Agmatine protects against scopolamine-induced water maze performance impairment and hippocampal ERK and Akt inactivation

Maryam Moosavi*, Golnaz Yadollahi Khales, Leila Abbasi, Asadollah Zarifkar, Karim Rastegar
Shiraz Neuroscience Research Center and department of Physiology, Shiraz University of Medical Sciences, Zand Street, Shiraz, Iran

Abstract
Cholinergic brain activity plays a significant role in memory. Scopolamine a muscarinic cholinergic antagonist is known to induce impairment in Morris water maze performance, the task which is mainly dependent on the hippocampus. It is suggested that hippocampal ERK and Akt activation play roles in synaptic plasticity and some types of learning and memory. Agmatine, a polyamine derived from l-arginine decarboxylation, is recently shown to exert some neuroprotective effects. This study was aimed to investigate if agmatine could reverse scopolamine-induced memory impairment and possible hippocampal ERK and Akt activity alteration. Adult male Sprague-Dawley rats weighing 200–250 g were randomly assigned into 5 groups. The animals were trained for 3 days in Morris water maze and in day 4 their memory retention was assessed in probe trial which was consisted of a 60 s trial with no platform. Scopolamine (1 mg/kg/ip) or saline were injected 30 min and agmatine (20 or 40 mg/kg/ip) was administered 60 min before each session. The hippocampi were isolated after behavioral studies and western blotting studies on hippocampal lysates were done to determine the levels of activated ERK and Akt. Scopolamine treatment not only impaired water maze learning and memory, but also decreased the amount of phosphorylated (activated) ERK and Akt. Agmatine pre-treatment prevented both the learning impairment and hippocampal ERK and Akt inactivation induced by scopolamine. It seems that agmatine may act as a candidate substance against amnesia.

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1. Introduction

One of the most prominent characteristics of Alzheimer's disease (AD) is the neurodegeneration of basal forebrain cholinergic neurons (Bartus et al., 1982). Acetylcholine (Ach) concentration is decreased in cerebro-spinal fluid of dementic patients; its concentration relates inversely with the severity of dementia (Tohgi et al., 1996). Therefore one of the most current treatments of AD is administrating acetylcholine esterase inhibitors like tacrine and donepezil (Lahiri et al., 2004). In animal studies the muscarinic receptors antagonist, scopolamine, causes learning and memory impairment, in a way that scopolamine administration is considered as a pharmacologic model of dementia (Sarter and Bruno, 1997; Sunderland et al., 1986).

One of the molecular signaling pathways involved in different kinds of learning, like spatial learning, is extracellular regulated kinases 1/2 (ERK 1/2). Also for long-term-potentiation (LTP), as a neuronal plasticity model, ERK activation is necessary. ERK activation (phosphorylation) is secondary to cholinergic neurons activation during and immediately after acquisition. It is suggested that Ach release leads to ERK activation of the hippocampus. In LTP experiments ERK activation leads to learning-related proteins expression (Giovannini, 2006).

Another signaling molecule which recently suggested being involved in learning and memory is Akt/PKB. Akt is a serine/threonine kinase which is involved in many biologic responses. Its active form exists in the cytoplasm while to get activated it is recruited to lipid bilayer and phosphorylated (Alessi et al., 1996). Some experiments suggest that Akt is involved in synaptic plasticity and also in some types of learning and memory. For example PI3 kinase activation is shown to be necessary for hippocampal LTP induction (Horwood et al., 2006; Karpova et al., 2006; Raymond et al., 2002). PI3/Akt inhibition causes impairment in passive avoidance learning (Barros et al., 2001), spatial learning (Mizuno et al., 2003) and fear-related learning (Chen et al., 2005; Lin et al., 2001). There is no document exploring if scopolamine-induced amnesia is associated with hippocampal Akt activity alteration.

Agmatine is a polycationic amine synthesized through decarboxylation of l-arginine by arginine decarboxylase (ADC). For a long
it had been known to act as an intermediate in polyamine metabolism of various bacteria, plants and a range of invertebrates (Tabor and Tabor, 1984). Later it was discovered that agmatine, ADC and agmatinase exist in mammalian tissues (Li et al., 1994; Raasch et al., 1995). In mammalian brains agmatine is expressed in some regions like as hypothalamus, hippocampus, cortex, locus ceruleus, Raphe nucleus and forebrain (Halaris and Plietz, 2007). Because agmatine is synthesized in the brain, stored in synaptic vesicles, accumulated by uptake, released by depolarization and inactivated by agmatinase, it is considered to be a putative neurotransmitter (Uzbay, 2012).

Agmatine has been reported to have some neuroprotective effects against MPTP neurotoxicity (Gilad et al., 2005), spinal cord ischemia (Gilad and Gilad, 2000), restraint-induced structural changes in the brain (Zhu et al., 2008) and LPS-induced memory impairment and apoptosis (Zarifkar et al., 2010). Additionally it is known to exert antidepressant, anxiolytic, anti-tumor cell proliferative and anticonvulsive effects (Halaris and Plietz, 2007).

Considering the impairing effects of scopolamine on learning and memory and suggested neuroprotective effect of agmatine, this study was designed to investigate if agmatine can prevent scopolamine-induced water maze performance deficit and possible alterations in hippocampal ERK and Akt.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats weighing 200–250 g were used. The animals were maintained at room temperature (25 ± 2 °C) under standard 12–12 h light–dark cycle with lights on at 7:00 AM. Food and water were available ad libitum. The experimental protocols were approved by the ethics committee of Shiraz University of Medical Sciences and the animal care was according to the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Materials

Scopolamine and agmatine sulphate were purchased from Sigma, USA. Western blot antibodies (phospho-ERK, phospho-Akt, beta-actin and secondary HRP-conjugated) were purchased from Cell Signaling Technology Company, USA. ECL advanced reagent kit was purchased from Amersham Bioscience, UK. Protease and phosphatase inhibitor cocktail was purchased from Pierce. Other reagents were obtained from usual commercial sources.

2.3. Drug administration

Agmatine and scopolamine were administered intraperitoneally, 60 min and 30 min respectively, before each block of training. The animals were divided into groups of 8 and received saline (ip) as vehicle, scopolamine (1 mg/kg), a combination of scopolamine (1 mg/kg) and agmatine (20 or 40 mg/kg) and agmatine (40 mg/kg).

2.4. Behavioral testing

2.4.1. Morris water maze apparatus

The water maze has been described previously (Rastegar et al.; Zarifkar et al., 2010). Briefly it was a black circular pool with a diameter of 140 cm and a height of 70 cm, filled with 20 ± 1 °C water to a depth of 25 cm. The maze was divided geographically into four equal quadrants and release points that were designed at each quadrant as N, E, S, and W. A hidden circular platform (11 cm in diameter), was located in the center of the southwest quadrant, submerging 1.5 cm beneath the surface of the water. Fixed, extra maze visual cues were present at various locations around the maze (i.e. computer, a door, a window, bookshelves, posters). A CCD camera was mounted above the center of the maze so that the animal motion could be recorded and sent to the computer. The path of animal’s swimming was automatically recorded by a computerized system (Noldus EthoVision, 3.1 version) and then analyzed by computing several parameters, e.g. latency to find the platform, traveled distance as well as the swimming speed.

2.4.2. Procedure

The rats were trained in a protocol consisting of 4 days training session. During the first three days a hidden platform, submerged about 1.5 cm below water surface was put in the center of southwest quadrant. The platform position was fixed during those 3 days. A block session consisted of four trials with four different starting positions. Each rat was placed in the water facing the wall of the tank at one of the four designated starting points (north, east, south and west) and allowed to swim and find the hidden platform. During each trial, the rat was given 90 s to find the hidden platform. After mounting the platform, the animals were allowed to remain there for 20 s until the start of the next trial. After completion of training, the animal was dried by a towel and returned to its home cage. On day 4 the hidden platform was removed and the retention testing (probe trial) was performed. The probe trial consisted of a 60 s free swim period without a platform and the time spent in the target quadrant was recorded.

2.5. Tissue preparation

Immediately after the completion of behavioral tests, the animals were decapitated after anesthesia with CO2 inhalation. The hippocampi were quickly isolated on ice and were transferred to liquid nitrogen and then stored in −70 °C.

2.6. Western blot analysis

The hippocampi of the rats evaluated for behavioral studies were homogenized on ice in cold RIPA lysis buffer containing protease and phosphatase inhibitor cocktail (n = 5 randomly assigned in each group). The lysates were centrifuged at 14,000 × g for 30 min at 4 °C to remove debris. Samples with equal amounts of protein were then separated by 12% polyacrylamide gel electrophoresis, and transferred to PVDF membranes. After blocking in 5% ECL advanced blocking reagent kit, the membranes probed with primary antibodies (ERK, Akt and β-actin) overnight in 4 °C. After washing, the membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated anti-rabbit antibody. Blots were revealed by ECL advanced kit (Moosavi et al., 2011).

2.7. Data analysis

Data were analyzed by a one-way analysis of variance (ANOVA) in most cases and a two-way ANOVA when specified followed by Tukey’s test for multiple comparison. All results have been shown as means ± S.E.M. In all statistical comparisons, P < 0.05 considered as significant difference.

3. Results

Fig. 1 shows the results obtained from pre-training administration of saline, scopolamine or (and) agmatine administration on water maze spatial learning and memory. This figure shows that there is a negative linear correlation between escape latency and the training sessions in all groups. This means that all groups have learnt the platform location; however scopolamine administration has slowed down learning speed from the first learning session. Two-way ANOVA of the escape latency revealed significant difference between groups (p value <0.001, F(4,122) = 12.3). Post hoc analysis by Tukey’s test showed that scopolamine treatment significantly increased escape latency in all training days.

![Fig. 1](image-url)
Pre-administration of agmatine (40 mg/kg but not 20 mg/kg) prevented scopolamine-induced escape latency increment. Agmatine (40 mg/kg) administration by itself did not change escape latency in comparison to saline treated group.

Fig. 2 shows the results obtained from pre-training administration of saline, scopolamine or (and) agmatine administration on animal’s swimming speed during days 1–3 of training. Two-way ANOVA of the swimming speed showed significant differences between groups (p value < 0.001, F(4,122) = 6.9). Post hoc analysis by Tukey’s test showed that scopolamine treatment significantly increased the swimming speed in all training days. Pre-administration of agmatine (40 mg/kg but not 20 mg/kg) prevented scopolamine-induced swimming velocity increment. Agmatine (40 mg/kg) administration by itself did not change swimming speed in comparison to saline treated group.

The effect of pre-probe saline, scopolamine or (and) agmatine administration on the time percent spent in target zone during probe trial is depicted in Fig. 3. One-way ANOVA revealed significant difference between groups (p value = 0.0002, F(4,31) = 7.991). Post hoc by Tukey’s test showed that scopolamine treatment decreased the time percent which animal swam in target zone, while agmatine pre-treatment in dose 40 mg/kg reversed that memory impairment.

Western blot analysis showing effects of saline, scopolamine or (and) agmatine (40 mg/kg as the effective dose) administration on phosphorylated ERK protein in the hippocampi of rats is depicted in Fig. 4. Antibody against phosphorylated ERK, detected two bands at 42 and 44 kDa. One-way ANOVA showed significant difference between groups (p = 0.0006, F(3,16) = 10.053). Post hoc by Tukey’s test showed that Scopolamine treatment suppressed ERK activation while Agmatine pre-treatment reversed scopolamine-induced decrement of ERK activation.

Western blot results showing the amount of phosphorylate Akt in the hippocampus is depicted in Fig. 5. Antibody against phosphorylated Akt (threonine 308), detected a band at 60 kDa. One-way ANOVA showed significant difference between groups (p = 0.0014, F(3,16) = 8.339). Post hoc by Tukey’s test showed that scopolamine treatment suppressed Akt activation but Agmatine pre-treatment reversed scopolamine-induced decrement of Akt activation.

4. Discussion

The finding of the present study revealed that scopolamine treatment not only deteriorates Morris water maze learning and memory but also suppressed hippocampal ERK and Akt activation. Agmatine treatment prevented both scopolamine-induced memory impairment and the decrement of hippocampal ERK and Akt activation.

In this study scopolamine produced spatial learning and memory deficit even with a higher swimming speed. Scopolamine is reported to induce hyperactivity when is injected centrally or peripherally. The cholinergic signaling in the hippocampus, striatum and/or frontal cortex is shown to be correlated to scopolamine-induced hyperactivity (Klinkenberg and Blokland, 2020).
This increased thigmotaxis in the water maze sometimes is suggested to be an indicator of anxiety (Wolfer et al., 1998).

Our results confirm previous data implicating scopolamine pre-training administration impairs learning and memory. It is documented that muscarinic receptor blockade impairs spatial learning and memory, showing that Ach is necessary for learning and memory process (Deiana et al., 2011). Ach is synthesized by enzyme cholinacetyl transferase (ChAT) and accumulated in synaptic vesicles. Its quantal release is stimulated by Ca²⁺ influx. Ach binds to muscarinic Ach (mAch) receptors which are G-protein coupled receptors exist in the hippocampus, cerebral cortex and amygdala. It is reported that during learning or exploration, the amount of Ach released to the hippocampus is increased (Mitsushima, 2011). The hippocampus plays an essential role in spatial learning and memory. It is suggested that cholinergic forebrain neurons are the first population neurons which are damaged in AD (Whitehouse et al., 1982). Recent evidence implies that beta amyloid impairs brain cholinergic system because it induces LTP in CA1 region of the hippocampus (Opazo et al., 2003) and also is involved in amygdale fear conditioning (Lin et al., 2001). Recently it is reported that acetylcholiestrase inhibitors, the drugs which increase cholinergic tone, increase the phosphorylation of hippocampal Akt (Autio et al., 2011). Similarly in human coroanry artery endothelial cells, it is suggested that muscarinic receptors stimulation promote activation of Akt (Smedlund et al., 2011). It is plausible that in the hippocampus muscarinic receptors stimulation causes Akt activation as scopolamine treatment increased Akt phosphorylation. Then this event might be considered as one of the other factors involved in scopolamine-induced learning and memory impairment.

This study showed that agmatine pre-treatment in dose 40 mg/kg both reversed hippocampal function deterioration by scopolamine, and ERK and Akt activity changes in the hippocampus. The administration of the same dose of agmatine 1 h before training did not affect water maze performance by itself, suggesting that the improvement described, is not the direct effect of agmatine on memory. Although some studies indicate that agmatine could improve memory performance (Liu and Collie, 2009), there are some other studies showing that exogenously administered agmatine does not affect water maze place learning (Rastegar et al.; Zarifkar et al., 2010). These differences might be related to different routes of administration or the time delay after injection. Since agmatine can cross blood-brain-barrier (Piletz et al., 2003) and the hippocampus is the critical structure involved in spatial learning and memory, it is speculated that agmatine protects the hippocampus from scopolamine-induced hippocampal function deterioration. How might agmatine improve memory in the scopolamine-treated rats? If agmatine is considered as a neurotransmitter, does scopolamine decrease hippocampal agmatine concentration? It does not seem to happen as recently Scopolamine treatment is shown not to affect agmatine level in the hippocampus (Knox et al., 2011). One of the putative mechanisms which are suggested to be involved in scopolamine-induced learning and memory impairment is the increased levels of glutamate or NMDA receptor activity (Barber and Haggarty, 2010). Although glutamate and NMDA receptors are necessary to induce learning and memory it is shown that too little or too much activity of the glutamate system leads to memory impairment (Parsons et al., 2007). Agmatine which carries the guanidine group blocks heterometric NMDA receptor channels (Askalany et al., 2005; Wang et al., 2006; Yang and Reis, 1999). The selective antagonism of NMDA receptors by memantine has been reported to prevent scopolamine-induced amnesia in chicks trained on taste-avoidance learning (Barber and Haggarty, 2010) and scopolamine-induced water maze memory impairment.
deterioration in mice (Drever et al., 2007). Then the antagonism of NMDA receptors by agmatine may act as one of the possible mechanisms which agmatine prevents scopolamine-induced learning and memory impairment. Additionally there are some documents showing that scopolamine reduces sustained attention (Hodges et al., 2009; Thienel et al., 2009). If scopolamine increases glutamate concentration, this excess glutamate can induce stress and loss of attention because when stress and anxiety increases attention decreases (Tsushima and Watanabe, 2009). Also stress itself results in excess glutamate at the synapse (Wang and Wang, 2009) which can impair memory. If we consider animals’ hyperactivity as a result of anxiogenic effect of scopolamine (Woller et al., 1998) then there is another possibility that agmatine could improve memory in scopolamine-treated rats by increasing attention as agmatine treatment prevented scopolamine-induced hyperactivity. Consistent with this view there are some studies indicating that agmatine has some anxiolytic and antidepressant effect (Halaris and Plietz, 2007). Agmatine has nitric oxide synthase (NOS) inhibitory property besides NMDA inhibition. In addition, NOS inhibitory effects result in NMDA antagonism in CNS (Tayfun Uzbay and Oglesby, 2001). Another possibility involved in neuroprotective action mechanism of agmatine in scopolamine-induced anxiety could be its direct NOS inhibitory effects or an interaction with NO-NMDA pathway.

Agmatine is also considered as one of the endogenous ligands for imidazoline receptors (Wu et al., 2008). It is shown that agmatine prevents morphine-induced memory impairment via imidazoline receptors (Lu et al., 2010) which may indicate imidazoline receptors are involved in agmatine’s protective effect. Also there are some documents indicating that imidazoline receptors stimulation lead to anxiety and stress suppression (Smith et al., 2009; Taksande et al., 2010); then there is another possibility that agmatine prevents scopolamine-induced anxiety through imidazoline receptor’s activation.

This study showed that agmatine prevented hippocampal ERK and Akt activity decline besides memory impairment by scopolamine. As mentioned before, Akt and ERK are suggested to be involved in learning and memory processes. Two possibilities might be considered in this regard: The first view implies that agmatine may affect scopolamine-induced memory impairment via inhibiting its direct effect on ERK and Akt. There are some documents supporting this claim for example it is reported that imidazoline agonists can activate ERK signaling for example in CHO cells (Li et al., 2006) and PC12 cells (Edwards et al., 2001). It is also reported that imidazoline agonists act through Akt activation in heart and aorta (Santhanam et al., 2007; Yamanaka et al., 2010). According to this view agmatine increases ERK and Akt level at least via imidazoline receptors and thereby increases learning ability in scopolamine-treated group. Also some immunohistochemical studies have revealed that ERK activation can induces muscarinic receptor expression and activation in CA1 pyramidal neurons of the hippocampus (Berkeley et al., 2001) meaning that even ERK activation by agmatine may increase muscarinic receptor activation which counteracts scopolamine blocking effect on these receptors. One of the problems about this view is that agmatine by itself induce no significant difference in hippocampal ERK and Akt levels comparing to control group which may guide us to the second view which implies that the effect of agmatine on ERK and Akt is indirect rather than direct, meaning that agmatine reverses scopolamine-induced impairment and because those signaling molecules (p-ERK and p-Akt) are involved in LTP and some types of learning and memory processes, agmatine indirectly affects them. If we consider this idea, the activation of hippocampal ERK and Akt in the animals with good water maze learning and not in scopolamine group, might support the hypothesis implying that these two signaling molecules are involved in water maze learning and memory. Definitely more research work will help to elucidate agmatine effect mechanism.

Compatible with our results agmatine is reported to protect against LPS-induced neuroinflammation (Zariakar et al., 2010). Neuroinflammation is implicated in several neurodegenerative diseases, like Alzheimer’s disease and HIV dementia. It might contribute to the learning and memory deficits associated with these disorders. During the early stages of Alzheimer’s disease, the greatest degree of neuroinflammation is found within temporal lobe regions involved in learning and memory (Cagnin et al., 2001). It has also been shown that systemic administration of lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, produces neuroinflammation, hippocampal apoptosis, cognitive impairment, learning deficits and even beta amyloid plaques generation in the hippocampus (Lee et al., 2008). Some studies have shown that beta amyloid-induced cell death is accompanied by PI3/Akt or ERK suppression (Townsend et al., 2007; Yin et al., 2005; Zeng et al., 2010). It will remain to be elucidated if agmatine protects against neuroinflammation-induced memory deficits through PI3/Akt or ERK.

In conclusion our study showed for the first time, that agmatine can reverse the memory impairment and the hippocampal ERK and Akt changes resulted from scopolamine treatment. As cholinergic neuronal loss is associated with memory deficits, agmatine seems to act as a candidate substance against amnesia. More investigations are necessary to know agmatine’s effect and its cellular and molecular mechanisms.

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References
