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Chemogenetics, Induced Neurons and Pluripotent Stem Cells: Towards Advanced Gene and Cell Therapies Targeting Epilepsy

Avaliani, Natalia

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Chemogenetics, Induced Neurons and Pluripotent Stem Cells:

Towards Advanced Gene and Cell Therapies Targeting Epilepsy

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Title and subtitle Chemogenetics, Induced Neurons and Pluripotent Stem Cells: Towards Advanced Gene and Cell Therapies Targeting Epilepsy

Abstract: The complexity of the central nervous system and existence of the blood-brain barrier often causes difficulties for traditional pharmacological treatments of neurological diseases. This thesis explores the feasibility and potential for novel gene and cell therapy approaches, which hold better promise for neurological disorders, while particularly targeting epilepsy. Epilepsy is a multifactorial neurological disorder affecting 1% of the population. Available pharmacological therapies are merely symptomatic, and have severe side effects, while failing to adequately control seizures in one third of the patients, predominately in those with temporal lobe epilepsy (TLE). Targeted silencing of the pathological circuits by expressing therapeutic genes, or increasing the inhibition by introducing new populations of GABA-releasing neurons, might prove therapeutic for epilepsy, by counteracting seizures and even modifying the disease.

Gene therapy offers localized, cell-type specific alteration of neuronal excitability, but on-demand seizure suppression can only be achieved by tools allowing external temporal control. One such recently developed chemogenetic technology is based on viral expression of modified muscarinic G-protein coupled receptors, specifically activated by otherwise inert clozapine-N-oxide (CNO). In *paper I*, we explored if such modified receptor, hM4Di, which selectively activates Gi pathway, thereby causing neuronal inhibition, could suppress epileptiform activity upon CNO application. This approach proved effective for localized suppression of neuronal excitability and seizure-like events in an *in vitro* model of TLE, organotypic hippocampal slice cultures (OHSC), without altering intrinsic properties of the hM4Di-expressing neurons, demonstrating the therapeutic potential of this technology.

In *papers II and III* we characterized two different cell sources with the prospect of cell replacement therapy: induced Pluripotent Stem cells (iPS) and induced Neurons (iN). These two patient specific alternative cell sources offer a solution to ethical and immunogenicity issues, related to embryonic stem cell use.

Already six weeks after grafting in OHSCs, iPS-derived neuroepitelial-like stem cells (lt-NES), predominately differentiating to GABA-ergic neurons, displayed functional neuronal properties and certain rate of synaptic integration. *In vivo* studies showed that longer differentiation time (up to 24 weeks) was needed for the grafts to fully mature and extensively integrate into the host synaptic network. The grafted cells still retained some of the distinct electrophysiological features, however, such as high input resistance.

Next, we studied long-term survival of human foetal fibroblast-derived induced neurons (iN) in rodent hippocampus. Human iNs survive and maintain neuronal profile up to six months post-grafting, developing more elaborate neuronal morphology and complex dendritic arborisation over time. Graft-derived neurons with mature neuronal properties could be observed at six months, although a portion of non-converted fibroblasts, as well as asynchronous neuronal conversion was apparent among the grafts. Improvements in conversion, survival and integration rate of iN cells are required before these cells can offer a better alternative to iPS or stem cells. While showing potential as candidates for cell replacement therapy, both characterised cell types have to be further tested in relevant epilepsy model systems to demonstrate their therapeutic effect.

In summary, this thesis adds new knowledge and experimental basis for development of gene- and cell-based therapies for neurological disorders.

Key words Chemogenetics, iPS, iN, electrophysiology, gene and cell therapy, Epilepsy

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To the memory of my father
Alexander Avaliani

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Original Papers

Avaliani N, Andersson M, Woldbey D, Kokaia M. DREADDs suppress seizures in mouse model of pharmacoresistant epileptic brain tissue. Manuscript.

Avaliani N, Sorensen AT, Ledri M, Bengzon J, Koch P, Brustle O, Deisseroth K, Andersson M, Kokaia M. Optogenetics reveal delayed afferent synaptogenesis on grafted human-induced pluripotent stem cell-derived neural progenitors. Stem cells. 2014 Dec;32(12):3088-98.

Avaliani N, Pfisterer U, Heuer A, Parmar M, Kokaia M, Andersson M. Directly converted human fibroblasts mature to functional neurons and show long-term survival in rodent hippocampus. Manuscript.

Summary

The complexity of the central nervous system and existence of the blood-brain barrier often causes difficulties for traditional pharmacological treatments of neurological diseases. This thesis explores the feasibility and potential for novel gene and cell therapy approaches, which hold better promise for neurological disorders, while particularly targeting epilepsy.

Epilepsy is a multifactorial neurological disorder affecting 1% of the population. Available pharmacological therapies are merely symptomatic, and have severe side effects, while failing to adequately control seizures in one third of the patients, predominately in those with temporal lobe epilepsy (TLE). Targeted silencing of the pathological circuits by expressing therapeutic genes, or increasing the inhibition by introducing new populations of GABA-releasing neurons, might prove therapeutic for epilepsy, by counteracting seizures and even modifying the disease.

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In summary, this thesis adds new knowledge and experimental basis for development of gene- and cell-based therapies for neurological disorders.

რეზიუმე

ცენტრალური ნერვული სისტემის კომპლექსურობა და ჰემატოენცეფალური ბარიერის არსებობა ხშირად აბრკოლებს ტრადიციული ფარმაკოლოგიური მეთოდების ეფექტურობას. წინამდებარე ნაშრომი იკვლევს გენური და უჯრედული თერაპიის, როგორც პოტენციურად უფრო ეფექტური მიდგომის შესაძლებლობებს ნეიროლოგიური დაავადებებისთვის, კერძოდ ეპილეპსიისთვის.

ეპილეპსია არის მრავალფაქტორული ნეიროლოგიური დაავადება, რომელიც მსოფლიო მოსახლეობის 1%-ში ვლინდება. დღეისათვის არსებული ფარმაკოლოგიური საშუალებები მხოლოდ სიმპტომურია და საკმაოდ მძიმე გვერდით მოვლენებს იწვევს. ამასთან, პაციენტების ერთ მესამედზე მეტში ეს პრეპარატები ეპილეპსიური კრუნჩხვების ადეკვატურ კონტროლს ვერ ახერხებენ. ასეთი ფარმაკორეზისტენტული შემთხვევების უმეტესობა საფეთქლის წილის ეპილეპსიაზე მოდის. პათოლოგიური ნეირონული ქსელის აქტივობის მიზანმიმართული დათრგუნვა თერაპიული გენის ექსპრესიის საშუალებით, ან ინჰიბირების ამაღლება ახალი, გადანერგილი, გაემ-ერგული ნეირონების პოპულაციით შესაძლებელია ეფექტური აღმოჩნდეს ეპილეპსიის მკურნალობისთვის, კრუნჩხვების დათრგუნვის, თუ დაავადების მექანიზმის მოდიფიკაციის გზით.

გენური თერაპია გვთავაზობს ნეირონული აგზნებადობის ლოკალურ, უჯრედ-სპეციფიურ მოდიფიცირებას, თუმცა ეპილეპსიური შეტევის მოთხოვნილებისამებრ დათრგუნვა მხოლოდ გარეგანი ტემპორალური კონტროლის არსებობის შემთხვევაშია შესაძლებელი. ასეთ კონტროლს ერთ-ერთი თანამედროვე ტექნოლოგია, ქემოგენეტიკა, გვთავაზობს, რომლის მოქმედებაც დამყარებულია მოდიფიცირებული მუსკარინული რეცეპტორების ჩვეულებრივ ინერტული მოლეკულით, კლოზაპინ-N-ოქსიდით აქტივაციაზე. პირველ სტატიაში გამოყე-

ნებულია ერთ-ერთი ამგვარი მუსკარინული რეცეპტორი, hM4Di, რომელიც კლოზაპინ-N-ოქსიდთან დაკავშირებისას სელექციურად რთავს Gi სასიგნალო გზას და ამგვარად იწვევს ნეირონულ ინჰიბირებას. აღწერილი მექანიზმით შესაძლებელი გახდა საფეთქლის წილის ეპილეპსიის *in vitro* მოდელში, ჰიპოკამპუსის ორგანოტიპურ კულტურებში, ნეირონული აგზნების და კრუნჩხვისმაგვარი აქტივობის დათრგუნვა, ნეირონების ნორმალური ელექტროფიზიოლოგიური პარამეტრების ცვლილების გარეშე, რაც ამ მეთოდის თერაპიულ პოტენციალზე მიუთითებს.
მეორე და მესამე სტატიაში დახასიათებულია უჯრედული თერაპიის ორი სხვადასხვა კანდიდატი: ინდუცირებული პლურიპოტენტური ღეროვანი უჯრედები (iPS) და ინდუცირებული ნეირონები (iN). ეს ორი ალტერნატიული პაციენტსპეციფიური უჯრედების წყარო ღეროვანი უჯრედების გამოყენებასთან დაკავშირებული ეთიკური პრობლემების გადაწყვეტას და მასპინძლისგან სავარაუდოდ დაბალ იმუნურ რეაქციას გვთავაზობს.
ორგანოტიპურ კულტურებზე დანერგილ, iPS-უჯრედული წამოშობის ნეიროეპითელიალურის მსგავს უჯრედებს, რომლებიც მეტწილად გაემ-ერგულ ნეირონებად დიფერენცირდებიან, უკვე ექვსი კვირის შემდეგ ძირითადი ნეირონული ფუნქციები გააჩნიათ და სინაფსურ ქსელში ინტეგრაციის გარკვეულ დონესაც ამყარებენ. თუმცა, *in vivo* კვლევების მონაცემების მიხედვით მათ დაახლოებით ექვს თვემდე სჭირდებათ სრულად მომწიფებული ფუნქციური ნეირონების ფორმირებისთვის და მასპინძელ ქსოვილთან ვრცელი სინაფსური კავშირების დასამყარებლად. გადანერგილ უჯრედებს ამ დროისთვისაც ზოგიერთი განსაკუთრებული ნიშან-თვისება აქვს შემორჩენილი, როგორიცაა, მაგალითად, უჯრედული მემბრანის წინაღობის მაჩვენებელი.
რაც შეეხება iN უჯრედებს, ჩვენ შევისწავლეთ ადამიანის ემბრიონული ფიბრობლასტების რეპროგრამირებით მიღებული ნეირონული უჯრედების გადარჩენის ხარისხი ვირთაგვას ჰიპოკამპუსში, გადანერგვიდან ექვსი თვის განმავლობაში. გადანერგილი უჯრედები სტაბილურად ინარჩუნებენ ნეირონულ მორფოლოგიას და დროის განმავლობაში უფრო რთულ დენდრიტულ დატოტვას ივითარებენ. აღსანიშნავია, რომ ექვსი თვის შემდეგ შესაძლებელი იყო ელექტროფიზიოლოგიურად

სრულად ჩამოყალიბებული ნეირონების პოვნა ტრანსპლანტანტში, თუმცა მათთან ერთად არარეპროგრამირებული და ნაწილობრივ კონვერტირებული უჯრედებიც კვლავ გვხვდებოდა. ამგვარად, საჭიროა რეპროგრამირების, გადარჩენისა და სინაფსური ინტეგრირების ხარისხის გაუმჯობესება iN-ისათვის, სანამ მათ iPS-თან და ღეროვან უჯრედებთან თანაბარწილად განვიხილავთ ტრანსპლანტაციისთვის. თუნცა აღწერილი ორივე სახის უჯრედები თერაპიისთვის პოტენციურად კარგი კანდიდატები არიან, საჭიროა მომავალი კვლევები შესაბამისი დაავადებების მოდელ სისტემებში მათი თერაპიული ეფექტის დასადასტურებლად.

წინამდებარე თეზისში წარმოადგენილ, ექსპერიმენტულ კვლევებზე დაფუძნებულ აღმოჩენებს მნიშვნელოვანი კონტრიბუციის შეტანა შეუძლია ნეიროლოგიური დაავადებებისთვის გენური და უჯრედული თერაპიის განვითარების მიმართულებით დღეისათვის არსებულ სამეცნიერო ცოდნაში.

Abbreviations

CNS	central nervous system
BBB	blood-brain barrier
LV	Lentivirus
HSV	herpes simplex virus
AV	adenovirus
AAV	adeno-associated virus
GPCR	G protein-coupled receptor
CNO	Clozapine-N-oxide
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
hM receptor	human Muscarinic receptor
ES	Embryonic Stem cells
iPS	induced Pluripotent Stem cells
lt-NES	long-term expandable neuroepithelial-like stem cells
GABA	γ-aminobutyric acid
BAM	Brn2, AsclI and Myt1b
AP	Action Potentials
iN	induced Neurons
SE	Status Epilepticus
TLE	temporal lobe epilepsy
AEDs	Anti-Epileptic
CA	Cornus Ammonis

DG	Dentate Gyrus
OHSC	Organotypic Hippocampal Slice Cultures
aCSF	artificial cerebrospinal fluid
MOI	multiplicity of infection
SM	small molecules
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
VPA	Valproic Acid
CBZ	Carbamazepine
MK	801(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogen malea
D-AP5	(2R)-amino-5-phosphonovaleric acid
PTX	picrotoxin
TTX	tetrodotoxin
RMP	Resting Membrane Potential
Ri	input resistance
PSCs	Postsynaptic Currents
NMDA	N-methyl-D-aspartic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxa- zolepropionic
STIB	Stimulation Train-Induced Bursting
ChR	Channelrhodopsin
PHT	Phenytoin

Introduction

Gene and cell therapies are overlapping fields of biomedical research aimed at repairing the direct cause of genetic diseases in the DNA or in cellular population, respectively. These strategies are also being focused on modulating specific genes and cell subpopulations in acquired diseases in order to reestablish the normal equilibrium. In many diseases, gene and cell therapies are combined in the development of promising treatment strategies. Additionally, these two fields have helped provide reagents, concepts, and techniques that are elucidating the finer points of gene regulation, stem cell lineage, cell-cell interactions, regenerative capacity, reprogramming etc.

Gene therapy in neurological disorders

Gene therapy was traditionally defined as an approach to replace the defective copy of a gene with a functional one and restore normal function in a cell population. In its classical form, gene therapy is a therapeutic delivery of genetic material to the patient's cells to treat disease. The development of gene therapy through introduction of therapeutic genes in neurons is an attractive strategy for neurological disorders, since they are among the most difficult to treat. Traditional pharmacological approaches often fail in the central nervous system (CNS) diseases due to the complexity of the system and the physical barriers, such as the blood-brain barrier (BBB). The distribution of many compounds to the CNS is further limited since most of the therapeutic compounds are metabolized before reaching the target area after peripheral administration (1). Neural gene therapy has the advantage of bypassing these issues and offers physiologically regulated release of the therapeutic substance in response to neural activity, or other endogenous stimuli. In addition, by choosing specific promoters and targeting injection sites for gene delivery vectors the therapy can be applied extremely localized, confined to specific cell types, or synapses, within

defined areas of the brain. This offers another advantage over conventional chemical drugs that cannot be delivered exclusively to the localized compartments within the brain and commonly cause unwanted side effects by influencing healthy brain circuits, or even peripheral structures.

Usually, gene therapy for CNS involves stereotactic injection of a vector, which introduces a gene, coding for a therapeutic substance in the cells. The therapeutic substance can subsequently be released by the transfected cells in response to neural activity (2). Such direct delivery of therapeutic gene into the host tissue, where host cells will be transfected is called *in vivo* gene therapy, as opposed to *ex vivo* gene therapy, or indirect introduction of the therapeutic gene in cells cultured and transfected *in vitro* in the form of cell transplant (3), or encapsulated implant (4). Viruses have the inherent ability to deliver genetic material to cells, which makes them excellent vectors. Lentivirus (LV), herpes simplex virus (HSV), adenovirus (AV), and adeno-associated virus (AAV) are some of the commonly used vectors for gene delivery to the CNS (5). The CNS has proven quite permissive to viral vector gene transfer, enabling successful expression for many of the conventional delivery vectors. While each confers unique strengths and weaknesses, many of these viral vectors support long-term non-toxic delivery of foreign genetic information to host cells.

There is a growing number of clinical successes (6, 7) and promising safety, tolerability and longevity findings in human (8) and non-human (9) primate trials, solidifying gene therapy as a realistic alternative to small molecule treatments for the disorders of CNS, although in majority of cases patient improvement levels have been limited so far (5). A major limitation in all of these trials was the available vector technology and the ongoing challenge of how to widely, efficiently, and safely transduce cells within the CNS. Vector technology, both for *in vivo* and *ex vivo* gene transfer, has advanced rapidly in the last few years compared to what has been used in the clinic so far. New approaches have been developed to significantly improve the scope and efficiency of gene transfer and these improvements are poised to greatly increase treatment efficacy (5). It must be mentioned that, following all the advancements in gene delivery technology, the first gene therapy, Glybera, has been approved by European Medicines Agency in late 2012 and is already a commercially available treatment for a rare inherited disorder, lipoprotein lipase deficiency, causing severe pancreatitis (10).

Obviously, gene therapy is not an acute therapy, and the preparatory work identifying and designing useful constructs is a long process, as will be the clinical implementation and application in individual patients (11). Once gene therapy is applied, it is aimed to function more or less autonomously, regulated by the host neural circuitry, and it can be difficult to intervene in the therapy at later stages. Recent advancements in this direction, namely, opto- and chemogenetics, offer a new gene therapy approach, by allowing external temporal control over neuronal excitability, upon transduction of targeted cells. Optogenetics allows for temporal control in millisecond resolution, but is technically more challenging and requires delivery of a light source inside the targeted brain region (12), while chemogenetics is based on delivering the effector molecule orally, or systemically (13), but lacks the temporal precision of optogenetics. The perspectives of the chemogenetic approach will be discussed in more details bellow, and is the major focus of the *paper I*.

Chemogenetics

Chemogenetics was first used as a term to describe the observed effect of mutations on enzyme chalcone isomerase activity on substrate specificities in the flowers of *Dianthus caryophyllus* (14). It is now used to describe the process by which macromolecules can be engineered to interact with previously unrecognized small molecule. Such engineered macromolecules include nucleic acid hybrids (15), kinases (16, 17), a variety of metabolic enzymes (18-20), and G protein-coupled receptors (GPCRs). In the field of GPCR research, numerous chemogenetic platforms have been described (21), of them the ones selectively activated by the inactive analog of clozapine, clozapine-N-oxide (CNO) have emerged as a widely adopted technology and were dubbed DREADDs – Designer Receptors Exclusively Activated by Designer Drugs, in the laboratory of B. L. Roth (13).

DREADDs are engineered human Muscarinic (hM) receptors, subjected to random mutagenesis, expressed in genetically engineered yeast (22). First DREADD created by this method was hM3Dq, mutated human M3 muscarinic receptor, relatively insensitive to the native ligand, acetylcholine and activated by CNO with nanomolecular potency. The name hM3Dq also indicates specificity of this DREADD to Gq-mediated signaling pathway (Fig.1). Since then, entire family of DREADD-based muscarinic receptors has

been created, all of which are potently activated by CNO, insensitive to acetylcholine and devoid of constitutive activity (13). To date, most known and commonly used DREADDs are: 1. The already mentioned hM3Dq, leading to burst firing in the neurons, via Gq pathway; 2. hM4Di, that selectively activates Gi signaling and attenuates/silences neuronal firing; 3. GsD, a chimeric muscarinic-adrenergic receptor, that selectively activates Gs pathway, resulting in cAMP-mediated signaling, and 4. Rq(R165L) that specifically activates β-arrestin signaling, the pathway widely implicated in the action of many psychoactive drugs (23).

To date, DREADDs have successfully been used in a number of therapeutically relevant animal models (24), allowing for chemogenetic activation of both canonical (e.g., Gq, Gi, Gs) and noncanonical (e.g., β-arrestin) signalling pathways in essentially any cellular context (Fig. 1). Activation of GsD and arrestin-biased DREADD in neurons modulates neuronal activity context (25-27), although the use of these two is more limited in vivo, due to the need of high concentration of CNO required to achieve a full activation of Rq(R165L) (28), and observed constitutive activity of GsD, although at modest degree, which can lead to basal phenotypes in some cellular contexts (29).

Chemogenetics based on techniques similar to DREADDs holds a good promise for developing into therapeutic tools for neurological diseases. The great advantage of this approach is readily available CNO that has long been administered to humans and is a known metabolite of widely prescribed medication – clozapine (30), it can be administered orally and noninvasively, activating DREADDs without a need of any specialized equipment for relatively prolonged (from minutes to hours) duration. Although, the lack of precise temporal control, as for example, is achieved by light for optogenetics, still remains a main drawback for the chemogenetic approach. It must be mentioned, that there is an ongoing work to solve this problem, as well as expanding the tools for chemogenetic control of various aspects of neuronal function, and integrating different technologies (e.g. chemo- and optogenetics) for addressing the scientific questions of neurophysiology and neuropathology (31).

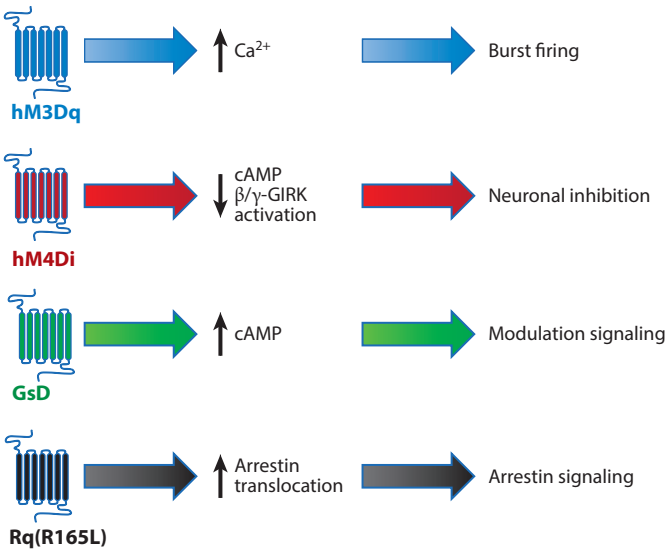

Figure 1. Currently available main DREADD-based tools and their typical uses in neuroscience. Adapted from (31).

Cell therapy for neurological disorders

Cell therapy is defined as therapeutic use of cellular material, generally, live cells. The aim of cell therapy is to replace, repair or enhance the biological function of damaged tissue or organ. This can be achieved by the transplantation of isolated and characterized cells to the target organ, in sufficient number and quality, for them to survive long enough to restore function (32). Since the first successful bone marrow transplantation in 1968 (33), cell therapy has emerged as a promising strategy for the treatment of many human diseases, including neurological disorders. Stem cells of various origin and commitment level have been in the spotlight as a major source for this approach over the past few decades.

Stem cells are self-renewing cells that can give rise to phenotypically and genotypically identical daughters as well as at least one other final cell type.

Zygote is the ultimate totipotent stem cell, giving rise to the placenta and all other cells of all the tissues of the body. During development, the pluripotency of stem cells narrows to more determined stem cells, so that, for example, neural stem cells only give rise to neural tissue. Cells, still able to divide but restricted to develop into only one, or few defined cell types are often referred to as progenitors, although discrimination between stem and progenitor cells is not always clear (34).

Several sources of neural stem cells and neural progenitors have been explored for treating neurological disorders, including ischemic stroke (35-37), Parkinson's disease (38-40), Huntington's disease (41, 42), amyotrophic lateral sclerosis (43, 44), spinal cord injury (45, 46) and epilepsy (47-51), offering a proof of principle that cell replacement is a feasible strategy for neurological diseases. However, being of an embryonic stem (ES) cell origin, the transplanted cells face ethical as well as a number of practical issues. There is no standardized protocol for deriving and expanding pure populations of neural stem cells that will form a neuron-rich graft after transplantation, containing a high fraction of the specific neurons with desired phenotype and function, without continuing to proliferate and form a tumorigenic-, or other undesired cell types. Poor survival due to immune rejection is another major challenge of stem cell therapy.

After discovery that somatic cells could be reprogrammed back to a pluripotent state (52), or even directly to another somatic cell type (53-56), new opportunities have opened for the regenerative medicine, providing more available, easily expandable, patient specific cell sources and addressing a number of issues related to ES cell use, among them ethical and immune rejection problems (Fig.2).

Induced pluripotent stem cells

In 2006, Yamanaka and Takahashi published a groundbreaking work demonstrating that somatic cells, specifically mouse fibroblasts, can be reprogramed back to the pluripotent stage by introduction of only four transcription factors: Oct4, Sox2, Klf4 and cMyc (52). They named the reprogrammed cells induced Pluripotent Stem (iPS) cells and a year later managed to do the same with human adult fibroblasts (57). The iPS cells exhibit morphology and properties of ES cells, express ES marker genes, and in addition, they possess the capacity to generate cells from all three germ layers.

Human iPS cells offer great potential to create patient-specific cells of all lineages disease modeling, drug screening and for regenerative medicine, eliminating the concerns of immune rejection and ethical issues associated with the use of human ES and fetus-derived cells.

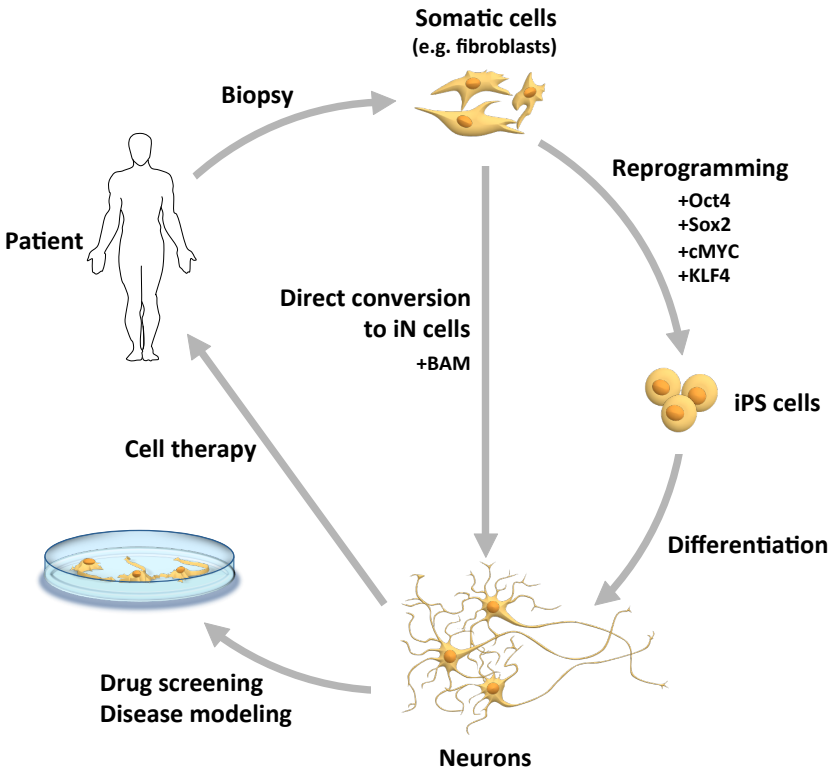

Figure 2. Potential use of reprogrammed iPS and iN cells for neurological disorders. *In vitro* cultured cells can be used both for cell replacement therapy, as well as for disease modeling and drug screening.

Shortly after the discovery, patient-derived iPS cells were created from number of individuals with different neurological disorders, amyotrophic lateral sclerosis (58), Parkinson's and Huntington's diseases (59, 60) and spinal muscular atrophy (61) among them. The subsequent differentiation into neurons *in vitro* showed that these cells recapitulate disease-specific features and mimic the

pathology in a dish that makes iPS cells strong research tools for studying the genetic and epigenetic mechanisms of the pathologies. iPS cells appear useful to describe characteristics of even more complex genetic and late-onset neuronal disorders, such as schizophrenia (62).

In addition to disease modeling iPS cells hold a good promise for therapeutic use for number of diseases and neurological disorders among them. iPS cells have been successfully differentiated into specific neuron types such as dopaminergic (63, 64) and motor neurons (58). There are number of animal transplantation studies showing promising results with good cell survival, neuronal differentiation and certain level of functional integration (64-67). Before transplantation, neural progenitor stem cell lines are usually generated from iPS cells, making them more specialized and determined toward the final desired fate. One example is long-term expandable neuroepithelial-like stem (lt-NES) cells, explored in *paper II*. These cells exhibit extensive self-renewal, clonogenicity, stable neurogenesis and high neurogenic potential that makes them suitable for cell replacement approaches (68). These cells predominantly differentiate into γ-aminobutyric acid(GABA)-ergic neurons, if not directed to any other specific cell type. This may become an advantage for cell replacement therapy for epilepsy, where one of the hallmarks of pathology is degeneration of a specific population of GABA-ergic interneurons. lt-NES cells also show high lineage plasticity, and while fated to the cortical phenotype *in vitro*, they have been shown to efficiently convert and mature into functional cortical neurons following transplantation into the stroke damaged rodent brain (69).

Despite all the advantages of iPS cells, there are several concerns and limitations when considering them for cell replacement therapies. The generation of iPS cells is a rather long process (70), which will be accompanied by additional differentiation *in vitro* and/or *in vivo* to gain the desired cell phenotype, making this technology a rather time consuming approach. In addition, owing to their pluripotency, iPS cells carry certain risk of tumour formation (64, 71). This observation is consistent with the insight that neural cells derived from ES cells also retain tumorigenic potential even after transplantation (72).

Induced neurons

Another way of acquiring patient specific cells from the somatic cell source for regenerative therapy is a direct conversion. In contrast to iPS cells, direct

conversion of one cell type to another bypasses the pluripotent state, which decreases the probability of uncontrolled growth and tumour formation, as well as shortens the differentiation times in culture. In 2010, the laboratory of M. Wernig demonstrated that by using only three neuronal lineage-inducing transcription factors – Brn2, AsclI and Myt1b (BAM) mouse fibroblasts were efficiently converted into neuronal cells (56). The resulting induced neurons (iN) showed neuronal marker expression and morphology, as well as functional neuronal properties – ability to fire action potentials (APs) and integrate into existing network *in vitro*. Interestingly, the majority of iNs resembled cortical neurons and exhibited excitatory traits. Since then, functional neurons with mature electrophysiological properties have been obtained from human somatic cells, ranging from embryonic and postnatal to adult fibroblast cell populations (73). A number of groups have demonstrated that with the BAM approach and by using varied combination of factors it is possible to direct human iN cells to the specific neuronal subtype, namely dopaminergic (74, 75), cholinergic (76), or spinal motor neurons (77). Recently, animal transplantation studies showed that human iN cells survive and express neuronal markers in the striatum up to six weeks (78), although long-term studies are needed to follow up the fate of the grafts. *Paper III* addresses this issue, showing long-term survival and fate of iN cells, transplanted in rodent hippocampus, up to six months *in vivo*.

Collectively, these and a growing number of ongoing studies further advance this fast-moving field, and demonstrate that direct conversion can be used to generate diverse human iNs for disease modeling, and potentially for future cell replacement therapies. However, being still in its infancy, there are many open questions and issues to be overcome. One of the problems is a low conversion rate. Further identification of optimal combinations of additional factors is needed to improve the efficiency of specific neuronal conversions. Furthermore, while direct conversion strategy does not involve pluripotent state and offers clinically safer alternative, it may limit the final number of cells that can be obtained and the number of transplanted cells surviving to be sufficient for the therapeutic effect. Little is known about whether iN cells acquire a long-term sustainable genetic program during the conversion that will function both *in vitro* and post-transplantation *in vivo*. There are, however, few studies showing that direct conversion is not dependent on continuous transgene expression and expression of exogenous reprogramming factors is later followed by activation of their endogenous counterparts (74, 79). Another major limitation of human iN approach is the generation of neurons that only partially gain functional

properties, or exhibit a very slow pace of maturation. These cells often lack appropriate membrane excitability, sustained long action potential firing and synaptic drive. However, recent technical advancements promise to minimize these issues, accelerating neuronal maturation and functional development (80).

Inspired by initial finding that somatic cell nucleus can be reprogrammed, there are few other new technologies being developed in parallel to iPS and direct *in vitro* conversion (e.g. iN). Direct *in vivo* conversion is one of them. In the past few years, neurons have been induced *in vivo* from residing astrocytes and reactive glia in mouse brain by overexpressing singular key transcription factors (81-85), providing an alternative approach for the repair of injured or diseased brain.

Epilepsy

One of the diseases where novel treatment strategies, like gene- and cell therapy can prove useful and is highly needed is epilepsy. Epilepsy is a family of devastating multifactorial neurological disorders, unified under one name simply by occurrence of more than one seizure. According to International League Against Epilepsy (ILAE) and International Bureau of Epilepsy (IBE),

> ''An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain,'' and
>
> ''Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by neurobiological, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure'' (86).

Epilepsy is one of the most common neurological disorders with the prevalence of 1% of the general population, predominantly diagnosed in children and elderly, although it can develop and effect individuals of any age (87). Epilepsy, as a group of various diseases, can have several different oetiologies, it can develop from head trauma, brain tumours, stroke, infections and degenerative conditions, like Alzheimer's disease, as well as due to inherited genetic defects. In some cases it might not have any obvious cause and is referred to as idiopathic epilepsy (87). Regardless of the initial cause, epilepsy is characterized by appearance of epileptic seizures that can have a diverse symptomatic

manifestation, from momentary loss of awareness, or short periods of unconsciousness, to highly generalized convulsions. If the seizures last longer than 30 minutes without full recovery of consciousness, a life-threatening condition, called status epilepticus (SE) may arise. The classification of seizures is a continuously evolving topic, but two main categories are generally recognized, partial and generalized (88). Partial seizures involve one confined brain area, at least initially, while in generalized seizures entire brain is involved.

The most common form of partial epilepsy, and epilepsies in humans in general, is temporal lobe epilepsy (TLE), characterized by appearance of partial complex seizures, with focus located in the temporal lobe structures, such as amygdala, temporal neocortex and hippocampal formation. As TLE progresses, seizures can eventually generalize and involve the entire brain (89).

Currently, the main choice for epilepsy treatment is anti-epileptic drugs (AEDs). In general, AEDs are designed to counteract hyperexcitability, developed in epileptic environment and to restore a normal balance between excitation and inhibition of the network by increasing the net inhibitory drive. Main mechanisms of their action includes limiting GABA uptake, increasing GABA-ergic inhibitory, or decreasing glutamatergic excitatory transmission, interfering with the function of sodium channels, responsible for AP generation, or blocking calcium channels and with this, reducing general neurotransmission. The main problems with using the AEDs is that they are symptomatic, rather than disease modifying, and they inevitably give rise to several side effects due to systemic administration, while being unable to address neuropsychiatric comorbidities, significantly affecting the quality of life of the patients (90). Recent advancements in drug development has provided new generation of AEDs with simpler pharmacokinetics, and thus, fewer interactions and better tolerance, but with no advancement in the direction of efficacy, leaving a significant amount of patients still with intractable epilepsy. Strikingly, the portion of such drug refractory cases is 30-40% of all epileptic patients, with TLE patients constituting the majority of these cases (91). The therapeutic alternative for the patients with drug refractory epilepsy is resection of epileptic focus, however, this high-risk surgery is limited to the subjects with clearly identifiable and easily removable focus. Even then, complete recovery is not guaranteed and psychiatric comorbidities are expected to occur in most of the cases (92). Therefore, development of more effective treatment strategies and uncovering the mechanisms of epilepsy and pharmacoresistance is highly needed.

Ideally, novel treatment should be more targeted, addressing only the specific regions involved in seizure generation, or propagation, and only the specific cell types responsible for seizure control, leaving others unaltered in order to minimize side effects. In this respect, gene therapy is an attractive approach and several genes have proven interesting in experimental studies, including neurotropic factors, adenosine, cholecystokinin, neuropeptide Y, galanine, GAD and $GABA_A$ receptor encoding genes (2, 93). A major consideration in gene therapeutic approach in general is the irreversibility of the transgene expression and activation. Recent advancements in gene technology might partially address this problem, by giving an external control over the activity of the expressed gene. One such approach could be chemogenetics. Recently, it has been shown, that by using DREADDs, systemic CNO administration can significantly attenuate focal neocortical seizures in the animals expressing modified hM4Di muscarinic receptor (94). The same approach proved effective in suppressing electrically induced epileptiform activity in *in vitro* model of TLE, organotypic hippocampal slice cultures (*paper I*).

Another potential treatment alternative for epilepsy is cell therapy. One of the hallmarks of this disease is degeneration of specific populations of GABA-ergic interneurons, causing increase of general network excitability and, thereby seizure susceptibility. Therefore, replacement of the lost or damaged interneurons with the new GABA releasing neurons might help restore the normal excitability levels and perhaps even modify or reverse the disease by substantially decreasing seizure susceptibility. Intrahippocampal transplantation of rodent neural stem cells that differentiate into GABA-ergic inhibitory neurons in the host brain has been shown to be able to counteract spontaneous seizures in animal model of TLE (51). Additionally, dramatic reduction of seizure occurrence was accompanied by recovery of behavioral deficits, spatial learning, hyperactivity and aggressive response to handling (50). However, poor survival due to immunogenicity still remains a major challenge of stem cell transplantation. Patient specific cell sources, such as iPS and iN might be a better choice in this respect. Initial steps have been made in this direction, and *paper II* and *III* show the proof of principle that both iPS-derived neuron precursors and iNs, respectively, can survive and form fully functional mature neurons in rodent hippocampus up two 6 month post transplantation.

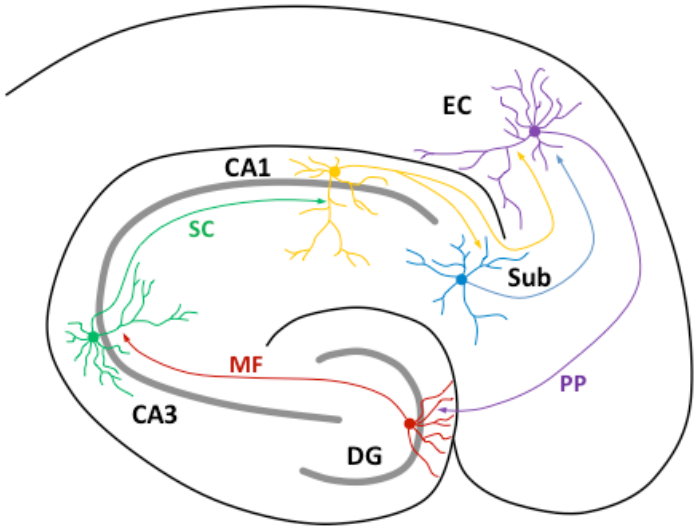

Figure 3. Schematic overview of organization of hippocampal formation. Main excitatory pathways connecting principal cell subfields are shown. DG – Dentate Gyrus; CA – Cornus Ammonis; Sub – Subiculum; EC – Entorhinal Cortex; MF – Mossy Fibers; SC – Schaffer Collaterals; PP – Perforant Pathway.

Hippocampus in epilepsy

One of the most suitable targets for the novel therapy approaches is TLE, as a predominant type of epilepsies in the adults with high percent of drug refractoriness and with the seizure focus mostly confined to one specific brain region, e.g. hippocampus. Approximately two third of the patients with TLE have hippocampal sclerosis as pathological substrate, consisting of cell loss in certain regions of the hippocampal formation (89). Being a key structure in TLE, it is necessary to understand the basic architecture of the hippocampus when exploring the disease both experimentally and clinically.

Hippocampus is an important part of the limbic system, involved in learning and memory. It is located in the medial temporal lobe in both hemispheres, and

comprises the hippocampus proper, or Cornus Ammonis (CA), Dentate Gyrus (DG) and subiculum (Fig. 3). Cornus Ammonis is farther divided in three subfields, CA1, CA2 and CA3, while DG also includes the hilar region. The principal cells in CA and subiculum are pyramidal cells, while in the DG there are granule cells (95). Excitatory input from various brain regions enter hippocampus predominantly from entorhinal cortex via perforant path fibers, terminating on the granule cells of the DG. DG cells project to CA3 pyramidal cells via mossy fibers, CA3 pyramidal cells then in turn send out Schaffer collateral to CA1 pyramidal neurons, which connect to pyramidal neurons of subiculum via the alveus. Finally, projections from the subiculum exit the hippocampal formation and reach the deep layers of entorhinal cortex, completing the circuit, referred to as the feed-forward tri-synaptic circuit (96). Moreover, the hippocampus also receives different extrinsic inputs from cholinergic, noradrenergic, serotonergic and dopaminergic projections (95). The inputs to hippocampus are mediated by various kinds of GABA-ergic interneurons and are believed to be essential for controlling proper function of this structure, responsible for spatial coding, learning and formation, storage and processing of memory (97).

Hippocampus is a plastic structure and undergoes number of changes following the epileptic seizure (98). One common structural change in TLE patients is hippocampal sclerosis, with extensive cell loss and activation of immune response. Due to the complex aetiology it is difficult to isolate a single cause underlying the cell loss, but it is thought that the original insult triggers a cascade of events that result in network reorganization and development of hyperexcitability, reducing the threshold for seizure generation, ultimately making the network more prone to develop abnormal activity. Prolonged seizure activity in turn, can cause excitotoxicity, where excessive levels of glutamate can damage and kill the hippocampal neurons (99). On the other hand, cell death has also been proposed to cause epileptic seizures (100). Another structural change characteristic to epileptic hippocampus is synaptic reorganization, particularly mossy fiber sprouting. Granule cell axons grow out into the inner third of the molecular layer, forming recurrent connections to other granule cells, contributing to an increased excitability in DG. This short-circuiting of tri-synaptic loop might initiate a self-amplifying cascade of increased excitatory drive, promoting abnormal activity and seizure occurrence (101). In parallel to the sprouting of excitatory connections, various subtypes of GABA-ergic

interneurons have also been reported to undergo cell death and synaptic reorganization in TLE hippocampus (102).

Interestingly, the subgranular zone of DG is one of the two unique places where adult neurogenesis occurs (103), together with olfactory bulb in rodents and other mammals (104) and lateral ventricle wall in humans (105). Largely because of the methodological challenges, the fate of neuroblasts, born in lateral ventricle is still debatable (106) and only recently it has become possible to acquire quantitative data on the extent of the hippocampal neurogenesis in humans (107). It is now estimated that approximately 700 new neurons are added in each hippocampus per day. This means that compared to rodents larger fraction of the DG neurons is exchanged in humans and with aging decline in neurogenesis in this region is also less dramatic. This level of neurogenesis is believed to be sufficient to contribute to brain function and judging by animal studies, it might be important in cognition and new memory formation, as well as underlie neurological or psychiatric diseases, if altered (108). Moreover, the potential of resident neural stem cells can be harnessed to promote a generation of new neurons for cell replacement, or create a hospitable environment for the transplanted cells.

As hippocampus plays a central part in the pathogenesis of TLE, it is a suitable structure for targeting the TLE treatment. The increase of local inhibition by targeted gene therapy or GABA-ergic cell transplantation could suppress the epileptic seizures and counteract pathological structural changes.

Organotypic hippocampal slice cultures

A prerequisite for successful development of any kind of new therapy is establishment of a good experimental model system that will closely resemble the pathological situation of the epileptic patients. In spite of the fact that there are a number of animal models for different kinds of epilepsy and seizures, experimental models for refractory epilepsy are few. One model system, closely resembling the features of clinical resection specimens of TLE patients and is experimentally established as a relevant *in vitro* model for TLE, is organotypic hippocampal slice cultures (OHSCs) (101, 109-111).

Organotypic slice cultures are 200-400 μm thick slices of young experimental animal brain, cultured for days to months (112, 113). Under the right conditions the slices sustain the organotypic architecture on microstructural,

cellular and synaptic level, allowing experimental interrogation, therapeutic intervention, electrophysiological or immunohistological evaluation etc. Obviously, preparation of these cultures involves certain degree of denervation, as projections are cut, which causes the divergence from the physiological conditions *in vivo*, but the same factor creates conditions closely resembling the pathological situation in the epileptic brain. Typically, isolated from the brain of 3-9 days old rodents, there is considerable cell death, mossy fiber sprouting, synaptic reorganization and formation of aberrant connections in the hippocampus during the first week of culturing, rendering it hyperexcitable (109, 110). Following initial reorganization, slices develop in a way resembling the development of age-matched animals *in vivo* (113).

Though implying several compromises compared to *in vivo* conditions, OHSCs offer several advantages as well. Slices derived from the same animal brain, or even from the same hippocampus, can be exposed simultaneously to several stimuli or challenges, avoiding the inter-animal variability (114, 115). In addition, organotypic cultures allow much higher throughput for fast drug screening, or more efficient utilization of animals, e.g. for electrophysiology. Compared to acute hippocampal slices from epileptic animals, OHSCs are not subjected to immediate slicing insult before electrophysiological measurements. Additionally, owing to hyperexcitability, spontaneous seizure-like bursts are often observed in OHSCs and epileptiform activity can easily be induced in various ways e.g. simply by electrical stimulation (*paper I*).

OHSCs can prove useful for initial screening of novel AEDs and treatment strategies, followed by validation in human epileptic post-resection tissue, where seizure-like events are also possible to induce (116, 117). Recent studies suggest that OHSCs are prone to pharmacoresistance, depending on the way of epileptiform activity provocation (115, 118). All of the above-mentioned offers OHSCs as useful model system for intractable epilepsy, which is extremely laborious to investigate *in vivo*.

Aims of the Thesis

The overall aim of the thesis was to study therapeutic potential of novel advanced gene and cell therapy approaches, such as chemogenetics and iPS- and iN- based cell therapies for neurological disorders, targeting epilepsy.

Specific aims where:

1. To establish OHSCs as an *in vitro* model of TLE for fast and efficient screening of AEDs and novel therapeutic approaches, such as chemogenetics.
2. To study survival, functional differentiation, maturation and synaptic integration of iPS-derived neuron precursor, lt-NES cells in the host hippocampus.
3. To study long-term survival and functional properties of human iN cells in rodent hippocampus, and the potential of these cells to become better alternative to human iPS and ES cells.

Methods

Animals

The following animal strains were used for the experiments: Balb/c, ChR2-YFP (Thy1-ChR2/EYFP, Jackson laboratories) and GAD65-EGFP (Glutamate Acid Decarboxylase 65-Enhanced Green Fluorescent protein (119)) mice were used for organotypic cultures (*papers I* and *II*) and immune deficient nude rats (NIH Nude rat, Charles River) were used for *in vivo* cell grafting (*papers II* and *III*).

All animals were housed under a 12/12-h light cycle with *ad libitum* access to water and food. Mice pups, used for organotypic cultures, were housed in standard cages with the mother, while nude rats were housed in individually ventilated cages. The Lund Ethical Committee for Experimental Animals approved all experimental procedures.

Organotypic Hippocampal Slice Cultures

OHSC were prepared as 250 µm thick sections of postnatal day 6–8 mice hippocampus as previously described (114). After decapitation, brains were removed and the two hemispheres were separated. Hippocampi were removed from each hemisphere and embedded in agar to offer mechanical support while slicing the sections in +3°C modified artificial cerebrospinal fluid (maCSF), containing in mM: sucrose 75, NaCl 67, $NaHCO_3$ 26, glucose 25, KCl 2.5, NaH_2PO_4 1.25, $CaCl_2$ 0.5, $MgCl_2$ 7 (all from Sigma-Aldrich), equilibrated with carbogen (95% O_2/5% CO_2), with pH 7.4 and osmolarity 300-305 mOsm. The hippocampi were cut on a vibratome (VT1200S, Leica Microsystems) and sections were kept for 15 min in ice-cold washing medium containing Hanc's Balanced Salt Solution (HBSS) with HEPES 20 mM, glucose 17.5 mM, NaOH 0.88 mM and penicillin/streptomycin (all from Gibco) and then moved

individually to membrane inserts (Millipore, PICM01250). The membrane inserts were placed in 240 µl culturing medium in 24-well dishes and preincubated at least for an hour for the medium to equilibrate. The culturing medium contained 50% MEM, 25% horse serum, 18% HBSS and 2% B27 supplemented with 0.5% penicillin/streptomycin solution (Life Technologies), glutamine 2 mM, glucose 11.8 mM, sucrose 20 mM. Slices were cultured as interface cultures at 37°C, 5% CO_2 and ambient O_2 in 90% humidity. Medium was changed on day one of culturing and three times per week thereafter.

In the cultures for STIB experiments (*paper I*), after one week, the medium was gradually changed to serum free medium, containing DMEM/F12 supplemented with 1% N2 supplement, 2% B27, 1% penicillin /streptomycin solution and 2mM glutamine. In the cultures with lt-NES cell grafts (*paper II*), concentration of B27 in the medium was decreased to 0.1% after one week.

Viral Transfections

Following LV or AAV constructs were used to deliver the gene of interest to the hippocampal tissue:

Paper I – AAV8-hSyn-hM4D(Gi)-mCherry (University of North Carolina, UNC, Vector Core) was expressed in OHSCs by direct application of the virus on top of the culture on the day of slice preparation (Titer: 8.3x10^12 vg/ml, 3µl/slice).

Paper II – LV-Syn-hChR2(H134R)-EYFP (Addgene plasmid #20945). Viral vector, produced by Lund University Vector Core facility following conventional protocols, was applied directly on the top of the hippocampal slice cultures on the day of culture preparation (Titer: 3.1x10^7 particle/ml, 3µl/slice) and 24h before cell grafting.

Paper II and *III* – AAV5-hSyn-hChR2(H134R)-EYFP (Addgene, plasmid #26973) was injected bilaterally in isoflurane anaesthetized rats to express ChR2 in Nude rat hippocampus. The total amount of virus suspension injected in each hippocampi was 4.5 µl with the titer of 2x10^12 particle/ml. With the rats' head fixed in a stereotaxic frame, viral vector was injected through a glass capillary at 0.1 µl/min rate in the following coordinates: anterior-posterior (AP) -6.2 mm, medial-lateral (ML) ±5.2 mm, dorsal-ventral (DV) -6.0, -4.8 and -3.6

mm, 1.5 µl at each location in DV plane. The reference points were bregma for the AP, midline for the ML and dura for the DV axis. The glass capillary was left in each DV point for 5 min after injection to prevent the backflow of the viral particles through the injection track.

Cell Cultures

lt-NES

Human iPS cell-derived lt-NES cells were produced as previously described (68). Long-term expandable cell cultures were maintained as monolayer rosettes plated on Poly-L-Ornithine + Laminin (Sigma-Aldrich) coated T25 flasks in proliferation medium, based on DMEM-F12 supplemented with N2 supplement (1:100), L-glutamine (1:100), glucose (1.6 g/l) and penicillin/streptomycin (1:100) (all from Invitrogen). The growth factors FGF (10 ng/ml), EGF (10 ng/ml) (both from R&D Systems) and B27 (1:1000) (Invitrogen) were added to the medium every day. Cells were passaged at a ratio of 1:2 to 1:3 every second to third day, using trypsin (Sigma-Aldrich).

Human iN

Foetal human lung fibroblast cells (HFL1; ATCC-CCL-153, ATCC®, USA), obtained from the American Type Culture Collection, were cultured in DMEM medium (Life Technologies), supplemented with 10% of FBS (Sigma), 2mM L-glutamine and 1% of penicillin/streptomycin (both from Invitrogen) and transduced with doxycycline-regulated LV vectors, expressing mouse cDNAs for BAM and co-transduced with the TET-ON transactivator (FuWmrtTA-SM2, Addgene) during conversion, as previously described (56). The LVs were used at a multiplicity of infection (MOI) of 5 (BAM) and 10 (mrtTA-FUW). Doxycycline (2 µg/mL) was added to the culture medium 5 days after transduction and given 1mg/ml in drinking water to the cell-transplanted rats one week before and 12 weeks post grafting. Two days after transgene activation, MEF medium was replaced by neural differentiation medium (N2B27; Stem Cells), supplemented with small molecules (SMs) with the

following concentrations: 2 µM CHIR99021 (Axon), 10µM SB431542, 100 ng/ml Noggin (R&D Systems) and 0,5 µM LDN-193189 (Axon) and growth factors at the following concentrations: 2 ng/mL LM4A22 (R&D Systems), 2 ng/mL GDNF, 10 ng/mL NT3 (R&D Systems) and 0.5 mM db-cAMP (Sigma). Every 2nd–3rd day, three quarters of the medium in wells was changed till the day of transplantation.

Cell Grafting

In vitro

For grafting on organotypic tissue cultures, human iPS cell-derived It-NES cells transduced with lentivirus carrying enhanced green fluorescent protein (GFP), or retrovirus carrying red fluorescent protein (RFP) (both produced by Lund University Vector Core facility following conventional protocols), were trypsinized and spun down at 300 g. The cells were resuspended in DMEM/F12 medium to reach a concentration of 5 million cells/ml and applied on the hippocampal slices with a pipette in the amount of 10 000 cells/slice. Grafting of the cells was done a few hours after the slice culture preparation, except for when ChR2 was expressed in the hippocampal slices, then the cells where grafted 24 h after virus application.

In vivo

For intrahippocampal and intrastriatal transplantation, both lt-NES and human iN cells were resuspended (in cytocon buffer for lt-NES and in culturing medium without SMs for human iN) to reach a concentration of 100 000 cells/µl and injected stereotaxically. In the right striatum the coordinates were: AP -0.5 mm, ML 3.0 mm and DV -5.0 mm 1 µl and in both hippocampi in the same coordinates as the virus, 3 µl in total per hippocampus (1 µl at each DV coordinate). This was performed one week after AAV virus injection in hippocampi, to ensure extracellular virus clearance (120).

Drugs

Drugs were bath perfused as an aCSF solution to the recording chamber, both for OHSCs and acute hippocampal slices, prepared from cell-injected rat hippocampi, with the following concentrations: 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) 5 µM (Abcam Biochemicals); Valproic Acid (VPA) Sodium Salt 1,5 mM (Sigma); Carbamazepine (CBZ) 100 µM (Sigma); (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogen maleate (MK 801) 40 µM (Tocris); Clozapine-N-oxide (CNO) 5µM (Tocris), (2R)-amino-5-phosphonovaleric acid (D-AP5) 50 µM (Abcam Biochemicals), picrotoxin (PTX) 100 mM (Tocris), tetrodotoxin (TTX) 1 µM (Tocris).

Electrophysiology

For electrophysiological recordings, hippocampal organotypic cultures were excised on their culturing membrane and transferred to the recording chamber (*Papers I* and *II*), while acute rat hippocampal and human neocortical slices were freshly prepared on the day of recordings (*papers II* and *III*). All recordings were done using a HEKA EPC10 amplifier (HEKA Elektronik, Germany) and sampled at 10 kHz.

Acute hippocampal slice preparation

For acute hippocampal slice preparation nude rats were anaesthetized and decapitated, the brains where removed from the skulls and rapidly immersed in ice-cold maCSF. The cerebellum was removed and the two hemispheres were divided with a razor blade, positioned on the medial side and a ”magic cut” (121) was performed on the dorsal cortex. The hemispheres where then glued onto a cutting pedestal with ”magic cut” side down and transferred to the cutting chamber containing maCSF maintained at 3°C and constantly oxygenated with carbogen (95% O_2/5% CO_2). Transverse slices of 300 µm thickness comprising hippocampus as well as entorhinal cortex were immediately transferred to an incubation chamber containing artificial

cerebrospinal fluid (aCSF) containing (in mM): NaCl 119, KCl 2.5, $MgSO_4$ 1.3, $NaHCO_3$ 26.2, NaH_2PO_4 1, glucose 11, $CaCl_2$ 2.5 (300 mOsm, pH7.4), oxygenated with carbogen and maintained at 34°C for 20 mins. After cutting, slices were allowed to rest for one hour at room temperature before whole-cell patch-clamp recordings were performed. Individual slices were then placed in the recording chamber, continuously perfused at a rate of 3 ml/min with carbogen-equilibrated aCSF.

Acute human neocortical slices

Human neocortical resections were obtained from Department of Neurosurgery at Lund University Hospital. Informed consent was obtained from the patients, in accordance to the Declaration of Helsinki, and local Ethical Guidelines. Tissue was immediately placed in carbogenated sucrose solution, containing in mM: sucrose 200, $NaHCO_3$ 21, glucose 10, KCl 3, NaH_2PO_4 1.25, $CaCl_2$ 1.6, $MgCl_2$ 2, $MgSO_4$ 2. The tissue was as coronal slices of 500µm thickness, cut in the same solution. Slices were allowed to rest for 3 h in carbogenated human artificial cerebrospinal fluid (haCSF) at 34°C, containing in mM: NaCl 129, $NaHCO_3$ 21, glucose 10, KCl 3, NaH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 1.6, (300 mOsm and 7.4 pH), and then transferred to the recording chamber perfused with carbogenated haCSF at a flow rate of 3 ml/min for whole-cell patch-clamp recordings.

Whole-cell patch-clamp recording

One of the best ways to characterize functional properties of neurons is to measure its electrophysiological parameters using whole-cell patch-clamp recording. Although it might seem complicated at a glance to those unfamiliar with the field, all aspects of the technique can be explained by Ohm's law: V (Voltage) = I (Current) x R (Resistance). For example, if we wish to measure the resistance of a cell, we can induce voltage change of e.g. 5 mV, measure the current that travels across the membrane in response and put the variables in the equation above.

The classic whole-cell recording is based on formation of a tightly isolated seal between the cell membrane and the recording pipette, reaching electrical resistance in the giga-ohm range. Subsequently, controlled amount of suction is

applied and the membrane inside the pipette tip ruptures, while leaving the seal intact, making the cell a functionally active part of the recording pipette. The electrode in the pipette is now also in contact with the interior of the cell, thus conducting electrical activity to an amplifier that converts the signals into recordable data. Because the cytosol gradually equilibrates to the pipette solution during recordings, it is important to carefully choose the composition of this solution, and adjust it to the osmolarity and ionic composition of the recorded cell cytosol, to prevent washout and artificial change of the electrophysiological properties of the cell.

Two main configurations can be used to record different electrical signals from the neuron: voltage clamp and current clamp. In voltage clamp, the voltage is kept constant (e.g. at -70 mV) while observing the current (i.e. ion flow) passing through the cell membrane; while in current clamp changes in membrane potential is observed on a constant current. In this way it is possible to measure intrinsic membrane properties of the recorded cell, such as resting membrane potential (RMP), AP threshold and current-voltage relationship, as well as study the nature of incoming synaptic inputs. Using different intracellular (pipette) and extracellular (bath) solutions it is possible to discriminate between different types of synaptic transmission and manipulate the environment to study the effect on the determined cell population.

For the whole-cell recordings, first step is identifying the desired cell. For this reason, using GFP- or RFP fluorescent labeling is extremely useful. In our slice preparations, labeled cells were identified using a wide-band excitation filter and visualized for whole-cell patch-clamp recordings using infrared differential interference contrast video microscopy (BX50WI; Olympus). Recordings were performed at 32-34 °C using a glass pipette filled with internal solution containing in mM: K-gluconate 122.5, KCl 17.5, NaCl 8, KOH-HEPES 10, KOH-EGTA 0.2, MgATP 2 and Na_3GTP 0.3 (295 mOsm, pH 7.2; all from Sigma-Aldrich), except for recordings for inhibitory connectivity in OHSCs, where the pipette solution contained (in mM): 135 CsCl, 8 NaCl, 10 HEPES, 0.2-1 EGTA, 2 MgATP, and 0.2 GTP. Average pipette tip resistance was between 3-5 MΩ. Pipette current was corrected online before gigaseal formation while fast capacitive currents were compensated for during cell-attached configuration. Only experiments with series resistance values of less than 20 MΩ were selected for analysis. Biocytin was included in the pipette solution at 0.5-1 mg/ml to retrospectively identify recorded cells.

Intrinsic properties

RMP was recorded in current clamp mode at 0 pA immediately after establishing whole-cell configuration. Series resistance and input resistance (Ri) was calculated from a 5 mV pulse and monitored throughout the experiment. AP threshold was determined by 500 ms square current step injections at RMP. Ramp injection of 1 s current was used to determine action potential threshold in addition to step depolarization. AP amplitude was measured from threshold to peak and duration was measured as the width at the threshold. The duration of the afterhyperpolarization (AHP) was measured as the entire duration of the potential below RMP, immediately following the action potential. Whole-cell currents were measured in voltage-clamp mode at a holding potential of -70 mV and voltage steps were delivered in 10 mV (200 ms) increments. Voltage-gated sodium channels were blocked with 1 µM TTX.

Spontaneous and evoked synaptic currents

Spontaneous excitatory postsynaptic currents (sEPSCs) in OHSCs were measured at -70 mV in the presence of 100 mM PTX, the $GABA_A$-receptor blocker. Evoked EPSCs in OHSCs were elicited with two square-wave pulses (100 µs, 150–300 µA, 20 Hz) delivered by a DS3 Constant Current Isolated Stimulator (Digitimer Ltd) connected to a single silver-silver chloride wire inserted in a glass pipette filled with aCSF (1.5-2 MΩ tip resistance). MiniAnalysis software (Synaptosoft Inc.) was used for detection and analysis of spontaneous postsynaptic currents (sPSCs). Their rise-time was measured as the time between 10 and 90% of the maximum amplitude, while the decay time was first fitted with a single exponential and then measured as above. N-methyl-D-aspartic acid (NMDA) and a-amino-3-hydroxy-5-methyl-4-isoxa-zolepropionic (AMPA) receptors were blocked using 50 µM D-AP5 and 5 µM NBQX, respectively.

STIB

In addition to single cell recording, electrophysiological measurements can also be done in a field of a cells. Field recording measures the orchestrated response of a small population of cells by placing the recording pipette extracellularly

among the cells of interest. In this case, it is common to use the same solution in the recording pipette as for the bath (e.g. aCSF). Field recordings are usually done in the areas with high cell density, and it is common to stimulate fibres terminating at the recorded cell population, to induce a large simultaneous response in the cells. With a stimulation electrode, synaptic response can be evoked and field postsynaptic potentials studied in the cells without altering their intracellular content. Field recordings can also be used to study pathological events, spontaneously occurring, or artificially induced in the tissue. One such example is a electrically induced ictal-like activity, or stimulation train-induced bursting (STIB). STIB represents an in vitro model of seizure-like activity and it can be readily induced in OHSCs.

In our experiments, STIB was induced in 3-4 weeks old OHSCs by a train of 1-msec square pulses of 20-50 μA delivered in the hylar region of dentate gyrus (DG) at 20 Hz for 2 sec by DS3 Constant Current Isolated Stimulator (Digitimer Ltd) connected to a single silver-silver chloride wire inserted in a glass pipette filled with aCSF. Field recordings were obtained through a glass capillary backfilled with aCSF, having a tip resistance of 1 to 2 MΩ, placed in the CA3 area of hippocampal tissue. Threshold of STIB corresponded to the current at which single 1-msec pulse stimulation would elicit a maximum population spike. STIB was defined as 2 or more consecutive field discharges with amplitudes of at least 2 times the amplitude of mean signal noise following immediately after the stimulation, and individual discharges not being temporally separated by more than 2 s. The interstimulation interval for STIB induction was 5 min.

Optogenetics

Optogenetics as a term refers to the combination of optical and genetic methods and is designed to offer a fast and specific control of the activity of a targeted cell population in living tissue. This kind of precision and speed was one of the major challenges to achieve in neuroscience by previously available methods, until 2005, when optogenetic tools could be introduced into neurons and proved effective in controlling neuronal excitability (12). Optogenetics requires introduction of light sensitive membrane proteins in the targeted cells, for example neurons, to make them easily controllable by exposure to light of the

specific wavelength. The most widely used optogenetic tools are the engineered versions of natural opsins, capable of translocating ions across the membrane upon exposure to light. Depending on the type and direction of ion movement, the potential of the neuronal membrane can either be depolarized (activating), or hyperpolarized (silencing).

In *Paper II* and *III*, we exploited this technique to study host-to-graft synaptic connections and expressed depolarizing opsin, Channelrhodopsin (ChR) in the hippocampal tissue before the cell grafting. ChR is a non-selective cation channel that, upon exposure to blue light (activation max. at 470 nm), depolarizes the expressing neuron by allowing a passive transport of sodium, potassium and calcium ions and protons. This results in generation of APs even by brief (1-2 ms) light exposure thanks to a very rapid activation/deactivation kinetics of ChR. In our experiments, by specifically activating ChR2 expressing neurons of the host tissue we could record the synaptic response of the grafted cells, dissecting the afferent nature of the synaptic inputs.

For optogenetic depolarization of ChR2 expressing cells, blue light was applied at 460 nm wavelength with a LED light source (Prizmatix, Modiin Ilite, Israel) and delivered through a water immersion 40x microscope objective. Stimulation of ChR2 expressing cells was done either by continuous application of the blue light for 5-15s or 1ms pulses, paired or as a train of 10, separated by 100 ms intervals. Spontaneous postsynaptic current frequencies in graft-derived neurons were analyzed before, during and after optical activation of the host tissue, to evaluate synaptic connectivity between host and grafted cells.

Immunohistochemistry and Imaging

All slice preparations were fixed in 4% paraformaldehyde solution (for 12 to 24 h) after patch-clamp recordings, then rinsed with phosphate buffer solution and stored in Walter's antifreeze medium at -20^{0}C. For concentration of the primary antibodies used, see the Table 1. Primary antibodies and biocytin, infused into the cells during recordings, were detected with appropriate fluorescent secondary antibodies: fluorophore-comjugated streptavidin, Cy2, Cy3 or Cy5 (all from Jackson immunolabs) at the concentration of 1:400. For DAB staining, brain sections were incubated with biotinylated secondary antibody, followed by streptavidine-horseredish peroxidase (Vector Laboratories) and then exposed to

DAB (0.5mg/ml Sigma) with 0.01% hydrogen peroxide for 5 min. Post-staining images were acquired either by confocal microscopy (Inverted Nikon Eclipse Ti microscope Csi) or by epifluorescence microscopy (Olympus BX61).

Data Analysis

Analysis of electrophysiological data was performed using Fitmaster (HEKA elektronik), Igor Pro (Wavemetrics) and MiniAnalysis (Synaptosoft Inc.). Statistical analysis of the data was done by Student's t-test or ANOVA, followed by Tukey-Cramer post-hoc analysis, and the level of significance was set at $p<0.05$, except for the analysis of spontaneous currents (*paper II*). Here, the Kolmogorov-Smirnov test was used and the significance was set to $p<0.01$. All data are presented as mean ± standard error of the mean (SEM).

Table 1. List of the primary antibodies

Antigen	Host	Dilution	Producer
mCherry	Rabbit	1:500	Abcam
RFP	Mouse	1:1000	Abcam
GFP	Rabbit	1:10 000	Abvam
Human Nuclei	Mouse	1:300	Chemicon
GAD65/67	Rabbit	1:1000	Abcam
hNCAM	Mouse	1:100	Santa Cruz
TE-7	Mouse	1:1000	Millipore
RFP	Rabbit	1:500	Abcam

Results and Comments

Exploring OHSCs as a Model of Intractable Epilepsy – *Paper I*

Diverse effect of AEDs on STIB in OHSCs

Organotypic hippocampal slice cultures are experimentally established as a relevant *in vitro* model of temporal lobe epilepsy. Studies suggest that chemically induced epileptiform activity in rat OHSCs are pharmacoresistant to a majority of AEDs, and thereby offers a model for intractable epilepsy (115); however, high-frequency electrical stimulation-induced ictal-like afterdischarges – STIB are responsive to two standard AEDs: carbamazepine (CBZ) and phenytoin (PHT), indicating that pharmacoresistance in this model may depend on the mode of seizure provocation (118).

We further investigated whether drug refractoriness of OHSCs could also depend on species, and/or specific mechanisms of AED action. We prepared OHSCs from P5-8 days old mouse pups and studied effect of chosen drugs on STIB, induced in 3-4 weeks old cultures. First, we confirmed that stable STIB could be repetitively induced with 5 min intervals in the same slice, without a significant alteration in the burst duration. Then, as a positive control, we made sure that AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) glutamate receptor antagonist, NBXQ, was able to almost completely block STIB in these cultures. This effect could be partially washed out, and STIB returned to 42% of initial duration (Fig.1 of *paper I*).

After establishing the working experimental model, we tested effect of three AEDs with different pharmacological target and mechanism of antiepileptic action: CBZ, an anticonvulsive drug of benzodiazepine family, which mainly acts by stabilizing the inactivated state of voltage-gated sodium channels (122);

valproic acid (VPA), one of the most widely used AEDs for the past five decades, with a broad spectrum of antiepileptic efficacy (123); and MK801, a non-competitive NMDA-receptor antagonist, known to have seizure-suppressant effect in kindled rodents (124). Interestingly, all three drugs showed different effect on STIB duration (Fig. 2 of *paper I*): with CBZ fully blocking it, MK801 decreasing the burst duration by 88% of baseline, and VPA completely failing to affect STIB induction. The effect of CBZ was fully washed out, while in the case of MK801, the STIB duration returned to 24% of baseline. These data suggest that pharmacoresistance in OHSCs not only depends on the mode of epileptiform activity provocation, but also on the pharmacological agent used.

Blocking STIB with DREADs in OHSCs

Next, to substantiate our hypothesis that drug refractoriness in OHSCs may depend on the mechanism of action and the pharmacological target of the drug used, we decided to explore a novel chemical-genetic approach, DREADD (24). It has been shown that modified muscarinic receptor, hM4Di, while specifically activated by normally inactive CNO, can substantially attenuate focal neocortical seizures in rodents (94). We tested whether DREADDs could also be effective in suppressing more acute epileptic-like activity, induced by high frequency electric stimulation – STIB.

First, we explored if viral expression of hM4Di transgene had any effect on intrinsic properties of transduced neurons. We observed no significant change in any of the electrophysiological parameters of the hM4Di-expressing CA3 pyramidal neurons recorded with whole-cell patch-clamp technique (Fig.3 of *paper I*).

As previously described, being a modified human muscarinic receptor 4, binding to the specific effector molecule, CNO should have initiated the Gi-coupled cascade in the cells, resulting in hyperpolarization, and therefore silencing of the targeted neurons (13). Indeed, we observed hyperpolarization in all four recorded hM4Di expressing neurons in current clamp mode; also reflected in decreased frequency and amplitude of post-synaptic currents (Fig. 3 of *paper I*).

Finally, we tested whether we could suppress epileptiform activity induced by electric stimulation in hM4Di-expressing OHSCs. CNO application showed potent attenuation of STIB, with 93% decrease in the burst duration on average. Partial return of STIB was observed after CNO washout.

Taken together, these findings suggest that drug resistance of STIB in OHSCs depends on the mechanism of AED action, and that this model, together with chemically-induced epileptiform activity, as shown previously, could be a useful *in vitro* model for fast and efficient screening of novel antiepileptic and anti-seizure drugs, as well as novel gene therapeutic approaches, e.g. chemogenetics. Using this model, we demonstrated that DREADDs may be effective in regulating the hyperexcitability, and supressing seizure-like events, in the epileptiform tissue.

Characterization of iPS-Derived lt-NES Cells for Potential Cell Replacement Therapy – *Paper II*

Electrophysiological characteristics of lt-NES cell-derived neurons in OHSCs

After establishing mouse OHSCs as in vitro model of TLE, we decided to explore if it could also be employed as a tool for exploring the potential of cell replacement therapies for epilepsy. For this purpose, we grafted human iPS-derived long-term expandable neuroepithelial-like stem (lt-NES) cells on hippocampal slices of the mouse pups, and cultured these as OHSCs for up to 6 weeks.

Human skin fibroblast-derived lt-NES cells represent a neural progenitor cell line, reported to predominantly differentiate into GABA-ergic neurons upon growth factor withdrawal (68). This makes them interesting targets for cell therapy approach in epilepsy patients, as replenishing the lost GABA-ergic interneurons, could counteract seizures. For this to occur, the transplanted cells should develop into mature neurons and synaptically integrate into the host neuronal circuitry.

Whole-cell patch-clamp recordings, performed on the grafted lt-NES cells at one, three and six weeks post-grafting, showed temporal maturation of these cells; and at the latest time-point they could already generate TTX-sensitive inward sodium currents and APs, both spontaneously and upon depolarizing current injection (Fig. 1 of *paper II*). However, the grafted cells had a distinct electrophysiological profile compared to the host cells in the OHSC: much higher input resistance, and slower APs with lower amplitude. They also

appeared more depolarized than host cells, and although both spontaneous and evoked postsynaptic currents could be observed, they were rather sparse (Fig. 2 and 3 and Suppl. table 1 of *paper II*).

To investigate the origin of synaptic afferents to the grafted lt-NES cell-derived neurons, the host neurons were transduced with Channelrhodopsin-2 (ChR2) and optogenetically activated by blue light. Simultaneous whole-cell recordings of synaptic currents in the grafts revealed limited synaptic afferents from host neurons (Fig. 4 of *paper II*). With all these properties, lt-NES cell-derived neurons appear immature at six weeks post-grafting, resembling the features of early neurons, derived from human ES cells *in vitro* (125), suggesting that despite differences in origin and apparent proteomic profile, human ES and iPS cell-derived neurons may share some initial developmental pathways and the differentiation pace.

Electrophysiological characteristics of It-NES cell-derived neurons transplanted in vivo

We hypothesized that the immature properties and lack of synaptic afferents of It-NES cell-derived neurons in OHSCs could be due to insufficient time for differentiation and synaptogenesis. Since it is challenging to keep OHSCs in good conditions beyond six weeks, we transplanted It-NES cells *in vivo*, into the hippocampi of immunodeficient rats, in order to test our hypothesis, and proceeded to characterize graft-derived neurons up to six months after transplantation. Electrophysiological recordings revealed that It-NES cell-derived neurons were significantly more mature, after six months of grafting, compared to what was observed in OHSCs after six weeks. After six months post-transplantation, the grafted cells did not differ with the majority of parameters from either host hippocampal neurons, or from the cortical neurons of the human post-resection tissue. The graft-derived neurons still sustained a number of distinct characteristics, namely higher input resistance and smaller soma size, compared to that of the human cortical neurons (Table 1 and Fig. 5 of *paper II*). However, the *in vivo* transplanted lt-NES cell-derived neurons had more elaborate neuronal morphology than those differentiated only for six weeks, with extensive dendrite branching and various dendritic spines (Suppl. Fig. 5 and 6 of *paper II*).

In line with the more mature intrinsic neuronal properties, *in vivo* grafted It-NES cells also exhibited increased synaptic integration into the neuronal network of the host hippocampus. When using similar optogenetic approach as *in vitro*, we observed significant increase in both frequency and amplitude of the postsynaptic currents in approximately 70% of the grafted cells, in response to blue light activation of ChR2-expressing host hippocampal neurons (Fig. 6 of *paper II*).

Taken together, we showed that It-NES cell-derived neurons do acquire mature neuronal phenotype, although with a relatively long differentiation time of up to six months, or even more. Considering that they mostly differentiate into GABA-ergic phenotype, these cells may prove useful for cell replacement therapy in epilepsy, by normalizing brain network impaired by degeneration of GABA-ergic neurons. The host derived afferent connections provide a possibility of regulated GABA release from the transplanted cells. Comprehensive *in vivo* studies in models of neurodegenerative diseases are necessary to provide further proof for this hypothesis.

Characteristics of Human iN Cells Transplanted *in vivo – Paper III*

Another cell source we explored for cell therapy potential for neurological disorders is induced neurons (iN). These cells, in contrast with iPS, are derived by direct conversion of one cell type, e.g. fibroblasts, to neuronal phenotype, thus bypassing the pluripotent state, and with this decreasing the probability of uncontrolled growth, as well as shortening the differentiation time needed in culture.

To date, functional neurons with mature properties have been derived from direct conversion of both rodent and human fibroblasts *in vitro* (73), and human iNs have been shown to survive and express neuronal markers, when transplanted to rodent striatum up to six weeks *in vivo* (78). It is not known, however, if the converted cells can survive for prolonged periods, or whether they keep their neuronal characteristics after transplantation.

To address this question, iN cells – derived from human foetal fibroblasts via BAM factor combination, using doxycycline-dependent lentiviral vectors – were transplanted into adult rat hippocampi.

We carried out immunohistochemical and electrophysiological characterization of the transplanted cells after one and six months, to observe their development throughout the post transplantation period. The transplanted cells formed grafts with neuron-rich populations, present up to six months in the host brain. They showed morphological properties of neurons with neurites stretching out to all the areas of the host hippocampus from the injection site. After six months, arborisation was more developed, although there was no significant difference in the soma size, or the distance to the first branching, between the two time-points. This indicates that even after removing doxycycline three months post-grafting, converted cells were capable of keeping stable gross morphology for months, without exogenous conversion factors. Staining for fibroblast marker TE7, showed that a portion of transplanted cells remained unconverted throughout the six month experimental period. These unconverted cells formed a high resistant extracellular matrix sheath around the converted cells, making them difficult to access for the patch-clamp recordings.

It has been shown that functional maturation of human iN cells is asynchronous *in vitro*, and relatively long time might be necessary for the complete and efficient conversion (56). To explore this *in vivo*, we performed whole-cell patch clamp recordings on iN cells with neuronal morphology, six months post-grafting. Even though the six recorded cells had passive membrane properties within the borders of mature neuronal phenotype, such as resting membrane potential (RMP=-63.6±6.1mV), we observed that asynchrony still prevailed even after rather long conversion time. With the ability to generate APs, we identified three major profiles of iNs: 1. non-firing, 2. firing broad, immature, non-repetitive AP, and 3. firing fast, repetitive APs. The two cells in the last group, furthermore expressed higher maturation level by displaying delayed-rectifier outward potassium- and inward sodium currents, as well as spontaneous postsynaptic currents, indicating their synaptic integration. Similar to *paper II*, ChR2 was expressed in the host tissue, but we were unable to observe any synaptic responses in the recorded iNs during light illumination.

This study shows that iN cells can become mature functional neurons post transplantation, and survive long term in the host brain. However, improvements in conversion, survival and integration rate of transplanted cells

are needed, before these cells can truly offer a superior alternative to human iPS or ES cells.

General Discussion

This thesis is a step forward in the search for novel therapies for complex neurological disorders that have proven difficult to treat with traditional pharmacological approaches. We particularly targeted epilepsy, employing chemogenetics and iPS- and iN-based cell replacement strategies, in order to investigate the potential of gene- and cell therapies.

In *paper I*, using OHSCs we confirmed and extended the earlier findings, that pharmacoresistance of this hyperexcitable epileptic-like tissue is not absolute (Fig.1-2 of *paper I*) (115, 118). Furthermore, we showed that pharmacoresistance of OHSCs not only depends on the mode of epileptiform activity provocation, but also on the distinct mechanisms of various drug action. The three AEDs tested in this study, have disparate pharmacological targets, and therefore different mechanisms of antiepileptic action, which might account for their effect on STIB observed in OHSCs. These findings present STIB in OHSC as a good model for screening novel AEDs with known pharmacological mechanisms of action.

Another advantage of this *in vitro* model is that OHSCs can also be used for testing novel therapeutic approaches based on genetic modifications. The therapeutic gene can easily be expressed in OHSCs using viral vectors, and targeted to a particular cell population with a specific promoter, which enables us to study the effect of the transgene on the targeted cell population in the hippocampal network. As proof-of-concept, we have shown the therapeutic potential of chemogenetics based on DREADD technology. Expression of hM4Di transgene was specifically targeted to the neurons of the hippocampal culture using synapsin promoter and CNO application showed efficient suppression of STIB in these slices. Earlier, OHSCs have also been used for a similar approach, when testing potential of optogenetic tools, namely inhibitory effect of halorhodopsin (NpHR) on STIB, while NpHR was specifically expressed in principal neurons, using CamKIIα promoter (126). These findings later served as a proof of principle for designing an *in vivo* study (127). All this

demonstrates that OHSCs can be used for screening drugs with delayed onset of action, or for testing novel therapeutic approaches emerging in the field, e.g. gene therapy.

One of the biggest challenges in epilepsy research is the difficulty in predicting the efficacy, tolerability and impact of potential new treatments not only on the disease itself, but also on its comorbidities in humans, using the available animal models. These models are usually quite far from the patient situation, both because of the species difference and also, mostly by being a model of one specific kind of seizure, rather than of epilepsy, or epileptogenesis. There is a growing number of studies showing that OHSC is a very accurate *in vitro* model of temporal lobe epileptic tissue, where all major neuronal types and connections are preserved and at around DIV 20 develop mature properties (128-131); together with this, due to the reorganization of the nerve connections and sprouting, additional connections are formed, similar to the epileptic environment, increasing overall network excitability (110). Spontaneous seizure-like burst are often observed in these cultures, and epileptiform activity can easily be induced in various ways. Taken together, we propose that OHSCs can be useful for initial screening of novel AEDs and treatment strategies, followed by validation in human epileptic post-resection tissue (Fig. 5 of *paper I*), where seizure-like events are also possible to induce (116, 117). Acute effects of antiepileptic and anti-seizure agents can be tested and mechanisms of action studied in these human slices (132). Furthermore, with some advancement in the direction of human brain tissue culturing, effects of the drugs with delayed therapeutic action, or gene therapies, which have proven to be potentially useful in rodent organotypic cultures, can also be tested in human cultured tissue (133). This approach will hopefully increase the probability for successful clinical translation, significantly reducing, or minimising the laborious and costly *in vivo* animal testing.

In *paper II* we went further and investigated if OHSCs can prove useful for characterizing neuronal differentiation and integration of the cells grafted on epileptic tissue. It has been reported that the epileptic brain environment can accelerate neuronal differentiation of newborn granule cells in rodent hippocampus (134). Since OHSCs share many features of epileptic tissue, we speculated that grafted human lt-NES cell-derived neurons might exhibit fast differentiation and synaptogenesis. However, lt-NES cell-derived neurons sustained characteristics of young immature neurons, with their intrinsic

properties and sparse synaptic integration, up to six weeks post-grafting on OHSCs (135).

Several factors might have contributed to the slow synaptogenesis and maturation of lt-NES cells in OHSCs, such as lack of necessary factors *in vitro*, xenografting, or the short timespan of the experiment in relation to the longer time needed for human cells to mature. To address this question, we transplanted the lt-NES cells into the rat hippocampus. *In vivo* electrophysiological data indicated that maturation of the transplanted human cells to fully functional neuronal phenotype takes about five to six months in healthy rat brain. Taking an advantage of optogenetic techniques that allowed selective activation of the host neurons, we also studied postsynaptic responses in the lt-NES grafts. In previous studies, it has been impossible to distinguish between synaptic afferents arising from the host and grafted cells (69, 136). By using an optogenetic approach, we unequivocally demonstrate that transplanted It-NES cell-derived neurons, which originate from human iPS cells, receive functional synaptic connections from host neurons, but host-to-graft synaptogenesis may take up to six months and even longer. This was in line with the timespan needed for the maturation of intrinsic electrophysiological properties. It must be mentioned, that five months after transplantation *in vivo*, afferent synapses were still sparse and increased dramatically at six months. So, there may be a sensitive period somewhere between five and six months, when afferent synapses become well-established and lt-NES cell-derived neurons get integrated into the host circuitry.

While all other features of lt-NES-derived neurons were similar compared to the human cortical neurons (Table 1 and Suppl. fig. 7 of *paper II*) at six months post-transplantation, they still retained higher input resistance and lower AP firing rate. This suggests that at least some of the particular features of lt-NES cell-derived neurons, may require even longer maturation time, compared to rodent neurons (137); or it might be caused by other factors, such as the iPS origin of the grafts. The temporal profile of differentiation, maturation and synaptogenesis of lt-NES cell-derived neurons may be different and speeded up in pathological, epileptic conditions. It has been shown that human iPS-derived medial ganglionic eminence cells, transplanted into TLE mouse hippocampus, become functional GABA-ergic neurons already at three months post-transplantation, with synaptic integration of the grafts into the host neuronal network, and significant behavioral recovery of the epileptic animals (67). It must be mentioned, though, that certain electrophysiological parameters of these

neurons also required up to five months to mature. The human brain may still be different from what is observed in rodents, and will be a challenge to explore in future studies.

Finding an alternative, patient specific source, offering faster differentiation times and higher safety in terms of uncontrolled proliferation, might be another option. To this direction, in *paper III* we characterized human foetal fibroblast-derived iN cells as a potential candidate for cell replacement therapy in neurological diseases.

As a result, we showed for the first time that human iNs can survive long-term in adult rodent hippocampus, and that they keep the converted neuronal morphology up to six months post-transplantation. We could not detect any differences in gross morphology measures, such as soma size, distance to first branching and cell number (Fig.1 of *paper III*) neither at early and later time-points, one and six months, respectively; nor between hippocampus and striatum – suggesting that the initial conversion is largely unaffected by the surrounding environment, as well as by removal of the transgene activator, doxycycline after three months.

Directly converted cells require time to change phenotype, a process, which includes the development of neuronal processes, rearrangement of the cell membrane, and expression of various ion channels. This was revealed when comparing neuronal morphology at one and six months *in vivo*: where more extensive branching of the neuronal tree was observed at the later time-point (Fig.2 of *paper III*). The process of full neuronal conversion is asynchronous within a cell population, and it has been shown to take three to four weeks *in vitro* for human iNs to display mature neuronal properties (138). Similarly, when recording intrinsic membrane properties with whole-cell patch-clamp technique after six months *in vivo*, we found that the population of cells are still heterogeneous (Fig. 4 of *paper III*), indicating that it is the initial rate of conversion that decides phenotype not time after transplantation. This is further supported by the presence of unconverted TE7-expressing cells at both one and six months after transplantation.

Despite the described asynchrony, the cells that fired fast and repetitive APs, were able to display, if small, postsynaptic currents (Fig. 5 of *paper III*), suggesting that converted cells that reach basic neuronal maturity also have the ability to synaptically integrate. We were also unable to elicit postsynaptic current by optogenetic activation of host cells, suggesting that the grafts mostly

make synapses with each other, rather than with the host. However, as the number of recorded cells was low, we did not have the opportunity to fully investigate this.

Based on all these considerations, human iN cells may function as a candidate for cell replacement therapy. Improvements in conversion, survival and integration rates of transplanted cells are needed, before these cells can truly offer a superior alternative to human iPS or ES cells.

Concluding Remarks

There are still many open questions about gene- and cell therapies in epilepsy. For gene therapy to be used as a possible treatment, certain technical advancements have to be made, e.g. safe delivery of the therapeutic genes to the preferred anatomical site. This means choosing an appropriate vector system, along with both promoter and encoding sequence, ensuring stable expression in the target cell population. The newest generations of AAV viral vectors appear to be the safest candidates for the therapeutic gene delivery, at this moment.

As for chemogenetics, although DREADD technology presents a powerful tool to modulate neuronal activity on demand, the use of CNO might become problematic in humans. On one hand, CNO has already been administered to humans without apparent pharmacological effect, but it is also known that, unlike in rodents, the human organism can metabolize CNO to the antipsychotic drug clozapine (139). The rate of this conversion is quite low in few percentage range, but might become a dose-restricting factor for CNO-based DREADD use. Alternatively, non-CNO-based DREADDs, or non-CNO ligands, could be developed to minimize the potential problems with current technology.

As for cell therapy, it is tempting to speculate that transplanted cells, capable of giving rise to neurons with GABA-ergic phenotype, may prove therapeutic by replacing lost GABA-ergic interneurons in epileptic tissue. Functional host-derived afferent synapses onto the grafts can lead to depolarization and release of GABA from these neurons, in a regulated manner, which would thereby exert an inhibitory effect on seizures. Gene- and cell therapies can also be combined, and developed into *ex vivo* gene therapy, where cells carrying therapeutic gene are transplanted to the patient. Functionally integrated grafts can then ideally exert therapeutic effect by releasing the neurotrophic factors, neuropeptides or neurotransmitters, e.g. GABA. These hypotheses would be important to be address in future epilepsy research to unravel the full potential of gene- and cell-based therapies, and their combinations.

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