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Research report

# Alterations in brain neurotrophic and glial factors following early age chronic methylphenidate and cocaine administration

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## HIGHLIGHTS

- Cocaine administration was associated with increased locomotor activity.
- There are age-dependent alterations in GLT1 and GFAP mRNA expression levels.
- GDNF mRNA levels decrease with age progression.
- GDNF mRNA levels increase after 21 withdrawal days from cocaine and MPH treatments.
- Cocaine or MPH treatments and age affect prefrontal BDNF protein expression.

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## ABSTRACT

Attention deficit hyperactivity disorder (ADHD) overdiagnosis and a pharmacological attempt to increase cognitive performance, are the major causes for the frequent (ab)use of psychostimulants in non-ADHD individuals. Methylphenidate is a non-addictive psychostimulant, although its mode of action resembles that of cocaine, a well-known addictive and abused drug. Neuronal- and glial-derived growth factors play a major role in the development, maintenance and survival of neurons in the central nervous system. We hypothesized that methylphenidate and cocaine treatment affect the expression of such growth factors. Beginning on postnatal day (PND) 14, male Sprague Dawley rats were treated chronically with either cocaine or methylphenidate. The rats were examined behaviorally and biochemically at several time points (PND 35, 56, 70 and 90). On PND 56, rats treated with cocaine or methylphenidate from PND 14 through PND 35 exhibited increased hippocampal glial-cell derived neurotrophic factor (GDNF) mRNA levels, after 21 withdrawal days, compared to the saline-treated rats. We found a significant association between cocaine and methylphenidate treatments and age progression in the prefrontal protein expression of brain derived neurotrophic factor (BDNF). Neither treatments affected the behavioral parameters, although acute cocaine administration was associated with increased locomotor activity. It is possible that the increased hippocampal GDNF mRNA levels, may be relevant to the reduced rate of drug seeking behavior in ADHD adolescence that were maintained from childhood on methylphenidate. BDNF protein level increase with age, as well as following stimulant treatments at early age may be relevant to the neurobiology and pharmacotherapy of ADHD.



## 1. Introduction

A major concern arising from overdiagnosis of attention deficit hyperactivity disorder (ADHD) [1–4] is the frequent use of psychostimulants in subjects who are not properly diagnosed. Another major problem is the psychostimulants usage as neuroenhancers in non-ADHD patients. As stated by Normann and Berger [5], neuroenhancement is a pharmacological attempt to increase cognitive performance in healthy humans. Methylphenidate, a common treatment for ADHD, is extensively misused, especially by students [6,7].

Psychostimulants are controlled substances, aimed to enhance monoaminergic transmission in the brain. Amphetamine, cocaine and methylphenidate are examples of psychostimulants, who

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differ in their mode of action [8–11]. Psychostimulants modulate the monoamine transporters function, thereby increase the monoaminergic signaling, in one of two mechanisms; inhibition of monoamine reuptake, like cocaine, methylphenidate and amphetamine, or substance-type releasers, as amphetamine [12,13]. Methylphenidate is a non-addictive psychostimulant although it is important to mention that its mode of action resembles that of cocaine, a well-known addictive and abused drug. Both methylphenidate and cocaine inhibit the reuptake of dopamine, resulting in an increase in dopamine levels at the synaptic cleft [14]. Dopamine transporter is a known molecular target which mediates the abuse-related effects of cocaine. The ability of cocaine to inhibit dopamine reuptake correlates with its potency to maintain drug self-administration [13].

Neurotrophic factors play a major role in the development, maintenance and survival of neurons in the central nervous system [15,16]. Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family; it is secreted as pro-BDNF and cleaved into its mature form. Its action is mediated through the tyrosine kinase receptor B (TrkB), which leads to a signaling cascade of the ERK pathway. Glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor β (TGF-β) superfamily. GDNF is a secreted protein, who acts through the receptor tyrosine kinase, which also leads to the stimulation of ERK pathway [17,18].

In this study we investigated the involvement of neurotrophic factors (BDNF and GDNF), as well as glial parameters, in neuroplasticity following chronic cocaine or methylphenidate treatment in young rats. We hypothesized that chronic exposure of young rats to dopamine stimulation induced by either methylphenidate or cocaine will be associated with increased locomotor activity (as assessed by the open field test) and improvement in spatial working memory (as assessed by the Y maze test), parallel to elevation in the expression of brain BDNF and GDNF.

## 2. Materials and methods

### 2.1. Animals

Male Sprague Dawley rats (SD strain) at the age of 10 days (30 g) with nursing mothers were purchased from Harlan laboratories (Jerusalem, Israel). The rats were housed 10 per cage, with a nursing female, at 22 ± 2 °C and a 12 light:12 dark hours cycle (lights at 05:00 h). At postnatal day (PND) 21 the rats were separated from their nursing mothers and housed four per cage, with unlimited access to commercial pellet food and tap water. All animal procedures were approved by the Animal Care Committees of Tel-Aviv University (approval numbers: M-11-012 and M-13-012).

### 2.2. Materials

Cocaine hydrochloride was purchased from Sigma–Aldrich (St. Louis, MO, USA). Methylphenidate hydrochloride (Ritalin®) was synthesized by Novartis Pharma AG (Basel, Switzerland). TRIzol was purchashed from Invitrogen, Life Technologies (Carlsbad, CA, USA). Chloroform, iso-propanol, sodium metavanadate, NP-40, glycerol, triton X-100, PMSF, aprotinin and leupeptin were of highest purity available from Sigma–Aldrich (St. Louis, MO, USA). Ultra-pure water (DNAse and RNAse free water) was obtained from Biological Industries (Beit-Haemek, Israel). High capacity cDNA RT kit, fast SYBR green master mix, TaqMan gene expression assays (Assay ID: GDNF: Rn00569510_m1, GAPDH: Rn01775763_g1) and TaqMan gene expression master mix were purchased from Applied Biosystems – Life Technologies (Foster city, CA, USA). Pierce BCA protein kit was purchased from Thermo scientific (Rockford, IL, USA). BDNF and GDNF ELISA kits and Substrate reagent pack (stabilized hydrogen peroxidase and stabilized tetramethylbenzidine) were purchased from R&D systems (Minneapolis, MN, USA). All other chemicals were of highest purity obtainable through regular commercial sources.

### 2.3. Cocaine and methylphenidate treatment protocols

Beginning on PND 14, three groups of rats ($N = 32$/group) were administered saline, cocaine (15 mg/Kg) or methylphenidate (3 mg/Kg) intraperitoneally for 21 days; drug administration was discontinued at PND 35. The doses of methylphenidate and cocaine were determined according to our previous reports on these agents [19,20]. Eight rats from each treatment group assembled an age group ($N = 24$/age group) and were examined once (at the corresponding age) during the study. There were separate saline control group for each cocaine and methylphenidate conditions. The rats in the first group, at PND 35, were examined behaviorally (open field and Y maze) 20 min after the drugs administration. 24 h later they were sacrificed (decapitation by guillotine) and their brains were dissected on ice. All other animals were sacrificed at three different time points (PND 56, 70 and 90; 24/age group), after behavioral examination.

### 2.4. Open field

This test was chosen since it is sensitive to changes in locomotor activity. The test was conducted as described previously by Hall [21]. A rat was placed in a 100 cm × 100 cm rectangular black-colored apparatus divided into 20 $cm^2$, in the middle of one wall near a black-colored box known as home. PND 35 rats were examined 20 min following administration. After 5 min observation and video recording the animal was removed, and the apparatus was cleaned using ethanol and allowed to dry in order to eliminate any odor cues before the recording of the next animal. Activity, zone crossing events, track length and velocity were analyzed using Bio-observe computer software.

### 2.5. Y maze

This test was chosen since it is sensitive to alteration in spatial working memory. The Y shaped maze was made of three identical black colored arms, each arm of 50 cm long, 20 cm wide and 30 cm height with a 120° angle separating the arms. The test was conducted as described previously by Dellu et al. [22]. The test consisted of two phases separated by a 2 min interval. In the first phase, one arm of the maze was blocked. The rat was placed facing the back wall of one arm, and had 5 min to explore the two unblocked arms. In the second phase the rats had free excess to all arms for another 2 min. The apparatus was cleaned using ethanol and allowed to dry in order to eliminate any odor cues before the recording of each new examined rat. Scoring was calculated as entrance of the rat into each of the arms. Discrimination ratio (relative exploration time spent at the novel arm out of total time spent in the maze) was used to compare between the groups.

### 2.6. RNA extraction

In order to yield RNA from a brain sample we used the TRIzol reagent, a monophasic solution of phenol and guanidine isothiocyanate, which was first introduced by Chomczynski and Sacchi [23,24]. Following homogenization of the tissue in TRIzol reagent, chloroform was added, and a 15 min 12,000 × *g* centrifugation separated the homogenate into 3 layers. The upper aqueous layer contained RNA. Precipitation of the RNA was made using isopropanol followed by 75% ethanol wash of the RNA pellet formed

after a 10-min 12,000 × *g* centrifugation. The RNA was resuspended in RNase-free water and incubated at 60 °C for 10 min. Total RNA concentration extracted from each sample was assessed using ND-1000 spectrophotometer (Nanodrop).

### 2.7. Real-time polymerase chain reaction (RT-PCR)

cDNA was generated from total RNA according to the High-capacity cDNA RT kit's instructions, using random primers and in the presence of RNase inhibitors. Three genes were examined: glial-derived neurotropic factor (GDNF), glial glutamate transporter 1 (GLT1) and glial fibrillary acidic protein (GFAP). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Quantitive gene expression analysis was performed in two different ways: for GDNF gene expression we used TaqMan gene expression assay (assay ID: GDNF: Rn00569510_m1, GAPDH: Rn01775763_g1) with the TaqMan gene expression master mix and for GLT1 and GFAP we used the fast SYBR green master mix (primers designed using PRIMER EXPRESS software). Real-time PCR reactions were carried out in triplicate according to the manufacturer's protocol using ABI STEPone device and software (Applied Biosystems, Foster city, CA, USA). Calculations of relative expression used the ddCt method, and processed using the DATAssist software. Product specificity was confirmed routinely by melting curve analysis.

| Gene | Primer sequence 5′–3′ | |
|---|---|---|
| GLT1 | Sense | ggctgctggatagaatgagaactt |
| | Antisense | cggtgttgggagtcaatggt |
| GFAP | Sense | gcggctctgagagagattcg |
| | Antisense | tgcaaacttggaccgatacca |
| GAPDH | Sense | gaaacctgccaagtatgatgacat |
| | Antisense | caaaggtggaagaatgggagtt |

### 2.8. Protein extraction for ELISA

BDNF: the tissue was homogenized 1:10 (w/v) in cold extraction buffer: Tris-buffered saline (137 mM NaCl and 20 mM Tris, pH 8) with 5 mM sodium metavanadate, 1% NP-40, 10% glycerol, 1 mM PMSF, 10 μg/ml aprotinin and 1 μg/ml leupeptin [25,26]. Homogenates were acidified using 1 N HCl to pH 2.5 according to Okragly and Haak–Frendscho method [27]. The samples were incubated for 15 min in room temperature and then neutralized to pH 7.5 using 1 N NaOH. The samples centrifuged for 10 min in 13,000 × *g* and the supernatant was separated and collected into a new tube. GDNF: the tissue was homogenized 1:5 (w/v) in cold extraction buffer: 100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin and 1 μg/ml leupeptin [28]. The samples were sonicated for 20 s on ice and acidified to pH 2.5 using 1 N HCl. After 15 min incubation in room temperature, the samples were neutralized to pH 7.5 using NaOH and centrifuged for 15 min in 13,000 × *g*. The supernatant was separated and collected into a new tube.

### 2.9. ELISA

Samples were assayed for BDNF protein levels using mouse anti-human BDNF antibody as a capture antibody and the biotinylated mouse anti-human BDNF as detection antibody. In this study, in contrast to our previous study (Simchon-Tenenbaum et al., submitted), we used ELISA to assess BDNF protein levels since it is a more accurate analysis for this purpose. Mouse anti-human GDNF was used as capture antibody and biotinylated goat anti-human GDNF was used as detection antibody for determining GDNF protein levels. Total protein levels were determined using the BCA protein assay kit.

### 2.10. Statistical analysis

Two-way Anova, using SPSS software version 17.0 (SPSS Inc. Chicago, IL), was used as appropriate. Results are expressed as means ± S.D.

## 3. Results

### 3.1. Behavioral tests

#### 3.1.1. Open field

The PND 35 rats (controls and drug treated) were significantly more active than the PND 56, 70 and 90 rats, irrespective of the drug treatment, thus average values of each age group was used for statistical analysis (65 ± 13 vs. 44 ± 4 and 47 ± 6 and 42 ± 7% activity; $F = 60.985$, $df = 3$, $p < 0.005$). Examining the PND 35 rats 20 min after the last drug administration showed that the activity of cocaine-treated rats was significantly higher compared to the saline and the MPH-treated rats of the same age (79 ± 9 vs. 54 ± 8 and 62 ± 7% activity; $F = 10.229$, $df = 2$, $p < 0.005$) (Fig. 1). Corresponding

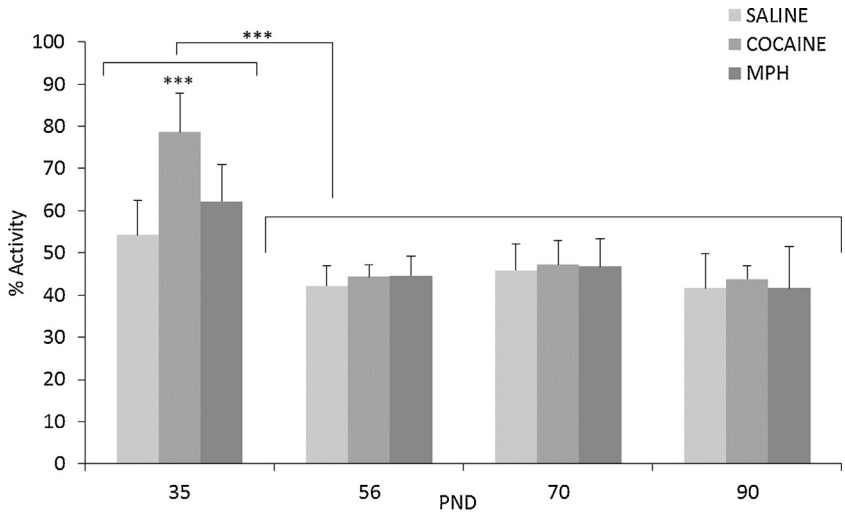


**Fig. 1.** Activity (percent of total experiment time) in an open field of PND 35 ($n = 27$), 56 ($n = 24$), 70 ($n = 27$) and 90 ($n = 27$) rats treated chronically (21 days) with saline, cocaine (15 mg/Kg) or MPH (3 mg/Kg). At the PND 35 group the test was performed 20 min after the last drug administration. Results are expressed as means ± S.D. (***$p < 0.005$, PND 35 vs. PND 56, 70 and 90 rats, ***$p < 0.005$, cocaine-treated vs. saline- and MPH-treated PND 35 rats).

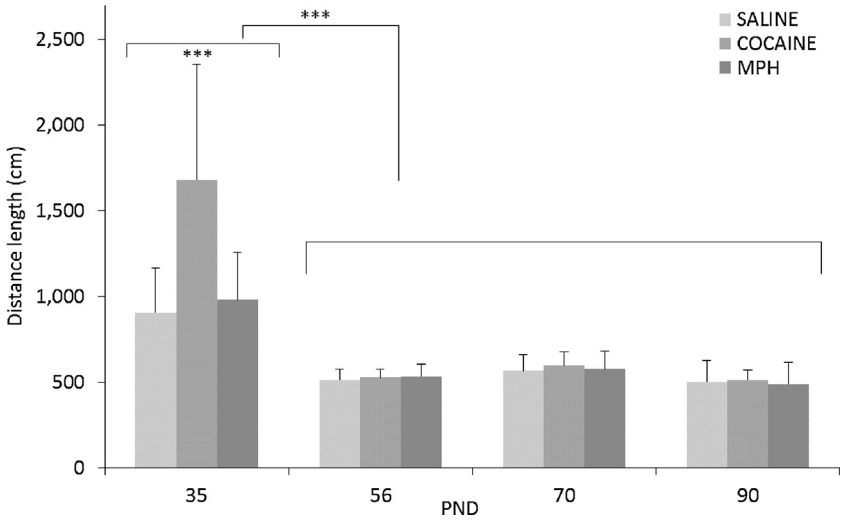


**Fig. 2.** Distance length attained in an open field by PND 35 ($n = 27$), 56 ($n = 24$), 70 ($n = 27$) and 90 ($n = 27$) rats treated chronically (21 days) with saline, cocaine (15 mg/Kg) or MPH (3 mg/Kg). At the PND 35 group the test was performed 20 min after the last drug administration. Results are expressed as means ± S.D. (***$p < 0.005$, PND 35 vs. PND 56, 70 and 90 rats, ***$p < 0.005$, cocaine-treated vs. saline- and MPH-treated PND 35 rats).

higher scores were observed at the distance length (1190 ± 560 vs. 525 ± 60 and 580 ± 90 and 500 ± 100 cm; $F = 50.118$, $df = 3$, $p < 0.005$) (1680 ± 675 vs. 910 ± 255 and 980 ± 275 cm; $F = 7.662$, $df = 2$, $p < 0.005$) (Fig. 2), as well as in the velocity (3.99 ± 1.8 vs. 1.73 ± 0.2 and 1.9 ± 0.3 and 1.67 ± 0.3 cm/s; $F = 51.762$, $df = 3$, $p < 0.005$) (5.62 ± 2.2 vs. 3.03 ± 0.9 and 3.32 ± 0.9 cm/s; $F = 7.725$, $df = 2$, $p < 0.005$) (Fig. 3).

### 3.1.2. Y maze

The PND 35 rats (controls and drug treated) spent significantly less time (lower discrimination ratios) exploring the new arm of the Y maze than did the PND 56, 70 and 90 groups (0.42 ± 0.3 vs. 0.66 ± 0.2 and 0.60 ± 0.2 and 0.73 ± 0.1; $F = 9.487$, $df = 3$, $p < 0.005$) (Fig. 4). We did not see any effect related to the cocaine or methylphenidate treatment in any of the ages.

## 3.2. Biochemical analyses

### 3.2.1. mRNA level determination using real-time PCR

*3.2.1.1. GDNF mRNA levels.* GDNF mRNA expression levels in the hippocampus and in the prefrontal cortex decreased by 50% and 60% from the age of PND 35 to 90, respectively (0.89 ± 0.30 vs. 0.65 ± 0.20 vs. 0.63 ± 0.20 vs. 0.53 ± 0.10; $F = 20.801$, $df = 3$, $p < 0.005$ and 0.98 ± 0.40 vs. 0.78 ± 0.40 vs. 0.56 ± 0.20 vs. 0.42 ± 0.20; $F = 11.889$, $df = 3$, $p < 0.005$) (Fig. 5). GDNF mRNA expression levels in the prefrontal cortex were not altered in response to the treatments, but a significant increase of GDNF mRNA expression levels in the hippocampus of the cocaine and methylphenidate treated rats was observed (40% and 25%, accordingly) at the age of PND 56 compared to the PND 56 saline-treated rats (0.54 ± 0.2 vs. 0.75 ± 0.2 vs. 0.67 ± 0.1; $F = 4.253$, $df = 2$, $p = 0.023$) (Fig. 5).

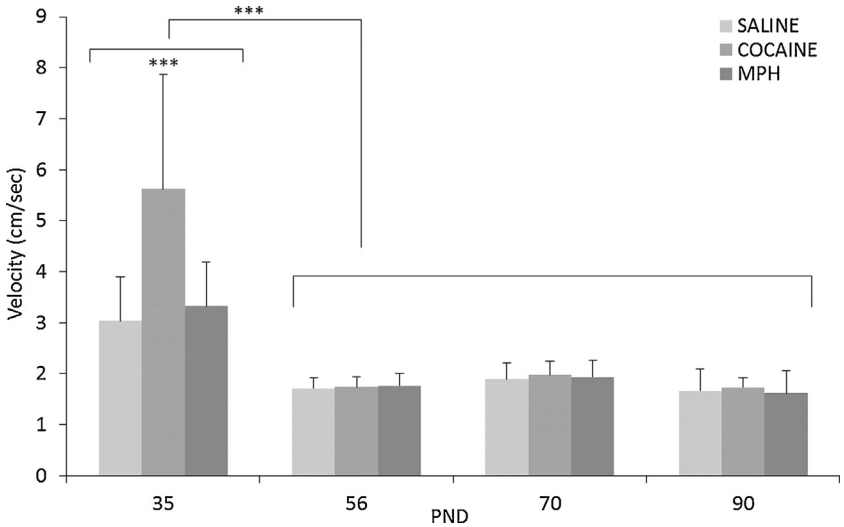


**Fig. 3.** Average velocity in the open field of PND 35 ($n = 27$), 56 ($n = 24$), 70 ($n = 27$) and 90 ($n = 27$) rats treated chronically (21 days) with saline, cocaine (15 mg/Kg) or MPH (3 mg/Kg). At the PND 35 group the test was performed 20 min after the last drug administration. Results are expressed as means ± S.D. (***$p < 0.005$, PND 35 vs. PND 56, 70 and 90 rats, ***$p < 0.005$, cocaine-treated vs. saline- and MPH-treated PND 35 rats).

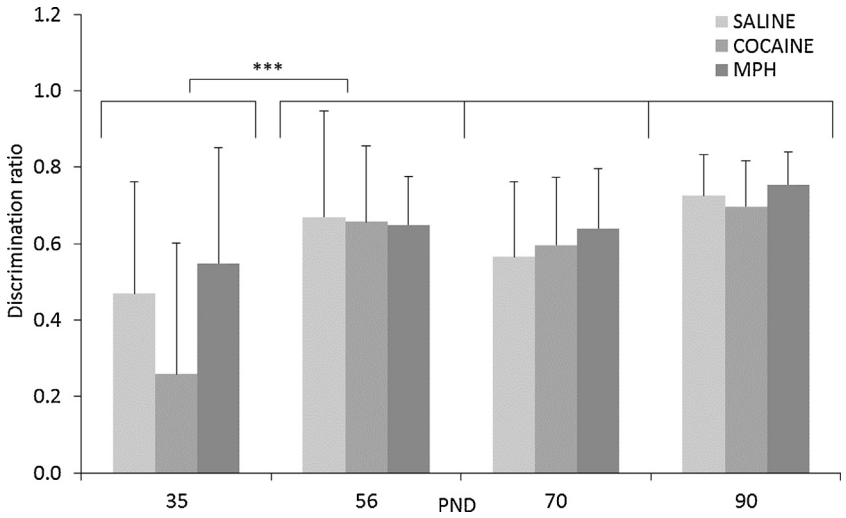


**Fig. 4.** Discrimination ratio (relative exploration time spent at the novel arm of total time spent at both arms) in the Y maze of PND 35 ($n = 27$), 56 ($n = 24$), 70 ($n = 27$) and 90 ($n = 27$) rats treated chronically (21 days) with saline, cocaine (15 mg/Kg) or MPH (3 mg/Kg). At the PND 35 group the test was performed 20 min after the last drug administration. Results are expressed as means ± S.D. (***$p < 0.005$, PND 35 vs. PND 56, 70 and 90 rats).

*3.2.1.2. GLT1 and GFAP mRNA levels.* Glial glutamate transporter 1 (GLT1) and glial fibrillary acidic protein (GFAP) mRNA levels following chronic cocaine or methylphenidate treatment, at an early age, are presented in Table 1. Neither GLT1 nor GFAP mRNA expression levels were altered following the cocaine or methylphenidate treatments in the hippocampus or in the prefrontal cortex. In the right and left hippocampus we observed minor but significant alterations in the expression of GLT1 mRNA levels which were related to the age of the rats (right: PND 35, 56, 70, 90; left hippocampus: PND 35, 56, 70, 90: 0.94 ± 0.10 vs. 1.03 ± 0.10 vs. 0.9 ± 0.10 vs. 1.00 ± 0.10; $F = 5.640$, $df = 3$, $p = 0.002$ and 0.99 ± 0.10 vs. 1.02 ± 0.10 vs. 0.95 ± 0.10 vs. 0.92 ± 0.10; $F = 3.603$, $df = 3$, $p = 0.019$, respectively). In the prefrontal cortex, we detected minor but significant alterations in the expression of GFAP mRNA levels which were also related to the age of the rats (PND 35, 56, 70, 90: 0.99 ± 0.20 vs. 1.04 ± 0.30 vs. 1.00 ± 0.20 vs. 0.74 ± 0.10; $F = 7.844$, $df = 3$, $p < 0.005$) (Table 1).

### 3.3. Protein levels determination using ELISA

#### 3.3.1. BDNF protein levels

There were no changes in BDNF protein levels following cocaine or methylphenidate treatments or in response to age progression in the hippocampus or in the striatum. In the prefrontal cortex, a significant association was found between BDNF protein levels to the age of the rats and the treatment they received. BDNF protein levels decreased in the saline-treated group with the progression of age, from PND 35 to 90. On the contrary, cocaine and methylphenidate treated rats showed increased BDNF protein levels as they age ($F = 2.345$, $df = 6$, $p = 0.042$). We also observed that as a response to the chronic cocaine and methylphenidate treatments, the BDNF protein levels decreased significantly by 30% and 35% respectively, at the age of PND 35 as compared to the PND 35 saline-treated rats (0.17 ± 0.04 vs. 0.12 ± 0.02 vs. 0.11 ± 0.02; $F = 5.658$, $df = 2$, $p = 0.015$) (Fig. 6).

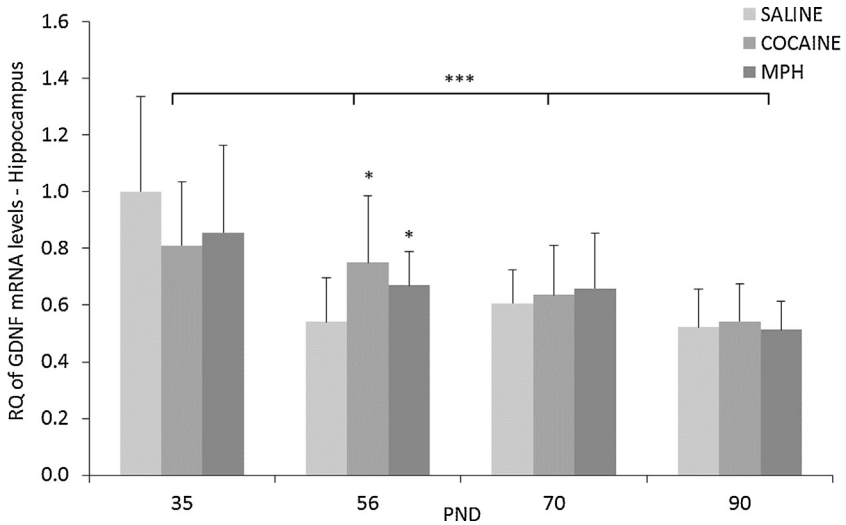


**Fig. 5.** The effect of chronic cocaine and MPH treatments on GDNF mRNA levels in the hippocampus as measured by real-time PCR. Results are expressed as relative means ± S.D. to the PND 35 saline-treated rats (*$p < 0.05$, saline-treated vs. cocaine and MPH-treated PND 56 rats; ***$p < 0.005$, PND 35 vs. PND 56 and 70 vs. PND 90 rats).

**Table 1**
Relative GLT1 and GFAP mRNA expression (to GAPDH).

| Gene | Treatment | PND | Hippocampus | | Prefrontal cortex |
|---|---|---|---|---|---|
| | | | Right | Left | |
| GLT1 | Saline | 35 | 1.00 ± 0.13[a,b] | 1.00 ± 0.08 | 1.00 ± 0.14 |
| | | 56 | 1.05 ± 0.10[c] | 1.05 ± 0.13[d] | 0.92 ± 0.12 |
| | | 70 | 0.90 ± 0.09[a] | 0.91 ± 0.09 | 0.88 ± 0.23 |
| | | 90 | 0.99 ± 0.12[b,c] | 0.89 ± 0.08[e] | 0.77 ± 0.10 |
| | Cocaine | 35 | 0.92 ± 0.06[a,b] | 0.99 ± 0.08 | 0.93 ± 0.12 |
| | | 56 | 1.03 ± 0.14[c] | 0.99 ± 0.08[d] | 0.98 ± 0.31 |
| | | 70 | 0.91 ± 0.08[a] | 1.04 ± 0.10 | 0.91 ± 0.05 |
| | | 90 | 1.00 ± 0.13[b,c] | 0.92 ± 0.09[e] | 0.85 ± 0.16 |
| | MPH | 35 | 0.89 ± 0.06[a,b] | 0.98 ± 0.15 | 0.97 ± 0.13 |
| | | 56 | 1.00 ± 0.10[c] | 1.03 ± 0.12[d] | 0.79 ± 0.04 |
| | | 70 | 0.89 ± 0.12[a] | 0.93 ± 0.06 | 0.92 ± 0.05 |
| | | 90 | 1.02 ± 0.07[b,c] | 0.95 ± 0.08[e] | 0.89 ± 0.13 |
| GFAP | Saline | 35 | 1.00 ± 0.15 | 1.00 ± 0.10 | 1.00 ± 0.09 |
| | | 56 | 1.05 ± 0.17 | 1.02 ± 0.18 | 1.07 ± 0.29 |
| | | 70 | 0.92 ± 0.11 | 0.96 ± 0.08 | 0.99 ± 0.29 |
| | | 90 | 0.94 ± 0.11 | 0.89 ± 0.05 | 0.68 ± 0.07[f] |
| | Cocaine | 35 | 0.93 ± 0.11 | 0.91 ± 0.11 | 0.96 ± 0.25 |
| | | 56 | 1.04 ± 0.17 | 0.96 ± 0.11 | 1.22 ± 0.29 |
| | | 70 | 0.90 ± 0.13 | 0.95 ± 0.04 | 1.02 ± 0.27 |
| | | 90 | 0.90 ± 0.11 | 0.93 ± 0.12 | 0.79 ± 0.16[f] |
| | MPH | 35 | 0.92 ± 0.11 | 0.93 ± 0.11 | 1.00 ± 0.10 |
| | | 56 | 0.93 ± 0.15 | 0.99 ± 0.18 | 0.85 ± 0.14 |
| | | 70 | 0.99 ± 0.13 | 0.99 ± 0.10 | 0.99 ± 0.21 |
| | | 90 | 0.92 ± 0.18 | 0.91 ± 0.14 | 0.75 ± 0.12[f] |

The effect of chronic cocaine or methylphenidate treatment, at early age, on RQ (relative quantitation) of mRNA levels of GLT1 and GFAP. Results are expressed as relative RQ means ± S.D.

[a] $p = 0.002$, PND 35 and 70 vs. PND 56 and 90 rats.
[b] $p = 0.002$, PND 35 and 90 vs. PND 56 and 70 rats.
[c] $p = 0.002$, PND 56 and 90 vs. PND 35 and 70 rats.
[d] $p = 0.0019$, PND 56 vs. PND 35, 70 and 90 rats.
[e] $p = 0.0019$, PND 90 vs. PND 35, 56 and 70 rats.
[f] $p < 0.005$, PND 90 vs. PND 35, 56 and 70 rats.

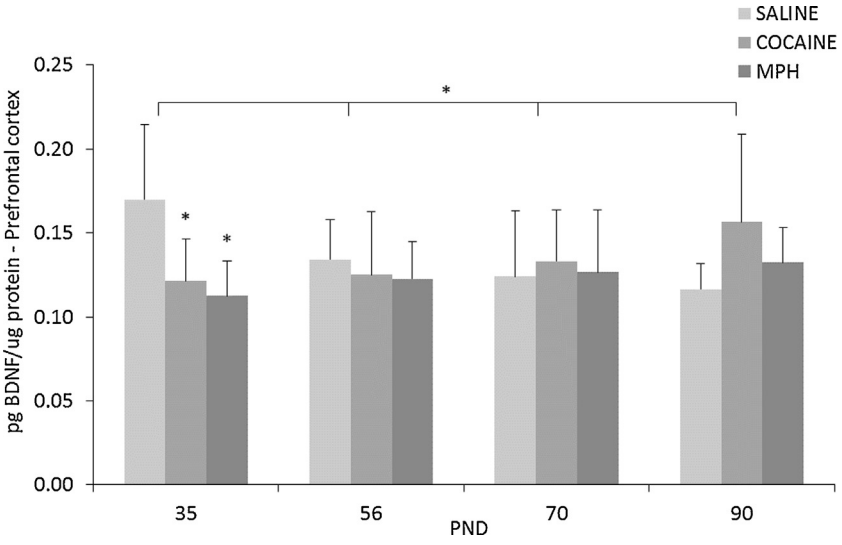


**Fig. 6.** The effect of chronic cocaine and methylphenidate treatment on BDNF protein levels in rat prefrontal cortex as measured by ELISA. Results are expressed as means ± S.D. (*$p < 0.05$).

### 3.3.2. GDNF protein levels

GDNF protein levels were not altered in the striatum following cocaine or methylphenidate treatments, nor in response to age progression (data not shown).

## 4. Discussion

The aim of this study was to examine the long-term effects of cocaine and methylphenidate administration at early age on behavioral and brain biochemical parameters in Sprague Dawley intact rats.

### 4.1. Behavioral effects

#### 4.1.1. Open field test

Regardless of the treatments, increased locomotor activity was detected in the younger group (PND 35) compared to all other age groups. Rats at the age of PND 35 treated with cocaine, but not

with methylphenidate, showed increased locomotor activity, distance lengths and velocity, consistent with predicted effects for cocaine. These results are consistent with Zubrycki et al. [29], Broderick et al. [30] and Damianopoulos and Carey [31], who showed increased locomotion in an open field test following cocaine administration compared to saline-treated rats. McFadyen et al. [32], and Ferguson and Cada [33] exhibited similar results to ours, showing no methylphenidate effect on open field performance. On the contrary, Yang et al. [34] showed increased locomotion 20 min after methylphenidate administration. Thus it appears that in the open field test there is acute drug effect, namely, cocaine administration is associated with better performance in this test 20 min after the exposure to the drug.

#### 4.1.2. Y maze test

To our surprise, the methylphenidate and cocaine-treated rats did not spend more time at the novel arm than the saline-treated group. The lack of effect may be related to the short duration of follow-up, 20 min after drug administration. It is possible that longer follow-up duration is needed to obtain pro-cognitive effects of methylphenidate in the Y maze. With regard to age, in our study PND 35 saline-treated rats exhibited lower discrimination ratios compared to all group ages (PND 56, 70 and 90 rats). These results are consistent with Kolyaduke et al. [35], who demonstrated that PND 35 rats showed lower discrimination ratios compared to the PND 45 saline-treated rats. Thus it seems that in the Y maze there is no drug effect but an age effect on the spatial working memory.

### 4.2. Biochemical effects

#### 4.2.1. mRNA expression

GDNF mRNA levels significantly decreased in the hippocampus and in the prefrontal cortex by 50% and 60% respectively, from the age of PND 35 to 90, regardless of the treatments. In the hippocampus, but not in the prefrontal cortex, at the age of PND 56, 21 days after the last administrations, GDNF mRNA levels were 40% and 25% higher in the cocaine and methylphenidate treated groups compared to the saline-treated group. Green-Sadan et al. [36] showed that cocaine self-administrated SD rats had a reduction in GDNF mRNA levels in the striatum but not in the nucleus accumbens, 24 h after last administration. Sadasivan et al. [37] showed decreased GDNF mRNA levels in the substantia nigra of mice treated chronically with methylphenidate, after a seven-day withdrawal. Although not significant, our results also showed a tendency toward lower GDNF mRNA levels 24 h after the last administration, of both cocaine and methylphenidate. Unfortunately there is no data concerning the impact of cocaine or methylphenidate treatments on GDNF mRNA after longer withdrawal times.

GLT1 and GFAP mRNA levels also did not alter in response to the cocaine and methylphenidate treatments. We observed minor, but significant, age-related alterations in hippocampal GLT1 mRNA expression and prefrontal GFAP mRNA expression. The relevance of these alterations to neurodevelopment and cognitive functioning is as yet unclear.

#### 4.2.2. Protein expression

BDNF protein levels decreased significantly in the prefrontal cortex 24 h after the last administration of the cocaine and methylphenidate. In addition, a significant association between age and treatment was observed. BDNF protein levels decreased gradually from age PND 35 to 90 in the saline treated group. In contrast, in the cocaine and methylphenidate treated groups the levels of BDNF increased with age. Fumagalli et al. [38,39], in accordance with our results found that 5-days cocaine treatment led to decreased BDNF protein levels, 2 h and 72 h after last administration. Similar to our results, Scherer et al. [40] described unchanged BDNF protein levels in the hippocampus of SD rats, but decreased BDNF levels in the prefrontal cortex 24 h following last administration of chronic methylphenidate treatment. In contrast to our results, Andersen et al. [41] failed to demonstrated alterations in BDNF protein levels in PND 20 rats treated with methylphenidate for 15 days, and assessed at PND 60.

We found treatment-dependent and age-dependent GDNF mRNA level alterations in the hippocampus and prefrontal cortex, but did not find alterations in GDNF protein levels in the striatum.

Our results suggest that stimulants treatment at early age, leads to some neuronal alterations, detected at the molecular level but not in the behavioral level. We found stimulant-dependent (both methylphenidate and cocaine) GDNF mRNA level increase in the hippocampus after a withdrawal period (21 days), and a significant association between age and treatment in the expression levels of BDNF protein in the prefrontal cortex. GDNF plays a major role in survival of dopaminergic neurons, as well as in the attenuation of addictive behavior [18]. Thus it is possible that the increased hippocampal GDNF mRNA levels, may be relevant to the reduced rate of drug seeking behavior in ADHD adolescence that were maintained from childhood on methylphenidate treatment [42]. BDNF is a known neurotrophic factor, with an important role in development and maturation of the brain. We found that BDNF protein level increases with age progression, as well as following stimulant treatments, at early age. Our observation may be relevant to the neurobiology and pharmacotherapy of ADHD, as well as, for the impact of dopamine transporter inhibitors on working memory and the expression of neurotrophic factors.

## Contributors

Yaarit Simchon-Tenenbaum performed the entire research as part of her Ph.D. thesis. She participated in designing the experiments, treating the rats and performed all of the behavioral and biochemical assays. Abraham Weizman participated in writing the final draft and the statistics. Moshe Rehavi designed the project and was Yaarit Simchon-Tenenbaum's mentor in the project, and he wrote the first draft. All authors contributed to and have approved the final manuscript.

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There is no involvement of the founding source.

## Conflict of interest

There are no conflicts of interest.

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