Neural stem cell transplantation in mouse model of juvenile neuronal ceroid-lipofuscinosis

MASTER'S THESIS

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PREFACE

This Master's thesis was done in the Institute of Medical Technology at the University of Tampere. The practical work was carried out in the Institute of Regenerative Medicine (Regea) at the University of Tampere and in the Disease Mechanisms group at the National Public Health Institute of Finland.

First of all, I wish to sincerely thank my supervisor Susanna Narkilahti, PhD, from Regea for always being there when she was needed, and for helping and advicing me to become a better researcher. I want to acknowledge Prof. Outi Hovatta (Regea) for giving her valuable and constructive opinions on this project. In addition, I am deeply grateful to people at the Disease Mechanisms group, especially Doc. Anu Jalanko, Tuula Manninen, Senior lab technician, and Essi Kaiharju, bioanalyst, for their invaluable co-operation and support during the work. I also wish to thank Prof. Markku Kulomaa from Institute of Medical Technology (IMT), University of Tampere, for his guidance during the end of this Master's thesis.

I want to address my warmest thoughts to my fellow-students at Regea and IMT for their friendship and stimulating discussions. Last but not least, I am priviledged to be able to thank my dear family Satu, Olavi, and Juhani, for their everlasting support, and my fiancé Jyri for being by my side throughout this long but rewarding project.

At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us.
(Albert Schweitzer)

Tampere, March 2008

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ABSTRACT

Background and Aims: This thesis investigates the suitability of neural stem cell transplantation in a mouse model of juvenile neuronal ceroid-lipofuscinosis (JNCL). JNCL is a childhood neurodegenerative disease leading to premature death in young adolescence. This hereditary disease has unfortunately no cure. Neural stem cells (NSCs) are under vigorous research for they have proven to be beneficial in many animal models of human disorders. This has been seen also in many lysosomal storage diseases, a group to which JNCL belongs. The aims of the thesis were to observe the survival, migration, and differentiation of NSC graft in the mouse model of JNCL.

Methods: GFP-expressing mouse neural stem cells were transplanted into 24 adult diseased mice and 24 wild type mice of the same strain. The cells were injected either into the cortex or the hippocampus and let to survive there for either one or four months. Control injections of PBS were performed on 48 mice. The differentiation capacity of the neural stem cells was analyzed by immunohistochemical stainings *in vitro*. Extensive microscopical analyses using endogenous fluorescence, immunohistochemistry, and Prussian blue stainings were performed to the brain sections. Relevant statistical analyses were done for the results.

Results: The survival rate of the transplanted cells was low in both diseased and wild type mice, ranging from 0 to 3 %. The transplanted cells were, however, able to survive for four months in the recipient brain with no statistical differences between the diseased mice and the wild type mice. The migration seemed not to be active, and the distribution of the cells was most prominent in the corpus callosum. The cells had the capability to produce neuron-like processes *in vivo* but did not show a clear differentiation profile.

Conclusions: The environment in the JNCL mouse model did not set limitations for the survival of the neural stem cells, and the cells were able to stay alive there for several months. However, a lot of optimization for the transplantation procedure needs to be done to accomplish better survival of the cells. With diminishing the early hostile reaction in the brain, the migration and differentiation of the cells will most likely become more active. As a conclusion, no reason is seen to avoid further testing of NSC transplantation in animal models of JNCL.

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TIIVISTELMÄ

Tutkimuksen tausta ja tavoitteet: Tämän pro gradu -työn tarkoituksena oli tutkia hermoston kantasolusiirteen sopivuutta nuoruusiän neuronaalisen seroidi-lipofuskinoosin (JNCL) hiirimallissa. JNCL on keskushermoston rappeumasairaus, joka johtaa ennenaikaiseen kuolemaan yleensä potilaan varhaisaikuisuudessa. Tälle perinnölliselle lysosomaaliselle kertymäsairaudelle ei valitettavasti ole olemassa parannuskeinoa. Hermoston kantasoluja tutkitaan ahkerasti, sillä niiden on havaittu olevan hyödyllisiä monien sairauksien, kuten lysosomaalisten kertymäsairauksien, eläinmalleissa. Työn tavoitteena oli käydä läpi kantasolujen selviytyminen, leviäminen ja erilaistuminen JNCL-taudin hiirimallissa.

Tutkimusmenetelmät: Aineisto koostui 48 aikuisesta sairausmallin hiirestä ja 48 villityypin hiirestä. Puolet kummankin hiirityypin yksilöistä saivat vihreää väriä fluoresoivan kantasolusiirteen ja puolet kontrolli-injektiona PBS:a. Kantasolut siirrettiin aivoissa joko korteksille tai hippokampukseen ja niiden selviämistä tutkittiin yhden ja neljän kuukauden kuluttua. Saaduille aivoleikkeille tehtiin laaja mikroskooppinen analyysi käyttäen hyväksi endogeenista fluoresenssia, immunohistokemiallisia värjäyksiä ja Prussian blue -värjäystä. Tuloksille tehtiin sopivat tilastolliset testit.

Tutkimustulokset: Kantasolusiirteen selviytyminen oli heikkoa sekä sairausmallin että villityypin hiirissä. Parhaimmillaan oli havaittavissa 3 % selviytymisaste. Osa siirretyistä soluista kykeni kuitenkin selviytymään neljä kuukautta eikä niiden määrässä tällöin ollut eroa hiirityyppien välillä. Kantasolujen leviäminen ei näyttänyt noudattavan aktiivista migraatiota ja suurin osa soluista havaittiin aivokurkiaisessa. Morfologisesti kantasoluilla oli kyky muodostaa hermosolun kaltaisia soluja, mutta vain muutama solu värjäytyi käytetyillä erilaistumista havaitsevilla vasta-aineilla.

Johtopäätökset: JNCL-hiirimallin taudinkuva ei ollut esteenä hermoston kantasolujen selviämiselle aivoissa ja solut kykenivät selviämään kyseisessä ympäristössä useita kuukausia. Tarvitaan kuitenkin paljon siirrostusvaiheiden kehittämistä, jotta saadaan aikaiseksi kantasolujen parempi selviytymisosuus kohdekudoksessa. Siirrettyjen solujen leviäminen ja erilaistuminen ovat mitä todennäköisimmin myös aktiivisempia mikäli alun vihamielistä reaktiota saadaan pienennettyä. Tässä työssä ei nähty esteitä kantasolujen jatkotutkimuksille JNCL:n eläinmalleissa.

TABLE OF CONTENTS

ABBREVIATIONS

AD	Anterodorsal nucleus of the thalamus
ANCL	Adult neuronal ceroid-lipofuscinosis
BBB	Blood-brain barrier
Br	Bregma level of the mouse brain
BSA	Bovine serum albumin
CC	Corpus callosum
CD	Cluster of differentiation
CLN1-10	Ceroid-lipofuscinosis, neuronal 1-10
CNCL	Congenital neuronal ceroid-lipofuscinosis
CNS	Central nervous system
DAPI	4',6-diamidino-2-phenylindole, nuclear stain
EGFP	Enhanced green fluorescent protein
EEG	Electroencephalogram
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
hESC	Human embryonic stem cell
hFSC	Human fetal stem cell
ICM	Intracellular mass of blastocyst
Ig	Immunoglobulin
INCL	Infantile neuronal ceroid-lipofuscinosis
HC	Hippocampus
HCl	Hydrogen chloride
JNCL	Juvenile neuronal ceroid-lipofuscinosis
KO	Knockout
LINCL	Late infantile neuronal ceroid-lipofuscinosis
MAP-2	Microtubule associated protein 2
MRI	Magnetic resonance imaging

NCL	Neuronal ceroid-lipofuscinosis
NDS	Normal donkey serum
NeuN	Neuronal nuclei
NF	Neurofilament
NSC	Neural stem cell
NT	Needletract
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PIR	Piriform area
PPT1	Palmitoyl protein thioesterase 1
PSA-NCAM	Polysialic acid – neural cell adhesion molecule
RMS	Rostral migratory stream
RT	Room temperature
SGZ	Subgranular zone
SOX-1	SRY (sex determining region Y)-box containing gene 1
SVZ	Subventricular zone
TBS	Tris-buffered saline
TPP1	Tripeptidyl peptidase 1
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
USPIO	Ultra-small paramagnetic iron oxide
vATPase	Vacuolar ATPase complex
vLINCL	Variant late infantile neuronal ceroid-lipofuscinosis
WT	Wild type

1. INTRODUCTION

Neural stem cells, NSCs, have an exciting ability to self-renew and differentiate into various types of neural cells, that is, neurons, astrocytes, and oligodendrocytes. This capacity has aroused a great deal of interest in the therapeutic potential of NSCs. Further, NSCs can be used to study the development of the nervous system, and the different effects of drugs on neurogenesis and mature neural cells. NSCs have been reported to alleviate neuronal deficits in many animal experiments (Lee et al., 2007; Meng et al., 2003; Snyder et al., 1995; Taupin, 2006). Still, the actual mechanism of NCSs in disease alleviation is not sufficiently known. Whether the potential lies in their functional integration into existing neural networks, secretion of neuroprotective signals, sharing of missing constituents with the affected cells, or a combination of these all, remains to be elucidated.

In the present Master's thesis work, the behavior of NSCs in a mouse model of juvenile neuronal ceroid-lipofuscinosis, JNCL, was studied. JNCL is a childhood neurodegenerative syndrome belonging to a group of hereditary diseases called neuronal ceroid-lipofuscinoses, NCLs. The ten different classes of NCLs have as a unifying feature progressive lipopigment accumulation in cells throughout the body (Haltia, 2006). Although the lysosomal lipopigment storage material is also present in peripheral tissues, the tissue destruction and cell loss are almost entirely restricted to the central nervous system, CNS (Dyken, 1988). After the accumulation of lipopigment, called lipofuscin and ceroid, the cells in the brain undergo degeneration through an unknown mechanism. Eventually, this leads to devastating clinical symptoms and to premature death.

There are no treatments available to cure JNCL. This thesis hypothesizes the possibility of NSCs as a part of a treatment for JNCL in the future. Diseases like JNCL will be most effectively treated by an early, widespread, and long-term delivery of a therapeutic agent. Although neural stem cells have shown to be effective as long-term gene delivery vehicles (Meng et al., 2003; Taupin, 2006), little is understood about their migration in the developed brain. The term "developed brain" is important, since most human patients with JNCL are not diagnosed until the corticogenesis is completed. At this point, the brain no longer offers developmental cues that are important enhancers for the transplanted NSCs. Therefore, the aim of this Master's thesis was to investigate the survival, migration, and differentiation capacity of the transplanted neural stem cells in an adult mouse model of JNCL. There are no previous published attempts to observe the potential of NSCs in JNCL.

2. REVIEW OF THE LITERATURE

2.1. Neuronal Ceroid-Lipofuscinoses

Neuronal ceroid-lipofuscinoses, NCLs, are inherited diseases classified into ten different forms according to the age of onset, clinical features, ultra-structural findings, and genetic linkage analyses (**Table 2.1**) (Haltia, 2006). The four main categories, based on the age of onset, are infantile (INCL), late-infantile (LINCL), juvenile (JNCL), and adult neuronal ceroid-lipofuscinosis (ANCL). Except for ANCL (Parry disease), all types are recessively inherited.

All NCLs are characterized by neurological symptoms. Most prominent features are visual failure leading to blindness, progressive epileptic seizures, cognitive decline, and finally premature death (Williams et al., 2006). The age of death varies depending on the type of NCL, occurring during first decade in INCL and around patient's fifth decade in some late onset forms (Haltia 2006). Currently, there are no cures for these devastating diseases.

Table 2.1 Molecular classification of NCLs (modified from Haltia, 2006; Siintola et al., 2007).

Disease	Other name	Onset of disease	Gene product	Protein form	Main storage material
CLN1	INCL	Infantile	PPT1	Soluble	Saposins A, D
CLN2	LINCL	Late-infantile	TPP1	Soluble	Subunit c
CLN3	JNCL	Juvenile	CLN3	Transmembrane	Subunit c
CLN4	ANCL	Adult	?		Subunit c
CLN5	vLINCL Finnish	Variant late-infantile	CLN5	Transmembrane	Subunit c
CLN6	vLINCL	Variant late-infantile	CLN6	Transmembrane	Subunit c
CLN7	vLINCL Turkish	Variant late-infantile	CLN7	Transmembrane	Subunit c
CLN8	Northern epilepsy	Variant late-infantile	CLN8	Transmembrane	Subunit c
CLN10	Congenital NCL	Congenital	Cathepsin D	Soluble	Saposins A, D
Parry disease	Parry	Adult	?		Saposins A, D

Abbreviations: ANCL = adult NCL, CLN1-8 = ceroid-lipofuscinosis, neuronal 1-8 (name for both the disease and the affected protein), CNCL = congenital NCL, INCL = infantile NCL, JNCL = juvenile NCL, LINCL = late-infantile NCL, NCL = neuronal ceroid-lipofuscinosis, PPT1 = palmitoyl protein thioesterase 1, Subunit c = mitochondrial ATP synthase subunit c, TPP1 = tripeptidyl peptidase 1, vLINCL = variant late-infantile NCL.

The estimated carrier frequency of NCLs is 1 % of the population (Scriver et al., 2001). The prevalence is around 1 in 12 500 live births, depending on the geographical location (Mole, 1998). Even though the prevalence seems to be low, neuronal ceroid-lipofuscinosis is the most common group of neurodegenerative disorders in childhood. The pathologically most distinctive features of these diseases are the progressive neural cell death and the accumulation of autofluorescent, lipofuscin-like lysosomal storage material called ceroid abundantly into the CNS (Boldrini et al., 2001). Lipofuscin is a term used for the aging pigment that accumulates during normal senescence, whereas ceroid has been used to describe the lipopigment seen in pathological conditions (Seehafer and Pearce, 2006). The major component of the storage material in most types of NCLs (see **Table 2.1**) is a protein called mitochondrial ATP synthase subunit c (Palmer et al., 1992) and the reason for this is unknown. Its accumulation is not a result of increased expression nor is its transportation into mitochondria affected in the diseased cells (Ramirez-Montealegre and Pearce, 2005). Despite of extensive research, the questions about which mechanisms are driving the above-mentioned cellular alterations and how they relate to the neurodegeneration still remain unanswered.

The function of the affected protein is known in only three of the ten different forms of NCL. INCL, the most aggressive form of the common subtypes, is caused by a mutation in palmitoyl protein thioesterase 1, PPT1, a soluble lysosomal enzyme with esterase activity (Vesa et al., 1995). Another class having onset in childhood, LINCL, is a result of mutations in tripeptidyl peptidase 1, TPP1, also a soluble lysosomal enzyme functioning as a serine protease (Vines and Warburton, 1999). The only congenital form of NCLs, CNCL, is a consequence of mutations in a gene encoding for cathepsin D which is an intracellular aspartic protease (Siintola et al., 2006). All of these proteins function to cleave their substrates, which could explain the accumulation of storage material when their function is defective.

Despite the striking similarities in the neuropathology and symptoms between the forms of NCL, there are only few remarks on the interactions between different NCL-proteins (for the proteins and their corresponding diseases, see **Table 2.1**). PPT1, TPP1, and CLN3 proteins did not show interaction with each other in a yeast two-hybrid model (Zhong et al., 2000). In another study, CLN5 was shown to interact directly with TPP1 and CLN3, whereas PPT1 was found not to have any interaction with the NCL-proteins (Vesa et al., 2002). Interestingly, TPP1 activity is increased in samples derived from patients lacking functional CLN3 and CLN5, and in the *Cln3*$^{-/-}$ mouse model (Mitchison et al., 1999; Sleat et al., 1998; Vesa et al., 2002), suggesting a common pathway.

2.2. Juvenile Neuronal Ceroid-Lipofuscinosis

The most common form of NCLs is the juvenile neuronal ceroid-lipofuscinosis (JNCL), also known as Batten disease or Spielmeyer-Vogt disease. The mean prevalence of JNCL is 1:21 000 (Santavuori et al., 2000), with a higher prevalence in northern European populations (International Batten Disease Consortium, 1995). Currently, there are around 60 JNCL-patients in Finland (Docent Taina Autti, MD, personal communication).

JNCL is a recessively inherited disease, which means that the heterozygous carriers are unaware of the gene defect they have. The disease is typically diagnosed around the age of four to seven years (Williams et al., 2006). Therefore, in unfortunate situations, the family may have several young children suffering from this disease. What makes it even harder for the families is that the disease is steadily progressive and no treatment, except for the ameliorative medication, is available.

2.2.1. The defective protein, CLN3

Traditional JNCL results from the autosomal recessive inheritance of mutations in a gene called *Cln3* (International Batten Disease Consortium, 1995) which is located in the chromosome region 16p12.1 (Mitchison et al., 1994). The function of the *Cln3* encoded protein is still unclear but research to solve this is actively ongoing. *Cln3* is conserved among eukaryotes indicating that it has a fundamental role in normal cellular metabolism (Gachet et al., 2005).

The most common mutation found in JNCL patients consists of a small 1.02 kb deletion comprising of exons seven and eight of *Cln3*. This deletion, found in almost 85 % of patients, results in a frameshift mutation, a premature stop codon, and finally, in a truncated dysfunctional protein (Phillips et al., 2005). In addition, 40 other mutations in *Cln3* have been found in patients with JNCL (NCL resource database, http://www.ucl.ac.uk/ncl/cln3.shtml, data visited on 9th January, 2008).

CLN3 protein is expressed throughout the body but most abundantly in the CNS (Margraf et al., 1999). There are many controversial reports on the cellular localization of CLN3 ranging from mitochondria, lysosomes, the Golgi apparatus, and cell membrane to nucleus (Järvelä et al., 1998;

Katz et al., 1997; Kremmidiotis et al., 1999; Margraf et al., 1999). The current view is that CLN3 travels through the endoplasmic reticulum and the Golgi apparatus to the plasma membrane and finally resides in the lysosomal/endosomal compartment as a transmembrane protein that undergoes many post-translational modifications (Kyttälä et al., 2006). Interesting findings came out from studies with neuronal cells, where the protein was shown to be co-localized with synaptophysin, a protein of the presynaptical vesicle (Haskell et al., 2000), and was associated with synaptosomes (Luiro et al., 2001). This could explain the unique role of CLN3 in the nervous system and the more dramatic disease manifestation in the CNS. On the other hand, CLN3 function may be the same in all cells, but neurons can simply be more sensitive to its dysfunction and possible mistargeting.

In addition to different localizations, also varying functions for CLN3 have been implied. Btn1, a CLN3 homologue of Saccharomyces cerevisiae, has been reported to function in the vacuolar (structure analogous to the lysosomes) pH homeostasis (Pearce et al., 1999) and in the vacuolar arginine transport (Kim et al., 2003). Introduction of *Cln3* vector in *Btn1* knockout yeast has been shown to result in a rescue phenotype, suggesting a functional overlap between these proteins (Kim et al., 2003; Pearce and Sherman, 1998). CLN3 may also have a role in neuronal endocytic/exocytic pathways (Luiro et al., 2004). CLN3 is not homologous to any known protein, but the six-transmembrane-domain topology (Kyttälä et al., 2004) is suggestive for a transporter function and CLN3 has been shown to share a distant homology to nucleoside transporters (Baldwin et al. 2004). Other proposed functions for CLN3 include lysosomal acidification, lysosomal arginine import, membrane/organelle fusion, vesicular transport, cytoskeleton-linked function, autophagy, apoptosis, and proteolipid modification (reviewed in Kyttälä et al. 2006).

Recently, Narayan and co-workers published that CLN3 is a novel palmitoyl-protein Δ-9 desaturase based on the findings in sequence analysis and fatty acid desaturase assay. They claim that this novel palmitoyl-protein desaturase likely has multiple physiological substrates involved in several different pathways impaired in JNCL. This was the first paper to state a precise function for CLN3, and it still needs more general acceptance. (Narayan et al., 2006.)

2.2.2. Pathology at the molecular and histological level

Various reports have described molecular and histological dysfunctions in JNCL. Some of them, particularly those that are interesting for the survival of stem cells, are discussed in this chapter.

First, a decrease in the activity of vacuolar (v) ATPases is reported in lysosomes isolated from JNCL patients' lymphoblasts (Ramirez-Montealegre and Pearce, 2005). These enzymes are proton pumps that are thought to be responsible for the acidification of intracellular compartments, such as lysosomes, and are important in CNS synapses and in concentrating the neurotransmitters (Morel, 2003). In addition, lysosomes have been shown to have increased intra-lysosomal pH in JNCL (Golabek et al., 2000; Holopainen et al., 2001). It remains to be studied whether the decrease in the vATPase activity could be 1) caused by the lack of CLN3, and 2) further cause the increased pH value, thus creating dysfunction at the synaptic level and lead to imbalance in neurotransmitters.

Second, the disruption of arginine levels in JNCL were first observed in a yeast model of JNCL (Kim et al., 2003). Defective arginine transport is also demonstrated in lysosomes isolated from lymphoblast cell lines that are established from JNCL patients but not from e.g. INCL cells (Ramirez-Montealegre and Pearce, 2005). Furthermore, depletion of arginine in cells derived from JNCL patients has been documented (Ramirez-Montealegre and Pearce, 2005). Arginine is the main substrate for nitric oxide (NO) production which impacts numerous cellular events including those important in immune response (Wiesinger, 2001). Intriguingly, imbalance in NO levels has been reported to cause seizures (Urbanska et al., 1996), one of the hallmarks in JNCL. However, the direct involvement of CLN3 to arginine import is still lacking and the dysregulated arginine import may be a secondary phenomenon, resulting from the disease pathology.

Third, there has been a report on the presence of an autoantibody to GAD65 (glutamic acid decarboxylase 65) in a *Cln3*$^{-/-}$ mouse model (Chattopadhyay et al., 2002a). The tested sera of several JNCL patients also show a varying amount of this autoantibody whereas in other known lysosomal storage diseases it has not been observed (Chattopadhyay et al., 2002b). GAD65 plays a role in converting the excitotoxic glutamate into inhibitory γ-aminobutyric acid (GABA). There are higher levels of glutamate in the brain of the *Cln3*$^{-/-}$ mouse most probably due to the inhibitory effect of the autoantibody to GAD65 (Chattopadhyay et al., 2002a; Pears et al., 2005). Preferential GABAergic neuron loss has been shown in mouse models of JNCL (Pontikis et al., 2004), which could be a direct consequence of the elevated levels of glutamate and its reduced conversion into GABA. Also, the role of glutamate receptor overactivation and the preceding excitotoxicity in cerebellar granule cells leading to motor ataxia is described in a recent study (Kovacs et al., 2006).

Both arginine and glutamate transport systems are dependent on the vATPase activity (Ramirez-Montealegre and Pearce, 2005; Thompson et al., 2005). Interestingly, all of these three players are

altered in the JNCL. What is the primary cause for the cascade and do these factors jointly affect the neurodegeneration, is an intriguing question looking for an answer. It is clear that they most likely have an impact on the cellular environment in the brain and might therefore also have an impact on the transplanted stem cell survival.

Very little quantitative information exists about the regional changes in the human brain of JNCL patients, and studies have been mainly restricted to autopsy material (Pontikis et al., 2004). The most affected population in the brain is the GABAergic interneurons (Tyynelä et al., 2004). In knock-in mouse model of JNCL the pathology of the brain shows different degrees of cortical thinning and significant loss of the afferent thalamic neurons and their target neurons in the somatosensory cortex with highly localized hypertrophy of astrocytes and microglia (Pontikis et al., 2005). In knockout mouse model there is a widespread loss of hippocampal interneurons and, prior to that, reactive gliosis (Pontikis et al., 2004). Changes in the number of interneurons in cerebral cortex and the hippocampus, the two sites targeted in this study, are documented in knockout mouse model at the age of seven months (Mitchison et al., 1999).

The autofluorescent storage material is known to be present in JNCL patients before birth (Munroe et al., 1996). The widespread accumulation of the storage material is also an early clinical sign in mice, and a diagnostic feature in human biopsies. Studies in knockout mouse revealed that the storage material is particularly prominent in the cortex, hippocampus, basal ganglia, and the reticular formation of the brain stem (Mitchison et al., 1999).

Pronounced gliosis is observed in the autopsy samples of NCLs (Tyynelä et al., 2004). According to the studies on *Cln3*$^{-/-}$ mouse model, the neurodegeneration is preceded by subtle reactive gliosis already at the presymptomatic age of five months, raising a possibility of an early glial-mediated component in the JNCL pathogenesis (Pontikis et al., 2004). The findings in this study suggested a gradual and slowly developing sequence of events in JNCL pathogenesis, including prolonged neuronal dysfunction leading to glial response. Recent analyses of the NCL mouse models, including the *Cln3*$^{-/-}$ mice, have highlighted the selective nature of neurodegeneration, glial responses prior to neuronal degeneration, and identification of thalamocortical circuits as important pathological targets in NCL disease progression (Cooper et al., 2006).

Finally, at the end-state of the disease, the brain looks macroscopically nearly normal, with a moderate decrease in weight. The remaining neurons have become rounded, being most prominent

in the cortical neurons of the forebrain and cerebellum. These changes are less obvious in the midbrain, medulla oblongata, and spinal cord. In addition, a wide variety of extraneural tissues, for example the myocardium, liver, and spleen, show lipopigment accumulation. (Scriver et al., 2001).

2.2.3. Symptoms and diagnosis

The major clinical symptoms in JNCL are the progressive visual failure, epileptic seizures, and decline in motor and cognitive capacity. The disease progression ultimately leads to premature death usually in the third or fourth decade of life (see **Figure 2.1** for the disease progression).

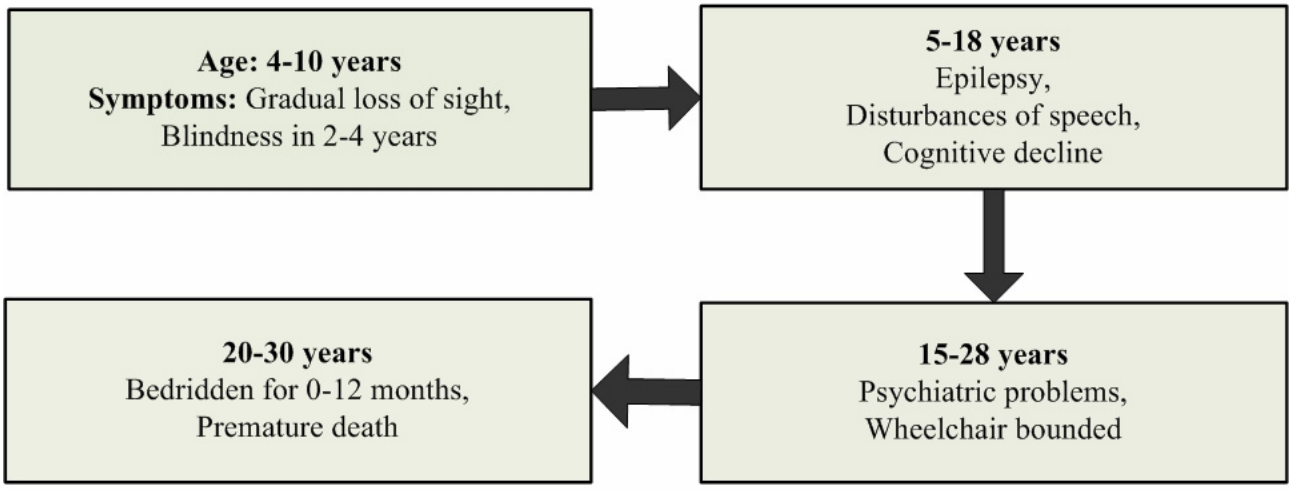

Figure 2.1 Disease progression in JNCL. (Figure is drawn on the basis of Wisniewski, 2006).

The first symptom is impaired vision, although some patients may have minor psychological disturbances years before that. Mental retardation develops slowly and is often noticed first at school. Dysarthia, the difficulty to talk, is evident later, and dementia is profound late in the course of the disease. First epileptic seizures occur around the age of ten and worsen as the disease progresses. Extrapyramidal symptoms, leading to impaired ability to move around, are noticed in about half of the patients between the ages of 12 and 15 years, and in the rest at a later stage (Järvelä et al., 1997). The EEG (electroencephalogram) shows slow and nonspecific deterioration, and MRI (magnetic resonance imaging) changes are detectable in the brain after the first decade of life. It has to be marked that some patients have a slow disease course, while others evolve more rapidly. (Scriver et al., 2001.)

JNCL is diagnozed on the basis of the ultra-structural findings of the autofluorescent storage material in the peripheral blood leukocytes or other accessible tissue like skin. Numerous lymphocytic vacuoles with inclusions resembling fingerprint patterns are a hallmark feature of classical JNCL (Scriver et al., 2001). Ultra-structural findings can be combined to DNA-based diagnostic tests, where mostly the major 1,02-kb deletion mutation of *Cln3* is screened for (Williams et al., 2006).

2.2.4. Treatment

Sadly, no effective therapy exists for JNCL, and treatment is only supportive. Anticonvulsant medication to control seizures is a mainstay treatment for many patients. Some therapies are used to minimize behavioral problems, sleep disturbances, and depression. In addition, physical therapy is used to aid in the retention of physical problems. (Hobert and Dawson, 2006.)

Clinical trials have been conducted with new treatment options but none have proven to prolong the duration of life. A combination of two antioxidants, namely vitamin E and sodium selenite, were tried in several Finnish patients and minor slowing in the disease progression was reported (Santavuori et al., 1989). Bone marrow transplantation (Lake et al., 1997) has also been performed in JNCL patient, but no clear benefits were reported. Recently, a company called StemCells Inc. announced an approval from Food and Drug Administration (FDA) to move into phase I clinical trials using human fetal NSC transplantation for INCL and LINCL patients. The first treatments have already been started and it remains to be seen what the outcome of these treatments is (StemCells Inc., http://www.stemcellsinc.com/news/, data visited on 15th March 2008).

2.2.5. Mouse models

With animal models, there is a great advantage that every step of the disease development can be studied in contrast to the rare patient material where only the end-stage samples are available. The first JNCL mouse models, *Cln3* knockouts, were reported in 1999 (Katz et al., 1999; Mitchison et al., 1999). These models exhibit a JNCL-like tissue pathology but the disease phenotype is mild displaying widespread accumulation of autofluorescent pigment material, and a selective loss of GABAergic interneurons (Katz et al., 1999; Mitchison et al., 1999; Pontikis et al., 2004). These

Cln3$^{-/-}$ mice also have an altered threshold for seizure generation and other neuropathological features seen in JNCL patients (Kriscenski-Perry et al., 2002). Later on, also a knock-in model, *Cln3*$^{\Delta ex7/8}$ was created. This model reproduces the actual major deletion of *Cln3* found in over 80 % of patients and these mice present with a clear, late onset NCL-like phenotype (Cotman et al., 2002). Although *Cln3* is known to exhibit widespread, low-level expression during embryonic development, it is not essential for the development of the embryo. The knockout mice are viable, fertile, and do not show obvious clinical signs by the age of 12 months (Mitchison et al., 1999).

2.3. Stem cells

The abilities to self-renew and to produce differentiated daughter cells are two hallmark properties of stem cells. Stem cells have, at least in theory, capacity to divide indefinitely and at the same time give rise to more differentiated cells (Reubinoff et al., 2000). The potency of stem cells varies from totipotent cells that can create the entire human being, to quite strictly committed cells like blood-forming hematopoietic stem cells. The restriction in the potency of stem cells is gradual and is slowly lost during the differentiation cascade (illustrated in **Figure 2.2** for neural stem cells**)**.

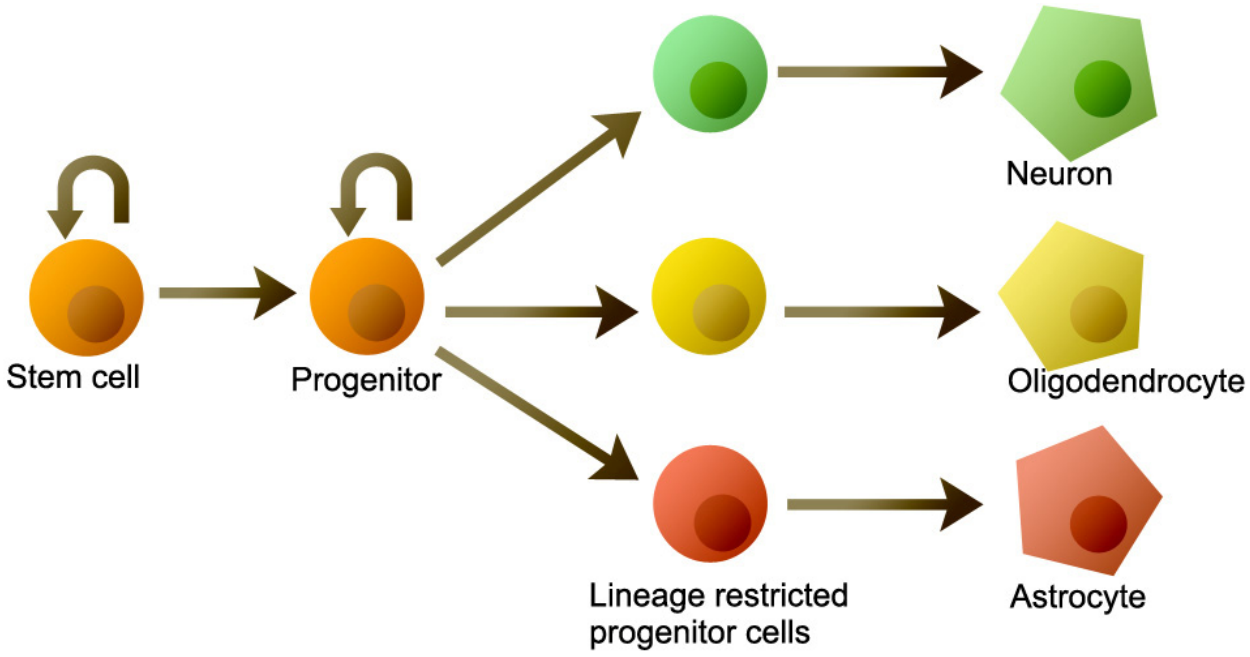

Figure 2.2 Restriction of potency in neural stem cell differentiation. Figure is drawn on the basis of the current knowledge from multiple articles.

Stem cells can be found in the human embryo, fetus, and adult. More and more stem cells are found throughout the adult human tissues. The knowledge about, for example, neural, endothelial, mesenchymal, and adipose-derived stem cells is building up constantly, and giving hope of using these cells in a wide variety of cell replacement therapies. With human embryonic and fetal stem cells (hESCs and hFSCs, respectively) many ethical, philosophical, religious, and technical obstacles have to be overcome before heading towards therapeutical applications. At present, the legislative regulations considering, for example, hESCs differ between nations and they need to be addressed in an exact manner before hESC therapy can be considered as a widely approved method of choice in the future (Matthiessen-Guyader, 2004).

2.4. Neural stem cells

The nervous system as a whole is intriguingly complex and fascinating environment harboring more cell types than any other organ. Neural cells originate from the ectodermal germ layer through a process called neurulation, and slowly develop into different lineages according to the interactions with the surrounding neighborhood and depending on their own intrinsic signals (Sariola et al., 2003). Neural stem cells, NSCs, offer fantastic opportunities for cell therapies, drug screening, modelling diseases, and for studying basic developmental processes *in vitro*.

NSCs can differentiate both *in vivo* and *in vitro* into three different cell types, that is, into neurons, astrocytes, and oligodendrocytes (Reubinoff et al., 2001). However, defining protocols for the generation of pure NSC cultures and for their controlled differentiation further into specified neuronal populations and glial cells represents an important challenge to overcome. During embryonic development, neural stem cells are differentiated in a sequence in which neurons are generated first, followed later by glial maturation (Temple, 2001). The same sequence of differentiation has also been observed *in vitro* (Itsykson et al., 2005). Glial cells of the CNS, namely astrocytes and oligodendrocytes (and microglia, derived from another developmental lineage), are cells that help the neuronal circuits to function properly. For example, oligodendrocytes form the ensheathing myelin layers around the neuronal axons, thus facilitating the speed of the neural impulse (Bear et al., 2001). Astrocytes have an essential role in regulating the chemical content of the extracellular space, and they function in synaptic junctions (Bear et al., 2001). Glial cells were long thought to be only supporting cells of the CNS but the recent studies have shown their

activities to be much more important to the CNS function than thought before (Kettenmann and Ransom, 2005).

NSCs can be cultured *in vitro* in two main ways. In the neurosphere culture, neural progenitors proliferate as free-floating aggregates on non-adherent culture dishes (Reynolds and Weiss, 1992; Watanabe et al., 2005). The neurosphere cultures are known to be heterogenous, containing both multipotent stem cells as well as more restricted progenitor cell populations at different states of differentiation (Reynolds and Weiss, 1992; Suslov et al., 2002). In the adherent culture system, neural progenitors grow as a monolayer on coated substrates, such as laminin (Ying et al., 2003). What is the best culture method considering transplantations, is still to be determined.

Neural stem cells can be divided based on their origin into three groups, namely embryonic, fetal, and adult derived neural stem cells. For the remaining part of chapter 2, the different types of NSCs, their behaviour in different transplantation studies, and their potential benefit in neurological disorders are discussed more carefully. Special attention is given to NSC transplantations in lysosomal storage diseases with neurological deterioration, a disease category including also JNCL.

2.4.1. Embryonic stem cell -derived neural stem cells

Embryonic stem cells, as their name indicates, are stem cells derived from an embryo. ESCs have been obtained from many species including mouse and human, and although being in many ways similar, cross-species differences exist (Ginis et al., 2004). Human ESCs are at their undifferentiated state pluripotent and capable of creating all three germ layers, endo-, meso-, and ectoderm (Thomson et al., 1998). They have an intrinsic capacity to produce neural cell types and this capacity can be directed and expanded with the help of different cultivation methods and substances such as growth factors (Reubinoff et al., 2001).

First human ESC (hESC) lines were established in 1998 (Thomson et al., 1998). Typically, the inner cell mass (ICM) is taken from a blastocyst stage embryo left over after *in vitro* fertilization treatments (Hovatta, 2006). This is done around day five, while, for instance, neurulation begins near the end of the third week in the embryo. There are many ways to collect the ICM cells. One of the most advantageous ways is by mechanically isolating the ICM (Skottman and Hovatta, 2006). ICM cells are transferred to a culture dish and can be propagated for extended periods of time on

feeder cells such as human foreskin fibroblasts in a defined culture medium (Skottman and Hovatta, 2006). A lot of effort has been invested into the optimization of the cultivation methods for hESCs and progress is being made towards clinical-grade culture systems that are free of animal substances (Skottman et al., 2007). This will be a major step towards using hESCs in medical applications.

2.4.2. Fetal neural stem cells

Fetal neural stem cells are derived from aborted fetuses. Neuroepithelial cells were explanted from five to 12 week old human fetal brain and cultured successfully already in 1995 (Buc-Caron, 1995). These cells were able to give rise to neurons, astrocytes, and oligodendrocytes. From then on, fetal neural stem cells have been studied as treatment choice in clinical applications and also as study material in pharmaceutical and toxicological research (Chalmers-Redman et al., 1997).

Fetal NSCs can be isolated from dissociated fetal brain tissue by flow cytometry based on specific cluster of differentiation (CD) marker -detection on the cell surface (Morrison et al., 1999; Uchida et al., 2000). After plating, these isolated cells are able to form neurospheres and produce differentiated neural cells (Morrison et al., 1999; Uchida et al., 2000). Fetal NSCs can also be accessed from developing human or rodent brain by dissecting the brain tissue, such as the lateral ventricular wall, and culturing the formed neurospheres after clonal division (Piltti et al., 2006). Rodent fetal NSCs isolated from different regions of the rostro-caudal axis and at various developmental stages exhibit differences in differentiation potential, growth factor dependence, and gene expression (Haydar et al., 2000; Reynolds and Weiss, 1996).

Human fetal progenitor cells can be cultured *in vitro* without losing their multipotency at least for a year, most likely even much longer (Carpenter et al., 1999). This result was shown in a study using neural progenitor cell cultures established from first-trimester (from five to eleven weeks old) human fetal forebrain. Human fetal neural tissues have been used in treatment of Parkinson's disease patients and shown to improve clinical status for over ten years (Bjorklund et al., 2003). There are, however, ethical concerns and limit to the supply of this tissue, as well as safety guarantee issues.

2.4.3. Adult neural stem cells

The adult central nervous system was long thought as an entity with very restricted renewal capacity and repair mechanisms. Repair was suggested to occur only in a post-mitotic manner involving axon sprouting, neurotransmitter-receptor expression changes, and synaptic plasticity (Lie et al., 2004). However, in restricted areas of the mammalian brain, new neurons are generated from neural stem cells throughout the life. The pioneering work was done over four decades ago by Altman & Das (Altman and Das, 1965) and adult neurogenesis was first demonstrated in humans in the late 1990s (Eriksson et al., 1998).

Adult neurogenesis, a process of generating functionally integrating neurons from progenitor cells, has been shown to persist in two structures of the brain: in the subgranular zone (SGZ) of the hippocampus and in the subventricular zone (SVZ) of the lateral ventricles (**see Figure 2.3**) (Alvarez-Buylla and Garcia-Verdugo, 2002). The majority of neurons born in the hippocampus die within the two weeks of their existence and the surviving ones migrate only a short distance within the hippocampal region (Hastings and Gould, 1999). These cells become electrically active in four to seven weeks and change their process organization within first few months (Lie et al., 2004).

From the SVZ of the lateral ventricles, the immature neuroblasts can migrate an extensively long way in a pathway called rostral migratory stream, RMS (Rousselot et al., 1995). This migration takes about six days in rats (Carleton et al., 2003) but is nearly absent in humans (Sanai et al., 2004). The final destination for the generated neurons is the olfactory bulb, a structure in vertebrate forebrain that is involved in the sense of smell (Ricard and Liebl, 2004).

The magnitude of adult neurogenesis is an interesting field of study. Young adult rats seem to generate quite a large amount of roughly 9000 new cells each day in the SGZ (Cameron and McKay, 2001). Regulation of the magnitude depends on many things. For example, enriched environment and pathological stimulation act as boosting, and aging and high stress level as reducing factors in adult neurogenesis (Ming and Song, 2005). Pathological stimulations have not only been seen to affect the adult neurogenesis in neurogenic regions, but are also thought to have an impact in otherwise non-neurogenic regions (Ming and Song, 2005).

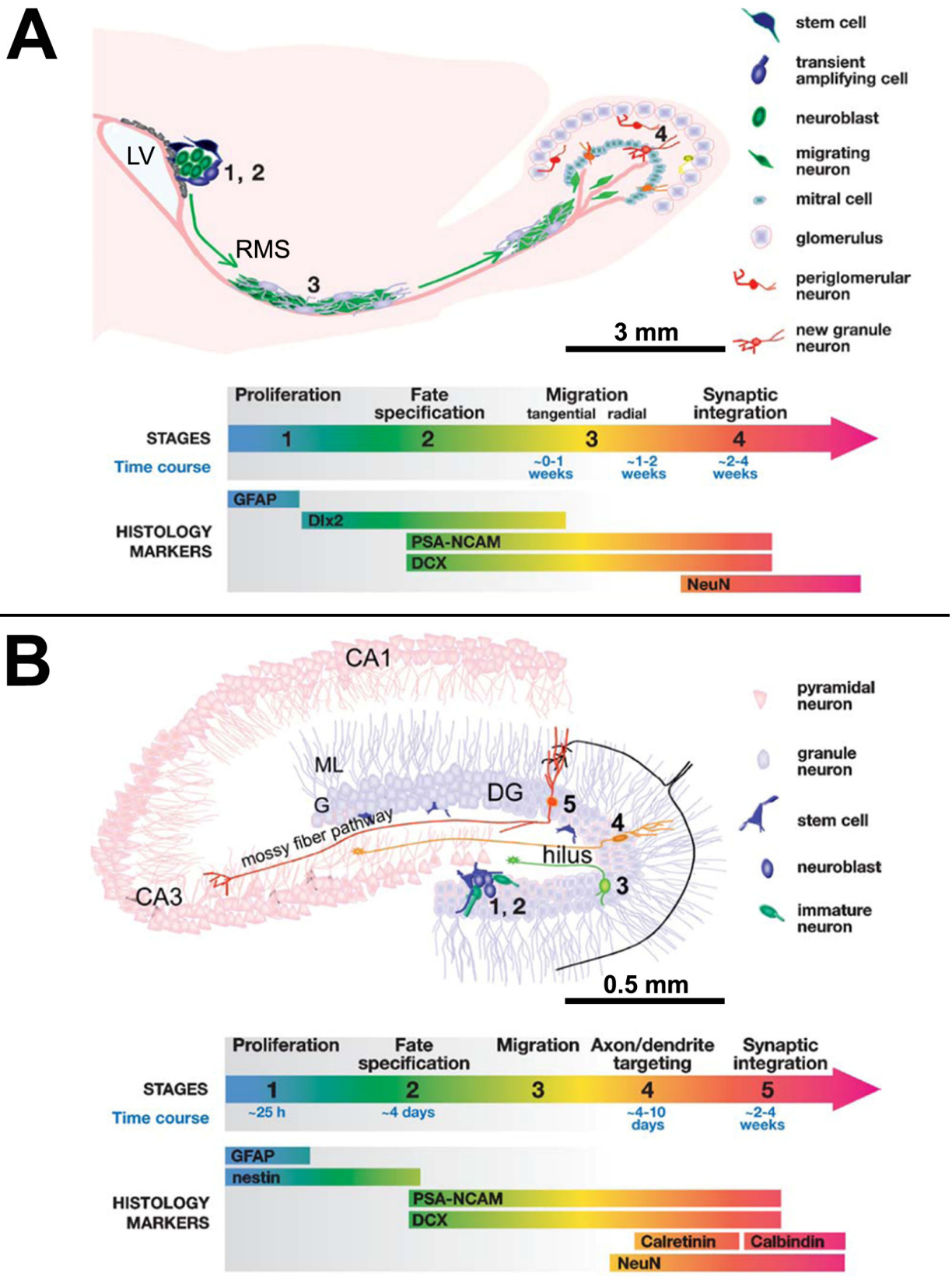

Figure 2.3 Adult neurogenesis in A) SVZ and B) SGZ. A) Sagittal view: precursor cells migrate from the SVZ via RMS to the olfactory bulb. B) Coronal view: Precursor cells migrate only a short distance inside the hippocampus. Abbreviations: DG = dentate gyrus, LV = lateral ventricle, ML = molecular layer, RMS = rostral migratory stream, SGZ = subgranular zone, SVZ = subventricular zone. Figure is modified from Ming and Son, 2005.

The adult neural stem/progenitor cells are not fate-determined intrinsically, but have a rather wide differentiation capability controlled by the extrinsic factors of the surrounding local environment (Lie et al., 2004). Factors that have been postulated to determine the neurogenic niche are vasculature- and blood-derived factors: interestingly, angiogenesis and neurogenesis have been interlinked in some studies (Jin et al., 2002; Louissaint et al., 2002). Glial cells also have an impact, namely local astrocyte populations and ependymal cells of the SVZ are different in their capacity to promote neurogenesis. Finally, there is a vast and ever growing list of different molecules that play a role in the fate-determination, survival, migration, and timing of endogenous neural stem cells (Lennington et al., 2003; Lie et al., 2004).

2.4.4. Behavior of transplanted neural stem cells

Many transplantation studies have been conducted for testing the NSC behavior *in vivo*. Cell transplantation therapies for neurological disorders require above all a good survival of the graft. Many aspects affect the survival and engraftment of transplanted neural stem cells. These include among others the neural stem cell origin, the hostility of the environment, the age of the recipient, and the timing and site of transplantation (Johann et al., 2006; Muraoka et al., 2006; Sheen et al., 1999). The percentages of survived transplanted NSCs vary between studies, but are on average clearly below 50 % and often only on the scale of a few percents (Burns et al., 2006; Muraoka et al., 2006; Sheen et al., 1999; Taupin, 2006). Large differences have been noted in same study groups and between different transplantation dates (Eriksson et al., 2003). One of the longest and most promising studies has shown that transplanted cells can survive for at least 22 months post-engraftment (Snyder et al., 1992). One of the main concerns in using stem cell transplantations is the question whether the cells really integrate into the recipient brain and mature to function properly. Functional integration is a sequential process starting from proliferation of the neural stem cell which generates rapidly amplifying progenitor cells that differentiate into immature neurons migrating to the final location (Lie et al., 2004). At the final location these immature cells need to proceed into growth of axons and dendrites, and formation of synapses with other neurons in the pre-existing circuits, finally leading to mature, fully functional neurons (Lie et al., 2004).

Transplanted neural stem cells have been found to migrate widely in the developing brain (Brustle et al., 1998; Flax et al., 1998; Ourednik et al., 2001; Snyder et al., 1995), within the RMS at all ages (Fricker et al., 1999; Suhonen et al., 1996), and towards areas of severe local pathology such as

injury or ischemia (Belmadani et al., 2006; Jin et al., 2005; Snyder et al., 1997). Most of the existing succesful studies have been performed in neonatal animals and with transplantation into the lateral ventricles. However, the widespread migration capability of NSCs in the adult brain is of outmost importance in treating global neurodegenerative diseases, where the environmental signalling for migration might be remote. Few studies addressing this question have shown that NSCs migrate most extensively in adult brain only along white matter tracts, such as the corpus callosum, and are often confined into one anatomical region (Ben-Hur et al., 2007; Bulte et al., 2003; Englund et al., 2002; Watson et al., 2006). The migration distance from the injection site is usually in the scale of 100-1500 μm (Ben-Hur et al., 2007; Watson et al., 2006). More research needs to be done to reveal the true migration capacity of NSCs inside the adult CNS where the architecture of the host is not disrupted and developmental cues are generally absent.

Differentiation of the transplantated cells is usually analyzed by different immunohistochemical stainings. Transplantation experiments using cells from mouse-derived neurospheres have reported differentiation predominantly into glial cells (Ader et al., 2000; Carpenter et al., 1997), but neural differentiation has also been observed (Eriksson et al., 2003). In one study, mouse neurospheres were able to differentiate into cortical cell types when transplanted *in utero*, but failed to do so under adult conditions (Sheen et al., 1999). State of the differentiation prior to transplantation has an effect on the differentiation capability *in vivo*. In one case, undifferentiated NSCs failed to show MAP-2 immunopositivity, whereas about 23 % of predifferentiated cells developed into MAP-2 immunopositive cells in spinal cord of adult mice (Ding et al., 2006). A common observation in stem cell transplantation experiments is that the majority of cells remain immature without distinct glial or neuronal characteristics, whereas only a small percentage acquire more differentiated phenotypes (Englund et al., 2002; Eriksson et al., 2003).

2.4.5. Neural stem cell treatment in neurological disorders

Neural stem cells offer hope for a wide range of neurological diseases. In addition to cell replacement, NSCs can deliver therapeutic gene products, blunt the toxic components of the microenvironment, replace neural elements, and act as trophic factor releasers (Flax et al., 1998; Snyder et al., 1995). Delivery of neural stem cells is more complicated than, for example, in the hematopoietic system, since cells must be transplanted directly into brain as they cannot cross the blood-brain barrier that is often still intact in the recipient's brain (Barker and Widner, 2004).

Another difficulty is the large number of neuronal phenotypes present in the CNS, making the actual replacement of a specific cell phenotype challenging to engineer. Indeed, nowadays more thought has been given to the concept of stem cells providing neuronal protection rather than actual cell replacement.

Two main strategies for cell therapies using NSC have been suggested. The first involves the activation of endogenous neural stem cells, the second deals with stem cell transplantation based therapy. The former approach requires attraction of newly generated neuroblasts to the damaged areas by manipulating migration, proliferation, and differentiation. In rodents, increased endogenous neurogenesis has been reported after damage, such as trauma, ischemia, or epilepsy (Ke et al., 2006; Nakatomi et al., 2002; Overstreet-Wadiche et al., 2006). Unfortunately, the amount of accelerated endogenous neurogenesis is thought to be too small to have a therapeutic effect in pathological conditions. The advantage of using endogenous stem cells is nevertheless tremendous. By avoiding immunological, ethical, and political concerns people could proceed more easily from the basic study to treating actual patients.

The use of hESCs offers a great deal of benefit as well. They have a remarkable developmental potential and replication capacity, and they promise an almost unlimited supply of specific neural cell types *in vitro* (Zhang, 2006). In addition, hESC as well as fetal NSCs can be genetically engineered prior to transplantation (Buchet et al., 2002; Yates and Daley, 2006) and could offer at their best a "from the shelf" -approach for treatment. NSCs derived from hESCs have been used in numerous animal experiments and been reported to survive, migrate, and differentiate in the CNS (Muotri et al., 2005; Reubinoff et al., 2001; Tabar et al., 2005; Zhang et al., 2001). Human ESCs have, however, also been shown to cause teratomas if transplanted in an undifferentiated state (Przyborski, 2005; Reubinoff et al., 2000). Thus, it is crucial to aim for pure, partly differentiated neural stem cell populations before using them in clinical trials.

Neural stem cell transplantations have been conducted with success in animal models of neurodegenerative diseases, including lysosomal storage diseases. Already in 1995, Snyder et al. reported a widespread correction of lysosomal storage accumulation in a mouse model of Sly disease after an intracerebral transplantation with mouse fetal neural precursor cells (Snyder et al., 1995). In 2003, human fetal NSCs were shown to relieve the disease manifestation in the same disease model (Meng et al., 2003). In both of these studies, the cells were genetically engineered to

overexpress β-glucuronidase, the soluble enzyme genetically defected in Sly disease, and were transplanted into the lateral ventricles of neonatal mice (Meng et al., 2003; Snyder et al., 1995).

In mouse model of Sandhoff disease, which is a progressive neurodegenerative disorder resulting from an abnormal accumulation of ganglioside in lysosomes, transplanted mouse, human fetal, and hESC-derived NSCs significantly improved motor deficits, reduced storage material, and prolonged the survival of the mice. Even though the transplanted cells had become electrophysiologically active, the authors concluded that neuronal replacement was not the predominant mechanism for the rescue. In fact, NSCs were also able to increase the dimished enzyme (Hex, β-hexosaminidase) levels and abate microglial activation, a hallmark of Sandhoff disease. These factors were thought to be crucial for the favorable effect of NSCs upon the disease manifestation. (Lee et al., 2007.)

Human NSC transplantation was found to lead to a better improvement than mouse NSCs in a classical lysosomal storage disease (Niemann-Pick) mouse model. Again, SCs were transplanted neonatally into the lateral ventricles of the cerebrum and were followed for four months. The transplantation of both SC types led to wide migration pattern of the cells and was associated with lesser vacuolization and cholesterol accumulation than in non-injected controls. (Sidman et al., 2007.)

Recently, the first studies on neural stem cell treatment in NCLs have also been published. In the mouse model of Northern epilepsy (CLN8), mouse ESC -derived NSCs were transplanted into the vitreous of an eye at an early stage of retinal degeneration (Meyer et al., 2006). Retinal degeneration is one of the first signs in NCLs and, in addition, the retina is an excellent model for studying SC transplantation since its organization is well understood. NSCs were found to differentiate into neural cells, exert neuroprotective effects, and retard both the accumulation of storage material and photoreceptor degeneration (Meyer et al., 2006). In 2006, Taupin published the convincing results that had led to the approval of phase I clinical trials in treating INCL and LINCL patients with NSCs from human fetal (16 to 20 weeks) brain tissue (Taupin, 2006). NSCs isolated by fluorescence-activated cell sorting (FACS) were used as a cellular delivery vehicle for PPT1 and transplanted into the cerebral ventricles of INCL mouse model. Data demonstrated a reduced storage material and a larger number of surviving neurons in transplanted mice compared to the control group. Transplanted cells were able to survive and secrete enzymes for up to six months at a level that was thought to be above the assumed threshold level for asymptomatic disease (Taupin, 2006). Both of these studies revealed a promising future for the NSCs in treatment of NCLs.

3. AIMS OF THE RESEARCH

The aims of this Master's thesis were,

1. To determine the survival of the neural stem cell graft in a mouse model of JNCL
2. To find out the migration capacity of the grafted cells
3. To observe the differentiation of the grafted cells

4. METHODS

4.1. Research sites

This Master's thesis was done in a co-operation project between the Neuro group, lead by Susanna Narkilahti, PhD, of the Institute for Regenerative Medicine, Regea (University of Tampere), and the Molecular Medicine – Disease mechanisms –group lead by Doc. Anu Jalanko (the National Public Health Institute, Helsinki). The author was responsible for the characterization of neurospheres *in vitro*, the optimization of the histological staining methods, and for all the post-mortem analyses of the grafted cells and the disease manifestation. The author also participated in a transplantation procedure at the National Public Health Institute. Culturing of neurospheres, animal work, MRI, and the processing of the brains were done by the group of Doc. Anu Jalanko.

4.2. Neurosphere culture

The transplanted cells used in this study were mouse neurospheres derived from mouse fetus. They were a kind gift from Kirmo Wartiovaara's group (Developmental Biology, University of Helsinki). Neurospheres were isolated by dissecting the lateral wall of embryonic day 11.5 (E11.5) mouse brain. The used transgenic mouse strain (NMRI, UBI-EGFP/BL6, 129/Sv-Trp53^{tm1Tyj} from Jackson Laboratory, USA), expressed enhanced green fluorescent protein, EGFP, under the control of the human ubiqutin C promoter. Cells derived from this strain will emit green light when exposed to 488 nm wave-length. The derived cells formed aggregates of free-floating heterogenous neural cells called neurospheres *in vitro* and were able to proliferate (Piltti et al., 2006).

Neurospheres were cultivated in a defined Neuromedium consisting of DMEM supplemented with 25 % Nutrient mixture F-12 Ham (Sigma-Aldrich, St. Louis, MO), 2 % B27 supplement (Gibco/Invitrogen, Carlsbad, CA), 1 % Glutamax (Gibco), 1 % Penicillin-Streptamycin (Gibco), 250 μg/ml Fungizone (Gibco) supplied with 20 ng/ml EGF (Sigma-Aldrich) and 40 ng/ml FGF2 (Sigma-Aldrich). They were able to form multiple types of neural cells including neural progenitor cells, mature neurons, and glial cells (see section 5.1).

4.2.1. Immunocytochemical staining of neurospheres *in vitro*

Immunocytochemical staining of neurospheres was performed after the incubation of cells on poly-DL-ornithine coated wells. The wells were coated by incubating them in 0,001 % poly-DL-ornithine (Sigma) diluted in PBS for 30 minutes in +37 °C. After the incubation, wells were washed once with PBS and single-cell suspension of neurospheres was plated in Neuromedium containing 1 % FCS (HyClone). The neurospheres were allowed to differentiate on poly-DL-ornithine coated wells in 37 % for 25 hours. During the prolonged overnight incubation the cells had attached and showed elongated processes. Cells were fixed with PFA (Sigma) and stained using different antibodies listed below. The immunocytochemical protocol is shown in **Table 4.1**.

Table 4.1 Immunocytochemical staining protocol for neurospheres.

1	Cells were washed 2 x 5 min with PBS (0,01 M, pH 7.4)
2	Fixation with 4 % PFA (Sigma) for 20 min
3	Washing 2 x 5 min with PBS
4	Blocking in 10 % NDS (Chemicon), 0.1 % TritonX-100 (Sigma), 1 % BSA (Sigma) in PBS for 45 min
5	Washing with 1 % NDS, 0.1 % TritonX-100, 1 % BSA in PBS
6	Incubation with primary antibodies (1 % NDS, 0,1 % TritonX-100, 1 % BSA in PBS) at +4 °C overnight
7	Washing 3 x 5 min with 1 % BSA in PBS
8	Incubation with secondary antibodies (1 % BSA in PBS) 1 h at room temperature, light protected
9	Washing 2 x 5 min in PBS
10	Washing 2 x 5 min in Tris-HCl
11	Mounting with Vectashield including DAPI (Vector Laboratories)

The primary antibodies were β-tubulin III (mouse IgG, 1:1000, Sigma); Doublecortin (goat IgG, 1:400, Santa Cruz); GFAP, glial fibrillary acidic protein (sheep IgG, 1:400, R&D systems); GFP, green fluorescent protein (rabbit IgG, 1:400, Santa Cruz); MAP-2, microtubule associated protein 2 (rabbit IgG, 1:400, Chemicon), Nestin (mouse IgG, 1:200, Chemicon); and PSA-NCAM, polysialic acid - neural cell adhesion molecule (mouse IgM, 1:400, Chemicon). The secondary antibodies used were anti-goat IgG, anti-mouse IgG, anti-mouse IgM, anti-rabbit IgG, and anti-sheep IgG, all conjugated with Alexa 568 dye (1:300, Molecular Probes).

4.3. Animal work

Animal model used in this study was a *Cln3* knockout mouse (Mitchison et al., 1999) that was backcrossed for ten generations in C57/Bl6 strain. Wild type (wt) mouse with the same congenic background was used as a control. Between two and six mice were kept at a same cage and had nest building material, refreshments, water, and food available. The mice had a light-dark cycle of 12 hours and they were checked visually on every day of the experiment. All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Public Health Institute (KTL 2005-26; ESLH-2005-9155/Ym23/Decision STU 1359A).

4.4. Transplantation

Prior to transplantation, NSCs were labeled with USPIO, ultra-small paramagnetic iron oxide, particles (Guerbet, Sinerem). The USPIO-particles were incubated with 1 mg/ml Lipofectamin for 30 minutes and thereafter the 1:10 diluted solution was added onto the neurospheres. A final concentration of 1.2 mg/ml of USPIO-label was used in the incubation medium. The neurospheres were incubated for the delivery of the label inside the cells at +37 °C for 24 hours. After the incubation, the neurospheres were centrifuged in an eppendorf tube, washed three times, counted, and suspended in PBS that was manufactured according to accredited laboratory protocol.

At the animal institute of the National Public Health Institute, USPIO-labeled GFP-expressing neural stem cells (50 000 cells / injection) were transplanted into four week old animals (± three days) (**Table 4.2**). The used anesthetic was Hypnorm-Dormicum: Hypnorm (fentanly/fluanisone 0.315 mg fentanyl/ml; 10 mg fluanisone/ml) and Dormicum (midazolam 5 mg/ml) each diluted 1:1 with sterile water and mixed in 1:1. This solution was injected to mice in the following quantities: 0.05-0.075 ml/10g and was given subcutaneously prior to the transplantation. The anesthetized animal was carefully placed into a Small Animal Stereotactic Instrument (Kopf Instruments) and two holes were drilled into the skull. Intrathecal transplantations were made bilaterally using Hamilton 75N syringe and a 26s-gauge needle into determined anatomical regions of the brain. These anatomical structures were either the cortex or the hippocampus (**Figure 4.1**). The stereotactic coordinates were as follows (distance from the bregma): for the cortex -1, 3, -1 mm, and for the hippocampus -2, 0.75, -2 mm (anterior-posterior, lateral, and ventral distances, respectively).

Table 4.2 Transplantation paradigm. Mice received GFP-expressing stem cell graft or control medium (PBS) either into the cortex or the hippocampus (HC). The mice were sacrificed one and four months post-transplantation.

MOUSE	INJECTION SITE	TIMEPOINTS			
		1 MONTH after injection		4 MONTHS after injection	
		CELL GRAFT	CONTROL	CELL GRAFT	CONTROL
Cln3$^{-/-}$	CORTEX	n = 6	n = 6	n = 6	n = 6
	HC	n = 7	n = 6	n = 6	n = 6
WILD TYPE	CORTEX	n = 7	n = 6	n = 5	n = 3
	HC	n = 9	n = 6	n = 5	n = 6

Abbreviations: *Cln3*$^{-/-}$ = mouse model of JNCL, HC = hippocampus, n = the number of mice per group.

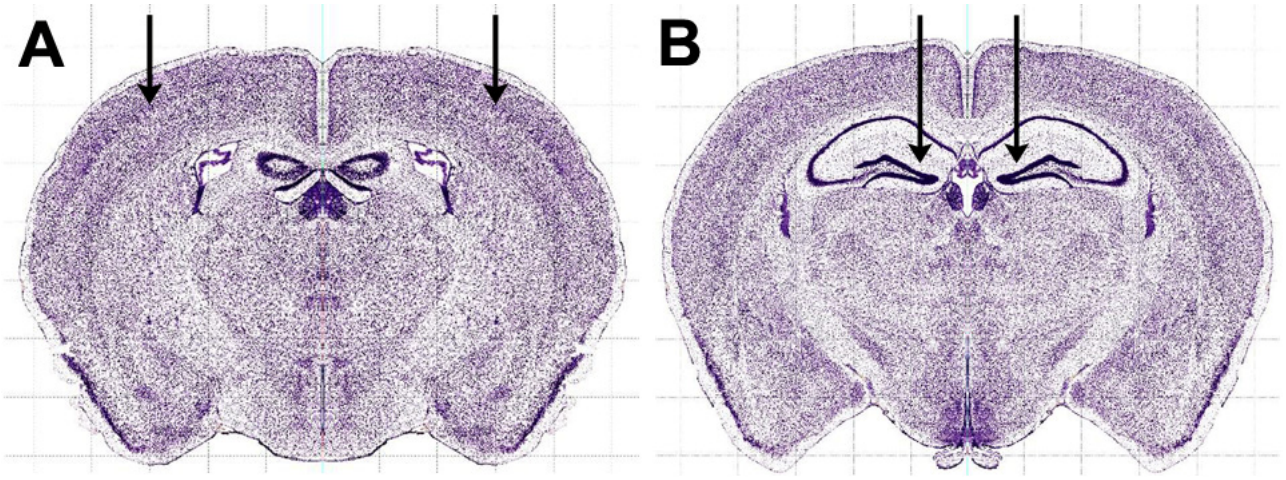

Figure 4.1 Transplantation routes for A) cortical injections, and B) hippocampal injections. Arrows represent the transplantation routes for the stem cell grafts. Coronal atlas pictures of the brain are taken and modified with permission from Allen Brain Atlas, www.brain-map.org.

Single-cell suspension of neurospheres was distributed in PBS and was kept on ice during the procedure. The cells were injected slowly, at a speed of 0.3 µl in a minute, to make up the total volume of 1 µl. No more than four animals were transplanted per day and cells which were left over were taken back to laboratory to ascertain their viability and proliferation. As a control, PBS was injected in the same manner as described for the stem cells. During the operation, good care was taken to keep the mice warm. When waking up, the mice were given analgesic Temgesic (buprenorfin) injected 0.05-0.1 mg/kg and were kept under intense observation post-operation. Injected mice were not immunosuppressed.

4.5. Processing of the brains

The mice were sacrificed one and four months after transplantation (**Figure 4.2**). Prior to transcardial perfusion, the mice were anesthetized using Mebunat vet (60 mg/ml) with 1:10 dilution, and about 400 µl per animal was injected intraperitoneally according to the weight of the mouse. After confirmed anesthetization, the thorax was opened and 60 ml of PBS was injected via the left ventricle to the blood circulation. Thereafter, 60 ml of 4 % PFA was used for fixation. The brain was removed, placed into 4 % PFA for two hours, and after that cut at the midsagittal line. The left side of the brain was placed into 2 % glutaraldehyde for immunohistochemical analysis. The right side of the brain was put into 30 % sucrose. The left side of the brain was changed to 30 % sucrose + 0.05 % NaN_3 in TBS for storage in -70 °C freezer. It was further processed into 40 µm thick sections using cryostat, and sections were stored in 3 x 96-well plates in the same solution. These plates were kept in -70 °C freezer and brain sections did not melt during the transportation from Helsinki to Tampere.

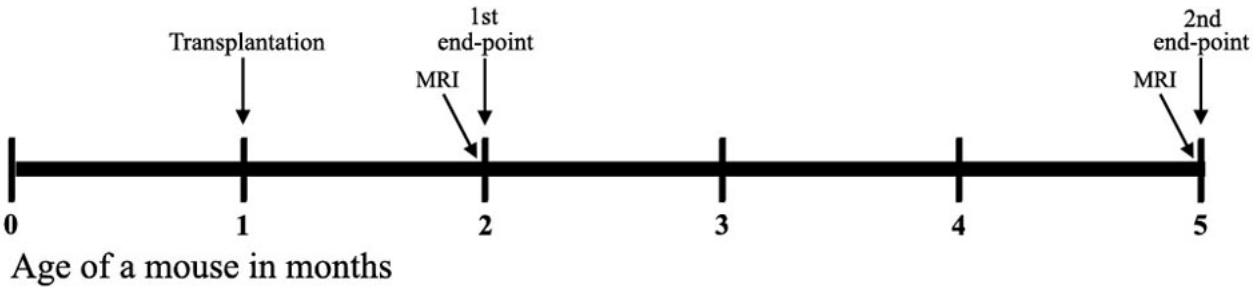

Figure 4.2 Timescale of animal experiments. Abbreviations: MRI = magnetic resonance imaging

4.6. Analyses of transplanted cells

The analyses of the grafted cells were done using only the hippocampus-injected brain samples. The cortex-injected mice were left out of the study based on the MRI-findings, which showed no survived cells in these animals. Three of the cortex-injected mice were microscopically analyzed and this confirmed the statement that none of the transplanted cells had survived. All microscopical analyses were carried out in a blinded fashion concerning the nature of the experimental manipulation.

4.6.1. Magnetic resonance imaging

MRI was used for the detection of the transplanted, USPIO-particles containing cells. Two animals per group receiving stem cells were taken to MRI prior to perfusion (**Figure 4.2**). The imaging was done under anesthesia (Hypnorm Dormicum) with Bruker 4.7 Tesla superconductive magnetic imaging equipment, which is especially good for imaging small vertebrates.

4.6.2. Analysis of cell survival

In the large-scale microscopical analysis, every sixth section of the cerebrum was taken to study the presence of the transplanted cells expressing GFP. The distance between the sections was 240 µm (**Figure 4.3**). Depending on the size of the brain, around 20 sections per each animal were analyzed for cell survival. These sections were lifted from the 96-well plate and washed two times ten minutes with TBS and two times ten minutes with Tris buffer to get rid of salt precipitates disturbing the fluorescence imaging. After washing, sections were placed carefully onto a Superfrost+ glass (Menzel) and mounted with Vectashield containing nuclear stain DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories). Expression of GFP was detected using the Olympus IX51 fluorescence microscope.

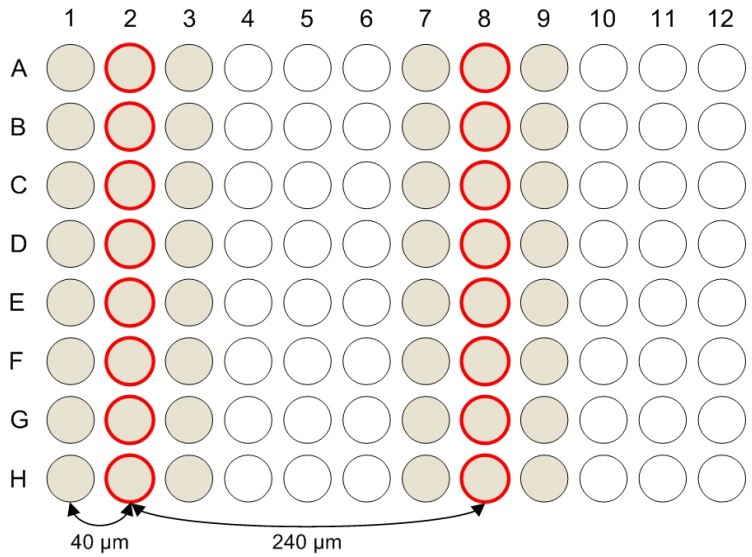

Figure 4.3 Plate (96-well) used in storing brain sections. Every sixth section (marked with red circle) was used in GFP-analysis and sections next to it (marked with brown filling only) were used in immunohistochemical stainings. The sections marked with white were stored in Docent Anu Jalanko's group.

Transplanted cells could be detected without using primary antibodies. The presence and location of GFP expressing transplanted cells were first determined by looking throughout the brain section with 20x objective. Cell counting was done using 40x objective. The results were documented into a result form (**Table 4.3**), one for each animal. In this form, the brain had been divided into eight regions (the corpus callosum medial, middle and lateral, needletract, the hilus of the hippocampus, the cortex, the lateral ventricle, and "other brain region"), and the corresponding cell counts were assigned. The amount of "golden cells" (see section 5.6) was also counted with precision and separated based on the appropriate anatomical region. Bregma level and the plate well -code were also documented for each section.

Some cerebellar sections were also analyzed for the presence of transplanted cells by both GFP signal and Prussian blue staining.

4.6.3. Analysis of cell differentiation

All the immunohistochemical staining protocols using different antibodies needed to be optimized before starting the actual stainings. 19 different primary antibodies were tested of which seven gave reliable and essential staining results for the purposes of this study. These seven (**Table 4.4**) were used in the stainings for the analysis. Stainings were performed to sections documented to be next to a section containing transplanted cells. For the immunohistochemical protocol, see **Table 4.5**.

The used primary antibodies were β-tubulin III (mouse IgG, 1:100, Sigma); Doublecortin (goat IgG, 1:400, Santa Cruz); GFAP, glial fibrillary acidic protein (sheep IgG, 1:750, R&D systems); MAP-2, microtubule associated protein 2 (rabbit IgG, 1:250, Chemicon); NF-200, neurofilament 200 (mouse IgG, 1:200, Sigma); NeuN, neuronal nuclei (mouse IgG, 1:1000, Chemicon); and PSA-NCAM, polysialic acid - neural cell adhesion molecule (mouse IgM, 1:3000, Chemicon). The secondary antibodies were anti-goat IgG, anti-mouse IgG, anti-mouse IgM, anti-rabbit IgG, and anti-sheep IgG, all conjugated with Alexa 568 dye (1:200, Molecular Probes).

Analysis was made by merging images acquired with fluorescence microscope using wavelengths: UV (nuclei of all the cells), 488 nm (GFP positive and golden cells, see section 5.6), and 568 nm (corresponding to the specific cell type being stained for). Images were overlapped using Olympus DP Controller & Manager software, and the double positive cells (488 + 568 nm) were counted.

Table 4.3 Result sheet from analysis of cell survival. The presented sheet is from the analysis of mouse H20, where both transplanted cells and unknown autofluorescent material, termed as golden cells, were apparent in regions such as the medial corpus callosum and needletract.

MOUSE **H20** **DATE OF LIFTING:** **181106** **ANALYSIS** **181106**

CC=corpus callosum, ME=medial, MI=middle, L=lateral, NT=needletract, HC=hippocampus, CTX=cortex LV=lateral ventricle, O=other, Br=bregma (from Mouse brain atlas, Paxinos), well=plate,row,column

Well	Br		CC ME	CC MI	CC L	NT	HC	CTX	LV	O
1E2	1.18	GFP-cells								
		Golden cells								
1E8	1.1	GFP-cells								
		Golden cells								
1F2	0.86	GFP-cells								
		Golden cells								
1F8	0.62	GFP-cells								
		Golden cells								
1G2	0.38	GFP-cells		1						
		Golden cells								
1G8	0.14	GFP-cells								
		Golden cells								
1H2	-0.22	GFP-cells								
		Golden cells								
1H8	-0.58	GFP-cells		14						
		Golden cells	34	160						
2A2	-0.94	GFP-cells	4	2		2				
		Golden cells	30	60		210				
2A8	-1.34	GFP-cells	7	1						
		Golden cells	60	47		28				
2B2	-1.46	GFP-cells	7							
		Golden cells	85							
2B8	-1.58	GFP-cells	6							
		Golden cells	90	20						
2C2	-1.7	GFP-cells							1	
		Golden cells		30						
2C8	-1.94	GFP-cells					2			
		Golden cells		9						
2D2	-2.6	GFP-cells								
		Golden cells								
2D8	-2.7	GFP-cells								
		Golden cells								
2E2	-2.8	GFP-cells							9	
		Golden cells							47	
2E8	-2.92	GFP-cells								
		Golden cells								
2F2	-3.16	GFP-cells								
		Golden cells								
2F8	-3.4	GFP-cells								
		Golden cells								
2G2	-3.88	GFP-cells								
		Golden cells								

Table 4.4 Antibodies used in differentiation analysis of transplanted neural stem cells *ex vivo*. The expression patterns of known markers may vary between cells *in vivo* and *in vitro*.

Neuronal markers	**DESCRIPTION**	**REFERENCES**
β-tubulin III	Marks early neurons as well as mature neurons; cytoskeletal staining	Roskams et al., 1998 Menezes et al., 1994
Doublecortin	Migrating neurons; localized in growing processes	Friocourt et al., 2003
MAP-2	Microtubule associated protein 2: Post-migratory, differentiating neurons; expressed in the cell body, dendrites, and in developing axons	Dehmelt et al., 2004 Menezes et al., 1994
NF-200	Neurofilament-200: Neuron-spesific intermediary filament; expressed especially in axonal processes	Lee et al., 2005
NeuN	Neuronal nuclei; Post-mitotic neurons; nuclear stain	Wolf et al., 1996
PSA-NCAM	Polysialic acid – neural cell adhesion molecule: Mostly expressed in immature neural precursor cells, abundant in adult neurogenic regions; cell surface marker	Bonfanti, 2006
Glial markers		
GFAP	Glial fibrillary acidic protein: Intermediate filament of mature and developing astrocytes; cytoskeletal protein	Messing et al., 2003

Table 4.5 Immunohistochemical staining protocol for cell differentiation analysis.

1	Washing 3 x 10 min with TBS (0,05 M, pH 7.4)
2	Blocking in 10 % NDS (Chemicon), 0,5 % TritonX-100 (Sigma) in TBS for 40 min
3	Washing 15 min with 1 % NDS, 0,5 % TritonX-100 in TBS
4	Incubation with primary antibodies in 1 % NDS, 0,5 % TritonX-100 in TBS at +4 °C o/n
5	Washing 3 x 15 min with 1 % NDS, 0,5 % TritonX-100 in TBS
6	Incubation with secondary antibodies (1 % NDS, 0,5 % TritonX-100 in TBS) at room temperature 2 h, light-protected from this step forward
7	Washing 2 x 15 min with TBS
8	Washing 2 x 15 min with Tris (0,05 M, pH 7.4)
9	Lifting of the sections onto Superfrost+ glass
10	Mounting with Vectashield including DAPI (Vector Laboratories)

4.6.4. Prussian blue staining

The Prussian blue staining was performed to part of the samples for detection of the iron oxide particles (USPIO), which had been incorporated into the neural stem cells prior to transplantation. For the staining, a mixture of 20 % HCl and 10 % potassium ferrocyanide (Sigma) was freshly made. The brain sections were incubated in this staining solution for 20 minutes and rinsed four times with water. For the nuclear detection, sections were further incubated in Nuclear Fast Red (Vector Laboratories) for five minutes and washed three times with water. Sections were then dehydrated once in 95 % and two times in 100 % alcohol, and cleared twice for three minutes in Xylene. Finally, the sections were mounted with VectaMount (Vector Laboratories) and analyzed with Nikon bright-field microscope.

4.7. Analysis of neuropathological progression

Semi-quantitative analysis of the neuropathological progression was made by examining the degree of background autofluorescence accumulation in three distinct structures of the brain in all of the analyzed mice. These structures were the piriform area (PIR) in the neocortex, anterodorsal nucleus of the thalamus (AD), and the CA2 region of the hippocampal pyramidal cell layer. They were chosen because they were all easily distinguishable, showed background autofluorescence, and were located in anatomically different regions of the brain. The intensity of background autofluorescence was semi-quantitatively scored as: 0 = none, 1 = faint, 2 = observable, 3 = clearly observable, and 4 = massive.

4.8. Statistical analysis

Statistical analyses were performed with SPSS version 14.0 using a non-parametric Kruskall-Wallis test for comparing population medians among several groups and a non-parametric Mann-Whitney U-test for testing differences between two groups. A p-value of < 0.05 was considered significant.

5. RESULTS

5.1. Characterization of neurospheres *in vitro*

The neurospheres demonstrated good proliferation capabilities *in vitro*. When viewed under fluorescence microscope, the great majority of the transgenic neural stem cells expressed endogenous GFP signal.

Neurospheres used in this study were a heterogeneous mixture containing both progenitor-stage cells and more differentiated cells that showed clear processes after overnight differentiation *in vitro* (**Figure 5.1**). The characterization of cultured NSCs was performed by immunocytochemical staining. Most of the cells were positive for neural marker MAP-2 (**Figure 5.2 A-C**), while some expressed neural precursor markers PSA-NCAM (**Figure 5.2 D-E**) and Nestin, and some the astrocyte marker GFAP. A few cells also expressed Doublecortin, which is a marker for migrating neurons. Thus, the cells were able to differentiate both towards neurons and astrocytes. However, most of the NSCs had neuronal rather than astrocytic characteristics. The β-tubulin staining for young and mature neurons was negative, most likely due to the partial mismatch between the human antibody and the mouse antigen.

5.2. Animals

The treated animals showed no post-operational deficits and were normal until the end-date of the study. *Cln3*$^{-/-}$ mice showed no visually observable symptoms during the study period and behaved as their wild type age-matches. Three mice (one five months old wild type mouse with NSCs and two five months old knockout mice with NSCs) were lost in the MR imaging. The number of mice analyzed is illustrated in **Table 5.1**. In addition, three cortex-injected animals were analyzed and no GFP-cells or golden cells were found as the MRI suggested. Thus, only hippocampus-injected mice were studied.

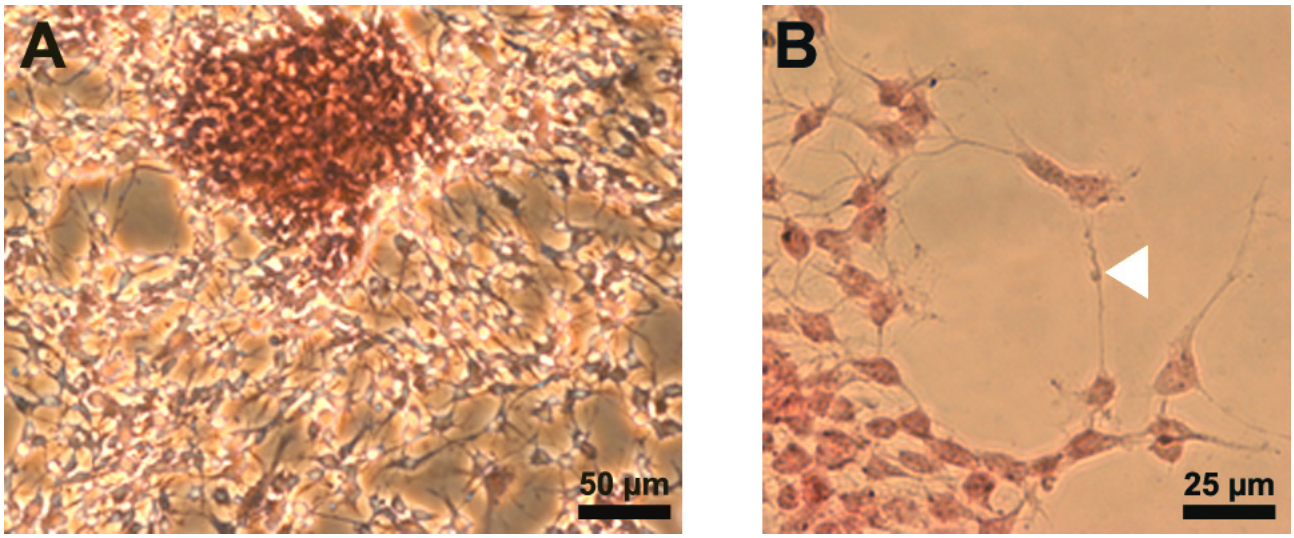

Figure 5.1 NSC culture after overnight differentiation. **A)** Adherent NSCs grew networks of processes *in vitro*. **B)** Larger magnification of a process (white arrow head). Nuclei are stained with Nuclear Fast Red.

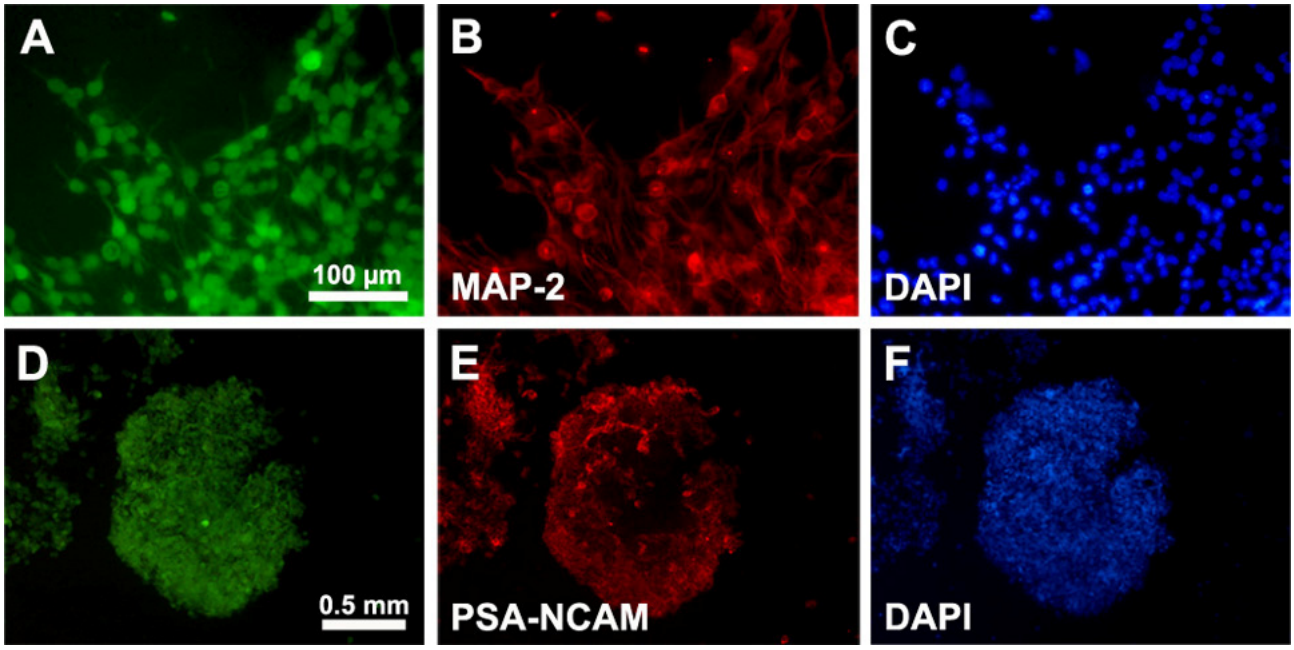

Figure 5.2 Characterization of neurospheres *in vitro*. A-C) Most of the cells expressed neural marker MAP-2. **D-F)** A large portion of the cells were positive for the neural precursor marker PSA-NCAM. **A)** and **D)** represent the endogenous GFP fluorescence. GFP signal was found in nearly all of the cells. Abbreviations: DAPI = nuclear stain, GFP = green fluorescent protein, MAP-2 = microtubule associated protein 2, PSA-NCAM = polysialic acid – neural cell adhesion molecule.

Table 5.1 Number of analyzed mice in hippocampal transplantation groups.

MOUSE	TIMEPOINTS			
	1 MONTH after transplantation		4 MONTHS after transplantation	
	CELL GRAFT	CONTROL	CELL GRAFT	CONTROL
CLN3$^{-/-}$	n = 6	n = 6	n = 4	n = 6
WILD TYPE	n = 6	n = 6	n = 5	n = 6

Abbreviations: Control = mice receiving PBS; n = number of animals analyzed per group.

5.3. Survival of transplanted cells

MRI findings revealed survival of stem cells in mice with hippocampal transplantations. The signal was most prominent in the corpus callosum. The signal was absent in mice with transplantations into the cortex.

The survival of the transplanted cells was studied histologically in a series of sections 240 µm apart from each other. For detection of endogenous GFP in transplanted cells, few antibodies against GFP were tested but the best result was observed by detection of GFP signal without any extra labeling. Antibodies against GFP were nevertheless used in some of the optimizations to determine that all of the transplanted cells could be found without the use of an antibody staining.

In general, graft survival was poor and no clear graft core was detected in any of the animals studied. GFP expressing cells were dispersed mostly as single cells (**Figure 5.3**). The amount of survived transplanted cells varied from 0 to 310 cells (0 to 3 %, respectively) in one hemisphere. This result was gotten with extrapolating the number of cells from every sixth section. No GFP positive cells were falsely detected in the 24 mice that had received PBS as a control vehicle.

Cell survival varied between the different groups. There were no transplanted cells observed in the *Cln3*$^{-/-}$ mice that had received the transplantation one month before. However, numerous transplanted cells had survived in the four months follow-up group of the *Cln3*$^{-/-}$ mice and showed the best survival rate of all the groups (**Figure 5.4 A**). In wild type mice, the transplanted cells were

present in both follow-up groups. The mean survival of transplanted cells dropped by 75 % in the wild type mice from the age of two to five months. This difference was, however, not significant (p-value = 0.24). The visually clear difference between the cell survival in five months old knockout and wild type mice (see **Figure 5.4 A**) was as well not statistically significant (p-value = 0.063).

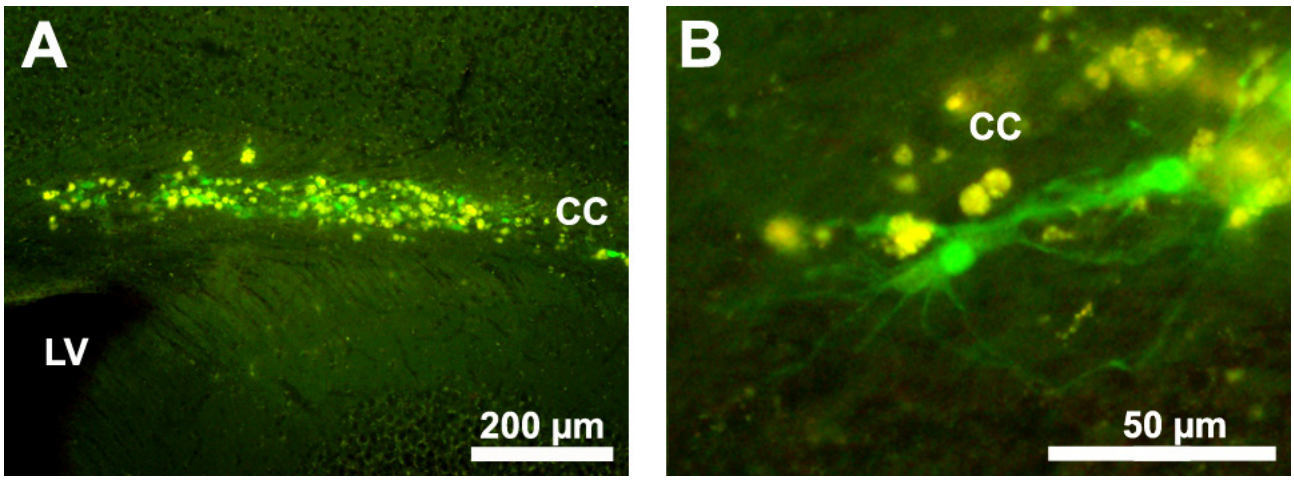

Figure 5.3 Survival of transplanted cells. A) The survival was most prominent along the corpus callosum (numerous bright green cells), and was nearly always accompanied by the presence of yellow autofluorescent material (in both **A** and **B**). **B)** A survived GFP-expressing cell with clear processes. Abbreviations: CC = corpus callosum, LV = lateral ventricle.

Cell survival varied also among the same group. The only group where all the mice had surviving cells was the knockout mice group with four months follow-up period.

In addition to the sections from cerebrum, some sections from the cerebellum were analyzed for the presence of transplanted cells by both GFP signal and Prussian blue staining. This was done after MRI pictures had shown some signal in the cerebellum. No transplanted cells were detected in the cerebellum and thus, immunohistochemical stainings were omitted in cerebellar sections.

5.4. Distribution of transplanted cells

The survived transplanted cells were mostly detected along the corpus callosum (**Figure 5.5**). On average, 90 % of the survived cells were found in the corpus callosum in wild type mice, whereas the corresponding number was 64 % in the knockout mice (one mouse per group was excluded since there was only one cell surviving in those mice). The cells had dispersed inside the corpus

callosum in small groups along the entire way, mostly accumulating near the midsagittal level of the brain (on average 74 % of the cells within the corpus callosum). Several GFP-cells were seen in the wall of the lateral ventricle in half of the knockout mice at the four months follow-up point (**Figure 5.6**). A few cells were also found in the hilus of hippocampus, in the needletract, and in the superficial layer of the neocortex. None of the cells migrated a long way into the brain parenchyma, for example, into the thalamus or the other neocortical layers.

There was no clear difference between the distribution distance in rostro-caudal direction when comparing the two time points, one month and four months post-transplantation (p-value = 0.792). In addition, neither was there any clear difference in the distribution distance in the four months follow-up point between the knockout and the wild type mice (p-value = 0.800) (**Figure 5.4 B**). This comparison could not be done in the one month time point, since transplanted cells had not survived in the knockout mice. The maximal distance that the cells had spread in the rostro-caudal direction was 2.96 mm (the distance from the most rostral cells to the most caudal cells). The average distance for the distribution of cells was 1.2 ± 0.8 mm in rostro-caudal direction.

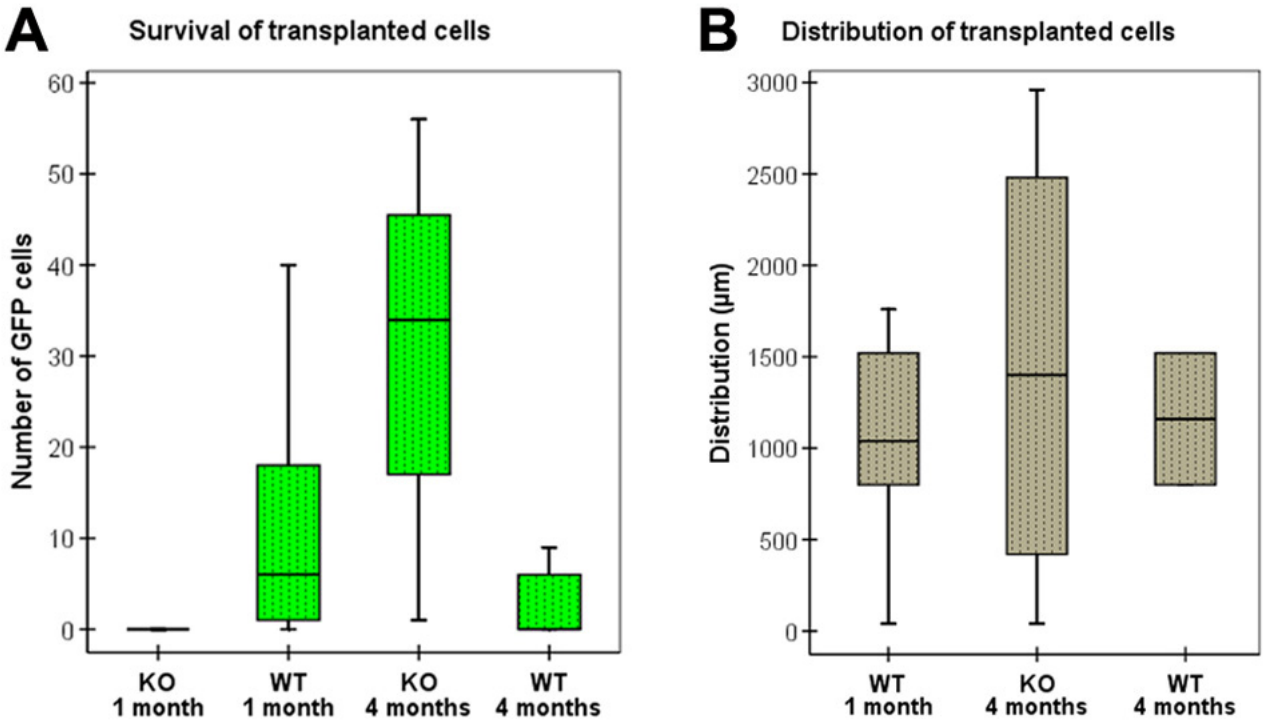

Figure 5.4 Survival (A) and distribution (B) of transplanted cells in box plot graphics. A) The scale on y-axis depicts the total amount of cells in the approximately 20 sections 240 µm apart from each other. The knockout mice aged five months show the best survival rate of the transplanted cells. **B)** Distribution is measured in the rostro-caudal axis. There were no significant differences in the distribution distance. Abbreviations: KO = hippocampus, WT = wild type.

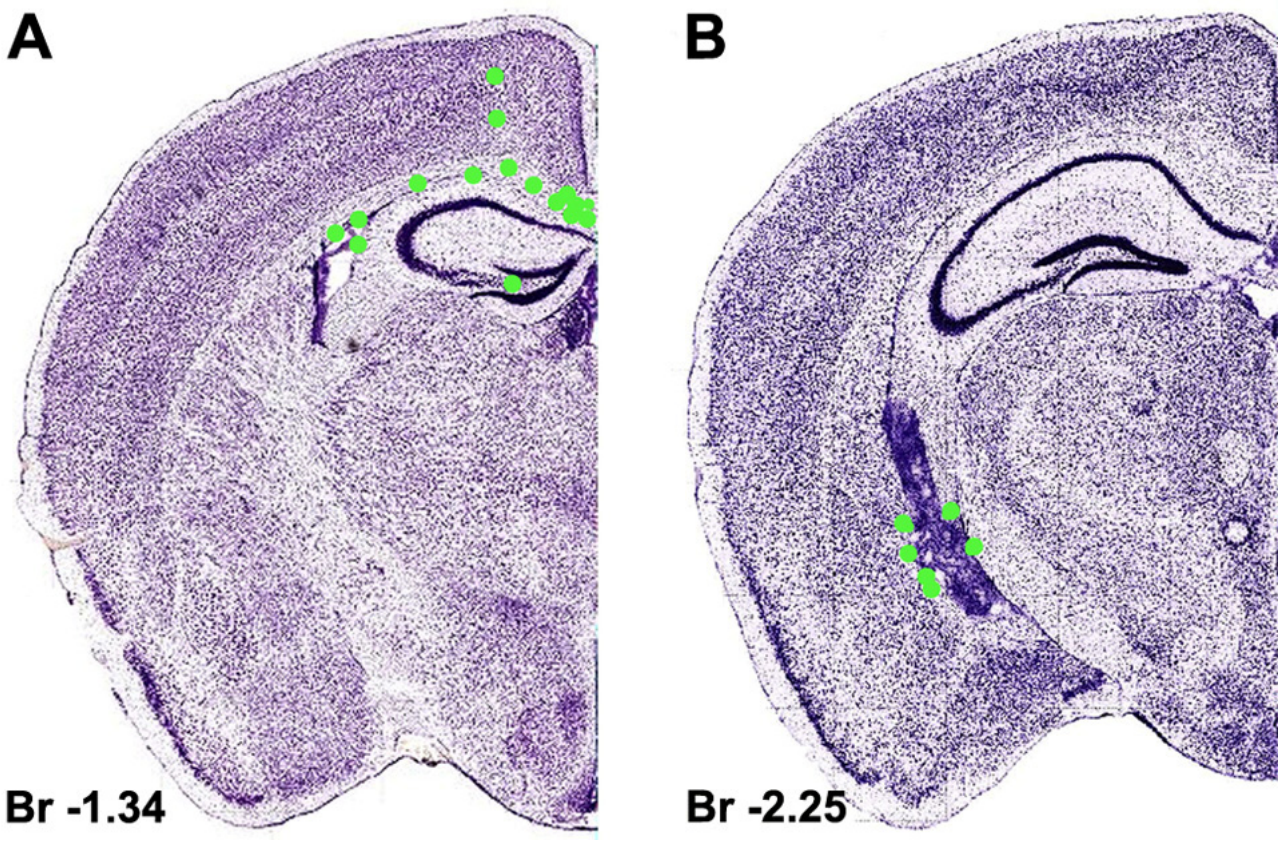

Figure 5.5 Major distribution patterns of survived NSCs. **A)** Most of the cells were found inside the corpus callosum and few also in the wall of the lateral ventricle, inside the hippocampus, and in the needletract. **B)** Few cells were found far away in the wall of the caudal lateral ventricle. Brain atlas pictures are taken and modified with permission from Allen Brain Atlas, www.brain-map.org. Abbreviations: Br = bregma level according to Paxinos: Mouse brain atlas, 2001.

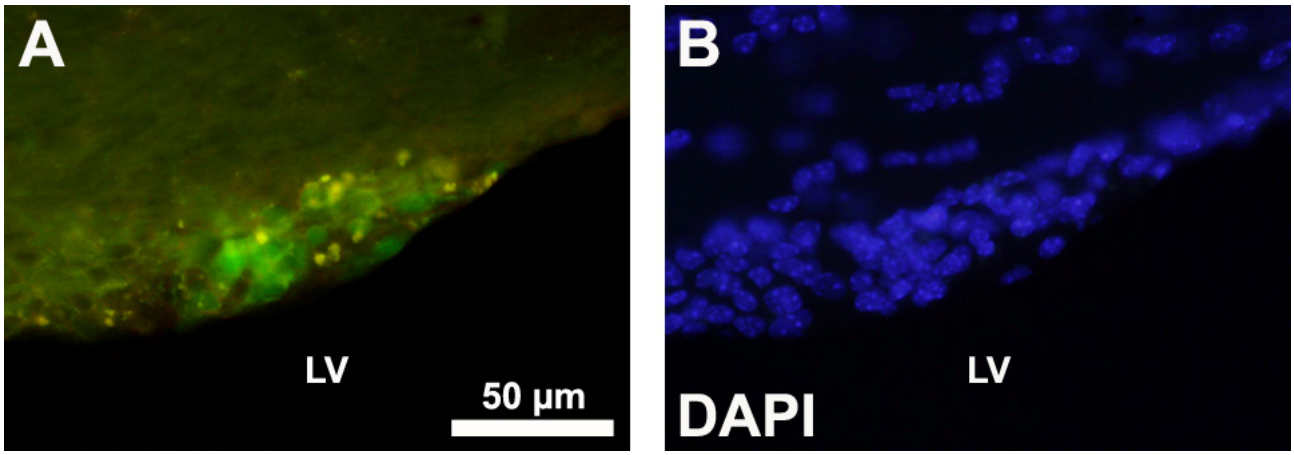

Figure 5.6 Distribution of transplanted cells into lateral ventricle. Some of the cells were seen in the wall of the lateral ventricle. Endogenous GFP fluorescence is shown in **(A)** and corresponding view with nuclear stain DAPI in **(B)**. Abbreviations: LV = lateral ventricle.

5.5. Differentiation of transplanted cells

The differentiation of transplanted cells was studied by immunohistochemical stainings. Surprisingly, almost none of the antibodies used (see **Table 4.4**) showed clear overlapping when studying the merged images of the specific staining and GFP-signal of the transplanted cells (**Figure 5.7 A-C**). Only once in the studied samples (with over 30 cells) one differentiation marker, a neurofilament marker NF-200, was found in a cell that had remained inside the hilus of the hippocampus (**Figure 5.7 D-F**).

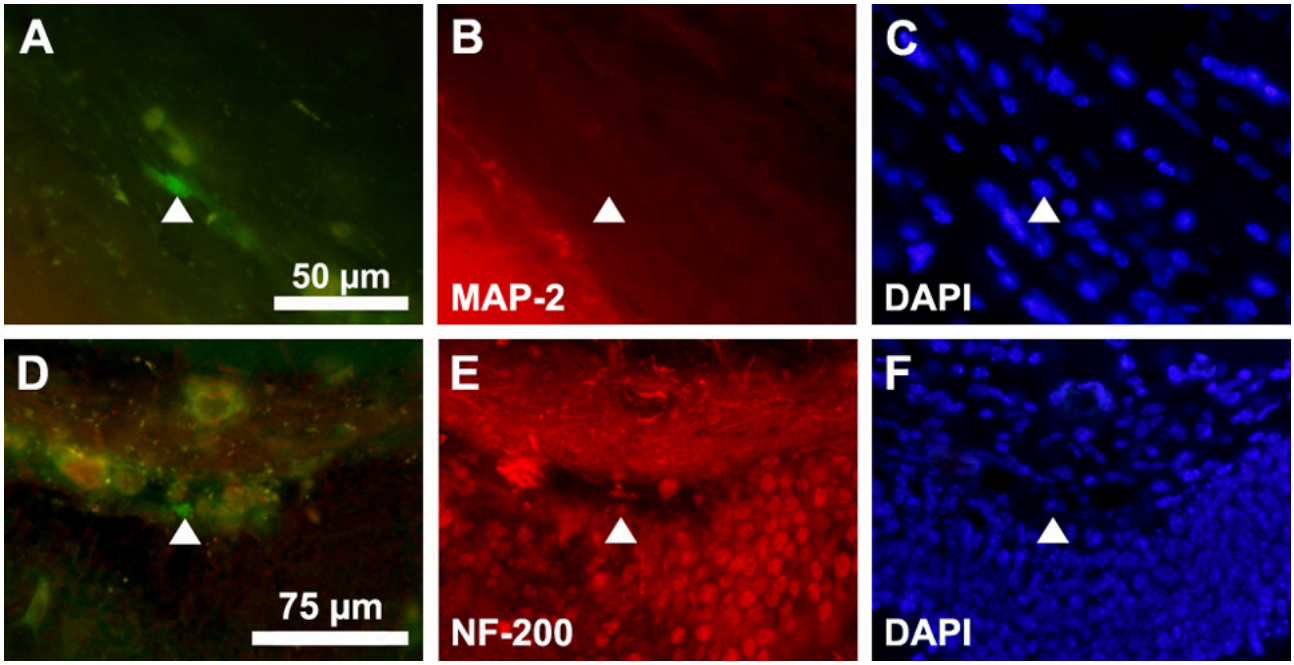

Figure 5.7 Differentiation of transplanted cells. A-C) Survived cell, showing a process, did not stain for MAP-2 (top apex of the white arrow head). The picture is taken from the corpus callosum. **D-F)** One transplanted cell showed overlapping with NF-200 staining (top apex of the white arrow head). The cell did not show any processes. The picture is taken from the hippocampus. Abbreviations: DAPI = nuclear stain, MAP-2 = microtubulus associated protein 2, NF-200 = neurofilament 200.

The morphological appearance of a small part of the cells showed clear features of neuronal morphology with long processes extending from one cell to another (**Figure 5.8**). This neuronal phenotype was mostly observed in the corpus callosum and the lateral ventricle, while in the hippocampus the morphology of surviving cells was always roundish with no clear processes.

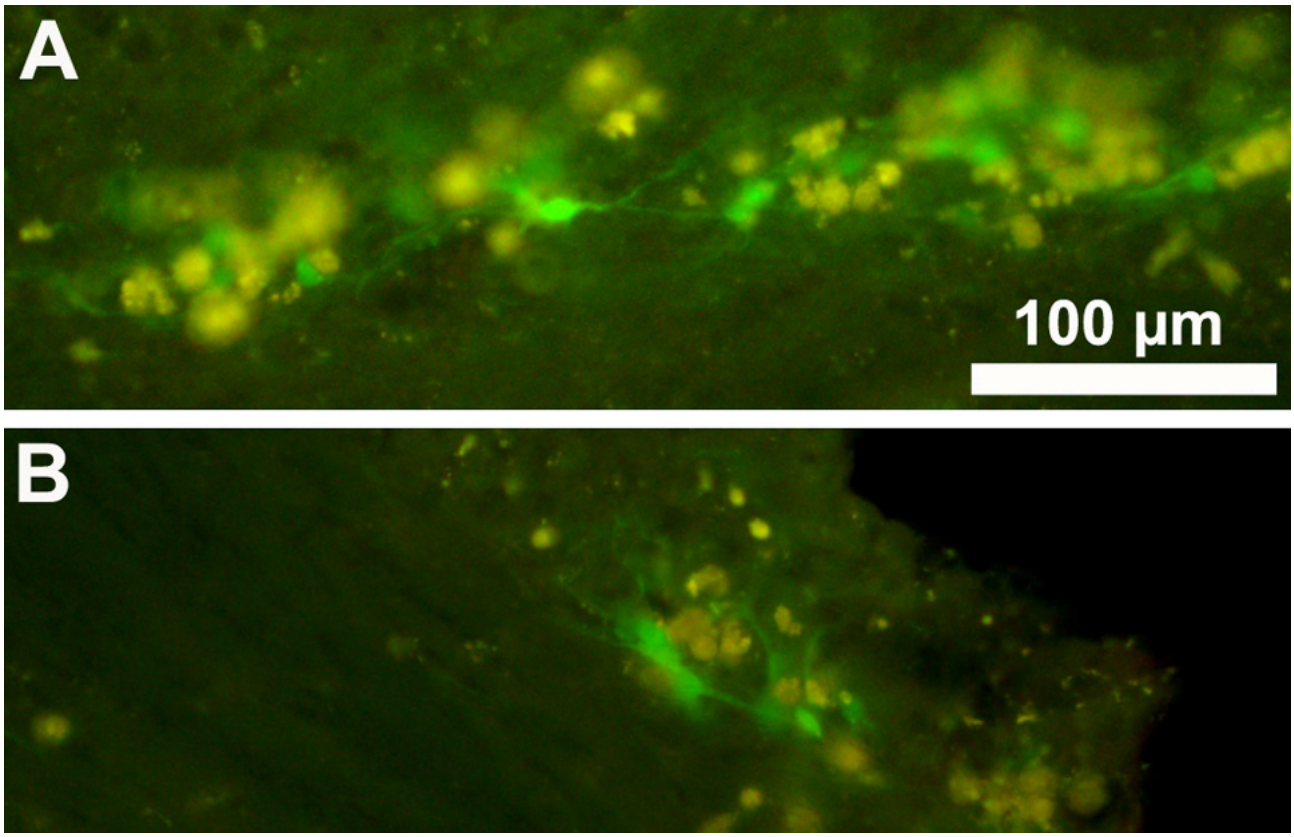

Figure 5.8 Morphological differentiation of transplanted neural stem cells. **A - B)** A part of the transplanted cells developed clear cellular lengthenings reminiscent of neural processes. Both pictures are taken from the corpus callosum.

5.6. Autofluorescent material next to transplanted cells

While studying the presence of GFP positive transplanted cells, clear additional autofluorescent material was observed in 46 out of the 48 cases analyzed. This material was in clumps and had the size and shape of roundish cells. It emitted yellow autofluorescence when excited with 488 nm wavelength. The material was mostly located throughout the corpus callosum, in the hilus of the hippocampus, in the first layer of neocortex, and in the needletract (**Figure 5.9**). It was nearly always associated with surviving transplanted cells. In addition, these cell-like objects were also found in mice that had received only PBS as a control injection. The additional autofluorescent material was named as golden cells and this term is used from this point onwards to depict the above described material.

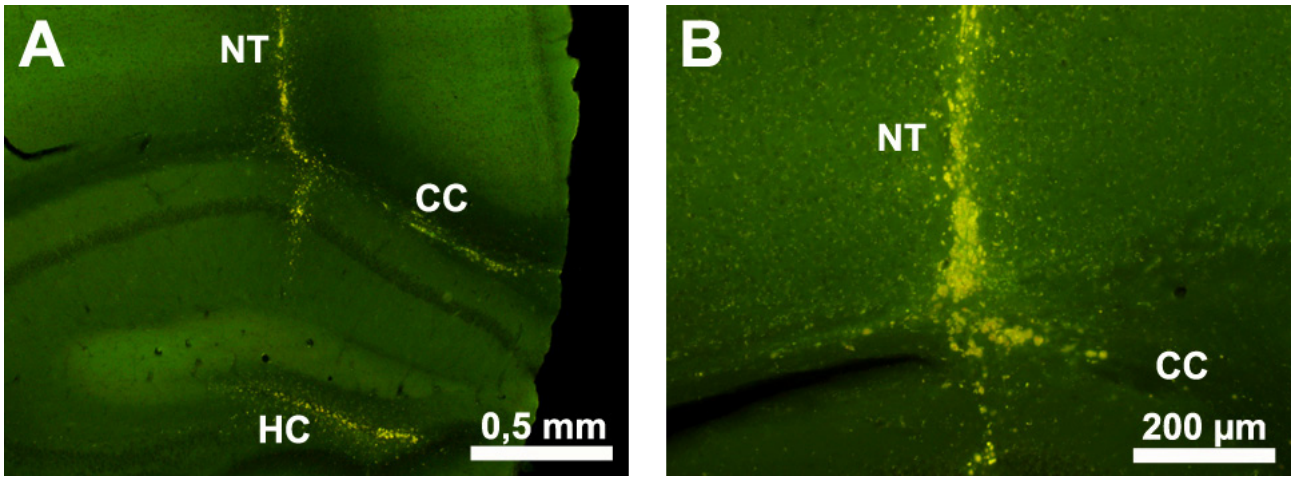

Figure 5.9 Presence of golden cells in **A)** knockout mouse that received neural stem cell transplantation, and in **B)** knockout mouse with only PBS injection. Abbreviations: CC = corpus callosum, HC = hippocampus, NT = needletract.

The amount of golden cells (see **Figure 5.10 A**) did not differ between the *Cln3*$^{-/-}$ mice strain and wild type mice, and between the age of two and five months, end dates of the study (data not shown). There was a significant increase in the golden cell number (p-value 0,000) in the mice (n = 15) belonging to a group with survival of the grafted cells compared to the mice (n = 24) receiving PBS (**Figure 5.10 B**). The knockout mouse group at one month time point receiving NSCs was excluded since there were no cells surviving.

The golden cells did not stain for markers GFAP (astrocytes), Ox-42 (microglia), or CD11b (macrophages, this staining was done by Anu Jalanko's group), nor did they show clear co-localization with markers used to study stem cell differentiation (**Table 4.4**). They did autofluoresce quite strongly also with 568 nm wavelength. Golden cells were often found to lack the nuclear stain DAPI, as seen in some areas of **Figure 5.11**. Prussian blue staining performed on some of the sections stained material reminiscent of golden cells and thus it could not be used for its proper meaning for detecting the transplanted cells (data not shown). The presence of golden cells was always associated with strong astrogliosis and this did not depend on the transplantation substance (**Figure 5.12**).

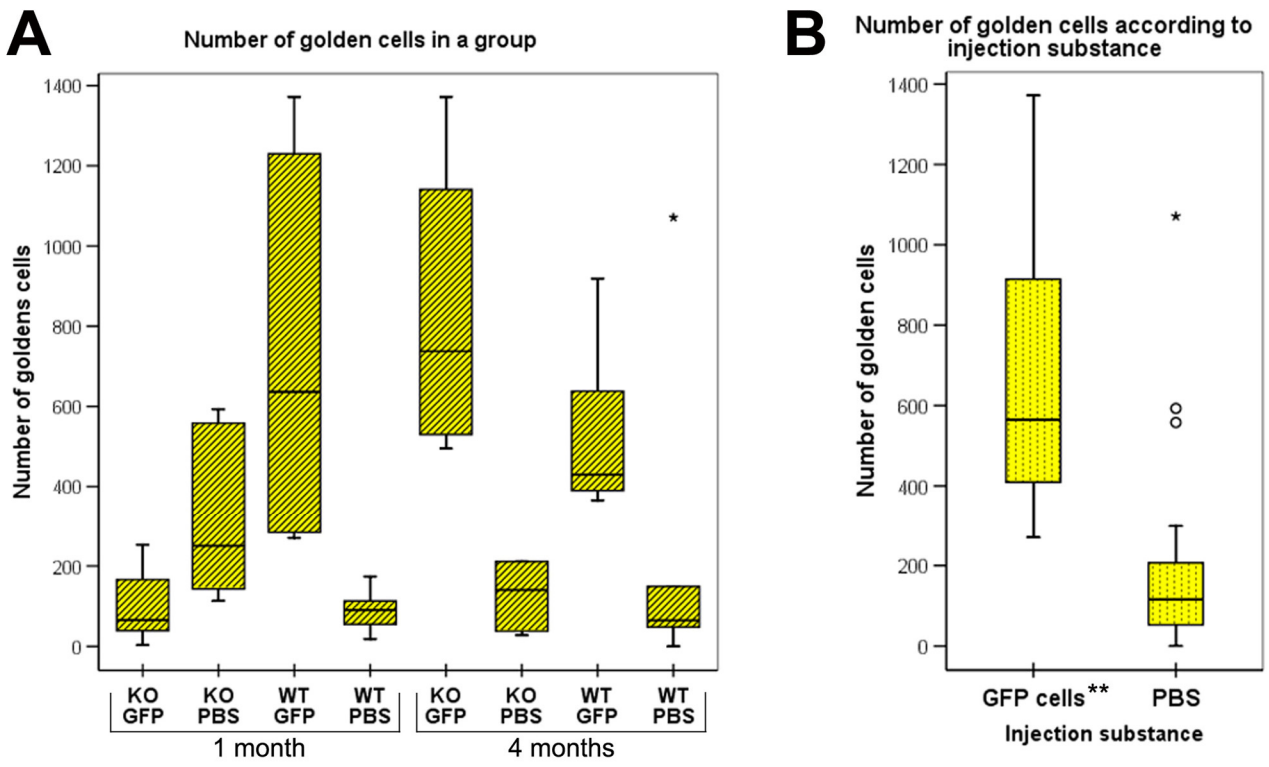

Figure 5.10 Statistics on number of golden cells as box plot graphics. A) Amount of golden cells per group. **B)** Amount of golden cells per injection substance: transplanted cells or control PBS. Abbreviations: KO = knockout, WT = wild type, * and ○ = outliers, ** = knockout group at the one month time point was excluded since there were no cells surviving.

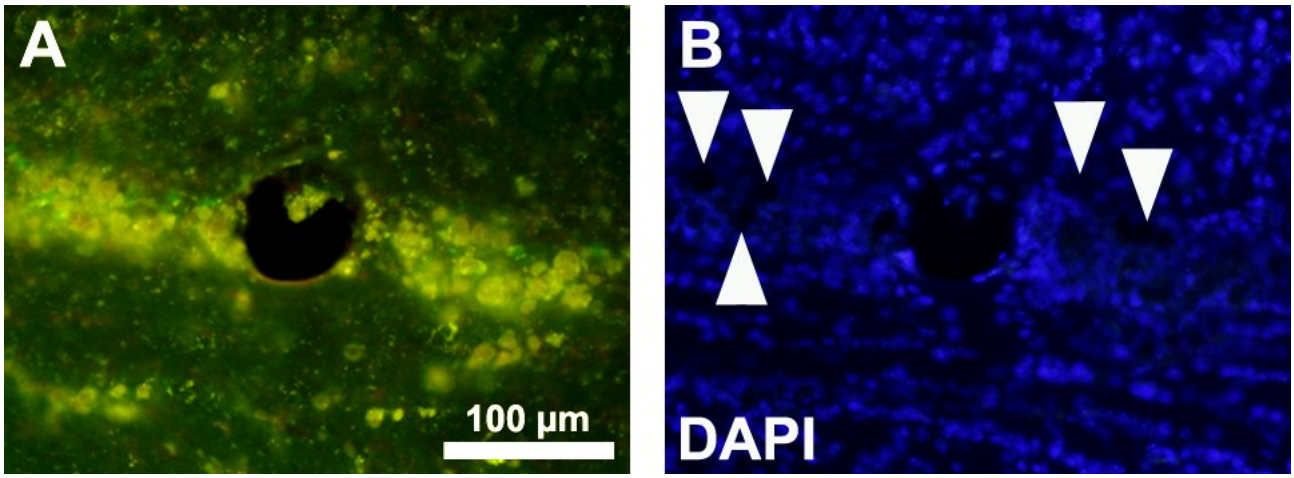

Figure 5.11 Appearance of golden cells. A - B) Golden cells near a vessel. Golden cells were on the size scale of cells, and areas of golden cells were often seen lacking DAPI (some of these areas shown by white arrow heads). Abbreviations: DAPI = nuclear stain.

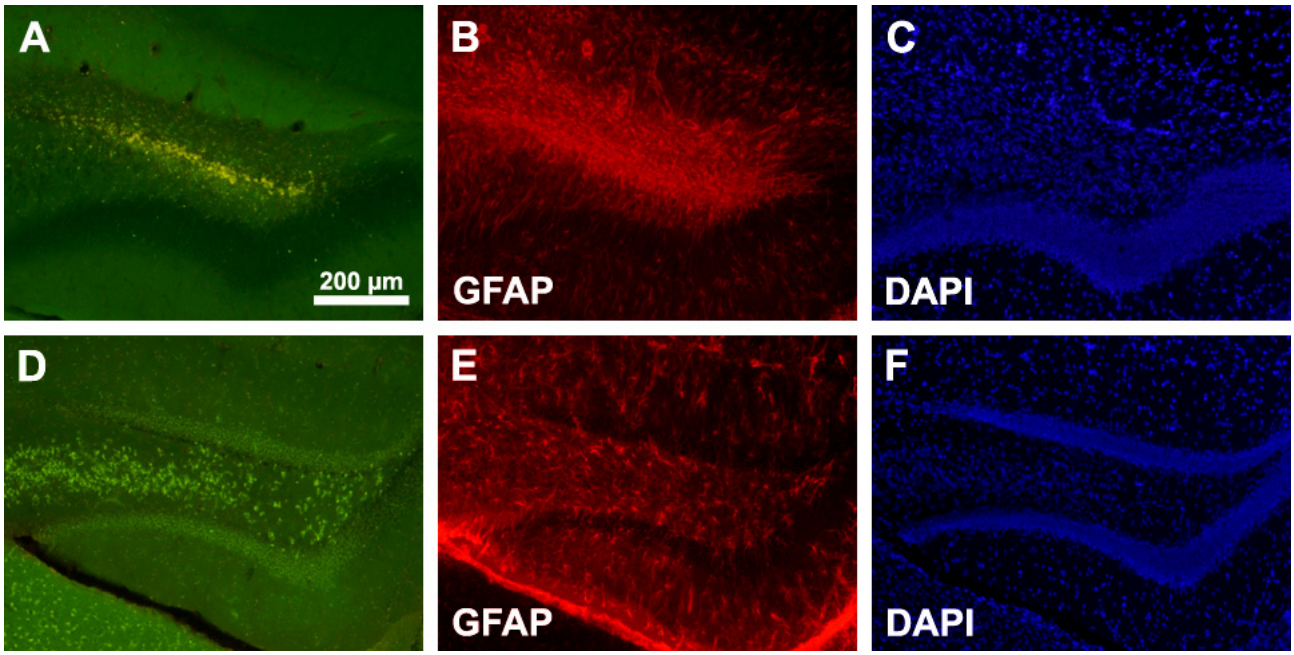

Figure 5.12 Strong astrogliosis accompanies the presence of golden cells. A - C) A clear accumulation of golden cells with the presence of strong astrogliosis is noted in the hilus of the hippocampus in a wild type mouse that had received GFP-cells. **D - F)** Staining for astrocytes is at its normal level in a knockout mouse with GFP-cells where no accumulation of golden cells is present in the hippocampus (yellow background autofluorescence is due to the disease manifestation). Both pictures are taken from hippocampus transplanted mice. The mouse background did not have an impact on the amount of astrogliosis. Abbreviations: GFAP = glial fibrillary acidic protein, an astrocytic marker; DAPI = nuclear stain.

5.7. Neuropathological findings

Semi-quantitative analysis of the neuropathological progression was made by examining the degree of background autofluorescence accumulation in piriform area (PIR), anterodorsal nucleus of the thalamus (AD), and the CA2-CA1 region of the hippocampal pyramidal cell layer.

The intensity of background autofluorescence was divided into five categories: 0 = none, 1 = faint, 2 = observable, 3 = clearly observable, 4 = massive (**Figure 5.13**). The category was given to each animal by first taking pictures for each mouse from the three above mentioned structures, and then comparing the acquired pictures with the category level pictures illustrated in **Figure 5.13**.

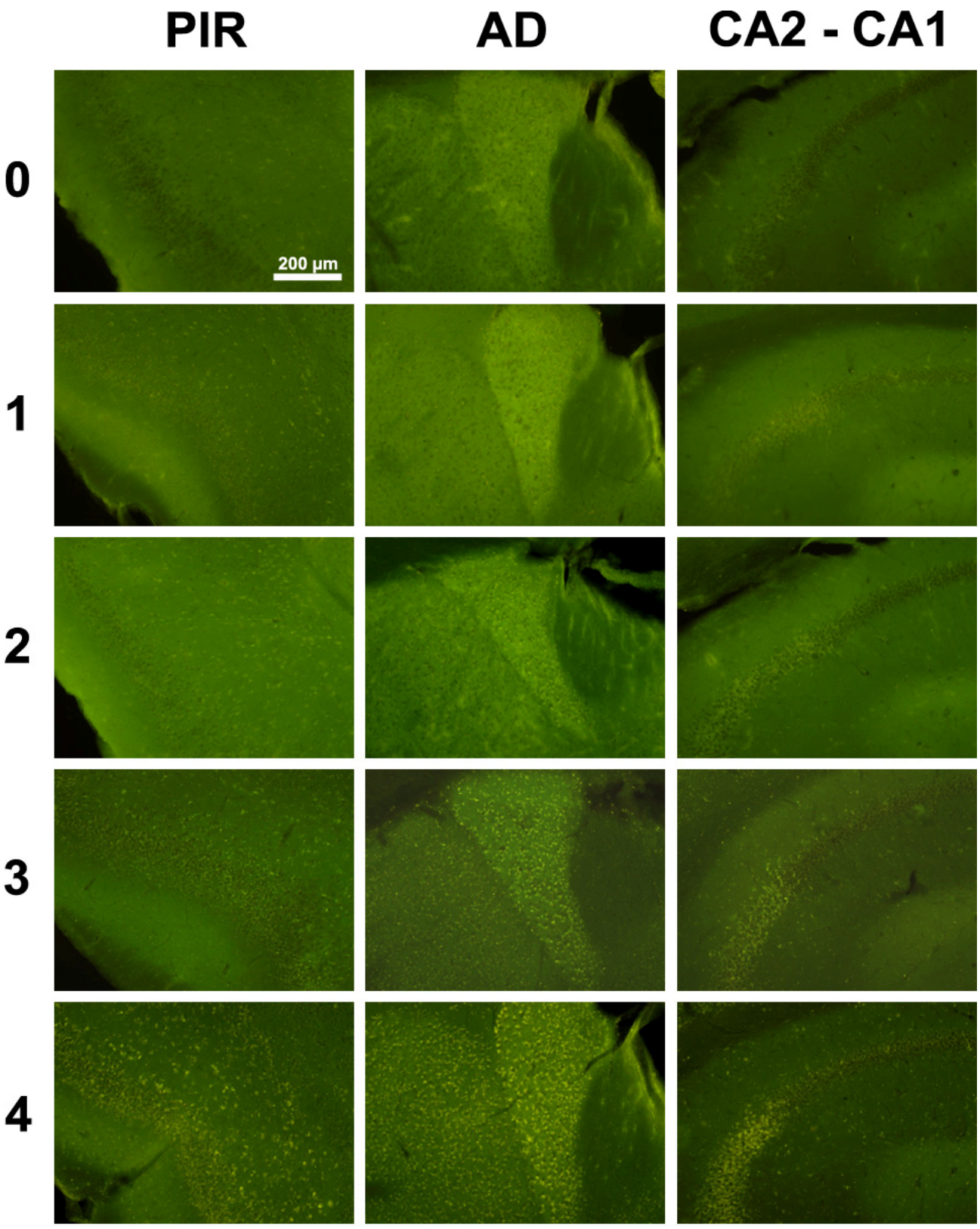

Figure 5.13 Categorization of the accumulation of autofluorescent storage material. Accumulation of the background autofluorescent material is categorized into five classes (shown on the rows): 0 = none, 1 = faint, 2 = observable, 3 = clearly observable, and 4 = massive. Abbreviations: AD = anterodorsal nucleus of the thalamus, CA2-CA1 = part of the pyramidal layers of the hippocampal formation, PIR = piriform area.

The categorization of accumulation resulted in a clear distinction between the two mouse groups (**Figure 5.14**). Already at the first time point, when the animals were two months old, there was a prominent background autofluorescence present in the knockout mouse. This accumulation was not ubiquitous throughout the brain, but was clear in the three structures studied and in many other distinct regions of the brain. By the age of five months, the second time point of the study, this accumulation had progressed in the knockout mice and was prominent in many structures of the brain. There was no visible reduction of background autofluorescence in the mice treated with stem cell transplantation.

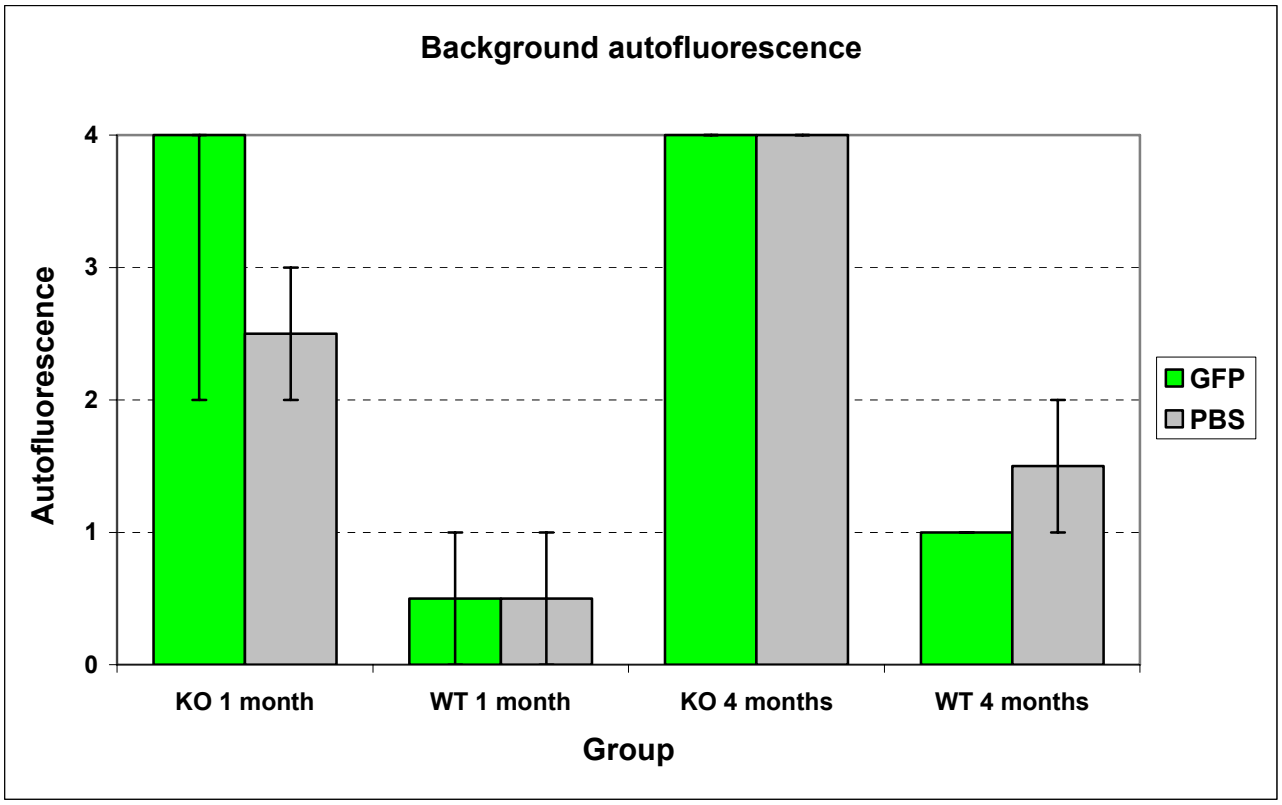

Figure 5.14 Background autofluorescence. The background autofluoresence was clearly stronger in the knockout mice than in wild type mice. Transplanted cells (GFP) did not reduce the accumulation of the autofluorescent material inside the brain. Bars represent the median amount of background autofluorescence and the error bars the minimum and maximum values inside the groups. KO = knockout, WT = wild type, 0 = none, 1 = faint, 2 = observable, 3 = clearly observable, 4 = massive.

6. DISCUSSION

6.1. Detection of transplanted cells

The reliable detection of transplanted cells is an essentially important issue in all transplantation studies. Thorough planning and optimization should be done on this issue to produce the most authentic results possible. Thus, a lot of attention was given also in this study for the confidence of the detection.

Magnetic resonance imaging, MRI, is a powerful tool for following the migration of the transplanted, iron labeled cells for a long time scale *in vivo*. The animals need to be only anesthetized during the detection and thus, the migration can be studied in one animal for multiple times at different time points. The resolution of MRI is fairly good but, of course, not on the scale of basic histochemical stainings. The sensitivity minimum of MRI detection is in the scale 500 labeled cells having the 4.7 T magnet used in this study (Magnitsky et al., 2005).

In the present study, MRI was used for two mice from groups receiving transplanted cells, and mice were sacrificed after the imaging. The results were on part convincing, on part troubling. In mice receiving hippocampal transplantation, MRI signal was detected in many areas of the brain, most strongly in the corpus callosum. This was also confirmed in histological analysis. The signal was also found in the cerebellum of two study animals *ex vivo*, but this was found to be a false positive finding since no signal was seen there in the stained sections. In these two mice, the perfusion was partly unsuccessful, and perhaps intracerebellar hematoma could have explained the false positive signal. All in all, MRI was found to be a good, fairly rough method for following the survival and migration of the transplanted cells. The procedure requires a lot of optimization of the parameters and can in fact result in false positive findings.

Green fluorescent protein –based labeling is a common method in the biomedical research field. The GFP-tag can be incorporated into the genome of an embryo to create transgenic animals, or transfected into cells *in vitro*. When using cells taken from a GFP-transgenic animal, as was done in the present study, the possibility of the molecule leaking to other cells is fairly small. GFP signal is

found in a majority of the cells derived form GFP-transgenic animals *in vitro* (Eriksson et al., 2003; observed also in this study). GFP-signal based detection has been used in numerous cell transplantation studies and is found to be valid for long periods of time, ranging to over one year (Priller et al., 2001). However, a disturbing comment is that the GFP-transgene is capable of showing only a portion of the transplanted cells in histological sections and can be down-regulated significantly between the two and four weeks post-grafting (Eriksson et al., 2003). GFP-based detection is, all in all, a widely used and trusted method.

In this case, the fact that GFP was visible in brain tissue without the use of antibody reduced the amount of work. The usage of antibodies can create false positive, unspecific staining which could have had great impact on the results, taking into account that the numbers of surviving cells were fairly small. GFP-signal was seen throughout the transplanted cells and was able to show the structure of processes, as well as the cell body, soma. Because the tissue sections were quite thick, namely 40 µm, the imaging of the cells was difficult, since the overall good focus level for the target area was hard to obtain. Use of a confocal microscope would have enabled more precise analysis.

Prussian blue staining is a method to verify the findings made with MRI. This staining is able to recognize the iron particles inside the cell and to produce a blue color for detection purposes. However, the staining proved to be an unreliable method of detection, since it showed clear staining in even those mice that had not received stem cell transplantation (data not shown). A clear blue color was seen inside what it appears to be golden cells, and if it had not been for the GFP label and good control cases, this finding would have been interpreted as a positive finding and the result would have looked totally different (the blinded fashion of the study). Thus, Prussian blue staining results should be always interpreted with the utmost care and rigorous controls should be employed in the transplantation studies.

Also many other staining methods are used to detect transplanted stem cells in the host CNS (Cai et al., 2003). Some have been documented to be partly unreliable, such as thymidine analogs (e.g. BrdU) that are incorporated into the DNA as the cells divide. Thymidine analogs have recently been found to be transferred from pre-labeled transplanted cells to host cells, thus making the identification between a host cell and a transplanted cell impossible (Burns et al., 2006). This finding has put a troubling question mark on the transplantation studies made with thymidine analog detection.

Taken all this together, the reliability of the detection method is highly important and cannot be highlighted enough. In this study, the use of multiple detection methods eased the interpretation and raised the confidence level of the results. Also the vast amount of controls was an important point to catch the false positive findings.

6.2. Survival of transplanted cells

Although there are promising reports on neural stem cell treatments in animal experiments, there are also few reports about the unsuccessful trials. In addition, most of the unsuccessful studies will never be published. The term "neural stem cells" covers a wide range of cells with different origins, characteristics, and intrinsic differentiation capabilities. Thus, it is clear that there will be a variety of results when using these cells in transplantation studies. The comparability between the published reports is quite poor already because of the different starting materials and the different recipients used.

The most significant limitation for neural stem cell transplantations and cell replacement therapies is that huge amount of transplanted cells are often lost very quickly after transplantation. The phenomenon was also apparent in this study where more than 95 % of the cells were lost within one month post-transplantation. Thereafter, there was no significant decrease in the survival of the cells. For stem cells to have an impact on disease progression, the level of destruction needs to be lowered drastically.

But what is the reason that the graft survival was so low? Clearly it was not the short life-time of neural stem cells, since it became obvious that NSCs can in fact survive for at the very least four months in the host brain. Neither was it because of the possible hostility in the brain of *Cln3*$^{-/-}$ mice, since there were no statistical differences between the survival in the wild type and the knockout mice at four months follow-up period. Unfortunately the comparison couldn't be made within the one month groups, since no cells had survived in the knockout mice. The graft destruction in this knockout group was more likely to occur from a technical rather than from a biological basis. The biological basis is unconvincing since the cells were able to survive for four months in the same brain environment transplanted at the same age. All of the animals in this group went through the

transplantation procedure at the same day, so most likely there was something wrong with the viability of the cells used at that day.

The cause of graft destruction as a whole is most likely due to the strong hostile environment where the cells are placed into. The brain was long thought to be an immunologically privileged organ, but recently this statement has been revised. The brain is actively under the control of immunological surveillance (Hickey, 2001). Perivascular microglia cells can function as antigen presenting cells in the brain and are probably critically important in graft rejection (Hickey, 2001). Pronounced microgliosis was not found in the animals in this study (data not shown), however, this step in the graft destruction could have settled down by the first observation point. Strong astrogliosis was detected accompanying the distribution of golden cells (see **Figure 5.12**), speaking on behalf of a strong immunological response after transplantation. Immunosuppression was not utilized in this study because of positive results in studies performed without immunosuppression (for example Lepore et al., 2006; Shihabuddin et al., 2004, Lee et al., 2007) and in addition, most of the immunosuppressive drugs are reported to be associated with different complications such as drug-related toxicities (Wobus and Boheler, 2005). However, in this study the advantages of immunosuppression might have been bigger than the disadvantages if it would have diminished the early graft destruction reaction.

The capillary blood-brain barrier (BBB) is a major factor in isolating the brain from the rest of the body. However, when a needle is placed through the brain parenchyma, the adjacent BBBs will inevitably be damaged, leaving the transplanted cells in contact with the circulating immune defense. The outside diameter of the needle used in this study was 0.47 mm, whereas in many other transplantation studies the diameter has been only half of that or less. Thus, the mechanical trauma to the brain parenchyma and BBB was quite big in this study compared to other studies.

Another possible explanation for the poor survival of the cells in this study might have been the quality of the cells. Data to back this up is that the cells were not able to survive well in any of the mice. The viability of the cells was checked prior to transplantation *in vitro* but this does not tell about the survival quality of the cells *in vivo*. A totally different survival capacity could have been found just by changing the origin of the neural stem cells for example to human embryonic stem cells.

The reason that the transplanted cells were not visible in the cortex-injected mice could lie, in addition to the immunological defense, in a pressure phenomenon. Even though the needle was withdrawn carefully and slowly, most of the cells could have escaped through the short and wide needletract outside the brain tissue (**Figure 4.1 A**), when the flexible tissue itself creates pressure to close the tract. Pronounced JNCL pathology in the cortex does not explain the absence of GFP-cells, since these cells were also not detected in the wild type mice. Because the preliminary results of cortex-injected mice looked discouraging, the focus was turned into the hippocampus-injected mice, where graft survival was detected.

It is important to find out why the majority of cells died, but it is also as important to know why a small portion of the cells survived. Are these survived cells somehow different in their capability to deal with the environmental hostility? The neurosphere culture produces a heterogeneous population of cells with different differentiation state and potential, and perhaps one population of these cells is the strongest to survive. Another explanation for this is partly covered in a publication from Johann et al. in 2006 (Johann et al., 2006). They show that NSCs survive much better when they are transplanted as neurospheres rather than single cell suspension. Perhaps the cells in the core of the neurosphere will be spared from the initial and fast hostility after injection, and only the cells on the surface of the sphere will be destroyed. When NSCs are transplanted as single cell suspension, they hardly have any support from the neighboring cells. In the present study, a small portion of the cells might have stayed in a neurosphere formation, thus, surviving better. All in all, for creating the best outcome in NSC treatments, the most endurable cell population able to differentiate and integrate functionally into the network of the CNS must be found and purified in sufficient amounts.

A lot of attention has been given to the stem cell nature of cancer cells in the brain (Singh et al., 2003). No teratoma formation was observed in this study and has extremely randomly been noticed in stem cell transplantation studies. Nevertheless, the capability of stem cells turning into cancer cells has to be studied with utmost precision before using them as a therapy.

The pathology in the *Cln3*$^{-/-}$ mice discussed in the literature review did not seem to have any effect on the survival of transplanted cells at the four months follow-up point. This gives confirmation that the neural stem cells could survive in the *Cln3*$^{-/-}$ brain and could thus be used as a part of a treatment option. However, based on this study, no benefit seemed to come out of the preliminary treatment with pure NSC transplantation.

6.2.1 The presence of golden cells

The presence of autofluorescent dense material in nearly all of the mice was obvious. This accumulation material was detected in the hilus of the hippocampus, throughout the corpus callosum, in the superficial layer of cortex, and in the needletract. The material, sized of a cell/cell clump, did not stain for markers for macrophages, astrocytes, or microglia, but did show autofluorescence with 488 nm and 568 nm wavelengths. The actual identity of this material remains unclear, since they were also seen not to contain the nuclear stain DAPI. The appearing absence of nuclei in the golden cells can result from the fact that the nuclei are pushed to the borders of the cells since the cytoplasm is filled with the autofluorescent material. The absence can also be due to the fact that golden cells are really not cells at all, but are some kind of necrotic debris.

The golden cells were always accompanied by the presence of reactive astrogliosis (**Figure 5.12**). Reactive astrogliosis with hypertrophy and possible glial scar formation is usually a consequence of trauma or inflammation, leading to assume that the golden cells could also be the result of a hostile event (Alonso, 2005). One possible explanation for the accumulation of golden cells is the mechanical trauma caused by the penetrating needle at the transplantation. The fact to back up this assumption is that golden cell accumulation is not progressive (**Figure 5.10 A**), speaking on behalf of a one-time event. However, if the golden cells were a product of the mechanical insult caused purely by the penetration of a needle, then one would assume the amount to be equal in all the mouse groups. The amount of golden cells was increased in mice treated with NSCs (p-value <0.05) and more specifically in those mice where the transplanted cells showed survival. Cells of the immune system have been found to produce neurotrophic factors and have neuroprotective effects (Hohlfeld et al., 2006). So whether the larger amount of golden cells is a reason for, or a consequence of the stem cell survival remains unclear.

The presence of accumulated autofluorescent material has been stated also in other transplantation studies, but is only briefly mentioned and has not been studied carefully. It seems that the accumulation of golden cells usually goes hand in hand with the rejection of the transplanted graft (Coyne et al., 2006; personal communication with Anna Hicks, MSc).

6.3. Distribution and differentiation of transplanted cells

One of the three primary goals in this study was to follow the migration of the transplanted NSCs. However, since the survival rate of the NSCs was so low and their expansion did not seem to represent a form of an active migration, a word distribution instead of migration is being used throughout the thesis.

Most of the distribution was observed along the corpus callosum, a track that connects the left and right cerebral hemispheres. The corpus callosum is a large fatty white matter structure filled with axonal projections. It has been shown that transplanted cells can travel within white matter tracks purely based on loaded pressure, such as that coming from the transplantation procedure (Lu et al., 2006). Most likely the distribution inside the corpus callosum in this study is also due to the fact that the cells have been pressured there where it is the easiest (the sparsest) for them to go. Serving as a proof for non-active migration is the fact that golden cells, most likely composed of inert debris, have the same kind of distribution pattern as the survived stem cells. However, the rostro-caudal distribution of the cells was actually rather good, compared to another study of migration done in an adult mouse (Watson et al., 2006).

Some of the cells in the knockout mice had interestingly dispersed, or in this case even migrated, into the lateral ventricle and more specifically into the subventricular zone, SVZ. This is a place for endogenous NSC production and can thereby offer a favorable environment for the transplanted NSCs. Unfortunately, even the environment of SVZ was not able to guide sufficient proliferation of the transplanted NSCs and the number of detected cells observed there was low. In addition, none of the surviving cells were able to migrate from the SVZ deeper into the brain tissue, again reflecting their impaired capability for migration.

For endogenous stem cells born in the SGZ of the hippocampus, the migration pathway is very short and happens within the dentate gyrus (Ming and Song, 2005). Thus, when NSCs are transplanted into the hippocampus, one would assume the highest probability of migration to happen inside the hippocampus, following the cues of the surrounding environment. However, only a few single cells remained in the hippocampus and they stayed inside the hilus.

Incomplete migration can be due to the non-permissive environment in an adult mouse and/or the lacking intrinsic properties of the transplanted cells. Migration has also been mentioned to be affected by the proteolysis of the cell surface proteins. Showed by Watson et al. in 2006, the trypsinized cells migrated further than the non-trypsinized cells still having their cell surface proteins and adhesion molecules on the plasma membrane (Watson et al., 2006). Even though the active migration in this study seems to be missing, it cannot be excluded. NSCs have been shown to migrate in chains sliding along each other and this might well be possible via the oriented processes inside the corpus callosum (Bonfanti, 2006). Migration within the corpus callosum has been stated in many other published studies but it remains to be shown if this migration is actually due to the pressure phenomenon as well.

The seemingly clear lack of differentiation of the cells in this study has been observed also elsewhere. In one example, differentiation of neural precursor cells was detected in animals transplanted *in utero*, but found to be missing when transplanted in adult animals (Sheen et al., 1999). The differentiation seems to be dependent on intrinsic developmental state and receptor competence to allow the responsiveness to the surrounding environmental cues. The cells used in this study seemed to differentiate into neurons based on their morphological appearance, although over half of the cells remained in a precursor-like state with no clear processes. However, when staining with different antibodies against known cell type specific markers, the transplanted cells showed no overlapping *ex vivo*. It was clear from the stainings *in vitro* that the cells used had the capability to differentiate into neuronal and glial lineages when exposed to permitting conditions. Also, the histological staining procedure could have failed but this was not the case since the endogenous neurons, neural progenitors, and astrocytes did stain very clearly when staining with NeuN, MAP-2 and NF-200, PSA-NCAM and Doublecortin, and GFAP, respectively, *ex vivo*. The obvious discrepancy between the staining results and the morphological features is surprising. This is, however, not exceptional because often only a small portion of the cells has been stated in the literature to stain for different markers. Further, because in this case only very few cells were surviving, the staining data was left negligible since it was hard to find even ten transplanted cells to study with a specific staining.

6.4. Suitability of stem cell treatment in JNCL

The suitability of NSC transplantation into a mouse model of JNCL was the key question in this study. The transplantations were targeted into the hippocampus and neocortex because they are the two regions mostly involved in mental retardation, which is also one of the symptoms in JNCL. In addition, they are both pathologically affected in JNCL, clear study targets histologically, and fairly easily accessible by transplantation. The hippocampus is also a place for endogenous neurogenesis in the adult brain and, interestingly, involved in the memory creation. The cortex is of great importance in many kinds of specialized functions. They proved, however, not to be ideal places for transplantation in JNCL, since the transplanted cells did not show sufficient survival or migration.

For global neurodegenerative diseases such as lysosomal storage diseases, the neural stem cells must be widely distributed throughout the CNS. This migration must take place in the absence of an overt pathological lesion. In addition, the survival of the cells must take place in a slightly hostile environment. This hostility did not seem to play a role in JNCL and the transplanted cells could survive equally well in knockout and in wild type mice for four months.

The storage material of JNCL is already identifiable in the prenatal period and the typical inclusion profiles have been reported in the first trimester (Scriver et al., 2001). This finding suggests that abnormal levels of accumulated material can be tolerated by the cells and tissues to a certain extent. If the process of accumulation could be halted or even reversed, the cells could sustain their functionality and the patients stay in an asymptomatic state. Stem cells could offer, in addition to neuroprotection, a way to get these accumulation halting agents in close proximity to neurons and to produce these agents for long periods of time. It would therefore be critical to start the NSC treatment as soon as possible so that the accumulation of the autofluorescent material has not exceeded the level when it becomes neurotoxic to the cells.

The comparison of autofluorescence accumulation between the treated and untreated disease-model showed that treatment did not end up with an improved situation in the brain. This comparison was nevertheless done on an unspecific and large scale, and was not able to detect any small changes possible in the treated animals. In addition, the neuropathological findings were only judged based on the accumulation of autofluorescence in three anatomically easily distinguishable brain structures and could not reveal any changes other than the ones being measured. In addition, the

amount of cells surviving was so negligible, that it would have been a miracle if there were a clear improvement in the histological status of the brain. A significantly improved level of survival needs to be attained to draw final conclusions on the suitability of stem cell treatment in JNCL. However, this study did reveal that the environment inside the *Cln3*$^{-/-}$ brain is as permissive as that of a wild type mouse, speaking on behalf of the possible suitability of stem cell transplantations in JNCL.

6.5. Future possibilities

When this project was first set out to take place, no previous reports existed on the neural stem cell transplantation experiments into animal models of neuronal ceroid-lipofuscinoses. While this project was ongoing, few promising articles on the subject were published shedding a light into the future of stem cell therapies in NCLs (Meyer et al., 2006; Taupin, 2006).

To improve the results gotten in this Master's thesis, one could use several different approaches and their combinations. The choice for the neural stem cell type can be revised. Human embryonic stem cells offer one interesting possibility since they provide an appropriate cell type for treatment also in humans, a goal that must be kept in mind. They have, for example, been found to differentiate and form synapses even after nine months of transplantation in immunodeficient mice when transplanted neonatally (Guillaume et al., 2006)

The seemingly better way of grafting the cells might be in small neurospheres rather than as a single cell suspension as shown by Johann et al. (Johann et al., 2006). PBS has been successfully used in many transplantation studies as the medium which the cells are given in, but perhaps it was the one that caused rejection in this study. After all, the golden cells were clearly visible also in mice receiving control injections with pure PBS. An earlier time point for transplantation could improve the survival and migration of the transplanted cells. In that case, the results would get harder to interpret since the transplantation in humans could be made only after the diagnosis which is usually done when the brain has already grossly developed. The neonatal brain is much more plastic and stimulatory for the NSCs. Thus, the result would most likely be much more promising than what would be expected from real treatments. However, one always has to start from somewhere and this would perhaps be the best way to do that.

The transplantation procedure could also be changed. First of all, the needle used should be thinner to diminish the mechanical trauma caused in the brain. This would most certainly mean better survival chances for the NSCs, but, at the same time, difficulties in the transplantation procedure since the cells would easily clog the flow in the thin needle. Second, the transplantation could be made into the lateral ventricles as many have shown them to function well as a transplantation place. From there, the cells have an excellent possibility to spread throughout the brain. The penetration into the brain parenchyma from the liquid filled cavities can, however, be more difficult than if the cells were placed straight into the parenchyma. Third, the number of cells grafted can be altered, and, in this case, increasing the number would be most beneficial.

As seen from above, a lot of optimization can be made to improve the results gotten in this research project. On the other hand, even though one could increase the survival of the grafted cells, what is the likelihood that it will relieve the status of the disease? Cell replacement therapy alone is hardly to be sufficient in JNCL since the destruction inside the brain is so far spread. The missing functional CLN3 protein can not be transported from the genetically engineered stem cells to diseased cells because it is a transmembrane protein, not a soluble one. This is by far the biggest difference in the prospective benefits of NSC treatment between the JNCL, and INCL and LINCL, where the missing enzymes are soluble and have been successfully transferred from the genetically engineered neural stem cells to the endogenous neurons *in vivo* (Taupin, 2006). One of the best future prospects of the NSCs in JNCL is for them to function as a genetically engineered vehicle for an agent capable of stopping or reversing the disease progression while at the same time offering neuroprotective signals for the surrounding cells.

7. CONCLUSIONS

This study described for the first time the transplantation of neural stem cells into a mouse model of juvenile neuronal ceroid-lipofuscinosis. The thesis was set out to answer three main questions that dealed with the survival, migration, and differentiation capability of the transplanted cells *in vivo*.

1. The cortex-grafted cells showed no survival. The survival of the hippocampus-grafted cells was poor, with the best survival rates ranging up to about 3 %. The survival of the cells was not dependent upon the mouse strain. Thus, the environment inside the *Cln3*$^{-/-}$ brain is not a constraint on the survival of the NSCs.

2. Most of the surviving cells were detected in the corpus callosum, while occasional cells were seen in the hippocampus, in the needletract, in the superficial layer of the cortex, and in the wall of the lateral ventricle. The expansion of the cells reminded more of a passive distribution pattern rather than an active migration. The migration capacity of the cells was considered therefore to be low.

3. The NSCs had capability to differentiate both into neurons and astrocytes *in vitro*. The differentiation of the grafted cells was studied with numerous markers *in vivo*, but they did not show overlapping with the transplanted stem cells. Morphologically, the cells were suggestive of both neurons and precursor cells. In conclusion, the transplanted cells did not have all the characteristics of normal CNS cells, but did seem to be capable of forming neuron-like cells on a morphological basis.

The NSC graft did not affect the rough development of the neuropathological situation. However, a lot better survival rate needs to be attained to make a conclusion about the effect of NSC transplantation on the neuropathological progression. As a conclusion, the disease model environment did not impair the behavior of transplanted cells, and the cells were able to stay alive there for several months. This gives hope for the future treatment of JNCL with stem cell -based therapies.

8. REFERENCES

Ader M, Meng J, Schachner M, Bartsch U. Formation of myelin after transplantation of neural precursor cells into the retina of young postnatal mice. Glia 2000; 30: 301-10.

Allen Brain Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science. © 2004–2008 [cited 30th December 2007]. Available from: http://www.brain-map.org

Alonso G. NG2 proteoglycan-expressing cells of the adult rat brain: possible involvement in the formation of glial scar astrocytes following stab wound. Glia 2005; 49: 318-38.

Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 1965; 124: 319-35.

Alvarez-Buylla A, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. J Neurosci 2002; 22: 629-34.

Baldwin S, Beal P, Yao S, King A, Cass C, Young J. The equilibrative nucleoside transporter family, SLC29. Pfugers Arch 2004; 447: 735-743.

Barker RA, Widner H. Immune problems in central nervous system cell therapy. NeuroRx 2004; 1: 472-81.

Bear MF, Connors BW, Paradiso MA. Neuroscience: exploring the brain. Baltimore, Md.: Lippincott Williams & Wilkins, 2001.

Belmadani A, Tran PB, Ren D, Miller RJ. Chemokines regulate the migration of neural progenitors to sites of neuroinflammation. J Neurosci 2006; 26: 3182-91.

Ben-Hur T, van Heeswijk RB, Einstein O, Aharonowiz M, Xue R, Frost EE, et al. Serial in vivo MR tracking of magnetically labeled neural spheres transplanted in chronic EAE mice. Magn Reson Med 2007; 57: 164-71.

Bjorklund A, Dunnett SB, Brundin P, Stoessl AJ, Freed CR, Breeze RE, et al. Neural transplantation for the treatment of Parkinson's disease. Lancet Neurol 2003; 2: 437-45.

Boldrini R, Biselli R, Santorelli FM, Bosman C. Neuronal ceroid lipofuscinosis: an ultrastructural, genetic, and clinical study report. Ultrastruct Pathol 2001; 25: 51-8.

Bonfanti L. PSA-NCAM in mammalian structural plasticity and neurogenesis. Prog Neurobiol 2006; 80: 129-64.

Brustle O, Choudhary K, Karram K, Huttner A, Murray K, Dubois-Dalcq M, et al. Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats. Nat Biotechnol 1998; 16: 1040-4.

Buc-Caron MH. Neuroepithelial progenitor cells explanted from human fetal brain proliferate and differentiate in vitro. Neurobiol Dis 1995; 2: 37-47.

Buchet D, Serguera C, Zennou V, Charneau P, Mallet J. Long-term expression of beta-glucuronidase by genetically modified human neural progenitor cells grafted into the mouse central nervous system. Mol Cell Neurosci 2002; 19: 389-401.

Bulte JW, Ben-Hur T, Miller BR, Mizrachi-Kol R, Einstein O, Reinhartz E, et al. MR microscopy of magnetically labeled neurospheres transplanted into the Lewis EAE rat brain. Magn Reson Med 2003; 50: 201-5.

Burns TC, Ortiz-Gonzalez XR, Gutierrez-Perez M, Keene CD, Sharda R, Demorest ZL, et al. Thymidine analogs are transferred from prelabeled donor to host cells in the central nervous system after transplantation: a word of caution. Stem Cells 2006; 24: 1121-7.

Cai J, Limke TL, Ginis I, Rao MS. Identifying and tracking neural stem cells. Blood Cells Mol Dis 2003; 31: 18-27.

Cameron HA, McKay RD. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol 2001; 435: 406-17.

Carleton A, Petreanu LT, Lansford R, Alvarez-Buylla A, Lledo PM. Becoming a new neuron in the adult olfactory bulb. Nat Neurosci 2003; 6: 507-18.

Carpenter MK, Cui X, Hu ZY, Jackson J, Sherman S, Seiger A, et al. In vitro expansion of a multipotent population of human neural progenitor cells. Exp Neurol 1999; 158: 265-78.

Carpenter MK, Winkler C, Fricker R, Emerich DF, Wong SC, Greco C, et al. Generation and transplantation of EGF-responsive neural stem cells derived from GFAP-hNGF transgenic mice. Exp Neurol 1997; 148: 187-204.

Chalmers-Redman RM, Priestley T, Kemp JA, Fine A. In vitro propagation and inducible differentiation of multipotential progenitor cells from human fetal brain. Neuroscience 1997; 76: 1121-8.

Chattopadhyay S, Ito M, Cooper JD, Brooks AI, Curran TM, Powers JM, et al. An autoantibody inhibitory to glutamic acid decarboxylase in the neurodegenerative disorder Batten disease. Hum Mol Genet 2002a; 11: 1421-31.

Chattopadhyay S, Kriscenski-Perry E, Wenger DA, Pearce DA. An autoantibody to GAD65 in sera of patients with juvenile neuronal ceroid lipofuscinoses. Neurology 2002b; 59: 1816-7.

Cooper J, Russell C, Mitchison H. Progress towards understanding disease mechanisms in small vertebrate models of neuronal ceroid-lipofsucinosis. Biocim Biophys Acta 2006; 1762: 873-889.

Cotman SL, Vrbanac V, Lebel LA, Lee RL, Johnson KA, Donahue LR, et al. Cln3(Deltaex7/8) knock-in mice with the common JNCL mutation exhibit progressive neurologic disease that begins before birth. Hum Mol Genet 2002; 11: 2709-21.

Coyne TM, Marcus AJ, Woodbury D, Black IB. Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. Stem Cells 2006; 24: 2483-92.

Ding S, Messam CA, Li P, Selzer ME, Dichter MA, Haydon PG. Murine brain progenitor cells have the ability to differentiate into functional neurons and integrate into the CNS. Cell Transplant 2006; 15: 699-710.

Dyken PR. Reconsideration of the classification of the neuronal ceroid-lipofuscinoses. Am J Med Genet Suppl 1988; 5: 69-84.

Englund U, Bjorklund A, Wictorin K. Migration patterns and phenotypic differentiation of long-term expanded human neural progenitor cells after transplantation into the adult rat brain. Brain Res Dev Brain Res 2002; 134: 123-41.

Eriksson C, Bjorklund A, Wictorin K. Neuronal differentiation following transplantation of expanded mouse neurosphere cultures derived from different embryonic forebrain regions. Exp Neurol 2003; 184: 615-35.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, et al. Neurogenesis in the adult human hippocampus. Nat Med 1998; 4: 1313-7.

Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billinghurst LL, et al. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. Nat Biotechnol 1998; 16: 1033-9.

Fricker RA, Carpenter MK, Winkler C, Greco C, Gates MA, Bjorklund A. Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. J Neurosci 1999; 19: 5990-6005.

Friocourt G, Koulakoff A, Chafey P, Boucher D, Fauchereau F, Chelly J, et al. Doublecortin functions at the extremities of growing neuronal processes. Cereb Cortex 2003; 13: 620-6.

Gachet Y, Codlin S, Hyams JS, Mole SE. btn1, the Schizosaccharomyces pombe homologue of the human Batten disease gene CLN3, regulates vacuole homeostasis. J Cell Sci 2005; 118: 5525-36.

Ginis I, Luo Y, Miura T, Thies S, Brandenberger R, Gerecht-Nir S, et al. Differences between human and mouse embryonic stem cells. Dev Biol 2004; 269: 360-80.

Golabek AA, Kida E, Walus M, Kaczmarski W, Michalewski M, Wisniewski KE. CLN3 protein regulates lysosomal pH and alters intracellular processing of Alzheimer's amyloid-beta protein precursor and cathepsin D in human cells. Mol Genet Metab 2000; 70: 203-13.

Guillaume DJ, Johnson MA, Li XJ, Zhang SC. Human embryonic stem cell-derived neural precursors develop into neurons and integrate into the host brain. J Neurosci Res 2006; 84: 1165-76.

Haltia M. The neuronal ceroid-lipofuscinoses. J Neuropathol Exp Neurol 2003; 62: 1-13.

Haltia M. The neuronal ceroid-lipofuscinoses: from past to present. Biochim Biophys Acta 2006; 1762: 850-6.

Haskell RE, Carr CJ, Pearce DA, Bennett MJ, Davidson BL. Batten disease: evaluation of CLN3 mutations on protein localization and function. Hum Mol Genet 2000; 9: 735-44.

Hastings NB, Gould E. Rapid extension of axons into the CA3 region by adult-generated granule cells. J Comp Neurol 1999; 413: 146-54.

Haydar TF, Wang F, Schwartz ML, Rakic P. Differential modulation of proliferation in the neocortical ventricular and subventricular zones. J Neurosci 2000; 20: 5764-74.

Hickey WF. Basic principles of immunological surveillance of the normal central nervous system. Glia 2001; 36: 118-24.

Hobert JA, Dawson G. Neuronal ceroid lipofuscinoses therapeutic strategies: past, present and future. Biochim Biophys Acta 2006; 1762: 945-53.

Hohlfeld R, Kerschensteiner M, Stadelmann C, Lassmann H, Wekerle H. The neuroprotective effect of inflammation: implications for the therapy of multiple sclerosis. Neurol Sci 2006; 27 Suppl 1: S1-7.

Holopainen JM, Saarikoski J, Kinnunen PK, Jarvela I. Elevated lysosomal pH in neuronal ceroid lipofuscinoses (NCLs). Eur J Biochem 2001; 268: 5851-6.

Hovatta O. Derivation of human embryonic stem cell lines, towards clinical quality. Reprod Fertil Dev 2006; 18: 823-8.

International Batten Disease Consortium. Isolation of a novel gene underlying Batten disease, CLN3. The International Batten Disease Consortium. Cell 1995; 82: 949-57.

Itsykson P, Ilouz N, Turetsky T, Goldstein RS, Pera MF, Fishbein I, et al. Derivation of neural precursors from human embryonic stem cells in the presence of noggin. Mol Cell Neurosci 2005; 30: 24-36.

Jin K, Peel AL, Mao XO, Xie L, Cottrell BA, Henshall DC, et al. Increased hippocampal neurogenesis in Alzheimer's disease. Proc Natl Acad Sci U S A 2004; 101: 343-7.

Jin K, Sun Y, Xie L, Mao XO, Childs J, Peel A, et al. Comparison of ischemia-directed migration of neural precursor cells after intrastriatal, intraventricular, or intravenous transplantation in the rat. Neurobiol Dis 2005; 18: 366-74.

Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci U S A 2002; 99: 11946-50.

Johann V, Schiefer J, Sass C, Mey J, Brook G, Kruttgen A, et al. Time of transplantation and cell preparation determine neural stem cell survival in a mouse model of Huntington's disease. Exp Brain Res 2006.

Järvelä I, Autti T, Lamminranta S, Aberg L, Raininko R, Santavuori P. Clinical and magnetic resonance imaging findings in Batten disease: analysis of the major mutation (1.02-kb deletion). Ann Neurol 1997; 42: 799-802.

Järvelä I, Sainio M, Rantamaki T, Olkkonen VM, Carpen O, Peltonen L, et al. Biosynthesis and intracellular targeting of the CLN3 protein defective in Batten disease. Hum Mol Genet 1998; 7: 85-90.

Katz ML, Gao CL, Prabhakaram M, Shibuya H, Liu PC, Johnson GS. Immunochemical localization of the Batten disease (CLN3) protein in retina. Invest Ophthalmol Vis Sci 1997; 38: 2375-86.

Katz ML, Shibuya H, Liu PC, Kaur S, Gao CL, Johnson GS. A mouse gene knockout model for juvenile ceroid-lipofuscinosis (Batten disease). J Neurosci Res 1999; 57: 551-6.

Ke Y, Chi L, Xu R, Luo C, Gozal D, Liu R. Early response of endogenous adult neural progenitor cells to acute spinal cord injury in mice. Stem Cells 2006; 24: 1011-9.

Kettenmann H, Ransom BR. Neuroglia. Oxford ; New York: Oxford University Press, 2005.

Kim Y, Ramirez-Montealegre D, Pearce DA. A role in vacuolar arginine transport for yeast Btn1p and for human CLN3, the protein defective in Batten disease. Proc Natl Acad Sci U S A 2003; 100: 15458-62.

Kovacs AD, Weimer JM, Pearce DA. Selectively increased sensitivity of cerebellar granule cells to AMPA receptor-mediated excitotoxicity in a mouse model of Batten disease. Neurobiol Dis 2006; 22: 575-85.

Kremmidiotis G, Lensink IL, Bilton RL, Woollatt E, Chataway TK, Sutherland GR, et al. The Batten disease gene product (CLN3p) is a Golgi integral membrane protein. Hum Mol Genet 1999; 8: 523-31.

Kriscenski-Perry E, Applegate CD, Serour A, Mhyre TR, Leonardo CC, Pearce DA. Altered flurothyl seizure induction latency, phenotype, and subsequent mortality in a mouse model of juvenile neuronal ceroid lipofuscinosis/batten disease. Epilepsia 2002; 43: 1137-40.

Kyttälä A, Lahtinen U, Braulke T, and Hofmann SL. Functional biology of the neuronal ceroid-lipofuscinoses (NCL) proteins. Biochim Biophys Acta 2006; 1762: 920-933.

Lake BD, Steward CG, Oakhill A, Wilson J, Perham TG. Bone marrow transplantation in late infantile Batten disease and juvenile Batten disease. Neuropediatrics 1997; 28: 80-1.

Lee HY, Hwang IK, Koh US, Lee JC, Kim DH, Kim JH, et al. Chronological changes of neurofilament 200 kDa immunoreactivity in the lateral olfactory tract after transient forebrain ischemia in gerbils. Brain Res 2005; 1036: 202-7.

Lee JP, Jeyakumar M, Gonzalez R, Takahashi H, Lee PJ, Baek RC, et al. Stem cells act through multiple mechanisms to benefit mice with neurodegenerative metabolic disease. Nat Med 2007; 13: 439-47.

Lennington JB, Yang Z, Conover JC. Neural stem cells and the regulation of adult neurogenesis. Reprod Biol Endocrinol 2003; 1: 99.

Lepore AC, Neuhuber B, Connors TM, Hans SS, Liu Y, Daniels MP, et al. Long-term fate of neural precursor cells following transplantation into developing and adult CNS. Neuroscience 2006; 142: 287-304.

Lie DC, Song H, Colamarino SA, Ming GL, Gage FH. Neurogenesis in the adult brain: new strategies for central nervous system diseases. Annu Rev Pharmacol Toxicol 2004; 44: 399-421.

Louissaint A, Jr., Rao S, Leventhal C, Goldman SA. Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron 2002; 34: 945-60.

Lu P, Yang H, Culbertson M, Graham L, Roskams AJ, Tuszynski MH. Olfactory ensheathing cells do not exhibit unique migratory or axonal growth-promoting properties after spinal cord injury. J Neurosci 2006; 26: 11120-30.

Luiro K, Kopra O, Lehtovirta M, Jalanko A. CLN3 protein is targeted to neuronal synapses but excluded from synaptic vesicles: new clues to Batten disease. Hum Mol Genet 2001; 10: 2123-31.

Luiro K, Yliannala K, Ahtiainen L, Maunu H, Järvelä I, Kyttala A, Jalanko A. Interconnections of CLN3, Hook1 and Rab proteins link Batten disease to defects in the endocytic pathway. Human Mol Genet 2004; 13: 3017-3027.

Magnitsky S, Watson DJ, Walton RM, Pickup S, Bulte JW, Wolfe JH, et al. In vivo and ex vivo MRI detection of localized and disseminated neural stem cell grafts in the mouse brain. Neuroimage 2005; 26: 744-54.

Margraf LR, Boriack RL, Routheut AA, Cuppen I, Alhilali L, Bennett CJ, et al. Tissue expression and subcellular localization of CLN3, the Batten disease protein. Mol Genet Metab 1999; 66: 283-9.

Matthiessen-Guyader. Survey of opinions from National Ethics Committees or similar bodies, public debate and national legislation in relation to human embryonic stem cell research and use (European Commission), 2004. Vol 1,
http://europa.eu.int/comm/research/biosociety/pdf/mb_states_230804.pdf.

Menezes JR, Luskin MB. Expression of neuron-specific tubulin defines a novel population in the proliferative layers of the developing telencephalon. J Neurosci 1994; 14: 5399-416.

Meng XL, Shen JS, Ohashi T, Maeda H, Kim SU, Eto Y. Brain transplantation of genetically engineered human neural stem cells globally corrects brain lesions in the mucopolysaccharidosis type VII mouse. J Neurosci Res 2003; 74: 266-77.

Messing A, Brenner M. GFAP: functional implications gleaned from studies of genetically engineered mice. Glia 2003; 43: 87-90.

Meyer JS, Katz ML, Maruniak JA, Kirk MD. Embryonic stem cell-derived neural progenitors incorporate into degenerating retina and enhance survival of host photoreceptors. Stem Cells 2006; 24: 274-83.

Ming GL, Song H. Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 2005; 28: 223-50.

Mitchison HM, Bernard DJ, Greene ND, Cooper JD, Junaid MA, Pullarkat RK, et al. Targeted disruption of the Cln3 gene provides a mouse model for Batten disease. The Batten Mouse Model Consortium [corrected]. Neurobiol Dis 1999; 6: 321-34.

Mitchison HM, Taschner PE, O'Rawe AM, de Vos N, Phillips HA, Thompson AD, et al. Genetic mapping of the Batten disease locus (CLN3) to the interval D16S288-D16S383 by analysis of haplotypes and allelic association. Genomics 1994; 22: 465-8.

Mole SE. Batten disease: four genes and still counting. Neurobiol Dis 1998; 5: 287-303.

Morel N. Neurotransmitter release: the dark side of the vacuolar-H+ATPase. Biol Cell 2003; 95: 453-7.

Morrison SJ, White PM, Zock C, Anderson DJ. Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. Cell 1999; 96: 737-49.

Munroe PB, Rapola J, Mitchison HM, Mustonen A, Mole SE, Gardiner RM, et al. Prenatal diagnosis of Batten's disease. Lancet 1996; 347: 1014-5.

Muotri AR, Nakashima K, Toni N, Sandler VM, Gage FH. Development of functional human embryonic stem cell-derived neurons in mouse brain. Proc Natl Acad Sci U S A 2005; 102: 18644-8.

Muraoka K, Shingo T, Yasuhara T, Kameda M, Yuan W, Hayase H, et al. The high integration and differentiation potential of autologous neural stem cell transplantation compared with allogeneic transplantation in adult rat hippocampus. Exp Neurol 2006; 199: 311-27.

Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, et al. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 2002; 110: 429-41.

Narayan SB, Rakheja D, Tan L, Pastor JV, Bennett MJ. CLN3P, the Batten's disease protein, is a novel palmitoyl-protein Delta-9 desaturase. Ann Neurol 2006; 60: 570-7.

NCL Resourse database, www.ucl.ac.uk/ncl. Data visited on 14th January 2008.

Ourednik V, Ourednik J, Flax JD, Zawada WM, Hutt C, Yang C, et al. Segregation of human neural stem cells in the developing primate forebrain. Science 2001; 293: 1820-4.

Overstreet-Wadiche LS, Bromberg DA, Bensen AL, Westbrook GL. Seizures accelerate functional integration of adult-generated granule cells. J Neurosci 2006; 26: 4095-103.

Palmer DN, Fearnley IM, Walker JE, Hall NA, Lake BD, Wolfe LS, et al. Mitochondrial ATP synthase subunit c storage in the ceroid-lipofuscinoses (Batten disease). Am J Med Genet 1992; 42: 561-7.

Paxinos G, Franklin KBJ. The mouse brain—in stereotactic coordinates. San Diego: Academic Press; 2001.

Pearce DA, Ferea T, Nosel SA, Das B, Sherman F. Action of BTN1, the yeast orthologue of the gene mutated in Batten disease. Nat Genet 1999; 22: 55-8.

Pearce DA, Sherman F. A yeast model for the study of Batten disease. Proc Natl Acad Sci U S A 1998; 95: 6915-8.

Pears M, Cooper J, Mitchison H, Mortishire-Smith R, Pearce D, Griffin J. High resolution 1H NMR-based metabolomics indicates a neurotransmitter cycling deficit in cerebral tissue from a mouse model of Batten disease. J Biol Chem 2005; 280: 42508-42514.

Phillips SN, Benedict JW, Weimer JM, Pearce DA. CLN3, the protein associated with batten disease: structure, function and localization. J Neurosci Res 2005; 79: 573-83.

Piltti K, Kerosuo L, Hakanen J, Eriksson M, Angers-Loustau A, Leppa S, et al. E6/E7 oncogenes increase and tumor suppressors decrease the proportion of self-renewing neural progenitor cells. Oncogene 2006; 25: 4880-9.

Pontikis CC, Cella CV, Parihar N, Lim MJ, Chakrabarti S, Mitchison HM, et al. Late onset neurodegeneration in the Cln3-/- mouse model of juvenile neuronal ceroid lipofuscinosis is preceded by low level glial activation. Brain Res 2004; 1023: 231-42.

Pontikis CC, Cotman SL, MacDonald ME, Cooper JD. Thalamocortical neuron loss and localized astrocytosis in the Cln3Deltaex7/8 knock-in mouse model of Batten disease. Neurobiol Dis 2005; 20: 823-36.

Priller J, Persons DA, Klett FF, Kempermann G, Kreutzberg GW, Dirnagl U. Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells in vivo. J Cell Biol 2001; 155: 733-8.

Przyborski SA. Differentiation of human embryonic stem cells after transplantation in immune-deficient mice. Stem Cells 2005; 23: 1242-50.

Ramirez-Montealegre D, Pearce DA. Defective lysosomal arginine transport in juvenile Batten disease. Hum Mol Genet 2005; 14: 3759-73.

Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001; 19: 1134-40.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000; 18: 399-404.

Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992; 255: 1707-10.

Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 1996; 175: 1-13.

Ricard J, Liebl DJ. Neurogenesis: is the adult stem cell young or old? IUBMB Life 2004; 56: 1-6.

Roskams AJ, Cai X, Ronnett GV. Expression of neuron-specific beta-III tubulin during olfactory neurogenesis in the embryonic and adult rat. Neuroscience 1998; 83: 191-200.

Rousselot P, Lois C, Alvarez-Buylla A. Embryonic (PSA) N-CAM reveals chains of migrating neuroblasts between the lateral ventricle and the olfactory bulb of adult mice. J Comp Neurol 1995; 351: 51-61.

Sanai N, Tramontin AD, Quinones-Hinojosa A, Barbaro NM, Gupta N, Kunwar S, et al. Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature 2004; 427: 740-4.

Santavuori P, Heiskala H, Autti T, Johansson E, Westermarck T. Comparison of the clinical courses in patients with juvenile neuronal ceroid lipofuscinosis receiving antioxidant treatment and those without antioxidant treatment. Adv Exp Med Biol 1989; 266: 273-82.

Santavuori P, Lauronen L, Kirveskari E, Aberg L, Sainio K, Autti T. Neuronal ceroid lipofuscinoses in childhood. Neurol Sci 2000; 21: S35-41.

Sariola H, Frilander M, Heino T, Jernvall J, Partanen J, Sainio K, et al. Kehitysbiologia: Kustannus Oy Duodecim, 2003.

Scriver C, Beaudet A, Valle D, Sly W. The Metabolic and Molecular Bases of Inherited Disease. Vol 3: The McGraw-Hill Companies, 2001.

Seehafer SS, Pearce DA. You say lipofuscin, we say ceroid: defining autofluorescent storage material. Neurobiol Aging 2006; 27: 576-88.

Sheen VL, Arnold MW, Wang Y, Macklis JD. Neural precursor differentiation following transplantation into neocortex is dependent on intrinsic developmental state and receptor competence. Exp Neurol 1999; 158: 47-62.

ShihabuddinLS, Numan S, Huff MR, Dodge JC, Clarke J, Macauley SL, et al. Intracerebral transplantation of adult mouse neural progenitor cells into the Niemann-Pick-A mouse leads to a marked decrease in lysosomal storage pathology. J Neurosci 2004; 24: 10642-51.

Sidman RL, Li J, Stewart GR, Clarke J, Yang W, Snyder EY, et al. Injection of mouse and human neural stem cells into neonatal Niemann-Pick A model mice. Brain Res 2007; 1140: 195-204.

Siintola E, Partanen S, Stromme P, Haapanen A, Haltia M, Maehlen J, et al. Cathepsin D deficiency underlies congenital human neuronal ceroid-lipofuscinosis. Brain 2006; 129: 1438-45.

Siintola E, Topcu M, Aula N, Lohi H, Minassian BA, Paterson AD, et al. The novel neuronal ceroid lipofuscinosis gene MFSD8 encodes a putative lysosomal transporter. Am J Hum Genet 2007; 81: 136-46.

Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003; 63: 5821-8.

Skottman H, Hovatta O. Culture conditions for human embryonic stem cells. Reproduction 2006; 132: 691-8.

Skottman H, Narkilahti S, Hovatta O. Challenges and approaches to the culture of pluripotent human embryonic stem cells. Regen Med 2007; 2: 265-73.

Sleat DE, Sohar I, Pullarkat PS, Lobel P, Pullarkat RK. Specific alterations in levels of mannose 6-phosphorylated glycoproteins in different neuronal ceroid lipofuscinoses. Biochem J 1998; 334 (Pt 3): 547-51.

Snyder EY, Deitcher DL, Walsh C, Arnold-Aldea S, Hartwieg EA, Cepko CL. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. Cell 1992; 68: 33-51.

Snyder EY, Taylor RM, Wolfe JH. Neural progenitor cell engraftment corrects lysosomal storage throughout the MPS VII mouse brain. Nature 1995; 374: 367-70.

Snyder EY, Yoon C, Flax JD, Macklis JD. Multipotent neural precursors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex. Proc Natl Acad Sci U S A 1997; 94: 11663-8.

StemCells Inc. News archive, www.stemcellsinc.com/news. Data visited on 14th January 2008

Suhonen JO, Peterson DA, Ray J, Gage FH. Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. Nature 1996; 383: 624-7.

Suslov ON, Kukekov VG, Ignatova TN, Steindler DA. Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. Proc Natl Acad Sci U S A 2002; 99: 14506-11.

Tabar V, Panagiotakos G, Greenberg ED, Chan BK, Sadelain M, Gutin PH, et al. Migration and differentiation of neural precursors derived from human embryonic stem cells in the rat brain. Nat Biotechnol 2005; 23: 601-6.

Taupin P. HuCNS-SC (StemCells). Curr Opin Mol Ther 2006; 8: 156-63.

Temple S. The development of neural stem cells. Nature 2001; 414: 112-7.

Thompson CM, Davis E, Carrigan CN, Cox HD, Bridges RJ, Gerdes JM. Inhibitor of the glutamate vesicular transporter (VGLUT). Curr Med Chem 2005; 12: 2041-56.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998; 282: 1145-7.

Tyynelä J, Cooper JD, Khan MN, Shemilts SJ, Haltia M. Hippocampal pathology in the human neuronal ceroid-lipofuscinoses: distinct patterns of storage deposition, neurodegeneration and glial activation. Brain Pathol 2004; 14: 349-57.

Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, et al. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci U S A 2000; 97: 14720-5.

Urbanska EM, Drelewska E, Borowicz KK, Blaszczak P, Kleinrok Z, Czuczwar SJ. NG-nitro-L-arginine, a nitric oxide synthase inhibitor, and seizure susceptibility in four seizure models in mice. J Neural Transm 1996; 103: 1145-52.

Watanabe K, Kamiya D, Nishiyama A, Katayama T, Nozaki S, Kawasaki H, et al. Directed differentiation of telencephalic precursors from embryonic stem cells. Nat Neurosci 2005; 8: 288-96.

Watson DJ, Walton RM, Magnitsky SG, Bulte JW, Poptani H, Wolfe JH. Structure-specific patterns of neural stem cell engraftment after transplantation in the adult mouse brain. Hum Gene Ther 2006; 17: 693-704.

Vesa J, Chin MH, Oelgeschlager K, Isosomppi J, DellAngelica EC, Jalanko A, et al. Neuronal ceroid lipofuscinoses are connected at molecular level: interaction of CLN5 protein with CLN2 and CLN3. Mol Biol Cell 2002; 13: 2410-20.

Vesa J, Hellsten E, Verkruyse LA, Camp LA, Rapola J, Santavuori P, et al. Mutations in the palmitoyl protein thioesterase gene causing infantile neuronal ceroid lipofuscinosis. Nature 1995; 376: 584-7.

Wiesinger H. Arginine metabolism and the synthesis of nitric oxide in the nervous system. Prog Neurobiol 2001; 64: 365-91.

Williams RE, Aberg L, Autti T, Goebel HH, Kohlschutter A, Lonnqvist T. Diagnosis of the neuronal ceroid lipofuscinoses: an update. Biochim Biophys Acta 2006; 1762: 865-72.

Wisniewski KE. Neuronal Ceroid-Lipofuscinoses. GeneReviews, 2006. www.genetests.org. Data visited on 14th January 2008.

Vines DJ, Warburton MJ. Classical late infantile neuronal ceroid lipofuscinosis fibroblasts are deficient in lysosomal tripeptidyl peptidase I. FEBS Lett 1999; 443: 131-5.

Wobus AM, Boheler KR. Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev 2005; 85:635-78.

Wolf HK, Buslei R, Schmidt-Kastner R, Schmidt-Kastner PK, Pietsch T, Wiestler OD, et al. NeuN: a useful neuronal marker for diagnostic histopathology. J Histochem Cytochem 1996; 44: 1167-71.

Yates F, Daley GQ. Progress and prospects: gene transfer into embryonic stem cells. Gene Ther 2006; 13: 1431-9.

Ying QL, Stavridis M, Griffiths D, Li M, Smith A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol 2003; 21: 183-6.

Zhang SC. Neural subtype specification from embryonic stem cells. Brain Pathol 2006; 16: 132-42.

Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001; 19: 1129-33.

Zhong NA, Moroziewicz DN, Ju W, Wisniewski KE, Jurkiewicz A, Brown WT. CLN-encoded proteins do not interact with each other. Neurogenetics 2000; 3: 41-4.