# Regulation of subventricular zone stem cell proliferation and differentiation by agmatine

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# Regulation of subventricular zone stem cell proliferation and differentiation by agmatine

Directed by Professor Jong Eun Lee

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This certifies that the Master's Thesis of Sa Hyun Kim is approved.

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June, 2006 *Sa Hyun Kim*

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## Abstract

# Regulation of subventricular zone stem cell proliferation and differentiation by agmatine

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Subventricular zone (SVZ) cells proliferate spontaneously *in vivo* in the telencephalon of adult mammals. It has been reported that the transplantation of SVZ neural stem cells (NSCs) into the brain infarction *in vivo* might be effective on stroke cure, as a therapeutic adult stem cells recently. It is useful as research theme, but SVZ neural stem or progenitor cell still have fatal troubles to grow over or form teratoma. Agmatine (Agm) is an endogenous primary amine, synthesized from L-arginine decarboxylase and is able to modulate the cellular concentration of polyamines which are essential molecules required for cell growth and proliferation. Recent studies showed that Agm has significant inhibitory effect on transplanted tumor growth *in vivo* and proliferation of tumor cells *in vitro*, and the mechanism might be associated

with intracellular polyamine concentration.

In this study, we examined the regulatory effect of Agm on proliferation and differentiation of SVZ NSCs. Subventricular NSCs were cultured and to characterize NSC and progenitor compartments further, the nestin expression was examined. Strong Nestin expression was also observed in SVZ NSCs culture. Agm was administrated as varied concentrations (0~500μM) daily for 3.5 days. The number of neurospheres (NPs) was diminished in all Agm administration groups. In the group of 100μM, the number and the diameter of NPs were less and smaller than NC group as about 30%. In addition, L-arginine, putrescine, spermidine, spermine, and polyamine mixture (same concentration of agmatine, L-arginine, putrescine, spermidine, spermine were mixed) were administrated in the culture medium, and all of them inhibited NSCs proliferation like as Agm. The anti-BrdU immunofluorescence staining and DAPI counterstaining was performed and the change of cell number was compared. To examine the effect on differentiation process, agmatine, L-arginine, putrescine, spermidine, spermine, and polyamine mixture were treated on cells from 4 DIV (days *in vitro*) to 12DIV. On 12 DIV, differentiated neural cells were immuno-fluorescence stained by making use of primary antibodies (Tuj1, MAP2 for neuron, GFAP for astrocyte, and anti-BrdU). Undifferentiated NPs were remained more in NC group, but in L-arginine and Agm group, we could find Tuj1-positive mature neurons and GFAP-positive mature astrocytes. We harvested differentiated mature neural cells to measure the differentiation ability by counting BrdU positive cell number.

We suggest that Agm inhibits the proliferation of SZV NSCs on the other hand, aids SVZ NSCs to differentiate to neural cells. Agm may be a novel therapeutic strategy to regulate the proliferation and differentiation of stem cell.

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Key Words : agmatine, polyamine, SVZ, neural stem cell, proliferation, differentiation

# Regulation of subventricular zone stem cell proliferation and differentiation by Agmatine

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## I. INTRODUCTION

In eukaryotic cells, putrescine, spermidine and spermine play an essential role in proliferation, differentiation and neoplastic transformation. The classic pathway for polyamine biosynthesis proceeds from L-arginine to putrescine by the action of arginase and ornithine decarboxylase. Arginase cleaves the terminal guanidine moiety of L-arginine to produce ornithine and urea, and ornithine decarboxylase in turn converts ornithine into putrescine by

decarboxylation (Figure 1).[2] Bacteria, plants, and other lower species synthesize polyamines via a second distinct pathway where arginine is first decarboxylated to agmatine (1-amino-4-guanidinobutane) by arginine decarboxylase followed by the removal of urea to form putrescine by agmatinase (agmatine ureohydrolase) (Figure 1)[3, 4].

Reports on the arginine decarboxylase activity in kidney[4] and brain[5] and the agmatinase activity in rat brain[7] and a mouse macrophage cell line[8] suggest that this alternative pathway for polyamine biosynthesis is also functional in mammals. Agmatine is widely distributed in a number of mammalian tissues including brain, kidney, stomach, intestine, and aorta[9]. Agmatine is the product of arginine decarboxylation and can be hydrolyzed by agmatinase to putrescine, the precursor for biosynthesis of higher polyamines, spermidine, and spermine. Besides being an intermediate in polyamine metabolism, recent findings indicate that agmatine may play important regulatory roles in mammals[17]. Polyamines are also scavengers of reactive oxygen species, thereby protecting DNA, protein, and lipids from oxidative damage.

Recent discoveries suggest that, in mammals, agmatine may possess functions other than that of a metabolic intermediate for polyamines[9-12] and that it has potential as a treatment of chronic pain, addictive states, and brain injury[17]. Its effects include inhibition of cell proliferation, stimulation of glomerular filtration rate in kidney, activation of constitutive nitric-oxide synthase, and inhibition of inducible nitric-oxide synthase[2, 9, 25, 26]. Agmatine has also been proposed to act as a possible neurotransmitter / neuromodulator. It binds to 2-adrenoreceptors and imidazoline-binding sites and blocks N-methyl-D-aspartate receptor channels and other ligandgated cationic channels.[12, 13] Changes in activity or expression of agmatinase could play an important role in regulating the physiological actions of agmatine. Agmatinase is a binuclear manganese metalloenzyme and belongs to the ureohydrolase superfamily.[14, 15, 16] Available evidence shows that polyamines are key

regulators of angiogenesis, early mammalian embryogenesis, placental trophoblast growth, and embryonic development.[31] The cellular polyamines are thought to be supplied through *de novo* synthesis from L-arginine and transport, while the mammalian polyamine transporter gene has not been identified[17, 18], unlike bacterial and/or fungal polyamine transporter.[19, 20] *De novo* polyamine synthesis is governed in part by the activity of ornithine decarboxylase (ODC), which catalyzes the conversion of the amino acid, L-ornithine to the diamine and putrescine. Other members of this superfamily include arginase, formiminoglutamase, and proclavaminate amidinohydrolase (PAH).[10,16]

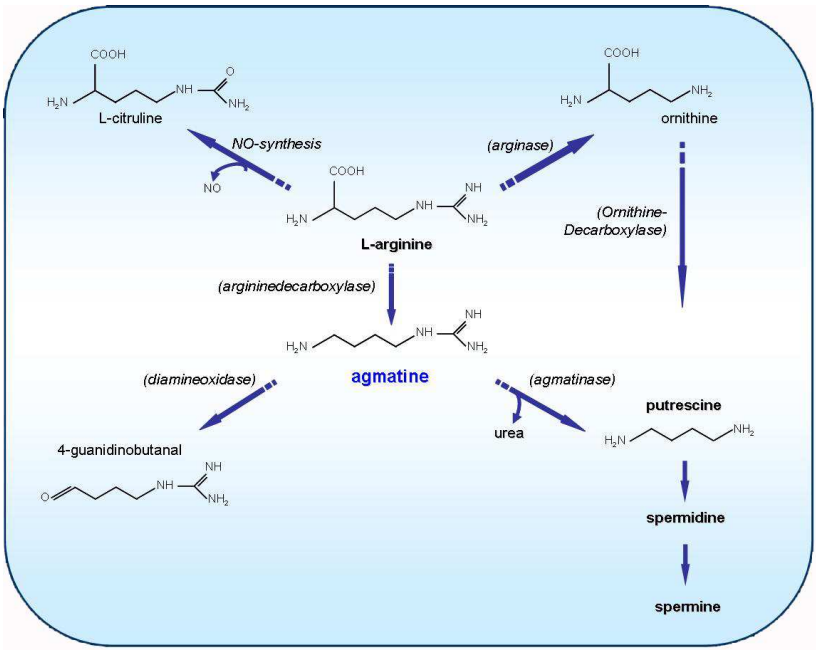


**Figure 1. Agmatine metabolism**. Arginase cleaves the terminal guanidine moiety of L-arginine to produce ornithine and urea, and ornithine decarboxylase in turn converts ornithine into putrescine by decarboxylation. Arginine is first decarboxylated to agmatine (1-amino-4-guanidinobutane) by arginine decarboxylase followed by the removal of urea to form putrescine by agmatinase

We suggest using the term “ureohydrolase superfamily,” because it is more suited to describe a variety of substrates that its members hydrolyze than the term “arginase superfamily.” An analysis of the evolutionary relationship

among ureohydrolase superfamily enzymes indicates that the sequence similarity trees separate agmatinases from arginases including Deinococcus radiodurans (DR) agmatinase.[34, 36, 37] It was suggested that the arginase pathway of polyamine biosynthesis is likely to have evolved later than the pathway involving arginine decarboxylase and agmatinase.

The discovery of neural stem cells was rooted in classic studies of haematopoiesis and of invertebrate neural development, which inspired examination of single neural progenitor cells. The early studies led to the isolation of stem-like cells from the embryonic mammalian central nervous system (CNS)[30, 31, 32, 33] and the peripheral nervous system (PNS).[33] Since then, stem cells have been isolated from many regions of the embryonic nervous system, indicating their ubiquity. After the discovery of neural stem cells in the embryo, the first isolation of stem-like cells from adult brain[32, 33, 38, 39, 40] began yet another chapter of neuroscience. Adult neural stem cells have now been found in the two principal adult neurogenic regions, the hippocampus and the subventricular zone (SVZ) (Figure 2), and in some non-neurogenic regions, including spinal cord.[35, 36, 37] These studies provided a cellular mechanism for adult neurogenesis, which was well-established in birds and becoming accepted in mammals, and raised the possibility that the most intractable of tissues — the CNS — might have regenerative powers. It is useful as research theme, on the other hand, stem cells including subventricular zone neural stem or progenitor cell have fatal troubles. There were reports that stem cell which is not differentiated for therapy have tends to be “cancer like” stem cell.[39] Like stem cells, cancer cells are widely thought to be able to proliferate indefinitely through a deregulated cellular self-renewal capacity. This raises the possibility that some of the clinical properties of tumor cells may be due to transformed stem cells.[40]

Regarding some reports which the agmatine was related to the regulation (inhibition) of kidney cell line and cancer cell line proliferation, we can imagine that the regulation of stem cell proliferation and differentiation by agmatine may

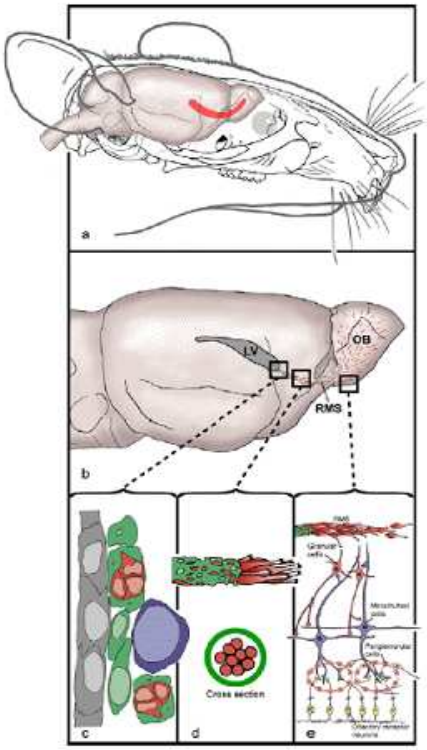


**Figure 2. Subventricular zone neural stem cell** (a) Head of a mouse showing the location of the brain and the rostral migratory stream, RMS, (in red) along which newly generated neuroblasts migrate from the SVZ of the lateral ventricle into the olfactory bulb (OB). (b) The migration of newly generated neuroblasts begins at the lateral ventricle, continues along the RMS and terminated in the OB, where mature interneuron populations are generated. (c) Schematic based on electron microscopy showing the cytoarchitecture of the SVZ along the ventricle. Ependymal cells (gray) form a monolayer along the ventricle with astrocytes (green), neuroblasts (red) and transitory amplifying progenitors (TAP) (purple) comprising the SVZ. (d) Schematic showing the migration of neuroblasts along the RMS. Astrocytes (green) ensheath the migrating neuroblasts (red) and are thought to restrict and contain the neuroblasts to their specific pathway. (e) Migrating neuroblasts enter the OB, migrate radially and give rise to granule or periglomerular cells.
Figure from ***Jessica B Lennington, et al. Neural stem cells and the regulation of adult neurogenesis. Reproductive Biology and Endocrinology. 2003.***

be possible. Usually the proliferous cells begins their differentiation process when the proliferation is inhibited. Agmatine itself doesn't only have a toxicity on cells[1], but it also inhibits tumor cell growth by suppressing NO synthesis, polyamindecarboxylases, and ODC synthesis. Agmatine is transported into hepatocytes in competition with putrescine.[41] Agmatine taken up shows several effects: inhibition of NOS,[42] marked decrease of polyamine concentration, due to SSAT induction,[43] and decrease of ODC activity and ODC protein content,[41, 21] in some instances following induction of ODC-antizyme.[28] These direct effects of agmatine, and also those deriving from the formation of $H_2O_2$ during its catabolism, induce apoptosis in non-proliferating cells.[43] In addition, it was

reported that stem cells generally have higher polyamins concentration. Accordingly in the present study, we investigated the possibility of regulation of neural stem cell proliferation and differentiation by agmatine, L-arginine, and polyamine.

# II. MATERIALS AND METHODS

### 1. Subventricular zone neural stem cell primary culture

The cerebral cortex was dissected and removed from newborn (P0–P2) or embryo day 14 limited mice. The dissection included all of the dorsal part of the ventricular zone and the three types of cell layers in subventricular zone of ICR (Crj : CD-1) mice was processed following well known method. (Arsenijevic et al., 2001, etc.)

Dissociated cells were grown in the presence of epidermal growth factor (EGF; 20 ng/ml; GIBCO), basic fibroblast growth factor (bFGF; 20 ng/ml; GIBCO), and B-27 supplement minus vitamin A (20μL/mL; GIBCO) in 24-well and 6-well plates (Nunc, Naperville, IL) at a concentration of $3.0\times10^4$ cells/mL, corresponding to a density of over 15,000 cells/cm2. The number of spheres to examine the proliferation was counted after 3.5 days *in vitro* (DIV). Differentiation assays was evaluated after 12 DIV.

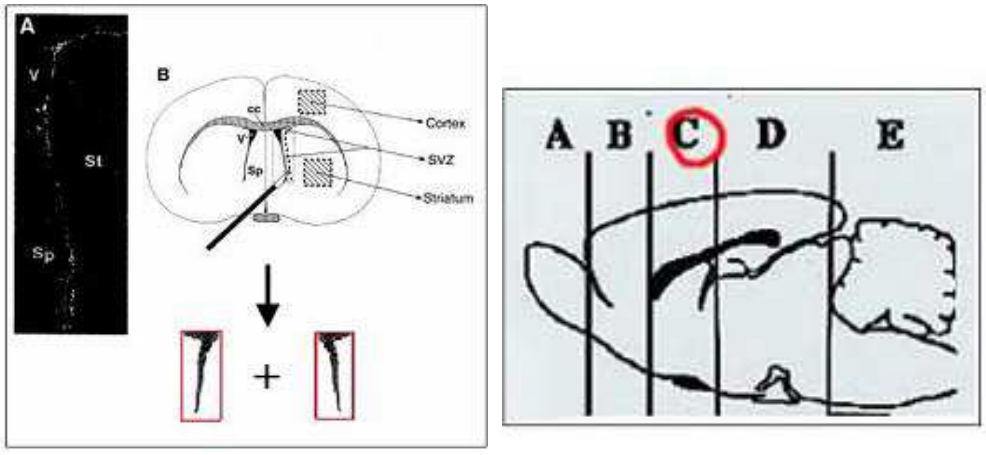


**Figure 3. Microdisection of ICR mouse subventricular zone.** Select lateral ventricle region containing ependymal cell layer and three types layers. Dissected tissue fragment is about 500μm-width and 1000μm-length.

**2. Treatment of chemicals**

Agmatine, L-arginine, putrescine, spermidine, spermine, and mixture of them were administrated daily to the culture medium of SVZ NSCs as various concentrations (0~500 μM) for 3.5 days for proliferation assay. And when the proliferation was completed, after 3.5~4 DIV and we treated them as same method till 12 DIV for differentiation assay.

**3. Thymidine incorporation with BrdU**

A cell proliferation marker, BrdU (Sigma, St Louis, MO, USA), was dissolved in saline. First, to determine the level of SVZ cell proliferation and differentiation with the effect of L-arginine, agmatine, putrescine, spermidine, and spermine we administrated BrdU with final concentration of 20 μM/mL. Next, to evaluate the of cell proliferation and differentiation of SVZ, the cells were immuno-fluorescence stainined using anti-BrdU (Chemicon, Temecula, CA, USA) and counted after 4 DIV for proliferation assay, and 12 DIV for differentiation assay respectively.

**4. Analysis of cell cycle by Flow Cytometry (FACs)**

We applicated the cell cycle analysis of FACs method to evaluate the cell proliferation quantitatively. For cell cycle analysis, cells were harvested, washed with ice-cold PBS and fixed in ice-cold 70% Ethanol. Propodium iodide (50mg/mL in PBS, containing 100mg/mL RNase A) was added for 5 minutes. Flow cytometry analyses were performed on a FACScan (Becton Dickinson, Mountain View, CA). For each time point, 20×103 cells were analysed and PI fluorescence was displayed on a linear scale using the FL2 photodetector. The DNA profile indicates the relative abundance of G0-G1, S and G2-M phase populations. Cell distribution into cell cycle phases were calculated using the Cell Quest (Becton Dickinson Immunocytometry Systems) sotfware.

**5. Immunocytochemistry**

Differentiated cells on culture plates which were coated with poly-L-lysine and laminine were washed three times with PBS, fixed with 90% Ethanol for 30 minutes on ice, and then washed three times with TBS. Cells were permeabilized with 1.6% H2O2 in TBS for 30 minutes at room temparature, washed two times for 5 minutes with TBS plus 0.025% triton, and blocked for 1 hour at room temperature with 0.5% bovine serum albumin and 6% normal goat serum in TBS. Primary antibodies were then added in TBS containing 3% normal goat serum and left overnight at 4°C. Primary antibody was made up in TBS with 1% BSA. Incubate overnight at 4℃ and primary antibody was removed, cells were washed three times for 5 minutes with TBS, and the appropriate secondary antibody conjugated to CY3 or fluorescein isothiocyanate was added in TBS containing 1% BSA for 2 hour at room temperature (FITC, Zymed, 1:1000 / TRITC, Zymed, 1:1000). Cells were washed three times for 5 minutes with TBS, conterstained with 4'-6-Diamidino-2-phenylindole (1μg/mL, DAPI, Sigma) for 30 minutes at room temperature, and visualized using a Olympus D-70 upright fluorescent microscope.

Primary antibodies were used neuronal class III beta-tubulin monoclonal (1:2000, Tuj1, Chemicon), microtubule-associated protein 2 monoclonal (1:500, MAP2, Sigma), Glial fibrillary acidic protein polyclonal (1:1000, GFAP, Chemicon), 5-bromo2'-deoxy-uridine (1:500, anti-BrdU, Sigma)

To obtain an estimate of the percentage of cells adopting neuronal and glial phenotypes, random fields were selected and photographed, and for each field the total number of cells (as determined by counting Hoechst stained nuclei) and the total number of cells positive for neuronal or glial markers were determined. This analysis was performed for three different differentiation experiments, and the mean and standard deviation were determined.

# III. RESULTS

## 1. Agmatine inhibits the proliferation of subventricular zone neural stem cells.

There are two important steps in the growth of stem cells, that is to say, proliferation and differentiation. At first, to study the proliferation step, we examined the diameter and the number of neurosphere which are the standards of the completion of proliferation. Because polyamine have been reported to inhibit the proliferation of proliferous cells like cancer cell, we studied whether agmatine inhibits the proliferation of neural stem cells by measuring of the diameter of and the number of neurospheres.

### A. Measurement of the neurosphere diameter

We cultured NSCs with agmatine but keep neural stem cell growth factors (EGF, bFGF, and B-27 supplement munus vitamin A) in the medium to make cells healthy *in vitro*, and they were used in the present experiment as positive normal control. Although growth factors clearly induced the proliferation and differentiation, yet agmatine did not (Figure 5, 6). First, to find treatment concentration of agmatine, we administrated to the medium of SVZ NSCs as various concentrations (0~200μM) for 3.5~4 days except for the high-dose group. Cells were cultured with EGF, FGF-2 and B-27 minus vitamin A (Gibco) and no FBS.

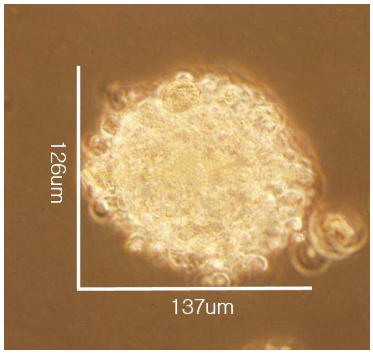


**Figure 4. How to Measurme the diameter of neurosphere.** For quantification of the size of neural spheres grown with different agmatine density, the diameter of spheres was measured along both the x and y axes, because spheres were not uniformly spherical. The average of these two measurements was then used as the diameter of the sphere. The mean sphere diameter was 134.52 ± 12.28 μm for NC group. Cells were cultured with EGF, FGF-2 and B-27 minus vitamin A and no FBS. The single cell size was in the range of 10 ~ 15 μm in diameter.

For quantification of the size of neural spheres grown with different density of agmatine, the diameter of spheres was measured along both the x and y axes, because spheres were not uniformly spherical. To evaluate the proliferation of SVZ NSCs, we measured the diameter of neurospheres (Figure 4) and the number of neurospheres (Figure 5). The average value of the length of x and y were used as the diameter of the sphere. The mean of sphere's diameter was 134.52 ± 12.28 μm on NC group and the other groups was dose-dependently decreased. These data suggest that agmatine inhibited the proliferation of SVZ NSCs. (Figure 6). And the size of single cell was in the range of 10~15 μm in diameter. These data were handled by statistical analysis of SAS syatem and Mini tab. Set independent variable as 'agmatine Concentration' and dependent variable as 'mean of the neurosphere diameter'. Ten independent experiments were repeated. On each experiments, 'pearson correlation' was -0.943 on an average. *p*-value was 0.005<0.05 and it means strong negative (inhibition) aspect of the 'mean of the neurosphere diameter' following the increasing of 'Agmatine Concentration' (Figure 7).

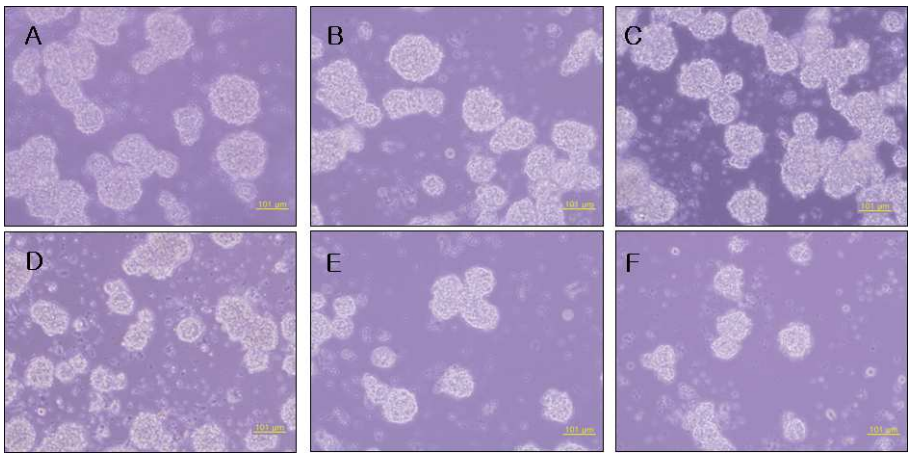


**Figure 5. The diameter of neurospheres was diminished agmatine dose-dependently.** NSCs were cultured with agmatine but keep neural stem cell growth factors (EGF, bFGF, and B-27 supplement munus vitamin A) in the medium to make cells neuronal growth *in vitro*. **(A)** : used in the present experiment as positive normal control (no agmatine treatment), the diameter was 130.855μm on a average. **(B)** : 20μM agmatine treated, the diameter was 113.753μm on a average. **(C)** : 50μM agmatine treated, the diameter was 94.160μm on a average. **(D)** : 100μM agmatine treated, the diameter was 89.575μm on a average. **(E)** : 150μM agmatine treated, the diameter was 84.452μm on a average. **(F)** : 200μM treated, the diameter was 64.025μm on a average.

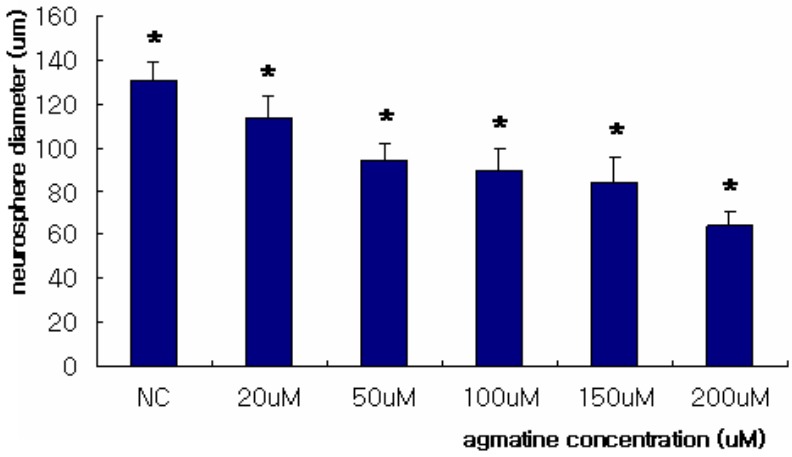


**Figure 6. The effect of agmatine on neurosphere diameter of subventricular zone neural stem cells.** NSCs were seeded in 24-well plates at a density of $3\times10^4$ cells/mL and incubated in culture medium with or without agmatine for 3.5~4days. NC (normal control) group was cultured with growth factors only. We could find the inhibition aspect. 6 independent experiments were repeated.

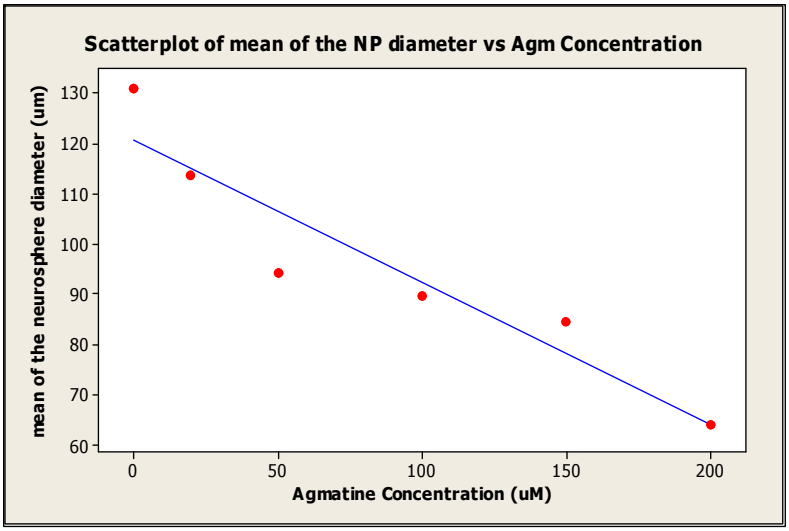


**Figure 7. The change of neurosphere diameter by increasing of agmatine concentration.** Examine the scatter plot graph and following the increasing of the independent variable, 'Agmatine Concentration', dependent variable, 'mean of the neurosphere diameter' was decreased. (a=-0.943)

### B. Counting the number of neurospheres

We could find the inhibition aspect by decreasing the number of neurospheres following the increasing of agmatine concentration (Figure 8). Set independent variable as 'Agmatine Concentration' and dependent variable as 'mean of the neurosphere number', 10 independent experiments were repeated. On each experiments, 'Pearson correlation' was -0.929 on an average. p-value was 0.007<0.05 and it means strong inhibition aspect of the 'mean of the neurosphere number' by the increasing of 'Agmatine Concentration' (Figure 9).

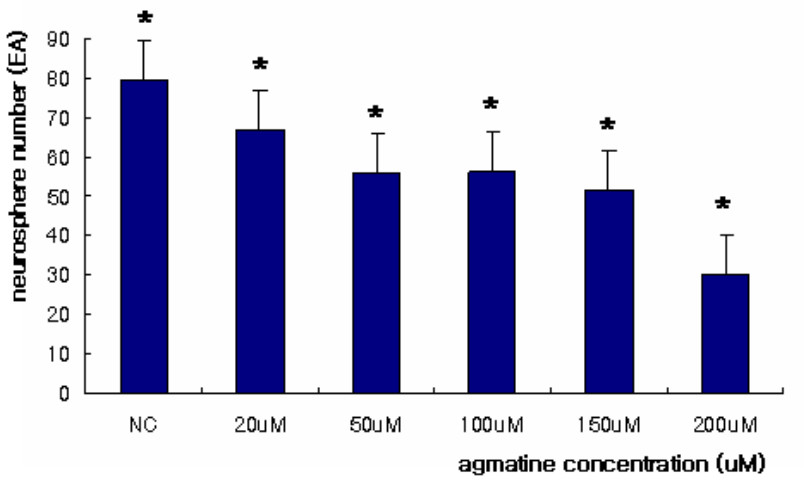


**Figure 8. The effect of agmatine on the number of neurosphere.** NSCs were seeded in 24-well plates at a density of $3\times10^4$ cells/mL and incubated in culture medium with or without agmatine for 3.5~4days. NC (normal control) group was cultured with growth factors only. We could could find the inhibition aspect

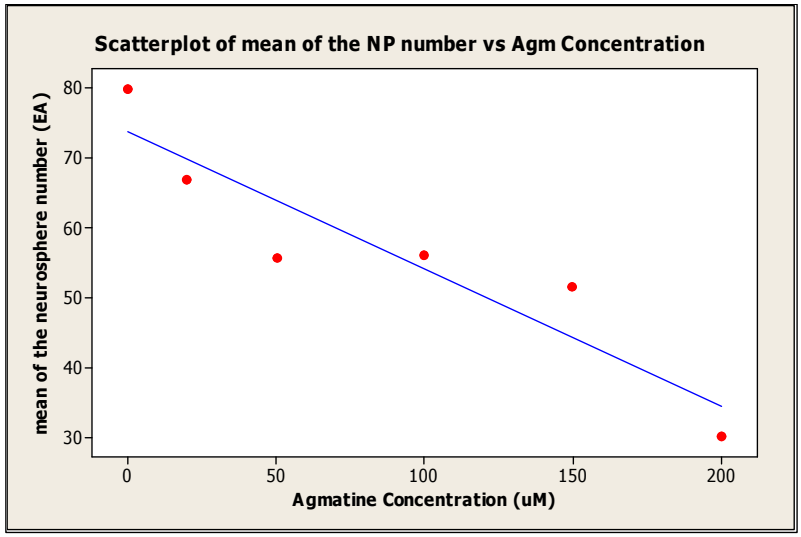


**Figure 9. Agmatine dose-dependent inhibition aspect of neurosphere formation.** Examine the scatter plot graph and following the increasing of the independent variable, 'Agmatine Concentration', dependent variable, 'mean of the neurosphere number' was decreased. (a=-0.929)

### C. BrdU incorporation assay and DAPI double staining

We examined how many cells of neurosphere were diminished by agmatine administration. To evaluate the rate of the inhibition, we counted the number of cells which have BrdU-positive nuclei, nuclei by DAPI stained and then measured BrdU/DAPI (%) (Figure 10).

$$\frac{\text{BrdU-positive cells}}{\text{DAPI stained cells}} \times 100\ (\%)$$

These data showed similar aspects that agmatine dose-dependently decreased the diameter and the number of neurospheres (Fighure 6, 8), (Figure 11). Set independent variable as 'Agmatine Concentration' and dependent variable as 'mean of the BrdU/DAPI' by SAS system statistically. 10 independent experiments were repeated. On each experiments, 'Pearson correlation' was -0.91 on an average. *p*-value was 0.012<0.05 and it means strong inhibitory aspect of the 'mean of the BrdU/DAPI' following the increasing of 'Agmatine Concentration' (Figure 12).

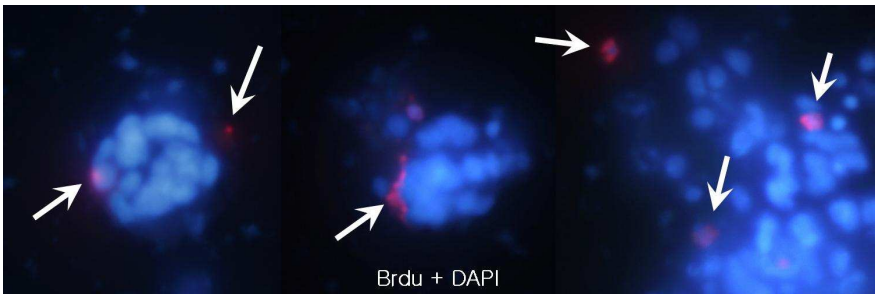


**Figure 10. BrdU incorporation.** BrdU incorporation transfer thymidine of DNA to Uracil during cell dividing. So, using anti-BrdU, newly proliferated cells could be detected. We counted BrdU positive cells (white arrows) among all of cells (TRITC red). And then evaluated BrdU positive cells / the number of DAPI. Data showed a tendency that agmatine inhibit the proliferation of neural stem cells going high concentration.

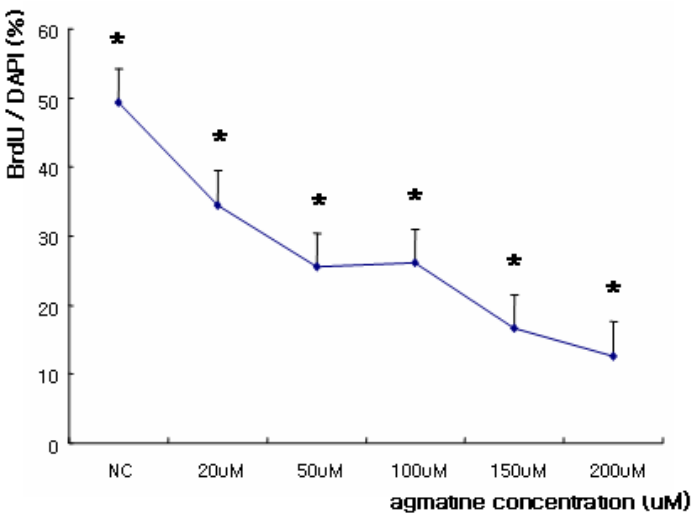


**Figure 11. The percentage of BrdU/DAPI was decreased dose-dependently.** Micrographs of neurospheres treated with agmatine (20, 50, 100, 150, 200μM) for 3.5~4 days. BrdU (20μM) was added at culture day. Data are expressed as the means + SEM of Diameters of 450 neurospheres obtained in three independent experiments. The percentage of BrdU-labelled cells in 30 microscopic fields counted in three independent experiments.

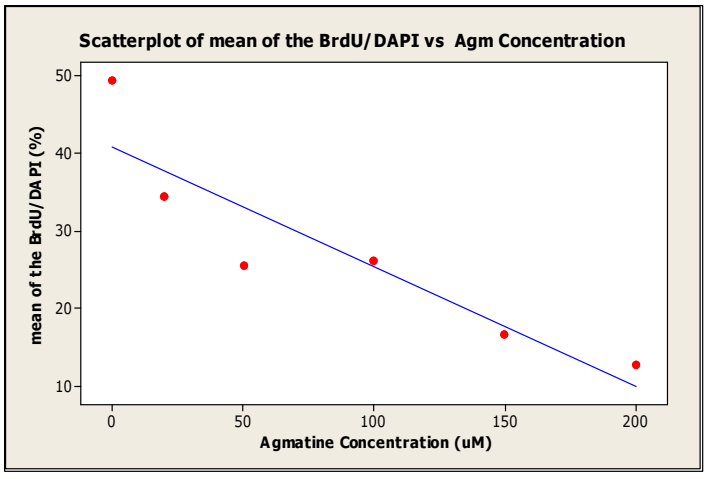


**Figure 12. Decreasing aspect of the cell number which forms neurosphere.** Examine the scatter plot graph and following the increasing of the independent variable, 'Agmatine Concentration', dependent variable, 'mean of the BrdU/DAPI' was decreased. (a=-0.91)

**2. L-arginine and polyamines inhibited the proliferation of SVZ stem cells.**

Cell proliferation was not increased by any of polyamines in this experiment, while L-arginine increased the cell proliferation on 100μM group than on 20μM group, but it was slight, moreover, it could not overcome normal control group (Figure 13-A, B). Putrescine group was also increased the cell proliferation, which did not overcome normal control group, between A and B, but it was decreased between B and C. General data was shown that polyamines suppressed the proliferation of SVZ NSCs. In the case of spermidine group and spermine group, cells did not grow at high concentrations (Figure 13-D). Polyamine mixture group was also did not showed ('Mix' on Figure 13) was produced by agmatine, L-arginine, putrescine, spremidine, spermine as mixing same concentrations.

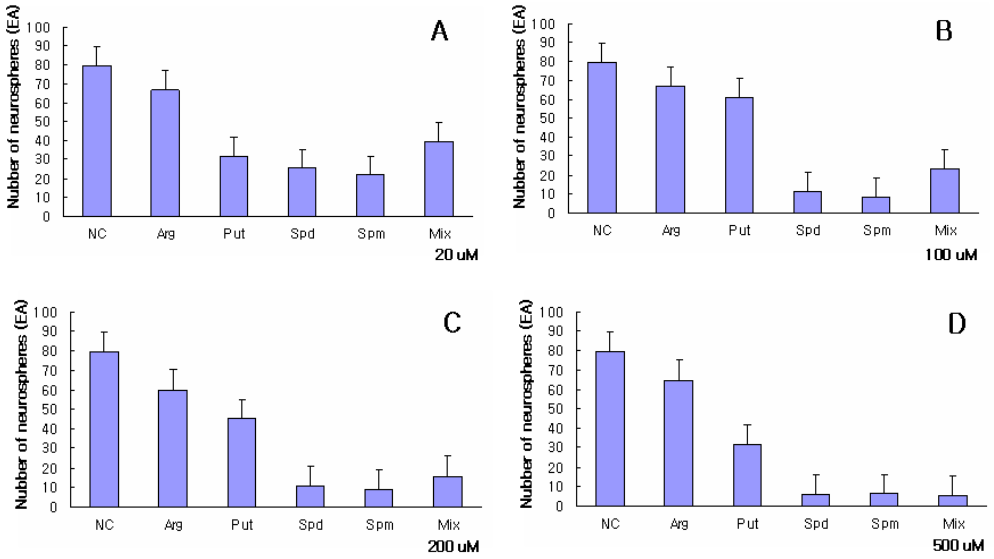


**Figure 13. The effect of polyamine on the proliferation of subventricular zone neural stem cells in various concentrations.** NSCs were seeded in 24-well plates at a density of $3\times10^4$ cells/mL and incubated in culture medium with L-arginine and polyamin for 3.5~4 days at a concentration of 20 **(A)**, 100 **(B)**, 200 **(C)**, 500 **(D)** μM. NC (normal control) group was cultured with growth factors only. Data represent mean and standard errors of 6 independent experiments. Data evaluated after DAPI staining.

**3. Agmatine affects the SVZ stem cell cycle distribution.**

Agmatine is regulated the neurosphere formation and the proliferation of subventricular zone stem cells, and a number of studies have shown an association between cell cycle arrest and disription of agmatine biosynthesis. Therefore, we investigated the effect of agmatine on cell cycle repartition in freely cycling growing cells duruing proliferation. Because we thought that such an inhibition effect was fatally enough in 200μM. Agmatine-treated SVZ NSCs were compared with the normal control group showed different proportions in the S phase by FACs. The cells treated with 200μM agmatine at '24 h' showed a significant increase in the number of cells in the S and G2-M phases and a decrease in the G0-G1 phase when compared with the control experiments (Table 1). The agmatine-induced modification in distribution of SVZ stem cell cycle was also evident after '90 h' treatment (Table 1). These results indicated that agmatine alters the cell cycle in colonic SVZ stem cells.

| Added agents | Cell cycle phases (%) | | |
|---|---|---|---|
| | G0-G1 | S | G2-M |
| ***24 hour treatment*** | | | |
| Control | 62.9±0.5 | 27.1±1.8 | 9.9±2.2 |
| Agmatine 200μM | 51.7±2.7 | 32.1±0.7 | 16.1±2.1 |
| ***90 hour treatment*** | | | |
| Control | 72.5±1.4 | 18.0±0.4 | 9.4±1.9 |
| Agmatine 200μM | 45.5±1.1 | 27.6±0.4 | 26.8±1.3 |

**Table 1. Agmatine affects the SVZ stem cell cycle distribution.** SVZ stem cells were cultured in the absence or in the presence of 200 uM agmatine for 24 or 90 h and then analysed for cell cycle distribution using flow cytometry after staining by propidium iodide. Results are provided as percentage of total cells and represent mean ± S.E.M. of three to six independent experiments.

From '24 h' to '90 h', the effect of agmatine was even more pronounced and the cells were found to be accumulated both in the S and G2-M phases when the G0-G1 phase was expended ($P<0.05$). Because almost of normal SVZ NSCs which have strong self renewal character in the phase of G0-G1, the diminish of G0-G1 phase and the increase of S and G2-M phase mean NSCs stopped the proliferation and begin the differentiation process.

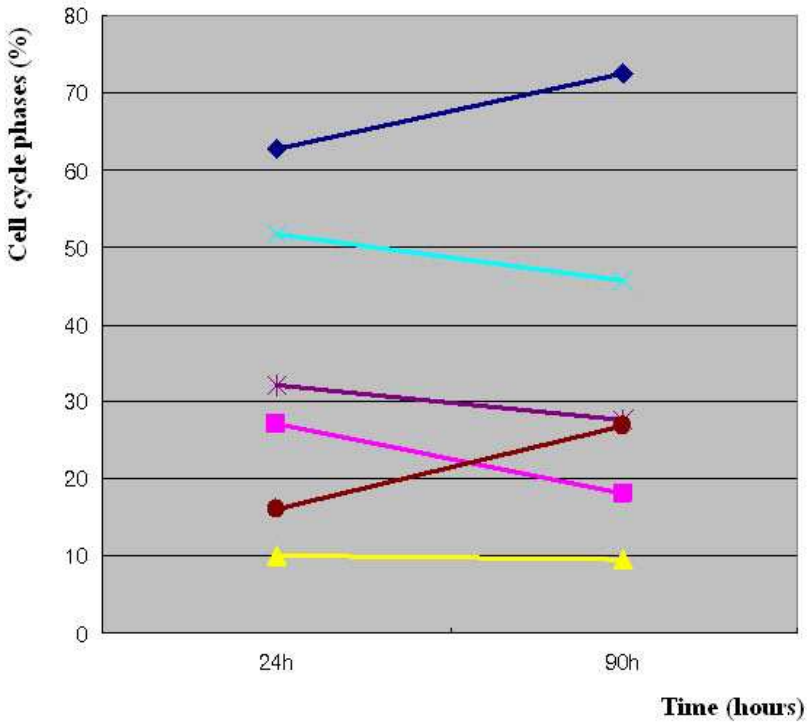


**Figure 14. Kinetics of agmatine effects on the cell cycle of SVZ stem cells.** SVZ stem cells were synchronised in the G0-G1 phase after 24 h culture in serum-free medium. They were then cultured in the absence (control) or in the presence of 200 uM agmatine sulfate (Agm) from period of time ranging from 6 to 48 h. The cell cycle distribution was analysed using flow cytometry after staining with propidium iodide. The results are provided as percentages of total cells and represent mean ± S.E.M. of three to six independent experiments. : **control G0-G1**. : **control S.** : **control G2-M**. : **Agm G0-G1**. : **Agm S**. : **Agm G2-M**.

Moreover, we need to concentrate on the accumulated portion of G2-M phase of 90 h agmatine group (Figure 14 —●—). The G2-M phase portion of normal control group was showed similar aspects both '24 h' group and '90 h' group, but cells were treated with agmatine increased at '90 h' group.

**4. The effect of agmatine and polyamine on the differentiation of SVZ stem cells in various concentrations.**

Agmatine and the most of polyamines inhibited the proliferation of neural stem cells, on the other hand, agmatine and polyamine have been reported to

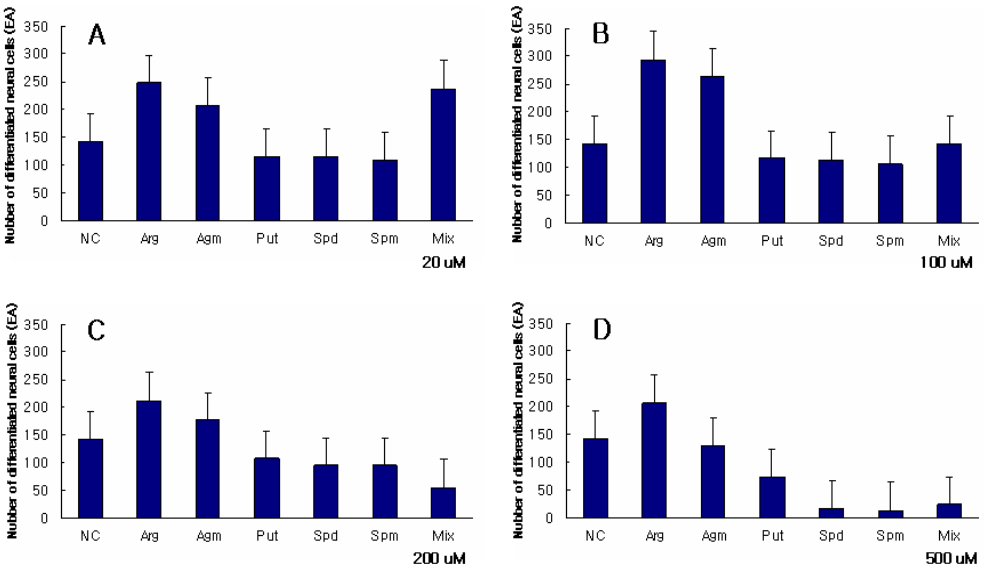


**Figure 15. The effect of polyamine on the differentiation of subventricular zone neural stem cells in various concentrations.** **n**eural stem cells were seeded in 24-well plates at a density of $3\times10^4$ cells/mL and incubated from 4 days *in vitro* to 12 days with L-arginine, agmatine, putrescine, spermidine, spermine at a concentration of 20μM **(A)**, 100μM **(B)**, 200μM **(C)**, 500μM **(D)**. The number of differentiated neurons and astrocytes which were stained by immunocytochemistry and counted on a fluorescent microscope. Data represent mean of 6 independent experiments.

induce differentiation. We examined whether polyamines could regulate the proliferation and differentiation of neural stem cells. Various concentrations (50 to 500 μM) of putrescine, spermidine, and spermine were used as polyamines, and also L-arginine, agmatine was treated. Agmatine and L-arginine induced the differentiation of subventricular zone neural stem cells (Figure 16 ; NC : normal control, Agm : agmatine, Arg : L-arginine, Put : putrescine, Spd : spermidine, Spm : spermine, Mix : polyamine mixture).

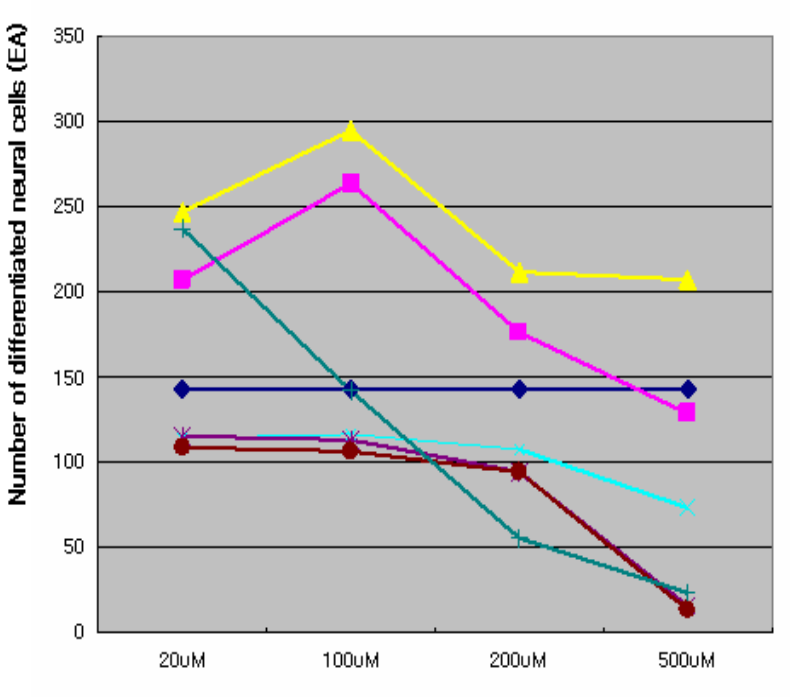


**Figure 16. The aspect analysis of the polyamine effect on the differentiation of SVZ NSCs in various concentrations. n**eural stem cells were seeded in 24-well plates at a density of $3\times10^4$ cells/mL and incubated from 4 days *in vitro* to 12 days in vitro with L-arginine, agmatine, putrescine, spermidine, spermine at a concentration of 20μM, 100μM, 200μM, 500μM. The number of differentiated neurons and astrocytes which were stained by immunocytochemistry and counted on a fluorescent microscope. Data represent mean of 6 independent experiments. : normal control. : agmatine. : L-arginine. : putrescine. : spermidine. : spermine. : polyamine mixture.

In these data, agmatine and L-arginine group showed inducing effect on the differentiation of SVZ NSCs definitely. Agmatine elevated the neural differentiation rate as 185.36% than normal control group (, on Figure 16) and L-arginine recorded as 206.60% than normal control in the group of 100μM (, on Figure 16) respectively. On the other hand, polyamine mixture increased as 166.69% than normal control in the group of 20μM (, on Figure 16), but suddenly diminished the number of neural differentiated cells over 100μM and finally recorded -83.48% than normal control at 500μM. Putrescine, spermidine, spermine could not affect the increase of differentiation and inhibited preferably. Putrescine decreased the rate of neural differentiated cells as -18.26% (, on Figure 16), spermidine as -20.75% than normal control (, on Figure 16), and spermine as -25.45% than normal control (, on Figure 16) in the group of 100μM. They finally recorded -48.99%, -88.77%, -90.57% at 500μM respectively.

In addition, almost of cells were differentiated from SVZ NSCs to mature neural cells in agmatine group, but a few undifferentiated neurospheres were also observed in normal control group. Because there are cells which are on various cell-stages in culture environment, it is possible that some neurospheres are processing the proliferation step till 12 DIV. From former results, we checked the fact that agmatine induced the differentiation of SVZ NSCs (Figure 14 ), and it suggests that there may be more undifferentiated neurospheres especially in normal control group. Neurospheres and differentiated cells were stained by microtubule-associated protein 2 monoclonal (1:500, MAP2, Sigma), Glial fibrillary acidic protein polyclonal (1:1000, GFAP, Chemicon) (Figure 17). In normal control group, we observed far more neurospheres than agmatine group indeed (Figure 17-A compared to B). On the basis of these difference between groups, we could convinced that agmatine have inducing effect on differentiation of subventricular zone neural stem cells.

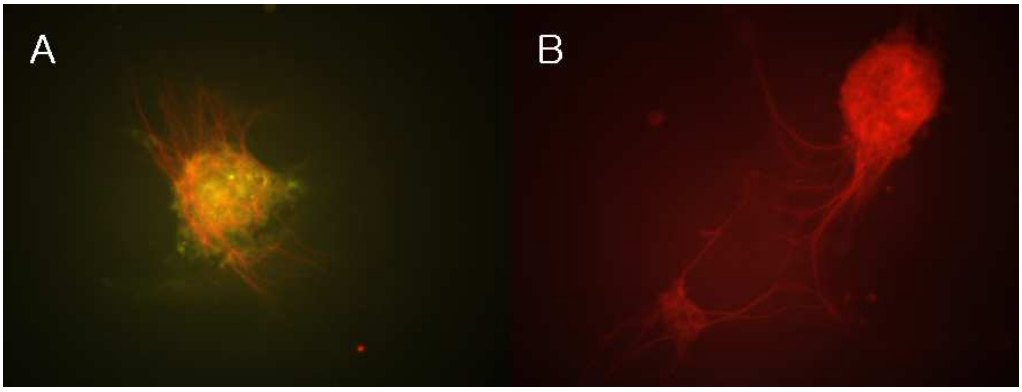


**Figure 17. Inducing effect on neural differentiation by agmatine. (A)** In normal control group, it remained many uniformal undifferentiated neurospheres. MAP2 with TRITC red + GFAP with FITC green. **(B)** In agmatine group, agmatine induced neural differentiation, cell to cell synaptic strategies were found. MAP2 with TRITC red. Neurospheres and cells were stained at over 12 days *in vitro*, culture plates were coated with poly-D-lysine, laminine. Cells were seeded $3\times10^4$ cells/mL and cells were cultured with growth factors, EGF, FGF2, B-27 supplement minus vitamine A in serum free basic DMEM.

Differentiated neural cells from SVZ NSCs were imunofluorescence stained by III beta-tubulin monoclonal (1:2000, Tuj1, Chemicon), microtubule-associated protein 2 monoclonal (1:500, MAP2, Sigma), Glial fibrillary acidic protein polyclonal (1:1000, GFAP, Chemicon). Nuclei were counter stained by 4'-6-Diamidino-2-phenylindole (1ug/mL, DAPI, Sigma). And genuine neurons, astrocytes were selected by checking anti-Tuj1-positive, anti-GFAP-positive cells. Stained cells were counted on the Olympus D-70 uplight fluorescent microscope. The representative neuronal marker, Tuj1 and GFAP antibodies were double stained, and then we counted total cells of Tuj1 positive, and GFAP positive (Figure 18).

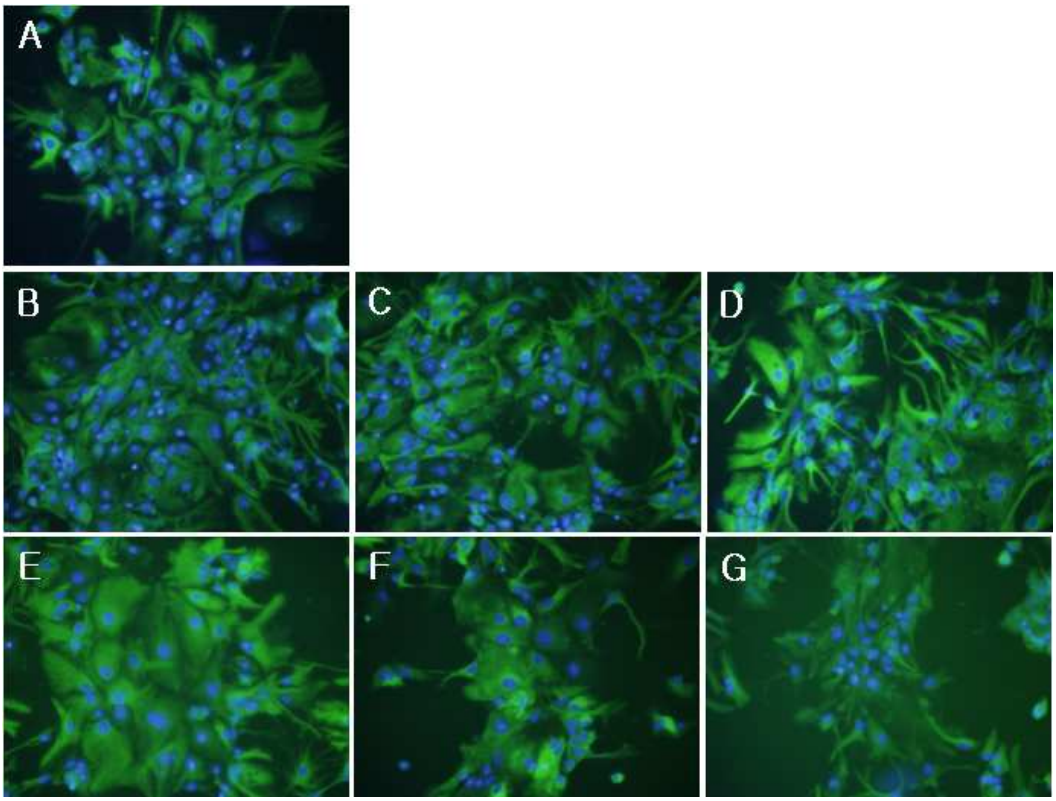


**Figure 18. Immunofluorescence staining : the neural differentiation was induced in the group of agmatine, L-arginine, and polyamine mixture at 100 uM agmatine concentration.** Neural cells differentiated from subventricular zone stem cells were imunofluorescence stained by III beta-tubulin monoclonal (1:2000, Tuj1, Chemicon), Glial fibrillary acidic protein polyclonal (1:1000, GFAP, Chemicon). Nuclei counter stained by 4'-6-Diamidino-2-phenylindole (1ug/mL, DAPI, Sigma). And genuine neurons, astrocytes were selected by checking anti-Tuj1-positive, anti-GFAP-positive cells. Stained cells were counted on the Olympua D-70 uplight fluorescent microscope. These data were from 100μM agmatine concentration. B, C, D showed eminent inducing effect on differentiation of SVZ NSCs as 206.60%, 185.36%, 166.69% respectively. **(A)** normal control group. **(B)** L-arginine group. **(C)** agmatine group. **(D)** polyamine mixture group. **(E)** putrescine group. **(F)** spermidine group. **(G)** spermine group

# IV. DISCUSSION

Agmatine, which is one of the metabolites of L-arginine has been implicated in the regulation of both proliferation and differentiation of the cells[23] and we and others showed that L-arginine is also essential for erythropoiesis[2, 3]. Among several intracellular products from L-arginine, the present study examined the effect of agmatine, L-arginine, and polyamines on the proliferation and differentiation of subventricular zone neural stem cells.

In this study, we examined the effect of agmatine on proliferation of SVZ NSCs which was found to be negative. Proliferating SVZ NSCs ought to form neurospheres, and then they start differentiation[30]. So, neurosphere formation is essential process on neural stem cell bios. We found that agmatine could not only reduce the number of neurospheres but also make them smaller. The proliferation of neural stem cells was affected by agmatine dose-dependently in this condition and we suggest that the change of the cells number which form neurosphere were decreased like as the experiments of diameter and number of neurosphere. It has reported that agmatine inhibits the tumor cells which has strong self renewal ability[21]. We also definite the result that agmatine is regulated the neurosphere formation and the proliferation of subventricular zone stem cells by these studies. Now, we had a question whether agmatine itself could regulates the proliferation of SVZ neural stem cell indeed. Following these results, we suggest that agmatine itself may regulates the proliferation and differentiation of SVZ NSCs. But, because it had been reported that agmatine is an endogenous primary amine, synthesized from L-arginine decarboxylase[2, 3, 4] and is able to modulate the cellular concentration of polyamines[14, 15, 21, 43] which are essential molecules required for cell growth[2, 3] and proliferation[23], we thought that there might be a synergic effect with something on polyamine metabolism. In the result, additional study about L-arginine and polyamines was needed.

Next, we investigated the effect of L-arginine, polyamines whether

polyamines regulated the proliferation of SVZ NSCs. Agmatine, L-arginine, putrescine, spermidine, and spermine all of them reduced the proliferation of neural stem cells as expected. Because it had already reported that agmatine, L-arginine and polyamine themselves don't have a toxicity on cells, but they inhibit proliferous cells growth by suppressing NO synthesis, polyamindecarboxylases, and ODC synthesis[21].

Agmatine treated SVZ NSCs which were compared to the control group showed different proportions in the S phase. The cells were treated with 200μM agmatine for '24 h' showed a significant increase in the number of cells in the S and G2-M phases and a decrease in the G0-G1 phase when compared to the control group. From '24 h' to '90 h', the agmatine effect was even more pronounced the accumulation portion of S and G2-M phases when the expense of the G0-G1 phase. It had been reported that the accumulation portion of S and G2-M phases means that the diploid cells and the cells preparing diploid were diminished, and are going to stop the proliferation process, to begin the differentiation process[51]. And, there was a study that stem cells are in a quiescent state for self-renewal, the immature progenitor population actively cycles for expansion, and terminally-differentiated cells are arrested in G0-G1 phases[52]. Thus, the result of the accumulation portion of the S and G2-M phase when the portion of G0-G1 phase was diminished explain SVZ NSCs stopped the proliferation and began differentiation by agmatine. Additionally, an important observation should be refered in this result. After '90 h', the G2-M phase accumulation of agmatine group increased than control group. It means that the inhibition of proliferation by agmatine promoted the differentiation of SVZ stem cells. We further investigated the differentiation-triggering effect of agmatine and polyamine.

Agmatine showed the inhibition effect on proliferation process of subventricular zone neural stem cell in all experiments, but on the differentiation process, agmatine induced neural differentiation of SVZ NSCs as 185.36% than NC, and also L-arginine recorded as 206.60% than NC in the

group of 100μM respectively. polyamine mixture (agmatine + L-arginine + putrescine + spermidine + spermine, ) increased neural differentiated cells as 166.69% in the group of 20μM, but suddenly diminished the number of neural differentiated cells over the group of 100μM and finally recorded -83.48% at 500μM. We suggest that putrescine, spermidine, spermine could not affect the increase of differentiation without agmatine, L-arginine but preferably inhibited in high dose groups. If SVZ NSCs do not make teratomatous over-proliferation, but differentiate to neural cells, we may be able to use agmatine on stem cell research usefully.

Let us look around our opinion again. According to the thesis, agmatine inhibited the proliferation of SVZ NSCs dose-dependently. L-arginine and polyamines also inhibited the proliferation of SVZ NSCs following the increasing agmatine concentration. On the other hand, agmatine and L-arginine promoted the neural differentiation about two times than NC respectively at the concentration of 100μM. However polyamines did not induceed any neural differentiation in the absence of agmatine and L-arginine rather reduced the cell differentiation depending on the examined concentration. Three possibilities are suggested in explanation of these results. (i) Polyamines themselves are not associated with differentiation of neural stem cells. (ii) External polyamines cannot be incorporated into neural stem cells. And (iii) polyamines alone cannot induce the differentiation.

At last, in the result which agmatine induced the differentiation of SVZ NSCs, we should think whether the inducing effect is by agmatine alone indeed. In the present study, the uptake of agmatine was affected by the deprivation of L-arginine in astrocytes, suggesting that intracellular level of L-arginine could regulate content of polyamines in the cells, but it may not sufficient to supply over 12 days *in vitro*, taking into consideration that agmatine and polyamines are synthesized *de novo* from L-arginine and polyamines, as is reported that there were feedback mechanism between L-arginine and agmatine. Therefore, another explanation is necessary for that

agmatine alone may could induce or not differentiation of neural stem cells.

# V. CONCLUSION

Our study demonstrates that various concentrations (0~500uM) of agmatine accumulated in subventricular zone neural stem cells without being metabolised and reduced cell proliferation without exerting any cytotoxic effect. We have demonstrated that agmatine modified the progression of the cell cycle in subventricular zone neural stem cells by accumulating the colonic cells in the S and G2-M phases, by reducing the rate and the speed of DNA synthesis and by delaying cyclin expressions. By these metabolic process, the diameter and the number of neurospheres were shrinked and diminished. The cells also proliferated newly were diminished. Finally the effect of agmatine on SVZ stem cells resulted in inhibition of proliferation.

Although we know the fact that stem cell is hilighted to research in many divisions approaching the 21th century, why we focus in the "inhibition" of stem cell proliferation. If we considerate the problems which stem cells have a character of strong self-reproduction, it is a merit to use stme cell for stroke cure[46], Alzhei-mer's disease cure[44, 45], spinal cord regeneration[47], heart infarction cure[48], bone formation and tissue engineering[49], and so on, but the strong self-reproduction character of stem cell causes the teratoma formation at times[50]. When the proliferous cells like as stem cells inhibited the proliferation, cells stop the division for proliferation and start differentiation process biologically. Now, we can see "although run after two hares, will catch either". If agmatine reduce the proliferation, that is to say, are able to induce the differentiation.

In recent study, it may be needed to regulate the overgrowth of proliferous cells like stem cells and cancer cells by agmatine to develop cell therapy methods. We suggest that agmatine inhibits the proliferation of SZV NSCs on the other hand, aids SVZ NSCs to differentiate to neural cells. Agmatine may be a novel therapeutic strategy to regulate the proliferation and differentiation of stem cell.

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국문요약

# 아그마틴에 의한 성체신경줄기세포 증식과 분화 조절

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본 연구는 증식성세포가 증식이 억제되면 분화를 시작한다는 생물학적 배경에 기초하여 그 결과를 확인하였으며, 아그마틴이 줄기세포 연구에서 가지는 의미를 제고하고자 시행하였다.

아그마틴은 생체대사에 필수적인 폴리아민과 L-아르기닌의 생합성과 분해 과정의 중간대사 물질이기 때문에 이를 이용한 동물세포의 증식조절이 가능하다는 최근의 보고가 있다. 또한, 뇌 허혈 손상 시 뇌경색 크기를 줄이는 등 신경 보호효과가 있음이 최근 보고되고 있는 생체 내 일차 아민이다. 이와 같은 아그마틴은 L-아르기닌으로부터 아르기닌탈탄산화효소에 의하여 합성되며, 세포 성장과 증식에 필수 물질인 폴리아민의 세포 내 농도를 조절하는 것으로 알려져 있다. 최근의 연구는 아그마틴이 특히 증식성 세포, 즉 암세포와 같이 증식이 왕성하게 일어나는 특징을 가지는 세포의 증식과 분화를 억제한다는 보고가 있다. 그것은 동물모델에서 형성된 종양의 성장과, 세포실험을 통해 배양한 종양 세포의 증식을 아그마틴이 각각 억제한다는 결과였으며, 이것은 과량의 아그마틴이 오히려 폴리아민 대사를 방해하여 세포사를 유도하기 때문인 것으로 보고되었다. 최근, 뇌

허혈 손상과 같이 비가역적인 뇌손상 시 줄기세포의 이식을 통해 분화가 끝난 신경세포를 새로운 신경세포로 대체하려는 시도가 활발하게 진행되고 있다. 그러나 이와 같은 줄기세포 이식과 같은 세포치료시 분화능력이 높은 줄기세포를 이용하기 때문에 이들의 과다한 증식으로 인한 기형종(teratoma)의 형성이 문제점으로 남아 있는 상태이다.

본 연구는 아그마틴이 종양세포의 증식을 억제한다는 연구배경에 기초하여 아그마틴이 종양세포와 유사하게 증식능력이 뛰어난 뇌실아래구역 성체줄기세포의 증식과 분화에 미치는 영향을 세포실험을 통하여 조사하였다. 우선, 뇌실아래구역의 신경 성체줄기세포를 생후 3일 이내의 ICR 생쥐 뇌로부터 일차 배양을 통해 얻었다. 배양이 완료된 배아줄기세포와 성체 신경줄기세포는 그것이 Nestin 항체에 양성반응을 띄는지 조사하여 순수성을 확인한 후 사용하였고, 트리판 블루 시약을 사용하여 살아남은 세포수를 정해둔 수치에 맞추어 사용하였다. 세포배양 시행 이후 4일 동안, 아그마틴을 다양한 농도(0~500μM)로 처리하여 신경세포구의 형성과정 상에서 신경세포구 크기와 수를 농도별로 비교 조사하고, BrdU 표지, FACS 분석을 통한 세포주기 분석방법을 통하여 아그마틴의 뇌실 하 구역 성체신경줄기세포 증식 억제 효과를 관찰하였다. 그 결과 아그마틴 농도 증가에 따라 세포 증식이 100μM 처리군에서 약 30% 억제되는 것을 확인하였다. 또한, L-아르기닌과 퓨트레신, 스퍼미딘, 스퍼민, 그리고 폴리아민혼합물(아그마틴, L-아르기닌, 퓨트레신, 스퍼미딘, 스퍼민을 균일 농도로 제조)을 다양한 농도로 처리하여(0~500μM) 증식억제를 확인하였다. 분화에 미치는 영향 조사는 증식이 완성되는 시점(배양 후 3.5일) 이후부터 아그마틴, L-아르기닌, 퓨트레신, 스퍼미딘, 스퍼민을 다양한 농도로 처리(0~500μM)하여 관찰하였다. 분화된 신경세포들은 각각의 표식 단백질 항체를 통한 면역염색을 시행하였다. 뉴런세포는 Tuj1, 별아교세포는 GFAP 항체를 이용하여 면역염색하였으며, DAPI를 이용하여 핵염색을 시행하였다. 농도 별로 염색된 세포 수를 비교하는 방법을 통하여 아그마틴을 비롯한 L-아르기닌, 그리고 폴리아민이 세포 분화에 미치는 영향을 조사하고, 이를 형광 현미경으로 분석하였다. 그 결과, 100μM 처리군에서 아그마틴과 L-아르기닌은 증식을 약 두 배 가량 촉진시켰으며, 폴리아민은 분화에 영향

을 주지 못하였다. 폴리아민혼합물의 경우, 20μM 처리군에서 일시적으로 약 1.7배 가량의 분화 촉진 효과를 보였으나, 농도가 증가함에 따라 급격하게 그 효과가 감소하여 약 -80% 정도의 억제 결과를 나타내었다.

핵심되는 말 : 아그마틴, 폴리아민, 뇌실 하 구역 성체신경줄기세포, 증식, 분화