

Atomoxetine enhances memory and proliferation in adult male rat hippocampus

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SUMMARY

Atomoxetine (ATX) is a noradrenaline reuptake inhibitor used to treat Attention deficit hyperactive syndrome (ADHD), or improve cognition in normal subjects. The cognitive effects of ATX require inputs from the hippocampus. Moreover, proliferation is said to be located in the dentate gyrus (DG) of the hippocampus.

In the present study, we hypothesised that ATX improves memory and proliferation of the adult rat hippocampus. To test this hypothesis, 5 intraperitoneal injections of ATX (30 mg/kg/day) over 5 consecutive days were delivered to rats. 30 minutes after the last injection, spatial memory was tested using the Novel location recognition (NLR) test. Proliferation of hippocampal cells was quantified using immunohistochemistry for the proliferative marker Ki67. ATX-treated rats showed cognitive enhancement in the NLR task and increase in cell proliferation in the Subgranular zone (SGZ) of the DG, compared to saline-treated controls. The results demonstrate that ATX is able to enhance cognition through increasing the levels of proliferation in the adult rat brains.

Key words: Atomoxetine – Memory – Proliferation – Hippocampus – Object location – Recognition

INTRODUCTION

Noradrenaline re-uptake inhibitors are classified according to the neurotransmitter systems they affect (Lucki and O'Leary, 2004). These drugs are

commonly used in various psychiatric disorders, such as depression and attention deficit hyperactivity disorder (ADHD) (Kelsey et al., 2004; Garnock-Jones and Keating, 2009; Fournier et al., 2010). Both noradrenergic and serotonergic activities have been associated with cognitive functions, including learning and memory (Fitzgerald, 2011; Pringle et al., 2013). Moreover, it has been suggested that noradrenaline plays a crucial role in memory processes (Sara, 2009) and that optimal levels of noradrenaline improve these and are necessary for memory consolidation (Tully and Bolshakov, 2010). Limited studies have so far investigated the effects of atomoxetine (ATX) (Tzavara et al., 2006; Epperson et al., 2011; Warner and Drugan, 2012; Tamburella et al., 2013) on memory. ATX is a selective noradrenaline re-uptake inhibitor, and is used for the treatment of cognitive impairments in children and adolescents suffering from ADHD (Kelsey et al., 2004; Garnock-Jones and Keating, 2009). Besides, ATX has been recently reported to have an effect on improving memory deficit in animals (Tzavara et al., 2006; Tamburella et al., 2013). Clinically, it has been suggested that ATX also has a beneficial effect on memory in pre- and post-menopausal women (Epperson et al., 2011).

The cellular effects of NE (Norepinephrine) are modulatory in nature, allowing synaptic transmission to be more effective, and thus presumably enhancing cognition and behavioural responses in brain areas innervated by NE (Woodward et al., 1991). The hippocampus has one of the denser regions of adrenergic terminals, supporting the role of NE in learning and memory in this region (Korz and Frey, 2007). It has been

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proposed that NE innervation is particularly dense in areas receiving mossy fibres inputs, including the DG and stratum lucidum (Moudy et al., 1993). Several studies have demonstrated the importance of NE signalling for consolidation of emotional events, especially for aversively trained animals, which may involve the amygdala and hippocampus (Ferry and McGaugh, 2000). Other studies have begun to investigate the role of NE in spatial navigation tasks and have found that NE is important for consolidation and retrieval of spatial memories (Murchison et al., 2004; Korz and Frey, 2007). Animals with reduced cortical NE levels performed significantly worse in the spatial memory task compared to controls (Collier et al., 2004). Furthermore, enhancing NE tone prior to retention testing showed significantly less "forgetting" in a spatial memory task of the Morris water maze (MWM) (Sara, 1989).

There is considerable evidence suggesting that NE manipulations can modulate early Long term potentiation (LTP) in several hippocampal pathways (Munro et al., 2001; Harley, 2007). These mechanisms have focused on beta-adrenergic receptors. For example, NE enhances LTP in the mossy fiber-CA3 synapse via beta-adrenergic mechanism, and pharmacological activation of beta- adrenergic receptors can rescue CA1 LTP in genetic strains with impaired LTP.

The mechanism by which ATX improves memory may also play an important role in neuroplasticity in the hippocampus (Harley, 1998; Izumi and Zorumski, 1999).

In the present study, we hypothesised that ATX increases memory in adult male rats through increasing level of proliferation in the hippocampus.

Previously, it has been demonstrated that Methylphenidate (MPH), a drug used to treat ADHD can enhance cell proliferation and differentiation in the sub granular zone (SGZ) of DG (Lee et al., 2012). In addition, ATX-treated Sprague-Dawley rats showed improved performance in the

water maze task, as well as increased levels of hippocampal plasticity as detected by increased Brain-derived neurotrophic factor (BDNF) levels (Spalding et al., 2013).

To test our hypothesis, 5 intraperitoneal injections of ATX (30 mg/kg/day) over 5 consecutive days were delivered to rats. 30 minutes after the last injection, spatial memory was tested using the novel location recognition (NLR) test, and proliferation of hippocampal cells was quantified using immunohistochemistry for the proliferative marker Ki67. ATX-treated rats showed cognitive enhancement in the NLR task and increase in cell proliferation in the SGZ of the DG, compared to saline-treated controls. The results demonstrate that ATX is able to enhance cognition through increasing the levels of proliferation in the adult rat brains.

MATERIALS AND METHODS

Animals and preparations

Male Lister hooded rats (150-200 g; The University of Jordan, animals home office) were randomly allocated to vehicle (n=7), ATX (n=7) groups. Animals were allowed to habituate for 2 weeks prior to drug administration.

Rats in the ATX group were administered ATX (30 mg/kg, 5 intraperitoneal doses), Manufacturer (Lilly S.A., Avda.de la Industria, Madrid, Spain) and rats in vehicle group were given an identical volume of 0.9% sterile saline (intraperitoneal). Throughout the experiment, rats were maintained with a 12-h light/dark cycle (7.00/19.00 h) with "ad libitum" food and water. Principles of laboratory animal care were in accordance to the University of Jordan Home Office Guidance regulations and with local ethical committee approval.

Behavioural testing

Novel location recognition (NLR)

The NLR test used here is a spatial variant of a

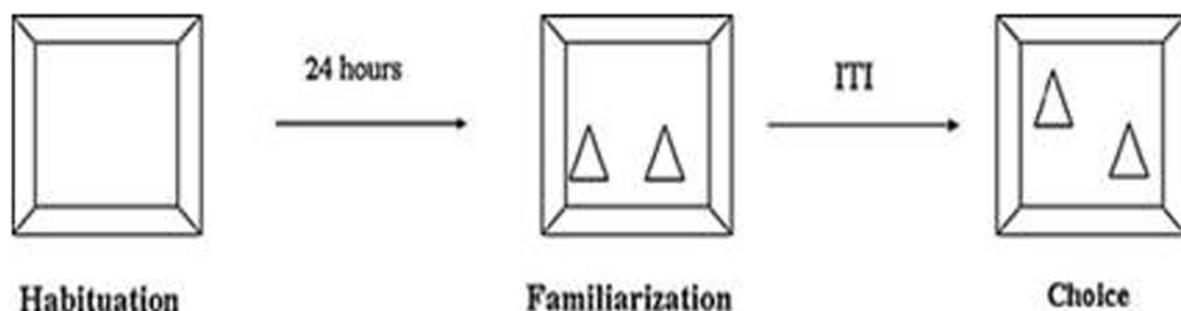


Fig 1 A. The Novel location recognition test protocol was carried out over two days. On the first day, animals were allowed to habituate to the test arena for 1 h. On the following day, two identical objects were placed in different locations of the box with the animal to explore for 3 min (Familiarization trial). Animals were then removed outside the box for 5 min inter-trial interval (ITI) after which the animal was put in the same box after changing the objects location for 3 min (choice trial).

two trial object recognition task adapted from Dix and Aggleton (1999) (Fig. 1A). The apparatus consisted of an arena (a semi-transparent Perspex box, dimensions; 49 cm wide × 66 cm long × 40 cm high) and pink, weighted water bottles (replicas, 15 cm high, 7 cm diameter). Arenas and water bottles (objects) were cleaned with 20% ethanol prior to each experiment and between trials to remove olfactory cues. A black square of card was on the wall of the room during trials to provide prominent cues for spatial orientation.

This was modified from a previous protocol (Dix and Aggleton, 1999) and was recorded by video camcorder as done previously in our past laboratory tests (Mustafa et al., 2008). The test apparatus consisted of plastic boxes (39 × 23.5 × 30 cm). The procedure consisted of habituating the animals for 1 h in the box on the day prior to testing. The following day, two identical objects (water bottles) were placed in separate locations in the box and the animals allowed 3 min to explore (Familiarisation trial). Animals were returned to their home cage for 5 min (inter-trial interval) during which the box was cleaned with 20% ethanol. For the choice trial, the animals were returned to the box for 3 min, where one object remained in its original position (familiar location), while the other object was moved to a new position (novel location) see (Fig. 1A).

Exploration of the object was scored when the animal sniffed, licked, chewed or directed its nose at a distance ≤1 cm from the object (Mustafa et al., 2008). Exploration was scored as the total time spent on each object (familiar and novel locations), and a discrimination index was calculated as the difference between the time spent on each object divided by the total exploration time (Dix and Aggleton, 1999; Bruel-Jungerman et al., 2005; El Beltagy et al., 2008).

Histology and immunohistochemistry

The brains were extracted, trimmed and fixed in 3% glutaraldehyde overnight. Next day brains were sectioned using Leica vibrating microtome. Sections (4 μm) were placed onto positively-charged slides for routine staining with haematoxylin and eosin (HE) and for IHC. The tissues were dewaxed with xylene and rehydrated through a series of graded ethanol. For antigen retrieval for Ki67, the samples were autoclaved in 0.01 M sodium citrate, pH6.0, at 121°C for 20 min and then heated in a microwave oven (800 W) for 5 min (Pisamai et al., 2017). Endogenous peroxidase activity was performed by incubating the slides in 3% H₂O₂ in methanol at room temperature for 20 min. Non-specific immunoglobulin binding was blocked with 3%(w/v) bovine serum albumin (Merck, Rockland, Massachusetts, USA) at 37°C for 20 min.

The polyclonal antibody against Ki67 (RB-9043-P0) was from Dako (Glostrup, Denmark). The

primary antibody was diluted in phosphate buffered saline (PBS) at a dilution of 1:50. The antibodies were incubated at 4°C for 16 h. The slides were counterstained with haematoxylin stain (Pisamai et al., 2017).

A systemic random sampling technique (Mayhew and Burton, 1988) was used to choose every 21st section throughout the length of the DG (overall 10 sections) Using a Zeiss Primo Star microscope (Zeiss, Oberkochen, Germany) equipped with a Canon EOS 550D camera (Canon, Tokyo, Japan).

Ki67 positive cells were counted within the SGZ, defined as within three cell diameters of the inner edge of the DG. Counts from all sections of one DG were averaged to provide a number per section (El Beltagy et al., 2008).

Statistical analysis

Statistical analysis and graphs were created using GraphPad Prism 4.0 and significance was regarded as $p < 0.05$. Student's paired t-tests were used to compare exploration times of animals in the familiarisation and choice trials. Preference indices (PI) were created by expressing time spent exploring the object in the novel location as a percentage of the sum of exploration time of novel and familiar locations in the choice trial, to create a single value to compare between groups (Bruel-Jungerman et al., 2005). PI was compared to 50% chance using t-test. Proliferating cell counts were also compared using t-test.

RESULTS

Effect of treatment on the Novel location recognition (NLR) task

The NLR test measures interactions with objects either in familiar or novel locations within a test arena. During the familiarisation trial, when animals explore two identical objects, both saline- and ATX-treated groups showed no preference for either object or the total exploration time (Fig. 1B). Following a 5 min inter-trial interval,

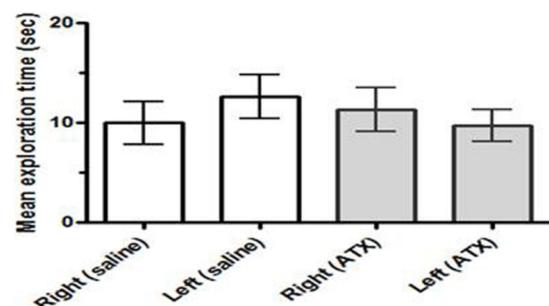


Fig 1 B. The figure shows that there was no preference for either object locations during familiarization trial in both saline and ATX-treated groups. The analysis was done using Paired T test (two-tailed) to compare between the mean exploratory time of the old and the new location during the familiarization trial before treatment in each group separately.

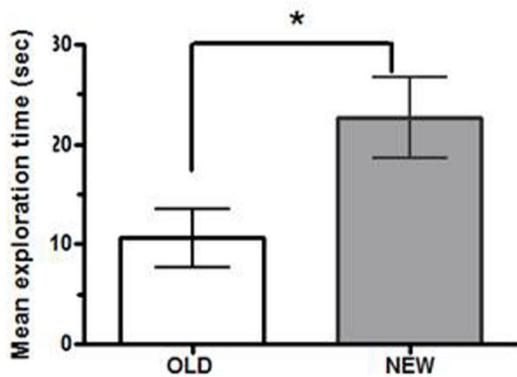


Fig 2. Saline-treated animals explored the new location significantly more than the old location ($P = 0.03$). Difference between means (-11.95 ± 4.991). The analysis was done using Paired T test (two-tailed) to compare between the mean exploratory time of the old and the new location in the choice trial after treatment.

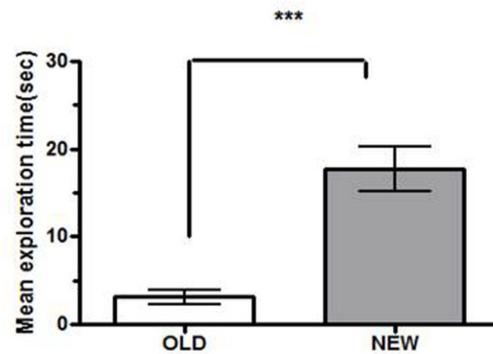


Fig 3. ATX-treated animals explored the new location significantly more than the old location ($P=0.003$). Difference between means (-15.35 ± 2.332). The analysis was done using Paired T test (two-tailed) to compare between the mean exploratory time of the old and the new location in the choice trial after treatment.

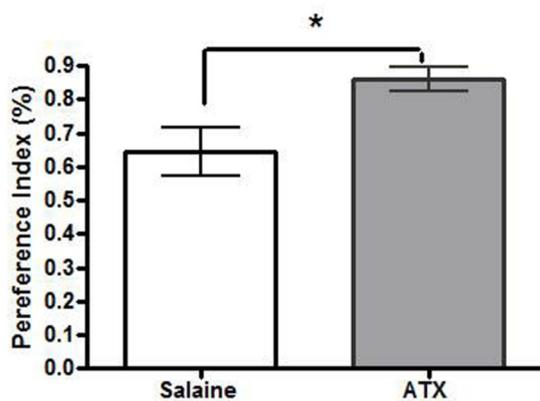


Fig 4. Difference in the preference index between the two groups. Preference index is defined as the calculated exploration time of the new location, represented as a percentage of the combined exploration time of both novel and familiar locations of an object. The preference index showed a significant increase in the ATX-treated rats in comparison to the saline-treated group ($p=0.026$). Difference between means (-0.2153 ± 0.08380). The analysis was done using paired T test (two-tailed).

one object is moved to a new location (choice trial) and object preference is recorded (Fig. 1A). Saline-injected controls explored the novel object significantly more than the old one ($P = 0.03$) (Fig. 2). Animals treated with ATX by Intraperitoneal injection significantly explored the novel location more than the old one ($P= 0.003$) (Fig. 3).

Further analysis using the preference index (PI) was done to compare between the two groups (see Fig. 4). Comparing the PI between the control and the ATX-treated group demonstrated a significant increase after ATX treatment ($p=0.02$). These findings indicate that animals treated with ATX improved memory more than those treated with saline.

Effect of treatments on proliferating cell counts

There was a significant increase in the total

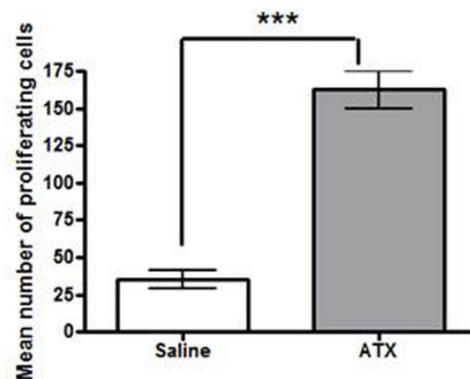


Fig 5. Significant increase in the total number of Ki67 positive cells of the ATX-treated group compared to saline-treated ($P < 0.0001$). Difference between means (-127.5 ± 12.92) which is correlated with the results obtained from the NLR test.

number of Ki67 positive cells in the ATX treated group compared to saline treated ($P < 0.0001$) – see Figs. 5 and 6 (A,B and C) – which correlated with the results obtained from the NLR test.

DISCUSSION

The present study addressed the effect of ATX on memory and proliferation in the adult rat hippocampus.

Attention-deficit hyperactivity disorder (ADHD) is among the most common neurobehavioral problems affecting children between 6 and 17 years of age. Its prevalence in the United States is believed to range from 2% to 18% in this age group, and affecting approximately 5% of children worldwide (Faraone et al., 2003; Polanczyk et al., 2007). ADHD heritability is similar across Europe, North America and Australia (Hawi et al., 2015). While lower prevalence rates have been observed in Europe (4.6%) and North America (6.2%) versus Africa (8.5%) and South America (11.8%)



Fig. 6 A (Ki67 positive cells in saline treated rat)

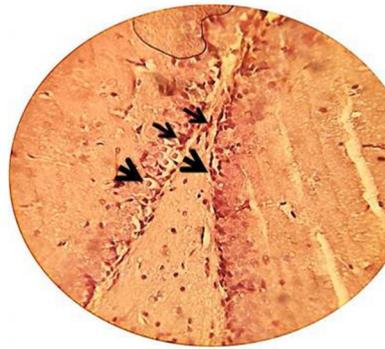


Fig. 6 B (Ki67 positive cells in ATX treated rat)

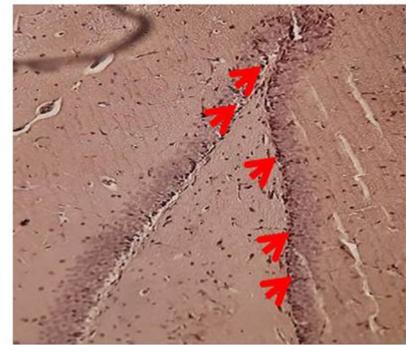


Fig. 6 C (Ki67 positive cells counterstained with Haematoxylin stain, ATX treated rat)

Fig 6. Dentate gyrus of adult rat brains. Ki67 positive proliferating cells (arrows) appear dark indicating neurogenesis. (A) Saline treated rat with few proliferating cells. (B) ATX treated with many proliferating cells. (C) Ki67 positive cells counterstained with haematoxylin stain, ATX treated rat.

(Polanczyk et al., 2007). ADHD leads to impairments in social, familial, emotional, academic and behavioural functioning. Functional impairments in childhood, such as academic underachievement and poor peer relationships, are also predictors of poor outcomes in adulthood. In addition to the primary diagnostic criteria of inattention and impulsivity–hyperactivity, functional impairments in the home and school environment distinguish children and adolescents with ADHD from those without (Harrison et al., 2010). Catecholaminergic activity in the cortex and sub-cortex is central to current pathophysiological models of ADHD. The present results indicate that enhancing hippocampal proliferation could also contribute to the mechanism of action of drugs that effectively treat ADHD. ATX was first licensed to treat ADHD in children and adolescents in the US in 2002 (Savill et al., 2015). Data suggest that an adequate ATX dose for sufficient duration is important for ADHD symptom improvement (Clemow, 2014). It has been reported that there was significant improvement in pattern recognition memory and spatial recognition memory as measured by the CANTAB (Cambridge Neuropsychological Test Automated Battery), sustained attention and response inhibition as measured by the CPT (Continuous Performance Test) and reaction time as measured by the CANTAB after treatment with ATX for 4 or 12 weeks. In addition, ATX significantly enhanced school functioning in children with ADHD (Shang and Gau, 2012).

It is nowadays unambiguously accepted that the SGZ of the hippocampal DG and the subventricular zone of the lateral ventricles are two discrete regions of the mammalian brain that continue to give rise to new neurons throughout life (Bekiaris et

al., 2015). However, there is some controversy as to whether human neurogenesis persists in the hippocampus with age or not (Sorrells et al., 2018). Some workers report a huge decrease of adult hippocampal neurogenesis (Knoth et al., 2010; Dennis et al., 2016), while others are supportive of the persistence of neurogenesis with age (Spalding et al., 2013; Boldrini et al., 2018). Neural stem cells (NSCs), originating from the ventral germinative ventricular zone, migrate during development along the temporo-septal axis to form the hippocampal SGZ in its full extent (Liagkouras et al., 2008), and retain their ability for self-renewal (Bonaguidi et al., 2011). Newly-generated neurons in the DG are closely involved in learning and memory (Abrous et al., 2005). On the other hand, it has been well known that neurogenesis in the brain can be altered by various factors, such as pharmacological drugs, diet, exercise and aging (Van Praag et al., 1999; Meng et al., 2006; Beltz et al., 2007; Lee et al., 2010; Lloyd et al., 2010; Schiavon et al., 2010; Park and Lee, 2011).

Persisting proliferation in the adult hippocampus highly reflects the continuous interaction between brain structure and function. Correlation of the above-presented findings with the change in hippocampal proliferation could further contribute to our current understanding of the mechanism by which ATX affects memory. Besides, it was suggested that cognitive brain functions, such as learning and memory, involve increased proliferation in the hippocampus (Kempermann et al., 2004). Moreover, the hippocampal circuit is essential for episodic and contextual memory formation, storage, and retrieval. Many models have stressed the importance of the hippocampus subregions in distinguishing similar patterns

(pattern separation) and in completing partial patterns (pattern completion) (Bakker et al., 2008).

In our study, we showed that ATX improves memory of the adult rat brain through increasing levels of proliferation in the DG of the hippocampus (see Fig 5). Spatial memory was tested using the NLR test. Rats that received 5 intraperitoneal injections of ATX spent significantly more time exploring the novel object than did rats in vehicle groups that were given an identical volume of 0.9% sterile saline (i.v.); see Figs. 2, 3 and 4.

In agreement with our findings, Tzavara et al. (2006) have reported that ATX improved memory deficit in object recognition tests and the radial arm-maze test. They investigated the precognitive properties of ATX in two distinct behavioural assays, the 8-arm radial arm maze and the object recognition test, which are used to test memory in animals. Their results showed that ATX increased the time spent by rats interacting with the novel object in the object recognition test, and decreased errors in spatial pattern recognition in the radial arm maze, suggesting improved memory performance in both tests (Tzavara et al., 2006).

Furthermore, Sasaki et al. (2015) have demonstrated that NMDA (N-methyl-D-aspartate) receptor agonists have the potential to reverse down-regulated NMDA receptor signalling, cognitive abnormalities and decreased neurogenesis in hippocampal cKO mice. In the study of Lee et al. (2012), they compared the effect of Methylphenidate (MPH) and ATX on neurogenesis in the SGZ of the DG of the hippocampus.

They concluded that MPH, not ATX, could enhance cell proliferation and neuroblasts differentiation in the SGZ of the DG via increasing BDNF level. However, a previous study showed that there was no difference in the number of Bromodeoxyuridine (BrdU) positive cells in the SGZ between saline- and 2 mg/kg MPH-treated rats (Lagace et al., 2006). Another study showed that MPH has no effect on survival of new-born cells in gerbils (Schaeffers et al., 2009). Previous researches have shown that ATX increased BDNF levels in the hippocampus, whereas MPH increased BDNF gene expression in other areas of the brain, such as the nucleus accumbens and caudate-putamen. Moreover, opposite effects were seen in the prefrontal cortex, a critical region in attention disorders, where ATX increased, while MPH reduced, total and exon IV BDNF mRNA levels (Fumagalli et al., 2010).

Besides, studies have shown that increased NE and DA neurotransmission can increase neuronal BDNF expression within the hippocampus (Ivi et al., 2003; Ramos-Quiroga et al., 2014; Liu et al., 2015). It has been documented that Neurotrophins, a family of neurotrophic factors, are a kind of proteins that are specific to the nervous

system and play an essential role in neuron survival, differentiation and proliferation during the development of the central and peripheral nervous system, and also reductions in BDNF levels in the plasma of adult patients with ADHD have been reported (Fumagalli et al., 2010). Furthermore, it has been documented that BDNF increases proliferation of neuroprogenitor cells (Izumi and Zorumski, 1999; Spalding et al., 2013). Recently, it has been shown that BDNF levels were up regulated by ATX treatment (Banerjee et al., 2009). One of the possible mechanisms by which ATX increased hippocampal proliferation in our experiment could be the elevation of BDNF levels in the hippocampus, and this should be further assessed. To our knowledge, the study of Lee et al. (2012) is the only one that investigated the effect of ATX on hippocampal proliferation. Although there are some discrepancies between their study and our present findings, it is possible that variations in experimental design, such as the dose and route of administration, were the causes behind this (they gave ATX orally in a low dose over long period and we gave 5 high doses intraperitoneally for 5 days). In conclusion, ATX has shown the ability to improve memory and number of proliferating cells in the SGZ of the DG of the hippocampus, as measured by the number of proliferative marker Ki67 positive cells.

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