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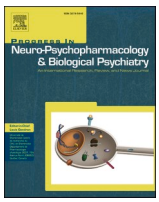

Recency memory is altered in cocaine-withdrawn adolescent rats: Implication of cortical mTOR signaling

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ABSTRACT

In humans, cocaine abuse during adolescence poses a significant risk for developing cognitive deficits later in life. Among the regions responsible for cognitive processes, the medial prefrontal cortex (mPFC) modulates temporal order information via mechanisms involving the mammalian-target of rapamycin (mTOR)-mediated pathway and protein synthesis regulation. Accordingly, our goal was to study the effect of repeated cocaine exposure during both adolescence and adulthood on temporal memory by studying the mTOR pathway in the mPFC. Adolescent or adult rats underwent repeated cocaine injections for 15 days and, after two weeks of withdrawal, engaged in the temporal order object recognition (TOOR) test.

We found that repeated cocaine exposure during adolescence impaired TOOR performance, while control or adult-treated animals showed no impairments. Moreover, activation of the mTOR-S6-eEF2 pathway following the TOOR test was diminished only in the adolescent cocaine-treated group. Notably, inhibition of the mTOR-mediated pathway by rapamycin injection impaired TOOR performance in naïve adolescent and adult animals, revealing this pathway to be a critical component in regulating recency memory.

Our data indicate that withdrawal from cocaine exposure impairs recency memory via the dysregulation of protein translation mechanisms, but only when cocaine is administered during adolescence.

1. Introduction

Adolescents are known to be more sensitive to the rewarding effects of commonly abused drugs and, conversely, less sensitive to adverse consequences following drug abuse (Casey et al., 2008; Spear, 2000). Because they are more susceptible to feeling the rewarding effects and less susceptible to the adverse consequences, interfering with an actively developing brain during adolescence may be detrimental to the individual. We have already demonstrated that exposure to cocaine during adolescence leads to structural and functional alterations of medial prefrontal cortex (mPFC) following both acute (Caffino et al., 2018a; Caffino et al., 2017b; Giannotti et al., 2015) and repeated exposure (Caffino et al., 2018b; Caffino et al., 2015b; Caffino et al., 2015a; Giannotti et al., 2016; Giannotti et al., 2013; Mottarlini et al., 2020). This finding is highly relevant given that the mPFC continues to develop during adolescence in addition to and maintains a critical role in mediating cognition via integrating inputs from different cortical and subcortical subregions (Caballero et al., 2016). In conjunction with our work, Gourley's lab has shown that cocaine administration during adolescence negatively affects decision-making skills by studying complex and long-term changes in the orbitofrontal cortex (DePoy et al., 2017). These lines of evidence are reinforced by the notion that cocaine exposure alters cognitive processes in humans (Bolla et al., 2003; Verdejo-García et al., 2007), an effect that may contribute to continued drug use (Torregrossa et al., 2011).

While it may be difficult to fully model complex cognitive processes using animal subjects, such model systems provide a means to evaluate specific forms of cognition as well as cause-effect relationships. Hence, we have recently studied the effect of developmental cocaine exposure on recognition memory by employing the so-called Novel Object Recognition test (Bevins and Besheer, 2006), which is known to be affected following THC or alcohol exposure during adolescence (Marco et al., 2017; Prini et al., 2018). We found that exposure to cocaine during adolescence, but not adulthood, impaired recognition memory primarily

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through impairments in the compartmentalization of the neurotrophin BDNF in the perirhinal cortex (Mottarlini et al., 2020).

This study focused on recency memory (otherwise called temporal order memory). Recency memory, defined as a type of memory specific to the order in which items/events are experienced, is primarily mediated by the mPFC (Kesner et al., 1994; Mitchell and Laiacona, 1998) and can be measured through the temporal order object recognition (TOOR) test. This memory task is based on the rat's ability to remember a specific object seen at different moments during the test. By measuring the time rats spend exploring one object or the other (from sample phase 1 or 2, see methods for details), this test allows us to measure their recency memory when inspecting the objects. Deficits in both the animals' and humans' ability to respond to cortical-dependent tasks have been observed even months after cessation of cocaine exposure (Kantak, 2020; Liu et al., 2008), with poorer outcomes detected in early-onset (adolescent) cocaine users (Lopes et al., 2017; Vonmoos et al., 2014).

The consolidation phase of new memories require de novo synaptic protein synthesis at dendrites via the activity of the mammalian target of Rapamycin (mTOR) signaling pathway (Costa-Mattioli and Monteggia, 2013; Hoeffer and Klann, 2010) in addition to the following initiation and elongation steps of protein translation processes through the modulation of downstream targets (Laplante and Sabatini, 2012; Zhang et al., 2021). Evidence suggests that cocaine not only regulates mTOR signaling (Bailey et al., 2012), more specifically, its main downstream effector, the S6 protein (Wu et al., 2011), but the elongation factor 2 (eEF2) as well, which is an essentialprotein required for ribosomal translocation during protein elongation (Sossin and Costa-Mattioli, 2019). Moreover, mTOR signaling is implicated in the development of cocaine craving (Werner et al., 2018), cue-induced reinstatement of cocaine-seeking behavior(James et al., 2014; Wang et al., 2010), and cocaine-induced locomotor sensitization and conditioned place preference (Bailey et al., 2012), further confirming the pivotal role of mTOR in cocaine-related mechanisms. However, little is known about mTOR's contribution to temporal memory in adolescent vs. adult rats with a history of repeated cocaine exposure.

In this study, we combined behavioral and pharmacological approaches with molecular analyses to elucidate the role of mTOR signaling in the mPFC during cocaine withdrawal and following a temporal memory task in adolescent vs. adult cocaine-exposed rats. To this end, adolescent and adult rats were injected daily with cocaine (5 mg/kg/day, subcutaneously) or saline and sacrificed two weeks after the last drug exposure, immediately after the end of the temporal order object recognition (TOOR) test. mTOR signaling was analyzed in the infralimbic (IL) and prelimbic (PL) cortices, two mPFC subregions in close proximity within the brain that serve individually distinct functions in response to cocaine. For instance, initiation of cocaine seeking appears to rely primarily on the PL, whereas extinction learning to block cocaine seeking primarily depends on the IL (Gass and Chandler, 2013; Peters et al., 2008; Van den Oever et al., 2010). Of note, very little is known about cocaine-induced developmental changes in the IL and PL, especially regarding protein synthesis mechanisms.

2. Experimental procedures

2.1. Animals and housing

Adolescent [postnatal day (PND) 28] and adult (PND 65) male Sprague–Dawley rats were used (Charles River, Calco, Italy), maintained under standard conditions of temperature (21 ± 1 °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 was taken from each litter in order to reduce "litter effects" and upon their arrival were housed in groups (Chapman and Stern, 1978). Animals were fed with standard rat chow (ssniff Spezialdiäten GmbH, Soest, Germany) with tap water ad libitum. All animal procedures were conducted at the Department of Pharmacological and Biomolecular Sciences (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 6 March 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 (914–2017-PR). The experiments have been reported in compliance with the ARRIVE guidelines.

2.2. Drugs

Cocaine (Space Import Export srl, Milan, Italy) was dissolved in saline 0.9%. Rapamycin (DivBio Science Italia, Italy) was dissolved in saline 0.9% + DMSO 10% + Tween-80 5%.

2.3. Experimental design and procedures

Fig. 1A shows a schematic representation of the experimental paradigm. Briefly, adolescent rats (experiment 1) ($n = 40$) arrived at PND 24, they were housed in four animals per cage, in classical home cages, with food and water ad libitum, and left undisturbed until PND 28. From PND 28, 20 animals received subcutaneous cocaine injections daily for 15 consecutive days (dose 5 mg/kg/day) up to PND 42, a period that roughly approximates adolescence in humans (Collins and Izenwasser, 2004) while other 20 animals received saline injections. This repeated treatment was followed by a 15-day drug-free period till PND 57, in which 24 of the treated animals performed the TOOR behavioral task as described below (Fig. 1B). Immediately after the test, animals were sacrificed by decapitation, and whole brains were frozen on dry ice for the microdissection procedure.

Adult animals (experiment 2) ($n = 24$) arrived at PND 60 and were housed three animals per cage, in classical home cages, with food and water ad libitum and left undisturbed until PND 65. Room conditions and treatment were exactly as used previously for the adolescent group. Cocaine or saline injections were administered from PND 65 to PND 79; the abstinence period took place from PND 80 to PND 94. At PND 94 TOOR task was performed, and animals were sacrificed for tissue collection.

In experiments 3 and 4 (Fig. 1C), adolescent and adult rats ($n = 40$, 20 adolescents, 20 adults) arrived at PND 24 (adolescents) and PND 60 (adults) respectively, and they were housed in the same conditions as the other groups. At PND 30 or PND 67, 12 adolescent and 12 adult animals received the first i.p. rapamycin injection (15 mg/kg) to prevent mTOR activation. Twenty-four hours later rats were trained in the TOOR task using the same protocol as described below and another rapamycin injection (15 mg/kg) was administered immediately after the second sample phase. The test was made as mentioned above 3 h later (Fig. 1C).

2.4. Tissue collection

Bilateral punches of PL and IL (from Bregma +4.20 mm to Bregma +2.52 mm) were microdissected according to (Giannotti et al., 2016) with minor modifications. Briefly, the frozen brains were acclimatized for 1 h in the cryostat at the temperature of −15 °C before being sliced. Coronal slices of 220 μm were placed on histological slides (4–5 brain sections per slide) and a microdissection needle of 1 mm of inner diameter was used to dissect the PL and IL. The dissected tissue pellets were blown out of the needle in centrifuge tubes, immediately frozen on dry ice, and stored at −80 °C until being processed for molecular analysis.

2.5. Behavioral protocol: temporal order object recognition test

The TOOR task was performed in an open field arena of 60x60x60 cm^3, in which animals had been habituated for 10 min the day prior to

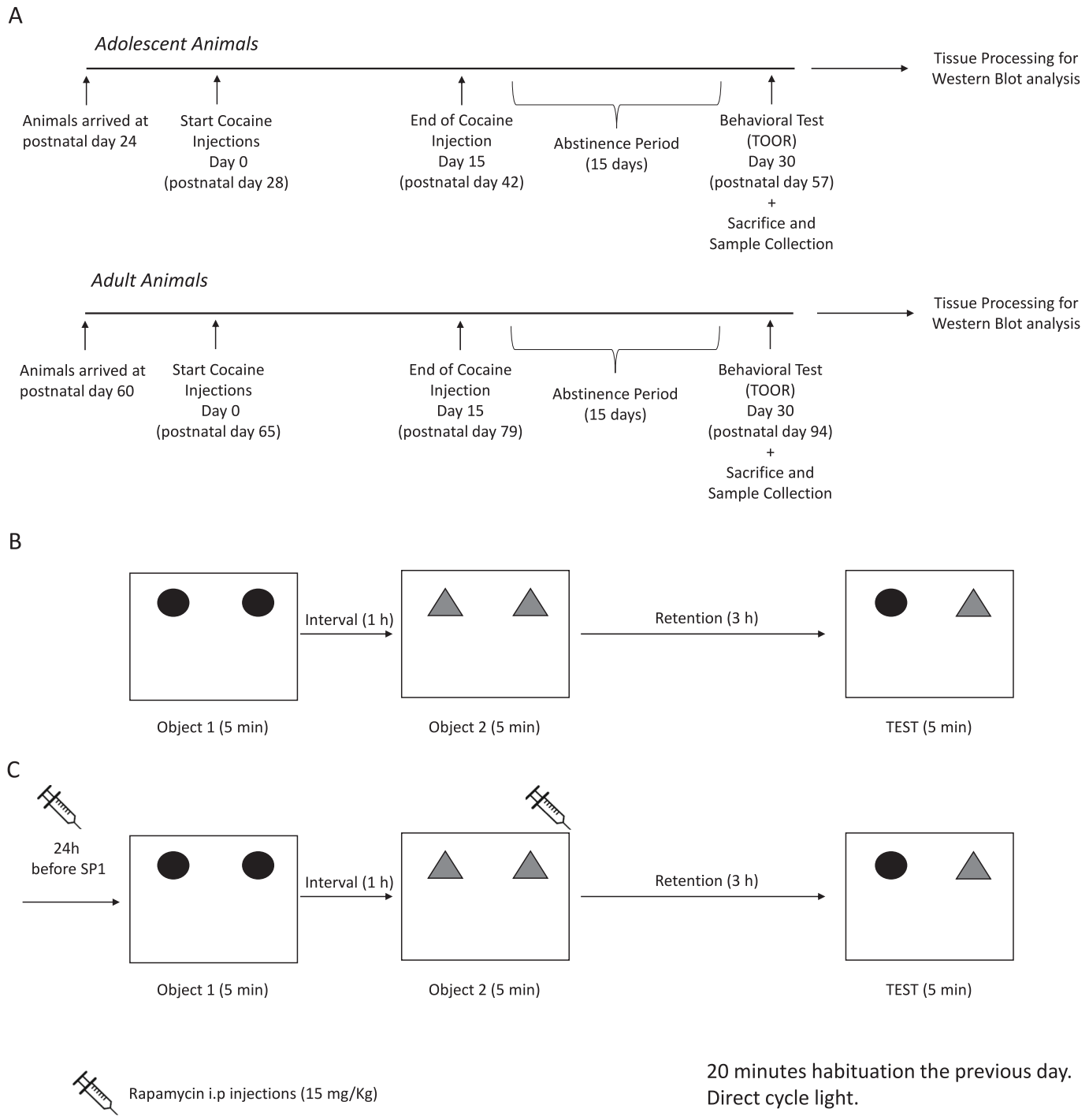

Fig. 1. Scheme of the experimental protocol. (A) Experimental schedule for both adolescent and adult animals. (B) TOOR task protocol. Animals were exposed to a 5 min sample phase (SP), followed by 1 h interval and a second SP for another 5 min. After a 3 h retention interval a 5 min test was performed. (C) TOOR task protocol in rapamycin-treated animals.

the test. During the TOOR protocol animals are allowed to explore two pairs of identical objects during two sample phases of 5 min separated by an interval of 1 h. After 3 h of retention, we tested the animals in a test phase of 5 min during which they have the possibility to explore one object of the first sample phase and one object of the second sample phase. Objects are located in two non-opposite corners of the arena and the objects used in the two sample phases were characterized by different colors, shapes, and materials, to facilitate their distinction by rats. The use of objects during the two sample phases and object placement during the test phase was counterbalanced. The arena and the objects were cleaned with 0.1% acetic acid between each phase. A total of 3 out of 24 tested animals were excluded as outliers after statistical analysis.

The discrimination index (DI) is calculated as follows: (Object 1 exploration time – Object 2 exploration time) / total exploration time. Control animals are expected to explore the object that they have seen during the sample phase 1 more time than the object they have seen during the second sample phase. This means that their recency memory is intact, and they remember better what they have seen more recently. Results were filmed and analyzed using ANY-maze software (Any-Maze

v7.15).

2.6. Preparation of protein extracts and western blot analysis

The IL and PL were homogenized in a glass-glass potter using a cold buffer containing 0.32 M sucrose, 1 mM Hepes solution, 0.1 mM EGTA, 0.1 mM PMSF, pH = 7.4, in the presence of a complete set of protease inhibitors and a phosphatase inhibitor cocktail. Total proteins were measured in the whole homogenate according to the Bradford Protein Assay procedure (Bio-Rad, Milan, Italy), using bovine serum albumin as the calibration standard.

Western Blot on the whole homogenate was run as previously described (Caffino et al., 2017a) The conditions of the primary antibodies were the following: anti-p-mTOR (1:1000 Cell Signaling Technology Inc., RRID: AB_330970), anti-p-S6 (1:1000 Cell Signaling Technology Inc., RRID: AB_2716873), anti-peEF2 (1:1000 Cell Signaling Technology Inc., RRID: AB_10015204), anti-mTOR (1:1000 Cell Signaling Technology Inc., RRID: AB_330978), anti-S6 (1:1000 Cell Signaling Technology Inc., RRID: AB_331355), anti-eEF2 (1:1000 Cell Signaling Technology Inc., RRID: AB_10693546).

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 1:4000 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. S1 and S2). 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., 2020).

2.7. Data analysis and statistics

Data were collected in individual animals (independent determinations) and are presented as means and standard errors. 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. However, when no interaction between cocaine exposure and the cognitive test was observed, the significant main effects were reported and differences among individual groups of rats were evaluated using the Bonferroni post-hoc test. The discrimination index was analyzed by unpaired *t*-test and one-sample t-test. Subjects were eliminated from the final dataset if their data deviated from the mean by 2

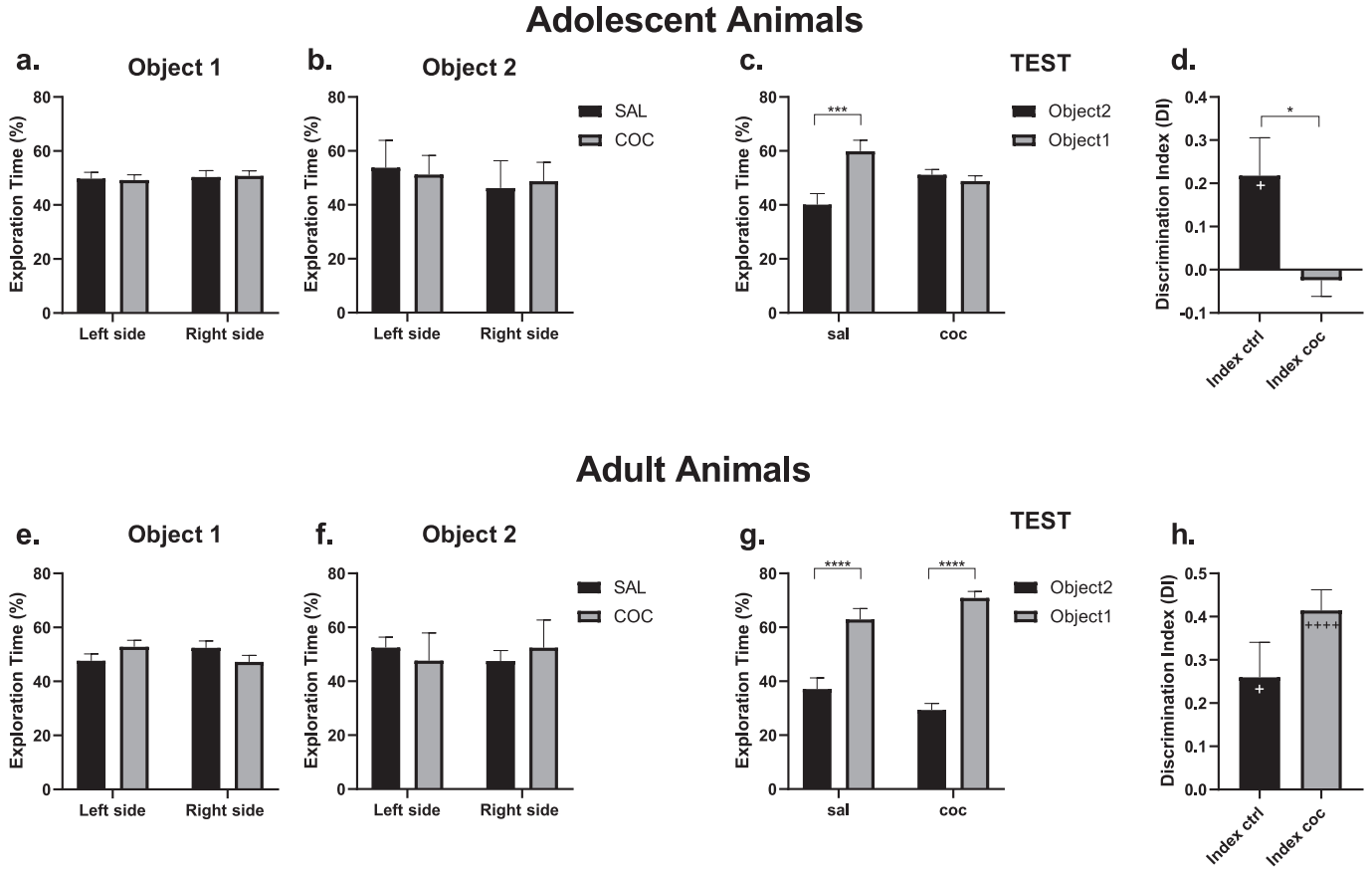

Fig. 2. Exposure to repeated cocaine during adolescence, but not during adulthood, impairs the temporal order memory. (A, B) Percentage of the exploration time for each object during the sample phase 1 (panel A) and sample phase 2 (panel B) of saline- and cocaine-treated animals during adolescence. No preference for any side of the arena was found for the two objects. (C) Percentage of the exploration time for each object during the test phase of saline- and cocaine-treated animals during adolescence. (D) Discrimination index (DI) for the two groups of adolescent animals. (E, F) Percentage of the exploration time for each object during the sample phase 1 (panel E) and sample phase 2 (panel F) of saline- and cocaine-treated animals during adulthood. No preference in the exploration was found between the sides of the arena for any of the objects. (G) Percentage of the exploration time for each object during the test phase of saline- and cocaine-treated animals during adulthood. (H) DI for the two groups of adult animals.
Asterisks indicate significant statistical differences between groups, Tukey *post-hoc* after two-way ANOVA *$p < 0.05$; ***$p < 0.001$, ****$p < 0.0001$; unpaired student's *t*-test (panel D) *p < 0.05; one-sample t-test +p < 0.05; ++++$p < 0.0001$. SAL: saline; COC: cocaine; ctrl: control.

SDs. 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. Long term cocaine-exposure impairs adolescent temporal order memory after a 2-week abstinence period

At the end of the 2-week cocaine abstinence period (PND 57), recency memory was tested in the TOOR task. No differences were found in the exploration time for both objects during the sample phases, indicating that animals had no natural preferences for any of them, and discarding any possible preference for the right or the left side of the arena (Fig. 2A. $F_{(1,44,\ interaction)} = 0.045$; $p = 0.83$, $F_{(side)} = 0.23$, $p = 0.63$; $F_{(treatment)} = 0.0003$; $p = 0.98$; Fig. 2B. $F_{(1;44,\ interaction)} = 0.98$; $p = 0.32$, $F_{(side)} = 3.951$, $p = 0.053$; $F_{(treatment)} = 0$; $p = 0.99$). During the test, as expected, control animals explored more time the object seen during the sample phase 1 in comparison to the object seen during the sample phase 2 (Fig. 2C. $F_{(1,40,\ interaction)} = 12.11$, $p = 0.0012$; $F_{(treatment)} = 0.00$; $p = 0.99$; $F_{(object)} = 7.50$; $p = 0.0092$). In this sense, they were able to distinguish the temporal order in which the objects were presented, recognizing the former object as a novelty and the latter object as a familiar one, indicating that their recency memory was intact. Conversely, animals that received cocaine for 15 days during adolescence showed an impairment in the performance of the TOOR task. These animals were not able to distinguish the objects temporal presentation leading to an equally distributed exploration time on both of them during the test (Fig. 2C. $F_{(1,40,\ interaction)} = 12.11$, $p = 0.0012$; $F_{(treatment)} = 0$; $p = 0.99$; $F_{(object)} = 7.50$; $p = 0.0092$). This result is also reflected in the discrimination index (DI). DI is statistically different from 0 for the control group, and statistically different from the cocaine-treated group (Fig. 2D. Control group: one-sample *t*-test $_{(t\ =2.475;\ df=9)}$, $p = 0.0353$; Treated group: one-sample t-test$_{(t\ =0.6442;\ df=10)}$; $p = 0.05340$; Unpaired t-test$_{(t\ =2.614;df=19)}$, $p = 0.0171$).

Next, we performed the same protocol for the adult group of animals. Contrary to the adolescent animals, no effects of the cocaine treatment and abstinence period were found in the TOOR task performance. During the test, both control and cocaine-treated groups performed correctly, showing a higher exploration time for the object seen during the sample phase 1 in comparison to the object presented in the sample phase 2 (Fig. 2G. $F_{(1;30,\ interaction)} = 5.821$; $p = 0.022$; $F_{(treatment)} = 0.001$; $p_{(treatment)} = 0.97$; $F_{(object)} = 105.5$; $p = 0.0001$). This means that animals were able to distinguish between the two objects. As it occurs for adolescent animals, no natural preferences were found for the adult group during the sample phases 1 and 2 (Fig. 2E. $F_{(1,41,\ Interaction)} = 3.43$, $p = 0.08$, $F_{(side)} = 0.07$, $p = 0.78$, $F_{(treatment)} = 0.19$, $p = 0.65$; Fig. 2F. $F_{(1,32,\ interaction)} = 3.22$, $p = 0.082$, $F_{(side)} = 0.001$, $p = 0.97$, $F_{(treatment)} = 0$, $p > 0.99$). In addition, a positive and different from 0 DI value was found for both groups (Fig. 2H. Control group: One sample t-test$_{(t\ =3.204;\ df=7)}$; $p = 0.0150$; Cocaine group: One sample t-test$_{(t\ =8.609;\ df=8)}$; $p = 0.0001$; Unpaired t-test$_{(t\ =1.685,\ df=1)}$; $p = 0.2185$). This means that adults' recency memory was not affected by the repeated exposure to cocaine and the subsequent withdrawal period, pointing out the vulnerability and susceptibility to the psychostimulant effects during adolescence.

3.2. Temporal order memory activates the mTOR signaling in the prefrontal cortex

In order to understand the molecular basis of the cognitive impairment found in adolescent animals after repeated cocaine treatment and abstinence period, PL and IL cortical tissues were processed and the mTOR-protein signaling was analyzed.

First, phosphorylated and total mTOR protein levels were quantified in the IL and PL of both adolescent and adult animals. 2-weeks of withdrawal from repeated cocaine exposure during adolescence did not alter mTOR phosphorylation either in IL or in PL (Fig. 3A. IL: $F_{(interaction,\ 1,33)} = 6.963$, $p = 0.0126$; $F_{(treatment)} = 10.84$, $p = 0.0024$; $F_{(test/notest)} = 8.614$, $p = 0.006$; Fig. 3C. PL: $F_{(interaction,\ 1,32)} = 18.92$, $p = 0.0001$; $F_{(treatment)} = 14.61$, $p = 0.0006$; $F_{(test/notest)} = 4.757$, $p = 0.0366$). Adolescent saline-exposed animals, with no alterations in their recency memory, have shown an increased mTOR phosphorylation state in both IL and PL cortices. On the contrary, adolescent cocaine-treated animals, which manifested an impaired temporal order memory, showed no alteration in the phosphorylation of mTOR in any of the areas evaluated (Fig. 3A and C). Interestingly, no changes in the total levels of mTOR were found in both brain areas (Fig. 3B. IL: $F_{(1,34,\ interaction)} = 0.83$, $p = 0.36$; $F_{(treatment)} = 0.096$, $p = 0.75$; $F_{(test/notest)} = 0.03$, $p = 0.85$; Fig. 3D. PL: $F_{(1,34interaction)} = 0.78$, $p = 0.38$; $F_{(treatment)} = 0.003$, $p = 0.95$; $F_{(test/notest)} = 0.11$, $p = 0.73$).

As observed for adolescent rats, 2-weeks of withdrawal from repeated cocaine exposure during adulthood did not alter mTOR phosphorylation either in IL or in PL (Fig. 3E. IL: $F_{(1,25,\ interaction)} = 0.69$, $p = 0.41$; $F_{(treatment)} = 0.14$, $p = 0.70$; $F_{(test/notest)} = 23.41$, $p < 0.0001$; Fig. 3G. PL: $F_{(1,24,\ interaction)} = 1.17$, $p = 0.29$; $F_{(treatment)} = 0.78$, $p = 0.38$; $F_{(test/notest)} = 27.67$, $p < 0.0001$). As is shown, increased phosphorylation levels of mTOR were observed in both saline- and cocaine-treated animals in both brain areas evaluated. Similar to adolescent animals, no differences were found for mTOR total protein levels neither in the IL nor in the PL within adult animals (Fig. 3F. IL: $F_{(1,23,\ interaction)} = 0.84$, $p = 0.36$; $F_{(treatment)} = 0.28$, $p = 0.59$; $F_{(test/notest)} = 1.53$, $p = 0.22$; Fig. 3H. PL: $F_{(1;22,\ interaction)} = 0.0003$, $p = 0.98$; $F_{(treatment)} = 0.008$, $p = 0.92$; $F_{(test/notest)} = 3.216$, $p = 0.086$).

mTOR downstream targets were also analyzed. mTOR activation is expected to phosphorylate the S6 (p-S6) protein which will result in the consequent phosphorylation of the S6 downstream proteins. Accordingly, 2-weeks of withdrawal from repeated cocaine exposure during adolescence did not alter S6 phosphorylation either in IL or in PL (Fig. 4A. IL: $F_{(1,34,\ interaction)} = 10.84$, $p = 0.0023$; $F_{(treatment)} = 9.11$, $p = 0.004$; $F_{(test/notest)} = 4.07$, $p = 0.05$; Fig. 4C. PL: $F_{(1,33,\ interaction)} = 2.72$, $p = 0.10$; $F_{(treatment)} = 0.51$, $p = 0.47$; $F_{(test/notest)} = 14.57$, $p = 0.0006$). TOOR test performance increased S6 phosphorylation state only in the adolescent animals exposed to saline treatment in the two areas evaluated. In addition, as it occurred for mTOR expression, no changes were found in the S6 total protein levels in both areas of adolescent treated animals (Fig. 4B, IL: $F_{(1,34,\ interaction)} = 0.43$, $p = 0.51$; $F_{(treatment)} = 1.66$, $p = 0.20$; $F_{(test/notest)} = 0.25$, $p = 0.61$; Fig. 4D. PL: $F_{(1,33,\ interaction)} = 0.53$, $p = 0.46$; $F_{(treatment)} = 0.24$, $p = 0.62$; $F_{(test/notest)} = 0.005$, $p = 0.94$).

Interestingly, as observed for mTOR phosphorylation, following TOOR test performance adult animals showed an increase in the phosphorylation of its downstream protein S6 in both IL and PL cortices independently from the previous treatment history (Fig. 4E. IL: $F_{(1,25,\ interaction)} = 0.69$, $p = 0.41$; $F_{(treatment)} = 0.14$, $p = 0.70$; $F_{(test/notest)} = 23.41$, $p < 0.0001$; Fig. 4G. PL: $F_{(1,24,\ interaction)} = 1.17$, $p = 0.29$; $F_{(treatment)} = 0.78$, $p = 0.38$; $F_{(test/notest)} = 27.67$, $p < 0.0001$). While S6 expression was not altered in IL cortex (Fig. 4F, $F_{(1,22,\ interaction)} = 2.61$, $p = 0.12$; $F_{(treatment)} = 0.94$, $p = 0.34$; $F_{(test/notest)} = 4.89$, $p = 0.03$), in PL cortex S6 levels were, first, reduced following cocaine exposure and two weeks of withdrawal and, then, TOOR test significantly increased its levels only in cocaine-exposed rats (Fig. 4H. $F_{(1,22,\ interaction)} = 4.97$, $p = 0.03$; $F_{(treatment)} = 5.79$, $p = 0.02$; $F_{(test/notest)} = 18.81$, $p < 0.001$).

Finally, we studied the eEF2 regulation in order to understand how the protein translational machinery could be affected. Interestingly, cocaine exposure during adolescence followed by two weeks of withdrawal reduced the eEF2 phosphorylation levels in both IL and PL cortices (Fig. 5A. IL: $F_{(1,33,\ interaction)} = 15.88$, $p < 0.001$; $F_{(treatment)} = 43.99$, $p < 0.0001$; $F_{(test/notest)} = 27.97$, $p < 0.0001$; Fig. 5C. PL: $F_{(1,33,\ interaction)} = 7.16$, $p = 0.01$; $F_{(treatment)} = 6.82$, $p = 0.01$; $F_{(test/notest)} = 2.23$, $p = 0.14$). Exposure to the TOOR test reduced peEF2 levels only in saline-treated animals in both IL and PL. No changes were found in the

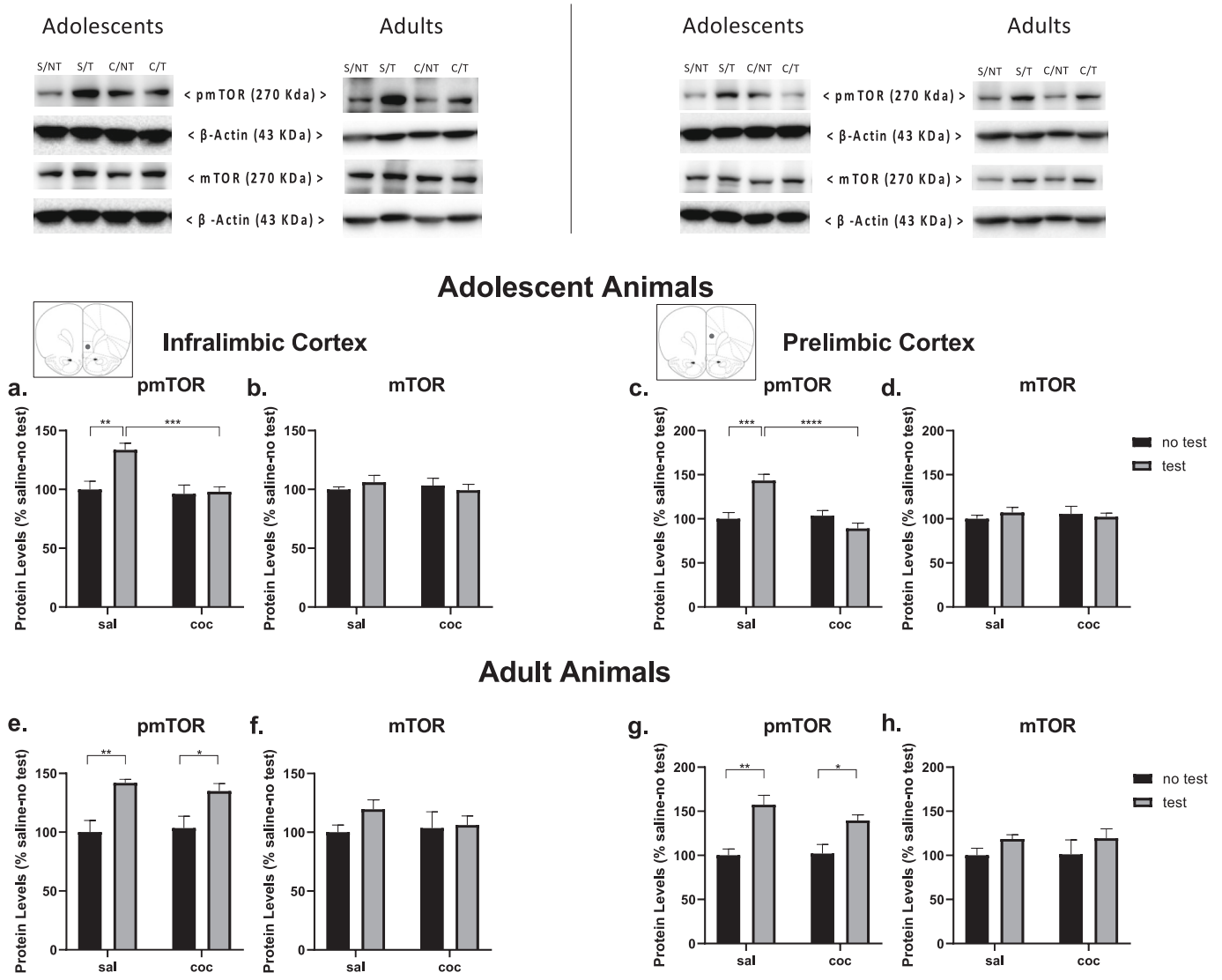

Fig. 3. mTOR phosphorylation and expression in IL and PL cortices after TOOR task performance in rats previously exposed to saline or cocaine.
Protein levels of mTOR phosphorylation and expression are expressed as percentages of adolescent saline-treated rats not exposed to TOOR (sal-no test) in IL (panel A, B) and in PL (panel C, D) and of adult saline-treated rats not exposed to TOOR (sal-no test) in IL (panel E, F) and in PL (panel G, H) cortices. In the upper panel, representative immunoblots are shown for pmTOR S2448 and mTOR proteins in the homogenate of IL and PL cortices. Histograms represent the mean ± SEM of 8 to 11 rats per group. Asterisks indicate significant statistical differences between groups, Tukey or Bonferroni *post-hoc* analysis after two-way ANOVA *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001.
sal or S: saline; coc or C: cocaine; NT: no test; T: test.

eEF2 total protein levels (Fig. 5B. IL: $F_{(1,34,\ interaction)} = 0.94$, $p = 0.33$; $F_{(treatment)} < 0.0001$, $p = 0.99$; $F_{(test/notest)} = 0.90$, $p = 0.34$; Fig. 5D. PL: $F_{(1,32,\ interaction)} = 1.370$, $p = 0.25$, $F_{(treatment)} = 4.04$, $p = 0.06$, $F_{(test/notest)} = 2.058$, $p = 0.16$).

Notably, adult animals showed no significant changes in the p-EEF2 protein levels neither in IL nor in PL (Fig. 5E. IL: $F_{(1,24,\ interaction)} = 0.36$, $p = 0.55$; $F_{(treatment)} = 0.31$, $p = 0.57$; $F_{(test/notest)} = 0.72$, $p = 0.40$; Fig. 5G. PL: $F_{(1,22,\ interaction)} = 0.82$, $p = 0.37$; $F_{(treatment)} = 1.87$, $p = 0.18$; $F_{(test/notest)} = 3.68$, $p = 0.07$). In addition, total eEF2 protein levels were increased in saline- and cocaine-treated animals in both studied areas as a consequence of TOOR test performance (Fig. 5F. IL: $F_{(1,25,\ interaction)} = 0.39$, $p = 0.53$; $F_{(treatment)} = 0.15$, $p = 0.69$; $F_{(test/notest)} = 128.9$, $p < 0.0001$; Fig. 5H. PL: $F_{(1,25,\ interaction)} = 4.35$, $p = 0.047$; $F_{(treatment)} = 5.55$, $p = 0.02$; $F_{(test/notest)} = 222.3$, $p < 0.001$), indicating a different control of this mechanism in adult animals in comparison to the adolescent group.

3.3. *mTOR systemic inhibition impairs the physiological recency memory performance*

To find direct evidence that mTOR signaling could be responsible for the control of the recency memory, we performed a new behavioral experiment using rapamycin to modulate this pathway. Two rapamycin i.p. injections were applied to adolescent and adult naïve animals to prevent mTOR signaling activation: one injection was made the day prior to the test, and a second injection was made immediately after the sample phase 2, as shown in Fig. 1C. Rapamycin-injected animals, either adolescents or adults, were not able to distinguish between the two objects during the test, thus exploring both objects the same amount of time.

In fact, while vehicle-exposed rats during adolescence explored more the object previously seen, no differences were found between the two objects exploration time in rapamycin-treated rats (Fig. 6C. $F_{(1,28,\ interaction)} = 5.43$, $p = 0.02$, $F_{(treatment)} = 0.90$, $p = 0.34$, $F_{(test/notest)} = 5.65$, $p = 0.02$). Moreover, they showed a reduction in the DI in comparison to

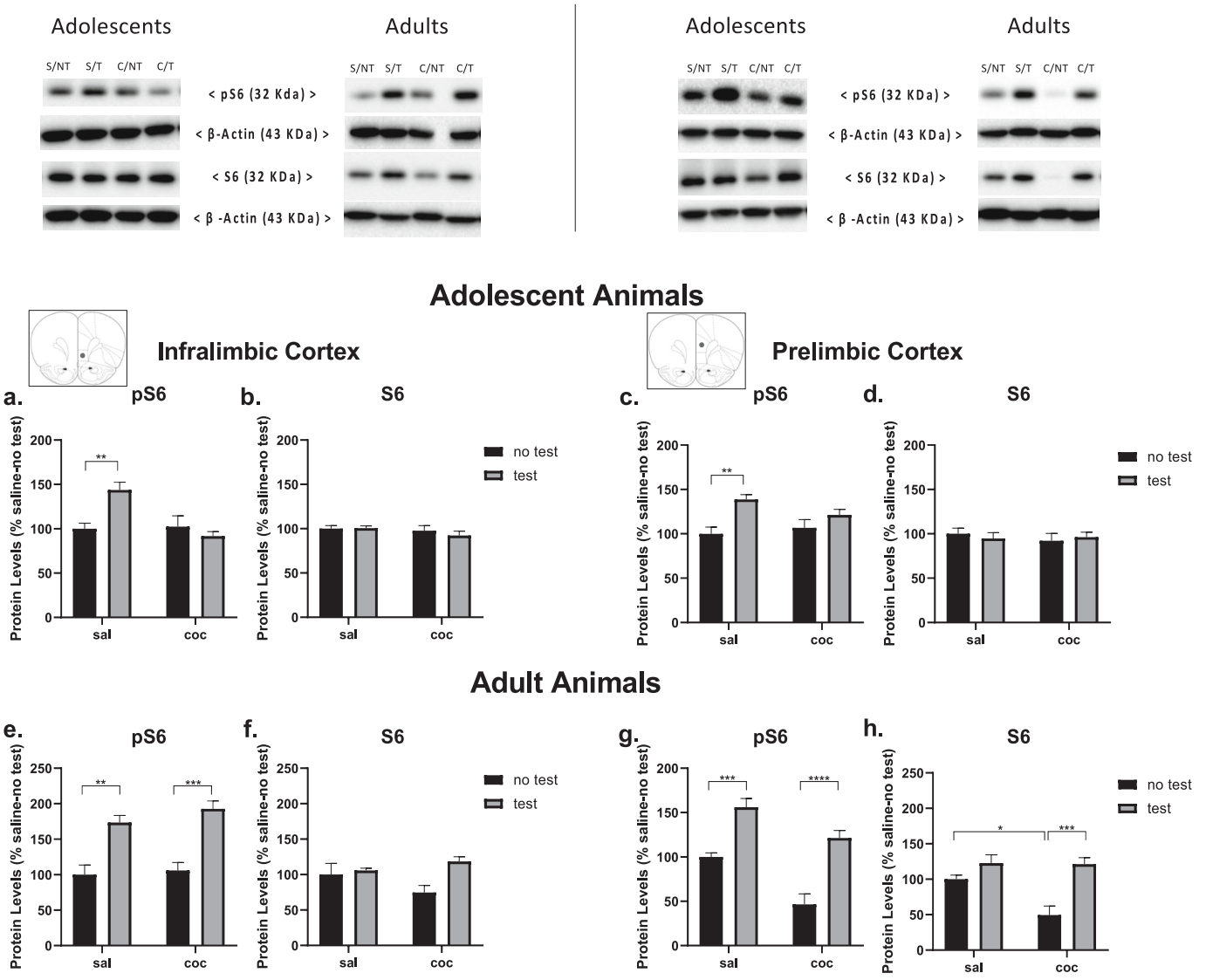

Fig. 4. S6 protein changes in the IL and PL cortices after TOOR task performance. Protein levels of S6 phosphorylation and expression are expressed as percentages of adolescent saline-treated rats not exposed to TOOR (sal-no test) in IL (panel A, B) and in PL (panel C, D) and of adult saline-treated rats not exposed to TOOR (sal-no test) in IL (panel E, F) and in PL (panel G, H) cortices. In the upper panel, representative immunoblots are shown for pS6 Ser240/244 and S6 proteins in the homogenate of IL and PL cortices. Histograms represent the mean ± SEM of 8 to 11 rats per group. Asterisks indicate significant statistical differences between groups, Tukey or Bonferroni *post-hoc* analysis after two-way ANOVA *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001.
sal or S: saline; coc or C: cocaine; NT: no test; T: test.

the control (vehicle) group (Fig. 6D. Control group: one-sample *t*-test$_{(t=5;df=6)}$, $p = 0.0023$; Treated group: one-sample t-test$_{(t=0.38.50;df=8)}$; $p = 0.71$; Unpaired t-test$_{(t=2.413;df=14)}$, $p = 0.030$). As previously observed, no differences were found in the exploration time of objects 1 and 2 during the sample phases indicating again that the animals do not have any natural preference for the objects or side (Fig. 6A. Object 1 exploration time: $F_{(1,36,\ interaction)} = 2.33$, $p = 0.13$, $F_{(side)} = 1.19$, $p = 0.28$, $F_{(treatment)} = 0.33$, $p = 0.56$; Fig. 6B. Object 2 exploration time: $F_{(1,36,\ interaction)} = 0.04$, $p = 0.83$, $F_{(side)} = 0.09$, $p = 0.75$, $F_{(treatment)} = 2.57$, $p = 0.11$).

In adults rats, as observed in adolescents, vehicle-exposed rats explored more the object seen firstly, while no differences were found between the two objects exploration time in rapamycin-treated rats (Fig. 6G. $F_{(1,30,\ interaction)} = 14.26$, $p = 0.0007$, $F_{(treatment)} = 0.00$, $p > 0.9999$, $F_{(test/notest)} = 4.46$, $p = 0.04$). In addition, adult rats treated with rapamycin showed a reduction in the DI in comparison to the control (vehicle) group (Fig. 6H. Control group: one-sample t-test$_{(t=3.050;df=5)}$, $p = 0.0284$; Treated group: one-sample t-test$_{(t=0.9407;df=10)}$; $p = 0.37$; Unpaired t-test$_{(t=2.676;df=15)}$, $p = 0.02$). Alike adolescents, no differences were found in the exploration time of objects 1 and 2 during the sample phases confirming that also adult rats do not have any natural preference for the objects or side (Fig. 6E. Object 1 exploration time: $F_{(1,30,\ interaction)} = 4.72$, $p = 0.04$, $F_{(side)} = 0.23$, $p = 0.64$, $F_{(treatment)} = 0.00$, $p > 0.9999$; Fig. 6F. Object 2 exploration time: $F_{(1,30,\ interaction)} = 0.99$, $p = 0.33$, $F_{(side)} = 11.41$, $p = 0.002$, $F_{(treatment)} = 0.00$, $p > 0.9999$).

4. Discussion

The main finding of the present study is that recency memory is impaired in adolescent, but not adult, rats after two weeks of cocaine withdrawal via the dysregulation of mTOR-related signaling in the mPFC. Because mTOR pathway inhibition by rapamycin blocks recency memory in both adolescent and adult naïve rats, cocaine interference with mTOR signaling during adolescence but not adulthood highlights an effect of the psychostimulant that strictly relies on the maturational stage of the mPFC, further indicating that adolescence is an extremely

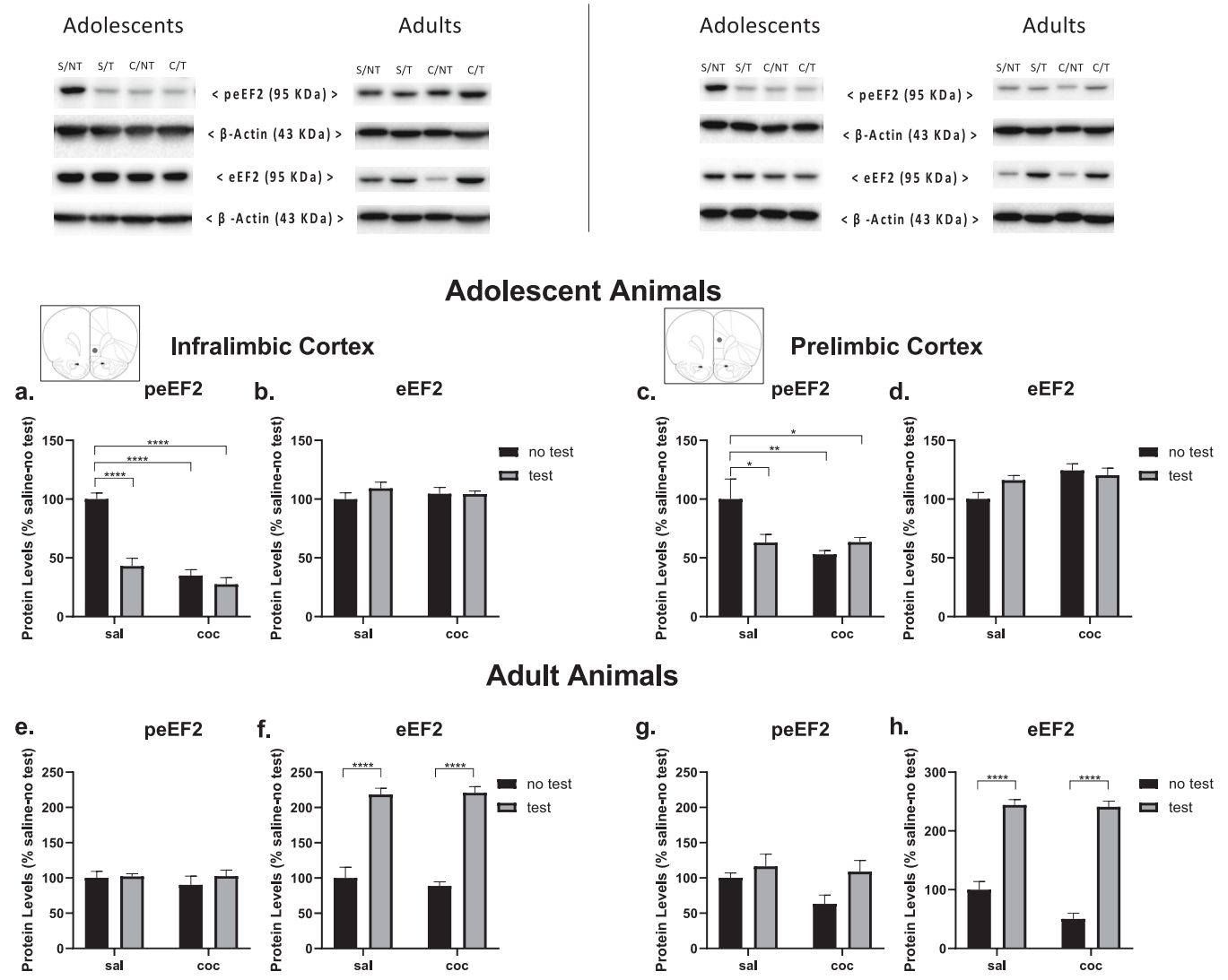

Fig. 5. eEF2 protein changes in the IL and PL cortices after the TOOR task. (A, C) Protein levels of eEF2 phosphorylation and expression are expressed as percentages of adolescent saline-treated rats not exposed to TOOR (sal-no test) in IL (panel A, B) and in PL (panel C, D) and of adult saline-treated rats not exposed to TOOR (sal-no test) in IL (panel E, F) and in PL (panel G, H) cortices. In the upper panel, representative immunoblots are shown for peEF2 Thr56 and eEF2 proteins in the homogenate of IL and PL cortices. Histograms represent the mean ± SEM of 8 to 11 rats per group. Asterisks indicate significant statistical differences between groups, Tukey or Bonferroni *post-hoc* analysis after two-way ANOVA *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$.
sal or S: saline; coc or C: cocaine; NT: no test; T: test.

sensitive and critical period for drug use.

We previously found that adolescent, but not adult, cocaine exposure causes a significant impairment in the NOR test, a measure of recognition memory (Mottarlini et al., 2020). In the present study, we sought to provide more evidence for the effects produced by adolescent exposure to cocaine on memory by adding experiments releated to recency memory, a cognitive domain in which items or situations that came last are recalled more clearly than those that came first (Barker et al., 2007; Warburton and Brown, 2015). Since protein translation has been found dysregulated during cocaine withdrawal in adult animals (Werner et al., 2018) and we previously found that the mTOR-S6 pathway is activated in the mPFC of adolescent cocaine-withdrawn animals (Giannotti et al., 2014), the lack of mTOR activation in our cocaine-exposed group might be due to the brain area selected for analysis (nucleus accumbens in previous manuscripts vs. mPFC in the current study), the duration of the withdrawal period (48 days in our previous work vs. 15 days in the present study) or the regimen of cocaine exposure (contingent vs. non-contingent). The reduced phosphorylation of eEF2 observed in the mPFC of adolescent rats withdrawn from cocaine exposure was similarly observed in adult animals following 50-days withdrawal from extended access to cocaine self-administration in the nucleus accumbens core (Werner et al., 2018), an effect elegantly correlated to the incubation development of cocaine craving that requires intact protein translation for its expression (Scheyer et al., 2014). Moreover, recently, an acute pretreatment with everolimus, an allosteric mTOR inhibitor, reduced cocaine craving incubation and reversed related protein adaptations in the ventromedial prefrontal cortex (vmPFC) (Chiu et al., 2021). This analogy in the molecular profile suggests that the increased protein synthesis that supports the progression of cocaine craving in adult rats occurs after a mere 15-days of withdrawal from a low dose of cocaine administration (5 mg/kg) in developing animals, further corroborating adolescence as a vulnerability window for drug abuse.

Our findings confirm this evidence and deem cortical mTOR-dependent signaling necessary for recency memory. To perform an in-depth study on the cortical regions involved in this type of memory, we divided the mPFC into the two main sub-regions known as the IL and PL cortices. Although these two brain regions serve distinct functions, the regulation of mTOR signaling and its downstream targets is indeed

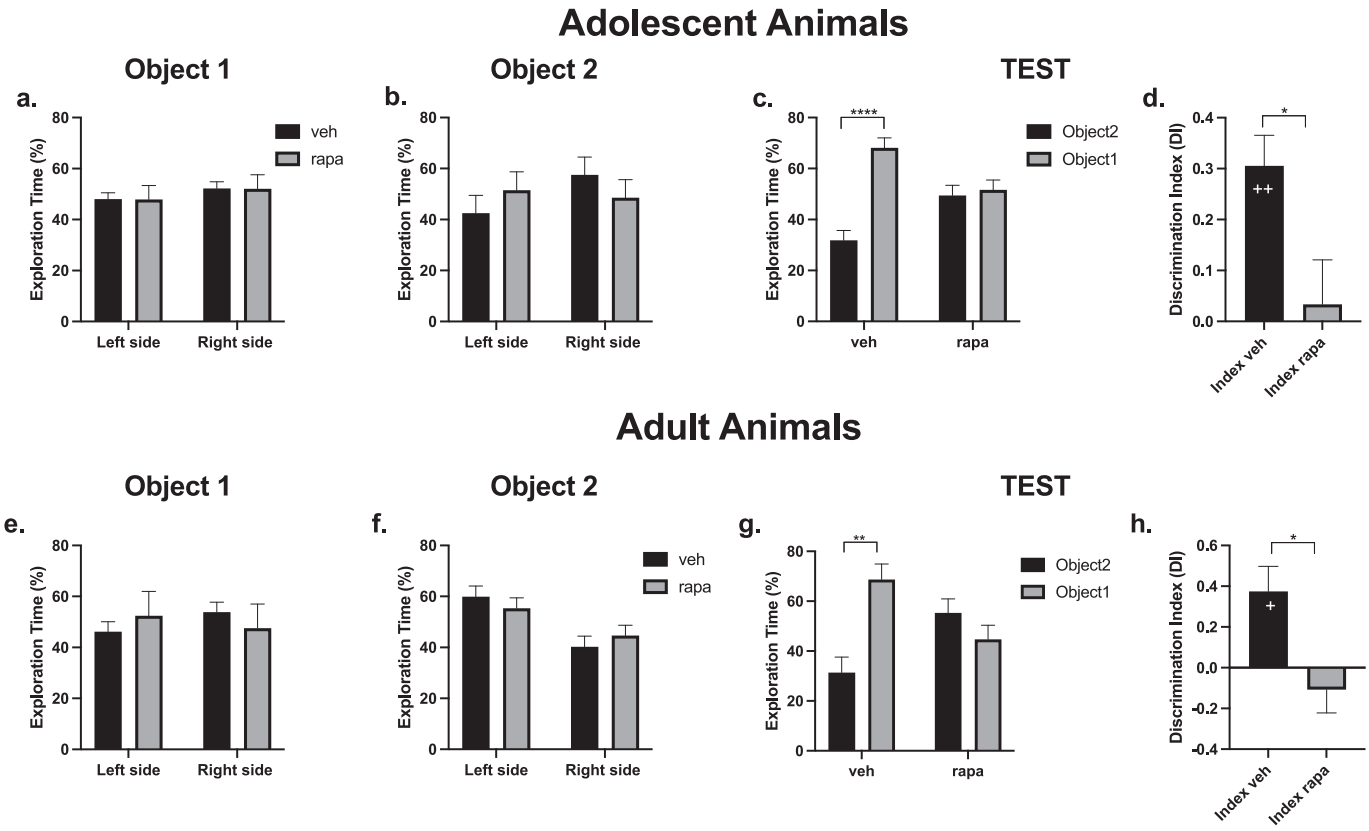

Fig. 6. mTOR-induced blockade by systemic rapamycin injection impaired TOOR task performance. Exploration time (%) for each object during sample phase 1 (panel A) and sample phase 2 (panel B) of vehicle- and rapamycin-treated adolescents (top) or adults (bottom) animals. Exploration time for object 1 (panel A, E) or object 2 (panel B, F) either in left or right side of the arena. Exploration time for each object during the test phase of vehicle- and rapamycin-treated animals (C, G). Discrimination index (DI) for the two groups of adolescent (D) and adult (H) animals.
Asterisks indicate significant statistical differences between groups: Tukey *post-hoc* analysis after two-way ANOVA **p < 0.01, ****p < 0.0001; for unpaired student's t-test in panel D and H *p < 0.05; **$p < 0.01$.
veh: vehicle; rapa: rapamycin.

superimposable, suggesting that, as far as this type of memory is concerned, there are no subregional differences in its regulation. Moreover, according to mTOR phosphorylation induced by test performance, the phosphorylation of S6 is significantly enhanced only in saline-treated animals during adolescence, whereas, in adulthood, such up-regulation is also observed in cocaine-treated rats in both the IL and PL cortices. Intriguingly, the specific requirements for mTOR signaling in the cognitive domain of temporal recognition is extremely sensitive to external stimuli, specifically in adolescent animals. Their recency memory is altered through (1) cocaine-induced impaired activation of the mTOR-S6 pathway and (2) rapamycin-induced inhibition of the mTOR cascade. Conversely, in adult animals, the role of mTOR in cognitive processing is preserved regardless of cocaine exposure. The herein-shown increased phosphorylation of mTOR and S6 is indeed interesting since the activation of these two factors can drive the initiation of translation, contributing to enhanced protein synthesis and, consequently, the promotion of neuronal plasticity (Browne and Proud, 2004; Taha et al., 2013).

A critical step in protein synthesis is elongation, a process controlled by several elongation factors, including eEF2. Of note, the phosphorylation of eEF2 results in reduced mRNA translation rates, whereas its reduced phosphorylation leads to the activation of protein synthesis (Kenney et al., 2014; Ryazanov and Davydova, 1989). Under our experimental conditions, we found that cocaine withdrawal in adolescent animals caused a significant reduction of eEF2 phosphorylation with no effects on its total levels; however, the response to the TOOR test revealed that while control animals can down-regulate eEF2 phosphorylation, thus activating protein translation, cocaine-withdrawn animals are no longer able to activate this process. Again, this mechanism was observed in both cortical subregions of the mPFC, further validating the superimposable mechanism. These findings support the hypothesis that the impaired recency memory observed in cocaine-withdrawn rats might be due to their inability to activate further protein synthesis, which is known to be necessary for the consolidation of long-term memory (Costa-Mattioli et al., 2009). Although we know that other signaling pathways, such as MEK/ERK, could also be implicated, our data suggest that the mTOR-S6-eEF2 protein synthesis pathway, at least in part, may mediate recency memory. This notion is further supported by the finding that adult animals from both experimental groups exhibit a typical performance in the TOOR test. Our data support the possibility that adult rats have the capacity to enhance the elongation phase of translation via dephosphorylation of eEF2, a step that occurs, with variance in adolescent rats, through increased eEF2 levels.

Indeed, our data show, albeit indirectly, that a proper performance in the TOOR test needs the activation of the mTOR-S6-eEF2 pathway; this is confirmed by the evidence that cocaine-withdrawn adult rats perform the TOOR test similarly to adolescent and adult controls, as adult rats maintain proper regulation of this pathway. In order to find direct evidence between our behavioral results and mTOR-S6-eEF2 pathway regulation, we introduced rapamycin, a widely used mTOR inhibitor. Undoubtedly, we found that adolescent and adult rapamycin-treated rats exhibit impaired recency memory, supporting our hypothesis of a tight relationship between protein synthesis and this type of memory. These results suggest that adolescent and adult rats use the same pathway for proper performance in the TOOR test and, therefore, since cocaine does not inhibit the mTOR pathway during the TOOR test in

adult rats, we can infer that it is, in fact, cocaine that differentiates adolescent from adult rats.

We are aware that our experimental approach does not cover all the temporal dynamics that memory processing could involve, meaning that different behavioral outcomes could be found when the TOOR test is performed at shorter or longer time intervals. For instance, it would be intriguing to test whether adult cocaine-exposed rats using a TOOR test with longer intervals would show impaired memory recency similar to adolescent rats. In addition, it would be interesting to investigate whether the mTOR pathway is involved due to cocaine exposure or, rather, as a consequence of memory retrieval. These critical questions will be addressed in future manuscripts.

5. Conclusion

Our results show the critical role that activation of mTOR-S6-eEF2 signaling plays in the performance of the TOOR test, which is indicative of recency memory. The previously unappreciated contribution of protein translation in regulating cortical functionality by early delivery of, and withdrawal from, adolescent cocaine may provide novel potential targets for treating psychostimulant abuse. Our results also provide further means to consider adolescence as a critical age for the long-term effect of cocaine as this pathway is selectively altered in the mPFC of cocaine-withdrawn adolescent, but not adult, rats. In addition, our data indicate the similar contribution of the IL and PL cortices in the mechanisms underlying recency memory, despite the well-known notion that they play different roles in response to the psychostimulant cocaine, indicating the global involvement of this brain region in the modulation of recency memory.

Even though we are aware that new studies along these lines of evidence are necessary to identify novel treatment strategies to reduce the risk of relapse, even after a long period of abstinence, the described results set the stage for a better understanding of the mechanisms regulating cocaine-induced translation and their contribution in inducing cognitive deficits in cocaine abusers.

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Author statement

The study was developed by FCD, FM and LC. The behavioral studies were conducted by FCD, FM and GT. The molecular analysis was done by FCD, FM, and BR. The statistical analysis was carried out by FCD, supervised by LC. All authors supported the results and assisted in the data analysis and interpretation. The first draft of the manuscript was written by FCD under guidance of all authors. LC and FF critically supervised the whole study and corrected the final version of the manuscript.

Ethical statement

All animal procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE) and received approval from the University of Milan Animal Care Committee. All efforts were made to minimize animal suffering and to keep the lowest number of animals used. The experiments have been reported in compliance with the ARRIVE guidelines.

The manuscript has been read and approved by all authors in the present form. Authors declare no conflict of interest with the current experimental work. The work described in the submitted manuscript has not been published previously and is not under consideration for publication elsewhere.

This work was supported by the Dipartimento delle Politiche Antidroga (Rome, Italy) through the ERANID Grant “STANDUP” awarded to FF as well as by grants from MIUR Progetto Eccellenza. Dr. Francesca Mottarlini is recipient of a fellowship from the Zardi-Gori Foundation.

The Dipartimento Politiche antidroga, MIUR or Zardi-Gori Foundation had no role in the collection, analysis and interpretation of data; in the writing of the report as well as and in the decision to submit the article for publication.

The study was developed by FCD, FM and LC. The behavioral studies were conducted by FCD, FM and GT. The molecular analysis was done by FCD, FM, and BR. The statistical analysis was carried out by FCD, supervised by LC. All authors supported the results and assisted in the data analysis and interpretation. The first draft of the manuscript was written by FCD under guidance of all authors. LC and FF critically supervised the whole study and corrected the final version of the manuscript.

Declaration of Competing Interest

The authors of the manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2023.110822.

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