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Impact of muscarinic receptor activation on neural stem cell differentiation

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The University of Toledo

A Thesis

Entitled

Impact of Muscarinic Receptor Activation on Neural Stem Cell Differentiation

by

Shufan Ge

Submitted to the Graduate Faculty as partial fulfillment of the requirements

for the Master of Science Degree in Pharmaceutical Sciences

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An Abstract of

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Since neurogenesis was demonstrated in adult mammalian brain, the field of neural stem cell (NSC) research has been in a state of rapid growth. However, it is still not very clear when and how NSCs begin to differentiate into other cell types and express different proteins. Recent studies suggest that some neurotransmitters such as acetylcholine (Ach) are involved in the proliferation as well as the differentiation process of NSCs. The aim of the present study was to determine the role of muscarinic receptors in the process of differentiation in NSCs.

In the present studies, we used NSCs from the hippocampus of the adult rat brain and cultured them in differentiation media. The expression of different protein markers such as nestin and GFAP was observed by cell imaging. There was a strong expression of both nestin and GFAP in astrocytes, but not in cells developing in neuronal pathways. The expression of GFAP was significantly increased after NSCs were differentiated into astrocytes.

A study was then done to understand the effects of muscarinic receptor activation on NSCs during differentiation. The expression of mRNA for the five muscarinic

receptor subtypes was determined using the RT-PCR technique in different types of cells. The m3 mAChR mRNA was the only subtype detected in each cell type. Carbachol was used as a cholinergic agonist to stimulate muscarinic receptors in NSCs in the present studies. Under the conditions we used, carbachol had no significant effect on the expression of nestin or GFAP in the process of differentiation, which suggests that carbachol does not promote differentiation into neuronal or astrocytic cells in NSCs.

Since extracellular signal-regulated kinase (Erk 1/2) phosphorylated after treatment with carbachol, and it is a marker for proliferation in many cell types, we examined the stimulation of ERK1/2 by carbachol in neural stem cells and astrocytes. Through immunoblotting and immunohistochemistry, we observed a rapid phosphorylation of ERK1/2 in stem cells as well as astrocytes after carbachol treatment for 5 minutes. The maximal stimulation was achieved at 0.1 mM carbachol in both cell types, accompanied by a decrease in total ERK(1/2) expression. Additional studies are necessary to understand the physiological significance of inducing ERK(1/2) activation.

Acknowledgments

I greatly appreciate my advisor Dr. William S. Messer's support and encouragement during the last two years. I would like to thank Dr. Linda Dokas for her guidance and advice. I could not have completed this project without their help.

I would like to thank my thesis committee members Dr. Surya Nauli and Dr. Youssef Sari for their valuable comments and suggestions

I am thankful to Dr. Ming-Cheh Liu and Dr. Frederick E. Williams, who were very helpful and generous in offering suggestions as well as equipments.

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I finally would like to thank my family and friends for their love and encouragement.

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Chapter 1

Introduction

Neural stem cells in the adult brain

Neural stem cells represent a special somatic cell type that have the capacity for long-term self-renewal and generate different neural lineages (Duan et al.,2008). Since neural progenitor and stem cells were isolated from adult mice brain tissue for the first time (Reynolds et al., 1992), scientists have successfully isolated neural progenitor and stem cells from various regions in the adult brain and from different species including human (Taupin et al., 2002). In the adult brain, neural stem cells are found mainly in two regions: subventricular zone (SVZ) of the lateral ventricles (LV) and subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (Lois C et al., 1994).

Subventricular Zone

The SVZ is a layer of dividing cells that extends along the lateral walls of the lateral ventricles. It is the adult brain region with the highest rate of neurogenesis. Proliferation of stem cells in the SVZ has been confirmed by [^{3}H]thymidine and bromodeoxyuridine (BrdU) incorporation into dividing cells. In labeling experiments, cellular progeny have been demonstrated to migrate through the rostral migratory

stream (RMS) to reach the olfactory bulb, where they differentiate into two kinds of inhibitory neurons: granule and periglomerular cells (Doetsch, et al., 2001; Altman, 1969). There are several cell types in the SVZ: migrating neuronal precursor cells (type A cells), GFAP-positive astrocytes (type B cells), rapidly dividing transit-amplifying cells (type C cells), and the ependymal cells (type E cells) (Doetsch, et al., 1997; Garcia-Verdugo et al., 1998). There has been considerable debate as to which cells in the SVZ are the multipotent 'stem cells' that give rise to the migrating neuronal type A cells. And some recent discoveries suggest that type B cells, the astrocytes, give rise to new type C cells which in turn differentiate into the migrating neural cells (Doetsch et al., 1999).

Subgranular Zone

The SGZ is located in a thin lamina between the granule cell layer and the hilus in the dentate gyrus of the hippocampus. In the adult SGZ, cells that express sex determining region Y-box 2 (Sox2) have the differentiation potentials to give rise to both neurons and astrocytes. (Suh et al., 2007). Experiments using BrdU to label dividing cells in the SGZ show that after BrdU treatment, about half of the cells in which BrdU is detectable after four weeks develop a neuronal phenotype. In contrast to the distant migration under taken by olfactory bulb neurons, hippocampal granule neurons move a relatively short distance into the granule cell layer and incorporate into the hippocampal neural circuit. About 15% of the cells differentiate into glial cells, and the remaining 35% of cells do not show a detectable neuronal or glial phenotype (Kempermann et al., 1997).

Development of Neural Stem Cells

Although *in vivo* NSCs appear to generate almost exclusively neuronal cells, their developmental potential is very broad. Cultured NSCs have the ability not only to give rise to neurons, but also to generate astrocytes and oligodentrocytes.

The neurons, also known as nerve cells, are electrically excitable cells that process and transmit information by electrical and chemical signaling. They are the core components of the nervous system. A typical neuron consists of a cell body, dentrites, and an axon. At the majority of synapses, signals are sent from the axon of one neuron to a dentrite of another. Generally, neurons in the adult brain do not undergo cell division, and usually cannot be replaced after being lost. In most cases, they are generated by stem cells. After being born, new neurons can form networks with other existing neurons. The functional relevance of adult neurogenesis is still not clear, but there is some evidence that hippocampal adult neurogenesis is important for learning and memory (Neves et al., 2008). And neurogenesis may also play a role in reversing cognitive deficits in mouse models of Alzheimer's Disease (Wang et al., 2010).

Astrocytes are stellate glial cells with multiple fine processes in the brain as well as the spinal cord. They are the most numerous cell type in the central nervous system and make up about 50% of human brain volume. Astrocytes are divided into three major types according to morphology and spatial organizaiton: radial astrocytes surrounding ventricles, protoplasmic astrocytes in gray matter, and fibrous astrocytes located in white matter (Privat et al., 1995). Astrocytes perform many functions that

are important for normal neuronal activity, including glutamate uptake, glutamate release, K^+ and H^+ buffering, and water transport. They provide structural, trophic, and metabolic support to neurons and modulate synaptic activity as well. The interactions between neurons and astrocytes are essential for normal brain function (Chen et al., 2003).

Oligodendrocytes are a type of neuroglia. They originate from migratory, mitotic progenitors that mature progressively into postmitotic myelinating cells. In the central nervous system, their main function is the isolation of axons. And in recent years, it has been revealed that oligodendrocytes have important functions beyond those related to myelin formation and maintenance, including participation in neuronal survival and development, as well as neurotransmission and synaptic function (Baumann et al., 2001).

Muscarinic acetylcholine receptors

Muscarinic acetylcholine (Ach) receptors (mAChRs) are members of the 7 membrane-spanning, guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR) superfamily. There are five subtypes of mAChRs, and these mediate the actions of Ach in the central nervous system as well as in the periphery. The five muscarinic receptor subtypes are designated as M_1 – M_5. The odd-numbered receptors (M_1, M_3, M_5) couple to $G_{q/11}$ and activate phospholipase C, which initiates the phosphatidylinositol trisphosphate cascade leading to intracellular Ca^{2+} mobilization and activation of protein kinase C. On the other hand, the even-numbered receptors (M_2, M_4) are coupled to the $G_{i/o}$ class of G proteins and inhibit adenylate cyclase and

reduce the intracellular concentration of cAMP.

Muscarinic acetylcholine receptors in the brain

All five muscarinic receptor subtypes are expressed in the brain. They activate many signaling pathways important for the modulation of neuronal excitability, synaptic plasticity and feedback regulation of Ach release (Volpicelli et al., 2004).

The predominant mAChR in the CNS is the M_1 subtype. They are abundantly expressed in all major regions within forebrain, including the cerebral cortex, hippocampus, and corpus striatum (Oki et al., 2005). Consistent with this distribution, muscarinic M_1 receptors are implicated in learning and memory processes. The activation of cholinergic receptors improves cognitive decline in preclinical and clinical studies (Terry et al., 2003). Furthermore, selective activation of M_1 receptors has been suggested as a therapeutic approach in dementia, including Alzheimer's disease as well as age-associated cognitive impairments or memory impairments associated with schizophrenia (Fisher et al., 2003).

M_2 receptors are located throughout the brain. They are mainly found in the brainstem and thalamus, though also in the cortex, hippocampus and striatum where they are thought to control Ach release (Rouse et al., 1997; Raiteri et al., 1990). Like M_1 receptors, M_2 receptors in the central nervous system also play an important role in cognitive function. M_2 knockout mice exhibit deficits in working memory, hippocampal plasticity, and behavioral flexibility (Seeger et al., 2004). Furthermore, in M_2 receptor-deficient mice, the oxotremorine (muscarinic receptor agonist)-mediated antinociceptive response was greatly attenuated in both tail-flick

and hot plate test, implicating M_2 receptors in muscarinic agonist-induced analgesia (Gomeza et al., 1999).

M_3 muscarinic receptors are widely distributed in the brain, although the expression level is markedly lower as compared to levels of the M_1 and M_2 receptors (Wess, 2004). Generally, M_3 receptors are expressed in smooth muscle and glandular tissues (Levey, 1993). And there are no obvious behavioral or cognitive defects observed in M_3 receptor-deficient mice (Yamada et al. 2001).

Muscarinic M_4 receptors are found in many brain regions including the cortex and hippocampus, although they are most abundant in the striatum (Levey, 1993). Consistent with this observation, locomotor activity was significantly increased in M_4 receptor-deficient mice, suggesting these receptors may play a role in controlling dopamine release and locomotor activity (Gomeza et al., 1999).

M_5 receptors have a discrete localization in the substantia nigra pars compacta, a structure in the midbrain providing dopaminergic innervation to the striatum. M_5 receptors are expressed in dopamine-containing neurons, and activation of muscarinic M_5 receptors facilitates striatal dopamine release. Concordantly, there are experiments showing that oxotremorine-mediated dopamine release in the striatum was markedly reduced in M_5 receptor-dedicient mice (Yamada et al., 2001). M_5 knockout mice exhibit reduced reward responses to opioid administration and fewer naloxone-induced withdrawal symptoms as compared to wild-type animals (Basile, 2002). Blockade of M_5 receptors thus might be useful in the treatment of drug abuse.

Cholinergic activation of hippocampal neural stem cells

Adult hippocampal neurogenesis has been demonstrated in rodents as well as in mammalian species, including humans (Altman et al., 1965; Kempermann et al., 1997). The process of adult hippocampal neurogenesis includes the proliferation of NSCs, migration to a target area, differentiation of progenitor cells, and the survival of immature new neurons. New neurons are generated in the subgranular zone of dentate gyrus in hippocampus throughout adult life. About half of the newly generated neurons die between 10 days and 1 month after their birth. It is still unclear what factors determine the fate of these newly formed neurons. The functional role of neurogenesis in the hippocampus is not yet defined conclusively, but there are some studies showing a relationship between hippocampal neurogenesis and the performance of hippocampus-dependent learning and memory tasks. For example, after being trained on learning tasks which require an intact hippocampus, there is an increase in adult-generated neurons in the rat dentate gyrus (Gould et al., 1999). On the other hand, the suppression of neurogenesis by treatment with an antimitotic agent causes impairment of hippocampus-dependent learning (Shors et al., 2001, 2002). Therefore, hippocampal neurogenesis might be associated with cognitive function. However, the regulatory mechanisms underlying the activation of NSCs have remained unclear.

The hippocampus receives abundant regulatory inputs from the basal forebrain acetylcholine system. And it is generally accepted that the cholinergic system is important for normal cognitive function. Drugs that block central acetylcholine

muscarinic receptors impair learning (Ogura et al., 1993). Furthermore, the degeneration of acetylcholine-releasing neurons has been observed in Alzheimer's disease patients (Coyle et al., 1983).

Therefore, it is considered that the cholinergic system is involved in the regulation of adult hippocampal neurogenesis. Several studies have examined the role of cholinergic pathways in neurogenesis. After selective lesions of basal forebrain cholinergic neurons, which project to the two main regions of adult neurogenesis, neurogenesis declined significantly in the granule cell layers of dentate gyrus in hippocampus (Cooper-Kuhn et al., 2004; Mohapel et al., 2005). Conversely, the administration of acetylcholine esterase (AchE) inhibitors promoted hippocampal neurogenesis (Mohapel et al., 2005). These studies indicate that NSCs can be stimulated by cholinergic activation. The cholinergic system primarily promotes the proliferation or the short-term survival rather than the long-term survival of new neurons (Mohapel et al., 2005). The present study determined the effects of activating cholinergic systems on NSCs in the process of differentiating into different cell types.

Chapter 2

Materials and Methods

Materials

Cryopreserved adult rat hippocampal neural stem cells were purchased from Chemicon (Temecula, CA). Both rat neural stem cell expansion medium and astrocyte differentiation media were obtained from Millipore (Temecula, CA). The neural stem cell marker characterization kit, which includes anti-Nestin, anti-Sox2, anti-GFAP, mouse IgG and rabbit IgG antibodies, was purchased from Millipore (Temecula, CA). Phospho-p44/42 MAPK(Erk1/2) mouse mAb was purchased from Cell Signaling technology (Beverly MA). Anti-MAP Kinase1/2(Erk1/2) antibody was bought from Millipore (Temecula, CA). Accutase, neural stem cell freezing medium, and mouse laminin purified protein were all from Millipore. Poly-L-omithine hydrobromide was purchased from Sigma Aldrich (St. Louis, MO).

The polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Temecula, CA), and SeeBlue Plus2 Pre-Stained Standard came from Invitrogen (Carlsbad, CA). Texas anti-mouse/anti-rabbit IgG, fluorescein anti-mouse/anti-rabbit IgG, and Vectashield/DAPI were all bought from Vector Laboratories (Burlingame, CA). Bradford Assay Kit was purchased from Pierce Biotechnology (Rockford, IL).

The TRIZOL Reagent was purchased from Invitrogen (Carlsbad, CA). First-Strand cDNA Synthesis Kit was obtained from GE Healthcare (Piscataway, NJ). TaKaRa Ex Taq was purchased from TAKARA Biotechnology. All primers came from Integrated DNA Technologies (San Diego, CA). The primers were as follows:

m1, 5'-AGCTCAGAGAGGTCACAGCCAGGGGCTGAAGGCT-3' and 5'-AGTCAAGAGGCCCACCAAGAAAGGCCGAGACCGA-3';

m2, 5'-CACGAAACCTCTGACCTACCCAGTTAAGCGGACC-3' and 5'-ACCACTGTAGAACTAGTTGGGTCGTCGGGTCAGA-3';

m3, 5'-GTGACAACTGTCAGAAGGATTTCACCAAGCTTC-3' and 5'-CCAGGACCATGATGTTGTAGGGGGTCCACGTGAT-3';

m4, 5'-AGCCGCGTTCACAAGCATCGACCTGAGGGCCCCA-3' and 5'-ACAGAGCTGTCCACCGCAGAGGCCACCACTCCAG-3';

m5, 5'-CCCGTAGAAGCACCTCAACAACAGGAAAGACAAC-3' and 5'-ACAAAAGGTCCGGATCCCAACCTCA GTCATCAAA-3'.

Instruments and Software

Trans-Blot SD semi-dry electrophoretic transfer cell was purchased from Bio-Rad Laboratories (Hercules, CA). The centrifuge 5403 was from Eppendorf (Hauppauge, NY). A Eppendorf Mastercycler Gradient instrument was used for PCR. The Metamorph version 7.0 software and Nikon Ti-U microscope coupled with Photometrics Coolsnap ES2, 12 bit, 20 MHz Digital Monochrome Camera with IEEE-1394 interface were used for the cell imaging. GraphPad Prism 5 software for Windows was purchased from GraphPad software (San Diego, CA). UN-SCAN-IT

gel™ digitizing software (version 6.1) was from Silk Scientific Corporation (Orem, UT).

Methods

Culture of neural stem cells

All tissure culture plasticware were coated with poly-L-ornithine (10mg/mL) and laminin (6ug/mL) in advance. Neural stem cells were cultured in plastic plates at 37℃ in a humidified atmosphere (5% CO_2) in neural stem cell expansion medium containing 20 ng/mL of FGF-2. Culture plates were exchanged with fresh medium containing FGF-2 every other day after plating.

When the cells were 80% confluent, they were considered ready for subculturing. After removing the medium from 10-cm tissue culture plates, 5ml of Accutase was applied to dissociate cells. Cells were incubated with Accutase for 5 min in the 37℃ incubator. Then, 5 ml of neural stem cell expansion medium was applied to the plate. The dissociated cells were transferred to a 15 ml conical tube and centrifuged at 300 x g for 3 minutes. After discarding the supernatant, cells were resuspended in neural stem cell expansion medium and plated at the desired density into appropriately coated plates.

Differentiation of neural stem cells into neurons

Neural stem cells were plated in poly-L-ornithine/laminin coated plates and incubated at 37℃ in a 5% CO_2 humidified incubator overnight. On the next day, the medium was exchanged with fresh neuron differentiation medium (1 μM retinoic acid and 5 μM of forskolin). Thereafter the medium was replaced with fresh neuron

differentiation medium every other day for four days.

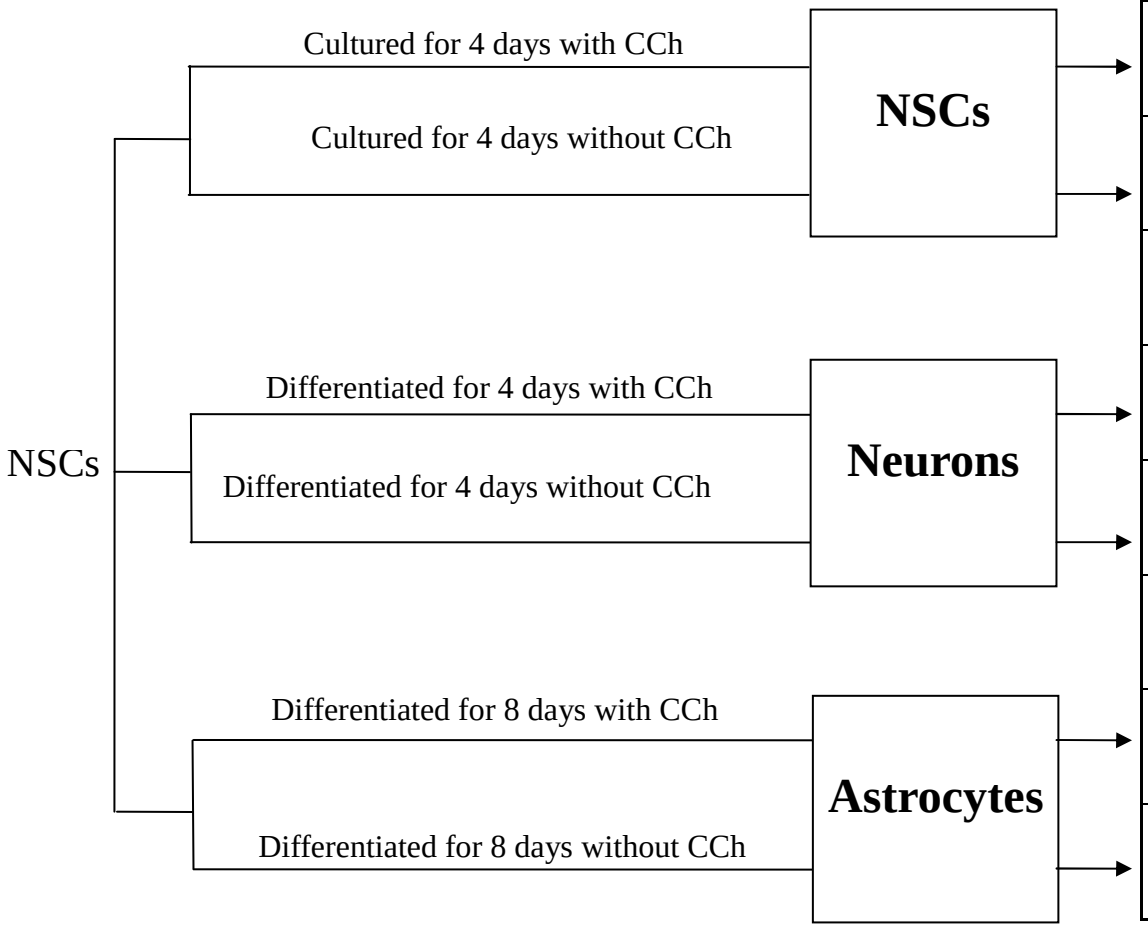
Differentiation of neural stem cells into astrocytes

After plating , at least three hours was required for the neural stem cells to settle and adhere to the surface. Three or four hours later, the medium was aspirated and astrocyte differentiation medium (containing BMP-4 and LIF) was applied. The medium was replaced with fresh astrocyte differentiation medium every other day for six days. After the sixth day, the medium was replaced with fresh astrocyte differentiation medium containing 20 ng/mL of FGF-2 to induce the growth of astrocyte processes. Cells were incubated at 37℃ for an additional three days before use in experiments.

Cell imaging

About 10,000-40,000 cells were plated and then cultured or differentiated on each cover slip according to the protocols described above. Cells at about 70% confluence were considered ready for the experiments. CCh was added at a concentration of 1 mM at the beginning of plating or just before the experiments. At the end of each treatment, medium was removed carefully and cells were washed with PBS. Freshly prepared 4% paraformaldehyde in PBS was used to fix the cells. After 40 minutes incubation at room temperature, cells were washed with PBS three times and permeabilized in a blocking solution (PBS-10%FBS-1% Triton-X-100) for at least two hours at room temperature. The primary antibodies were diluted to working concentrations in the blocking solutions and applied to the cells after permeabilization. Dilutions were as follows: nestin, 1:200; GFAP, 1:250; phospho-p44/p42 ERK1/2:

1:2000; ERK1/2: 1:5000. In control wells, only blocking solution was used. Cells were incubated with or without primary antibodies overnight at 4℃. On the second day, cells were washed twice with PBS and twice with the blocking solution. At the completion of the last wash, cells were left in blocking solution for at least 30 minutes. Anti-mouse or anti-rabbit IgG secondary antibodies conjugated to fluorescein (1:1000) were added for at least two hours at room temperature. Finally, following five washes with PBS, cells were adhered to microscope slides with DAPI. The cells were visualized with a Nikon Ti-U microscope. All images in each group were taken with the same exposure time.



Number of experiments for different proteins			
Nestin	GFAP	PO_4-ERK	ERK
3	-	3	3
3	3	3	3
3	-	-	-
3	3	-	-
-	3	2	2
3	3	-	2

Figure 1: Diagram showing the experimental design and number of experiments for cell imaging

Immunoblotting

Cell lysates were prepared using 1X PLB according to the manufacturer's specifications and stored at -20℃ prior to immunoblotting. Samples containing equal amounts of protein (as determined by a Bradford Assay) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, proteins were transferred to polyvinylidene fluoride (PVDF) membranes. In consideration of the relative sizes of proteins, a 30 min transfer was used for ERK1/2. The membranes were subsequently blocked with a solution of 2.5% dry milk-0.1% Tween-20 for 1 hour. Primary antibodies were diluted to working concentrations and applied to the membranes. Blots were incubated at 4℃ in the primary solutions overnight. Immunoreactive bands were visualized using anti-mouse or anti-rabbit alkaline phosphatase-conjugated secondary antibodies. Equal loading of protein was confirmed by staining the high molecular weight proteins remaining on gels after transfer to immunoblots.

RNA extraction and reverse transcriptase-PCR (RT-PCR)

For analysis of muscarinic receptor subtype mRNA expression in neural stem cells, astrocytes, as well as neurons, cells were cultured and differentiated according to the protocols described above. Total RNA was isolated using TRIZOL Reagent and then reverse-transcribed by First-Strand cDNA Synthesis Kit. Amplification was performed for 45 cycles at 94℃ for 30 sec, at 68℃ (for m1 and m4 subtypes) or 62℃ (for m2, m3, and m5 subtypes) for 40 sec, and at 72℃ for 30 sec. All samples were loaded on a 1.5% agarose gel. The PCR products were then visualized on a U.V.

trans-illuminator by staining the DNA with ethidium bromide.

Bradford Assay

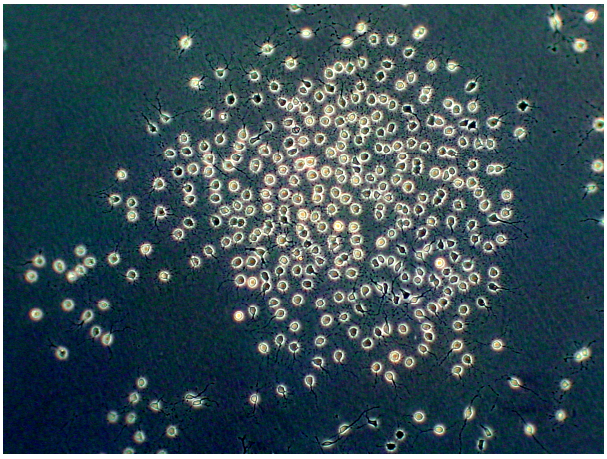
10 μl of each protein sample was combined with the assay reagent in 96-well microplates. After mixing and incubation for 10 minutes, the absorbance was read at 595 nm. Protein concentrations were estimated by reference to the absorbance levels obtained for a series of BSA standard protein dilutions, which were assayed alongside the unknown samples.

Chapter 3

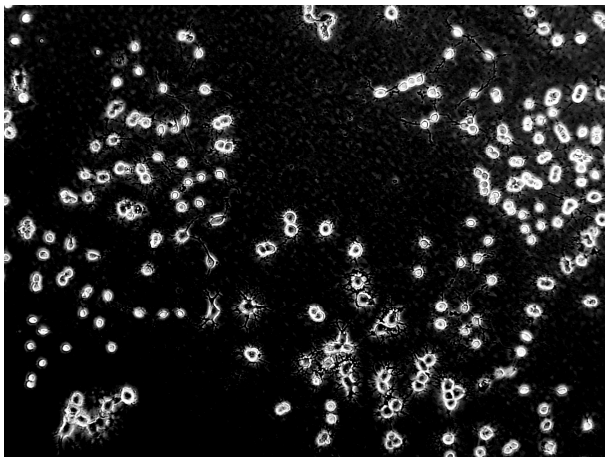
Results

1. Morphology of neural stem cells before and after differentiation:

(A) Most of the neural stem cells are spherical and devoid of obvious neurites.



(B) Neural stem cells have been treated with medium containing forskolin and retinoic acid for 4 days according to the instruction provided by Millipore, although they resemble neural stem cells closely.



(C) Neural stem cells are differentiated in astrocyte differentiation medium. In the medium containing LIF, BMP4, and fetal bovine serum, the majority of the cells exhibit an astrocyte phenotype.

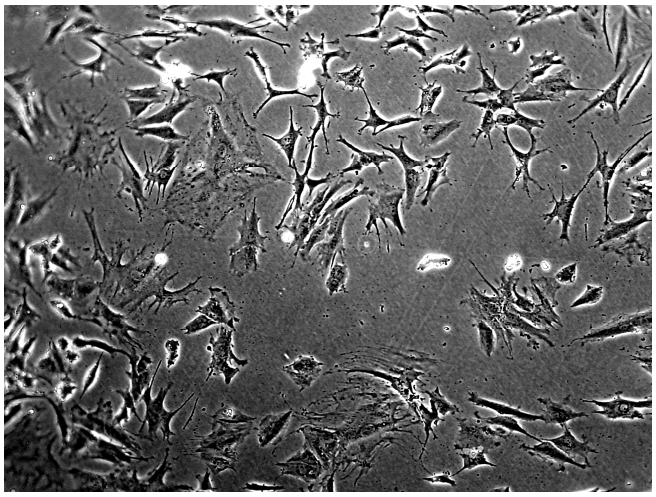


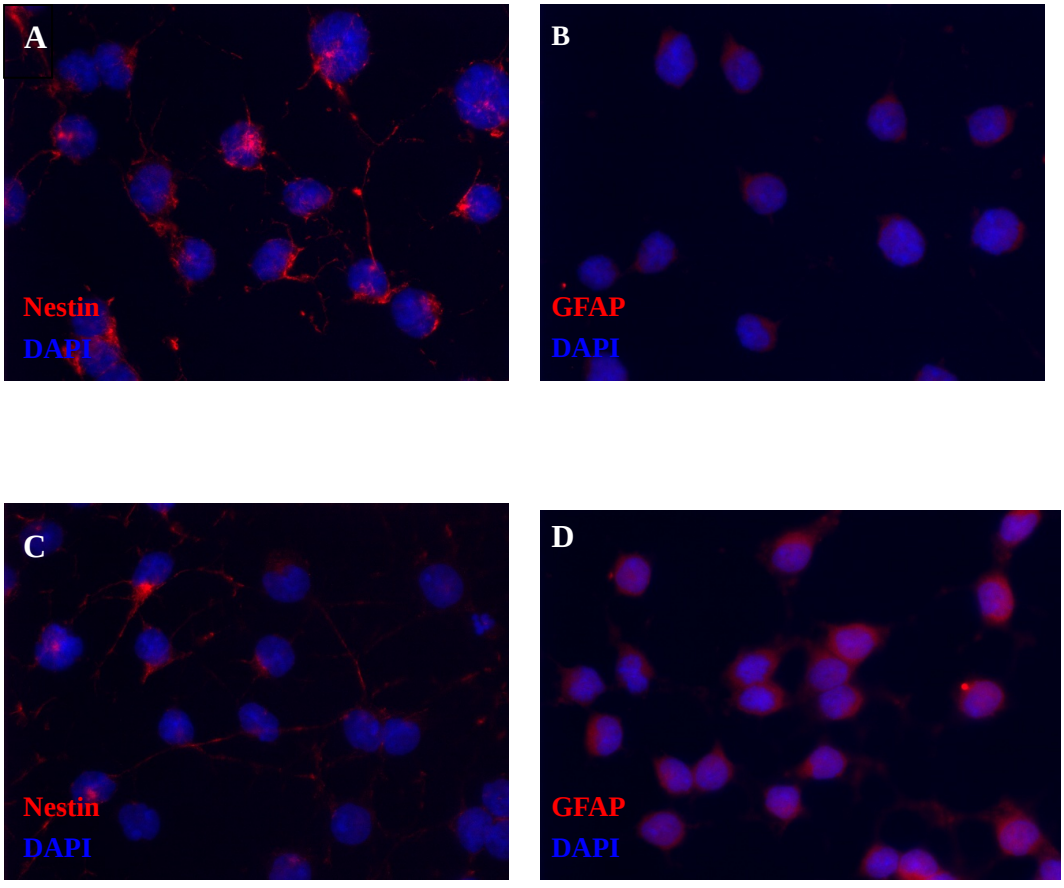
Figure 2. Morphology of neural stem cells before and after differentiation.

2. Expression of nestin and glial fibrillary acidic protein (GFAP) in different cell types:

Nestin is a type VI intermediate protein. It is expressed in many types of cells during development, but its expression is usually transient. In adults, nestin is mainly

expressed in neuronal precursor cells in the subventricular zone. After differentiation, nestin is downregulated and replaced by other cell type-specific intermediate filaments. Therefore, nestin was used as a protein marker for neural stem cells.

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein. It is specific for astrocytes in central nervous system. GFAP is thought to play an important role in maintaining the shape and structure of astrocytes, and it is also involved in many cellular functions. In the experiments described here, GFAP is considered as a protein marker for astrocytes.



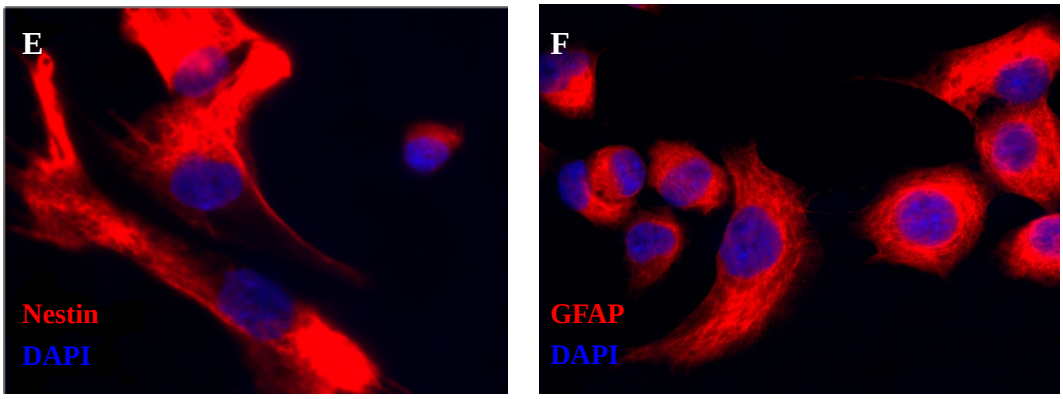


Figure 3. The expression of nestin and GFAP in NSCs, neurons, and astrocytes. Staining with antibodies to GFAP (red) and nestin (red) showing expression of these markers in different cells: (A-B) Rat hippocampal neural stem cells cultured in expansion medium. (C-D) NSCs cultured in medium containing retinoic acid and forskolin for 4 days. (E-F) NSCs cultured with astrocyte differentiation medium for 9 days.

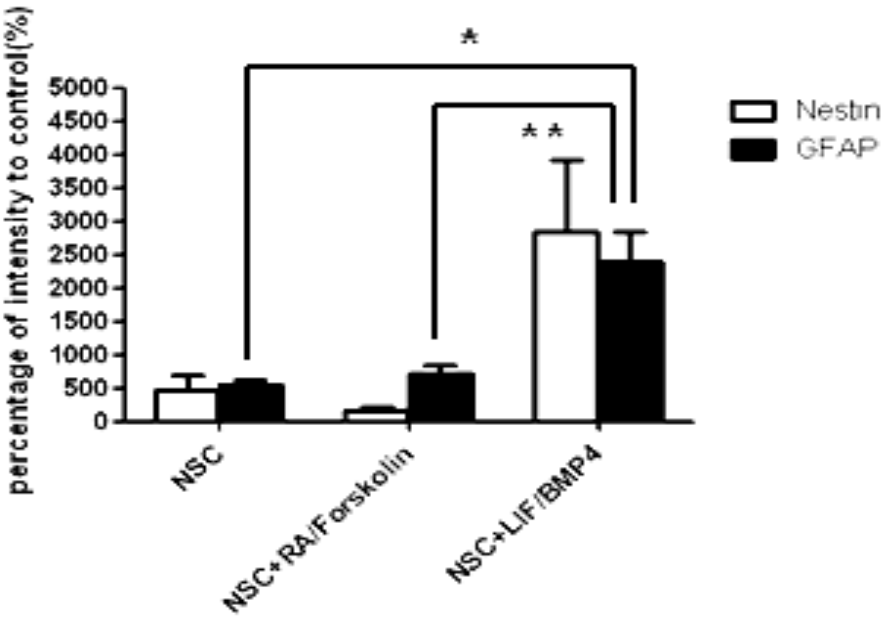


Figure 4: Quantitative Immunochemistry for different markers. Quantitative analysis of immunocytochemical staining was performed using Metamorph version 7.0 software. In each series of experiments, a control sample was prepared by applying blocking solution instead of the primary antibody; therefore the average intensity of control represents the fluorescence produced by secondary antibodies conjugated to Texas Red. The percentage of intensity relative to control was calculated using the formula:

(average intensity – background) * 100 / (average intensity of control – background) * 100%

Data are presented as the mean ± standard error of the mean (SEM). P values less than 0.05 were considered statistically significant (* : $P < 0.05$).

3.Expression of muscarinic receptor subtypes in neural stem cells before and after differentiation. The expression of five subtype mRNAs was determined using an RT-PCR technique in different types of cells. The m3 mAChR mRNA was expressed in neural stem cells, neurons, and astrocytes, whereas the other subtypes were not.

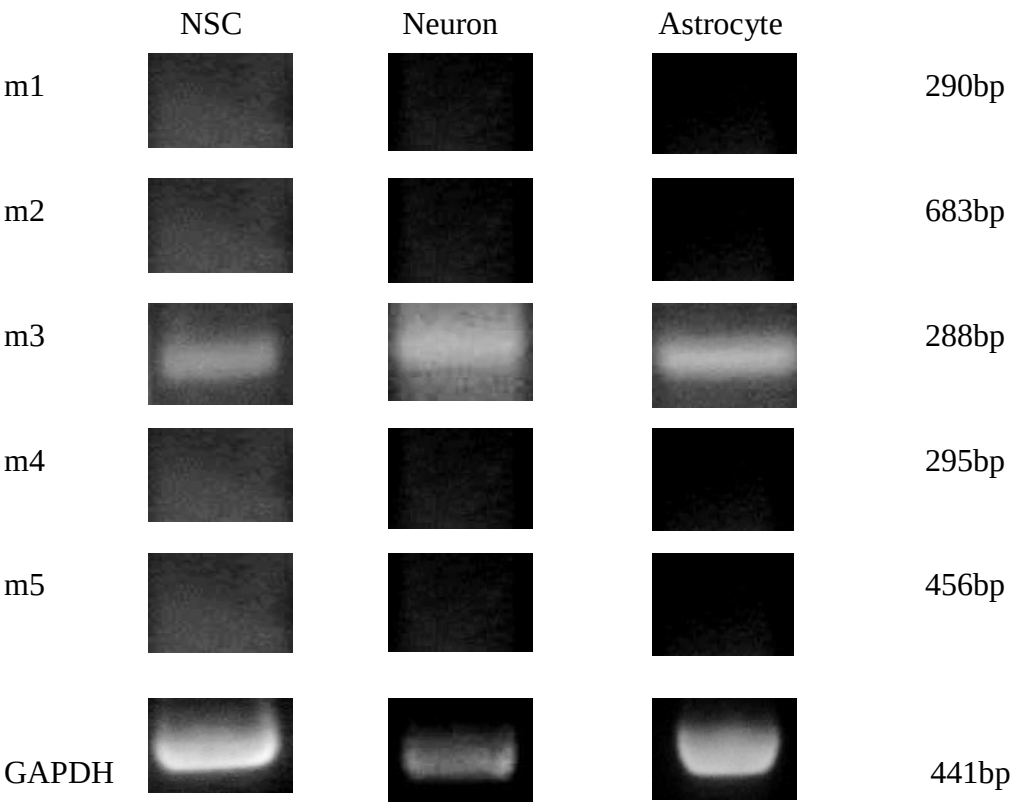
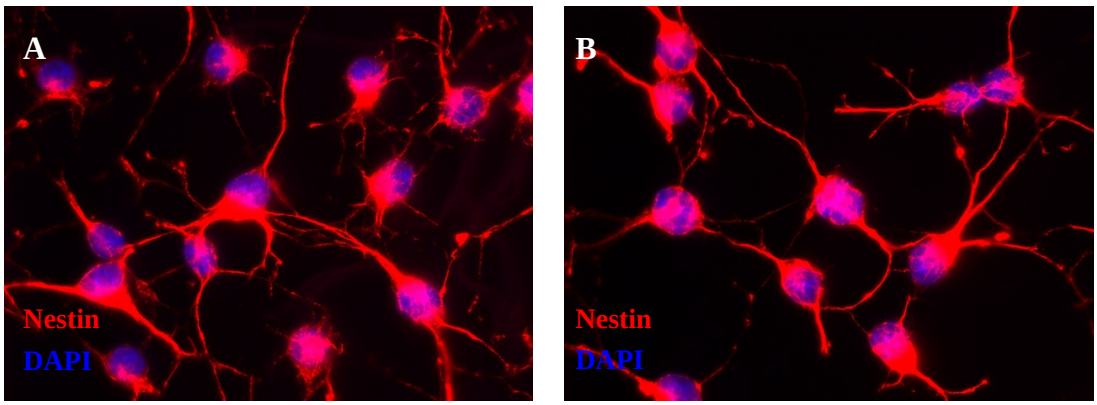


Figure 5: RT-PCR analysis of mAChR gene expression in neural stem cells, neurons, and astrocytes. Total RNA isolated from neural stem cells, neurons (neural stem cells cultured in the presence of retinoic acid and forskolin for 4 days), and astrocytes (neural stem cells differentiated in the astrocyte differentiation medium for 9 days) was reverse-transcribed and analyzed by PCR as described in Materials and Methods. As indicated at left, oligonucleotides primers corresponding to the following

gene products were used: m1, m2, m3, m4, m5 and GAPDH. The size of the amplified products are indicated in bp.

4. Effects of carbachol (CCh) on the expression of protein markers in neural stem cells, neurons, and astrocytes.

To determine the role of muscarinic receptors in the process of differentiation, CCh was used during the culture or differentiation periods. In the control group, cells were cultured or differentiated in the medium without CCh.



(C).

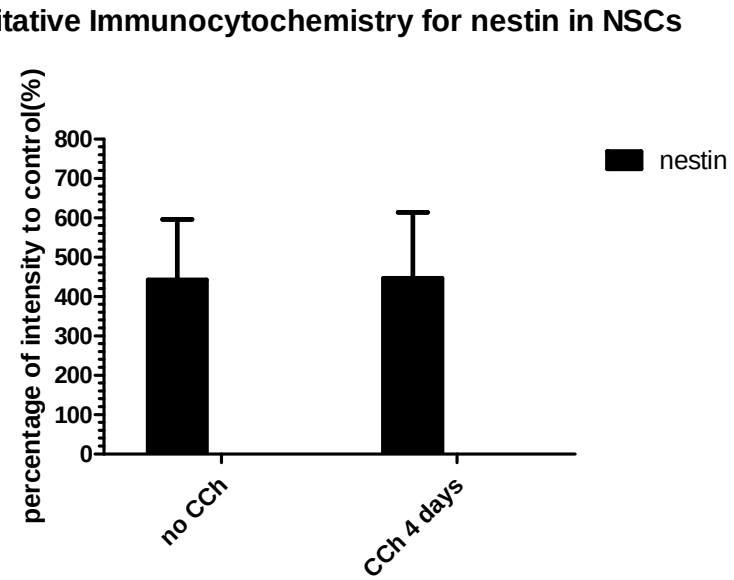
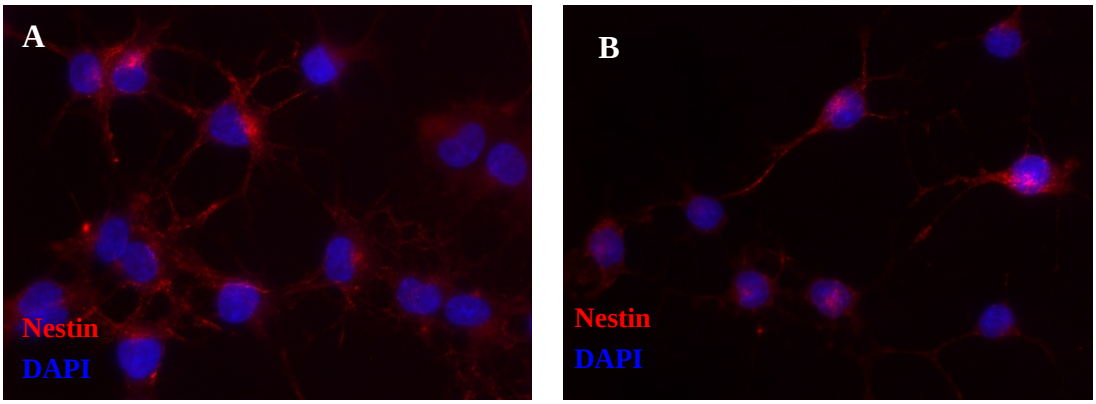


Figure 6. Effects of CCh on the expression of nestin in neural stem cells. (A) Rat hippocampal neural stem cells cultured in expansion medium for 4 days. (B) Neural stem cells cultured in expansion medium containing 1 mM CCh for 4 days. (C) Quantitative immunocytochemistry. There was no significant difference observed after CCh treatment.



(C)

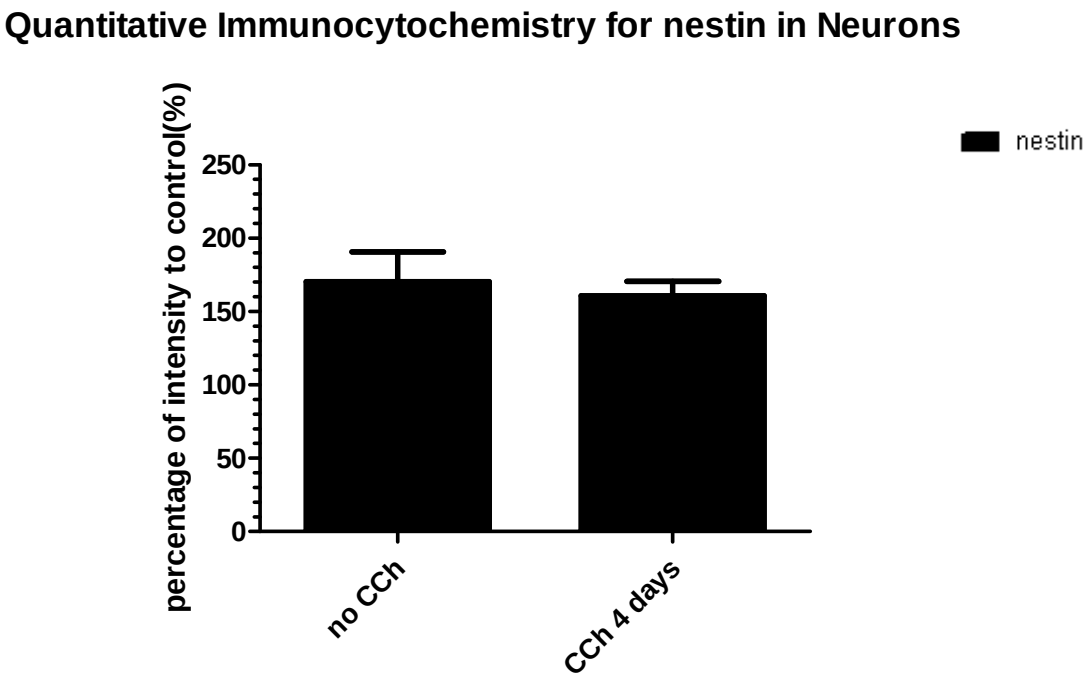
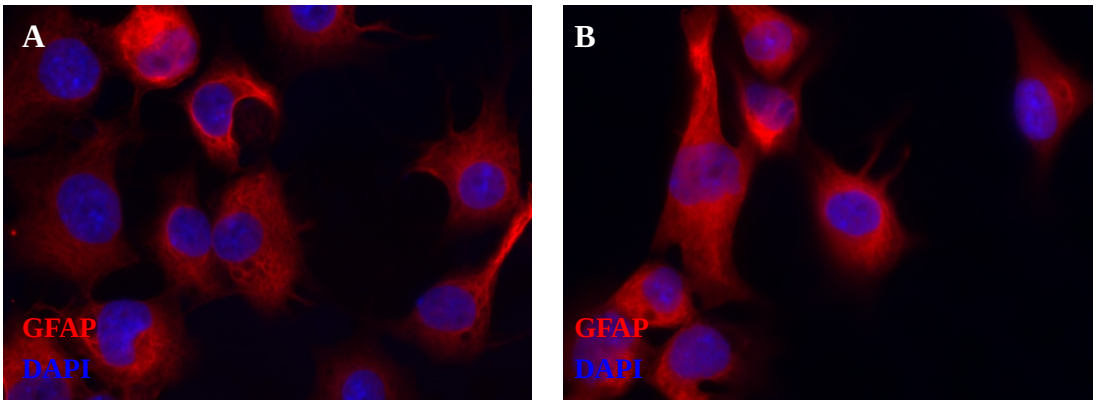


Figure 7. Effects of CCh on the expression of nestin in the cells cultured in

neuronal differentiation medium. (A) Neural stem cells cultured in the presence of retinoic acid and forskolin for 4 days. (B) Neural stem cells cultured in the medium containing retinoic acid, forskolin, and 1 mM CCh for 4 days. (C) Quantitative immunocytochemistry. There was no significant different observed after CCh treatment.



(C)

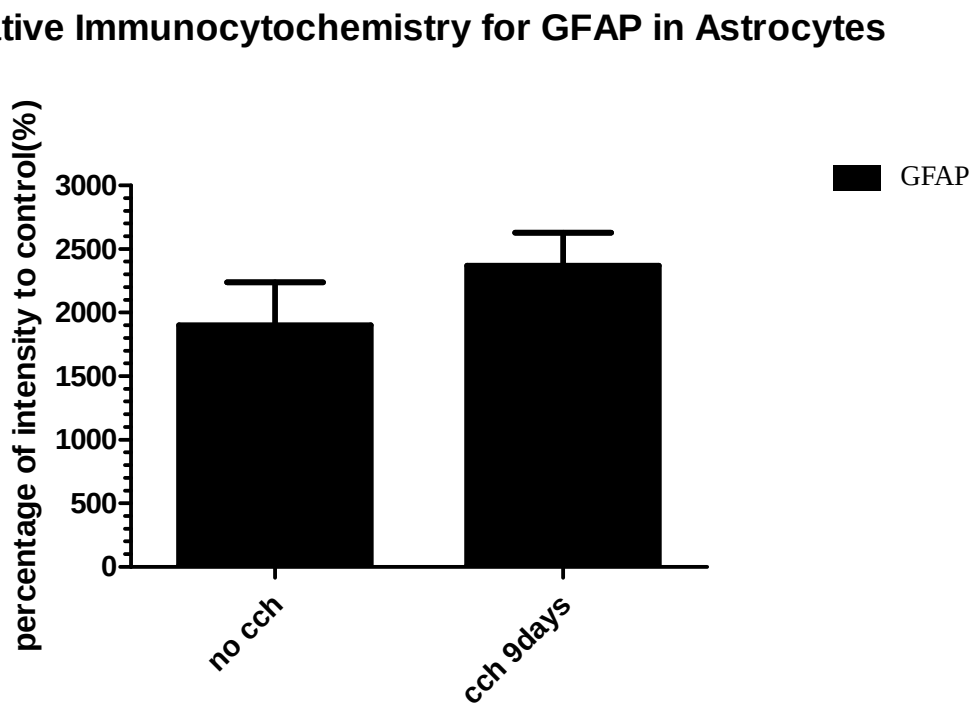


Figure 8. Effects of CCh on the expression of GFAP in astrocytes. (A) Neural stem

cells cultured in astrocyte differentiation medium for 9 days. (B) Neural stem cells cultured in astrocyte differentiation medium containing 1 mM CCh for 9 days. (C) Quantitative immunocytochemistry. There was no significant difference observed after CCh treatment.

5. The stimulation of extracellular regulated kinases (ERK1/2) by carbachol in neural stem cells and astrocytes.

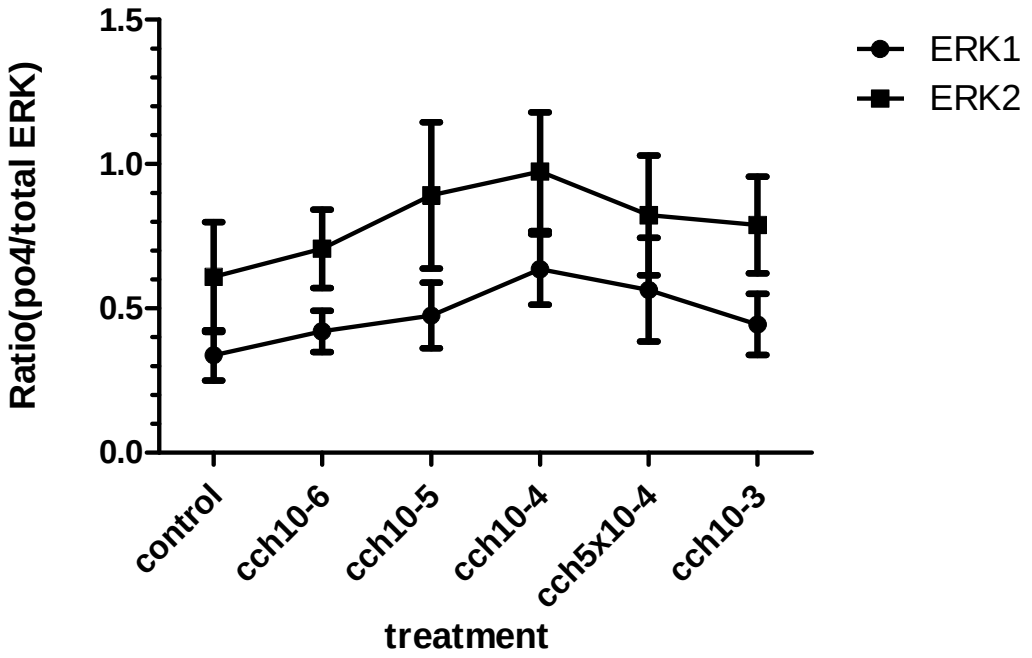
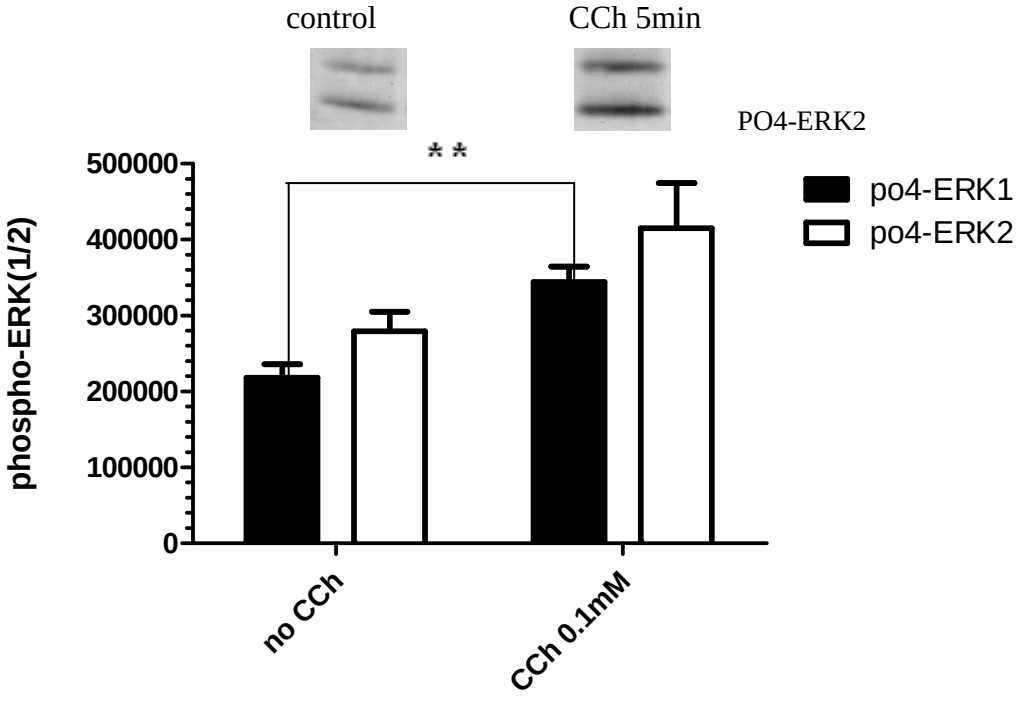


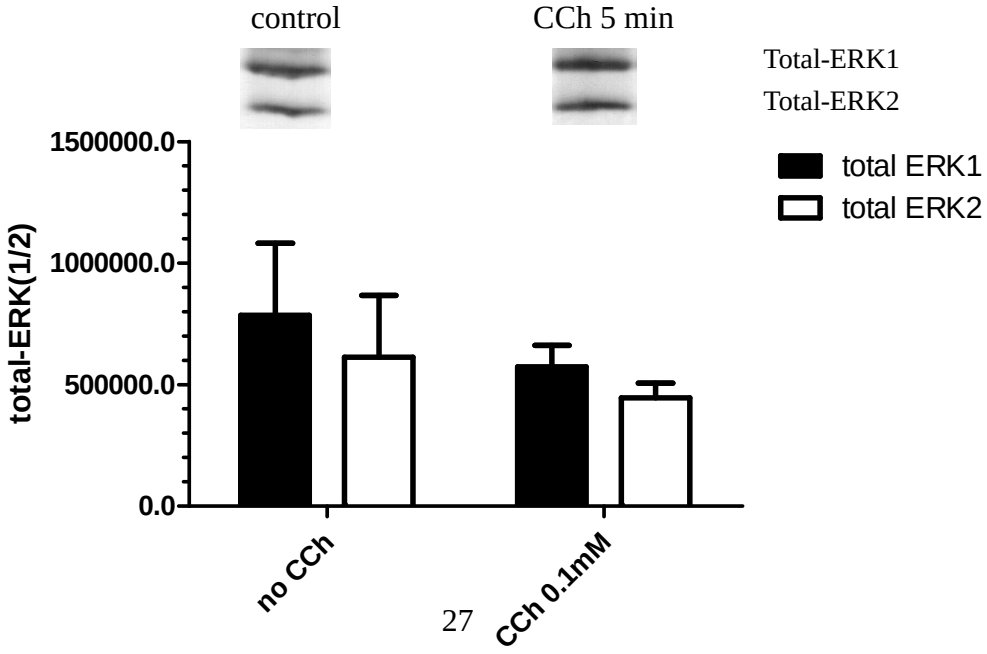
Figure 9. Dose-response curve for the ratio of phospho-ERK to total ERK in NSCs. To examine the activation of ERK1/2 by carbachol, neural stem cells were cultured in expansion medium for 4 days and incubated with different concentrations (1 μM, 10 μM, 100 μM, 500 μM, 1 mM) of CCh for 5 minutes at 37℃. Cell lysates were prepared and subjected to immunoblot analysis for total ERK(1/2) and

phospho-ERK(1/2). The bands detected by phospho-ERK(1/2) and ERK(1/2) antibodies lie in the region of 42-44 kDa. Data represent the mean ± standard error of the mean (SEM) from three independent experiments expressed as the intensity of these bands on the membranes. The effects of CCh reach a peak at the concentration of 100 μM.

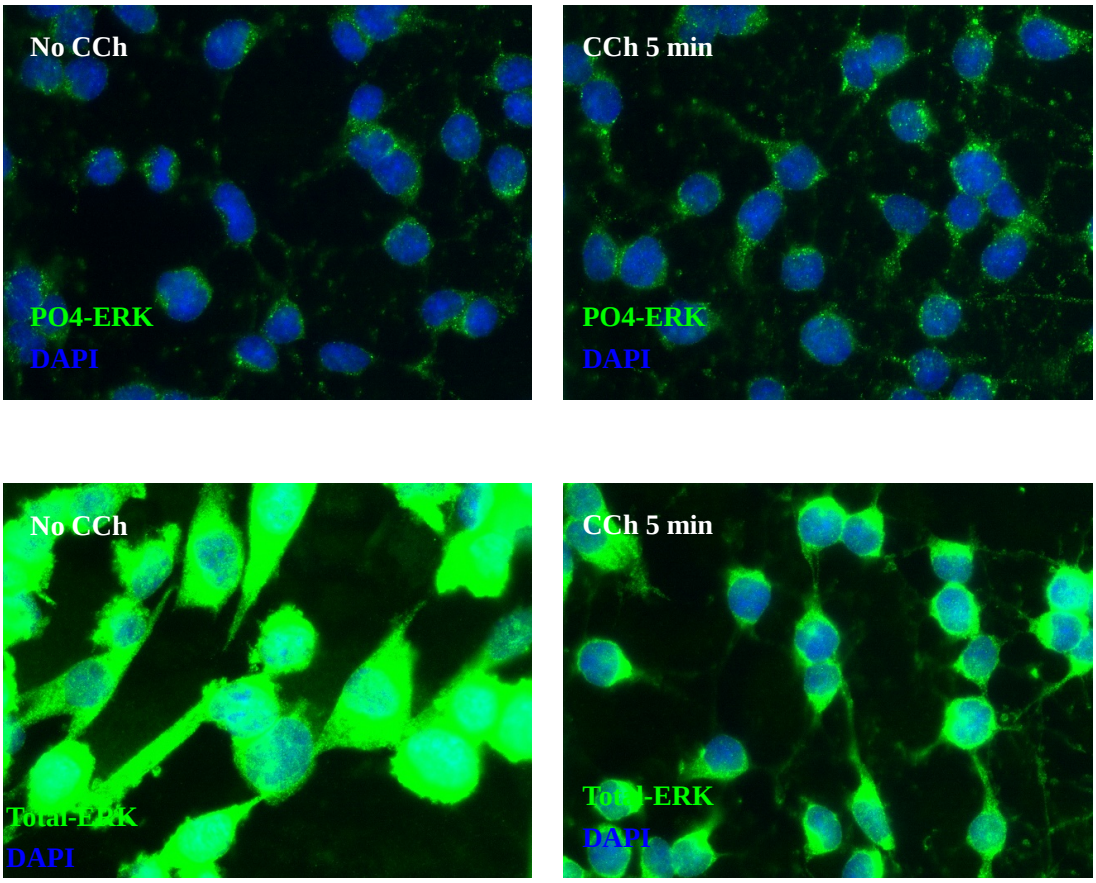
(A)



(B)



(C)



(D)

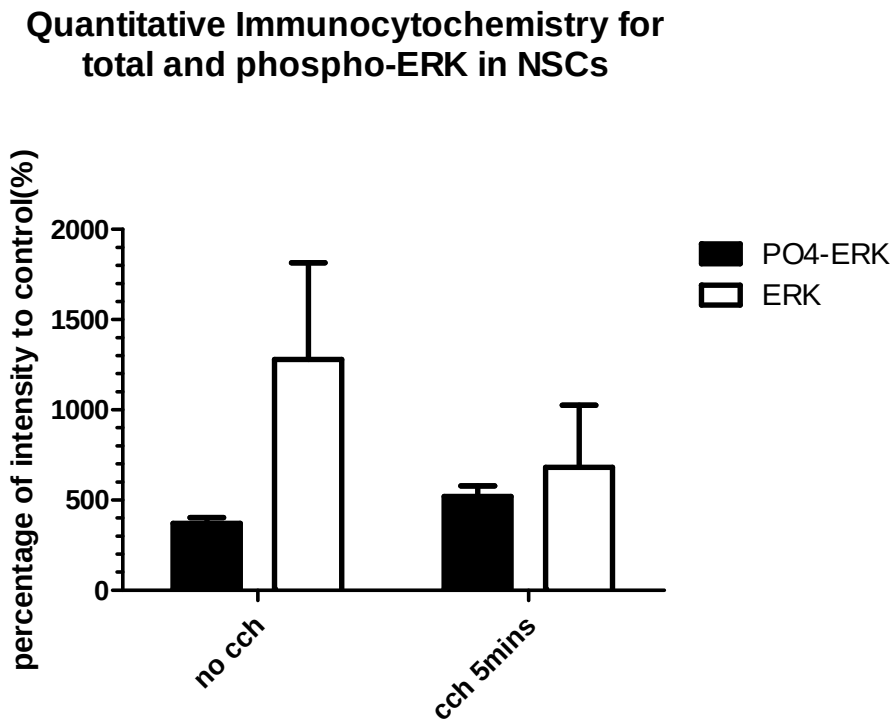
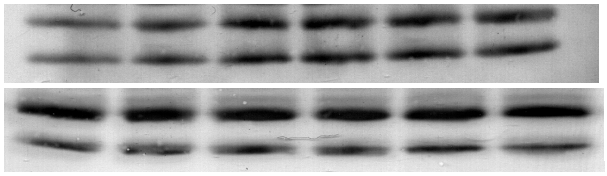


Figure 10. Expression of phospho-ERK and total-ERK in NSCs following

treatment with 100 μM CCh for 5 minutes. (A-B) Immunoblotting was performed for phospho- and total ERK(1/2). The expression of phospho-ERK(1/2) was significantly increased after treatment with CCh. (C) Immunostaining showing an overlay of total or phospho-ERK labeled (green) neural stem cells with DAPI (blue). (D) Quantitative immunocytochemistry. There was no significant different observed after CCh treatment.

(A) control 10^{-6}M 10^{-5}M 10^{-4}M $5x10^{-4}$M 10^{-3}M



(B)

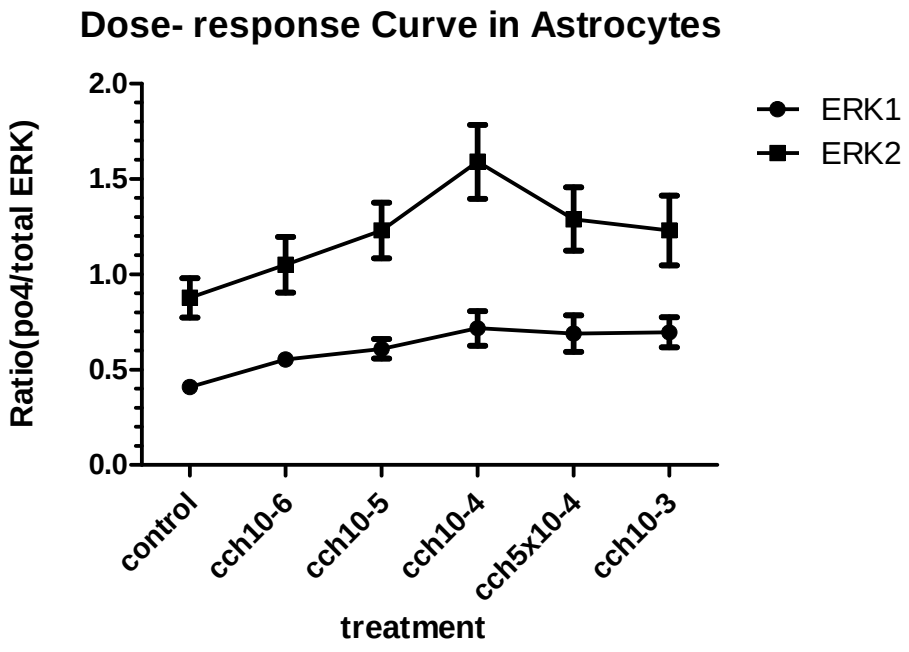


Figure 11. Dose-response curve for the ratio of phospho-ERK to total ERK in Astrocytes. (A) Neural stem cells were differentiated in astrocyte differentiation medium for 9 days. Following incubation with different concentrations (1 μM, 10 μM,

100 μM, 500 μM, 1 mM) of CCh for 5 minutes at 37℃, cells were lysed and immunoblotting was performed for total and phospho-ERK(1/2). (B) Results are expressed as the mean ± SEM of the ratio of the phospho- to total ERK(1/2) from 3 experiments. The effects of CCh reach the peak at the concentration of 100 μM.

Chapter 4

Discussion

1. Neural stem cell differentiation

(1) Differentiation of neural stem cells into neurons

The process of neuronal differentiation can be influenced by a variety of extracellular signaling molecules that act through nuclear receptors or through cell surface receptor-mediated signal cascades. For example, the nuclear receptors for thyroid hormone, retinoids, or glucocorticoids are known to effect the development of the central nervous system (Barres et al., 1994; Sucov et al., 1995). One of the early events in neuronal differentiation involves alterations in cell-cycle protein expression and exit from the cell cycle.

Retinoic acid (RA), a biologically active derivative of vitamin A, plays an important role in maintaining normal cellular growth and development. It is present in various tissues especially in the nervous system, where it promotes neuronal differentiation (Takahashi et al., 1999). Previous studies have demonstrated that although withdrawal of FGF-2 was sufficient to stimulate limited neuronal differentiation in neural stem cells, treatment with RA was able to potentiate this process. After treatment, a greater number of neurites and increased neurite length

were observed (Maden, 2001). The cellular responses to RA include stimulation of NeuroD expression followed by up-regulation of p21, which is an indicator of changes in cell-cycle status. Some treated cells thus commit to a neuronal lineage and exit the cell cycle (Takahashi et al., 1998).

Forskolin is a labdane diterpene extracted from plants. It can re-sensitize cell receptors by activating the enzyme adenyl cyclase and increasing intracellular levels of cyclic AMP (cAMP). Regulation cAMP levels is involved in the control of many biological functions such as intermediary metabolism, cellular proliferation, and neuronal signaling. It also provides survival and differentiation signals for neurons. It has been demonstrated that cAMP could sustain survival and promote neurite outgrowth of neurons in culture for one week under serum-free conditions. The differentiation induced by cAMP in neuronal cells is generally believed to involve the activation of Protein Kinase A (PKA) and phosphorylation of transcription factors binding to a cAMP responsive element (Montminy, 1997).

In this study, we intended to differentiate neural stem cells into neurons in the medium containing 1 μM of retinoic acid and 5 μM of forskolin. However, neither the morphology of cells nor the expression of protein markers was significantly different from neural stem cells after four days of differentiation. To obtain more efficient differentiation, these stem cells might need higher concentrations of retinoic acid and forskolin, and other neurotrophins might need to be applied at the same time.

(2) Differentiation of neural stem cells into astrocytes

Bone morphogenetic protein 4 (BMP4) and leukemia inhibitory factor (LIF) both

promote the differentiation of neural stem cells into astrocytes, as specified by glial fibrillary acidic protein (GFAP) expression. LIF activates the janus kinase / signal transducer and activator of transcription (JAK-STAT) pathways (Rajan et al., 1998). LIF signaling generates $GFAP^+$ cells, and also promotes re-entry into the cell cycle and maintenance of progenitor cell properties. In contrast, BMP4 signaling activates Sma-MAD homolog (SMAD) proteins which belong to a distinct family of transcriptional regulators. BMP4 signaling generates stellate $GFAP^+$ astrocytes. It also causes $GFAP^+$ cells to exit the cell cycle and lose progenitor cell potential. Figure 12 depicts a model for the effects of LIF and BMP on astrocyte differentiation (Lagna et al., 1996; Sun et al., 2001).

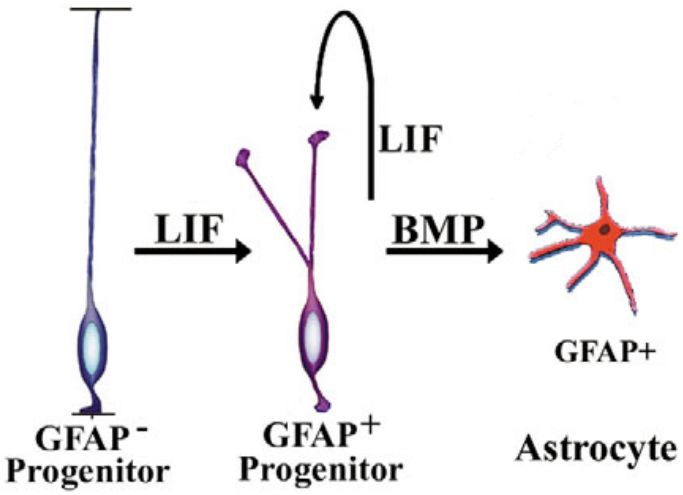


Figure 12. Effects of LIF and BMP on differentiation of neural stem cells into astrocytes (from Bonaguidi et al., 2005).

In the experiments described above, neural stem cells were cultured in differentiation medium containing LIF and BMP4 for 9 days. After differentiation, the

morphology of cells became more like that of astrocytes, and very different from neural stem cells. Also the expression of GFAP was significantly increased compared to stem cells.

2. Nestin expression

Nestin is an intermediate filament protein first identified in neuroepithelial stem cells of rat (Lendahl et al., 1990). This 240-kDa protein contains a short N-terminus and an unusually long C-terminus. Nestin forms mainly heterodimers with vimentin, the latter assembling into homodimers (Marvin et al., 1998). During embryogenesis, nestin is expressed in migrating and proliferating cells, whereas in the adult, it is mainly restricted to regions of regeneration.

Although it is well established that nestin is expressed in undifferentiated CNS progenitor cells, there are some observations suggesting that nestin may not always be associated with stem cells in the CNS. For example, nestin was detected in a number of pathological conditions including neoplastic processes and cerebral injury. Nestin expression is also re-induced in reactive astrocytes following hippocampal lesions or spinal cord injury (Dahlstrand et al., 1992; Frisen et al., 1995). Other studies demonstrated that nestin was co-expressed with glial markers in cells developing in glial pathways. This suggests that astrocytic lineage cells could go through a transitional period during which they express both progenitor and astrocyte markers, which are nestin and GFAP respectively. In contrast, the transitional neuronal progenitor cells, positive for both progenitor and neuronal markers, were rarely observed (Messam et al., 2001). Because nestin is expressed differentially in cells

developing in glial and neuronal pathways, it is possible that there are different mechanisms regulating nestin expression in these two cell types. Consistent with these previous studies, we found a strong expression of both nestin and GFAP in astrocytes, but not in cells developing in neuronal pathways. Therefore, the presence of nestin alone may not determine an undifferentiated stem cell. Some cells express nestin, but they may have already initiated differentiation.

3. GFAP expression

The glial fibrillary acidic protein (GFAP) is the principal 8-9 nm intermediate filament protein in mature astrocytes of the CNS. As a member of the cytoskeletal protein family, GFAP is important in modulating astrocyte motility and shape by providing structural stability to extensions of astrocyte processes. Some studies also suggest that GFAP might play a role in normal white matter architecture and blood-brain barrier integrity (Liedtke et al., 1996).

Although GFAP has been accepted as a marker for differentiated astrocytes, various cell types inside and outside the CNS express GFAP, including cells in the liver, gut, kidney, lung, and other tissues during development as well as in adults (Eng et al., 2000). And like other intermediate filaments, GFAP is regulated dynamically and differentially in cells during different stages of maturation. Some NSCs in adult brain also express GFAP (Doetsch et al., 1999; Imura et al., 2003). Our findings support these observations by showing that the NSCs and cells developing in neuronal pathways also expressed GFAP to a certain degree, albeit at a much lower level as compared to astrocytes.

4. Muscarinic receptors in neural stem cells

G protein-coupled receptors, such as muscarinic cholinergic receptors, have been identified in neural stem cells. Furthermore, some reports suggest that they are involved in proliferation and differentiation of neural stem cells (Li et al., 2001). In order to better understand the effects of muscarinic receptor activation on NSCs, we examined the expression of mRNA for the five subtypes of muscarinic receptor using the RT-PCR technique in NSCs, cells developing in neuronal pathways, and astrocytes. The M_3 muscarinic receptor was the only subtype expressed in each cell type. To examine the effects of muscarinic receptors on the expression of different protein markers, carbachol was used as a cholinergic agonist to activate acetylcholine receptors during the differentiation process. Our studies showed that carbachol had no significant effect on the expression of nestin or GFAP in the process of differentiation, which suggests that carbachol does not promote neuronal or astrocytic pathways in NSCs under the conditions we used.

Stimulation of muscarinic receptors has been shown to activate mitogen-activated protein kinase (MAPK) in different cellular systems. MAPK is a family of protein kinases that play an important role in signal transduction and are thought to mediate many processes in cells (Davis, 1993; Derkinderen et al., 1999). The best-studied MAPKs are ERK1 and ERK2 (p44 and p42 MAPK), which are activated by mitogens and play an essential role in cell proliferation (Cobb, 1999). In the studies described here we observed a rapid phosphorylation of MAPK (ERK1/2)

in both stem cells and astrocytes after carbachol treatment for 5 minutes. Dose-response experiments in NSCs and astrocytes showed that maximal phosphorylation was achieved at 0.1 mM in both cell types, accompanied by a decrease in total ERK(1/2) expression. The results from western blotting and immunohistochemistry in NSCs were generally consistent. However, the effects of carbachol were even more noticeable in the immunoblotting experiments, probably because of the variations in protein expression observed using immunohistochemical techniques. Unlike immunoblotting, by using immunohistochemistry it is possible to observe the expression of different proteins in each cell. The response to carbachol treatment may be very different from one cell to another, which could result in the variations in the expression of cell markers and signaling molecules. In order to determine whether or not the phosphorylation of MAPK was due to activation of muscarinic receptors, inhibitors such as atropine will need to be applied. Further studies are necessary to understand the physiological significance of inducing MAPK activation.

Chapter 5

Conclusions

1. For commercial adult rat hippocampal NSCs, culturing in neuronal differentiation media containing 1 mM of retinoic acid and 5 mM of forskolin is not an effective method for differentiation into neurons. After four days differentiation, neither the morphology of cells nor the expression of protein markers was significantly different from NSCs. Cells differentiated with retinoic acid and forskolin appeared to express less nestin but more GFAP compared to NSCs, although the differences were not statistically significant.

2. After culturing NSCs in astrocyte differentiation media (containing LIF and BMP4) for 9 days, the majority of the cells exhibited an astrocyte phenotype. The expression of GFAP was significantly increased compared to stem cells. Cells also expressed much more nestin than NSCs.

3. The M3 muscarinic receptor was the only subtype expressed in NSCs before and after differentiation.

4. According to the results from western blotting, the expression of phospho-ERK(1/2) in NSCs was significantly increased after treatment with 100 μM CCh for 5 minutes, accompanied by a decrease in total ERK(1/2) expression.

5. According to the results from immunocytochemistry studies, the expression of phospho-ERK(1/2) in NSCs was also increased after treatment with 100 μM CCh for 5 minutes, accompanied by a decrease in total ERK(1/2) expression. But the effects were less noticeable compared to the results from western blotting. In dose-response experiments, the ratio of phospho-ERK to total ERK in astrocytes reached a peak after treatment with carbachol at dose of 100 μM for 5 minutes.

6. Carbachol had no effect on the expression of cell markers. There was no effect of carbachol (100 μM) on the expression of nestin in NSCs or cells developing in neuronal pathways after four days of treatment. There was no significant effect of carbachol (100 μM) on the expression of GFAP in astrocytes after nine days of treatment during differentiation.

Chapter 6

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