlation between the amygdala volume and the years of cocaine consumption, indicating that the volumetric decrease arose as a side effect of cocaine use [12].

In this study, cocaine exposure could not explain the anxiety-like behavior, as it was measured prior to the administration of cocaine. It is also unlikely to explain the histological outcomes because both the HighCPP- and the LowCPP-mice received equal amounts of the drug. However, we cannot completely rule out the possibility that different neuroadaptations emerged in each group after cocaine was administered. The investigation of a group of animals expressing a similar anxiety dimension that were never exposed to the drug and a deeper description of the amygdala and other anxiety-related brain areas would improve future animal research aimed at characterizing the neurobiological basis of the anxiety-related cocaine vulnerable phenotype.

Conflict of interest

The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1. The ‘Anxiety’ dimension in the EPM predicts cocaine-induced conditioned place preference behavior (n = 16 mice). For the sake of sampling adequacy, some variables that reported redundant information were not included in the PCA. a) The PCA of the EPM measures revealed three independent behavioral dimensions (factors): ‘Anxiolysis’, ‘Caution’ and ‘Exploration’. A behavior was considered to be included in a factor when its loading was > 0.7 in absolute value (highlighted in bold). The behaviors best representing each factor were those that loaded high in that factor but low in the others. The ‘open arms entries’ measure partially loaded in all three factors and thus did not accurately represent any of them. Please note that in the ‘Anxiolysis’ factor, the measures indicating a reduced anxiety (i.e. time in open arms calculated as the Anxiolysis-Score and head-dipping) loaded with a positive sign, while the measures indicating high anxiety (i.e. grooming) loaded negatively. This means that the higher the Factorial Score in the ‘Anxiolysis’ factor is, the more reduced the anxiety-like behaviours are (i.e. mice with lower scores in the ‘Anxiolysis’ factor are more anxious). a, b) Importantly, mice’s Factorial Score in the ‘Anxiolysis’ dimension was significantly correlated with the CPP-Score as assessed by Pearson’s correlation.

Fig. 2. Low and high CPP-expressing mice (n= 5/group) show structural differences in the basolateral amygdala. a-c) Behavioral features of mice selected for histology. HighCPP- and LowCPP-mice differed in both their 'anxiolysis' dimension in the EPM (a) and in the magnitude of cocaine-seeking (b), which persisted across extinction sessions (c). d-f) Histology results of the right hemisphere. There were not evident differences in the total number of neurons in the mPFC (d) or in the number of dopaminergic TH+ neurons in the VTA between groups (f), but HighCPP-mice had an increased number of neurons in the BLA compared to the LowCPP-group (e). Volume and cell density estimates for each structure, as well as for each mFPC region (Cg: cingulate, PrL: prelimbic, IL: infralimbic cortex), are included in the Supplementary material. Arrows indicate a positive neuron.

Table 1. Calculations of the behavioral scores.

Elevated Plus Maze	
Anxiolysis-Score	[time in open arms / (time in open arms + time in closed arms)]
Y-Maze	
Novelty-Score	[(time in the novel arm – mean time in the familiar arms) / (time in the novel arm + mean time in the familiar arms)].
Object recognition	
What-Score	(average exploration time for the old objects / average exploration time for the recent objects)
When-Score	(time spent exploring the old-static object / average exploration time for the recent objects)
Where-Score	[(time exploring the old-displaced object - time exploring the old-static object) / total time exploring both objects]
Cocaine-induced CPP	
CPP-Score	[(seconds spent in the cocaine-paired compartment - seconds spent in the saline-paired compartment) / seconds spent in both compartments]*100

Table 2. Results of the behavioral assessment in a sample of 16 mice and Pearson's correlations between behaviors and cocaine-seeking (the CPP-Score) in the test session.

	Mean ± SEM	Correlation with CPP-Score in test session
Elevated Plus Maze		
Time in open arms (s)	40.83 ± 4.70	**-0.687; *P* = 0.003***
Anxiolysis-Score	0.18 ± 0.02	**-0.605: *P* = 0.013***
Open arm entries (nº)	9.88 ± 0.91	0.351; *P* = 0.182
Open arm latency (s)	14.38 ± 3.50	0.152; *P* = 0.574
Locomotion in closed arms (cm)	834.13 ± 53.24	0.096; *P* = 0.724
Locomotion in open arms (cm)	171.56 ± 21.46	**-0.599; *P* = 0.014***
Total locomotion (cm)	1176.91 ± 63.27	-0.200; *P* = 0.458
Rearing (s)	11.28 ± 1.71	-0.031; *P* = 0.907

Grooming (s)	23.72 ± 2.27	0.432; *P* = 0.095
Head-Dipping (s)	12.81 ± 2.03	**-0.862; *P* = 0.000****
Risk-assesment (s)	3.79 ± 0.66	0.365; *P* = 0.164
Hole-Board		
Time in centre (s)	114.30 ± 9.21	-0.082; *P* = 0.768
Centre entries (nº)	28.86 ± 2.53	0.697; *P* = 0.797
Locomotion in centre (cm)	401.67 ± 23.25	-0.090; *P* = 0.739
Locomotion in periphery (cm)	841.01 ± 47.98	-0.132; *P* = 0.626
Total locomotion (cm)	1242.69 ± 54.67	-0.154; *P* = 0.568
Rearing (s)	10.86 ± 1.92	**-0.514; *P* = 0.042***
Grooming (s)	9.83 ± 1.06	0.216; *P* = 0.422
Hole-exploration (s)	24.25 ± 2.26	-0.355; *P* = 0.177
Risk-assesment (s)	1.38 ± 0.31	0.255; *P* = 0.340
Y-Maze		
Time in novel arm (s)	144.18 ± 9.06	-0.197; *P* = 0.464
Novelty-Score	0.23 ± 0.06	0.172; *P* = 0.525
Latency to enter the novel arm (s)	18.73 ± 7.65	-0.097; *P* = 0.721
Episodic-like object recognition memory		
What-Score	0.20 ± 0.04	-0.013; *P* = 0.962
When-Score	0.23 ± 0.05	0.040; *P* = 0.884
Where-Score	-0.10 ± 0.07	-0.081; *P* = 0.766
Object exploration in test trial (s)	48.58 ± 2.12	0.317; *P* = 0.232
Forced Swimming Test		
Latency of first immobility (s)	63.21 ± 2.82	-0.073; *P* = 0.788
Total immobility (s)	91.55 ± 6.03	-0.016; *P* = 0.953
Struggling (s)	55.22 ± 2.21	0.165; *P* = 0.542
Cocaine-induced CPP		
CPP-Score (habituation session)	-7.93 ± 3.03	
CPP-Score (test session)	31.90 ± 4.08	

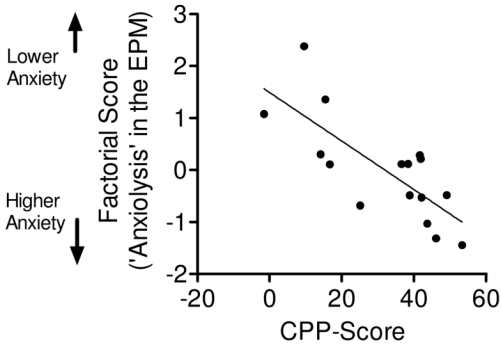
*$P < 0.05$, **$P < 0.001$

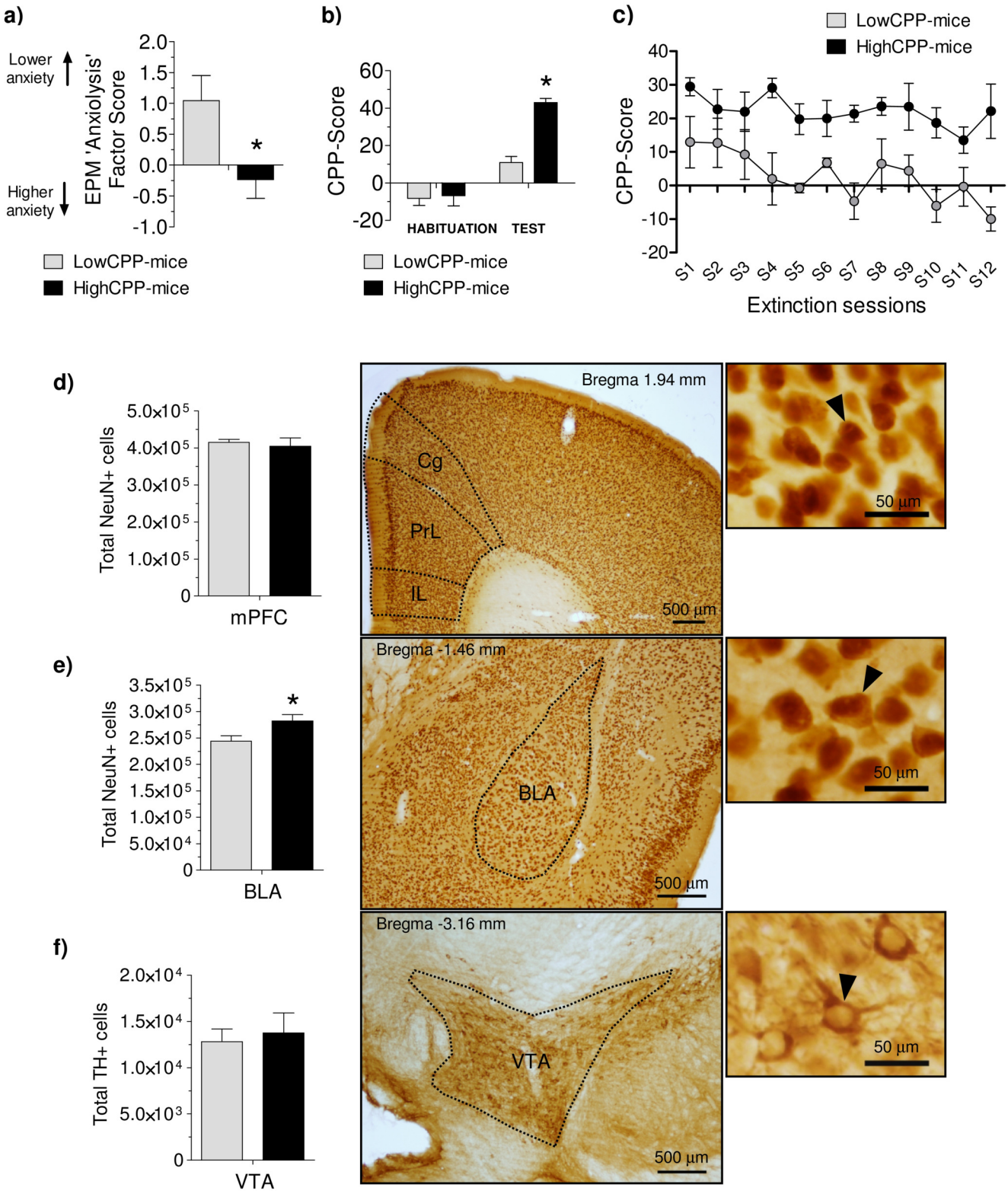
According to the false discovery rate method to control for multiple comparisons, significant correlations found in the EPM had less than a 5% (for correlations with $P = 0.003$ and 0.000) or less than 10% (for correlations with $P = 0.013$ and 0.014) probability to being attributed to chance. However, the significant correlation found in the HB task ($P = 0.042$) had as much as a 22% probability of being a false positive and thus should be interpreted with caution.

a)

Behaviour in the elevated plus maze	Factor 1 'Anxiolysis'	Factor 2 'Caution'	Factor 3 'Exploration'
Anxiolysis-Score	**0.928**	-0.052	-0.222
Open arm entries	0.429	-0.523	0.418
Open arm latency	0.243	**0.918**	-0.022
Rearing	-0.200	0.129	**0.891**
Grooming	**-0.748**	0.268	-0.122
Head-dipping	**0.879**	0.183	0.148
Risk assessment	-0.185	**0.714**	-0.030
Eigenvalue	2.796	1.871	1.309
Variance explained	34.94 %	23.39 %	16.36 %
r (Factor-Score and CPP-Score)	-0.762 *P* = 0.001*	-0.026 *P* = 0.923	-0.162 *P* = 0.548

b)




a)
Lower anxiety
Higher anxiety
EPM 'Anxiolysis' Factor Score
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
*
LowCPP-mice
HighCPP-mice
b)
CPP-Score
60
40
20
0
-20
*
HABITUATION
TEST
LowCPP-mice
HighCPP-mice
c)
LowCPP-mice
HighCPP-mice
CPP-Score
40
30
20
10
0
-10
-20
S1 S2 S3 S4 S5 S6 S7 S8 S9 S10 S11 S12
Extinction sessions
d)
Total NeuN+ cells
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
mPFC
Bregma 1.94 mm
Cg
PrL
IL
500 µm
50 µm
e)
Total NeuN+ cells
3.5x10^5
3.0x10^5
2.5x10^5
2.0x10^5
1.5x10^5
1.0x10^5
5.0x10^4
0
*
BLA
Bregma -1.46 mm
BLA
500 µm
50 µm
f)
Total TH+ cells
2.0x10^4
1.5x10^4
1.0x10^4
5.0x10^3
0
VTA
Bregma -3.16 mm
VTA
500 µm
50 µm