Agmatine enhances neurogenesis by increasing ERK1/2 expression, and suppresses astrogenesis by decreasing BMP 2,4 and SMAD 1,5,8 expression in subventricular zone neural stem cells

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Abstract

Aim: Our study aimed to demonstrate whether agmatine (Ag) could regulate proliferation and cell fate determination of subventricular zone neural stem cells (SVZ NSCs).

Main methods: SVZ NSCs were grown in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (20 ng/ml) until 4 days in vitro (DIV) and later the culture medium was replaced without EGF and bFGF until 11 DIV in the absence (EGF/bFGF+/+/Ag−) or presence of agmatine (EGF/bFGF+/+/Ag+). Another set SVZ NSCs were maintained with EGF and bFGF until 11 DIV without (EGF/bFGF+/+/Ag−) or with agmatine treatment (EGF/bFGF+/+/Ag+). Agmatine’s effect on proliferation and cell death (H and PI staining and Caspase-3 immunostaining) was examined at DIV 4 and 11. Agmatine’s (100 μM) effect on cell fate determination was confirmed by immunostaining and Western blot at 11 DIV.

Key findings: Agmatine treatment reduced the neurosphere size and total cell count number dose-dependently in all the experimental groups both at DIV 4 and 11. Immunoblotting and staining results showed that agmatine increased the Tuj1 and Microtubule-associated protein 2 (MAP2) and decreased the Glial fibrillary acidic protein (GFAP) with no change in the Oligo2 protein expressions. This neurogenesis effect of agmatine seems to have a relation with Extracellular-signal-regulated kinases (ERK1/2) activation and anti-astrogenesis effect is thought to be related with the suppression of Bone morphogenetic proteins (BMP) 2,4 and contraction of Smo and Mad (SMAD) 1,5,8 protein expression.

Significance: This model could be an invaluable tool to study whether agmatine treated SVZ NSC transplantation to the central nervous system (CNS) injury could trigger neurogenesis and decrypt the full range of molecular events involved during neurogenesis in vivo as evidenced in vitro.

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Introduction

The discovery of neural stem cells (NSCs) and their contribution to continuous neurogenesis remains as an elusive goal of cell therapy. It was not until the 1990s, with the isolation of multi-potential NSCs from adult brain that Reynolds and Weiss (1992) and Reis and Regunathan (2000) demonstrate that the dividing cells in the subventricular zone (SVZ) could migrate and become neurons (Kazanis et al., 2008; Lois and Alvarez-Buylla, 1993; Rousselot et al., 1995). Differential regulation of neurogenesis in adult mammalian brain has been described in the dentate gyrus of the hippocampus (Altman and Das, 1965; Bayer et al., 1982; Epp et al., 2007) and subventricular zone (SVZ) (Bayer et al., 1982; Kaplan and Hinds, 1977). Adult neural progenitor cells in the SVZ express epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) receptors (Gritti et al., 1999) and can respond to EGF and bFGF signals with increased proliferation and neuronal differentiation in vitro (Reynolds and Weiss, 1992; Vescovi et al., 1993). These studies suggest that EGF and bFGF can serve as the extra positive cellular signals that enhance the regenerative capacities of NSCs. Some of the cells that survived in that culture condition could expand and undergo many passages maintaining an undifferentiated status (self-renew). In the absence of growth factors or in the presence of inducing factors, the NSCs differentiate to neurons, astrocytes, or oligodendrocytes (Liu et al., 2010; Palmer et al., 1995). More recently, a greater understanding of the functional significance of adult neurogenesis has gained much importance.

Agmatine, an endogenous primary amine, was reported to function as a vasodilator (Reis and Regunathan, 1999), a neurotransmitter in the brain (Reis and Regunathan, 2000), a modulator of several excitatory synapses and also as a potent protector from ischemic injury (Kim et al.,
2004). Agmatine has been shown to exert anti-proliferative effects in the brain and the periphery (Li et al., 1995; Love, 1999; Morrissey et al., 1995), and was reported to induce an anti-proliferative effect in various cancer cell lines (Hahn et al., 2001; Ishizuka et al., 2000), in Ras/3T3 and Src/3T3 cell lines (Isome et al., 2007). However, recently it was reported that agmatine increases proliferation and neurogenesis in hippocampal progenitor cells in chronically stressed mice (Li et al., 2006). Strong proliferation capacity is a peculiar behavior that is common in both cancer and stem cells (Asano et al., 2006) and it would be interesting to see the agmatine’s regulatory effect on proliferation and differentiation of these rapidly proliferating cells. This present study investigated the effects of agmatine on proliferation both at DIV 4 and 11. The agmatine’s effect on cell fate determination was confirmed by checking the MAP2, GFAP and Oligo2 expressions for confirming neurogenesis, astrogenesis and oligogenesis in SVZ NSCs maintained with EGF/bFGF+/− and EGF/ bFGF+−/− growth supplements until 11 DIV. Moreover, experiments were performed to elucidate the molecular signaling induced by agmatine treatment for the neural differentiation of SVZ NSCs at DIV 11.

**Experimental procedures**

**Animals**

Pregnant mice (Institute of Cancer Research strain) were obtained from Coatech in Seoul, South Korea, and housed under constant conditions of light, temperature, and humidity. All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care and Use Committee in accordance with the National Institutes of Health (NIH) guidelines.

**Drugs and reagents**

Agmatine, tetramethyl rhodamine iso-thiocyanate (TRITC), 4',6-diamidino-2-phenylindole (DAPI), goat serum, and Triton X-100 were purchased from Sigma (St. Louis, MO); Dulbecco’s Modified Eagle Medium/F-12 media from HyClone Laboratories (South Logan, UA); epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) from Invitrogen (Carlsbad, CA); rabbit normal serum, rabbit antiseraum, and avidin–biotin-peroxidase complex were bought from Vector bio–tech (Burlingame, CA). Cell cycle determination by using flow cytometry analysis (FACS) was done by counting 100 μM of cytoplasm treated until 4 and 11 DIV. Cells were grown as neurospheres in tissue culture flasks in the presence of EGF and bFGF (20 ng/ml) growth supplements until 4 DIV and later the culture medium was replaced freshly without adding EGF and bFGF in the absence of agmatine (group 1) or in presence of agmatine (group 2) until 11 DIV. In groups 3 and 4 SVZ NSCs were maintained with EGF and bFGF until 11 DIV in the absence of agmatine (group 3) or in the presence of agmatine (group 4). Among the experimental groups, results were compared between groups 1 and 2, and groups 3 and 4. Agmatine’s effect on cell proliferation and cell death (Hoechst/PI staining) in SVZ NSCs was determined by counting the total number of cells, measuring the neurosphere diameter and determining the PI positive cells (dead cells) with different concentrations of agmatine (0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM and 200 μM) treatment until 4 and 11 DIV. Caspase-3 immunostaining and the Western blots of all the considered proteins were performed with 100 μM of cytoplasm treated until 11 DIV in SVZ NSCs (Fig. 1).

**SVZ NSC primary culture**

New born ICR mice (P0–P2) were sacrificed for SVZ NSC primary culture according to the method described by Gritt et al. (1996). Briefly, a brain slice extending from the crossing of anterior commissure to the rostral opening of the third ventricle (approximately 2 mm thick) was removed. The SVZ, which lies next to the lateral ventricles, was dissected out from the brain slice using fine forceps under a dissecting microscope (Olympus, Japan) at 30× magnification. The resulting tissue had no striatal contamination and retained ependymal lining overlying tissue of interest. Dissociated cells were grown in the DME/F-12 medium at a concentration 3.0 × 10⁴ cells/ml with EGF, bFGF (20 ng/ml) and B27 (2%) supplement until 11 days in vitro (DIV). Culture conditions of each experimental group were described below.

**Culture conditions and experimental design**

SVZ NSCs cultured with EGF and bFGF supplements were harvested and analyzed to determine the agmatine’s regulatory effect on the early and late stage of proliferation i.e. at 4 and 11 days in vitro (DIV).

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Schematic representation of the experiment

**Western blot analysis**

Western blots were performed to confirm the various effects and elucidate the molecular mechanism of agmatine on the differentiation of SVZ NSCs. Briefly, SVZ NSCs cultured until 11 DIV from each group were washed with ice-cold PBS and were collected by scraping the neurospheres from the plate for analysis because most of the agmatine treated spheres from the plate for analysis because most of the agmatine treated

SVZ NSCs. Brieﬂy, SVZ NSCs cultured until 11 DIV were washed three times with PBS for immunostaining and were then blocked for 20 min. Equal amounts of protein (20 μg) from the resulting supernatants were separated on a 10% acrylamide gel and proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline with Tween-20 (TBST) (20 mM Tris·HCl pH 7.5, 50 mM sodium chloride, and 0.05% Tween-20) containing 5% nonfat dry milk for 2 h and then incubated with primary antibodies at 4 °C overnight. Primary antibodies used were: β-actin, active-ERK1/2 (mouse anti-rabbit, 1:2000), active-SAPK/JNK antibodies at 4 °C overnight. Primary antibodies used were: β-actin, active-ERK1/2 (mouse anti-rabbit, 1:2000), active-SAPK/JNK, whereas anti-MAPK antibodies recognize all forms of ERK1/2, p38, and SAPK/JNK. For MAPK Western blots, results were expressed as a ratio of arbitrary density time area units and then normalized to vehicle-treated controls (β-actin) run in parallel. Western blot was performed for detecting the total ERK1/2 protein expression and dual phosphorylated ERK1/2 (Thr202/Tyr204 of ERK1, 44 kDa protein; and Thr185/Tyr187 of ERK2, 42 kDa protein) protein expression by two different antibodies which recognize the respective protein bands. The protein bands representing the total ERK1/2 expression showed a similar pattern of expression. The results of Western blotting for all three Mitogen-activated protein kinase (MAPKs). The anti-active-MAPK antibodies recognize phosphorylated forms of ERK1/2, p38, and SAPK/JNK, whereas anti-MAPK antibodies recognize all forms of ERK1/2, p38, and SAPK/JNK. For MAPK Western blots, results were expressed as a ratio of arbitrary density time area units between anti-active-MAPK and anti-MAPK blots and then normalized to vehicle-treated controls (β-actin) run in parallel. Western blot was performed for detecting the total ERK1/2 protein expression and dual phosphorylated ERK1/2 (Thr202/Tyr204 of ERK1, 44 kDa protein; and Thr185/Tyr187 of ERK2, 42 kDa protein) protein expression by two different antibodies which recognize the respective protein bands. The protein bands representing the total ERK1/2 expression (both active and non active) and the dual phosphorylated ERK1/2 (Thr202/Tyr204); and ERK2 (Thr185/Tyr187) were migrated separately showing the band size of 42 (non-active ERK) and 44 (active ERK) kDa. The densitometry results for the total ERK1/2 and dual phospho ERK1/2 expression showed a similar pattern of expression. The results of phospho active ERK 1/2 were calculated by taking the percentage of total ERK1/2 and Phospho ERK1/2 protein expression. For all Western blots, proteins were extracted from three independent primary cultures and the obtained data are expressed as mean ± s.e.m.

**Immunocytochemical staining of SVZ NSCs**

To determine the agmatine's effect on SVZ NSCs, immunocytochemistry was performed. SVZ NSCs at 11 DIV were washed three times with PBS for immunostaining and were then blocked for 20 min.
in PBS containing 10% FCS, 5% normal goat serum, and 0.1% Triton X-100. After blocking, SVZ NSCs were incubated with primary antibodies prepared in PBS containing 10% FCS and 0.1% Triton X-100, overnight at 4°C. Primary antibodies used were: anti-MAP2 (mouse monoclonal, 1:300), anti-GFAP (rabbit monoclonal, 1:300), anti-Oligo2 (goat polyclonal, 1:200), anti-ERK1/2 (rabbit monoclonal, 1:200) and anti-Caspase-3 (rabbit polyclonal, 1:200). After incubating the SVZ NSCs with primary antibodies, they were washed three times with PBS for 5 min each and were then incubated with secondary antibodies which were individually prepared in PBS containing 10% FCS and 0.1% Triton X-100 for 1 h each separately at room temperature. The secondary antibodies used were: rhodamine rabbit anti-mouse IgG (1:300), fluorescein isothiocyanate mouse anti-rabbit IgG (1:600), or CY5 mouse anti-goat IgG (1:300). NSCs were counterstained with DAPI for 10 min at room temperature. Immunostained SVZ NSCs were visualized using Olympus D-70 upright fluorescent microscope (Melville, NY), and confocal microscope (Leica DMLB upright microscope, Bannockburn, IL). Images from fluorescence and confocal microscope were captured with a Spot RT color Charge Coupled Device (CCD) camera (Diagnostic Instruments) and confocal 1024 ES software (BioRad, Hercules, CA), respectively. Images were generated at 200× magnification visual fields.

Data analysis

Statistical comparisons were performed by independent t-test for two groups or analysis of variance and multiple comparisons were done with Newman–Keuls post hoc tests. Values are expressed as

Fig. 2. Effect of agmatine on the mean diameter and total cell count number in the subventricular zone neural stem cells (SVZ NSCs) cultured until 4 days in vitro (DIV). (A) SVZ NSCs were seeded in 24-well plates (3.0×10⁴ cells/ml) and cultured in medium containing EGF/bFGF+/+ growth supplements with different concentrations of agmatine (μM): (a) 0, (b) 0.1, (c) 1.0, (d) 10, (e) 100, and (f) 200 until 4 DIV. (B) Graph representing the mean diameter of the neurospheres at all concentrations of agmatine treatment. (×100). (C) Graph representing the total cell count number in all the experimental groups. *represents \( p < 0.05 \). Values were presented as mean±sem of six independent experiments.
mean ± s.e.m of 3–6 experiments. Differences were considered significant at \( p < 0.01 \), and 0.05.

**Results**

**Agmatine treatment decreases the proliferation of subventricular zone neural stem cells (SVZ NSCs)**

Increase in agmatine concentrations resulted in significant decrease \( (p < 0.05) \) in the total number of cells and the mean diameter of neurospheres with the same start of cell seeding number \((3.0 \times 10^4 \text{ cells/ml})\) of the SVZ NSCs both at DIV 4 and 11.

The average diameter of SVZ NSCs was measured about 50–85 \( \mu \text{m} \) at DIV 4. At DIV 11 with EGF/bFGF+/− and EGF/bFGF+/+, growth supplements the diameter of the neurospheres were measured around 95–115 \( \mu \text{m} \) and 150–170 \( \mu \text{m} \) respectively. A dose dependent decrease of the average mean diameter of the neurospheres was observed with agmatine treatment both at DIV 4 and 11 but the values were significant from 100 \( \mu \text{M} \) concentration at DIV 4 (Fig. 2A and B), 1 \( \mu \text{M} \) (EGF/bFGF+/−, Fig. 4A and B) and 1 \( \mu \text{M} \) (EGF/bFGF+/+, Fig. 5A and B) concentrations at DIV 11 compared with the control group.

Total cell count using tryphan blue staining showed that agmatine treatment decreased the total number of cells at all the concentrations dose dependently and the decrease was found to be statistically significant from 10 \( \mu \text{M} \) at DIV 4 (Fig. 2C). But, at DIV 11 the values were significant from 0.1 \( \mu \text{M} \) (EGF/bFGF+/−, Fig. 4C) and 1 \( \mu \text{M} \) (EGF/bFGF+/+, Fig. 5C) concentrations compared with the control group.

**Agmatine treatment shortens S phase duration and traps more NSCs in Go–G1 phase**

To determine whether the growth inhibition was attributable to cell-cycle arrest and/or apoptosis, flow cytometric analysis was performed in 4 and 11 days agmatine treated SVZ NSCs. 20,000 cells were recorded and results were presented as percentage of cells in each phase both at 4 DIV (Fig. 3) and 11 DIV (Suppl. Fig. 2). Results strongly suggest that more cells were trapped in Go–G1 phase when agmatine was added in the medium. Percentages of cells in the Go–G1 phase were 88.53% (Fig. 3A), 90.80% (Fig. 3B), 66.15% (Fig. 3C), and 83.83% (Fig. 3D) respectively at DIV 4 and 79.165% (Suppl. Fig. 2A), 84.74% (Suppl. Fig. 2B), 81.78% (Suppl. Fig. 2C), 87.15% (Suppl. Fig. 2D) respectively at DIV 11. Results for 4 (Fig. 3) and 11 DIV (Suppl. Fig. 2) samples almost showed the same pattern of cell cycle arrest suggesting that when agmatine was added in the media, (Fig. 3B, D and Suppl. Fig. 2B, D) number of cells in S phase decreased indicating that agmatine traps NSCs in Go–G1 phase both at 4 and 11 DIV.

**Agmatine treatment to SVZ NSCs showed no cell death/apoptosis**

To investigate whether agmatine induced inhibition of cell proliferation was due to apoptosis, the immunostaining of the apoptosis-related protein Caspase-3 expression and Hoechst/Propidium Iodide (PI) staining was done. The antiproliferative effect of agmatine was not paralleled by a measurable increase in Caspase-3/PI positive cells (dead cells) in SVZ NSCs depicting that agmatine treatment to SVZ NSCs does not induce apoptosis until 11 DIV (Fig. 4 and Suppl. Fig. 1).

![Fig. 3. Agmatine treatment shortens S phase duration and traps more NSCs in Go–G1 phase. SVZ NSCs were cultured in four different medium conditions until 4 DIV and then analyzed by FACS. 20,000 cells were counted for FACS analysis. Results strongly suggest that more cells are trapped in Go–G1 phase when agmatine was added in the media. (A) SVZ NSCs cultured without EGF/bFGF growth supplements and agmatine treatment. (B) SVZ NSCs cultured without EGF/bFGF growth supplements with agmatine treatment. (C) SVZ NSCs cultured with EGF/bFGF growth supplements without agmatine treatment. (D) SVZ NSCs cultured with EGF/bFGF growth supplements with agmatine treatment.](image-url)
Agmatine treatment enhances neurogenesis and suppresses astrogenesis

Immunostaining results showed that agmatine treatment (groups B and D) increased the MAP2 immunopositive cells (neuronal marker, red) and decreased the GFAP positive cells (astrocyte marker, green) at 11 DIV. However, the number of Oligo2 positive cells (oligodendrocyte marker, blue) was barely seen during merging in all the experimental groups (groups A, B, C and D). We furthermore confirmed the expression of all the cell fate markers by Western blot. The Western blot results showed that agmatine treatment (groups 2 and 4) significantly increased Tuj1 and MAP2 protein expressions indicating the neurogenesis effect of agmatine. But, agmatine treatment significantly decreased the expression of GFAP depicting the role of agmatine in suppressing astrogenesis. However, no change in oligodendrocytes (Oligo2) expression was seen between agmatine treated group and control group (Fig. 7).

Agmatine treatment regulates ERK1/2 activation

Among the three MAPKs (ERK1/2, SAPK/JNK, and p38), agmatine treatment seems to have modulation in ERK1/2 pathway (Fig. 8). Activation of ERK1/2 pathway is known to be related with enhancement of neurogenesis of SVZ NSCs and agmatine treatment triggered the ERK1/2 activation depending on the culture conditions. Western results showed that agmatine treatment increased \( p<0.01 \) the ERK1/2 protein expression and the immunostaining results also showed the same pattern at 11 DIV compared with the control group (Fig. 8A and B). The obtained results demonstrate that agmatine activated neurogenesis via ERK1/2 activation and contributed for the early differentiation of SVZ NSCs.

Agmatine treatment suppresses astrogenesis by decreasing BMP2,4 and SMAD1,5,8 protein expressions

BMP2 and BMP4 are well known astrogenesis inducing substances. Agmatine treatment suppressed the expression of BMP2 \( p<0.01 \), BMP4 \( p<0.01 \), and SMAD1,5,8 \( p<0.05 \) in SVZ NSCs at 11 DIV. However, SMAD2,3 protein expression levels did not show significant difference when agmatine was added in the media. The obtained results depict that agmatine treatment may suppress astrogenesis by down-regulating BMP 2,4, and SMAD 1,5,8 protein expressions (Fig. 9).

Discussion

Neural stem cells (NSCs) have the potential of self-renewal and differentiate into neurons, astrocytes and oligodendrocytes under certain conditions. Our present study aimed at the ability of agmatine's
regulatory effect on proliferation and cell fate determination of subventricular zone neural stem cells (SVZ NSCs) under controlled EGF and bFGF growth factor supplements.

The effect of agmatine on proliferation of SVZ NSCs was confirmed by counting the total number of cells, measuring the mean diameter and FACS analysis. The proliferation assay results showed that agmatine treatment significantly decreased the total cell count number and the average diameter of SVZ NSCs dose dependently (from lower concentration to higher concentration) in early and late phase of proliferation i.e. both at DIV 4 and DIV 11 (Figs. 2, 4 and 5). The FACS results showed that most of the agmatine treated SVZ NSCs were trapped in the G0/G1 phase and less number of cells in the S phase showing agmatine’s role in blocking the proliferation (Fig. 3 and Suppl. Fig. 2). Agmatine has been reported to inhibit tumor cell proliferation (Ishizuka et al., 2000) and few researchers have already reported that the inhibition of cell proliferation by agmatine treatment is associated with reduction in the intracellular polyamines levels in different cell lines (Babal et al., 2001; Dudkowska et al., 2003; Satriano et al., 1998; Vargiu et al., 1999). In contrast to the effects of agmatine on cell lines, Li et al. (2006) reported that lower doses of agmatine treatment (0.1, 1, and 10 μmol/l) from 7 days to 10 days after the culture i.e. for 3 days increased the proliferation of cultured hippocampal cells in vitro. Our other recent studies showed that agmatine treatment increased the proliferation in neural stem cells (cortical derived) transfected with retrovirus carrying Arginine decarboxylase (ADC) gene which can synthesize agmatine endogenously under different culture conditions (Bokara et al., 2010). However, agmatine treatment from the day of culture until 11 DIV decreased the proliferation of SVZ NSCs in our present study. Increasing evidence suggests that SVZ is not a homogenous region, but rather the precursors in different areas of the SVZ give rise to different types of neurons (Young et al., 2007) mostly “astrocyte like cells” function as neural stem cells (Garcia et al., 2004). A number of factors have been implicated in the control of proliferation in SVZ NSCs by acting on GFAP expressing cells (Palma et al., 2005) such as Galectin-1, (Sakaguchi et al., 2006), beta-catenin (Adachi et al., 2007), p27KIP (Li et al., 2009), and agmatine seems to be one of the factors which could regulate proliferation of SVZ NSCs. Our current study showed that agmatine treatment to SVZ NSCs blocked the proliferation until 11 DIV under controlled conditions of EGF/bFGF treatment. From the results it was assumed that agmatine seems to regulate proliferation depending on the duration of treatment and cell types (subventricular zone and hippocampal/cortical derived neural stem cells) and these differential effects of agmatine on proliferation could not be compared in this present study and further studies have to be carried out to know how agmatine regulates the proliferation differently in specific types of cells depending on the source of origin.

Previously, it was reported that agmatine requires cellular import for antiproliferative effects and is a multifunctional molecule with both receptor dependent and independent functions (Ishizuka et al., 2007) and the inhibitory effect of agmatine on proliferation (Cattaneo and McKay, 1990) led us to investigate the mechanism by which agmatine stimulates the differentiation process and tends to drive SVZ NSCs for early differentiation. The Caspase-3 immunostaining and Hoechst/PI staining results showed less/no Caspase-3 immunopositive cells and PI positive cells respectively (representing dead cells) in all the samples i.e. both in agmatine treated and non-treated samples cultured until 4
and 11 DIV (Fig. 6 and Suppl. Fig. 1). The results from the cell death analysis suggest that suppression of proliferation (Figs. 2, 4 and 5) by agmatine treatment is not due to apoptosis but because of change in cell cycle (Fig. 3 and Suppl. Fig. 2) leading to inhibition of proliferation in SVZ NSCs. Our results are well corroborated with the earlier findings suggesting that agmatine treatment to SVZ NSCs showed no evidence of cell death and markedly shifted the cell population into G0–G1 phase (Eto et al., 2006; Haenisch et al., 2011) and promoted for early differentiation of SVZ NSCs in this present study (Figs. 7, 8 and 9).

It has been reported that BMP signaling is well known for its function to induce astrocyte differentiation in embryos (Wilson and Edlund, 2001) and embryonic stem cells (ESCs) (Tropepe et al., 2001; Ying et al., 2003a) are reported to be involved in inducing the lineage fates of mesoderm, endoderm, and trophoblast (Li et al., 2001; Nakayama et al., 2000; Pera et al., 2004; Xu et al., 2002; Ying et al., 2003b). Signaling by bFGF through ERK1/2 and p38 regulations favors neural differentiation and inhibits non-neural differentiation (Xu et al., 2005). The ERK1/2 and p38 which are subjected to regulation either by LIF or bFGF signaling pathways play critical roles in the induction of ESC differentiation (Burdon et al., 2002). Moreover, bFGF signaling through activation of MEK/ERK has been shown to induce mouse ESCs to neural differentiation (Ying et al., 2003b). In this study we intended to investigate the agmatine’s regulatory effect on cell fate determination and define the underlying mechanism involved in the process of SVZ NSC differentiation. Western blot and immunostaining results showed that agmatine treatment triggered neurogenesis by up-regulating the Tuj1 and MAP2 expressions through ERK1/2 protein activation (Figs. 6 and 7). Moreover, agmatine treatment down-regulated GFAP, BMP2, BMP4 and SMAD 1,5,8 protein expressions (Figs. 6 and 8) in SVZ NSCs showing its role in suppressing astrogenesis. BMP signaling represses the activation of ERK and p38 expressions thereby inhibiting neural differentiation (Paling et al., 2004). SMAD

Fig. 6. Immunostaining for detection of active Caspase-3 activity in SVZ NSCs. Immunostaining with active Caspase-3 antibody was performed in all the experimental groups at DIV 11. Results showed less/no active Caspase-3 immunopositive cells in agmatine treated/non treated samples. (A) SVZ NSCs cultured with EGF/bFGF+/− growth supplements with no agmatine treatment until 11 DIV. (B) SVZ NSCs cultured with EGF/bFGF+/- growth supplements with agmatine treatment until 11 DIV. (C) SVZ NSCs cultured with EGF/bFGF+/- growth supplements with no agmatine treatment until 11 DIV. (D) SVZ NSCs cultured with EGF/bFGF+/- growth supplements and treated with agmatine until 11 DIV. Scale bars 200 μm.
pathway activation and MAPK pathway inhibition were also involved in BMP-mediated maintenance of stem cells (Qi et al., 2004). Recently it was demonstrated that bFGF signaling through the MEK/ERK pathway can inhibit SMAD activation through phosphorylation of the link region of SMAD1 (Aubin et al., 2004) thus suppressing astrogenesis. Reciprocally, ERK and p38 activity can be inhibited by the BMP-Transforming growth factor-β-activated kinase 1 (TAK1) cascade (Goswami et al., 2001). BMP2 and BMP4 are known for inducing astrocyte formation (Gross et al., 1996). Earlier findings indicate that balanced inhibition of stem cell lineage is essential for stem cell differentiation (Zhang and Li, 2005). In our current investigation Western blot results confirmed that agmatine reduces BMP2, BMP4, SMAD 1,5,8 and SMAD 2/3 protein expressions except in the SVZ NSCs treated with agmatine cultured with EGF/bFGF+/−/− growth supplements (EGF/bFGF+/−/−/Ag+ group-2). It was reported that SMAD 3 activity does not induce an overall differentiation but instead the preferential differentiation of certain neuronal subtypes was seen at the expense of other cell types mediated through Transforming growth factor-β (TGF-β) activity (Garcia-Campmany and Marti, 2007). In our present study the differential expression of Smad 2/3 may be because of the regulated expression of TGF-β receptor that culminates during differentiation at two different culture conditions but the exact factor responsible for this differential expression could not be identified.

![Fig. 7. Agmatine treatment enhances neurogenesis and suppresses astrogenesis. At DIV 11 agmatine treatment induced more neurogenesis in experimental groups (B, D) compared to the agmatine non-treated groups (A, C). MAP2 (Rhodamine, red), GFAP (FITC, green), and Olig2 (Cy 5, blue) were used as cell-specific markers. (A) SVZ NSCs cultured with EGF/bFGF+/− growth supplements with no agmatine treatment until 11 DIV. (B) SVZ NSCs cultured with EGF/bFGF+/− growth supplements with agmatine treatment until 11 DIV. (C) SVZ NSCs cultured with EGF/bFGF+/− growth supplements with no agmatine treatment until 11 DIV. (D) SVZ NSCs cultured with EGF/bFGF+/− growth supplements and treated with agmatine until 11 DIV. Scale bars 200 μm. Western blot results showed that agmatine treatment significantly lowered GFAP expression level (p<0.05), increased Tuj1 and MAP2 expressions (p<0.05), and did not cause any change in Olig2 expression (E, F). Values are expressed as mean±s.e.m of three independent experiments.](image)

![Fig. 8. Agmatine treatment enhances neurogenesis by increasing ERK1/2 activation. (A) Immunostaining results showed that agmatine treatment induced more neurogenesis by increasing the expression of ERK1/2 in experimental groups (b, d) compared to the agmatine non-treated groups (a, c) at DIV 11. (a) SVZ NSCs cultured with EGF/bFGF+/− growth supplements with no agmatine treatment until 11 DIV. (b) SVZ NSCs cultured with EGF/bFGF+/− growth supplements with agmatine treatment until 11 DIV. (c) SVZ NSCs cultured with EGF/bFGF+/− growth supplements with no agmatine treatment until 11 DIV. (d) SVZ NSCs cultured with EGF/bFGF+/− growth supplements and treated with agmatine until 11 DIV. Scale bars 200 μm. (B) SVZ NSCs were cultured in four different medium conditions until 11 DIV and then harvested for Western blot analysis. At 11 DIV agmatine treatment increased ERK1/2 activation (p<0.05) in the SVZ NSCs cultured with EGF/bFGF+/− growth supplements but did not seem to have effects on SAPK/JNK and p38 activation compared to agmatine treated and non treated SVZ NSCs cultured with EGF/bFGF+/−. Values are expressed as mean±s.e.m of three independent experiments.](image)
investigated in this study. BMP signaling is critical during neurogenesis and was reported to be regulated by the SMAD 1,5,8 expression (Gajera et al., 2010) and our findings also showed evidence that agmatine treatment to SVZ NSCs reduced the expressions of BMP 2,4 and SMAD 1,5,8 and promoted SVZ NSCs for early neurogenesis. Considering the fact that growth factors enhance the proliferation of NSCs and ERK1/2 activation augments proliferation activity of stem cells (Elia et al., 2007) we demonstrate that agmatine treatment increased ERK1/2 protein activation in the presence of growth factors (EGF and bFGF), thereby induced neurogenesis of SVZ NSCs. As mentioned earlier, many researchers reported that both ERK1/2 and p38 up-regulate neural differentiation of ESCs. However, our data suggest that only ERK1/2 activation is regulated by agmatine treatment and this activation may be sufficient to induce the differentiation of SVZ NSCs (Figs. 8 and 9). Recent findings suggest that the neuronal differentiation was mediated through Wnt/β-catenin signaling in the adult dentate gyrus. Kuwabara et al. (2009) and Fernando et al. (2011) demonstrated the P38K-related kinase signaling pathway and histone H2AX phosphorylation following GABA receptor activation in subventricular zone neurogenesis (Fernando et al., 2011). Our results demonstrated a previously non-described BMP 2,4 and SMAD 1,5,8 mediated regulatory mechanism that simultaneously co-ordinates the activation of neurogenesis in agmatine treated SVZ NSCs.

Previous report suggests that agmatine treatment regulates both proliferation and differentiation in specific types of cells (Morrisey et al., 1995) showing similarity with our results. Our studies demonstrated that agmatine treatment significantly decreased astrocytosis by down-regulating BMP2 and BMP4 expressions indicating that the SVZ NSCs transforming to astrocytes were suppressed and that proportion of the astrogenesis was replaced by neurogenesis with the same start of cell seeding number at the time of experiment. However, BMPs have also been shown to support the neuronal and the astrocytic differentiation differentially in a stage and cell specific manner (Mehler et al., 2000).

Although neurons have been shown to rise from the SVZ, both in vitro and in vivo (Gritti et al., 1996; Kirschenbaum and Goldman, 1995; Lois et al., 1996; Morshed and van der Kooy, 1992) cultures of SVZ explants do not give rise solely to neurons from a dividing precursor population. During in vivo experiments, the precursor cells within the SVZ have been shown to give rise to neurons, which migrate rostrally along the rostral migratory stream (RMS) and repopulate in the areas of the olfactory bulb throughout life (Lois and Alvarez-Buylla, 1994; Luskin, 1993). However, the exact regulation of the differentiation is still unknown. The fact that portion of the neurons derived from SVZ-derived differentiated cells is too small is thought to be the major disadvantage when it comes to cell therapy for recovering brain damages including strokes. The efficiency of cell therapy may depend on the technique to set up a method which can increase the yield of neurons. Our data suggest that agmatine is a potent mediator of SVZ NSCs neurogenesis and transplantation of agmatine treated SVZ NSCs to CNS injury model could trigger early neurogenesis and undergo the full range of molecular events involved during neurogenesis in vitro as evidenced in vivo.

Conclusion

Evidence for neurogenesis in the SVZ and the elements of the signaling pathways that control this process are beginning to be revealed, but a complete or even consistent picture of the process at the molecular level is incomplete. We reported that agmatine treatment enhances neurogenesis by activating ERK1/2 expression and decrease astrogenesis through suppression of BMP 2,4 and SMAD 1,5,8 expressions in this in vitro study. Supplementary materials related to this article can be found online at doi:10.1016/j.lfs.2011.07.003.

Conflict of interest

The authors declare that there are no conflicts of interest.

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