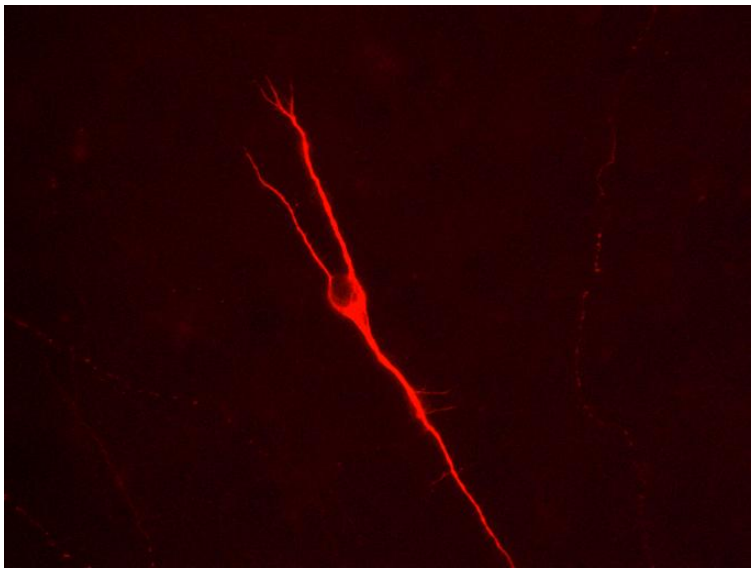
T-TYPE CALCIUM CHANNEL – FROM PHYSIOLOGICAL FUNCTION TO THERAPEUTICAL TARGETING:



EXPLORING THE NEURONAL DEVELOPMENT

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Tiivistelmä – Referat – Abstract

The aim of this study was to explore the functions of *T-type calcium channels*, and their possible role in *neuronal stem cells migration*. The role *of T-type calcium channel* in mature brain is known to be in producing electroencephalographic oscillations. This action in turn is the key factor in some neuronal physiological and pathophysiological functions, like non-REM sleep, memory, learning and absence epilepsy. In addition, *T-type calcium channels* have peripheral actions, but this study concerns on its neuronal functions. This low-voltage activated channels functions in *neurogenesis* is less known than its role in mature brain. It is known to promote neuronal proliferation and differentiation, but what comes to its possible actions in *neuronal migration*, is poorly studied. This study shows some evidence of *T-type calcium channel* taking part in *neuronal migration* in mice embryonic subventricular zones progenitor cells. Selective T-type antagonists, ethosuximide, nickelchloride and a scorpion peptide toxin kurtoxin, decreased the rate of migration in differentiating *progenitor cells*.

This study consists of a literature review and an experimental part. Another aim of this study is to consider an alternative approach to *stem cell* therapies based on invasive transplantation of the cells. This other attempt is *non-invasive* manipulating of endogenous *stem cells* to proliferate and *migrate* to the injured or depleted area in the brain, differentiate into a desired phenotype and stop their division after they have completed their mission. *Non-invasive* altering of the stem cells is awaiting *pharmacological* solutions to resolve the problems being faced in this effort. There are some *non-invasive therapies* already being used successfully to cure pathological conditions such as spinal cord injury. These methods could be used as well in *stem cell* based therapies in the treatment of neurodegenerative diseases and brain injuries. These methods are still in the beginning of their way and lacking the full understanding of the key factors that affect *neuronal development*. These factors include some important endogenous inducing and inhibiting substances. One of the most important inducing substances is calcium ion regulating a variety of events in *neurogenesis*. *T-type calcium channel*, as being widely expressed during early *brain development*, and decaying by neuronal maturation, might have a pivotal role in conducting *progenitor cells*.

Avainsanat – Nyckelord – Keywor

T-type calcium channel, neuronal stem cells migration, non-invasive therapies, pharmacology, neurogenesis, progenitor cells, neuronal development

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Tiivistelmä – Referat – Abstract

Tämän tutkimuksen tarkoitus oli tutkia *T-tyypin kalsiumkanavan* toimintaa ja sen mahdollista roolia *neuronaalisten kantasolujen migraatiossa*. *T-tyypin kalsiumkanavan* tehtävän kehittyneissä aivoissa tiedetään olevan elektroenkefalografisten oskillaatioiden tuottaminen. Nämä taas ovat eräiden fysiologisten ja patofysiologisten tapahtumien säätelyssä avainasemassa. Tällaisia tapahtumia ovat uni, muisti, oppiminen ja epileptiset poissaolokohtaukset. Näiden lisäksi *T-tyypin kalsiumkanavalla* on myös periferaalisia vaikutuksia, mutta tämä tutkielma keskittyy sen neuronaalisiin toimintoihin. Tämän matalan jännitteen säätelemän kanavan toiminta *neurogeneesin* aikana on vähemmän tutkittua ja tunnettua kuin sen vaikutukset kehittyneissä aivoissa. T-tyypin kalsiumkanavan tiedetään edistävän *kantasolujen* proliferaatiota ja erilaistumista *neurogeneesiksen* aikana, mutta vaikutukset niiden *migraatioon* ovat vähemmän tunnetut. Tämä tutkimus näyttää *T-tyypin kalsiumkanavan* todennäköisesti osallistuvan *neuronaaliseen migraatioon* hiiren alkion subventrikkeli alueelta eristetyillä *kanta- tai progeniittorisoluilla* tehdyissä kokeissa. Selektiiviset *T-tyypin kalsiumkanavan* antagonistit, etosuksimidi, nikkeli ja skorpionitoksiini, kurtoxin hidastivat *migraatiota* erilaistuvissa progeniittorisoluissa.

Tämä tutkimus koostuu kirjallisuuskatsauksesta ja kokeellisesta osasta. Tämän tutkimuksen toinen tarkoitus oli esitellä vaihtoehtoinen lähestymistapa invasiiviselle kantasoluterapialle, joka vaatii kantasolujen viljelyä ja siirtämistä ihmiseen. Tämä toinen tapa on endogeenisten kantasolujen *ei-invasiivinen* stimulointi, jolla ne saadaan *migratoitumaan* kohdekudokseen, erilaistumaan siellä ja tehtävänsä suoritettuaan lopettamaan jakaantumisen. *Non-invasiivinen kantasoluterapia* on vasta tiensä alussa, ja tarvitsee *farmakologista* osaamista kehittyäkseen. Joitain onnistuneita *ei-invasiivisia* hoitoja on jo tehty selkärangan vaurioiden korjaamisessa. Vastaavanlaisia menetelmiä voitaisiin käyttää myös keskushermoston vaurioiden ja neurodegeneratiivisten sairauksien hoidossa. Näiden menetelmien kehittäminen vaatii endogeenisten *kantasoluja* inhiboivien ja indusoivien mekanismien tuntemista. Yksi tärkeä kantasolujen erilaistumista stimuloiva tekijä on kalsiumioni. Jänniteherkät kalsiumkanavat osallistuvat kaikkiin neurogeneesiksen eri vaiheisiin. *T-tyypin kalsiumkanava*, joka ekspressoituu suuressa määrin keskushermoston kehityksen alkuvaiheessa ja vähenee *neuronaalisen kehityksen* edetessä, saattaa olla oleellisessa asemassa *progeniittorisolujen* ohjaamisessa.

Avainsanat – Nyckelord – Keywor

T-tyypin kalsiumkanava, neuronaalisten kantasolujen migraatio, non-invasiivinen terapia, farmakologia, neurogeneesi, progeniittorisolu, neuronaalinen kehitys

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Literature part

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Abbreviations

Experimental part

Abbreviations

ACI- rats	Augustus and Copenhagen-Irish- rats
ATP	adenosine triphosphate
BDNF	brain-derived neurotropic factor
BMP	bone morphogenic protein
BrdU	bromodeoxyuridine
CaMK	calcium/calmoduline kinase
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CNTF	ciliary neurotrophic factor
C-chain	carboxylacid chain
DCX	doublecortin
DG	dentate gyrus
DNA	deoxyribosome nucleotide acid
DPBP	diphenybutylpiperidine
EEG	electroencephalographia
EGF	epithelial growth factor
EPO	erythropoietin
ESC	embryonic stem cells
ETX	ethosuximide
FGF	fibroblast growth factor
GA	generalized absence
GABA	gamma-aminobutyric acid
GAERS	genetic absence epilepsy rat form Strasbourg
GCL	granule cell-layer

GCSF	granulocyte colony stimulatory factor
GDNF	glial –derived neurotropic factor
GFAP	glial fibrillary acidic protein
GLAST	glial glutamate transporter
GTP	guanosine triphosphate
HEK	human embryonic kidney
HERG	human ether-a-go-go- related gene
HTB	high threshold burst
HVA	high-voltage activated calcium channel
IGF	insulin-like growth factor
IPC	intermediate progenitor cell
IL	interleukine
I_T	voltage-activated T-type calcium channel
IUPAC	International Union of Pure and Applied Chemistry
KTX	kurtoxin
LIF	leukemia inhibitory factor
LTD	long-term depression
LTP	long-term potentiation
LTS	low threshold calcium spikes
LVA	low-voltage activated calcium channel
MPS	methyl-propylsuccinimide
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSC	mesenchymal stem cell/marrow stromal cell
NeuN	neuronal nuclear antigen
NGF	neuronal growth factor
NMDA	N-methyl-D-aspartic acid

NREM	nonREM
NRG	neuregulin
NRT	nucleus reticularis
NSC	neuronal stem cells
NSE	neuron-specific enolase
N-chain	amino-acid chain
OB	olfactory bulb
OEC	olfactory ensheating cell
ω-CgTx	omega-conotoxin
ω-AgTx	omega-agatoxin
PDGF	platelet-derived growth factor
PF	parallel fibre
PKA	protein kinase A
PSA-NCAM	highly polysialylated neural cell adhesion molecule
RG	radial glia
REM	rapid eye-movement
RT-PCR	reverse transcription polymerase chain reaction
SC	stem cell
SDF-1	stromal cell-derived factor 1
SGZ	subgranular zone
Shh	Sonic Hedgehoc
SNX-482	synthetic peptide toxin- 485
SVZ	subventricular zone
TC	thalamocortical
TGF-β	transforming growth factor- β
VEGF-α	vascular endothelial growth factor- α

VZ	ventricular zone
WAG/Rij	
-rats	Wistar Albino Glaxo from Rijswijk- rats
Wt	wild-type

LITERATURE PART

1. Introduction

Until the beginning of last century, the cells of the central nervous system were thought to be incapable of regeneration (Stahnisch and Nitsch, 2002). In 1928, a Nobel-prized researcher Santiago Ramón y Cajal wrote: "In adult centres, the nerve paths are something fixed, ended, immobile. Everything may die, nothing may be regenerated" (Ramón y Cajal, 1928). Over the last decade various studies have shown that there is plasticity and regeneration in the adult brain (Cotman and Nieto-Sampedro, 1985). These findings give rise to a possibility of regenerative cell therapy.

The strategies to investigate novel stem cell therapies begin with understanding their mechanisms of division, proliferation, migration and differentiation of neuronal stem cells (Morest and Silver, 2003). These events can be understood by studying the key modulators of these events (Barkho *et al*, 2006). One important factor in neurogenesis is genes dictating the fate of cells on their way differentiating from stem cells into a variety of mature cells. One could conclude that the genetics is the main thing controlling this scenario. Nevertheless, neuronal differentiation is influenced by several signaling molecules in addition to the control of gene expression (Greenberg *et al*, 1992). It is proven in a number of studies that some environmental factors also can affect stem cells life cycle (Schofield, 1978; Fuchs *et al*, 2004; Barkho *et al*, 2006). This study concentrates on the latter factor.

Environmental factors can be of different biochemical groups containing growth factors, neurotransmitters and some external factors including toxic compounds and pharmacological substances, for example (Emerit and Riad, 1992; Nguyen *et al*, 2001; Hagg, 2005). Elevated intracellular calcium is also a typical trigger of plastic changes in

neurons (Spitzer *et al*, 2004; D'Ascenzo *et al*, 2006; Deisseroth *et al*, 2004). Changes in calcium concentration may thus represent a common target for factors and signals that regulate neuronal progenitor cells differentiation and acquisition of specific neuronal phenotypes.

When investigating the effect of intracellular calcium in this study, the issue of interest is in particular the neuronal T-type calcium channel. This is partly due to the lack of studies of this type in this field of research. More precisely, the aim of this study is to explore the T-type calcium channels role in neuronal migration. One of the most poorly studied events in stem cells differentiation is their migration (Morest and Silver, 2003). Some essential questions remain unanswered: What makes them move? How do they know where to go? Why do some of them stay close to the sphere and others migrate far away from the beginning?

It is evident to study the function of T-type calcium channel when exploring its role in stem cells migration. While studying the physiological function of the T-type calcium channel, we gain some important information on its action, which might be useful also in designing other therapies than those based on stem cells. Yet, these theories need to be confirmed with studies on the relevant cell lines in question.

The capacity of adult neurons to regenerate offers intriguing possibilities which are nowadays being better understood, yet still beyond the reach of healthcare.

2. Neural progenitor cells

During the early brain development in the vertebrate embryo, a structure called neural plate is formed (Spemann and Mangold, 1924). That consists of a thick epithelium of cells, which transforms into a tube called the neural tube. This neural tube is an early development stage of the brain and spinal cord. The cells of this neural tube with the capacity to differentiate into neurons and glial cells are known as neural precursor cells (Sauer, 1935). These precursor cells are often reffered to as dividing neural stem cells (NSC). The neural stem cells give rise to neurons, astrocytes and oligodendroglial cells (Alvarez-Buylla and Lim, 2004). Neural progenitor cells form a heterogeneous population of glial and neural cells with varying multipotency. Differentiation happens gradually by the cells dividing and giving birth to new daughter cells, while some of them undergo an apoptotic cell death. The line between a stem cell and a differentiated cell is rather obscure. The difference between a stem cell and a progenitor cell is described with terms omnipotency and pluripotency. What then actually is a stem cell? There is a precise definition for a stem cell. The common features of stem cells are the ability of self-renewal by dividing and multi-potency to differentiate into any kind of a cell in the body, i.e., is omnipotent. This concerns a limited group of progenitor cells, which have lost some of their multi-potency, and have adapted features of a tissue type, i.e., is pluripotent. A neural progenitor cell or a precursor cell might differentiate as well into a neuron as a glial cell, while a stem cell has the ability to differentiate into any kind of a cell in the body. This makes the term progenitor cell useful concerning a large group of cells during their differentiation.

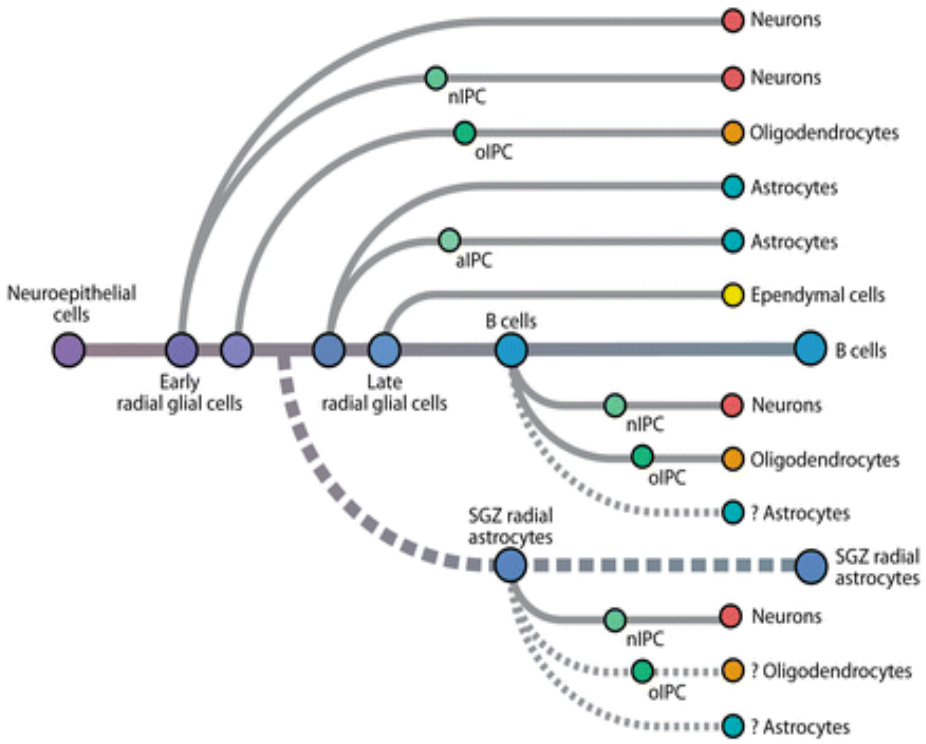
Distinguishing the states of differentiation is not problematic just because of the complexity of biology, but also because of the definitions being arguable (Morest and Silver, 2003). The often used term “glia” has lost its previous meaning of being a hyperonym for the glial cells. This is because of the different kind of glial cells can have very little in common with each other. Even less useful it is as a definition for a non-neuronal cell, since the neurons arise from the same progenitors and change constantly their phenotype during the differentiation. The term “glial” is more often used to describe the glial nature of the cells retaining the traditional glial functions as being the

supporting cells and also those that maintaining the capacity to differentiate (Kriegstein and Alvarez-Buylla, 2009).

As the multipotent neural precursor cells divide and differentiate, they generate an event known as neurogenesis which occurs both in the developing, and in the adult brain (Imayoshi *et al*, 2008). Neurogenesis in mammals begins with the induction of the neuroectoderm, which forms the neural plate at embryonic day 7.5 in mice (Temple, 2001). That gives rise to the neural tube (E8.5 in mice). These structures are made up by the neuroepithelial progenitors, which are a complex and heterogenous population (Boulder, 1970). This is followed by a progressive thickening of the cortex, and neuroepithelial progenitors obtaining features associated with radial glial cells (Choi and Lapham, 1978). The continuous dividing of these radial glial cells leads to a dynamic formation of intermediate progenitor cells (IPCs) producing mature neurons and such glial cell subtypes as astrocytes and oligodendrocytes (Haubensak *et al*, 2004). The same process can be seen also as a balance between symmetrical and asymmetrical division (Alvarez-Buylla, and Lim, 2004). Radial glial cells divide mostly asymmetrically, while the IPCs divide symmetrically. Symmetrical division is the cell's way of self-renewing by making similar daughter cells. Asymmetrical cell division produces fate-determined differentiated cells. Symmetrical division takes place in the beginning of neurogenesis. As the cells divide, they form a symmetrical round sphere, called neurosphere. The cells forming neurospheres happens *in vitro*. Neurosphere is a free floating cell aggregate (Reynolds and Weiss, 1992). It is possible to derive pure NSC lines that exhibit features of neurogenic radial glia (RG) progenitors (Conti and Cattaneo, 2005). *In vitro* they form neurospheres in a two-dimensional growth environment by symmetrical division. They can be considered as tripotent as they can give rise to neurons, astrocytes and oligodendrocytes. The extrinsic factors fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF) are proven to be sufficient for derivation and continuous expansion by symmetrical division of pure cultures of neural stem cells.

At the end of a developmental period of neuroepithelial division, the radial glial cells lose their ventricular attachment, proliferate and start migrating towards the cortical plate (Morest 1970; Choi and Lapham, 1978). In mammals, most radial glial cells

transform into astrocytes. The exact timings of division, proliferation, migration and differentiation are not specified yet, and these developmental phases are dynamic, overlapping processes (Kriegstein and Alvarez-Buylla, 2009), rather than chronological and rigid events, as it can be seen in picture 1.



Picture 1 Lineage tree of NSCs (Adapted and modified from Kriegstein and Alvarez-Buylla, 2009)

2.1 Postnatal stem cells

Before 1962 it was believed that after birth the CNS was structurally stable, with no neurogenesis happening in the postnatal brain. After publication of a study using the newly introduced methods of ^{3}H-thymidine autoradiography challenged this view (Altman, 1962). ^{3}H-thymidine is taken up by cells undergoing DNA synthesis in preparation for mitosis, and thus can be used as a marker for proliferating cells and their progeny.

Neurogenesis continues in the adult hippocampus, specifically in the subventricular zone (SVZ) in the walls of the lateral ventricles (Gage, 2000). In adult rodents the neurogenesis occurs mainly in the hippocampal subgranular zone (SGZ) and in the subventricular zone (SVZ), whereas in man it is restricted to SVZ. The young neurons from this area migrate to the olfactory bulb where they continually replace local interneurons (Imayoshi *et al*, 2008).

Adult neurogenesis happens also in normal conditions at some level, and is being induced in the cases of brain injury or degeneration (Kriegstein and Alvarez-Buylla, 2009). There is evidence of induced adult neurogenesis under certain circumstances. It can be activated by stress (Gould *et al*, 1997). Social stress in tree shrews is an animal model of depression (Raab and Storz, 1976). Both social stress and treatment with NMDA- receptor agonist MK-801 induced cell division in dentate gyrus of hippocampus of a tree shrew, and these cells migrate into the hippocampal granule cell layer (Gould *et al*, 1997). It was suggested that these results could be extrapolated to other mammalian species as well.

Also enriched environment induced neurogenesis in mice dentate gyrus of hippocampus and enlarged hippocampal granule cell layer (Kempermann *et al*, 1997). An enriched environment is an experimental setting that differs from standard laboratory housing conditions (Praag *et al*, 2000). It contains much more stimulus and exercise than that of a control group. The stimulus can be a larger group of animals in larger cages with varying and complex environment with for example tunnels and toys. The circumstances are changed frequently. Although increasing neurogenesis in mice, this study also questioned the beneficial effect of an enriched environment. There are many

factors that affect this method. Some of them are motivation and voluntary of the animal to interact with the environment. This method is questionable also for another reason. It cannot be said whether the enriched environment actually causes stress to the animal, and thereby induces neurogenesis, stimulates CNS cells and increases neuronal connections. More evidence of the effect of stimulation is gained with an opposite method. It is known that sensory deprivation withers neuronal networks.

Several forms of brain damage are shown to trigger neurogenesis in the postnatal brain (Tanaka *et al*, 2004). Seizure and stroke are shown to promote neurogenesis in the dentate gyrus. In an adult gerbil animal model the three steps of neurogenesis were detected with chemical markers after ischemia (Iwai *et al*, 2002). Three steps of neurogenesis, proliferation, migration and differentiation were elucidated with bromodeoxyuridine (BrdU), highly polysialylated neural cell adhesion molecule (PSA-NCAM), and neuronal nuclear antigen (NeuN) and glial fibrillary acidic protein (GFAP), respectively. A peak in cell number was detected 10 days after ischemia, with a seven-fold increase in BrdU-labeled cells and a three-fold increase in PSA-NCAM-positive cells in SGZ. The cell number of all markers increased gradually, and the BrdU- labeled cells with NeuN continued dividing in GCL (granule cell layer) until 2 months after ischemia. These results demonstrate that neural stem cell proliferation begins in SGZ and continues with the cells migrating into the GCL where they differentiate into mature neurons. It should be noted at this point, that in man the proliferation occurs mainly in the SVZ (Gage, 2000). This study also shows that brain damage in the form of ischemia induces neurogenesis.

Another study supports the findings described above. In a chemoconvulsant model of temporal lobe epilepsy the contribution of newly differentiated dentate granule cells was detected (Parent *et al*, 1997). Pilocarpine-induced status epilepticus in rats was followed by neurogenesis. BrdU- labeled cells had colocalized from SGZ to granular cell layer, GCL. The BrdU-labeled cells in the SGZ increased up to 13 days and were found from granular cell layer 56 days after the seizure. Also the ratio between the BrdU- labeled cells in SGZ and GCL gradually changed as an increase in the cells found in GCL. This indicates the level of migration being higher than in the control group. The cells that had migrated into GCL also expressed neuronal markers.

The role of adult neurogenesis seems to be the replacement of neurons. This still does not explain why this takes place in varying conditions. For an unknown reason, neurogenesis does not happen decently in neurodegenerative diseases, and not always in the case of brain injury, not at least at a sufficient level that is needed for the brain to function normally. To be optimistic, the evidence of adult neurogenesis gives hope of non-invasive stem cell treatment based on stimulation of the endogenous repair processes.

2.2. Stem cell therapies in neuronal regeneration

The therapeutic interest of the stem cells is their ability to generate cells and tissues to replace those that may have been lost due to disease or injury (Purves *et al*, 2008). Although there has been some promising work done in mice and some other experimental animals, there is no clinically validated use of stem cells for human therapeutic applications in the nervous system. The main problem is to introduce stem cells into mature tissue and control their division and stop it when needed.

Sources of used stem cell lines should be validated depending on the purpose (Hess and Borlongan, 2008). There is a significant difference between the mature somatic stem cells and embryonic stem cells (ES cells). They both have the potential for infinite self-renewal, but the ES cells can give rise to all tissue and cell types of the organism, while the somatic stem cells generate only tissue-specific cell types. Mesenchymal stem cells, MSCs, are multipotent, embryonic stem cells derived from the mesoderm. Confusing, yet, is a finding that bone marrow cells can adopt a neuronal phenotype when grown on a soft matrice, and a bone phenotype while on a hard matrice (Engler *et al*, 2006) and are also termed as MSC, marrow stromal cells. In addition, these cells can differentiate to haematopoietic stem cells (Spangrude *et al*, 1988). Olfactory receptor neurons that are replaced throughout life in mammals send axonal projections into the olfactory bulb. The cell that ensheaths these axons, known as an olfactory ensheathing cell (OEC), has same characteristics as astrocytes (Doucette, 1984). Also adult and embryonic neural stem cells derived from hippocampal areas are used in treatment of neural diseases. The best alternative for a stem cell source would be the patient's own cells. That would

avoid the problems faced with the immune system. On the other hand, in the case of disease having a genetic background, there is still a risk of the same diseased DNA being imported to the body.

Neural stem cells possess therapeutic potential because of their ability to migrate towards distant areas of injury (Aboody *et al*, 2000; Benedetti *et al*, 2000; Herrlinger, *et al*, 2000). More amazingly, they can migrate to CNS also from the peripheral tissues. This is a huge advantage, considering the problems of intra-cerebral injecting. In a rat Parkinson's disease animal model neural progenitor cells can survive and differentiate into neurons and glial cells (Svendsen *et al*, 1996).

There are some strategies in stem cell therapy that have been used to cure neurological diseases and injuries in humans with more or less success. These strategies can be divided into invasive and non-invasive methods (Barreiro-Iglesias, 2010). Invasive methods can be carried out by delivering the cells intracerebrally, or by an intravenous or intra-arterial route (Hess and Borlongan, 2008). Non-invasive methods include endogenous stem cells mobilization approaches in which stem and progenitor cells are mobilized by delivering into the brain cytokines such as granulocyte colony stimulatory factor (GCSF) or chemokines such as SDF-1; trophic and growth factor support, such as brain-derived neurotrophic factor (BDNF) or glial-derived neurotrophic factor (GDNF), to list some examples.

2.2.1 Problems with invasive therapies

Problems with current invasive methods have kept stem cell therapies out of the reach of healthcare. Of great clinical significance would be a solution of a scalable and easily commercialized therapy (Hess and Borlongan, 2008).

All invasive, cell based therapies involve *in vitro* culturing and manipulation of the stem cells before transplantation into the patient (Barreiro-Iglesias, 2010). Other disadvantages include the potential for teratoma formation, aberrant reprogramming and the presence of transgenes in cell populations.

Mesenchymal stem cells have gained some clinical interest because of their potential use in regenerative medicine and tissue engineering thus MSCs safety in clinical use remains questionable (Yu *et al*, 2008). In a study MSCs derived from human adipose tissues together with tumor cells were transplanted subcutaneously or intracranially into nude mice to observe tumor outgrowth. The results indicated that human adipose stem cells with tumor cell lines promoted tumor growth in nude mice. These findings indicate that MSCs could favor tumor growth *in vivo* (Yu *et al*, 2008). Some studies show that also the putative epithelial progenitor cells derived from bone marrow also contributes to tumor angiogenesis (Asahara *et al*, 1999; Poulsom *et al*, 2002; Rabitti, 2004). Once introduced to the body, the cell division is uncontrolled.

Immunity is another thing to consider. In the worst case, the transplant will be rejected by the host immune system (Griffin *et al*, 2010). If succeeded, a life-long immunosuppressing therapy might still be needed. Optimistically there is some promising work done with allogenic mesenchymal stem cells. MSCs are shown to induce immune system by stimulating multiple targets in the immune system including T-cells (Batten et al, 2001) and even in the absence of additive immunosuppressive therapy (Kahan, 2003).

In addition to the problems described above, one essential challenge is to control the differentiation of transplanted stem cells into a desired phenotype. Transplantation of human embryonic stem cell- derived neural progenitors into rodent striatum led to partial behavioural recovery in a rodent Parkinson's disease model (Ben-Hur *et al*, 2004). In this experiment the cells did not acquire a full dopaminergic phenotype in the rodent brain which made the researchers conclude that the differentiation into neurons should take place before transplantation. In a 1 methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) primate model of Parkinson's disease, transplantation of embryonic stem cell-derived enriched dopaminergic neurons resulted in survival of less than 1 % of transplanted neurons during a 14 weeks period (Takagi *et al*, 2005).

However, the ethical questions remain in what comes to using of fetal and embryonic stem cells. Two randomized controlled trials using dopaminergic neurons from aborted

fetuses failed to show much benefit and also there were reported troublesome dyskinesias in both studies (Freed *et al*, 2001). These poor results combined with ethical concerns led to abandonment of this approach. Ethics upon the use of embryos depend a lot on the source of embryonic cell used. Currently, there are three main sources of embryonic stem cells: already existing embryonic stem cell lines, embryos that are left unused after *in vitro* fertilization procedures and embryos created by means of somatic cell nuclear transfer technique (cloning) (Hug, 2004). Acceptance of these methods is variable, but the most commonly accepted source is already existing stem cell lines.

The outcome of these problems is that the stem cell therapies current situation is desperate. Although succeeding in culturing stem cells from the donor being the same as receiver, and thereby avoiding ethical and immunity problems, differentiating the cells into a desired phenotype and transplanting them into the body, several problems still remain. Those include the stem cells survival, differentiation and not turning into tumorgenic cells. In addition to all this, a cost-effective method must be developed before implementation into healthcare.

2.2.2 Possibilities in non-invasive therapies

The knowledge about adult neurogenesis and the factors inducing it offer us a possibility to create strategies of manipulating endogenous mechanisms of regeneration. First thing to find out before that can happen is to examine the key factors that affect this phenomenon.

The neuronal stem cells are able to migrate towards areas of injury mediated by some agents as a chemokine called SDF-1 (stromal cell-derived factor-1) that is up-regulated in astrocytes and endothelial cells in injured tissue (Imitola *et al*, 2004) although this does not happen in uninjured tissue (Abbott *et al*, 2004). This substance is classified as inflammatory mediators (Martino and Pluchino, 2006), and other agents of this group are cytokines as rantes (Owens *et al*, 2005) other chemokines as fractalkine, also including some interleukins as IL-1β and IL-6 (Aloisi *et al*, 2006). Inflammation is associated in many pathological conditions, such as cancer and some neurodegenerative

diseases. In addition to migration, these factors induce cell proliferation and differentiation. Conducting the differentiation into dopaminergic neurons is partly affected by IL-1β (Storch *et al*, 2001).

Growth factors are reported to conduct differentiation, proliferation and survival of neuronal progenitor cells (Martino and Pluchino, 2006). Glial-derived neurotrophic factor, GDNF, has shown neuroprotective and regenerative effects in a variety of animal Parkinson's disease models (Björklund *et al*, 1997; Behrstock *et al*, 2006). In human clinical studies this promising approach has failed due to significant side-effects (Kordower *et al*, 1999; Nutt *et al*, 2003). Brain-derived neurotrophic factor, BDNF, is reported to enhance the differentiation (Ahmed *et al*, 1995), but not in that specific way as GDNF. Of a great significance to laboratory *in vitro* propagation of neuronal precursors has been a discovery, that epidermal growth factor (EGF) and fibroblast growth factor (FGF-2) stimulate the division of embryonic or adult CNS precursors (Ciccolini and Svendsen, 1998). This is evident for maintaining a continuous undifferentiated cell line where a single cell divides and forms a cluster called neurosphere. Leukemia inhibitory factor (LIF) is also used for the same purpose (Tropepe *et al*, 2001). Other growth factors taking part in neurogenesis are ciliary neurotrophic factor, CNTF, erythropoietin, platelet-derived growth factor, PDGF, transforming growth factor-β, TGF-β and vascular endothelial growth factor-α, VEGF-α (Martino and Pluchino, 2006).

Some therapeutic strategies have already been investigated with trophic factors and cytokines to promote endogenous SVZ cell mobilization. They have been delivered to the brain either by viral vectors, infusions or via intranasal administration (Cayre *et al*, 2009). In Parkinson disease mouse models neuronal progenitors have been attracted toward the lesion with GDNF or TGFα infusion, and improved functional deficits. This has raised hope for the development of new strategies in neurodegenerative diseases, but clinical trials have failed so far. Also an infusion of FGF-2 and EGF has had the same effect in increasing directed migration toward the lesioned area (Winner *et al*, 2008). EGF is also shown to be useful in other studies, but presents some limitations. One of them is that it favors astrocytic differentiation over neuronal differentiation,

which may not have beneficial effects. The other concern is that it may facilitate the development of tumors. BDNF is another growth factor that has properties for progenitor cell mobilization. An injection of adenovirus expressing BDNF triggers increased olfactory neurogenesis and induces the migration of newborn neurons from SVZ to otherwise non-neurogenic regions (Benraiss *et al*, 2001). The hematopoietic cytokine erythropoietin (EPO) injection after a stroke increases the levels of VEGF and BDNF, which in turn leads to angiogenesis, neurogenesis and functional recovery (Wang *et al*, 2007).

The specific stem cell regulators form one group of the mediators in neurogenesis (Martino and Pluchino, 2006). Two major stem cell fate regulators are Sonic hedgehog, Shh and Notch-signaling membrane protein. Other regulators of this group are bone morphogenetic protein 4 (BMP 4), noggin-polypeptide and an extracellular matrix protein called tenascin C (Martino and Pluchino, 2006). Sonic hedgehog is a glycoprotein molecule that plays a critical role in the proliferation of endogenous neural precursor cells (Alvarez-Buylla *et al*, 2001). Administration of an intravenous hedgehog agonist induced endogenous cell proliferation after contusion in the adult rat spinal cord (Bambakidis *et al*, 2009). No side-effects were detected in the animals during the experiment. The data gained in study also provides evidence that direct injection of the agonist into the CNS is unnecessary to induce a proliferative response. Another study corroborates the effect of an Shh agonist. Intraperitoneal injection of cyclopamine, a sonic hedgehog antagonist, reduces ventricular proliferation and motor neuron regeneration in zebrafish (Reimer *et al*, 2009). Furthermore, one study shows, that activation of the Shh signaling pathway, using an orally administered Hh agonist, leads to proliferation of endogenous progenitor cells (Machold *et al*, 2003). Notch signaling regulates stem cell numbers both *in vitro* and *in vivo* (Androutsellis-Theotokis *et al*, 2006). It activates expression of genes hairy and enhancer of split 3 (*Hes3*) as well as the expression of sonic hedgehog, by activating cytoplasmic signals that lead to stem cell expansion. Hypoxia-inducible factor 1 is shown to be crucial for embryonic stem cell survival (Iyer et al, 1998). As a homeostatic O_2- regulator, it activates in response to physiologically relevant levels of hypoxia. It is activated also in progenitor cells niche. A related finding is that the number of progenitor cells in spinal cord increases in

response to injury or physical activity in adult rats (Cizkova *et al*, 2009). Both of these states represent a condition where hypoxia occurs.

Some common inductive neurotransmitters such as acetylcholine, glutamate and receptor activating ions play also an important role in differentiation (Kärkkäinen *et al*, 2009). Neural progenitor cells show metabotoropic responses (Ca^{2+} -discharge from intracellular stores) to glutamate, norepinephrine, acetylcholine and ATP during the first days of in vitro differentiation (Kärkkäinen *et al*, 2009). One of the most important inductive signals is retinoic acid that activates a class of transcription factors that modulate the gene expression. That is interesting regarding the fact that retinoic acid is also a teratogen. Not all the signals are inductive, to maintain the regulation and homeostasis, there are also some inhibitory signals, such as noggin and chordin, endogenous antagonists that modulate signaling via the TGF-β family.

A variety of studies suggest that environmental enrichment and exercise enhance brain plasticity. In physiological conditions, running and enrichment increase the levels of growth factors such as BDNF, IGF (insulin-like growth factor), NGF (neuronal growth factor) and FGF-2 (Gomez-Pinilla *et al*, 1997) and dentate gyrus (DG) neurogenesis (Kempermann *et al*, 1997). Environmental enrichment in clinical trials is much alike compared to the animal experiments. It includes physical and cognitive exercise and completing of demanding tasks in an environment that changes every day. Enrichment and exercise can be considered as a complementary therapy for brain repair.

These results demonstrate the possibility of stimulating the endogenous signaling procedures as a regenerative treatment. Although the recent approaches in non-invasive stem cell therapies seems to be a promising alternative to invasive technologies, the previous achievements in the latter one should not be abandoned. Taking into account that the only successful stem cell therapies in humans so far are invasive therapies, they still have enormous potential to be a therapeutic approach to such pathological conditions that lack clinical treatments. One method in therapeutic treatment does not exclude another, thus a combination of, for instance, pharmacological non-invasive treatment and cell transplantation could be used in order to improve regeneration. To

achieve the control over stem cells requires the understanding of them and their complex mechanisms of dividing, migrating and differentiating, and the forces that drive them to complete this diversity of reactions.

2.3 Identification of the multipotent stem cells

Stem cells expression of neuronal features or ion channels depends on the timing and the area of extraction of the cells and the *in vitro* circumstances they are grown in. This makes the question of the existence of these features in an experimental set up legitimate. Especially, when studying the effects of a specific ion channel blocker, one way of validating the experiment is to indicate the ion channel and the phenotype of the cell.

As described before, the difference between a neuron and a glial cell is not that straightforward. Fully understanding the phenotype of maturating neural cells involves a range of analyzing methods (Zigova *et al*, 2002). There are several analytical approaches to determine the phenotype of a maturating cell. This chapter introduces some of the most commonly used *in vitro* methods. Methods other than chemical labeling and morphology include electrophysiology, northern- and dot blots, *in situ* hybridization, RT-PCR (reverse transcription polymerase chain reaction) techniques and pharmacology (Lory *et al*, 2006), which is reviewed in chapter 4.3.

2.3.1 DNA labeling

Neuroblasts and neurons are unable to divide, while the multipotent glial stem cells undergo the continuous cell cycle (Zigova *et al*, 2002). When the cells divide, they take up nucleotides, the building blocks of DNA. A labeled nucleotide, for example a tritium-labeled thymidine can be used to separate the dividing cells from neurons. The labeled probe is available form minutes to hours after being injected. If a stem cell continues to divide, the levels of labeled probe in the cell's DNA are quickly diluted. If a cell undergoes only a single division the neuron retains high levels of labeled DNA. A

widely used marker of the newborn cells is 5-bromodeoxyuridine, BrdU. It is a halogenated thymidine analog that is permanently integrated into the DNA of dividing cells during DNA synthesis in cell cycles S-phase. It indicates the cells that are dividing during the period of BrdU exposure. The methods based on DNA labeling are useful when the intension is to distinguish the multipotent progenitor cells from differentiated cells.

2.3.2 Immunostaining

Immunocytochemical cell identification is an approach to label specific markers that are expressed by a certain phenotype (Zigova *et al*, 2002). Some proteins are specific to neurons and some are expressed by different types of glial cells. By immunostainig the proteins with antibodies one can detect the morphological and metabolic characteristics of the cells and quantify the phenotypes in the assay. The primary antibody attaches to the epitope, which is usually a phenotype specific protein on the cell's surface. The secondary antibody binds to the primary. The secondary antibody is used to label the bound primary antibody. In the experiments of this study, fluorescent secondary labels have been used. It is also important to examine literature, and choose the relevant antibodies and labels. Using more than one primary antibody is necessary for making sure that the findings are exactly of the desired phenotype. That is, because of the binding is not inevitably specific. Reducing the background staining is also used to prevent the non specific binding. That can be done by incubating the cells with serum of the species that the secondary antibodies are derived. One more issue to consider is the cross-reacting of the antibodies. For example a secondary anti mouse antibody can cross-react with a primary mouse antibody, which causes problems in detecting the images with microscopy. Here are some examples of antibodies to label a certain kind of proteins in the cells.

An example of a neuronal marker is β- III tubulin, Tuj-1. It is a neuron specific protein that the cells express during their development into mature neurons (Jiang and Oblinger, 1992). Tubulin isotypes induce axonal growth and the expression of them increases also when adult neurons are injured (Hoffman and Cleveland, 1988).

To identify glial cells, one can use a phenotype specific protein glial glutamate transporter, GLAST (Shibata *et al*, 1997). It is localized on the cell membrane of mature astrocytes and is marker for radial glial cells in the developing brain.

For identifying calcium channels there is, for instance, a commercially available synthetic peptide, which is an antibody produced in rabbit that binds to calcium channels α_1 subunit (Westenbroek *et al*, 1995). Therefore, it binds to all calcium currents.

There are also some T-type specific antibodies on the market that binds to T-type calcium channel subunit α_1G.

2.3.3 Morphology

It is difficult to identify developing stem cells by their morphology because it is changing constantly during the migration. Yet some characterization is been made and the typical morphological features of the cells are well known and agreed. In 1992 neurons and glial cells were distinguished by their morphology and the results were confirmed with chemical labels. The proliferating cells that expressed nestin and were immunoreactive for γ-aminobutyric acid and substance P had a neuronal morphology (Reynolds and Weiss, 1992). Also the cells that responded to NSE, neuron specific enolase, had small rounded somas with long and fine processes. In contrast, GFAP (glial fibrillary acidic protein) immunoreactive cells were stellate, with large somas and several thick processes. In the study of Reynold and Weiss the expression of phenotypes was restricted. The cells were not immunoreactive for glutamate, serotonin, tyrosine hydroxylase, methionine-enkephalin, neuropeptide Y or somatostatin. This is because of the other phenotypes appearing at different times or under different culture conditions. That is why one cannot expect to find all the possible phenotypes in a one cell culture.

2.3.4 Electrophysiology

Electrophysiological methods can be used to explore the functions of ion channels and ion conductances through these channels. Measuring the voltage of the whole cell membrane, results in excessive background noise. To diminish that a method of measuring a single voltage activated channel was developed and called the patch-clamp method (Neher and Sakmann, 1976), as a refinement from its predecessor, voltage-clamp technique. Nowadays measuring one or multiple channels (whole-cell patch clamp) with high resolution is possible (Hamil *et al*, 1981). Patch clamp recording uses, as an electrode, a glass micropipette that has an open tip diameter of about one micrometre (Kandel *et al*, 2000). The tip of the pipette encloses a membrane surface area or "patch" that often contains just one or a few ion channel molecules. The interior of the pipette is filled with a solution matching the ionic composition of the bath solution where the cell is, or in some methods, the cytoplasm. A chlorided silver wire is placed in contact with this solution and conducts electric current to the amplifier. The amplifier is connected to a computer and the software shows the data on the screen. The investigator can change the composition of the bath solution or add drugs to study the ion channels under different conditions.

2.4. Migration of stem cells

Migration and differentiation of the cells is controlled not only by genes, but also some external and endogenous factors (Emerit and Riad, 1992). The cells that express certain types of transcription factors are determined to differentiate into certain types of cells. Regardless, it has been shown in many studies (Kärkkäinen *et al*, 2009), that there are some environmental factors that can regulate the process. Thus, stem cells migration is closely related to the modulators of overall neurogenesis, described in the chapter 2.2.

2.4.1 Factors that affect migration

To begin their migration, the neural progenitor cells must undergo an essential transition from an epithelial to neuronal or glial cell (Hay, 1995). This transformation is induced by intrinsic and extrinsic factors. Intrinsic factors are the genes that conduct the changes from inside the cell. Extrinsic factors include for example growth factors, such as the transforming growth factor β (TGF-β) which affects the very first transformal changes in the cell (Sandford *et al*, 1997). In addition to morphological changes, other processes are required for a cell to start moving away from the epithelium of the spinal tube. Disassembling of the cell-cell contacts by expression of adherin molecules and reorganization of actin cytoskeleton are the very first changes in the phenotype of the cell (Burdsal *et al*, 1993; Hay, 1995). The cells must downregulate expression of adhesive genes so that they can move and migrate freely. Other factors associated with cell detachment are proteins reelin, tenascin-R and prokineticin 2 (Hack *et al*, 2002; Saghatelyan *et al*, 2004; Nguyen *et al*, 2001).

As essential as the detachment from other cells is to a single cell, it is equally important to the cell to attach to its environment. One change in extracellular matrix proteins is the expression of integrins. Especially alphaV integrin seems to be important for migration since interfering with its function inhibits migration in cell cultures (Blaschuk *et al*, 2000). These provide appropriate levels of adhesion to promote cell motility. Extracellular matrix proteins known to interact with integrins are chondroitin sulphate, tenascin-C, laminin and proteoglycans (Thomas *et al*, 1996).

Several growth factors regulate migration. Platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) keep the cells in migratory and immature state (Assanah *et al*, 2006; Ivkovic *et al*, 2008). In addition, PDGF seems to have chemoattractive features, as the cells migrate towards higher concentrations of PDGF in a transfilter migration assay (Armstrong *et al*, 1990). Neural growth factors BDNF and GDNF are also considered as chemoattractants in what comes to their roles in migration (Paratcha *et al*, 2006; Chiaramello *et al*, 2007). Many agents that serve some other purpose have a chemoattractive nature. A pure chemoattractive compound is a chemokine, SDF-1 (Imitola *et al*, 2004).

Polysialylated NCAM (PSA-NCAM) that belongs to the immunoglobulin superfamily and doublecortin (DCX) which is a microtubule-associated protein facilitate the translocation of the cell in migration (Ono *et al*, 1994). Translocation is a term describing the cells movement.

There are a number other factors that control not only the migration, but other stages of neurogenesis, proliferation and differentiation. These mentioned factors are not limited to migration. These phases are sometimes difficult to separate because they occur partly at the same time in an overlapping manner, and a certain factor clearly connected to migration or some other developmental period is not that evident.

2.4.2 Physical movement of the cells

The morphological changes in neuroepithelial cells were already described in 1977 by Domesick and Morest. The cell body moves forward within a leading process (Domesick and Morest, 1977). A variety of patterns occur in this movement within the cells. There are differences in speed and in the manner of moving either constantly forward or in a saltatory way. The latter manner is intermittent moving in bursts with little or no movement between the bursts.

Two main migration modes are described during development: radial and tangential migration (de Carlos *et al*, 1996; Anderson *et al*, 2001). Radial glial cells of the ventricular zone (VZ) provide tracks along which immature neurons migrate into the cortex. This type of migration is called radial migration because the cells migrate perpendicular to the ventricular surface. Neuronal progenitors born in the SVZ migrate in a characterized tangential migration forming chains towards the olfactory bulb. Newborn neurons integrate into the pre-existing neuronal network and traverse along the chains made by radial glial (RG) cells. This three-dimensional movement causes problems in two-dimensional *in vitro*- circumstances. In the growth medium cells can grow in a limited amount of layers.

2.4.3 Migration in pathological conditions

Cell proliferation and migration are almost always disturbed in a diseased brain (Cayre *et al*, 2009). Developmental cell migration disruption causes neurological disorders, not only in developing, but also in the adult brain. Disruptions in the migration can lead to many psychiatric disorders: autism, retardation, schizophrenia, epilepsy and neurodegenerative diseases. The role of neurogenesis in chronic neurodegenerations is rather uncertain. In Alzheimer's disease the hippocampal neurogenesis is increased (Jin *et al*, 2004). This happens supposedly because the newborn neurons replace the dead ones. Conversely, dopamine depletion impairs precursor cell proliferation in Parkinson disease (Höglinger *et al*, 2004). This seems logic according to the fact that dopamine positively regulates adult neurogenesis (Baker *et al*, 2004). Research in epilepsy affords contradictory findings. Dentate gyrus neurogenesis is stimulated by pilocarpin-induced seizures (Parent *et al*, 1997; Gong *et al*, 2007). Recent work challenges these results performed on hippocampal biopsies of patients with temporally lobe epilepsy. This study suggests that epileptic activity does not stimulate neurogenesis in the human dentate gyrus (Fahrner *et al*, 2007).

In the case of ischemia and acute injury the role of progenitor cell activity and migration is clearer. Similar observations are made in a variety of studies (Miles and Kernie 2006). In rodent models ischemia leads to proliferation of dentate gyrus progenitors together with migration towards the injured area (Liu *et al*, 1998).

In schizophrenia, hippocampal stem cell proliferation is impaired in patients (Reif *et al*, 2006) as well as in animal models (Liu *et al*, 2006). Several molecules that play crucial roles in cell migration are disrupted in schizophrenia patients (Stefansson *et al*, 2003). One of them is Reelin that takes part to cell detachment (Hack *et al*, 2005). Also a chemoattractant protein NRG1 (neuregulin-1) and its receptor Erb4 are disrupted (Steffanson *et al*, 2003).

2.4.4 The role of voltage gated ion channels in neurogenesis

Researchers Hitoshi Komuro and Pasko Racik have made a lot work studying the role of calcium fluctuations and calcium ion channels in neurogenesis. In 1992 they reported that the rate of granule cell movement depends on both extracellular calcium concentrations and calcium influx through N-type calcium channel (Komuro and Rakic, 1998). They have proven the movement inducing effect of intracellular calcium fluctuations in many studies (Komuro and Rakic, 1992; 1993; Rakic and Komuro, 1995; 1996; 1998). These findings were gained with newborn progenitor cells starting their migration and differentiation. N-type calcium channel seemed to have a specific effect on migrating cells (Komuro and Rakic, 1998). A statistically significant decrease in the rate of migration was gained with a 30 nM concentration of a specific N-type antagonist, omega-conotoxin, ω-CgTx. This concentration of toxin decreased the rate by 24 %. The effect was dose-dependent, lowering the rate by 50 % at 300 nM of ω-conotoxin, 65 % at 1000 nM and >78 % at 3000 nM. They also studied the effect of L- and T-type calcium channels and reported no significant effect. A T-type channel blocker nickel was used at 100 µM and to block L-type channels they used a specific blocker nifedipine at a concentration of 5 µM. Also Na^+-channels were blocked with its specific antagonist tetrodotoxin at the concentration of 10 µM and with no significant effect.

Another study reports a response to L-type calcium channel antagonist nifedipine in olfactory bulb progenitor cells. Compared to the studies of Komuro and Rakic, these data were gained from cells after three days growing *in vitro* (Cigola *et al*, 1998). A significant change in the cells phenotype and their expression of ion channels happens during the very first days of differentiation (Kärkkäinen *et al*, 2009). In the study of Cigola et al., after one day of differentiation a small population of immunostained neurons was detected (Cigola *et al*, 1998). After additional two days the number had doubled. The study demonstrated that L-type calcium channels mediate the regulation of the dopamine phenotype of neurons.

Another study presents similar findings. In neural progenitor cells derived from postnatal mice cortex, stimulation of L-type calcium channel with its specific agonist

BAY K 8644, induced the neuronal differentiation significantly (D'Ascenzo *et al*, 2006). Treatment with an antagonist nifedipine at 5 µM produced opposite effects. These experiments were made with cells after 12 days growing *in vitro*. All this data shows that calcium has a significant role in progenitor cells development, in migration and differentiation. The role of T-type calcium channel is discussed in chapter 3.2.

3. Classification of voltage activated calcium channels

A ligand, which is a receptor binding molecule, regulates transmembrane ion channels (Koulu and Tuomisto, 2007). Some endogenous ligands are acetylcholine, GABA, glysine and glutamate. In addition to these ligand activated channels, there are voltage activated ion channels. Conversely to ligand activated channels, voltage gated ion channels do not function as receptors for physiological transmitters. Instead, there are many pharmacological substances that can bind also to voltage gated ion channels. Such compounds are for instance, calcium blockers that are used to treat hypertension, majority of anti-arrhythmic agents and beta-blockers.

Calsium ion is a common intra- and extracellular messenger, which takes part to neurotransmission and actionpotential. Over the past years the voltage-gated calcium channels have gained attention, due to their role as activators of gene expression and cell fate by regulating the downstream signaling to the nucleus (Spitzer *et al*, 1994). Voltage-gated calcium channels are known to mediate calcium influx in response to membrane depolarization (Yu *et al*, 2005). They also regulate intracellular processes such as contraction, secretion and neurotransmission in many different cell types. They belong to a gene superfamily of transmember ion channel proteins that includes voltage-gated potassium and sodium channels.

The voltage-gated calcium channels can be divided into two groups, low-voltage activated (LVA) and high-voltage activated (HVA) channels (Koulu and Tuomisto, 2007). HVA -channels consist of L-type channels situated mostly in the cardium, as

well as P/Q, N and R- type channels. The LVA- channels are also known as T-type calcium channels. All these channel types can be further divided into α_1- subunits.

Table 1 Classification of voltage-activated calcium channels

L-type	P/Q-type	N-type	R-type	T-type
• $Ca_V1.1$ (α_{1S}) • $Ca_V1.2$ (α_{1C}) • $Ca_V1.3$ (α_{1D}) • $Ca_V1.4$ (α_{1F})	• $Ca_V2.1$ (α_{1A})	• $Ca_V2.2$ (α_{1B})	• $Ca_V2.3$ (α_{1E})	• $Ca_V3.1$ (α_{1G}) • $Ca_V3.2$ (α_{1H}) • $Ca_V3.3$ (α_{1I})

There are altogether ten members of the voltage-gated calcium channel family (Catterall, 2005). Calcium currents have diverse physiological and pharmacological properties, distribution in different cell types and therefore they stand for distinct classes named among an alphabetical nomenclature (Tsien *et al*, 1988). High-voltage activated channels L, N, P/Q and R require strong depolarization for activation, whereas the low-voltage activated T-type currents are activated by weak depolarization and are transient. The rational nomenclature, based on the well defined potassium channel nomenclature was created in 1997 (Ertel *et al*, 1997). The name of calcium channels, Ca, stands for the chemical symbol of the principal permeating ion, calcium, with the principal physiological regulator (voltage) indicated as a subscript (Cav). The numerical identifier corresponds to the α1 subunit gene subfamily (1 to 3, see table x).

Dividing channels into subtypes is based on the α1 subunit, which is the largest subunit of the calcium ion channel (Hofmann *et al*, 1994). It consists of four homologous domains with six transmembrane segments. Encoded by multiple genes, the α1 subunits differ by pharmacological and electrophysiological activities. The complete amino acid

sequences of these α1 subunits are more than 70 % identical within a subfamily but less than 40 % identical among the three subfamilies.

3.1 High-voltage activated calcium channels

L-type calcium channels are localized mainly in vascular endothelial cells and in the heart (Koulu and Tuomisto, 2007). Specific antagonists for these channels are dihydropyridines, phenylalkylamines and benzothiazepines (Hockerman *et al*, 1997). In muscle and endocrine cells the L-type calcium channels are the main calcium currents. Their function in those tissues is to initiate contraction and secretion. Dihydropyridines are commonly used to treat hypertension by blocking L-type channels in the myocardium and vascular endothelium. Their main effect *in vivo* is vasodilatation (Koulu and Tuomisto, 2007). Other physiological functions that L-type channels take part in are hormone release, regulation of transcription, synaptic regulation, hearing and neurotransmitter release from sensory cells (Hockerman *et al*, 1997). $Ca_V1.4$ channel subtype's specific function is to release neurotransmitters from photoreceptors.

Both P/Q- and N-type calcium currents are distributed in nerve terminals and dendrites, as well as in neuroendocrine cells (Catterall *et al*, 2005). In these cells they are involved in neurotransmission at most fast synapses and mediate calcium entry into cell bodies and dendrites. This Ca_V2 subfamily of calcium channels is relatively insensitive to dihydropyridines, but these channels are specifically blocked with high affinity by peptide toxins from spiders and marine snails (Miljanich and Ramachandran, 1995). The $Ca_V2.1$ channels are blocked specifically by ω-agatoxin from funnel web spider venom. A specific blocker of $Ca_V2.2$ channels is ω-conotoxin, which is a snail toxin. The $Ca_V2.3$ channels are antagonized by a synthetic peptide toxin SNX-482 which is derived from tarantula venom.

3.2 Low-voltage activated calcium channels

The T-type calcium currents, Ca_V3 channels, are insensitive to both dihydropyridines and the spider and snail toxins that block HVA channels. There are no widely useful pharmacological agents that block T-type calcium currents (Perez-Reyes, 2003). There are somewhat selective antagonists for T-type channel. One of them is mibefradil which is 3- to 5-fold more selective to T-type than L-type calcium currents. A peptide toxin kurtoxin inhibits the activation gating of $Ca_V3.1$ and $Ca_V3.2$ channels. Such a chemical agent that is more specific and binds to T-type calcium channels with high-affinity would be beneficial for therapy and more detailed analysis of the physiological roles of these channels (Catterall *et al*, 2005).

4. T-type calcium channels

4.1 T-type calcium channels electrophysiology

The T-type calcium channel family consists of three alpha subunits, α1G, α1H, and α1I (Fox *et al*, 1987). These low voltage activated channels, LVAs, can activate at significantly lower activating potentials than the high voltage activated channels. Under physiological recording conditions, the apparent activation threshold for I_T is near resting membrane potential -50 mV to -60 mV (Huguenard and Prince, 1992; Umemiya and Berger, 1994). High threshold calcium channels require activating potentials more positive than -40 mV to open (Sundgren-Andersson and Johansson, 1998). In CNS neurons, the resting potential is lower than – 60 mV. Several studies have been made using isolated neurons from a variety of brain regions (White *et al*, 1989; Allen *et al*, 1993; Kuo and Yang, 2001). In these studies the membrane potential was measured by holding the cell at – 90 mV and then increasing depolarizing pulses in -10 mV increments. T-type channels begun to activate when the membrane potential depolarized above -60 mV, while the HVA currents peaked around 0 mV. T-type

currents also depolarized and decayed slowly, the whole action potential lasting for hundreds of milliseconds. In contrast, the HVA channels activate fast and recovery in relation to activation is slow. Another specific function of LVA currents in neuronal excitability is that they mediate low-threshold burst discharges throughout the brain.

Low-threshold calcium spikes, LTS, have been described in slices and in isolated neurons from several parts of the brain (see for review, Perez-Reyes E, 2003). These bursts or spikes appear under physiological and pathological conditions. LTS cannot be triggered by depolarization from the resting membrane potential, which is typically between -60 and -65 mV (Kim *et al*, 2001). These spikes occur during the hyperpolarization. LTS begun to appear after the neuronal cells membrane was hyperpolarized below -69 mV and full-amplitude spikes were observed when the membrane potential reached -73 mV. As a conclusion, the role of these spikes is considered as a pacemaker. LTS can trigger a burst of action potentials that are brief and happen in high frequency, especially in some thalamic neurons (Sundgren-Andersson and Johansson, 1998). This can also be called burst firing. T-type calcium channels play a role in neuronal rhythmogenesis also referred to as oscillations, such as the sleep-related slow electroencephalographic (EEG) waves, rhythms related to motor coordination, learning and hyper-synchronous oscillations during epilepsy (Huguenard, 1996).

4.2 T-type calcium channels functions

Although being expressed throughout the body, T-type calcium channels are widely expressed in the nervous system, and sometimes at very high densities (Talley *et al*, 1999). In CNS, T-type channel's have a very specific role in neuronal rhythmogenesis (Huguenard, 1996). T-type channel's peripheral functions include taking part in muscle contraction, hormone secretion and pain (Perez-Reyes, 2003). This thesis concentrates on the most documented CNS functions of T-type calcium channel. Neuronal rhythmogenesis, operated by T-type channel, include sleep-related EEG waves, rhythms related to motor coordination, learning, memory and epilepsy, in addition to sensory transmission, dendritic integration, neurotransmitter release and neural development

(Cueni *et al*, 2009). From the viewpoint of this study it is interesting to note some similarities in what comes to all of these functions. There is a connection between sleep, brain development and learning. How is T-type calcium channel related to the forming of synaptic connections? Does the malfunction of this channel manifest in a form of absence epilepsy? Some answers can be found in the literature and the conclusions that can be made according to it.

4.2.1 Electric oscillations during the sleep

Sleep in mammals comprises two distinct states, REM (rapid eye movement) and nonREM (NREM) sleep, which alternate at fairly regular intervals throughout each sleep period (Carskadon and Dement, 2000). During a natural night sleep the EEG activity of the brain is characterized by regular oscillations at different frequencies (Horne, 1988; Niedermeyer and Lopes da Silva, 1993). These EEG waves can be divided into groups by their frequencies. The so-called beta (10–20 Hz) and gamma (20–80 Hz) waves are detected when a person is awake and in a fully attentive state. Relaxed wakefulness is characterized by alpha (8-13 Hz) waves and NREM sleep by theta (3-7 Hz) waves. As the sleep deepens, the oscillations slow down to frequencies of delta waves (1-4 Hz) and deeper delta waves (< 1 Hz).

The rhythmic action potential bursts underlying alpha/theta waves have been named high threshold bursts, HTBs. These are made by thalamo-cortical, TC neurons that fire repetitive, relatively short bursts of action potentials in synchrony with EEG waves at these frequencies (Hughes *et al*, 2004). Logically, original observations suggested that HTBs were mediated by high threshold calcium channels (Hernandez-Cruz and Pape, 1989; Jahnsen and Llinas, 1984). However, the spikes that underlie HTBs are more effectively blocked by relatively small concentrations of Ni^{2+} than Cd^{2+} (Hughes *et al*, 2004). Nickel ion is a relatively specific antagonist of T-type calcium currents (Biagi and Enyeart, 1991). This observation led to a conclusion of T-type channels being more involved in TC neurons high threshold bursts (Hughes *et al*, 2004). Similar activity of HTBs that involve a contribution by T-channels have been shown in many other

neuronal types including hippocampal CA1 pyramidal neurons (Magee and Carruth, 1999), cortical (Gibson *et al*, 1999) and thalamic interneurons (Zhu *et al*, 1999) and NRT neurons (Landisman *et al*, 2002). Since HTBs are also moderately suppressed by nifedipine, it is unlikely that T-channels are solely responsible for their generation (Hughes *et al*, 2004).

The slow sleep rhythm (< 1 Hz) is present in almost all stages of NREM sleep, underlies the sleep K-complex and groups together periods of delta waves (Amzica and Steriade, 1997) and sleep spindles (Amzica and Steriade, 2002). The K-complexes are an EEG manifestation of repeatedly varying membrane potentials from depolarization to hyperpolarization, called as UP and DOWN states respectively. The membrane potentials switching from depolarization to hyperpolarization and back are mediated by I_{Twindow} in TC and NRT (nucleus reticularis) neurons (Crunelli *et al*, 2005). The writers named the combination of recombinant T-type Ca^{2+} channels ($Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$) as I_{Twindow}.

Sleep spindles are a burst of EEG waves, that occur during the stages 2 and 3 of natural NREM sleep in humans, has a frequency of 12-15 Hz, lasts for a few seconds and has a feature of increasing and waning waveform (Niedermeyer and Lopes da Silva, 1993). Since the low threshold calcium potentials, LTCPs, are present at almost every cycle of the spindle wave in TC and NRT neurons, the role of T-type channels is quite evident (Steriade *et al*, 1985; von Krosigk *et al*, 1993; Contreras and Steriade, 1996).

To summarize, the T-type channels constitute the single, most crucial voltage-dependent conductance that permeates all major non-REM sleep oscillations in thalamocortical and NRT neurons, including oscillations that stand for theta waves, K-complex, slow sleep rhythm, sleep spindles and delta waves. In the light of current views on the critical function of NREM sleep in learning and memory (Bennington *et al*, 2003), one could expect that T-type channel has also some other patho-physiological significance.

4.2.2 Memory and learning

The dominant model of activity-dependent synaptic plasticity was represented already in 1949 as the Hebbian synapse when Hebb presented his finding of neural networks having learning-related properties (Hebb, 1949). His theory was that synaptic activity enhances learning, if the synaptic inputs that activate the postsynaptic neurons are strengthened. On the other hand, some other postsynaptic neurons need to be silent. In other words, neuronal transmission strengthens the synapse. The experimental methods of learning are based on this theory. There are several studies that have investigated the relevance of cellular phenomenons to learning, by genetic or pharmacological manipulations that affect LTP (long-term potentiation) and LTD (long-term depression) in learning abilities in experimental animals (e.g. Miller and Mayford, 1999; Yin and Tully, 1996). The outcome of these studies has been that stimulation of neurons leads to nuclear transcription and protein synthesis in neuronal networks. The stimulation does not always necessarily lead to long-term potentiation, but can also trigger LTD, long-term depression, which leads to decay or silencing of synaptic interactions. The necessary condition for inducing LTD is activation of a specific synapse *without* action potential in the postsynaptic neuron (Kemp and Bashir, 2001).

It has been suggested, that REM sleep could influence synaptic plasticity producing LTP (Aston-Jones and Bloom, 1981). However, the neuronal activity during REM sleep is substantially similar to that of being awake (Hobson and Steriade, 1986). This questions the specific role of REM sleep in neuronal plasticity. The main difference between REM sleep and wakefulness is the dramatic decrease in release of norepinephrine and serotonin in REM sleep. Reduced norepinephrine release could stand for LTP, but does not explain it thoroughly (Bramham and Srebro, 1989). Synaptic plasticity has also been related to NREM sleep being a homeostatic regulator due to the minimal neurotransmission happening in it (Benington, 2000).

Putting the question in other order has resulted with more clear effects. Instead of asking “Does the sleep enhance synaptic plasticity?” The question can be posed as “Does sleep-deprivation impair learning and synaptic plasticity?” Sleep deprivation does profoundly interfere with learning and memory in mice and rats, especially in

demanding tasks that presumably require more complex cognitive processing (Block *et al*, 1981; Smith, 1985, 1995). A range of studies suggest the same causes of sleep deprivation. Since the neuronal activities of NREM sleep are produced largely by two types of ion channels: the T-type calcium channel and a hyperpolarization activated cation channel (I_h) (Steriade et al., 1993b), one might conclude that T-type has a role in synaptic plasticity and thus in learning and memory.

In mature CA1 hippocampal pyramidal cells, a contribution of T-type channels to NMDA-receptor –dependent long-term potentiation was reported (Thomas *et al*, 1998). Another study reported that increase in postsynaptic calcium concentration, resulting from subthreshold excitatory synaptic activity in cerebellar Purkinje neurons, led to synaptic integration (Eilers *et al*, 1995). Before that it was thought, that synaptic integration results from the simple summation of electrical signals produced by each active synapse innervating a given neuron. The finding of some non-electrical, subthreshold synaptic integration caused by a second-messenger, calcium, occurs, revealed another function of low-threshold calcium channels. One form of cerebellar LTP is parallel-fibre (PF)- Purkinje cell stimulation (Lev-Ram *et al*, 2002). T-type calcium channels are the main component of the LVA calcium input in dendritic spines (Isope and Murphy, 2005).

Surprisingly, PF-long term depression still provides the most convincing example available showing that alterations in synaptic strength can mediate behavioral learning (Jörntell and Hansel, 2006). The creation of new synaptic connections requires the decay of older ones, with a complexity of mechanisms fortifying and silencing synaptic interactions. Other studies yet indicate that both LTP and LTD are the main mechanisms of synaptic plasticity (Bliss and Collingridge, 1993; Bear and Malenka, 1994). Both of these activities are dependent on Ca^{2+} entry through NMDA receptors (Bear and Malenka, 1994). The increases in Ca^{2+} associated with LTD are both smaller and longer lasting than those associated with LTP (Cho *et al*, 2001; Cornier *et al*, 2001). Since the critical difference between LTP and LTD is indeed that smaller increases in Ca^{2+} produce LTD, the involvement of T-type Ca^{2+} channels in LTD is reasonable (Benington and Frank, 2003). A range of other studies show that T-type calsium

channels have been implicated in long-term depression, LTD, induced by metabotropic glutamate receptors (mGluRs) (Oliet *et al*, 1997; Birtoli and Ulrich, 2004; Bender et al, 2006; Nevian and Sakmann, 2006). As both LTD and LTP are uniquely important for neuronal plasticity, the *hebbian* thesis still seems to apply.

4.2.3 Epilepsy

Absence seizures in epilepsy are characterized by a brief loss of consciousness associated with an EEG recording of 3 Hz bilaterally synchronous spike-and-wave discharges (SWDs) (Niedermeyer, 1996; Williams, 1953). These discharges are burst-like action potentials, the so-called burst firing, in thalamo-cortical neurons, TC neurons (Crunelli and Leresche, 1991). This characteristic firing pattern of TC neurons is evoked by low-threshold Ca^{2+} potentials (LTCPs) (Deschénes et al, 1984; Jahnsen and Llinás, 1984). Therefore, it has been proposed, that low-threshold T-type calcium channels are involved in the genesis of absence seizures in the thalamocortical network (Coulter *et al*, 1989; Crunelli and Leresche, 1991). More evidence of T-type channels dysfunction in absence epilepsy is gained in studies with genetically modified animals. T-type calcium channels were moderately increased in the thalamic neurons of the genetic absence epilepsy rat from Strasbourg (GAERS), a model of spontaneous absence epilepsy (Tsakiridou *et al*, 1995). In addition, one group generated knockout mice for the α_{1G} subunit of the T-type channel. It revealed, that the mice lacked burst firing in thalamocortical relay neurons, and thereby were resistant to absence seizures, that were caused by systemically injected γ-butyrolactone or (RS)-baclofen. Absence seizures induced by systemic injecting of these drugs were demonstrated before (Snead *et al*, 2000). These findings have supported development of a specific T-type antagonist, ethosuximide (Coulter *et al*, 1989; Kostyuk *et al*, 1992).

5. T-type calcium channel's role in neurogenesis

The question of T-type calcium channel's actual role in neurogenesis remains yet unanswered. However, a few studies have led the researchers closer to finding an answer. The role of Ca^{2+} being a key regulator in the process of neural induction is well established (Webb *et al*, 2005). In spite of that, there is a gap in our knowledge, in what comes to the exact functions of each calcium channel subtype during the neurogenesis. During the neurogenesis, T-type calcium channels appear first, are widely expressed, until later the HVA calcium currents appear by the maturating of neurons (Yaari *et al*, 1987). After this takes place, the ratio starts changing constantly towards a dominating population of HVA currents. *In vitro* this effect is more marked at differentiation days 3 to 5 (D3-D5) (D'Ascenzo *et al*, 2006).

It has been shown, that T-type calcium channel α_{1H} participates in the morphological and electrical differentiation of neuroblastoma (tumor-cells) NG108-15 cells (Chemin *et al*, 2002; 2004). Pharmacological inhibition of T-channel activity impairs HVA channel expression and neuritogenesis, i.e. maturation of neurons, indicating that T-type channels have a crucial role in both morphological and electrical changes during the early stage of neuronal differentiation (Chemin *et al*, 2002). Treatment with the T-channel blockers Ni^{2+} and mibefradil significantly decreased the number of cells with neurites. Percentage of cells with neurites was 65 % of the number in control cells when treated with Ni^{2+}, and 78 % when treated with mibefradil, whereas HVA blockers had no effect. Authors concluded that T-type calcium channels trigger the onset of differentiation and regulate the expression of HVA channels. Soon after birth, the expression of T-type channels collapses in visual cortex and hippocampal neurons and early action potential possibly depends on T-type channels (Nowycky *et al*, 1985). This early development of T-type channels is widely reported. In embryonic *Xenopus* young spinal neurons T-type channels are present during the calcium-sensitive period (Gu and Spitzer, 1993). Later on, they disappear, and in mature neurons only half of them express T-type calcium channels, in contrast to HVA current, which persists in all neurons. The same developmental change occurs also in other embryonic systems, including rat hippocampal neurons, chick dorsal root ganglion cells and ciliary

ganglion neurons, chick motoneurons, rat hippocampal pyramidal neurons, and neurons of the rat neostriatum. Gu and Spitzer conclude that the early appearance of T-type calcium channel in different embryonic cells suggests that it may play an important role in their development. T-type channel's function in mature neurons is better known than its role in neurogenesis. It appears that they trigger activation of HVA currents in young neurons and lower the threshold of action potentials (Gu and Spitzer, 1993). Interestingly, T-current has no effect on the threshold of mature neurons.

Conducting neurogenesis into neuritogenesis is controlled by HVA channels, especially by L-type calcium channels, and it plays a key role in promoting neuronal differentiation (D'Ascenzo *et al*, 2006). Ca^{2+} influx through Ca_v1 current promotes neuronal differentiation, which was reduced by 77 % compared to control cells, after blocking the channel with 5 µM nifedipine. In contrast, treatment with an L-channel activator BAY K 8644 significantly increased the percentage of cells expressing neuronal markers. Also increasing free internal calcium with 100 nM ionomycin, leads to increased neuritogenesis by 15 % compared to the control cells (Chemin *et al*, 2002).

It is claimed that T-type calcium channels contribute to the secretion of autocrine differentiation factor(s) that promote neuritogenesis (Chemin *et al*, 2003). This self-regulating system appears also as a positive feedback loop, as the enhancement of calcium channel's activity during differentiation produces increases in Ca^{2+} influx, which in turn appears to promote further neuronal differentiation (D'Ascenzo *et al*, 2006). Authors of this study suggest that their findings of the calcium playing a pivotal role in NSC differentiation raise possibilities for pharmacologic enhancement of the neurogenic potential of NSCs *in situ*. This means that modulation of Ca^{2+} influx might be a useful tool to increase differentiation toward the neuronal lineage when NSCs are manipulated and expanded *in vitro* prior to transplant *in vivo*.

6. T-type calcium channel subtypes

6.1 $Ca_V3.1$ channel subtype

Between T-type calcium channels, there are differences and similarities in their biophysical properties (Klöckner *et al*, 1999). $Ca_V3.1$ channel subtype, also marked as α_{1G} subunit, shows the fastest activation and inactivation kinetics. It activates at -46 mV in 1 millisecond and inactivates at -73 mV in 11 ms.

α_{1G} subunit has no known activators nor radioligands. Instead, there are some antagonists known to block the channel, although no subtype-specific blocker exists (Heady *et al*, 2001). Overall T-type channel antagonists as mibefradil (Martin *et al*, 2000), U92032 (Avery and Johnston, 1997), penfluridol and pimozide (Santi *et al*, 2002), amiloride (Lacinova *et al*, 2000), ethosuximide (Gomora *et al*, 2001) and nickel (Lee *et al*, 1999) antagonize also this channel subtype. A gating modifier, peptide toxin named kurtoxin, affects this channel (Chuang *et al*, 1998).

$Ca_V3.1$ channel subtype is widely expressed over the brain. It is distributed especially to soma and dendrites of neurons in olfactory bulb, amygdala, cerebral cortex, hippocampus, thalamus, hypothalamus, cerebellum and brain stem (Perez-Reyes *et al*, 1998). Peripherally this channel subtype is expressed in ovaries, placenta and heart (Catterall *et al*, 2005). The interesting point in channel distribution is that it is closely related to the channel subtypes' physiological functions. In the case of α_{1G} subunit, its main physiological function in producing thalamic oscillations, correlate with its expression in the thalamic areas (Perez-Reyes, 2003).

6.2 $Ca_V3.2$ channel subtype

T-type calcium channel subunit $Ca_V3.2$, or α_{1H}, has the second lowest activation and inactivation kinetics of the three T-type calcium channel subtypes. It activates at -46 mV in 2 milliseconds and inactivates at -72 mV in 16 ms (Klöckner *et al*, 1999).

$Ca_V3.2$ subtype has no known activators, nor radioligands, but instead there are many substances that antagonize it (Catterall *et al*, 2005). It is significantly more sensitive to the block of nickel than $Ca_V3.1$ and $Ca_V3.3$ subtypes (Lee *et al*, 1999). α_{1H} subunit is antagonized also by the other overall T-type channel antagonists, mibefradil, U92032, penfluridol and pimozide, ethosuximide and amiloride. In addition, it is sensitive to nonselective antagonists, nimodipine (Williams *et al*, 1999) and some anesthetics (Lee *et al*, 1999). Kurtoxin is a gating modifier of this channel as well as the $Ca_V3.1$ channel (Chuang *et al*, 1998).

This channel subtype is expressed in several brain areas, especially in olfactory bulb, striatum, cerebral cortex, hippocampus and reticular thalamic nucleus (Talley *et al*, 1999). $Ca_V3.2$ channel takes part in thalamortical dysrhythmias, which are related to the pathology of absence epilepsy (Llinas *et al*, 1999). It is widely expressed also in peripheral tissues including kidney, smooth muscle, liver, adrenal cortex and heart. Its peripheral physiological actions go together with these findings. They are smooth muscle contraction, smooth muscle proliferation, aldosterone secretion and cortisol secretion (Catterall *et al*, 2005). It looks like this calcium channel subtype has more important peripheral functions than CNS functions. T-type calcium channels have also been suggested to be potential drug targets in hypertension and angina pectoris, due to their cardiac and vascular functions, which are quite similar to those of L-type calcium channels (Ertel *et al*, 1997). This effect is probably mostly due to the $Ca_V3.2$ channel subtype.

6.3 $Ca_V3.3$ channel subtype

Calsium channel subtype $Ca_V3.3$, or α_{1I} subtype, has the slowest activation and inactivation kinetics of the three T-type calcium channel subtypes. It activates at -44 mV in 7 milliseconds and inactivates at -72 mV in 69 ms (Klöckner *et al*, 1999). The activation and inactivation potentials are very similar among the three channel subtypes.

Similarly to other T-type channel subtypes, it has no known activators or radioligands (Catterall *et al*, 2005). α_{1I} channel has no subtype-specific blocker (Heady *et al*, 2001), but it is antagonized by the same compounds as $Ca_V3.1$ and $Ca_V3.2$, mibefradil, U92032, penfluridol, pimozide, ethosuximide and nickel. Kurtoxin does not affect this channel subtype, in contrast to the other two subtypes (Chuang *et al*, 1998).

α_{1I} subunit is distributed to brain, especially olfactory bulb, striatum, cerebral cortex, hippocampus, reticular nucleus, lateral habenula and cerebellum (Talley *et al*, 1999), and its main function is causing thalamic oscillations (Perez-Reyes, 2003). It does not seem to have any significant peripheral roles and it is mostly expressed in CNS. For a summary of all the T-type calcium channel subtypes, see Table 2.

Table 2, T-type calcium channel subtypes characters

Channel subtype	Blockers	Activators	Gating modifiers	Distribution	Physiological functions	Special features
$Ca_V3.1$	mibefradil, U92032, penfluoridol, pimozide, nickel, amiloride	not established	kurtoxin	CNS, ovary, placenta, heart	thalamic oscillations	kurtoxin has a high affinity to this channel: IC_{50} = 15nM
$Ca_V3.2$	mibefradil, amiloride, U92032, penfluridol, pimozide, nickel (nimodipine and anesthetics)	not established	kurtoxin	CNS, kidney, liver, adrenal cortex, heart	smooth muscle contraction and proliferation, aldosterone and cortisol secretion	nickel is the most selective blocker of this channel: IC_{50} = 12μM
$Ca_V3.3$	mibefradil, U92032, penfluridol, pimozide, nickel	not established	none	CNS	thalamic oscillations	distribution mainly in CNS

7. Pharmacology of T-type calcium currents

Pharmacological substances have been important in the identification of voltage-gated Ca^{2+}-channels (Tsien et al, 1988; Olivera et al, 1994; Randall and Tsien, 1995; Mishra and Hermsmeyer, 1994). Compounds showing specificity towards T-type calcium channels include drugs from diverse therapeutic groups such as antihypertensives, antiepileptics, antipsychotics and anesthetics (Perez-Reyes, 2003). In addition to therapeutic compounds, there are some T-type-specific di- and trivalent cations and peptide toxins for the use of physiological experiments.

In this study, the action of T-type channels is detected as the cells response to some known T-type calcium channel antagonists. Therefore, to validate the method, it is crucial to find out the other actions of these antagonists. If it appears, that these compounds share the same functions, other than blocking of T-type channel, the effect on the cells might as well be caused by interaction with some other receptor than T-type calcium channel. One must also consider the affinity towards the receptor in question. Five compounds: ethosuximide, mibefradil, nickel, pimozide and kurtoxin, were chosen for experiments because of their relative specificity towards T-type calcium channel. Pimozide is the least specific of the substances, and therefore it is interesting to compare its action to the other compounds actions.

7.1 Ethosuximide

Inhibition of T-type Ca^{2+} channels has been proposed to play a role in the therapeutic action of succinimide antiepileptic drugs (Coulter *et al*, 1989). It is being used extensively in the treatment of absence epilepsy. Ethosuximide is known to block human T-type currents in a state-dependent manner. It is classified as a 2-ethyl-2-methylsuccinimide (see picture 9). The block of a certain current depends on the concentration. According to a study, the detected affinity K_I for α1G and α1I was 0.3 to 0.5 mM and for α1H was 0.6 to 1.2 mM (Gomora JC, et al. 2001). IC_{50} was 0.6 mM.

This study was made on HEK (human embryonic kidney) -293 cells expressing human T-type calcium channel subtypes.

The concentration response curves suggest that there is a biphasic function due to two apparent binding sites: a high-affinity (approximately 0.1 mM) site and a low-affinity site (approximately 10 mM) (Huguenard, 2002). Anti-absence drugs, such as ethosuximide and its active metabolite, MPS (methyl-propylsuccinimide), are known to block T-type calcium channels (Coulter *et al*, 1989). Several electrophysiological studies have confirmed these findings (Huguenard and Prince, 1994; Gomora *et al*, 2001; Broicher *et al*, 2007).

Absence seizures during epilepsy are generated by a rhythmic burst firing between the thalamic and cortical neurons. This spike-wave activity is the pathology behind generalized absence (GA) epilepsy (Williams, 1953). During periods of slow-wave sleep and absence seizures the regular linearly appearing spike-waves in the human EEG turn into non-linear burst firing (Livingstone and Hubel, 1981; Hirsch *et al*, 1983). This pattern of activity results from Ca^{2+}- dependent low-threshold spikes generated by a transient calcium current, I_T, (T-type calcium channel) (Coulter *et al*, 1989c). There are firm evidence that the blocking of Ca^{2+} channels is the primary target for seizure protection in epilepsy treatment and specifically the block of T-type Ca^{2+} channels is the main action of succinimide antiepileptic drugs, such as ethosuximide (Gomora *et al*, 2001; Huguenard, 2002).

There is one study that completely denies the action of ethosuximide on T-type calcium channels (Leresche *et al*, 1998). It is claimed in this study that ethosuximide acts by inhibiting the voltage-gated sodium current and the calcium-activated potassium current, and that these effects underlie the decrease in burst firing of action potentials. Other studies reviewed in here do not have any discrepancies with each other. After all, the experimental conditions in all of these studies differ in many ways, although all of them being electrophysiological experiments.

There are several factors that affect the study results listed in Table 3. Different methods are being used, and the cells are excited with different voltage potentials. As one could guess, also the cell type used in the study has its affection on the results. Concentration

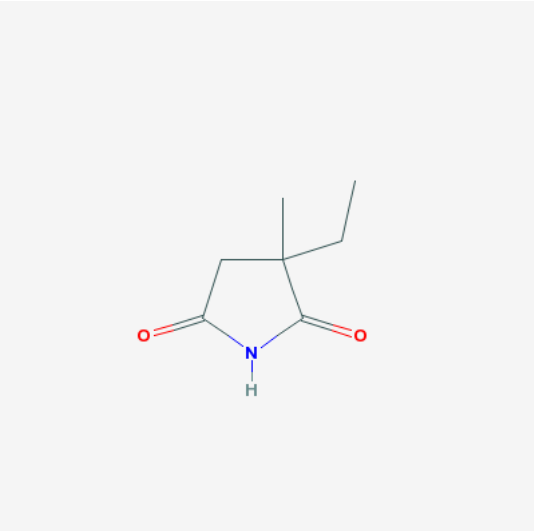
is another thing to note when comparing the results. Among these studies ethosuximide's concentrations vary from 0.7 mM to 3.05 mM. Varying results gained with ethosuximide are discussed in an article by Gomora et al. They emphasize that a notable difference in these electrophysiological studies is the holding potential (Gomora *et al*, 2001). Since the studies that reported no effect of ethosuximide on T-type channels used very negative holding potentials (-110 mV). They suggest that these experiments tested block of rested channels, which are less sensitive to block than inactivated channels.

One interesting view is presented in a study of Broicher and his co-workers. Their study offers a solution to a current controversy in ethosuximide's antiepileptic action. Combined effects of ethosuximide on several membrane currents (I_T, I_{NaP} and I_{KCa}) may account for the full anti-absence action of therapeutically relevant ethosuximide concentrations (Broicher *et al*, 2007).

Ethosuximide's antagonistic action is shown in a number of studies with varying methods, cell types and concentrations, as seen in Table 3.

Table 3, Ethosuximide's antagonistic action

Cell type	**Method**	**ETX concentration**	**T-type Channel**	**Other than t-type ca-channels**	**Publisher**
WAG/Rij-rats thalamic neurons	Whole-cell patch-clamp	EC_{50} 0,21 mM	I_T	I_{NaP}, at 10 mM,	Broicher et al. 2007
ACI- rats thalamic neurons	Whole-cell patch-clamp	EC_{50} 3,05 mM	I_T	I_{NaP}, at 10 mM,	Broicher et al. 2007
Wt- rats thalamic neurons	Voltage-clamp	0,25-0,75 mM	I_T	not studied	Coulter et al. 1989
HEK-293-cells expressing human α1G-, and α1H-subunits	Ruptured patch-method	K_1 2,5 mM K_1 1,9 mM	α1H- subunit (inactivated) α1G- subunit (inactivated)	not studied	J.C. Gomora et al. 2001
HEK-293-cells expressing murine α1I-subunit	Whole-cell patch-clamp	35 % reduction at 3 mM	α1I- subunit (-60 mV)	not studied	Lacinová L. et al. 1999
Wt- rats thalamic neurons	Whole-cell patch-clamp	33 % reduction at 0,7 mM	I_T	not studied	J.R. Huguenard and D.A. Prince, 1994
Rats and cats thalamic-cortical cells	Sharp-and-patch-method	1 mM	no effect	60 % reduction of I_{NaP} at 1mM 39 % reduction of $I_{K(Ca)}$ at 0,5 mM	Leresche et al. 1998



Picture 2, Chemical structure of Ethosuximide. Ethosuximides IUPAC- name is 3-ethyl-3-methylpyrrolidine-2.5-dione. It is classified as a succinimide, which belongs to a heterocyclic compound group of pyrrolidines. More precisely, it belongs to a subgroup of pyrrolidines, called pyrrolidinones. (PubChem electric database)

7.2 Mibefradil

Another low-threshold activated calcium channel blocker is mibefradil, which doesn't have clinical implications anymore (Krayenbühl *et al*, 1999). Although it seemed to be a promising cardiovascular medicine with its ability to decrease blood pressure and heart rate with a minimal negative ionotropic effect, as it was observed in clinical trials. This non-dihydropyridine inhibitor was approved for use in essential hypertension and stable angina pectoris until it was withdrawn from the market because of drug-drug interactions leading to irregular heart rhythms.

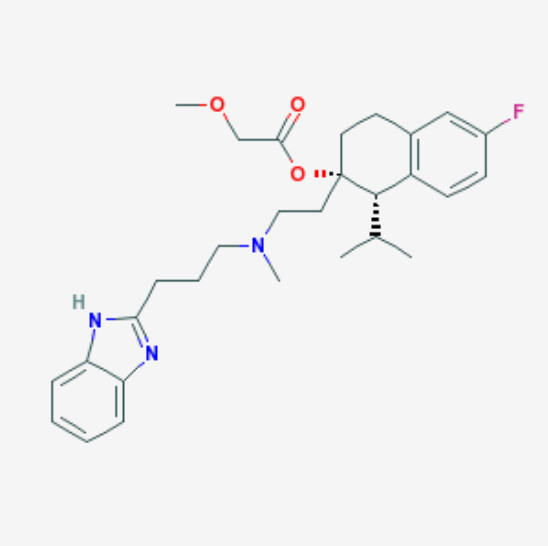
Mibefradil's function as an antihypertensive agent was to antagonize T-type calcium channels in vascular smooth muscle cells (Mishra and Hermsmeyer, 1994). T-type channels in those cells are very sensitive to Mibefradil. 50 % inhibition of T-type channels was obtained at 0.1 µM. Mibefradil inhibits both high- and low-voltage activated calcium currents, but it has a higher affinity for neuronal T-type over cardiac L-type channels. It prefers T-type channels to L-type channels with 10- to 20-fold

selectivity. This compound appears to be very effective in blocking calcium channels in tissues with less negative resting potential, such as vascular smooth muscle and cardiac sinoatrail node (Bezprozvanny and Tsien, 1995; Gomora *et al*, 1999). However, it cannot be used to discriminate between the T-type calcium channel subtypes (Strege, 2005). Mibefradils action is also dose- and state- dependent (Bezprozvanny and Tsien, 1995), as in the case of ethosuximide.

Mibefradil's toxicity is probably produced by blocking also potassium channels (Gomora *et al*, 1999). It is a potent K^+ channel blocker, but less selective than as a T-type calcium channel blocker. Mibefradil is shown to inhibit ATP activated potassium channels in adrenal cells with an IC_{50} value of 0.50 µM, a concentration that is 2-fold lower (IC_{50} = 1.0 µM) than that required to inhibit T-type calcium channels in the same cells under similar conditions. As a pharmacological agent, mibefradil is problematic for two more reasons. It is an inhibitor of cytochrome P-450 (CYP-450) 3A4 enzyme, which can result in toxic drug interactions. It is also shown to prolong QT interval with other drugs having the same mechanism. This in turn may lead to severe arrhythmias. This effect on the cellular level is blockage of potassium channels.

Table 4, Mibefradils antagonism in electrophysiological studies

Cell Type	Method	Mibefradil concentration	T-type channel	Other than T-type channel	Publisher
Rats Vascular muscle cells	Whole-cell patch-clamp	~100 % block at 1 µM	I_T	28 % block L-type at 1 µM	Mishra S.K. & Hermsmeyer K., 1994
Gene transfected *Xenopus laevis* oocytes	Whole-cell patch-clamp	K_d 3 µM K_d 8 µM K_d 7 µM	α_1B α_1A α_1E	L-type (α_1C) K_d 22 µM	Bezprozvanny I. & Tsien R.W., 1995
Gene transfected HEK-293 cells	Whole-cell patch-clamp	IC_{50} 2,9 µM	$\alpha_1$3.3b + β_2	Na^+ channel (SCN5A) IC_{50} 0,98 µM L-type (α_1C + β_2) IC_{50} 2,7 µM	Strege P. et al., 2005
WAG/Rij rats TC neurons	Whole-cell patch-clamp	100 % block at 2 µM	I_T	18 % HVA Ca^{2+} current at 2 µM 28 % Na^+ current at 2 µM	Broicher at al., 2007
Gene transfected HEK-293 cells	Whole-cell patch-clamp	IC_{50} 0,12 µM	α_1G	not studied	Lacinová L. et al., 1999
Bovine adrenal zona fasciculata cells	Whole-cell patch-clamp	-	not studied	IC_{50} 0,50 µM I_{AC} (ATP-activated K^+-channel)	Gomora J.C. et al., 1999



Picture 3, Mibefradil's chemical structure. Mibefradil's IUPAC- name is [(1S, 2S)-2-[2-[3-(1H-benzimidazol-2-yl)-propyl-methylamino]ethyl]-6-fluoro-1-propan-2-yl-3,4-dihydro-1H-naphthal en-2-yl] 2-methoxyacetate. It belongs to heterocyclic compounds, and can be classified as a benzimidazole, as well as a tetrahydronaphtalene, due to its complex chemical structure. (PubChem electric database)

7.3 Nickel

Inorganic divalent and trivalent cations were some of the first chemicals used to block T-type currents (Hagiwara *et al*, 1988). Comparison of the concentrations of drugs necessary to inhibit half-maximal T-type currents suggested different rank orders of inhibition by different polyvalent cations. The orders were different in varying brain areas. This in turn suggests that there is heterogeneity between the subtypes, because of their division throughout the CNS varies. Several cations, such as La^{3+}, Zn^{2+}, Cd^{2+}, Ni^{2+} (Ye and Aikaike, 1993) inhibit T-type channels.

It has been known for over a century that di- and trivalent cations act on cells' electrophysiology. Nickel has been considered as a T-type specific calcium channel antagonist. Some studies cast doubts on its specificity (Gloding *et al*, 1999), while others state that the specificity is dose-dependent. T-type specificity has been recorded at a concentration range from 5 to 50 µM (Biagi and Enyeart, 1991), and HVA-affection

from 216 to 250 µM (Lee *et al*, 1999). There can also be differences between the three cloned T-type receptor subtypes. It is shown, that nickel is specific only to the subunit α_1H, and the higher concentrations needed for blocking the two other subtypes block also HVA- channels (Lee *et al*, 1999).

One study revealed that also Ni^+ has interaction with potassium channels (Golding *et al*, 1999). In this study >0.5 mM nickel was needed to block calcium spikes completely in CA1 pyramidal neurons. Conversely effect was seen in local application of nickel to the soma and adjacent dendrites. Nickel increased reversibly the number and/or width of calcium spikes compared to control cells. The authors suspected that this increased excitability could be explained by a reduction in the activation of calcium-activated potassium channels through blockade of calcium influx by Ni^+.

What comes to nickels selectivity among T-type calcium channel subtypes, only α_{1H} subunit is blocked by micromolar concentrations of Ni^+ (Lee *et al*, 1999). The higher concentrations that are needed to block the other T-type calcium channel subtypes also blocked HVA calcium channels. Similar observations were made in another study, where T-type current was selectively blocked by Ni^+ at concentrations between 5 and 50 µM (Biagi and Enyeart, 1991) (see Table 3). In this study, trivalent cations lanthanum La^{3+} and yttrium Y^{3+} blocked T current at 10- to 20-fold lower concentrations in thyroid C-cell line. However, these cations are not selective for T-type channels, as they also block L-type calcium channels.

Table 5, Nickels antagonism in electrophysiological studies

Cell Type	Method	NiCl-concentration	T-type channel	Other than t-type channel	Publisher
Rat medullary thyroid carcinoma cells	Whole-cell patch-clamp	T-type selective at 5-50 µM	I_T	not studied	Biagi B. & Enyeart J., 1991
Gene transfected HEK-293 cells	Whole-cell patch-clamp	IC_{50} 13 µM IC_{50} 216 µM IC_{50} 250 µM	α_1H α_1I α_1G	HVA-channels at 216 and 250 µM	Lee J. et al., 1999
Wistar rats hippocampal cells	Whole-cell patch-clamp	100 % block at 500-1000 µM	I_T	$I_{K(Ca)}$ 500 µM HVA channels at 500-1000 µM	Gloding N. et al., 1999
Gene transfected HEK-293 cells	Whole-cell patch-clamp	IC_{50} 470 µM	α1G	not studied	Lacinová L., et al., 1999

7.4 Pimozide

Antipsychotic drugs' selectivity has been an issue for recent decades. Since the first antipsychotic drugs were invented, their effect of action was mostly based on a dopaminergic D_2- receptor antagonism (Koulu and Tuomisto, 2007). This effect has lately been related to their typical side-effects worsening the so-called negative symptoms of schizophrenia; social exclusion, passivity and poverty in speech. The newer antipsychotic drugs' effect of action is based on antagonism on more than just one receptor.

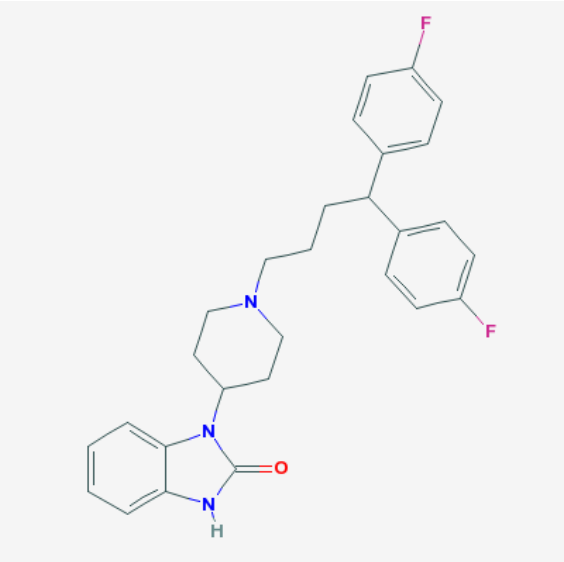
An antipsychotic drug, Pimozide belongs to a chemical group of diphenylbutylpiperidines, DPBPs (Santi *et al*, 2002). Its mechanism of action is believed to be mainly blocking the dopaminergic receptor D_2. One of the more recent

studies show that its mechanism of action is as much of blocking the receptor D_2 as blocking of the T-type calcium channel. This might explain the drug's clinical improvement to the negative symptoms of schizophrenia, compared to the classical antipsychotics potently blocking the dopaminergic receptor subtype D_2.

Pimozide blocks cardiac HERG potassium channels with a high affinity, as well as the neuronal HERG potassium channels (Jiesheng *et al*, 2001). Drugs that block cardiac HERG potassium channels are known to prolong the QT interval on the electrocardiogram via this channel blockage (Brown and Rampe, 2000). In addition to pimozide, some other drugs also have this effet. These drugs include certain antihistamines, antibiotics and a few other antipsychotics.

Table 4, Pimozides antagonism in electrophysiological studies

Cell Type	**Method**	**Pimozide concentration**	**T-type channel**	**Other than T-type channel**	**Publisher**
Gene transfected HEK-293 cells	Whole-cell patch-clamp	*K*d 43,5 nM *K*d 57,7 nM *K*d 39,2 nM	α_1G α_1H α_1I	not studied	Santi C. et al., 2002
		*K*d 29 nM	not studied	D_2 (dopamine)	Richelson & Souder, 2000
Gene transfected Chinese hamster ovary cells	Whole-cell patch-clamp	IC_{50} 103 nM	not studied	erg_3 (human neuronal K^+ channel)	Kang J. et al., 2001



Picture 5, Pimozide's chemical structure. Pimozide's IUPAC- name is 3-[1-[4.4-bis-(4-fluorophenyl)-butyl]-piperidin-4-yl]-1H-benzimidazol-2-one. It belongs into a benzimidazoles group of heterocyclic compounds. (PubChem electric database)

7.5 Kurtoxin

Kurtoxin is a 63 amino-acid protein toxin from the venom of a South African scorpion (*Parabuthus transvaalicus*) (Chuang et al, 1998). It blocks specifically the α_{1G} T-type calcium channel. In the study of Chuang et al., kurtoxin inhibited α_{1G} T-type calcium channels by over 95 % and α_{1H} channels by 85 % at a concentration of 350 nM. Kurtoxin distinguished between α_{1G} and the high-voltage activated calcium channels with over 600-fold selectivity. It also interacts with voltage-gated sodium channels and slows their inactivation. This is also noted in another study reporting that ≥300 nM kurtoxin inhibited the peak amplitude of voltage activated Na^+ channel at −10 mV (Zhu *et al*, 2009), which is approximately ten times higher than the concentration of kurtoxin that is specific for T-type calcium channel. The authors emphasized that many channel blockers are non-specific, if used at high enough concentrations. Kurtoxin can be considered as a high-affinity blocker of T-type calcium channel (Chuang *et al*, 1998).

In a study made with thalamic neurons, 500 nM kurtoxin resulted in almost complete inhibition of T-type calcium currents (Sidach and Mintz, 2002). 90.2 % of the currents

were inhibited. Its selectivity was lower than the researchers expected because it also reduced the composite high-threshold calsium channel current in the cells by 46.1 %. According to the studies described above, this was probably because of the relatively high concentration used in the study.

Peptide toxins are valuable tools for studies of voltage-gated ion channels (Sidach and Mintz, 2002). Although the origin of these proteins may be diverse, their modes of action fall within two major categories. Pore-blocking toxins bind to the external vestibule of the channel pore and physically obstruct the movement of ions (MacKinnon and Miller, 1988). Gating modifiers bind to the channel voltage sensor and alter the energetics of voltage-dependent gating (Cahalan, 1975). Kurtoxin is a gating modifier that stabilizes the closed state of the channel, so that it requires larger depolarizations in order to gate into the open state. It binds to T-type channels extracellular protein chains S3-S4 linker of domain IV close to the voltage sensor (Chuang et al, 1998). The conclusion of kurtoxin being a gating modifier is strengthened by the sequence homology between kurtoxin and the alpha-scorpion toxins, a group of well studied toxins that modify the gating of sodium channels. Chuang and his co-workers suspected that the voltage-sensing domain in repeat IV of T-type calcium channel's quaternary structure contains a competent kurtoxin binding site. The researchers got the clue of their experiments indicating a 1:1 stoichiometry between toxin and channel.

In the study of Sidach and Mintz, also some problematic aspects of kurtoxin appeared. Accurate measurements of kurtoxin rate constants were impaired because of toxin nonspecific binding to glass capillaries, or its nonhomogeneous application into the minichamber used in the measurement. The authors advice that all the glass material, including vials and recording chamber, that is in connection with kurtoxin, should be siliconized to minimize toxin loss via nonspecific binding.

Table 6, Kurtoxin's antagonistic action in electrophysiological studies

Cell Type	**Method**	**Kurtoxin-concentration**	**T-type channel**	**Other than t-type channel**	**Publisher**
Xenopus oocytes	Patch-clamp	95 % block at 350 nM	α_{1G}	I_{Na}	Chuang R. et al. 1998
Rat thalamic neurons	Patch-clamp	90,2 % block at 500 nM	I_T	HVA-calcium channels	Sidach S. and Mintz I. 2002
Mice smooth muscle cells	Patch-clamp	≤300 nM	not studied	I_{Na}	Zhu H-L. et al. 2009

As seen in the Table 1, not all the studies agree on the ethosuximide's specificity on the low-voltage activated calcium channels. These differences could be explained by the experimental procedures. The action of ethosuximide is shown to be both dose-dependent and state-dependent (Gomora et al, 2001). The channel's state depends on the holding potential in electrophysiological studies. T-type channel can be activated with small depolarizations into the range near -60 mV that did not activate other currents (Huguenard and Prince, 1994). There are certain differences in electrophysiological methods. For example, the whole-cell measurements differ in many factors from the sharp-electrode recordings (Staley *et al*, 1992). Such a specific and selective drug hardly exists, that it wouldn't have side effects due to its affinity to a substrate other than its desired pharmacological target.

As seen in the Table 1, 2, 3 and 4, all these T-type specific substances have affinity to the voltage gated potassium channel, I_K. One reason for a drug to be rejected from the pre-clinical trials is that it binds to cardiac HERG (human ether-a-go-go-related) potassium channels. That is because there is a high risk of prolonged QT- time and severe antiarrhythmias if a drug binds to this receptor, which was the case of

ethosuximide being withdrawn from the market (Leresche *et al*, 1998). Binding to cardiac HERG potassium channels is dangerous, especially when there is a drug-drug interaction of two different drugs used at the same time that bind to this channel. This, in turn, was the reason for mibefradil being withdrawn from the market. Paradoxically, these cardiac HERG potassium channels are one primary target for the pharmacological management of arrhythmias (Kiehn *et al*, 1999). The class III antiarrhythmic drug amiodarone is used to block these potassium channels. The patients are being controlled regularly and any usage of other drugs that are known to affect this channel is forbidden.

Since these compounds listed above seem to have some kind of affinity to potassium receptors, it was evident to apply one more substance to the experiments. Kurtoxin, a peptide toxin, does not seem to have interaction with potassium channels, according to the published research.

All of these pharmacological substances act on T-type calcium channels, but affect also other voltage-activated ion channels. Most of the substances: ethosuximide, mibefradil, pimozide and nickelchloride are also known to block potassium channels. Mibefradil, nickel and kurtoxin also block high-voltage activated calcium channels at higher concentrations. T-type channels seem to be not only low-voltage activated, but also affected by lower concentrations of ligands. The reason why the substances that bind to T-type channels affect also other channels is probably the genetic similarity of these channels (Perez-Reyes, 1999). Voltage-gated calcium channels are part of a superfamily that includes the highly related sodium channels and more distantly related potassium channels (Jan and Jan, 1990). Not one of these pharmacological or toxicological compounds are considered as specific T-type channel blockers. Many studies confirm their selectivity while some others disagree. This contradiction raises a question of what exactly is specificity. In the light of the fact, that there are no absolutely specific pharmacological substances that would not affect any other thing in the body than their desired target, the essence of specificity is, if not outright a dogma, then at least a relative term. In spite of that, if all of these T-type blockers listed above are used in the experiments of this study, it is likely that the results are valid to show a tendency of the

pattern, in which the cells are affected by blockage of T-type calcium channels. This is even more likely if the concentration of the substance to be used is relevant.

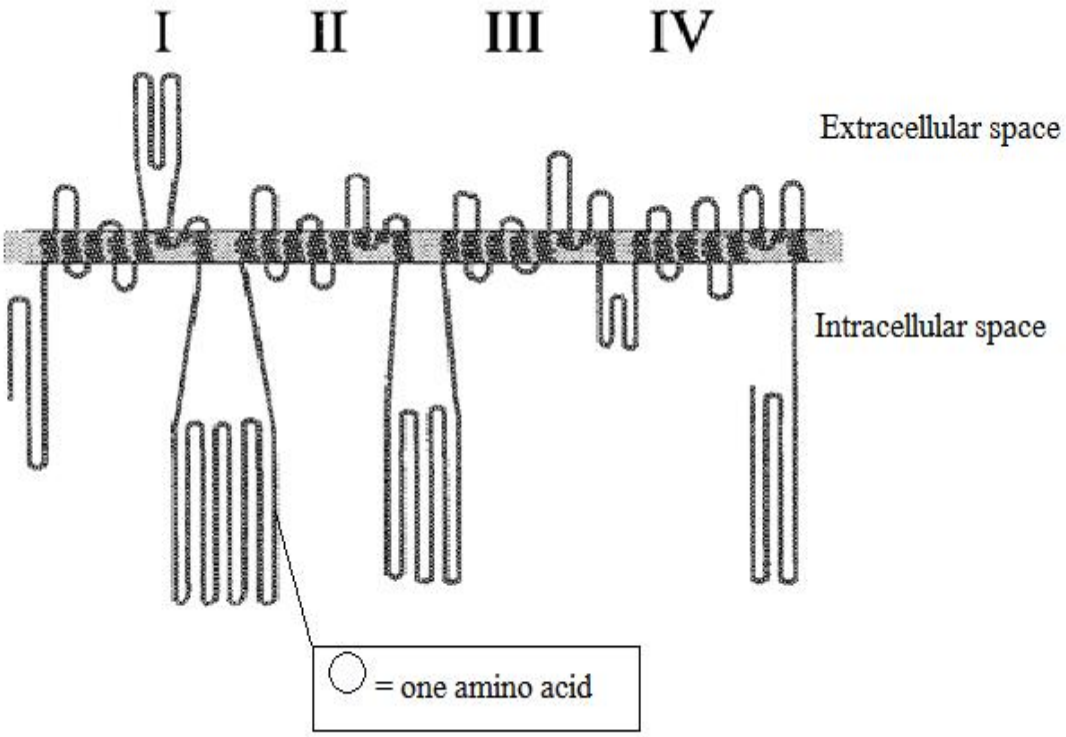
8. Structure of T-type channel

Conservation of amino acid sequences and predicted secondary structure indicate that T-type channels are evolutionarily related to potassium, sodium and HVA channels, and together they form an ion channel superfamily (Jan and Jan, 1990). Although the sequences are genetically alike, and the secondary structures are quite similar, the level of homology is actually rather low. Many amino acid substitutions differ as changes in hydrophobic residues such as leucine, which is often replaced by other hydrophobic residues, such as isoleucine, valine or phenylalanine.

Structural motifs, which are three-dimensional structural elements within the chain, are repeated four times in T-type channels. They are polypeptides of four homologous domains (I-IV), each of them containing six transmembrane spans (S1-S6), and cytoplasmic N- and C-termini. These are also the four voltage-sensing domains in the ion channel. Three intracellular linkers (I–II, II–III and III–IV) connect the four domains. Repeats are usually marked with roman numbers. Ion channels are often represented by snake diagrams, where the protein is shown to snake its way through the membrane (Picture 6).

As seen in the picture 6, clearly the most of the protein is situated in the cytoplasm and the only significant extracellular portion is at the pore loop in repeat I. Also the terminal chains N and C are located in cytoplasm. A conclusion of this physiological position is that T-type channels have also important intracellular activities. It has indeed multiple putative phosphorylation sites, which are determinant for the modulation by several protein kinases, including protein kinase A (PKA), protein kinase C, tyrosine kinase and Ca2+/calmodulin kinase II (CaMKII) (Yunker and McEnery, 2003). Furthermore, many

intracellular molecules can modulate endogenous T-type currents, including GTP, cGMP dependent protein kinase and protein tyrosine kinases, suggesting activation of many pathways can alter T-type currents. Also there is a great deal of heterogeneity in the responses of different T-type channels to many of these hormones and neurotransmitters. For example, T-type currents may be increased, decreased, or not affected by the beta-adrenergic receptor agonists, norephinephrine and isoproterenol. In addition, GABA, substance P, and serotonin can either attenuate or potentiate T-type currents recorded in a variety of neurons (Yunker, 2003).



Picture 6, Snake diagram of CaV_3 transmembrane protein with its four repeats (Adapted and modified from Perez-Reyes, 1999)

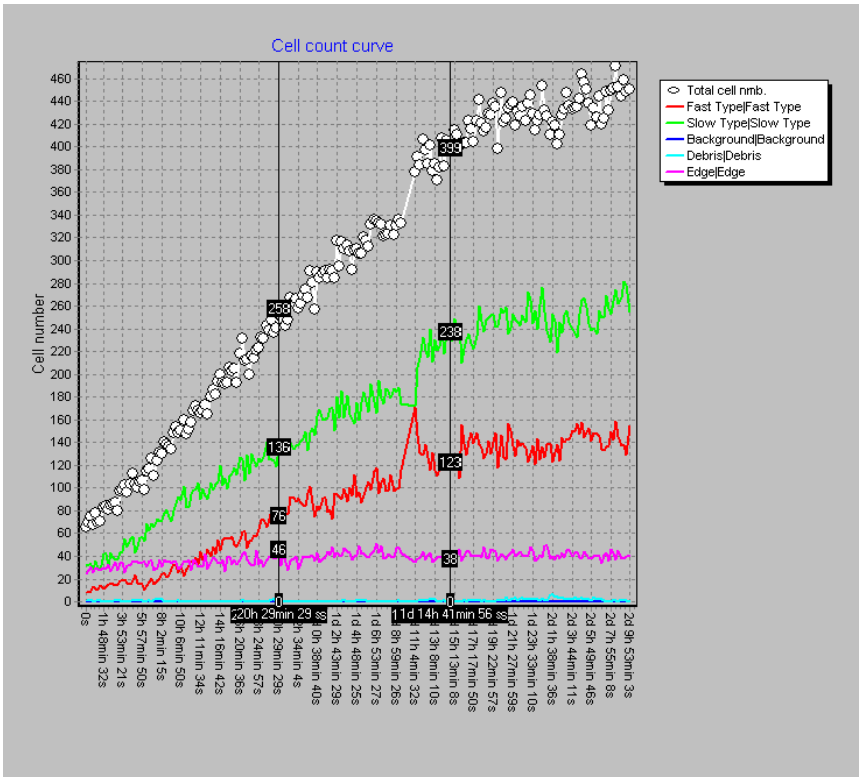
9. Building of the Cell-IQ library

When the key interests in this study are the movement and morphology of the cells, in addition to all the other possible phenomenons that can be detected, Cell IQ provides an excellent tool for performing the experiments. Cell IQ is a cell incubator equipped with a camera (Korhonen *et al*, www.smts.fi, 2008). It is made in cooperation with Chip-Man Technologies Oy, VTT (Valtion teknillinen tutkimuskeskus) and the University of Tampere's Center of Cell Research. With this hardware, one can perform picture and video analysis using imaging software. The temperature and gas composition inside the incubator can be regulated with the additional software. In addition to the videos and pictures one can make with Cell IQ, it can also be used as an analyzing tool. By defining the properties of the cells and their background, the software is able to count the number of the cells and export the data to Excel-software (Cell IQ user manual, Chipman-Technologies, 2010). Cell-IQ can recognize, not only the total number of the cells, but also different cell populations, when they are defined and saved to the protocol. Analyzing software is also commercially available for a range of different cell and tissue types. Building an analyzing protocol by oneself, is reasonable when considering the economical savings and the selectivity of the protocol for your own cell culture and experimental set up. Cell IQ Analyzer is able to count cells in thousands of pictures taken during several days only in a few hours. One can just imagine how long it would take for a researcher to count that number of cells.

The process of building a library begins with building a protocol. In a protocol four parameters will be set to suit best the experiment in question. Those are segment gradient threshold, cell distance, maxim cell diameter and cell symmetry. With segment gradient threshold, the contrast of the image can be set either to reduce background or increase the threshold so that the analyzer counts also the cells that fade into the background. The numbers of these parameters depend on the cells and the matrix they grow in. Solving out the appropriate value of the parameter just requires trying different values and comparing the results. An easy way to validate the results counted by the program is to count them manually, and see if the numbers are equal. When the population of differentiating stem cells increases, the cells form connections and networks in multiple layers. In this case, the cell distance should be set low, so that

analyzer counts the cells that are closely connected to each other. By setting maxim cell diameter big enough and the value of cell symmetry high, one can avoid analyzer counting debris as cells.

After making the protocol, gathering samples for the library can start. Introducing these samples to the analyzer teaches it to recognize certain kind of similarities, and count them into a same group, as seen in picture 7.

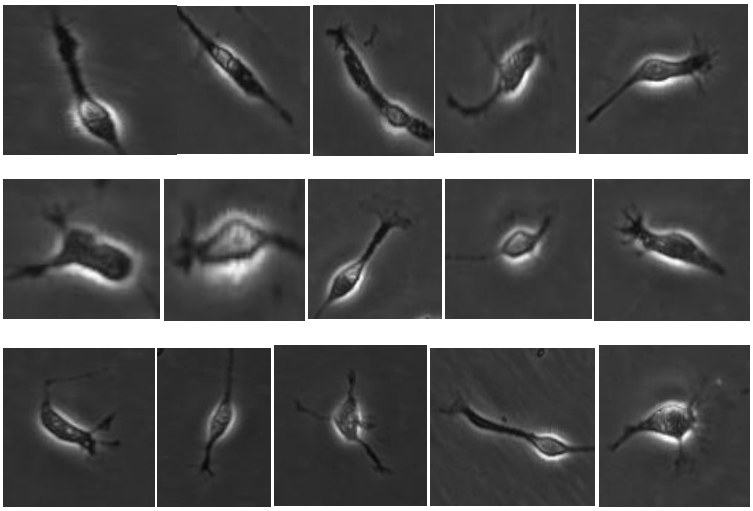


Picture 7, Example of cell count curve made by the analyzer. Cell number is on the y-axis and time on the x-axis. This analysis is made of 2 days and 10 hours period. Total cell number is marked with a white line, slow-type cells with a green line, fast-type cells with a red line, edge of the sphere with a purple line, background with a blue line and debris with a turquoise line.

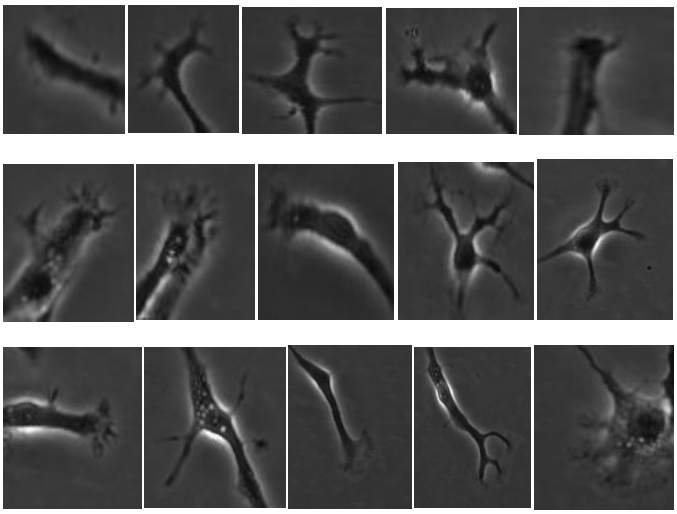
There is a possibility to create as many groups of items as needed. In this experiment six different groups were formed for cell counting. These are debris, edge, background, dividing cells, fast type and slow type. Fast and slow type cells were created to

distinguish two different cell populations, one of them being smaller and faster, and the other clearly slower and glia-like. Naming them neurons and glial cells solely according to their morphology would be misleading. In a good library the samples are alike, the pictures resolution is decent and the item is set in the middle of the picture. Below there are listed some samples from the library.

Fast type-cells



Slow type-cells

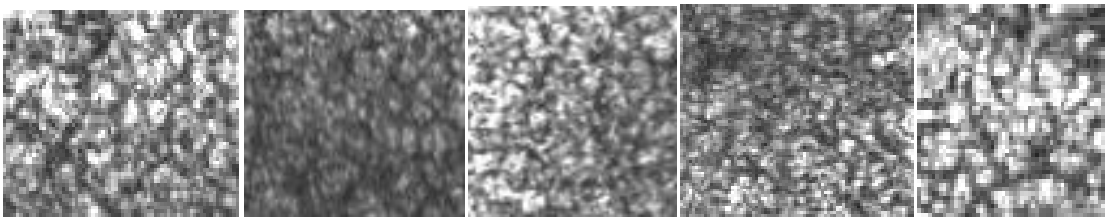


The edge of the sphere was defined to separate the sphere from the migrating cells. If the aim is to count migrating cells, it is reasonable to leave the sphere out of counting.

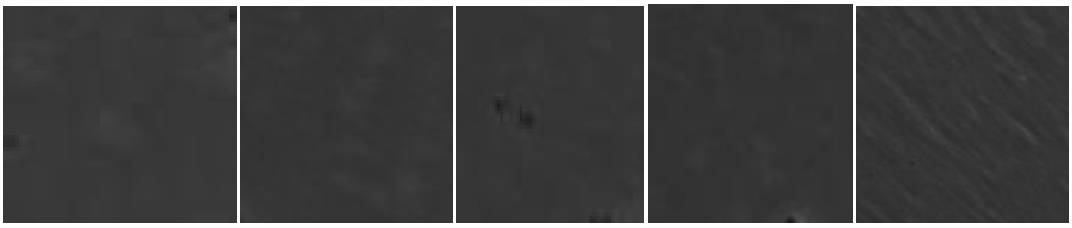
Another thing to be set is the background, so that it differs from the cells. Also all the debris can interfere with cell counting and should therefore be defined. One thing of interest was to detect cells division. Dividing cells were set as a one separate group. There are some picture samples of these four groups, edge of the sphere, background, debris and dividing cells.

The more pictures there are in the library, the better is the software's ability to recognize cells. On the other hand, every picture changes the whole library, and all the differences between the cells make more parameters for the program to count. This in turn creates a risk of making the library disorderly only by adding too many samples. The manufacturer recommends the number of samples in each group to be set around twenty.

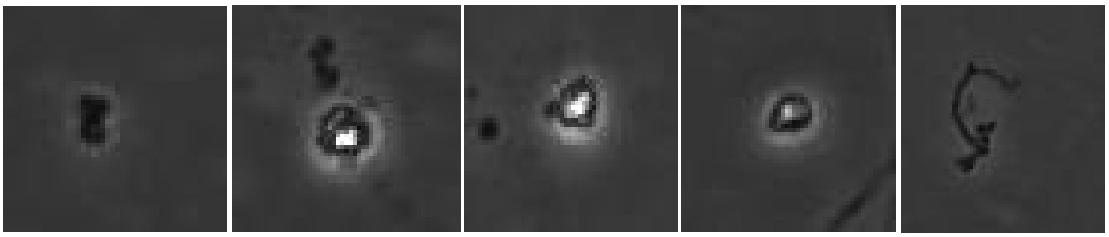
Edge of the sphere



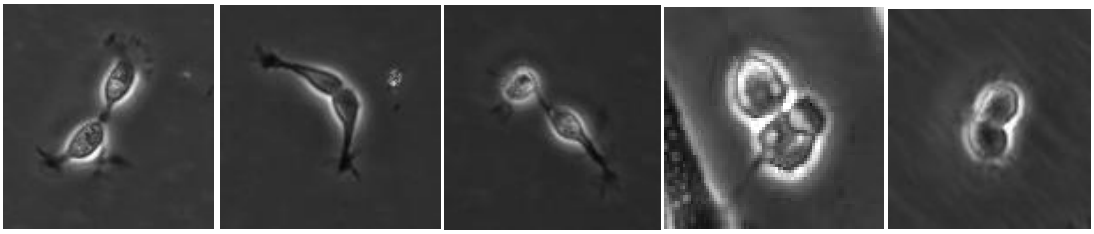
Background



Debris



Dividing cells



9.1 Optimizing the method

Creating the first version of the protocol resulted in an error rate of 27 % in counting the cells. This means that the number of cells counted by the analyzer differed 27 % from the number of cells that was counted manually. Because of these rather poor results, some development of the protocol had to be done. Analyzer counted too many cells, so it could be concluded that it counted as cells something else than the actual cells. It could be seen on the screen that it counted debris as cells. This is easy to notice, because the program marks the counted cells with colored dots. Introducing more samples of debris to the analyzer teaches it to recognize different kind of debris. After increasing the number of debris samples by 28, the correspondence of the cells counted by the analyzer compared to the cells counted manually, increased. The percentage of correspondence reached 82 %, meaning it was now 9 % more accurate than before.

A crucial move was decreasing moderately the number of gradient threshold, when the analyzer separates fewer objects from the background than before. Usually this means that it counts less of the background objects, which is the intension. This change decreased the error rate to 7 %, although with a standard deviation of 6.29.

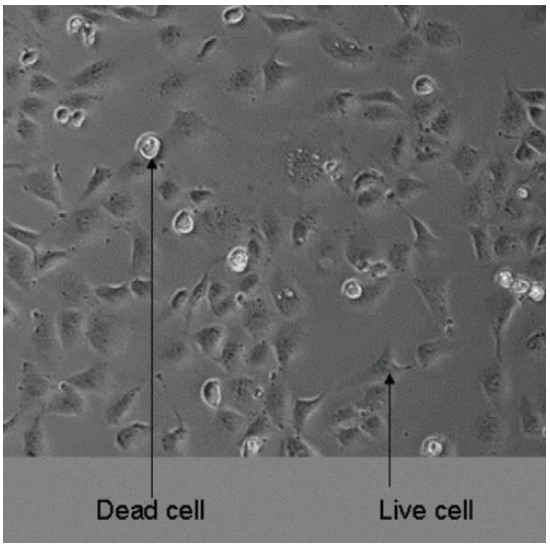
This accuracy was sufficient for the experiments considering that the changes do not always result in a better protocol. They might even have opposite effects. However, whether the error rate is high or low, the tendency of the cell count curve is always the same. For example, if the analyzer counts 7 % more cells than there actually are the increasing cell number results in the same percentage or ratio of cell growth.

9.2 Cell IQ in studying stem cells

When starting to study the stem cells differentiation, it is important to identify the various cell types that are developing (Kolb, 2007, www.biotech-online.com). During the very first hours, it is essential to detect and record data from the culture. At this time, some crucial information is gained at the moment when the different cell types

appear and the time each event begins, and how long they last for. This is easy with on-line recording, which is possible with Cell IQ. Videos can be watched afterwards, so this is really a time saving method.

All kinds of events can be monitored with the help of Cell IQ. Practically, any morphological parameter that can be detected by naked eye can be analyzed and quantified by Cell-IQ (www.chip-mantechnologies.com, page browsed on 11/2010). These parameters can be for example cell number, cell viability, cell division, cell death, morphological parameters, cell movement, analysis of selected single cells in the cell culture, and analysis of structures composed of groups of cells in cell culture. All these parameters listed above can be defined to the program, analyzed by the analyzer and then put to a form of quantitative data with plots and diagrams. Another advantage of this method is that no labels are needed to gain all the data. Toxic and pharmacological compounds can be added to the culture to study their effect on the cells. The software counts the cells dead and living, as long as they are listed in the library.



Picture 8, an example of quantitating cell viability of a cell population with Cell IQ. In this picture the number of dead cells is approximately 18 and the number of living cells is approximately 134. These numbers are counted by the analyzing software. (Adapted and modified from www.chipmantechnologies.com)

10. Research questions

One aim is to indicate the functional significance of T- type calcium channel in what comes to the migration of NSC's. The cells that were examined in this study were derived from the mice brains' SVZ at the embryonic day 14, which is known to be the climax of the neurogenesis. This study focuses on the migration and T-type calcium channels as possible modulators of this episode.

Finding specific information about the functions of stem cells and what conducts them in their differentiation leads us closer to benefit from this self-renewing and multipotent source of reserve in all of us. When the transplantation and other invasive strategies in the stem cell therapies come to their end, due to the laborious and costly implement to healthcare, pharmacology takes place. This need is described in an article of Meletis et al as: "The development of pharmacological strategies to modulate endogenous stem cells and their progeny may be an attractive alternative to cell transplantation for the treatment of spinal cord injury." (Meletis *et al*, 2008). Since stem cells construct all the tissues and parts of the body, they can be used to reconstruct any cell type in the cases of injury or degeneration, or just a genetic lack of a certain cell type.

Conducting stem cells to the way we want and differentiating them to the types needed is definitely a challenge. Taking into account the variety of factors that affect SCs, the question is: What their role in the process is, and whether there is a hierarchy between them. Even though this abundance of factors is a challenge, we must keep in mind that it is also a possibility.

The role of calcium channels in neurogenesis has been proven to be essential. The specific role of T-type calcium channel is interesting for several reasons. Firstly, it is widely expressed at the beginning of neuronal development, decreasing constantly by the maturation of neurons. It is shown in a few studies that it also has functional significance in the early development. Secondly, it is one of the least studied calcium channels, so the opportunity for fundamental research exists. T-type channels play important roles in a range of neurological actions such as sleep, learning, memory and different kinds of electrophysiological oscillations in CNS. Therefore, there is a reason

to assume they would also possess an essential place in neurogenesis. One intriguing property of T-type channel is that it is already a pharmacological target with some antagonists commercially available, although they were later withdrawn.

The aim of this study is to indicate the role of T-type calcium channel during the migration, and consider the pharmacological possibilities it brings. This study together with the literature review is in its place to raise the interest towards the action of T-type channel and the pharmacological potential that it shows.

EXPERIMENTAL PART

11. Materials and methods

11.1 Cell cultures

The central nervous system's stem cells were derived from mice brains at embryonic day 14, from the area known as the subventricular zone. The cells were incubated for 3 days in the culture medium consisting of 2 mM l-glutamine, 15 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin and 1:50 B27 supplement in DMEM-F12 medium (Life Technologies), at 37 ˚C in 5 % CO_2 in an air ventilated humified incubator. In the medium the cells formed free floating neurospheres. As soon as the size of the diameter of neurospheres increased to approximately 200 µm, they were dissociated mechanically by pipetting back and forth. Then the cells were centrifuged for 5 minutes at 800 rpm. After that, they were resuspended and plated in the culture medium. Neurospheres were passaged every 7-9 days.

Every third day two growth factors were added to the culture medium, epidermal growth factor, EGF (Life Technologies) and fibroblast growth factor, FGF (PeproTech EC Ltd., London, UK), in concentrations of 20 ng/ml and 10 ng/ml, respectively. The growth medium was refreshed once a week by changing half of it to a new medium, and was changed completely approximately once a week, depending on the condition of the medium.

11.2 Immunocytochemistry

A series of neural stem cells were differentiated for this experiment for 7 days on cover slips. Three of them were fixed on differentiation day 1, 3 on day 3, 3 on day 5 and 3 of them on day 7. NSC cells on cover slips were fixed for 10 minutes at room temperature (RT) with 4% paraformaldehyde (PFA) in PBS (pH 7.4) and then rinsed twice with PBS for 5 minutes. The cells were then permeabilized with ice-cold methanol for 20 minutes and rinsed twice for 5 minutes with PBS. To prevent the non-specific binding of the antibodies, the cells were treated for 20 minutes in RT with 20% normal goat serum (NGS, Chemicon International, Temecula, CA, USA) in PBS. Next the cells were incubated with β- tubulin III specific antibody, Tuj-1 and T-type specific antibody (both from Sigma-Aldrich) over night in +4˚C. The cells were then washed three times for 5 minutes with PBS and stained with goat anti-rabbit Alexa 488 and 568 secondary antibodies (Molecular Probes, Invitrogen, 1:500) in 37˚C for 1 hour. After that the washings with PBS were repeated. The nuclei of the cells were counterstained with 4'6-diamino-2-phenylindole (DAPI) (Boehringer Mannheim Biochemica, Germany, 1:10000). The cover slips were rinsed again with PBS and mounted with Gel Mount™ Aqueous Mounting Medium to glass slides. T-type calcium channels were detected with a laser scanning confocal microscopy was performed with LSM 5 Pascal system (Zeiss). Brightness and contrast of the obtained images was adjusted with Corel Draw Graphics Suite X3 (Corel). Tuj-1 was detected with a fluorescence microscope (Olympus) using objectives of 10x, 20x and 40x. To control the specificity of the antibodies primary or secondary antibody were left out from some of the samples.

11.3 Blocking of the T-type calcium channels

Three experiments were made to block the T-type calcium channels of the cells, to detect what happens when the cells are grown in the presence of a T-type channel

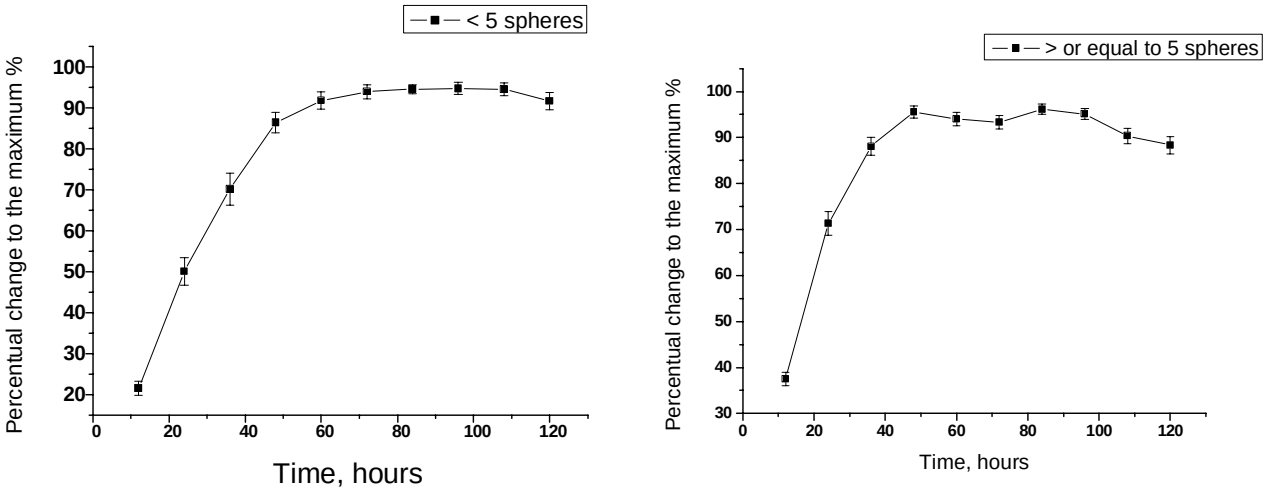
blocker and after two days of differentiation. Three pharmacological substances were used that are known to block T-type channels. 5 mM ethosuximide, 1 mM nickelchloride, $NiCl_2$, and 300 nM kurtoxin (all from Sigma-Aldrich) were used to block the channels. Ethosuximide was diluted in alcohol, $NiCl_2$ in *aqua purificata* and kurtoxin in HEPES. Concentrations of the substances were determined according to the literature review in Literature part, chapter 5.2.

Before plating the cells to the 6- well-plate, the bottom of the plate was covered with a protein called poly-l-ornithin. This fibre-like protein makes the surface uneven, and therefore beneficial for the cells to attach to the plate. To prevent the protein from drying out, it was covered with Elliot's salt solution. Cell incubation assay with Cell-IQ® (Chip-Man Technologies Ltd., version 2010) equipped with CCD-camera (Nikon), 10x objective, 0,7 µm resolution, was always carried out using the same method. A 6-well plate was filled with 4 milliliters of medium, and the substance to be examined was added to two wells. The plate was coated with Poly-l-ornithine the day before the experiment. Cells were plated on each well of the plate. The cells were left to attach to the plate for 30 minutes. After that the plate was inserted to the Cell-IQ- imager that started continuously imaging pictures with a five minutes interval in between of each cycle. Cell IQ took pictures from every 6 well and every 9 determined position. After two days, the medium was refreshed by changing half of it and the pharmacological substances were added, so that the final concentration was approximately the same as in the beginning of the experiment. To see if the pharmacological substance had any effect on the cells that already had started their migration, the compound was added to two more wells after two days of incubation, leaving two wells free of treatment. The same concentration was used as in the beginning of the incubation. Each experiment continued for at least four days.

All the experiments were made by controlling the parameters that might affect the results. The same fixed protocol was used at every experiment. The amount of spheres in a well was 15-20 and the diameter of one sphere was approximately 200 µm. It is important to select spheres with a bright center that marks the viability of the cells. Center of the sphere being dark is a sign of apoptotic cell death in the middle of the sphere. When the stem cells divide symmetrically they produce identic daughter cells

with an ability of self-renewal and multipotency. Symmetrical dividing leads to a symmetric round sphere. Therefore choosing symmetric and round spheres is essential for maintaining a culture of multipotent undifferentiated cells.

Another thing to consider was the number of the spheres in a selected imaging position. That number was defined to be <5 or ≥5 spheres, that were comparable with each other. This is because of the growth curve is similar in each group of spheres. When grown and differentiated in medium, cells begin to divide and migrate rapidly, but after the first two days the fast growth exceeds its limit. During those first days the growth follows a logarithm or even a sigmoidal scale ending at a lag-time followed later by apoptotic cell death. That is why the first hours and the first days are so critical and interesting to examine. In the case when there are several spheres growing near to each other, the growth curve turns to decline, simply because of the lack of space for the cells to live in and migrate, as seen in the picture 10. This is the reason why the number of spheres in a position was set to <5 after the nickel experiment. If that was not possible for some reason, then the positions with <5 spheres and the positions with ≥5 spheres were pooled together.



Picture 9, the growth curve of positions with <5 spheres compared to positions with ≥5. Growth is described as a percentual change to the maximum cell number during the incubation. Growth curve on the left presents the positions with spheres <5.

Although the main parameters are controlled, there are still several uncontrolled variables when examining living organisms. To obtain reliable results, the number of migrating cells is announced as a procentual or a relative change compared to the beginning. This is made to normalize the data. Counting the number of the cells outside the sphere indicates the number of migrating cells. The raw data was analyzed with Excel, and Origin™ 7.0 was used for further analysis. Results were expressed as mean ± standard error (SEM). Student's *t* test was used in analyzing data for cell incubation measurements. A p-value less than 0.05 was regarded as statistically significant.

11.4 HEK-cells

HEK-cells, human embryonic kidney cells, offer an excellent assay system as a negative control to T-type calcium channels, since they do not express voltage gated calcium channels, and contain very little background currents under appropriate assay conditions (Gomora *et al*, 2001). The cells were grown in standard Dulbecco's modified Eagle's cell culture medium (DMEM; Invitrogen) supplemented with 10 % fetal bovine serum (Invitrogen), 100 U/ml penicillin-streptomycin solution (Invitrogen), at 37 ˚C in 5 % CO_2 in an air ventilated humified incubator in 260-ml culture flasks (Nunc A/S, Roskilde, Denmark).

12. Results and performing of the experiments

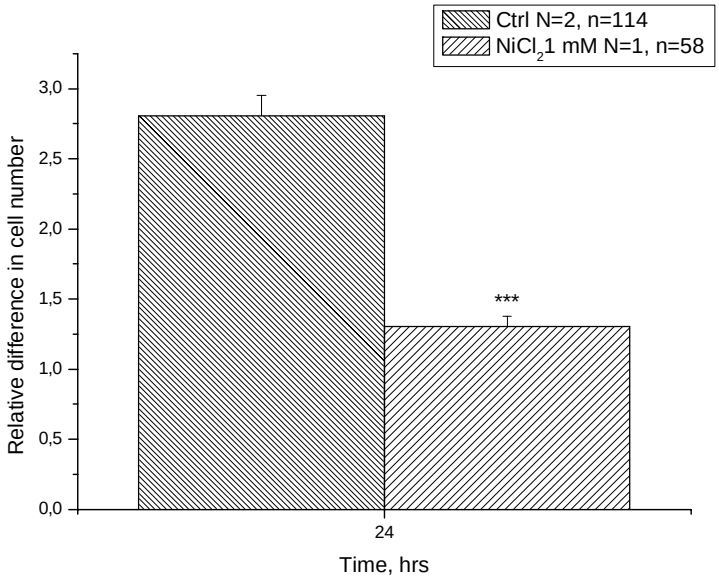
12.1 Blocking of the T-type calcium channel

12.1.1 Experiment 1: Examining the effect of nickel on the cells

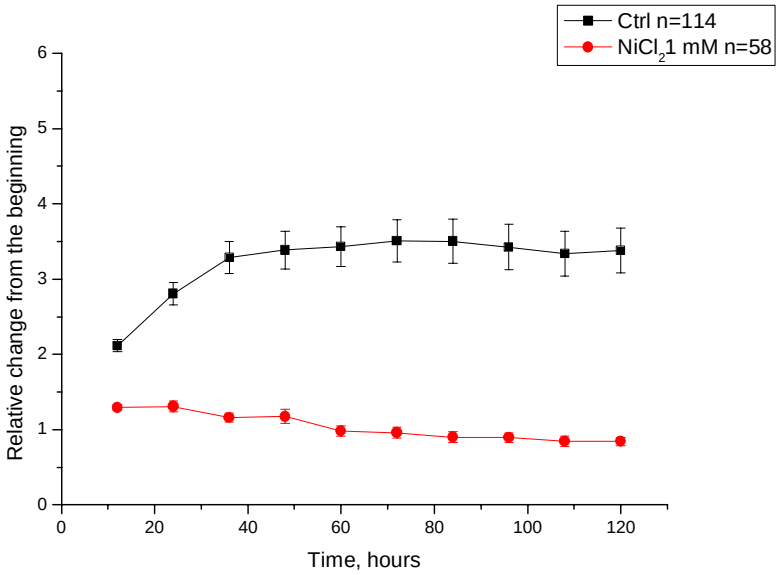
In this experiment, 1 mM nickelchloride was used to examine its effect on the cells' migration. Movements and morphology were detected with Cell-IQ. The events were recorded with Cell IQ video camera during a 6 days period.

On the first day, a 6-well plate was filled adding nickelchloride to only one well, otherwise the procedure was peformed according to the manner described in Materials and methods (Blocking of the T-type calcium channels). In this experiment, passage 9 cells were used.

On the second day, the results were examined online, while Cell-IQ continued the imaging. In the nickel-well, a clear change had taken place in comparison with the control wells. The cells were set on the edge of the sphere, and hadn't started migrating away from the sphere, like the cells in the control plates did. The control cells had migrated far and achieved different developmental stages on their way. After 24 hours, there was a statistically significant difference ($p < 0.05$) between the migrating control cells and the nickel-treated cells (picture 11).



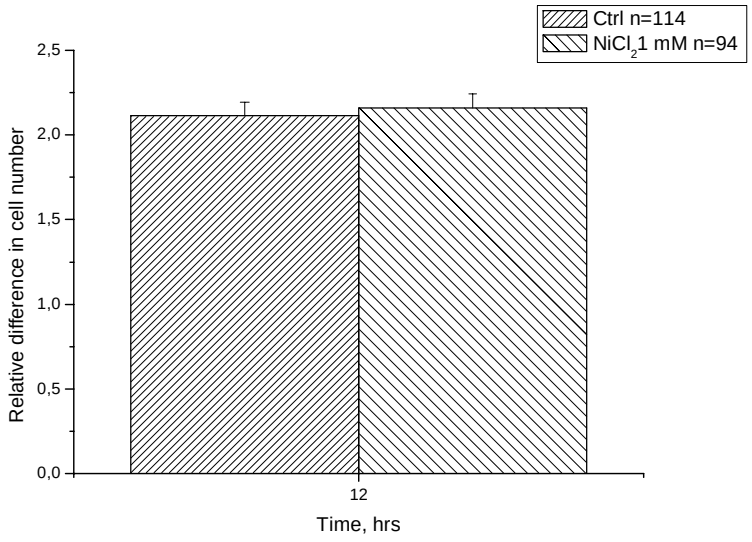
Picture 11, relative difference in cell number compared to the beginning after 24 hours of incubation. N = number of wells, n = number of spheres. The cells grown in the presence of nickelchloride were significantly lower in their number ($p < 0.05$) than those grown in the control wells.



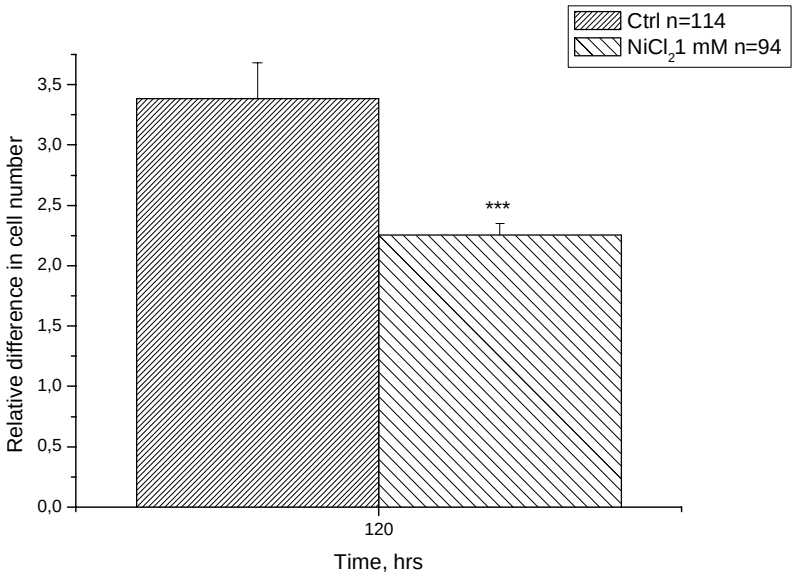
Picture 12, cells grown in the presence of nickelchloride compared to the growth of control cells. The growth in the presence of nickelchloride is significantly slower.

To see if nickelchloride had an effect on the divided and migrated cells, 1 mM nickelchloride was added to two more wells, on the third day of the experiment. The wells were taken out of the Cell-IQ- incubator, 2 milliliters of new medium was added to the wells with nickelchloride, so that the final concentration of it was the same 1 mM as before, as described in Materials and methods.

On the day 4 the cells were observed and it was noticed that after adding nickelchloride, all cells in the nickel-wells seemed to be set on their places, and they had stopped moving. If the cells stay still for too long, they die. In this case there were no signs of acute cell death. There were no detectable signs of cell death. The number of migrating cells outside the sphere was equal in the control- and nickel wells before adding nickelchloride (picture 13), but significantly higher ($p < 0.05$) in the control well, than in the nickel well after adding the substance (picture 14).

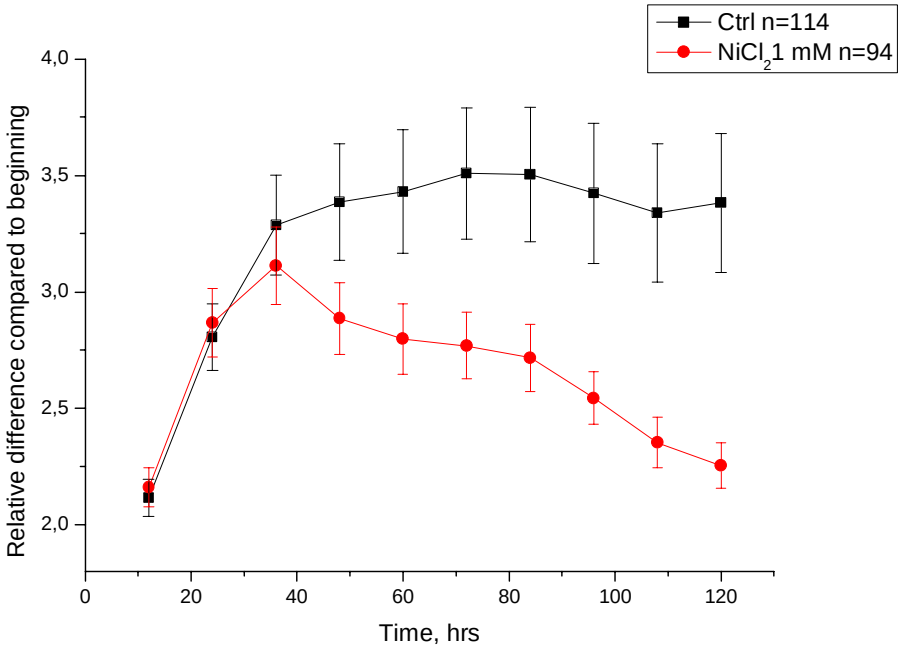


Picture 13, relative difference in the cell number before adding nickelchloride to the wells, at 12 hours of incubation. There is no significant difference between the controls.



Picture 14, cell number outside the sphere was significantly lower when grown in the presence of nickelchloride added after 2 days of incubation. At the final measure point, (120 hrs) there was a statistically significant difference (p-value <0.05) between the control and the nickel wells concerning the number of the cells migrating outside the sphere.

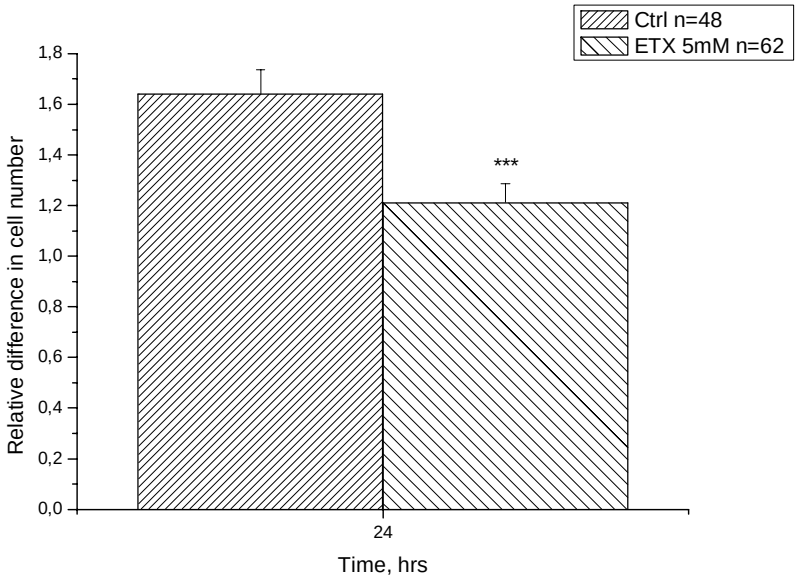
The same results can also be demonstrated as a cell growth curve during the whole experiment (picture 15).



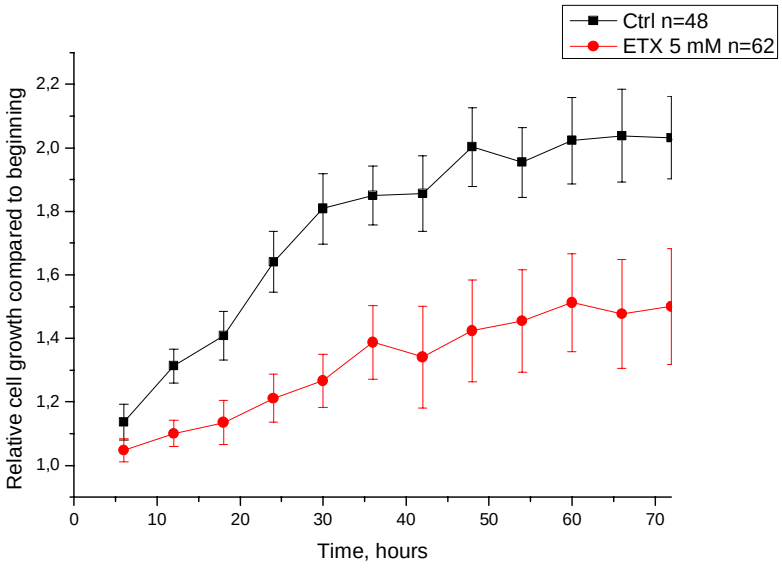
Picture 15, the number of cells outside the sphere during 120 hours of incubation. Nickelchloride was added on the second day, and after that the growth slowed down.

12.1.2. Experiment 2: Examining the effect of ethosuximide on the cells

When the cells were grown in the precesence of ethosuximide, the migration of the cells was significantly slower than in the control wells (picture 17). After 24 hours, the number of cells was increased 1.6-fold in the control wells, and 1.2-fold in the ETX wells ($p < 0.05$) (picture 16).

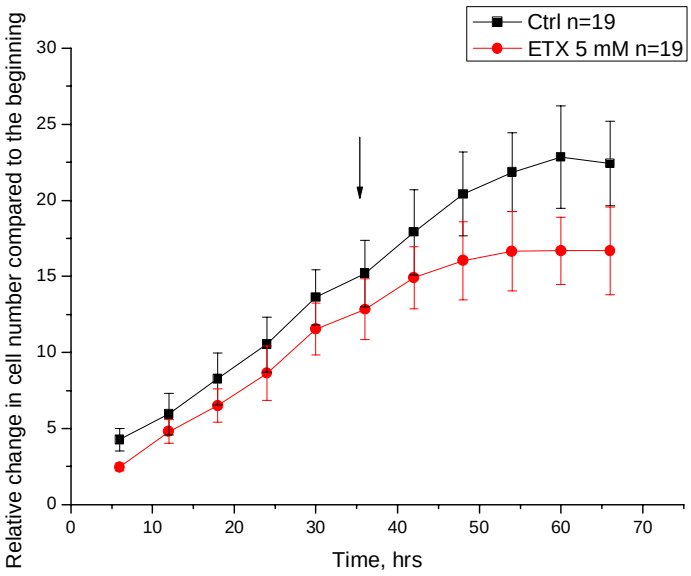


Picture 16, cells grown in the presence of 5 mM ethosuximide compared to the control. Number of cells migrating after 24 hours as a relative change in cell number compared to the beginning. N = 2, n(Ctrl) = 48, n(ETX) = 62

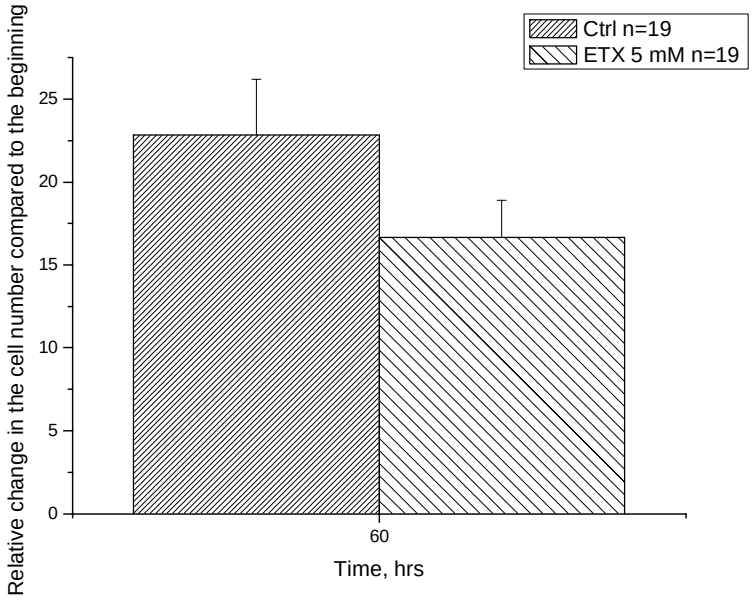


Picture 17, cells grown in the presence of ETX during 70 hours time. Cell growth expressed as a relative change compared to the beginning.

Ethosuximide, 5 mM was added after 36 hours of incubation. The effect of ethosuximide was not as clear as when grown in the presence of this substance, as seen in the picture 18. When the relative growth was compared after 24 hours since adding ETX to the cells, there was no statistical difference between control and ETX wells $p = 0{,}17703$ (picture 19).



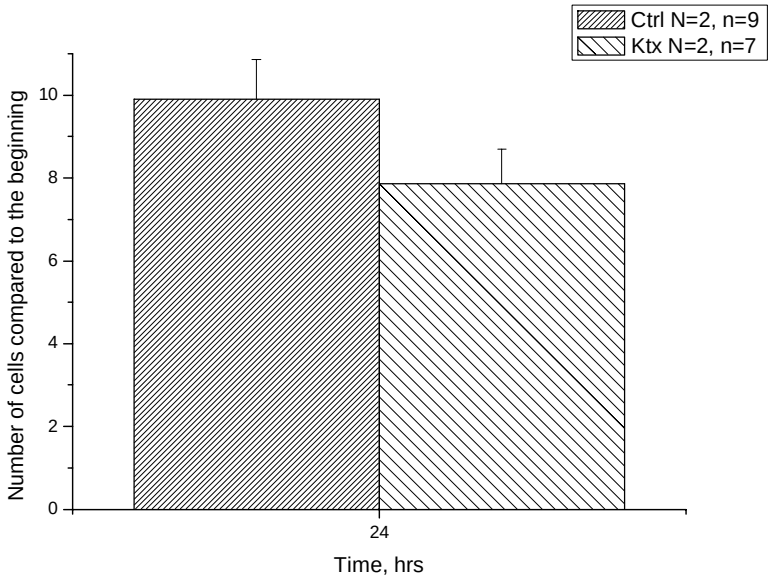
Picture 18, relative change in the cell number compared to the beginning during 70 hours of incubation. ETX was added to the wells at 36 hours of incubation, black arrow.



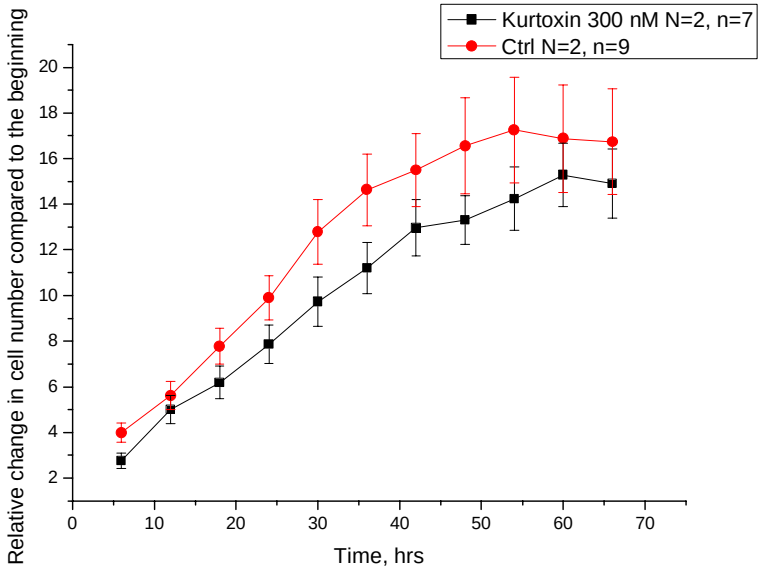
Picture 19, relative change in the number of migrating cells after 24 hours of adding ETX to the wells. There is a difference between the groups, but it is not statistically significant. N = 2, n = 19

12.1.3. Experiment 3. The effect of kurtoxin on the cells

Cells were grown in the presence of a T-type calcium channel specific blocker, scorpion toxin named Kurtoxin at a concentration of 300 nM. There was a slight difference between the kurtoxin and the control wells after 24 hours of incubation, but it was not statistically significant, p = 0.14261 (picture 20). During the 70 hours incubation the cells in the kurtoxin wells seemed to migrate slower than the cells in the control wells, but that was not significant either (picture 21).



Picture 20, Relative change in the number of migrating cells in kurtoxin wells compared to the control after 24 hours of incubation when grown in the presence of 300 nM kurtoxin.

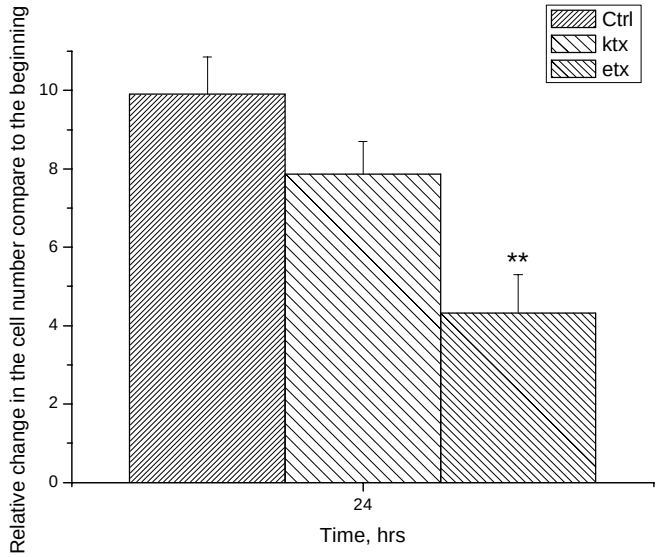


Picture 21, relative change in the cell number compared to the beginning when grown in the presence of 300 nM kurtoxin.

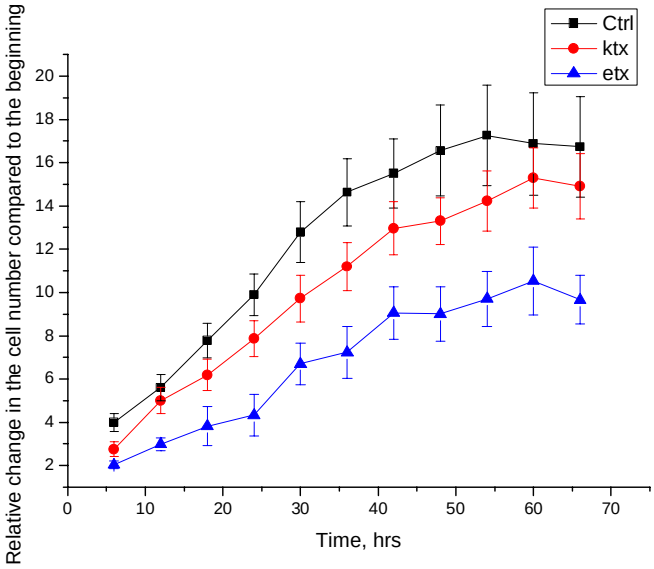
Kurtoxin at 300 nM was added to 2 of wells of a 6-well plate after 2 days of incubation. Unfortunately, the differentiation of the cells in these wells was already poor from the beginning, so the results were unreliable, and therefore excluded. As it can be seen in the picture 24, kurtoxin was added after 48 hours, but the growth curve is showing very slow migration rate for many hours before that.

12.1.4 Comparing kurtoxin and ethosuximide to the control

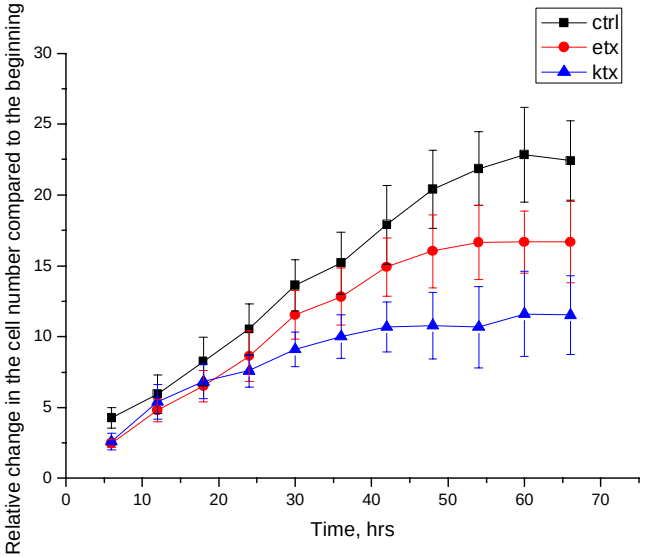
When the cells were differentiated in the presence of kurtoxin at 300 nM and ethosuximide at 5 mM, the outcome was that ethosuximide had a significant effect in decreasing the migration rate of the cells, when measured at 24 hours after starting the experiment. (p =0.05). There was also a difference between kurtoxin and the control wells, which was not statistically significant (picture 22). Growth curves of the cells show the same tendency (picture 23.)



Picture 22, Relative change in the number of cells migrating after 24 hours of incubation in the presence of 300 nM kurtoxin and 5 mM ethosuximide compared to the control.



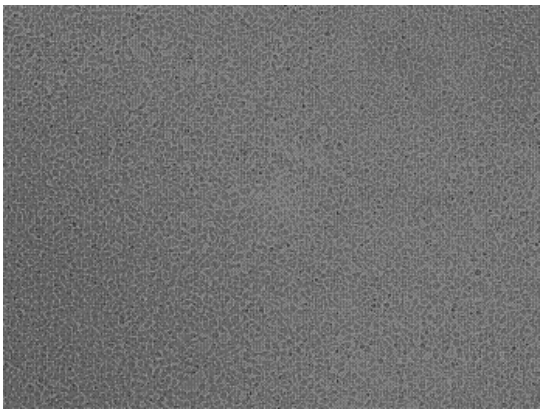
Picture 23, Cells grown in the presence of 5 mM ETX and 300 nM KTX during 70 hours of incubation.



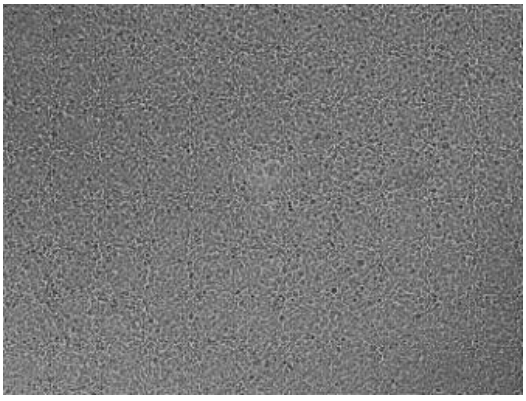
Picture 24, Cells count curves. Ethosuximide was added at 36 hours of incubation and kurtoxin 48 hours after incubation. Differentiation was disturbed in the kurtoxin wells already before adding the substance.

12.1.5 HEK- cells as a negative control

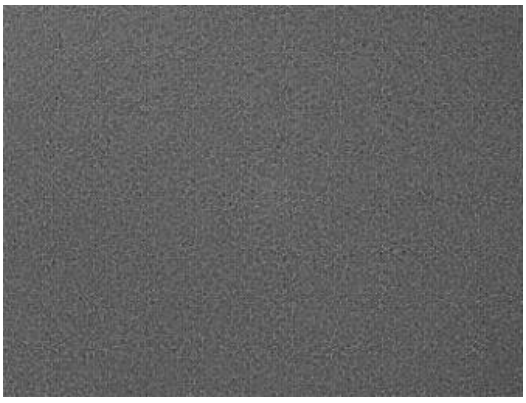
HEK- cells were used as a negative control because they do not express voltage-activated calcium channels. By adding the substances to be examined to these cells, some information of unspecific cytotoxicity can be gained. The HEK- cells were plated on 30 5 milliliters petri- plates containing DMEM- growth medium. 6 plates were used as control plates. Ethosuximide 5 mM was added to 6 other plates, as well as mibefradil hydrochloride (Tocris Bioscience, USA) 1 µM, manganese (Mn) 10 µM and pimozide 100 nM. By the time this experiment was made, nickelchloride was already shown to have unspecific toxicity on the HEK- cells. First, all the three substances were added to three separate plates. The confluence was detected after 24 hours incubation at 37 ˚C. It was noted that the confluence was 100 % in the control plates (picture 25) and in the ethosuximide (picture 26) and mibefradil (picture 27) plates.



Picture 26, control cells after 24 hours of incubation

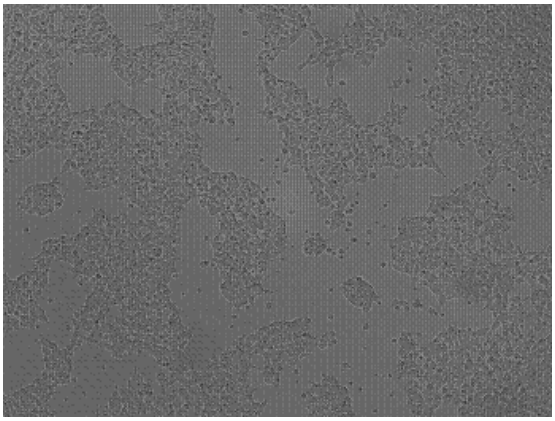


Picture 27, ethosuximide treated cells after 24 hours of incubation

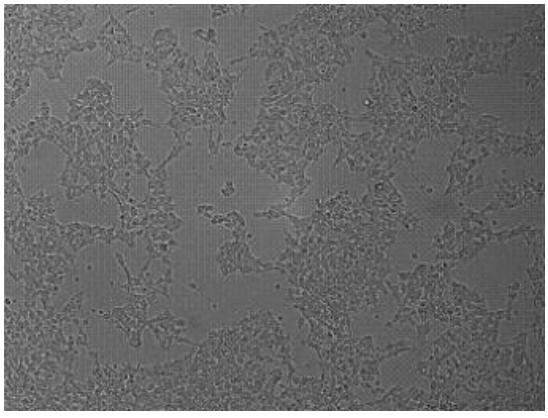


Picture 28, mibefradil hydrochloride after 24 hours of incubation

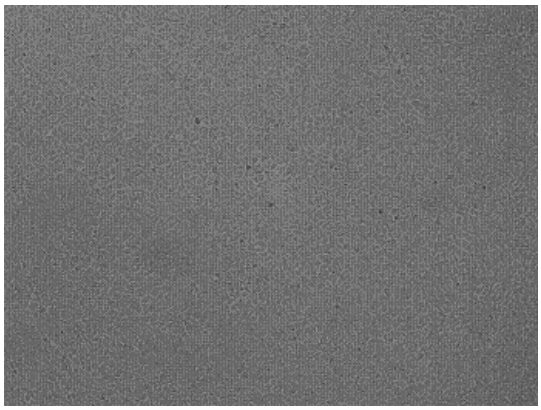
Cells in the pimozide (picture 30) and manganese (picture 29) plates were disturbed and detached from the bottom of the plate. This shows that these substances have unspecific cytotoxicity and effects on something else than voltage-activated calcium channels. After incubating the cells 24 hours more, the situation did not change. Control, ethosuximide and mibefradil plates were still alike and confluence was not disturbed (pictures 31, 32, 33).



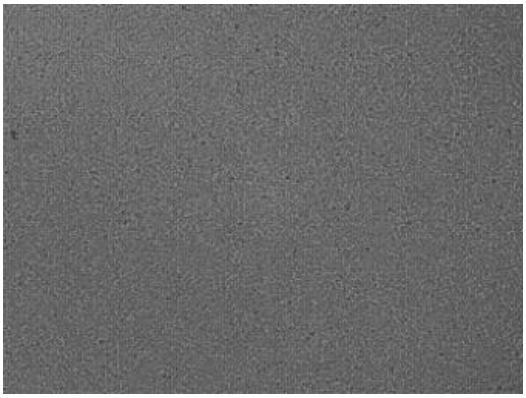
Picture 29, manganese after 24 hours of incubation



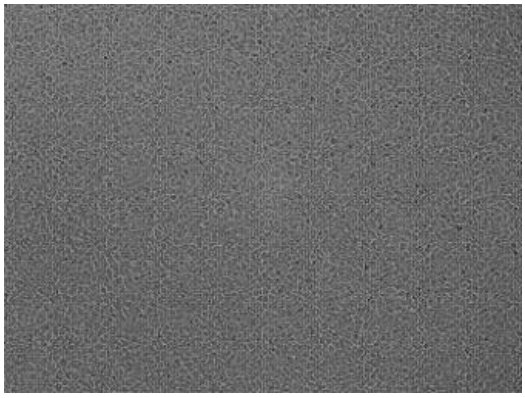
Picture 30, pimozide after 24 hours of incubation



Picture 31, control cells after 48 hours of incubation



Picture 32, ethosuximide treated cells after 48 hours of incubation

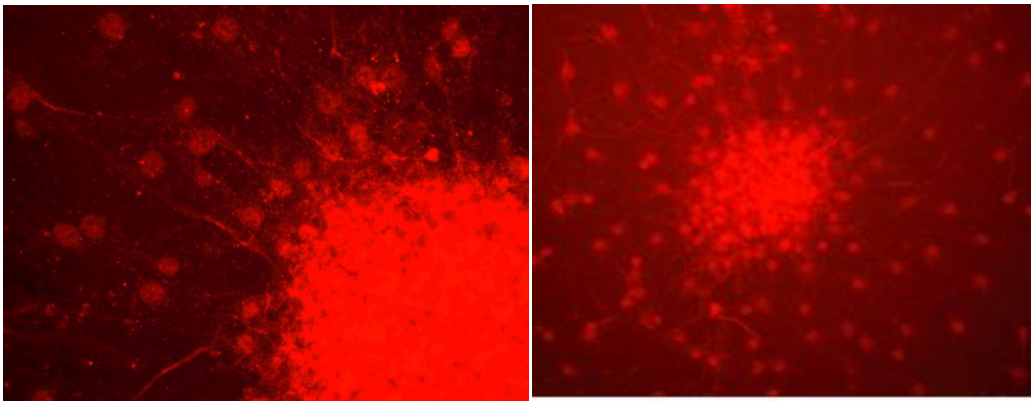


Picture 33, mibefradil treated cells after 48 hours of incubation

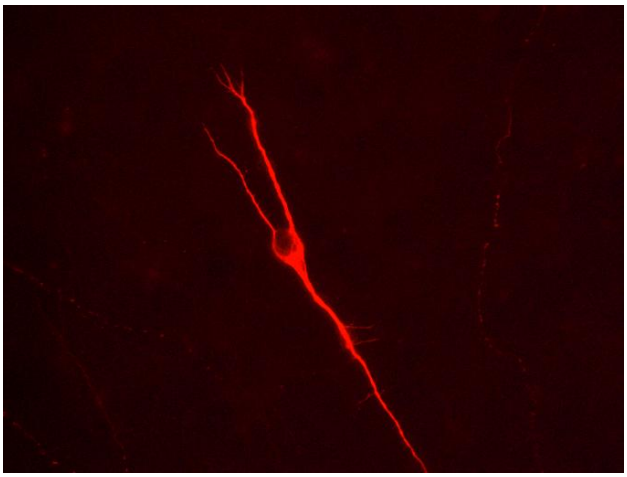
12.2 Immunostaining

12.2.1 Experiment 4: detecting the cells expressing neuronal markers

Immunostaining the cells with Tuj-1, as described in Materials and methods, chapter 10.2, showed that the cells used in these experiments differentiate and gain some neuronal features. This is shown in pictures 35 and 36 that are taken from the cells that were differentiated for 5 days (picture 35) and for 7 days (picture 36). In picture 37, there is one cell from the spheres that were differentiated for 7 days. In addition to the expression of β-tubulin III, which shows as a red color, it has clear morphological features of a neuronal cell.



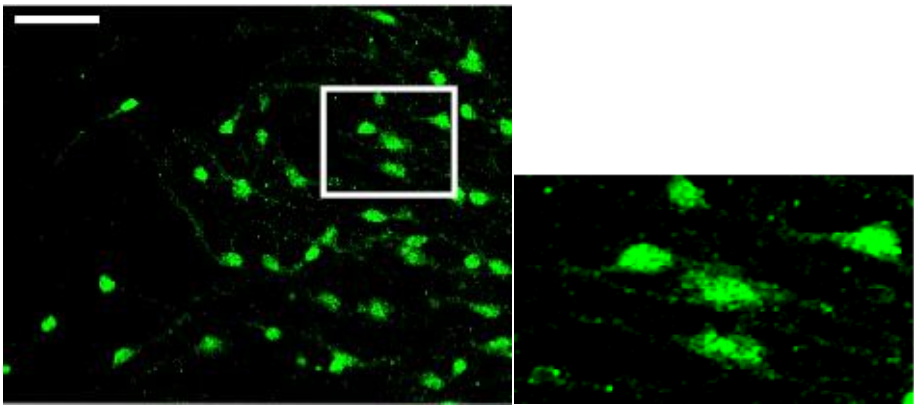
Pictures 35 and 36, cells differentiated for 5 days are on the left, and the cells differentiated for 7 days are on the right. Red colored cells in the pictures express β-tubulin III, a marker of neural maturation.



Picture 37, a neuronal cell in the culture differentiated for 7 days

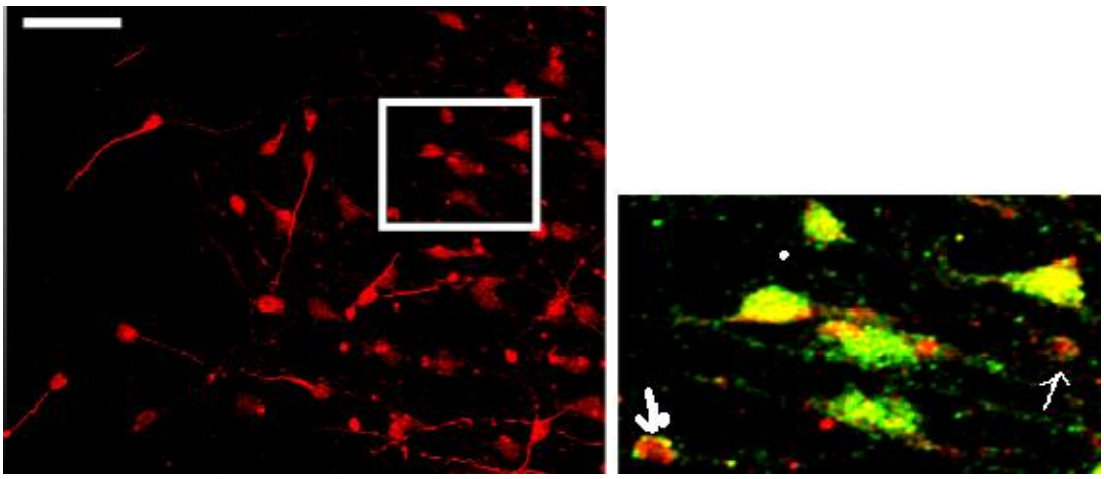
12.2.2 Experiment 5: Detecting the cells expressing T-type calcium channels

Another essential thing to prove was that the cells used in these experiments express T-type calcium channels. Labeling the cells with a T-type specific antibody and imaging with confocal microscope revealed that the 87.5 ± 5 % of the cells differentiated for 3 days, express T-type calcium channels (picture 38). Pictures 38-41 are published with permission.



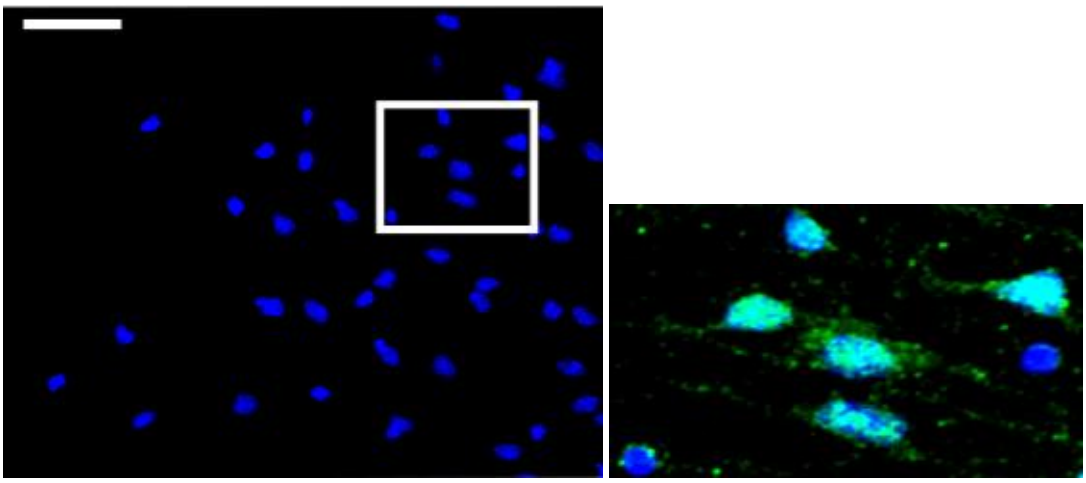
Picture 38, 87.5 ± 5 % of the cells differentiated for 3 days expressed T-type calcium channels. The image limited with the white bordered box is on the right.

The same picture with Tuj-1 antibody shows that the cells also express neuronal elements (picture 39). On the right side of picture 39, there is an overlap of T-type antibody expressing picture and Tuj-1 picture. That overlap shows two cells with smaller somas, which do not express T-type calcium channels as much as the other five cells in the overlap picture.

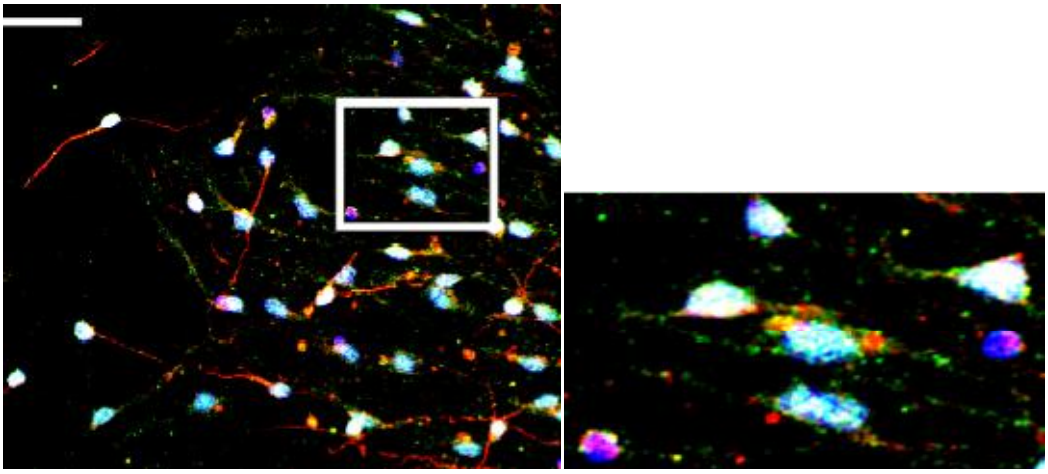


Picture 39, cells expressing β-tubulin III after 3 days of incubation. On the right there is an overlap of Tuj-1 and T-type antibody pictures. The cells that express both neuronal markers and T-type calcium channels are mostly yellow in their color. Two of the cells that do not express T-type calcium channels are marked with white arrows.

DAPI, which dyes all the cells somas blue, is shown in the picture 36. On the right side of the picture there is an overlap of the pictures with T-type antibody and DAPI. In this overlap the cells expressing T-type calcium channels are light blue in their color. Picture 40 shows all the cells in the picture. Picture 41 is an overlap of all these pictures.



Picture 40, DAPI dyes all the cells somas blue. An overlap of all the pictures where T-type channels and other cells are on the right side.



Picture 41, an overlap of all the previous pictures showing the cells expressing β-tubulin III, T-type calcium channels and all the cells that have somas.

13. Conclusions

The results show that all the known blockers of T -type calcium channels added to the growth medium decreased the number of cells migrating from the neurospheres. This effect was even clearer when the cells were grown in the presence of nickel chloride and ethosuximide than when the differentiation was started without pharmacological treatment. These two different treatments were conducted to see the difference in timing. This reflects the role of low-voltage activated calcium channels in neuronal differentiation. It can be assumed that if the role of T-type calcium channel is crucial in the very beginning of migration, the specific blockers would have a clearer effect on the cells than if added two days later. In the case that T-type calcium channel's role would appear later on, one could expect to see no significant effect of the blockers when added right in the beginning of the differentiation.

The results gained in our studies demonstrate that nickel chloride had the most significant effect, although it might also be less selective. The number of the cells when they were grown in the presence of nickel chloride was 1.5-fold lower compared to the control than when it was added afterwards. The ratio when grown in the presence of ethosuximide and added afterwards compared to the control was not so clear, with 1.5 and 1.4-fold difference in the cell number, respectively. Considering that the difference in the cell number between the control and ethosuximide treatment when ethosuximide was added 24 hours afterwards was not statistically significant, makes the effect more questionable at this specific time point. The effect of these substances decreasing the cell number when grown in the presence of them might indicate that T-type calcium channel has an important role in the early state of the migration. When the antagonists are added afterwards, the effect on the cells is not as strong. On the other hand, in our experiments kurtoxin produced different effects at this specific time point, although the results gained with kurtoxin are not very reliable for two reasons. Firstly, differentiation of the cells in the kurtoxin wells, as wells as in the control wells, was disturbed even before adding the compound, and there was no statistical difference compared to the control. Secondly, kurtoxin is a peptide toxin, and relatively instable compared to the non-peptide compounds. Also other studies have disputed over the results and performing of kurtoxin experiments. Kurtoxin has produced very different sets of gating

modifications in different calcium channel subtypes in one experiment (Sidach and Mintz, 2002). The authors describe these findings as unexpected, and therefore rule out the use of kurtoxin in functional studies of T-type calcium channel. At this point it must be noted that although the compound is complicated, probably mostly due to its peptide structure, failures in one study should not completely exclude the use of this substance in functional studies, in my opinion. In another study it was noted that kurtoxin interacts with voltage-gated sodium channels (Chuang *et al*, 1998). After all, in our experiments made with kurtoxin, the tendency of the toxin inhibiting cells' migration was the same as in the results gained with nickel and ethosuximide.

Previous studies done by Komuro and Rakic show that N-type calcium channel would play the key role in neuronal migration. In this study we investigated LVA calcium channels and saw an effect of T-type calcium channel blockers interfering with the migration. Komuro and Rakic showed that blocking of T-type channels did not have a significant effect. However, they studied the migration of purkinje cells which are postnatal. Furthermore, the concentrations used in their studies were different. Komuro and Rakic investigated the effect of calcium channel blockers on neuronal migration as movements of single cells, while we studied the effect on the whole cell population as a number of migrating cells. They showed that the blockage of N-type calcium channel significantly decreases the rate of cell movement (Komuro and Rakic, 1998). They also claimed that blocking other types of voltage-gated calcium channels, such as L- or T-type calcium channels had no significant effect. This might be due to the low concentrations of antagonists they used in the experiment. Nickel chloride was used at a concentration of 100 µM, while the concentration of nickel chloride in this study was ten times higher, 1 mM. This is simply because of the growth medium we used contains phosphates that buffer most of the heavy metals. It is clear that the experimental procedures being so different in our study compared to the ones of Komuro and Rakic, makes the comparison very difficult.

In two other studies L-type calcium channel is shown to promote neuronal differentiation (Gicola et al. 1998; D'Ascenzo et al. 2006). There are similar results gained in the research of T-type calcium channel (Chemin J. et al. 2002; 2003). At this point, it must be kept in mind that these results do not disagree with the results of

Komuro and Rakic, considering that migration and differentiation are classified as different events in the neurogenesis, and they are altered mostly by different reactions. It is likely that an endogenous factor does not affect a certain phase of neurogenesis but promotes another. In the study of Chemin *et al*, 2002, the authors concluded that T-type calcium channels trigger the onset of differentiation and regulate the expression of HVA calcium channels. When comparing all these studies to each other a question arises: Is there in fact a controversy after all or do we just face the complexity of neuronal developmental events and their overlapping nature? Undoubtedly one problem is comparing the different experimental set ups to each other. Comparison of the studies made with cell cultures and electrophysiological single-cell studies is not entirely impossible, but challenging. There are several factors that have to be taken into account, including the cell type, time and origin of extraction of the cells and the day of differentiation when the records are made, in addition to the concentration and selectivity of the test compound, just to list some of the most critical issues.

In this study, the selectivity of the compound was tested with a negative control, HEK-cells, which do not express voltage-gated calcium channels. The results were as expected. The drugs known to be more selective, ethosuximide and mibefradil had no effect on them, while the less selective compounds manganese and pimozide disturbed the growth of the HEK-cells. In pictures 29 and 30 this can be seen as detached cell aggregates on an above layer of the cells attached to the plate.

14. Discussing the results and questions to analyze further

This study shows that T-type calcium channel is likely to take part in neuronal progenitor cells' migration. This finding is in line with other studies that show T-type calcium channel promoting neurogenesis (Gu and Spitzer, 1993; Chemin *et al*, 2002; 2003) and calcium ion being a key regulator in the process of neural induction (Webb *et al*, 2005). T-type selective antagonists, ethosuximide, nickelchloride and kurtoxin decreased the rate of migration in mice embryonic subventricular zone progenitor cells. The effect was clearer when differentiation of the cells was started in the presence of these substances. In contrast, an L-type specific blocker nifedipine and an N-type specific blocker ω-conotoxin did not have a significant effect under the same conditions (data under inspection).

There are yet some problems this study could not overcome. One of them is the lack of a specific T-type channel antagonist, and these results are based in a statistical likelihood of the function of different T-type channel antagonists. The results do not conclude that T-type channel's role in migration would be the most dominant. If assumed that T-type channel has a significant role in promoting migration, one would conclude, that the more specific the T-type blocking antagonist is, the more it inhibits the migration. This study shows conversely results. This in turn gives us a reason to consider that the previous assumption made about T-type channel having the biggest role in migration, might be wrong. More likely it seems to be that the T-type calcium channel works together with some other endogenous factors, like for example other voltage-activated channels, integrins, and external signals, without any kind of hierarchy between them.

Hence, further studying should be made to confirm the results gained in this study, and point out the exact role of T-type calcium channel in migration, and the significance of this role. Also the roles of each subtype of the T-type channel remain unsolved. These studies could include experiments with gene-experssed cells overexpressing a certain subtype. Three-dimensional cell growth conditions would be the most favorable, mimicking the cells' natural environment in the brain. *In vivo* labeling and detecting of

the progenitor cells would give some valuable information of the cells' migration. An urgently needed investigation would be a highly specific T-type calcium channel antagonist. In addition to that it would be useful in the research of T-type channel's functions it would also be of a great clinical profit in treatment of absence epilepsy.

What comes to the therapeutic relevance of these findings is that a lot more fundamental studying has to be made to achieve the "pharmacological toolkit" to manipulate the endogenous stem cells. To generalize, the main problems in stem cell therapies are the reciever's immunity, stem cells' survival, differentiation, targeted migration, and not turning into tumors. One of these barriers is already crossed with the help of pharmacology. Namely, the reciever's immunity can be stopped from rejecting the cells with immunosuppressive medication.

The challenge, that Santiago Ramón y Cajal casted nearly a century ago for the future researchers is overcome. Now that we know that neurogenesis continues in the adult brain, has brought us a new question to answere. How can the mankind benefit from the stem cells in a cost-effective way that is available for everyone?

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ATTACHMENT 1: SOLUTIONS

Poly-l-ornithin

- is used to coat the wells and plates, NSCs attach to the coating material and start their differentiation
- ordered from Sigma

Poly-l-ornithin is diluted in 25 mg/ 50 ml of PBS and sterile filtered. This solution is used in a concentration of 1:10 of PBS, or even more diluted. The solutions are stored in -20˚C.

1 M HEPES

- is added in growth medium in 0.75 ml/ 50 ml as a buffer solution
- the solution has to be autoclaved before use
- the salt powder ordered from Sigma-Aldrich is added in *aqua sterilisata* in a concentration of 1 M (mol/l):

M = 238g/mol

V = 500 ml

$m = MCV$ = 119g

PBS

1 litre of PBS consists of:

- $NaCl_2$		80.0
- KCl_2	80.0	
- Na_2HPO_4 x 2 H_2O	14.0	
- KH_2PO_4	2.0	

ad 1 l *aqua sterilisata*

- pH of the solution needs to be adjusted to 7.3
- solution must autoclaved
- the solution is stored in the fridge

ATTACHMENT 2: B27 SUPPLEMENT

B27 supplement is prepared in distilled water. The components of the solution are:

- d-biotin
- BSA
- l-carnithine
- corticosterone
- ethanolamine HCl
- d-galactose (anhydr.)
- insulin (human, Zn)
- linoleic acid
- progesterone
- putrescine 2HCl
- sodium selenite
- T3/albumin complex
- transferring (human, iron-)

The solution is stored in -20˚C