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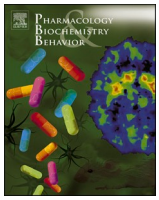

IGF2 modulates behavioral and hippocampal changes induced by chronic cocaine exposure during adolescence in mice

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ABSTRACT

Adolescence is a period of heightened neuroplasticity and vulnerability to environmental insults, including drug exposure. In this study, we investigated the short- and long-term behavioral effects, as well as the long-term hippocampal effects, of chronic cocaine administration during adolescence, along with the potential neuroprotective role of insulin-like growth factor 2 (IGF2) in male C57BL/6J mice. Over 21 days, mice received daily intraperitoneal injections of saline, cocaine, IGF2, or a combination of cocaine and IGF2. Behavioral assessments were conducted immediately after treatment and following a 30-day abstinence period, using a battery of tests including marble burying, nest building, elevated plus maze, open field, novel place and object recognition, and forced swim. Cocaine-treated mice exhibited persistent compulsive-like behaviors and altered risk perception, effects that were attenuated by IGF2 co-administration. At the cellular level (after 40 days of abstinence), chronic cocaine reduced the density of parvalbumin-positive interneurons in the CA1 and CA3 hippocampal regions, an effect not mitigated by IGF2 co-administration. IGF2 treatment also increased expression of the presynaptic marker synaptotagmin, without altering postsynaptic proteins (PSD-95) or neurotrophic factors (BDNF, pro-BDNF). However, IGF2 downregulated IGF2R expression and impaired performance in hippocampus-dependent spatial memory, suggesting that receptor downregulation may underlie cognitive side effects. No significant differences were observed in markers of oxidative stress, neurogenesis, or basal corticosterone levels. These findings indicate that IGF2 partially counteracts behavioral and cellular alterations induced by adolescent cocaine exposure but may also impact specific cognitive domains. Overall, this study supports further investigation of IGF2 as a therapeutic strategy to mitigate long-term neurobehavioral consequences of adolescent drug use.

1. Introduction

Adolescence is a critical developmental stage marked by profound physical, cognitive, and emotional changes in both humans and nonhuman animals (Lees et al., 2020; Moore et al., 2011; Reynolds and Flores, 2021). During this period, rodents show increased locomotor and exploratory behavior, impulsivity, novelty-seeking, and reduced anxiety (Casey and Jones, 2010; Laviola et al., 2003), which may be linked to anatomical and neurochemical differences between the adolescent and adult brain (Spear, 2000). The adolescent brain undergoes dynamic remodeling, including synaptic pruning (Andersen, 2003; Rice and Barone, 2000), a decline in cortical gray matter, particularly in the prefrontal cortex (PFC), before stabilizing at adult levels, and continued increases in white matter volume and axonal myelination until early

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adulthood (Lees et al., 2020; Reynolds and Flores, 2021). Neurogenesis in the adolescent hippocampus is markedly higher than in adults, though its mechanisms and functional significance remain poorly understood due to limited research (Kozareva et al., 2019). Additionally, during adolescence, neurotransmitter systems undergo marked changes, including dopaminergic fluctuations in the PFC and mesolimbic regions, alterations in NMDA-mediated glutamatergic signaling, and maturation of GABAergic inhibitory circuits, particularly PV-positive interneurons (Larsen and Luna, 2018). These processes underlie the staggered maturation of brain regions, with sensorimotor areas maturing first, followed by limbic and then frontal regions (Lees et al., 2020). These maturational processes render the adolescent brain especially vulnerable to environmental insults, including drug exposure, which can disrupt normal development and elevate the risk of psychiatric disorders later in life (Andersen, 2003; Caffino et al., 2022; Marco et al., 2010). Compared to adults, adolescents show increased sensitivity to the rewarding effects of drugs such as alcohol, nicotine, cocaine, amphetamine, and delta-9-tetrahydrocannabinol, with attenuated aversive effects, potentially contributing to greater vulnerability to addiction (see Spear, 2016 for review). Particularly, the effects of cocaine exposure during adolescence have yielded inconsistent findings, possibly due to differences in exposure duration, timing of evaluation, and brain regions studied (Caffino et al., 2022).

Acute adolescent cocaine exposure increases extracellular dopamine more than in adults, alter the expression of fibroblast growth factor (FGF-2) (Giannotti et al., 2015) and impairs synaptic plasticity by reducing dendritic spine density in the PFC, along with a decrease in NMDA and AMPA glutamatergic receptors, the postsynaptic density protein 95 (PSD-95), and the cytoskeletal protein Arc/Arg3.1 (Caffino et al., 2018). Chronic exposure elevates neurotrophins (FGF-2, BDNF, GDNF) and CREB, but only after prolonged abstinence (Giannotti et al., 2013, 2014; Simchon-Tenenbaum et al., 2015; Valzachi et al., 2013). Furthermore, adolescent cocaine exposure reduces hippocampal neurogenesis, affecting proliferation and survival (García-Fuster et al., 2017), increases GABAergic transmission in the prelimbic cortex (Shi et al., 2019), alters dopamine levels in several brain regions including the hippocampus (Alves et al., 2014), and causes gray and white matter changes in dopamine-innervated areas (Wheeler et al., 2013).

At the behavioral level, adolescent rodents exhibit reduced sensitivity to the locomotor effects of psychostimulants (Laviola et al., 2003; Spear, 2000); however, repeated cocaine exposure during this period induces locomotor sensitization in early adulthood (Marin et al., 2008; Wheeler et al., 2013). Regarding anxiety-related behaviors, chronic adolescent cocaine exposure generally produces persistent anxiogenic effects, although some studies report anxiolytic outcomes instead (Caffino et al., 2022). Adolescent rats also show greater cocaine self-administration than adults, reflected in higher intake at low doses, greater escalation, and stronger resistance to "price" increases, although this depends on the age at exposure (Wong et al., 2013). Cognitive deficits from adolescent cocaine exposure include impairments in perseverance, impulsivity, threat assessment, reinforcement processing, and memory. However, their onset and persistence vary with abstinence length (reviewed in Caffino et al., 2022; Spear, 2016). Interestingly, despite substantial neuronal alterations in PFC circuits, adolescent cocaine exposure does not appear to impair executive function tasks dependent on these regions (Spear, 2016). A similar mismatch is seen in the hippocampus, where cellular changes occur without behavioral deficits (García-Cabrerizo et al., 2015; Zhu et al., 2016).

Insulin-like growth factor 2 (IGF2) is a widely distributed growth factor in the central nervous system, working independently or synergistically with IGF1 in energy homeostasis, growth, development, and cognitive functions, especially hippocampus-dependent learning and memory. It contributes to neurotransmitter release, adult neurogenesis, and synaptic plasticity in the hippocampus (Stern et al., 2014a; Ziegler et al., 2019). IGF2 also shows neuroprotective effects under both physiological conditions, like aging (Castilla-Cortázar et al., 2011), and pathological states characterized by neurodegeneration and oxidative stress (Wang et al., 2017; Werner and LeRoith, 2014), including Alzheimer's disease (Pascual-Lucas et al., 2014), schizophrenia (Ouchi et al., 2013; Yang et al., 2020), autism spectrum disorder (Steinmetz et al., 2018), and Parkinson's disease (Arcos et al., 2023; Martín-Montañez et al., 2021). Despite these known neuroprotective properties, IGF2's role in drug exposure remains largely unexplored, with, to our knowledge, only one study reporting its ability to reverse long-term cognitive deficits from prenatal cocaine exposure (Zhao et al., 2015).

In this study, we hypothesized that adolescent cocaine exposure may induce persistent behavioral deficits into adulthood, potentially via neurotoxic effects, and that IGF2 co-administration might mitigate these outcomes. To test this, adolescent mice received repeated cocaine and/or IGF2 treatments, aiming to assess their impact at two levels. First, we examined cocaine's effects on multiple behavioral domains (i.e., exploratory activity, emotional behavior, and cognitive performance), evaluating whether any impairments persisted into adulthood, resolved spontaneously, or improved with IGF2 co-administration. Behavioral assessments were conducted at two critical time points: immediately after treatment (late adolescence) and following a prolonged abstinence (adulthood). Second, given the critical role of the hippocampus in emotional processing, spatial memory, and addiction (Fanselow and Dong, 2010; Kutlu and Gould, 2016) and its limited investigation compared to the PFC in adolescent research, we evaluated the long-term impact of adolescent cocaine exposure on hippocampal integrity and function, focusing on oxidative damage, neuroplasticity, neurogenesis, and neuronal populations, and whether IGF2 could attenuate these effects. Our findings may offer insights into the interaction between drug exposure and neuroprotection during adolescence, contributing to a better understanding of therapeutic strategies to mitigate the long-term impact of substance abuse on the developing brain.

2. Materials and methods

2.1. Animals and ethical statement

Fifty-six adolescent male C57BL/6J mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Mice were acclimated to the animal facilities and the experiments began at post-natal day (PND) 30, a period identified as early adolescence (Laviola et al., 2003). Animals were individually housed in cages equipped with nesting materials. They were maintained under standard conditions (temperature of 22 ± 2 °C and a 12-h light/dark cycle, with the lights turning on at 8:00 AM) and ad libitum access to water and food.

Procedures were performed according to the ARRIVE guidelines and the European Directive 2010/63/EU and Spanish regulations for animal research (Real Decreto 53/2013 and 1386/2018 and Ley 32/2007). The experimental protocols were approved by the Ethic and Research Committee of the University of Málaga (CEUMA 133-2021-A) and the regional Government (Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible, Junta de Andalucía; N° 14/03/2022/034).

2.2. Pharmacological treatments

The mice were randomly assigned to four experimental groups (n = 14 per group) and received three daily intraperitoneal (i.p.) administrations for 21 days, from PND 30 to 50. At 9 AM, each mouse received two i.p. injections, one on each side of the abdominal midline, administered consecutively without releasing the mouse, and at 5 PM, a single injection was given. The treatment groups were as follows: (1) Saline group: three daily administrations of 0.9 % NaCl (two injections AM and one injection PM); (2) Cocaine group: one cocaine injection on one side and one NaCl injection on the other side in the morning, plus one NaCl injection in the afternoon; (3) IGF2 group: one IGF2 injection on one side and one NaCl injection on the other side in the morning, plus one IGF2 injection in the afternoon; and (4) Cocaine + IGF2 group: one cocaine

injection on one side and one IGF2 injection on the other side in the morning, plus one IGF2 injection in the afternoon. Cocaine was administered at a dose of 20 mg/kg (Alcaliber S.A., Madrid, Spain), diluted in a 10 mL/kg volume of 0.9 % NaCl saline solution, following previous studies in which behavioral impairments and hippocampal dysfunction were observed in adult mice (Ladrón de Guevara-Miranda et al., 2016; Mañas-Padilla et al., 2021a, 2021b, 2023). The saline group received an equivalent volume of saline solution. IGF2 was administered twice daily at a dose of 7.5 μg/kg, dissolved in phosphate-buffered saline (PBS, 0.1 M, pH 7.4), based on previous studies demonstrating its neuroprotective properties (Martín-Montañez et al., 2021). To ensure balance, the order of administration varied daily among groups, and the injection site (right/left abdomen at PM) was alternated. Animal weights were recorded daily, and all efforts were made to minimise animal suffering and reduce the number of animals used.

2.3. Behavioral testing and timeline

Behavioral testing was conducted following previously established protocols, using a battery of tests to evaluate exploratory activity, emotional and motivational behavior, and cognitive performance. To increase sensitivity to treatment effects and to capture both immediate and delayed behavioral consequences of adolescent cocaine exposure, we employed repeated behavioral assessments separated by a prolonged abstinence period (as shown in Fig. 1), consistent with previous studies (Santucci and Madeira, 2008). The first evaluation (Evaluation 1) began one day after the final pharmacological treatment, on experimental day 22 (PND 51), and was carried out over a 9-day period. Following the completion of the first behavioral assessment, all animals remained undisturbed for 20 days before undergoing a second identical battery of tests (Evaluation 2). This approach allowed for the assessment of behavioral outcomes at two distinct developmental stages: late adolescence, immediately following treatment completion, and adulthood, 30 days post-treatment cessation, in accordance with developmental classifications proposed by Laviola et al. (2003). On experimental day 61 (PND 90), one day after the final behavioral test, all animals were euthanized, and brain and serum samples were collected and stored for further analysis.

The behavioral test battery was administered in the following sequence: The marble burying test (MBT) was conducted on days 22 and 52 to assess compulsive and/or anxiety-like behavior. The nest building test (NBT) (days 23 and 53) and sucrose preference test (SPT) (days 24–25 and 54–55) were used to evaluate motivated natural behaviors and overall welfare. The elevated plus maze (EPM) (days 26 and 56) and open field test (OF) (days 27 and 57) assessed exploratory activity and anxiety-related behavior. Episodic-like recognition memory was evaluated using the novel place recognition (NPR) and novel object recognition (NOR) tests (days 28 and 58). Finally, the forced swim test (FST) was performed on days 29–30 and 59–60 to assess depression-/despair-like behavior. Each day, mice were acclimated to the testing room for at least 30 min before the assessment began. To eliminate odour cues, all apparatuses were thoroughly cleaned with a 30 % alcohol solution after each session and between assays. The tests were administered between 9:00 a.m. and 4:00 p.m. in a noise-isolated room illuminated at 200 lx.

2.3.1. Marble burying test

On the first day, the MBT was conducted following previously established (Dixit et al., 2020; Sampedro-Piquero et al., 2022). The test was performed in a clean laboratory cage for each mouse (20 × 35 cm surface area and a methacrylate lid), with a bedding thickness of 5–6 cm. Initially, mice were allowed to explore and dig freely for 10 min (habituation phase). After this, they were removed from the cage, and 15 marbles were arranged in a 5 × 3 grid on the bedding surface. The mice were then reintroduced into the cage for 30 min, after which the number of buried marbles (covered by at least two-thirds with bedding) was recorded. A high number of buried marbles indicates compulsive-like behavior.

2.3.2. Nest building test

The NBT was performed following a previously described protocol with modifications (Deacon, 2012). Upon arrival, mice were provided with shredded paper strips as nesting material, which was removed before testing. A compressed cotton nestlet (2–3 g, 5 cm/side, 5 mm thick) was then introduced as nesting material. Nestlet shredding was measured (g) at 30, 60, 90, and 240 min, and at 24 h, following Angoa-Pérez et al. (2013) Additionally, nest quality was visually scored after 24 h without disturbing the mice, using a scale where higher scores indicated better nest construction (reflecting motivated behavior and welfare), while lower scores represented poor nest quality, according to the following criteria (Gaskill et al., 2013; Hess et al., 2008): 0 = undisturbed nesting material; 1 = disturbed nesting material, but no nest site; 2 = flat nest without walls; 3 = cup-shaped nest with walls less than half the height of a complete dome; 4 = Incomplete dome with walls at least half the height of a full dome; 5 = fully enclosed dome with walls higher than half the height of a complete dome.

2.3.3. Preference sucrose test

Mice were first habituated for 24 h to two identical drinking bottles containing tap water. Then, these bottles were removed, and the mice were deprived of food and water for 12 h. Subsequently, two bottles were reintroduced: one containing 1 % sucrose solution and the other containing tap water for a period of 24 h, in accordance with the

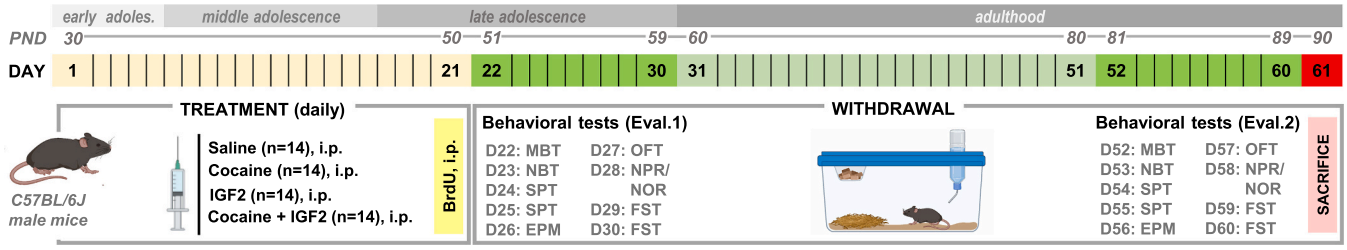

Fig. 1. Experimental design. The experiment began when mice were 30 days old (early adolescence). Four experimental groups were established and received daily intraperitoneal injections of either saline, cocaine, IGF2, or a combination of cocaine and IGF2 for 21 consecutive days (see Section 2.2 for details on the injections). On the final day of treatment, BrdU was administered. Over the following 9 days (Evaluation 1, late adolescence), a battery of behavioral tests was conducted. Mice then remained undisturbed in their home cages for 21 days. On experimental day 52, the same behavioral battery was repeated (Evaluation 2, adulthood), and animals were sacrificed one day after the completion of testing, at which point brain tissue and a blood sample were collected. Abbreviations: PND, postnatal day; i.p., intraperitoneal; BrdU, bromodeoxyuridine; Eval., evaluation; MBT, marble burying test; NBT, nest building test; SPT, sucrose preference test; EPM, elevated plus maze; OFT, open field test; NPR, novel place recognition; NOR, novel object recognition; FST, forced swim test.
Figure partially created in BioRender.com.

protocol by Song et al. (2020). To prevent position bias, the placement of bottles was counterbalanced among mice and switched after 12 h. At the end of the 24-h test, sucrose and water consumption were measured (ml). Higher sucrose preference rates suggest strong motivation for natural rewards, and the sucrose preference rate was calculated as:

$$\text{Sucrose preference} = \frac{\text{sucrose intake}}{\text{sucrose intake} + \text{water intake}} \times 100$$

2.3.4. Elevated plus maze

On the fourth day of behavioral testing, the EPM was performed according to previously published protocols (Carola et al., 2002; Ladrón de Guevara-Miranda et al., 2019). The apparatus consisted of two open arms positioned opposite each other (30 × 10 cm), two closed arms also in opposite positions (30 × 10 cm with 15-cm-high opaque walls), and a central platform (10 × 10 cm) connecting the arms in a cross shape. It was elevated 56 cm above the floor. Mice were placed on the central platform facing a closed arm and allowed to explore freely for 10 min. Each session was videotaped and analyzed using Ethovision XT, 12 software (Noldus, Wageningen, The Netherlands). The following parameters were automatically recorded: time spent (s) in the open arms, closed arms, and center platform, number of entries and latency (s) to the open arms and distance traveled (cm). Time spent in both open and closed arms was integrated into an open arm exploration ratio, calculated as follows:

$$\text{Open arm exploration ratio} = \frac{\text{Time in open arms}}{\text{Time in open arms} + \text{Time in closed arms}}$$

Higher values of this ratio indicate increased time spent in the open arms, suggesting greater exploratory interest and/or lower anxiety levels. Additionally, manual scoring was used to assess the frequency and duration of spontaneous behaviors: rearings (standing on hind paws), risk assessment (stretching forward and retracting), grooming (self-cleaning: licking, washing, scratching) and head dipping (peering over the maze edges).

2.3.5. Open field

The OF was conducted on the fifth day of behavioral testing, following previously published protocols with minor modifications (Carola et al., 2002; Ladrón de Guevara-Miranda et al., 2019). The apparatus was a 40 × 40 square arena surrounded by 40-cm-high opaque walls. Mice were placed in one corner and allowed to explore freely for 10 min. For data analysis, the test was videotaped and analyzed using Ethovision XT, 12. The arena was divided into two zones: center (considered a central area of 20 × 20 cm) and periphery (defined as the remaining edge of the arena). The behaviors evaluated included distance traveled (cm) in the center and periphery — as a measure of exploratory activity, time spent (s) in each zone, and the number of entries into the arena center. Spending more time in the center or showing a higher number of center entries is interpreted as reduced anxiety-related behavior. The test was performed twice in one day, with a 2-h interval, serving both as an open field assessment and as the habituation phase for the NPR/NOR test. Conducting two open field test sessions spaced 2 h apart before the object recognition task habituates the animals, reducing novelty-induced anxiety and ensuring that differences in exploration reflect memory performance rather than stress or locomotor changes (Antunes and Biala, 2012; Leger et al., 2013).

2.3.6. Novel place recognition and novel object recognition

The NPR and NOR tests were performed according to Denninger et al. (2018). These tests consisted of multiple stages conducted over two consecutive days. On Day 1, mice underwent habituation to the OF arena, as previously described (see OF test). On Day 2, each mouse completed three trials, each lasting 10 min. (1) Training trial: mice were placed in the OF arena and allowed to explore two identical objects positioned according to spatial environmental cues. (2) NPR trial (2-h delay): one of the previously explored objects was relocated to a new position within the arena. Mice were reintroduced and allowed to explore both objects—one in its original position and the other in a novel location. (3) NOR trial (2-h delay): One of the previously explored objects was replaced with a novel object, and mice were allowed to explore both. All objects used in this study had been validated in previous research conducted by our group (Mañas-Padilla et al., 2021a, 2021b, 2023) and all trials were videotaped for further analysis. Total object exploration time (s) was automatically quantified using Ethovision XT 12 software. Object exploration was defined as the mouse actively touching an object with its nose or forepaws, or orienting its nose toward the object. Additionally, all videos were manually reanalyzed by a trained observer to confirm the accuracy of the automated analysis. The object Discrimination Index (ODI) and Place Discrimination Index (PDI) were calculated using the following formulas: [ODI = (time exploring the novel object – time exploring the familiar object) / total time exploring both objects] and [PDI = (time exploring the object in the novel location – time exploring the object in the original location) / total time exploring both objects].

Higher index values indicate better performance in episodic-like recognition memory tasks, including both object memory (ODI) and place memory (PDI).

2.3.7. Forced swimming test

The FST was conducted on the last two days of behavioral testing, with one trial per day. Mice were gently placed in a clear cylinder (27 cm high, 10 cm diameter) filled with 23 ± 1 °C water to a height of 15 cm. Each trial lasted 5 min, after which mice were dried and returned to their home cages. Sessions were videotaped, and behaviors were manually scored, following Cryan et al. (2002) and Moreno-Fernández et al. (2017): (1) immobility time (s), (2) latency to first immobility (s), (3) climbing time (s) and (4) swimming time (s). Typically, longer immobility times and shorter climbing times are associated with increased depressive-like behaviors and behavioral despair. Repeating the forced swimming test on two consecutive days is used to distinguish between acute stress responses, habituation, and is commonly employed to evaluate depressive-like behaviors, thereby improving the reliability of the assessment and the interpretation of pharmacological effects (Becker et al., 2023; Cryan et al., 2005).

2.4. Tissue processing

2.4.1. Tissue and blood sample collection

Twenty-four hours after the last behavioral test, mice (PND = 90) were anesthetized with sodium pentobarbital (200 mg/kg, i.p.). From each experimental group, half of the animals were randomly assigned to either transcardial perfusion with PBS (0.1 M, pH 7.4) followed by 4 % paraformaldehyde (PFA) (n = 7 per group), or decapitation (n = 7 per group). The order of sacrifices was balanced across experimental groups. In perfused mice, brains were collected and post-fixed in 4 % PFA at 4 °C for 48 h. The hemispheres were then separated along the midline and sectioned into 40 μm coronal slices (1/12 serial sectioning) using a vibratome (Microm H650V; Thermo Fisher Scientific, MA, USA). These sections were used for immunohistochemical studies. In decapitated mice, brains were removed, and the hippocampus was macrodissected, collected in a tube, drop-frozen on dry ice, and stored at −80 °C. The tissue was used for western blot (WB) analysis and spectrophotometric assays. Blood samples were collected from trunk blood after decapitation and centrifuged (10 min, 3000 rpm, 4 °C), and the supernatant (serum) was stored at −80 °C for corticosterone assays.

2.4.2. Immunohistochemistry and cell quantification

For immunohistochemical analysis, a random series of coronal sections from the left hemisphere was arbitrarily selected to represent the hippocampus along the anteroposterior axis. Free-floating immunohistochemistry (IHC) was performed following previously well-established

protocols (Avila-Gámiz et al., 2023; Ladrón de Guevara-Miranda et al., 2019), with detailed methodology provided in the Supplementary Material. Cellular markers were analyzed in four sections per animal by an experimenter blinded to the experimental conditions, including two sections from the dorsal hippocampus and two from the ventral hippocampus. The boundaries of hippocampal regions were defined according to anatomical criteria (Paxinos and Franklin, 2001), and focused on the dentate gyrus (DG) and cornu ammonis (CA1 and CA3).

To quantify neuronal density in adulthood, immunostaining for the neuron-specific nuclear protein (NeuN) was performed using mouse anti-NeuN (1:500, Abcam, 104224). NeuN serves as a marker for postmitotic neurons, labeling both mature and newly generated postmitotic neurons (Von Bohlen und Halbach, 2011). Following immunohistochemical processing, sections were scanned at 20× magnification using VS-ASW software linked to an Olympus VS120 virtual microscope (Olympus, Tokyo, Japan). The number of NeuN+ cells in the granular layer of the DG and the pyramidal layers of CA1 and CA3 was automatically quantified using an extension of the free software Fiji (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, Maryland, United States). Data for each animal and region were expressed as the number of positive cells per unit area (mm^2).

The analysis of parvalbumin (PV)-expressing interneurons, which represent the most abundant subtype of GABAergic interneurons and play a key role in excitatory/inhibitory balance, was also performed. These neurons contribute to emotional regulation, cognition, and information processing and are implicated in neurodevelopmental and neuropsychiatric disorders (Woodward and Coutellier, 2021) IHC for PV+ cells was performed using rabbit anti-parvalbumin (1:3000, Swant, PV25), and sections were scanned at 20× magnification. The number of PV+ cells was automatically quantified using the free software QuPath, v. 0.5.1 (University of Edinburgh), which was also used to outline and measure the regions of interest (DG, CA1, and CA3). Data were expressed as the number of PV+ cells per unit area (mm^2).

To assess adult hippocampal neurogenesis, we quantified bromodeoxyuridine (BrdU) incorporation during the abstinence period and measured the expression of the endogenous markers doublecortin (DCX) and phosphorilated-histone H3 (pH 3) in the DG. BrdU, a thymidine analog widely used to study neurogenesis, is integrated into the DNA of dividing cells during the S-phase of the cell cycle (Hayes and Nowakowski, 2000; Taupin, 2007). It was administered on the last day of cocaine/IGF2 treatment (i.e., experimental day 21), prior to the start of behavioral testing. BrdU was injected twice: first, 1 h after cocaine/IGF2 administration, and again 3 h later (two injections in total, each at a dose of 100 mg/kg, i.p., Sigma, St. Louis, MO, diluted in 0.9 % NaCl saline solution). Its incorporation into the cell nucleus was assessed after the abstinence period, 40 days post-administration, as an indicator of cell survival. To evaluate cell proliferation at the time of sacrifice, pH 3 was used a well-established marker, given its expression during the early stages of chromatin condensation, from late G2 phase through anaphase (Taupin, 2007; Von Bohlen und Halbach, 2011). From the birth of neuroblasts and throughout the first month, they express DCX, a microtubule-associated protein widely recognized as a specific marker of newly generated neurons. (Brown et al., 2003). DCX+ cells were quantified in the DG and classified based on their dendritic morphology, which reflects their stage of neuronal differentiation, ranging from less to more mature as follows: (1) proliferative stage, characterized by the absence of processes or the presence of only short ones; (2) intermediate stage, where medium-length processes may extend into the molecular layer; and (3) postmitotic stage, defined by dendritic branching within the molecular layer or the formation of a dendritic tree in the granule cell layer (Plümpe et al., 2006). For immunohistochemical analysis, BrdU+ cells in the granular layer of the DG were detected using a rat anti-BrdU antibody (1:500, Abcam, 6326), a rabbit anti-pH 3 antibody (1:200, Millipore, 06–570) for pH3+ cells and a rabbit anti-doublecortin (1:400, Abcam, 18723) for DCX+ cells. The number of BrdU and pH 3 positive cells, and DCX subtypes, were manually quantified using an Olympus BX41 microscope (Olympus, Solms, Germany) equipped with a 100× oil immersion lens. High-resolution images (10×) were captured with an Olympus DP70 digital camera (Olympus, Glostrup, Denmark), and the regions of interest (ROIs) were outlined and measured using QuPath, software to express the number of BrdU+, pH3+ and DCX+ cells and subtypes per unit area (mm^2).

2.4.3. Western blotting

Hippocampal tissue obtained from decapitated mice was used for WB studies. Four independent experiments were conducted for each experimental condition, with each hippocampal homogenate formed by pooling tissue from three to four mice. Blots were performed following previously described protocols (Romero-Zerbo et al., 2025) with additional details provided in the Supplementary Material. Rabbit anti-β-actin (1:1500, Cell Signaling Technology, USA) was used as a control. Additionally, multiple primary antibodies were used to detect and quantify specific proteins in hippocampal samples. To assess the expression of the IGF2 receptor (IGF2R, mannose 6-phosphate receptor -M6PR-), which plays a key role in mediating IGF2 functions and its regulation (Alberini, 2023), a rabbit anti-IGF2R antibody (1:150,000) was used. To determine the levels of Brain-Derived Neurotrophic Factor (BDNF) and its precursor pro-BDNF, the following primary antibodies were used: rabbit anti-BDNF (1:1000, Santa Cruz Biotechnology, TX, USA) and mouse anti-pro-BDNF (1:300, Santa Cruz Biotechnology, TX, USA). BDNF is a growth factor essential for synaptic plasticity and long-term potentiation (LTP), key mechanisms for learning and memory, and also plays a neuroprotective role in neurodegenerative diseases. Conversely, pro-BDNF has been associated with apoptosis induction and long-term depression (LTD) (Eggert et al., 2022; Foltran and Diaz, 2016). To evaluate synaptic plasticity, the presynaptic marker synaptotagmin I and the postsynaptic marker PSD-95 were analyzed (Zhornitsky et al., 2023), using rabbit anti-PSD-95 (1:2000, Abcam, USA) and mouse anti-synaptotagmin (1:6000, Abcam, USA). Protein-specific signals were detected using chemiluminescence with the ChemiDoc™ XRS+ Imaging System (Bio-Rad Laboratories, USA), and band intensities were quantified using Image Lab™ Software (Bio-Rad Laboratories, USA).

2.4.4. Spectrophotometry assays

In the same mice used for WB analysis, oxidative damage in the hippocampus was assessed using spectrophotometric methods (see details in Supplementary Material). Lipid hydroperoxides (LOOH) were quantified as an indicator of general lipid oxidative damage (García-Fernández et al., 2008a; García-Fernández et al., 2008b) while total glutathione (GSH) was measured to estimate the cellular redox environment (Schafer and Buettner, 2001). Their levels were spectrophotometrically determined following previously validated protocols (Aller et al., 2008; Boraldi et al., 2013).

2.4.5. Corticosterone assay

Serum obtained from blood samples was used to quantify corticosterone using a commercially available Enzyme Immunoassay Kit, following the manufacturer's instructions (Assay Designs/Stressgen, Ann Arbor, Michigan, USA; cat. number K014-H; sensitivity 20.9 pg/mL).

2.5. Statistical analysis

To assess the effects of treatment on behavioral tests, statistical analysis were performed using a two-way ANOVA with repeated measures (RM), with adolescent drug treatment and behavioral assessment time point (Evaluation 1: adolescence; Evaluation 2: adulthood) as factors, followed by post hoc Newman-Keuls (N-K) tests when significant differences were found. Data from the OF and FST were analyzed using a two-way repeated-measures ANOVA, with the two trials conducted during each evaluation included as a repeated measure. For the analysis of biological markers in tissues, a one-way ANOVA was performed to

evaluate differences between the administered pharmacological treatments, followed by Tukey's HSD post hoc test when required. Statistical analyses were conducted using Statistica 8 (StatSoft, Software Inc.). All data were reported as mean ± SEM, with a statistical significance threshold set at $p \leq 0.05$. The effect size was calculated using partial eta-squared (η_p^2), following Cohen's criteria (Cohen, 1977). Subjects were excluded from the final dataset if their values deviated by two or more standard deviations (SDs) from the mean. GraphPad Prism 8.02 was used for graphical representation.

3. Results

3.1. Behavioral results

3.1.1. Marble burying test

To estimate compulsive behaviors, the number of marbles buried was evaluated at two different time points (evaluation time): during adolescence (Evaluation 1, immediately after treatments) and during adulthood (Evaluation 2, following an abstinence period). The two-way ANOVA with RM revealed no significant effects of evaluation time or the interaction between evaluation time and pharmacological treatment [evaluation time: $F_{(1,51)} = 3326$, $p = 0.074$, $\eta_p^2 = 0.061$; interaction: $F_{(3,51)} = 0.662$, $p = 0.579$, $\eta_p^2 = 0.037$]. However, a significant main effect of treatment was detected [$F_{(3,51)} = 7534$, $p \leq 0.001$, $\eta_p^2 = 0.307$], indicating that cocaine-treated mice exhibited higher levels of compulsive behavior compared to the other groups (N-K: $p \leq 0.05$). This effect emerged during adolescence and persisted into adulthood despite the the prolonged abstinence period. Notably, this behavioral alteration was not observed in mice co-treated with cocaine and IGF2 (Fig. 2A).

3.1.2. Nest building test

Nest-building behavior was analyzed over a 24-h period by evaluating both nestlet shredding and nest quality. A two-way ANOVA with RM revealed significant differences in nestlet shredding between treatments groups across both evaluations [$F_{(3,52)} = 9345$, $p \leq 0.001$, $\eta_p^2 = 0.350$]. Post hoc *comparisons* indicated that mice treated with IGF2, either alone or in combination with cocaine, shredded less nestlet than the groups that did not receive IGF2 (N-K: $p \leq 0.05$). Additionally, a significant effect of evaluation time was observed [$F_{(1,52)} = 17{,}612$, $p \leq 0.001$, $\eta_p^2 = 0.253$], showing that adult mice shredded more nestlet than adolescent mice. However, the interaction between treatment and evaluation did not yield significant differences [$F_{(3,52)} = 1635$, $p = 0.193$, $\eta_p^2 = 0.086$] (Fig. 2B). When assessing nest complexity on a 0–5 scale after 24 h, two-way ANOVA with RM indicated no significant effects of treatment, evaluation time, or their interaction [treatment: $F_{(3,52)} = 2603$, $p = 0.062$, $\eta_p^2 = 0.131$; evaluation: $F_{(1,52)} = 0{,}052$, $p = 0.821$, $\eta_p^2 = 0.001$; interaction: $F_{(3,52)} = 0.158$, $p = 0.748$, $\eta_p^2 = 0.023$] (Fig. 2C). Additional results on nestlet shredding at 30, 60, 90, and 240 min are provided in the Supplementary Material.

3.1.3. Sucrose preference test

The intake of a sucrose solution relative to water over a 24-h period was measured, and the sucrose preference rate, which serves as an indicator of motivated natural behaviors, was calculated (Fig. 2D). A two-way ANOVA with RM was conducted to compare the results obtained during evaluations 1 and 2. The analysis showed no significant differences between treatment groups across both evaluations [$F_{(3,51)} = 1192$, $p = 0.322$, $\eta_p^2 = 0.066$]. However, a significant effect of the evaluation time was observed [$F_{(1,51)} = 10{,}915$, $p \leq 0.01$, $\eta_p^2 = 0.176$], indicating that adolescent mice exhibited lower motivation for sucrose consumption compared to adults. Notably, this difference may be attributed to specific groups, as a significant interaction effect between evaluation time and treatment was detected [$F_{(3,51)} = 6061$, $p \leq 0.01$, $\eta_p^2 = 0.263$]. Post hoc comparisons showed that mice exposed to cocaine during adolescence exhibited lower sucrose consumption than the same mice in adulthood, and a similar pattern was observed in animals treated with saline (N-K: $p \leq 0.05$). This effect was not observed in the IGF2 or cocaine + IGF2 groups, which maintained high sucrose consumption in both evaluations (N-K: $p > 0.05$).

3.1.4. Elevated plus maze

Anxiety-related behavior was assessed in the EPM using the open arm exploration ratio, as described in the Methods section. A two-way ANOVA with RM was used to analyze the data. The analysis revealed no significant differences in the open arm exploration ratio between treatment groups [$F_{(3,49)} = 1{,}92$, $p = 0.139$, $\eta_p^2 = 0.105$]. However, significant differences were found between evaluations, showing that values were higher during adolescence than in adulthood [$F_{(1,49)} = 117{,}41$, $p \leq 0.001$, $\eta_p^2 = 0.706$], and in the interaction between treatments and evaluation [$F_{(3,49)} = 4{,}54$, $p \leq 0.01$, $\eta_p^2 = 0.218$]. Post hoc comparisons revealed that adolescent mice treated with cocaine exhibited significantly higher open arm exploration ratio values compared to those treated with saline, IGF2 or cocaine + IGF2 (N-K: $p \leq 0.05$) (Fig. 2E). High values reflect a disinhibited behavior in adolescent mice exposed to cocaine, which was also evidenced by an increased time spent peering into the void (dipping). These behavioral changes were not associated with increased locomotor activity. Data on dipping, locomotion, and the remaining analyzed variables are provided in the Supplementary Material.

3.2. Open field test

Behavior in the OF was assessed across two trials conducted on the same day, with a 2-h intertrial interval. Data were analyzed using a two-way ANOVA with RM for each evaluation period (adolescence and adulthood), with trial as the within-subjects factor and treatment group as the between-subjects factor.

One of the primary indicators of exploratory behavior in mice is the distance traveled in the OF, reflecting locomotor activity. Analysis of this variable during the first evaluation period (adolescence) revealed statistically significant differences between groups, with both IGF2-treated groups being less active compared to the saline and cocaine-treated groups [$F_{(3,47)} = 11{,}36$, $p \leq 0.001$, $\eta_p^2 = 0.420$, N-K: $p \leq 0.05$]. Nevertheless, all groups exhibited a significant reduction in distance traveled during the second trial compared to the first, with a similar magnitude of change across groups [$F_{(1,47)} = 135{,}96$, $p \leq 0.001$, $\eta_p^2 = 0.743$], indicating normal adaptation to the environment upon re-exposure to the OF (Fig. 2F). A similar behavioral pattern was observed during the second evaluation period (adulthood), with reduced distance traveled during the second trial, although the magnitude of this decrease was smaller compared to adolescence [$F_{(1,45)} = 11{,}42$, $p \leq 0.01$, $\eta_p^2 = 0.202$], and this effect was independent of treatment group [$F_{(3,45)} = 1{,}88$, $p = 0.147$, $\eta_p^2 = 0.111$].

Another relevant variable analyzed was the time spent in the center of the maze, a commonly used indicator of anxiety-like behavior in rodents, where increased time reflects reduced anxiety levels. Statistical analysis revealed a significant group x trial interaction during the first evaluation period (adolescence), with saline and cocaine-treated groups spending less time in the center of the OF in the second trial, compared to both IGF2-treated groups [$F_{(3,46)} = 4{,}65$, $p \leq 0.01$, $\eta_p^2 = 0.233$, N-K: $p \leq 0.05$]. In the second evaluation period (adulthood), statistical analysis also revealed a significant treatment x trial interaction, with IGF2 and cocaine + IGF2 groups spending less time in the center of the OF during the second trial [$F_{(3,47)} = 4{,}67$, $p \leq 0.01$, $\eta_p^2 = 0.230$, N-K: $p \leq 0.05$] (Fig. 2G).

3.2.1. Novel place recognition and novel object recognition test

To assess episodic-like recognition memory (both place memory and object memory), the time that mice spent exploring each object was analyzed, and a discrimination index was calculated. A two-way ANOVA with one RM was used to analyze each task, in accordance with the other tests. In the NPR test (Fig. 2H), the ANOVA showed significant

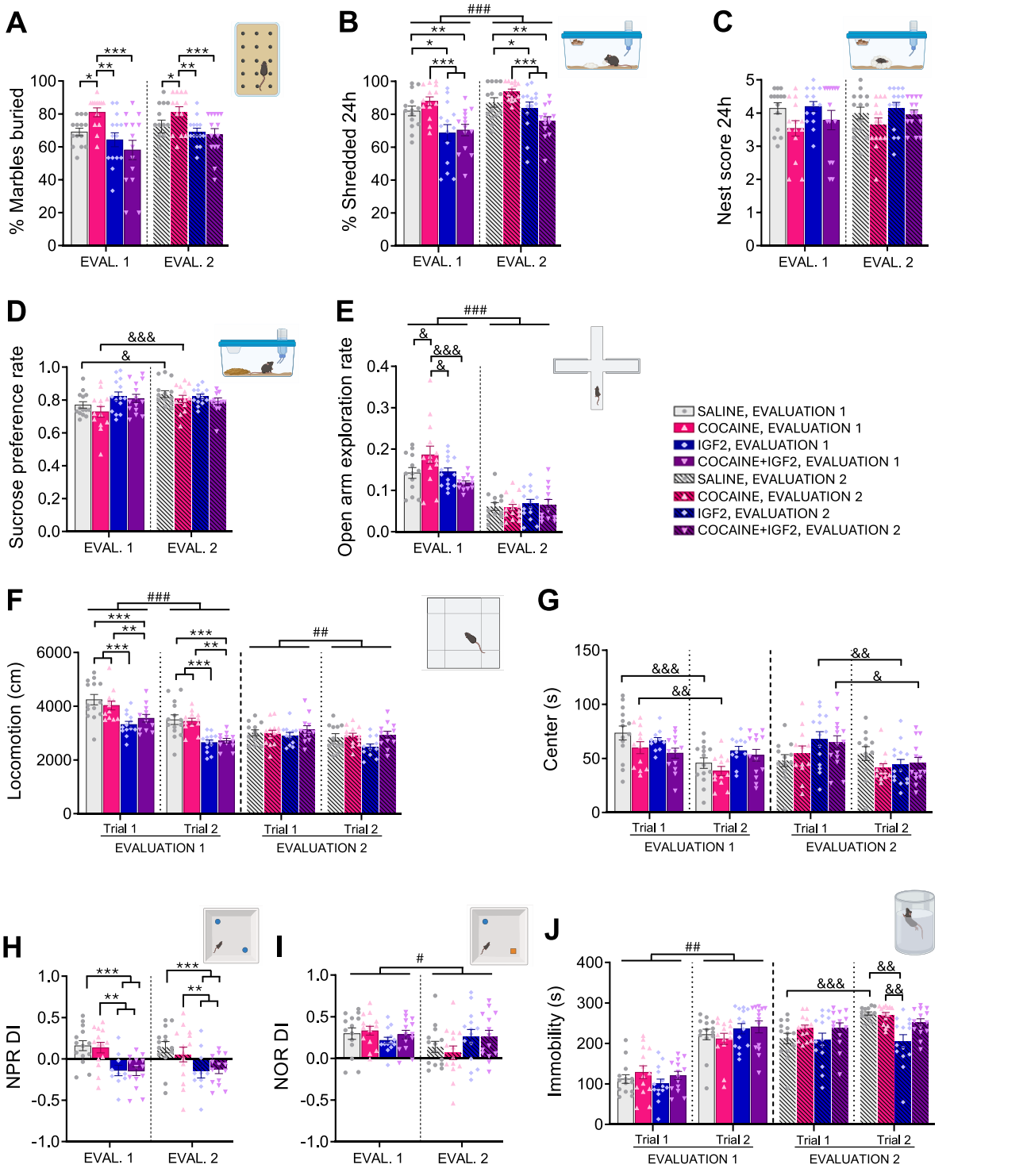

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Fig. 2. Effect of adolescent cocaine and/or IGF2 administration on a battery of behavioral tests. Behavioral assessments were conducted during adolescence (Evaluation 1) and repeated in the same animals during adulthood (Evaluation 2). (A) In the marble burying test, animals treated with cocaine buried more marbles in both evaluations; this effect was abolished by co-administration of IGF2 (n = 13–14 per group). (B) In the nest building test (24 h), adult animals shredded more nesting material than adolescents. Moreover, animals treated with IGF2 or IGF2 + cocaine shredded less nesting material than the other groups (n = 14 per group). (C) However, no significant differences were found between treatment groups when evaluating nest quality (n = 14 per group). (D) In the sucrose preference test, animals treated with saline or cocaine increased sucrose consumption in adulthood, while those treated with IGF2 or IGF2 + cocaine maintained high and stable consumption across both evaluations (n = 13–14 per group). (E) The open arm exploration ratio, calculated from the time spent in the open arms of the elevated plus maze, was higher in cocaine-treated adolescents (indicating lower anxiety), an effect that was prevented by IGF2 co-administration (n = 12–14 per group). (F) In the open field test, animals underwent a double exposure per evaluation. In the first trial of each evaluation, animals traveled a greater distance than in the second. Additionally, during adolescence, IGF2-treated groups showed lower locomotor activity than saline and cocaine groups (n = 12–14 per group). (G) Saline- and cocaine-treated animals spent less time in the center of the open field during the second trial of the first evaluation, whereas this behavior was observed in IGF2-treated groups during adulthood (n = 12–14 per group). (H) In the novel place recognition task, animals treated with IGF2 (alone or with cocaine) showed a reduced discrimination index for the displaced object, a long-lasting effect (n = 10–14 per group). (I) However, no such deficits were observed in the novel object recognition task. In this case, discrimination index values were generally higher during adolescence than adulthood (n = 14 per group). (J) The forced swim test was performed over two consecutive days in each evaluation. During adolescence, all animals showed increased immobility time in the second trial compared to the first. In adulthood, this effect was only observed in the saline group. Moreover, animals treated with IGF2 alone spent less time immobile in the final trial (n = 14 per group). Data are presented as mean ± SEM, with dots indicating individual values. Two-way repeated-measures ANOVAs were performed, followed by Newman-Keuls (N-K) post hoc tests when significant differences were detected. Symbols: (*) indicate significant treatment effects (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$); (#) indicate effects of evaluation time (or trials in F, G and J); (&) indicate treatment × time interaction effects. One-way ANOVAs were also conducted to independently evaluate the treatment effects in each evaluation; see Supplementary Material for detailed analyses. Abbreviations: Eval., evaluation; NPR DI, novel place recognition discrimination index; NOR DI, novel object recognition discrimination index.
Figure partially created in BioRender.com.

differences between treatments [$F_{(3,48)} = 10{,}338$, $p \leq 0.001$, $\eta^2_p = 0.393$]. Post hoc analysis revealed that animals treated with IGF2 (either alone or in combination with cocaine), spent significantly less time exploring the displaced object than the one that remained in its original position (N-K: $p \leq 0.05$). However, evaluation time did not have a significant effect, nor did the interaction between treatment and evaluation time [evaluation: $F_{(1,48)} = 0{,}213$, $p = 0.647$, $\eta^2_p = 0.004$; interaction: $F_{(3,48)} = 0.232$, $p = 0.874$, $\eta^2_p = 0.014$].

Regarding the NOR test (Fig. 2I), a significant effect of the evaluation time was observed [$F_{(1,52)} = 4630$, $p \leq 0.05$, $\eta^2_p = 0.082$], showing that adolescent mice performed better in the object memory task than adults. However, neither treatment nor the interaction between evaluation time and treatment had a significant effect on object recognition memory [treatment: $F_{(3,52)} = 0{,}463$, $p = 0.709$, $\eta^2_p = 0.026$; interaction: $F_{(3,52)} = 2247$, $p = 0.094$, $\eta^2_p = 0.115$].

3.2.2. Forced swimming test

Behavior in the FST was assessed across two trials conducted on two consecutive days at the end each evaluation period. A two-way ANOVA with RM was performed for each evaluation (adolescence and adulthood), with trial as the within-subjects factor and treatment group as the between-subjects factor.

Analysis of immobility during the first evaluation period (adolescence) showed that all groups exhibited a statistically significant increase in immobility time during the second trial compared to the first, with a similar magnitude of change across groups [$F_{(1,52)} = 789{,}402$, $p \leq 0.001$, $\eta^2_p = 0.785$], suggesting that none of the treatments affected the animals' adaptation to the inescapable situation. During the second evaluation period (adulthood), statistical analysis revealed a significant group x trial interaction, with saline group increasing the immobility time in the second trial compared to the first. In addition, analysis showed that in the second trial, saline and cocaine-treated groups spend more time immobile, compared to IGF2-treated group [$F_{(3,52)} = 4{,}30$, $p \leq 0.01$, $\eta^2_p = 0.199$, N-K: $p \leq 0.05$] (Fig. 2J).

Additional FST variables (climbing and swimming) were also analyzed, and the corresponding results are provided in the Supplementary Material.

3.3. Tissue analysis results

3.3.1. Immunohistochemistry and cell quantification

The number of neurons in adulthood, measured by the quantification of NeuN+ cell density, did not differ significantly across the four experimental groups in any of the hippocampal regions studied: CA1 [$F_{(3,24)} = 0{,}591$, $p = 0.627$, $\eta^2_p = 0.069$], CA3 [$F_{(3,23)} = 2613$, $p = 0.076$, $\eta^2_p = 0.254$], and DG [$F_{(3,24)} = 0{,}346$, $p = 0.793$, $\eta^2_p = 0.041$] (Fig. 3A and B). In contrast, quantification of the PV+ subpopulation of GABAergic interneurons revealed a significant reduction in PV+ cell numbers in both the CA1 and CA3 regions of animals treated with cocaine, either alone or in combination with IGF2 (CA1: [$F_{(3,24)} = 8721$, $p \leq 0.001$, $\eta^2_p = 0.522$, Tukey's HSD: $p \leq 0.05$], CA3 [$F_{(3,24)} = 7763$, $p \leq 0.001$, $\eta^2_p = 0.493$, Tukey's HSD: $p \leq 0.05$]. However, this treatment effect was not observed in the DG [$F_{(3,24)} = 1933$, $p = 0.151$, $\eta^2_p = 0.195$] (Fig. 3C and D).

Analysis of BrdU incorporation revealed that the survival of newly formed cells during the withdrawal period did not differ significantly between groups. [$F_{(3,23)} = 2665$, $p = 0.0717$, $\eta^2_p = 0.258$] (Fig. 3E). Similarly, the analysis of pH 3 immunostaining (marker of cell proliferation), revealed no significant differences between groups in the number of $pH3^+$ cells [$F_{(3,24)} = 0{,}731$, $p = 0.5439$, $\eta^2_p = 0.08368$] (Fig. 3F). Consistently, the analysis of DCX (marker for young neurons), showed no significant differences in the DG, either in the total number of DCX+ cells [$F_{(3,22)} = 2324$, $p = 0.103$, $\eta^2_p = 0.241$], or when analyzed according to their maturation stage (postmitotic DCX+ cells [$F_{(3,22)} = 2196$, $p = 0.117$, $\eta^2_p = 0.230$]; proliferative [$F_{(3,22)} = 1105$, $p = 0.368$, $\eta^2_p = 0.131$]) (Fig. 3G).

3.3.2. Western blotting analysis of neurotrophic and synaptic markers

WB analysis showed that the expression levels of BDNF and its precursor (proBDNF) was not affected by the different treatments ([$F_{(3,28)} = 1870$, $p = 0.158$, $\eta^2_p = 0.167$] and [$F_{(3,28)} = 1474$, $p = 0.243$, $\eta^2_p = 0.136$], respectively) (Fig. 4A and B). Similarly, no significant differences were detected in the expression of the postsynaptic marker PSD-95 [$F_{(3,28)} = 1409$, $p = 0.261$, $\eta^2_p = 0.131$] (Fig. 4C). However, the presynaptic marker synaptotagmin exhibited significant variations among groups [$F_{(3,28)} = 16{,}280$, $p \leq 0.001$, $\eta^2_p = 0.636$], with its expression being consistently higher in animals treated with IGF2, either alone or in combination with cocaine (Tukey's HSD: $p \leq 0.05$) (Fig. 4D). In addition, the expression of the IGF2R was lower in animals that received IGF2 compared to those treated with saline or cocaine. When IGF2 was co-administered with cocaine, receptor expression showed a less pronounced decrease but remained significantly lower than in the saline-treated group [$F_{(3,28)} = 11{,}270$, $p \leq 0.001$, $\eta^2_p = 0.547$, Tukey's HSD: $p \leq 0.05$] (Fig. 4E).

3.3.3. Spectrophotometric analysis and corticosterone assay

The quantification of LOOH (Fig. 4F) and total GSH levels (Fig. 4G) indicated that none of the treatments induced oxidative damage in the

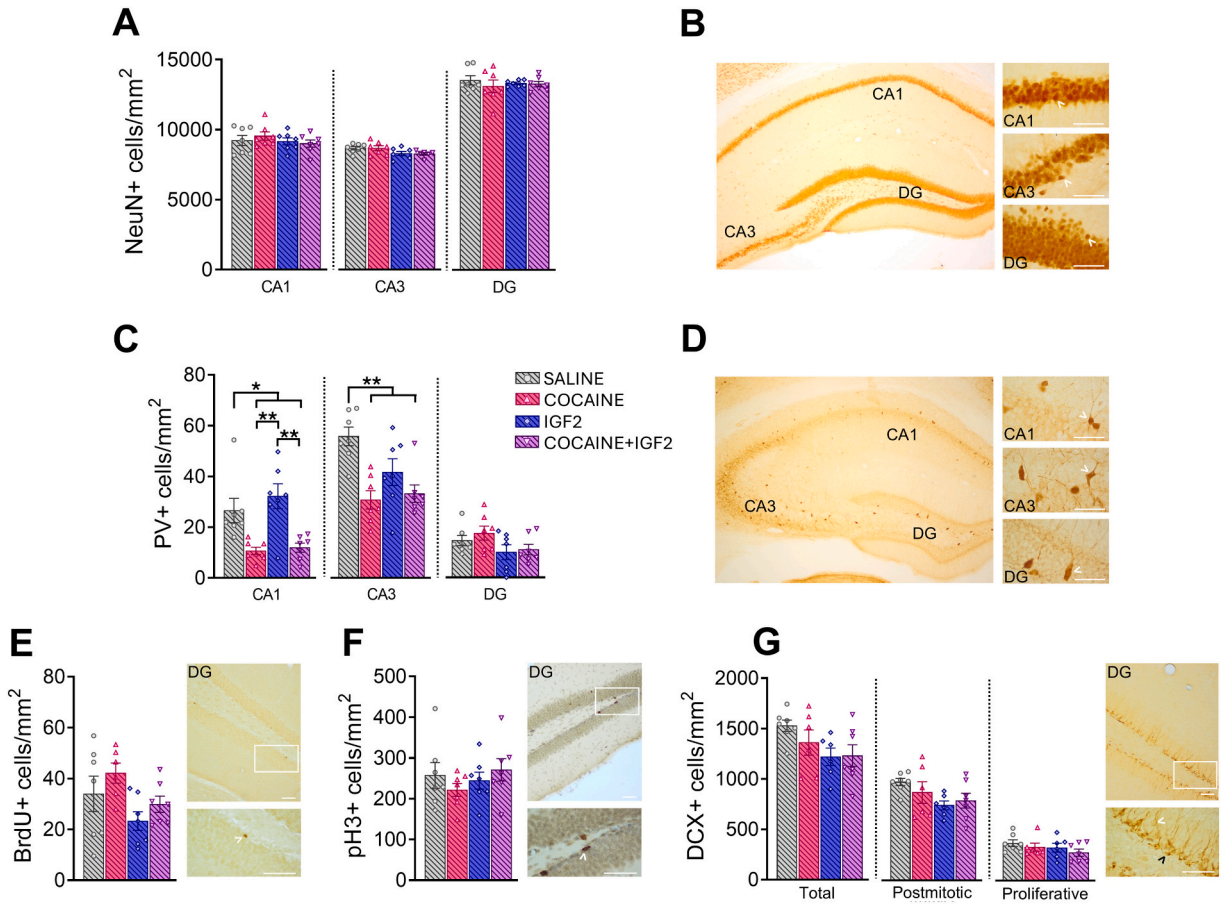

Fig. 3. Effect of adolescent cocaine and/or IGF2 administration on hippocampal cellular markers. Brains were collected 24 h after the second behavioral evaluation. Coronal sections were processed for immunohistochemical analysis. (A) The number of mature neurons labeled as $NeuN^+$ was similar across all groups in the CA1, CA3, and dentate gyrus (DG) regions. (B) Representative NeuN immunostaining in CA1, CA3, and DG for saline group, with examples of positive signals indicated by arrows. (C) The number of GABAergic interneurons labeled as parvalbumin $(PV)^+$ was reduced in CA1 and CA3 in cocaine-treated animals, and this effect was not reversed by IGF2 co-treatment. (D) Representative PV immunostaining in CA1, CA3, and DG for saline group, with positive cells indicated by arrows. No significant differences were found between experimental groups in hippocampal neurogenesis markers in the DG: (E) bromodeoxyuridine $(BrdU)^+$ cells, (F) phosphorylated histone 3 $(pH\ 3)^+$ cells, (G) doublecortin $(DCX)^+$ cells. (E, F, G) Representative BrdU, DCX, and pH 3 (respectively) immunostaining in the dentate gyrus (DG) for saline group, with examples of positive signals indicated by arrows. In DCX immunostaining, the white arrow indicates a cell in the postmitotic stage and the black arrow indicates a cell in the proliferative stage. Scale bars indicate 50 μm. One-way ANOVAs were performed, followed by Tukey's HSD post hoc tests when significant differences were detected: $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Abbreviations: NeuN, neuron-specific nuclear protein; PV, parvalbumin; BrdU, bromodeoxyuridine; pH 3, phosphorylated histone 3; DCX, doublecortin; DG, dentate gyrus.

hippocampus, as no significant differences were observed among groups ($[F_{(3,24)} = 0{,}832, p = 0.490, \eta_p^2 = 0.094]$ and $[F_{(3,24)} = 1.322, p = 0.290, \eta_p^2 = 0.142]$, respectively). Similarly, serum corticosterone levels remained unchanged across experimental conditions, with no significant differences observed between groups $[F_{(3,24)} = 1047, p = 0.390, \eta_p^2 = 0.116]$ (Fig. 4H).

4. Discussion

The results of this study contribute to the understanding of how repeated cocaine exposure during adolescence impacts behavior and brain physiology in mice, both in the short and long term, following a prolonged period of abstinence and into adulthood. Additionally, the potential neuroprotective role of IGF2 is explored. Our findings show that chronic cocaine administration during this critical developmental period induces certain behavioral and cellular alterations, while co-administration of IGF2 attenuates some of these effects.

The most evident effects of cocaine administration during adolescence were observed in the MBT, which revealed an increase in compulsive-like behaviors both 24 h and 30 days after drug abstinence, when the animals had reached adulthood. These findings indicate that chronic cocaine exposure induces compulsive behaviors beyond the early abstinence phase, as demonstrated in previous studies on ethanol (Sampedro-Piquero et al., 2022; Umathe et al., 2008). Interestingly, coadministration with IGF2 reversed the effect of cocaine on these behaviors, both in the short and long term, suggesting a neuroprotective role of IGF2 against the drug's effects. A study using BTBR mice, an autism model that exhibits repetitive behaviors, also reported a reduction in marble-burying activity following IGF2 administration (Steinmetz et al., 2018). In our study, the reduction in compulsive behaviors following IGF2 administration was also evident in the NBT, where IGF2-treated groups consistently exhibited lower amounts of shredded nesting material across different time points, including the 24-h mark. However, no significant differences in nest quality were found across treatments or ages, although there was a non-significant trend toward poorer quality nests in cocaine-treated mice. This difference between the amount of shredded material and the complexity of nest construction, suggests that these two variables assess distinct behavioral phenomena: the former may reflect anxiety-related compulsive behaviors (Albelda and Joel, 2012; Angoa-Pérez et al., 2013), while the latter reflects the quality of nest construction and its functionality, which could be interpreted as a motivational aspect related to welfare (Gaskill

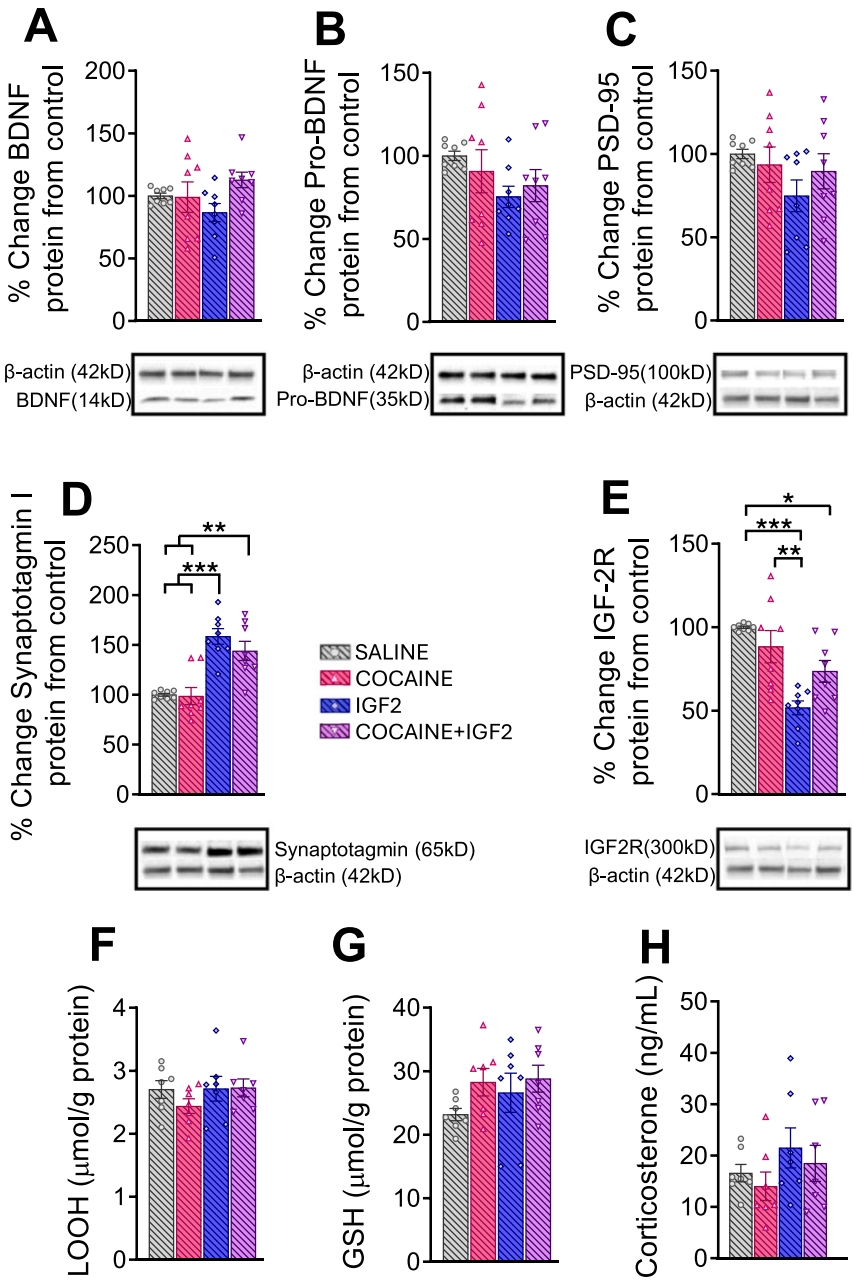

Fig. 4. Effect of adolescent cocaine and/or IGF2 administration on neurotrophic and synaptic markers, oxidative stress, and corticosterone levels. Hippocampal tissue and blood samples were collected 24 h after the second behavioral evaluation. Western blotting, spectrophotometric analyses, and corticosterone assays were performed. Brain-derived neurotrophic factor (BDNF) and its precursor (proBDNF) showed no significant differences between groups. Synaptic neuroplasticity was affected by the treatments: levels of the postsynaptic protein PSD-95 did not differ among groups, whereas IGF2-treated groups exhibited higher levels of the presynaptic protein synaptotagmin I compared to saline- or cocaine-treated groups. Analysis of IGF2 receptor (IGF2R) levels revealed a reduction in IGF2-treated animals, especially when IGF2 was administered without cocaine. No significant group differences were observed in oxidative stress markers, including lipid hydroperoxides (LOOH) and total glutathione (GSH). Corticosterone levels were also similar across all experimental groups. One-way ANOVAs were performed, followed by Tukey's HSD post hoc tests when significant differences were detected: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Abbreviations: BDNF, brain-derived neurotrophic factor; PSD-95, postsynaptic density protein 95; IGF2R, IGF2 receptor; LOOH, lipid hydroperoxides; GSH, glutathione.

et al., 2013; Gjendal et al., 2019; Kraeuter et al., 2019). In humans, a hallmark of the transition from recreational cocaine use to addiction is the development of compulsive drug-seeking bahavior, which is driven by dysregulated brain mechanisms controlling impulse control and reward processing (Sun and Yuill, 2020). Given that IGF2 seems to reduce compulsive-like behaviors, it would be interesting to further investigate the underlying neurobiological mechanisms involved.

The results from the EPM showed that adolescent mice spent more time in the open arms of the maze, suggesting that they were more likely to explore new or potentially threatening environments than adults. This pattern is consistent with previous research demonstrating that, as the brain matures, impulsivity and novelty-seeking behaviors decrease, while anxiety responses stabilize (Casey and Jones, 2010; Laviola et al., 2003; Spear, 2000). The reduction in exploratory anxiety was even more pronounced in the cocaine-treated mice, which also exhibited an increased frequency of peering into the void, independent of locomotor activity. However, these cocaine-induced effects were not long-lasting, as they disappeared after a prolonged abstinence period when the

animals were evaluated in adulthood. These results are consistent with some previous literature but also contrast with others. Caffino et al. (2022) suggested that discrepancies in the literature may result from differences in exposure paradigms, specific vulnerability windows for anxiety symptoms, and the timing of symptom evaluation, particularly the duration of withdrawal. Interestingly, in our study, when IGF2 was coadministered, the effect of cocaine on adolescent risk-taking bahavior was abolished, suggesting that IGF2 might counteract the disinhibitory effects or the increased propensity for risk-taking induced by cocaine exposure.

Together, the results obtained from the MBT, NBT, and EPM indicate that cocaine exposure during adolescence led to an increase in compulsive behaviors and a decrease in risk perception. In our study, this compulsive behavioral response was lower in animals treated simultaneously with IGF2, suggesting that IGF2 could mitigate the impact of cocaine on these behaviors. Since reducing anxiety symptoms has been associated with a lower risk of cocaine (Buffalari et al., 2012), future research should further explore IGF2's potential therapeutic applications in addiction models.

An unexpected finding was obtained in the NPR test. Both this test and the NOR test rely on rodents' innate preference for novelty. We observed that animals treated with IGF2, alone or with cocaine, showed lower discrimination indices, spending less time exploring the displaced object than the static one. Given the negative index in these groups, it seems that IGF2 promotes a greater preference for the object located in a familiar position (bias toward familiarity). This finding suggests that IGF2 may impair performance in a spatial memory task highly dependent on hippocampal function, such as NPR (Barker and Warburton, 2011). Interestingly, this result contradicts previous literature, where IGF2 has been described as a molecule that enhances memory, both when administered directly into the hippocampus (Agis-Balboa et al., 2011; Steinmetz et al., 2018), and when administered systemically (Stern et al., 2014b). This hippocampus-dependent memory-enhancing effect, including performance in the NPR test, has also been reported by other research groups (Lee et al., 2015; Stern et al., 2014b; Yu et al., 2020). However, in all of these studies, IGF2 was administered for a shorter period than in ours, and the route of administration was often intracerebral microinfusion. In addition, it has been demonstrated that this ability to enhance memory is only evident when IGF2 is administered within a specific time window (Lee et al., 2015) and are mediated through its binding to the IGF2R (Beletskiy et al., 2021; Chen et al., 2011; Stern et al., 2014b; Yu et al., 2020). IGF2R plays a key role in growth and development, but is also widely expressed in specific regions of the mature nervous system, including hippocampal neurons, where it plays a crucial role in long-term memory consolidation. It likely exerts its function by regulating the sorting, recycling, synthesis, and degradation of proteins involved in long-term plasticity and memory, possibly through the modulation of endosomal trafficking networks via molecular mechanisms directly or indirectly linked to the activation of downstream G-protein signaling pathways (Alberini, 2023). In our study, IGF2-treated animals displayed a significant decrease in IGF2R expression, particularly in the group receiving IGF2 alone but also in combination with cocaine. This result was expected as part of a down-regulation mechanism associated with chronic administration of this growth factor. Given that IGF2R is directly involved in hippocampus-dependent spatial memory, it is not surprising that animals with lower receptor levels exhibited impaired performance in the NPR task. This potentially detrimental effect of chronic IGF2 exposure at specific doses is a critical factor that should be further explored in future studies.

Interestingly, the effect observed in the NPR test was not replicated in the NOR test, where no significant differences were observed across treatment groups. As expected, adolescents exhibited a greater interest in exploring novel objects compared to adults, a widely documented phenomenon reflecting age-dependent novelty-seeking bahavior (Spear, 2000). Although a trend was observed in IGF2-treated groups toward maintaining interest in the novel object over time, the difference did not reach statistical significance. Previous studies administering IGF2 under conditions of brain injury have also failed to report memory enhancement in this task (Redell et al., 2021; Vafaee et al., 2018), and another study (using an autism model) reported improvements in NOR, but only when IGF2 was administered within a specific time window (Steinmetz et al., 2018). This divergence between NPR and NOR results suggests that IGF2's effects may be specific to certain cognitive domains or brain regions. While the hippocampus plays a well-established role in spatial memory tasks such as NPR, its involvement in object recognition memory tasks remains unclear, as other brain structures may play a more decisive role in these cognitive processes (Cohen and Stackman, 2015).

SPT and FST are two commonly used tests to evaluate depressive-like behaviors, such as anhedonia and despair, in animal models (Song et al., 2020), including those focused on adolescence and substance abuse administration (Ribeiro-Carvalho et al., 2011). In our study, the SPT revealed that adolescent animals exhibited slightly lower motivation for sucrose consumption compared to adults. This difference was primarily observed in both cocaine- and saline-treated (control) animals, suggesting that exposure to cocaine during adolescence does not significantly alter motivation for natural rewards. In contrast, animals treated with IGF2, either alone or in combination with cocaine, maintained high levels of sucrose preference at both evaluation stages. In the FST, immobility progressively increased with consecutive days of exposure to the test, indicating habituation to the task. Notably, cocaine exposure did not increase immobility behavior in mice compared with controls, either during adolescence or adulthood. Thus, our study did not reveal a clear effect of cocaine on depressive-like behaviors, reflecting a normal habituation pattern to the task, in line with reports from other groups (Bis-Humbert et al., 2021; García-Cabrerizo et al., 2015) In contrast, IGF2-treated animals showed increased immobility across successive trials during adolescence, similar to the other experimental groups, but in adulthood they exhibited reduced immobility during the final exposure compared to non-IGF2-treated groups, with an even more pronounced reduction compared to controls. This specific effect of IGF2 could be interpreted as mitigating depressive-like behaviors, considering the antidepressant action of IGF2 described by other groups (Guo et al., 2023; Luo et al., 2015). IGF2 has been implicated in depressive symptomatology and in mechanisms of antidepressant action, both in animal models (Malysheva and Ordyan, 2022), and in humans (Fernández-Pereira et al., 2023; Ye et al., 2024), although its role has not yet been fully elucidated.

On the other hand, the adolescent hippocampus is still in development, undergoing significant structural and functional changes (Fuhrmann et al., 2015; Hueston et al., 2017; Lynch et al., 2018) and is highly sensitive to drug abuse during this critical period (Blest-Hopley et al., 2020; Wooden et al., 2021). Consequently, exposure to drugs such as cocaine during adolescence may disrupt hippocampal maturation, leading to long-lasting alterations in neuronal structure and function within this brain region, which may in turn affect hippocampus-related behavioral processes.

To assess whether adolescent cocaine and IGF2 exposure affects adult neuronal density, we analyzed NeuN expression in hippocampal subregions (CA1, CA3, DG). Cocaine exposure did not reduce overall neuronal density in adulthood, though long-term cellular damage cannot be ruled out, as reported in rats exposed to cocaine during adolescence (Zhu et al., 2016). We also examined PV+ GABAergic interneurons, critical for hippocampal function and linked to behavioral disorders (Nahar et al., 2021; Woodward and Coutellier, 2021). Our results indicate that the number of PV+ GABAergic interneurons in the DG of the hippocampus remained unchanged following a prolonged period of abstinence from cocaine, consistent with previous reports in adult animals (Ladrón de Guevara-Miranda et al., 2017). However, a significant reduction in PV+ cell density was observed in the CA1 and CA3 regions. Notably, this effect was not reversed by IGF2, suggesting that certain cocaine-induced alterations are resistant to IGF2 treatment

and persist despite extended abstinence. Cass et al. (2013) showed that adolescent cocaine disrupts GABAergic network maturation in the PFC, modulated by ventral hippocampal glutamatergic input and dopamine. PV+ interneuron dysfunction may lead to PFC disinhibition, contributing to deficits in working memory, decision-making, and impulse control. Our results extend this finding to the hippocampus. Since PV+ interneurons synchronize neural networks, their loss may also disrupt hippocampal–PFC bidirectional communication. These interneurons originate in the ganglionic eminences and migrate to regions like the hippocampus, which stabilizes late, making it vulnerable to migration disturbances (reviewed in Ruden et al., 2021). Thus, the reduction in PV+ interneurons observed in our study may result from altered migratory patterns caused by cocaine exposure.

Previous studies, including those from our group, have demonstrated that cocaine negatively affects adult neurogenesis in the DG of the hippocampus, which plays a crucial role in cognitive and behavioral functions, potentially contributing to the behavioral consequences of drug exposure (reviewed in Castilla-Ortega et al., 2016, 2017). However, our study did not replicate this effect, likely because we administered cocaine during adolescence, when neurogenesis is at its peak (Kozareva et al., 2019), rather than in adulthood, or possibly due to the timing of our analysis, conducted 40 days after drug abstinence. This suggests that the impact of cocaine on the proliferation and survival of young neurons (as assessed by pH 3, BrdU and DCX studies) spontaneously recovers during this period, according with previous research (reviewed in Castilla-Ortega and Santín, 2020). Consistent with our findings, Noonan et al., 2008) reported that a four-week cocaine withdrawal period in rats did not significantly alter the numbers of DCX+, BrdU+, or Ki-67+ cells. Interestingly, they observed that BrdU+ neurons exhibited enhanced maturity following abstinence. Taking these findings into account, our study cannot rule out the possibility that cocaine may influence the maturation of newly generated cells during the abstinence period, potentially contributing to the behavioral outcomes. On the other hand, although previous studies have reported that IGF2 administration enhances hippocampal neurogenesis in adulthood (Agis-Balboa et al., 2011; Iwamoto and Ouchi, 2014; Ouchi et al., 2013), we did not observe an increase in either mature or young neurons following IGF2 treatment. However, our analysis was performed after 40 days of abstinence, and it is possible that the pro-neurogenic effects of IGF2 are transient, occurring only during earlier phases of neuronal development and differentiation. This raises the possibility that IGF2 exerts its neuroprotective role primarily during the initial stages of neurogenesis, promoting neuronal proliferation and survival shortly after administration, rather than maintaining long-term effects on neuronal maturation and integration. Notably, in a separate study using a similar protocol (where adolescent mice received systemic IGF2 and/or cocaine for 21 consecutive days and were sacrificed the day after the final administration, without behavioral testing or abstinence) we observed a significant impact on hippocampal neurogenesis. In that study, cocaine reduced hippocampal neurogenesis, an effect partially counteracted by IGF2, which appears to exert protective actions primarily during the early stages of neuronal development (unpublished data). Future studies examining the time-dependent impact of IGF2 on different phases of neurogenesis will be essential to elucidate its therapeutic potential in mitigating drug-induced deficits.

Existing literature have shown that chronic cocaine exposure during adolescence alters hippocampal expression of neuroplasticity-related proteins, linked to behavioral deficits persisting after prolonged abstinence (Fole et al., 2015; García-Cabrerizo et al., 2015; Giannotti et al., 2013; Valzachi et al., 2013; Zhu et al., 2016). In our study, cocaine had no significant effect on cellular plasticity markers, an unexpected finding given prior evidence. However, IGF2, alone or with cocaine, significantly increased long-term expression of the presynaptic marker synaptotagmin in the hippocampus. Despite this, the postsynaptic marker PSD-95 remained unchanged, suggesting IGF2's effects may be presynaptic without major impact on postsynaptic density. In cell culture, both synaptic plasticity proteins increase with IGF2 (Martín-Montañez et al., 2017), but in vivo effects in hippocampus remain poorly explored. No significant differences were observed in BDNF or pro-BDNF levels among groups. Although prior studies link adolescent cocaine exposure to altered BDNF after prolonged abstinence (Caffino et al., 2022), we did not replicate this effect under our conditions.

Finally, given that cocaine is associated with oxidative damage (Cunha-Oliveira et al., 2008, 2013; Kovacic, 2005) and IGF2 has antioxidative properties in neurotoxic conditions (Martín-Montañez et al., 2021; Romero-Zerbo et al., 2025), we analyzed lipid oxidative damage (LOOH) and redox status (total GSH) in the hippocampus after long-term abstinence following adolescent cocaine exposure. The absence of differences in LOOH and total GSH among groups suggests that neither cocaine nor IGF2 significantly altered the hippocampal redox environment. These results contrast with studies linking chronic cocaine use to oxidative stress in adults (Lipaus et al., 2019; Muriach et al., 2010; Pomierny-Chamioło et al., 2013), but align with findings of unchanged oxidative damage and antioxidant capacity (MDA and GSH levels respectively) after prolonged abstinence from adolescent cocaine exposure (Zhu et al., 2016). This may reflect a restoration of redox homeostasis during the abstinence period. As cocaine did not induce measurable oxidative damage, the antioxidant effects of IGF2 reported in the literature could not be evaluated. We also assessed corticosterone, since elevated glucocorticoids have been linked to hippocampal oxidative stress and neuronal vulnerability (Sato et al., 2010). and cocaine-related dysregulation of the HPA axis may underlie long-term neural and behavioral effects (Manetti et al., 2014; Sinha et al., 2006). In our study, serum corticosterone levels were unchanged following long-term withdrawal from adolescent cocaine and/or IGF2, consistent with prior reports showing no alterations even after short abstinence (Caffino et al., 2015a; Caffino et al., 2015b; Mottarlini et al., 2020). However, we cannot rule out the possibility of dysregulation in this endocrine system in response to environmental challenges, such as exposure to stressors or drugs after long-term withdrawal from adolescent cocaine exposure (Alves et al., 2014).

This study provides new insights into the long-term consequences of adolescent cocaine exposure and the potential modulatory effects of IGF2, highlighting both its protective role in compulsive-like behaviors and its possible detrimental effects on spatial memory. These findings underscore the complexity of IGF2 as a neuromodulator and emphasize the need for further research to elucidate its precise mechanisms of action, optimize its therapeutic potential, and determine its long-term safety. Despite these valuable findings, certain limitations should be considered. First, this study focused exclusively on the hippocampus, yet other brain regions, such as the CPF and striatum, play crucial roles in the cognitive and behavioral consequences of cocaine exposure. Future research should expand the scope of analysis to these regions to provide a more comprehensive understanding of IGF2's effects on neural circuits involved in addiction-related behaviors. Second, our study exclusively used male mice, limiting the generalizability of the findings to females. Given the growing evidence of sex differences in the neurobiological effects of cocaine and IGF2 signaling, future studies should explore these findings in female subjects to determine potential sex-dependent differences in response to cocaine and IGF2 treatment, which could have important implications for personalized therapeutic strategies. Third, while IGF2 showed a protective effect on some behaviors, its potential negative impact on spatial memory function raises concerns about its therapeutic application. Future studies should investigate whether modifying the dose or duration of IGF2 administration could optimize its beneficial effects while minimizing potential cognitive drawbacks. Additionally, research should focus on whether IGF2R downregulation following prolonged IGF2 exposure plays a role in these cognitive alterations and whether alternative administration strategies could prevent receptor desensitization. Finally, the timing of the molecular analysis (after a long abstinence period) may have influenced the results. The fact that neither oxidative damage nor neurogenesis alterations

were detected does not necessarily mean that these changes did not occur at earlier stages. Future studies should include multiple time points of analysis to track the evolution of neurobiological alterations throughout different phases of drug withdrawal and IGF2 treatment.

CRediT authorship contribution statement

Sara Gil-Rodríguez: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Mario Berdugo-Gómez:** Investigation, Formal analysis. **Silvia Claros:** Investigation, Formal analysis. **Silvana-Yanina Romero-Zerbo:** Investigation, Formal analysis. **M. Carmen Mañas-Padilla:** Investigation. **María del Carmen Gómez-Roldán:** Investigation. **Eduardo Blanco-Calvo:** Project administration, Funding acquisition. **María García-Fernández:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Luis J. Santín:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbb.2025.174095.

Data availability

Data will be made available on request.

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