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To Helena

ABSTRACT

Cells exploit calcium (Ca^{2+}) signaling to transmit information. In a multi-cellular organism, each cell must be able recognize, process, and respond to information received from the surrounding environment. In this thesis I investigate molecular mechanisms and cellular consequences of Ca^{2+} signaling.

Ouabain is an endogenous hormone, and ligand of the Na,K-ATPase, that has previously been shown to induce Ca^{2+} oscillations in renal cells. Here we report that the N-terminal tail of the Na,K-ATPase α-subunit binds directly to the N-terminus of the inositol 1,4,5-trisphosphate receptor ($InsP_3R$). Three amino acid residues in the Na,K-ATPase N-terminal tail, LKK, conserved in most species, are essential for this binding to occur. Over-expression of a peptide encoding for the N-terminal tail impaired ouabain-triggered Ca^{2+} oscillations. Thus we have identified a well conserved Na,K-ATPase-motif that binds to the $InsP_3R$ and is vital for intracellular Ca^{2+} signaling.
The role of Na,K-ATPase signaling during dendritogenesis was examined by treating embryonic cortical rat neurons with ouabain. We report that Na,K-ATPase signal transduction triggers dendritic growth as well as a transcriptional program dependent on CREB and CRE-mediated gene expression, primarily regulated via Ca^{2+}/calmodulin-dependent protein kinases. This signaling cascade also involves intracellular Ca^{2+} oscillations and sustained phosphorylation of mitogen-activated protein kinases. These results suggest a novel role for the Na,K-ATPase as a modulator of dendritic growth in developing neurons.

We explored the Ca^{2+} signaling properties of differentiating mouse embryonic stem cells. Spontaneous Ca^{2+} activity was shown to be present in neural progenitors derived from mouse embryonic stem cells. This Ca^{2+} activity was dependent on influx of extracellular Ca^{2+} through plasma membrane channels, since removal of extracellular Ca^{2+} from the medium and inhibition of voltage-dependent channels blocked the signaling event. Cross-correlation analysis revealed that the spontaneous Ca^{2+} activity was more synchronous in sub-populations of the neural progenitors than in the undifferentiated mouse embryonic stem cells. A significant reduction Ca^{2+} activity was observed when cells were challenged with gap junction blockers. Inhibiting the spontaneous Ca^{2+} activity significantly reduced the number of dividing cells.

In conclusion, this thesis presents novel data on a conserved Na,K-ATPase-motif important for the N-terminal signaling activity and demonstrates a role of Na,K-ATPase in dendritic growth in developing cortical neurons. Further, spontaneous Ca^{2+} activity in neural progenitors derived from embryonic stem cells is dependent on extracellular Ca^{2+} and is important for cell division.

PUBLICATIONS

I. Zhang S*****, **Malmersjö S***, Li J, Ando H, Aizman O, Uhlén P, Mikoshiba K, and Aperia A
Distinct Role of the N-terminal Tail of the Na,K-ATPase Catalytic Subunit as a Signal Transducer
J Biol Chem 281, 21954-21962 (2006)

II. Desfrere L*****, Karlsson M*****, Hiyoshi H*****, **Malmersjö S**, Nanou E, Estrada M, Miyakawa A, Lagercrantz H, El Manira A, Lal M, and Uhlén P
Na,K-ATPase Signal Transduction Triggers CREB Activation and Dendritic Growth
Under revision

III. Edman L C, Mira H, Erices A, **Malmersjö S**, Andersson E, Uhlén P, and Arenas E
Alpha-chemokines Regulate Proliferation, Neurogenesis, and Dopaminergic Differentiation of Ventral Midbrain Precursors and Neurospheres
Stem Cells 26, 1891-1900 (2008)

IV. **Malmersjö S**, Rebellato P, Liste I, Arenas E, and Uhlén P
Spontaneous Calcium Activity Regulates Proliferation in Neural Progenitors Derived from Embryonic Stem Cells
Manuscript

***** denotes equal contribution

Additional publications not included in this thesis

Scott L, Zelenin S, **Malmersjö S**, Kowalewski J M, Markus E Z, Nairn A C, Greengard P, Brismar H, and Aperia A
Allosteric Changes of the NMDA Receptor Trap Diffusible Dopamine 1 Receptors in Spines.
Proc Natl Acad Sci U S A 103, 762-767 (2006)

CONTENTS

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
CaM kinase	Ca^{2+}/calmodulin-dependent protein kinase
CBP	CREB binding protein
Chemokine	Chemotactic cytokine
CREB	cAMP response element binding
CREST	Calcium-responsive transactivator
CTS	Cardiotonic steroids
DAG	Diacylglycerol
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ES cells	Embryonic stem cells
FRAP	Fluorescent recovery after photobleaching
GFP	Green fluorescent protein
GST	Glutathione S-transferase
$InsP_3$	Inositol 1,4,5-trisphosphate
$InsP_3R$	Inositol 1,4,5-trisphosphate receptor
MAP kinase	Mitogen-activated protein kinase
NCX	Na^+/Ca^{2+} exchanger
NF-AT	Nuclear factor of activated T-cells
NMDA	N-methyl-D-aspartate
$PI(4,5)P_2$	Phosphatidylinositol (4,5)-biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMCA	Plasma-membrane Ca^{2+}-ATPase
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SOCE	Store operated Ca^{2+} entry
STIM1	Stromal interaction molecule 1
TH	Tyrosine hydroxylase
TRP	Transient receptor protein
TTX	Tetrodotoxin

1 CALCIUM SIGNALING

Cellular signaling via calcium ions (Ca^{2+}) is governed by two principles encoding and decoding. Encoding is how cells translate environmental cues into intracellular Ca^{2+} signals. Decoding is how cells interpret the Ca^{2+} signals and thereby execute appropriate physiological responses. The encoding process is dependent on the timing of the incoming stimuli. During development, as the expression pattern of proteins changes, the way stimuli are encoded can change dramatically. For example, a depolarizing stimulus will not affect intracellular Ca^{2+} until voltage-dependent Ca^{2+} channels are expressed. In this thesis I will discuss both the encoding of Ca^{2+} signaling and how the decoding leads to different cellular consequences.

Ca^{2+} is a ubiquitous intracellular signal messenger that regulates many diverse cellular processes. (Berridge et al., 2003; Clapham, 2007). Fast processes such as exocytosis and muscle contraction are regulated by Ca^{2+} within the microseconds to millisecond range. Transcription, proliferation and differentiation are examples of slower cellular processes where Ca^{2+} is driving events within the scale of minutes and hours. Ca^{2+} is just a simple ion, not a complex molecule that can be phosphorylated or manipulated in other ways. A question arises - How can such a simple messenger like Ca^{2+} regulate such a wide variety of cellular functions?

Ca^{2+} binding triggers changes in a molecules shape and charge, which may alter its functions. The cell uses Ca^{2+} as a signaling messenger by changing the local and global concentration of the ion over time. This is achieved by using a sophisticated Ca^{2+} signaling toolkit. The level of intracellular Ca^{2+}

is determined by regulating the activity of processes allowing Ca^{2+} entry into the cytoplasm and reactions facilitating its removal.

1.1 CALCIUM SIGNALING TOOLKIT

In order to control the spatial and temporal properties of intracellular Ca^{2+} the cell has an elaborate Ca^{2+} signaling toolkit (see Box 1). The basal intracellular Ca^{2+} level is low (~100 nM) whereas the intracellular stores and the extracellular Ca^{2+} levels are 1,000 to 10,000 times higher. This means that reactions leading to intracellular Ca^{2+} elevations involve channels, where Ca^{2+} ions diffuse in the direction of the gradient. The reactions leading to removal of Ca^{2+} from the cytoplasm depend on pumps, exchangers and buffer molecules (see Box 1). Many of the toolkit components exist in multiple isoforms, each with special properties, further expanding the versatility of Ca^{2+} signaling.

Entry of extracellular Ca^{2+} to the cytoplasm is one of the main sources of Ca^{2+}. The electrochemical gradient across the plasma membrane drives the entry of Ca^{2+} through channels. Voltage-dependent Ca^{2+} channels are found in excitable cells and generate rapid Ca^{2+} fluxes that control fast cellular processes such as muscle contraction or exocytosis in synapses. The plasma membrane contains many other channels that have the ability to conduct Ca^{2+}, such as the receptor-operated channels, for example the NMDA (N-methyl-D-aspartate) receptor that responds to glutamate. There is also the large family of transient receptor protein (TRP) ion-channels, which includes the thermosensor and stretch-activated channels (Clapham, 2003). Another type of channels that has recently received a large amount of attention are the store operated Ca^{2+} channels which will be discussed

further in the next section (Feske et al., 2006; Liou et al., 2005; Yeromin et al., 2006).

The other principal source of Ca^{2+} is the internal stores that are located primarily in the sarco/endoplasmic reticulum. There are two types of channels responsible for the release of Ca^{2+} from the internal stores, the Inositol 1,4,5-trisphosphate receptor ($InsP_3R$) and the ryanodine receptor

Box 1: Ca^{2+} signaling toolkit

Increase of intracellular Ca^{2+}			
	Type	Action	Example
Voltage-dependent channels	Channel	Extracellular Ca^{2+} entry	L-type
Receptor-operated channels	Channel	Extracellular Ca^{2+} entry	NMDAR
Ryanodine receptors	Channel	Intracellular Ca^{2+} release	RYR1-3
Inositol-1,4,5-trisphophate receptors	Channel	Intracellular Ca^{2+} release	$InsP_3R1$-3
Transient receptor potential	Channel	Extracellular Ca^{2+} entry	TRPC1-7
Second-messenger-operated channels	Channel	Extracellular Ca^{2+} entry	CNGA1-4
Store-operated Ca^{2+} channels	Channel	Extracellular Ca^{2+} entry	Orai1

Decrease of intracellular Ca^{2+}			
	Type	Action	Example
Plasma membrane Ca^{2+}-ATPases (PMAC)	Pump	Ca^{2+} out of cell	PMCA1-4
Sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCA)	Pump	Ca^{2+} into internal stores	SERCA1-3
Na^{+}/Ca^{2+} exchangers	Exchanger	Ca^{2+} out (Na^{2+} in)	NCX1-3
Golgi pumps	Pump	Ca^{2+} into internal stores	SPCA1-2
Cytosolic Ca^{2+} buffers	Buffer	Ca^{2+} buffer	Parvalbumin
ER/SR Ca^{2+} buffers	Buffer	Ca^{2+} buffer	Calnexin
Mitochondrial Ca^{2+} uniporte	Channel	Ca^{2+} into mitochondria	MiCa

(Berridge et al., 2003; Mikoshiba, 2007). Both channels are sensitive to Ca^{2+} and are thus contributing to Ca^{2+} induced Ca^{2+} release. The $InsP_3R$ will be discussed in more detail in a separate section.
The intracellular Ca^{2+} homeostasis is balanced between processes for entry and removal of Ca^{2+} (see Box 1). Once that intracellular Ca^{2+} levels raise the pumps, exchangers and buffers are used to lower the Ca^{2+} concentration to avert toxic effects. The pumps are driven by ATP, the main energy source of the cell, and are pumping ions against the electrochemical gradient. The main pumps are the plasma-membrane Ca^{2+}-ATPase (PMCA) and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Berridge et al., 2003). The Na^+/Ca^{2+} exchanger (NCX) removes Ca^{2+} from the cytoplasm by exchanging one Ca^{2+} ion for three Na^+ ions. Mitochondria is also an important compartment for taking up Ca^{2+} (Kirichok et al., 2004).

1.2 CALCIUM OSCILLATIONS

Ca^{2+} oscillations, repeated sets of Ca^{2+} transients, have been implicated in the control of many biological processes, including oocyte activation at fertilization (Miyazaki et al., 1993), development of neurotransmitter cell phenotypes (Borodinsky et al., 2004; Ciccolini et al., 2003; Root et al., 2008), release of cytokines from renal epithelial cells (Uhlen et al., 2000), growth-cone turning (Gomez et al., 2001; Robles et al., 2003), cortical development (Weissman et al., 2004) and neuronal migration (Komuro and Rakic, 1996; Kumada and Komuro, 2004).

It has been suggested that Ca^{2+} oscillations resemble radio communication by applying frequency modulation (FM) and amplitude modulation (AM) (Berridge, 1997). Dolmetsch and colleagues showed how downstream

effectors can decode information contained in the amplitude and duration of Ca^{2+} signals (Dolmetsch et al., 1997). Other studies have pointed out the importance of the frequency of Ca^{2+} oscillation in activating transcription and signaling cascades (Aizman et al., 2001; Dolmetsch et al., 1998; Kupzig et al., 2005).

Internal stores are often the Ca^{2+} source during Ca^{2+} oscillations and the stores must hence be refilled. More than 20 years ago it was suggested that the emptying of Ca^{2+} stores itself activates Ca^{2+} channels in the plasma membrane to help refill the stores (Putney, 1986). The phenomenon was named store operated Ca^{2+} entry (SOCE). However, the research field had struggled to find the molecules involved though TRP channels were often suggested as candidates (Boulay et al., 1999; Putney, 2001). In 2005, siRNA screens were used by two independent research groups who identified stromal interaction molecule 1 (STIM1) as an ER Ca^{2+} sensor (Liou et al., 2005; Roos et al., 2005). One year later the plasma membrane channel Orai1 was found to be responsible for SOCE (Feske et al., 2006; Vig et al., 2006; Yeromin et al., 2006; Zhang et al., 2006b). Although the precise mechanism is not clear, it is generally believed that STIM1 senses store depletion and that Orai1 acts as a store operated Ca^{2+} channel (Lewis, 2007).

The mechanisms for generating Ca^{2+} oscillations vary in different cell types and depend on the specific Ca^{2+} toolkit expressed in the cell (see figure 1 and Box 1). Different agonists have been shown to trigger Ca^{2+} oscillations, including ouabain (Aizman et al., 2001), testosterone (Estrada et al., 2006), alpha-haemolysin (Uhlen et al., 2000), glutamate acting on mGluR5, gonadotropin-releasing hormone and ATP (Berridge et al., 2003)

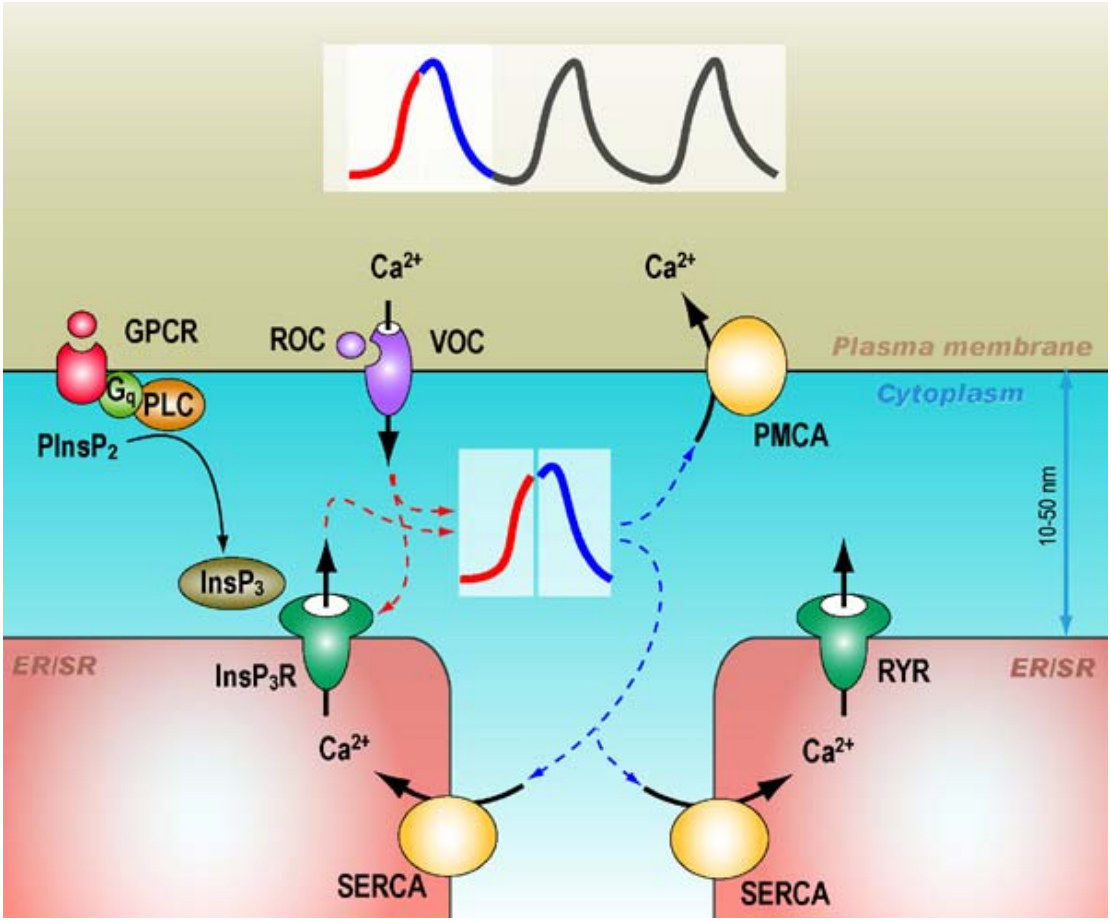

Figure 1. Ca^{2+} signaling toolkit generates Ca^{2+} oscillations. Channels in the plasma membrane and in ER/SR contributes to the up-slope (red) and pumps contributes to the down-slope (image by Per Uhlén).

Release of Ca^{2+} from intracellular stores via the $InsP_3R$ and the ryanodine receptor is important for Ca^{2+} oscillations in many different systems, though other channels have also been identified to play major roles. In T-cells TRPM4 was shown to regulate Ca^{2+} oscillations. Knock down of TRPM4 transformed a Ca^{2+} oscillatory response into a sustained elevation in Ca^{2+} (Launay et al., 2004). Ca^{2+} oscillations will be discussed further in the section "Spontaneous calcium activity during development" below.

1.3 CELLULAR CONSEQUENCES OF CALCIUM SIGNALING

The basic rule governing downstream effects of Ca^{2+} signaling is that the binding of Ca^{2+} triggers changes in the shape and charge of molecules. Ca^{2+} binds to a wide variety of molecules in the cell and can hence affect many processes.

Calmodulin functions as an intracellular Ca^{2+} receptor and is found ubiquitously in eukaryotic cells. It has four EF-hand motifs, which allow sensing of the cytoplasmic Ca^{2+} concentration. Many proteins have EF-hand motifs, but calmodulin is unique because it interacts with numerous highly diverse target enzymes involved in a wide variety of intracellular signaling pathways. This functional versatility is largely a result of the structural flexibility of calmodulin. When the intracellular Ca^{2+} concentration rises to about 1 - 10 μM, four Ca^{2+} ions bind to the EF-hand motifs inducing conformational changes that expose hydrophobic amino acids on the surface. These hydrophobic clusters are then used to bind peptide sequences in target enzymes. Although calmodulin binding sites typically have little sequence identity, the positions of several hydrophobic residues are often conserved, allowing for classification of calmodulin recognition motifs (Chin and Means, 2000).

Many of the highly characterized targets of calmodulin are directly or indirectly involved in protein phosphorylation, though proteins with other functions are also regulated such as the PMCA, the L-type Ca^{2+} channels, the $InsP_3Rs$ and the ryanodine receptors.

One of the most widely studied targets for calmodulin is the family of Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases). CaM kinase II is one of the most prominent protein kinases, present in essentially every

tissue but most concentrated in brain. CaM kinase II regulates important neuronal functions, including neurite outgrowth, neurotransmitter synthesis, synaptic plasticity, learning and memory, and gene expression. The transcription factor cAMP response element binding (CREB) is activated through phosphorylation by CaM kinase II. CREB binds to a specific DNA sequences called CRE and requires CREB binding protein (CBP) as a co-activator (Mayr and Montminy, 2001).

Calmodulin also regulates calcineurin which is known to activate the transcription factor nuclear factor of activated T-cells (NF-AT). Binding of calcineurin dephosphorylates NF-AT and the complex is then imported into the nucleus, where NF-AT activates transcription (Uhlen et al., 2006). The transcription factor NF-κB has been shown to be activated by intracellular Ca^{2+} oscillations (Aizman et al., 2001; Dolmetsch et al., 1998). Other transcription factors known to be activated by Ca^{2+} signaling are MES-2, LMO4, NeuroD2 and CREST (Kashani et al., 2006; McKinsey et al., 2002).

The MAPK/ERK pathway is known to influence many cellular functions, including cell survival, gene transcription and dendritic growth. This pathway is influenced by Ca^{2+} and can activate the transcription factors CREB and MEF-2 (Bonni et al., 1999; Dolmetsch et al., 2001; Kupzig et al., 2005; Wu et al., 2001).

Protein kinase C (PKC) is activated by diacylglycerol (DAG) (figure 2) and Ca^{2+}. PKC has various targets for phosphorylation and is thought to be a decoder of Ca^{2+} oscillations (Boulware and Marchant, 2008; Reither et al., 2006).

Recently an activity-regulated transcription factor, Npas4, was identified as a key regulator of GABAergic synapse development. Npas4 is induced in neurons by depolarization mediated Ca^{2+} influx (Lin et al., 2008).

2 NEURAL DEVELOPMENT AND CALCIUM SIGNALING

In general, brain development has been viewed as a process largely driven by genetic programs, whereas electrical activity has been thought to be important for refinement of connections, such as spines. Neuronal development begins with the generation of a large number of neurons differentiating from stem cells. The neurons then migrate to their final destinations where they mature and establish connections with other cells.

Electric activity has previously been viewed as the activity generated by sodium channels, leading to action potentials that can be blocked with tetrodotoxin (TTX). Other types of electrical activity, insensitive to TTX, have been recognized to play an important role during development (Spitzer, 2006). This activity often leads to intracellular Ca^{2+} elevations, but does not involve synaptic activity and generation of action potentials.

2.1 SPONTANEOUS CALCIUM ACTIVITY DURING DEVELOPMENT

Spontaneous Ca^{2+} activity has been described in many parts of the developing nervous system including the spinal cord (Borodinsky et al., 2004; Gomez and Spitzer, 1999), cortex (Cossart et al., 2003; Weissman et al., 2004), hippocampus (Ben-Ari et al., 1989; Crépel et al., 2007) and retina (Firth et al., 2005; Pearson et al., 2005).

The mechanisms for achieving spontaneous Ca^{2+} activity are diverse and depend not only on the developmental stage, but are also region and cell type specific. Ultimately the Ca^{2+} activity depends on both the Ca^{2+} toolkit

present in the cell and the environmental stimulus. Mechanisms of spontaneous Ca^{2+} activity related to gap junctions will be discussed in the next section.

Different types of spontaneous activity were observed in the mouse hippocampus. At embryonic stages (E16-E19) few cells have activity and the detected activity did not seem to correlate between cells. Around birth more cells generate Ca^{2+} activity which is characterized by a fast onset followed by a slow plateau. The activity is nonsynaptic, but is thought to be mediated by voltage-dependent signaling and gap junctions (Crépel et al., 2007). At later postnatal stages (P6-P10) strongly correlated synaptic activity has previously been observed (Ben-Ari et al., 1989).
Kriegstein and colleagues showed that spontaneous Ca^{2+} waves that propagate through embryonic rat radial glia cells are important for neuronal proliferation in the ventricular zone (Weissman et al., 2004). In contrast, it has been shown that release of GABA from differentiating neuroblasts depolarizes progenitor cells in the postnatal mouse subventricular zone inhibiting proliferation (Liu et al., 2005).
Spontaneous Ca^{2+} activity in the visual cortex has been widely studied. Activity in the mouse visual cortex was shown to be inhibited by TTX, as well as by high concentration of Ni^{2+} (2 mM, inhibitor of voltage-dependent Ca^{2+} channels) (Cossart et al., 2003).
Synchronized Ca^{2+} oscillations were observed in newborn rats, starting in the posterior cortex and propagating slowly towards the anterior. These oscillations required activation of AMPA and NMDA receptors and continue until around postnatal day 7 (Garaschuk et al., 2000).
Metabotropic glutamate receptors (group I) were suggested to trigger spontaneous Ca^{2+} oscillations in immature neurons of the mouse cortex (Flint et al., 1999).

Blockade of $GABA_B$ or metabotropic glutamate receptors (group III) suppresses Ca^{2+} spikes in developing neurons in the Xenopus neural tube (Root et al., 2008).

Spontaneous Ca^{2+} activity is present in many parts of the developing brain and is likely to play a role in the formation of neural networks. Ca^{2+} affects gene expression, which in turn can change the Ca^{2+} signaling properties of the cell by altering expression of pumps and channels. In this way Ca^{2+} provides a feedback loop for the cell to regulate the balance of pumps, channels and receptors. Ca^{2+} signaling is also a way for the cell to respond to different kinds of environmental cues.

2.2 GAP JUNCTIONS DURING DEVELOPMENT

Developing neurons and other embryonic cell types are often connected by gap junctions, intercellular channels that allow the direct transfer of electrical signals and small molecules (<1 kDa) between coupled cells. Each gap junction is made up of two hemichannels on opposing membranes that join through hydrophobic interactions.
Given the wide variety of gap junction proteins (at least 20 genes in rodents and humans), each with different properties and differential expression pattern in the developing brain, it is likely that cell coupling serves different functions in specific neuronal populations and developmental stages. Developing neurons in the vertebrate spinal cord, retina, and cortex are interconnected by gap junctions that are lost later in life (Elias and Kriegstein, 2008; Kandler and Katz, 1995; Montoro and Yuste, 2004).

Release of ATP through gap junction has been demonstrated as a mechanism for spontaneous Ca^{2+} activity in various systems. For example, gap junction hemichannels can mediate Ca^{2+} waves in the developing ventricular zone through the release of ATP that binds to P2Y1 receptors in neighboring cells. When extracellular ATP binds to P2Y receptors Ca^{2+} is released through $InsP_3R$ (Weissman et al., 2004). The same mechanism has also been reported in the developing retina and in human bone marrow-derived mesenchymal stem cells (Kawano et al., 2006; Pearson et al., 2005).

Spontaneous Ca^{2+} activity in the mouse hippocampus was shown to be dependent on gap junctions. Around birth Ca^{2+} activity, characterized with a fast onset followed by a slow plateau, was inhibited by gap junction blockers. The activity is nonsynaptic, but is thought to be mediated by voltage-dependent signaling and gap junctions (Crépel et al., 2007).
Gap junctions are important for adhesion between migrating neurons and radial fibers in rat cortex. In this system gap junctions did not mediate cell-cell communication through an aqueous channel, but instead they provide dynamic adhesive contacts between cells during migration (Elias et al., 2007).
Gap junctions have been proposed to control left-right asymmetry in C. Elegans (Chuang et al., 2007).
Yuste and colleagues, performing Ca^{2+} imaging on cortical slices from rat and mice in the first postnatal week, revealed neurons that spontaneously increased intracellular Ca^{2+} concentration in synchrony. These groups of neurons were named ‘‘neuronal domains’’. The spontaneous activity of neuronal domains was insensitive to TTX, but was inhibited by gap junction blockers (Montoro and Yuste, 2004; Yuste et al., 1995).

The expression of functional gap junctions in undifferentiated embryonic stem (ES) cells is well documented (Huettner et al., 2006), but their role in Ca^{2+} signaling or differentiation is not known.

2.3 NEUROTRANSMITTER SPECIFICATION

The specification of neurotransmitter phenotype is a process depending on early electrical activity. Spitzer and colleagues showed that alterations in electrical activity can change the numbers of neurons expressing excitatory and inhibitory transmitters in the Xenopus spinal cord. Increasing spontaneous Ca^{2+} activity decreases the incidence of neurons expressing excitatory transmitters and increases the incidence of neurons expressing inhibitory transmitters. Conversely, the inhibition of spontaneous Ca^{2+} activity increases the incidence of excitatory transmitter expression and decreases the incidence of inhibitory transmitters (Borodinsky et al., 2004; Root et al., 2008; Spitzer et al., 2005). In differentiating mouse neurosphere-derived precursors Ca^{2+} signaling has also been shown to correlate with the onset of GABAergic neurotransmitter phenotype (Ciccolini et al., 2003). Electrical stimulations were shown to induce expression of tyrosine hydroxylase (TH) through activation of N-type Ca^{2+} channels in rat primary neurons (Brosenitsch and Katz, 2001).

2.4 DENDRITIC OUTGROWTH

The establishment of dendrites is a dynamic process characterized by initial extension and retraction of branches, followed by stabilization and growth. Dendrites serve a critical role in detecting and processing incoming information. A large body of evidence indicates that Ca^{2+} has

important and diverse roles in the control of axonal and dendritic growth and guidance.

Elevations of growth cone Ca^{2+} have been associated with slow down, stop or retract responses in a Ca^{2+}-dependent manner. This seems to be the case when growth cones are exposed to stimuli that produce a large, sudden Ca^{2+} elevation (Gomez and Spitzer, 1999; Gomez and Zheng, 2006). However, other studies have found that elevating Ca^{2+} in growth cones promotes neurite outgrowth (Ciccolini et al., 2003). One possible explanation for this contrast in Ca^{2+} mediated actions may be due to sustained Ca^{2+} signaling controlling gene expression. Sustained Ca^{2+} signaling would then alter cellular differentiation independent of the local effects of Ca^{2+} on the motility machinery of the growth cones.

Ghosh and colleagues have studied the mechanisms of dendritic outgrowth and the role of Ca^{2+} in this process. Constitutive active CaM kinase IV was found to stimulate neurite growth, whereas CaM kinase II inhibited growth. The dendritic growth was mediated through the transcription factor CREB, which is a known target of CaM kinase IV (Konur and Ghosh, 2005; Redmond et al., 2002; Wong and Ghosh, 2002). Another transcription factor shown to be important in dendritic growth is calcium-responsive transactivator (CREST) (Aizawa et al., 2004).

2.5 DIFFERENTIATION OF EMBRYONIC STEM CELLS

ES cells are an in-vitro population of pluripotent cells, meaning that they have the potential to give rise to any cell type of the adult organism. It is possible to differentiate ES cells to more committed progenitors characterized by an extensive proliferative capacity, though their

differentiation potential is then restricted to specific cell types. Neural progenitors for instance constitute the foundation for all lineages in the adult central nervous system. Different neural cell types such as dopamine neurons have been successfully induced from neural progenitors derived from mice and human ES cells by the means of different protocols (Andersson et al., 2006; Barberi et al., 2003; Perrier et al., 2004). The clinical use of ES derived cells is one of the most intriguing prospects of today's medicine, but progress has been hindered by several factors, particularly the need to produce pure populations of cells at the required quantities for transplantation. This fact highlights the importance of investigating the mechanisms that underlie the proliferation and differentiation of ES cells.

3 $InsP_3R$

Inositol 1,4,5-triphosphate receptor ($InsP_3R$) is an intracellular Ca^{2+} channel that has been widely studied. A number of recent reviews have been written about the functional and molecular properties of $InsP_3R$ (Bezprozvanny, 2005; Foskett et al., 2007; Mikoshiba, 2007; Patterson et al., 2004). The $InsP_3R$ channel is a tetramer of four subunits, where each subunit has 2749 amino acids ($InsP_3R1$ in mouse). There are three different subtypes of $InsP_3R$, $InsP_3R1$-R3. Multiple $InsP_3R$ subtypes are expressed in most cell types but the physiological significance of this heterogeneity is poorly understood. $InsP_3R1$ is for example abundantly expressed in purkinje cells in the cerebellum and in CA1 pyramidal cell layer of the hippocampus. Knocking out $InsP_3R1$ in mice resulted in ataxia and epileptic seizures, followed by a premature death (Matsumoto et al., 1996). $InsP_3R2$-R3 double knockout mice exhibited growth abnormalities and severely impaired saliva secretion (Futatsugi et al., 2005).

3.1 REGULATION OF $InsP_3R$

Activated G-proteins stimulate the plasma-membrane-bound enzyme phospholipase C (PLC). The active PLC then hydrolyses an inositol phospholipid called $PI(4,5)P_2$, which is present in small amounts in the inner half of the plasma membrane, see figure 2. $PI(4,5)P_2$ is cleaved into DAG, which remains in the membrane, and $InsP_3$ which diffuses to the ER where it binds to the $InsP_3R$ stimulating Ca^{2+} release into the cytosol.

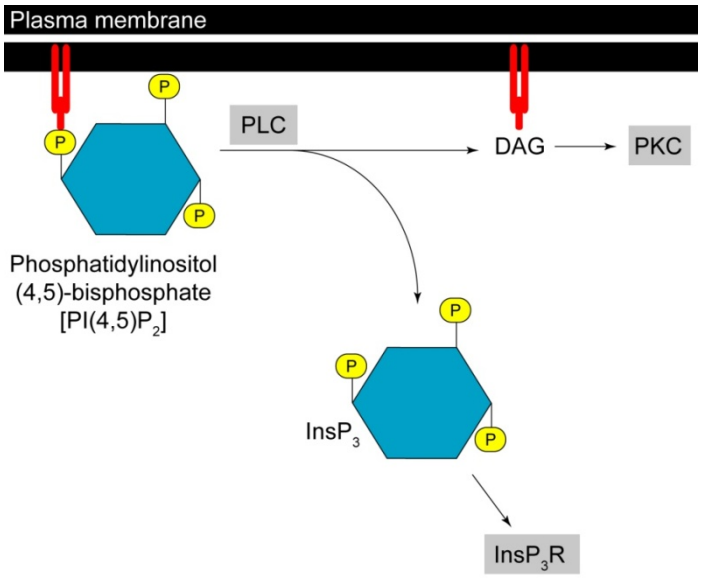

Figure 2. Stimulation of a G protein-coupled receptor leads to activation of Phospholipase C (PLC), which cleaves $PI(4,5)P_2$ into DAG and $InsP_3$. DAG stays in the membrane and is one of the activators of PKC. $InsP_3$ diffuses through the cytosol and triggers Ca^{2+} release through $InsP_3R$.

$InsP_3$ is necessary for the activation of $InsP_3R$, but Ca^{2+} also regulates $InsP_3R$. The opening probability of the channel shows a bell-shaped dependency on Ca^{2+}, meaning that both low and high Ca^{2+} has an inhibitory effect on the channel (Bezprozvanny et al., 1991). In this way a moderate increase in cytosolic Ca^{2+} can trigger the release of Ca^{2+} through $InsP_3R$ (Ca^{2+} induced Ca^{2+} release), but once the cytosolic Ca^{2+} levels reaches a threshold the $InsP_3R$ becomes inhibited. The different subtypes of $InsP_3R$ have different sensitivities for $InsP_3$ and Ca^{2+} (Iwai et al., 2007). Cytosolic ATP and calmodulin are also important modulators of $InsP_3Rs$. Several phosphorylation sites are present in the $InsP_3R$ (Bezprozvanny, 2005; Foskett et al., 2007).

In recent years numerous proteins have been found to interact with $InsP_3R$ and the receptor could thus be viewed as a scaffold protein forming a macro signal complex. Examples of proteins known to interact with $InsP_3R$ are IRBIT, RACK1, cytochrome c, 4.1N, Na,K-ATPase, TRPC3 and ankyrin (Bezprozvanny, 2005; Mikoshiba, 2007; Patterson et al., 2004).

Mutations in the protein family presenilins are the major cause of familial Alzheimer's disease and have been associated with impaired Ca^{2+} homeostasis. Recently mutated presenilins were shown to interact with $InsP_3R$ resulting in exaggerated cellular Ca^{2+} signaling in response to agonist stimulation (Cheung et al., 2008).

3.2 STRUCTURE OF $InsP_3R$

The $InsP_3R$ has been divided into five functional domains: N-terminal coupling/suppressor domain, $InsP_3$-binding core domain, internal coupling domain, transmembrane/channel-forming domain and gatekeeper domain (Mikoshiba, 2007). As the names indicate, $InsP_3$-binding core domain contains the binding site for $InsP_3$ and the suppressor domain suppresses the binding of $InsP_3$. When expressed in isolation, the core domain has a high affinity for $InsP_3$ (k_d = 2.3 nM), but together with the suppressor domain the binding affinity is attenuated to a physiological range (k_d = 45 nM) (Yoshikawa et al., 1996). It is the suppressor domain that is responsible for the difference in $InsP_3$ affinity between the different subtypes of the $InsP_3R$ (Iwai et al., 2007).

Nobody has yet been able to publish a high resolution structure for the whole $InsP_3R$. The $InsP_3$-binding core domain and the suppressor domain have been crystallized separately (Bosanac et al., 2002; Bosanac et al., 2005). Visualizing the $InsP_3R$ using electron microscopy revealed a reversible transition between two distinct structures with fourfold symmetry: a windmill structure and a square structure. It was further demonstrated that Ca^{2+} promotes the transition between the two structures (Hamada et al., 2002).

4 Na,K-ATPase

In 1997 Jens C. Skou was awarded the Nobel prize in chemistry for the discovery of the ion-transporting enzyme, Na,K-ATPase (Skou, 1957). The Na,K-ATPase belongs to the P-type ATPase group. The pump catalyzes active transport of cations across the cell membrane and maintains ionic gradients through hydrolysis of ATP. A significant fraction (up to around 30 %) of the ATP generated by cell metabolism is dedicated to this active transport process (Kaplan, 2002; Lingrel and Kuntzweiler, 1994; Sweadner, 1989). The minimal functional unit is a heterodimer of a catalytic α-subunit and a β-subunit that is required for protein folding. An auxiliary subunit, FXYD (also known as γ-subunit), modulates substrate affinity but is not required for enzymatic activity (Geering, 2006). The catalytic α-subunit of Na,K-ATPase exists in four isoforms encoded by different genes (Shamraj and Lingrel, 1994; Shull et al., 1986). All four isoforms have a high degree of amino acid identity, but differ in substrate affinity (Segall et al., 2001).

Three α-isoforms are found in the brain but are expressed in different cell types. The α1-isoform is ubiquitously expressed, whereas the α2-isoform is predominantly expressed in glia and the α3-isoform is expressed in neurons. Interestingly, the α2-isoform is found to be expressed in neurons during early development, suggesting a developmental role of the different isoforms of Na,K-ATPase (McGrail et al., 1991; Moseley et al., 2003).

4.1 CARDIOTONIC STEROIDS - OUABAIN

The Na,K-ATPase has an evolutionarily conserved binding site for cardiotonic steroids (CTS) and ligand binding to this site inhibits the pump activity. Digitalis Purpurea or commonly called Foxglove is the source of digoxin and has been used as a heart medicine to treat congestive heart failure for more than 200 years (figure 3). Ouabain, an analog of digoxin, has been found endogenously in the human body (Hamlyn et al., 1991; Kawamura et al., 1999). Endogenous ouabain is believed to be synthesized in the adrenal gland, although there are indications that it could be synthesized in the hypothalamus or in the heart (Schoner and Scheiner-Bobis, 2008).

Figure 3. Digitalis Purpurea - Foxglove.

It has been hypothesized that inhibition of the Na,K-ATPase by ouabain would lead to a decrease in the Na^+ gradient, lowering or even reversing the activity of the Na^+/Ca^{2+} exchanger and thus increasing intracellular Ca^{2+} levels. This would lead to an increase in the amount of Ca^{2+} ions available for contraction of the heart muscle and improves cardiac output (Zhang et al., 2005).

The evolutionary conserved CTS binding site of the Na,K-ATPase can be eliminated by mutating two amino acids in the extracellular loop between the first and the second transmembrane helixes (Price et al., 1990). In rodents each α-isoforms has a specific sensitivity to ouabain, for example the α1-isofrom is insensitive to ouabain. However, in humans the α1-isofrom is sensitive to ouabain. The functional consequences of the

differences in ouabain sensitivity among α-isoforms in rodents is hence not clear (Crambert et al., 2000).

Recently the CTS binding site of the Na,K-ATPase was shown to play a role in blood pressure regulation. Chronic administration of adrenocorticotropic hormone (ACTH) is known to cause elevated ouabain levels and hypertension in mice. When genetically engineered mice, expressing an ouabain insensitive α2-isoform, were challenged with ACTH they did not develop hypertension, but the treatment did induce elevated levels of ouabain (Dostanic et al., 2005). This finding indicates that the CTS binding plays an important role in blood pressure regulation and underlines the importance of ouabain as an endogenous hormone.

Recently agrin, a heparan sulphate proteoglycan, was found to bind specifically to the Na,K-ATPase α3-isorform inhibiting the pump activity at elevated concentrations (Hilgenberg et al., 2006). Agrin has previously been implicated in synapse formation and accumulation of acethylcholine receptor in the neuromuscular junction.

4.2 Na,K-ATPase AS A SIGNAL TRANSDUCER

In addition to its classical ion transporting function, Na,K-ATPase has more recently been shown to have a role as a signal transducer. Xie and colleagues have studied the signaling mechanisms and downstream effects of the Na,K-ATPase. Ouabain treatment induces activation of Src and stimulates tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) and activation of mitogen-activated protein kinase (MAP kinase) (Dmitrieva and Doris, 2003; Haas et al., 2002). In LLC-PK1 cells the Na,K-ATPase and Src colocalized in the plasma membrane.

Src binds to Na,K-ATPase constitutively and this binding inhibits the Src kinase activity. Addition of ouabain to the purified Na,K-ATPase/Src complex released the kinase domain and restored the Src activity (Liang et al., 2006; Tian et al., 2006). Recently Na,K-ATPase was shown to regulate membrane trafficking of caveolin-1. Knockdown of Na,K-ATPase decreases the plasma membrane pool of caveolin-1, which leads to a significant reduction in the number of caveolae on the cell surface (Cai et al., 2008).

Ouabain was shown to induce slow Ca^{2+} oscillations and activation of the transcription factor NF-κB in renal proximal tubule cells. The mechanism for the generation of the Ca^{2+} signal has been shown to involve a protein-protein interaction between Na,K-ATPase and $InsP_3R$ (Aizman et al., 2001; Aperia, 2007; Miyakawa-Naito et al., 2003; Zhang et al., 2006a). The adaptor protein ankyrin B has also been suggested to play a role in the interaction between Na,K-ATPase and $InsP_3R$ (Kline et al., 2008; Liu et al., 2008).

4.3 STRUCTURE OF THE Na,K-ATPase

The Na,K-ATPase transports three Na^+-ions out of the cell and in exchange takes two K^+-ions into the cell. The pump adopts two main conformations, known as E1 and E2. In the E1 state the ion binding sites are accessible to the cytoplasm and have a high affinity for Na^+-ions. The binding of three Na^+-ions promotes the ATP dependent phosphorylation of the pump at a highly conserved aspartate residue. The pump is then in the phosphorylated E1 state (E1P) and ADP remains associated. In the E1P state the bound ions are inaccessible from either side of the membrane. The pump then releases the ADP and relaxes to the E2P conformation. In

this confirmation the ion binding sites are then exposed to the extracellular environment and demonstrate a higher affinity for K^+-ions, allowing the escape of Na^+-ions. The binding of two K^+-ions triggers dephosphorylation of the pump, resulting in the E2 state where the ions are inaccessible from either side of the membrane. The pump then relaxes back to the E1 state and releases the K^+-ions to the cytoplasmic side.

The catalytic α-subunit consists of 1023 amino acids forming 10 transmembrane helices. In 2007 the crystal structure of the Na,K-ATPase was published (Morth et al., 2007). Previously structural conclusions were based on homology between Na,K-ATPase and the SERCA pump (Sweadner and Donnet, 2001; Toyoshima et al., 2000). The crystal structure of the Na,K-ATPase by Morth et al. is in the E2 confirmation with two Rb^+-ions bound, mimicking the binding of two K^+-ions (figure 4). As predicted by the homology analysis, the structural differences between SERCA and the α-subunit of the Na,K-ATPase are small. The presence of the β- and γ-subunits have little influence on the structure of the α-subunit of the Na,K-ATPase, when compared to the structure of SERCA pump which lacks β- and γ-subunits.

4.4 Na,K-ATPase AND GENETIC DISEASES

In 2003 came the first report that a mutation of the Na,K-ATPase was involved in a human genetic disease. Familial hemiplegic migraine is a hereditary form of migraine characterized by aura (a perceptual disturbance, usually visual) and some hemiparesis. It was shown that mutations in the gene encoding for Na,K-ATPase α2-isoform were associated with familial hemiplegic migraine type 2. The mutants had

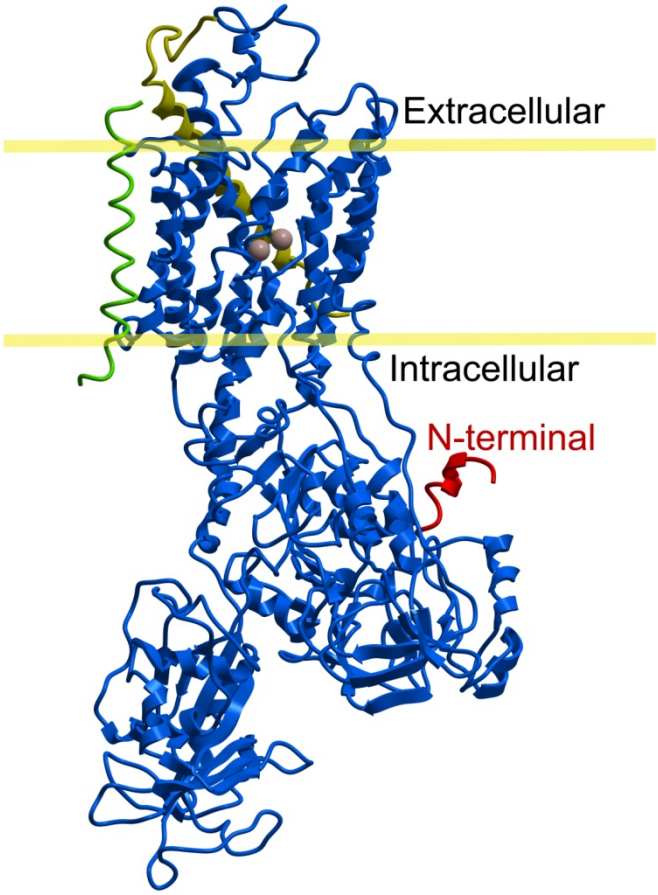

Figure 4. Crystal structure of the E2 confirmation of Na,K-ATPase. Two Rb^+-ions (mimicking K^+-ions) are seen in the middle of the membrane. β-subunit is shown in yellow, γ-subunit in green and the N-terminal tail in red (Morth et al., 2007).

proper folding and membrane insertion, but lacked pump activity (De Fusco et al., 2003).

Mutations in the gene encoding for Na,K-ATPase α3-isoform have been shown to be involved in Rapid-onset dystonia-parkinsonism. Six different mutations from seven unrelated families affected by the disease were studied. Rapid-onset dystonia-parkinsonism is an autosomal-dominant disease characterized by the sudden onset of dystonia and parkinsonism. Symptoms usually start in late adolescence or early adulthood. All six of the α3 mutants examined lacked functional pump activity, but it is not clear from this studied whether it was a result of protein misfolding or loss of enzymatic activity (de Carvalho Aguiar et al., 2004). In drosophila mutations of the catalytic α-subunit leads to neurodegeneration (Palladino et al., 2003).

5 AIMS

The overall aim of this thesis was to investigate molecular mechanisms and cellular consequences of Ca^{2+} signaling. The specific aims were:

- To investigate the signaling properties of the N-terminal tail of Na,K-ATPase and its role in ouabain induced Ca^{2+} oscillations.

- To explore the impact of Na,K-ATPase signaling for dendritic growth in embryonic cortical neurons.

- To test whether chemokine ligands and their receptors can regulate ventral midbrain development.

- To investigate molecular mechanisms and downstream effects of spontaneous Ca^{2+} activity in differentiating ES cells.

6 METHODOLOGICAL CONSIDERATIONS

This section covers some of the experimental methods used in this thesis. For specific descriptions of the materials and methods, see Paper I-IV.

6.1 CELL IMAGING

Different microscopy techniques based on fluorescence are widely used in medical and biological sciences. The basic concept of fluorescence is that some molecules absorb light at a particular wavelength and subsequently emit light of longer wavelength from electronically excited states. The shift in wavelength between absorption and emission, due to loss of vibrational energy, is termed Stokes shift.

Dr. Roger Tsien has greatly contributed to the field of cell imaging and was awarded the Nobel Prize in Chemistry 2008 for his work on the green fluorescent protein (GFP). His laboratory has also developed Ca^{2+} indicators, such as Fura2 (the article describing Fura-2 has been cited over 17,000 times) (Grynkiewicz et al., 1985). Ca^{2+} indicators change their spectral properties upon Ca^{2+} binding, as shown in figure 5. By modifying carboxylic acids with acetoxymethyl (AM) ester groups, the molecule becomes uncharged and can permeate cell membranes. Once inside the cell, the lipophilic groups are cleaved by nonspecific esterases, resulting in a charged molecule that is less prone to leak out of cells. When cells have been loaded with an AM Ca^{2+} indicator, intracellular Ca^{2+} can be measured simultaneously in hundreds of cells for several hours.

The Ca^{2+} indicators used in this thesis are Fura2/AM and Fluo3/AM. Fura2/AM is a ratiometric dye, excited at 340 and 380 nm, and is hence less sensitive to focus shifts and bleaching. With Fura2/AM it is possible to estimate the absolute Ca^{2+} concentration by performing a calibration using Ca^{2+} ionophores. Since Fura2/AM is excited with UV-light it can be

combined with many other dyes and fluorescent proteins which are excited with visual light. However, excessive UV-light exposure is toxic to many cell types and Fluo3/AM (excitation maximum around 506 nm) can thus be a good alternative. Ca^{2+} measurements with Fluo3/AM can be performed faster, compared to Fura2/AM, since only one wavelength is needs to be acquired for each image.

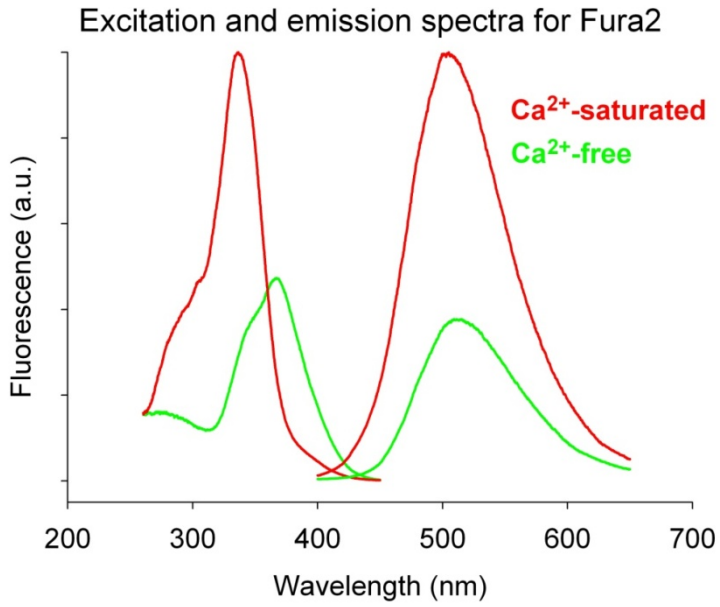

Figure 5. Excitation (left) and emission (right) spectra for Fura2. Ca^{2+}-saturated in red and Ca^{2+}-free in green. Excitation spectra detected at 510 nm and emission spectra was excited at 340 nm.

Ca^{2+} imaging data was recorded with custom built fluorescence microscopes equipped with cooled CCD cameras. Two different systems were used, one equipped with a filter wheel as a light sources and another one with a monochromator. After conducting a Ca^{2+} imaging experiment the collected data consists of multiple digital images. In these images individual cells can be identified and changes in fluorescent correspond to changes in intracellular Ca^{2+} concentrations. From the traces of each cell different data can be extracted, such as percentage of cells responding to a specific treatment, peak amplitude or full duration at half maximum. Other Analysis methods for extraction of data from Ca^{2+} recordings will be further discussed below.

Laser scanning confocal microscopy uses a pinhole to eliminate out-of-focus light and can thus be used to construct a 3D image of a specimen. In the past 15 years confocal microscopy has evolved from custom built systems to mass-produced instruments used in thousands of laboratories all over the world. The strong progress in this field is mainly due to development in computers and electronics, as well as high demand from customers. In this thesis confocal microscopy was used for all different kinds of cell imaging, such as fluorescent recovery after photobleaching (FRAP), immunocytochemistry and Ca^{2+} imaging. Since confocal microscopy is such a well know technique it will not be discussed any further in this thesis.

6.2 CROSS-CORRELATION

Cross-correlation is a measure of similarity of two signals as a function of a time-lag applied to one of them. The cross-correlation for two discrete functions *f* and *g* is defined by:

$$\xi_{fg}[m] = \sum_{n=1}^{N} f[n]g^{*}[n+m]$$

In Paper IV we used cross-correlation analysis to determine if the detected spontaneous Ca^{2+} activity in each cell was independent or correlated within clusters of cells. A custom MATLAB program was written for the pair-wise comparison of all cells in each experiment. These comparisons can be time consuming for large data sets, for example an experiment of n = 250 cells will result in $n^2 - n = 62{,}250$ pair-wise comparisons.

Data was prepared by extracting single cell traces from the Ca^{2+} recordings and followed by binarization of the data. Binarization was performed by assigning the onset of each Ca^{2+} transient, exceeding a certain threshold, to 1 and set all other data points to zero (figure 6). With this binarization method all peaks are given the same weight independent of duration and amplitude.

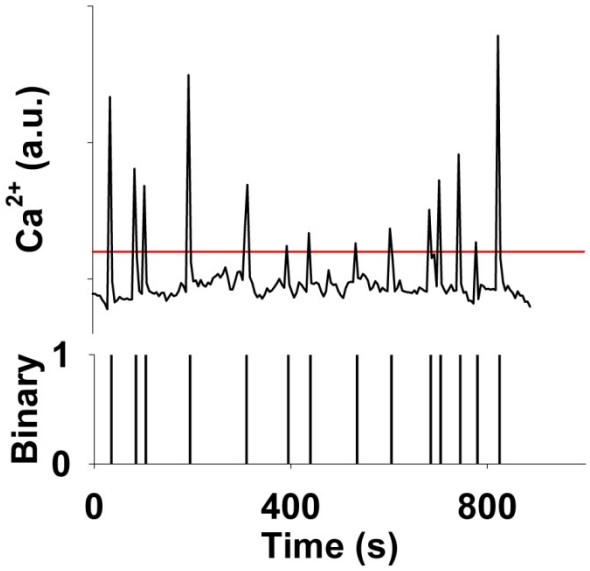

Figure 6. Binarization of a calcium trace from a single cell. The leading edges of all transients exceeding a threshold (horizontal line) are assigned to 1.

All binarized cell traces in one experiment were compared pair-wise and the cross-correlation coefficients were calculated with the MATLAB function *xcorr*. To compare correlation coefficients between cells and across separate experiments we normalized the coefficients with the root mean square of the number of peaks. Then the normalized correlation coefficient Ψ with zero lag time was calculated, according to:

$$\Psi_{fg} = \frac{\xi_{fg}[0]}{\sqrt{\frac{(\sum_{n=1}^{N} f[n])^2 + (\sum_{n=1}^{N} g[n])^2}{2}}}$$

The normalized cross-correlation coefficient has a value between 0 and 1, representing none or 100 % concurrent peaks respectively. This method

finds cells with many concurrent peaks and thus highly correlated Ca^{2+} traces. If two cells have a significantly higher correlation compared to two random signals, they are probably connected in a cellular network.
At a sampling frequency of around 1 Hz we are unable to determine the mechanisms of the Ca^{2+} activity. We simply see pairs of cells as active at the same time, but are unable to conclude if one cell is triggering the other. Nevertheless, this is a good tool to simultaneously examine hundreds of cells and determine if they are interconnected in subnetworks.
Here we calculated the cross-correlation coefficient at zero lag and only the leading edges of the transients are taken into account. Instead a certain lag of the signals could be allowed or each peak could be represented by its total duration.

6.3 SPECTRAL ANALYSIS

Ca^{2+} signals can be encoded in amplitude and frequency. Spectral analysis can be a useful tool to extract the underlying frequencies of a complex signal. If a signal only contains one frequency, it is fairly simple to analyze. However, signals containing more than one frequency can be impossible to analyze manually. Biological signals, such as Ca^{2+}, usually consist of many signals superimposed on each other.
A MATLAB program was used to analyze oscillation in Ca^{2+} recordings (Uhlen, 2004). The Ca^{2+} traces are transformed to the frequency domain by using Fourier transform, which is defined as:

$$F(\omega) = \int_{-\infty}^{\infty} f(t)\, e^{-i\omega t} dt$$

In this way a complicated periodic function is written as the sum of simple waves mathematically represented by sines and cosines and the dominant frequency can be determined.
It is important to have a high enough sampling rate when recording Ca^{2+} oscillations. The Nyquist rate is the minimum sampling rate required to avoid aliasing and is defined as twice the highest frequency contained within the signal. This means that if we are doing a Ca^{2+} recording at a rate of one image every second (1 Hz), the highest frequency component we find when using spectral analysis is 0.5 Hz.

In summary, the data usually extracted from Ca^{2+} recordings are percentage of responding cells, full duration at half maximum, amplitude, up-slope and down-slope of a transient. Here I have discussed two other methods to analyze Ca^{2+} data, cross-correlation and spectral analysis. Cross-correlation can be used to find similarities between cells and to analyze networks of cells. Spectral analysis is used to determine how the frequency of Ca^{2+} oscillations changes after specific treatments or to extract underlying frequencies from superimposed signals.

7 RESULTS AND DISCUSSION

7.1 PAPER I

The aim of this study was to elucidate the mechanism of slow Ca^{2+} oscillations triggered by ouabain and to get a more detailed understanding of the functions of Na,K-ATPase as a signal transducer. It has previously been shown that ouabain can induce Ca^{2+} oscillations in renal proximal tubule cells and that this leads to activation of the transcription factor NF-κB (Aizman et al., 2001). It has further been demonstrated that Ca^{2+} oscillations depend on the interaction between Na,K-ATPase and $InsP_3R$ (Aperia, 2007; Miyakawa-Naito et al., 2003).
In this study we report that the N-terminal tail of the α-subunit of the Na,K-ATPase binds to the N-terminus of the $InsP_3R$. This interaction was shown to modulate the Ca^{2+} oscillatory signal.

To screen for an interaction between the Na,K-ATPase and the $InsP_3R$, we incubated glutathione S-transferase (GST) fused proteins encoding for various lengths of the N-terminus of the $InsP_3R$ type 1 with lysates from whole rat brain or with lysates from COS-7 cells transfected with GFP-fused Na,K-ATPase α-subunits. An interaction was found between the Na,K-ATPase α1-subunit and the N-terminus of the $InsP_3R$, residues 1–604. This fragment of $InsP_3R$ type 1 consists of the suppressor domain and the $InsP_3$-binding domain and shares high homology with other subtypes of $InsP_3R$.

Various lengths of the N-terminus of $InsP_3R$ were tried, $InsP_3R$ (1–604) and $InsP_3R$ (1–343) were found to interact with Na,K-ATPase, whereas $InsP_3R$ (1–225) (suppressor domain) and $InsP_3R$ (226–604) ($InsP_3$-binding domain) did not interact. This is interesting and gives us two hypotheses:

(1) the first 118 residues of the $InsP_3$-binding domain together with a part of the suppressor domain are important for the binding to occur or (2) the first 118 residues of the $InsP_3$-binding domain changes the folding of the suppressor domain so that the binding can occur. The first hypothesis suggests that the amino acids important for the binding with Na,K-ATPase are situated in both the suppressor domain and the $InsP_3$-binding domain. The second hypothesis would imply that the two crystallized structures by Bosanac et al., the $InsP_3$-binding domain and the suppressor domain, actually changes conformation when put together, exposing residues important for protein-protein interaction (Bosanac et al., 2002; Bosanac et al., 2005).

We next demonstrated that the N-terminus of $InsP_3R$ binds to the N-terminal tail of Na,K-ATPase. By taking away the first 32 amino acids of the N-terminal of the Na,K-ATPase, binding to $InsP_3R$(1–604) was inhibited, but the pump activity was not altered. In contrast, a peptide of the first 32 amino acids of the Na,K-ATPase, named α-subunit N-terminal tail (αNT-t), was sufficient for the binding with $InsP_3R$(1–604) to occur.

These experiments demonstrated that the αNT-t, consisting of 32 amino acids, did bind to the $InsP_3R$(1-604). To further narrow down the residues important for the binding of αNT-t to $InsP_3R$(1-604) the binding of smaller versions of αNT-t were tested. The last amino acids of αNT-t are LKK (one leucine and two lysine) and were found to be conserved in all species examined. Interestingly, when these three amino acids were cut off from αNT-t, resulting in αNT-tΔLKK, the binding to $InsP_3R$(1-604) was abolished. This was shown not only for the rat α1-subunit, but also for human α1 and rat α3. Furthermore, it was also shown that the binding was

direct, not mediated through adaptor proteins, by determining that purified $InsP_3R$(1-604) associated with purified αNT-t.

We then characterized the ouabain induced Ca^{2+} oscillations in COS-7 cells. The frequency of the oscillations was analyzed by spectral analysis. The period was found to be 3.9 ± 0.2 and 3.3 ± 0.1 min for 200 nM and 1 μM ouabain respectively. Significantly less Ca^{2+} oscillation was observed when overexpressing αNT-t compared to cells on the same plate not expressing the peptide. Whereas the peptide lacking the three amino acids LKK, αNT-tΔLKK, did not differ from the control cells. These results suggests that by disturbing the binding between Na,K-ATPase and $InsP_3R$ less cells respond with Ca^{2+} oscillations. Less Ca^{2+} oscillation was also observed when overexpressing a truncated form of Na,K-ATPase, where the first 32 amino acids had been cut off. This truncated version of Na,K-ATPase lacks the ability to bind to $InsP_3R$ and overexpression of it would thus make less endogenous Na,K-ATPase available for binding to $InsP_3R$.
Expression of the peptide αNT-t did not change the cellular Ca^{2+} homeostasis, since intracellular Ca^{2+} response to thapsigargin (SERCA pump blocker) and bradykinin (trigger of $InsP_3$ production) was not altered.

It has been shown that a low dose of ouabain protects from serum deprivation-triggered apoptosis and stimulates cell proliferation via activation of NF-κB (Li et al., 2006). Here we showed that overexpression of αNT-t abolished the ouabain induced protection from apoptosis. Conversely, the peptide lacking the three amino acids LKK, αNT-tΔLKK, did not differ from control cells.

7.2 PAPER II

The establishment of dendrites is a dynamic process that is characterized by extension and retraction of branches, followed by stabilization and growth. Dendrites serve a critical role in detecting and processing incoming information.

In this study we report that signal transduction activated by the Na,K-ATPase modulates dendritogenesis in developing cortical neurons from rat. This process involves activity of the transcription factor CREB, MAP kinase phosphorylation, CaM kinases activity as well as intracellular Ca^{2+} signaling. These data provide evidence for a novel signaling pathway by which the Na,K-ATPase stimulate dendritic growth in the developing brain and may thus play an important role in circuits wiring.

In addition to its central role in establishing the electrochemical gradient, the Na,K-ATPase has recently been reported to function as a receptor and signaling mediator (Aperia, 2007; Xie and Askari, 2002).

Both the length and number of dendrites significantly increased when rat cortical neurons were exposed to 1μM ouabain. The dendritic lengths were extended almost two-fold and the number of dendrites increased by ~100 % following treatment of ouabain for 48 h. Whole-cell patch-clamp recordings demonstrated that this concentration of ouabain had no significant effect on the resting membrane potential of cortical neurons. Ca^{2+} signaling is known to regulate dendritic growth through activation of a transcriptional program (Konur and Ghosh, 2005). Ca^{2+} oscillations were observed in cortical neurons challenged with 1μM ouabain for 6 h and were suppressed by the L-type Ca^{2+} channel blocker nifedipine.

In neurons, MAP kinases have been shown to regulate dendritic growth and morphology (Wu et al., 2001). Ouabain treatment was found to activate MAP kinase in cortical neurons, demonstrated by monitoring ERK1/2 (extracellular signal-regulated kinases) phosphorylation using Western blotting. Cortical neurons exposed to ouabain for 6 h, showed a sustained ERK1/2 phosphorylation, which was abolished using the selective MEK inhibitor U0126. Nifedipine (L-type Ca^{2+} channel blocker) was used to determine the effect of Ca^{2+} signaling on ERK1/2 phosphorylation. Cortical neurons treated with nifedipine exhibited a reduced level ouabain-induced ERK1/2 phosphorylation.

The transcription factor CREB has been implicated in neuronal differentiation where it is strongly regulated via CaM kinases (Redmond et al., 2002). Ouabain was shown to activate CREB after 6 h of treatment, using an antibody specific for Ser133-phosphorylated CREB both by immunocytochemistry and Western blotting. Phosphorylation of Ser133 is required for inducing CREB-dependent transcription, however it may not be sufficient for target gene activation (Shaywitz and Greenberg, 1999). We hence transfected a CRE-luciferase reporter gene which is activated by endogenous CREB. A significant 3-fold increase in CRE-luciferase activity was observed when cells were exposed to ouabain for 6 h. The CaM kinases inhibitor KN93 blocked CRE-luciferase activity, whereas nifedipine and U0126 did not.

We then examined the impact of Ca^{2+} signaling, MAP kinases and CaM kinases on Na,KATPase signal transduced dendritic growth in cortical neurons. The inhibitors nifedipine (Ca^{2+} signaling), U0126 (MAP kinases) and KN93 (CaM kinases) all decreased ouabain-induced dendrite outgrowth.

Our results altogether suggest that Na,K-ATPase receptor activation leads to CREB activation and dendritic growth via crosstalk between the MAP kinase and CaM kinase pathways.

7.3 PAPER III

Chemokines (chemotactic cytokines) constitute a family of small protein ligands that are expressed by all major cell types in the central nervous system (Bajetto et al., 2001). The chemokine receptors are seven transmembrane G protein-coupled receptors and are known to trigger release of intracellular Ca^{2+}, mediated through PLC cleavage of PIP_2 into $InsP_3$ and DAG.

In this study we examine how the chemokine ligands CXCL1, CXCL6 and CXCL8, and their receptors, CXCR1 and CXCR2, regulate ventral midbrain development.

We first examined the function of two chemokine ligands CXCL6 and CXCL8, and their receptors, CXCR1 and CXCR2, in the developing rat ventral midbrain. mRNA for CXCR2 and CXCL6, but not CXCR1, was found to be expressed in the developing rat ventral midbrain by using quantitative-PCR.

Both CXCL6 and CXCL8 increased the number of Nurr1+ and TH+ cells in a dose-dependent manner. CXCL6, but not CXCL8, increased the proportion of TH+ neurons in the Nurr1 population, suggesting that CXCL6 has a predominant effect on the conversion of dopaminergic precursors into dopaminergic neurons. Pulse chase experiments with BrdU treatment and immunocytochemistry for TH showed that CXCL8, but not CXCL6, induced an increase in newborn TH+ cells. These results indicate that CXCL8 promotes the neurogenic division of progenitors and that

CXCL6 promotes the differentiation of Nurr+ precursors into dopaminergic neurons.

Next we examined CXCL1, the murine ortholog to the human CXCL8, which also binds both CXCR1 and CXCR2. Quantitative -PCR analysis confirmed that CXCL1 is highly expressed in the ventral midbrain between E11.5 and E14.5 in mouse. The number of cells incorporating BrdU and the number of TH+ cells were increased by CXCL1treatment. Moreover, we found that CXCL1 exerts a selective effect on TH+ neurons, since it did not increase the overall number of neurons.
We carried out intracellular Ca^{2+} measurements to validate that CXCL1, CXCL6, and CXCL8 enhanced the number of functional dopaminergic neurons. The glutamate receptor agonist AMPA is known to evoke a robust intracellular Ca^{2+} increase in neurons. After Ca^{2+} imaging, the cells were immunostained for TH and back-traced/correlated to their respective Ca^{2+} response to AMPA. A similar transient Ca^{2+} increase in response to AMPA was observed in TH+ cells induced by CXCL1, CXCL6, CXCL8, or control treatments.
Our study supports the idea that chemokines regulate ventral midbrain development. In this context, our report identifies CXCL1, CXCL6, and CXCL8 as novel regulators of distinct aspects of dopaminergic development, including proliferation, neurogenesis and differentiation.

7.4 PAPER IV

As discussed previously in this thesis, Ca^{2+} has been shown to be important for neural development (Berridge et al., 2003; Spitzer, 2006). In this study we examine spontaneous Ca^{2+} activity in mouse ES cell-derived neural progenitors and its role in proliferation. Mouse ES cells

were plated on PA6 stromal cells and differentiated towards neural cells and dopaminergic neurons. After 8 days in culture, cell colonies started to express the neuronal markers TuJ1 and TH, expressed by dopaminergic neurons. Cells were loaded with Ca^{2+} sensitive dye and subjected to time-laps recordings to investigate the intracellular Ca^{2+} homeostasis in neural progenitors. Spontaneous Ca^{2+} activity was observed between 7 to 14 days of ES cells differentiated in neural culture conditions.
To determine if the detected spontaneous Ca^{2+} activity in each cell was independent or if neighboring cells were connected, we performed cross-correlation analysis. Cross-correlation analysis is a well-established mathematical method and statistical tool to analyze multiple time series. Cross-correlation analysis of Ca^{2+} traces from undifferentiated ES cells showed low correlation between neighboring cells, whereas neural progenitors seemed to connect to neighboring cells forming local cell networks.

To elucidate the source of Ca^{2+} and the signaling mechanism in the neural progenitors we challenged the cells with a wide range of pharmacological blockers. ATP receptor antagonists (suramin and PPADS) had no effect on observed spontaneous Ca^{2+} activity, nor did hexokinase (consumption of extracellular ATP). Depletion of the intracellular stores with the SERCA pump blocker cyclopiazonic acid also failed to inhibit the Ca^{2+} activity. In addition, blocking synaptic transmission (TTX) and blocking neurotransmitter receptors for NMDA and AMPA (D-AP5 and NBQX) had no effect on the observed spontaneous Ca^{2+} activity.
When neural progenitors were subjected to Ca^{2+}-free extracellular medium virtually all spontaneous Ca^{2+} activity was inhibited and the activity reappeared when Ca^{2+} was added back to the medium.

The gap junction blockers octanol and flufenamic acid effectively inhibited most spontaneous Ca^{2+} activity. The importance of extracellular Ca^{2+} was confirmed and further strengthened by exposing the neural progenitors to Ni^{2+}, which blocks voltage-dependent Ca^{2+} channels in the cell plasma membrane.

We then wanted to examine if functional gap junctions are expressed in neural progenitors. Immunostaining for Connexin43, a major gap junction protein, showed expression in cell colonies with neural progenitors positive for Nestin and Doublecortin. We then used FRAP to assess if the gap junctions were functional. Cells were loaded with the dye 6-CFDA and the dye was then photo-bleached in a single cell with a laser scanning confocal microscope. Following the bleaching the recovery of the fluorescence was imaged. The recovery is mainly by means of influx of fluorescent molecules via gap junction channels connected to adjacent cells. Cells treated with the gap junction blocker octanol did not recover to the same extent as control cells. Together this indicates that functional gap junctions are expressed in neural progenitors and that octanol is acting as a gap junction blocker.

Intracellular Ca^{2+} signaling has previously been implicated in cell cycle transition between the G1 and S phase. It has been suggested that elevated Ca^{2+} may push G1 cells towards S phase rather than exiting from the cell cycle (G0) (Weissman et al., 2004).

We examined whether inhibition of the spontaneous Ca^{2+} activity in neural progenitors would influence cell proliferation. Using BrdU staining, which reflects S phase entries, we monitored DNA synthesis in neural progenitors with a single 1 hour pulse of BrdU. Both octanol and Ni^{2+} significantly lowered the number of BrdU positive cells when compared to control cells.

We next did equivalent BrdU experiments for undifferentiated ES cells. The undifferentiated ES cells do not express voltage-dependent Ca^{2+} channels and gap junction blockers do not inhibit Ca^{2+} signaling. The number of BrdU positive cells was not affected with octanol or Ni^{2+} treatment. These experiments indicate that spontaneous Ca^{2+} activity enhances proliferation of ES cell-derived neural progenitors.

In this study we demonstrate that spontaneous Ca^{2+} activity is present in neural progenitors derived from mouse ES cells. This Ca^{2+} activity is dependent on influx of extracellular Ca^{2+} through plasma membrane channels, since removal of extracellular Ca^{2+} from the medium and exposure to Ni^{2+} block the signaling event. Depletion of ER Ca^{2+} stores had no effect suggesting that intracellular Ca^{2+} release do not significantly contribute to this signaling event. We further show that neural progenitor cells are connected in sub-networks with highly synchronized spontaneous Ca^{2+} activity and that gap junction blockers significantly inhibit the activity in virtually all cells. By inhibiting the spontaneous Ca^{2+} activity we decreased the entry of cells into S phase, suggesting that Ca^{2+} signaling promote neural progenitor proliferation.

We hypothesized that membrane depolarization could be involved in spreading the Ca^{2+} activity through gap junctions. A depolarizing current could travel between cells through gap junctions leading to activation of voltage-dependent Ca^{2+} channels. The spontaneous Ca^{2+} oscillations are likely to be related to membrane depolarization, since the observed activity appears approximately at the same time point in differentiation as when these cells start to respond to depolarization treatment with high KCl. When cells are exposed to 50 mM KCl a rapid and distinct Ca^{2+} increase is observed which show that these cells express voltage-dependent Ca^{2+} channels. When studying spontaneous Ca^{2+} oscillations in neural

progenitor cells we observe that the Ca^{2+} activity is inhibited both by zero extracellular Ca^{2+} and gap junction blockers. Together these data suggest that Ca^{2+} enters the cell through Ca^{2+} permeable channels in the plasma membrane.

8 CONCLUSIONS

The studies in this thesis present new insight in the mechanism of Ca^{2+} signals and their down-stream effects.

We identify a conserved Na,K-ATPase-motif important for the N-terminal signaling activity. This motif is necessary for the interaction of the Na,K-ATPase with the N terminus of $InsP_3R$ and important for ouabain-induced Ca^{2+} oscillations. The role of Na,K-ATPase signaling was further examined in embryonic cortical rat neurons. We found that the endogenous hormone ouabain, a ligand of the Na,K-ATPase, triggers dendritic growth as well as a transcriptional program dependent on CREB and CRE-mediated gene expression, primarily regulated via CaM kinases.

We showed that the chemokine ligands CXCL1, CXCL6 and CXCL8 regulate ventral midbrain development, enhancing TH+ cell numbers in rodent precursor and neurosphere cultures. TH+ cells, induced by chemokine treatment, were demonstrated to have a functional Ca^{2+} homeostasis.

We found that spontaneous Ca^{2+} activity is present in neural progenitors derived from mouse ES cells. This Ca^{2+} activity is dependent on influx of extracellular Ca^{2+}. We further showed that neural progenitor cells are connected in sub-networks with highly synchronized spontaneous Ca^{2+} activity and that gap junction blockers significantly inhibit the activity. By inhibiting the spontaneous Ca^{2+} activity we decreased the percentage of dividing cells, suggesting that Ca^{2+} signaling promote neural progenitor proliferation.

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