Epigenetic regulation of neuronal differentiation related genes expression by amine analogues

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Directed by Professor Jong Eun Lee

A Master's Thesis
Submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of
the requirements for the degree of
Master of Medical Science

Yoon Mi Nho

December 2009

This certifies that the Master's Thesis of Yoon Mi Nho is approved.

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The Graduate School
Yonsei University

December 2009

Acknowledgement

Special Thanks: 태초부터 지금까지 함께하시고 내 반석과 피난처 되신 하나님께 사랑과 감사로 이 논문을 드립니다.

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전공이 달라 부족함이 많고 실수도 많은 저를 잘 적응할 수 있도록 배려해 주시고 지도해주신 이종은 교수님, 바쁘신 와중에도 논문에 대해 심사를 맡아주신 박경아 교수님과 박국인 교수님, 사진에 대한 조예를 가지신 이원택 교수님께도 감사를 드립니다. 제가 이 자리에 있을 수 있도록 학문적 흥미를 깨워주신 장원철 교수님, 감사합니다.

마음이 많이 여리고 예민한 저를 위해서 항상 기도해주시고 적절한 때에 좋은 말씀을 해주시는 큰 외숙(한상현 목사님), 큰 외숙모 (이기옥 사모님) 그리고 날 이 세상에 태어나게 해준 분이자 가장 친한 친구인 우리 엄마 한명숙 여사님과 아빠, 너무 예쁘고 자랑스런 동생 윤아, 절대 줄지 않은 10 년 계약 관계 이면서 언제나 주님의 마음을 깨닫게 해주는 사랑하는 동기오빠, 가족의 힘을 보여주는 우리 사촌 언니, 오빠, 동생들 그리고 형부들, 첫 조카 지은이 (이모가 지은이 때문에 많이 웃어^^), 사랑의 교회 목사님과 사모님께 감사의 마음전합니다. 이분들의 기도가 버틸 수 있는 힘이 되었습니다.

또 다른 "나"이기도 한 Dezoro 송이와 민정이, 선화, 진화, 희선, 진철, 정임이, 예쁜 후배 고은이와

현주, 정순이에게 고맙다는 말을 전합니다. 의(義)오라방 선호오빠, 성격이 닮은 진호오빠, 생방 기둥 영창오빠, 선배님 감사합니다^^.

석사를 처음 시작하던 무렵, 적응을 잘 할 수 있게 도와주시고 좋은 이야기를 해주신 너~무 (백만 번) 감사한 너무 좋은 그래서 편한 종열선생님 그리고 미란언니, 석사 입학 동기인 생리학 교실의 형아, 신경외과교실의 진형이. 그리고 비타민에게도 고맙습니다.

마지막으로 인사를 반갑게 맞아주시던 옆 실험실(사람해부학 실험실, 발생학 실험실) 선생님들과 교수님, 제가 실험할 때 가장 가까이에서 함께해준 조직학실험실 식구들에게 감사의 마음을 전합니다.

맛있는 것들을 챙겨주시던 매점 아주머니와 언제나 사고의 처리를 해주신 그래서 죄송한 마음이 더 드는 청소해주시던 아주머니까지 "연세" 라는 추억으로 소중히 간직하겠습니다

석사를 마치는 겨울에

노 윤 미 씀.

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Methylation of genomic deoxyribonucleic acid (DNA) is an epigenetic modification that has key role in gene expression by gene silencing or activation. In mammals, DNA methylation occurs mainly at carbon-5 position of cytosine residues in CpG island, typically having more than 500 base pairs in size and guanosine and cytosine content greater than 55%. This epigenetic regulation plays an important role during embryogenesis of mammals. During early postnatal development of the brain DNA methylation is believed to play a key role in regulating the proliferation of neural stem cells (NSCs) and their differentiation into neurons and glial cells.

Polyamines (putrescine, spermidine, and spermine) that are pivotal for cell growth and are synthesized *in vivo* accelerate proliferation in actively growing cells by inhibiting specifically gene expression thought DNA methylation during early embryonic development. Agmatine is an endogenous amine generated by decarboxylation of L-arginine catalyzed by arginine decarboxylase (ADC). At the

intercellular, agmatine inhibits cell proliferation by decreasing intercellular levels of the polyamines. L-arginine, the precursor of polyamine, agmatine, and nitric oxide (NO), is also associated with cell proliferation and differentiation. 5'-deoxy-5' (methylthio) adenosine (MTA) influences numerous cells process, including regulation of gene expression, proliferation, differentiation and apoptosis. Also, MTA synthesize the high rate of polyamines during cellular proliferation.

This present study investigated the regulation of neural stem cell differentiation by agmatine, L-arginine, and MTA through DNA methylation on the bone morphogenetic proteins (BMPs), as well as Noggin, SOX2 and Neurogenin2. NSCs were cultured from embryonic day 14 (E14) ICR mouse cortex and then divided normoxic and hypoxic group. Cells were maintained in neural stem cell proliferation media. NSCs were treated with L-arginine, agmatine, MTA at active proliferation (day *in vitro* 5) and before differentiation (day *in vitro* 10) stage and maintained in humidified incubator at 37 ºC with 5% CO_2. Amine analogues mediated DNA methylation was confirmed by performing methylation specific polymerase chain reaction, Western blotting and flow cytometry analysis were done for the above two groups.

The obtained results showed that agmatine decreased methylation of CpG islands upstream of the first exon of Noggin and Neurogenin2, and increased DNA methylation of BMP2 and SOX2 CpG islands in cortex-derived neural stem cells. The effect of MTA was the opposite of agmatine. In conclusion, amine analogues induce neurogenesis by regulating the expression of BMP2, SOX2, Neurogenin2 and Noggin through DNA methylation.

Key word: DNA methylation, agmatine, L-arginine, 5'-deoxy-5'- (methylthio) adenosine (MTA), neural stem cells (NSCs) differentiation

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I. INTRODUCTION

Two fundamental properties of stem cells are the ability to self-renew, and the ability to differentiate. Self-renewal requires integration of proliferative control with the maintenance of the undifferentiated state [13-17]. Multipotent neural stem cells (NSCs) give rise to the neurons, astrocytes and oligodendrocytes of the mammalian central nervous system (CNS) [15, 18]. Although the three cell types are all derived from NSCs, their differentiation is spatially and temporally regulated during development. NSC fate determination is controlled by both extracellular cues, including cytokine signaling, and intracellular programs such as epigenetic gene regulation [13, 19].

The epigenetic mechanisms of deoxyribonucleic acid (DNA) and histone modifications result in the heritable silencing of genes without changing their coding sequence [2, 16]. Gene suppression by DNA methylation is thought to occur either by the blocking of transcription factor binding and/or by formation of an inactive chromatin state [1, 16]. The bulk of the mammalian genome lacks cytosine-guanosine (CpG) sequences, but short stretches of CpG-dense DNA and clusters of CpGs called CpG islands, are often found associated with genes, and occur most often in the promoters, first exons (regions more toward the 3'end), 5' promoter end of all housekeeping genes and many tissue-specific genes at the 3' end. The CpG island in most promoters are unmethylated, a state associated with active gene transcription in a normal cell. CpG islands often function as strong promoters and have also been proposed to function as replication origins [20-23]. In higher eukaryotes, DNA is methylated only at the cytosine that are 5' to the guanosine in the CpG dinucleotide [2, 24], and the modification to 5-methylcytosine (5mC) is the only nucleotide modification that is heritable and reversible [21]. This modification plays an important role in the regulation of gene expression during embryogenesis and cell differentiation of mammals [20].

Sex determining region of Y chromosome-related high mobility group box 2 (SOX2) is a development gene that is highly expressed in the neuroepithelium of the developing central nervous system. SOX2 is a neural stem cell marker, because it is expressed in proliferating neural progenitors, and has been shown to function in NSCs. Constitutive expression inhibits neural differentiation, and results in the maintenance of progenitor characteristics [25-29]. SOX proteins contain a characteristic high-mobility group (HMG) domain that facilitates their interaction with DNA. In NSCs, SOX2 is part of general transcriptional program of neurogenesis and regulated by proneural and neurogenic basic helix-loop-helix (bHLH) transcription factors [25-28, 30, 31].

Transcription factors with bHLH motifs modulate critical events in the development of the mammalian neocortex from NSCs. NSCs are maintained in a proliferative state by bHLH factors from the Id and Hes families. The transition from proliferation to neurogenesis involves a coordinate increase in the activity of the

proneural bHLH factors, Mash1, Neurogenin1, and Neurogenin2 (Neurog2), and a decrease in the activity of the Hes and Id factors [29, 32]. Neurog2 is essential for the generating several classes of neurons [18].

Bone morphogenetic proteins (BMPs) are in the transforming growth factor β superfamily. Their signaling has a critical role *in vitro* stem cell differentiation, in the negative regulators of neural determination and in the development of heart, neural and cartilage. BMP antagonists include Noggin, Chordin, Gremlin and Dan, of which Noggin is a secreted polypeptide that binds and inactivates BMP2, 4, and 7, to prevent signal activation [13, 33-35].

BMP2 is regulated by heterotetrameric serine/threonine kinase receptors and the downstream transcription factors Smad1, 5, or 8. BMP2 has anti-neurogenic functions [36], and is reported to inhibit neuronal differentiation and promotes the generation of non-neural ectoderm, and regulate mesoderm and neural crest cell fates [34]. In contrast, Noggin enhances the production of neural progenitors from human embryonic stem cells (ESCs) mouse brain cells from mouse ESCs [37].

Agmatine is a naturally occurring guadino compound that is abundant in bacteria and plants, and was recently also found in the mammalian brain. It is an endogenous cationic polyamine formed by the enzymatic decarboxylation of L-arginine, by arginine decarboxylase (ADC). Agmatine is hydrolyzed to putrescine and urea by agmatinase. Recent studies have shown that agmatine may be neuroprotective in neurotrauma events, using both neonatal ischemia models and cultured neurons [6-10]. Also, agmatine has antiproliferative effects that correlate with the rate of cellular proliferation. Agmatine has a unique ability to arrest proliferation by depleting intracellular levels of polyamines such as putrescine, spermidine, and spermine, which are small cationic molecules required for cellular proliferation [38]. Polyamines are synthesized using s-adenosylmethionine (SAM) as a methyl donor to spermidine and spermine (Figure 1). SAM is a common substrate for polyamine synthesis and DNA methylation [9, 39].

5'-deoxy-5'- (methylthio) adenosine (MTA) is the metabolic precursor for SAM. MTA is required for a high rate of polyamine synthesis during cellular proliferation, and provides methionine for protein synthesis. MTA also is inhibits protein methylation [11].

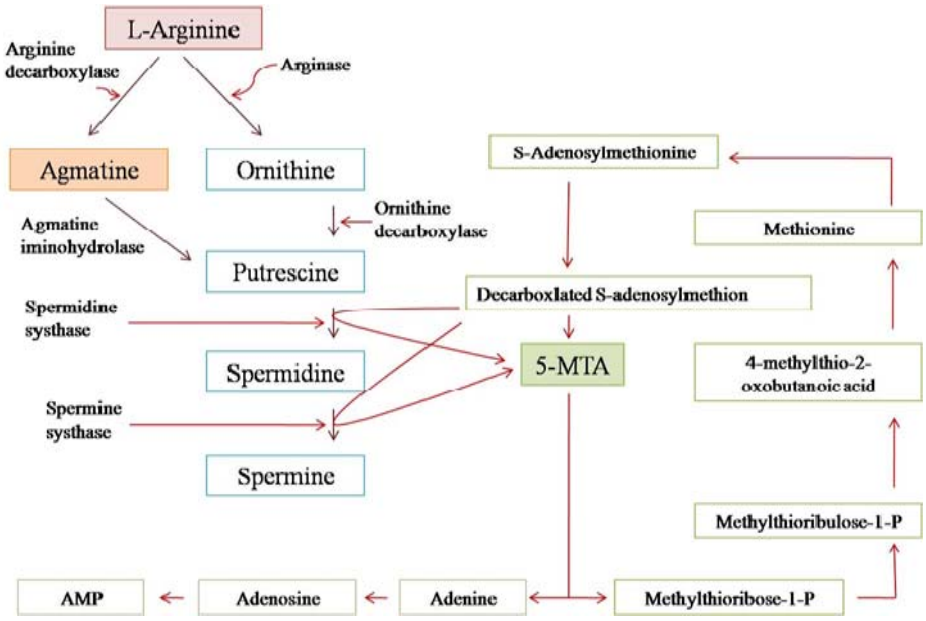

Figure 1. Metabolism of polyamines and amine analogues

In vivo, human embryos grow naturally in an oxygen (O_2) tension environments than is lower that atmospheric O_2 (~21%) tension [40]. Changes in DNA methylation of developmental genes after NSCs treatment with exogenous amine analogues in hypoxic conditions were also investigated.

Hypoxia (1% O_2) has been shown to inhibit self-renewal and induce early differentiation of mouse ESC through the induction of hypoxia-inducible factors-1α (HIF-1α) and subsequent suppression of leukemia inhibitor factor (LIF) - signal

transducers and activators of transcription protein 3 (STAT 3) signaling *in vitro* leading to growth arrest and finally to apoptosis. Interestingly, an O2 tension higher than atmosphere conditions (40%) slows ESC differentiation. However, a low O2 (3-5% O_2) inhibits human ESC differentiation, and exerts positive effect on the establishment and maintenance of mouse ESCs [40]. CNS tissue partial pressure of oxygen (pO_2) values is conserved among mammalian species, and ranging from as low as 0.55% in the midbrain, to 8.0% in the pia. The pO_2 of cortical grey matter in rodents is 2.53-5.33%, while measurements in human brain show mean pO_2 values from 3.2% at 22-27 mm above the dura to 4.4% at 7-12 mm below to dura. These numbers suggest that a steadily decreasing oxygen gradient is formed as blood reaches the brain tissues. Tissue oxygen perfusion is often disrupted in pathological states, such as ischemia-reperfusion and head injury and may be altered in hyperbaric therapy [41]. Moreover, low O_2 tension is known to induce angiogenesis and glycolysis for cell growth, and to increase the percentage of specific neuronal types, and represses neuronal differentiation [40]. Hypoxia regulates differentiation in stem- and precursor cells in culture. In the CNS, increased proliferation, reduced apoptosis, promote survival, and lead to inhibition of differentiation [42].

This study investigated the regulation of DNA methylation and differentiation, and the regulation of BMP2, SOX2, Noggin, and Neurog2 in normoxic and hypoxic conditions when treated with L-arginine, agmatine, and MTA. Differentiation patterns were altered by amine analogue treatment. Agmatine triggered differentiation, while MTA had the opposite effect.

This study provides information relevant to the possible recovery of ischemia related lesions, by implantation of NSCs at the injury site.

II. MATERIALS AND METHODS

1. Primary Neural stem cell (NSC) culture

Time-pregnant ICR mice (Coatech) were used for NSCs, which were prepared from the cortex of embryonic day 14 (E14) mice. Briefly, cortical tissues were isolated and placed in Hanks' balanced salt solution (Gibco, 14170). The isolated tissue was dissociated into mono-cells in neural stem cell media by gentle pipetting. Dissociated cells were cultured in a neural stem cell basal medium (Stem Cell Technologies, 05700), 20 ng/ml EGF (Invitrogen, 13247-051) with a proliferation supplements (Stem Cell Technologies, 05701) on T75 plates at a seeding density of 3.0 x 10^4 cells/ml.

2. Treatment of L-arginine, agmatine and 5'-deoxy-5'-(methylthio) adenosine (MTA) to neural stem cells

A 10 mM stock solution's of L-arginine (Sigma, A5131), agmatine (Sigma, A7127), and MTA (Sigma, D5011) were prepared in distilled water and kept at -20ºC until use. NSCs were incubated in culture medium with 20, 50, 100, and 200 μM of L-arginine, agmatine and MTA in normoxia at day *in vitro* (D.I.V) 5 and 100 μM L-arginine, agmatine, and MTA for normoxia D.I.V 10 groups, hypoxic-injured D.I.V 5 and D.I.V 10 group were also simultaneously processed.

3. Atmosphere-controlled incubation

Normoxic group, NSCs were grown in a humidified chamber with 5% CO_2, at 37 ºC. For hypoxic-injured groups, NSCs were transferred to an anaerobic chamber (Forma Scientific co.) and maintained at 0.2% O_2, 5% CO_2, and 94.8% N_2. Cells were dissociated into mono-cells in deoxygenated culture medium, seeded on a T25 plate (2.0×10^5 cells/ml) and incubated for 5 min in the chamber. After hypoxic injury, NSCs were grown in the same conditions as normoxic groups.

4. Genomic DNA extraction and Sodium bisulfate modification

Genomic DNA was extracted from NSCs according to the manufacturer's protocol (Exgene Tissue SV Mini kit, Geneall, 104-101). Briefly, harvested cells were washed in phosphate-buffered saline (PBS) and centrifuged at 14,000 g for 20 sec. Pellets were treated with lysis buffer, with 20 μL of 2 mg/mL poteinase K solution, vortexed, and heated at 56 ºC for 30 min vortexing every 10 min. Tissue binding buffer was add, moved to a column and washed with washing buffer by centrifuging at 6,000 g for 1 min. Elution buffer was added to the column and centrifuged at 14,000 g for 1 min. The eluted DNA was stored at -20 ºC until sodium bisulfate modification.

Sodium bisulfate modification of genomic DNA the methylation-specific polymerase chain reaction (PCR) was performed using an Epitect Bisulfate kit (Qiagen, 59104), which converts all unmethylated cytosine residues to uracil. The thermal cycle reactions were 12 μL genomic DNA, 8 μL ribonulease (RNase) -free water, 85 μL sodium bisulfate, and 35 μL DNA protectant. Modified DNA was quantified by ultra violet (UV) spectrophotometer (Phatmacia Biotech) at 260 nm and stored at -20 ºC.

5. Methylation specific polymerase chain reaction (MSP)

MSP primers were designed using Methprimer program to contain CpG sites. The primer sets used to amplify each gene incorporated one CpG site specific for a methylated sequence, and one for an unmethylated sequence (Table 1; U= unmethylated sequence, M= methylated sequence).

Table 1. Designed Primer Sequences by methprimer program

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Size (bp)
BMP 2 -U	TTTTTTATTATTTAAAATAGAAGTGT	AAATATAAAACCAACCCCCAAA	223
BMP 2 -M	TTTTTTATTATTTAAAATAGAAGCGT	AAATATAAAACCGACCCCCG	223
Noggin -U	AAATTTAAAGGTTTGGATTTTGTGA	TCTCTCTACCTACTCCAAACAACAC	135
Noggin -M	TTAAAGGTTTGGATTTTGCGA	TCTACCTACTCCGAACAACGC	127
SOX 2 -U	GGGGGATATAAAGGTTTTTTAGTG	AAAAAATAAATAAATTTCCAACAAC	196
SOX 2 -M	GGGGGATATAAAGGTTTTTTAGC	AAAAAATAAATAAATTTCCGACGAC	196
Neurog 2 -U	TTTTTTTATTAATAAATTAGTGGTATGT	CAAACTACAACCCAAACTACCAAA	132
Neurog 2 -M	TTTTTATTAATAAATTAGCGGTACGT	AAACTACAACCCAAACTACCGAA	131

MSP performed according to manufacturer's protocol (Epitect MSP kit, Epitect, 59305). Briefly, PCR for each gene was in 50 μL containing 2x Master mix buffer, 19 μL RNase-free water, 2 μL each primer (forward and reverse) and 2 μL of sodium bisulfate modified DNA. Amplification was 95ºC for 10 min, 94ºC for 15 sec, 50ºC for 30 sec, and 72ºC for 30 sec for 40 cycles, followed by 72ºC extension for 10 min. PCR products were resolved by 6% agarose electrophoresis in tris-acetate ethylenediaminetetraacetic acid buffer (TAE) and stained with ethidium bromide.

6. Cell cycle analysis

NSCs were harvested by centrifugation at 800 rpm for 5 min. Cells were gently resuspended using a Neurocult chemical dissociation kit (Stem Cell Technologies, 05707), according to the manufacturer's instructions. Cells were fixed by adding 1.0 mL cold ethanol (70%) drop-wise to a tube containing 0.5 mL of cell suspension in PBS while gently vortexing. Fixed cells were keeping at -20ºC for overnight, then washed once with PBS and centrifuged at 1680 rpm for 5 min before adding 100 μL of 1mg/mL RNase (Bioworld, Korea). Cells were incubated at room temperature for 20 min, and then stained with propidium iodide (Sigma, P4170) at 1 μg/mL for 5 min at room temperature. Cells were analyzed by flow cytometry (Becton Dickinson, Facscaliber) at 488 nm. Histogram plots were created using Cell Quest program.

7. Western blot analysis

NSCs were harvested by centrifugation at 800 rpm for 5 min and washed once with PBS then centrifuged at 13000 rpm for 2 min. Washed cells were treated in RIPA buffer containing 150 mM sodium chloride (NaCl, Sigma, S7653), 1% NP-40 (Sigma, I3021), 0.5% sodium deoxycholate (Sigma, D6750), 0.1% sodium dodecyl sulfate (SDS, Sigma, l3770), 50 mM tris-hydrochloric acid (tris-HCl, pH 8.0, Sigma, T3253) and protease inhibitors of 1 μg/ml pepstain A (Sigma, P5381), 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma, T9011), 5 mM ethylenediaminetetra acetic acid (EDTA, Sigma, E5134), 5 mM ethylene glycol bis (-aminoethylether) -N, N, N', N'-tetraacetic acid (EGTA, Sigma, E3889), 5 mM sodium fluoride (Sigma, S6521) and 1 mM sodium orthovanadate (Sigma, S6508) and incubated at 4ºC for at least 2 hr. Extracted protein suspensions were centrifuged at 13200 rpm at 4ºC for 30 min and the supernatant collected for Western analysis. Protein concentration was determined using a BCA kit (Thermo Scientific, 23209) and measured using an

enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Spectra Max 340) at 562 nm.

Equal amounts of total protein were loaded on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto an Immobilon P polyvinylidenedifluoride (PVDF, Milipore, IPVH00010). Non-specific binding was blocked by a blocking solution containing 5% skim milk (BD, 20121127) and 0.05% tween-20 in tris-buffer saline (TBS). Blots were incubated with primary antibodies anti-Noggin (1:500, Abcam, ab16054), anti-BMP2 (1:500, Abcam, ab6285) diluted in TBS with 0.05% tween-20. GAPDH (1:1000, Abcam, ab8245-100) was detected for normalization of protein loading. After secondary antibody reaction (1:3000, Abcam), binding was detected with an ECL detection reagent kit (Thermo Scientific). Target protein expression was calculated as the optical density ratio of the target protein to the housekeeping protein GAPDH. The expression of BMP2 and Noggin was measured by Sconic NIH Image Analysis Software (Image J) version 3.5 (USA).

8. Hoechst and propidium iodide (PI) nuclear staining

Cell death was evaluated by staining the non-viable cells with PI (Sigma, P4170), and living cells with Hoechst 33258 dye (Sigma, H6024).

To determined hypoxic injury time, NSCs at D.I.V 9 in a 24-well plate were treated as described above for 5 min, 30 min, 1 hr, and 4 hr, then stained with 5 mg/mL Hoechst in a humidifying incubator at 37ºC, 5% CO_2 for 30 min, and 2 mg/mL PI in the same conditions for 5 min. Stained cells were observed using epifluorescence with a UV filter block microscope (Olympus).

9. Statistics

Results are presented as mean ± standard deviation (SD). Statistical significances for the flow cytometry data and Western blot data were determined by Student's t-test. $P < 0.05$ was considered a significant difference.

III. RESULTS

1. Neurosphere formation when amine analogues were treated

Neural stem cells (NSCs) cultured from E14 mouse cortex, were treated with arginine, agmatine, and 5'-deoxy-5'- (methylthio) adenosine (MTA) with dosage of 20, 50, 100 and 200 μM under normoxic condition at D.I.V 5. Normally at D.I.V 3~3.5, NSCs formed neurosphere by aggregation of mono- cells.

The results showed that mono-cells were dissociated from neurosphere dose-dependently. However, reverse pattern was observed in arginine and MTA treated NSCs showing dissociation of the neurosphere into mono-cells at lower dosage only (Figure 2).

2. Effect of amine analogues on differentiation-inhibiting genes under normoxia at D.I.V 5

NSCs were obtained in a stage of active proliferation at D.I.V 5. BMP2 and SOX2 were selected as differentiation-inhibiting genes, and primers designed, for the regulatory region containing a CpG island in front of the first exon. Two primer sets one for methylated and one for unmethylated and MSP was performed on genomic DNA.

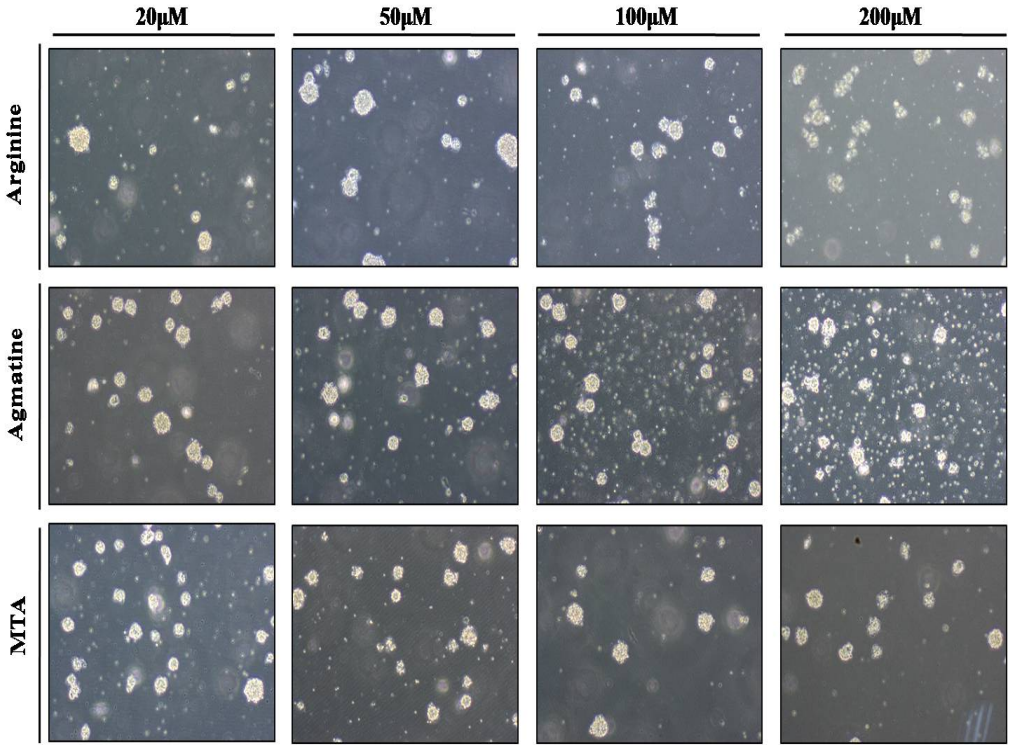

Figure 2. Photographs of growth of neural stem cells after amine analogues treatment. NSCs formed neurosphere were dissociated to mono-cells at low dosage in arginine and MTA treatment. But, NSCs with agmatine treatment showed opposite effect compared to arginine and MTA (x20).

In agmatine-treated NSCs, DNA methylation of BMP2 and SOX2 genes increased compared to untreated NSCs, in dose-dependent manner. However, methylation of BMP2 and SOX2 genes decreased dose-dependently in the MTA-treated group. Thus, MTA and agmatine caused opposite responses. Methylation in the arginine-treated group increased up to 100 μM (Figure 3, 4).

This result suggested that agmatine acts to accelerate NSC differentiation in the active proliferation stage. In contrast, MTA and arginine reduced NSC differentiation.

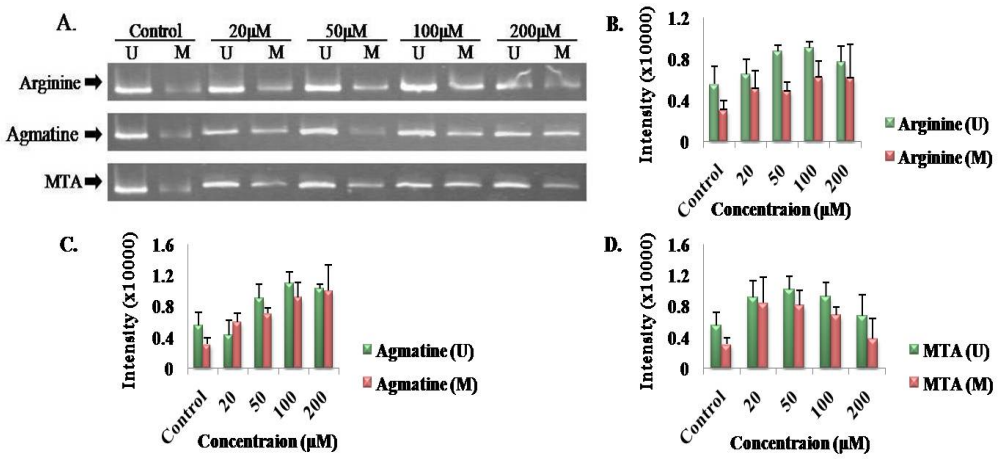

Figure 3. MSP analysis of BMP2 gene in neural stem cells under normoxic condition at D.I.V 5. (A, B, and C) BMP2 methylation band intensified in arginine- and agmatine-treated groups in a dose-dependent manner compared to the control. (A, D) MTA- and arginine-treated groups showed the opposite effect, also dose-dependently.

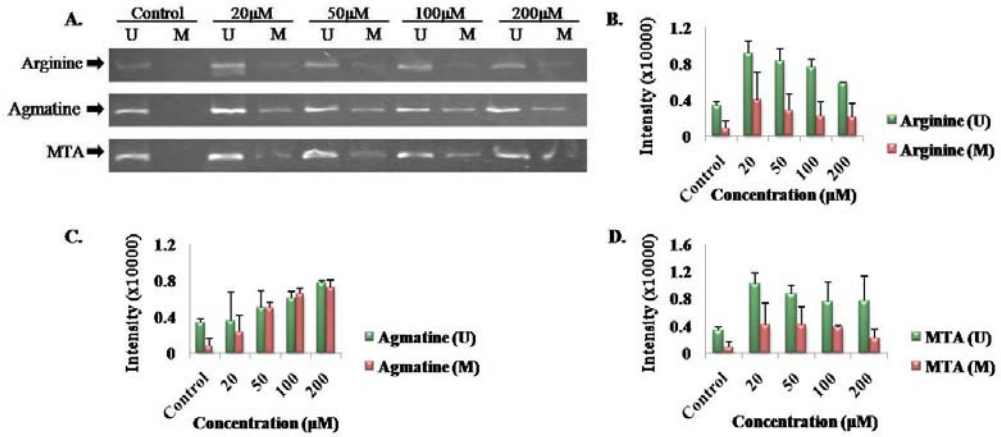

Figure 4. MSP analysis of SOX2 gene in neural stem cells under normoxic condition at D.I.V 5. (A, C) SOX2 in NSCs indicated suppressed differentiation with agmatine. (A, B, and D) MTA- and arginine-treated groups showed a methylation band that was reduced compared to the agmatine-treated group.

3. DNA methylation of differentiation-inhibiting genes with amine analogues in neural stem cells under normoxia at D.I.V 10

To understand the changes in DNA methylation of BMP2 and SOX2 during active NSCs differentiation, MSP was performed using 6% agarose gels, showing a threshold limit of 100 μM for all the amine analogues tested under normoxic condition at D.I.V 5.

DNA methylation at BMP2 and SOX2 was observed in agmatine-treated NSCs. In contrast, no methylation of these genes was seen in MTA-treated cells. SOX2 methylation was reduced in MTA-treated NSCs (Figure 5).

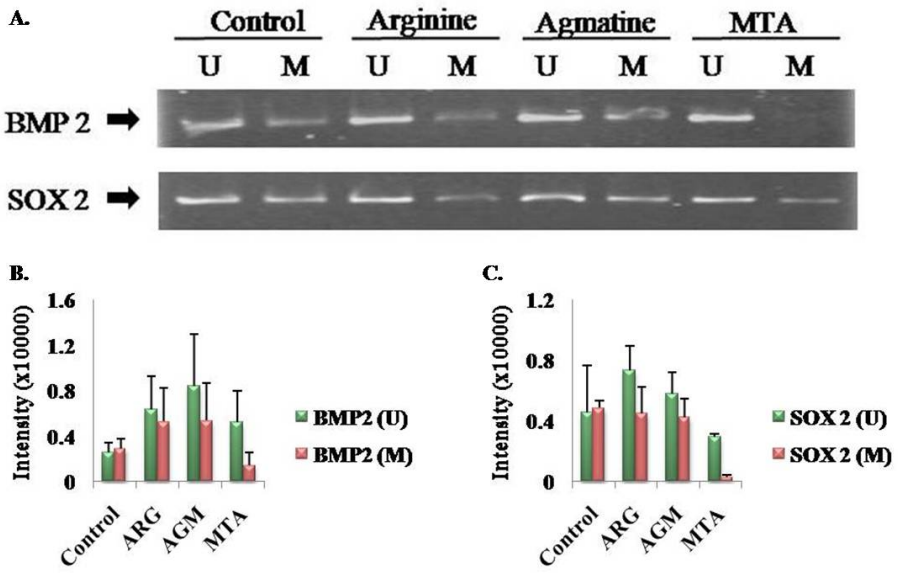

Figure 5. MSP analysis of BMP2 and SOX2 under normoxic condition at D.I.V 10. Agmatine-treated groups showed methylation of BMP2 (A, B) and SOX2 (A, C) compared to other amine analogue-treated groups. MTA- and arginine-treated groups showed less methylation for BMP2 and SOX2 compared to the control (all amine analogue treatments were 100 μM).

4. Regulation of DNA methylation in differentiation-promoting genes by amine analogues under normoxia at D.I.V 5

The effect of amine analogue treatment on NSC differentiation through regulation of Noggin and Neurog2 was examined by treating NSCs with arginine, agmatine, and MTA at 20, 50, 100, and 200 μM.

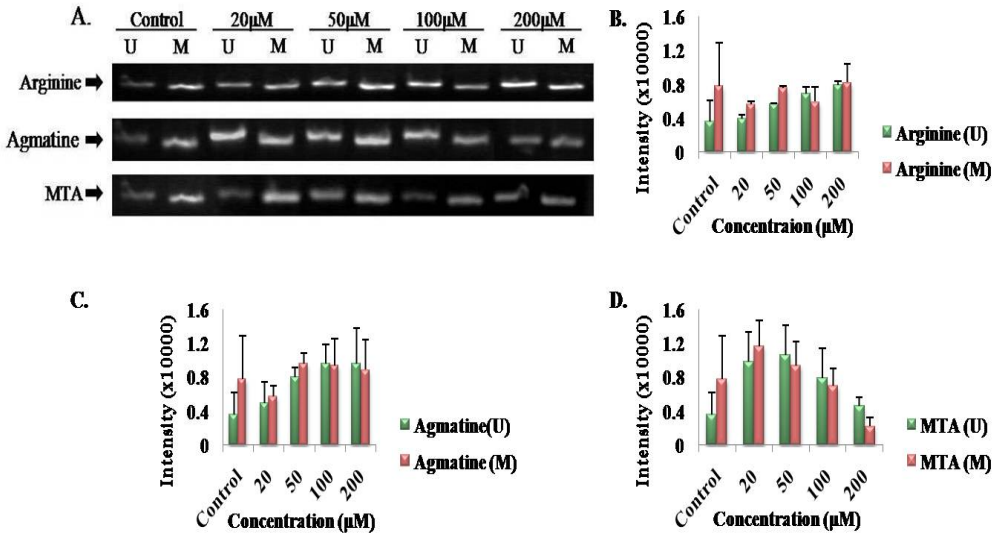

Figure 6. MSP analysis of Noggin gene in neural stem cells after amine analogues treatment under normoxic condition at D.I.V 5. (A, C) Noggin, a BMP2 antagonist, showed decreased methylation and increased unmethylation in the agmatine-treated group. (A, B, and D) MTA- and arginine-treated groups showed decreased unmethylated bands.

An unmethylated band from Noggin was detected in the agmatine-treated group and increased dose-dependently up to 100 μM (Figure 6A, C). However, arginine and MTA treatment caused effects that were the opposite of agmatine treatment (Figure 6A, B, and D). Arginine and MTA had no effect on the methylation pattern of Neurog2 until 50 μM treatments with arginine and 100 μM for MTA respectively (data not clearly shown). However, nonmethylated of Neurog2 was seen for all amine analogue treatment groups (Figure 7).

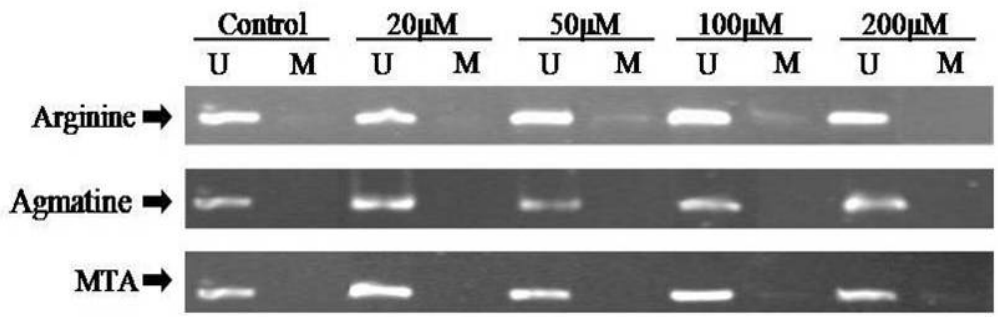

Figure 7. MSP analysis of Neurog2 in neural stem cells after amine analogues treatment under normoxic condition at D.I.V 5. The agmatine-treated group showed no unmethylated Neurog2, while the methylated band increased. MTA- and arginine-treated cells showed unmethylated Neurog2 with low methylated Neurog2.

5. DNA methylation of differentiation-promoting genes with amine analogues under normoxia at D.I.V 10

From above results prompted to investigate how DNA methylation changes at the Neurog2 and Noggin genes at D.I.V 10, when cell differentiation begins using 100 μM amine analogue treatments of NSCs.

Under normoxic conditions at D.I.V 10, agmatine treatment resulted in reduction of methylation at both Noggin and Neurog2, with the unmethylated band from Noggin increasing relative to the control, and the methylation band decreasing in intensity. DNA methylation did not change in arginine-treated cells (Figure 8).

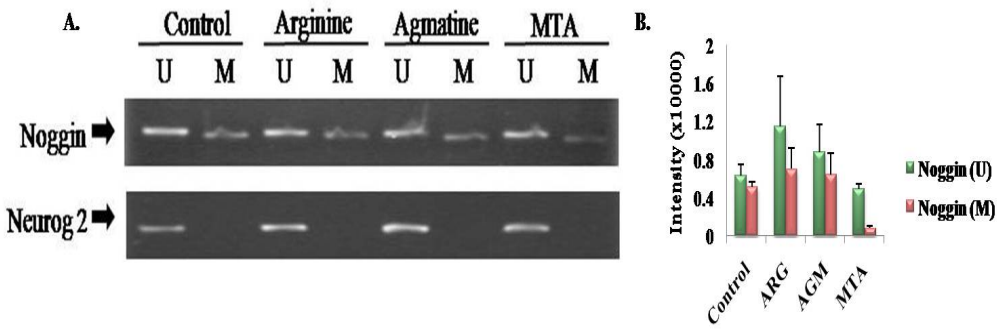

Figure 8. MSP analysis of Noggin and Neurog2 after amine analogues treatment under normoxic condition at D.I.V 10. By both Noggin (A, B) and Neurog2 (A), agmatine accelerated differentiation in NSCs. MTA treatment showed the opposite effect as the agmatine-treated group. Arginine-treated NSCs showed no changes compared to the control (all amine analogue treatments were 100 μM).

6. Protein expression of BMP2 and Noggin in neural stem cells after amine analogues treatment under normoxia

Based on the MSP results, further studies were conducted to know that DNA methylation has an effect on protein expression of the transcription. NSCs were treated with 100 μM amine analogues and extracted proteins analyzed by Western blot with anti-BMP2 and anti-Noggin antibodies. Normoxia D.I.V 5 and D.I.V 10 groups were studied (N=3).

Arginine-treated NSCs showed decreased expression of BMP2 and Noggin up to 50 μM of treatment, and BMP2 and Noggin expression increased with 100 μM

arginine. Noggin expression was higher than BMP2 at 20 μM treatment, suggesting that arginine may accelerate cell proliferation. The expression of BMP2 and Noggin decreased in arginine-treated NSCs at 20 μM and 50 μM, but Noggin expression increased with 100 μM (Figure 9A, B).

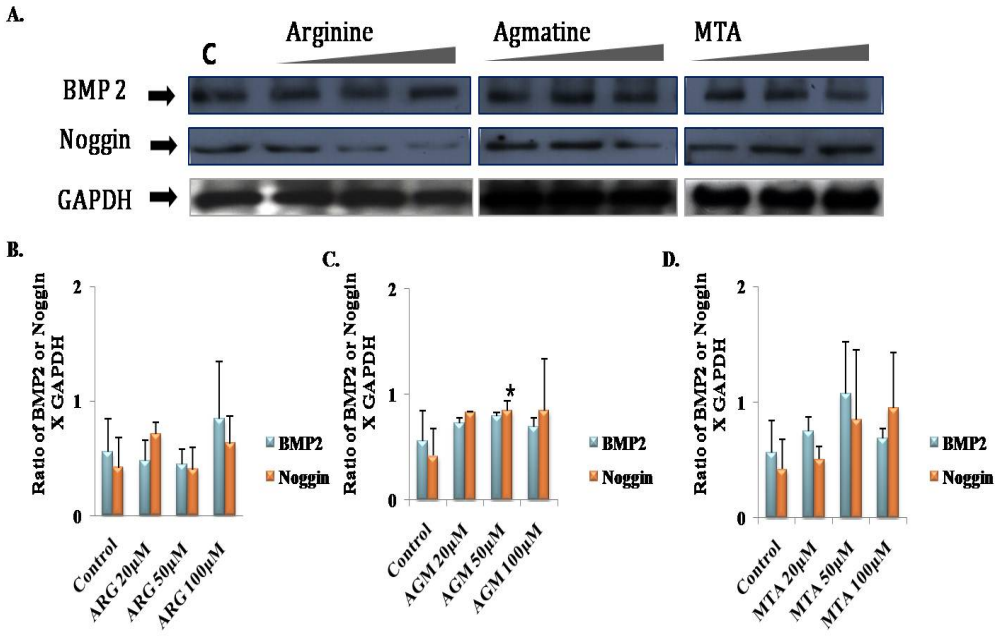

Figure 9. Western blot analysis of BMP2 and Noggin with amine analogues in neural stem cells under normoxic condition at D.I.V 5. (A, B) In the arginine-treated group, BMP2 expression decreased with 50 μM treatment. The ratio of BMP2 and Noggin expression was reversed at 100 μM. (A, C) In the agmatine-treated group, Noggin and BMP2 expression were higher than the control. Noggin expression rapidly increased initially and then leveled. (A, D) In the MTA-treated group, BMP2 expression increased up to 50 μM treatment. At 100 μM, the ratio of BMP2 and Noggin expression was reversed compared to the control the other treatments. (*: $P < 0.05$)

Agmatine-treated NSCs exhibited higher Noggin expression than BMP2 expression, although the results were significant only at 50 μM. In particular, Noggin expression rapidly increased initially and was steadily maintained in the agmatine-treated group, indicating that agmatine enhances the differentiation of NSCs under normoxic condition at D.I.V 5. This was in accordance with the MSP results (Figure 9A, C). In contrast to the agmatine results, MTA-treated NSCs showed higher expression of BMP2 than Noggin up to 50 μM treatment (Figure 9A, D).

All amine analogue-treated groups showed high expression of Noggin and low expression of BMP2 in NSCs in normoxia at D.I.V 10. MTA appeared to play a role in cell differentiation acceleration, similar to agmatine (Figure 10).

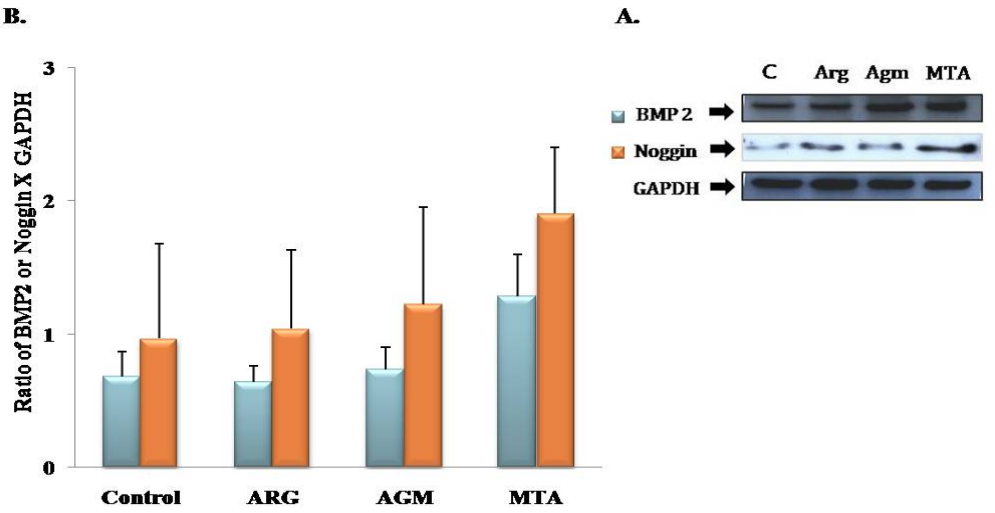

Figure 10. Western blot analysis of BMP2 and Noggin with amine analogues in neural stem cells under normoxic condition at D.I.V 10. BMP2 expression increased in the MTA-treated groups, but expression decreased slightly with arginine treatment. Noggin expression increased in all groups, especially the MTA-treated group (all amine analogue treatments were 100 μM).

7. Cell cycle analysis of neural stem cells after amine analogues treatment under normoxia

Above experiment result confirmed that agmatine up-regulates NSCs differentiation at the DNA level. NSCs were grown for 5 days proliferation duration and 10 day differentiation span, and stained with PI. Differentiation of stained NSCs was determined by cell cycle analysis using flow cytometry and the results were in accordance with the MSP results. The cell cycle is divided into G1/G0, S, and G2/M phases. G1/G0 represents NSCs in proliferation stage, S and G2/M indicated NSCs in differentiation stage.

Agmatine-treated groups showed a linear increase in proliferation up to 100 μM treatment (Figure 11B), supporting the hypothesis that agmatine plays an important role in differentiation regulation at the DNA level. NSC proliferation was significantly reduced compared to the control, for agmatine treatment at 50 and 100 μM. However, the arginine and MTA-treated groups showed an opposing trend to agmatine treatment. The G1/G0 data were corresponding with S+G2/M data (Figure 11C), and the results were consistent with the MSP and Western blot data.

G0/G1 phase cells decreased with agmatine and arginine treatment under normoxic condition at D.I.V 10 (Figure 12A). Unexpectedly, MTA treatment increased the S+G2/M phase cells significantly. This suggested that MTA helps stimulate differentiation of NSC differentiation. Agmatine was an up-regulator of NSC differentiation at both the activation of proliferation, and the differentiation stages. MTA treatment down-regulated NSC differentiation at the proliferation stage, but was a better activator of differentiation over time agmatine.

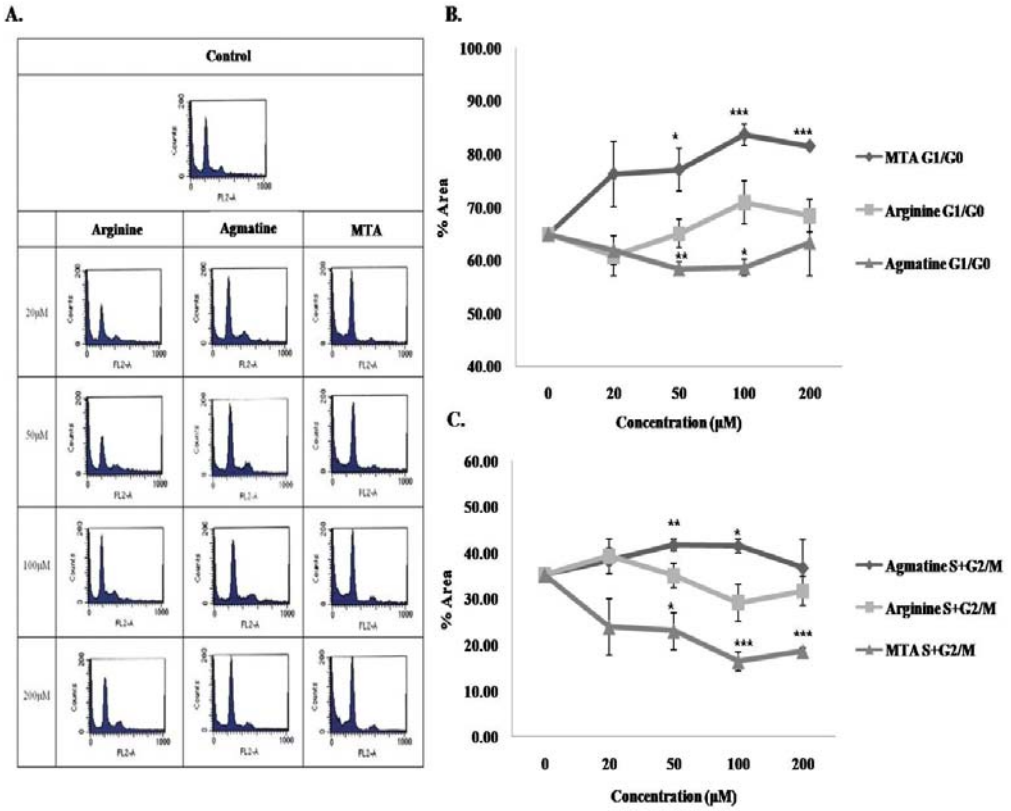

Figure 11. Flow cytometry analysis of neural stem cells after amine analogue treatment at D.I.V 5 under normoxia. (A) All amine analogue treatments at D.I.V 5 changed the cell cycle phase, dose dependently. The first peak represents G1/G0, the second G2/M, with S phase between the peaks. (B, C) NSCs showed decreased proliferation rates as shown by the G0/G1 stage, up to 100 μM agmatine. Agmatine treatment accelerated NSC differentiation by activating S+G2/M, while NSCs showed the opposite effect with other amine analogues. (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$)

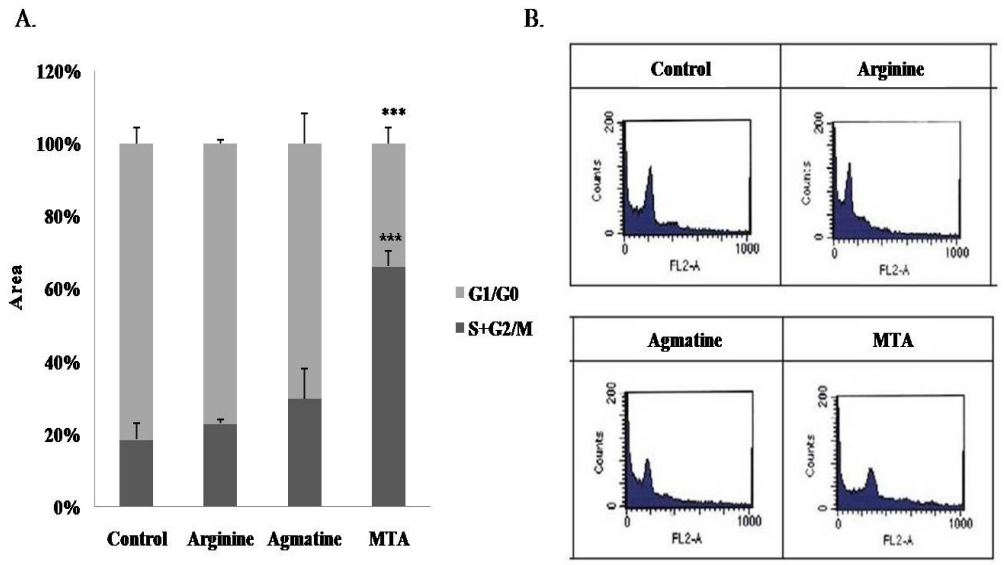

Figure 12. Flow cytometry analysis of neural stem cells after amine analogues treatment at D.I.V 10 under normoxia. (A) S+G2/M phases increased in arginine, agmatine, and MTA treatment at D.I.V 10. MTA treated cells showed a significant increase in S+G2/M compared to the control. (B) All amine analogue treatments at D.I.V 10 changed the cell cycle phase, dose dependently. The first peak represents G1/G0, the second G2/M, with S phase between the peaks. (***: $p < 0.001$; all amine analogues treatments were 100 μM)

8. Determining the survival of neural stem cells under hypoxic condition

To determine the time for hypoxic injury, NSCs were stained with Hoechst and PI. Hoechst visualizes the nucleus of live cells under a blue UV fluorescence microscope, and PI stains the nuclei of dead cells, which appear red in color under the UV. At 5 min of hypoxia, death occurred in a few cells, with the neurosphere

disaggregating into mono-cells, and death occurring after 30 min (Figure 13). Based on this result, hypoxic injury was determined at 5 min.

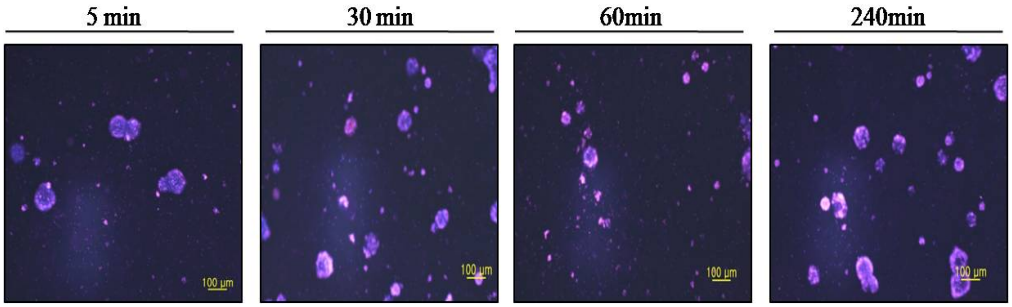

Figure 13. Photographs of survival of neural stem cells stained with Hoechst and propidium iodide (PI). Hoechst stained live cells (blue) and PI stained dead cells (red). As hypoxic injury time increased, cell survived was profoundly declined.

9. DNA methylation of BMP2 and SOX2 promoter region after amine analogues treatment under hypoxic condition

Cells were treated with arginine, agmatine, and MTA treatment at 100 μM, and BMP2 and SOX2 examined to determine inhibition of NSC differentiation.

At D.I.V 5 with hypoxic injury, unmethylation of BMP2 gene was higher than control after treatment with arginine, agmatine, and MTA, indicating that the self-renewal properties of NSCs are more active in hypoxic conditions. Unmethylated SOX2 was also observed after arginine and agmatine treatment of NSCs, but methylated SOX2 was seen only after MTA treatment, suggesting that hypoxic conditions led to strong activation of NSCs proliferation (Figure 14A).

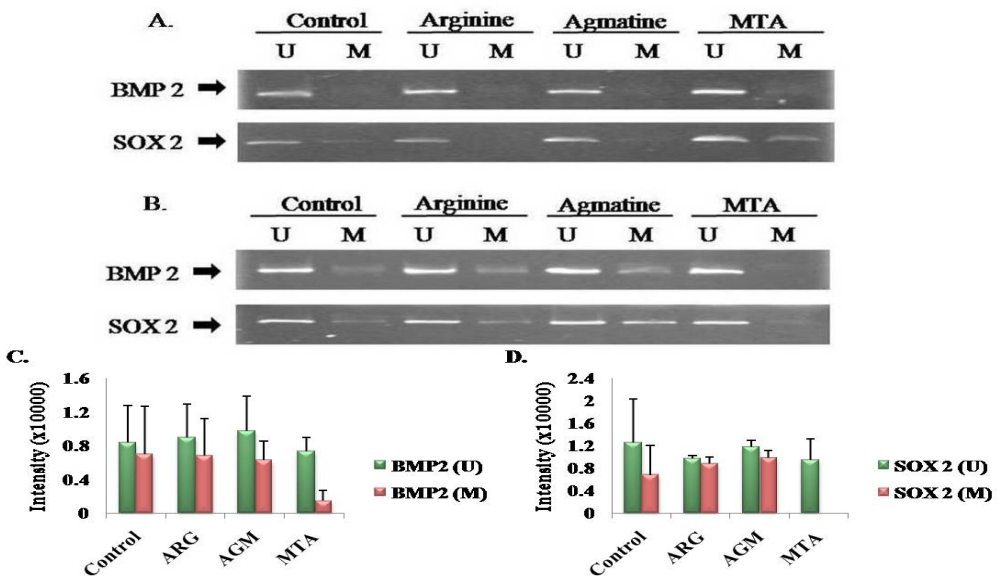

Figure 14. MSP analysis of BMP2 and SOX2 after amine analogues treatment under hypoxic condition. (A) At D.I.V 5, agmatine- and arginine-treated groups did not show methylation in the NSC- differentiation-repressive genes BMP2 and SOX2. MTA-treated cells showed methylation of SOX2 and BMP2, indicating differentiation was not suppressed. (B, C, and D) At D.I.V 10, methylation of BMP2 and SOX2 genes was strong for the agmatine-treated group compared to the control. After arginine treatment, methylation of BMP2 and SOX2 genes was weak. Methylation of BMP2 and SOX2 was not seen in the MTA-treated group.

Under hypoxic condition at D.I.V 10, methylation of the BMP2 and SOX2 decreased after arginine and agmatine treatment, while MTA treatment caused an increase (Figure 14B, C, and D). These results support the hypotheses that hypoxic conditions lead to NSCs proliferation, and that agmatine controls NSC differentiation through expression of BMP2 and SOX2.

10. DNA methylation of Noggin and Neurog2 promoter regions after amine analogues treatment under hypoxic condition

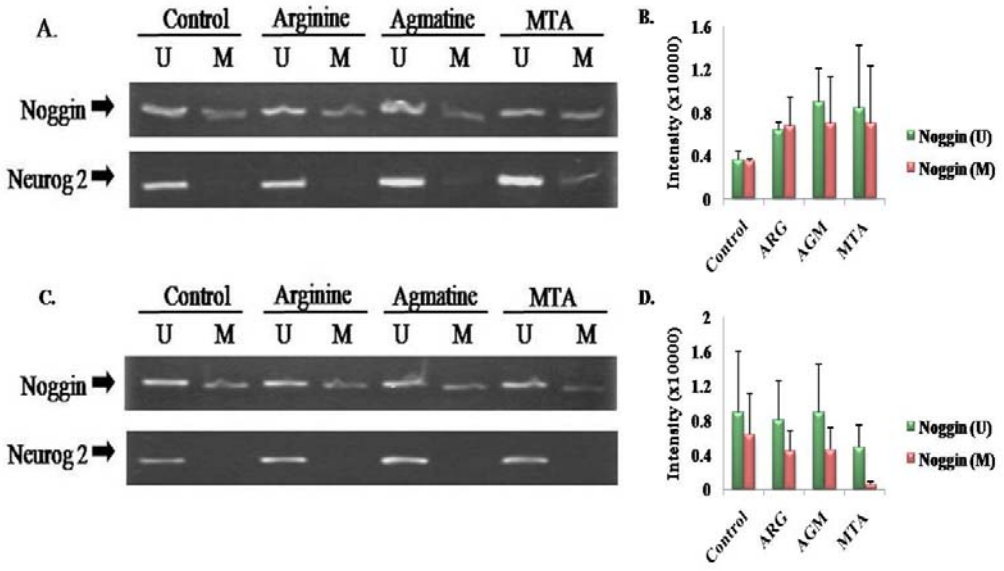

Figure 15. MSP analysis of Noggin and Neurog2 after amine analogues treatment under hypoxic condition. (A, B) At D.I.V 5, Agmatine treatment did not change methylation in Noggin and Neurog2. MTA-treated cells showed methylation of Noggin and Neurog2. (C, D) At D.I.V 10, methylation of Noggin was weak after agmatine treatment. The unmethylated Neurog2 band did not appear after agmatine treatment.

The effect on the differentiation-enhancing Noggin and Neurg2 genes was also examined by MSP. Unmethylated Noggin and Neurog2 genes were clearly observed in agmatine-treated cells under hypoxic condition at D.I.V 5 but it had a weaker effect than D.I.V 5 cells in the normoxic condition. Methylation of the Noggin and Neurog2

genes was seen after MTA treatment under hypoxic conditions at D.I.V 5 (Figure 15A, B), but this effect disappeared under hypoxic conditions at D.I.V 10. NSCs with arginine treatment were not different than control cells (Figure 15C, D).

11. Protein expression of Noggin and BMP2 after amine analogues treatment under hypoxia

NSCs cultured from E14 mice were subjected to low O_2 tension, and treated with amine analogues at 100 μM. At D.I.V 5 and 10, protein was extracted and equal amounts analyzed by Western blot.

In hypoxic D.I.V 5 cells, BMP2 expression was higher than Noggin for all groups, indicating 5 days after hypoxic injury conditions, greater activation of proliferation was seen than under normoxia conditions. However, expression of BMP2 decreased in the agmatine-treated group relative to Noggin expression. In the arginine treated group, Noggin expression decreased relative to BMP2, and in the MTA treatment group, BMP2 and Noggin expression decreased compared to control (Figure 16A, B).

The results of Western blot analysis, of cell under hypoxic conditions at D.I.V 10 showed similar results for all amine analogue treatments. Specifically, all amine analogue-treated groups expressed higher levels of BMP2 than Noggin (Figure 16C, D).

This is because hypoxic injury activates the proliferation phase. Noggin expression was slightly increased after arginine treatment, but no difference was seen in BMP2 expression after this treatment. Expression of Noggin increased compared to BMP2 in agmatine-treated cells, which supports the hypothesis that agmatine is involved in NSC differentiation. Conversely, MTA tend to cause slightly more Noggin expression than BMP2

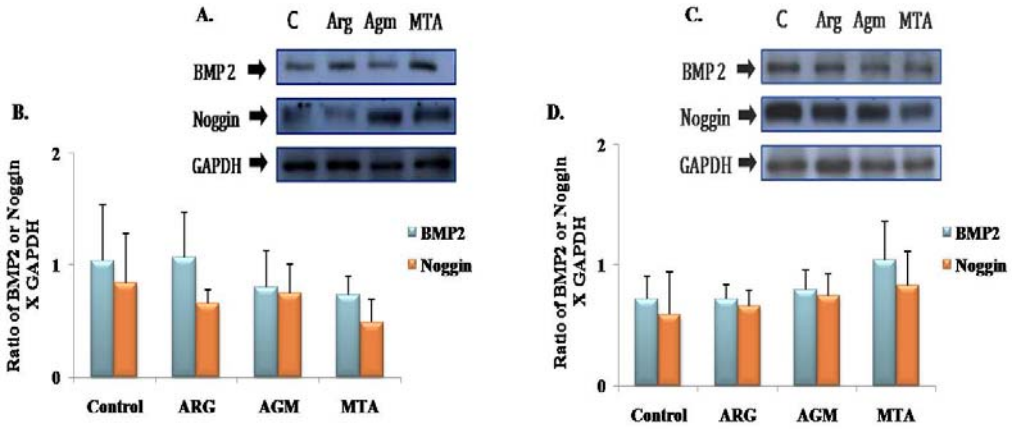

Figure 16. Western blot analysis of BMP2 and Noggin with amine analogues in neural stem cells under hypoxic condition. (A, B) BMP2 expression was reduced compared to the control after arginine and MTA treatment. Agmatine-treated NSCs did not show differences in BMP2 and Noggin expression under hypoxic condition at D.I.V 5. Noggin expression was lower than BMP2 in the control group. (C, D) Under hypoxic conditions at 10 days, Noggin expression was higher than BMP2 after agmatine or arginine treatment, compared to the control. BMP2 expression increased more than Noggin expression. (all amine analogue treatments were 100 μM.)

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IV. DISCUSSION

The investigation of stem cell proliferation and differentiation has been rapidly growing since the 1980s, when ESCs were first isolated [47]. NSCs have a self-renewing ability and can transform into neurons, oligodendrocytes, and astrocytes [13-18]. These unique properties have been promising for stem cell therapy for brain diseases such as stroke or cerebral infarction. Amine analogues participate in cell proliferation and differentiation. For example, agmatine blocks cell proliferation, while MTA and arginine, a precursor of agmatine and ornithine, control polyamine synthesis, which promotes cell proliferation [5, 11, 12]. This research aimed to investigate whether amine analogue treatment promotes differentiation in NSCs via DNA methylation.

MSP result of BMP2 and SOX2 gene expressions revealed that in normoxia group of agmatine treatment at D.I.V 5, DNA of SOX2 showed no methylation (Figure 3). This supports the model that SOX2 is a SOXB1 transcription factor that universally marks neural pregenitors and stem cells. SOX2 shares more than 90% amino acid identity with the HMG DNA binding domain [25-29]. Previous studies reported that SOX2 is down-regulated as cells exit their final cell cycle, and that SOX2 acts on an upstream signaling molecule of the Notch signaling pathway. This pathway appears to play an essential role in the maintenance of a stem cell-progenitor cell pool. During embryogenesis and in adulthood, expression of Notch1 or its downstream regulators, such as HES-1, inhibits neuronal differentiation, and results in the maintenance of the progenitor state. However, the exact mechanism by which Notch signaling regulates cell fate has not completely determined. SOX2 interacts with the minor groove of the DNA helix, causing a transcriptional permissive change in chromatin structure. Moreover, it controls the expression of several developmentally important genes, including Oct4, Nanog, nestin, δ-crystalline, fibroblast growth factor 4, undifferentiated embryonic cell

transcription factor 1 and F-box containing protein 14, which play crucial roles in embryonic development. Thus, NSCs might differentiate into neurons when SOX2 is inhibited or degraded, and Notch signaling is repressed [25-28, 30, 31]. This study found that the control cells under normoxic conditions at D.I.V 10 showed methylation of SOX2, revealing that D.I.V 10 is the time-point for differentiation (Figure 5). Based on these and previous findings, we propose that agmatine accelerates NSC differentiation by methylating the BMP2 and SOX2 genes at the 5th day and 10th day of primary culture. However, MTA and arginine treatment reduced NSC differentiation as seen by their effects on these genes, although MTA treatment did not cause methylation of BMP2 under normoxic condition at D.I.V 10 (Figure 3, 4, and 5).

Control cells showed low amounts of unmethylated Noggin (Figure 6, 8), which is a differentiation-enhancing gene that encodes a BMP2 antagonist. BMPs promote cellular differentiation in non-neural tissues; including bone, bone marrow, kidney, and lung. But BMPs sustain self-renewal and pluripotency in mouse ESCs. They have also been reported to promote a neural crest phenotype in CNS precursors isolated from the embryonic and adult brain and spinal cord [34]. Furthermore, BMP2 acts synergistically to induce astrocytogenesis of fetal mouse telencephalic neuroepithelial cells, although it does not trigger neurogenesis [48]. The BMP antagonist Noggin increases the number of dopamine neurons generated *in vitro* from human and mouse ESCs derived from mouse PA6 stromal cells, promotes neural differentiation from ectoderm, and serves as a rostral induction factor [49].

However, all amine analogue-treated groups show strongly unmethylated Neurog2 (Figure 7, 8), indicating that NSCs were already differentiating into neural system cells. This explains why NSCs express Neurog2 [18, 29, 32], which is a number of a large family of basic helix-loop-helix (bHLH) transcription factors, many of which are encoded in the human genome. A small set of bHLH factors have important roles in cell fate decisions during corticogenesis, including members of the NeuroD, Neurogenin, Mash, Olig, Id, and Hes families [29, 32]. The MSP results for Noggin and

Neurog2 were same as for BMP2 and SOX2. Thus, agmatine treatment appeared to promote differentiation by up-regulating the Noggin and Neurog2 genes in NSCs, while arginine and MTA treatment inhibited differentiation (Figure 6, 7, and 8).

Based on MSP results, Western analysis was performed using NSCs treated with 100μM polyamines. The data showed clear expression of BMP2 and Noggin proteins relative to SOX2 and Neurog2. The protein levels in amine analogue-treated NSCs were in accordance with the DNA methylation results. The normoxia D.I.V 5 control cells showed increased expression of Noggin compare to BMP2, while normoxia D.I.V 10 control cells showed increased BMP2 compared to Noggin. Thus this study proposes that normoxia D.I.V 5 represents a phase of active proliferation, and normoxic D.I.V 10 represents an active differentiation period (Figure 9, 10). Little change was in the total DNA of NSCs (Figure 11, 12).

NSC differentiation is dependent on the action of agmatine at the DNA level. Moreover, agmatine has been shown to have several functions in the brain and the periphery [50-56], including anti-proliferative effects. In this present study exogenously administered agmatine enhanced NSC differentiation by suppressing intracellular polyamine and inducing antizyme expression via a programmed +1 ribosomal frame shift. It is the only known endogenous molecule, other than the canonical polyamines, with this capacity. Agmatine also induces spermidine/spermine acetyltransferase, which promotes the conversion of higher-order, more highly charged molecules to low-order polyamines. The anti-proliferative effects of agmatine thus appear to be due to polyamine depletion, and not caused by agmatine functionally replacing or displacing the canonical polyamines [38].

MTA controls NSC differentiation. Results from MSP, Western analysis, and FACS, revealed that MTA prevents NSC differentiation in the active proliferation period, but promotes it during the active differentiation period. This might be why MTA is mainly produced during the biosynthesis of polyamines in mammalian tissues (Figure 1), when a high rate of polyamine synthesis occurs during cell proliferation.

MTA is also produced mainly through the polyamine biosynthetic pathway. However, MTA inhibits the function of ornithine decarboxylase, which synthesizes putrescine from ornithine. Therefore, if the rate of accumulation of MTA inside the cell is faster than the rate of MSP phosphorylase, MTA blocks polyamine biosynthesis [11, 38]. This may explain why MTA powerfully accelerated NSC differentiation in normoxic conditions at D.I.V 10, an active differentiation phase.

L-arginine is a precursor for the synthesis of not only proteins but also nitric oxide (NO), polyamines, and agmatine [57]. Previous studies have reported that NO is increased in neural stem cell differentiation by introducing nitric oxide synthase (NOS) inhibitors such as N^G-monomethyl-L-arginine, N^G-iminoethyl-L-ornithine, aminoguanidine, N^G-nitro-L-arginine, and N^G-nitri-L-argininemethylester, or by NOS-enhancer treatment [58-62]. However, this study found that when L-arginine was exogenously added to NSCs, proliferation was activated, suggesting that excess arginine availability triggered the hydrolytic cleavage of arginine to urea and ornithine by arginase, resulting in excess polyamine synthesis, leading to proliferation. Arginine analogues are potent inhibitors of NOS, and NO is a metabolite in the intracellular citrulline-NO cycle. Therefore, if L-arginine is high, it may influence the polyamine to NO pathway through L-arginine catabolism [57, 63].

Hypoxic injury induces glycolysis for cell growth, inhibits differentiation, and improves morphologic scores [40-42]. This found it led to arrest of NSC differentiation at the DNA and protein level. MSP and Western blots (Figure 14, 15, and 16) showed that NSCs at hypoxia D.I.V 5 showed more active cell proliferation than normoxia D.I.V 5 cells. Supporting to this current previous study reported polyamine biosynthesis was activated at hypoxia D.I.V 5 suggested the accumulation rate of MTA is faster than its degradation [11]. MTA treatment enhanced NSC differentiation, although the rate of NSC proliferation decreased in hypoxic injury conditions at D.I.V 10. Results for DNA and protein were consistent with the results for normoxia D.I.V 5.

This study suggests that agmatine treatment up-regulated NSC differentiation, and MTA treatment also promoted NSCs differentiation at the 5th day after hypoxic injury. However, MTA down-regulates differentiation in NSCs at hypoxia D.I.V 10. Arginine treatment had no effect on cell differentiation and proliferation. One notable finding was that agmatine treatment played an important role in accelerating NSC differentiation by controlling DNA methylation of BMP2, SOX2, Noggin, and Neurog2 genes, and expression of their proteins. However, MTA treatment of NSCs resulted in effects that were opposite to the other amine analogues, enhancing NSC proliferation at the active cell proliferation stage, and increasing NSC differentiation at the active cell differentiation period.

V. CONCLUSION

Arginine, agmatine and MTA treatment resulted in transcriptional regulation of differentiation genes via DNA methylation in NSCs, both in normoxic and hypoxic conditions.

1. Agmatine treatment enhanced NSC differentiation by methylation of BMP2 and SOX2, and unmethylation of Noggin and Neurog2.
2. Arginine treatment suppressed NSC differentiation.
3. MTA treatment inhibited NSC differentiation during active cell proliferation but enhanced differentiation during the differentiation phase.

From these facts this study concludes that DNA methylation is the mechanism by which amino analogue treatment regulates NSC differentiation. This may lead to a useful therapeutic approach for using neural stem cell transplantation at brain damage regions *in vivo*.

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Abstract (in Korean)

아민 유도체에 의한 신경줄기세포 분화 관련 유전자들의 후성체 조절

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노 윤 미

Deoxyribonucleic acid (DNA) 메틸화는 유전자 발현을 억제하거나 일부 유전자의 발현을 활성화하기도 한다. 포유류에서는 첫 번째 엑손 앞에 존재하고 시토신과 구아닌이 밀집되어 있는 CpG island 라는 곳에서 5'방향에 위치한 시토신에서 DNA 메틸화가 흔히 발생하게 된다. 이 유전자 조절은 배아형성과정에서 특정 세포에 특이적인 유전자 발현 조절에 중요한 역할을 한다. 뇌의 발생 초기 동안에는 신경선조줄기세포 들이 신경계 세포로 분화 하는 과정에서 DNA 메틸화 과정을 겪게 된다. 특히 생체 내에서 합성되는 폴리아민은 뇌의 발생 초기 과정에서 특정 유전자의 발현을 DNA 메틸화를 통하여 억제 시킴으로써 증식이 빠른 세포의 성장을 가속화 시킨다. 아그마틴은 알기닌에서 알기닌탈탄산효소에 의해 생성되는 일차 아민으로 세포의 생존 및 내성을 강화시키는 물질로 알려져 있으며, 이는 폴리아민이 세포막 안으로 이동되는 것을 막음으로써 증식을

억제시키며, 그 전구체인 알기닌은 폴리아민과 결합하여 세포의 증식을 촉진시키는 것으로 알려져 있고, 5'-deoxy-5'- (methylthio) adenosine (MTA)는 폴리아민의 대사를 촉진시킨다.

이와 같은 다양한 아민 유도체들이 세포의 증식 및 분화조절에 관여하는 사실을 바탕으로 증식이 활발한 시기와 분화되기 바로 전 시기의 신경선조줄기세포를 이러한 시기에서 뇌의 발생이 일어나는 환경과 유사한 저산소 자극을 준 뒤, 알기닌, 아그마틴, MTA 를 처리하여 증식과 분화관련 유전자인 BMP2, SOX2, Noggin, Neurogenin2 에서의 DNA 메틸화를 조사하고자 하였다. 이를 위해 Methylation specific polymerase chain reaction (MSP), Western blotting, Flow cytometry 를 시행하였다.

그 결과, 아그마틴은 분화 억제 유전자인 BMP2 와 SOX2 에서 DNA 메틸화를 시키고 분화 촉진 유전자인 Noggin 과 Neurogenin2 에서는 DNA 메틸화가 일어나지 않게 함으로써 분화를 촉진하지만, MTA 는 그와 반대작용을 하는 것으로 나타났다. 이는 아민유도체인 아그마틴이 후성유전적 과정에 관여함으로써 신경줄기세포의 분화에 기여하는 것으로 보인다.

핵심 되는 말: DNA 메틸화, 알기닌, 아그마틴, 신경줄기세포 분화, MTA (5'-deoxy-5'- (methylthio) adenosine).