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ION HOMEOSTASIS IN CONTROL OF STEM CELL PROLIFERATION

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To my beloved mom, dad and brother...

ABSTRACT

The ion homeostasis is critically maintained in all cells by a combined effort from various ion channels, transporters, pumps, and gap junctions. Ion fluxes are essential in excitable cells as they regulate the excitability of the cells and initiate and propagate action potentials. However, in non-excitable cells they are also involved in a number of cellular functions including proliferation, differentiation, migration, and cell volume regulation. This thesis focuses on the various mechanisms through which ion channels and transporters regulate cell cycle progression in stem cells.

Stem cells are characterized by their ability to self-renew, which entails the ability to proliferate continuously while maintaining pluripotency. Embryonic stem cells (ES cells) are pluripotent cells derived from the inner cell mass of the blastocyst and they have the ability to differentiate into any cell type originating from the three germ layers. Neural stem cells (NSCs) are multipotent adult stem cells that reside in the subventricular zone (SVZ) and differentiate to give rise to new neurons.

Previous work showed that γ-aminobutryic acid (GABA) acts on the chloride (Cl^-) ion channel, $GABA_A$ receptor, to negatively regulate ES cell proliferation. We show that NSC proliferation in the adult mouse brain is regulated by a similar mechanism, where $GABA_A$ receptor activation induces the DNA damage response (DDR) pathway and the phosphorylation of histone H2AX. This results in a reduction of NSC proliferation and in long-term changes of the stem cell niche and neuronal output.

Cancer cells and stem cells share a common characteristic in their ability to proliferate extensively. We show that the main regulator of ion homeostasis in cells, the Na,K-ATPase, regulates quiescence in neuroblastoma cells. Inhibition of Na,K-ATPase with the endogenous cardiac glycoside, ouabain, induced the DDR pathway and caused a reversible cell cycle arrest. Ouabain treated cells had increased levels of the cell cycle regulator p21 and upregulated the quiescence-specific transcription factor HES1. Upon removal of ouabain, neuroblastoma cells re-entered the cell cycle and resumed proliferation.

An important role of ion homeostasis is to regulate cell volume changes over the cell cycle. The GABA signaling pathway that was shown to regulate ES cell proliferation, also altered cell volume. We found that the voltage-gated potassium (K^+) channel ether-a-go-go-related gene (Erg) was differentially expressed in G1. Inhibiting Erg channel in ES cells caused a decrease in cortical stiffness and led to cell swelling followed by cell death in an apoptosis independent manner. Increasing the extracellular osmotic pressure or blocking K^+ flux into ES cells resorted cell viability, suggesting that Erg channel inhibition caused cell death by increasing intracellular osmotic pressure by blocking K^+ efflux from ES cells.

In summary, we show that ion homeostasis, which is maintained by ion channels and transporters, is critical for cell cycle progression in proliferating cells through various mechanisms, and that perturbation in ion homeostasis results in disrupted cell proliferation.

LIST OF SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. Fernando, R. N., B. Eleuteri, **S. Abdelhady**, A. Nussenzweig, M. Andang and P. Ernfors (2011). "Cell cycle restriction by histone H2AX limits proliferation of adult neural stem cells." Proc Natl Acad Sci U S A **108**(14): 5837-5842.

II. Hiyoshi, H.*, **S. Abdelhady***, L. Segerstrom, B. Sveinbjornsson, M. Nuriya, T. K. Lundgren, L. Desfrere, A. Miyakawa, M. Yasui, P. Kogner, J. I. Johnsen, M. Andang and P. Uhlen (2012). "Quiescence and gammaH2AX in neuroblastoma are regulated by ouabain/Na,K-ATPase." Br J Cancer **106**(11): 1807-1815.

III. **Abdelhady, S**., S. S. Kitambi, V. Lundin, R. Aufschnaiter, P. Sekyrova, I. Sinha, K. T. Lundgren, G. Castelo-Branco, S. Linnarsson, R. Wedlich-Soldner, A. Teixeira and M. Andang (2013). "Erg channel is critical in controlling cell volume during cell cycle in embryonic stem cells." PLoS One **8**(8): e72409

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I. Malmersjo, S., P. Rebellato, E. Smedler, H. Planert, S. Kanatani, I. Liste, E. Nanou, H. Sunner, S. **Abdelhady, S**. Zhang, M. Andang, A. El Manira, G. Silberberg, E. Arenas and P. Uhlen (2013). "Neural progenitors organize in small-world networks to promote cell proliferation." Proc Natl Acad Sci U S A **110**(16): E1524-1532.

CONTENTS

LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
BK	Big-conductance Ca^{2+} activated potassium channels
BrdU	Bromodeoxyuridine
Ca^{2+}	Calcium
CaM	Calmodulin
CDK	Cyclin dependent kinase
Cl^-	Chloride
ClC	Chloride channels
DNA	Deoxyribonucleic acid
DDR	DNA damage response
Eag	Ether a-go-go
Erg	Ether-a-go-go related gene
ES cell	Embryonic stem cell
GABA	γ-aminobutyric acid
$GABA_AR$	$GABA_A$ receptor
GFP	Green fluorescent protein
IK	Intermediate-conductance Ca^{2+} activated potassium channel
H^+	Hydrogen
HES1	Hairy and enhancer of split1
K^+	Potassium
K2P	Two-pore domain potassium channels
K_{Ca}	Ca^{2+}- activated potassium channels
KCC	$K^{+/}Cl^-$ co-transporter
K_{ir}	Inward-rectifying potassium channels
K_V	Voltage-gated potassium channels
MAPK	Mitogen-activated protein kinase
NCC	Na^+/Cl^- co-transporter
NHE	Na^+/H^+ exchanger

NKCC	$Na^+/K^+/2Cl^-$ co-transporter
NSC	Neural stem cell
PI3K	Phosphoinositide 3-kinase
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
Rb	Retinoblastoma protein
RVD	Regulatory volume decrease
RVI	Regulatory volume increase
SK	Small-conductance Ca^{2+} activated potassium channel
SVZ	Subventricular zone

1 INTRODUCTION

Even the simplest organisms needs to maintain a barrier between itself and its immediate external environment. In an aquatic environment, a hydrophobic lipid layer makes an excellent barrier to the outside world. However, the organism will need to regulate and control transport of various substances across this lipid barrier. It needs to allow molecules that are necessary for growth, development and function to travel across the barrier while at the same time excluding other molecules, in other words, it needs to be selectively permeable to specific molecules. If the barrier is completely made up of lipid, it will be impermeable to most water soluble molecules, many of which are necessary for proper organism function. Therefore, there needs to be alternative ways for water soluble molecules to cross the lipid barrier, while at the same time, the passage of ions and solutes should be balanced, as an accumulation of solutes within the barrier may lead to cell rupture due to osmotic water influx.

The cell is surrounded by a plasma membrane which is consists of a lipid bilayer. The plasma membrane contains protein molecules that form channels and transporters which allow the diffusion and transport of various molecules and solutes across the cell membrane. The channels, also called ion channels, are made up of a hydrophobic exterior which is integrated into the plasma membrane, and a hydrophilic center which allows water and other water-soluble molecules to passively pass through into the cell. The transporters, also called ion transporters or ion pumps, use energy to actively transport molecules and solutes across the plasma membrane.

Ion channels and transporters are fundamental in many essential cellular functions. This is evident by their existence in all living organisms such as animal cells, microorganisms, and even plant cells. They are involved in very diverse processes, ranging from the controlling of the leaf-closing response in the Mimosa plant (Stoeckel and Takeda 1993) to memory and learning in humans (Voglis and Tavernarakis 2006).

The most fundamental function for ion channels and transporters is to establish and maintain the ion homeostasis inside the cell. Ion homeostasis is crucial for proper cell function including maintaining the resting membrane potential, initiating and propagating action potentials, regulating the pH balance, and regulating cell volume. Ions also function as second messengers in signal transduction, regulate transportation and secretion and even serve as the energy source for transporting solutes into the cell.

Stem cells are characterized by an ability to self-renew, which entails maintaining the ability to proliferate in an undifferentiated state and preserving potency. During development and regeneration of damaged tissue, the balance between self-renewal and differentiation is strictly controlled. This occurs through a highly regulated cell cycle progression where a tightly controlled sequence of events guides the cell through the different phases of cell cycle. Besides having various checkpoints that ensure the correct transcription and duplication of

the DNA, cells also need to regulate and control the cell volume as they progress through the cell cycle.

In 1984, Decoursey et al reported about the relationship between potassium (K^+) ion channels and cell proliferation when they showed that inhibition of voltage-gated K^+ channels inhibited T-lymphocyte proliferation (DeCoursey, Chandy et al. 1984). Since then, many studies have implicated the role of ion fluxes and ion channels in cell cycle progression both in normal cells and cancer cells. However, the precise mechanisms of how ion channels and transporters regulate the cell cycle remains elusive.

1.1 ION CHANNELS AND TRANSPORTERS

The asymmetrical concentration of ions and solutes across the plasma membrane is critical for normal cell function. The main proteins responsible for this distribution are ion channels and transporters. Each cell type has a repertoire of ion channels and pumps that suits its specific needs and functions, and the expression and activity of the various ion channels and transporters changes within the same cell during different processes. Because their movement creates currents, ions underlie all electrical activity within the cell and between cells. The location of ion channels and transporters in the plasma membrane makes them very effective in transducing signals and events at the surface of the cell into cellular responses.

A lot of our knowledge about ion channel function is due to the development of tools for electrophysiological measurements, which were developed by Erwin Neher and Bert Sakmann in the late 1970s and early 1980s. Their patch clamp technique allowed minute and precise measurement of currents arising from a single or multiple channels, and similarly those arising from the whole cell. In 1963, Alan Lloyd Hodgkin and Andrew Huxleys received the Nobel Prize in physiology/medicine, for describing a model of ion currents that initiate and propagate action potentials in excitable cells using the squid giant axon as a model.

1.1.1 Ion channels

Ion channels are found in all cells and are essential for many cellular processed, and ion channel dysfunction lies at the base of many diseases. Ion channels are transmembrane proteins that form a hydrophilic pore through which specific ions can pass. These pores are gated, and can be open or closed in response to various stimuli. When open, they allow the diffusion of ions down their electrochemical gradient through passive transport. The electrochemical gradient is the result of uneven distribution of ions and electric charge across the cell membrane created through active transport (more on this in section 1.1.2).

There are more than 300 different genes encoding ion channels in the human genome (Venter, Adams et al. 2001). Classified based on their ion selectivity, there are four major families of ion channels; K^+ channels, sodium (Na^+) channels, calcium (Ca^{2+}) channels and chloride (Cl^-) channels. Most ion channels are either voltage-gated or ligand-gated, however some respond to other cues such as mechanical stimuli or temperature.

1.1.1.1 Potassium channels

Potassium channels are the most common and diverse family of ion channels. There are 77 genes encoding K^+ channels (Pardo and Stuhmer 2014) and they play a major role in maintaining the resting membrane potential and, in electrically active cells, repolarizing the plasma membrane after an action potential. There are four major classes of K^+ channels; Ca^{2+} activated K^+ channels (K_{Ca}), inward rectifying K^+ channels (K_{ir}), two pore domain K^+ channels (K2P) and voltage-gated K^+ channels (K_V). K^+ channel subunits contain a certain number of membrane spanning segments and a pore forming domain, that together form the pore of the channel when they come together and form a tetramer. In 2003, Rod McKinnon received the Nobel Prize in chemistry for solving the three dimensional structure of the selectivity filter in K^+ channels, which is responsible for selectively letting K^+ ions pass through the channel. This high selectivity is achieved by a unique residue sequence that is shared between all K^+ channels. The carbonyl oxygens from the main chain of the amino acids are aligned toward the center of the pore and their side chains point outwards (Figure 1)(Armstrong 1998). As the K^+ ions passes through the pore, it becomes progressively dehydrated, and the carbonyl oxygens act as substitutes for the water molecules (red spheres in Figure 1) that the K^+ ion sheds in order to pass through the channel. This specific electrochemical properties and physical dimensions of the filter make it more energetically favorable for K^+ ions than other monovalent cations, such as Na^+, to pass through (Doyle, Morais Cabral et al. 1998).

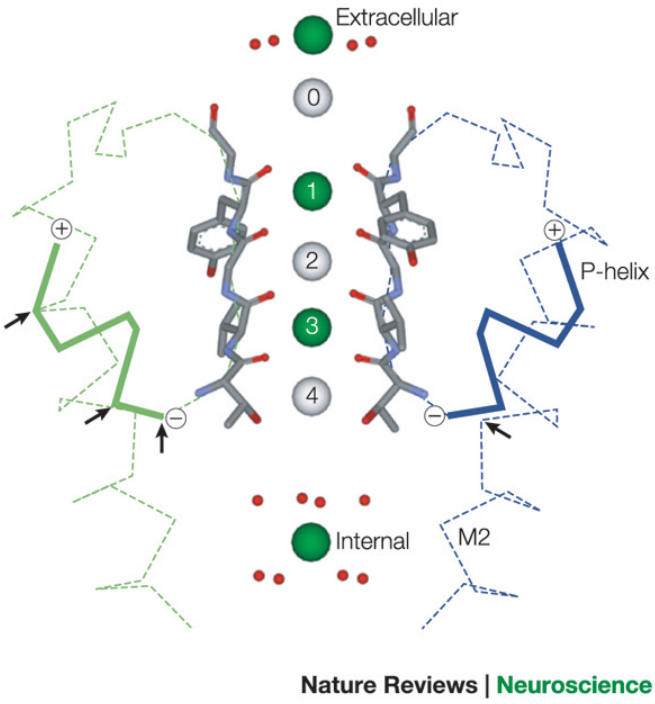

Figure 1. Selectivity filter of K^+ channels

As the K^+ ion enters the channel, the carbonyl oxygens substitute the water molecules making it energetically favorable for K^+ to pass through the selectivity pore. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Bichet et al (2003). Copyright © 2003

Calcium-activated potassium channel

Ca^{2+} activated K^+ channels are activated by an increase in intracellular Ca^{2+}. Like many other K^+ channels, they have a tetrameric structure where each monomer consists of six transmembrane spanning helices (S1-S6) and one pore-forming domain between S5 and S6. Calcium does not bind directly to the channel, rather their Ca^{2+} sensitivity is due to a calmodulin (CaM) unit that is constitutively associated with the channel (Xia, Fakler et al. 1998). Activation of K_{Ca} leads to an efflux of K^+ and thus hyperpolarizes the plasma membrane. This in turn leads to decrease of intracellular Ca^{2+} by deactivating voltage-gated Ca^{2+} channels (Berkefeld, Fakler et al. 2010). There are three major subfamilies of K_{Ca}; the large/big conductance (BK) channel, the intermediate conductance (IK) channel and the small conductance (SK) channel (Vergara, Latorre et al. 1998). BK channels are unique in that they are both voltage-dependent and Ca^{2+} dependent and furthermore consist of seven transmembrane spanning domains instead of the characteristic six (Meera, Wallner et al. 1997). K_{Ca} are expressed in neuronal and non-neuronal tissues, for instance epithelia, smooth muscle cells and sensory cells (Petersen and Maruyama 1984, Fettiplace and Fuchs 1999).

Two pore domain potassium channels

Two-pore domain K^+ channels (K2P), also known as leak channels, are expressed throughout the central nervous system (Talley, Solorzano et al. 2001, Aller, Veale et al. 2005). They are responsible for the high permeability of the plasma membrane to K^+ ions, bringing the membrane potential towards the equilibrium potential of K^+. In excitable cells, like neurons, this entails stabilizing the membrane potential at hyperpolarized voltages and suppressing excitability (Goldstein, Bockenhauer et al. 2001). They also modulate the duration and frequency of the action potential (Goldstein, Bayliss et al. 2005) K2P are unique among the K^+ channels in that they contain two pore domains in each subunit instead of one, which is typical of K^+ channels and four transmembrane segments instead of six (Figure 2).

Inward rectifying potassium channels

Inward rectifying potassium channels (K_{ir}) are important in setting the resting membrane potential for cells due to their facilitation of inward K^+ currents at potentials that are more negative than their reversal potential. K_{ir} channels form homotetramers where each subunit consists of two membrane-spanning segments linked by an extracellular pore-forming region (Figure 2). Since K_{ir} channels lack the voltage sensor, they are insensitive to membrane voltage and their conductance capacity is due to the block of outward K^+ flow by intracellular substances (such as magnesium and polyamines) at certain voltages. K_{ir} channels are expressed in a variety of cell types such as cardiac myocytes (Rougier, Vassort et al. 1968, Beeler and Reuter 1970), neurons (Williams, North et al. 1988, Takahashi 1990), endothelial cells (Silver and DeCoursey 1990) and kidney epithelial cells (Greger, Bleich et al. 1990, Hebert, Desir et al. 2005).

Voltage-gated potassium channels

Voltage gated potassium channels (K_V) belong to a family that consists of 12 subfamilies, K_V1-12, and are encoded by 39 genes. Each subunit consists of six transmembrane domains and one pore-forming domain. They can form heteromeric complexes within the same subfamily. In the fourth transmembrane domain, which is also the voltage sensing domain, a lysine or arginine appears in every third or fourth position in an otherwise hydrophobic stretch. These positive amino acids are repelled by the positive charge in the membrane as cells depolarize, and their upward movement leads to a conformational change and opening of the ion channel (Papazian, Schwarz et al. 1987, Pongs, Kecskemethy et al. 1988).

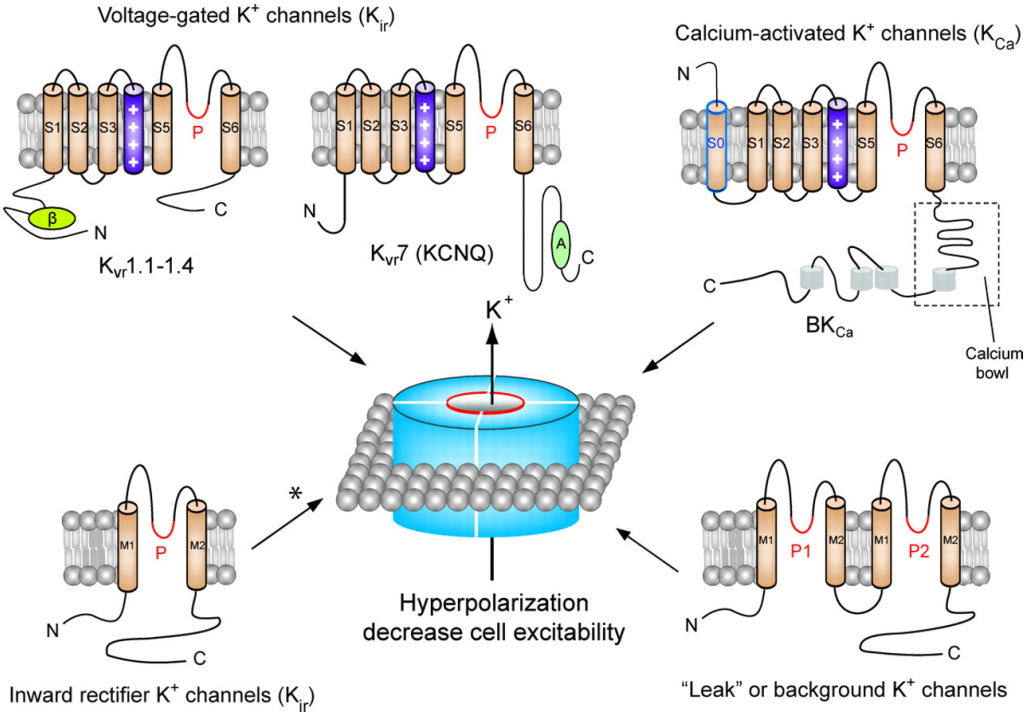

Figure 2. General structure of K^+ channels α subunits.

K^+ channels form tetramers of a-subunits that contain a pore (P) forming segment. K^+ channels can have 2, 4 or 6 transmembrane segments. For K_v channels, the fourth transmembrane segment contains positively charged residues and constitutes the voltage sensor. Reprinted by permission from Wolters Kluwer Health Lippincott Williams & Wilkins: Neurology, Benarroch E et al (2009). Copyright © 2009

Ether-a-go-go-related channel

The ether-a-go-go (Eag) related gene (Erg) encodes a voltage-gated K^+ channel that was first described in 1994 as a homolog to the Drosophila Eag gene (Warmke and Ganetzky 1994). Erg channels are essential for ending the plateau phase and repolarization of the action potential in cardiac myocytes. Inherited or acquired dysfunction of Erg channels leads to a prolonged cardiac action potential causing heart arrhythmias and possibly death in a condition called the long QT syndrome (Curran, Splawski et al. 1995). In some cells, that lack classical K_{ir} channels, the resting membrane potential is partially maintained by Erg

channels due to their inward rectifying properties (Schwarz and Bauer 2004). For example, blockage of erg channels in oesophageal smooth muscle cells induced spontaneous contractions (Akbarali, Thatte et al. 1999) and in gallbladder smooth muscle cells, inhibition of erg channel depolarized muscle fibers and increased contractility (Parr, Pozo et al. 2003).

1.1.1.2 Chloride channels

Chloride channels (ClC) are one of the less studied families of ion channels. They play a role in cell volume regulation, ligand-gated post-synaptic transmission, stabilization of the plasma membrane, and fluid secretion (Zifarelli and Pusch 2010). In most cells, Cl^- is actively transported into the cell by the Cl^-/HCO^{3-} exchanger, the $Na^+/K^+/2Cl^-$ co-transporter (NKCC) and by Na^+/Cl^- co-transporter (NCC). In epithelial cells, the Cl^- gradient is used to drive fluid secretion when Na^+ and water exit the cell. In other cells, such as neurons, Cl^- influx causes membrane hyperpolarization and inhibition of excitability.

The $GABA_A$ receptor

The γ-aminobutyric acid (GABA)-A receptor ($GABA_AR$) is a ligand-gated Cl^- channel that consists of a heteromeric pentamer. The pentamer is formed by subunits from 8 different subclasses (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) where different subunit compositions give rise to receptors with different kinetics and properties (Henschel, Gipson et al. 2008). Despite the potential for diversity, the majority of $GABA_ARs$ is composed of α1–6, β1–3 and γ1–3 or δ subunits. There are five binding sites localized in or near the pore of the channel where GABA, benzodiazepines, barbiturates, picrotoxin, anesthetic steroids, and others can bind. The GABA binding site is at the border between the α and β subunits, and upon GABA binding, the channel opens and Cl^- flows into or out of the cell, depending on the intracellular Cl^- concentration, leading to a depolarization or hyperpolarization (Jacob, Moss et al. 2008). $GABA_ARs$ are expressed in stem cell niches in the CNS (Stewart, Hoge et al. 2002, Wang, Krueger et al. 2003, Bolteus and Bordey 2004, Liu, Wang et al. 2005), the liver (Erlitzki, Gong et al. 2000), the pancreas (Borboni, Porzio et al. 1994, Bailey, Ravier et al. 2007) and the prostate (Erdo, Nemet et al. 1983, Napoleone, Bronzetti et al. 1990).

1.1.2 Ion transporters

While ion channels facilitate the passive diffusion of ions down their electrochemical gradient in an energy free process, ion transporters actively transfer ions against their electrochemical gradient in a process that requires energy. The energy source can be the hydrolysis of ATP or the concentration gradient of another ion. The most important transporter for maintaining ion homeostasis is the Na,K-ATPase.

The Na,K-ATPase

The electrochemical gradient arising from the asymmetrical distribution of ions across the membrane is maintained in all eukaryotic cells, mainly by the Na,K-ATPase. Through primary active transport, it pumps out 3 Na^+ ions from the cell and pumps in 2 K^+ ions using

hydrolysis of ATP as an energy source. This process is so fundamental for cellular function that 30% of the cell's ATP production goes to the Na,K-ATPase (Kaplan 2002). The electrochemical gradient that is established is used for various cell functions. It maintains the osmotic balance of the cell and the resting membrane potential, as well as allowing membrane excitability in neurons and muscle cells. Furthermore, the Na^+ gradient that is created by the Na,K-ATPase is also used to facilitate transport of other ions, such as H^+, Ca^{2+}, Cl^-, as well as substrates, such as glucose and amino acids. The Na,K-ATPase belongs to the P-type ATPase family and is a heterodimeric protein consisting of one α-subunit and one β-subunit. The catalytic α-subunit has ten membrane spanning segments that carry out the transport function of the ATPase and contains the binding site for the cardiac glycoside ouabain (Karlish, Goldshleger et al. 1993), while the β-subunit, which has one single transmembrane crossing segment, has a regulatory function and is required for proper folding and integration of the Na,K-ATPase into the cell membrane. There are four different isoforms of the α-subunit (α1-α4) and four different β subunit isoforms (β1-β4), with some of these subunits expressed in a tissue-specific manner (Blanco and Mercer 1998).

Besides maintaining the ion homeostasis, the Na,K-ATPase also functions as a signal transducer independent of its pump activity. Binding of ouabain to the Na,K-ATPase causes a conformational change and forms the ouabain/Na,K-ATPase complex that is involved in signaling cascades involving Ca^{2+}, PI3K/Akt, Ras/Raf and MAPK pathway (Schoner and Scheiner-Bobis 2007).

In paper II, we show that the ouabain/Na,K-ATPase complex phosphorylates H2AX and induces quiescence in neuroblastoma cells.

1.2 CELL PROLIFERATION AND CELL CYCLE

All organisms require a strictly regulated cell cycle in order to develop, grow, repair tissue damage, and reproduce. Normal cell cycle progression is a highly controlled and complex process involving several regulatory proteins that direct the cell through a specific sequence of events leading to the production of two daughter cells. Dysregulation in the cell cycle process may lead to abnormal proliferation and oncogenesis.

1.2.1 The cell cycle

Generally, the cell cycle is divided into four different phases with various checkpoints ensuring proper cell cycle progression. With the exception of G0, which is absent in some cells and is not part of active cycling, cells sequentially go through all phases in a predetermined sequence. For most cells, early G1 progression requires the increase in cyclin D levels, which occurs as a result of mitogen signaling (for example, by growth factors) through the mitogen-activated protein kinase (MAPK) signaling pathway (Cheng, Sexl et al. 1998). Cyclin D interacts with cyclin-dependent kinases (CDK) 4 and 6 to form cyclin D-CDK4 and cyclin D-CDK6 complexes, which hyperphosphorylate the retinoblastoma tumor suppressor protein (Rb) preventing its interaction with the E2F family of transcription factors (Kato, Matsushime et al. 1993). This allows the E2F family of transcription factors to activate the expression of genes required for S phase progression (for example cyclin E) (DeGregori, Kowalik et al. 1995). Once the cell has advanced past this point, called the restriction (R) point, it is committed to go through the cell cycle and no longer needs mitogenic stimuli (Zetterberg, Larsson et al. 1995). When cyclin E levels are sufficient to allow the necessary level of cyclin E-CDK2 activity, the cell goes through G1/S checkpoint and enters the DNA synthesis (S) phase. DNA replication involves several mechanisms ensuring the correct duplication of DNA in order to avoid genetic abnormalities and maintain chromatin integrity. If DNA damage is detected, the DNA damage response pathway is activated through the PI3K kinase ATM and ATR, and cell cycle progression is stalled in the S phase checkpoint (Elledge 1996). After successful DNA duplication, the cell enters the second gap phase, G2, and prepares for mitosis. The G2 phase is characterized by cell growth and protein synthesis (Smits and Medema 2001). The final stage of the cell cycle is mitosis which is regulated by cyclin B-CDK1 activity. During M phase, chromosome segregation occurs and two daughter cells are produced.

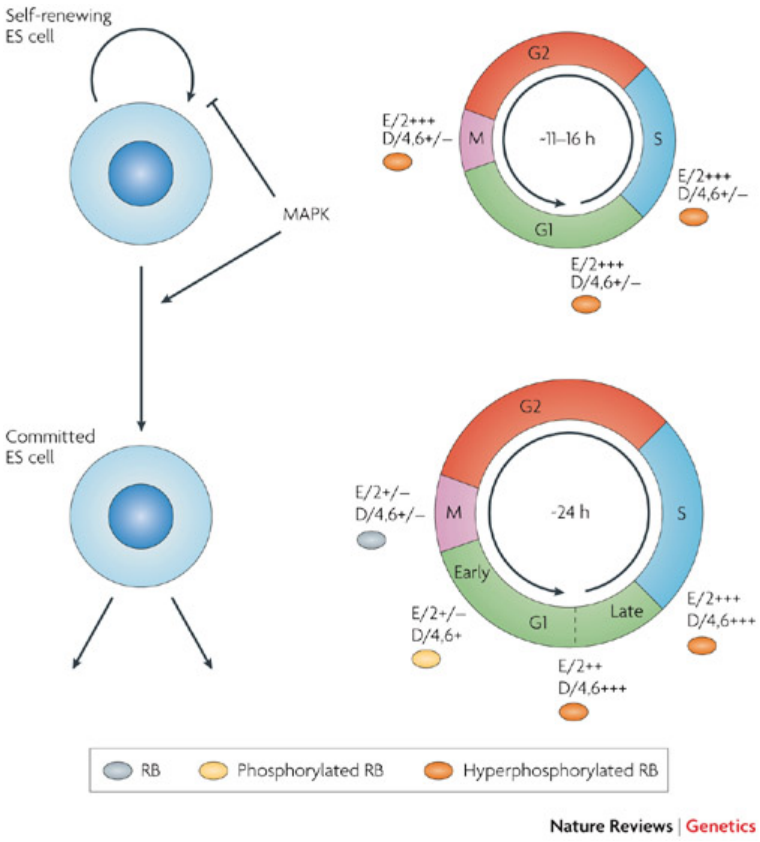

Figure 3. The cell cycle in ES cells.

Embryonic stem cells have a shorter cell cycle than most other cells. A hyperphosphorylated Rb and a constitutively active cyclinE-CDK2 complex allows the transition from M phase directly to late G1. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, Orford et al (2008). Copyright © 2008

1.2.2 Cell cycle in stem cells

One of the hallmarks of a stem cell is self-renewal, meaning that the cells maintain their undifferentiated state through multiple rounds of proliferation. This is critical, as stem cells are essential for replacing differentiated cells after tissue damage or cell loss. A depletion of the pool of stem cells in an adult organism would lead to an insufficient capacity to regenerate and heal wounds, and leads to developmental abnormalities in an immature organism. The unique ability of stem cells to self-renew means that the cell cycle in embryonic stem (ES) cells and adult stem cells differs in some aspects from the cell cycle in somatic cells. A difference between ES cells and adult stem cells is that adult stem cells have a relative proliferative quiescence. For instance, about 75% of long-term haematopoietic stem cells are in G0 (Cheshier, Morrison et al. 1999), but following transplantation, these cells are capable of reconstituting the haematopoietic system.

In ES cells, rather than being hypophosphorylated, the Rb protein levels are decreased after mitosis, and the cyclin E-CDK2 complex is constitutively active, which means ES cells are past the R point as soon as they enter G1 (Savatier, Huang et al. 1994, Burdon, Smith et al. 2002, White and Dalton 2005). This shortens the G1 phase and increases the proliferation rate in ES cells (Figure 3) (Orford and Scadden 2008). The MAPK signaling pathway and ERK

phosphorylation is not essential for ES cell cycle progression from G1 to S (Jirmanova, Afanassieff et al. 2002) and ES cells express low cyclin D1 levels, no cyclin D2 or D3 (Savatier, Lapillonne et al. 1996). However, ES cells are dependent on PI3 kinase activity for entry into S phase and regulation of cyclin D1 (Jirmanova, Afanassieff et al. 2002).

1.3 THE ROLE OF ION CHANNELS IN CELL CYCLE PROGRESSION

Many studies showed that ion channels function is not limited to maintaining ion homeostasis or regulate excitability of cells, but that they are also involved in cellular processes such as migration, apoptosis, differentiation and proliferation. Not only are some ion channels differentially expressed over the cell cycle, they also seem to regulate cell cycle progression. These studies have been performed on both normal and neoplastic cells.

There are several ways in which ion fluxes can influence the cell cycle. Among other things, they can modulate the membrane potential, modify intracellular Ca^{2+} concentration and pH, and regulate cell volume and secretion of autocrine/paracrine factors.

K^+ channels have been of particular interest when it comes to studying how ion channels control cell cycle progression. The first detailed study on ion channel activity over the cell cycle was done by Day et al in 1993. They showed that large conductance K^+ channels were activated right after zygote fertilization but deactivate during S and G2 phase, only to activate again during mitosis (Day, Pickering et al. 1993). This process was independent of a cycling nucleus, indicating that it was regulated by a cytoplasmic oscillator (Day, Johnson et al. 1998). Since then, several studies have indicated the K^+ modulate cell progression through G0/G1 and that K^+ channels have differential expression over the cell cycle (Wonderlin and Strobl 1996, Pardo 2004).

In oligodentrocyte progenitor cells, proliferation was inhibited following treatment with K^+ channel blockers (Attali, Wang et al. 1997) and the downregulation of $K_V1.5$ reduced astrocyte proliferation (MacFarlane and Sontheimer 2000). This effect was explained by a constitutively phosphorylated K_V 1.5 by Src family protein kinase. Inhibition of Src reduced K^+ currents, dephosphorylated K_V 1.5 and arrested astrocyte proliferation in G0/G1 cell cycle phase. MacFarlane et al. also showed that there is a cell cycle dependent expression of these K^+ channels over the cell cycle. A cell cycle dependent differential expression of K_V was also shown in tumor cells (Crociani, Guasti et al. 2003).

Even in non-neuronal stem cells K^+ channels seem to regulate cell cycle progression. In mesenchymal stem cells, K_V 1.2, K_V 2.1, and K_{Ca} mediate currents that vary during progression from G1 to S phase, and downregulation of these channels inhibited proliferation (Deng, Lau et al. 2007).

There have also been some studies on ClCs and proliferation. In 2003, Andäng et al. showed a novel signaling mechanism regulating proliferation of ES cells. GABA acted on the $GABA_AR$ to regulate cell proliferation in ES cells and neural crest cells. Activation of $GABA_AR$, lead to an influx of Cl^- into the cell, causing hyperpolarization and a cell volume increase. This in turn led to an activation of the PI3K kinases ATM and ATR, which phosphorylated histone variant H2AX, causing a reversible cell cycle arrest in S phase. This effect was dependent on the phosphorylated H2AX, as knockdown of H2AX abrogated the restrictive effects on proliferation by GABA signaling (Andang, Hjerling-Leffler et al. 2008). In paper I, we show a similar role for the $GABA_AR$ in NSC proliferation *in vivo*.

1.3.1 Membrane potential and cell cycle progression

It is widely accepted that there is a transient hyperpolarization of the cell membrane as some cells progress from G1 to S phase, and that proliferating cells are more depolarized than non-dividing cells. Furthermore, the membrane potential changes throughout the cell cycle (Wonderlin and Strobl 1996, Blackiston, McLaughlin et al. 2009). Since one of the major functions of ion channels and transporters is to regulate the membrane potential, it is tempting to imagine that ion channels may control cell proliferation by modulating the membrane potential throughout the cell cycle. Freedman et al showed in 1992 that the depolarization of lymphocyte membranes inhibited their proliferation in a similar way that blocking the K_v.1.3 channel did (Freedman, Price et al. 1992). Increasing the extracellular K^+ levels, thus depolarizing Schwann cells also inhibited mitogen-stimulated proliferation (Wilson and Chiu 1993). Membrane potential also regulates Ca^{2+} influx into the cell, and the Ca^{2+} flow will in turn trigger cell proliferation by initiating the Ca^{2+}-PKC pathway.

Ca^{2+} also regulates the cell cycle is by modifying the activity of transcription factors that regulate cyclins, in addition to having a direct effect on cyclins, cyclin kinases and associated proteins (Roderick and Cook 2008). A prominent target to Ca^{2+} is the CaM dependent pathway, where CaM-dependent kinase II and calceiurin mediate the regulation of cyclin related proteins (Kahl and Means 2003, Skelding, Rostas et al. 2011). In fibroblasts, inhibiting CaM causes cell cylcle arrest in G1. The arrest is caused by inhibition of CDK4 and CDK2, a loss of cyclin D1, and the upregulation of the CDK inhibitor p27 (Morris, DeLorenzo et al. 1998).

1.3.2 Cell volume as a regulator of cell cycle progression

Besides the proper duplication and segregation of DNA, cells must have tight and precise monitoring and regulation of its size and volume as it goes through the cell cycle. The two daughter cells that are produced need to be the same size as the mother cell and therefore cell size is greatest at M and smallest in early G1. The main contributor to cell volume change is water due to its high permeability over the plasma membrane compared with inorganic ions like Na^+, K^+ and Cl^-. Cell swelling is compensated through a process called regulatory volume decrease (RVD), where a number of ion channels and transporters enable the outward movement of K^+ and its counter-ion, Cl^-, leading to water efflux and cell volume decrease (Koivusalo, Kapus et al. 2009). RVD is enabled through KCCs that are activated by cell swelling through phosphorylation (Jennings and Schulz 1991, Kaji and Tsukitani 1991). In addition, multiple K^+ channels and volume-regulated anion channels are activated by cell swelling which leads to efflux of K^+, Cl^- and osmolytes (Stutzin and Hoffmann 2006) In contrast, upon cell shrinkage an opposite process called regulatory volume increase (RVI) is initiated and is mainly dependent on transport of Na^+ and Cl^- into the cell, with the accompanying influx of water. The main mechanism for RVI occurs via the Na^+/H^+ exchanger (NHE), which exchanges Na^+ ions for H^+ and NKCC1 and NKCC2 which are activated by shrinkage via phosphorylation (Wehner, Bondarava et al. 2006). In addition, there is an accumulation of organic osmolytes, such as taurine, inositol and sorbitol, over time

following cell shrinkage. This accumulation is due to uptake from extracellular media or novel synthesis of osmolytes, and serves to counteract the potential harmful effect of high influx of inorganic ions (Burg, Ferraris et al. 2007).

In relation to the cell cycle, it is still unclear the exact mechanisms linking cell volume and cell cycle progression, and which ion channels that are involved. Huang et al. showed that a K_V channel, Eag2, controls entry into mitosis by regulating cell volume in medulloblastoma (Huang, Dubuc et al. 2012). In paper III we show that a delayed rectifying K_V channel, Erg, is critical for cell volume control during cell cycle in ES cells.

2 AIM

The general aim of this thesis is to study how ion homeostasis is involved in the regulation and control of cell cycle progression and stem cell proliferation.

Specific aims include:

I. As $GABA_AR$ activity regulates ES cell proliferation *in vitro*, we aim to study the effects of $GABA_AR$ activity on NSCs *in vivo* in adult mice.

II. Given that the Na,K-ATPase is one of the main regulators of ion homeostasis, we examen its role in cell proliferation and if it regulates cell cycle progression.

III. As K^+ channels have been indicated to regulate cell proliferation, we aim to study whether they are regulated over the cell cycle and their role in regulating cell cycle progression in ES cells.

3 RESULTS AND DISCUSSION

3.1 PAPER I – CELL CYCLE RESTRICTION BY HISTONE H2AX LIMITS PROLIFERATION OF ADULT NEURAL STEM CELLS

Adult neural stem cells have the capacity to self-renew and generate new neurons, however the precise mechanism by which this occurs is not fully understood. Andäng et al showed a novel mechanism that regulate cell cycle progression in embryonic stem cells and neural crest cells *in vitro* and *in vivo* (Andang, Hjerling-Leffler et al. 2008). We used a glial fibrillary acidic protein (GFAP)-GFP mouse to investigate whether $GABA_AR$ activity had a similar role in adult mouse NSCs.

3.1.1 $GABA_AR$ activity phosphorylates H2AX and regulates NSC proliferation *in vitro*

We performed mRNA analysis and immunohistochemistry on GFP^+ NSCs from the subventricular zone (SVZ) and found that they express all necessary subunits and enzymes required for synthesis of and response to GABA. Among the most highly expressed subunits was the $GABA_AR$ β3 subunit, which is the same subunit that Andäng et al identified to be critical for response to GABA in ES cell proliferation.

To test the effect of GABA signaling on NSC proliferation, we inhibited or activated $GABA_AR$ signaling by treating cultured GFP^+ cells the with $GABA_AR$ agonist muscimol or antagonist bicuculline. Following acute (2.5h) treatment with muscimol the number of dividing cells decreased as assessed by increased EdU incorporation, while treatment with bicuculline had the opposite effect, increasing the number of dividing cells.

As $GABA_AR$ activity has been shown to induce phosphorylation of histone variant H2AX by PIKKs ATM and ATR (Andang, Hjerling-Leffler et al. 2008), we used immunohistochemistry to analyze the levels of phosphorylated H2AX (γH2AX) after administering muscimol or bicuculline to adult mice. After 1.5h of muscimol treatment, the levels of γH2AX increased in $GFAP^+$ cells in the SVZ as well as in some $Tuji1^+$ neuroblasts. This increase of γH2AX was also observed on cultured NSCs treated with muscimol.

These results show that $GABA_AR$ activity can modulate the number of NSCs *in vitro* and that $GABA_AR$ signaling induces H2AX phosphorylation *in vitro* and *in vivo*.

3.1.2 $GABA_AR$ regulation of NSC proliferation is dependent on H2AX

To test if H2AX was critical for $GABA_AR$ modulation of NSC proliferation, we obtained H2AX-null mutant mice. We stained for the proliferative marker Ki67, and bromodeoxyuridine (BrdU) in the SVZ and found that there was an increase in mitotically active and dividing cells in H2AX knockout mice compared to wild type mice upon bicuculline treatment. Similar results were obtained *in vivo* after administering H2AX mutant mice with $GABA_AR$ agonist or antagonist. Examining this effect *in vitro*, we treated H2AX deficient cells with muscimol or bicuculline, which did not affect their proliferation, as the

number of $BrdU^+$ cells was not significantly altered. This indicates that, similar to ES cells, $GABA_AR$ regulation of NSC proliferation is dependent on H2AX.

3.1.3 $GABA_AR$ regulation of transient amplifying cells

The increase in γH2AX after muscimol treatment was limited to cells adjacent to the lateral wall of the lateral ventrical. However, comparing the number of $BrdU^+GFP^+$ cells to the total number of $BrdU^+$ cells in the SVZ revealed that $GABA_AR$ activity was not limited to $GFAP^+$ cells but also included type C transient amplifying progenitors. Modulating $GABA_AR$ activity affected the cell cycle of NSCs such that the number of cells in G2/M phase were increased.

3.1.4 GABA signaling has a long-term regulatory effect *in vivo*

We next examined if long-term modulation of GABA signaling resulted in changed numbers of NSCs and niche size by staining for Ki67 following an initial long-term treatment with muscimol and bicuculline. Our results showed a significant increase or decrease of $Ki67^+$ cells in the SVZ following muscimol or bicucilline treatment respectively. This effect was not seen to the same extent in H2AX deficient mice, indicating that it was largely dependent on H2AX. To specifically examine the effect on the pool of NSCs, we used GLAST-CreERT2;CAG-GFP mice that would label NSCs upon tamoxifen administration. Comparing GFP^+ cells with GFP^+Ki67^+ cells after long-term treatment with bicuculline, showed an increase in both the number of NSCs and niche size up to 4 weeks after treatment. These results suggest that $GABA_AR$ activity can modulate NSC number and niche size long-term.

Taken together, our results show that $GABA_AR$ activity modulates NSC proliferation and niche size via an epigenetic mechanism *in vivo*. Inhibiting $GABA_AR$ activity leads to larger stem cell number and greater neuronal output. The histone variant H2AX seems to be critical for this mechanism as evident by experiments performed on H2AX-null mutant mice.

3.2 PAPER II – QUIESCENCE AND γH2AX IN NEUROBLASTOMA ARE REGULATED BY OUABAIN/NA,K-ATPASE

The Na,K-ATPase has previously been implicated in control of cell proliferation. It has the dual function of acting both as a transporter and as a signal transducer independent of its pumping function. In this paper we investigated the role of Na,K-ATPase on cell proliferation in human neuroblastoma cells.

3.2.1 Ouabain/Na,K-ATPase inhibition leads to reversible cell cycle arrest and quiescence

To study the effect of Na,K ATPase inhibition on neuroblastoma cells, we treated human SH-SY5Y cells with 50 nM of the cardiac glycoside ouabain. This concentration inhibits the Na,K-ATPase by approximately 12.7% and BrdU was used to asses cell proliferation 2 days and 7 days after treatment. A seven days treatment with ouabain caused a complete cell cycle arrest in neuroblastoma cells as most cells were negative for BrdU. This effect was not due to apoptosis as there was no increase of cleaved caspase-3.

A cell cycle arrest can either be reversible, if cells are in a quiescent state, or irreversible, if there is irreparable DNA damage. To investigate if ouabain treatment induced quiescence, we washed out ouabain after 7 days treatment and cells were left for 2 days to recover, before being treated with BrdU to evaluate proliferation. Even after prolonged ouabain treatment, the neuroblasotma cells were able to re-enter the cell cycle suggesting that the inhibition of Na,K-ATPase caused them to go into quiescence. This was also confirmed by the increased expression of the HES1 gene, which is required for quiescence (Sang, Coller et al. 2008).

3.2.2 Ouabain causes increase in γH2AX independent of DNA damage

To investigate the pathway by which ouabain induced quiescence in neuroblastoma cells, we stained for phosphorylated H2AX to see if the DDR pathway was activated. Already 2 days after treatment with ouabain, neuroblastoma cells showed an increase in γH2AX levels, indicating an activation of the DDR pathway. However, using comet assay we showed that no DNA damage was induced by inhibiting the Na,K-ATPase with ouabain.

Similar results were obtained *in vivo*, where we xenografted neuroblastoma cells in immune-deficient mice, and then treated the mice orally with ouabain for 12 days. Our results showed a reduction of tumor volume by 54%. Immunohistochemistry of the tumors showed an increase in γH2AX and no cleaved caspase 3, indicating a similar mechanism as observed *in vitro*.

3.2.3 Inhibition of Na,K ATPase reduces levels of cell cycle regulators

Treating neuroblastoma cells with ouabain for 7 days showed a downregulation of cyclin A, B1, D3, and E as well as CDK1, 2, and 4 in western blot experiments. The CDK inhibitor p21 was increased indicating that cells had entered a quiescent state. This effect was independent of Ca^{2+} signaling as voltage gated Ca^{2+} channel inhibitors did not prevent quiescence upon

ouabain treatment. We also investigated if ouabain-induced quiescence was achieved through the PI3K/Akt, MAPK and/or Src pathway, by inhibiting these signaling cascades during ouabain treatment. Our results show that these pathways were not involved in inducing quiescence in ouabain treated neuroblastoma cells, as there were no effects on the cell cycle regulators.

In summary, this study showed that the ouabain/Na,K-ATPase complex induced quiescence in neuroblastoma cells by an, as yet, unclear pathway. We showed that activation of the DDR pathway by H2AX phosphorylation was induced without DNA damage.

3.3 ERG CHANNEL IS CRITICAL IN CONTROLLING CELL VOLUME DURING CELL CYCLE IN EMBRYONIC STEM CELLS

3.3.1 K^+ channels differentially expressed over the cell cycle

Previous studies have shown how ion channels change expression over the cell cycle in spinal cord astrocytes (MacFarlane and Sontheimer 2000) and in tumor cells (Crociani, Guasti et al. 2003). To identify differential expression of K^+ channels over the ES cell cycle, we sorted ES cells according to their cell cycle phase, G1, S, and G2, using flow cytometry and subsequently performed Illumina sequencing to analyze mRNA expression in the various cell cycle phases. Several K^+ channels showed a preferential expression in G1, such as $K_V11.1$ and $K_V3.3$, while TASK2, $K_V9.3$ and $K_{ir}3.1$ had an increased mRNA expression in G2.

To confirm that mRNA expression correlated with protein levels, we immunostained the cell cycle sorted ES cells for Erg channel protein using an antibody towards an extracellular epitope. We also ran flow cytometry on ES cells stained with the same antibody. Both flow cytometry and immunostaining showed that only ES cell in G1 expressed Erg at the cell surface.

3.3.2 Erg inhibition caused cell death independent of apoptosis

To investigate the effect of Erg channels on ES cell cycle progression, we treated ES cells with an Erg channel inhibitor and analyzed cell viability and cell cycle distribution after 24 hours. Flow cytometry results showed that blocking Erg channels leads to a decreased number of ES cells in G1 and early S phase compared to late S and G2/M phase. Using cell viability assay, we tested whether cell death induced by Erg inhibition was caused by apoptosis, by co-administering an inhibitor for caspase 3 activity with the Erg channel inhibitor. Our results showed that inhibition of apoptosis only slightly prevented ES cell death following Erg channel inhibition.

3.3.3 Erg inhibition induced cell swelling and decreased cortical stiffness

Using time-lapse microscopy, we observed ES cells that were treated with an Erg inhibitor for 24 hours. Upon Erg inhibition, ES cells increased in cell size and the cell membrane lost its ability to bleb. This eventually led to cell rupture possibly as a consequence of the cells inability to regulate its cell volume.

We used atomic force microscopy to measure the effect of Erg inhibition on cortical actin and cell membrane stiffness as indicator of the osmotic pressure inside the cell. After treatment with an Erg inhibitor for 24 hours, ES cells had a decreased cortical stiffness, implying a re-organization of the actomyosin and the cytoskeleton.

3.3.4 Erg channel is critical for ion homeostasis and cell volume regulation over the cell cycle

If the observed cell volume increase was a result of increased intracellular pressure due to blocking of K^+ efflux and retention of water inside the cell, then adding sucrose to the media

would create a hyperosmolar environment that would decrease the osmotic pressure by promoting water efflux from the cell. Using cell viability assay, we showed that cell death induced by Erg inhibition was abolished when extracellular osmolarity was increased, indicating that it was caused by an inability to maintain cell volume due to high intracellular osmotic pressure.

Another way to test whether Erg inhibition caused cell death through disruption of ion homeostasis, was to inhibit the main regulator of the ion homeostasis in the cell, namely the Na,K-ATPase. This would lead to a reduction in intracellular K^+ levels, and thus reduced intracellular osmotic pressure. Our results showed that inhibiting Na,K-ATPase with ouabain while co-administering an Erg inhibitor restored cell viability, and ES cells acquired a normal volume.

Our results suggest that inhibiting the Erg channel led to an accumulation of intracellular K^+ ions and creating an osmotic pressure that led to cell volume increase, and eventually to cell rupture. Therefore, we suggest that Erg channel is critical in regulating cell volume during cell cycle progression by maintaining the ion homeostasis.

4 CONCLUSIONS

This thesis presents three studies where the role of ion homeostasis in relation to cell cycle and cell proliferation was examined by modulating ion channels and transporters.

In paper I we showed that the Cl^- channel $GABA_AR$ modulates NSC proliferation *in vivo* via an H2AX dependent mechanism. These changes in proliferation have long-term effects on stem cell number, niche size and neuronal output.

In paper II we demonstrated that the ouabain/Na,K-ATPase complex regulates quiescence in neuroblastoma and induces phosphorylation of H2AX via a pathway that is yet to be identified.

In paper III we showed that the Erg K^+ channel is critical for ES cells to regulate their cell volume as they progress through the cell cycle. This is possibly achieved by facilitating efflux of K^+ and, thereby, decreasing the intracellular osmotic pressure.

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