Psychopharmacology (2011) 214:877–889
DOI 10.1007/s00213-010-2098-y

ORIGINAL INVESTIGATION

The intriguing effects of ecstasy (MDMA) on cognitive function in mice subjected to a minimal traumatic brain injury (mTBI)

Shahaf Edut · Vardit Rubovitch · Shaul Schreiber · Chaim G. Pick

Received: 24 May 2010 / Accepted: 4 November 2010 / Published online: 1 December 2010

Abstract
Rationale The use of ecstasy (MDMA) among young adults has dramatically increased over the years. Since MDMA may impair the users' driving ability, the risk of being involved in a motor vehicle accident (MVA) is notably increased. Minimal traumatic brain injury (mTBI) a common consequence of MVAs—produces short- and long-term physical, cognitive, and emotional impairments.
Objectives To investigate the effects of an acute dose of MDMA in mice subjected to closed head mTBI.
Methods Mice received 10 mg/kg MDMA 1 h prior to the induction of mTBI. Behavioral tests were conducted 7 and 30 days post-injury. In addition to the behavioral tests, phosphorylation of IGF-1R, ERK, and levels of tyrosine hydroxylase (TH) were measured.
Results mTBI mice showed major cognitive impairments in all cognitive tests conducted. No additional impairments were seen if mTBI was preceded by one dose of MDMA. On the contrary, a beneficial effect was seen in these mice. The western blot analysis of TH revealed a significant decrease in the mTBI mice. These decreases were reversed in mice that were subjected to MDMA prior to the trauma.
Conclusions The presence of MDMA at the time of mTBI minimizes the alteration of visual and spatial memory of the injured mice. The IGF-1R pathway was activated due to mTBI and MDMA but was not the main contributor to the cognitive improvements. MDMA administration inverted the TH decreases seen after injury. We believe this may be the major cause of the cognitive improvements seen in these mice.

Keywords Mice · Minimal traumatic brain injury (MTBI) · MDMA (3,4-methylenedioxymethamphetamine) · Novel object recognition · Ecstasy · Spatial learning · Cognitive test · Behavioral assessment

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Introduction

Driving under the influence of drugs is a well-established cause of motor vehicle accidents. Evidence shows that up to 25% of car accidents involve drivers affected by drugs (Drummer et al. 2003; Nochajski and Stasiewicz 2006). One of the most common consequences of car accidents is a traumatic brain injury (TBI) (Alexander 1995; Bazarian et al. 2005; Cassidy et al. 2004). Most of these accidents involve alcohol use, but recently, other drugs such as marijuana and amphetamines have become a major problem as well (Darke et al. 2004; Hooft and Vandevoorde 1994; Movig et al. 2004; Smink et al. 2008). This terrible cascade, drugs–car accidents–head injuries have become a significant burden on the economy of the western world.

Ecstasy (methylenedioxymethamphetamine, MDMA) is a synthetic drug, popular among young people for its euphoric and energizing effects (Green et al. 2003; Morton

2005). Studies have shown that ecstasy users are not able to estimate their objective impairment accurately when they are under the influence of the drug. Their lack of judgment during intoxication puts them at high risk of a crash when engaged in traffic (Hooft and Vandevoorde 1994; Kuypers et al. 2009; Morgan 2000; Weinbroum 2003). Epidemiological studies have shown that MDMA can impair judgment and lead to reckless behavior such as speeding and running red traffic lights (Brookhuis et al. 2004; Drummer et al. 2003; Hooft and Vandevoorde 1994; Kuypers et al. 2009; Logan and Couper 2001).

Traumatic brain injury (TBI) is a leading cause of death and lifelong disability in individuals under the age of 50 (Fleminger 2008; Fujimoto et al. 2004; Morales et al. 2005). Most TBI cases are a result of motor vehicle accidents, but there are other causes including accidental falls or sports injuries. TBI occurs when an external force is applied to the head. The brain is then damaged either from skull penetration, rubbing, or colliding with the rough surfaces. Brain acceleration, deceleration, or uneven rotation may cause additional injury (Bullinger 2002; Gennarelli et al. 1994; Sosnoff et al. 2008).

In contrast to TBI in which a brain morphological alteration is detectable (Graham et al. 2000), minimal traumatic brain injury (mTBI) lacks diagnosable objective structural brain damage and presents as a number of imprecise perceptual cognitive symptoms (the so-called "post-concussion syndrome") (Hamm et al. 1993; Margulies 2000). This type of injury accounts for 80–90% of total brain injuries (Vos et al. 2002). The symptoms of mTBI include headache, dizziness, fatigue, irritability, various degrees of memory loss, attention and concentration problems, and emotional liability (Kushner 1998; Ryan and Warden 2003; Schreiber et al. 2008). We have previously reported the use of a modified weight drop model in order to produce a non-invasive closed-head minimal traumatic brain injury (mTBI) in mice (Milman et al. 2005; Milman et al. 2008; Tashlykov et al. 2007; Tashlykov et al. 2009; Tweedie et al. 2007; Zohar et al. 2003). In these studies, we showed that our mTBI model induces cognitive and emotional short- and long-term deficits. These deficits in mice mimic the persistent post-concussion syndrome that occurs in humans as well.

MDMA and mTBI share some physiological and cellular destructive mechanisms including hyperthermia, oxidative stress, and apoptotic cell death (Brown and Kiyatkin 2004; Capela et al. 2009; Colado et al. 2001; Fantegrossi et al. 2008; Warren et al. 2006). MDMA was shown to cause acute dose-dependent hyperthermia in many laboratory animals, including mice. In both clinical and experimental studies, hyperthermia in the acute phase of TBI caused additional deterioration (Carvalho et al. 2002; Morales et al. 2005; Piper et al. 2005). Several studies suggested that MDMA use causes oxidative stress (Cadet et al. 2001; Colado et al. 2001; Gudelsky and Yamamoto 2008). In TBI, oxidative stress plays a key role both in the primary and the secondary damage (Bayir and Kagan 2008; Chong et al. 2005; Shein et al. 2007; Shohami et al. 1999). Finally, an acute dose of MDMA up-regulated and activated calpains and caspases, traumatic brain injury (TBI), and ischemia had similar effects on these pathways (Warren et al. 2006; Warren et al. 2007).

As a result of these mutual processes, this study was designed to investigate the possible role of these shared destructive mechanisms in the behavioral, cognitive, and biochemical changes following mTBI in mice that were exposed to MDMA before injury.

Experimental procedures

Mice

Male ICR mice weighing 25–30 g were kept five per cage under a constant 12-h light/dark cycle, at room temperature (23°C). Food (Purina rodent chow) and water were available ad libitum. The lighting during the light phase was kept constant, and all experimental manipulations were conducted during the light phase of the cycle. Each mouse was used for one experiment and for one time point only. The Ethics Committee of the Sackler Faculty of Medicine approved the experimental protocol (M-08-040). The minimum possible number of animals was used, and all efforts were made to minimize their suffering.

MDMA administration

MDMA (±3,4-methylene-dioxy-metamphetamine hydrochloride, generously supplied by NIDA) was dissolved in 0.9% saline and injected intraperitoneally (IP) at a dose of 10 mg/kg in a volume of 1 ml/100 g body weight. All other mice were injected with 0.9% saline. This 10 mg/kg IP dose was chosen according to the literature (Green et al. 2009) and fairly imitates a human MDMA dosage. One hour following injection, the mice were placed in the weight-drop device for the brain injury procedure.

Brain injury

Experimental mTBI was induced using the concussive head trauma device described previously (Darke et al. 2004; Milman et al. 2005; Zohar et al. 2003). Slightly anesthetized mice were placed under a device consisting of a metal tube (inner diameter 13 mm) placed vertically over the animal's head. The injury was induced by dropping a metal weight (30 g) from 80 cm height down the metal tube,

striking the skull. Immediately after the injury, mice were placed back in their cages for recovery. The sham mice were slightly anesthetized and put in the head trauma device without receiving any weight drop. The behavioral and cognitive effects of the injury were studied at 7 and 30 days following the trauma. Biochemical measurements were taken 24 h post-injury.

Physiological and behavioral tests

Temperature measurements Rectal temperature of the mice was measured by a mice thermometer. Baseline values (in degrees Celsius) were performed 30 min before MDMA administration. The effects of MDMA and mTBI on the mice's rectal temperature were measured at 1 and 4 h post-injury.

Staircase test Normal motor skills were assessed (Milman et al. 2006; Weizman et al. 2001). Five steps made of black Plexiglas are enclosed, each step is 3×11×7 cm, and the height of the walls was 12 cm above the stairs. Each mouse was placed onto the staircase floor individually, facing the wall. The number of steps ascended, and the number of rearing events were counted for 3 min. Before each session, the staircase was cleaned with 70% ethanol solution (*v*/*v*).

Hot plate test Changes in nociceptive threshold were assessed. The Apparatus consists of a metal platform (30×30 cm), capable of being uniformly heated by an electrical current, and is surrounded by a transparent Plexiglas wall (28 cm) (Pick et al. 1991; Pick et al. 1997). Each mouse was individually placed on the hot-plate with the temperature adjusted to 52°C (±1°C). The latency to the first jump response was measured; the cut-off time was 40 s in order to avoid damage to the paws.

Elevated plus maze Anxiety level was assessed (Alcalay et al. 2004; Baratz et al. 2010). The apparatus consisted of two open arms (30×5×15 cm) and two closed arms (30×5×15 cm) with an open roof, arranged such that the two arms of each type were opposite each other (in a "+" shape). The maze was elevated 60 cm above the floor level (Hogg 1996). On the test day, mice were placed in the center of the plus-maze, facing one of the open arms. The time spent in the open arm was measured during 5 min of observation. The maze was cleaned between animals with 70% ethanol solution (*v*/*v*).

Cognitive tests

Novel object recognition test The novel object recognition (NOR) task was used to evaluate visual memory (Hammond et al. 2004; Tang et al. 1999). The NOR apparatus consisted of a black open field box (59 cm width× 59 cm length×20 cm height). The day before training, mice were allowed to explore the experimental apparatus for 5 min in the absence of objects. During the training phase, mice were placed in the experimental apparatus for 5 min with two identical objects. After a retention interval of 24 h, mice were placed back into the arena in which one of the familiar objects was replaced with a novel one for the test trial. As our objects, we used a plastic bottle (diameter, 7 cm, height, 20 cm) and a tin can (diameter, 8 cm, height, 15 cm). The kind of object presented during the training as well as its position during the test trial were counterbalanced and randomly permuted. Time near each of the objects was manually measured. A mouse was scored as exploring an object whenever it was within 1 cm from the object and facing it. The new object preference index (PI) was calculated as follows $PI = (\text{time near new object} - \text{time near familiar object})/(\text{time near new object} + \text{time near familiar object})$. Objects were cleaned with 70% ethanol between each animal. Animals that did not explore both objects for more than 30 s over the course of the 5-min test session (less than 10% of the time) were excluded from the analysis.

Y maze test Spatial memory was assessed by using the Y-maze, which was first described by (Dellu et al. 1992) and then subsequently validated as a task requiring hippocampal function and spatial memory (Conrad et al. 1996). The procedure was carried out as described before (Baratz et al. 2010; Rubovitch et al. 2010). Briefly, the first run (familiarization) was 5 min with two arms open (the start arm and the arm called "old" arm), the third arm was blocked by a door (the novel arm). After the first run, the mouse was put back into its home cage for 2 min. The second run lasted 2 min when all three arms were open. Time spent in each of the arms was measured. Between each run and between each mouse, the maze was cleaned with 70% ethanol. The new arm preference index was calculated: $PI = (\text{time at novel arm} - \text{time at old arm})/(\text{time at novel arm} + \text{time at old arm})$.

Dry maze test The dry maze test was used to assess the spatial learning ability of the mice. Dry maze task is a variation of the well-known Morris water maze (Morris 1981) that was designed for mice, which have less affinity for water than rats (Whishaw and Tomie 1996). The dry maze is comprised from a circular plastic arena on which 20 tiny wells (10 mm) are arranged in a circular manner. One week before the beginning of the test, mice were put under water restriction and were allowed to drink water for only 1 h a day. The dry maze test consists of three phases: *Training (pre-test) phase*: all 20 wells of the arena are filled with water (200 μl each); the mice were allowed to drink from the wells for 3 min (two trials a day for 3 days).

Learning (test) phase: only one chosen well was filled with water. Each mouse was given 2 min to find the well (3 trials a day for 7 days). *Adjustment phase (probe)*: the site of the chosen water well was replaced and similar to the learning phase mice were given 2 min to find the novel place of the well (three trials a day for 2 days). In all phases, mice were placed at a random starting position, facing the arena wall. Each mouse's latency to reach the water well was measured. The maze was cleaned between each trial, and each mouse with 70% ethanol solution (*v*/*v*), water inside the well was changed.

Biochemistry

Western blots Whole brains were removed 24 post-insult, and hippocampus and cortex (ipsilaterally and contralaterally) were immediately frozen in liquid nitrogen and homogenized with T-PER Tissue Protein extraction Reagent (Pierce, Rockford, IL), with appropriate protease inhibitors (Halt Protease Inhibitor Cocktail; Sigma-Aldrich). Samples were run on precast 10% Bis–Tris gels (Bio-Rad) and transferred to nitrocellulose membranes. Blots were blocked for 1 h with Tris-buffered saline containing 0.01% Tween-20, 5% powered milk, or 5% bovine albumin (Sigma-Aldrich). Membranes were incubated for 2 h at room temperature with antibodies against phospho-IGF-1R (95 kDa) (Cell Signaling Technology) diluted 1:1,000 and incubated with secondary horseradish peroxidase-linked antibodies (Jackson Immunoresearch, West Grove, PA) at room temperature for 1 h. For tyrosine hydroxylase (TH) and phospho-ERK levels, membranes were incubated overnight at 4 C with antibodies against TH (60 kDa) and phospho-ERK1/2 (42+44 kDa) (Santa Cruz Biotechnology) diluted 1:1,000 and incubated with secondary horseradish peroxidase-linked antibodies (Jackson Immunoresearch, West Grove, PA) at room temperature for 1 h. Bands were visualized by enhanced chemiluminescence (Pierce Rockford, IL) and exposed to an X-ray film. Protein band intensities were quantified by using the TINA software. Uniform loading was verified by stripping and reprobing with antibodies against total IGF-1R, total ERK1/2 (1:1,000; Cell Signaling Technology). Antibodies against tubulin (1:2,000; Santa Cruz Biotechnology) were used to verify the uniform uploading for the TH levels.

Data analysis

All results are given as mean±SEM and were analyzed with SPSS 13 software (Genius Systems, Petah Tikva, Israel). One-way analysis of variance (ANOVA) was performed to compare all groups, followed by least significant difference (Fisher LSD) post hoc tests. ANOVAs were used to analyze the results of all behavioral and cognitive tests and for western blot analysis results. For the dry maze, repeated-measures ANOVA (RMANOVA) was used followed by Fisher LSD post hoc tests.

Overall, 99% of the mice had survived the mTBI and MDMA exposure. Five mice died within 24 h following injury (two mice in the sham group, one mouse in the mTBI group, one mouse in the MDMA group, and one mouse in the MDMA+mTBI group).

Results

Evaluation of the mice for “basic wellbeing”

“Basic wellbeing” is a concept that underlies the combined health and wellness. Four parameters were evaluated in order to define the mices' “basic wellbeing”: rectal temperature, motor skills, pain threshold, and anxiety levels.

Rectal temperature measurements were used to assess the temperature changes caused by MDMA administration. Significant differences were found between the groups [$F_{(3,16)}=7.3$, $p<0.01$] and between time of measures (30 min before injury and 1 and 4 h post-mTBI) [$F_{(2,16)}=26.9$, $p<0.01$] post-injury. LSD post hoc analysis revealed that major temperature elevations were observed in the MDMA and the MDMA+mTBI mice compared to sham group when measured 1 h post-mTBI $p<0.01$. The mice subjected to mTBI alone had no elevations in rectal temperature, and their rectal temperature as measured at 1 h post-injury was normal. All groups had normal temperature at 4 h post-injury.

The Staircase test was used to assess normal motor skills. No significant differences were found between the groups as far as the number of steps ascended was considered, 7 days [$F_{(3,64)}=0.17$, N.S] and 30 days [$F_{(3,35)}=1.17$, N.S] post-injury. The number of rearing events (an indicator of agitation) did not differ between groups as well [7 days, $F_{(3,64)}=0.90$, N.S; 30 days, $F_{(3,35)}=0.09$, N.S].

When the hot-plate assay was used to measure the pain threshold of the mice, no differences were found between the sham mice and all other experimental groups at both 7 days [$F_{(3,83)}=0.83$, N.S] and 30 days [$F_{(3,38)}=0.24$, N.S] post-injury.

The elevated plus maze was used in order to examine anxiety level. Time spent in the open arm of the maze was measured. No differences were found between the groups at 7 and 30 days post-injury [$F_{(3,71)}=0.322$, N.S] [$F_{(3,76)}=2.00$, N.S], respectively.

The findings of no motor impairments, no changes in pain threshold, and no high anxiety levels stands for

healthy mice, with no major deficit caused by either the mTBI or MDMA exposure.

Cognitive tests

Novel object recognition (NOR) test was used to examine visual memory. Overall, the mTBI and the MDMA mice showed impairments in visual memory and spent less time near the new object (low preference index) compared with the sham mice. High preference for the new object, similar to the sham group, was seen in mice that were subjected to MDMA prior to injury (the MDMA+mTBI group) (Fig. 1). One-way ANOVA revealed group effect on both 7 and 30 days post-injury [$F_{(3,62)}$=3.53, p=0.02] and [$F_{(3,63)}$=3.29, p=0.02], respectively. Fisher LSD post hoc analysis revealed that at 7 days post-trauma, preference index of the mTBI mice was significantly low compare with all other groups (Fig. 1a). At 30 days, post-trauma mTBI as well as MDMA groups had low preference index compared to the sham or the MDMA+mTBI mice (Fig. 1b).

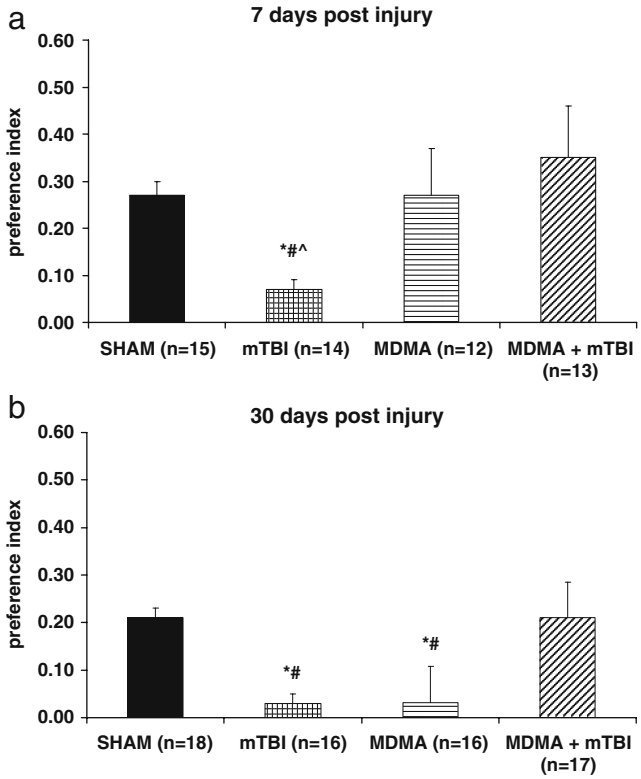

Fig. 1 Novel object recognition. **a** Low-preference index was seen in the mTBI mice compared with all other experimental groups ($F_{(3,62)}$= 3.53, p=0.02, LSD post hoc, p<0.05). **b** Low-preference index was seen in both mTBI mice and MDMA mice compared with sham and MDMA+mTBI group ($F_{(3,63)}$=3.29, p=0.02, LSD post hoc, p<0.01). *p<0.05 vs. sham #p<0.05 vs. MDMA+mTBI ^p<0.05 vs. MDMA

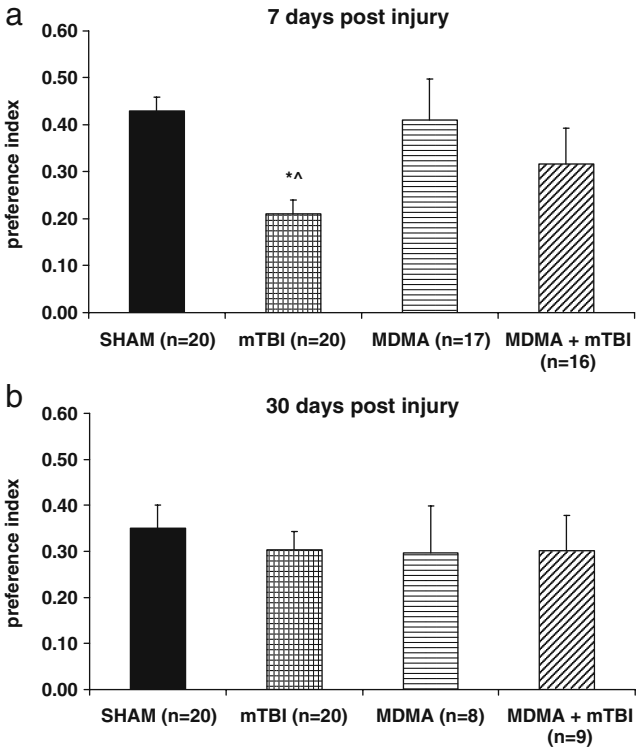

Fig. 2 Y maze test. **a** One-way ANOVA revealed that the mTBI was different from all groups and did not display preference to the novel arm [$F_{(3,69)}$=3.30, p=0.025], LSD post hoc (p<0.01). **b** Novel arm preference was seen in all tested groups including the mTBI mice. *p<0.05 vs. sham ^p<0.05 vs. MDMA

Short-term spatial memory was tested with the Y maze. One-way ANOVA followed by LSD post hoc test revealed group differences at 7 days post-injury [$F_{(3,69)}$=3.30, p= 0.025]. The mTBI mice had low novel arm preference (p< 0.01) and were different from sham mice. All other tested groups including the MDMA+mTBI group showed significant preference for the novel arm as seen in Fig. 2a. High novel arm preference was found in all experimental groups 30 days post-injury (Fig. 2b).

Long-term spatial learning was tested by the dry maze. The mean latencies to reach the water well during the test and probe phase are shown in Fig. 3. In the pre-test phase, no differences between the groups were found (data not shown), and all the mice drank from the water wells. In the test phase, 7 days post-injury, there were no differences between experimental groups [$F_{(3,55)}$=2.14, p=0.105 by RMANOVA], a significant effect of test day was found [$F_{(6,18)}$=16.84, p<0.001] with no interactions between the factors. At the test phase, 30 days post-injury, a RMANOVA revealed significant effects of group (sham, mTBI, MDMA and MDMA+mTBI) [$F_{(3,55)}$=4.62, p<0.01] and test day [$F_{(6,18)}$=23.63, p<0.001] but no significant interaction between these factors [$F_{(6,18)}$=1.22, p=0.241].

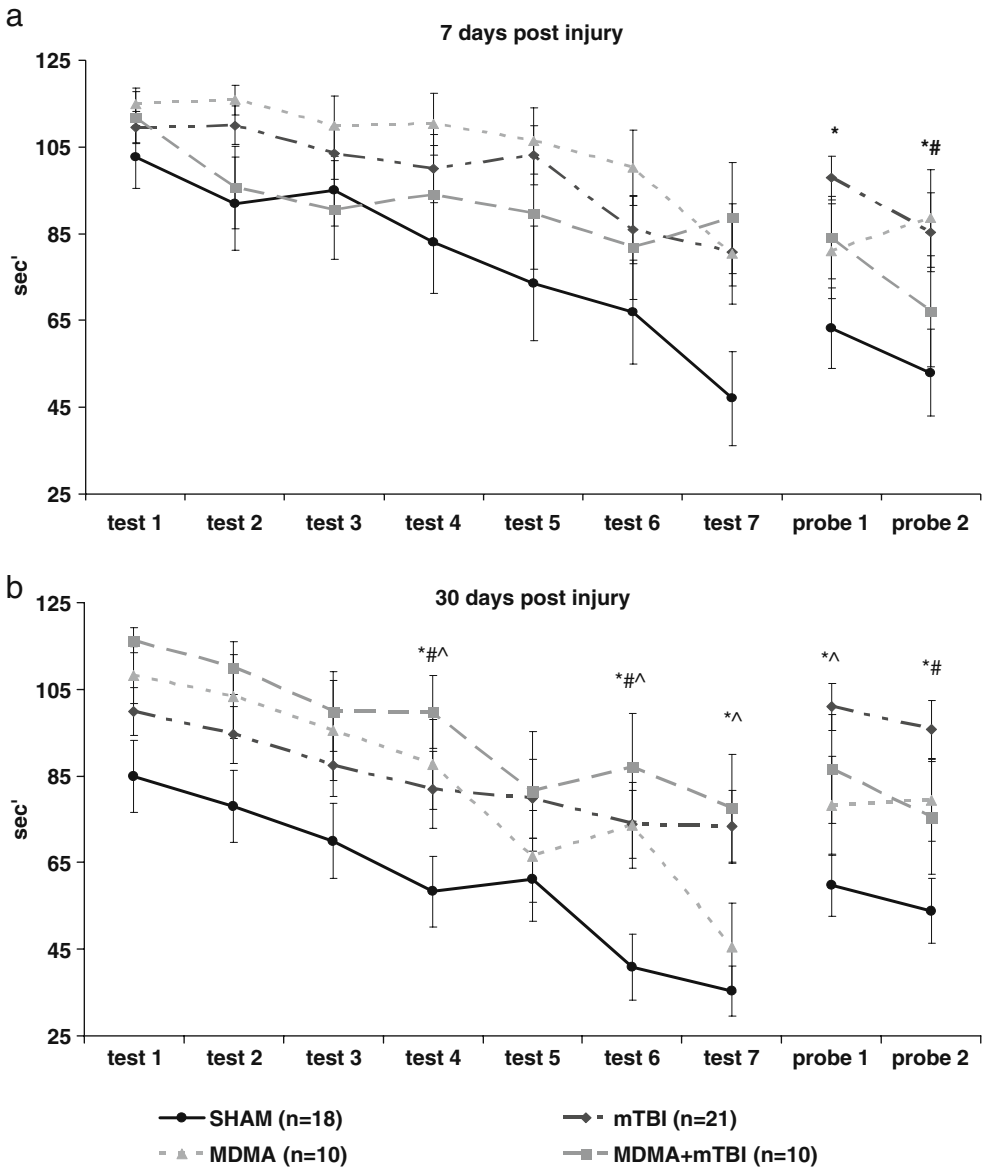

Fig. 3 Dry maze test. **a** No significant group effects were found in the test phase. In the probe phase, differences were between mTBI and MDMA mice vs. sham [$F_{(3,55)}$=3.38, p=0.02], LSD post hoc, p=0.002 and p=0.04. **b** RMANOVA revealed significant group effects in the test phase $F_{(3,55)}$=4.62, p<0.01. All groups were different from sham mice (p<0.001, p=0.035, and p<0.001, respectively). In the probe phase, differences were between mTBI and MDMA+mTBI mice compare to sham $F_{(3,55)}$=6.87, p<0.01. *p<0.05 mTBI vs sham #p<0.05 MDMA vs. sham ^p<0.05 MDMA+mTBI vs. sham

Further analysis (LSD post hoc) showed that all groups had impairments in their spatial learning and were different from the sham mice (mTBI p<0.01, MDMA p=0.03, and MDMA+mTBI p<0.01; Fig. 3b). On days 4 and 6, the latencies in the sham group were significantly shorter than those in all other groups (p<0.05) (Fig. 3b). At day 7, the latencies in the sham group were significantly shorter compared with the mTBI and MDMA+mTBI groups. The probe phase measures the mice's ability to override their previous learning and to change their exploration strategy to find the new location of the water well. Group effects were found at both 7 and 30 days post-injury [$F_{(3,55)}$=3.38, p=0.02] and [$F_{(3,55)}$=6.87, p<0.01], respectively. No significant day effect and no interactions were found. mTBI and MDMA groups had longer latencies in reaching the new water well compared with sham mice 7 days post-injury (LSD post hoc p=0.002 and p=0.04, respectively). At 30 days post-injury, the mTBI and MDMA+mTBI groups were impaired at finding the new water well compared with sham mice (LSD post hoc p<0.001 and p=0.035, respectively).

Biochemistry

In order to reveal the MDMA mechanism, we measured the phosphorylation of the known pro-survival IGF-1R. Previous studies from our lab found elevation in IGF-1R activation due to mTBI. In the present study, IGF-1R phosphorylation was elevated in all tested groups as indicated by one-way ANOVA ($F_{(3,20)}$=16.78, p<0.01) (Fig. 4). LSD post hoc analysis revealed that all tested groups were different from sham mice but not different from one another.

Phosphorylation of ERK1/2: The IGF-1R is known to activate the extracellular regulated kinase (ERK1/2). MDMA administration is also known to cause elevations in ERK1/2 phophorylation. Following mTBI and MDMA exposure, significant group effect was found in ERK1/2 phosphorylation [ERK 1, $F_{(3,20)}$=8.07, p<0.01] and [ERK 2, $F_{(3,20)}$=28.69, p<0.01]. Although all groups showed elevations in ERK1/2 phosphorylation compared with the sham mice, these differences reached statistical significance only in the MDMA and mTBI groups (Fig. 5). No differences were found between the mTBI group and the MDMA+mTBI group (Fig. 5b).

Tyrosine hydroxylase (TH) levels: TH is a key enzyme in the synthesis of catecholamines and its levels demonstrate the presence of DA. MDMA in mice is known to attenuate dopamine levels. Following mTBI, a significant group effect was seen regarding TH levels [$F_{(5,35)}$=2.61, p=0.041] (Fig. 6). The decrease in TH levels were seen at 1 h post-mTBI till 72 h, LSD post hoc analysis revealed significant decrease at 24 h post-injury, p<0.01 compared with sham mice (Fig. 6). After determining a significant decrease in TH levels, 24 h post-injury, we measured the effect of MDMA (10 mg/kg) on mTBI-induced TH decrease. Following administration of MDMA, 10 mg/kg prior to mTBI, we revealed normal TH levels. A significant group effect was seen [$F_{(3,19)}$=3.61; p=0.032] (Fig. 7). LSD post hoc analysis determined that differences were observed between mTBI mice compared with sham and MDMA+mTBI mice (p<0.01 and p=0.02, respectively).

Discussion

The present study reconfirms the observation that behavioral deficits, such as impaired visual memory and reduction in spatial memory performance, occur following mTBI. However, a novel and intriguing finding is that the significant differences seen between mTBI and sham mice disappeared almost completely following pre-mTBI induction MDMA administration. The restored cognitive abilities seen here may be related to the preserved TH levels found following the MDMA administration.

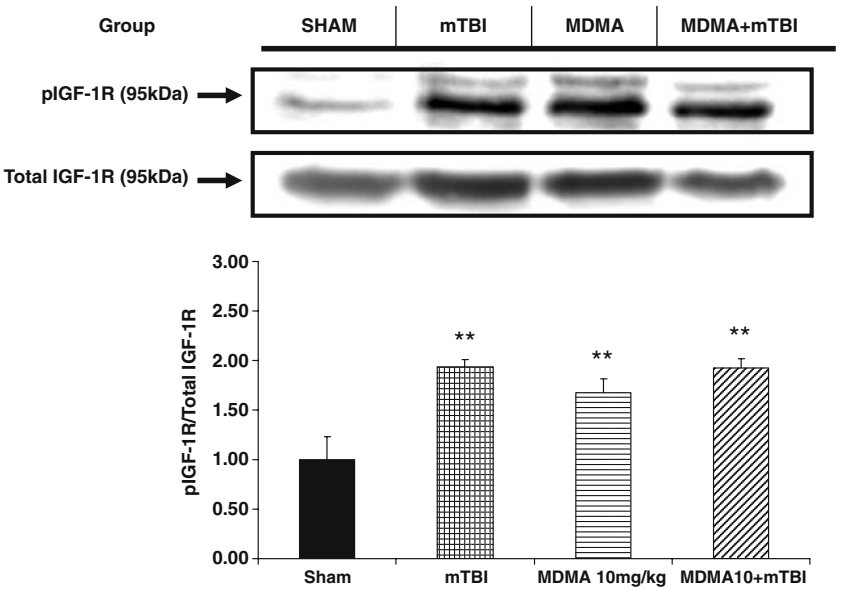

Fig. 4 Phosphorylation of IGF-1R. IGF-1R phosphorylation was significantly elevated after mTBI. Hippocampus protein extracts were subjected to gel electrophoresis, followed by immunoblot analysis using antibodies against pIGF-1R and total IGF-1R. The level of phosphorylation was evaluated by densitometry analysis. The sham values were set as 1, and the relative values of respective treatment were calculated accordingly. Results indicate that all tested groups induced an elevation in IGF-1R phosphorylation. One-way ANOVA revealed group effect [$F_{(3,20)}$=16.78, p<0.01], LSD post hoc test revealed that differences were between sham vs. all other tested groups p< 0.01. Results are mean±SEM, n=6 mice. **p<0.01 vs. sham

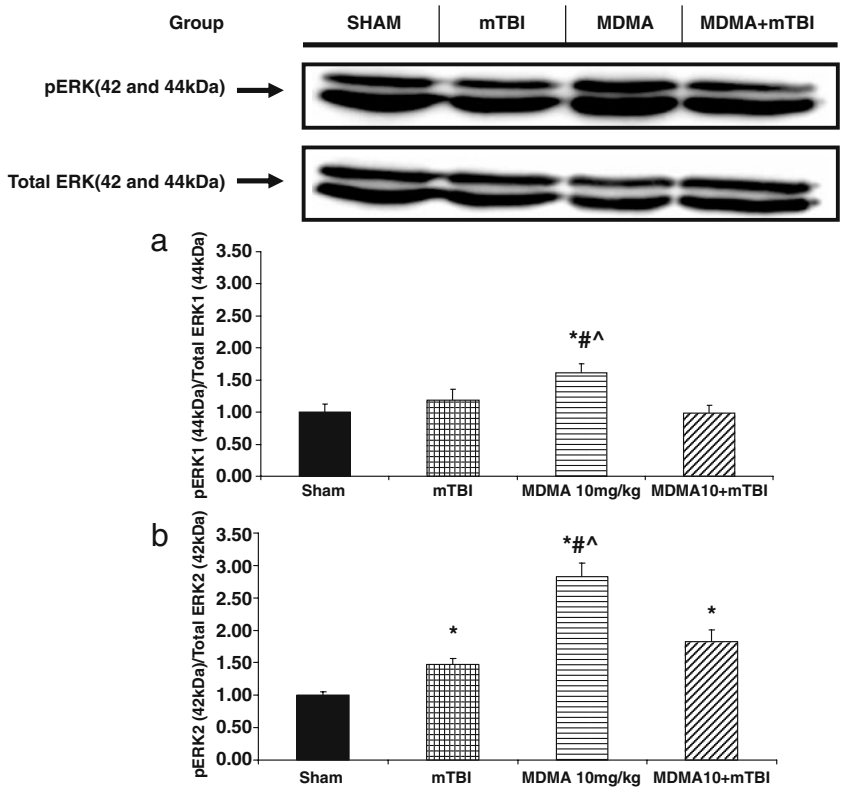

Fig. 5 Phosphorylation of ERK1/2. The level of ERK phosphorylation was evaluated by densitometry analysis. The sham values were set as 1, and the relative values of respective treatment were calculated accordingly. **a** Elevation in phosphorylated ERK1 was seen mainly in the MDMA group. One-way ANOVA showed significant group effect: [$F_{(3,20)}$=8.07, p<0.01], LSD post hoc, p<0.05. **b** All groups showed elevations in phosphorylated ERK2 compare with sham group, significant group effect [$F_{(3,20)}$=28.69, p<0.01]. The MDMA group was different from mTBI and MDMA+mTBI groups as well LSD post hoc p<0.001 and p<0.001, respectively. Results are mean± SEM of six mice. *p<0.05 vs. sham #p<0.05 vs. mTBI ^p<0.05 vs. MDMA+mTBI

Very few studies have investigated the effects of ecstasy on TBI and to the best of our knowledge, none in mice. The current research was aimed at describing the effects of MDMA on mTBI-induced behavioral and cognitive performance as well as changes in some biochemical markers. Our experimental procedure attempts to describe the scenario in which a "drug user" might experience a TBI in a motor vehicle collision while acutely intoxicated with MDMA.

The World Health Organization has predicted that by the year 2020, traffic accidents will be the third largest cause of the global burden of diseases and injuries (Maas et al. 2008). Many of these motor-vehicle accidents involve drivers under the influence of drugs. Epidemiological studies and case studies have shown that MDMA can impair driving abilities and cause reckless behavior such as speeding and ignoring red traffic lights (Brookhuis et al. 2004; Drummer et al. 2003; Hooft and Vandevoorde 1994; Kuypers et al. 2009; Logan and Couper 2001). The growing numbers of MDMA users and TBI incidents encourage this research.

Mice that experienced only the mTBI and did not receive MDMA showed deficits in behavior performance compared with the sham animals on the three cognitive tests utilized. Lower visual and spatial memory were found in the NOR and Y maze test. In the dry maze test, the impairments were found in the spatial learning and in the probe phase. These results support previous experiments performed in our laboratory, in which similar post-injury learning deficits were demonstrated (Milman et al. 2005; Zohar et al. 2003). Regarding the biochemical tests, mTBI was found to cause elevations in IGF-1R and ERK2 phosphorylation and a decrease in tyrosin hydroxylase (TH) levels. These results are in accordance with previous studies demonstrating time-depended IGF-1R, Akt, and ERK1/2 activation after mTBI (Rubovitch et al. 2010). The low levels of TH in the mice's cortex were found 24 h post-injury. These results indicate an alternation in the DA system following TBI as previously reported in rats (Henry et al. 1997; McIntosh et al. 1994; Wagner et al. 2009).

The mice that were subjected to MDMA alone showed visual memory impairments and had low preference index

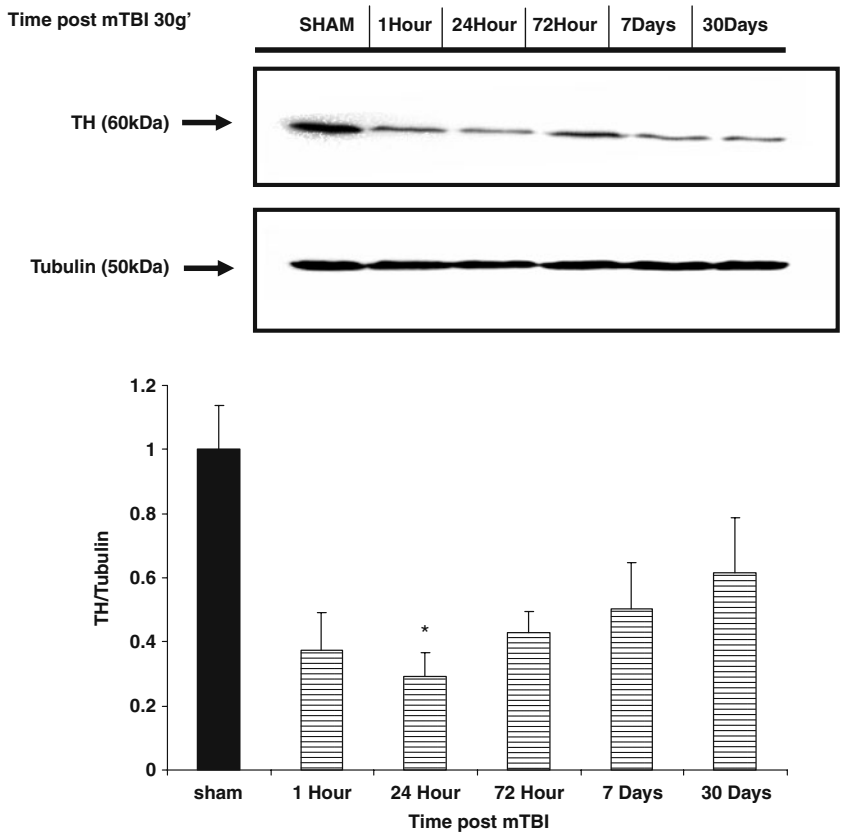

Fig. 6 Tyrosine hydroxylase after mTBI. The level of TH was evaluated by densitometry analysis. The sham values were set as 1, and the relative values of respective treatment were calculated accordingly. Decreases in TH levels were seen since 1 h post-injury; these decreases reached significance 24 h post-mTBI. One-way ANOVA showed significant group effect [$F_{(5,35)}=2.61$; $p=0.041$], LSD post hoc revealed significant differences $p<0.01$, 24 h post-injury, $p<0.05$. Results are mean±SEM of 6–8 mice. $^{**}p<0.01$ vs. sham

in the NOR test. According to the Y maze, these mice had no short-term spatial memory impairments (Figs. 1 and 2). Long-term visual memory deficits caused by MDMA were seen previously in rats but not in mice (Camarasa et al. 2008; McGregor et al. 2003; Piper and Meyer 2004). In the dry maze, the MDMA mice spent more time looking for the water well than the sham mice 7 days after administration; these deficits reached statistical significance at 30 days post-injection. Many previous studies used the Morris water maze to access spatial learning after MDMA treatments (Able et al. 2006; Skelton et al. 2008; Sprague et al. 2003). In most of these experiments, MDMA didn't cause any major impairment in rats' spatial learning. A possible explanation for these discrepancies is the different effects that MDMA has on mice compared to rats. MDMA in mice is predominately selective to dopamine release and dopamine transporter (DAT) inhibition, in the same time in rats, the major activity of MDMA is on the serotonergic system (Capela et al. 2009; Capela et al. 2007; Colado et al. 2001; Colado et al. 2004). Even though both neurotransmitters are related to learning and memory, impairments in spatial learning are more influenced by dopamine than serotonin (Granado et al. 2008; Kern et al.; Petrasek and Stuchlik 2009). Additionally, stimulation of dopamine receptors may play an important role in synaptic plasticity and memory storage of motor behaviors (Kleim et al. 2003; Simola et al. 2009).

When animals were given MDMA prior to the mTBI procedure, their behavioral performance was significantly improved compared with the mTBI mice. These mice showed intact visual memory and undamaged spatial memory on both the NOR test and the Y maze test (Figs. 1 and 2). No beneficial effect was seen following MDMA in the mTBI mice in the dry maze test. Currently, no other studies have been carried out combining MDMA and TBI in mice. Recent studies from our lab combined alcohol consumption before and during mTBI (Baratz et al. 2010) or morphine injection prior to mTBI (Zohar et al. 2006). In these experiments, both alcohol and morphine provided a neuroprotective effect in mTBI mice. In another study, administration of cocaine to pigs before fluid percussion TBI did not have any harmful effects on physiologic parameters such as cerebral blood flow or cerebral oxygen extraction ratio (McBeth et al. 2005).

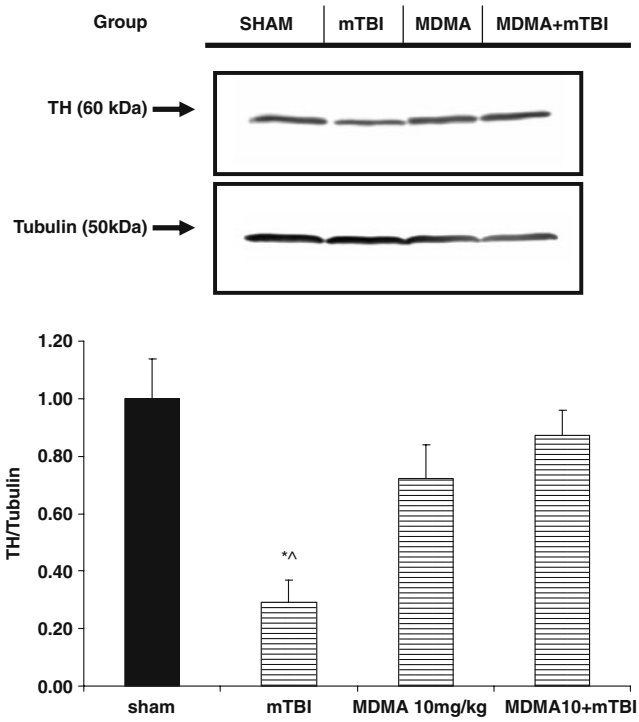

Fig. 7 Tyrosine hydroxylase 24 h after mTBI. Levels of TH were significantly decreased 24 h after mTBI. Cortex protein extracts were subjected to gel electrophoresis, followed by immunoblot analysis using antibodies against TH and tubulin. The level of TH was evaluated by densitometry analysis. Decreases in TH levels were abolished when MDMA 10 mg/kg were administrated prior to injury. One-way ANOVA showed significant group effect [$F_{(3,19)}$=3.61, p=0.032], LSD post hoc revealed that mTBI group was significantly different from sham and MDMA+mTBI groups (p<0.01 and p=0.02, respectively). Results are mean±SEM of 6–8 mice. **p<0.01 vs. sham ^p<0.05 vs. MDMA+mTBI

In order to understand the mechanism of this potentially beneficial effect seen after MDMA administration in mTBI mice, we measured the activation of IGF-1R and ERK. Given the role of IGF as a potential neuroprotective agent (Cheng et al. 2003; Guan et al. 2003), it was hypothesized that changes in its phosphorylation will underlie the behavioral changes seen in the NOR and Y maze. Even though cognitive improvements were seen following MDMA injection to mTBI mice, no differences in the activation of both IGF-1R and ERK were detected. This indicates that the improvements in the behavioral tests previously described may not be connected to IGF-1R or ERK activation.

The results of the cognitive tests still raised the question whether the presence of MDMA prior to mTBI exerts a real beneficial effect. In addition, if MDMA and mTBI separately cause cognitive impairments, and many previous studies suggested that MDMA and mTBI share neurodestructive mechanisms, why does the combination of the two factors not have a worsening effect? A report by Evans et al. (1987), who administered dextroamphetamine (an amphetamine analog) following closed head TBI, found that it enhanced processing speed time and improved memory. Previous case studies showed that "in hospital" mortality rates were lower in people who consumed amphetamines before accidental TBI (O'Phelan et al. 2008). Furthermore, amphetamine abuse in experimental setting of TBI has been shown to accelerate recovery (Dhillon et al. 1998). Studies on both animals and humans have identified alterations in DA neurotransmission that occur after TBI. These changes in DA may be crucial factors in cognitive and behavioral deficits seen after TBI (for review, see Bales et al. 2009).

In mice, the administration of MDMA produced a rapid increase in the extracellular levels of dopamine with a peak level occurring 1 h after injection (Camarero et al. 2002). Regarding this hypothesis, we measured the levels of tyrosine hydroxylase (TH) in the injured mice's cortex after MDMA administration. TH is the key enzyme for synthesizing dopamine (DA) in dopaminergic neurons and its terminals (Haavik and Toska 1998; Kumer and Vrana 1996) and was seen to be decreased in our model of mTBI. When mice were administered with MDMA prior to the brain injury, their TH levels were as high as sham mice, meaning, that the dramatic decrease in TH levels seen after mTBI (Fig. 6) was totally abolished after MDMA injection. This restoration of TH levels may be a key explanation to the cognitive improvements seen in these mice. Studies in both animals and humans have identified a series of temporal alterations in DA neurotransmission that occur after TBI (Donnemiller et al. 2000; Wagner et al. 2005a; Wagner et al. 2009; Wagner et al. 2005b; Yan et al. 2002; Yan et al. 2001). Decreases in DA overflow and alterations in both DAT and TH expression were found to occur after injury. Although in our study, the beneficial effect seen in cognitive performance was due to a dangerous recreational drug (MDMA), understanding the temporal alterations in DA and the mechanism of dysfunction at a cellular level will allow DAergic legal therapies to become potential candidates for clinical use.

In summary, the results of the present study suggest that MDMA has a protective effect on the cognitive impairment resulting from mTBI in mice, an effect observed in most of the cognitive tests. Furthermore, although the IGF-1R pathway is activated both as a result of MDMA administration or mTBI, it currently cannot explain the improvement of the cognitive abilities of the mice subjected to MDMA prior to mTBI. In contrary, the returning to normal TH levels in MDMA+mTBI mice seem to be the cause of the restored cognitive abilities demonstrated. Further research, looking mainly at the dopamine levels and its downstream agents is needed to confirm if this neurotransmitter system is responsible for the behavioral effects seen.

Acknowledgments This study was supported (in part) by a grant from the Dr. Herman Schauder Endownment Fund for Research and by a grant from The Dr. Miriam and Sheldon G. Adelson Center for the Biology of Addictive Diseases, Tel Aviv University, Tel Aviv, Israel.

Role of funding source None declared.

Contributors Shahaf Edut and Chaim Pick designed the study and wrote the protocol, as well as designed the behavioral experiments and contributed to the interpretations of the results. Shahaf Edut managed the literature searches and analyses. Shahaf Edut and Vardit Rubovitch undertook the statistical analysis, and Shahaf Edut wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest None.

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