Modelling the neurodegenerative disease amyotrophic lateral sclerosis using induced pluripotent stem cells

Doctoral Thesis

Maximilian Naujock

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Hannover Medical School, Department of Neurology, Hannover, Germany

Harvard Medical School, McLean Hospital, Molecular Neurobiology Laboratory, Boston (MA), US A
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Maximilian Naujock,

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Supervisors: Prof. Dr. Susanne Petri
            PD Dr. Florian Wegner

Supervision Group: Prof. Dr. Susanne Petri
                    PD Dr. Florian Wegner
                    Prof. Dr. Peter Claus
                    Prof. Dr. Evgeni Ponimaskin

1st Evaluation: Prof. Dr. Susanne Petri
                Department of Neurology
                Hannover Medical School, Germany
                PD Dr. Florian Wegner
                Department of Neurology
                Hannover Medical School, Germany
                Prof. Dr. Peter Claus
                Institute of Neuroanatomy
                Hannover Medical School, Germany
                Prof. Dr. Evgeni Ponimaskin
                Cellular Neurophysiology
                Hannover Medical School, Germany

2nd Evaluation: Prof. Dr. Ludo van den Bosch
                Experimental Neurology
                KU Leuven, Belgium

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**Cover image**

The cover image (Naujock et al. 2014, Stem Cells Dev, Naujock et al. 2015 Klin Neurophysiol, Naujock et al. 2016, Stem Cells) shows from upper left to lower right: iPSC colonies via brightfield microscopy, fura-2 loaded motor neurons (MN) during calcium imaging, iPSC derived MNs immunolabeled with DAPI (blue) the early neuronal marker TuJ1 (green) and the more mature neuronal marker MAP2 (red), iPSC colony stained for the pluripotency markers SOX2 (green) and SSEA4 (red), a single MN recorded during a patch clamp session and mature MNs that express the neuronal marker TuJ1 (green) and the MN specific neurofilament marker SMI32 (red).
Dedication

This dissertation is lovingly dedicated to my growing family, my faithful friends, and in particular to my Marie who was incredibly supportive throughout the whole time of my Ph.D. and with whom I spent some wonderful months in beautiful Boston, MA.
Parts of this thesis have been published previously in:


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List of abbreviations

4AP 4-aminopyridine
Aa ascorbic acid
Ach acetylcholine
AHP afterhyperpolarization
ALS amyotrophic lateral sclerosis
AMPA alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP action potential
BDNF brain-derived neurotrophic factor
BIC bicuculline
BMP bone morphogenetic protein
cAMP cyclic adenosine monophosphate
DAPI 4’,6-diamidino-2-phenylindole
DM dorsomorphin
DMSO dimethyl sulfoxide
ESC embryonic stem cells
FGF-2 fibroblast growth factor 2
GABA gamma-aminobutyric acid
GDNF glial cell-derived neurotrophic factor
GFAP glial fibrillary acidic protein
hCBiPSC human cord blood-derived induced pluripotent stem cells
hESCs human embryonic stem cells
iPSCs induced pluripotent stem cells
K potassium
KCl potassium chloride
Klf4 Krueppel-like factor 4
MAP2 microtubule-associated protein 2
MNs motor neurons
NBQX 6-nitro-sulfamoyl-benzo-quinoxaline-dione
Na sodium
NPCs neuronal progenitor cells
Pax6 paired box gene 6
PBS phosphate buffered saline
PFA paraformaldehyde
PMA purmorphamine
Oct4 octamer 4
SHH sonic hedgehog
Sox1/2 sex determining region Y box 1/2
TEA tetraethylammonium chloride
TTX tetrodotoxin
TuJ1 tubulin beta III
Summary

Maximilian Naujock

Modelling the neurodegenerative disease amyotrophic lateral sclerosis using induced pluripotent stem cells

For the study of functional deficiencies in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) patient-derived iPSC can be used as in-vitro disease models. What has been proposed as theoretical possibility several years ago has become relevant for academic and industrial research nowadays. Very recently, independent groups identified ALS-associated key pathologies as targets for the assessment of novel pharmacological treatments.

In the first part of the thesis project, we worked out an innovative differentiation protocol to specify human cord blood-derived induced pluripotent stem cells (hCBiPSC) into functional motor neurons (MN). First, we generated expandable neural precursor cells which enabled subsequent specification into mature MNs. Via immunocytochemical and molecular methods we identified MN identity and tested their functionality via path clamp recordings of passive membrane properties and via calcium imaging (NAUJOCK et al. 2014).

During the second part of this dissertation we applied the experience in differentiating iPSC into functional MNs to ALS patient-derived iPSC with mutations in the superoxide dismutase 1 (SOD1) or fused-in sarcoma (FUS) gene. During initial electrophysiological characterization we observed that both ALS-iPSC-derived MN-groups showed significantly less spontaneous activity and reduced excitability. As underlying mechanism of this hypoexcitability we identified imbalances in Na⁺/K⁺ ratios. In parallel, quantitative real-time PCR (qPCR) results backed up those functional data by showing markedly reduced levels in Na⁺ channel expression in the SOD1 and FUS group as well as elevated levels of K⁺ channels in FUS ALS cells. Based upon these findings we designed a pharmacological strategy to reverse the observed phenotype in order to provide neuroprotection. With 4-Aminopyridine (4AP) we chose a FDA-approved reversible potassium channel blocker that has the capacity to increase the excitability of our hypoexcitable ALS MNs. Since there is accumulating evidence that the induction of activity that could rescue ALS MNs from neurodegeneration (SAXENA et al. 2013; LEROY et al. 2014) we aimed to reduce cell stress via induction of

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activity. Incubation with 4AP indeed re-established neuronal excitability, reduced elevated endoplasmic reticulum stress levels of ALS MNs and decreased caspase activity.

The present dissertation project has therefore identified a novel therapeutic approach with the potential to rescue cells via the induction of activity, a concept that stands in direct contrast to conventional treatment options. Our studies support the usefulness of iPSC based disease models in the exploration and discovery of new potential therapeutic strategies for neurodegenerative diseases such as ALS.
Zusammenfassung

Maximilian Naujock

Induzierte pluripotente Stammzellmodelle der neurodegenerativen Erkrankung Amyotrophe Lateralsklerose

Für die Erforschung neurodegenerativer Erkrankungen wie der Amyotrophen Lateralsklerose (ALS) können induzierte pluripotente Stammzellen (iPSC) als in-vitro Krankheitsmodelle dienen. Was vor wenigen Jahren noch als abstrakte Idee galt, hat heute bereits breiten Einzug in die akademische und industriebezogene Forschung erhalten. Erst kürzlich gelang es unabhängigen Arbeitsgruppen wichtige Pathomechanismen der ALS zu modellieren um diese schließlich als pharmakologisches Ziel auszugeben und erfolgreich zu behandeln.

In dem ersten Teil der vorliegenden Dissertation wurde ein innovatives Differenzierungsprotokoll ausgearbeitet, das zuverlässig und reproduzierbar aus iPSC Motoneurone (MN) generiert. Aus Nabelschnurblut gewonnene iPSC wurden hierbei zu expandierbaren neuronalen Vorläuferzellen (smNPC) prä-differenziert und anschließend zu funktionellen motorischen Nervenzenellen spezifiziert um schließlich funktionell und molekular untersucht zu werden (NAUJOCK et al. 2014).


Zusammenfassend wurde ein möglicher Wirkstoff identifiziert, der das Potenzial hat durch das Induzieren von Aktivität MN vor zellulärem Stress zu schützen, was im direkten Widerspruch zu konventionellen Behandlungsmöglichkeiten steht. iPSC-basierte Krankheitsmodelle zeigen demnach einmal mehr ihr großes Potenzial in der Identifizierung neuer Wirkstoffe zur Behandlung neurodegenerativer Erkrankungen wie die der ALS.
Introduction

1.1 iPSC discovery and application

The Nobel prize-winning discovery of induced pluripotent stem cells (iPSC) by the Yamanaka group (Takahashi u. Yamanaka 2006; Takahashi et al. 2007) paved the way for the rapidly growing field of iPSC-based disease modelling. In a groundbreaking series of experiments, human fibroblasts were exposed to a cocktail of reprogramming factors which were known to play a pivotal role in the regulation of pluripotency and proliferative activity during early embryogenesis. Following a defined strategy, the researchers reduced down the necessary factors to a number of four (Takahashi u. Yamanaka 2006; Takahashi et al. 2007) which still allowed them to obtain iPSCs following retroviral transduction of the fibroblasts. In their basic characteristics, iPSC resemble human embryonic stem cells (Xu et al. 2001) which are highly proliferative and pluripotent, meaning they can be easily expanded and specified towards a cell type of meso-, endo- or ectodermal origin. Thanks to the work of the past decades, we nowadays can differentiate pluripotent stem cells into a variety of cell types such as cardiac muscle cells (Burridge et al. 2011), liver hepatocytes (Zhu et al. 2014) or cerebral neurons (Shi et al. 2012) according to a number of highly defined differentiation protocols. The importance of well-defined and robust differentiation protocols will be discussed in more detail later in this thesis.

iPSC have been a game changer for two different scientific fields in particular, the field of tissue engineering and regenerative medicine and the field of in-vitro disease modelling. As for the field of regenerative medicine, the use of iPSC raised the hope that artificially created cell types or even organs from patient-own material would bypass the major hurdle of immunorejection following transplantation (De Almeida et al. 2014). A landmark study was initiated in 2014 with the transplantation of retinal pigment epithelium cells into the retina of a 70 year old patient suffering from exudative age-related macular degeneration (Kamao et al. 2014). However, justified concerns about the tumorigenic potential of the proliferative iPSC interfered with the initial enthusiasm and still today is a serious threat to the clinical translation of the ideas and advances that have been made in the lab (A. S. Lee et al. 2013). Non-integrative methods such as the delivery of the Yamanaka factors by episomal vectors (Yu et al. 2009) or proteins (Kim et al. 2009) and the reduction of the Yamanaka factors to a minimum (Chou u. Cheng 2013) have been applied and hold the promise to further reduce the oncogenic risk.
With iPSC-based modelling of diseases another scientific field has emerged. Starting with the collection of skin biopsies from patients and healthy controls, fibroblasts can be isolated, expanded and finally exposed to a certain combination of the Yamanaka factors. After several weeks, those former fibroblasts will be reprogrammed with low but reliable efficiencies to iPSC which in turn will be expanded clonally. iPSC collected from patients with a specific disease can be differentiated into a cell type which is known to be affected in the patient. Often, as for any neurodegenerative disease, those cell types are hardly accessible for pre-mortem analysis. Patient-derived iPSC which have been differentiated into the cell type of interest should show disease related phenotypes which in turn can be target of therapeutic intervention. For neurodegenerative diseases such as Parkinson’s Disease (PD) or ALS the choice of the cell type to be investigated is rather easy since it is primarily dopaminergic (DA) neurons and motor neurons (MNs) which are affected respectively. Difficulties rise for modelling disease with a rather diffuse spread of pathologies such as Alzheimer’s Disease even though some very interesting insights have been gained from iPSC-based studies for those diseases as well (ISRAEL u. GOLDSTEIN 2011; ISRAEL et al. 2012; EHRLICH et al. 2015).

1.2 Motor neuron differentiation

The prerequisite for an iPSC-based ALS modelling as described above is a robust differentiation protocol that generates MNs reliably and in a reproducible fashion throughout the many rounds of differentiation that will be required to finish all experiments. To specify iPSC into rostroventral cells such as MNs there are different specification methods starting either from floor plate iPSC (ROELINK et al. 1995), embryoid bodies (HU u. ZHANG 2009; STOCKMANN et al. 2013) or expandable neural precursor cells (NPCs) (EBERT et al. 2013; REINHARDT et al. 2013a; NAUJOCK et al. 2014; SAREEN et al. 2014). Whereas protocols starting from iPSC usually take about 60 days before mature MNs can be observed the use of expandable NPCs has a major advantage. Since those NPCs are pre-differentiated they can shorten the maturation period by a few weeks. Also those NPCs tend to be more resistant to undesired differentiation in expansion culture, the biggest enemy to the work with pluripotent cells.

The majority of MN differentiation protocols have in common the ingredients that force the iPSC into the desired cell type. Key elements in every protocol are the actions of ventralizing sonic hedgehog agonists and caudalizing retinoic acids (LIU u. ZHANG 2011). During this patterning phase which usually lasts up to two weeks, the iPSC lose their
pluripotent characteristic and stop proliferating as strongly as before. Following this period of neutralization there will be a phase of maturation, guided by the application of mainly neurotrophic factors such as ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF) or glial cell line derived neurotrophic factor (GDNF) (HENDERSON 1996). During this time, neurons are allowed to form typical networking structures and to establish synaptic connections to surrounding cells.

1.3 iPSC-based modelling of ALS

ALS, also known as Lou Gehrig’s disease, is a fatal neurodegenerative disease caused by the death of spinal and cortical MNs. Depending on the specific subtype, patients typically have a life expectancy of 3-5 years (KIERNAN et al. 2011) and so far Riluzole, a presumed glutamate release inhibitor and sodium channel antagonist has been identified as only pharmacological treatment which minimally prolongs survival by 2-3 months (R. G. MILLER et al. 2007). Clinically, patients suffer from severe and progressively decreasing muscle weakness in limbs and bulbar regions resulting in total paralysis and death by respiratory failure. Whereas 5-10% of the cases are familial 90% are of sporadic nature. Amongst the more prevalent genetic causes of ALS are mutations in the TARDBP gene encoding for TDP43, fused-in-sarcoma (FUS) and superoxide dismutase 1 gene (SOD1). Very recently the C9Orf72 mutation has been identified as major genetic cause of ALS (DEJESUS-HERNANDEZ et al. 2011; RENTON et al. 2011; DAOUD et al. 2012).

Shortly after the discovery of iPSC in 2006, fibroblasts from patients with a variety of diseases ranging from Down Syndrome to PD were reprogrammed to iPSC serving as proof of principle for the mere reprogrammability of those patient-derived tissues (PARK et al. 2008). The first attempt to model ALS using iPSC from a patient was published in 2008 already showing feasibility of differentiation of iPSC from a 82 year old patient into MNs at all. ALS related phenotypes were not reported. Subsequently, many groups started working on ALS disease modelling with a seminal study published in 2012 (EGAWA et al. 2012). Working on 9 healthy control iPSC lines and 7 ALS patient iPSC lines with mutations in the transactive response DNA binding protein (TARDBP) Egawa et al. purified MN cultures which then were analyzed on transcriptional and translational levels for ALS-associated phenotypes. Knowing about the accumulation of the TDP-43 protein, encoded by the TARDBP gene, in post-mortem tissue of ALS patients (E. B. LEE et al. 2012), higher amounts of insoluble TDP-43 protein (ARAI et al. 2006; NEUMANN et al. 2006) were identified in the ALS MNs. Corresponding to these results immunocytochemical stainings of MNs
revealed mislocalized cytoplasmic TDP-43 aggregates. While different substances were tested on their ability to prevent misfolding, mislocalization and accumulation of the TDP-43 protein one substance was especially effective. Incubation with histone acetyltransferase inhibitor anacardic acid (DEKKER u. HAISMA 2009) decreased TDP-43 transcription and provided neuroprotection. As first of its kind, the study impressively demonstrated the power of ALS iPSC models to identify relevant pharmacological targets and even drug candidates to be further tested in pre-clinical trials.

While the majority of related ALS iPSC studies focused on the differentiation and analysis of iPSC-derived MNs (MITNE-NETO et al. 2011; BILICAN et al. 2012; BURKHARDT et al. 2013; SAREEN et al. 2013; ZHANG et al. 2013; KISKINIS et al. 2014) (CHEN et al. 2014; WAINGER et al. 2014; DEVLIN et al. 2015) some studies investigated different cell types as well which also are known to be affected in ALS (SERIO et al. 2013; JAPTOK et al. 2015). Japtok et al. (JAPTOK et al. 2015) reported mislocalized cytoplasmic fused in sarcoma (FUS) protein aggregates in cortical neurons which were differentiated from ALS patient iPSC with mutations in the FUS gene. In still another study (SERIO et al. 2013) TDP-43 mutant iPSC were differentiated into astrocytes. Focusing on folding capacity of those glial cells the same mislocalization phenotype as reported in spinal MNs was observed. To further highlight the importance of non-neuronal cells in the pathogenesis of ALS healthy MNs were co-cultured with mutant or healthy control astrocytes. Those MNs cultured on mutant astrocytes did show decreased viability.

1.3 Electrophysiological phenotypes
Whereas cellular and more molecular ALS-associated phenotypes were identified in the first published studies on iPSC based ALS models, disease-specific functional changes have not been described yet. Even though there are hardly any patient data on maladaptive changes in the excitability of spinal and cortical iPSC-derived MNs, those changes were described previously in many in-vitro and in-vivo ALS models (PIERI et al. 2003; VAN ZUNDEERT et al. 2008) and upon these findings the excitotoxicity hypothesis has been built (ROTHSTEIN 1995). According to this hypothesis, MN degeneration is caused by increased excitability of ALS MNs that in turn leads to increased calcium influx due to more pronounced activity of the respective cell. Once the cell cannot handle the calcium overshoot anymore it will undergo apoptosis. Therefore, in order to prevent MN degeneration in ALS it should be tried to reduce excitability levels of MNs, a strategy by which the only available drug Riluzole has been designed (CHEAH et al. 2010). Even though the exact mechanisms by which Riluzole tends
to increase the life span by a few months has still not been fully elucidated, it is assumed that it works as indirect glutamatergic antagonist and sodium channel blocker. Further evidence for the excitotoxicity hypothesis stems from the observation that spinal MNs which selectively die in ALS have limited calcium buffering capacities available. In comparison to highly resilient oculomotor MNs fura-2 based calcium buffer estimates (NEHER 1995) revealed a 5-6 fold lower capacity in spinal MNs (VANSELOW u. KELLER 2000). However, recent studies do not hold the described hyperexcitability accountable for the observed cell death. According to them, the increase in excitability is rather an endogenous protective mechanism that makes MNs cope with the disease situation (SAXENA et al. 2013; LEROY et al. 2014) possibly through the production of neurotrophic factors (MATTSON 2013). Saxena et al. (SAXENA et al. 2013) reported in direct contrast to the conventional treatment option for ALS that it is the induction of activity that exerts neuroprotective effects. In the transgenic SOD1 mouse model they observed a decrease of misfolded and insoluble SOD1 protein upon the application of the activity inducing glutamate agonists AMPA and NMDA. Furthermore, the induction of activity did inhibit cellular stress responses such as endoplasmic reticulum stress, which arises from the accumulation of misfolded proteins in the ER and finally causes cells to undergo apoptosis. Finally, they also reported improved survival data and better performance on behavioral tests of motor function. More doubts about the theory behind the excitotoxicity hypothesis arose from another set of experiments. In the same way as the presumed mechanism of Riluzole, Saxena et al. inhibited glutamatergic transmission by the incubation of several glutamatergic antagonists and observed a worsening of the observed disease phenotypes. More evidence against the excitotoxicity hypothesis comes from another study which explored electrophysiological profiles of spinal MNs. In primary MNs, Leroy et al. (LEROY et al. 2014) first distinguished between the different MN subtypes of the spinal cord. Knowing about the higher resilience of S-type MNs against neurodegeneration, those more resistant MNs were presented with higher excitability thresholds as well.

1.4 Aim of the thesis

The aim of the thesis was to establish a well-defined and robust differentiation protocol to specify iPSC into functional spinal MNs (manuscript I). Once this milestone had been achieved, the protocol was applied to ALS patient-derived iPSC and those from healthy controls with the goal to compare electrophysiological properties. The discovered pathological phenotypes in turn were target of pharmacological reversal experiments and potential neuroprotective effects were evaluated subsequently (manuscript II).
Manuscript I
Published in *Stem Cells Development*, December 2014 (doi: 10.1089/scd.2014.0180)

Running title: Rapid generation of functional hCBiPSC derived MNs

**Molecular and functional analyses of motor neurons generated from human cord blood derived induced pluripotent stem cells**

Maximilian Naujock\(^a,b,f^\ast\), Nancy Stanslowsky\(^a,b\), Peter Reinhardt\(^c\), Jared Sterneckert\(^c\), Alexandra Haase\(^d,e\), Ulrich Martin\(^d,e\), Kwang-Soo Kim\(^f\), Reinhard Dengler\(^a,b\), Florian Wegner\(^a,b,\#\), Susanne Petri\(^a,b,\#\)

\(^a\) Department of Neurology, Hannover Medical School, Hannover, Germany
\(^b\) Center for Systems Neuroscience, Hannover, Germany
\(^c\) Department of Cell and Developmental Biology, Max Planck Institute for Biomolecular Medicine, Münster, Germany
\(^d\) Leibnitz Research Laboratories for Biotechnology and Artificial Organs (LEBAO), Department of Cardiac, Thoracic, Transplantation, and Vascular Surgery, Hannover Medical School, Hannover, Germany
\(^e\) REBIRTH-Cluster of Excellence, Hannover, Germany
\(^f\) Molecular Neurobiology Laboratory, McLean Hospital/Harvard Medical School, Belmont, MA 02478, USA

\(^\ast\) Corresponding author: Maximilian Naujock, Department of Neurology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover; phone: +49/511/5323737, fax: +49/511/5323579, email: mnaujock@mclean.harvard.edu; naujock.maximilian@mh-hannover.de

\(^\#\) These authors contributed equally.

**Keywords:** Human induced pluripotent stem cells (hiPSCs), cord blood, small molecule neural precursor cells (smNPC), differentiation, motor neurons, calcium imaging, patch-clamp, electrophysiology
Authors’ contribution

MN: in vitro experiments, data analysis and interpretation, manuscript writing and final approval of the manuscript. NS: realization of functional analysis, data analysis and interpretation, critical revision and final approval of the manuscript. PR and JS: generation of small molecule derived neural precursor cells, critical revision and final approval of the manuscript. AH and UM: reprogramming of the iPSC, critical revision and final approval of the manuscript. KK and RD: conception and design of the experiments, critical revision and final approval of the manuscript. FW and SP: conception and design of the study, data analysis and interpretation, critical revision and final approval of the manuscript.

Abstract

Induced pluripotent stem cells (iPSC) have become the most promising candidates for in-vitro modeling of motor neuron diseases (MND) such as Amyotrophic Lateral Sclerosis (ALS) and possibly for future therapeutic implementation in regenerative medicine. We here present for the first time the differentiation of human cord blood derived iPSC (hCBiPSC) into motor neurons (MN), the cell type primarily affected in ALS. In contrast to iPSC generated from adult tissue the hCBiPSC used in this study hold the promise of lower genetic mutations burden or epigenetic alterations which makes them ideal candidates for transplantation studies.

After 18 days of in-vitro differentiation the MNs stained positive for neuronal and for MN specific markers accompanied by respective gene expression patterns. To demonstrate that hCBiPSC can be differentiated into functional MNs, the cells were characterized by calcium-imaging and patch-clamp analysis. Calcium-imaging detected the expression of functional voltage-dependent calcium and ligand-gated channels of several important neurotransmitters. Using whole-cell patch-clamp recordings, we observed functional neuronal properties like sodium-inward currents and action potentials. Some cells showed spontaneous action potentials and synaptic activity that are signs of essential functional maturation.

Having established a rapid and efficient method to generate functional MNs from hCBiPSC, we demonstrate the differentiation potential of genetically unbiased hCBiPSC as promising source for transplantation studies and also create a framework for future in-vitro modelling.
Manuscript II
Published in Stem Cells, March 2016 (doi: 10.1002/stem.2354. Epub ahead of print)

Running Title: 4AP rescues ALS iPSC derived motor neurons

4-Aminopyridine induced activity rescues hypoexcitable motor neurons from ALS patient-derived induced pluripotent stem cells

Maximilian Naujock1,2,*, Nancy Stanslowsky1, Sebastian Bufler1, Marcel Naumann3, Peter Reinhardt4, Jared Sterneckert4, Ekaterini Kefalakes1, Carola Kassebaum1, Xenia Lojewski1, Alexander Storch3,4,5, Marie Frickenhaus6, Tobias M. Boeckers7, Stefan Putz7, Maria Demstre7, Stefan Liebau8, Moritz Klingenstein8, Albert C. Ludolph9, Reinhard Dengler1, Kwang-Soo Kim2, Andreas Hermann3,5, Florian Wegner1,# and Susanne Petri1,#

1Department of Neurology, Hannover Medical School, Hannover, 30625, Germany
2Molecular Neurobiology Laboratory, McLean Hospital/Harvard Medical School, Belmont (MA), 02478, United States
3Division for Neurodegenerative Diseases, Department of Neurology, Dresden University of Technology, Dresden, 01069, Germany
4DFG Research Center for Regenerative Therapies, Dresden University of Technology, Dresden, 01069, Germany
5German Center for Neurodegenerative Diseases (DZNE), Dresden, 01069, Germany
6Max-Planck-Institute for Biomolecular Medicine, Münster, 48149, Germany
7Institute of Anatomy and Cell Biology, Ulm University, Ulm, 89081, Germany
8Institute of Neuroanatomy, Eberhard Karls University Tübingen, Tübingen, 72074, Germany
9Department of Neurology, Ulm University, Ulm, 89081, Germany

* Corresponding author: Maximilian Naujock, Department of Neurology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover; phone: +49/511/5323737, fax: +49/511/5323579, email: mnaujock@mclean.harvard.edu; naujock.maximilian@mh-hannover.de

# These authors contributed equally.

Keywords

Induced pluripotent stem cells; motor neurons; amyotrophic lateral sclerosis; hypoexcitability; 4-Aminopyridine
Authors’ contributions

MN: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. NS: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. SB: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. MN: provision of study materials or patients, final approval of manuscript. PR: provision of study materials or patients, final approval of manuscript. JS: provision of study materials or patients, final approval of manuscript. EK: data analysis and interpretation, final approval of manuscript. CK: collection and/or assembly of data, final approval of manuscript. XL: provision of study materials or patients, final approval of manuscript. AS: conception and design, provision of study materials or patients, final approval of manuscript. MF: provision of study materials or patients, final approval of manuscript. TMB: conception and design, provision of study materials or patients, final approval of manuscript. SP: provision of study materials or patients, final approval of manuscript. MD: provision of study materials or patients, final approval of manuscript. SL: conception and design, provision of study materials or patients, final approval of manuscript. MK: provision of study materials or patients, final approval of manuscript. ACL: provision of study materials or patients, final approval of manuscript. RD: conception and design, financial support, final approval of manuscript. KSK: conception and design, financial support, final approval of manuscript. AH: conception and design, provision of study materials or patients, final approval of manuscript. FW: conception and design, financial support, manuscript writing, final approval of manuscript. SP: conception and design, financial support, manuscript writing, final approval of manuscript.
Abstract

Despite decades of research on amyotrophic lateral sclerosis (ALS), there is only one approved drug, which minimally extends patient survival. Here, we investigated pathophysiological mechanisms underlying ALS using motor neurons (MNs) differentiated from induced pluripotent stem cells (iPSCs) derived from ALS patients carrying mutations in FUS or SOD1. Patient-derived MNs were less active and excitable compared to healthy controls, due to reduced Na⁺/K⁺ ratios in both ALS groups accompanied by elevated potassium receptor (FUS) and attenuated sodium receptor expression levels (FUS, SOD1). ALS iPSC-derived MNs showed elevated endoplasmic reticulum stress (ER) levels and increased caspase activation. Treatment with the FDA approved drug 4-Aminopyridine (4AP) restored ion-channel imbalances, increased neuronal activity levels and decreased ER stress and caspase activation. This study provides novel pathophysiological data, including a mechanistic explanation for the observed hypoexcitability in patient-derived MNs and a new therapeutic strategy to provide neuroprotection in MNs affected by ALS.
Electrophysiological characterization of induced pluripotent stem cells (iPSC) as in vitro models for motor neuron diseases

Maximilian Naujock, Florian Wegner, Susanne Petri
Klinik für Neurologie, Medizinische Hochschule Hannover, Hannover, Deutschland
Korrespondenzadresse: Prof. Dr. med. Susanne Petri, petri.susanne@mh-hannover.de, +49 511 532-3740

Keywords
Induced pluripotent stem cells – motor neurons – electrophysiology – ALS – drug screening

Authors’ contributions
MN, FW, SP: literature research, manuscript writing, final approval of manuscript.

Abstract
Efficient treatment options for the majority of neurodegenerative diseases have not been developed to date. The discovery of induced pluripotent stem cells (iPSC), awarded by the Nobel prize in 2012, paved the way for the development of innovative in-vitro disease models. Patient-derived iPSC nowadays can be differentiated towards the desired neural lineage depending on the respective disease context. As for the motor neuron disease amyotrophic lateral sclerosis (ALS), electrophysiological characterization of motor neurons from patient derived iPSC has revealed relevant disease pathomechanisms. These in turn could represent the target for potential novel pharmacological treatment concepts.
Discussion

Almost ten years after the discovery of iPSC and a decade accompanied by the stem cell hype, first conclusions about their usefulness and newly gained insights can be drawn. Whereas the field of regenerative medicine struggles with the clinical translation of the gathered knowledge, iPSC disease modelling has become an established in-vitro approach for the discovery of molecular and functional disease-related phenotypes with a focus on the exploration of new innovative targets via drug screens. However, several limitations still exist and have to be overcome to fully exploit the potential of iPSC based disease models.

In the first project of this thesis we developed an MN differentiation protocol to robustly differentiate iPSC into spinal MNs. In contrast to a majority of protocols we did not start every single differentiation batch from iPSC but from an expandable neural precursor population instead. The major advantage in using these smNPC lied in the rapid differentiation time. Whereas studies in MNs derived by more traditional protocols usually report the presence of prominent MN surface markers and MN-specific gene expression profiles around day 60 of differentiation (H. LEE et al. 2007; DIMOS et al. 2008; BILICAN et al. 2012; EGAWA et al. 2012; SAREEN et al. 2013), we were able to yield similar efficiencies as early as day 18-20 of differentiation (NAUJOCK et al. 2014). Even though this may sound highly advantageous and indeed markedly shortens differentiation time, one should not forget about the time it takes to generate those smNPCs. In our experience, passages up to 10 times were necessary to obtain a homogeneous neural precursor population ready for further differentiation. Also, the smNPCs had to be evaluated for their neural precursor properties via immunocytochemical stainings and gene expression profiling via RT-qPCR, consuming even more time. Having established a rapid and reliable MN differentiation protocol very timely, we could start with the second project to compare electrophysiologic phenotypes between healthy control and mutant ALS iPSC-derived MNs. Furthermore, the hCBiPSC derived smNPC could serve as optimal transplantation candidates as being demonstrated in a recent study (SAREEN et al. 2014).

For the second project we had the opportunity to work with multiple healthy control iPSC lines and multiple ALS iPSC lines from patients carrying mutations in either the FUS or SOD1 gene. We applied our MN differentiation protocol and started recordings during week 7. Very strikingly, we observed a pronounced hypoexcitability phenotype in both mutant ALS MN groups, corresponding to reduced synaptic activity and less spontaneously occurring APs. At about the same time, other electrophysiologic studies in ALS iPSC derived MNs were published that presented somewhat conflicting data on the electrophysiologic profile of ALS
iPSC-derived MNs. Whereas several studies observed, in line with our results, a hypoexcitability phenotype (SAREEN et al. 2013; ZHANG et al. 2013) in MNs differentiated from ALS patient-derived iPSC with mutations in the C9Orf72- or TARDBP gene, another study reported a hyperexcitability (WAINGER et al. 2014) phenotype in iPSC-derived MNs with mutant SOD1. Initially, people were struggling with the interpretation of these independent and contradicting datasets. A longitudinal study by Devlin et al. (DEVLIN et al. 2015) brought further clarity demonstrating temporal progression from early hyper- to late hypoexcitability in ALS iPSC-derived MNs with mutations in the TARDBP and C9Orf72 genes. Even though more healthy control MNs were recorded with repetitive APs as response to electrical stimulation, the mutant MNs fired APs in lower frequencies. However, still unanswered remained the question of the clinical correlates and therapeutic relevance of these two phenotypes. In the study by Wainger et al. (WAINGER et al. 2014) the observed hyperexcitability in the ALS MNs was inhibited with defined concentrations of Retigabine which rescued MNs from neurodegeneration. Based on our observation of hypoexcitability in ALS MNs, on the other hand, we treated them with an activity-inducing drug (4AP) and observed enhanced activity and reduced cell stress as well. For translation of these results into clinical application it will be crucial to understand the temporal changes of excitability of ALS motor neurons in vivo at onset and during disease progression. As expected, data about the excitability of MNs from the spinal cord or precentral gyri in ALS patients is very limited. A series of transcranial magnetic stimulation studies (BAE et al. 2014; MENON et al. 2014) indicated hyperexcitability in cortical MNs, thereby favoring an activity-decreasing treatment as proposed by Wainger et al. (WAINGER et al. 2014). In fact, based upon the iPSC generated in-vitro data, a clinical trial (NCT02450552) was initiated. Bypassing further in-vivo experiments was possible because Retigabine already is an FDA-approved drug for the treatment of epilepsy. This story very impressively highlights the power of iPSC disease models to identify new targets and compounds to be tested in clinical trials.

However, iPSC disease models do not come without weaknesses or limitations. One of the major concerns in the use of this in-vitro platform is the incomplete maturation grade of the analyzed cell types. The differentiation time of few weeks only mainly mimics early embryonic development with as consequence that identified targets for further treatment could be irrelevant since they do not represent the patient’s situation. Regarding MN differentiation to model neurodegenerative diseases such as ALS or spinal muscular atrophy (SMA) (BOZAMORAN et al. 2015; FRATTINI et al. 2015; HEESENN et al. 2015), a reliable indicator for maturation grade is the resting membrane potential (RMP) which typically lies around -70mV
in most adult neurons. Very commonly, differentiation protocols yield RMPs around -37mV (NAUJOCK et al. 2014) to -49mV (DEVLIN et al. 2015), not exactly reaching the -70mV of the adult physiology to be modelled. In our study, we recorded RMPs between -33 and -42mV and even though we successfully replicated the late hypoexcitability phenotype we cannot say for sure whether that corresponds to spinal MNs in ALS patients. Despite the quite extensive in-vitro cultivation times of up to several months (SAREEN et al. 2014) and the application of neurotrophic factors such as BDNF, CNTF and GDNF there still is a fair doubt of the real maturation grade of iPSC-derived MNs, even if they in turn do express key MN genes and cell surface markers. To overcome this hurdle Miller et al. who studied neuronal aging in the dish (J. D. MILLER et al. 2013) came up with an interesting approach. Studies about underlying molecular and genetic causes of the Hutchinson-Gilford-Syndrome identified the lamin A/C gene (LMNA) as being responsible for the observed clinical phenotype, the premature aging of the affected patient (RANKIN u. ELLARD 2006). LMNA mutations cause elevated nuclear progerin protein levels which in turn induce DNA damage and fastened telomere shortening, known to be a characteristic of natural aging. The experimental overexpression of progerin in healthy iPSC as expected caused accelerated maturation of the cells. In ALS iPSC models such as ours this could result in more mature MNs, which then more closely resemble the patients’ situation. Characterization of the electrophysiological phenotypes of those more mature neurons would allow us an advice of what therapeutic strategy to apply during a clinical trial.

Another major limitation in the use of iPSC disease models stems from inter-clonal variability that at least partly derives from imbalances in the generation of iPSC (VITALE et al. 2012) but also from their subsequent differentiation. In practical terms, this means that even in two different iPSC clones from the same patient different outcomes during phenotypic assays can be observed. To meet these challenges and to counteract the effects of unwanted variation we worked with a robust differentiation protocol and a high number of healthy control and patient lines. Therefore, we could reduce the observed inter-clonal variability to a minimum which did not interfere with the statistical evaluation and subsequent interpretation of our data.

To finally make sure that an observed phenotype such as the hypoexcitability phenotype really stems from the genetic disease background and not from undesired inter-clonal variability one could have implemented so called isogenic control lines. Thanks to state of the art genome editing methods such as the CrispR-Cas9 system it is possible to cut out a piece of mutated DNA and to replace it with a correct sequence. Hereby one directly can compare the ALS mutant iPSC line to its corrected version, excluding most effects that could stem from
inter-clonal variability. Most but not all effects, because the corrected isogenic control line of course is still another subclone of the former ALS iPSC line. So far, several studies incorporated isogenic control lines into their studies and were successful in eliminating the observed disease phenotype (REINHARDT et al. 2013b; RYAN et al. 2013; KISKINIS et al. 2014; WAINGER et al. 2014). To summarize, many efforts have been made to optimize existing iPSC in-vitro models of certain diseases and with increasing sophistication of available differentiation protocols and phenotypic screening assays iPSC based disease models will further contribute to elucidate pathomechanisms and to identify potential therapeutic approaches.

Taken together we provide a well-defined approach to identify and treat new targets in the fatal disease ALS. Based upon a robust differentiation protocol we identified with 4AP a FDA-approved drug that showed neuroprotective effects in-vitro and therefore holds the potential to be suitable for symptomatic and neuroprotective therapy in ALS.
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“Let us keep looking, in spite of everything. Let us keep searching. It is indeed the best method of finding, and perhaps thanks to our efforts, the verdict we will give such a patient tomorrow will not be the same we must give this man today.” (Charcot, 1889).
Declaration

I herewith declare that I autonomously carried out the PhD-thesis entitled “[title]”. No third party assistance has been used. I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis. I conducted the project at the following institutions:

Department of Neurology
Hannover Medical School

Molecular Neurobiology Laboratory/McLean Hospital
Harvard Medical School

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context. I hereby affirm the above statements to be complete and true to the best of my knowledge.

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