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ROLE OF CALCIUM IN DEVELOPING CELLULAR NETWORK

Ca^{2+} in human fetal neural stem cells and rat neonatal cardiomyocytes

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ABSTRACT

This thesis aimed to study Ca^{2+} homeostasis and its regulation in developing heart and brain cells. Cardiomyocytes rapidly developed a fast calcium machinery, (1) to regulate cytoskeletal protein interactions to the extracellular matrix of neighboring cells through gap junctions, and (2) to induce Ca^{2+}-induced Ca^{2+} release (CICR) from sarcoplasmic reticulum (SR), (3) to generate rapid energy supply of ATP from mitochondria. In contrast, the developing human brain cells undergo a slow nurturing process to proliferate and differentiate into various specific cell types with multi-functions through specialized endoplasmic reticulum (ER) and mitochondria. Although, I used two different developmental stages (fetal, postnatal) in two different cell types (brain cells, cardiomyocytes) from two different origins of lineages (ectoderm, mesoderm) both have regulation of Ca^{2+} homeostasis as a vital component for their development and function.

The fetal brain undergoes proliferation, migration, differentiation, region patterning and specification of cell morphology and function and Ca^{2+}signaling is involved in all developmental stages. Morphological, immunocytochemical and functional phenotypes were characterized over time in expanded and differentiated fetal (11-13 week old) human neural stem cells (hNSC) from three sources. During proliferation, gap junctions were involved in spontaneous calcium oscillations and coordinated Ca^{2+} waves between cells. The role of gapjunctions decreased during differentiation of hNSC but was still prominent in several cells. Purinergic receptor activation by ATP induced Ca^{2+} signals in proliferating hNSC that were independent from gapjunction signaling. In contrast both glutamate- and GABA-induced Ca^{2+} signals, partly depend on gap junction signaling. Purinergic signaling plays a crucial role in spontaneous development and neurotransmitter-induced Ca^{2+} regulation in proliferating and differentiating hNSC. I demonstrate that calcium homeostasis in three primary fetal hNSC is regulated through ATP and purinergic receptors during all stages of proliferation and differentiation studied. Glutamate-induced Ca^{2+} signals were dependent on purinergic receptors in BDNF+GDNF-differentiated cells. The role of GABAergic induced changes in calcium homeostasis increased with differentiation time and seemed to be independent of gapjunction and purinergic signaling.

Neonatal lupus is induced by maternal anti-Ro52 autoantibodies. Cardiac manifestations include AV-block. We found that the electrical signal transmission was delayed in neonatal hearts of pups born to female rats injected with Ro52 monoclonal antibodies specific for the p200 epitope. We studied the effect of the Ro52-p200 antibodies on cultured neonatal cardiomyocytes, and found disturbed Ca^{2+} regulation following application of anti-Ro52 monoclonal antibodies. The effects on calcium homeostasis were time- as well as dose-dependent and could be the initial mechanism for development of congenital cardiac pathology in fetuses to mothers with autoimmune disease and SSA antibodies.

LIST OF PUBLICATIONS

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*indicates equal contribution

CONTENTS

LIST OF ABBREVIATIONS

AA	Ascorbic acid
AD	Alzhemer's disease
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AV node	Atrioventricular node
APP	beta-amyloid precursor protein
BDNF	Brain-derived neurotrophic factor
bpm	Beats per minute
bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
CBX	Carbenoxolone
ChAT	Choline acetyltransferase
CHB	Congenital heart block
CICR	Ca^{2+} -induced Ca^{2+} release
CNS	Central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CP	Cortical plate
Cx	Connexin
DA	Dark Agouti
DS	Down syndrome
ECG	Ecocardiogram
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GA	Gestational age
GABA	Gamma-Amino-n-Butyric acid
GAD 65/67	Glutamic acid decarboxylase
GE	Ganglionic eminence
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
hNSC	Human neural stem cells
ICH	Intracerebral hemorrhage
IZ	Intermediate zone
KCC2	K^+, Cl^- -cotransporter 2
LIF	Leukemia inhibitory factor
MZ	Marginal zone
NF	Neurotrophic factor
NGF	Nerve growth factor
NLE	Neonatal lupus erythematosus
NMDA	N-Methyl-D-Aspartic acid
NSC	Neural stem cells
PDGF	Platelet-derived growth factor
P2X	Purinergic receptor 2 - ionotrophic
P2Y	Purinergic receptor 2 - metabotrophic

RA	Retinoic acid
RyR	Ryanodine receptors
SA node	Sino-Atrial node
Shh	Sonic hedgehog
SLE	Systemic lupus erythematosus
SR	Sarcoplasmic reticulum
SVZ	Subventricular zone
VOCC	Voltage-operated Ca^{2+} channels
VZ	Ventricular zone

1 BACKGROUND

1.1 GENERAL OVERVIEW OF CALCIUM

Calcium is the chemical element with atomic number 20, atomic mass of 40. Calcium is the 5th most abundant element by mass in the Earth's crust, and dissolved in sea water by both molarity and mass after sodium (Na^+), chloride (Cl^-), magnesium (Mg^{2+}) and sulfate (SO_4^{2-}). Mineralized form of calcium is the most abundant metal by mass in vertebrates and the 5th most abundant in human body.

In evolution, the original source of calcium to the Earth may be supplied from cosmic activities from hot stars and the Sun: ^{36}Ar (argon) + ^{4}He (helium) = ^{40}Ca (calcium) as the heaviest and stable isotope or decay from atmospheric ^{41}K (Russell, 1978). If this ^{40}Ca isotope were formed inside the Earth, it was presumably created during earlier periods of the hot primitive Earth. Earlier form of life could extensively utilize the source of atmospheric- and oceanic calcium for respiration and metabolism to survive and generate progeny or offspring.

In most bone-forming organisms, calcium is extensively used to form the skulls and skeletons that support the body structure. For example, 99% of calcium is stored in the human bones, only 1% is used in the organs and tissues. It is still not completely understood how calcium became to play a crucial role in cell physiology. Calcium is commonly found in the plants and foods as well as other mammalian animals. This indicates that calcium uptake might have contributed development of cell physiology. Calcium became essential for the organisms from the process of fertilization, development, proliferation, differentiation, and further to maintain homeostasis for survival as well as in the process of cell death including diseases.

1.1.1 Yin-Yang of free calcium ions Ca^{2+}

The balance (yin-and-yang) of calcium regulation is the key of calcium homeostasis. Calcium homeostasis is a dynamic and complicate phenomenon. At the *systemic* level of calcium regulation, calcium is stored in the bone as calcium phosphate or calcium sulfate. Parathyroid hormone in the parathyroid regulates calcium release from the bone into the blood circulation, called bone resorption: ionized calcium Ca^{2+} is dissolved or bound to serum protein albumin. Those Ca^{2+} do

not enter the blood circulation, it remains extracellular space or intercellular fluids. Calcium absorption is regulated by calcitriol (active form of vitamin D_3) in the intestines from the food intake. Some Ca^{2+} is reabsorbed by the kidney and restored into the bone.

At the *local cellular* level of calcium regulation, calcium is stored in the intracellular organelles, such as endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) and mitochondria. Intracellular Ca^{2+} level is tightly tuned with extracellular Ca^{2+}. How these Ca^{2+} are regulated is highly tissue- or cell-specific and dependent on the stage of development. There are several factors that determine the pattern of Ca^{2+} signalling: expression of specific Ca^{2+}–binding proteins and its binding affinity to Ca^{2+} determine, the expression of specific Ca^{2+} channels (voltage-operated, receptor-opearated) as well as type of ligands binding to G-protein. Intracellular Ca^{2+} is a result of synchronized activities between influx/efflux of Ca^{2+} from three compartments – extracellular spaces, ER/SR and mitochondria. Intracellular Ca^{2+} homeostasis is achieved by spatio-temporal balancing between Ca^{2+} - influx and – efflux. To reach the resting state, the removal of cytosolic Ca^{2+} is necessary by coordination of Ca^{2+}-ATPase in the cytoplasmic membrane to extrude Ca^{2+} from the cytosol; inositol-1,4,5-triphosphate receptors (IP_3-R), ryanodine receptor (RyR), Ca^{2+}-ATPase to restore Ca^{2+} in the ER/SR (Berridge, 1997).

Intracellular Ca^{2+} can be divided into two parts – global or local Ca^{2+} - within the cytoplasm (Cancela, 2002). L*ocal* Ca^{2+} is fast and spiky, which is locally generated near the cytoplasmic membrane, ER and mitochondria. *Global* Ca^{2+} is often shown to be slow and wave-like transients. How local Ca^{2+} is tranformed into global Ca^{2+} is not completely understood. Here is one of examples for the mechanism. Local Ca^{2+} spikes are genearted via cytoplasmic Ca^{2+} ion channels, and global Ca^{2+} are generated via Ca^{2+}-induced Ca^{2+} release (CICR) by ER (Berridge, 1997).

Wave-like global Ca^{2+} signaling is more common in early development - fertilization, gastrulation, dorsal-ventral patterning. During embryonic development, cell-specific differentiation is regulated by extracellular modulators. These external modulators trigger to generate specific Ca^{2+} signals for cell specification and eventually orchestrate genetic programming such as protein expressions in time- and space-dependent manners. When the balance of calcium regulation become out of order or altered at early stage of development, normal developmental programming cannot be achieved so that it leads to abnormality of body formation and malfunction.

1.1.2 Ca^{2+} in the brain cells

Calcium is very important in maintaining homeostasis of healthy brain cells and also involved in brain diseases. For example, Alzhemer's diseases (AD) and toxoplasmosis (cortex, basal ganglia) are caused by calcium deposit in the tissues. *Excessive calcium concentration* in the blood circulation with lack of absorption of calcium into the body tissues can lead excessive calcium to the brain, resulting in accumulation of calcium in the brain tissues.

Disturbance of calcium metabolism – for example, altered by aluminum absorption - was suggested to cause senile plaques and forms neurofibrillary tangles in the brain of Alzheimer's patients (Martyn, 1989). Calcium dysregulation in the brain cells was a also suggested to be related to brain aging and AD in coordination with the endocrine system – excessive glucocorticoid activates to increase calcium influx into hippocampal neurons through VOCCs (Landfield, 1992). Another theory about the causes of AD, *impaired neuroprotection* can induce altered β-amyloid precursor protein (APP), resulting in accumulation of β-amyloid peptide ('plaques'), eventually leading to destabilizing Ca^{2+} homeostasis (i.e., abnormally increased calcium uptake) in hippocampal neurons (Mattson, 1993; Barger, 1993; Zhang, 1993; Fukuyama, 1994). *Altered Ca^{2+} homeostasis* in these hippocampal neurons modulate expression of specific neurotransmitter receptors – i.e., high levels of glutamate receptors become vulnerable to neuro-excitotoxic or ischemic insults contributing to the pathogenesis of AD (Mattson, 1994). *Elevated Ca^{2+}* also modulates cytoskeletal changes such as forming neurofibrillary tangles in AD.

Ca^{2+} homeostasis is maintained by neuroprotection in the brain cells such as basic fibroblast growth factor (bFGF), nerve growth factor (NGF) and insulin growth factor (IGF). Neuroprotection can rescue vulnerable cells from damage – i.e., bFGF suppress over-expression of glutamate receptors (NMDA receptors) in excitotoxic hippocampal neurons (Mattson, 1993).

As earlier described, expression of specific Ca^{2+} channels is varied depending on the type of tissues/cells and on the developmental stage, causing various intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. L-type Ca^{2+} channels coupled with RyRs are involved in neurogenesis and neuronal differentiation *in vitro*, according to a compared study in two different types of stem cells, murine *embryonic*- and *adult* stem cells (Resende, 2010). The pattern of Ca^{2+} spikes or oscillations is often characterized as being action-potential-driven or ER-opearted during activation and differentiation. Spontaneous Ca^{2+} spikes are coupled with membrane depolarization

According to Spitzer, action potential-dependent spontaneous Ca^{2+} spikes are generated during the period of embryonic neuronal differentiation, and subsequently induce further Ca^{2+} release from the ER, and consequently release neurotransmitter via Ca^{2+} vesicles (Spitzer, 2004). However, his hypothesis of activity-dependent neurotransmitter release is not clear enough to distinguish how far neuronal maturity is achieved. There are still no striking evidences how much distinguishable or differ Ca^{2+} spikes and oscillations from activations by different types of neurotransmitters.

During neuronal differentiation, discriminative expressions of neurotransmitter receptors are related to functional specification of neurons. Inotrophic glutamate receptors (i.e., NMDA-Rs) are crucial in developing neurons in terms of facilitaing neural circuits, while coactivation of AMPA-Rs together with NMDA-Rs is necessary in mature neurons (Muth-Köhne, 2010). In the developing neurons, non-functional response to certain neurotransmitter receptors does not mean structural absence of receptor expression- this could be due to inactive form of or secretive form of receptors. This indicates that the interpretation from immunological detection is not always to represent functionality of proteins. Therefore, it is not quite conclusive that immunopositivity is equal to functionality. To show functionality of specific neurotransmitter receptors, measurement of Ca^{2+} activities upon activation of respective neurotransmitter or inhibition of its receptor is necessary.

1.1.3 Ca^{2+} in the heart muscle contraction

The importance of calcium in the contraction of the heart was pointed out by Sydney Ringer (Ringer, 1883). Ringer observed that 133 mM Na, 1.34 mM KCl, 2.76 mM $NaHCO^3$ and 1.25 mM $CaCl_2$ were able to sustain or restore heart beats from pipe water (0.2 mM K and 1 mM Na)-bathed non-beatable hearts (Ringer, 1883). This indicated that Na^+/K^+ were important together with Ca^{2+} in cardiac muscular contraction. Niedergerke measured extracellular forces by diffusion using 1.5 mM ^{45}Ca-labelled Ringer's solution as a mechanism of action of calcium ions into the cells to initiate the contraction of the heart (Niedergerke, 1957).

Free Ca^{2+} concentration in quiescent ventricular muscle were measured with microelectrodes at 0.26 µM, but increased as high as 10 µM in contractile ventricular muscle (Marban, 1980). The contraction of heart muscle is a coupled phenomenon between rhythmic electrical excitation and a rise of intracellular free

ionized calcium Ca^{2+} that binds to the cytoplasmic contractile proteins (actin and myosin) (Vaughan-Jones, 1986).

Fast inward Na^{+} currents generate action potential through voltage-operated Na^{+} channels (VONCs) – initial depolarization – membrane depolarization opens up voltage-operated Ca^{2+} channels (VOCCs) allowing the inward Ca^{2+} currents – then, membrane potential goes down to 0 mV. The outward K^{+} current re-polarizes heart muscle cells. The current paradigm of calcium regulation within the cells for contraction and relaxation of heart muscle is begun with intracellular Ca^{2+} influx into sarcoplasmic reticulum (SR) through sarcolemmal Ca^{2+} channels; secondly, Ca^{2+}-induced Ca^{2+} release from the SR; thirdly, ATP-dependent re-uptake of Ca^{2+} into the SR.

Ca^{2+}-induced Ca^{2+} release from the SR occurs as extracellular Ca^{2+} influx was coupled with excitation. As a result, cytoplasmic Ca^{2+} increases by ten-fold, and subsequently activates contractile filament proteins such as *actin* and *myosin*. Relaxation is achieved by Ca^{2+} re-uptake into the SR by coordination of Ca^{2+}-ATPase pump and Na^{+}-Ca^{2+} exchanger at the sarcolemma.

1.2 DEVELOPMENT OF HUMAN FETAL BRAIN

The question what made us the human is still not completely answered in the science, religion and philosophy. One of the wonders in the human brain is the cerebral cortex. Is the cerebral cortex extended part from the forebrain? However, the developing human cortex has its distinctive features such as the early expression of GFAP in radial glia at the onset of neurogenesis, the existence of non-dividing radial glia, the production of interneurons in the subventricular zone (SVZ), the distinctive subpial granular (SG) layer, the existence of entirely new classes of neurons (i.e., fusiform neurons), late-born subplate (SP) cells (Levitt, 1981; Kostovic, 2004; Zecevic, 2004; reviewed by Brystron, 2008). Whether these features made the human distinctive in cognition and behaviors from the other mammalian species still remains under discovered.

[Study I] I have investigated neurotransmitter-induced Ca^{2+} neurochemistry at the cellular levels during progressive differentiation. To understand how Ca^{2+} homeostasis is regulated in the developing human brain cells, the *in vitro* model was primary human fetal cerebral cortical cells and forebrain cells (gestational

age, GA 11-13 week) during proliferation and differentiation. These cells were utilized as neural stem cells (NSC) by inducing proliferation in bFGF and EGF, and further differentiated in serum FBS and serum-free condition with a combination of BDNF and GDNF, respectively.

Basic FGF, also known as FGF-2, is expressed in the brain neuroepithelium during embryonic development and in astroglial cells throughout life. bFGF plays a role in the proliferation of neural progenitors in the embryonic cerebral cortex and in maintenance of adult cortical progenitor cells (Raballo, 2000; Chen, 2008). The exon 3 of bFGF gene is conserved and has a binding site for heparin. Deletion of exon 3 on bFGF gene causes reduced number of neural progenitors, of excitatory neurons and of astrocytes in both the embryonic- and adult cerebral cortex (Chen, 2008).

EGF receptors (EGFR) are expressed in the infant, juvenile, young adults, and adult human rostral subependymal zone (SEZ), where migrating neuroblasts and early neurons residues and involve in neurogenesis during postnatal periods (Weickert, 2000). Neonatal brain injury increases protein expression of EGFR in both neural stem cells (NSC) and progenitors in the SVZ of the rat brain (Alagappan, 2009). Besides, over-expression of EGF-R increases in both number of progenitor cells in the SVZ and number of NSC at the cell cycle S-phase, which activation of EGFR eventually increased the size of neurospheres *in vitro*.

Activation of mitogens such as bFGF and EGF is crucial for the fetal brain development, and for maintaining the pool of regenerative progenitors in the adult brain. The study I was designed to do further investigation on the role of Ca^{2+} regulation in proliferative- and differentiating neural progenitors. To expand the understanding of human brain development together *in vitro* behaviors of primary human brain cells, I first reviewed literatures in the development of human fetal brain (i.e., cerebral cortex and forebrain) prior to further interpretation of *in vitro* proliferation of and differentiation of human brain cells.

1.2.1 Cerebral cortex

The understanding of human cerebral cortex at early fetal development enhances more knowledge about disorders in cerebral cortex such as epilepsy, mental retardation, developmental delay, neurological deficits in childhood, and further neurodegenerative diseases in adults. Abnormal development of human cerebral cortex during fetal

periods is often found in the fetus with Down Syndrome (DS) – 57 of 71 genes on the human chromosome 21 are up-regulated, 14 of 71 genes are down-regulated (Yu, 2007). These genetic characteristics may indicate an association with impairment of cognitive functions. Some infants (35%, 13/37) born with DS also have an increased risk of congenital heart defect (Narchi, 1999).

Tiu et al. suggested that innate programming seem to play a dominant role during embryonic & early fetal development of cerebral cortex (1st trimester of pregnancy), but developmental process is more influenced by extrinsic factors during later fetal development of cerebral cortex (2nd-3rd trimesters): expression pattern of neuronal- (i.e., GABA, choline acetyltransferase ChAT, excitatory amino acid transporter GLT-1, dopamine beta hydroxylase, dopamine receptor DR1 and synaptophysin) and glial cell proteins (GFAP) is various depending on time- and location (space)-dependent manners (Tiu, 2003).

The development of human cerebral cortex was well described (Marín-Padilla, 1998): The neocortex starts its development with a primary plexiform layer in the telencephalon, which precedes and is essential for formation of the cortical plaque. Layer I and the sublayer are derived from this primary plexiform layer. The other layers (II, III, IV, V and VI) are derived from the cortical plaque. During the development of the cortical plaque, migration, early differentiation and morphological and functional maturity of the neurons occur. The neurons, guided by the radial glia, reach layer I, develop an apical dendrite and establish contact with the cells of Cajal-Retzius, after which the migratory neurons assume a common pyramidal morphology. During ascending cortical maturity, controlled by the thalamus, the neurons acquire their specific morphology and function. The cortical plaque represents a biologically non-specific stratified nucleus which increases the number of pyramidal layers during the evolution of the mammal.

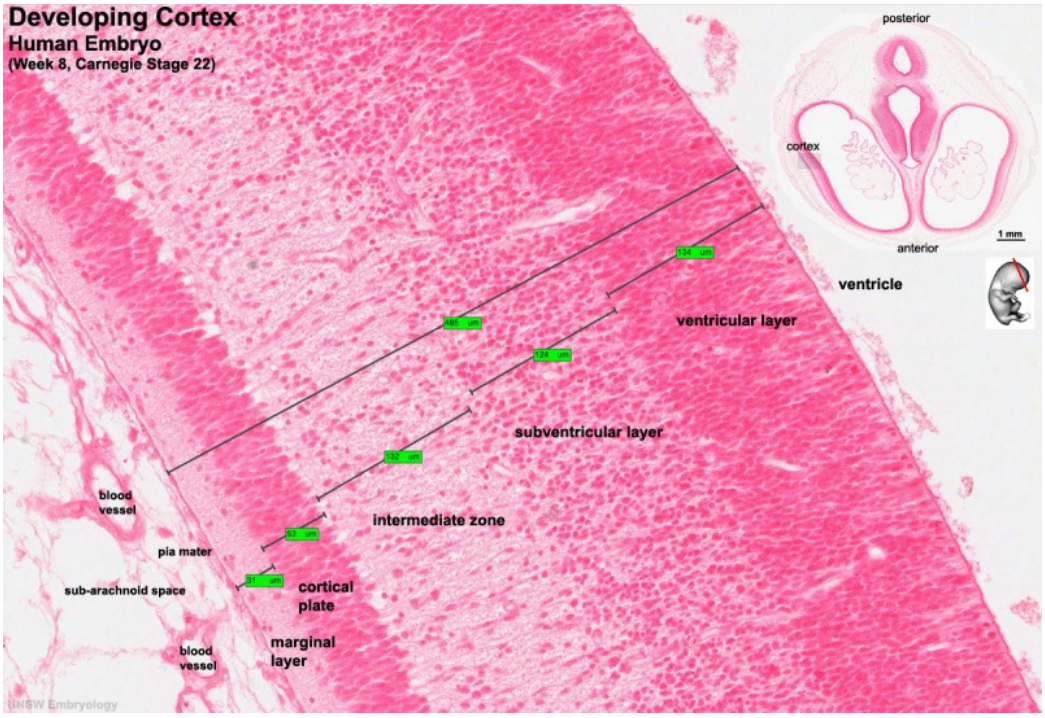

Figure 1 | Developing human cerebral cortex at gestational week 8. The entire cortical layer is 485 µm thick, which consists of the ventricular layer (134 µm), the subventricular layer (124 µm), the intermediate zone (IZ) (132 µm), the cortical plate (CP) (53 µm), the marginal layer (31 µm).

This figure is adapted from the online embryology lectures (by Dr Mark Hill) at the Univ. of New South Wales, Sydney, Australia. (The source is downloaded on 2011-12-20)
http://php.med.unsw.edu.au/embryology/index.php?title=Neural_-_Cerebrum_Development

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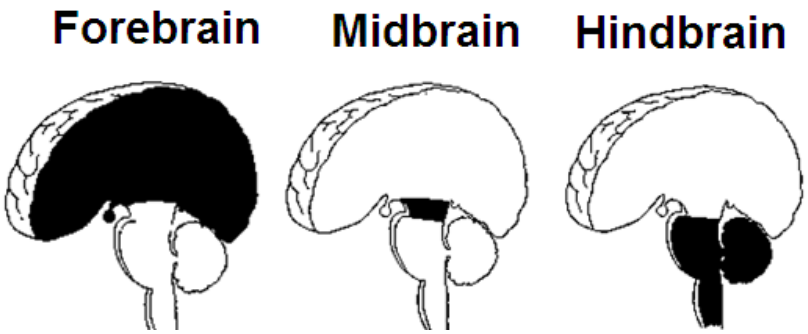
The cortical ventricular zone (VZ)/subventricular zone (SVZ) are cell reservoir of neural cells (neurons and marcroglia) for the developing brain. The lumen of cerebral ventricle, VZ, is the only proliferative zone in the human embryonic telencephalon at gestational age (GA) week 5-6. The cortical plate (CP) and the SVZ appear at GA 7-8 week (Zecevic, 2005). The significant increase and migration of cortical neurons exhibit at GA 10-25 week. All cortical neurons originate from the ganglionic eminence of the ventral forebrain (Andersson, 1997), while a subpopulation of interneurons, *neocortical GABAergic neurons* - 70% (at GA 14 week) / 62% (at GA 24 week) / 65% (at GA 25 week) of Mash1-expressing progenitors originate locally from the cortical VZ/SVZ of dorsal forebrain; 35% of Dlx1/2-expressing, *non-radial, migratory-like neurons* originate from the ganglionic eminence (GE) of ventral forebrain (Letinic, 2002). Letinic *et al.* also found that *radial, migratory* Dlx-expressing

GABAergic neurons residue in the intermediate zone (IZ), situated between the VZ/SVZ and the cortical plate (**Fig. 1**). Here two faces of GABAeric neurons are suggested that *non-radial GABAergic neurons* migrate within the VZ/SVZ, but are switched *to radial GABAergic neurons* in the IZ after exiting the VZ/SVZ. They characterized morphological phenotypes of *non-glial migratory cells* in the VZ (8%) /SVZ (85%) - *dividing progenitors* (52%) with a large (10-12 μm in diameter) round shape containing one or two processes; *post-mitotic migrating cells* (45%) with a small oval soma shape (4-6 μm in diameter) containing one or two leading processes. Interestingly, they observed the pattern of cell division of these dividing progenitors – 82% of these dividing cells undergo, first, asymmetric division into migratory-like cells and progenitor-like cells, and subsequently, symmetric division into their own identical daughters.

The appearance of neuronal synapses is the sign of emerging neuronal function. In the first CP, very few synaptic circuitries including establishment of few synaptic vesicles were formed at around 8 week of gestation (37 mm-long fetus). The first CP is located beneath of the marginal zone (MZ) in the lateral wall of the hemisphere dorso-lateral to the ganglionic eminence (Molliver, 1973). However, synapses are not found in the CP, but found in the marginal zone and the intermediate zone at 15 week of gestation (120 mm-long fetus) (Molliver, 1973). Interestingly, neuronal synapses are formed extensively during human postnatal periods, for example, neuronal synaptic density is shown a maximum at postnatal 8th month in human primary visual cortex.

1.2.2 Forebrain

The forebrain consists of diencephalon (optic vesicle and third ventricle) and telencephalon (cerebral hemisphere and lateral ventricle).



The ventricular zone (VZ) within the deep brain harbors heterogeneous populations of cells - neural progenitors, neurons and glia. The postmitotic neurons originated from the VZ migrates into the marginal zone (MZ), and further these postmitotic neurons generates a new layer, called intermediate zone (IZ), and the prospective white matter which is situated between the VZ and the MZ. Later, the subventricular zone (SVZ) is formed by the secondary proliferative cells originated from the VZ (Rakic, 1972; Levitt, 1981). The CP is formed from migratory young neurons along the radial glial cells toward the pial surface of the cerebral wall. At the beginning of the 2nd trimester, the subplate zone (SP) is formed between the IZ and CP. The SP reaches a maximal thickness at week 22 of gestation, and gradually starts decreasing after 35 week of gestation, and completely disappears at around 6 month of postnatal periods.

For the [study I], the primary human fetal forebrain cells are derived from the telecenphalic ventricular zone (VZ) at 13 week of gestation, and from the subcortical regions (SP, IZ, SVZ) at 11 week of gestation at around the end of the 1st trimester or the beginning of 2nd trimester of pregnancy. For the general features of fetal development at week 11-13 of gestation, the fetus develops teeth, finger nails, toe nails, hair follicles, the ear, red blood cells in the liver, clear genital formation, long limbs, and the heart and the spleen starts functioning.

The human brain seems to have region-specific phase of developmental programs. In the human forebrain, fast phase of development occurs at 10-18 week of gestation, while slower phase of development around from gestational age week 20 to the term (Dobbing and Sands, 1973). In contrast to previous findings, the exponential increase of fetal forebrain development occurs between week 13 and week 20 of gestation (Samuelsen, 2003).

1.2.3 Human neural stem cells

The sources of human neural stem cells (hNSC) can be obtained from the developing central nervous system (CNS) and the SVZ of adult brain. NSC are often characterized by their multipotency, capacity of self-renewal, and undifferentiated state for the extensive periods of life time *in vivo*. Multipotency of hNSC may be achieved through spontaneous differentiation into neurons, astrocytes, and oligodendrocytes following withdrawal of mitogens such as bFGF and EGF *in vitro* (Vescovi, 1999). However fetal brain-derived NSC may exhibit heterogeneity of cell types depending on the sources of

the brain region and the gestational age, therefore multipotency of fetal NSC can be limited as compared to NSC from the SVZ. In the study I, hNSC lines were established from isolations of the fetal brain tissues as close as possible to the VZ/SVZ.

The reasons to utilize hNSC in the [study I] are to characterize functionality of expanded- and differentiated hNSC in neurotrophic factors. Expanded hNSC often do not differentiate into specific cell types upon the region of transplantation – remain undifferentiated precursor cells or turn into astrocytes/glial cells (Wu, 2002). Therefore, *in vitro* differentiation seems necessary prior to transplantation into the injured area of the brain. The [study I] did neither aim for the transplantation in the rodent brain injury model nor for the clinical applications. hNSC behave differently when they are grafted into the host brain, because the microenvironment *in vivo* is completely different from the restricted *in vitro* environment. As an initial step towards stem cell treatment in the future, the functional behavior of differentiated-non-grafted hNSC is necessary to screen at present, depending on extracellular media.

It is important to investigate the neurochemistry of differentiated hNSC when the target brain diseases require for the specific neuronal cell types and functions. For example, to replace loss of motor neurons in the patients with amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), generation of cholinergic neurons is demanded from the graft of hNSC. In this strategy, either hNSC successfully differentiate into cholinergic neurons *in vitro* or expanded/differentiated hNSC can be transplanted along with neurotrophic factors (NF) that act paracrine on both endogenous cells and transplanted exogenous cells.

In vitro chemical manipulation of hNSC with a cocktail of factors produces the cholinergic neurons from cerebral cortex-derived hNSC: A cocktail of factors consists of either recombinant human basic fibroblast growth factor (bFGF), natural mouse laminin and heparin; or epidermal growth factor (EGF), leukemia inhibitory factor (LIF), mouse sonic hedgehog amino-terminal peptide (Shh-N), all-trans retinoic acid (RA) (Wu, 2002). However, the result from the graft of hNSC-derived cholinergic neurons raises us another question of the microenvironement within the host brain, as grafted hNSC undergo region-specific differentiation *in vivo* - glutamatergic neurons in the cortex, GABAergic neurons in the hippocampus, cholinergic neurons in the medial septum and the spinal cord (Wu, 2002).

In the [study I], a cocktail of neurotrophic factors, BDNF and GDNF was used to differentiate hNSC *in vitro*. BDNF is a mediator involved in neuronal survival

and plasticity of dopaminergic, cholinergic, and serotonergic neurons in the CNS and has neuroprotective effects against neurotoxicity in neurodegenerative diseases (Angelucci, 2005; Singh, 2006; Jourdi, 2009). BDNF promotes neuronal differentiation and survival of neurons. Overexpression of BDNF in non-neuronal lineage stem cells, mesenchymal stem cells induces neuronal differentiation *in vitro* (Lim, 2008). The effect of BDNF is mostly studied in the transplantation model. Transplantaion of BDNF-overexpressed hNSC promotes angiogenesis and functional recovery in a rat intracerebral hemorrhage (ICH) model (Lee, 2010). Earlier, transplantation of BDNF-overexpressed fibroblasts (i.e., producing 100 ng BDNF/106 cells/day) reduced neuronal death cerebral cortex in the rat stroke model (Ferrer, 2001). Infusion of BDNF itself in the rat model of spinal cord injury does not reduce inflammation, but increase regeneration of motor neurons and functional recovery (Kim, 2004). GDNF is involved in promoting neuronal survival such as dopaminergic neurons and cortical neurons (Pezeshki, 2003). GDNF also promotes differentiation of and migration of cortical GABAergic neurons (Pozas, 2005). The therapeutic potential of GDNF is studied using gene therapy in a rat model of traumatic brain injury (TBI): GDNF enhances faster recovery of forelimb and protects damaged cortical neurons. In the experimental design of [study I], a combination of BDNF and GDNF in cultures is to induce neuronal differentiation and survival, and to protect neuronal death.

1.2.4 Gap junction

One of critical features during embryonic- and fetal development of the human brain is gap junction-mediated regulation of signaling (i.e., electrical-, metabolic-, and ionic signal transmission) between neighboring cells. Gap junctions play a role in both maintaining homeostasis and the pathogenesis of diseases.

In the structural aspect, expression of gap junction proteins (connexin-26, -32, -43) is transient and tissue-/cell-specific. For example, connexin 26 (Cx26) is dominantly expressed in the early embryonic development of rodent brain the brain. Connexin 32 (Cx32) is not expressed at great extent, but more frequently expressed in the oligodendrocytes, some subtypes of differentiated neurons in the adult brain (Derrmietzel, 1989). Connexin 43 (Cx43) is expressed from the earlier embryonic development and consistently to the adult brain during neurogenesis of neuroepithelial cells or neural progenitors. Expression of gap junction proteins is also related to the progression of diseases depending on both their expression levels and cellular locations

such as human brain tumors: strong intensity of immunoreactivity of Cx32 was shown in gangliomas and oligodendroglioma, Cx43 in astrocytomas in the epileptic patients (Aronica, 2001). The localization of protein expression that is membranous or cytoplasmic indicates whether the cells are normal or malignant. Cx43 in the high-grade of astrocytomas is accumulated in the cytoplasm, while is located in the plasma membrane of low grade of astrocytomas and normal astrocytes (Aronica, 2001). The degree of gap junction protein expression is developmentally regulated in the normal brain and controlled by microenvironment. For instance, higher expression of Cx43 is found in the both the microvascular endothelial cells of the cortical plate (CP) within the telencephalon during fetal periods (18 week of gestation) and astroglioma in the adult brain, whereas the mature cerebral cortex does not express (Errede, 2002).

In the functional aspect, the gap junction is also of importance in the regulation of neurotransmitters from early corticogenesis to the periods of establishing functional neural circuits during the developing brain. In the [study I], it was hypothesized that electrical signal transmission via gap junction is coupled with neurotransmitter-regulated signal transmission via Ca^{2+} in the early human corticogenesis and differentiation of hNSC. This study compared the response of classical neurotransmitters (i.e., ATP, glutamate and GABA) with involvement of gap junction. It was first investigated the role of gap junction in the regulation of spontaneous Ca^{2+} signals depending on extracellular conditions - proliferation (bFGF+EGF) and differentiations (FBS and BDNF+GDNF, respectively).

1.2.5 Purinergic signaling

The role of ATP has been debated for many years. The recent evidences emphasize the role of ATP as a purinergic neurotransmitter or modulator in the peripheral and central nervous system. Four P1 receptors, seven P2X ionotrophic and eight P2Y metabotrophic receptor subtypes are recognized (reviewed by Burnstock, 2006). These receptor subtypes are differentially expressed depending on the tissue-/cell-types and their functions.

The poem below was presented by Samuel C. Silverstein at the meeting held by the New Your Academy of Sciences in 1989 – emphasizing many roles of ATP.

Oh tell me Lord how could it be,
That though our cells make ATP,
It's not all used for energy,
But sometimes is secreted free.
It puzzles you, it puzzles me,
While Geoffrey Burnstock smiles with glee
At the many roles of ATP.

In the brain, purinergic signaling is involved in nervous tissue remodeling following trauma, stroke, ischemia or neurodegenerative disorders (reviewed by Burnstock, 2006). Brain injury activates astrocytes via the actions of ATP on P2X-receptor subtype to release FGF, EGF, platelet-derived growth factors (PDGF) which stimulate proliferation of astrocytes, resulting in gliogenesis or reactive astrogliosis as a mechanism of neuroprotection (Neary, 1996).

Slow purinergic signaling is involved in proliferation, migration, differentiation, apoptosis during embryonic development, while *fast* purinergic signaling include neural transmission, neuro-modulation, exocrine-/endocrine-secretion, platelet aggregation, vascular endothelial cell-mediated vasodilatation and nociceptive mechano-sensory transduction (reviewed by Burnstock, 2006). In the synaptic neural signal transmission within the human cerebral cortex, ATP regulates Ca^{2+} signals by activating ionotrophic P2X receptors via L- and N-type Ca^{2+} channels in synaptic terminals (Pintor, 1999).

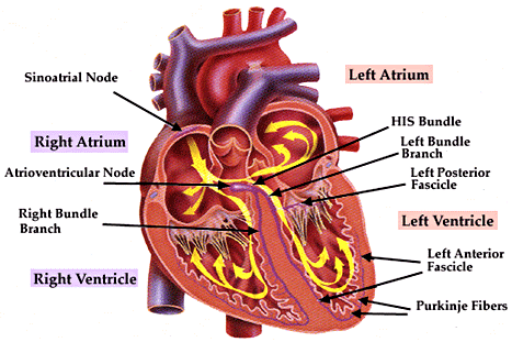
1.3 HUMAN FETAL CARDIAC DEVELOPMENT AND MATERNAL AUTOIMMUNITY

At 21 days after conception, five weeks after the last normal menstrual period (LMP), the human heart begins beating at 70 to 80 beats per minute (bpm), and increases linearly for the first month of beating, peaking at 165-185 bpm during the early 7th week. After peaking at about 9.2 weeks after the LMP, it decelerates to about 150 bpm during the 15th week after the LMP. After the 15th week the deceleration slows reaching an average rate of about 145 bpm at term (DuBose, 1990; Schats, 1990; Qasim, 1997).

The electrical depolarization triggers cardiomyocytes to contract spontaneously. Pacemaker cells develop in the primitive atrium and the sinus venosus to form the sinoatrial (SA) node and artrioventricular (AV) node. Conductive cells develop the bundle of His (or AV bundles) transmit the depolarization into the lower heart (ventricles).

1.3.1 Sinoatrial (SA)- and artrioventricular (AV) conduction system

In a normal (adult) heart, electrical impulses pace the rhythm at which the heart contracts and relaxes. The sinoatrial (SA) node triggers the electrical impulse, causing the upper chambers (atria) to contract. The rhythmic Ca^{2+} influx is commonly observed in the SA node, known as the heart pacemaker. The signal travels through the atrioventricular (AV) node to the AV bundle, which divides into the Purkinje fibers that carry the signal and cause the lower chambers (ventricles) to contract. The AV node consists of different cell types - transitional cells, midnodal (or typical nodal cells), lower nodal cells, and cells of the AV bundle (Meijer, 1988). The AV node elicits slow conduction via gap junctions, which delays ventricular excitation to allow the heart to function effectively as a pump (Habuchi, 1996). AV conduction is enhanced by β-adrenergic stimulation via L-type Ca^{2+} channels, but suppressed by acetylcholine (Ach) (Meijer, 1988).



The figure is adapted/downloaded from the website http://www.emergencymedicaled.com/215AED.htm

Neonatal lupus erythematosus (NLE) is developed in an infant who is born from a mother with systemic lupus erythematosus (SLE), one of autoimmune diseases. At the late 2nd trimester of pregnancy, maternal IgG antibodies (anti-Ro/SSA and -La/SSB antibodies) passively transport to the fetus, which develops fetal bradycardia (slow heart beating, <120 bpm) and myocarditis (Buyon, 1989). Ro/SSA-La/SSB ribonucleoprotein complex were found in 85% of sera from mothers of offspring with neonatal lupus (Buyon, 1989). A swedish population-based study showed the strong association of anti-Ro52 with congenital heart (AV) block: 95% (88/93) of mothers were positive to anti-Ro52 antibodies, 63% (59/93) of mothers were positive to anti-Ro60 antibodies, 58% (54/93) of mothers were positive anti-La-antibodies (Salomonsson, 2011).

1.3.3 Congenital heart failure

According to NEO ECG guidelines by the European Society of Cardiology in 2002, the heart rate of newborn babies (< 3 months) is between 123-150 bpm with PR interval is around 0.10 sec (range: 70-140 ms) (www.escardio.org). Normally, this interval for the adults is between 0.12 and 0.20 sec in the adult hearts.

The PR interval represents the time needed for an electrical impulse from the SA node to conduct through the atria, the AV node, the AV bundle, the bundle branches, and the Purkinje fibers. The PR interval of the surface ECG is measured from the onset of atrial depolarization (P wave) to the beginning of ventricular depolarization (QRS complex).

In the 1st degree AV block, the electrical impulses move through the AV node more slowly (or delay) than usual. First-degree AV block is diagnosed by electrocardiogram (ECG) showing a prolongation of the PR interval (> 0.20 sec) (John, 2006; Barold, 2006). Complete (3rd degree) AV block has no conduction from atria to ventricles.

2 AIMS

The main aim of this thesis was to compare the roles of Ca^{2+} in the developing human fetal brain cells and rat cardiomyocytes

Specific aims for

[Study I]

- To characterize morphology and function following *in vitro* proliferation of and differentiation of hNSC
- To elucidate the role of gap junctions in the spontaneous Ca^{2+} activities and regulation of neurotransmitter-mediated Ca^{2+} signals
- To emphasize the developmental regulation of purinergic signaling and neurotransmitters
- To quantify induction profile of neurotransmitters in extracellular environment- and culture time-dependent manners during *in vitro* differentiation of hNSC
- To screen functional expression of ionotrophic and metabotrophic receptors of glutamate and GABA

[Study II]

- To find out specific epitope within Ro52 protein for anti-Ro52 antibodies in relation to congenital heart block
- To investigate Ca^{2+} regulations on rat cardiomyocytes following induction of anti-Ro52 antibodies

3 METHODOLOGY

In this section, the brief description of methodology is written and more details can be found in the papers.

• In vitro expansion of hNSC	Paper I
• In vitro differentiation of hNSC	Paper I
• Primary cardiomyocytes cultures	Paper II
• Rodent neonatal lupus syndrome model	Paper II
• Ro52 protein	Paper II
• Anti-Ro52 antibodies	Paper II
• Passive antibody transfer and ECG recording	Paper II
• ELISA	Paper II
• Time-lapse Ca^{2+} imaging	Paper I-II
• Immunocytochemistry	Paper I
• Statistical methods	Paper I

Paper I of manuscript (Study I) and Paper II of published article (Study II)

This thesis is diverted into two different models - but compared Ca^{2+} regulation and its dynamics in the developing cardiac cells and brain cells. Ca^{2+} is in common as an important regulator in the most important organs in the body. Ca^{2+} is tissue-/cell-specific and regulated diversely depending on their stage of development. This thesis is to unlock the mystified role of Ca^{2+} in two different tissue types.

3.1 IN VITRO MODELS

For measuring Ca^{2+} and detecting protein expression, primary cortical-/forebrain cells from human fetal cerebral cortex- and forebrain (11-13 week of gestation) were used in the [study I], and primary cardiomyocytes from neonatal Dark Agouti (DA) rats (1-2 day-old) in the [study II].

3.1.1 In vitro expansion of hNSC

To induce proliferation of primary fetal brain cells (cerebral cortex and forebrain) *in vitro*, mitogens bFGF and EGF were used to establish primary NSC cell lines. These mitogens partly acts as a mimic of *in vivo* microenvironment in the model of normal developing brain or brain injury that both require proliferative neural precursors or progenitors from the SVZ to recruit the pool of young neurons into the target brain region. Both human cortical and forebrain cells were responsive to these mitogens and grew in cultures as a form of neurospheres. Neurosphere is characterized as a typical feature of neural stem cells (NSC). However, recent studies warn the formation of neurospheres *in vitro* as the cultured brain tumors from patients also form neurospheres with capacity of self-renewal and differentiation – this indicates cautious clinical approach of neurospheres, which likely become malignant glioma or glioblastoma upon transplantation (Laks, 2009; Panosyan, 2010). *In vitro* culture environment is not exactly nourished and protected as found *in vivo* environment. For example, oxidative stress and higher concentration of mitogens with lack of neuroprotection may cause chromosomal changes of NSC – forming tumor-like stem cells. Multi-passages of primary NSC change genetic stability such as length of telomeres. In the [study I], the chromosomal numbers were not found abnormal, but more detailed investigations on gene mutations were not performed as the tumor was not the scope of the aims.

3.1.2 In vitro differentiation of hNSC

To induce differentiation of hNSC, two differentiation conditions were used: serum-containing condition (FBS) and serum-free condition (BDNF and GDNF). In addition to these factors, 100 µM of ascorbic acid (AA, vitamin C) was supplied in the media as an aqueous antioxidant. AA is also a co-inducer of differentiation in neurogenesis from NSC. The effect of AA in differentiation is region-specific or lineage-dependent. For example, AA increases the yield of dopaminergic (DA) neurons derived from bFGF-expanded mesencephalic precursors (Yu, 2004). A combination of BDNF and AA increases DA neurons (tyrosine hydroxylase, TH-positive cells) derived from fetal

midbrain neural precursor cells (NPC) which are expanded in bFGF, EGF and leukemia inhibitory factor (LIF) (Maciazyk, 2008). The effect of AA in the [study I] may be involved in the generation of cortical neurons. Serum induces differentiation of neural progenitors into neurons and astrocytes and influences cell behaviors and morphological differentiation (Hung, 2006). Deprivation of serum induces apoptosis (Li, 2007).

3.1.3 Primary cardiomyocyte cultures

For the [study II], primary cardiomyocytes from the hearts of neonatal rats were cultured in a combination of factors such as serum, insulin, transferrin, selenin and Bromodeoxyuridine (BrdU). Insulin is used in the metabolism of cardiac cells to produce ATP necessary for contraction of cardiac cells (Bertrand, 2008). Insulin is also suggested to prevent cardiomyocytes from oxidative stress-induced apoptosis (Aikawa 2000). BrdU is a synthetic nucleotide that is an analog of thymidine, which incorporating into the DNA of replicating cells, causing mutation – this chemical treatment was to prevent over-growth of replicating cells (i.e., fibroblasts) in the cultures.

3.2 IN VIVO ANIMAL MODELS

To induce AV block in the heart of newborn pups, the monoclonal Ro52 IgG_1 antibodies were injected into the pregnant female DA rats (aged 10-12 week) at gestation age- and concentration-dependent manners. Anti-Ro52 monoclonal antibodies (IgG_1) were generated against different epitopes within the Ro52 protein.

3.2.1 Ro52 protein

Ro52 is an auto-antigen that is often found in immune cells (i.e., B lymphocytes) from the autoimmune patients such as SLE and Sjögren's syndrome (Espinosa, 2006). Ro52 is a member of the tripartite motif (TRIM) family of proteins. The TRIM motif includes three zinc-binding domains, a RING finger, a B-box type 1 and a B-box type 2, and a centre coiled-coil region (leucine zipper) (Ottosson, 2006; Hennig, 2008) (**Fig. 2**).

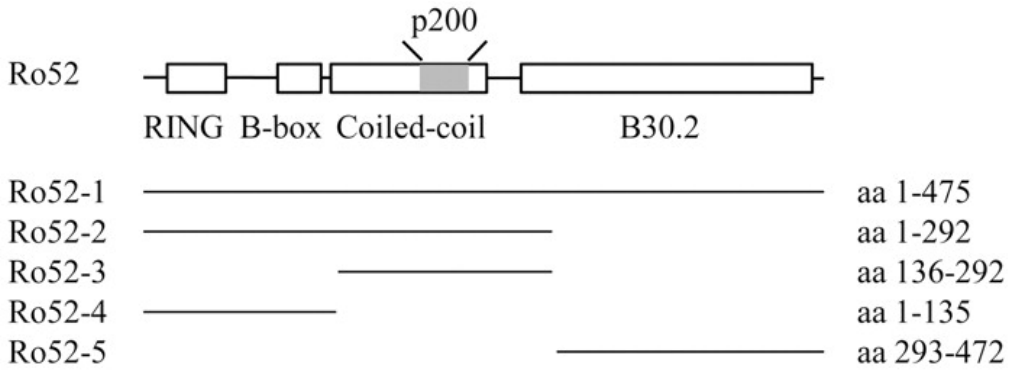


Figure 2 | The protein domain of Ro52 and its amino acid sequences.

The figure is adapted from Fig. 2A in the paper II.

3.2.2 Anti-Ro52 monoclonal antibodies

Anti-Ro52 autoantibodies are often found in maternal sera of autoimmune patients. Anti-Ro52 proteins are found in the fetal heart affected by complete congenital heart block (Reichlin, 1994). Circulating maternal autoantibodes are passively transferred across the placenta, which presumably injure the heart of the developing fetus, but never affect maternal heart (Eftekhari, 2000). Anti-Ro52 autoantibodies were suggested to interfere with L-type calcium channels on the heart in association with neonatal lupus (Garcia, 1994; Boutjdir 1997). Cross-activity of Ro52 protein with 5-HT_4 is found by inhibition enzyme immunoassay – suggesting that anti-Ro52 antibodies antagonize serotonin-induced L-type Ca^{2+} channel activation on human atrial cells (Eftekhari, 2000).

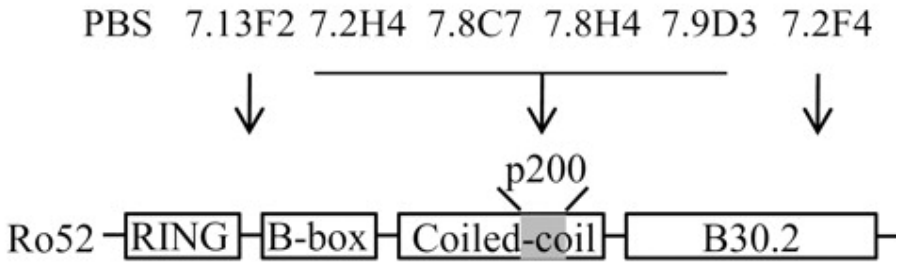


Figure 3 | The target motifs of anti-Ro52 monoclonal antibodies. Anti-Ro52 monoclonal antibodies were generated against respective motifs of Ro52 protein.

The figure is adapted from Fig. 3A in the paper II.

In the [study II], a panel of monoclonal antibodies was generated against the motifs within Ro52 protein (**Fig. 3**). 7.2H4, 7.8C7, 7.8H4 and 7.9D3 specifically bind to p200 in the coiled-coil leucine zipper motif. Antibody 7.13F2 was raised against non-coding region. Antibody 7.2F4 was raised against one of zinc-binding domain B-box motif.

3.2.3 Passive antibody transfer and ECG recording

To induce the heart block in the newborn pups, monoclonal antibodies were intraperitoneally injected to the pregnant female DA rats on gestation age (6- and 9 days) - and concentration (0, 2, 4, 6 mg) - dependent manners. Phosphate-buffered saline (PBS) was used as the control. Electrocardiograms (ECG) were performed in the newborn pups within 24 hours of birth at 4 intervals of 5 seconds /minute with a rate of 1000 Hz. QRS complexes (*mean*), PR interval and heart rate (bpm) were measured. It was observed whether the pups developed first-degree AV block or bradycardia.

3.2.4 ELISA

Anti-Ro52 monoclonal antibodies were generated from the BALB/c mice, which were immunized with human recombinant Ro52 protein (full sequence). Spleen cells from BALB/c mice were fused with SP 2/0 myeloma cells. Monoclonal antibodies from supernatant of hybridoma were screened for Ro52 IgG_1 antibodies using ELISA. Respective monoclonal antibodies (1 µg/mL) and serum from antibody-injected female rats were tested using ELISA.

Specific binding affinity of p200 or mutated p200 peptides to anti-Ro52 p200 monoclonal antibodies (7.2H4, 7.8C7, 7.8H4, 7.9D3) was measured using ELISA (**Fig. 4**).

```
        200       210       220       230
p200    LEKDERQLRILGEKEAKLAQQSQALQELISELD
pOUT          Q       N               K
pZIP     V  L            V
A233                                    A
```

Figure 4 | Peptide sequences of Ro52 p200 and mutated p200 (pOUT, pZIP, A233)

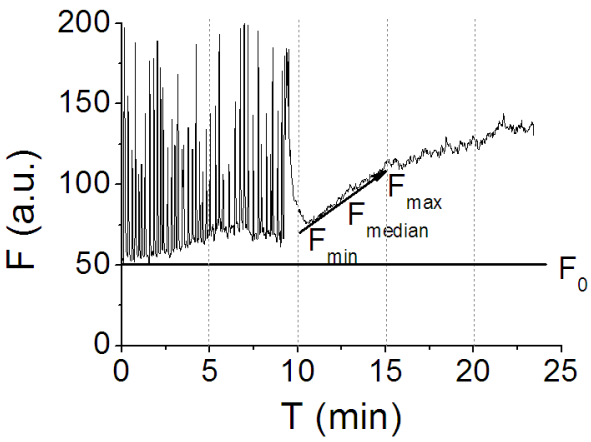
This figure is adapted from Fig. 4 in the paper II.

3.3 TIME-LAPSE CA^{2+} IMAGING

In the [study I and II], intracellular Ca^{2+} was measured using time-lapse imaging technique to investigate how Ca^{2+} is regulated in both cardiomyocytes and hNSC. For live Ca^{2+} imaging, the cells were cultured on poly-L-lysine [study I] or poly-D-lysine [study II]-coated glass slips. The cells were loaded Ca^{2+}-sensitive dye (Fluo 4-AM, Molecular Probes) with pluronic acid (F-128, Molecular Probes) that facilitates entry of Fluo 4 into the cytoplasm. Free Fluo 4 binds to free calcium ions within the cells. This process was achieved by incubating the cells for 30-45 min. at 37°C/5% CO_2 in a mix of conditioned medium and fresh medium (50:50). Subsequently, unbound Fluo 4 was removed and the fresh medium was replenished for 10 min. at 37°C/5% CO_2 for de-esterification. The glass slips were mounted in the chamber of the microscope. Calcium oscillations were recorded for approx. 30-40 min. on a laser-scanning confocal microscope Leica DM IRBE. Ca^{2+} signals were acquired at every 1.756 sec. using software Leica confocal software (LCS) (Leica Microsystems). The control recording lapsed for approx. 5 min., and subsequently the respective drug [study I] or antibody [study II] was slowly applied into the chamber of the microscope. To maintain pH and temperature, warm fresh medium (prepared at 37°C/5% CO_2) was routinely added.

Acquired data of Ca^{2+} signals (fluorescence intensity) were analyzed using imaging software LCS Lite ver. 2.61 (Leica Microsystems), mathematical software ORIGIN 8 (OriginLab) and Excel (MS Office 2007). Further conditional analysis was performed in statistical software Statistica 8 (Statsoft).

3.3.1 Median fluorescence intensity of Ca^{2+} in cardiomyocytes



Changes in median fluorescent intensity (median, F_m) were used as an indicator that the cardiomyocytes accumulates Ca^{2+} within the cells with loss of *oscillatory activities* (*regular Ca^{2+} influx/efflux with contraction*). Both maximal (F_{max}) and minimal (F_{min}) of fluorescence intensity were determined at every 5 min. of intervals during the control recording and after the application of antibody. F_{median} was determined between F_{max} and F_{min}. Relative F_{median} was calculated by relative increase of intensity against control F_{median}. To plot the graph of Ca^{2+} tracers (F-

F_0), the background basal level of fluorescence intensity (F_0) was subtracted. To produce the Ca^{2+} tracer images, 4-5 individual frames of images were selected and averaged in both the control and after the application of antibody.

3.3.2 Frequency of Ca^{2+} in hNSC

Frequency of Ca^{2+} was regarded as an encoder of neural signals that harbors translated signals transmitted from extracellular signals in the neural cells. Extracellular environment modulates the internal programs in the small organelles and the nucleus. We hypothesized that mitogens or factors for proliferation or differentiation in hNSC reprogram the pattern of Ca^{2+} signals, eventually representing developmental pattern of Ca^{2+} activities.

In the [study I], new approach in Ca^{2+} studies was employed: two types of neural stem cells by activity - quiescent cells (non-spontaneous cells) and active cells (spontaneous cells) at the time of observations. Cell size (μm^2) was defined by the total area of individual cell. It was to investigate whether cell size of proliferative hNSC *in vitro* is associated with response of neurotransmitter at different culture time (7- and 52-DIV) – is cell morphology associated with neurotransmitter? We also elucidated that duration of *in vitro* expansion and differentiation of hNSC affects functional behaviors of neurotransmitter. Frequency of neurotransmitter-induced Ca^{2+} was calculated how many transients were observed per minute on a culture time-dependent manner. Ca^{2+} frequency was used as a functional index to compare the response pattern of neurotransmitter, which indicates functional expression of receptors or ion channels for ATP, glutamate and GABA and their regulation in proliferative and differentiating hNSC (i.e. cerebral cortex).

Another interesting approach in the [study I] was the induction pattern of neurotransmitters (ATP, glutamate and GABA) and quantification of cell cultures depending on duration of cell culture time. These results will emphasize how culture environment affects in the neurochemistry of stem cells.

3.3.3 Cell population-based quantification of Ca^{2+} in hNSC

This thesis for the [study I] addressed some questions of the developmental pattern of neurotransmitter regulations – is there specific patterning of neurotransmitter during neural proliferation and differentiation? If so, what types of neurotransmitters are expressed? And what are the major orchestrators in Ca^{2+} signaling? To answer the questions, three typical neurotransmitters (ATP, glutamate and GABA) were chosen to

screen their regulatory patterns through gap junctions, purinergic signaling, glutamatergic signaling and GABAergic signaling via Ca^{2+} during proliferation and differentiation of hNSC. The quantitative method was used to quantify the cultures (cell population, %) depending on extracellular conditions (proliferative or differentiating).

3.4 IMMUNOCYTOCHEMISTRY

To detect structural expression of proteins, immunocytochemistry was performed. Primary antibodies were selected to detect morphological- and functional-differentiations. There are other candidates of markers to detect neural progenitors/stem cells, but nestin was the most common marker. Glial marker, GFAP, is the most debating marker with no clear cutline for cellular identity whether glial cells or astrocytes. However, in the paper I, we defined GFAP as a glial cell marker, which is often expressed in the early glial cells and later glial cells that differentiates into more like astrocytes. The full length protein S100 are often used to detect astrocytes, but co-expression of S100 with GFAP was often found in hNSC cultures. Therefore, S100 was defined as an astroglial cell marker. Secreted form of S100/S100β is somewhat used to detect reactive astrocytes in the diseases. Beta tubulin III, commercially known as Tuj1, was used as an immature neuronal marker. More mature neuronal markers NeuN, MAP2ab and NF200, were used. Olig2 was used to detect early oligodendrocyte marker. In some experiments, O4 was also used to detect more differentiated oligodendrocytes. To detect structural expression of functional proteins, K^+-Cl^- co-transporter (KCC2) was used as for an indication of developmental actions of GABA and formation of GABA synapse; GAD-65/67 for GABA production from glutamate; myelin basic protein (MBP) for myelination of nerves by oligodendrocytes.

3.5 STATISTICAL METHODS

In the study I, descriptive statistics or Kruskall-Wallis or ANOVA F-test were performed. The statistical significance was measured with p values: *$p<0.05$, **$p<0.01$, ***$p<0.001$. In the study II, A Mann-Whitney U test or Kruskall-Wallis test followed by Dunn's post tests (multiple comparision) were used.

4 RESULTS AND DISCUSSION

In general, Ca^{2+} oscillations were much slower in the developing fetal brain cells than neonatal cardiomyocytes. This phenomenon might be due to the following reasons (*from my insights*): contracting cardiomyocytes developed fast calcium machinery, (1) to regulate cytoskeletal protein interactions to the extracellular matrix of neighboring cells through gap junctions, and (2) to induce Ca^{2+}-induced Ca^{2+} release (CICR) from sarcoplasmic reticulum (SR), (3) to generate rapid energy supply of ATP from mitochondria. However, the developing human brain cells undergo slow nurturing process to proliferate and differentiate into various specific cell types with multi-functions. This thesis is focused on two different developmental stages (fetal, postnatal) in two different cell types (brain cells, cardiomyocytes) from two different origins of lineages (ectoderm, mesoderm). Two different stories from distinct cell types are connected into the one through the story of Ca^{2+} homeostasis. First, I would like to scrutinize the results from the study I, and then from the study II.

[Study I]

4.1 EXTRACELLULAR ENVIRONMENT AND NEUROMODULATION

Human fetal neural stem cells (hNSC) undergo proliferation and differentiation on region-specific and gestational age-dependent manners. Morphology of forebrain-and cerebral cortex-derived hNSC in the expansion media condition was similar, but hNSC-forebrain often exhibited extensive bridges between small neural clusters (neurospheres attached on the culture plate) (**Fig. S1**). This result indicated that neurite outgrowth was more extensive in hNSC-forebrain (11-13 week of gestation) than in hNSC-cortex during *in vitro* proliferation. Morphological differentiation was similar between hNSC-cortex and hNSC-forebrain during proliferation (bFGF+EGF), except increased number of immature neurons ($Tuj1^{+}$) in hNSC-forebrain (11-week, hFB11w-s6) during proliferation (FGF2+FGF8+Shh) (**Fig. S2**). Expression of S100 protein was higher during proliferation and lower during differentiation (**Table 1** and **Fig. S2**). Expression of both GFAP and to S100 in hNSC-cortex was reduced as duration of differentiation in vitro was longer in serum conditioned media (**Table 1**). Instead, expression of GFAP was increased in serum-free BDNF+GDNF conditioned media. BDNF+GDNF seemed to prevent rapid reduction of S100 expression (**Table 1**). These results indicated that a

cocktail of BDNF and GDNF enhanced generation of glial cells and of astrocytes, while serum FBS reduced the astroglial populations (**Fig. 1**).

One of great challenges in NSC differentiation is to generate large numbers of neurons with specificity of morphology and function. The aim of study I was to compare morphology and function in two differentiation conditions. In function, there was no structural expression of KCC2, GAD-65/67 and MBP in both differentiation conditions using a method of immunocytochemistry. Neither serum itself nor a cocktail of factors BDNF+GDNF was sufficient enough to induce the birth of mature neurons (**Table 1**). There were no other specific neuronal markers used to detect cholinergic neurons, GABAerigc neurons and glutamatergic neurons. To compensate weakness of the results from immunocytochemistry, functional identity of hNSC was determined by Ca^{2+} studies.

One of critical characteristics of NSC is able to sustain neurotrophic effect in both *in vitro* and *in vivo upon transplantation*. The differentiation conditions used for the [study I] maintained high expression of BDNF (**Table 1**). This result remained elusive whether endogenous expression of cultured primary hNSC-cortex (11 week) were induced by extracellular environment, or from properties of stem cells and/or characteristics of cortical progenitors from *in vivo* developing programs.

4.1.1 Culture time-dependent proliferation

The [study I] gave an emphasis on importance of expansion of NSC itself. Most stem cell researchers are likely to concern about larger quantity of expanded stem cells prior to differentiation. Haven't we missed out how long hNSC require to be expanded in cultures? We found that culture time (days-in-vitro, DIV) influenced the response pattern of neurotransmitters via Ca^{2+} (**Fig. S3**). Longer exposure (i.e., 52-DIV) of hNSC to mitogens bFGF+EGF decreased ATP-induced Ca^{2+} frequencies, but increased glutamate- and GABA-induced Ca^{2+} frequencies. These results might possibly indicate that neurite outgrowth between proliferative cells within the neurosphere presumably maintain their potential of self-renewal and survival from *in vitro* environment.

4.1.2 Culture time- and condition-dependent differentiation

Expanded hNSC were found two different types of cells by Ca^{2+} activities during the control recordings: quiescent cells (non-spontaneous, *non-spt*) and active cells (spontaneous, *spt*) in the populations (**Fig. 2**). Proliferative cells exhibited wave-like Ca^{2+} oscillations with longer transient time of wave – slow Ca^{2+} oscillations in hNSC-

cortex (**Fig. 2a**). Number of spontaneous cells in culture was decreased upon differentiation, but majority of cells were quiescent in proliferative- and differentiating hNSC (**Fig. 2d** and **h**). Most of these quiescent cells were responsive to ATP, but selectively responsive to glutamate and GABA. Culture time did not influence on neurotransmitter-induced Ca^{2+} frequencies in FBS-differentiated hNSC-cortex, but selectively increased Ca^{2+} frequencies by ATP and GABA were observed in BDNF+GDNF-differentiated hNSC-cortex (**Fig. S3-B** and **–C**). This may indicate that BDNF and GDNF up-regulated ATP- and GABA-dependent Ca^{2+} signals in the quiescent cells. How neurotransmitters were differentially regulated among spontaneous cells is followed in the section 4.2.

4.2 DIFFERENTIAL REGULATIONS OF NEUROTRASNMITTERS IN HUMAN NEURAL STEM CELLS

4.2.1 Functional neurotransmitters in the spontaneous cells of proliferative hNSC

A new approach to characterize the functional behaviors of proliferative- and differentiating hNSC was employed (**Fig. 6**). Over 90% of proliferative cells accelerated Ca^{2+} frequencies (faster induction) in response to ATP (**Fig. 6a**). Approximately 60% of proliferative cells accelerated Ca^{2+} frequencies, but 40% of cells slowed down Ca^{2+} frequencies in response to glutamate. About 30% of proliferative cells were involved in slow actions of GABA (slower induction) (**Fig. 6c**). There was no direct interpretation between the induction types of Ca^{2+} and morphology of cells grown in bFGF+EGF. From the results of immunocytochemistry, majority of cells were neural progenitors/stem cells (nestin+), and minority of cells were immature neurons (Tuj1+) (data not shown). To summarize the functional phenotype and morphological phenotype, the small numbers of spontaneous cells in bFGF+EGF were possibly astroglial cells (nestin+, GFAP+), which actively took part in regulating neurotransmitters (*excitatory*) at early neurogenesis and proliferation. In conclusion, mitogens bFGF+EGF enhanced the proliferation of neural progenitors/stem cells, but the epigenetic memory as cortical progenitors at that time of fetal development might maintain asymmetric divisions of cortical progenitors into cortical neurons, glial cells and progenitors.

4.2.2 Functional neurotransmitters in the spontaneous cells of differentiating hNSC

Differentiation of hNSC in FBS and BDNF+GDNF further decreased number of spontaneous cells in culture of hNSC-forebrain (**note: difficult to conclude due to large variation in hNSC-cortex*). Decreased expression of GFAP at later DIV coincided with decreased populations of faster induction by ATP and GABA in FBS-differentiated hNSC-cortex (**Fig.1** and **Fig 6b**). In contrast, increased expression of GFAP at later DIV coincided with increased populations of faster induction by GABA in BDNF+GDNF-differentiated cells. Even these results could not linearize that GABA induced faster Ca^{2+} regulation in progenitors/glia (GFAP+), it can be suggested that GABA takes active role in early differentiation of hNSC and possibly in the subcortical regions of forebrain and of cerebral cortex due to their origin. In short, BDNF and GDNF presumably modulated faster action of GABA in differentiating hNSC-cortex.

4.3 GAP JUNCTIONS IN DEVELOPING BRAIN CELLS

4.3.1 Gap junctions in spontaneous Ca^{2+} regulations

To investigate the developmental role of gap junction, a model of gap junction inhibition by carbenoxolone (CBX) in correlation with spontaneous Ca^{2+} regulations during *in-vitro*-proliferation of and differentiation of hNSC was used in the [study I]. The percentage of Ca^{2+}-mediated spontaneous cells was smaller in hNSC-cortex than in hNSC-forebrain. This difference might be caused by developmental morphology *in vitro* between them as described earlier – extensive neural networks between cells in hNSC-forebrain. Depression of spontaneous Ca^{2+} oscillations was induced by 100 µM CBX to great extent in proliferative hNSC-forebrain than hNSC-cortex (**Fig. 3**). Interestingly, a rapid decrease of spontaneous Ca^{2+} oscillations was observed in proliferating cells, while a transient increase of intracellular Ca^{2+} was observed in differentiating cells (**Fig. 3b-c** and **3f-g**). This transient increase of Ca^{2+} was accompanied with a transient accumulation of intracellular Ca^{2+} within the cells, leading to loss of active Ca^{2+}oscillations, and eventually Ca^{2+} homeostasis was no longer maintained – it could be speculated that this dysregulation might cause neural apoptosis. Further investigation on apoptosis was not performed in the [study I].

Neural apoptosis naturally occurs during embryogenesis and fetal development *in vivo*. It might be interesting further to investigate whether spontaneous neural apoptosis is also mediated through inactivation of gap junctions. Intercellular

spreading of apoptotic signals between cells can be prevented by metabolic depression through gap junctions as well (Nodin, 2005). Gap junctions play a role as a gate keeper for opening or closing for spreading endogenous- or exogenous signals by spreading such as apoptotic-, protective-, proliferative-, differentiating- and patterning signals. The neuroprotective effect of hNSC was one of big challenges in the stem cell therapy. If hNSC were manipulated to regulate the positive role of gate keeper, a possible therapy using hNSC may come into a step forwards to the clinical applications.

4.3.2 Gap junctions and neurotransmitters

In the brain development, uptake of neurotransmitters (i.e., glutamate, GABA, choline, dopamine and serotonin) by neural progenitors is a process of differentiation specification, which determines neural progenitors to proliferate and differentiate into specific morphology and function. It is not well studied how tightly neurotransmitter specification is regulated through gap junctions during proliferation of and differentiation of hNSC. Addition of exogenous neurotransmitter agonists into the cultures was a mimic of neurotransmitters secreted from other type of cells or transported/released from the other part of brain. We wanted to test the hypothesis that neurotransmitter-induced Ca^{2+} signals are facilitated with gap junctions. All three neurotransmitter agonists, ATP, glutamate and GABA, elicited Ca^{2+} oscillations or transient increase of intracellular Ca^{2+} (**Fig. 7**).

How subsequent blockade of gap junction by CBX influences on neurotransmitter-activated Ca^{2+} signals? Here I suggested the possible mechanism of gap junction-mediated Ca^{2+} regulation by neurotransmitter from the basis of results (**Fig. 7d, h, l**). ATP-generated Ca^{2+} signals were dependent on gap junctions in 50% of cells (bFGF+EGF), but about 25% of cells by induction of glutamate. Gap junction was independent from GABA-induced Ca^{2+} signals during proliferation of hNSC-cortex (bFGF+EGF). ATP generates regular Ca^{2+} oscillations through gap junctions: endogenous ATP is released through gap junctions – recycling of ATP might be the key factor that generates rhythmic oscillations. Extracellular ATP stimulates the cells to undergo metabolic/mitochondrial oxidative cycle for new production of endogenous ATP, which in turn, autocrine and paracrine actions of endogenous ATP are repeated.

However, differential regulation of Ca^{2+} signals was observed in differentiating hNSC. Gap junction-dependency of ATP-induced Ca^{2+} signals was reduced to large extent in both FBS and BDNF+GDNF, but more glutamate- and GABA-induced Ca^{2+} signals became gap junction-dependent with transient

accumulation of intracellular Ca^{2+}. This phenomenon of transient accumulation of intracellular Ca^{2+} probably indicates that either Ca^{2+} efflux through gap junctions were inhibited or other Ca^{2+} channels might presumably be affected by the side effect of CBX.

We further investigated with pre-blockade of gap junction whether glutamate- and GABA-elicited Ca^{2+} signals were dependent on the coordination of gap junctions during differentiation of hNSC-cortex. The results were conclusive that glutamate and GABA failed to increase Ca^{2+} oscillations/transients in the majority of differentiating cells, but ATP was independently able to elicit Ca^{2+} oscillations (**Fig. 8**). In conclusion, ATP-generated Ca^{2+} signals in proliferation of hNSC, while both glutamate- and GABA-generated Ca^{2+} signals, are dependent on gap junction.

4.4 PURINERGIC SIGNALING IN CALCIUM REGULATION

Extracellular ATP binds to purinergic receptors (P2X- and P2Y-receptors). P2Y-R is G-protein-coupled receptors and depending on Gq (i.e., P2Y1) or Gi (i.e., P2Y12). P2Y-R elicits phospholipase, adenylate cyclases as well as several cytoplasmic protein kinase signaling pathways (Dubyak, 1991; Zimmerman, 2011). P2Y receptors are involved in proliferation and differentiation of SV-derived neural progenitors, and purinergic inhibition by suramin decreased proliferation of neural progenitor cells without affecting phenotypic differentiation (Lin, 2007). Purinergic signaling also played a crucial role in the neurotransmitter-induced Ca^{2+} regulation in both proliferating and differentiating hNSC.

4.4.1 Purinergic signals in the spontaneous development

Spontaneous Ca^{2+} activities in BDNF+GDNF-differentiated hNSC-cortex were more susceptible to purinergic inhibition than in FBS-differentiated hNSC-cortex (**Fig. 4**). The interpretation of these results is that a combination of BDNF and GDNF might either increase expression of purinergic receptors in cortical progenitors. There was a transient increase of Ca^{2+} after application of suramin among FBS-treated spontaneous cells. This might indicate expression of ryanodine receptors (i.e., RyR3) on ~30% of differentiated cortical cells in FBS. Suramin also interacts with calmodulin-binding site on the ryanodine receptor 1 (RyR1) on skeletal muscles (Papineni, 2002). Suramin may activate RyR1-induced-voltage-gated Ca^{2+} release. I speculate that suramin might

activate RyR3-induced-voltage-gated Ca^{2+} release, so the similar effect was found in FBS-differentiated cortical cells.

4.4.2 Purinergic signals and neurotransmitters

Our finding demonstrated that Ca^{2+} signals of hNSC were affected by ATP and purinergic receptors during all examined stages of proliferation and differentiation. But glutamate-induced Ca^{2+} signals were affected in BDNF+GDNF-differentiated cells and GABA-induced Ca^{2+} signals were dependent on purinergic receptors in both proliferating cells (>50% of cells in bFGF+EGF) and differentiating cells (~50% of cells in FBS) (**Fig. S6**).

[Study II]

4.5 RO52-SPECIFIC ANTIBODIES AND CONGENITAL HEART BLOCK

Neonatal lupus syndrome (NLS) was rarely found in the infants born from the mothers with autoimmune diseases such as systemic lupus syndrome (SLE) and Sjögren's syndrome. Circulating maternal autoantibodies affect developing human fetal heart. 1st degree of AV block or sometimes acquired 3rd degree AV block following inflammation. Anti-Ro52 autoantibodies from sera of those mothers with autoimmune diseases were found. A panel of Ro52-specific monoclonal antibodies against human Ro52 was generated from the previous study: 7.13F2, 7.2H4, 7.8C7, 7.8H4, 7.9D3, 7.2F4 (Strandberg, 2008). In the study II, it was aimed to identify specific binding domain for autoantibodies to Ro52 protein in association with AV block.

4.5.1 Ro52-p200 specific binding motifs for antibodies

The results from ELISA in **Fig. 2** showed binding reactivity of respective Ro52 protein construct (full length or deleted) against individual monoclonal antibodies, which were raised against binding domain of Ro52 (non-coding region, exon; p200 peptide; Box B30.2). All four antibodies against p200 region exhibited strong binding affinity. In the *in vivo* animal model, the newborn pups developed congenital AV block from those mothers immunized with p200-specific monoclonal antibodies 7.8C7. These pups had a problem of conduction system with longer PR interval and lower heart rate (bpm), compared to the other antibodies (**Fig. 3**).

In addition, genetic variation within the Ro52-p200 peptide was a crucial factor that influenced binding reactivity between p200 and its antibodies (**Fig.**

4). Gene mutation D → A at the residue 233 of p200 (A233) was related to the strong reactivity. Genetic variation within the populations determines incidence of AV block. Further study in the correlation between genetic polymorphism in at a residue 233 and incidence of AV block in neonates or babies would also support the findings of the present study.

4.5.2 Gestational age and development of congenital heart block

The strongest candidate of antibodies (7.8C7) was selected to further investigate the timing of developing AV block in the fetal heart and the quantitative threshold of antibodies (concentration) (Fig. 1). Newborn pups exhibited longer PR interval and slower heart rate in monoclonal antibodies 7.8C7-affected pups, in comparison to PBS-affected pups. (Fig. 1A and B). Newborn pups that were affected by injection of antibodies 7.8C7 at gestational age day into pregnant female rats showed longer PR interval. This elongation of PR interval was also associated with higher concentration (4-6 mg) of antibodies 7.8C7. In conclusion, fetal AV block was strongly affected by specificity of Ro52 p200 antibodies, and the critical period of fetal heart development was around gestational age day 6. In addition, a dose-dependent (concentration of antibodies) effect on congenital AV block was seen.

4.5.3 Ro52-specific antibodies and disturbed Ca^{2+} homeostasis

To elucidate the mechanism of direct causes of congenital AV block in the fetal heart, primary rat cardiomyocytes were used to study Ca^{2+} regulation and effect of antobodies. Anti-Ro52-p200-specific (7.8C7) and anti-Ro52 monoclonal antibodies (7.13F2) against epitopes outside the P200 region were applied on primary cardiomyocytes. This was to investigate eventual alterations of Ca^{2+} homeostasis in the cardiomyocytes. Contracting cardiomyocytes were identified by eye observation of spontaneous contraction under the confocal microscope. These contracting cells maintained fast and regular Ca^{2+} oscillations. Upon application of antibodies 7.8C7, Ca^{2+} homeostasis of rat cardiomyocytes was disturbed initial alterations of frequency with loss of regular Ca^{2+} oscillations. The effects were dose and time dependent and higher concentration of anti-Ro52 p200 specific antibodies induced a rapid and non-reversible accumulation of intracellular Ca^{2+} (**Fig. 5**). In conclusion, monoclonal antibodies specific for the p200 region of Ro52 bound cardiomyocytes and interrupted Ca^{2+}-dependent contractions. After antibody application the cytosolic levels

of calcium steadily increased with an initial increase in calcium oscillations until basal calcium levels seemed to over-saturate the cardiomyocyte calcium regulatory mechanism and oscillations ceased and cells subsequently died often within 30 minutes at higher antibody concentrations. At present we speculate that mechanisms of dysregulation of Ca^{2+} could be either that antibodies bind to fast voltage-gated Ca^{2+} channels or gap junctions (e.g. Connexin 43) on the cytoplasm, opening windows for extra cellular calcium to enter the cells. An alternative explanation of the observed calcium dyshomeostasis might be that antibodies act in the cytoplasm directly or indirectly cytoplasmic molecules specifically bound to Ca^{2+}-ATPase on the sarcoplasmic reticulum (SR), so that Ca^{2+} efflux from SR by CICR are persistent, but Ca^{2+} influx into the SR is inhibited.

5 CONCLUSIONS

This thesis describes the role of Ca^{2+} in developing neural network and cardiomyocytes in association with congenital heart block.

In vitro proliferation of hNSC

- Expanded hNSC-cortex in bFGF+EGF were neural progenitors in majority and *immature* neurons in minority, but a cocktail of FGF2+FGF8+Shh increased number of *immature* neurons in hNSC-forebrain.
- Exposure of hNSC to mitogens bFGF+EGF for longer period (52 days-in-vitro, DIV) decreased ATP-induced Ca^{2+} frequency, while increased GABA-induced Ca^{2+} frequency. Culture time is an important factor that can modulate functional behaviors of proliferative hNSC.
- Spontaneous Ca^{2+} activities of hNSC-forebrain were more dependent on gap junctions than ones of hNSC-cortex during *in vitro* proliferation.
- ATP- and glutamate-generated Ca^{2+} signals in proliferative hNSC were dependent on gap junctions, but GABA-elicited Ca^{2+} signals were of independence.
- GABA-evoked Ca^{2+} signals were more sensitive to purinergic inhibition in proliferating hNSC-cortex (>50% of cells)

In vitro differentiation of hNSC

- Proliferative hNSC underwent condition-dependent morphological differentiation: FBS decreased number of GFAP-positive cells, while BDNF+GDNF increased number of GFAP-positive cells. A cocktail of BDNF and GDNF in differentiation of hNSC-cortex enhanced generation of glial cells and of astrocytes.
- Neither FBS nor a combination of BDNF and GDNF (BDNF+GDNF) induced mature differentiation of cortical neurons in morphology.
- Both FBS and BDNF+GDNF maintained high expression of BDNF, indicating neurotrophic effect.
- BDNF+GDNF up-regulated ATP- and GABA-dependent Ca^{2+} signals in the quiescent cells.

- Transient increase of intracellular Ca^{2+} with loss of oscillations upon gap junction blockade in differentiating hNSC.
- Spontaneous Ca^{2+} signals in BDNF+GDNF-differentiated hNSC-cortex were more susceptible to purinergic inhibition than in FBS-differentiated cells.
- FBS increased purinergic signaling-sensitive specification of GABA regulations, but BDNF+GDNF increased specification of glutamate regulations.

Ro52 p200-specific antibodies and congenital heart block

- Leucine zipper binding domain (p200) within the Ro52 protein was very crucial for developing congenital AV block.
- Genetic mutation at the residue 233of Ro52-p200 was associated with strong binding affinity to anti-Ro52 autoantibodies.
- There was a time window (gestational age 6^{th}-8^{th} day of rodent pregnancy) for cardiac cells to develop AV block.
- Ro52 p200-specific antibodies 7.8C7 induced dysregulation of Ca^{2+} homeostasis in the contracting cardiomyocytes at a concentration-dependent manner.

6 FUTURE PERSPECTIVES

This thesis for the [study I] has lack of comparative study between forebrain- and cortex-derived hNSC in functional Ca^{2+} studies: neurotransmitter-mediated Ca^{2+} regulations. To make a concise conclusion of functional differentiation, further experiments for antagonists against (i.e., glutamatergic receptors/ion channels, GABAergic receptors/ion channels, cholinergic receptors/ion channels, dopaminergic receptors/ion channels can be performed. In-depth analysis in Ca^{2+} oscillatory patterns and transient Ca2+ accumulation in the effect of CBX and suramin could be interesting.

For the clinical applications, firstly, defined expansion and differentiation conditions must be developed; secondly, functional recovery using in vivo animal model could be approached. Thirdly, the screening of tumor formation in cultured primary stem cells will be necessary depending on culture time, for example, chromosomal numbers, telomere length.

In the [study II], genetic variation within the populations determines incidence of AV block. Further epidemiological study in the correlation between genetic polymorphism in at a residue 233 and incidence of AV block in neonates or babies would also support the findings of the present study II. The mechanistic study in dysregulated Ca^{2+} homeostasis of cardiomyocytes can be further approached using antagonists of voltage-gate ion channels and gap junction inhibitors.

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