The interplay of JNK and SCG10 during cortical development and in excitotoxic responses in brain

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ISBN 978-952-12-3114-8
Painosalama Oy-Turku

"Nothing shocks me, I'm a scientist"
Indiana Jones

To my loved ones

Table of Contents

ABSTRACT

Initially identified as stress activated protein kinases (SAPKs), the c-Jun N-terminal kinases (JNKs) are currently accepted as potent regulators of various physiologically important cellular events. Named after their competence to phosphorylate transcription factor c-Jun in response to UV-treatment, JNKs play a key role in cell proliferation, cell death or cell migration. Interestingly, these functions are crucial for proper brain formation. The family consists of three JNK isoforms, JNK1, JNK2 and JNK3. Unlike brain specific JNK3 isoform, JNK1 and JNK2 are ubiquitously expressed. It is estimated that ten splice variants exist. However, the detailed cellular functions of these remain undetermined. In addition, physiological conditions keep the activities of JNK2 and JNK3 low in comparison with JNK1, whereas cellular stress raises the activity of these isoforms dramatically. Importantly, JNK1 activity is constitutively high in neurons, yet it does not stimulate cell death. This suggests a valuable role for JNK1 in brain development, but also as an important mediator of cell wellbeing.

The aim of this thesis was to characterize the functional relationship between JNK1 and SCG10. We found that SCG10 is a *bona fide* target for JNK. By employing differential centrifugation we showed that SCG10 co-localized with active JNK, MKK7 and JIP1 in a fraction containing endosomes and Golgi vesicles. Investigation of JNK knockout tissues using phosphospecific antibodies recognizing JNK-specific phosphorylation sites on SCG10 (Ser 62/Ser 73) showed that phosphorylation of endogenous SCG10 was dramatically decreased in *Jnk1-/-* brains. Moreover, we found that JNK and SCG10 co-express during early embryonic days in brain regions that undergo extensive neuronal migration. Our study revealed that selective inhibition of JNK in the cytoplasm significantly increased both the frequency of exit from the multipolar stage and radial migration rate. However, as a consequence, it led to ill-defined cellular organization. Furthermore, we found that multipolar exit and radial migration in *Jnk1* deficient mice can be connected to changes in phosphorylation state of SCG10. Also, the expression of a pseudo-phosphorylated mutant form of SCG10, mimicking the JNK1-phopshorylated form, brings migration rate back to normal in *Jnk1* knockout mouse embryos. Furthermore, we investigated the role of SCG10 and JNK in regulation of Golgi apparatus (GA) biogenesis and whether pathological JNK action could be discernible by its deregulation. We found that SCG10 maintains GA integrity as with the absence of SCG10 neurons present more

compact fragmented GA structure, as shown by the knockdown approach. Interestingly, neurons isolated from *Jnk1*-/- mice show similar characteristics. Block of ER to GA is believed to be involved in development of Parkinson's disease. Hence, by using a pharmacological approach (Brefeldin A treatment), we showed that GA recovery is delayed upon removal of the drug in *Jnk1*-/- neurons to an extent similar to the shRNA SCG10-treated cells. Finally, we investigated the role of the JNK1-SCG10 duo in the maintenance of GA biogenesis following excitotoxic insult. Although the GA underwent fragmentation in response to NMDA treatment, we observed a substantial delay in GA disintegration in neurons lacking either JNK1 or SCG10.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and manuscript, which are referred to in the text by their Roman numerical. The original publications have been reproduced with the permission of the copyright holders.

I. Tararuk T., Ostman N., Li W., Bjorkblom B., Padzik A., **Zdrojewska J.,** Hongisto V., Herdegen T., Konopka W., Courtney M.J., Coffey E.T.: JNK1 phosphorylation of SCG10 determines microtubule dynamics and axodendritic length. (2006) Journal of Cell Biology 173, 265–277.

II. Westerlund N., **Zdrojewska J*.**, Padzik A., Komulainen E., Bjorkblom B., Rannikko E., Tararuk T., Garcia-Frigola C., Sandholm J., Nguyen L., Kallunki T., Courtney M.J., Coffey E.T.: SCG10/Stathmin-2 phosphorylation determines neuronal exit from multipolar stage and subsequent migration speed. (2011) Nature Neuroscience 14, 305–313
* shared first authorship

III. **Zdrojewska J.,** Malm T., Koistinaho J, Pitkänen A, Coffey E.T.: JNK and SCG10 cooperate to regulate Golgi biogenesis and breakdown in response to excitotoxic stress. (Manuscript)

ABREVIATIONS

AD	Alzheimer's disease
AG	Golgi apparatus
ALS	amyotrophic lateral sclerosis
AP-1	activator protein -1
APP	amyloid precursor protein
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BBB	blood brain barrier
BSA	bovine serum albumin
ERK	extracellular signal regulated protein kinase
CNS	central nervous system
CP	cortical plate
DCX	doublecortin
DIV	days *in vitro*
DJNK1	D-*retroinverso* peptide inhibitor of JNK kinase
DLK	leucine zipper- bearing kinase
E15.5	embryonic day 15.5
ER	endoplasmic reticulum
FCS	fetal calf serum
GABA	γ-aminobutyric acid
HMW	high molecular weight
INM	interkinetic nuclear migration
JBD	JNK binding protein
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
LMW	low molecular weight
MAP2	microtubule associated protein 2
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MAPs	microtubule associated proteins
MCAO	middle cerebral artery occlusion
MEK	MAP/ERK kinase
MGE	medial ganglionic eminence
MLK	mixed lineage kinase

MT	microtubule
MTOC	microtubule organizing center
MZ	molecular zone (Cajal-Retzius cells)
ND	neurodegenerative diseases
NLS	nuclear localizing signal
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NRS	neural restrictive silencer
NRSF	neural restrictive silencer factor
NTD	neural tube defect
p-JNK	phosphorylated form of JNK
PFA	paraformaldehyde
PID	peri-infarct depolarization
PNS	peripheral nervous system
PVE	pseudostratified ventricular epithelium
SCG10	superior cervical ganglion 10
SCLIP	SCG10-like protein
shRNA	short hairpin RNA
siRNA	small interfering RNA
STMN	stathmin
SVZ	subventricular zone
VZ	ventricular zone

INTRODUCTION

The fundamental cellular unit of our nervous system is a neuron. It consists of soma, many thin extensions termed dendrites and an "output" neurite called an axon. In the brain, we have roughly 100 billion neurons, which provide approximately 100 trillion interneuronal connections. In addition, we have several times as many supporting cells. This is why the brain, in its complexity, is one of the most spectacular biological creations. Often it is described as the last frontier of biology. Neurons are significantly larger than non-neuronal cells. Along with size, their distinctive cell shape gives rise to particular requirements for neuronal cells. They strongly rely on protein and organelles transport from cell body to the axon and dendrites which makes them unique compared to other cell types. The Golgi apparatus and centrosome placement marks the destiny of proximal neurites. The extension of neurites, in turn, requires the continuous flow of membranes and microtubules. The Golgi and centrosome serve as the first source of those building blocks. In addition, they play a crucial role in the delivery of axonal and dendrite destined molecules.

Modern neuroscience faces the difficult task of finding answers to a wide array of questions ranging from the development of the nervous system, its organization, and the development of behavior. With this comes the challenge of combining these pieces of diverse knowledge to produce a clear understanding of the brain structure, but also most importantly, its function.

The family of stress-activated protein kinases (SAPKs/ JNKs) has already previously been shown to be involved in various life-threatening diseases like heart disorders, diabetes and stroke. Moreover, JNKs are key players in neural tube closure, differentiation of nerve cells or are otherwise involved in the mechanisms controlling cell death. Current reports also indicate their role in various neurodegenerative processes including Alzheimer or Parkinson's diseases, as well as some mental disorders like schizophrenia. Future normal brain function requires proper neuronal positioning during development. Cellular migration, which is a universal mechanism of development, promotes cell interaction by bringing cells together, in addition to movement. Misplacement of neurons ultimately leads to various neurological diseases, including epilepsy, lissencephaly and schizophrenia, but also to less severe disorders like dyslexia. Therefore, identification of key players involved in neuronal placement is crucial for future well-being. Along with various

modern molecular and cell biology techniques, the animal model of *Jnk* knockout mice serves as an invaluable source of information, allowing for better understanding of numerous pathologies. Without proper understanding of deep molecular processes taking place inside neurons, we may not be able to tackle the nature of the investigated problem correctly. The studies presented in this thesis shed new light on JNK1 function in the brain along with its interacting protein Superior Cervical Ganglion 10 (SCG10). A better interpretation of the JNK1-SCG10 interplay will improve our understanding of the importance of physiologically active JNK in neurons.

REVIEW OF LITERATURE

1. Introduction to brain development

1.1 Establishing cell polarity

When considering the brain we think of electrical signals that allow information to pass from one cell to another. However, the very early events that occur during brain development take place before synapses are formed and are mostly free from electrical activity. Complex brain architecture is a result of genetic instructions, ongoing signaling between cells, and finally communication between the growing child and the outside world. Events like the formation of the primordial nervous system in the embryo, the appearance of newly generated neurons from undifferentiated precursors, the establishment of key brain regions and finally, the proper neuronal migration precede development of axonal pathways and formation of synaptic connections. Disturbance of any of those processes by genetic mutations, drugs, or chemical abuse may lead to dramatic consequences (reviewed by Purves et al., 2008).

In vertebrates the initial distinction of nervous system appears in parallel with the formation of basic body axes. During a process called gastrulation, the endoderm, mesoderm and ectoderm that form the primitive germ layers are developing and being arranged. The ectoderm that forms the most outer layer gives rise to the nervous system, but also the skin and other external tissues (Solnica-Krezel, 2005).

Shortly after the neural tube is formed, four major brain areas become outlined: the prosencephalon, mesencephalon, rhombencephalon as well as the future spinal cord, which originates from the most posterior part of the neural tube. Once the first brain regions are formed they undergo further partitioning giving rise to future adult brain structures, which is briefly depicted in Figure 1.

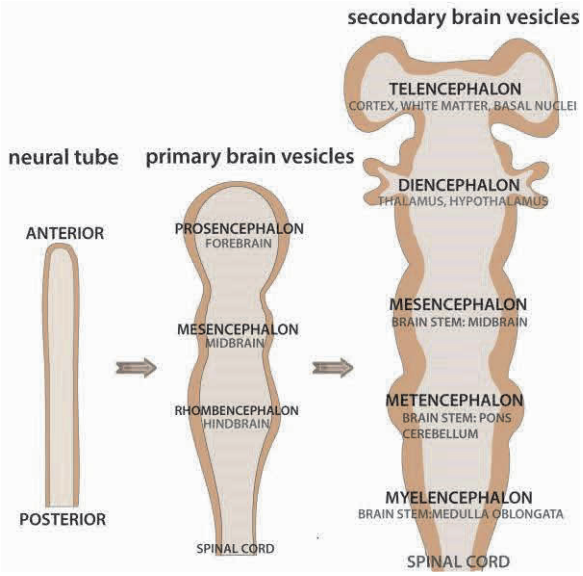


Figure 1. **A longitudinal section of the neural tube development**. At first, the neural tube segments into three areas: prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain). The future spinal cord originates from the more posterior part of the neural tube. Further on, the prosencephalon gives rise to telencephalon and diencephalon, which will form the cortex, white matter, basal nuclei as well as the thalamus and hypothalamus. The rhombencephalon subdivides into the metencephalon and myelencephalon. Those regions will form pons, cerebellum and medulla oblongata in the adult brain. (Modified from Purves et al., 2008)

The gross number of neurons is produced between weeks 12 and 18 of gestation with a speed of about 100 000 neurons per second (Linderkamp et al., 2009). That, in turn, means that there are at least 200 billion cells in the whole brain and 40 billion solely in the newly forming cortex. Roughly 50% of newborn neurons will be eliminated throughout the maturation process leading to a final amount of 100 billion at birth. This number represents roughly 100 trillion interneuronal connections (Linderkamp et al., 2009).

The majority of neocortical pyramidal neurons are generated from radial glia and intermediate progenitors (IPs) between E11-E17 in murine brain and week 12 to 18 of gestation in human, which refers to a neurogenic phase (Table 1) (Noctor et al., 2004). The neurogenic phase is followed by gliogenic phase when astrocytes and oligodendrocytes, in the ventral region are born (Hirabayashi and Gotoh, 2005).

Table 1. Fetal cortical development: human versus mouse

	Peak incidence	Major developmental stages
HUMAN	5-9 wk	1. Neural tube formation 2. Development of prosencephalon (forebrain)
MOUSE	E8.5-E10	
HUMAN	12-18wk	1. Neurogenesis
MOUSE	E11-E17	
HUMAN	12-20wk	1. Neuronal migration 2. Establishment of cell layers
MOUSE	E11-E17	
HUMAN	22-34wk	1. Ultimate migration of neurons 2. Regulation of axon formation between thalamus, cortex and subcortical structures
MOUSE	E17.5-E19	
HUMAN	24wk to 10mo	1. Extension of axons and dendrites 2. Synaptogenesis
MOUSE	E14- P30	
HUMAN	35 wk to 24mo	1. Myelination
MOUSE	P10-P60 mostly	

Abbreviations: wk- weeks of gestation, mo- months, E- embryonic day
Adapted from Linderkamp et al., 2009

The key to the generation of such a complex, yet extremely well orchestrated structure as the brain is provided by the ability of neuronal cells to polarize (Yoshimura et al., 2006). During development, neurons undergo dramatic morphological changes. Over 20 years ago, Craig and Banker provided a model for studying neuronal polarity *in vitro*. This model is based on cultured pyramidal neurons extracted from rodent hippocampus. Based on the detected alterations in cell shape these changes have been classified into 5 stages (Dotti et al., 1988). The process of neuronal polarization is briefly depicted in Figure 2. Initially (stage 1), freshly plated cells form short protrusions known as lamellipodia. These extensions further evolve into short immature neurites. This is recognized as stage 2. Interestingly, at this point the cells remain unpolarized. This phase is characterized by alternating periods of elongation and retraction of the neurites, which leads to an even length of protrusions. In stage 3, this highly dynamic period ends and one of the extensions initiates rapid and uninterrupted growth, later on acquiring axonal morphology. In

stage 4 the remaining neurites slowly elongate and acquire dendritic characteristics. Finally, in the fifth stage cells form a neuronal network and establish synaptic specializations (Yoshimura et al., 2006, Cáceres et al., 2012).

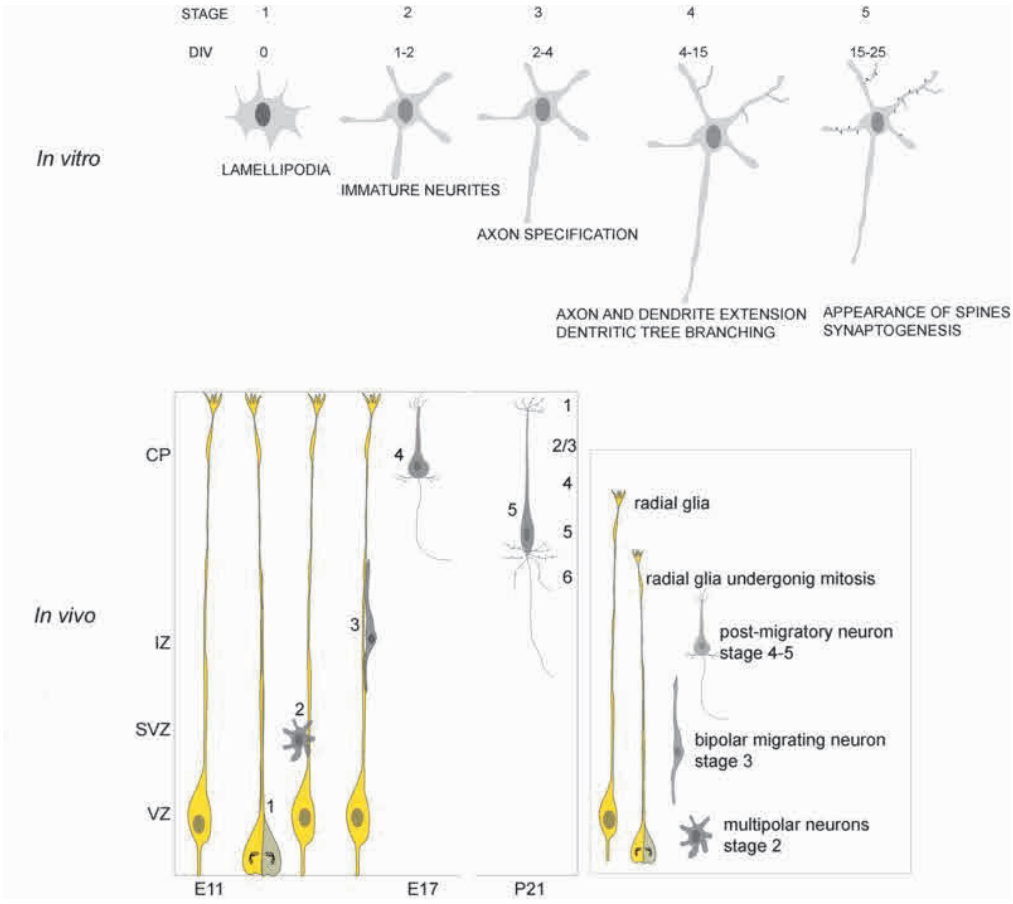


Figure 2. Polarization of cortical neurons *in vitro* versus *in vivo*. Freshly dissociated cultured neurons are present with many lamellipodial and filopodial protrusions (stage 1/DIV0). Next, protrusions progress into multiple immature neurites (stage 2/DIV 1-2). During stage 3/DIV 2-4, a single neurite acquires axonal morphology while others form dendrites. During stage 4/DIV 4-15, both axon and dendrites extend in length. At the last stage 5/DIV 15-25, cells are fully differentiated with dendritic spines and axon initial segment (AIS). Corresponding *in vivo* polarization stages are marked in the bottom panel. E11/17 embryonic day 11/17, P21, postnatal day 21. Brain layers are marked as follows ventricular zone (VZ), subventricular zone (SVZ), intermediate zone (IZ), cortical plate (CP). At P21 six distinctive layers are present in CP. These are labelled: 1, 2/3, 4, 5 and 6 starting from the most superficial (1) to the deepest layer (6) (Modified from (Polleux and Snider, 2010).

Creation of the first neurite has major repercussions: it gives the neurite a chance to enter into axon-like growth and outlines the place of origin for the second one. In practice, it reflects the first stage in bipolar organization, which is a crucial step for neuronal migration and final brain arrangement (Barnes and Polleux, 2009).

1.2 Reaching destiny: from a progenitor to a migrating cell

Cell migration is a key process in the life of nearly all organisms. It has a pivotal role during embryogenesis, but in adulthood it is also essential for tissue repair and immune response. Understanding the mechanisms behind initiation, preservation and finally, termination of migration is essential for obtaining knowledge about how alterations in those processes may be contributing to neurodevelopmental disorders. Early on during murine cortical development precursor cells of the pseudostratified ventricular epithelium (PVE) span the entire depth of the neural tube wall (Del Bene et al., 2008). At that point they present characteristics of regular epithelia that are highly polarized along their apical-basal membrane (Huttner and Brand, 1997, Chenn et al., 1998, Wodarz and Huttner, 2003). However, when neurogenesis starts some of the epithelial features like tight junctions or distribution of certain proteins associated with plasma membrane along the apical-basal axis become downregulated (Aaku-Saraste et al., 1997). The onset of the period of neurogenesis is followed by the emergence of radial glia and basal progenitors (Huttner and Brand, 1997, Götz, 2003, Noctor et al., 2004). The latter give rise to subventricular zone and during symmetrical cell division produce two neuronal daughter cells (Haubensak et al., 2004, Noctor et al., 2004). Interestingly, radial glia displays both neuroepithelial and astroglial properties. As a consequence most neurons in the brain originate from radial glia precursors, which are more fate-restricted progenitors than neuroepithelium (Williams and Price, 1995, Malatesta et al., 2000). Prior to cell division neuroepithelial cells undergo interkinetic nuclear migration (INM). This process, initially observed by Sauer in 1935, involves oscillatory movement of the nucleus along the advancing cell cycle (Del Bene, 2011). During S-phase the nucleus of the neuroepitheilum moves toward the basal side of ventricular zone, yet mitosis occurs only at the apical side close to ventricular surface (Caviness and Takahashi, 1995, Kosodo et al., 2011). Interestingly, in radial glia, INM is restricted to most ventricular part of the cell, unlike in the neuroepithelium where it stretches along the whole apical-

basal axis. At the same time basal progenitors show hardly any INM (Takahashi et al., 1993, Haubensak et al., 2004). Disruption of INM may result in reduction of neuronal precursors therefore leading to uneven determination of cell fate (Schenk et al., 2009).

When cell division is completed the newborn neurons are obliged to leave the ventricular zone and move to their final destinations. The distance that neurons must complete to reach their endpoint varies from 50-100 μm in the developing retina to approximately 2 cm in the cerebral cortex during radial migration. This length increases even more in the case of tangentially migrating neurons (Tsai and Gleeson, 2005, de Anda et al., 2010).

The neocortex is composed of 6 layers (I-VI) each of which contains a unique subdivision of neurons that are both functionally, as well as cyto-architecturally discrete (Bradke and Dotti, 1997, Kwan et al., 2012). The first cohort of postmitotic neurons leaves the ventricular zone (VZ) at E11 form the primordial plexiform layer, known also as the preplate (PP) right beneath the pial surface (Hatten, 1999, Nadarajah and Parnavelas, 2002). When the preplate is formed, the space between the PP and VZ is filled with newly forming axons and gives rise to the intermediate zone (IZ). This region will make up the white matter of the brain containing both afferent and efferent axons. The next wave of neurons migrates through the IZ and splits the PP into the marginal zone (MZ) and the subplate (SP) (Uylings, 2000). Cells located in the marginal zone will later differentiate into Cajal-Retzius cells and form layer I. These cells are the earliest born cells of the cerebral cortex and further layers II-VI are generated in an "inside out" manner within the cortical plate. The "inside out" term describes the phenomena where early born neurons occupy the deepest layers (eg. VI), while more recently born cells move across previously formed layers and set a new one (Rakic, 1972, Hatten, 1999, Nadarajah and Parnavelas, 2002).

To date, three modes of neuronal migration have been described in the literature: radial, tangential and multipolar. During migration, cells undergo morphological changes and the bipolar shape is typically an attribute of radially migrating neurons, where the leading and trailing processes are in opposition to each other. Interestingly neurons undergoing tangential migration (vertical to radial glia) present great diversity in morphology.

1.2.1 Radial migration

In the telencephalon region radial glia-based migration is the primary mechanism utilized by pyramidal neurons reaching their final positions in the developing brain (Angevine and Sidman, 1961, Rakic, 1974, Nadarajah et al., 2001). Neurons move perpendicular to the VZ gliding on radial fibers hence increasing thickness of the cortical plate (Marín et al., 2010). It is estimated that 80-90% of the precursors in the cortex once committed to neuronal fate move along the radial glia "backbone" (Anton et al., 1996). Bipolar oriented pyramidal neurons utilize two modes of migration known as locomotion and somal translocation (Nadarajah and Parnavelas, 2002). Importantly, the latter one is believed to be the major mode of migration during the first days of neuronal relocation (Nadarajah et al., 2003). Cells utilizing somal translocation are characterized by rather long processes stably connecting them either to the pial surface or to the marginal zone. In fact the length of the leading neurite spans the thickness of the growing cortical plate (Nadarajah et al., 2001, Kriegstein and Noctor, 2004). Interestingly, when radial migration is over, glial cells evolve into stellate astrocytes (Schmechel and Rakic, 1979, Culican et al., 1990). The leading processes of locomoting neurons are rather short in comparison to translocating cells. Extension of the leading process is followed by nucleokinesis and eventually elimination of the trailing process (Marín et al., 2010). Cells undergoing translocation present an uninterrupted, smooth forward movement, while neurons gliding on radial glia display a characteristic saltatory forward movement. This in turn leads to significant speed differences between translocating and locomoting cells (Nadarajah et al., 2001). Importantly, individual cells are not restricted to one mode of migration (Kriegstein and Noctor, 2004).

The nucleus is undoubtedly the largest cargo in neurons and moving it during migration requires remodeling of the cytoskeleton (Tsai and Gleeson, 2005). Prior to nucleokinesis the main cellular structures like the centrosome, microtubule organizing center (MTOC), Golgi apparatus (GA), endoplasmic reticulum (ER) or the mitochondria gather in front of the nucleus (Bellion et al., 2005). Nucleokinesis itself is critically dependent on the microtubule (MT) network (Lambert de Rouvroit and Goffinet, 2001), which forms a cage or a fork-like structure around the nucleus (Rivas and Hatten, 1995, Xie et al., 2003). The slow move of the centrosome into the leading process is followed by the nucleus (Solecki et al., 2004). Proper connection between centrosome and nucleus seems to be one of the critical elements in neuronal migration. There are many recognized mutations that may negatively influence

migration thus leading to severe developmental consequences. Selected syndromes present in humans are listed in Table 2 below.

Table 2. Mutations affecting neuronal migration in humans

Syndrome	Gene affected	Description	Reference
Miller-Dieker syndrome	14-3-3ε	A group of conditions which present failure in forebrain migration, fewer and smoother gyri. Characterised by deletion in PAFAH1B1 (platelet activating factor acetyldehydrogenase).	(Toyo-oka et al., 2003, Yingling et al., 2003)
Lissencephaly	LIS1, TUBA1A	Characterized by smooth brain.	(Pilz et al., 1998, Kumar et al., 2010)
X-Linked lissencephaly	Doublecortin	Females present with double cortex, due to mutation in doublecortin protein. Males suffer from lissencephaly.	(des Portes et al., 1998 Ross et al., 1997)
Zellweger syndrome	12 genes involved/ PEX1 mutation found in 70% cases	Impairment of cortical migration due to disturbance in peroxisomal biogenesis. Decreased number of gyri that are atypically large (stereotypic medial pachygyria) and present additional small gyri (lateral polymicrogyria).	(Baes et al., 1997)
Bilateral periventricular nodular band heterotopias	BPNH	Neurons in the forebrain form heterotopias.	(Dobyns et al., 1997, Fink et al., 1997a)
Microencephaly	ND	Though the lamination pattern seems to be unimpaired the observed thickness of the layers is reduced.	(McKusick, 1994)
Schizophrenia	DISC-1	Disturbance in intracellular transport, neurite architecture and migration observed in DISC-1 protein truncation may add on to ontogenesis of schizophrenia.	(Morris et al. 2003)
Fukuyama- type congenital muscular dustrophy	FUKUTIN	CNS migration disturbances that result in changes consistent with cobblestone (previously type II) lissencephaly with cerebral and cerebellar cortical dysplasia.	(Saito et al., 2003)
Muscle-eye- brain disease (MEB)	POMGnT1	An autosomal recessive disorder characterized by congenital muscular dystrophy, ocular abnormalities, and lissencephaly.	(Yoshida et al., 2001)
Periventricular heterotopia (PH)	Filamin A	PH is an X-linked dominant human disorder where many neurons fail to migrate and persist as nodules lining the ventricular surface. Females with PH present with epilepsy and patent ductus arteriosus and coagulopathy, while hemizygous males die embryonically.	(Fox et al., 1998)

Modified from Hatten et al., 1999

1.2.2 Tangential migration

In contrast to radial migration, tangential migration does not require communication with radial glia. Interestingly, these cells have been shown to be in close association with corticofugal axons, serving as a guide to the cortex (Métin and Godement, 1996, Denaxa et al., 2005). Tangentially moving neurons can migrate as a group of cells e.g. olfactory bulb interneurons, or individually like cortical interneurons or Cajal-Retzius cells (Marín et al., 2010). The process of tangential migration predominantly refers to GABA (gammaaminobutyric acid)-containing interneurons (Anderson et al., 1997) with an exception of the very first cortical neurons. These neurons reach the cortical primordium by tangential migration however they lack the phenotype of an interneuron (Bystron et al., 2006). It also applies to the population of oligodendrocytes generated in the subpallial telencephalon (Spassky et al., 1998, Olivier et al., 2001). Apart from neurons traveling from the subpallial telencephalon to the cortex, other routes of tangential migration have also been observed. One such example is provided by progenitor cells localized to subventricular zone in telencephalon, which generate two types of cells in the olfactory bulb. The granule and periglomerular cells of olfactory bulb are GABA interneurons and move along the rostral migratory stream (RMS) to their destination in the olfactory bulb (Luskin, 1993, Lois and Alvarez-Buylla, 1994). The process of tangential migration to the olfactory bulb continues until adulthood (Curtis et al., 2007). However, neurons that are predestined for the cortex derive from distinct proliferative regions like the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) anterior entopeduncular area (AEP) as well as the retrobulbar area of the olfactory bulb (Anderson et al., 2001, Cobos et al., 2001, Wichterle et al., 2001). MGE and AEP serve as the primary source of tangentially migrating neurons throughout initial days of migration (E11.5 in the mouse). Those neurons move mainly towards the striatum, marginal zone and subplate in the cortex (Lavdas et al., 1999, Anderson et al., 2001). At later developmental stages (E12.5-E14.5), MGE serves as the primary reservoir of tangentially moving interneurons. Interestingly, interneurons derived concurrently from the LGE migrate to the olfactory bulb but not to the cortex (Wichterle et al., 2001). Nevertheless at a late stage (E14.5-E16.5) both eminences provide interneurons to the developing telencephalon (Anderson et al., 2001). At the beginning of neurogenesis, early-generated interneurons maneuver through the IZ of the eminences, yet later on they migrate through the VZ and SVS in order to invade the cortex. Importantly,

interneurons that adopt their final position in the cortex can switch from tangential to radial mode of migration (Polleux et al., 2002, Tanaka et al., 2003).

1.2.3 Multipolar migration

The molecular mechanism underlying multipolar migration has lately come to the attention of the scientific community (Tabata et al., 2009, Pacary et al., 2011). Multipolar neurons present in the SVZ and IZ come from radial glia precursors, similarly to bipolar pyramidal neurons (Noctor et al., 2002, Tabata and Nakajima, 2003). During this stage, the centrosome is localized at the base of an axon (Kupijers and Hoogenraad, 2011). During radial migration neurons move towards the pial surface. In multipolar migration they frequently change the direction and rate of movement, or else transiently remain in approximately same localization (Tabata and Nakajima, 2003). These changes take place right before neurons start to take up a bipolar morphology and move in direction of the pial surface (LoTurco and Bai, 2006). Interestingly, this transient state applies also to tangentially moving interneurons in the vicinity of the cortical SVZ (Nadarajah et al., 2002) and neurons in auditory nuclei of the brainstem (Book and Morest, 1990). In terms of speed, this mode of movement is the slowest among all previously described and reaches only 1-6 μm/h (Tabata and Nakajima, 2003). During bipolar migration, and in particular throughout periods of locomotion, cells move at approximately 10 μm/h. However, during somal translocation, cells travel at the speed of 10-50 μm/h towards the pial surface (Nadarajah et al., 2001).

The improper transition from multi- to bipolar neurons leads to altered migration pattern, which is depicted in Figure 3. Several independent studies have pointed towards Filamin A (Flna), LIS1 and DCX as molecules involved in the transition from the multi to the bipolar phenotype (Nadarajah et al., 2001, Nagano et al., 2002, Bai et al., 2003, Nagano et al., 2004, Hatten, 2005).

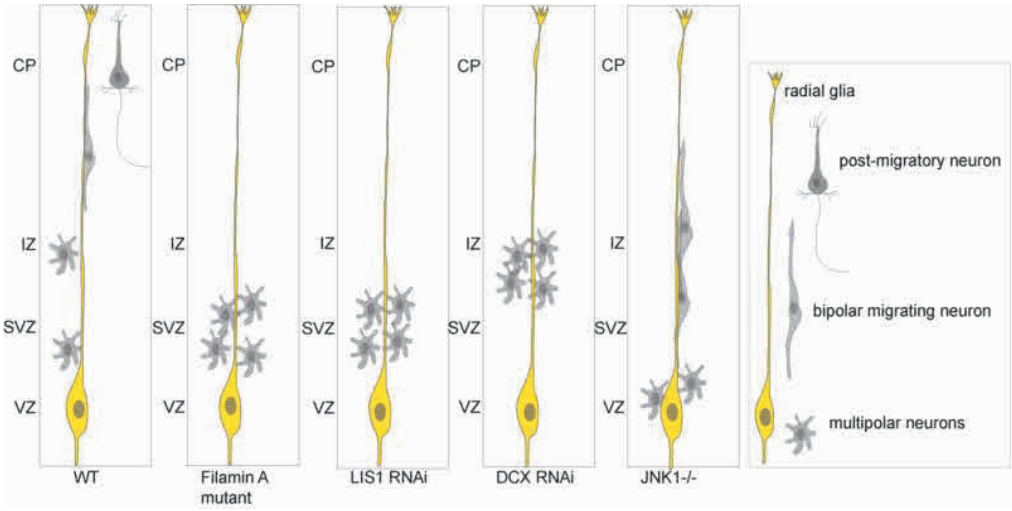


Figure 3. Multipolar migration is a critical step in neuronal movement. Disruption in various proteins including Filamin A, LIS1, DCX or JNK1 disrupts the normal migration pattern and may lead to severe neurological issues in the long-term perspective. In Filamin A, mutant multipolar neurons fail to exit from the multipolar stage and remain in VZ/SVZ. Similarly LIS1 RNAi treated neurons are retained in the VZ/SVZ region. DCX-silent cells remain in IZ zone, whereas *Jnk1-/-* neurons exit multipolar stage faster than wt and acquire bipolar morphology (Modified from (LoTurco and Bai, 2006)

Recently JNK1 and SCG10 have been shown to be key players in this process, which will be further discussed in results section of this thesis (Study II). Manipulating the function of Filamin A and DCX with either a RNAi approach or protein overexpression revealed that both proteins affect the amount of cells in the multipolar stage (Bai et al., 2003, Nagano et al., 2004). Likewise, LIS 1 is needed for exit from the multipolar stage since LIS1 RNAi-treated cells remain in the VZ and SVZ without commencing to bipolar morphology (Hatten, 2005, Tsai et al., 2005). Moreover, at the transcriptional level several factors have been appointed roles in each step of neurogenesis including a multipolar to bipolar shift. Recently, RP58, a transcriptional repressor that is highly enriched in the immature brain, has been shown to regulate the transition from a multi to a bipolar neuron. Moreover it was proposed by the authors that RP58 plays a valuable role in neuronal migration via repression of Ngn2 and Rnd2 pathway (Ohtaka-Maruyama et al., 2013).

2. The Golgi apparatus

Over 100 years ago Camillo Golgi found a new intriguing compartment in spinal ganglion cells, which he described as "the internal reticular apparatus". Today it is known by the name of its founder as the Golgi apparatus (GA). Observed in the most eukaryotic cells the GA is definitely a unique subcellular organelle that functions as a hub in the complex secretory pathway. The key features of the GA are summarized in Table 3 (Klute et al., 2011).

Table 3.

Summary of Golgi complex key features
• It functions as a hub of the secretory pathway and remains in close contact with the ER
• It is built from flattened, interconnected stacks of cisternae which form a ribbon-like structure
• Its structure is due to interactions with a unique cytoplasmic matrix
• It possesses enzymes that are in charge of post-translational modifications of newly synthesized proteins and lipids
• Contains additional enzymes required for sphingo- and glycolipids synthesis
• GA-processing enzymes are in a continuous flow between GA, ER, plasma membrane and endosomes
• Processed cargo is sorted for its destination at the *trans* Golgi compartment
• "Endosomal- lysosomal" pathway is used for molecules recycled from plasma membrane to GA complex
• It interacts with various components of the cytoskeleton: MTs, actin filaments and IFs
• Forms a platform for a large number of signaling cascades, which, in turn control GA function
• In the absence of cytoskeletal support (MTs) GA complex fragments into Golgi ministacks

In contrast to the fruit fly *D.melanogaster*, plants and fungi where GA stacks are spread throughout the cytoplasm, in vertebrates the stacks are connected creating a ribbon-like structure in the vicinity of the nucleus (Xiang and Wang, 2011). In spite of its defined organization, it is a very dynamic structure that goes through the rapid cycles of dismantling and

rebuilding during the course of the advancing cell cycle, as well as upon various chemical treatments and finally it fragments during apoptosis (Machamer, 2003).

Proteins and lipids newly synthesized in the endoplasmic reticulum (ER) are transported to the GA complex where various posttranslational modifications including phosphorylation, acylation, glycosylation, methylation and sulphation take place (Marsh and Howell, 2002). Then the final product is sent away to its destination. However, the GA recovers some of the proteins and lipids and sends them back to the ER, showing that the GA not only functions as a processing terminal but also as a screening system by which plasma membrane components are separated from those intended to remain in the ER (Mellman and Simons, 1992). Since the transport intermediates operating in biosynthetic-secretory trafficking may move across lengthy distances in cells such as neurons, it is widely acknowledged that the cytoskeleton is closely associated with the GA stacks (Donaldson and Lippincott-Schwartz, 2000).

In mammals, GA stacks are separated from endoplasmic reticulum exit sites (ERES) and are in close connection with the MTOC/centrosome (Saraste and Goud, 2007). Close association of the GA and microtubules is maintained, even when the cytoskeleton is undergoing major architectural changes, for instance upon migration events or during neuronal polarization (Kupfer et al., 1982, de Anda et al., 2005). In fact, MTs tightly accompany the cis-GA cisterna. In addition, the GA structure is preserved by MTs intersecting the tubules in noncompact areas and openings in the GA ribbon (Marsh et al., 2001). Moreover microtubules in the close vicinity of the GA contain detyrosinated and acetylated tubulin, which is a feature of stable MTs (Schulze et al., 1987, Burgess et al., 1991). However, the antimicrotubular drugs lead to microtubule depolymerisation, fragmentation of the system of interconnected GA stacks and fragments dispersal (Thyberg and Moskalewski, 1999). Interestingly, the GA stacks are not randomly dispersed in the cytoplasm but they get redistributed close to the ER exit sites instead (Cole, et al., 1996, Thyberg and Moskalewski, 1999)

2.1 Protein transport through the Golgi stacks

The cisternal stacks of GA are often compared with a pile of pancakes. Yet it is worth remembering that this is an oversimplification and does explain the

diversity of morphologies visible in a variety of cell types (Connerly, 2010). Cargo proteins travelling across GA subcompartments are altered by resident enzymes. Consequently, they receive sugars, sulfates or phosphate groups. Defects in those processes may result in inborn glycosylation disorders, muscular dystrophy or otherwise may be a significant factor in the development of diabetes or cancer (Ungar, 2009). To date two main models have been suggested in order to explain protein flow across GA.

The initial model proposed by Palade and colleagues in the 1960s was called the vesicular transport model. According to it, GA membranes are steady structures across which flow the cargo proteins. The vesicles that travel from the ER fuse with the *cis*-side of the GA providing newly synthesized proteins for further modifications. Enzymes that are meant to serve at the GA, remain in the stack unlike other secretory proteins that are packed in vesicles meant for the following cisternae. Once they reach the trans-side, the cargo is packed into diverse vesicles that will deliver them to their final destination. Importantly, structural elements like SNAP receptor proteins (SNAREs) or alleged cargo receptors that participate in ER-GA route are packed up into vesicles and send back to ER (Farquhar and Palade, 1998). The other model, named as cisternal maturation, proposes that vesicles originating in the ER contain all elements required for forming a brand new *cis*-Golgi cisterna. During the maturation process of the *de novo* created cisterna, various proteins and lipids are exchanged while they move from the ER to the GA and across the other GA stacks. Upon reaching the trans-face, the cisterna collapses and GA components are recycled. Cargoes are then enclosed in carrier vesicles that will ensure their delivery to their final destinations (Rothman and Wieland, 1996, Pelham, 1998). The following models are briefly depicted in Figure 4.

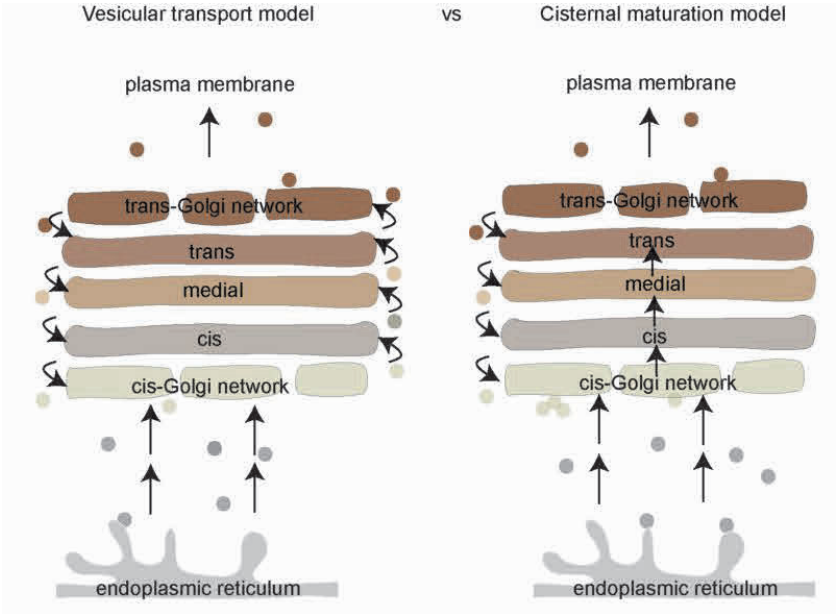


Figure 4. Transport of cargo across Golgi stacks; vesicular transport versus cisternal maturation model. In the vesicular transport model the ER-derived vesicle fuses with *cis*-Golgi and delivers the cargo. While the cargo undergoes various posttranslational modifications, ER-escaped proteins, SNAREs or other transport involved ER to GA components are packed into vesicles and travel back to ER. Upon reaching the *trans*-face the cargo is redistributed into appropriate vesicle for its destiny. In the cisternal maturation model the ER derived "new" Golgi *cis*-compartment matures by exchanging enzymes and lipids but not the cargo. Proteins are transported within cisternae, while vesicles take back resident proteins in a retrograde way. (Modified from Short and Barr, 2000).

The organization of GA membranes still remains to be addressed. Nevertheless, several factors affect Golgi membranes, including coat complex proteins I (COPI), conserved oligomeric Golgi (COG) tethering complexes, Rab GTPases, SNAREs, long coiled-coil tethering factors. The latter from the "golgin family" (Miaczynska and Zerial, 2002, Rein et al., 2002, Barr and Short, 2003, Duden, 2003, Ungar and Hughson, 2003, Marquardt, 2004). The family of golgins has also been linked to proper GA function. It has been shown that various members of the family play a role in cisternal stacking and tethering of vesicles, as well as acting as effectors of various Rab proteins (Gleeson et al., 1996, Short et al., 2001). Several golgins including giantin, golgin-230, GMAP210 or GM130 regulate the structure of the GA (Nakamura et al., 1997).

2.2 The Golgi apparatus as a crossroad of cellular trafficking

Eukaryotic cells are extraordinary structures able to respond to both extra- and intracellular ques. Beginning at the plasma membrane, which is rich in various receptors, they can transfer the incoming signal to different proteins present in the cytoplasm. This in turn triggers further partners of the signaling cascade triggering a final cellular response. It has been estimated that around 10-12% of human genes encode for proteins involved in signal transduction (Imanishi et al., 2004). GA membranes accommodate a great number of signaling particles including a variety of kinases, phosphatases, phospholipases, heterotrimeric G-proteins and phosphodiestrases (Mazzoni et al., 1992, Polizotto et al., 1999). Many of the regulatory proteins present at the GA have been shown to regulate its function. They can also alter the signaling lipids present in the GA membrane as well as manage its contact with the surrounding cytoskeleton (De Matteis and Morrow, 1998, Godi et al., 1999).

A recent RNAi screen revealed that eight members of the MAP kinase family and thirteen closely related genes were found to control GA function in human cells (Chia et al., 2012). It was previously shown that active ERK, H-ras as well as MEK are present at the GA (Torii et al., 2004). ERK can phosphorylate GA reassembly stacking protein (GRASP65) at Ser227 during interphase leading to the disassembly of the stacks (Yoshimura et al., 2005, Wei and Seemann, 2009). In 2003, Harada and co-workers found that Akr1p-like 1 and 2 (AKRL 1 and AKRL 2), both present at the Golgi, are potent activators of JNK. Moreover direct JNK activators MKK4 and MKK7 were also activated in the presence of AKRL1/2. In addition, DLK/MUK/ZPK known JNK activator was found on the periphery of GA in NIH 3T3 cells (Holzman et al., 1994, Douziech et al., 1999).

2.3 The role of the Golgi apparatus in health and disease

Over the years it has been observed that the GA may fragment under specific conditions. Those can be stimulated by physiological responses or conversely by pathological conditions (Gonatas et al., 2006). During mitosis GA stacks are known to disassemble and are equally divided between the two daughter cells. After the mitotic program is finished, the GA, together with the nuclear envelope, is re-building. Additionally, blocking GA disassembly inhibits cell entrance into mitosis (Sütterlin et al., 2002). A variety of kinases have been linked to promoting GA fragmentation during mitosis by phosphorylation of

a variety of GA proteins. Among those are Cdc2, RAF/MEK1/ERK1c, Plk1, Plk3 and Cdk5 (Acharya et al., 1998, Sütterlin et al., 2001, Xie et al., 2004a, Sun et al., 2008). Interestingly, it was also observed that during apoptosis GA membranes fragment into smaller tubulovesicular clusters, resembling those previously noted upon mitotic disassembly (Chiu et al., 2002, Lane et al., 2002, Machamer, 2003). Several structural proteins are cleaved in a caspase-mediated manner, and therefore apoptotic fragmentation, unlike the one observed during cell division, is irreversible. Golgin160, GRASP65, p115 and GM130 are primary targets of various caspases upon apoptotic stimuli (Mancini et al., 2000, Lane et al., 2002).

For the first time, GA fragmentation was observed *in vivo* in a disease model of sporadic amyotrophic lateral sclerosis. Although affected spinal cord motor neurons maintained intact nuclei as well as axons and dendrites, the secretory pathway was compromised as shown by morphometric measurements (Gonatas et al., 1992, Gonatas et al., 2006). Similar lesions were also found in the familial form of ALS (fALS), which is characterized by mutation in superoxide dismutase (SOD1). Interestingly, in the studied mouse model, GA fragmentation alongside the accumulation of mutant SOD1 was noticed already long before the signs of paralysis appeared (Gurney et al., 1994, Stieber et al., 2000). Several other neurodegenerative diseases involve the accumulation of misfolded proteins leading to impaired neuronal connectivity and cellular plasticity, which in turn initiates cell death (Bence et al., 2001). Among those are Alzheimer's (AD), Parkinson's (PD), corticobasal degeneration and Creutzfeldt-Jacob disease (Stieber et al., 1996, Sakurai et al., 2000, Gosavi et al., 2002). Alzheimer's and Parkinson's diseases are the two most common neurodegenerative disorders among people above the age of 65 years (Tanner and Ben-Shlomo, 1999, Gibrat et al., 2009). It was previously shown that accumulation of α-synuclein in cultured cells inhibits mitochondrial function, protein synthesis, proteasome function, exocytosis, and oxidative stress (Cookson and van der Brug, 2008). The study conducted by Cooper and collegues (Cooper et al., 2006) shed a new light on α-synuclein function. They found that the overexpression of α-synuclein suppressed the growth of yeast cells. The observed pathology was mainly due to the actions of α-synuclein on ER-to-Golgi vesicle transport. This however, could be rescued by overexpression of Rab1 (Ypt1p in yeast) protein (Cooper et al., 2006), which plays a key role in tethering/docking of the vesicles to GA compartments (Moyer et al., 2001, Cooper et al., 2006). A later study from Jesse C. Hay group showed that mild overexpression of human α-synuclein in normal rat kidney

(NRK) cells, wild type and disease-associated mutants strongly inhibited ER-to-Golgi transport. Upon expressions of SNAREs the transport inhibition was reversed without affecting α-synuclein expression, localization, aggregation or disposal (Thayanidhi et al., 2010).

It has been suggested that the size of GA may correlate with neuronal activity. Therefore readout of its condition can be indicative of ongoing pathology (Stieber et al., 1996). A morphometric study on tissue from AD brains showed that neurons lacking neurofibrillary tangles (NFTs) presented with fragmented and atrophic GA. Conversely, the NTFs positive cells had GA mostly displaced by NFTs and the remaining GA stacks were unbalanced and merged into bigger aggregates (Salehi et al., 1994). Moreover, in AD, GA fragmentation was similar to that observed in ALS but also the one seen *in vitro* when treating cells with microtubule depolymerizing agents (Robbins and Gonatas, 1964, Turner and Tartakoff, 1989). Interestingly, protein profiling performed on spinal cord neurons from a mouse model of human familial ALS revealed that stathmin protein accumulated in the cytoplasm of neurons alongside the fragmented GA. Moreover membrane–bound SCG10 and SCLIP accumulated in a fashion similar to stathmin in diseased cells (Strey et al., 2006). It was also shown that stathmin is downregulated when heavily phosphorylated (Strey et al., 2004). As a consequence of microtubule depolymerization GA stacks disassemble (Gonatas et al., 2006). This in turn may impair trafficking and the secretory pathway (Stieber et al., 1996, Strey et al., 2004).

Nakagomi and co-workers showed that the disassembly of GA came prior to neuronal cell death in response to various stressors, including excitotoxic, oxidative/nitrosative insult or ER stress. Interestingly, overexpression of the C-terminal fragment of GRASP65 impeded fragmentation, consequently postponing cell death. Likewise, blocking of the mitochondrial or ER death signaling pathways reduced the extent of fragmentation (Nakagomi et al., 2008). Until recently, it has been believed that GA fragmentation is an irreversible process eventually leading to cell death. A recent report suggests this process may be in fact reversible under special conditions. Lily Yeh Jan's group performed an *in vitro* study on hippocampal neurons cultured in hyperexcitable conditions to show that transiently fragmented GA may revert to its native state. They found that GA disassembles in the presence of elevated potassium, yet this process is reversible upon its removal. Moreover, they also found that extended treatment with bicuculline or withdrawal of APV leading to increased neuronal activity, results in GA

fragmentation. Importantly GA stacks reorganized back to their normal state once neuronal activity returned to basal levels (Thayer et al., 2013). In the light of the previously published reports, this study sheds new light on GA fragmentation emphasizing that the GA breakdown is recoverable. Nonetheless, much work still needs to be done to unravel the exact mechanism of GA fragmentation.

2.4 Neurons: specialized cells with particular demands

Neurons are considerably bigger than non-neuronal cells. The shape and size dictate particular demands for neurons. Distinctively, they rely on protein and organelle transport from cell body to axon and dendrites. Cellular polarization, as well as multifaceted morphologies, gives rise to additional challenges (Brady and Morfini, 2010). Nowadays, it is estimated that approximately 40-400 different mRNAs are employed in localized translation in dendrites (Eberwine et al., 2001, Steward and Schuman, 2001). This allows cells to respond quickly to localized signals. Nevertheless, most proteins are produced in the cell body and need to exit GA to reach their destination (Brady and Morfini, 2010).

2.4.1 Golgi outposts

It was shown in a non-neuronal system that microtubules might arise from other intracellular elements than the MTOC/centrosome. In a process referred to as acentrosomal nucleation, microtubules originate from varied sites including the nuclear envelope, melanosomes, the plasma membrane or the GA (Vinogradova et al., 2009). Several proteins have been listed as important in nucleation of microtubules. These include γ-tubulin, AKAP450, GM130 and CLASP (Baas and Yu, 1996, Ahmad et al., 1998, Efimov et al., 2007, Kollman et al., 2010). In neurons whose discrete and highly complex architecture is maintained by a dynamic array of microtubules, the process of the nucleation of microtubules needs to be well maintained (Desai and Mitchison, 1997, Hoogenraad and Bradke, 2009). Hence, several studies have undertaken the challenge of investigating this process in the neuronal system. The requirement of centrosomes, but not γ-tubulin, is lost when neurons get older and MTs arise from acentrosomal sites (Stiess et al., 2010).

Dendrites of mammalian neurons when observed *in vitro* present with mixed orientation of microtubules in the vicinity of the cell body. At the same

time are organized in a plus-end-out manner in more distal regions (Stepanova et al., 2003, Baas and Lin, 2011). Interestingly, in *Drosophila melanogaster* the array of microtubules in dendrites is highly polarized with a minus end situated away from the cell soma (Stone et al., 2008). Both in mammals and the fruit fly, dendrites can be discriminated from axons by minus-end-out microtubules (Nguyen et al., 2011). Importantly it was noticed in dendritic arborization (da) neurons in *D. melanogaster* that neurons grow complex dendritic arbors despite the absence of centrosomes (Grueber et al., 2003, Nguyen et al., 2011). Moreover, the centrosome serves as a primary site for microtubule nucleation only in young cortical neurons (DIV 2), but not in the mature culture (DIV 11-12), where γ-tubulin is depleted from the centrosome (Stiess et al., 2010). Likewise in fibroblasts it has been shown that the GA can serve as a site of microtubule nucleation independently of the centrosome (Chabin-Brion et al., 2001, Efimov et al., 2007). Although MTs do not require centrosome as an initiation point for their growth they still require the presence of γ-tubulin, AKAP450 and CLASP proteins (Chabin-Brion et al., 2001, Efimov et al., 2007, Miller et al., 2009).

In neurons, regardless of the origin, mammalian or the fruit fly's so-called Golgi outposts are present only in dendrites (Gardiol et al., 1999, Pierce et al., 2001, Horton and Ehlers, 2003). The incidence pattern, however, is slightly different. In the fruit fly Golgi outposts are present all over dendritic arbors while in hippocampal neurons they localize to a group of primary branches (Horton et al., 2005). Similar to mammalian the Golgi outposts found in class IV da neurons contain all components essential to start up the process of microtubule nucleation (Efimov et al., 2007, Ori-McKenney et al., 2012). They contain γ-tubulin and *Drosophila's* homolog of AKAP450, CP309 protein, both critical in Golgi-outposts microtubule nucleation process (Ori-McKenney et al., 2012). Presumably smaller outposts support dendrite branching and neurite outgrowth by delivering membranes. Nonetheless the bigger ones that are more immobile and present at dendrite branch points in hippocampal neurons could also serve additional functions such as regional protein translation (Eberwine et al., 2001, Horton et al., 2005, Ye et al., 2007). A recently published study by Garner and Green laboratory indicates that NMDA receptor trafficking engages both Golgi outposts and the dendritic ER subcompartment (Jeyifous et al., 2009). Interestingly, the majority of NMDA receptors were noted to employ Golgi outposts and this required assistance from COPI coated vesicles (Jeyifous et al., 2009).

3. C-Jun N-Terminal Kinases (JNKs)

3.1 MAP kinase pathways

Mitogen activated protein kinases have been well conserved during evolution. They are present from yeast to humans and share a similar activation scheme that comprises a three-tiered signaling cascade, known as the core-signaling module (Karin, 1996, Kyriakis and Avruch, 2012). Such a cascade involves sequential phosphorylation events targeting an activation loop, which is a typical structural element of all protein kinases (Goldsmith and Cobb, 1994). Phosphate transfer to the activation loop leads to conformational changes, which in turn make the catalytic site available to ATP and potential substrates (Goldsmith et al., 2004). Protein phosphorylation plays a crucial role in many cellular events. It controls metabolism, cell division, cell movement, but also determines cell survival or else death. Hence, any disturbance in ordinary phosphorylation events may result in signaling cascade malfunction resulting in a severe disease state in the whole organism (Cohen and Alessi, 2013). Based on conserved motifs, over 500 protein kinases have been identified in the human genome to date. Based on similarities within sequences among recognized kinases they are classified into 20 families (Manning et al., 2002).

The c-Jun N-terminal kinases belong to a larger group of serine/threonine (Ser/Thr) protein kinases. They are known as mitogen activated protein kinases (MAPK) a subgroup of CMGC protein kinase (*c*yclin dependent kinases, *M*APKs, *g*lycogen synthase kinase 3 (GSK3), and *c*asein kinase 2-related protein kinase) (Hanks and Quinn, 1991). The extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), stress and proinflammatory activated p38 kinase (p38 α,β,γ,δ) and JNK family of kinases are considered to be fundamental MAPK. All MAPKs are proline-directed, meaning that they target serine/threonine residues that are in the direct vicinity of a proline. Particular docking sites are those that further maintain high substrate specificity of MAP kinases, providing steady and selective interplay between MAP kinases and their genuine targets (Kyriakis and Avruch 2012).

JNKs act within a protein kinase cascade consisting of MAPKKKs (MKKKs), MAPKKs (MKKs) and MAPKs. The simplified MAPK signaling pathway is depicted in Figure 5 (Kyriakis and Avruch, 2001, Johnson and Nakamura, 2007). MKK4 and MKK7 control JNK phosphorylation within a conserved motif Thr-

Pro-Tyr. Both kinases show site directed specificity and MKK4 demonstrates a preference for Y185, whereas MKK7 targets T183 (Wada et al., 2001, Kishimoto et al., 2003). Importantly, MKK4 may also act via the p38 pathway. There are at least 20 MAPKK kinases, among which the majority may signal via activation of MKK4 or MKK7. Activation of both MAPKKs (MKK4 and MKK7) is mediated by MEKK1-4, mixed lineage kinases (MLK), dual leucine zipper kinase (DLK), apoptosis signal-regulating kinases (ASKs), transforming growth factor β-activated kinase (TAK), Cdc42 and Rac (Bogoyevitch and Kobe, 2006). JNKs are activated in response to mitogens. Nonetheless, they are also triggered in answer to a variety of environmental stress like heat shock, radiation, oxidants, ischemic reperfusion, mechanical shear stress, genotoxins, vasoactive peptides, and least but not last, cycloheximide and anisomycin (Dérijard et al., 1994, Kyriakis and Avruch, 2012).

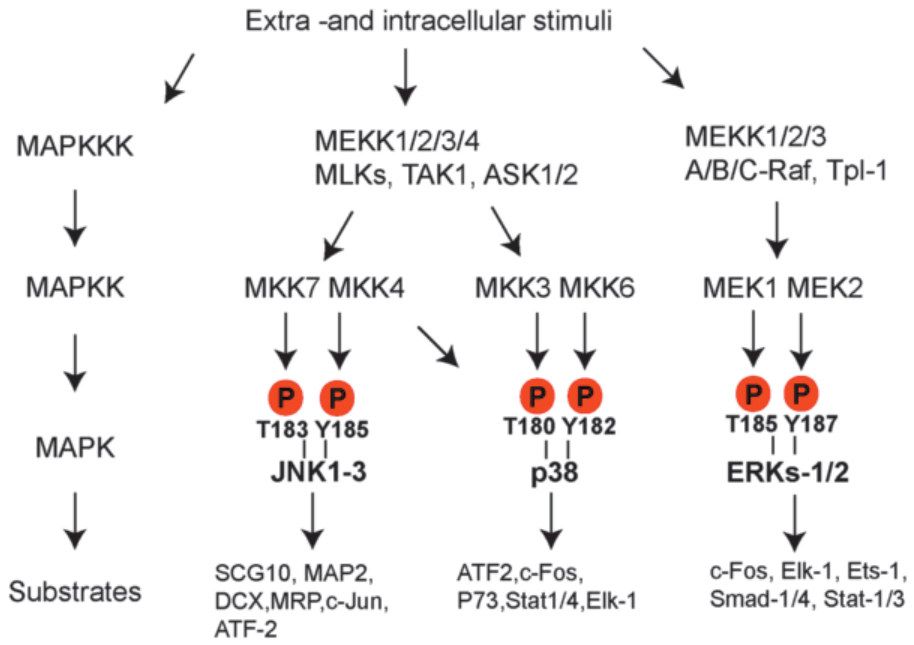


Figure 5. A simplified scheme of the JNK cascade. Diverse extra- and intracellular stimuli may activate the MAPK pathway. Among those are: UV, heat shock, hyperosmolarity, cytokines, interleukins, Toll-like receptors or ER stress, including protein misfolding. All MAPKs are controlled by direct phosphorylation by upstream MAP kinase kinases (MAPKKs or MAP2Ks), which are in turn phosphorylated by MAP kinase kinase kinases (MAPKKKs or MAP3Ks). JNK is activated by dual specificity MEK kinases (MKK4 and MKK7) on two sites T183 and Y185 within the activation domain. For the simplicity of the scheme only some MAPK targets are included.

Complexity of MAP kinase signaling provided by diversity of MAPKKK regulation permits responses to a huge range of diverse signals both intra- and extracellularly (Kyriakis and Avruch, 1990, 2012).

3.2 JNKs family

When originally discovered in the liver of cycloheximide-challenged rats, Jnk was classified as a stress-activated protein kinase (SAPKs) (Kyriakis and Avruch, 1990). In contrast to *D. melanogaster* that has only one JNK protein encoded by basket (bsk) (Sluss et al., 1996), the mammalian genome has three Jnk genes, namely *Jnk1* (*Mapk8*), *Jnk2* (*Mapk9*) and *Jnk3* (*Mapk10*) (Kyriakis and Avruch, 2012). In human *JNK* genes are located to chromosomes 10, 5 and 4 for *JNK1, JNK2* and *JNK3* respectively. In rodents they localize to chromosomes 14, 11 and 5 in mouse and chromosome 16, 10, 14 in rat (Haeusgen et al., 2011). While JNK1 and JNK2 can be found in a wide range of tissues, JNK3 is primarily expressed in the brain, heart and testis (Kuan et al., 1999, Manning et al., 2002). Structurally JNKs resemble any other protein kinase (Hanks et al., 1988). They contain two domains, and while the N-terminal domain is mainly formed by β-structures the C-terminus is rich in α-helices. Although JNK has an insertion in the C-terminus typical for MAP kinases, this extension is 12 residues longer among JNKs than in comparison to ERK or p38 kinases (Bogoyevitch and Kobe, 2006). As a result of alternative splicing within a region across subdomain IX and X (α and β JNK isoforms) and their C-terminus (JNK type 1 and 2) there are 10 JNK variants with a size range between 46 and 54kDa (Gupta et al., 1996). Although the functional significance of the two JNK types (1 and 2) still remains to be elucidated, the α and β isoforms seem to show differences in substrate preferences (Dérijard et al., 1994, Kallunki et al., 1994, Dai et al., 1995). A wide range of intracellular substrates and the complexity of the MAPK signaling pathway itself often evoke apparently opposing cellular responses. Thus it is essential to remember that cellular response to JNK activation will be a combination of two seemingly independent events. One of them is a physiological context that leads to JNK activation and the other one is the time it takes for the signal to last. And so transient activation of JNK within 1h may signal pro-survival events whereas a later phase, a continuous one gives rise to pro-apoptotic signaling (Ventura et al., 2006).

3.3 JNK input into brain development

Development of the neural tube, the precursor of the nervous system, occurs in two ways as a result of a primary and secondary neurulation. As soon as the neural plate is formed the primary process begins. This step is led by cells in the vicinity of the neural plate that stimulate cells of the neural plate to proliferate, infold and to finally form an empty tube. In the secondary neurulation neural tube is a result of a dense string of cells that penetrates into the growing embryo to mold a hollow tube. The degree to which these are used differ across vertebrates (Gilbert SF, 2000). In fish we deal exclusively with secondary mode of neurulation, while in amphibians like Xenopus, the neural tube is mostly formed by primary type neurulation, except in the tail where it arises from the secondary mode. In mammals, both types of neurulation have been observed and secondary form appears at the upcoming upper sacral level (Copp et al., 2003).

Neural tube defects (NTDs) are one of the most widespread inborn anomalies detected. About 300 000 children are born each year with NTD globally (Kondo et al., 2009). Excencephaly is a condition where cranial nerve tube remains open, and it is characterized by an outfolded appearance (Copp et al., 1990). The majority of mammalian cells tightly control JNK activation and only under very specific circumstances is its activity triggered. Conversely, in the brain, JNK kinase is highly and constitutively active. This elevated activity comes from JNK1 mainly, whereas JNK2/3 although someway active constitutively, have received more attention as a stress responsive JNKs (Coffey et al., 2002). Knockout studies provide invaluable information on gene function. Mice devoid of a single *Jnk* gene grow and breed normally like their wild type counterparts (Yang et al., 1997, Yang et al., 1998, Dong et al., 2001), whereas *Jnk1* and *Jnk2* double knockout mice die early during embryogenesis (E11.5) and present with defects in the neural tube closure (Kuan et al., 1999). Removal of both genes resulted in decreased apoptosis in the hindbrain, preceding the neural tube closure (excencephaly phenotype) and increased cell death accompanied by activation of caspase-3 in the forebrain (Kuan et al., 1999, Sabapathy et al., 1999). Although the process of neural tube closure requires cell migration no major defects in cell movement at this stage were observed in the double knockout mice. Interestingly, the number of proliferative cells also remained unchanged (Kuan et al., 1999, Sabapathy et al., 1999). Moreover analysis of various *Jnk* mutants revealed that $Jnk1^{-/-}; Jnk2^{+/-}$ show excencephaly, however $Jnk^{+/-}; Jnk2^{-/-}$ do not (Table 4). This suggests that the leading role of Jnk1 is in the regulation of neural tube closure (Sabapathy et al., 1999).

Table 4. Summary of JNK knockout phenotypes

	jnk1-/-	*jnk2-/-*	*jnk3-/-*	*jnk1-/- jnk2-/-*	*Jnk-/- jnk2+/-*	*Jnk1+/- jnk2-/-*
Phenotype	Viable. Disorganized neuron positioning in cortex, increased multipolar and bipolar motility rate[1] Decreased duration of multipolar stage[1] Anterior commissure lost by 3 months [2] Altered dendrite morphology [2,3]	Viable. No overt brain malformations[4]	Viable. No overt brain malformations[4]	E11.5 lethal. Impaired neural tube closure[4,5]	E16 lethal, impaired neural tube closure[5]	Viable. No overt brain malformati-ons.
Cell death proliferati on	A small decrease in cell cycle exit in embryonic brain[1]	Resistance to MPTP toxicity[6]	Resistance to MPTP and kainate induced neurotoxicity and seizures[6,7]	Increased death in hindbrain, decreased death in forebrain at E11.5[5]	Decreased apoptosis in the hindbrain[5]	
Tissue expression profile of respective MAPK	*jnk1* from E7 onwards in different tissues including brain, heart, liver, lung[5]	*jnk2* E7 onwards in brain, heart, lung, liver[5]	*jnk3* E11 onwards in brain and lower levels in heart and testis[5]	*jnk1/jnk2* E7 onwards in various tissues including brain, heart, liver, lung[5]		
Splice variants	**4**[8] JNK1β2-long JNK1α2-long JNK1β1-short JNK1α1-short	**4**[8] JNK2β2-long JNK2α2-long JNK2β1-short JNK2α1-short	**4**[8] JNK3β2-long JNK3α1-long JNK3β1-short JNK3α1-short			
Chromosome Localization	Ch. 10 in human Ch. 14 in mouse Ch. 16 in rat	Ch. 5 in human Ch. 11 in mouse Ch. 10 in rat	Ch. 4 in human Ch. 5 in mouse Ch. 14 in rat			

Modified from (Zdrojewska and Coffey, 2014)
[1]Westerlund et al., 2011; [2]Chang et al., 2003; [3]Björkblöm et al., 2005; [4]Sabapathy et al.,1999; [5]Kuan et al., 1999; [6]Hunot et al., 2004; [7]Yang et al., 1997; [8]Kyriakis and Avruch, 2012

Later on, it was shown that in *Jnk1*-/- mice the establishment of the anterior commissure tract is disturbed and also these mice increasingly loose microtubules within axons and dendrites (Chang et al., 2003). Moreover, a single knockout of *Jnk1* revealed its role in dendrite formation and axon maintenance at later stages of brain development (Chang et al., 2003, Björkblom et al., 2005). *Jnk2* and *Jnk3* knockout studies showed the importance of those in cell death-induced stress in mature brain. *Jnk2*$^{-/-}$ like *Jnk3*$^{-/-}$ single knockouts showed no overt brain malformations, contrary to *Jnk1*$^{-/-}$. Interestingly they showed resistance to 1-methyl-4phenyl-1,2,3,6-tetrahydropirydine (MPTP) which leads to death of dopaminergic neurons and reproduce symptoms of PD's (Dauer and Przedborski, 2003, Hunot et al., 2004). Moreover, *Jnk3*$^{-/-}$ mice present reduced cell death to kainate and neonates display resistance to ischemia-induced cell death (Yang et al., 1997, Kuan et al., 2003, Pirianov et al., 2007). Furthermore, Morishima et al., (2001) showed that *in vitro Jnk3*$^{-/-}$ cells are less sensitive to apoptotic death induced by β-amyloid in AD's. *Jnk3*$^{-/-}$ neurons moreover showed transient resistance to administration of 6-hydroxydopamine that under normal conditions leads to death of dopaminergic neurons in *substantia nigra pars compacta* (Brecht et al., 2005). Analogous to single deficiencies of JNK, dual null mice for *Jnk1*$^{-/-}$; *Jnk3*$^{-/-}$ and *Jnk2*$^{-/-}$; *Jnk3*$^{-/-}$ develop normally. Interestingly, genetic ablation of any of direct Jnk activators, namely Mkk4 and Mkk7 does not phenocopy defects in neural tube development (Ganiatsas et al., 1998, Wada et al., 2004). Single knockout mice of either *Mkk4* or *Mkk7* die between E.11.5-E13.5 due to liver-related pathogenesis. Double knockout embryos, however, do not survive past day E8.5 (Asaoka and Nishina, 2010).

To date, not many JNK substrates can provide further details on the role of JNK in brain morphogenesis. Some transcription factors, known as JNK targets could be of a great importance during early embryogenesis. Null mice for downstream Jnk targets, *c-Jun* or *Atf2*, do not share the *Jnk1*$^{-/-}$; *Jnk2*$^{-/-}$ phenotype. Notably, *cJun* $^{-/-}$ mice die due to hepatic defects similarly to *Mkk4* or *Mkk7* knockout mice whereas *Atf2*$^{-/-}$ animals die at birth because of respiratory failure (Maekawa et al., 1999, Behrens 2002). Interestingly, a novel Jnk substrate in the brain has been recently identified. It is a myristoylated Alanine Rich C-Kinase Substrate Like protein -1 (MARCKSL1) which is an actin regulatory protein (Björkblom et al., 2012). Disruption of MARCKSL1 gene in mice leads to neural tube defects resembling the phenotype of *Jnk1*$^{-/-}$; *Jnk2*$^{-/-}$ mice with regards to the observed excencephaly in those animals (Wu

et al., 1996b, Kuan et al., 1999, Björkblom et al., 2012). So far it is the only Jnk substrate known to cause isolated neural tube malformations.

3.4 JNK substrate specificity

In humans, it is predicted that each kinase has approximately 40 potential substrates (Johnson and Hunter, 2005). To date more than 50 JNK target proteins have been found and the search is not over (Kyriakis and Avruch, 2012). Apart from the most famous, giving its name to the kinase, transcription factor c-Jun, the diverse JNK substrates fit in all different groups of proteins. Moreover they are scattered across all cellular compartments (Haeusgen et al., 2011). The MAPK signaling cascade permits cells to understand the external signals and react accordingly. Only in the cell nucleus there are at least 26 substrates for JNK, among which many are transcription factors that modify gene expression following a specific stimulus (Bogoyevitch and Kobe, 2006). Yet the cellular functions behind JNK regulation range from differentiation and proliferation to movement and inflammation. Thus cells have acquired novel mechanisms that allow a particular signaling cascade to regulate various functions in response to a wide array of stimuli.

The specificity of the JNK pathway is achieved by a number of mechanisms that control components of MAP kinase module both temporally and spatially (Hibi et al., 1993, Brown and Sacks, 2009). Scaffold proteins are an essential element to this puzzle. By bringing together several components of the JNK cascade these multidomain proteins assemble into a signaling complex (Davis, 2000, Park et al., 2003). They allow signals to pass quickly within the module but also they may serve a catalytic role in some cases. Importantly, they isolate signaling cascades from each other hence reducing possible crosstalk (Whitmarsh and Davis, 1998). Scaffolding is just one of the mechanisms ensuring specificity to the JNK kinase cascade. Studies on c-Jun exposed the need of a distinct phosphorylation site sequence of amino acids on the substrate (Adler et al., 1994, Dérijard et al., 1994). This domain has been named as JNK-binding domain or δ domain (Dai et al., 1995). It is also known as common docking domain (CD), thanks to their role in facilitating communication between other MAPKs and their target proteins, various activators and scaffolds (Tanoue et al., 2000). The presence of CDs in a protein greatly improves the specificity and efficiency of target protein phosphorylation (Sharrocks et al., 2000).

Although today we understand the main mechanisms behind JNK pathway specificity, little is still known about various JNK isoforms and their substrate penchants. Despite a high degree of sequence similarities among JNK isoforms, there seems to be a predilection for different substrates. A good example has been provided by Gdalyahu et al. 2004, who showed that it is JNK2 but not JNK1 that preferably interacts with doublecortin (DCX). Furthermore based on *in vitro* and *in vivo* binding assays, JNK2 and not JNK1, has been concluded to be the interacting partner for human ATFa (Bocco et al., 1996) while the E3 ligase Itch is a preferred target for JNK1 (Gao et al., 2004). Moreover JNK1 among all JNKs shows preference for SCG10 phosphorylation (Tararuk et al., 2006). Therefore for future studies it seems of a high importance to continue the search for isoform-dependent roles of JNKs.

4. Neuronal cytoskeleton

The cytoskeleton is one of the fundamental elements that describe a eukaryotic cell. In neurons it refers to three distinct yet interacting complexes, each of which has discrete qualities. These are microtubules (MTs), intermediate filaments (IFs) and microfilaments (MFs) (Kirkpartick and Brody, 1999). The general basic structural elements of MTs are the same in neuronal and non-neuronal cells. However, brain MTs create quite a distinct pool with a variety of tubulin isotypes (some neuron-specific eg. class III, IVa), post-translational tubulin modifications (PTMs) and collection of microtubule-associated proteins (MAPs) than is present in other cell types. Moreover, organization of MTs differs significantly between axons and dendrites in at least two aspects. For instance, MTs present in axons are uniformly oriented, with a plus-end directed towards the tip of the neurite, while in dendrites MTs show mixed orientation, having plus-end aiming at either the tip of the dendrite or cell body. In addition these two cellular compartments differ in their complement of microtubule associated proteins with MAP2 protein mostly present in dendrites and tau in axons (Conde and Caceres, 2009). Furthermore, a variety of post-translational modifications (PTMs) including polyglutamylation, polyglycylation, acetylation, phosphorylation, palmitoylation and detyronisation may affect stability of MTs or their structure. In addition, they also play a role in the recruitment of specific effectors (Hammond et al., 2008). Some PTMs like detyrosination has been widely used to identify a stable pool of MTs (Schulze et al., 1987).

The IFs are uniformly distributed across the cell body. Within this large protein family are the neurofilaments (NF), the primary IF subtype expressed in neurons (Trojanowski et al., 1986). They consist of three subunits, known as a NF triplet: high, medium and low molecular weight, 180 to 200 kDa, 130 to 170 kDa and 60 to 70 kDa, respectively. Each subunit is a product of a separate gene (Fliegner and Liem, 1991, Lee and Cleveland, 1996). The NF triplet proteins are neuron specific and are classified as group IV IFs together with α-internexin, nestin and synemin. Other brain-related IFs belong to class III, including vimentin, GFAP and peripherin. The NF triplet proteins keep the central core domain of 40kDa, which structurally is a characteristic element of all IFs (Lewis and Cowan, 1985). Furthermore, they can be few hundreds micrometers in length. Although they look as ropelike fibers similarly to other IFs, they have a distinct side arm protruding from the surface. The projecting arm is a small 10kDa amino-terminal domain on one side and a carboxy tail domain on the other (Fliegner and Liem, 1991). In neurons IFs are important elements determining axonal caliber. This is particularly significant in large fibers (Lee and Cleveland, 1996).

Microfilaments are made of actin and resemble a string of pearls when two threads of actin get twisted around each other (Theriot, 1994). Microfilaments are found everywhere in neurons and glia, however they tend to concentrate at particular regions inside the cell. These are plasma membrane, presynaptic terminals, dendritic spines as well as growth cones (Hitt and Luna, 1994). Actin filaments are yet another set of highly dynamic elements of the neuronal cytoskeleton undergoing cycles of continuous polymerization and disassembly. The switch between G-actin (monomeric form) and F-actin (filamentous form) is regulated by the hydrolysis of ATP. Similarly to microtubules, F-actin polymers exhibit structural polarity with a minus (pointed) end at one side and plus (barbed) end at the other. The barbed end is known for its dynamic growth due to addition of G-actin-ATP bound monomers while ADP-bound monomers dissociate from the pointed end. The actin filament treadmilling process of continuous polymerization/depolymerisation is utilized in a variety of cellular processes including motility, adhesion, endocytosis and in regulation of cell morphology (Pollard and Borisy, 2003).

4.1 From simple tubulin dimers to complex filamentous structures

Microtubules consist of α/β tubulin heterodimers that joined end to end with alternating α and β subunits form a protofilament. These laterally associated building blocks form hollow cylinders about 25 nm wide. Protofilaments are bound to each other via noncovalent interactions (Desai and Mitchison, 1997). To serve their role microtubules need to be correctly nucleated. It was thought that this only takes place at the region of the centrosome where centrioles together with pericentriolar material create a microtubule friendly environment (Mandelkow and Mandelkow, 1995, Pereira and Schiebel, 1997). However, it came into attention that in some cells, like A498 kidney cell lines or retinal pigment epithelial cells, the process of nucleation can be centrosome independent (Yvon and Wadsworth, 1997, Efimov et al., 2007). As previously mentioned, Golgi outposts may serve as microtubule nucleation centers (Ori-McKenney et al., 2012). Moreover, during the development of hippocampal neurons the centrosome is no longer needed for nucleation, as neurites continue to expand even after laser ablation of the centrosome (Stiess et al., 2010). One of the key players in the process of nucleation is γ-tubulin which, when recruited, leads to microtubule assembly at their minus end (Raff et al., 1993, Zheng et al., 1995).

Microtubules present distinctive polarity, which reflects their role in a variety of cellular processes. Due to the arrangement of α/β tubulin heterodimers within the microtubule, one end of the cylinder always has α-tubulin exposed (minus end) whereas the other end, referred to as plus end, always has β uncovered. Present in all eukaryotic cells, these dynamic polymers take part in cell division or intracellular transport. In addition they maintain cell shape and are involved in cell motility (Steinmetz, 2007).

The fundamental feature of microtubules is their dynamic instability at the plus growing end (Desai and Mitchison, 1997). The concept of dynamic instability depicts a constant change between depolymerization/shrinkage and polymerization/growing phase. This process is tightly controlled by an internal equilibrium between stabilizing and destabilizing factors (Desai and Mitchison, 1997). Each α and β subunit of tubulin is bound to guanosine triphosphate (GTP). The GTP attached to a α subunit is neither hydrolysable nor exchangeable. Importantly, the GTP bound to a β subunit is both exchangeable, when the dimers are unassembled, and hydrolysable (Wang et al., 2007). Formation of MTs involves hydrolysis of β-bound GTP to GDP and P_i, yet it is not dependent on it (Hyman et al., 1992, Vandecandelaere et al.,

1999). However, the MT disassembly rate is regulated by GTP hydrolysis (Carlier et al., 1984). While the site for GTP hydrolysis is predominantly placed in the tubulin β subunit, some residues are also present in the α subunit. Hence hydrolysis is completed when both subunits form heterodimers upon head-to-tail assembly (Nogales et al., 1998). In neurons, microtubules are typically formed at the centrosome area. When ready, protofilaments are released by the MT-cutting protein katanin (Baas, 1999). At this point, stabilized by a variety of microtubule associated proteins, protofilaments can be transported to axons and dendrites (Nunez and Fischer, 1997).

4.2 Microtubule-associated proteins (MAPs)

Microtubule-associated proteins are divided into a couple of distinct groups. Some, like MAP1, MAP2, MAP4, tau or doublecortin (DCX), belong to structural MAPs, which stabilize and promote microtubule outgrowth. Others, like motor proteins, tightly yet transiently bind to tubulin which facilitates intracellular transport (Mandelkow and Mandelkow, 1995). The general organization of structural MAPs reveals their function in cross-linking microtubules in the cytosol. They consist of two domains: basic tubulin interacting domain and an acidic projection domain, expanding from the microtubule surface and binding it to a variety of cellular structures including membranes, IFs or other microtubules (Lodish et al., 2000). MAPs coat the microtubule lattice and prevent the dissociation of tubulin hence regulating the rate of MAPs binding which can control the length of microtubules (Lodish et al., 2000). They work as anchors for a number of cytoplasmic proteins like protein kinases (Rubino et al., 1989, Serrano et al., 1989) and other cytoskeleton related proteins (Selden and Pollard, 1983). Therefore the presence of a variety of different MAPs along the microtubule lattice may to some extent control structure and properties of microtubules at different stages during neuronal development (Chen et al., 1992).

Sequence analysis of MAPs has further divided them into two types. Type I, comprised of MAP1A and MAP1B, is characterized by the presence of a number of acidic sequence repeats Lys-Lys-Glu-X (e.g. KKEV KKEI KKEE) thought to facilitate interaction with tubulin (Avila, 1991). These proteins form filamentous structures present both in axons and dendrites as well as in non-neuronal cells. The second type of MAPs consists of MAP2, MAP4 and tau. These MAPs contain 3-4 repeats of an 18-residue long

sequence present in the carboxy-terminal microtubule-binding domain. These sequences are divided by a sequence of inter repeats (Al-Bassam et al., 2002). MAP4 was previously found to be present in glial cells and in a variety of other non-neuronal tissues e.g. sertoli cells, cardiac, smooth or skeletal muscle (Parysek et al., 1984). Recently MAP4 has also been shown to be present in brain-specific cells including Purkinje cells in the cerebellum, and pyramidal neurons of the hippocampus (Tokuraku et al., 2010). MAP2 and tau however, are brain specific MAPs enrolled in maintenance of microtubule dynamics in dendrites and axons, respectively (Drewes et al., 1998). Interestingly, the low molecular weight-MAP2 is expressed in axons and glial cells (Doll et al., 1993). *In vivo* studies revealed that the regulation of MAPs by phosphorylation influences their ability to bind to microtubules (Brugg and Matus, 1991). Moreover, phosphorylation affects the binding properties of MAPs in different ways. For instance, as shown by Itoh et al., (1997) when MAP2 is phosphorylated by protein kinase A (PKA) 70% of phosphates are found in the projection arm. However, when using cyclin B dependent kinase cdc2, over 60% of incorporated phosphates were distributed over the microtubule binding region. Importantly, the MAP2 protein, like MAP1B, is phosphorylated by JNK *in vitro* (Kyriakis and Avruch, 1990, Chang et al., 2003, Björkblom et al., 2005). Microtubule assembly is impaired in *Jnk1-/-* mouse brains where both MAP2 and MAP1B are hypophosphorylated, which in turn decreases their ability to bind and stabilize microtubules (Chang et al., 2003). It was moreover demonstrated that MAP2 is phosphorylated by JNK in intact cells and that this process plays a key role in maintenance of dendrite homeostasis (Björkblom et al., 2005).

Tau, on the other hand, as a specific microtubule stabilizer in axons, forms neurofibrillary tangles when hyperphopshorylated in patients suffering from AD. Accumulation of hyperphosphorylated tau is clinically linked to loss of cognitive functions, mood disturbance, debilitating language and motoric skills (Huang and Jiang, 2011). Doublecortin, known as DCX, is yet another important MAP which is JNK's pool of cytosolic substrates (Gdalyahu et al., 2004). Mutation in the X-linked DCX gene leads to abnormal brain lamination that results in smooth brain in males and double cortex in females, as a consequence of impaired neuronal migration (des Portes et al., 1998, Gleeson et al., 1999). Various mutations within the conserved region of Doublecortin domain impair DCX interaction with microtubules (Sapir et al., 2000). Three JNK phosphorylation sites (T331,

S334 and T321) found in DCX seem to play an important role in regulation of neurite length and cell migration (Bai et al., 2003, Gdalyahu et al., 2004). It was shown in PC12 cells that expression of unphosphorylable by JNK $DCX^{T331A,S334A}$ leads to neurite-like structures outgrowth inhibition. Conversely, the presence of pseudophopshorylated $DCX^{T331E, S334E}$ stimulated not only increased neurite length but also the total number of extensions (Gdalyahu et al., 2004).

5. Stathmin family proteins are potent microtubule destabilizers

Stathmin is a small (19kDa) cytosolic, ubiquitously expressed phosphoprotein (Sobel et al., 1989, Ozon et al., 1997). It binds tubulin heterodimers, which leads to microtubule depolymerization (Belmont and Mitchison, 1996, Jourdain et al., 1997). Its expression level profile is tightly controlled during development and tissue regeneration (Koppel et al., 1990, Pampfer et al., 1992, Koppel et al., 1993, Okazaki et al., 1993a). Interestingly, stathmin overexpression in human sarcoma has been associated with cell migration and invasion (Baldassarre et al., 2005). While stathmin plays a significant role in spindle formation during cell-cycle progression, the other members of the family were found to participate in structural and functional plasticity of neurons (Riederer et al., 1997, Tararuk et al., 2006, Poulain and Sobel, 2007). The stathmin family proteins comprise of stathmin, SCG10, SCG10- like protein (SCLIP), RB3 and its two splice variants RB3' and RB3'', which is schematically represented in Figure 6 (Sobel et al., 1989, Ozon et al., 1997, Ozon et al., 1998).

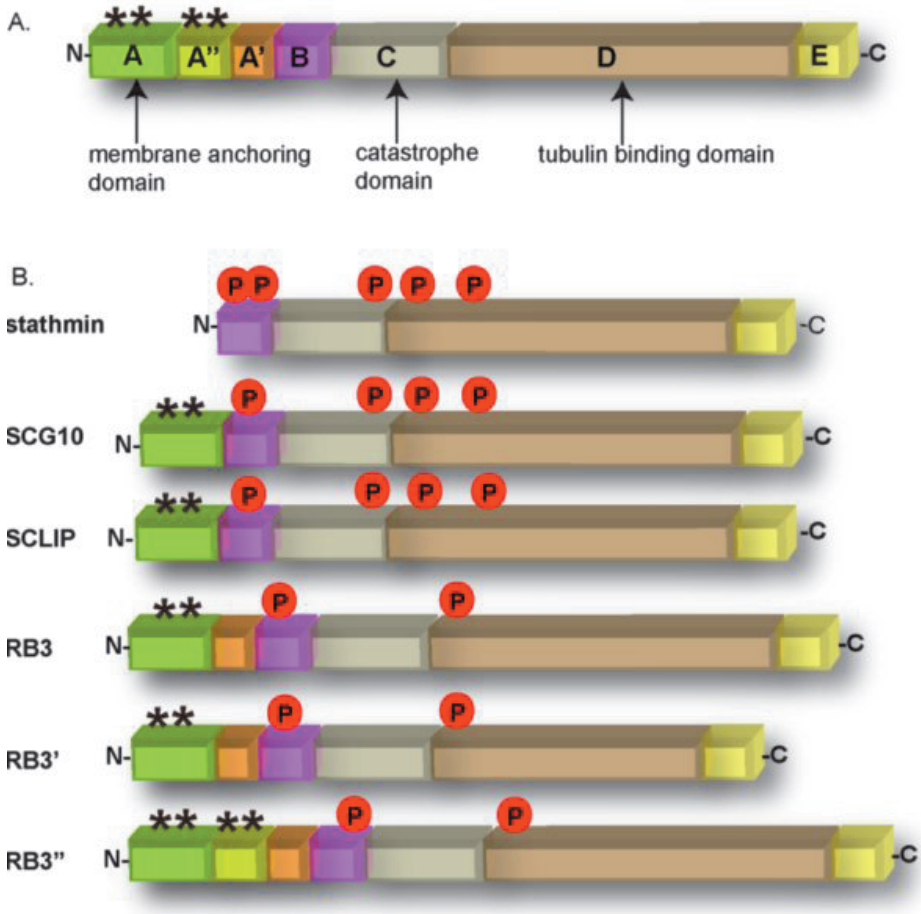


Figure 6. Schematic representation of stathmin family members. A. General scheme of all stathmin domains identified to date. Domain A and A'' contain cysteine residues (indicated with an asterisk sign) that provide an anchor to the membrane. Unlike other family members, stathmin lacks domain A and cannot bind to membrane and remain cytosolic. B. Unlike the N-terminus, the C-terminus that contains a tubulin binding domain is highly conserved among family members. RB3'' protein contains two additional A domains, namely A' and A''. Phopshorylation sites in all members are marked with letter P (Adapted from Mori and Morii, 2002).

Detailed analysis of evolutionary relationships between members of the stathmin family revealed that SCG10 and SCLIP (most closely related to each other) originate from a mutual ancestral gene, although stathmin and RB3 with its splice variants diverged earlier from the same gene (Ozon et al., 1998). Rat RB3 was initially discovered as a homologue of a Xenopus B3 (XB3) protein, whereas RB3' and RB3'' are the result of its alternative splicing

(Maucuer et al., 1993, Ozon et al., 1997). The RB3′ protein, due to an insertion of a stop codon within the tubulin biding domain, is approximately 13 aa smaller than RB3 (Okazaki et al., 1993b), whereas RB3′′ possess an additional A′′ 27 aa long sequence between domain A and A′ that is present also in RB3′ (Ozon et al., 1997, Ozon et al., 1998). Nevertheless they share a high degree of similarity with stathmin, which in fact is greater than with SCG10 (Ozon et al., 1997).

Stathmin is most abundantly (about 0.25% of total protein content) expressed in the brain, where it is equally present in different cellular compartments (Gavet et al., 2002). It contains five putative phosphorylation sites (Ser 16, Ser 25, Ser 38, Ser 46 and Ser 63). All apart from Ser 46 have been shown to be phosphorylated both *in vitro* and *in vivo* by various kinases (Beretta et al., 1993). Most of those sites are conserved in SCG10 as well as in SCLIP, whose consensus phosphorylation sites are identical to those in SCG10 (Ozon et al., 1997, Ozon et al., 1998). Interestingly, RB3 and XB3 protein contain only two conserved sites corresponding to Ser 16 and Ser 46 (Ozon et al., 1997, Mori and Morii, 2002). RB3/RB3′ and RB3′′ proteins, unlike soluble stathmin, are localized to the perinuclear region labeling medial compartment of GA and growth cones (Gavet et al., 1998, Gavet et al., 2002). Due to the N-terminal extension, SCG10 and SCLIP are also associated with membranes and similarly to RB3s are present in the GA region, GA associated vesicles and growth cones (Lutjens et al., 2000, Gavet et al., 2002, Tararuk et al., 2006). SCG10 is present in the trans-GA compartment, whereas SCLIP appear in medial to trans region of GA (Lutjens et al., 2000, Poulain et al., 2008). Remarkably, contrary to stathmin, SCG10, SCLIP and RB3 with its splice variants are present in a very small population of cells (Gavet et al., 2002).

Stathmin is highly enriched in the brain, yet its expression is the highest during development and decreases postnatally (Gavet et al., 2002). Similarly, SCG10 expression can be found during early embryonic stages (E10.5-E11.5), yet its transcript declines after birth with few specific regional exceptions e.g. the olfactory bulb, the hippocampus and the cerebellum (Anderson and Axel, 1985, Gavet et al., 2002, Mori and Morii, 2002). Conversely, SCLIP and RB3 are present at a rather low level during early development, but their expression level peaks postnatally and mostly in adulthood (Ozon et al., 1998).

The most prominent feature of stathmins is their role in the control of microtubule dynamics (Belmont and Mitchison, 1996, Jourdain et al., 1997, Curmi et al., 1999). They inhibit polymerization of tubulin or stimulate disassembly of microtubules in a process that is reliant on phosphorylation (Marklund et al., 1996, Steinmetz, 2007, Wang et al., 2007). It is well known that stathmins form a ternary T_2S complex with tubulin by sequestering two α/β heterodimers (T_2) per one molecule of stathmin (S) (Gigant et al., 2000, Wang et al., 2007). All family members contain a stathmin-like domain (SLD) that is responsible for interactions with tubulin and T_2S complex formation. The overall arrangement of the T_2S complexes is very similar among stathmins, however their stability is different (Jourdain et al., 2004, Wang et al., 2007). For instance, the T_2S complex formed by RB3 has been shown to be more stable than the one with stathmin (Jourdain et al., 2004, Ravelli et al., 2004, Steinmetz, 2007). There are two tubulin-binding sites within SLD and their positions are interchangeable and rearrangement of those greatly affects stability of the T_2S complexes (Jourdain et al., 2004). A great amount of stathmin remains either hypophosphorylated or even unphosphorylated in cultured rat neurons. This in turn leads to sequestration of a significant amount of free tubulin, inducing catastrophe events (Gavet et al., 2002).

Interestingly, knockout mice of stathmin develop normally and show no overt neurological or behavioral abnormalities when compared to the wild type cohort (Schubart et al., 1996). However with age, knockout mice develop axonopathy observed both in the central and the peripheral nervous system (Liedtke et al., 2002) similar to a condition noted in *Jnk1* knockouts mice (Chang et al., 2003). Although stathmin is enriched in the amygdala, no clear anomalies in morphology of pyramidal cells of the lateral amygdala were observed in the knockout mice. Similarly, CA1 hippocampal neurons seemed unaffected by depletion of stathmin (Shumyatsky et al., 2002, Shumyatsky et al., 2005). Additionally, in rat PC12 cells, depletion of stathmin prevented their differentiation into sympathetic-like neurons following NGF stimulation (Di Paolo et al., 1997b). Furthermore, the total dendrite length was decreased in Purkinje cells when stathmin expression was silenced with siRNA (Ohkawa et al., 2007). Unlike in *D. melanogaster,* where a single gene encodes the whole family of stathmins, in vertebrates some functional redundancy is expected among different family members. Hence the knockout phenotype seems rather mild in mice, but not in the fruit fly, where inactivation of stathmin with RNAi leads to cell migration arrest, loss of commissures, and other serious anomalies (Ozon et al., 2002).

5.1 JNK substrate Superior Cervical Ganglion 10 protein (SCG10)

Unlike ubiquitously expressed, soluble stathmin (Sobel et al., 1989), SCG10 is a neuron-specific and membrane-associated protein, although it is not an integral part of the membrane (Stein et al., 1988a). Initially, SCG10 was discovered during a screen of neural crest-derived cells (Anderson and Axel, 1985). Its expression is under control of neural restrictive silencer (NRS), which in non-neuronal cells binds to neural restrictive silencer factor/repressor element-1 silencing transcription (NRSF/REST), hence suppressing SCG10 expression (Mori et al., 1992). The NRSF/REST is predominantly expressed in non-neuronal cells as well as in undifferentiated neurons. Its expression however declines upon neuronal maturation (Armisen et al., 2002).

In the brain, it has been shown that SCG10 associates with punctuate structures accumulating in the perinuclear cytoplasm of developing neurons (Stein et al., 1988a). Its expression is developmentally upregulated and peaks during differentiation of central and peripheral neurons (E10-E11) and declines within a few weeks of postnatal development (Sugiura and Mori, 1995). Similarly, cultured cortical neurons present high level of SCG10 during neurite outgrowth, yet after the formation of synapses the amount of protein is dramatically decreased (Di Paolo et al., 1997a). In general, the level of SCG10 significantly declines in adulthood. However, it persists in the regions of synaptic plasticity, including long-distance projection neurons and neurons with extensive dendritic arborisation (Himi et al., 1994). SCG10 and stathmin share 70-80% sequence homology within the conserved region (Mori and Morii, 2002). Moreover, the SCG10 gene originated from stathmin by the duplication and additional modification of the ancestral stathmin gene (Okazaki et al., 1993b). In contrast to stathmin, SCG10 has an amino-terminal extension necessary for its membrane targeting and GA localization (Figure 6) (Di Paolo et al., 1997b, Di Paolo et al., 1997c). Two cysteine residues (Cys 22 and Cys 24) within the amino-terminal domain are essential for GA sorting and growth cone targeting of SCG10 (Lutjens et al., 2000). As a result of its distribution in the central domain of a growth cone, where population of highly dynamic microtubules accumulate, it is considered to be a regulatory factor of microtubule dynamics (Grenningloh et al., 2004). It has previously been shown that overexpression of SCG10 stimulates neurite outgrowth in neuronal cells, which is thought to be dependent on dynamic instability of microtubules (Riederer et al., 1997).

SCG10 contains several phosphorylation sites. It has been shown that protein kinase A (PKA) phosphorylates SCG10 on Ser 50 and Ser 97, while Ser 62 and Ser 73 are targets for MAP kinases. Additionally, Ser 73 can be utilized by the cyclin dependent kinase (Antonsson et al., 1998).

In contrast to the developing brain, the adult brain has very little capacity of regeneration. There are, however, a few regions where this ability has been retained. These are for instance the ventricular zone, the olfactory bulb, and the dentate gyrus (Cameron and McKay, 2001). It was found that SCG10 and JNK are present in the pyramidal neurons of CA1 and CA3 region of the hippocampus in four months old mouse brains. Furthermore, both proteins were found in two types of cells in the olfactory bulb. These are mitral and periglomerular cells (Westerlund et al., 2008).

Interestingly, it was shown that Ser 73 may be phosphorylated in response to kainate stimulation and might be a target for another MAP kinase (Morii et al., 2006). Injection of the stimulant led to NMDA receptor activation and induction of the Ras-Erk2 pathway, which in turn induced phosphorylation of SCG10 on Ser 73 (Morii et al., 2006). Importantly, activation of NMDA receptors in postsynaptic area results in dephosphorylation of MAP2 protein (Halpain and Greengard, 1990, Quinlan and Halpain, 1996). This shift could potentially lead to more stable microtubules; hence the synaptic connection could take on a firmer structure consequently intensifying synaptic efficiency (Morii et al., 2006). It is however important to mention that no other kinases were tested in the proposed model.

5.2 Microtubule destabilizing activity of SCG10 in the nervous system

Similar to stathmin, SCG10 can form a ternary complex with tubulin by binding its heterodimers (Fleury et al., 2000, Charbaut et al., 2001). Upon phosphorylation, this interaction can be abolished as shown both *in vitro* and in intact cells (Gavet et al., 1998). Like stathmins, SCG10 can influence microtubule stability by plus-end stabilization and minus-end catastrophe events (Riederer et al., 1997, Howell et al., 1999, Togano et al., 2005). However, it seems that the minus-destabilizing activity is more favorable than the stabilizing effect at the plus-end (Manna et al., 2007).

In an *in vitro* assay SCG10 hinders polymerization of microtubules in a dose dependent manner (Grenningloh et al., 2004). It can be as efficient as

stathmin in inducing catastrophe. It not only co-purifies with microtubules but in addition, it may neutralize the stabilizing effect of taxol (Riederer et al., 1997). It has been demonstrated that SCG10 is phosphorylated both *in vitro* and *in vivo* by JNK (Tararuk et al., 2006). The presence of JNK is crucial during embryonic development as well as in adulthood where it plays a role in defining neuronal cytoarchitecture (Kuan et al., 1999, Björkblom et al., 2005). Interestingly, Tararuk et al. (2006) found that when regulated by JNK, SCG10 is in control of axodendritic length. Expression of SCG10 $^{62A/73A}$, an unphosphoryable by JNK form of SCG10, hindered recovery of tubulin by 50% in photobleaching experiments. As previously mentioned, stathmins comprise of two separate tubulin-binding motifs and sequestration of free tubulin leads to depolymerisation of microtubules at the minus end (Gigant et al., 2000). It is indeed intriguing that SCG10 can stabilize microtubules at the plus-end (Manna et al., 2007). The GTP-bound tubulin is known to connect tightly to other subunits within the microtubule lattice; hence the GTPase activity plays an essential role in separating tubulin heterodimers from the plus-end. Importantly, stathmins decrease the GTPase activity of tubulin dimers when incorporated into the protofilament (Wang et al., 2007). Therefore, one may expect stathmins to stabilize the plus end of microtubules containing recently added, GTP-bound tubulin (Westerlund et al., 2008).

Moreover the connection formed between zippered and unzippered microtubule lattice revealed that protofilaments undertake an outward twist, which is similar to that formed by tubulin heterodimers when bound to stathmin (Gigant et al., 2000). Hence it may be advantageous energy-wise for SCG10 to bind to tubulin exposed at the zipper junction (Westerlund et al., 2008).

5.3 The role of SCG10 in the disease state and neuronal regeneration

Although under physiological conditions SCG10 is present only in cells of the central and peripheral nervous system, an anomalous expression of SCG10 mRNA was noticed in nonsmall lung carcinoma cell lines which are lacking NRS binding proteins Brm and BrG (Watanabe et al., 2006). Moreover, a recently published report indicates that SCG10 contributes to an aggressive phenotype of gastric cancer (Guo et al., 2013). A variety of diseases that involve architectural remodeling as in the case of Budd-Chiari syndrome, describe upregulation of SCG10 expression (Paradis et al., 2005). Budd-Chiari syndrome

is characterized by obstruction of the hepatic venous system in young adults and the character of the disease may be acute, subacute and chronic (Valla and Benhamou et al., 1999). Upregulation of SCG10 was observed in the chronic phase, associated with extensive liver fibrosis (Paradis et al., 2005). During axon regeneration in the olfactory epithelium not only SCG10 but also stathmin is upregulated following injury (Pellier-Monnin et al., 2001). In addition, high levels of SCG10 are observed following stroke during a period of axonal sprouting in the peri-infarct cortex (Carmichael et al., 2005). Moreover, SCG10 mRNA increases within 72 h of a post-sciatic nerve crush and remains elevated up to 2 weeks post injury. The decline in mRNA level in the PNS is associated with re-innervation of the target (Mason et al., 2002). Interestingly, in another model of nerve injury a marked increase in SCG10 mRNA was noticed (Iwata et al., 2002). Although other family members of mRNAs were also induced, SCG10 appeared the quickest but similarly to others declined to control levels after a few weeks (Iwata et al., 2002).

A recent report from Shin et al., (2012) further indicates a role for SCG10 in preventing degeneration of transected axons in dorsal root ganglion neurons. They suggest that preservation of SCG10 levels post injury in the distal part of axons endorses movement of mitochondria and therefore postpones degenerative processes. Consequently, SCG10 is likely to be a moderator of regenerative processes, although the definite proof is still missing. Nevertheless we know nowadays that SCG10 is one of the key molecules in growth cone progression. Blockage of SCG10 function induces redistribution of the growth cone cytoskeleton, followed by pauses in expansion of an axon (Suh et al., 2004). Moreover, it has been suggested that SCG10 may participate in the remodeling of presynaptic terminals, since long-term potentiation induced its upregulation in rat hippocampal neurons (Peng et al., 2003). Conversely, SCG10 mRNA was downregulated in answer to monocular lack of visual stimulus in macaque monkeys, when shown by in situ hybridization (Higo et al., 2000). Currently it is believed that both SCG10 and stathmin have crucial roles in axonal outgrowth throughout the early developmental stages and during regenerative processes in adulthood. Yet few studies have implicated SCG10 and stathmin in neurodegenerative disorders. The work done by Okazaki and co-workers showed that in Alzheimer's patients neither the mRNA nor protein level of SCG10 or stathmin is affected, nevertheless SCG10 immunoreactivity in neuronal cell bodies is increased in AD-affected brain regions, which futher suggests impairment in axonal transport. Furthermore correlation analysis showed

that there is a positive correlation between the number of neurofibrillary tangles (NFTs) and SCG10 protein levels. This in turn suggests that the changed SCG10 compartmentalization and metabolism may contribute to events involved in tangle formation (Okazaki et al., 1995).

Recently, processing of amyloid precursor protein (APP), which plays a crucial role in pathogenesis of AD, has been linked to SCG10. It has been shown that SCG10 may be involved in nonamyloidogenic processing of APP by interacting with APP's intracellular part. High levels of SCG10 were associated with APP accumulating in post-Golgi vesicles and the cell surface (Wang et al., 2013).

SCG10 abnormal gene expression has also been reported in neurospheres from Down syndrome fetuses (Bahn et al., 2002). It was observed that neuronal precursors from the diseased brains showed severe deficiency in regulation by NRSF/ REST genes. As a consequence, it led to almost complete loss of SCG10 expression and had a severe impact on neuronal differentiation. Notably, stathmin expression is also affected in Down's syndrome (Cheon et al., 2001).

6. Excitotoxic stress in the brain

Glutamate is the principal excitatory neurotransmitter in the mammalian brain. Its activation is essential for proper synaptic plasticity. It plays a key role in learning processes as well as development of the central nervous system. However, its action at the synaptic cleft needs to be well regulated. An excessive release and insufficient reuptake of excitatory neurotransmitters may lead to excitotoxicity, which ultimately results in cell death (Nicholls and Attwell, 1990). In 1957, for the first time, Lucas and Newhouse demonstrated neurotoxic properties of glutamate by systemic delivery of glutamate to newborn mice, which in turn led to retinal degeneration (Lucas and Newhouse, 1957). Glutamate is a non-essential amino acid that is unable to pass through the blood-brain barrier (BBB), hence it needs to be produced by neurons using available precursors e.g. glutamine or glucose. Excitatory amino acid transporters (EAAT) are responsible for proper management of glutamate amounts in the synaptic cleft. That results in tenfold higher concentrations of glutamate inside the cell than outside (Maragakis and Rothstein, 2001).

There are two types of glutamate receptors in the human brain, known as metabotropic (mGluR) and ionotropic (iGluR). The metabotropic receptors are part of the G-protein-coupled receptors (GPCR) superfamily. In contrast to iGluRs, mGluRs produce lengthier postsynaptic responses that either intensify or reduce the excitability of postsynaptic cells. Hence the physiological responses they elicit may differ (Niswender and Conn, 2010). iGluRs are nonselective cation channels that permit Na^+, K^+ and in some cases Ca^{2+} ions to pass. Named after their agonists: NMDA (N-methyl- D-aspartate), AMPA (*α*amino-3-hydroxyl-5-methyl.4.isoxazole-propionate) and kainate, iGluRs elicit excitatory responses (Madden, 2002). Upon release, newly synthesized glutamate may bind to iGluRs present in the postsynaptic membrane, which in turn leads to their activation. Often glutamate toxicity can result from excessive stimulation of NMDA receptors (NMDAR) in particular (Choi et al., 1988). It has been demonstrated that extreme activation of NMDAR is partly responsible for pathological processes associated with different forms of neuronal insult including stroke, *status epilepticus* and a variety of neurodegenerative disorders (Hynd et al., 2004). The excitatory postsynaptic potentials (EPSPs) created by overstimulation of NMDA receptors leads to Ca^{2+} influx which, when excessive, may cause excitotoxic death. The sudden entry of Ca^{2+} combined with excessive NMDAR activity is believed to play a crucial role in mediating neuronal cell death (Arundine and Tymianski, 2003).

6.1 Structure of NMDA receptors

To date, three subfamilies of NMDAR subunits have been described. These are i) the NR1 subunit, ii) NR2A, 2B, 2C, 2D and iii) two NR3 subunits A and B (Van Dongen, 2009). The NR1 subunit is associated with the functional NMDAR and has several splice variants, which may affect the properties of the channel. Structurally NMDAR are heterotetramers, comprised of two NR1 subunits and two NR2 or NR3 subunits (Papadia and Hardingham, 2007). Due to great variability in the electrophysiological profiles of NR2 subunit it potentiates the activity of the channel adjusting the functional properties of the receptor (Madden, 2002). The most recently discovered NR3 requires interaction with the NR1 subunit for correct membrane placement (Perez-Otano et al., 2001).

In general, all ionotropic receptors show a similar structural design. They consist of i) an extracellular N-terminal domain (NTD), a 400 aa long

sequence which plays a role in proper assembly of the receptor, trafficking and its function, ii) the S1S2 ligand-binding domain (LBD) which is followed by a re-entrant loop domain outlining the ion channel as well as iii) the intracellular C-terminal tail of NMDAR which connects the receptor to various signaling cascades (reviewed in Madden, 2002).

NMDARs possess unique properties. Apart from permitting entry of different ions they also bind extracellular Mg^{2+}, which under resting potentials blocks the pore of NMDAR. However, this blockage is current-dependent and depolarization forces ion out allowing passage of other cations (Popescu and Auerbach, 2004). Another interesting feature of NMDAR is that opening of the channel needs a co-agonist, e.g. glycine or D-serine, which can bind to the NR1 subunit (Shleper et al., 2005).

Table 5. Summary of the NMDAR expression pattern

Subunit	Expression time	Brain region
NR1	Starts E14 Peaks P21	• Homogenous expression throughout brain
NR2A	Starts P0 Peaks P14	• Hippocampus • Cortex • Throughout brain (P14 onwards)
NR2B	Starts E14	• Prenatal cortex • Thalamus • Spinal cord • Colliculi • Hippocampus • Hypothalamus
NR2C	Starts P0 Peaks P21	• Cerebellum • Forebrain
NR2D	Starts E14 Peaks P7	• Diencephalon • Mesencephalon • Spinal cord • Cortex • Hippocampus • Septum
NR3A	Starts E15 Peaks P8	• Spinal cord • Medulla • Pons • Tegmentum • Hypothalamus • Thalamus
NR3B	Peaks P14	• Motor neurons of the brain stem • Spinal cord

Based on Ewalds and Hollins , 2009

The development of glutamatergic synapses is essential for proper brain function. Although there are numerous components that modulate synapse growth, synaptic activity is one of the key elements. In the early stages of development this function is mainly governed by the NMDAR (Durand et al., 1996, Wu et al., 1996a, Aizenman and Cline, 2007). The expression time for NMDAR subunits starts at around E14-15 (Table 5). All members of the NMDAR family show diverse yet overlapping expression patterns during development, which is summarized in Table 5 (Cull-Candy and Leszkiewicz, 2004).

The NR1 subunit shows the highest expression level at around three weeks post delivery, and later on it drops, reaching adult levels (Paupard et al., 1997). Some isoforms are expressed equally throughout the brain whereas some show a more restricted pattern in the cortex or hippocampus (NR1-1), or thalamus and cerebellum (NR1-4) (Laurie and Seeburg, 1994, Lee-Rivera et al., 2003). Among the NR2 subunits only NR2B and NR2D have been detected in embryos. Unlike NR2B, which is present in the developing cortex, thalamus and spinal cord, NR2D is expressed in the diencephalon, mesencephalon and in the spinal cord. At P0 NR2A begins to appear in hippocampus and NR2C mRNA can be detected in cerebellum (Cull-Candy et al., 2001). Similar to NR1 subunits the expression levels of various NR2 isoforms starts to decline after the first three weeks of life (Monyer et al., 1994). For example, NR2B and NR2D, although highly expressed in embryonic brain during development, are supplemented with or even replaced by NR2A and 2C (Cull- Candy et al., 2001). The most recently discovered NR3A and NR3B subunits are present in embryos showing a differential expression pattern (Sucher et al., 1995, Nishi et al., 2001). Interestingly, NR3s reach the highest expression level about 2 weeks post delivery and remain high throughout later life (Matsuda et al., 2002).

6.2 Functional role and clinical risks of NMDA receptors activation

Transgenic animals lacking single NMDAR subunits show no overt brain malformation. However, they die briefly after birth for various reasons including respiration failure ($NR1^{-/-}$) or lack of suckling responses ($NR2B^{-/-}$) (Forrest et al., 1994, Kutsuwada et al., 1996). The synaptic activity, which is essential for the incorporation of new neurons into existing neural circuits, is diminished in these animals (Sakimura et al., 1995, Tashiro et al., 2006).

Interestingly, deletion of other NMDAR subunits does not alter viability and mice mature (Kadotani et al., 1996).

It has been shown that blocking NMDA receptors *in vivo* leads to an increased level of cell death, especially among cerebellar granule neurons (CBGs) (Monti and Contestabile, 2000). Moreover, the survival rate of CBGs in culture is severely impaired upon NMDAR blockage (Ciani et al., 1997). Furthermore, it was found that NMDAR antagonists in spinal cord cultures stimulate death rate by hindering electrical activity (Brenneman et al., 1990). Interestingly, the cAMP-response-element-binding-protein (CREB) is thought to mediate pro-survival responses in answer to NMDAR stimulation (Mantamadiotis et al., 2002). Following ischemia and exposure to glutamate, entry of Ca^{2+} triggers phosphorylation of CREB at Ser133, which in turn has neuroprotective implications (Walton and Dragunow, 2000). However, activation of extrasynaptic NMDAR have an opposing effect on CREB and stimulate cell death (Hardingham et al., 2002). During ischemia, the balance of glutamate, which is normally managed by glutamate transporters, is severely impaired. In addition, instead of removing the excess of glutamate, the transporters release it to the extracellular space leading to extrasynaptic NMDAR activation and cell death (Rossi et al., 2000). While entry of Ca^{2+} through activation of extrasynaptic receptors may lead to phosphorylation of CREB, it does not result in pro-survival gene stimulation but leads to cell death (Rossi et al., 2000). This implies that the spatial localization of NMDARs may influence their downstream action; promoting either survival or death (Hardingham et al., 2002).

During development, NMDAR excitatory neurotransmission plays a fundamental role in establishing synaptic connections and refinement of functional circuits. Their activity is critical for mechanisms involved in learning and memory; then again glutamate toxicity is an indisputable source of neural damage during stroke and neurodegenerative diseases including AD, ALS or Huntington's disease (Riccio and Ginty, 2002, Papadia and Hardingham, 2007). These contradictory functions of NMDARs may provide some explanation as to why there is poor tolerance and effectiveness of NMDAR antagonist used in clinical trials following excitotoxic insult. Furthermore, blocking NMDAR compromises normal brain function, leading to serious neurological and psychiatric consequences (Gilmour et al., 2012, Lakhan et al., 2013).

It is worth remembering that the character of various disorders in which excitotoxicity has been involved shows, that most likely there are variations in the basic excitotoxicity process. Since the starting events may be of a various nature including: vascular/ ischemic, as seen in stroke, genetic, metabolic or immunologic, then both the temporal and anatomical features may be significantly different (Lynch and Guttman 2002). The differences among excitotoxicity models may also reflect the activation of receptors other than NMDA, like AMPA/kainate receptors activated in multiple sclerosis (Pitt et al., 2000). Finding the main post-receptor signaling event responsible for cell death could therefore provide a target for potential therapeutic interventions and save the lives of many people suffering from various neurodegenerative diseases.

6.3 The role of JNK in excitotoxicity

Excitotoxocity has been linked to many brain injuries over the years, as has been the JNK kinase. A pivotal study assessing the role of JNK in excitotoxic death was based on studies done in *Jnk3* knockout mice. *Jnk3*-/- mice presented reduced seizures as well as decreased cell death in response to systemic administration of kainite (Yang et al., 1997). It has been shown that kainite stimulates expression of c-Jun and c-Fos, the AP-1 complex proteins, leading to activation of JNK in the hippocampus (Yang et al., 1997). In addition, mutant mice of c-jun, in which JNK phosphorylation sites are mutated to alanines, showed resistance to excitotoxic insult. This implies that blocking JNK from accessing c-Jun may have a neuroprotective effect (Behrens et al., 1999). Interestingly, the *Jnk3* knockout mice resemble the glutamate receptor 6 (GluR6) deficient mice phenotype very much (Mulle et al., 1998). It was thus hypothesized that JNK may take part in GluR6 mediated excitotoxicity (Mulle et al., 1998).

Furthermore, it was shown that post-synaptic density protein (PSD-95) may provide a link between JNK and GluR6 (Savinainen et al., 2001). Post synaptic density proteins act to arrange a signaling matrix. It was shown that PSD95 may anchor two upstream activators of JNK, MLK2 or MLK3 (Garcia et al., 1998, Savinainen et al., 2001), and deletion of the binding site for GluR6 on PSD95 significantly decreased activation of JNK and cell death. Moreover, the presence of dominant–negative MLK2 or MLK3 considerably reduced JNK activation (Savinainen et al., 2001). In cortical neurons, it was previously shown that activation of NMDAR leads to JNK activation already within 1

min of post-excitotoxic insult. Moreover, JNK activity remains high for the first 30 min, after which it slowly declines. This response was demonstrated to be dependent on flux of extracellular Ca^{2+} (Ko et al., 1998).

6.4 Animal models:

6.4.1 Middle Cerebral Artery Occlusion (MCAO) model of stroke

Stroke is one of the major causes of death and the foremost source of disability in the US (Rosamond et al., 2008). Although in a great majority of cases ischemic stroke is responsible, there is only one therapy approved for use during the acute phase. It is the thrombolytic tissue plasminogen activator (tPA) (Armstead et al., 2010). To date four main ischemic stroke models have been studied, namely complete global cerebral ischemia, incomplete global ischemia, focal cerebral ischemia (including MCAO) and multifocal cerebral ischemia (Graham et al., 2004).

The MCAO is most often used to mimic human condition in an animal. It is due to the fact that, in majority of cases, the ischemic stroke is a consequence of a thrombotic or else embolic occlusion found in the middle cerebral artery (MCA) (Durukan and Tatlisumak, 2007). The cerebral blood flow in the core of the infarct drops dramatically from 50-60mL/100g/min to less than 7mL/100g /min and so the damage in that region is permanent. However, the core is surrounded by so-called penumbra, where blood flow is retained at a level of 7 -17mL/100g/min. Although this region is electrically silent, metabolic activity can still be detected (Astrup et al., 1981, Baron, 1999). An excess of glutamate appears subsequently to cerebral ischemia (Budd, 1998). The core of the infarct emanates peri-infarct depolarizations (PIDs) towards the penumbra region. The PIDs can be a consequence of glutamate release or else K^+ and Ca^{2+} ions. The number of PIDs increases with the size of the infarct (Mies et al., 1993). In practice, their presence is useful as a tool to differentiate between the core and the penumbra (Ohta et al., 2001).

Calcium overload is an obvious consequence following release of glutamate, so are reactive oxygen species generated during an ischemic attack. They have been shown to act on several intracellular signaling pathways resulting in detrimental consequences. One such candidate is the p38 MAPK pathway, which is activated during stroke (Cao et al., 2005). Cerebral ischemia interrupts the blood-brain-barrier (BBB), damaging the

tight junctions of endothelial complexes (Mark and Davis, 2002). Modification in the structure of the BBB recruits leukocytes to the site, which in turn leads to activation of inflammatory cascades and additional cerebral damage (Huang et al., 2006). It used to be considered that necrotic death is a hallmark of ischemia. However, a few hours post ischemic attack, neurons suffer transient reversible damage, after which they undergo apoptosis (Linnik et al., 1993, Charriaut-Marlangue, 1998). The complexity of the responses elicited following an ischemic infarct makes further research indispensable in order to generate effective neuroprotective approaches.

6.4.2 Pilocarpine-induced *status epilepticus*

Approximately 50 million people around the globe suffer from epilepsy (Sander, 2003). There are several risk factors that account for development of epilepsy and these are: cerebrovascular diseases, brain cancer, alcohol, traumatic head injuries, defects in cortical development, genetic aspects as well as infections of the CNS, or malaria in less developed countries (Duncan et al., 2006). Spontaneous repeated seizures are a hallmark of epilepsy. They result from focal or general sudden variations in neurological functions caused by atypical electrical activity observed in the cortex (Shin and McNamara, 1994). This is due to lack of balance between the two most important neurotransmitters: GABA (inhibitory) and glutamate (excitatory) (Dalby and Mody, 2001). There are two types of convulsions: partial and general. In case of generalized seizures both hemispheres misfire at the same time, unlike in partial convulsions, where the local onset of epilepsy may spread to other brain regions (Duncan et al., 2006).

Administration of pilocarpine, which is a strong agonist of muscarinic receptors, leads to consecutive changes at both the behavioral and electrographic level in the rat model of epilepsy. Among those changes we can distinguish three different periods. The first one is an acute phase that evolves into a limbic *status epilepticus* lasting for about 24 h. It is followed by a silent period during which both electroencephalography (EEG) readout and behavioral changes normalize. The length of this period varies from 4 to 44 days. Finally the last, third period, is a chronic one and is characterized by spontaneous recurrent seizures (SRSs) (Cavalheiro, 1995). Systemic administration of pilocarpine acts on either M1 or M2 muscarinic receptors. Activation of the M2 receptor type leads to inhibition of adenylate cyclase, which in turn causes reduction of acetylcholine release and neuronal

activation. Conversely, activation of M1 leads to phospholipase C activation and production of diacyloglycerol (DAG) and inositol triphosphate (IP3). That leads to modifications in the Ca^{2+} and K^{+} current resulting in high excitability of the brain tissue (Segal, 1988). Increased levels of Ca^{2+} trigger glutamate release and induce *status epilepticus*. The excitotoxic insult triggers instant cell death occurring within minutes after application of pilocarpine, but also leads to neurodegeneration extended over weeks and months (Leite et al., 1990, Cavalheiro, 1995). The acute phase is characterized by increased level of glutamate, which in turn leads to activation of the NMDAR resulting in cell death (Costa et al., 2004). It has been shown that the level of neuronal damage correlates with the severity and duration of the seizures (Covolan and Mello, 2006). The neuronal damage observed after epileptic seizures has been found in various brain areas, including the hippocampus, thalamus, amygdala as well as various cortical regions (Fabene et al., 2007).

7. The role of JNK in neurodegeneration

The range of diseases that JNK is involved in is very broad and it encompasses various neurological, coronary, respiratory, hepato-biliary, autoimmune, inflammatory, metabolic disorders as well as multiple forms of cancer (Sabapathy, 2012). However, for the scope of this thesis, the attention will be brought to the role of JNK in selected neurodegenerative syndromes.

7.1 Stroke

Activation of the JNK signaling cascade is associated with ischemia. In a transient *in vivo* model of global ischemia, active JNK, p38, as well as ERK were found already active within 5 minutes in the CA1 and CA3 region of the hippocampus (Sugino et al., 2000). Following reperfusion, activity of JNK and p38 were enhanced for the next 72 h. Moreover, in the proposed model of transient ischemia, administration of the p38/JNK inhibitor SB203580 decreased cell death, unlike when MEK-ERK inhibitor PD98059 was administered (Sugino et al., 2000). In addition, administration of the JNK inhibitor SP600125 reduced apoptosis in the CA1 region of the hippocampus in a model of mild ischemia (Guan et al., 2005). In another model of transient focal cerebral ischemia, 60 min occlusion of the middle cerebral artery was sufficient to induce JNK activity in the MCA region. Administration of SP600125 prevented apoptotic death, as measured by TUNEL assay. It was

proposed that JNK death signals are transmitted via interaction with BimL and further downstream via Bax. The JNK-BimL interaction was blocked upon inhibitor treatment (Okuno et al., 2004). Importantly, these findings were further verified by another study using SP 600125. Presence of the inhibitor not only reduced JNK activity but also diminished the size of the infarct in a dose-dependent manner (Gao et al., 2005). In addition, inhibition of JNK resulted in decreased expression of Bim and Fas, but not Bcl-2 or FasL. In other words, inhibition of JNK stopped the ischemia-induced Bax and Bim translocation to mitochondria, thus preventing the release of cytochrome c that normally results in cell death (Gao et al., 2005).

So far, administration of tPA within a few hours following ischemic attack is recommended as a primary choice treatment. Interestingly, development of a peptide inhibitor called DJNKI1 has brought many promising results for JNK suppression in stroke models. DJNKI1 is a *D-retroinverso* peptide inhibitor of JNK kinase. It consists of a 20 aa sequence derived from the JIP1 protein, which is the JNK-binding domain of JIP1 (Centeno et al., 2007). The *in vitro* approach with cortical neurons treated with NMDA showed that DJNKI1 confers protection to cortical neurons treated with NMDA (Borsello et al., 2003). Moreover, in the *in vivo* transient model of stroke, DJNKI1 has significantly reduced the size of the lesion, despite late application (6 h after the lesion). The reduction of the lesion size corresponded with improved animal behavior (Borsello et al., 2003). This implies that the potential therapeutic time window could be twice that of tPA. It was further shown that late administration of DJNKI1 to hippocampal slices deprived of glucose and oxygen for 30 min significantly reduced the amount of cell death. In addition, when DJNK1 was used in the same study but in a permanent occlusion model of stroke, it substantially reduced the size of the infarct within 3 h (Hirt et al., 2004). Importantly, DJNKI1's function is not impaired in the presence of tPA and the peptide inhibitor was still providing neuroprotection both *in vitro* and *in vivo* (Wiegler et al., 2008). Nonetheless, following ischemic attack, the JNK cascade is also activated in other cell types apart from neurons, including glia or the brain endothelium (Xie et al., 2004b, Kacimi et al., 2011). This may in turn have an undesirable impact on neuronal survival. However, it was presented that DJNKI1, when delivered peripherally, may affect some of the inflammatory processes in non-neuronal population of cells (Benakis et al., 2010). Therefore, we should hope that DJNKI1 will pass all clinical tests required for future human use, and that it will be beneficial to all patients suffering from stroke.

7.2 Wallerian degeneration

Behind the pathology associated with traumatic, inflammatory or degenerative neurological diseases lies deterioration of the axon. Growing evidence indicates that axonal degeneration appears early on during the progression of the disorder. Therefore, it is an important target in terms of future therapies (Lingor et al., 2012). Originally discovered by Waller in 1850 in transected glossopharyngeal and hypoglossal nerves, Wallerian degeneration refers to degenerative processes initiated in the distal-to-the-injury part of an axon. Following injury, the parts of an axon which are unaffected by acute degeneration remain morphologically stable for about 24-72 h. After that, the distal part goes through gradual fragmentation resembling the acute axonal degeneration, eventually leading to its total elimination (Kerschensteiner et al., 2005).

Activation of various kinases including JNK plays an important role in progression of degeneration (Lingor et al., 2012). It is widely known that JNK, apart from its prominent role as a stress kinase, governs many physiological functions in neurons (Coffey et al., 2000, Coffey et al., 2002, Björkblom et al., 2005, Kyriakis and Avruch, 2012). Mice lacking *Jnk1* display abnormal axonal tracks (Chang et al., 2003). Moreover, JNK has been shown to control the transcriptional events involved in neurite outgrowth in PC12 cells as well as axon regeneration in dorsal root ganglion (DRG) neurons (Yao et al., 1997, Kenney and Kocsis, 1998). In addition, activated JNK is needed for axon formation. Blocking JNK also inhibits axogenesis but does not counteract dendrite formation (Oliva et al., 2006).

Nerve injury elicits many changes both in severed neurons and in the neighboring non-neuronal cells, which eventually lead to effective reinnervation or else commit cells to death (Cavalli et al., 2005). Transection of the sciatic nerve leads to activation of c-Jun and the presence of activated JNK in the cell body (Jenkins and Hunt, 1991, Kenney and Kocsis, 1998). In addition, it was shown that in the sciatic nerve, JNK is transported bidirectionally at high rates (Middlemas et al., 2003). A study from the Goldstein laboratory proposed that pJNK, via its interaction with Sunday Driver (syd), could be part of a vesicular damage surveillance system in severed peripheral nerves (Cavalli et al., 2005). Using olfactory receptor neurons as an axonal lesion model in *D. melanogaster* and transection of sciatic nerves in mice, it was proposed that JNK acts via activation by the dual leucine kinase (DLK) to promote axonal degeneration (Miller et al.,

2009). Moreover, it was suggested that JNK is needed at the early stage of degeneration before an axon is committed for final breakdown (Miller et al., 2009). Previously, it was reported that presence of p-JNK in the cell soma of the injured neuron is dependent on both time and the distance from the injury (Kenney and Kocsis, 1998).

Microtubule disassembly is one of the hallmarks of Wallerian degeneration (Coleman, 2005). The JNK substrate SCG10 has recently been described as an axonal maintenance factor, whose loss is associated with the early stage of axonal injury. Moreover, the absence of SCG10 allows progression of the axonal degeneration mechanism (Shin et al., 2012). Looking into a wide range of various neurological disorders, it becomes more obvious how devastating the loss of an axon is for maintaining normal life. Therefore it is crucial to seek for the mechanism behind the observed degeneration that in turn may reveal potential therapeutic targets in the future.

OBJECTIVES

My PhD project focused on defining the function of SCG10 phosphorylation by JNK in neurons. The questions we set to answer concerned both physiological and pathophysiological conditions.

The main goals established for studies I-III were:

1. To determine the role of JNK1 in regulation of its *bona fide* target protein SCG10 in regulating neurite growth. (Study I)
2. To elucidate the importance of the "JNK1-SCG10 duo" in orchestrating the early stages of neuronal migration. (Study II)
3. To investigate if JNK1 and SCG10 regulate GA biogenesis under resting conditions and following pathological excitotoxic stimuli. (Study III)

EXPERIMENTAL PROCEDURES

The following section contains a brief description of the materials and methods used during the thesis progress. More detailed information concerning specific experiments can be found in the original publications (I-III)

1. Plasmids

For preparation of shRNA constructs the oligonucleotide UCU AGC UGC UAU CAU UGA AUU CAA GAG AUU CAA UGA UAG CAG CUA GAU UUU UU containing sense and antisense siRNA targeting rat *scg10* was ligated into pSilencer 1.0 according to the manufacturer's instructions. Insensitive to silencing mutants of SCG10 WT (i) and SCG10$^{S62D/S73D}$ (i) were prepared using 2 step PCR reaction with following primers: rSCG10i (+) 5'-aagg aaaaccgtga ggct aac ctg gca gca ata ata gag cgtct gcaggaaaaggagag-3' rSCG10i (-) 5'ctctcctttt cctgcagacg ctctattatt gctgccaggt tagcctcacg gttttcctt-3' Final products were inserted into EcoRI site of pCAGGS. To create GA targeted chimera of active JNK a fragment of N-terminal domain of rat scg10 was ligated upstream to MKK7-JNK1 (a kind gift from Roger Davis). For short targeting sequence 10 aa was used (mayke kmkel) for long targeting sequence 33 aa were used (mayke kmkel smlsl icscf ypepr niniy tyd) based on (Lutjens et al., 2000). For visualization purposes chimera was cloned into pEGFP-N1 vector.

2. Antibodies

Antibody	Type	Vendor	Application
p-SCG10	Rabbit polyclonal	Coffey lab/raised against EAPRTLAS(PO3)PKKKDLSLEE	WB
SCG10	Rabbit polyclonal	Coffey lab/ raised against GST-SCG10	WB, IHC
GM130	Mouse monoclonal	Transduction Laboratories	WB, IHC
P115	Mouse monoclonal	Transduction laboratories	WB, IHC
pJNK (Thr183/T yr185)	Rabbit polyclonal	New England Biolabs	WB
JNK1	Mouse monoclonal	Pharmingen	WB
panJNK	Rabbit polyclonal	Upstate Biotechnology	WB
β-tubulin III (TuJ1)	Mouse monoclonal	Nordic BioSite	IHC
BrdU	Mouse monoclonal	Calbiochem	IHC
Ki67	Rabbit polyclonal	Novocastra	IHC
CSPG	Mouse monoclonal	Sigma; St.Louis, MO, USA	IHC
TbrI	Rabbit polyclonal	Millipore/Chemicon	IHC
p-c-Jun (KM-1)	Mouse monoclonal	Santa Cruz	WB
GFP (JL-8)	Mouse monoclonal	BD Biosciences Clontech	WB
Histone pS28	Rat monoclonal	Pharmingen	WB, IHC
BrnI	Guineapig polyclonal	Kind gift from Dr. A.Ryan	IHC
dsRed-1	Mouse monoclonal	Clontech	WB

WB-Western Blot, IHC- Immunohistochemistry

3. Special reagents

The NMDA receptors agonist N-methyl-D-aspartic acid was from Research Biochemicals Inc (RBI). JNK peptide inhibitor DJNK1 was purchased from Genecust. Kinase inhibitors SP600125 and SB203580 were purchased from Calbiochem (San Diego, CA). Hoechst 33342 nuclear dye together with mounting medium (Mowiol) was from Hoechst Marion Roussel (Frankfurt, Germany).

4. Cell culture

4.1 Primary cell culture

Cortical neurons were prepared from newly born Sprague Dawley rats (P0) or from C57/B6J *wt* and *Jnk1-/-* mice. Both rats and mice were decapitated and cortices were dissected in a medium containing kynurenic acid to prevent activation of NMDA and AMPA/kainic receptors. Dissected cortices were digested in a papain solution (100 U) from Worthington (#3119). The enzyme activity was blocked using Trypsin Inhibitor (100 mg) (Sigma, Type IIL). Cortices were gently triturated to avoid unnecessary cell damage. Cells were grown on glass coverslips coated with poly-D-lysine provided by BD Biosciences (#354210). Rat cortical neurons were plated in MEM medium containing 10% fetal calf serum, 30mM glucose, 2mM glutamine, and penicillin/streptomycin (50 U/50 μg/ml). Whereas mouse cortical neurons were grown in Neurobasal medium (Gibco) supplemented with B27 50x (Gibco ™ # 17504044), and 2 mM glutamine. 900.000 cortical neurons (rat) and 750.000 cells (mouse) per well were plated in 24 well plates. Within 48 h post plating 50% of growth medium was exchanged for a fresh medium supplemented with 2.5 μM cytosine arabinofuranoside (Sigma) to reduce non-neuronal cell proliferation.

4.2 Cell lines: COS7, HEK293T

COS7 cells were grown in Minimal Essential Medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS), 2mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. HEK293T were cultured in

Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% (v/v) FCS, 2mM glutamine, 50 U/ml peniclilin and 50 μg/ml streptomycin.

All cell cultures were grown in a humidified 5% CO_2 incubator at 37°C.

5. Transfection methods

5.1 Primary cell culture

Cortical neurons were transfected with a Lipofectamine 2000™ reagent (Invitrogen). Ratio of DNA: LF2000™ was 0.5 μg: 1μl to avoid increased toxicity. For experiments followed by NMDA treatment cells were transfected at 6 DIV, 48h prior to excitotoxic insult. For gene silencing experiments using shRNASCG10 cortical neurons were transfected 72 h prior additional treatment to ensure sufficient knockdown effect.

5.2 Cell lines: COS 7, HEK 293T

COS7 cells were transfected with a Lipofectamine 2000™ (Invitrogen) reagent. Cells were plated in a 24 well plate 24 h prior transfection. The expected cell confluency on the day of transfection was 60-80%. 400 ng of DNA and 1.2 μl of Lipofectamine was used in the assay maintained in 24 well plates. DNA-Lipofectamine mix was diluted in 40 μl MEM/10 mM Hepes and incubated for 30min at room temperature. Cells were prewashed with MEM/10 mM Hepes prior DNA mix addition. 6 hours post supplementing DNA complete COS 7 cells medium was added in an equal to transfection volume and cells were grown for additional 20-48hours.

HEK 293T cells were transiently transfected using calcium phosphate precipitation method. Similarly to COS 7 HEK 293T cells were re-plated 24 h prior transfection. The expected cell density on the day of transfection was 50-60%. DNA in the amount of 0.5 μg was diluted to 450 μl in water and mixed with 50 μl of 2.5 M $CaCl_2$. Prepared mix was pipetted into swirling HeBS buffer (274 mM NaCl, 10mM KCl, 1.4 mM $Na_2HPO4*7H_2O$, 15 mM D-Glucose, 42 mM Hepes-free acid, pH 7.2) and left for additional 30 min at room temperature to allow precipitate formation. Finally cells were grown for additional 24-48 h after DNA mix was dripped onto them.

6. Immunostaining

6.1 Immunohistochemistry of cultured cells

Cortical neurons grown on coverslips were fixed with 4% paraformaldehyde for 15 min at room temperature. Thereafter cells were permeabilised with phosphate buffer saline (PBS) containing 1% Triton for 2 min. Thoroughly washed with PBS cells were blocked for unspecific binding of the antibody with 10% fetal calf serum (FCS) containing 0.2% Tween 20 for 1h at room temperature. Primary antibody diluted in 1% FCS/PBS was incubated over night at +4°C (1:1000 for GM130, 1:1000 for SCG10, 1:1000 for p115, 1:5000 for TuJ1). Following thorough washing secondary antibodies (generally at 1:500 for Alexa 488 or Alexa 568) were incubated for 1h at room temperature. Afterwards nuclei were stained using Hoechst 33342 dye (1:3000). Samples were analyzed with Leica DMRE fluorescent microscope.

6.2 Immunohistochemistry of glass-mounted tissue sections

Brains pre-labelled with BrdU were extracted from C57B6 mice and fixed by immersion in 4% paraformaldehyde in phosphate buffer for 24 h at + 4°C. In order to prepare tissues for cryosectioning tissues were moved to 30% sucrose buffer for 3-4 days. Afterwards tissues were swiftly frozen in isopentane and on the day of sectioning embedded in O.C.T compound (Tissue-Tek, Zoeterwoude, Netherlands). Slices (40 μm) of sagittal sections were collected on gelatin coated glass slides and stained with antibodies for BrdU 1:50 (Calbiochem) and Ki67 1:100 (Novocastra). Anti-BrdU antibody was detected using anti- mouse IgG 1 specific A 488 (1:500) secondary antibody and Ki67 was detected using antirabbit IgG specific A 568. Samples stained for Tbr I (1:1000) were detected using A 568. Slides were counterstained with Hoechst 33342. For final analysis single plane images were collected with Zeiss LSM510 META confocal microscope using 20x air objective.

6.3 Immunohistochemistry of tissue sections: free-floating technique

Fixed brain tissue, cryoprotected either in 30% sucrose (for MCAO) or 20% glycerol was embedded in Optimum Cutting Temperature (O.C.T) compound (Tissue Tek) prior to cutting. Consecutive coronal (MCAO) and saggital (pilocarpine) 40 μm sections were cut on a cryostat (Microm HM500 OM) and stored in cryoprotectant (30% ethylene glycol, 25% glycerol, 0.05M Na-Phosphate buffer). On the day of analysis, slices were washed 3x 10min with 1xTBS (50 mM TrisHCl pH 7.4, 150 mM NaCl) to remove remaining cryoprotectant, treated with 1% sodium borohydrate for 15 min and washed 6 times with 1xTBS. For blocking 10% horse serum (40 min) was used. Primary antibody (GM130 1:1000, or p115 1:1000) was added in 1% horse serum. Slices were placed at +4°C in order to allow slow binding of the antibody. Next they were washed with 1% horse serum prior secondary antibody addition (A 488, Invitrogen) and left ON at +4°C. Nuclei were stained with Hoechst 33342. Sections were mounted on glass slides with Mowiol 4-88 (Hoechst). The MCAO tissues were a kind gift from the laboratory of Prof. Jari Koistinaho (University of Eastern Finland) and pilocarpine treated brains were kindly given by Prof. Asla Pitkänen (University of Eastern Finland).

7. SDS-PAGE and Western blot analysis

7.1 Harvesting and processing of brain tissue/cell culture

Protein lysates in 1x Laemmli sample buffer were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences). Membranes were blocked for 1h RT in TBST or PBST buffer containing 1-5% milk. Membranes were incubated with primary antibody ON at +4 °C. Next day membranes were subjected to several washes and incubated for 1h at RT with horseradish peroxidase conjugated secondary antibody. Secondary antibody was used at concentrations 1:50 000-1:200 000 in 1-5% milk TBST/PBST buffer. The signal was detected with West Femto SuperSignal (Pierce).

8. Animal experimentations

All animal procedures were conducted in accordance with the guidelines provided by European Community Council Directives 86/609/EEC replaced during the thesis progress by Directive 2010/63/EU. Each experiment had the approval of the National Animal Experiment Board (ELLA). In designing our experiments we followed the 3R's ethical principle to refine, reduce and replace animal use when possible. All embryos used in this study originated from matings between inbred C57BL6J mice. Mice were mated over night and early in the morning the following day female mice were examined for presence of vaginal plug. Mice positive for the plug were marked as 0.5 post coitum (p.c) and remained under strict observation for visible signs of pregnancy.

8.1 Labelling of progenitor cells with BrdU

For labeling of the progenitor cells pregnant mice (15.5 p.c) were injected intraperitoneally with 5 doses of BrdU (100mg/kg). The brains were extracted at P0 and fixed in 4% paraformaldehyde for 24 h, immersed in 30% sucrose buffer, frozen in iso-pentane and sliced sagitally with a cryostat (35 μm). Slices were postfixed with 70% ethanol for 30 min and RT and washed in phosphate buffered saline. DNA was denatured with 2M HCl for 10 min at RT and 30 min at 37°C after which acid was neutralised with 0.1 M sodium borate (pH.8.5) for 10 min at RT. The tissue permeabilisation was done in phosphate buffered saline containing 2% TX-100 for 15min. The unspecific binding of the antibody was prevented by incubation of the tissue slices in 1% goat serum in phosphate saline buffer for 1h. Anti-BrdU antibody (Calbiochem #NA61) 1:50 was detected using anti- mouse IgG 1 specific A 488 (1:500) secondary antibody. Tile images collected for analysis were captured with Zeiss LSM510 META confocal microscope using 40x oil objective.

8.2 *In Utero* Electroporation

Timed-pregant C57/B6J wt and *Jnk1-/-* mice 15.5 days post coitus were put to sleep with 4% isofluarane to induce deep anesthesia. 2% isofluarane was administered during the surgery to maintain stable surgical conditions. Tail and pedal reflexes as well as breathing rhythm were carefully observed

during each experiment. To provide analgesia mice were given buprenorphine injection (Temgesic ® 0.3 mg/ml) at dose 0.05-1 mg/kg (SC) 30 min prior surgery. Approximately 2 cm incision in the abdominal cavity allowed exposure of uterine horns and access to embryos. About 1-2 μl of DNA was injected using a microinjection needle from Eppendorf (Sterile Femtotips II) into the lateral ventricle. Electroporation was done using square wave pulse electroporator from NEPA Gene (CUY21E) using tweezer type electrodes (CUY650-P5). Five electrical pulses (45 V, 50 ms) at 1s interval were passed through embryonic brain tissue. To ensure targeting of the ventricular zone area electrodes were placed at 0° angle inclination in respect to the horizontal plane. With no additional delay uterus was placed back in the abdominal cavity and embryos were left to develop to later stages. Muscle and skin layer were sutured separately using Silkam sutures from Braun.

8.3 Organotypic cortical slice culture

In utero electroporated C57/B6J wt and *Jnk1*-/- embryos were extracted 48 h post electroporation for purposes of organotypic slice culture. Brains were dissected in cold PBS/glucose and transferred into liquid 3% low melting agarose (38 °C) (Bio-Rad #161-3112) and kept on ice for 1 h. Embedded brains were cut sagittaly (300 μm) with a vibratome (Leica VT 1000S). Brain slices were transferred onto culture plate inserts from Millipore (Millicell CM #PICMORG 50) containing Neurobasal medium (Invitrogen # 12348-017) supplemented with 1% N2 (Invitrogen # 17502-048), 1% B27 (Gibco ™ # 17504044), 2mM Glutamax (Invitrogen #35050-038) and penicillin/streptomycin (50U/50 μg/ml). Slices were cultivated under semi-dry conditions and recovered for 4 h in the cell culture incubator prior to imaging.

9. Pharmacological treatments of time course experiments

9.1 NMDA

N-methyl D-aspartate treatment was carried out by adding 60 μM NMDA to 8 days old cortical neurons (8 DIV) remaining in growth medium. Cells were

fixed (4% PFA) at designated times 15 min, 30 min, 60 min, 1 h, 4 h and 24 h. Samples were further subjected to immunochemistry analysis.

9.2 DJNKI1

The D- *retroinverso* peptide inhibitor of JNK kinase (DJNK1) was added to 8 DIV cortical neurons 1h prior NMDA addition. Cells were grown in the presence of serum hence two concentrations of DJNK1 were used 2 and 20 μM. Cells were fixed (4% PFA) at selected times 15 min, 30 min, 60 min, 1 h, 4 h and 24 h post NMDA treatment. Cells were further stained according to immunochemistry protocols.

10. In vitro model of cell migration

Neurons in Boyden chamber were transfected with g-LMKK7JNK1-GFP, pEGFP-GM130, gLJIPJBD-dsRed1, pEGFP-NES-JIPJBD, pEGFP-SCG10DD at the moment of plating using Lipofectamine 2000 (Invitrogen) protocol. Cells were co-transfected with pEGFP-C1 empty vector to allow better visualization of cells passing through the pore. After 24 and 48 h cells were fixed with 4% paraformaldehyde and nuclei were stained Hoechst 33342. Cells that remained on the inner surface (did not migrate) were removed using a cotton bud. The number of fluorescent cells that had migrated through the membrane were counted (blind) using Olympus BX100.

11. Microscopy

11.1 Confocal analysis and time lapse imaging

For timelapse experiments, C57/B6J wt/*Jnk1*-/- mice were electroporated at E15.5 with GFP and embryos were extracted at E17.5. Imaging was done with inserts placed on glass- bottomed dishes (MatTek #P35G-0-20-C) under environmental control maintained by Tempcontrol 37-2 digital (Zeiss). Timelapse movies were constructed from z-stack images captured at 30 min intervals using a Zeiss LSM510 META confocal microscope equipped with 20x air objective (N_A =0.5). Each stack was composed of approx. 25 single scans. Movies were recorded for 10h. Image processing was done with Image J free software by using Z projection with Average intensity.

RESULTS AND DISCUSSION

1. Identification and characterization of novel JNK effector protein SCG10 (Study I)

Initially, JNK's role was associated with stress functions and cell death (Ip and Davis, 1998, Kyriakis and Avruch, 2012). Over time however, the way JNK is perceived changed and it is nowadays acknowledged that JNK is also an important regulator of physiologically indispensable proteins (Gdalyahu et al., 2004, Xia and Kao, 2004, Xia and Karin, 2004, Björkblom et al., 2005). In the brain, JNK activity is constitutively high when compared to non-neuronal tissue (Hu et al., 1997, Coffey et al., 2000, Coffey et al., 2002). During early brain development it acts as a critical regulator of regional apoptosis (Kuan et al., 1999). Interestingly, an increasing JNK activity was observed during differentiation in cerebellar granule neurons (Coffey et al., 2000). In those cells, JNK predominantly localizes to the cytoplasm. Finally, the high basal activity among JNK isoforms was attributed to JNK1, which activity was reduced by 70% in brain tissues of *Jnk1-/-* mice (Björkblom et al., 2005). It was previously proposed that JNK controlled neuronal architecture (Coffey et al., 2000, Björkblom et al., 2005). Nevertheless, the exact mechanism remained undetermined. Therefore in study I we undertook an approach to elucidate potential effectors of JNK action on neuronal morphology.

In our search for JNK targets, we found that SCG10 interacts with JNK1 in a protein interaction screen using brain homogenate. SCG10 belongs to the stathmin family, known for their microtubule destabilizing potential (Antonsson et al., 1997). To check for interaction with other stathmins, all members of the family were cloned and subjected for pull down experiments with GST-JNK1. As assumed, we detected an interaction between most family members and JNK1 (Study I, Fig.2A). To further determine if SCG10-JNK1 interaction takes place under physiological conditions, SCG10 antiserum was generated and used for coimmunoprecipitation assay. Using this approach, we were able to demonstrate that SCG10 and JNK1 interact *in vivo* (Study I, Fig.2C).

Protein phosphorylation is a key process in cell signaling. Serine 62 (S62) and Serine 73 (S73) are among the known phosphorylation sites on SCG10. S62 and S73 are targeted by MAP kinases (Antonsson et al., 1998, Neidhart et

al., 2001, Tararuk et al., 2006). We have shown that S73 was more intensely phosphorylated by JNK *in vivo.* Therefore a p-S73-SCG10 antibody was raised against a synthetic phosphopeptide. It was affinity purified using phospho-SCG10 and dephopsho-SCG10 columns and tested against recombinant phosphorylated and non-phosphorylated SCG10 (Study I, Fig.5A). We showed that purified p-S73-SCG10 antibody recognized only the phosphorylated form of SCG10.

We reasoned that if JNK is indeed a key regulator of SCG10 function then JNK site specific phosphorylation of SCG10 should be diminished in *Jnk* deficient mice. Hence, we immunoblotted cortex from 7 days old *Jnk*-knockout mice (*Jnk1-/-, Jnk2-/-, Jnk3-/-* and *Jnk2-/3-/-*) with p-S73-SCG10, SCG10 and JNK1/2 antibodies (Study I, Fig.5B). Interestingly, phosphorylation of SCG10 was reduced by 50% in *Jnk1* deficient mice and to smaller extent in other knockouts (Study I, Fig.5C, D).

1.1 SCG10 and JNK expression patterns overlap in tissue and cell level

It has been known that SCG10 expression peaks during embryogenesis although its exact location and function remained unidentified (Stein et al., 1988b). We argued that for SCG10 to be a *bona fide* target protein for JNK1, the expression profiles of the two should overlap. We found that indeed JNK1 and SCG10 displayed a stunningly similar expression pattern, both were found in the midbrain roof, the olfactory epithelium, the inferior colliculus, the medulla oblongata and importantly in the telencephalon (developing cortex) (Study I, Fig.6A). Further careful examination revealed that JNK1 and SCG10 were enriched in the intermediate zone of the telencephalon, known for the highest density of postmitotic neurons (Study I, Fig.6C, D). Importantly, both proteins phosphorylated (pJNK, p-S73SCG10) and non-phosphorylated (JNK, SCG10) were present in exactly the same brain area, where early differentiating neurons undertake migration, shown by co-labeling with TuJI (class III β-tubulin) (Study I, Fig. 6D).

We have shown that JNK1 and SCG10 interact *in vitro*, while *in vivo* in the developing brain their expression profile overlaps. At the cellular level, SCG10 expression has been observed at the GA and in growth cones, with a conspicuous punctate staining along neurites (Lutjens et al., 2000, Gavet et al., 2002, Ozon et al., 2002, Tararuk et al., 2006). Immunofluorescent labeling

of cortical neurons showed that SCG10, JNK-phosphorylated SCG10 and active JNK displayed noticeably similar punctate labeling in neurites (Study I, Fig 7A). We wanted to know if pJNK and pSCG10 were colocalized in vesicles. We therefore applied crude cell fractionation to a postnatal day 7 rat brain. Using differential centrifugation, we separated cellular compartments permitting us to analyze protein distribution. We received several different fractions, which were characterized for their content. The P1 fraction contained non-homogenized parts of rat cortex, while the S1 fraction represented the pre-cleared supernatant comprised of all homogenized cell structures. The S1 was further subjected to next step centrifugation at which small vesicle and soluble proteins (S2) were separated from GA, lysosomes, ER, peroxisomes, endosomes and mitochondria (P2). Fractions S3 and P3 contained soluble proteins and plasma membranes, respectively. Equal amounts of fractions were separated by SDS-PAGE and immunoblotted for the proteins of interest. If SCG10 is an *in vivo* JNK target it should be present in the same cell compartments as JNK and *vice versa*. The total pool of JNK determined by pan-JNK antibody was present in most of the fractions (S1, S2, P2, S3) (Study I, Fig.7B). Conversely, active JNK (p-JNK) strictly co-localized with SCG10 in the vesicular fraction (P2) enriched in GA, lyzosomes, mitochondria and endoplasmic reticulum.

2. Absence of cytoplasmic Jnk1 regulates neuronal migration *in vitro* and *in vivo* (Study II)

Mice lacking a single *Jnk* gene grow and breed just like their corresponding wild type animals (Yang et al., 1997, Dong et al., 2001). Conversely, *Jnk1* and *Jnk2* double knockout mice die *in utero*, failing in neural tube closure (Kuan et al., 1999). Based on profound analysis of various combinations of *Jnk1*/2 double knockouts it was suggested that Jnk1 might have a leading role in regulation of neural tube closure (Sabapathy et al., 1999). We have shown that Jnk1 basal activity is high in developing telencephalon; moreover, it orchestrates dynamics of MTs and neurite length via SCG10 (Study I, Fig. 6D, 9, 10). Earlier studies in non-neuronal systems indicated that JNK activity is crucial for cell migration (Xia and Karin, 2004). JNK was shown to be necessary for dorsal closure in the fruit fly as well as the eye-lid closure in mice (Kuan et al., 1999, Takatori et al., 2008). Yet its role in migration of neuronal cells remained undetermined. Based on our earlier study, where we showed that Jnk1 controls microtubule dynamics, we undertook an approach

to unravel if Jnk1 function may expand into the process of radial migration and to which extend.

To address those questions we initially set two *in vitro* tests employing cerebellar granule neurons that are well recognized primary cell culture model of neuronal migration (Edmondson and Hatten, 1987). Results obtained from surface and transwell migration assay indicated involvement of Jnk1 in the process of migration (Study II, Fig.1C,D and Fig. 2 A-G). We further searched for lamination defects *in vivo,* since it may reflect prominent marking of migration defects (Reiner, 2013). Close inspection of E15 and E18 mouse brains cortex from both *wt* and *Jnk1-/-* animals revealed significant thickness difference between genotypes. At E15, the IZ and CP were considerably thicker in mutant mice (Study II, Fig.3A,B,C). At E18, VZ of *Jnk1-/-* mice was decreased in comparison to wt, which was in accordance with thicker CP (Study II, Fig. 3G, H). All mammals share a similar pattern of generating cortical neurons. When placed in the proliferative pseudo-stratified VZ, neuronal progenitors undergo massive expansion to exit the cell cycle, and finally become a cortical neuron (Caviness et al., 1995). Early proliferation and migration control both number and location of future cortical neurons, factors that are crucial for the functional future cortex (Rakic, 1988). Hence, we investigated the cell cycle exit from *wt* and *Jnk1-/-* mice. For that purpose we used BrdU, a thymidine analog, to label dividing neural precursors, and Ki67 to identify subpopulations of proliferating cells (Kuhn et al., 1996, Zambon, 2010). Cells that exited the cell cycle were labeled as BrdU+/Ki67- whereas those remaining in cell cycle were identified as BrdU+/Ki67+. Based on the ratio between cells leaving and remaining in active cell cycle we calculated a cell cycle index (Study II, Fig.S2). Importantly, in *Jnk1-/-* brains the cell cycle index was decreased by 13% in VZ and SVZ, indicating that more cells have exited cell cycle and committed to neuronal fate. This was in agreement with increased number of neurons in CP (Study II, Fig. 3H,I).

To investigate if detected changes in thickness are a consequence of advanced migration we employed an *in utero* electroporation gene delivery method. Similarly to other techniques like e.g. BrdU labeling, this pulse chase method can exactly label neurons present in different cortical lamina based on time of electroporation (Langevin et al., 2007). This method gives an opportunity to target a particular population of cells both in time and space. This technique allows for efficient transfer of DNA into cortical precursors present in the VZ of the telencephalon. Moreover, visualization of cells by the

use of DNA-carrying genes for fluorescently-tagged proteins permits precise observation of migration patterns (Tabata and Nakajima, 2001). Therefore in order to unravel JNK's role in migration, we electroporated E15.5 embryos with the GFP-tagged, compartment-specific JNK inhibitor (NLS-JIPJBD, NES-JIPJBD) or GFP *in utero* and analyzed their influence on migration at following days E17, E19 and P2 (postnatal day 2). Interestingly, blocking the cytoplasmic pool of JNK with NESJIPJBD speeded up cell migration. Those cells reached superficial cortical layers II/III already at day E19, whereas in control conditions (GFP-only labeled cells) neurons achieved the same position at P2 (Study II, Fig.4A, C,D). On the other hand, inhibition of the nuclear pool of JNK had an opposite result. Furthermore, at P2, half of the GFP-NLS-JIPJBD positive cells remained in the ventricular zone, whereas only 10% of control cells stayed in the VZ at that time (Study II, Fig.4A,B,D). We have shown that different pools of JNK may have opposing effect on neuronal migration, with the nuclear pool composed typically of JNK2 and JNK3 isoforms having mostly stimulatory effect and the cytoplasmic pool (JNK1) having inhibitory influence.

2.1 Knockdown of SCG10 speeds up neuronal migration in a similar fashion to *Jnk1* knockout

We have previously shown that p-Ser73-SCG10 is enriched in the IZ at E15 and so is active Jnk1 (Study I, Fig.6D). We previously postulated that Jnk1 controls microtubule dynamics via SCG10, and hence regulates axodentritic length. In *D. melanogaster,* which has only one stathmin gene (D-stathmin), it was demonstrated with a knockdown approach that the absence of stathmin leads to serious anomalies and migration irregularities (Ozon et al., 2002). Hence we undertook a similar approach to investigate SCG10's role in migration and transfected neurons with short hairpin RNA (shRNA) targeting SCG10. Interestingly, we observed a significant increase in migration rate in the absence of SCG10 (Study II, Fig. 5H). Moreover, this increase was strikingly similar to that observed when Jnk1 was downregulated (Study II, Fig.2C, E). Notably, there was no additional rise in migration rate upon inhibition of JNK with NES-JIPJBD (Study II, Fig.5H). Based on the results obtained, we concluded that both proteins JNK1 and SCG10 may share a common pathway involved in regulation of neuronal migration. Furthermore, expression of a double alanine mutant of SCG10 ($SCG10^{S62A/S73A}$), a mutant that cannot be phosphorylated by JNK, increased

migration rate in the *in vitro* assay (Study II, Fig.5I). Similarly, an increased pool of free tubulin, induced by overexpression, had comparable outcome (Study II, Fig.5H).

Therefore we proposed a tentative model explaining Jnk1 and SCG10's role in regulation of neuronal migration. It was previously suggested that SCG10 induces catastrophe at the minus end and growth at the plus end (Manna et al., 2007). Induced depolymerisation increases the pool of free tubulin, which then can be used for stabilization of the plus end. When MTs are linked to an immobile structure, then the observed stabilization at the plus end could cause protrusion of the growth cone. Because Jnk1 activity in the developing brain is high, SCG10 subsequently phosphorylated by Jnk1 could retard migration by inhibiting depolymerization of microtubules at the minus end, thus depleting the pool of free tubulin. The relationship between Jnk1 and SCG10 could stipulate for a system in which Jnk1 controls the forward movement of neurons (Study II, S1).

2.2 Pseudo-phosphorylated SCG10 can rescue migration defects in *Jnk1*-/- brains

Based on *in vitro* results indicating a role of Jnk1 and SCG10 in migration (Study II, Fig. 2C,E & Fig.5 H,I), we decided to perform a series of rescue experiments *in vivo.* If SCG10 is indeed downstream of Jnk1 in migration, then the pseudo-phosporylated form of SCG10 ($SCG10^{S62D/S73D}$) should restore the normal rate of migration. For that purpose we electroporated E15.5 mouse embryos with GFP-NES-JIPJBD *in utero*, previously shown to increase migration rate (Study II, Fig.2C & Fig 4A,C) along with $SCG10^{S62D/S73D}$ and without it. Careful inspection at E19 revealed that the presence of the pseudo-phosphorylated form of SCG10 led to complete restoration of advanced migration previously seen, when the cytoplasmic pool of JNK1 was inhibited by GFP-NES-JIPJBD (Study II, Fig.6A, B). In addition, electroporation of $SCG10^{S62A/S73A}$ increased migration rates (Study II, Fig. 6A, B) as previously observed in *in vitro* migration assays (Study II, Fig.5I). Moreover, the observed acceleration of migration in the presence of $SCG10^{S62A/S73A}$ was accompanied by a shorter leading process (Study II, Fig.6A) as previously noted in postnatal cortical neurons (Study I, Fig.9B).

Next, we investigated the role of SCG10 in migration by applying a gene knockdown approach *in vivo*. As expected, based on *in vitro* results (Study II,

Fig.5H), in the absence of SCG10, cells migrated faster reaching superficial layers (I-III) already by E19 in contrast to controls (GFP) (Study II, Fig.6C, D). For rescue purposes we used SCG10$^{S62D/S73D}$ insensitive to shRNA (SCG10 DDi). The functionality of the mutant was tested in COS7 cells in the presence and absence of shRNA (Study II, Fig. 6E). Importantly, electroporation of the insensitive to silencing mutant restored normal migration rate (Study II, Fig.6C, D). Moreover, introducing SCG10$^{S62D/S73D}$ into *Jnk1* deficient mice reestablished normal migration rate (Study II, Fig.6F).

The increased migration observed in *Jnk1-/-* brains (Study II, Fig.2E & Fig.3) was further investigated using BrdU labelling, a more traditional method for tracking radial migration. BrdU was administered to pregnant wt and *Jnk1-/-* mice, 15 days pc (post coitum). Embryos were sacrificed at E19 or else at P0. We observed strikingly similar advancement in migration after BrdU labelling in *Jnk1* deficient mice, as seen earlier, when using *in utero* electroporation approach (Study II, Fig.6H, I & Fig. 2E). Hence we believe that SCG10 is a key effector of Jnk1 in controlling neuronal migration.

2.3 Distorted cell arrangement is present in *Jnk1-/-* brains despite lack of layering defects

The structure of the neocortex reflects its complexity. It consists of six layers, each comprising a unique group of cells that are distinct in their function and cytoarchitecture (Bradke and Dotti, 1997, Kwan et al., 2012). Although we did not observe any clear layering defects in *Jnk1-/-*, cell organization was to some extent disordered. Immunofluorescent analysis of *wt* and *Jnk1* deficient brains revealed that at E17.5 neurons forming layer VI displayed an elongated and crowded appearance in the mutant mouse (Study II, Fig. 7A, B). Moreover, further investigation of subplate layer using chondroitin sulphate proteoglycan (CSPG) showed that in *Jnk1-/-* this layer is less clearly defined (Study II, Fig.S2A). We have furthermore checked if *Jnk1-/-* neurons reach the cortical plate before control cells in the *wt* brains. Indeed we were able to detect more neurons restricted to superficial layers (II-III) in *Jnk1-/-* than in *wt* using Brn-1, a specific layer II-III marker. This was again in agreement with higher migration rate seen earlier in *Jnk1-/-*.

The subplate forms early during development of the cortex and comprises the first generated neurons. Abnormalities in the subplate have been linked to some developmental disorders including schizophrenia, autism or

attention deficit/hyperactivity disorder and dyslexia (Kostovic and Rakic, 1990, Eastwood and Harrison, 2003, McQuillen and Ferriero, 2005). Neurons forming the subplate are crucial for establishing the correct wiring as well as functional maturation (Ghosh and Shatz, 1992, Kanold, 2009). Despite our general focus on late-born neurons (labeled at E15.5) the disruption of the subplate and layer VI in *Jnk1-/-* mice suggests that Jnk1 serves additional roles during earlier stages of migration, which in the long run may be connected to pathology in adulthood.

2.4 *Jnk1 -/-* neurons exhibit increased migration rate and faster multipolar exit

Upon completion of cell division, newborn neurons are obliged to move out of the VZ and migrate towards their final destination. In order to investigate the migration pattern of *Jnk1* deficient neurons we set time-lapse imaging on the electroporated *in utero* cortical neurons. The *Jnk1-/-* tissues subjected to real-time imaging derived from middorsolateral telencephalon. The GFP-positive cells were imaged continuously for 10 h. Interestingly, we observed that bipolar *Jnk1-/-* neurons migrated two times faster than control *wt* neurons imaged in parallel (Study II, Fig.7D,E).

Before undertaking a bipolar morphology and moving towards the pial surface, neurons go through a multipolar stage, during which they frequently change both direction and rate of the movement (Tabata and Nakajima, 2003, LoTurco and Bai, 2006). Morphologically they are quite distinctive, as they show no fixed cell polarity and keep extending and retracting thin processes in multiple directions (Tabata and Nakajima, 2003). Having discovered the significant difference in speed between radially migrating *Jnk1* deficient neurons and their *wt* counterparts, we examined the speed of multipolar cells. Importantly, the speed of multipolar neurons remained unchanged (Study II, Fig.7F). Hence we counted the proportions of multipolar to bipolar cells present in the IZ of both *Jnk1-/-* and *wt* mice. We observed a dramatic shift in proportions of multi- to bipolar neurons between the genotypes. In the *wt* brain, 75% of neurons presented multipolar morphology, in contrast to 25% observed in *Jnk1* deficient brains (Study II, Fig. 7G). Likewise, the presence of GFP-tagged JNK inhibitor NES-JIP-JBD dramatically decreased the number of multipolar cells in the IZ (Study II, Fig. 7H). Similarly, *in utero* introduction of the unphosphorylable-byJNK form of SCG10 ($SCG10^{S62A/S73A}$) led to the same distribution of multi- to bipolar

neurons as observed in the *Jnk1-/-* brain. Conversely, presence of the pseudophosporylated-by-JNK form of SCG10 ($SCG10^{S62D/S73D}$) reversed the phenotype previously observed in $SCG10^{S62A/S73A}$ electroporated cells (Study II, Fig. 7G). Furthermore, we counted proportions of cells, which left the multipolar state and acquired bipolar morphology during the 10 h recording. It was done in order to discriminate between cells, which transitioned more rapidly from the multipolar state, and those that bypassed it. Unlike *wt* multipolar cells, which in the great majority kept their multipolar morphology through the 10 h monitoring period, the *Jnk1-/-* multipolar neurons mostly transited into bipolar (Study II, Fig.7J).

These results indicate a key role of JNK1 in regulating both speed and multi- to bipolar transition, which in turn leads to advanced translocation of neurons during brain development. Moreover, it also implicates SCG10 in the regulation of multipolar transition.

3. JNK1 and SCG10 are important regulators of Golgi apparatus function (Study III)

Microtubules are key elements of neuronal morphogenesis in the developing brain. Both neuronal migration and polarization depend on proper function of MTs (Barnes and Polleux, 2009, Conde and Cáceres, 2009). The particular shape and size of neurons exerts unusual demands on them, hence the organization and nucleation of MTs has to be precisely regulated to maintain such complex arrays (Desai and Mitchison, 1997). The Golgi complex plays a central role in protein sorting and secretion, but also serves as a potential source of microtubule nucleation (Presley et al., 1998, Efimov et al., 2007). Interestingly, upon treatment with the cytoskeletal drugs nocodazole, fragmented GA cisternae can still actively promote nucleation of microtubules (Efimov et al., 2007, Rivero et al., 2009).

We have demonstrated that SCG10, a potent microtubule destabilizing protein, is an *in vivo* substrate for JNK1. Moreover, we have shown that JNK1 can regulate axodendritic architecture in cortical neurons by phosphorylating SCG10. Moreover, JNK1 acting *via* SCG10 negatively regulates radial migration. In addition, active SCG10 is highly concentrated at the GA, with p-JNK1 simultaneously being excluded from that area. To date the role of SCG10 in maintenance of GA stacks has remained undetermined. Therefore, we investigated a role for JNK and SCG10 in preservation of GA biogenesis.

In addition, since both JNK and SCG10 are involved in neurodegenerative processes (Anderson et al., 1994, Okazaki et al., 1995, Borsello et al., 2003, Wang et al., 2013) we examined if pathological JNK action may be manifested by deregulated GA structure.

Because the GA form is maintained by MTs we initially screened cultured cortical neurons for the most commonly appearing, representative GA morphologies detected under physiological conditions.

We observed a vast range of distinct GA shapes ranging from loose to highly elongated ones, constricted to one long tubule. Based on microscopic analysis we grouped observed morphologies into four different groups (I to IV). Group I neurons were characterized by somewhat elongated reticular Golgi, neurons in group II had (more) compact fragmented Golgi, group III neurons had very compact juxtanuclear Golgi, while group IV contained cells with characteristically highly elongated Golgi. In order to address a role for JNK1 in Golgi morphology, we used wt and *Jnk1*-/- mouse neurons and grouped cells accordingly. To our surprise we noticed that the number of neurons falling into group IV (highly elongated Golgi) was significantly decreased in *Jnk1*-/- in comparison to wt (Study III, Fig. 1 B). At the same time, to investigate the role of SCG10 in maintenance of GA morphology, we used a gene knockdown approach with shRNA targeting SCG10. The absence of SCG10 resulted in an apparent loosening of the GA complex, as more cells displayed very compact juxtanuclear type Golgi (group III) and fewer highly elongated Golgi (group IV) (Study III. Fig. 1 C). Similar to *Jnk1*-/- cells, the absence of SCG10 led to less compact GA morphology (Study III, Fig.1B, C).

In mammalian neurons and also in *D. melanogaster* neurons, the GA localized to the GA cisternae and the so-called Golgi outposts that exist in dendrites (Gardiol et al., 1999, Pierce et al., 2001, Horton and Ehlers, 2003). Their structure is maintained by a highly dynamic array of microtubules, serving as tracks for directed vesicular transport and sustaining both antero- and retrograde protein transport between the endoplasmic reticulum and the GA (Trucco et al., 2004, Horton et al., 2005, Ye et al., 2007, Hoogenraad and Bradke, 2009, Stiess et al., 2010). We have previously proposed that SCG10 is predominantly localizing to the GA compartment in its dephosphorylated state (Study I). As there is a continuous flow of vesicles between the ER and the GA, we wondered if SCG10 together with JNK could be involved in orchestrating ER to GA transport. To test this, we used Brefeldin A (BFA),

which acts by blocking ADP-ribosylation factor protein 1 (ARF1), a GA associated guanine nucleotide exchange factor (Helms and Rothman, 1992, Donaldson and Lippincott-Schwartz, 2000). ARF1 is a key player in vesicular traffic from the ER to the GA. Blocking its action eventually leads to redistribution of GA complex proteins into the ER exit sites (Dascher and Balch, 1994, Elazar et al., 1994). Importantly, removal of the drug reestablishes GA cisternae (Lippincott-Schwartz et al., 1989). Pretreatment of mouse cortical neurons with BFA resulted in different recovery responses in *wt* and *Jnk1-/-* neurons (Study III, Fig. 2A,B). Importantly, cells lacking *Jnk1* recovered structural integrity of the GA at a slower rate than *wt* neurons (Study III, Fig.2B). We have previously noted that *Jnk1-/-* neurons were characterized by less compact GA structure and as were SCG10 depleted cells (Study III, Fig. 1B,C). As SCG10 is a potent microtubule destabilizing protein we wanted to know if it plays a role in GA recovery, hence we transfected cells with shRNA SCG10 and repeated BFA treatment. Interestingly, SCG10- depleted cells were unable to complete recovery of GA stacks reaching their baseline of vesicular GA at 4 h, unlike control cells which recovered their stacks much more efficiently (Study III, Fig. 2C,D).

3.1 Golgi fragmentation in response to excitotoxic insult involves both JNK1 and SCG10

JNK has been well established as a stress-activated kinase leading to transcriptional regulation and cell death (Kyriakis and Avruch, 2012). Hence, we tested whether activation of JNK at the GA could underlie SCG10-mediated GA dysregulation and play a role in neuronal stress responses. It was previously observed that some upstream JNK activators (Akr1p-like 1 and 2, MUK/DLK) localize on the outer face of the GA (Douziech et al., 1999, Harada et al., 2003). Importantly, active JNK itself is excluded from the GA region. To test if disregulated JNK activity at the GA is sufficient to disturb GA integrity we brought active JNK (MKK7-JNK1) to the GA compartment. We used the Golgi-localizing sequence of SCG10 (Charbaut et al., 2005) and fused it upstream from MKK7-JNK1 (Lei et al., 2002). Targeting of active JNK to the GA resulted in a strong GA fragmentation (Study III, Fig. 3C).

Given that GA fragmentation has been reported in stroke and neurodegeneration (Vizi et al., 2012) and the important role of JNK in stroke (Borsello et al., 2003, Hirt et al., 2004, Gao et al., 2005, Borsello et al., 2007), we examined the time-course of NMDA-induced GA fragmentation in cultured

neurons. We treated cortical neurons with 60 μM NMDA to induce hyper-activation of NMDA receptors. In control cells, the GA was localized at the peri-nuclear region, organized as a collection of well-defined cysternal stacks. We observed a mild fragmentation as quickly as 15 min post NMDA addition. We noticed that GA fragmentation proceeded in a time-dependent manner, starting from a nicely structured compartment, the GA fragmented into tiny particles within 1 hour of NMDA treatment (Study III, Fig. 4A). At the same time as shown in Fig. 4B, Study III, around 80% of cells died within 24 h.

We further examined the role of JNK1 in NMDA induced fragmentation of the GA in particular. For that purpose we used cortical neurons from wild type and *Jnk1* knockout mice. NMDA treatment revealed partial but significant delay in GA fragmentation in *Jnk1* deficient cells compared to wild type (Study III, Fig. 5A). We have repeated NMDA treatment on cells depleted of SCG10 (shRNA SCG10 transfected cells) and noticed that the fragmentation of the GA was also significantly retarded, suggesting that SCG10 is instrumental in NMDA-induced disruption (Study III, Fig.4C). Together, these results imply that NMDA-induced JNK may trigger SCG10-induced GA fragmentation. It was previously suggested by Nakagomi and co-workers that GA may serve as a sensor for death signals and various types of cellular stress like excitotoxicity, reactive oxygen species or ER stress irreversibly fragment the GA (Nakagomi et al., 2008). At the same time blocking GA fragmentation in non-neuronal cells entering mitosis blocks cell division (Sutterlin 2002). Interestingly Thayer et al., (2013) proposed that GA fragmentation in response to stress can be a reversible process. In their study they used elevated potassium, which led to GA fragmentation in hippocampal neurons. However this process was reversed upon returning cells to low potassium medium. This raises a possibility that GA fragmentation could be a transitional state before commencing to death. The fact that SCG10 and JNK1 both delay GA fragmentation could suggest a role for them in promoting cell survival, however upon longer stimulation with NMDA the death promoting signaling pathway take over and neurons die in result of excitotoxic stimulation.

3.2. Hyperactivation of NMDA receptors leads to Golgi fragmentation *in vivo* in animal models of diseases involving JNK activation

It has recently been observed that the GA undergoes fragmentation in a variety of neurodegenerative disorders including ALS, AD, Creutzfeldt-Jacob's, Huntington's disease, corticobasal degeneration as well as spinocerebral ataxia type 2 (Mourelatos et al., 1990, Stieber et al., 1996, Sakurai et al., 2000, Huynh et al., 2003, Gonatas et al., 2006). Importantly, the disease onset is only noticed years later (Gonatas et al., 2006). Given that we detect a substantial disruption of the GA in cultured cortical neurons following NMDA treatment, we wanted to examine GA integrity in animal models of excitotoxicity, namely stroke and epilepsy. Therefore, we monitored GA fragmentation in the MCAO model of stroke and in the Pilocarpine-induced *status epilepticus* (SE), both of which mimic disease states resulting from hyper-activation of NMDA receptors (Rice and DeLorenzo, 1998, De Angeli et al., 2009). Microscopic analysis of mouse brain, where one MCA was electro-coagulated and the other remained unaffected, revealed profound GA fragmentation in the infarct region already within 30 min. Conversely, in the contralateral side where the middle cerebral artery was unaffected, so was the GA (Study III, Fig. 6A,B). The sham-operated animal showed no evidence of disturbed GA structure (Study III, Fig. 6C). We have further investigated the protein expression profile after MCAO. For that purpose a small part of brain tissue from the MCAO region and a matching piece from the contralateral side were excised and subjected to western blot analysis. By 4h post-occlusion, we observed increased levels of SCG10 protein as well as an additional band of about 90 kDa in a GM130 blot, possibly representing a proteolytic cleavage product (Fig.6D). Moreover, we have also noticed increased activity of JNK (pJNK blot) and phosphorylation of its target protein c-Jun. These data show that GA fragmentation occurs in a well-characterized stroke model, indicating that disassembly of the GA is a feature of hyperactivation of glutamate receptors *in vivo* in the cortex.

The first clear indication that JNK was involved in excitotoxicity came from *Jnk3* knockout mice, which presented with reduced seizures as well as decreased cell death in response to systemic administration of kainate (Yang et al., 1997). It is well known that the hippocampus, among other regions such as the endopiriform nucleus, piriform cortex and claustrum, is one of the most affected brain regions following excitotoxic insult (Scorza et al.,

2009). Therefore we investigated whether GA degradation occurred in the hippocampus following *status epilepticus*. The inspected brain tissues revealed profound GA fragmentation as soon as 30 min following pilocarpine treatment within the hilus area of the dentate gyrus, CA3 and CA1 region compared to control brains, where the GA remained unchanged (Study III, Fig. E). Although pilocarpine induces cell death within hours post administration, SE-animals live and behave normally (Schauwecker, 2012). The level of observed fragmentation indicates a general feature of cells from the inspected regions. Moreover, we have previously shown that both SCG10 and JNK1 show comparable localization in brain regions known for their regenerative capacity. Both are present in pyramidal cells of the CA1 and CA3 as well as granule cells of DG in the hippocampus (Westerlund et al., 2008). However, it remains to be seen if JNK1 or any JNK isoform contributes to GA restoration following excitotoxic insult *in vivo.*

CONCLUDING REMARKS

This thesis was dedicated to furthering our understanding of the JNK1 and SCG10 function in the brain. We provided evidence that SCG10 is a *bona fide* target for JNK1, although JNK3 was also shown to phosphorylate SCG10 (Neidhart 2001). Hence a new physiologically essential function in regulation microtubule dynamics could be attributed to a key kinase, JNK1.

Microtubules are key elements of the eukaryotic cytoskeleton. They take part in diverse cellular functions including cell migration, polariztion, cell division or else intracellular trafficking. They originate at the centrosome, which serves as a microtubule-organizing center. Microtubule are characterised by their dynamic instability as shown by alternating cycles of growth and shrinkage separated by catastrophe and rescue events (Desai and Mitchison, 1997). Interestingly, cellular structures like the nucleus or the GA can also function as sites for nucleation of specific microtubules subpopulations (de Forges et al., 2012).

For neurons to grow long axons and dendrites, it requires strict control over the dynamics of microtubules. Therefore, accidental disassembly would easily impede outgrowth (Dent and Getler, 2003). We believe that JNK1, by blocking SCG10 destabilizing activity via phosphorylation events, can provide this stabilizing effect to extending neurites and therefore regulate the axodentritic architecture of cortical neurons (Study I).

It has been shown that SCG10 expression levels peak in the embryonic brain and decline after birth (Stein 1988). Importantly, neurite extension is one of the characteristic features of migrating neurons in the developing brain (Rakic, 1972). While we have shown that SCG10 is an important regulator of neurite growth (Study I) we have furthermore shown that SCG10 is also a new and important player in the process of radial migration in the developing brain (Study II). Knockdown of SCG10 significantly speeded up radial migration, as did the absence of *Jnk1* in knockout animals. In addition, exogenous expression of pseudophopshorylated SCG10 restored the migration defect noticed in *Jnk1*-/- mice. So did the presence of the JNK-specific inhibitor JIP-JBD. We have furthermore disclosed an additional role for JNK1 and SCG10 during earlier stages of neuronal migration, namely their involvement in multipolar transition (StudyII).

Finally, we have shown that the JNK1-SCG10 duo regulates the biogenesis of the GA complex. Depletion of either JNK1 or SCG10 affects its morphology and significantly impairs restoration of GA cisternae following BFA treatment. Moreover, we showed that either knockdown of SCG10 or knockout of *Jnk1* results in delayed GA fragmentation upon excitotoxic insult. This implies that JNK1 and SCG10 may also act as important regulators of microtubule dynamics within the close vicinity of the GA compartment under specific conditions.

ACKNOWLEDGEMENTS

This work was carried out at Turku Centre for Biotechnology, University of Turku and Åbo Akademi University during years 2007-2014. I am very grateful to Professor Riitta Lahesmaa for providing excellent facilities and promoting creative atmosphere at CBT. I am also grateful to Finnish Graduate School of Neuroscience for accepting me in their Doctoral programme and providing financial support for substantial amount of time during my PhD. I am thankful to Professor John Eriksson for admitting me at the Department of Biosciences at Åbo Akademi. Your support especially during last years of my PhD has been absolutely invaluable.

To my supervisor Docent Eleanor T. Coffey I am grateful for sharing her expertise and guidance throughout the thesis process. Professor Michael J. Courtney is thanked for his interest and enthusiasm he shared during our projects meetings.

I am sincerely grateful to my reviewers Professor Jaakko Saraste and Dr. Vicki Waetzing for taking the time to critically review this thesis and giving their ideas, comments and suggestion to improve it.

I am very thankful to the staff at the Turku Centre for Biotechnology for always being ready to help and give good advices. Special thanks go to Mikael Wasberg, Mårten Hedman, Petri Vahakoski, Hannele Vuori, Virpi Korpiranta, Pasi Viljakainen and Juha Stranden for making everything run nicely and smoothly in the department. Sirkku Grönroos, Aila Jasmavaara, Eva Hirvensalo and Tuula Suvanto your help with administrative issues has been priceless. Jouko Sandholm and Markku Saari thank you guys for sharing your amazing expertise on imaging, and creating great atmosphere in CIC facilities.

Past and present colleagues from the Coffey lab are thanked for collaboration and creating lively atmosphere in the lab: Artur Padzik, Dani Flinkman, Emilia Komulainen, Raghavendra Mysore, Benny Björkblom, Vesa Hongisto, Jenni Vainio, Tanya Tararuk, Lihua Sun, Emmy Ranniko, IIris Nykanen, Minna Tuittila. My special gratitude goes to Nina Westerlund with whom I had pleasure to work on migration study. I am very grateful to Susanna Pyökäri and Terhi Huitila-Masala without whose help no bench and animal work could be done.

"The Boys" Prasannakumar Desphande, Hasan Mohammad, Patrik Hollos are acknowledged for being extraordinary lab mates. It has been great

pleasure to be working with you, having fun, sharing thoughts and ideas on crime TV series when preparing neuronal cultures ;).

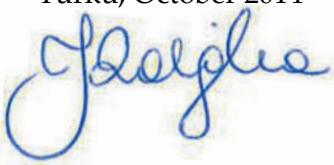
I could not reach this important day without absolutely the coolest and most amazing friends I have met and to whom I owe my deepest gratitude for being part of my life and notably letting me be part of theirs.

Especially I would like to thank the following; Sonia and Anna-Liisa you are my sisters you are my family! The Best Naapuris Jouko and Johanna for always letting me invite myself over ☺. Tomoko, Irina and Ghaffar our long-night discussions always made me keep up the spirit ;) and shed a new perpesctive on many "interesting life events". Hanna suuri kiitos for "completely spoiling" Nöffi, and sharing berries from your "secret" spots. Elli, Henna, Viveka, Maija and Francisco wining and dining with you guys each time have been great fun. Although away from Turku, yet it always feels easy to pick up where we have left; Dominique, Heidi, Jenny, Aurelie and Nathalie thank you girls! Saima and Eeva you are great travelmates and wonderful friends. Last but not least, big thank you goes to Taina Kovalainen and all the amazing people I met at her dance school. Thank you for keeping up the dancing spirit. In the moment of madness I always find peace of mind at your school Taina! Thank you for all the joy and happiness you share so generously ☺

Na koniec pragne podziekowac mojej rodzine i przyjaciolom w Polsce, ktorzy zawsze czekaja z niecierpliwoscia na moj przyjazd. Dziekuje Wam za to, ze nigdy nie przestaliscie we mnie wierzyc. Slowa szczegolnego uznania kieruje do mojej mamy, ktora w obliczu ciezkiej choroby kazdego dnia zaskakuje mnie hartem ducha.

Additional financial support from Magnus Ehrnooth Foundation, Oscar Öflund stiftelse, Åbo Akademi förskninginstitut, Rector of Åbo Akademi University, The Finnish Cultural Foundation, Kuistila Maud Muistosäätiö is gratefully acknowledged.

Turku, October 2014



Justyna Zdrojewska

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ISBN 978-952-12-3114-8

Painosalama Oy-Turku, Finland 2014