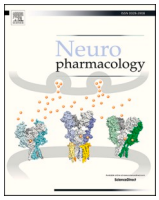
Neuropharmacology 273 (2025) 110453

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm



Repeated cocaine exposure and prolonged withdrawal induce spatial memory impairment and dysregulate the glutamatergic synapse composition in the dorsal hippocampus of male rats

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ARTICLE INFO

Handling Editor: Dr I. Spigelman

Keywords:
Adolescence
Cocaine
Hippocampus
Glutamate
Spatial memory

ABSTRACT

Adolescents are particularly susceptible to various forms of gratification, among which psychostimulants. During adolescence the hippocampus, a brain area relevant to spatial memory domain, undergoes maturational processes, such as structural and molecular reorganization of the excitatory synapses. Our goal was to reveal putatively enduring spatial memory deficits and molecular correlates in male rats exposed to repeated cocaine after a period of withdrawal.

Towards this goal, adolescent Sprague-Dawley male rats were exposed to chronic cocaine treatment (5 mg/kg/day, subcutaneously) for 15 days and, after 2 weeks of withdrawal, were subjected to spatial order object recognition (SOOR) test, a memory task based on the rat's ability to recognize objects displacement. Next, we investigated subcellular specific expression of markers of the glutamate synapse in the dorsal hippocampus.

Our findings show that withdrawal from repeated cocaine exposure during adolescence is associated with spatial memory impairment. Such deficit was correlated to a reduced expression and retention of NMDA receptor subunits, GluN1, GluN2A and GluN2B, at both synaptic and extra-synaptic sites, an effect indicative of impaired NMDA receptor trafficking. Analysis of endocytosis markers (Rab family of monomeric GTPase) revealed that cocaine-withdrawn rats favor the degradative pathway (Rab7-Rab9) over the recycling pathway (Rab11). In contrast, saline-treated rats primarily activate the recycling pathway. Our findings, mislocalization of glutamatergic receptors together with sorting of NMDA receptor towards degradation, rather than recycling, may contribute to the understanding of the mechanisms underlying the spatial memory deficits in male rats with an adolescent history of cocaine.

1. Introduction

Despite decades of public awareness and the recent introduction of a whole new plethora of synthetic drugs, the attraction of cocaine has not waned among adolescents and young adult (Fole et al., 2015). This is particularly concerning given that cognitive deficits have been observed in cocaine abusers (Bolla et al., 2003; Torregrossa et al., 2011; Verdejo-García et al., 2007) and brain functional alterations have been observed in the circuits subserving episodic memory (Tau et al., 2014).

Consistently, preclinical studies show that the interaction between psychostimulants and the adolescent brain can lead to significant cognitive dysfunctions (Caffino et al., 2022; Kantak, 2020). Acute and prolonged cocaine exposure during adolescence disrupts brain homeostasis (Caffino et al., 2015a, 2015b, 2018, 2019a, 2020a; Giannotti et al., 2015) and impairs recognition and recency memory, as assessed by a novelty discrimination task (Bevins and Besheer, 2006). Gourley's research group supports these findings, and found that adolescent cocaine exposure influences decision-making processes (DePoy et al.,

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https://doi.org/10.1016/j.neuropharm.2025.110453
Received 2 January 2025; Received in revised form 1 April 2025; Accepted 2 April 2025
Available online 3 April 2025

2017; Gourley et al., 2012).

During adolescence, the brain undergoes significant maturation (Andersen, 2003). The hippocampus experiences structural and functional refinement, as well as molecular reorganization of the synapses. These changes are crucial for shaping cognitive flexibility, adaptive responses to environmental stimuli, and decision-making (Arellano et al., 2024; Larsen and Luna, 2018; Naneix et al., 2012; Paus, 2024; Tan et al., 2017). At present, little is known on the impact of cocaine use during adolescence on the molecular reorganization of the excitatory synapses in the hippocampus and the consequent impact on spatial memory, a domain regulated by the activity of its dorsal subregion (dHip) (Basu and Nagel, 2024) and driven by NMDA-mediated glutamatergic neurotransmission (Baker and Kim, 2002; Barker and Warburton, 2015). Notably, several studies conducted in adult rats and mice already suggest that hippocampus is a critical brain region involved in addiction-related behaviors, especially in forming and recalling drug-related memories (Hernández-Rabaza et al., 2008; McGlinchey and Aston-Jones, 2018; Meyers et al., 2006; Shen et al., 2006; Wells et al., 2016) and facilitating drug relapse (Fuchs et al., 2005; Kutlu and Gould, 2016; Vorel et al., 2001).

This study adds to the existing body of literature addressed above, by investigating spatial memory and related hippocampal molecular alterations occurring following a period of withdrawal post a daily cocaine exposure in adolescence.

Specifically, adolescent rats [postnatal day (PND) 28] were injected daily with cocaine (5 mg/kg/day, subcutaneously) or saline for two weeks, and after two weeks of forced abstinence they were exposed to spatial order object recognition (SOOR) test, a memory task based on the rat's ability to recognize object displacements in a defined location (Barker and Warburton, 2011). Next, we focused our molecular analysis on the glutamatergic synapse of the rat dHip (Bliss and Collingridge, 1993). In particular, we investigated the expression and localization of the different subunits of the NMDA glutamate receptor (GluN1, GluN2A, and GluN2B) in the postsynaptic density and at extrasynaptic sites, as well as their major scaffolding proteins, such as Rabphilin3A, which anchors the GluN2A subunits (Yang et al., 2023), SAP102, which anchors the GluN2B subunits (Delgado et al., 2018) and PSD95, an index of post synaptic density integrity (Won et al., 2017). The rationale for this focus is as follows: 1) during development, the dynamic regulation of GluN2A and GluN2B accessory subunits of NMDA receptors is fundamental to cognitive processes. Alterations in this equilibrium have been strongly implicated in neurodegenerative and psychiatric diseases (Espana et al., 2021; Ladagu et al., 2023; Paoletti et al., 2013); 2) it has been demonstrated that even a single cocaine exposure influences synaptic transmission by altering NMDA subunit expression and composition in the ventral tegmental area, nucleus accumbens and prefrontal cortex, thus reopening a critical period of vulnerability (Bellone and Lüscher, 2012; Lüscher and Malenka, 2011). We here observed that adolescent exposure to cocaine followed by long-term withdrawal are associated with spatial memory impairments. Among the various mechanisms that could contribute to such behavioral effect, we suggest that it may occur by de-recruiting NMDA receptors from cell surface and by the activation of sorting mechanisms toward degradative, but not recycling, pathway.

2. Experimental procedures

Adolescent Sprague–Dawley male rats [postnatal day (PND) 22] were used (Charles River, Calco, Italy). Rats were maintained under standard conditions of temperature (21 °C) and humidity (50–60 %), and under a 12 h light/dark cycle (lights on/off: 7:00/19:00 h). A maximum of two siblings were taken from each litter to reduce 'litter effects', and upon their arrival, they were housed in groups (Chapman and Stern, 1978). Animals, housed in two per cage, were fed with standard rat chow (ssniff Spezialdiäten GmbH, Soest, Germany) and received tap water ad libitum. All animal procedures were conducted at the Department of Pharmacological and Biomolecular Sciences "Rodolfo Paoletti" (University of Milan, Milan, Italy), and carried out in accordance with the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive, 2010/63/UE). Authorization for animal use has been obtained from the Italian Ministry of Health (382/2023-PR). The experiments have been reported in compliance with the ARRIVE guidelines.

2.1. Experimental paradigm and tissue isolation

Adolescent rats (n = 40) were left undisturbed until PND 28. From PND 28, 22 animals received daily subcutaneous injections of cocaine (5 mg/kg/day in saline 0.9 %, Space Import Export srl, Milan, Italy) for 15 consecutive days (up to PND 42, a period that corresponds closely to adolescence in humans (Collins and Izenwasser, 2004) while other 18 animals received saline injections. Next, the animals were given a 15-day drug-free interval until PND 56. At PND 56, 12 saline- and 16 cocaine-treated rats performed the SOOR behavioral test as described below (Fig. 1B), while 6 animals per group were kept as non-tested counterpart (controls) randomly selected per treatment. To calculate group size, an effect size of Cohen's $d \geq 0.5$ with 95 % power at $p < 0.05$ has been applied; for the molecular analysis, 6 animals per group, as representative of the entire cohort of rats employed, have been used based on our previous experience and in line with the 3R principle. Immediately after the test animals were sacrificed by decapitation, and whole brains were rapidly removed. Dorsal hippocampi (dHip) were manually dissected from the entire brain indicatively from bregma −2.16 mm to bregma −6.12 mm according to The Rat Brain Atlas (George Paxinos, 2013), frozen on dry ice and stored at −80 °C.

2.2. Spatial order object recognition test (SOOR)

The SOOR task was performed in an open field arena of 60 ×60× 60 cm^3, in which animals had been habituated for 10 min the day prior to the test. The SOOR protocol comprised a sample phase of 10 min, and a test phase of 10 min performed 1 h after the sample phase (Fig. 1A). During the sample phase, animals were allowed to explore two identical objects, while in the test phase one of the objects was moved in the opposite corner and the object displacement was counterbalanced (Denninger et al., 2018). A total of 2 cocaine-tested animals out of 28 tested animals were excluded as outliers after statistical analyses.

The discrimination index (DI) was calculated as follows: (Exploration time of the Object in the familiar location – Exploration time of the object displaced)/total exploration time. Control animals were expected to spend more time exploring the displaced object during the test phase than the object that was not displaced. This means that their spatial memory is intact. Results were filmed and analyzed using ANY-maze software (Any-Maze v7.15).

2.3. Protein extraction and Western Blot analysis

Proteins from the whole homogenate, as well as the post-synaptic and extra-synaptic fractions of the dHip, were isolated from six animals per group as previously described (Piva et al., 2020). The total amount of proteins was measured according to the Bradford Protein Assay procedure (Bio-Rad, Milan, Italy), using bovine serum albumin as the calibration standard.

Western Blot on the isolated fractions were run as previously described (Mottarlini et al., 2022). The conditions of the primary antibodies were the following: anti-GluN1 (1:1000 Cell Signaling Technology Inc., RRID: AB_1904067), anti-GluN2A (1:1000 Cell Signaling Technology Inc., RRID: AB_2112295), anti-p-GluN2B (Y1472) (1:1000

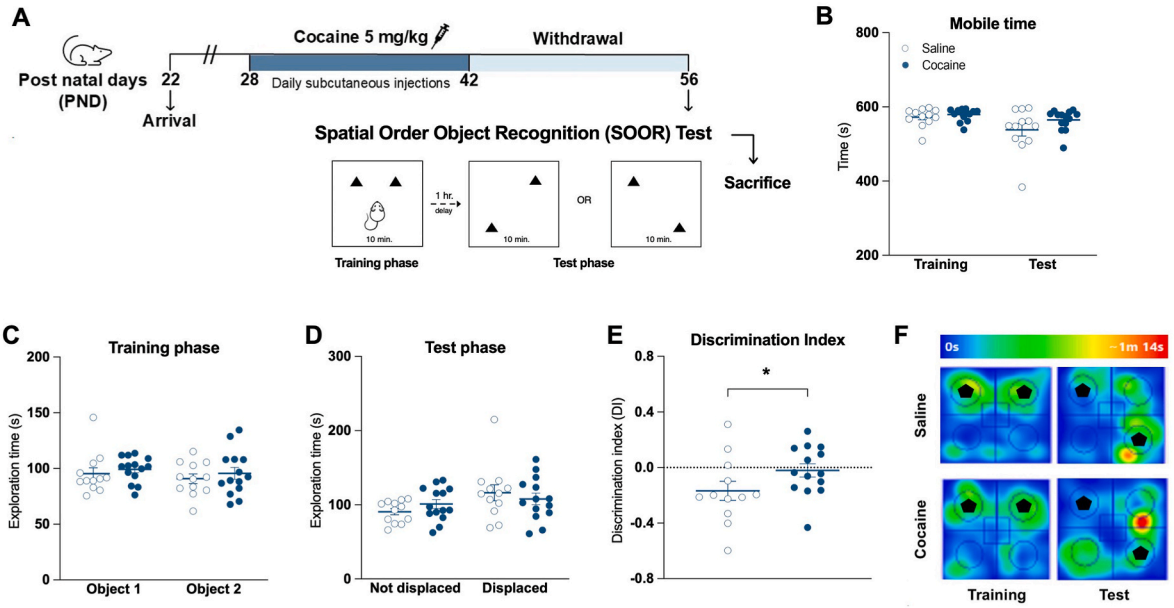

Fig. 1. Long-term developmental cocaine exposure followed by two weeks of withdrawal impairs spatial memory
Experimental paradigm and SOOR test design (**A**). Measure of the locomotor activity of saline and cocaine-withdrawn rats during the training and the test phase expressed as mobile time (s) (**B,** two-way ANOVA interaction *(treatment x test)*: F (1, 48) = 0.9996, p = 0.3224). Exploration time of the two objects during the training (**C,** two-way ANOVA interaction *(treatment x test)*: F (1, 48) = 0.0076, p = 0.9311) and the test (**D,** two-way ANOVA interaction *(treatment x test)*: F (1, 48) 1.538, p = 0.2209) phases. The discrimination index (DI) for the two experimental groups (**E,** unpaired student's t-test: t = 2.525, p = 0.0189) has been calculated as (exploration time of the object in the familiar location – exploration time of the object displaced)/total exploration time. Representative heatmaps of locomotor activity during training and test phase for both saline and cocaine-withdrawn groups (**F**). Data are expressed in histograms and represent the mean ± SEM (sal-test n = 12, coc-test n = 14). Two-way ANOVA followed by Tukey's multiple comparisons test (**D**); unpaired student's t-test (**E**). Statistical significance *p < 0.05.

Cell Signaling Technology Inc., RRID: AB_1549657), anti-GluN2B (1:1000 Cell Signaling Technology Inc., RRID: AB_2798506), anti-SAP102 (1:1000 Cell Signaling Technology Inc., RRID: AB_2799325), anti-Fyn (1:1000 Millipore, RRID: AB_310056), anti-Rab5 (1:1000 Cell Signaling Technology Inc., RRID: AB_832625), anti-Rab7 (1:1000 Abcam., RRID: AB_882241), anti-Rab9 (1:1000 Abcam, # ab179815), anti-Rab11 (1:1000 Cell Signaling Technology Inc., AB_10693925), anti-PSD95 (1:1000 Cell Signaling Technology Inc., RRID: AB_2292883), anti-Arc/Arg 3.1 (1:500 BD Transduction, RRID: AB_399886).

Results were standardized to β-actin control protein that was detected by evaluating the band density at 43 kDa after probing with a monoclonal antibody with a 1:10000 dilution (Merck Life Sciences Srl, Milano, Italy, RRID: AB_476744). Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories, Segrate (MI), Milan, Italy). Gels were run two or three times each, and the results represent the average from two/three different runs (the full-size cropped WB bands are presented in Supplementary Figs. 2 and 3). We used a correction factor to average the different gels: correction factor gel B = average of (OD protein of interest/OD β-actin for each sample loaded in gel A)/(OD protein of interest/OD β-actin for the same sample loaded in gel B) (Caffino et al., 2020b).

2.4. Statistics

Data were collected from individual animals as independent determinations and are presented as means and standard errors (SEM). Results were analyzed by two-way analysis of variance (ANOVA) with the factors treatment and exposure to the cognitive test as independent variables. When dictated by relevant interaction terms, Tukey's multiple comparisons test was used to characterize differences among individual groups of rats. The discrimination index was analyzed by unpaired Student's t-test and One-Sample *t*-test. F and p values of independent variables of two-way ANOVA and t and p values of unpaired Student's t-test were reported in Supplementary Tables 1–9. Data from three subjects were excluded from the final dataset because they deviated from the mean by 2 standard deviations (SDs) and were therefore considered outliers. Prism 8.2.1 (GraphPad Software, Prism v8.2.1, San Diego, CA, USA) was used to analyze all the data. Significance for all tests was assumed at $p < 0.05$.

3. Results

3.1. Withdrawal from adolescent cocaine exposure alters spatial memory

The object displacement recognition memory was tested in saline- and cocaine-withdrawn rats at PND 56 (Fig. 1A). During both the training and test phases, there were no significant differences in the total mobility (Fig. 1B) and distance travelled (Supplementary Fig. 1) of all animals. Specifically, during the training phase, both saline- and cocaine-withdrawn rats explored the objects placed in the arena without showing any significant difference between the objects (Fig. 1C), indicating no discernible preference for any particular object, thus providing a baseline level of exploration. During the test phase, the saline-treated animals exhibited a significantly longer duration of exploration on the displaced object (Fig. 1D and F). In contrast, cocaine-withdrawn rats did not exhibit any significant preference between the two objects (Fig. 1D and F). This effect is clearly represented by the discrimination index (DI), which is significantly different between the two experimental groups (Fig. 1E). Furthermore, the DI close to 0 in cocaine-withdrawn rats further underscores a failure in recognition of the displaced object.

3.2. Spatial memory deficits observed following 2-week withdrawal from cocaine are associated with a dysregulated glutamatergic synapse composition in the dorsal hippocampus

To explore a molecular target that may subserve the spatial memory

deficit, we investigated critical determinants of the glutamatergic synapse in the dHip. We focused our attention on the expression and localization of the NMDA receptor subunits, analyzing the obligatory subunit GluN1 and the two accessory subunits GluN2A and GluN2B in both the synaptic and extra-synaptic sites.

In the synaptic sites, we observed that cocaine-withdrawn rats show increased levels of GluN1 and GluN2A, compared to saline-treated animals (Fig. 2A, B left). Notably, the SOOR test induced an increase of GluN1 only in saline-treated animals, while in cocaine-withdrawn rats it significantly reduced both GluN1 and GluN2A levels (Fig. 2A, B left). The SOOR test reduced GluN2B protein expression regardless of the treatment (Fig. 2C left). In line with the effects induced by SOOR test in cocaine-withdrawn rats on the NMDA subunits, we observed a reduction in their relative scaffolding proteins Rabphilin and SAP102 (Fig. 2D and E), suggesting that the cognitive test reduced the localization and stabilization of those receptors in the postsynaptic site only in cocaine-withd.

In parallel, in the extra-synaptic site, withdrawal from cocaine exposure did not alter either GluN1 or GluN2B protein levels (Fig. 2A, C right). The SOOR test significantly increased the GluN2B subunit levels in saline-treated animals (Fig. 2C right), whereas, in cocaine-withdrawn rats, it reduced the expression of the GluN1 and GluN2B subunits compared to cocaine-no test animals (Fig. 2A, C right). The GluN2A subunit was not detected in the extra-synaptic site in line with the literature (Miwa et al., 2008).

Of note, Pearson's correlation and linear regression analyses show significant correlations between the DI and the protein levels of GluN1 (Fig. 4A), GluN2A (Fig. 4B), Rabphilin (Fig. 4C) in the post synaptic site, and with the GluN2B in the extra-synaptic site (Fig. 4F). Such effects further support a relation, even though not causative, between the failure of the test performance in cocaine-withdrawn rats and the reduced protein levels of the abovementioned molecular targets.

3.3. Spatial memory deficits observed following 2-week withdrawal from cocaine are associated with a dysregulated endocytic pathway in the dorsal hippocampus

To evaluate whether the reduced localization and stabilization of the NMDA receptor in both the postsynaptic density and the extra-synaptic sites induced by the exposure to the cognitive demanding test in cocaine-withdrawn rats could be ascribed to the involvement of endocytic pathways (Mignogna and D'Adamo, 2017), we first measured the expression levels of Rab proteins, responsible for the vesicular trafficking of receptors either towards a degradative or recycling pathways.

SOOR test increased Rab5 levels, the endosomal GTPase necessary for receptor internalization and formation of early endosomes (Bucci et al., 1992), in both saline and cocaine-withdrawn rats (Fig. 3B), thus pointing to the formation of early endosomes in response to the SOOR test regardless of the adolescent treatment. Interestingly, the test exposure significantly increased Rab11 protein levels, marker of recycling pathway, only in saline animals (Fig. 3C), suggesting that the correct performance in the test directs the early endosomes to recycling. Conversely, Rab9 and Rab7, critical intermediates of the formation of late endosomes directed toward a degradative pathway (Soldati et al., 1995), are significantly increased after the spatial task in cocaine-withdrawn rats (Fig. 3D and E), with no effects in saline or cocaine-withdrawn no test rats (Fig. 3D and E).

As a potential mechanism responsible for the differential

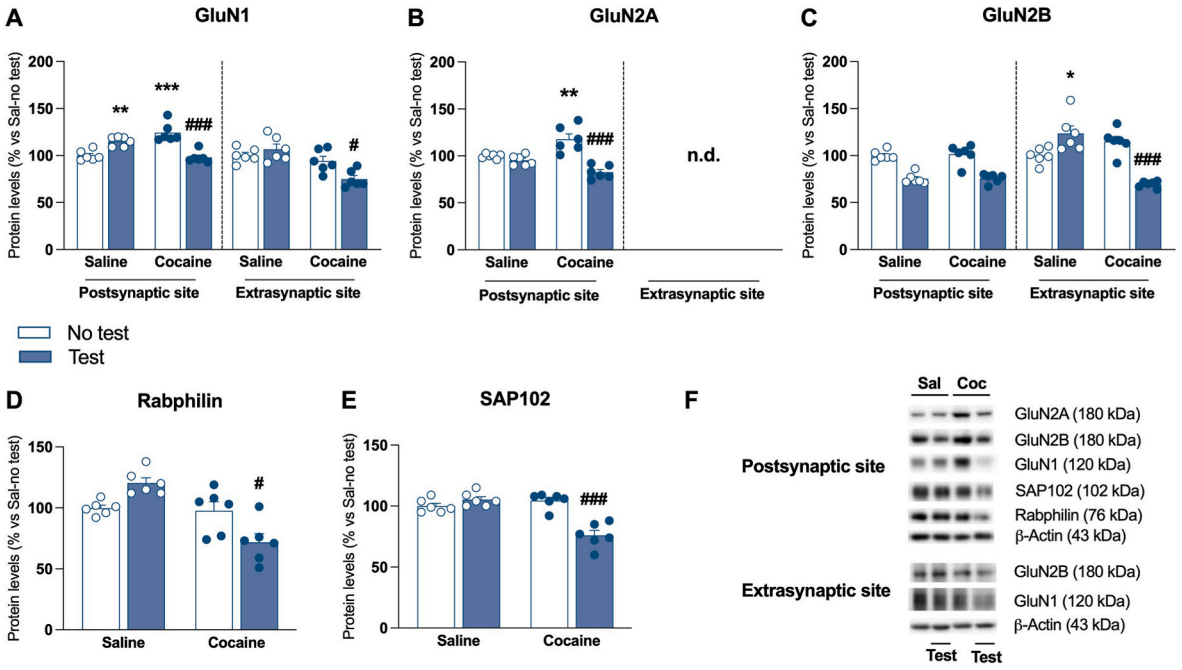

Fig. 2. Effects of a two-week withdrawal from repeated cocaine exposure during adolescence on NMDA receptor subunits and scaffolding proteins expression in the dorsal hippocampus of rats exposed to the SOOR test

Protein levels of GluN1 (**A,** postsynaptic site: two-way ANOVA interaction *(treatment x test)*: $F(1, 20) = 54.15$, $p < 0.0001$; extrasynaptic site: two-way ANOVA interaction *(treatment x test)*: $F(1, 20) = 8.750$, $p = 0.0078$), GluN2A (**B,** two-way ANOVA interaction *(treatment x test)*: $F(1, 20) = 20.43$, $p = 0.0002$) and GluN2B (**C,** postsynaptic site: two-way ANOVA interaction *(treatment x test)*: $F(1, 20) = 0.06684$, $p = 0.7986$; extrasynaptic site: two-way ANOVA interaction *(treatment x test)*: $F(1, 20) = 44.49$, $p < 0.0001$) subunits of NMDA receptors were measured in postsynaptic (left) and extra-synaptic (right) sites of the dorsal hippocampus (dHip). The expression levels of the scaffolding proteins Rabphilin (**D,** two-way ANOVA interaction *(treatment x test)*: $F(1, 20) = 15.11$, $p = 0.0009$) and SAP102 (**E,** two-way ANOVA interaction *(treatment x test)*: $F(1, 20) = 33.92$, $p < 0.0001$) were measured in the postsynaptic site. Representative immunoblots for each protein are shown in panel (**F**). Data are expressed in histograms as percentage of saline-treated rats not exposed to the behavioral test and represent the mean ± SEM of six rats per group.

Two-way ANOVA followed by Tukey's multiple comparisons test. Statistical significance $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs saline-no test; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs cocaine-no test.

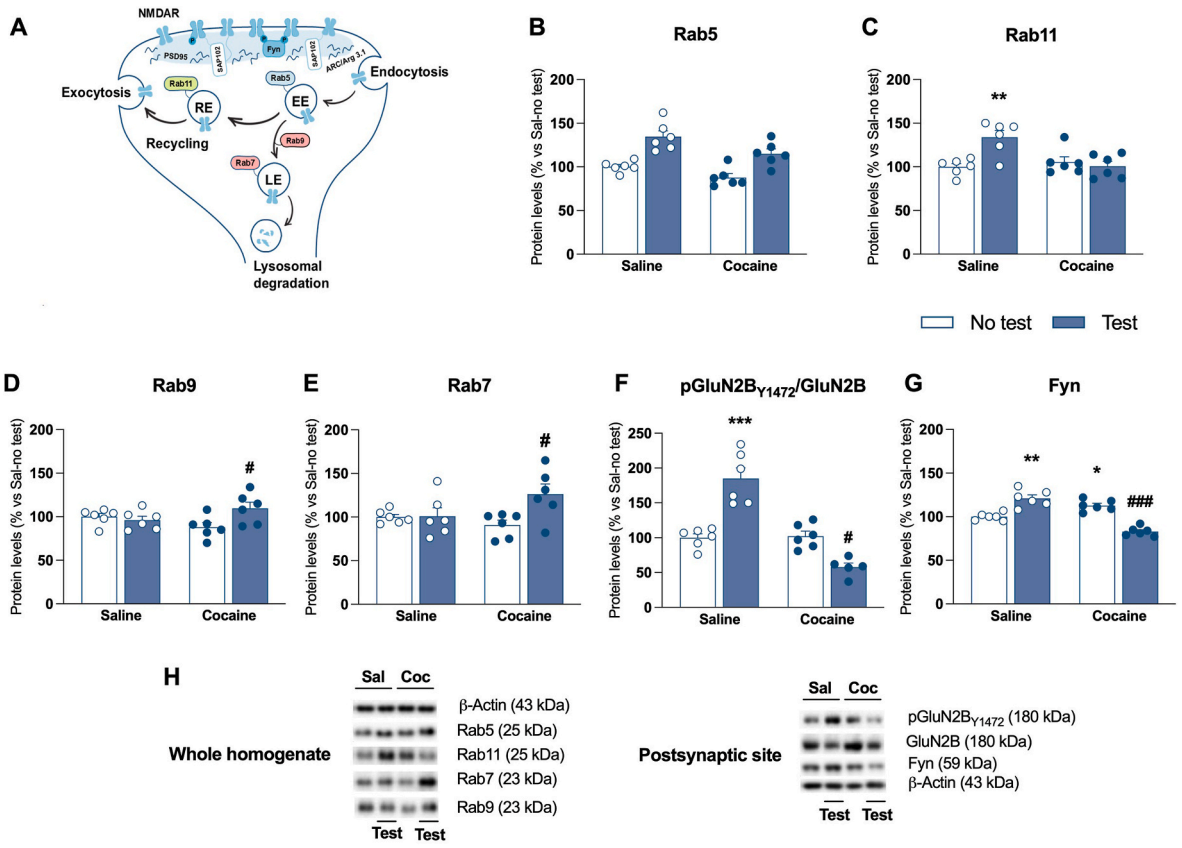

Fig. 3. Effects of a two-week withdrawal from repeated cocaine exposure during adolescence on the recycling and degradation pathway in the dorsal hippocampus of rats exposed to the SOOR test

Schematic representation of the internalization and trafficking of endosomes in the post-synapse. EE, early endosome; RE, recycling endosome; LE, late endosome (**A**). The expression levels of Rab5 (**B,** two-way ANOVA interaction *(treatment x test)*: F (1, 20) = 0.5364, p = 0.4724), Rab 11 (**C,** two-way ANOVA interaction *(treatment x test)*: F (1, 20) = 10.67, p = 0.0039), Rab 9 (**D,** two-way ANOVA interaction *(treatment x test)*: F (1, 20) = 5.872, p = 0.0250) and Rab 7 (**E,** two-way ANOVA interaction *(treatment x test)*: F (1, 20) = 4.527, p = 0.0460) were measured in the whole homogenate of the dHip. The levels of GluN2B phosphorylation in Tyr1472 (**E,** two-way ANOVA interaction *(treatment x test)*: F (1, 19) = 38.99, $p < 0.0001$) and of the Fyn kinase expression (**F,** two-way ANOVA interaction *(treatment x test)*: F (1, 20) = 87.79, $p < 0.0001$) were measured in the postsynaptic density fraction. Representative immunoblots for each protein are shown in panel (**F**). Data are expressed in histograms as percentage of saline-treated rats not exposed to the behavioral test and represent the mean ± SEM of six rats per group. Two-way ANOVA followed by Tukey's multiple comparisons test $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs saline-no test; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs cocaine-no test.

involvement of recycling or late endosome under these experimental conditions, we evaluated the phosphorylation site of the GluN2B subunits (i.e., Tyr1472, a phosphorylation mediated by Fyn) known to be involved in the formation and shuttling of endosomes. SOOR test increased the levels of GluN2B phosphorylation, here expressed as ratio between pGluN2B and its total form GluN2B, in saline animals, while reducing it in cocaine-withdrawn rats (Fig. 3 F). In parallel, the analysis of the Fyn kinase, responsible for the GluN2B phosphorylation, revealed similar effects after test exposure. In fact, Fyn levels in the postsynaptic density were increased in saline rats, whereas reduced in cocaine-withdrawn animals (Fig. 3 G). In addition, withdrawal from cocaine exposure per se increased Fyn levels (Fig. 3 G). This effect is indeed in line with the activation of the recycling pathway observed in saline animals, and with the activation of the degradation machinery in cocaine-withdrawn rats after the test exposure.

Interestingly, Pearson's correlation and linear regression analyses show significant correlations between the DI and the protein levels of pGluN2B/GluN2B (Fig. 4D) and Fyn (Fig. 4E) in the postsynaptic site, further supporting a strong relationship among the impaired spatial memory and the altered endocytic machinery.

3.4. Spatial memory deficits observed following 2-week withdrawal from cocaine are associated with an impaired synaptic structural stability in the dorsal hippocampus

To have further insight into the architecture of the synapses in our experimental condition, we measured the levels of the post synaptic density protein PSD95, an index of post synaptic density integrity, and of Arc/Arg3.1, as a marker of cytoskeletal stability. The withdrawal from cocaine exposure per sè induced an increase in PSD95 (Fig. 5B), while it did not change Arc/Arg3.1 protein levels (Fig. 5A). After test exposure, we observed that both protein levels are significantly increased in saline-treated rats (Fig. 5A and B), while reduced for Arc/Arg3.1 levels (Fig. 5A) or unaltered for PSD95 (Fig. 5B) in cocaine-withdrawn rats. Interestingly, Pearson's correlation analyses revealed a significant negative correlation between the DI and the levels of expression of Arc/Arg3.1 and PSD95 (Fig. 5C and D), further sustaining an opposite relationship between the abundance of those markers and the SOOR test outcome.

4. Discussion

Our main behavioral finding is the spatial memory deficit observed after two weeks of withdrawal from repeated cocaine exposure during

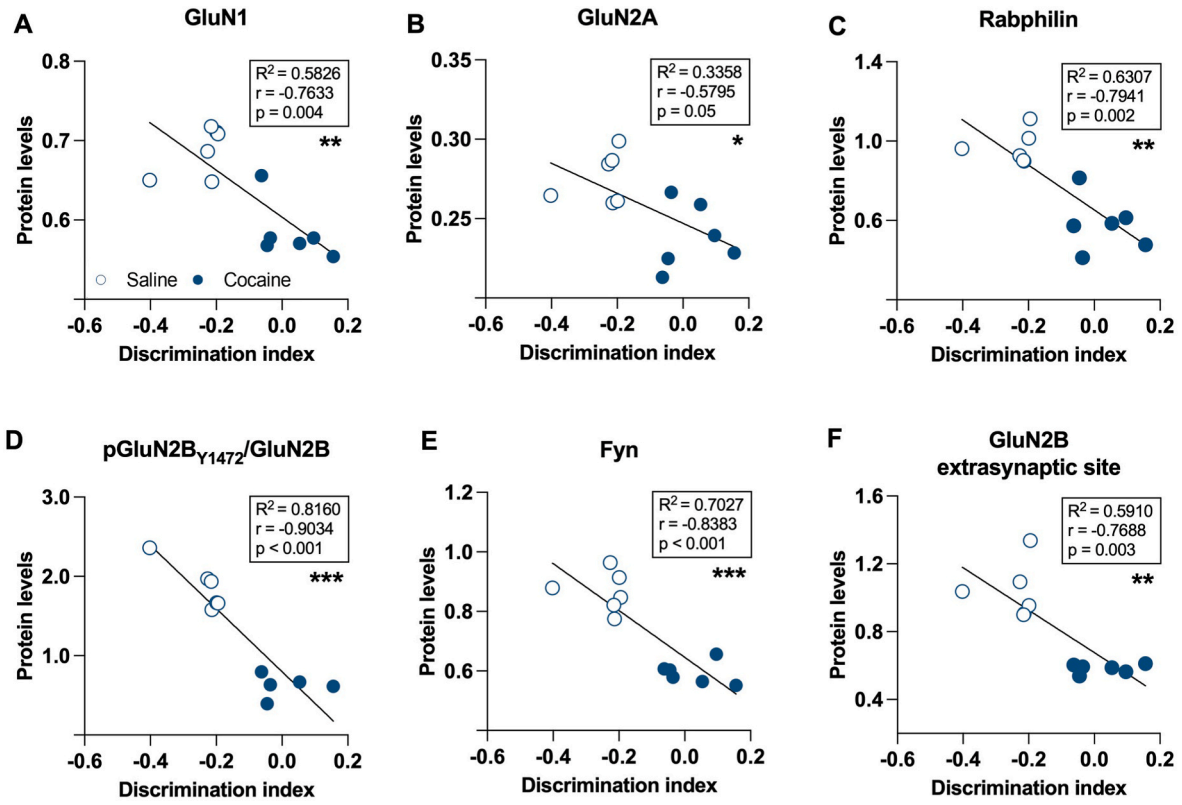

Fig. 4. Correlation analyses between the SOOR test performance and the protein levels of critical determinants of the glutamatergic synapse in saline and cocaine-withdrawn rats.

Pearson's product–moment correlation (r) analyses and linear regression (R2) analyses between the DI and their relative protein expression of GluN1 (**A**), GluN2A (**B**), Rabphilin (**C**), pGluN2B/GluN2B (**D**), Fyn (**E**) and GluN2B at extra-synaptic sites (**F**). Pearson's product–moment correlation (r) analyses and linear regression analyses (R2) statistical significance *p < 0.05, **p < 0.01, ***p < 0.001.

adolescence. We suggest that the reduced expression and retention of NMDA receptor subunits at both synaptic and extra-synaptic sites may contribute, at least in part, to such deficit. Furthermore, cocaine appears to disrupt the normal NMDA receptor redistribution in response to the cognitive challenge, favoring the degradative pathway over the recycling pathway. Taken together, our data offer a link to explain the spatial memory impairment observed after a protracted withdrawal (2 weeks) from adolescent cocaine exposure.

Spatial memory, in terms of recognition of an object in a novel location, together with the underlying neuronal circuitry, reaches maturity specifically during adolescence, since the preferential exploration of the object displaced in a novel location starts at PND 38, while peri-adolescent rats (PND 31) show no preference (Contreras et al., 2019). In line with these maturational processes, we here show that long-term exposure to a low dose of cocaine negatively interferes with spatial memory development. Notably, under the same experimental conditions of cocaine treatment and length of withdrawal, we have previously found impaired recognition memory (Mottarlini et al., 2020) as well as recency memory (Castillo Díaz et al., 2023). The present data expand our understanding of the impact of withdrawal from cocaine exposure during adolescence on different memory domains as it shows that also spatial memory is impaired, an effect in line with the impairment in the novel location recognition memory induced by chronic cocaine treatment during adolescence, and not adulthood, followed by a period of withdrawal. These findings also support the hypothesis that the impaired spatial memory might be due to the inability of cocaine-withdrawn rat to set in motion a physiological NMDA-mediated glutamate neurotransmission, which is necessary for memory processes to take place (Guidi et al., 2015; Nakazawa et al., 2004; Tsien et al., 1996). In addition, the present results, together with our previous findings, indicate that, despite its acute mechanism of action that targets the dopaminergic system in the mesolimbic pathway (i.e., blockade of the dopamine transporter), cocaine affects other brain regions of the limbic system and cerebral cortex such as the dHip, the perirhinal cortex (Mottarlini et al., 2020) or the prefrontal cortex (Castillo Díaz et al., 2023).

At molecular level we observed several changes indicative of a different response to the SOOR test between saline- and cocaine-withdrawn rats in the dHip. Following repeated cocaine treatment and long-term withdrawal, we observed an activation of the NMDA receptor system, as shown by increased expression of the mandatory GluN1 subunit and its accessory subunit GluN2A with no effect on the GluN2B subunit. This maladaptive neuroadaptation may underlie increased cocaine seeking and vulnerability to relapse as previously observed in adult rats following a prolonged abstinence from cocaine self-adminstration paradigm (Werner et al., 2020).

In our experimental conditions, the overactivation of the glutamate synapse in cocaine-withdrawn rats may oppose the ability of the cognitive demanding test to further recruit NMDA receptors in the membrane, and thus to further activate the glutamate system. In fact, the reduced expression of GluN1, GluN2A and GluN2B following the test at synaptic sites in the dHip of cocaine-withdrawn rats is corroborated by the reduced expression of the scaffolding protein SAP102 and Rabphilin, indicating not only their reduced synaptic availability but also that such subunits are less stable, and therefore less functional, at the synapse. Further, our analysis revealed that the synaptic reduction in cocaine-withdrawn rats is mirrored by similarly reduced levels of GluN1 and GluN2B receptor subunits at extra-synaptic sites, thus ruling out the possibility of a SOOR-induced receptor mislocalization and consequently hypofunctionality, in line with recent data demonstrating that

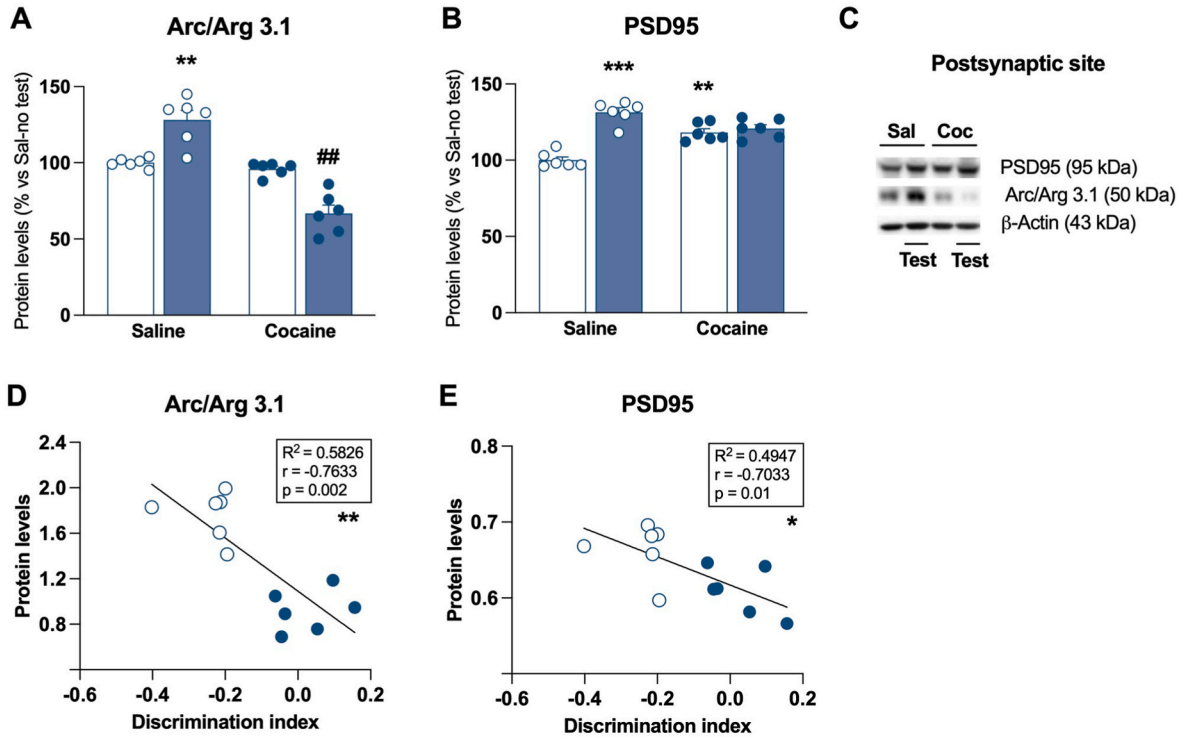

Fig. 5. Effects of a two-week withdrawal from repeated cocaine exposure during adolescence on synaptic integrity markers in the dorsal hippocampus of rats exposed to the SOOR test. Protein levels of the cytoskeletal marker Arc/Arg 3.1 (**A,** two-way ANOVA interaction *(treatment x test)*: $F(1, 20) = 43.90$, $p < 0.0001$) and of the post synaptic protein PSD95 (**B,** two-way ANOVA interaction *(treatment x test)*: $F(1, 20) = 33.95$, $p < 0.0001$) were measured in the postsynaptic density fraction. Pearson's product–moment correlation (r) analyses and linear regression (R2) analyses between the DI and their relative protein expression of Arc/Arg 3.1 (**C**) and PSD95 (**D**). Representative immunoblots for each protein are shown in panel (**E**). Data are expressed in histograms as percentage of saline-treated rats not exposed to the behavioral test and represent the mean ± SEM of six rats per group. Two-way ANOVA followed by Tukey's multiple comparisons test $^{**}p < 0.01$, $^{***}p < 0.001$ vs saline-no test; $^{\#\#}p < 0.01$ vs cocaine-no test. Pearson's product–moment correlation (r) analyses and linear regression analyses (R2) statistical significance $^{*}p < 0.05$, $^{**}p < 0.01$.

NMDA receptor blockade in juvenile male rats impairs the novel object location memory (Narattil and Maroun, 2024). These data raise the possibility that the SOOR test in cocaine-withdrawn rats may have activated specific endocytic pathways (Zhang et al., 2022). Accordingly, Rab5, a protein responsible for the formation of the early endosome (Zerial and McBride, 2001), is indeed increased in both saline- and cocaine-withdrawn rats exposed to the SOOR test, in line with the possibility that the SOOR test per sè recruits endosomes, regardless of the adolescent treatment. Further analysis revealed that whereas saline-treated rats exposed to the SOOR test showed increased expression of Rab11, which is responsible of the vesicular recycling to the post-synaptic membrane and fundamental for spatial memory acquisition (Siri et al., 2020), cocaine-withdrawn rats exposed to the SOOR test showed an increase in Rab9 and Rab7 expression, i.e., proteins mediating the lysosomal degradation pathway. These data suggest a different fate for NMDA receptor subunits in response to the SOOR test depending on the adolescent treatment (saline vs. cocaine). In fact, the performance of SOOR test in saline-treated rats increases the recycling of, at least, GluN1 in the membrane, while following withdrawal from cocaine exposure the SOOR test targets GluN1, GluN2A and GluN2B subunits at both synaptic and extra-synaptic sites directly to degradative pathways. As a potential process responsible for such different fate of NMDA receptors under these experimental conditions, we focused our attention on the GluN2B subunit, since we took advantage of the fact that such subunit holds a phosphorylation site (i.e., Tyr1472), a phosphorylation mediated by Fyn and known to be involved in the formation and shuttling of endosomes. Our findings reveal that, in saline-treated rats exposed to the test, the level of GluN2B phosphorylation are significantly increased in line with an increase of the kynase Fyn suggesting that the Glun2B subunit is indeed recycled and re-exposed to the membrane. Conversely, in cocaine-withdrawn rats exposed to the SOOR test, pGluN2B and Fyn are significantly reduced corroborating the previously seen effects on Rab 9 and Rab7, which is indicative of GluN2B internalization and degradation.

Together with these findings, we have also observed that the expression of Arc/Arg3.1, a marker of cytoskeletal stability critical in learning and memory processes and in response to drugs of abuse (Caffino et al., 2014, 2017; Fumagalli et al., 2006; Gao et al., 2018; Mottarlini et al., 2024), is not altered in cocaine-withdrawn rats. However, we observed a different response of Arc/Arg3.1 expression, which was enhanced in saline-treated rats while reduced in cocaine-withdrawn animals: this may be due to the fact that the previous history of adolescent cocaine exposure may have influenced the response to the cognitive test.This reduction might suggest not only the potential inability of cocaine-withdrawn rats to remodel the synaptic structural architecture, but also a reduced synaptic strenght, thus impairing the demanding process of spatial memory learning. Moreover, also PSD95, the main determinant of post synaptic density integrity at the excitatory synapse (Won et al., 2017), exhibits a different pattern of expression in saline or cocaine-withdrawn rats exposed to the test. The increased PSD95 levels after test exposure in saline-exposed animals further corroborates the necessity of stable synaptic integrity while performing the test. Cocaine-withdrawn animals show stable and significantly higher levels of PSD95, as we previously observed in the habenula and amygdala of rats exposed to a long-access protocol of cocaine self-administration (Caffino et al., 2019b, 2019c). The increased levels of PSD95 is suggestive of cocaine-induced reshaping of synaptic architecture and of increased formation of dendritic spines in the dHip, as it has previously observed in the prefrontal cortex or nucleus accumbens (DePoy et al., 2014; Robinson, 1999). We can hypothesize that this

increase blunts cocaine-withdrawn rats capability to further modulate the levels of PSD95 in response to the cognitive task strenghtening the hypothesis of their impaired ability to suistain the learning task, as we observed in cocaine-withdrawan rats exposed to the novel object recognition test (Mottarlini et al., 2020).

It is interesting to point out that the changes observed in the herein investigated determinants of glutamate synapse appear to be correlated with the SOOR test performance. In fact, the correlation analyses show a significant negative correlation between the discrimination index of the SOOR test and the protein levels of the different glutamate targets, confirming that the positive discrimination index of cocaine-withdrawn rats corresponds to reduced protein levels, as opposed to saline-treated rats.

Our study has potential limitations. First, our experimental design (cocaine exposure + withdrawal) does not allow us to discriminate the effects of cocaine treatment from those related to forced abstinence. Thus, our interpretation, relying on the combination of these two experimental conditions, may underestimate the contribution of each individual condition. We cannot infer a causal link between the cognitive impairment and the reorganization of the glutamate synapse; however the set of molecular changes we provide suggests a potential mechanism, albeit of a correlational nature, underlying the spatial memory deficit induced by 2-week withdrawal from repeated cocaine exposure. Second, we exclusively utilize male rats. Since we are aware that biological and hormonal differences between males and females can influence neurobiological and behavioral outcomes in addiction-related paradigms (Torres, 2022), we cannot generalize our findings to both sexes. Moreover, even though we employed only adolescent rats, we can reasonably suggest that the effects herein seen are limited to the adolescent population. We based this statement on previous work demonstrating an impairment of spatial discrimination following chronic exposure to cocaine in adolescence, but not adulthood (Fole et al., 2015). In addition, we have demonstrated that alterations of other memory domains such as recency memory and recognition memory occur selectively in adolescent, but not adult, rats following repeated cocaine exposure followed by prolonged withdrawal (Castillo Díaz et al., 2023; Mottarlini et al., 2020).

In conclusion we have observed a spatial memory impairment in rats exposed to repeated cocaine exposure followed by long-term withdrawal during adolescence. We hypothesize that such deficit may result, at least in part, from a complex mechanism that involves the de-recruiting of NMDA receptors from cell surface and the activation of lysosomal degradative pathways together with the concomitant blunting of recycling pathways. We propose that internalized NMDA receptors subunits are sorted to degradation rather than stored in recycling endosomes in cocaine-withdrawn rats, an effect that may subserve the rat's inability to correctly perform in the cognitive task.

CRediT authorship contribution statement

Francesca Mottarlini: Writing – review & editing, Funding acquisition, Conceptualization. **Paolo Miglioranza:** Validation, Methodology, Formal analysis, Data curation. **Beatrice Rizzi:** Methodology, Data curation. **Sofia Taddini:** Investigation, Data curation. **Susanna Parolaro:** Investigation, Data curation. **Daniele Caprioli:** Writing – review & editing, Funding acquisition. **Roberto Ciccocioppo:** Writing – review & editing, Funding acquisition. **Lucia Caffino:** Writing – review & editing, Supervision, Conceptualization. **Fabio Fumagalli:** Writing – review & editing, Funding acquisition, Conceptualization.

Role of funding source

This work was supported by MUR PRIN 20227HRFPJ (to FF and RC) as well as by grants from MIUR Progetto Eccellenza 2023–2027. We acknowledge financial support under the National Recovery and Resilience Plan (NRRP), Mission 4, Component 2, Investment 1.1, Call for tender No.1409 published on 14.9.2022 by the Italian Ministry of University and Research (MUR), funded by the European Union - NextGenerationEU - CUP B53D23031390001 (DC), G53D23007570001 (FF), J53D23017900001 (RC) - Grant Assignment Decr1369 adopted on 01/09/2023 by the MUR.

Declaration of competing interest

The authors declare that they have no known competing financial interests that could have influenced the work reported in this paper.

Acknowledgement

Dr. Francesca Mottarlini is recipient of a fellowship from the Zardi-Gori Foundation. Beatrice Rizzi is supported by cycle XXXVIII of the PhD programme in Theoretical and Applied Neuroscience, DM351 (Finanziamenti PNRR). Susanna Parolaro is supported by cycle XL of the doctorate in Pharmacological Biomolecular Sciences, Experimental and Clinical, Department of Pharmacological and Biomolecular Sciences "Rodolfo Paoletti", Università degli Studi di Milano

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2025.110453.

Data availability

Data will be made available on request.

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