The human NT2 cell line as *in vitro* test system for neuroprotection and developmental neurotoxicity

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Abstract

A large number of animal models have been established, which mimic the development of the human brain or the conditions of human CNS disorders. However, the use of laboratory animals is time consuming, expensive and creates ethical concerns. Thus, replacement of these models with relevant human *in vitro* systems appears attractive. In this study the well characterized human NT2 teratocarcinoma (NTera-2) cell line was used as *in vitro* model in two different assays to investigate potential neuroprotective and potential neurotoxic compounds. Postmitotic NT2 neurons were generated by rapid differentiation in sphere cultures as described by Paquet-Durand et al. (2003).

To analyze the efficacy of potential neuroprotective compounds, cell physiological experiments were performed with postmitotic neurons. In simulated ischaemic conditions in cultures of purified NT2 neurons the application of low doses of the antihypertensive drug and calcium channel blocker diltiazem protected against excitotoxic neuronal damage *in vitro*. Experiments with primary cortical mouse neuron cultures demonstrated a similar response to simulated ischaemia and confirmed the neuroprotective effect of diltiazem.

In a second approach potential developmental neurotoxic compounds [lead-acetate, methyl mercury, valproic acid (VPA), methylazoxy methanol acetate (MAM)] and non developmental neurotoxic compounds [paracetamol and glutamate] were analyzed in migration assays using differentiating human NT2 neurospheres. Neurospheres were formed under non-adherent conditions and cell migration was assayed by placing spheres onto an adherent substrate in the presence of test compounds. Cell migration out of these neurospheres was determined microscopically and compared to acute toxicity. The developmental neurotoxins inhibited specifically migration, whereas EC_{50} values for non developmental neurotoxins did not differ as expected from acute toxicity values.

In summary, these results demonstrate that cell cultures of human model neurons can provide an important initial test system for drug development in stroke therapy. Furthermore, they show that it is possible to perform *in vitro* tests for developmental neurotoxicity which are predictive for the effects of known developmental neurotoxins. Thus, the NT2 cell line offers a promising perspective towards establishing *in vitro* assays as replacement for drug and toxin screening *in vivo.*

Keywords: human NT2 neuronal cells, Neuroprotection, Developmental neurotoxicity

Zusammenfassung

Es wurde eine Vielzahl an Tiermodellen entwickelt, die die Entwicklung des menschlichen Gehirns oder die Bedingungen von Erkrankungen des menschlichen ZNS nachahmen. Jedoch ist der Gebrauch der Labortiere Zeit intensiv, teuer und verursacht ethische Bedenken. Darum ist ein Austausch dieser Modelle mit relevanten menschlichen *in vitro* Testsystemen vorteilhaft. In dieser Studie wurde die menschliche teratocarcinoma NT2 (NTera-2) Zelllinie als *in vitro* Modell in zwei verschiedenen Testsystemen benutzt, um potentielle neuroprotektive und potentielle neurotoxische Substanzen zu analysieren. Postmitotische NT2 Neurone wurden durch eine schnelle Differenzierungsmethode, wie von Paquet-Durand et al. (2003) beschrieben, erzeugt.

Um die Wirksamkeit von möglichen neuroprotektiven Substanzen zu analysieren, wurden physiologische Experimente mit postmitotischen NT2 Neuronen durchgeführt. Unter simulierten ischämischen Bedingungen in neuronalen NT2 Kulturen konnte gezeigt werden, dass niedrige Dosen des Bluthochdruckmittels und Kalziumkanalblockers Diltiazem die Zellen gegen exzitotoxische Schädigung schützt. Experimente unter anoxischen und hypoglycämischen Bedingungen mit primären kortikalen Mauszellkulturen zeigten einen ähnlichen Effekt und bestätigten die neuroprotektive Wirkung von Diltiazem.

In einem weiteren Ansatz wurden potentielle entwicklungsneurotoxische Substanzen [Bleiazetat, Methylquecksilber, Valproinsäure (VPA), Methylazoxymethanolazetat (MAM)] und nicht-entwicklungneurotoxische Substanzen [Paracetamol und Glutamat] in Migrationsassays mit differenzierenden NT2 Neurosphären untersucht. Die Neurosphären wurden unter nicht-adhärenten Bedingungen gebildet und die Migrationsassays wurden dann unter adhärenten Bedingungen in Anwesenheit der Testsubstanzen durchgeführt. Die Zellmigration aus diesen Neurosphären heraus wurde mikroskopisch ermittelt und mit den zytotoxischen Eigenschaften der Testsubstanzen verglichen. Für die Entwicklungsneurotoxine konnte eine spezifische Hemmung der Migration nachgewiesen werden, während die EC_{50} Werte für die nicht-entwicklungsneurotoxischen Substanzen sich erwartungsgemäß nicht von der Zytotoxizität unterschieden.

Zusammenfassend zeigen diese Ergebnisse, dass es möglich ist Zellkulturen von humanen NT2 Neuronen für ein Testsystem zu benutzen, mit dem neuroprotektive Substanzen für die Hirnschlagtherapie gefunden werden können. Außerdem besteht die Möglichkeit, *in vitro* Tests für Entwicklungsneurotoxizität durchzuführen, welche die Effekte von bekannten Entwicklungsneurotoxinen bestätigen. So bietet die NT2 Zelllinie eine viel versprechende Alternative zur Etablierung von *in vitro* Assays um *in vivo* Screening Testsysteme zu ersetzen.

Schlagwörter: humane NT2 Neuronen, Neuroprotektion, Entwicklungsneurotoxizität

Eidesstattliche Erklärung

Hiermit erkläre ich, dass die vorliegende Arbeit von mir selbständig verfasst wurde. Zur Erstellung wurden von mir keine anderen als die angegebenen Quellen und Hilfsmittel verwendet. Diese Dissertation wurde weder als Ganzes noch in Teilen für Master-, Diplom- oder ähnliche Prüfungsarbeiten verwendet.

Ludwigshafen, 09.06.2010

Andrea Gierse

List of used abbreviations

Abbreviation	Full name
Ara C	1-6-D-arabinofuranosylcytosine
BrdU	5-bromo-2'-deoxyuridine
BMBF	Federal Ministry for Education and Research
BSA	Bovines serum albumine
BSS	balanced salt solution
cAMP	Cyclic adenosine-mono-phosphate
ChAT	Choline acetyltransferase
CNS	Central nervous system
CY3	Cyanine dye 3
DIV	days in vitro
DAPI	4',6-Diamidin-2'-phenylindol- dihydrochlorid
DMEM	Dulbecco´s minimum essential medium
DMSO	Di-methyl-sulfoxide
EC	Embryonic carcinoma
ES	Embryonic stem
EG	Embryonic germ
EDTA	Ethylene-diamine-tetraacetate
EtOH	Ethanole
F12	Ham´s F12 medium
FBS	Fetal bovine serum
FGF-1	Fibroblast growth factor-1
FudR	2'-deoxy-5-fluorouridine
GABA	γ-amino-butyric-acid
HEPES	N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid
IBMX	3-isobutyl-1-methylxanthine
LDH	Lactate dehydrogenase
MAM	Methylazoxy methanole acetate
MAP2	Microtubuli associated protein 2
MeHgCl	Methylmercury (II) chloride
MeOH	Methanole

Abbreviation	Full name
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NHNP	Normal human neural progenitor
N.D.	not determinable
ns	not significant
NMDA	N-methyl-D-aspartate
NF	Neurofilament
NT2	NTera-2
PBS	Phophate buffered saline
PDL	Poly-d-lysine
Pb-ac	Lead-acetate
PGCs	Primordial germ cells
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
PKC	protein kinase C
PCBs	polychlorinated biphenyls
PTX	PBS with 0.1 % Triton X100
RA	Retinoic acid
SEM	Standard error of the mean
s.p.	synaptic punctae
SMBS	Sodium metabisulphite
TH	Tyrosine Hydroxylase
Urd	1-ß-D-ribofuranosyluracil
VPA	Valproic acid

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1. Introduction

In biomedical assays, the use of original human brain tissues is restricted because of availability and ethical concerns. Thus, cell based *in vitro* models are the most suitable alternative. The use of human nerve cells, generated and multiplied on a large scale in culture could provide alternative test systems for pharmacological studies as replacement for animal models. A relevant human *in vitro* system could facilitate and simplify drug and toxin screening as well as the study of disorders of the central nervous system (CNS) or could serve as a source for neural transplantation.

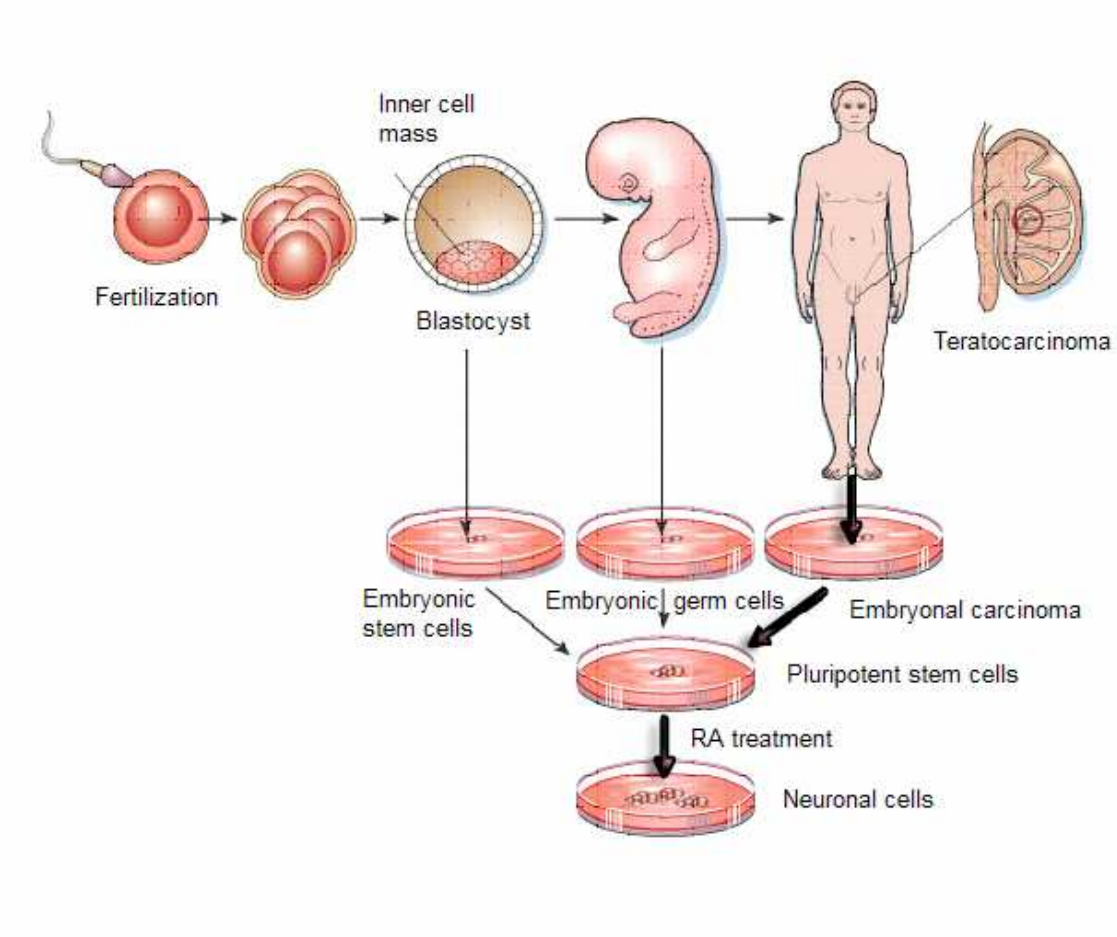


Fig. 1 *Origin of human pluripotent stem cells. Embryonic stem (ES) cells are derived from the inner cell mass of the pre-implantation embryo, Embryonic germ (EG) cells are derived from primordial germ cells (PGCs) isolated from the embryonic gonad and Embryonal carcinoma (EC) cells are stem cells of testicular tumours in the adult. Pluriopotent stem cells can be induced into neuronal cells by treatment with retinoic acid (RA). Figure modified from Donovan and Gearhart (2001)*

Sources for human neurons are pluripotent stemm cells which have the potential to generate any cell type in the body. They could derive from embryonic carcinoma (EC) cells, embryonic stem cells (ES) or embryonic germ (EG) cells. EC cells are stem cells of testicular tumours in the adult, ES cells are derived from pre-implantation embryos and EG cells are derived from primordial germ cells (PGCs) of the post-implantation embryo (**Fig. 1**). The disadvantage for the use of ES or EG cells is the fact that the sources of these cells are human embryos, which raise questions of ethical concerns.

This study is based on the well characterized human NT2 teratocarcinoma (NTera-2) EC cell line which can be induced with retinoic acid to differentiate into post-mitotic human neurons. Two different assays for neurobiological research were applied:

Differentiated NT2 neuronal cells were used as a model of the excitotoxic cascade during stroke and differentiating human NT2 neurospheres were used in migration assays as *in vitro* test systems for developmental neurotoxicity.

1.1 The NT2 cell line

In this study the human teratocarcinoma cell line Ntera-2 (NT2) which has been derived from a human testicular cancer (Andrews, 1984) was used to develop human neuronal *in vitro* model systems. NT2 cells can be induced to differentiate into post-mitotic neurons, upon exposure to retinoic acid (RA), which is a potent inducer of normal mammalian neural development (Lee and Andrews, 1986 / Pleasure et al., 1992). In culture, NT2 neuronal cells show neuronal morphology, migrate to form ganglion-like cell conglomerates and are immunoreactive to neuronal cytoskeletal markers such as MAP2, Tau, and NF-66 (Pleasure et al., 1992). These NT2 neuronal cells display a variety of neurotransmitter phenotypes (Guillemain et al., 2000) and have been used in a large number of biomedical investigations in the past 2 decades (reviewed by Paquet-Durand and Bicker, 2007). The neuronal differentiation of NT2 cells leads to increased expression levels of synapsins (Leypoldt et al., 2002) and under specific culture conditions, these neurons form functional synapses (Hartley et al., 1999). NT2 cell line can be used as a model for hypoxic-ischaemic cell injury (Munir et al., 1995) and they have been used to study the effects of anoxia and hypoglycaemia *in vitro* (Rootwelt et al., 1998). This cell type has also been used in several transplantation studies, which have demonstrated successful engraftment in experimental animals (Trojanowski et al., 1993) and human stroke patients (Nelson et al., 2002). In this context, the induction of a dopaminergic phenotype seemed interesting for transplantation studies of dopamine lesions, for instance caused by Parkinson disease. It has been reported that NT2 neurons can be

differentiated into a dopaminergic phenotype under exposure to lithium (Zigova et al., 1999; 2000), or a mix of growth factors, dopamine, and high intracellular cAMP levels by application of forskolin and IBMX (Iacovitti et al., 2001).

Moreover, NT2 neurons would allow for high throughput screening of chemical compounds both for possible therapeutical use and for risk assessment of developmental neurotoxins (Hill et al., 2008).

One disadvantage of the NT2 system has been the time-consuming process (up to two months) needed to generate purified postmitotic neurons. In this study a new method utilizing freely flowing aggregates, which significantly shorten the differentiation process up to one month (Paquet-Durand et al., 2003) was applied. This method is based on the observation that a high concentration of RA, together with the influence of cell-cell adhesion in neurosphere aggregates facilitates neurogenesis in comparison to the classical differentiation in surface attached monolayer culture (Pleasure et al., 1992).

1.2 Simulation of ischaemia

Ischaemic brain injury is a major cause of death and health problems in industrialized countries with a demographic shift towards an ageing population. Brain damage following acute ischemic stroke results from the obstruction of blood supply to nerve cells, setting off membrane depolarization, which causes the release of the excitatory neurotransmitter glutamate. The excessive extracellular accumulation of glutamate stimulates glutamate receptors of surrounding cells, inducing increases in intracellular Ca^{2+} levels and the production of free radicals, which orchestrate cell injury via the so-called excitotoxic cascade (Olney, 1974; Choi, 1985; Coyle and Puttfarcken, 1993; Kristián and Siesjö, 1998; Nichols and Ward, 2000). In addition, a chain reaction triggered by glutamate leakage from the dying brain cells endangers neurons in the penumbra, a much larger region surrounding the core infarct. Because cell death and the expansion of the penumbra occur over a period of hours to days, there are therapeutic windows for intervening at critical steps of the excitotoxic cascade. One approach of reducing the delayed death of brain cells following stroke (Dirnagl et al., 1999; Lee et al., 1999) involves the application of neuroprotective substances such as glutamate receptor blockers, free radical scavengers and inhibitors of cell death signalling pathways. However, despite much animal research into the cellular mechanisms of ischaemic brain injury, so far little of this work has translated into clinical treatments for stroke in human patients (Hoyte et al., 2004b). *In vitro* models are often used to investigate pathophysiological mechanisms of brain cell injury and to develop therapeutic approaches

(Melzian et al., 1999; Campbell et al., 2000; Melena and Osborne, 2001). In this study neurons generated by rapid differentiation in sphere cultures from the human teratocarcinoma cell line NT2 were used to investigate the excitotoxic cascade. These neurons integrate and survive in rodent brains (Kleppner et al., 1995) and promote functional recovery in a rat model of transient focal ischaemia (Borlongan et al., 2000). In a transplantation study for treating stroke in human patients, the therapeutic application of NT2 neurons has also been successfully demonstrated (Kondziolka et al., 2001). NT2 neurons respond to glutamatergic stimulation with increases in cytosolic Ca^{2+} levels. They express functional N-methyl-D-aspartate (NMDA) receptors, ionotropic glutamate receptor channels that are permeable to Ca^{2+} and Na^{+}. NT2 Neurons are vulnerable to combined anoxia/hypoglycemia, an effect which is exacerbated by the addition of glutamate (Paquet-Durand and Bicker, 2004).
In this study the protective effect against ischaemic damage of diltiazem, a licensed drug that is commonly used to treat heart arrhythmias, high blood pressure and angina pectoris (reviewed in Chaffman and Brogden, 1985), has been examined with human model neurons *in vitro*. To compare this model with an animal model the response to simulated ischaemia in primary cortical mouse neuron cultures has also been analysed.

1.3 Neuronal Migration

Besides differentiation and synaptogenesis, cell migration is an essential process during development of the central nervous system (CNS). Within the developing nervous system, cells migrate several centimeters before reaching their proper positions and forming the correct connections. Two different types of migration are known: radial migration, which describes the migration of neurons along glial fibers; and tangential migration, in which cells migrate orthogonal to the direction of radial migration, increasing cellular complexity and connectivity (Marin and Rubenstein, 2003). Defects in neuronal migration lead to several functional pathologies like autism, attention deficit disorder, mental retardation and cerebral palsy (Grandjean and Landrigan, 2006) or like lissencephaly and heterotopia (Pilz et al., 2002). Exposure to developmental neurotoxic compounds could lead to migration disorders as seen in the fetal alcohol syndrome (Miller, 1993 / Guerri, 1998) or mercury intoxication (Clarkson, 2002). Only few industrial chemicals (lead, methylmercury, polychlorinated biphenyls [PCBs], arsenic and toluene) are proven to cause human brain injury by exposure to these chemicals during early fetal development (Grandjean and Landrigan, 2006). The developing human brain is much more susceptible to injury and doses needed to affect are much lower than those affecting adult brain function. For that reason, the recognition of

developmental neurotoxicity in children exposed to industrial chemicals often ocured after recognition of neurotoxicity at high doses in the adult nervous system (**Fig. 2**). Exposure on childhood populations at low doses lead to subclinical toxicity and a pandemic of subclinical neurotoxicity is therefore supposable to be silent. Due to the fact that the documentation of most neurotoxicants is directed toward adults only, there is a great need to generate new approaches which are able to give clear evidences about the potential of chemicals to disturb human brain development.

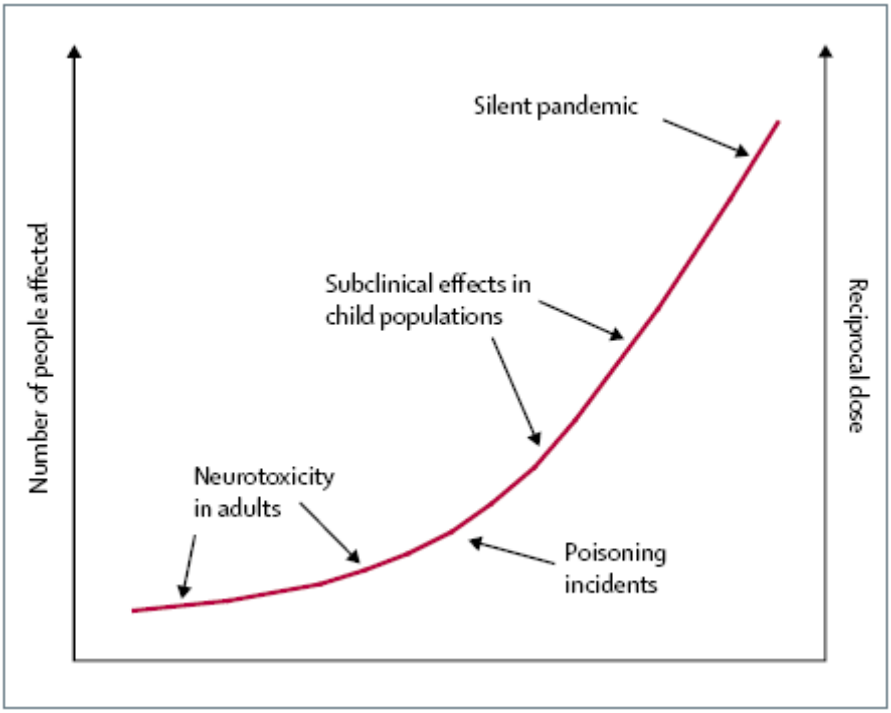


Fig. 2 *The effects of a neurotoxic chemical on a population over time. For identification of chemicals toxic to neurodevelopment, the first evidence dealt with adverse effects of high doses on the adult nervous system, and was followed by case reports and epidemiological evidence on developmental toxicity at successively lower doses, to which childhood populations of increasing magnitude were exposed. Figure from Grandjean and Landrigan (2006)*

For identifying potential developmental neurotoxic chemicals and discovering the mechanism that control neuronal migration, many different animal *in vivo* and *in vitro* assays have been used, like live cell imaging of granule mouse neurons (Fishman and Hatten, 1993), the technique of photographing murine cerebellar cells on a CELLocate grid coverslip (Sass et al., 2001) or microscopical observation of organotypic insect midgut cultures (Haase and Bicker, 2003). To study human neuronal migration Moors et al. (2007) established a human neurosphere-based migration assay using normal human neural progenitor (NHNP) cells, in

which the distance is measured that cells wander over time. Hill et al. (2008) examined with human NT2 cells endpoints of cell viability and neuronal protein marker expression, to identify developmental neurotoxins. Tegenge and Bicker (2009) showed that aggregates of human NT2 precursor cells are a useful tool to study regulatory mechanism of cell motility. According to these methods a migration assay for developmental neurotoxicity with differentiating human NT2 neurospheres seemed interesting. When one-week retinoic acid treated NT2 neurospheres were seeded on an adherent surface, cells started to migrate out of these spheres. The distances the cells transfer over time could be measured with and without the present of test substances to analyse potential developmental neurotoxic effects. With exposure to model substances like Ethanol, which inhibits migration, or Phorbol-12-myristate-13-acetate (PMA), which stimulates migration of neuronal cells (Sears and Ciment, 1988), a migration assay could be developed, which allows testing several potential developmental neurotoxic compounds.

1.4 Aim of this study

The intention of this study was to establish the human NT2 cell line as human neuronal *in vitro* model systems for replacement of animal models. Firstly, the terminally differentiated, postmitotic NT2 neurons generated by rapid differentiation in sphere cultures (Paquet-Durand et al., 2003) have been characterised using immunocytochemical methods. The expression of a particular neurotransmitter phenotype seemed to be interesting for transplantation studies. Examples would be the induction of a dopaminergic phenotype for transplantation studies of dopamine lesions caused by Parkinson disease or the cholinergic phenotype for replacement of degenerating motorneurons.

In cell physiological experiments with simulated ischaemic conditions in cultures of purified NT2 neurons the efficacy of potential neuroprotective compounds could be analysed *in vitro* (Paquet-Durand and Bicker, 2004). The protective effect against ischaemic damage of diltiazem should be examined and compared with the response to simulated ischaemia in primary cortical mouse neuron cultures.

Furthermore, a migration assay with differentiating human NT2 neurospheres should be established to analyse potential developmental neurotoxic compounds. Cell migration out of these neurospheres could be determined microscopically and compared to acute cytotoxicity measured with an Alamar Blue® viability assay.

2. Materials and Methods

2.1 NT2 cell culture and differentiation

The human Ntera2/D1 cell line (NT2) was obtained from ATCC (American Type Culture Collection, VA, USA). NT2 precursor cells were maintained and cultivated in DMEM/F12 culture medium (Gibco-Invitrogen, Karlsruhe/Germany) supplemented with 10% fetal bovine serum (FBS, Gibco-Invitrogen) and 1% penicillin/streptomycin (Gibco-Invitrogen) in the atmosphere of 5% CO_2 at 37°C (Andrews 1984). Generation of NT2 neurons was performed using the differentiation protocol in free-floating aggregates by Paquet-Durand et al. (2003). Briefly, the NT2 precursor cells were seeded in 80 mm, bacteriological grade Petri dishes (Greiner, Hamburg/Germany) at a density of 4×10^6–5×10^6 cells per dish. On the first day, 10 ml of cell culture medium was added to each Petri dish. On the second day and later on, retinoic acid (RA) was added to the medium to yield a final concentration of 10 µM. Every 2–3 days the medium was changed by transferring the cell suspension to centrifuge tubes. Remaining cell conglomerates were washed off with a few milliliters of medium. Cells were centrifuged at 200×g for 5 min, resuspended in 10 ml of medium containing 10 µM RA, and seeded on new Petri dishes. After 7–8 days the cell conglomerates from one Petri dish were seeded in T75 cell culture flasks (Falcon, Franklin Lakes, NJ, USA) and cultured for another 6– 8 days. Cells were trypsinized (Trypsin-EDTA (1X), Gibco- Invitrogen), transferred to T175 cell culture flasks and cultured in T175 flasks for 2 days to obtain conditioned medium. Then the cells were trypsinized, transferred to T75 flasks and supplied with inhibitor medium (1-6- D-arabinofuransylcytosine: 1 µM, 2′-deoxy-5-fluorouridine: 10 µM, 1-β-D-ribofuranosyluracil: 10 µM). After 7–10 days, neurons were completely differentiated and growing on a layer of non-neuronal cells. For that reason neurons were selectively trypsinized. After differentiation, NT2 neurons were plated on poly-D-lysine (PDL) and matrigel (Becton-Dickinson, Bedford, MA, USA / see 2.2) coated 96-well plates (Falcon) or cover glasses at a density of 10,000-50,000 cells per well and cultured for 1-5 weeks. To limit evaporation from outer wells in long-term cultures, cells were not seeded in the outermost wells. The concentric ring of empty wells around the neuronal cell cultures was filled with sterile phosphate buffered saline (PBS). Cell cultures were maintained in a humidified incubator at 37 °C and 5% CO_2.

To store undifferentiated precursor cells or neurons whenever needed, they were transferred to ice-cold 95% FBS / 5% DMSO medium and frozen in liquid nitrogen.

In order to induce a dopaminergic phenotype, differentiated NT2 neurons were incubated for up to one week with 1 mM lithium chloride and a mixture of fibroblast growth factor 1 (FGF1, 1 ng/ml), 12-O-Tetradecanoylphorbol 13-acetate (TPA, 200 nM), dopamine (20 µM], 3-isobutyl-1-methylxanthine (IBMX, 250 µM) und forskolin (50 µM).). As a positive control for dopamine immunostaining the cells were preincubated for 15 min in 1 mM dopamine prior to fixation. For the detection of cell proliferation, cells were incubated with 50 µM BrdU (5-bromo-2'-deoxyuridine) for 2 h prior to fixation.

2.2 Coating of cell culture ware

Plastic and glass ware was coated with PDL and matrigel before neuronal cells were plated. A stock solution containing 1 mg/ml poly-d-lysine in sterile distilled water was diluted 1:100 (final concentration: 10 µg/ml) and the surface to be coated was incubated with this solution for 2h. After incubation the liquid was removed and allowed to dry completely at room temperature. A matrigel coating followed this. A matrigel matrix solution (approx. 10 mg/ml) was diluted 1:30 in ice cold DMEM/F12 and was pipetted up and down with a fire polished Pasteur pipet 5-10 times to ensure even distribution of matrigel. To each well of a 96 well plate approx. 100 µl of this solution were added and incubated for 2 h. Afterwards the liquid was removed and the plates dried completely at room temperature. Larger surfaces (such as glass cover slips) were incubated with a volume of matrigel solution large enough to cover the entire surface.

2.3 Mouse and human brain tissue

Mouse brain tissue (a kind gift from the Institute of Physiology, University of Veterinary Medicine, Hannover) was used as a positive control for tyrosine hydroxylase immunostaining. Mouse brains were fixed in PBS containing 4 % paraformaldehyde (PFA) and placed in PBS with 30 % sucrose. After freezing the tissue in liquid nitrogen, the brain was sectioned at 20 µm on a Reichert-Jung Frigocut 2800E cryostat microtome at -20°C and collected on PDL coated slides. As a second positive control for tyrosine hydroxylase immunostaining a human brain tissue section microarray (Oligene, Berlin, Germany, Cat.-No.: 401 1210) was used. Immunocytochemical stainings were done as described in 2.4.

2.4 Immunocytochemistry

Cells were washed with PBS once and then fixed for 30 minutes in PBS containing 4 % PFA. For the detection of dopamine 1 % glutaraldehyde was added to the fixative. After fixation, cells were washed 3 times for 5 minutes in PBS containing 0.1 % Triton X100 (PTX). For the detection of dopamine reduction steps with 1 % sodium borohydride and 1 % sodium metabisulphite (SMBS) were required. Detection of BrdU required a 20-min incubation in 2 N HCl in PBS after fixation. Blocking solution containing PTX and 5 % normal serum from the host animal, from which the secondary antibody (**Table 1**) was obtained, was applied for one hour. Primary antibodies (**Table 1**) were diluted in PTX containing 5 % normal serum and applied for one hour. Wells were washed 3 times for 5 minutes in PTX and incubated with diluted secondary biotinylated antibody (Vector) directed against the host animal of the primary antibody for one hour. After 3 more washing steps in PTX immunofluorescence was detected by applying streptavidin bound with alexafluor 488 or CY3 for one hour. Finally, cells were incubated for 5-10 minutes in PBS containing 10 µM DAPI as a nuclear counterstain and washed 3 times in PBS. For visualisation of actin, a solution of phalloidin-Alexa Fluor-568 in PBS was applied for 15 min.

Antibody	***Provider***	***Dilution***
Monoclonal mouse anti-MAP2 (clone AP20)	Sigma, Deisenhofen/Germany	1:500
Polyclonal rabbit anti-Tau (AB1512)	Chemicon International, Temecula (CA)/USA	1:200
Monoclonal mouse anti-Tyrosine Hydroxylase (MAB318)	Chemicon International, Temecula (CA)/USA	1:500
Polyclonal rabbit anti-Tyrosine Hydroxylase (AB152)	Chemicon International, Temecula (CA)/USA	1:200
Polyclonal rabbit anti-Dopamin (AHP847)	Serotec GmbH, Oxford/UK	1:200
Polyclonal rabbit anti-GABA (A2052)	Sigma, Deisenhofen/Germany	1:5000
Polyclonal goat anti-ChAT (AB144P)	Chemicon International, Temecula (CA)/USA	1:100

Antibody	*Provider*	*Dilution*
Monoclonal mouse anti-Synapsin (106001, Clone 46.1)	Synaptic Systems GmbH, Göttingen/Deutschland	1:500
Monoclonal mouse anti-Nestin (MAB5326)	Chemicon International, Temecula (CA)/USA	1:1000
Monoclonal mouse anti-Vimentin (cloneVS; V6630)	Chemicon International, Temecula (CA)/USA	1:1000
Monoclonal mouse anti-acetylated-α-Tubuline (T6793)	Sigma, Deisenhofen/Germany	1:1000
Monoclonal mouse anti-BrdU	Sigma, Deisenhofen/Germany	1:200
Monoclonal mouse anti-β-Tubuline isotype III (cloneSDL.3D10; T8660)	Sigma, Deisenhofen/Germany	1:10000
Alexa Fluor 568-Phalloidin (200 units/ml)	Molecular Probes, Eugene (OR)/USA	1:200
Secondary horse anti-mouse-biotin	Vector, Burlingame (CA)/USA	1:250
Secondary horse anti-rabbit-biotin	Vector, Burlingame (CA)/USA	1:250
Secondary horse anti-goat-biotin	Vector, Burlingame (CA)/USA	1:250

Table 1 *List of primary and secondary antibodies used for the immunocytochemical characterisations.*

2.5 Microscopy, image processing, quantification and statistics

Morphological observations, routine light microscopy and fluorescence microscopy were performed on a Zeiss Axiovert 200 microscope equipped with an HBO 100 Halogen lamp or on a Zeiss Axioskop microscope equipped with an HBO 50 Halogen lamp. Images were captured with a CoolSNAP EZ camera by using MetaFluor 7.0r1 software rather with an Axio Cam HRc camera by using AxioVision 4.7 software. Image overlays and contrast enhancement were done using Adobe Photoshop 6.0.

To quantify fluorescence labeld cells, all cells in one image were counted using the DAPI staining and the proportion of positive cells was determined. To quantify synaptic punctae in synapsin stainings a mask was generated using the treshold function of ImageJ 4.2 (brightness:

+30; contrast: +50; treshold: 0-75) to remove the background staining. The proportion of synaptic punctae in relation to the number of cells (DAPI staining) was determined. Statistical comparisons among different experimental groups were done using a two-tailed Student's t-test. Error bars in the figures indicate the standard error of the mean (SEM), levels of significance are: *p<0.05, **p<0.01, ***p<0.001.

2.6 Anoxia

NT2 neurons were cultured in 96-well plates for 2–4 weeks prior to the anoxia/hypoglycemia experiment. Anoxia experiments were performed in Krebs HEPES buffer (anoxia medium; 1 mM NaH_2PO_4, 118.5 mM NaCl, 5.57 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.03 mM Na_2EDTA, 0.06 mM ascorbic acid, 20 mM HEPES and pH 7.4). The normal cell culture medium was replaced by 100 µl anoxia medium immediately before the experiment was started. 2 mM sodium dithionite was added to the anoxia medium to scavenge oxygen in the medium. An internal positive control (live control) was exposed to anoxia medium without sodium dithionite and these wells were closed with airtight caps to maintain normoxic conditions. In samples, the anoxia medium was supplemented with 500 µM glutamate and 500 µM glycine. Between 5 and 250 µM D-cis-diltiazem was added to these samples. The 96-well plates were placed in a sterile, anoxic chamber which was perfused with a mixture of 95 % argon and 5 % CO2 during the full duration of the anoxia. The chamber itself was transferred to a 37 °C warm water bath. Temperature inside the anoxic chamber was monitored constantly and did not exceed 38 °C. The duration of anoxia was measured from the time after the temperature inside the anoxic chamber had reached 37 °C. This was usually the case 10–14 min after the chamber was placed into warm water bath. After 2 h to 3 h 30 min, the anoxia experiment was stopped by reoxygenation and a viability assay was assayed several times after the anoxia experiment (see 2.8).

2.7 Mouse neuronal cell cultures

Newborn C3H mice from the BMC animal breeding facility were used. All procedures were performed in accordance with the Lund University ethics committee (permits #M213-03 and #M76-03). Animals were killed by decapitation, the eyes were collected for separate experiments, the brains were removed and primary cortical cell cultures were prepared essentially as described by Li et al. (1997). Briefly, cortices were placed in a balanced salt solution (BSS; 137 mM NaCl, 5.3 mM KCl, 0.168 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 10 mM HEPES, 33.3 mM Glucose, 43.8

mM Sucrose and pH 7.4), cut into small pieces (≈1 mm^2) and exposed to BSS containing 10 U/ml papain for 30 min at 37 °C. After centrifugation for 5 min at 100×g and resuspension in BSS containing 1 % bovine serum albumin (BSA; Boehringer, Mannheim/Germany) and 1 % trypsin inhibitor, the pieces were gently dissociated by trituration with a glass micropipette for 20 times and with a fire polished glass micropipette for another 10 times. Cells were centrifuged again and resuspended in D-MEM/F12 medium (Gibco-Invitrogen) supplemented with FBS and penicillin/streptomycine (see 2.1). To enhance the percentage of neuronal cells, the non-neuronal cells were allowed to adhere for 30 min to a plastic cell culture dish. The cells in the supernatant were then seeded in poly-D-lysine and matrigel matrix (Becton-Dickinson) pre-coated 96-well plates with a density of 30,000 cells/well. Proliferation of non-neuronal cells was inhibited by adding 1 μM 1-6-D-arabinofuransylcytosine and 10 μM 2′-deoxy-5-fluorouridine (Huettner and Baughman, 1986) to the growth medium 1 day after plating. This treatment was continued during the next 4 days until anoxia experiments were performed as described above.

2.8 Cell viability assay

The development of the viability of cell cultures was monitored using the Alamar Blue® assay. In this assay the dye – which is originally blue – is incorporated into the cells, reduced by the cells metabolism to a red form, and re-released to the surrounding medium. The viability of a cell culture was detected by measuring the absorbance (**Fig. 3a**) of the liquid medium in a photometric microplate reader (Tecan Spectra II) or by measuring the emission (**Fig. 3b**) of the liquid medium in a fluorescence microplate reader (Tecan infinite M200). The photometric microplate reader was equipped with 570 nm and 600 nm filters. The fluorescence reader measured the fluorescence intensity using an excitation wavelength of 530 nm and an emission wavelength of 590 nm and was controlled using i-control 1.3 software. The measured value allows to calculate the percentage of the dye that is present in the oxidised and in the reduced form. The higher the reduction rate, the higher the viability of the cell culture.

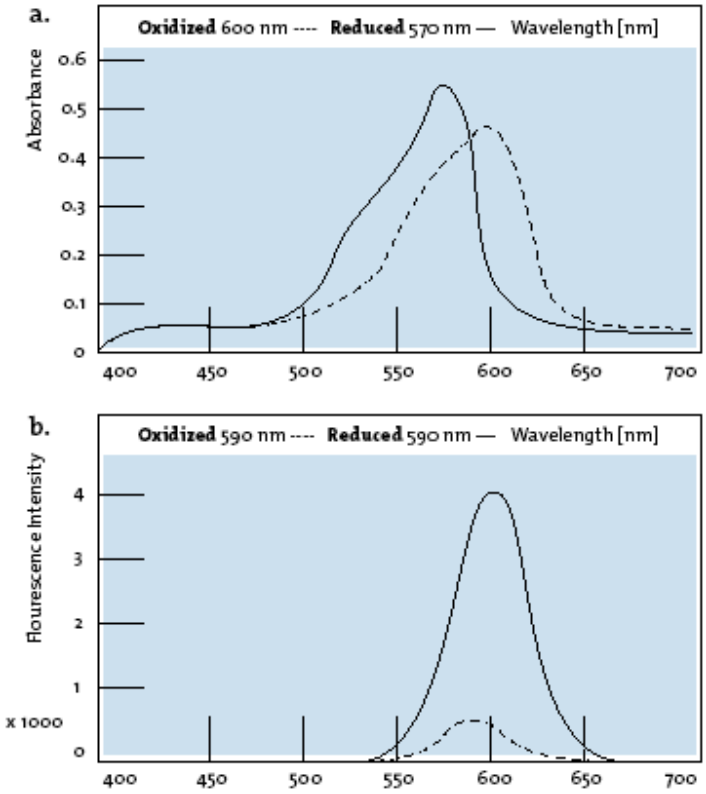


Fig. 3 *Absorbance- (**a**) and emission- (**b**) spektra of the oxidized and reduzed forms of Alamar Blue.*

For every experiment the viability was measured before and after the treatment of the cells. For each measurement the cell culture medium (or Krebs HEPES Buffer) was completely changed and replaced with culture medium with 3 % Alamar Blue® solution. The assay volume in 96 well plates was 100 µl per well and in 24 well plates 200 µl per well. The plates were read in the microplate reader and incubated for 4 h at 37 °C / 5 % CO_2 before the plates were read again. After the measurement the medium was changed to cell culture medium without Alamar Blue and cell cultures were maintained at 37 °C and 5 % CO_2 up to the next measurement.

The viabilities measured before the cells were treated were set to 100 % for each well in every sample group. Viabilities measured after treatment, were compared to the values obtained before and to the internal positive control. They are given as percentage of the positive control. To establish whether a distinctive treatment had a significant effect on viability, the samples were compared using Student´s t-test.

2.9 Migration assay with NT2 neurospheres

NT2 neurospheres were formed by one-week treatment of retinoic acid using the first step of the differentiation protocol in free-floating aggregates described above (2.1). These neurospheres were seeded on PDL and matrigel coated cover glasses (12 mm) in 24 well plates at a density of approx. 10 neurospheres in 40 µl medium per well. The neurospheres were allowed to adhere on the surface for one hour at 37 °C and 5 % CO_2 before adding 500 µl of RA medium to each well and incubate for further 48 h. To investigate the effects of several test compounds in different experiments the medium was supplemented with a wide spread of concentrations of these substances. A positive control was done without additions. The migration was observed under a Zeiss Axiovert 200 microscope with 10x objectives. To quantify the cell-migration for every sample eight photos, each with one neurosphere, were taken after 24 h and 48 h. The distance between the edge of the sphere and the furthest migrated cells were measured using ImageJ 1.41o at four distinct position per neurosphere. The cells were fixed after 48h for immunocytochemical stainings. For every experiment a viability assay with Alamar Blue after 24 h and 48 h (see 2.8) was performed that allowed to compare the migration values with cytotoxicity values. Dose/effect-profiles and resultant EC_{50} values were calculated using Sigma Plot 8.02.

2.10 Time lapse video microscopy of NT2 neurospheres

The neurospheres were seeded on 20 mm cover glasses coated with PDL and matrigel and incubated for 24 h at 37 °C and 5 % CO_2. The cover slip was transferred to an observation chamber with culture medium, covered with a 35 mm Petri dish and closed with Parafilm. The chamber was maintained at 37 °C. For a time lapse sequence every two minutes a picture was taken from a small section of a neurosphere under a Zeiss Axiovert 200 microscope with 10x objectives. The migration was observed over night for approx. 24 h and a video was created using MetaFluor 7.0r1 software.

2.11 List of chemical substances, biological agents and cell culture material used

Item	***Provider***
1-6-D-arabinofuranosylcytosine (Ara C)	Sigma, Taufkirchen/Germany
1-ß-D-ribofuranosyluracil (Urd)	Sigma, Taufkirchen /Germany

Item	*Provider*
2'-deoxy-5-fluorouridine (FudR)	Sigma, Taufkirchen /Germany
Acetaminophen	Sigma, Taufkirchen /Germany
Alamar Blue® Assay	Trek Diagnostic Systems, East Grinstead/UK
Argon/CO_2 gas (95%/5%)	Messer Griesheim, Duisburg/Germany
BrdU (5-bromo-2'-deoxyuridine)	Sigma, Taufkirchen /Germany
BSA (bovines serum albumine)	Sigma, Taufkirchen /Germany
$CaCl_2$	Aldrich, Gillingham/UK
Cell culture flasks (tissue culture grade)	Falcon, Franklin Lakes (NJ)/USA
$CuSO_4$	Fluka, Buchs/Switzerland
CY3 (streptavidin bound)	Vector, Burlingame (CA)/USA
DAPI	Sigma, Taufkirchen /Germany
Diltiazem	Sigma, Taufkirchen /Germany
DMEM/F12 Medium	Gibco, Paisley/UK
DMSO (dimethyl sulfoxide)	Sigma, Taufkirchen /Germany
Dopamine hydrochloride	Sigma, Taufkirchen /Germany
Ethanole	Roth, Karlsruhe/Germany
Fetal bovine serum (FBS)	Gibco, Paisley/UK
Fluorescein (streptavidin bound)	Vector, Burlingame (CA)/USA
FGF-1	Merck, Darmstadt/Germany
Forskolin	Sigma, Taufkirchen /Germany
Glutamine solution	Gibco, Paisley/UK
Glutaraldehyde	Sigma, Taufkirchen /Germany
Glycine	Sigma, Taufkirchen /Germany
HCl (1 N solution)	Roth, Karlsruhe/Germany
HEPES	ICN Biomedicals, Aurora (OH)/USA
Hoechst 33342 (bisBenzimide)	Sigma, Taufkirchen /Germany
IBMX	Sigma, Taufkirchen /Germany
$K_3Fe(CN)_6$	Roth, Karlsruhe/Germany
KCl	Sigma, Taufkirchen /Germany

Item	*Provider*
L - Ascorbic acid	Sigma, Taufkirchen /Germany
L-Glutamic acid	Sigma, Taufkirchen /Germany
Lead(II) acetate trihydrate (Pb-ac)	Sigma, Taufkirchen /Germany
LiCl	Sigma, Taufkirchen /Germany
MAM (Methylazoxy methanole acetate)	NCI, Kansas City/Missouri
Matrigel	Becton dickinson, Franklin Lakes (NJ)/USA
MeHgCl (Methylmercury (II) chloride)	Sigma, Taufkirchen /Germany
Methanole	Roth, Karlsruhe/Germany
$MgCl_2$	Fluka, Buchs/Switzerland
MK-801 (Dizocilpine)	Sigma, Taufkirchen /Germany
Na_2EDTA	Sigma, Taufkirchen /Germany
NaCl	Sigma, Taufkirchen /Germany
NADPH	Sigma, Taufkirchen /Germany
NaH_2PO_4	Merck, Darmstadt/Germany
NaN_3	Sigma, Taufkirchen /Germany
NaOH	Merck, Darmstadt/Germany
NMDA (N-methyl-d-aspartic acid)	Sigma, Taufkirchen /Germany
Normal horse, rabbit and goat sera	Vector, Burlingame (CA)/USA
NT2 precursor cells	Stratagene Corp., La Jolla (CA)/USA
Penicilline / streptomycine solution	Gibco, Paisley/UK
Petri dishes (bacteriological culture grade)	Greiner, Hamburg/Germany
PFA	Sigma, Taufkirchen /Germany
PMA	Alomone Labs, Jerusalem/Israel
Poly-d-lysine (PDL)	Sigma, Taufkirchen /Germany
Retinoic Acid (RA)	Sigma, Taufkirchen /Germany
Secondary biotinylated antibodies	Vector, Burlingame (CA)/USA
SMBS (Sodium metabisulphite)	Sigma, Taufkirchen /Germany
Sodium borohydride	Sigma, Taufkirchen /Germany
Sodium dithionite ($Na_2S_2O_3$)	Merck, Darmstadt/Germany

Item	*Provider*
D(+)-Sucrose	Sigma, Taufkirchen /Germany
Tissue-Tek	Sakura Finetek, Zoeterwoude/Netherlands
TRIS	Sigma, Taufkirchen /Germany
Triton X100	Sigma, Taufkirchen /Germany
Trypsine EDTA solution (1x)	Gibco, Paisley/UK
VPA (Valproic acid sodium salt)	Sigma, Taufkirchen /Germany

3. Results

3.1 Characterisation of NT2 neuronal cells

3.1.1 Neuronal differentiation

NT2 precursor cells were initially kept in adherent culture until 90 % confluency (**Fig. 4a**) and then differentiated according to the method of Paquet-Durand et al. (2003). When NT2 cells were seeded in the presence of retinoic acid onto non-adhesive bacteriological grade petri dishes, they formed free floating spheres which support cellular proliferation and neural differentiation (**Fig. 4b**). After replating to adherent substrate some of the cells which migrated out of the spheres showed already long processes, typical for neuronal morphology (**Fig. 4c**). During cell culture in medium containing mitotic inhibitors (**Fig. 4d**) the preferential adhesion of differentiating neurons to each other became apparent. After the inhibitor treatment and selective trypsinisation, purified neurons were cultured for at least 10 days on matrigel coated cover slips prior to immunocytochemical characterization. During this period, the initially dispersed NT2 neurons formed clusters and extended numerous long neurites (**Fig. 4e**). As reported by Pleasure et al. (1992), cell cultures contained more than 95% postmitotic neurons, less than 5% large (~100-300 µm) undifferentiated, but postmitotic epitheloid cells, and no proliferating precursor cells.

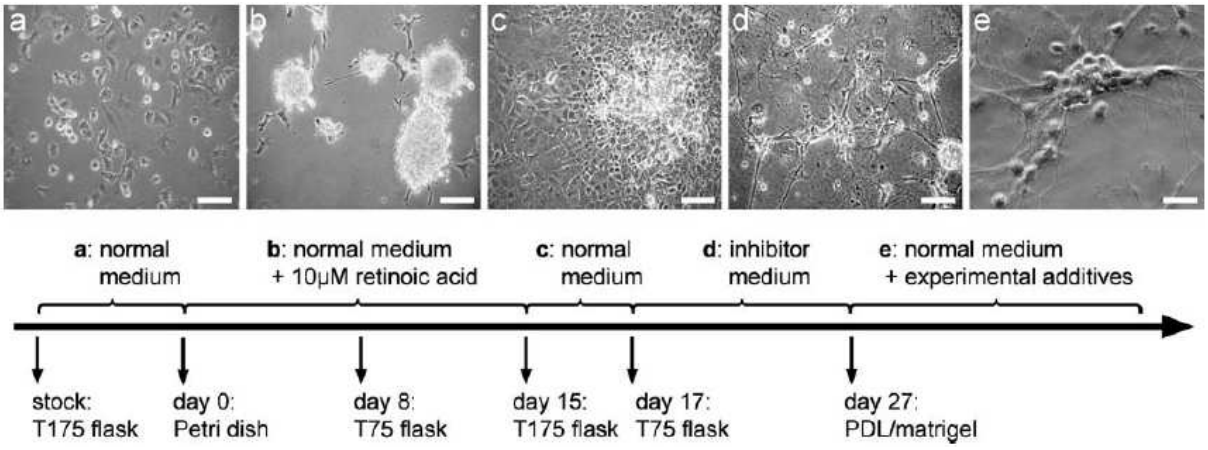


Fig. 4 *Differentiation of Ntera-2 (NT2) precursors into neurons.* ***a*** *Stock of undifferentiated precursor cells, grown on an adherent substrate.* ***b*** *Day 8: neurospheres after 1 week of differentiation in the presence of 10 µM retinoic acid in a non-adherent Petri dish.* ***c*** *Day 17: mixed culture of NT2 neurons and NT2 precursors after being replated.* ***d*** *Day 22: culture of NT2 neurons in inhibitor medium, with few precursor cells remaining.* ***e*** *Day 41: purified neurons, 14 days after final replating on PDL and matrigel. Bars 100 µm (**a–d**), 50 µm (**e**)*

3.1.2 Neuronal phenotype

To confirm neuronal differentiation in the aggregate culture the expression of cytoskeletal markers both for immature precursors and mature neurons were tested. NT2 precursor cells did not express type III ß-tubulin (**Fig. 5a**). They expressed the intermediate filaments nestin and vimentin (**Fig. 5b, c**) which are typical for neuronal precursor cells (Chu et al. 2006). NT2 neurons displayed immunoreactivity to type III ß-tubulin, MAP-2 and tau (**Fig. 5d, g, h**). Tau staining was weak in cultures stained eight days after replate, but became much stronger after prolonged outgrowth and maturation of the cell culture. **Fig. 5h** displays tau staining after 24 days in culture. The few large epitheloid cells that accompany NT2 neurons displayed only faint staining for type III ß-tubulin, and no immunoreactivity to antibodies against MAP-2 and tau (arrowheads in **Fig. 5d, g, h**). Instead, these cells retained cytoskeletal proteins characteristic for the precursor stadium such as the intermediate filaments vimentin and nestin (**Fig. 5e, f**) which were not expressed by neurons (arrowheads in **Fig. 5e, f**). They differed from the proliferating precursors, however, by their large size (diameter 100-300 µm as compared to precursors: 20-30 µm), and by the fact that they did not divide.

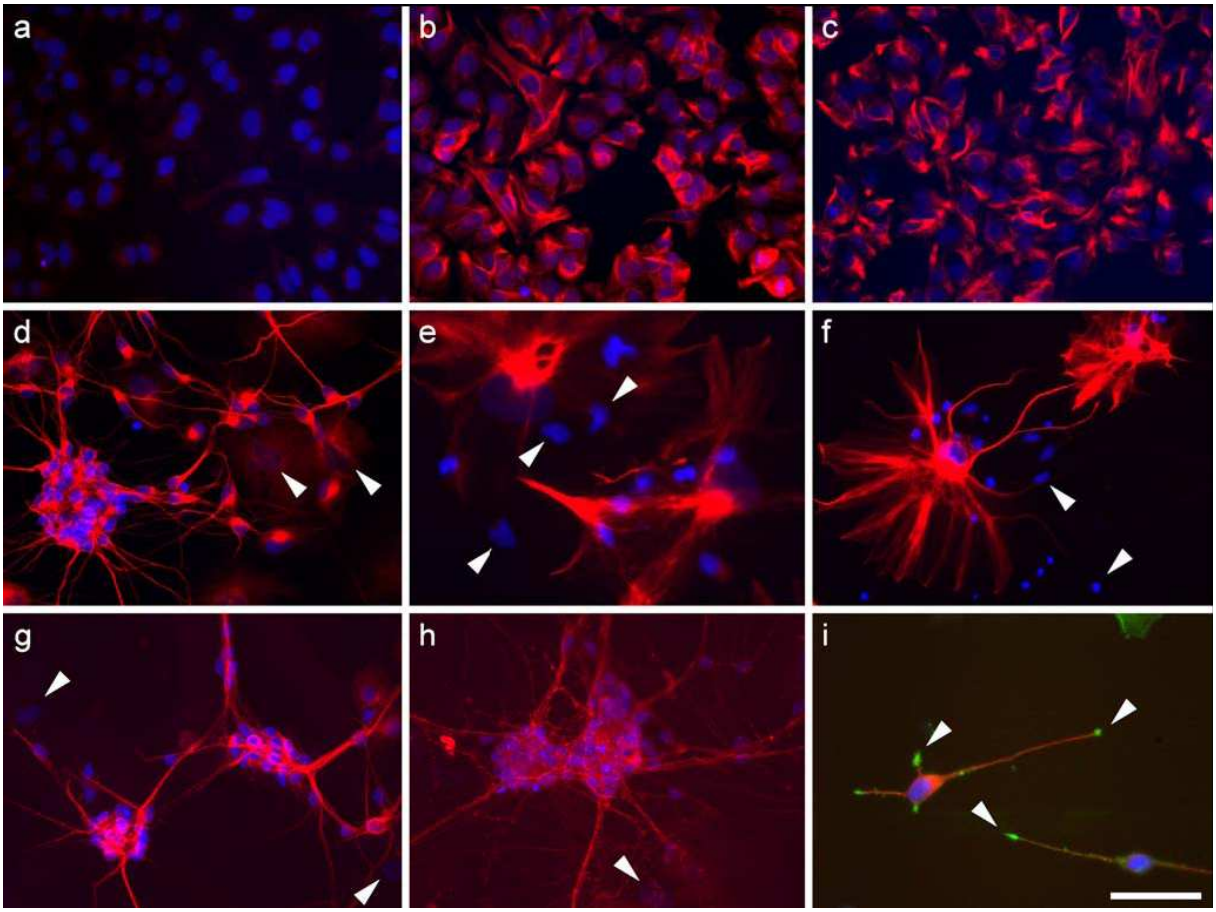


Fig. 5 *Cytoskeletal elements (red) and corresponding DAPI-labeled nuclei (blue) in NT2 precursor cells (**a–c**) at 24 h after seeding onto plastic and in purified NT2 neurons (**d–i**) cultured for 10 days on matrigel before fixation (except for h: 24 days).* ***a*** *NT2 precursor cells express only minimal amounts of β-tubulin type III.* ***b*** *Precursors express nestin.* ***c*** *Precursors express vimentin.* ***d*** *All neuronal cells display immunoreactivity for β-tubulin type III, whereas non-neuronal epitheloid cells (arrowheads) do not.* ***e*** *Nestin is expressed only by nonneuronal epitheloid cells, but not by neurons (arrowheads).* ***f*** *Vimentin is expressed only by non-neuronal epitheloid cells, but not by neurons (arrowheads).* ***g*** *MAP-2 is expressed by neurons, but not by non-neuronal epithelial cells (arrowheads).* ***h*** *Processes (but not perikarya) of neurons express tau, whereas non-neuronal epitheloid cells (arrowhead) do not.* ***i*** *Multiple growth cones (arrowheads) on NT2 neurons, at 24 h in culture, labeled with green-fluorescent phalloidin; the red staining is β-tubulin type III. Bar 100 μm (**a, b, d–h**), 50 μm (**c, i**)*

Within ten days of culture, NT2 neurons aggregated into clusters and formed multiple neurites with numerous contacts (**Fig. 5d, g, h**). To test whether some of these contacts might contain functional connections, a staining for synapsin, a soluble protein associated with the reserve pool of synaptic vesicles, was performed. A strong staining for synapsin (**Fig. 6a, d**)

predominantly in the neural processes was found. The staining appeared punctate, indicating local areas of high concentrations of the synaptic marker, as would be expected in presynaptic structures. From one week cultured neurons to two week cultured neurons the number of synaptic punctae (s.p.) increased. By generating an optical mask beyond a certain intensity threshold, the quantification of these synaptic punctae (**Fig. 6c, f**) revealed 37± 2.3 s.p./100 cells in one week and 67± 4.6 s.p./ 100 cells in two week cultured neurons.

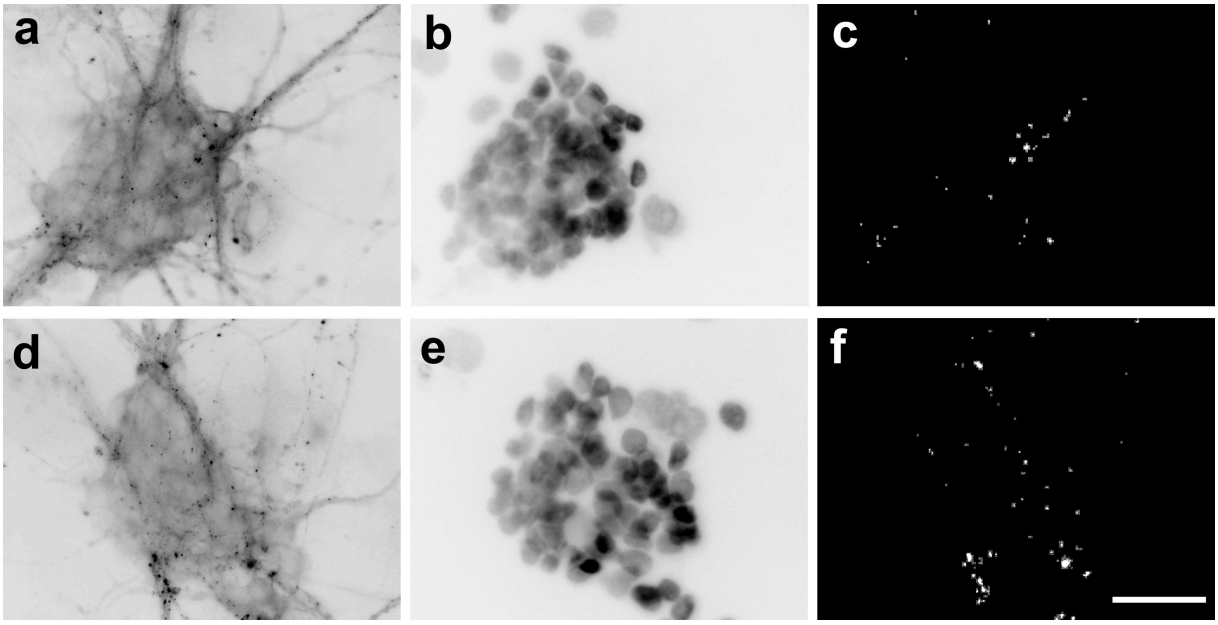


Fig. 6 *NT2 neurons stain for synapsin (inverted immunofluorescence staining) after 1 week (**a**) and after 2 weeks (**d**) culture; DAPI nuclear counterstaining (**b, e**). On the basis of the generated mask to remove the background staining (**c, f**, not inverted) the increase of synaptic punctae becomes apparent. Bar 50 µm*

3.1.3 Neurotransmitters

NT2 neuron cultures contained a strong immunostaining against GABA (**Fig. 7a**) in 14.5 ± 2.3 % of the neurons, that was above a weak background staining which also appeared in the non-neuronal epitheloid cells.

A large population of neurons were immunopositive for the cholinergic marker choline acetyl-transferase (ChAT, **Fig. 7c**). This marker was detected in the soma and processes; cell counting revealed 34.0 ± 6.7 % ChAT-positive neurons.

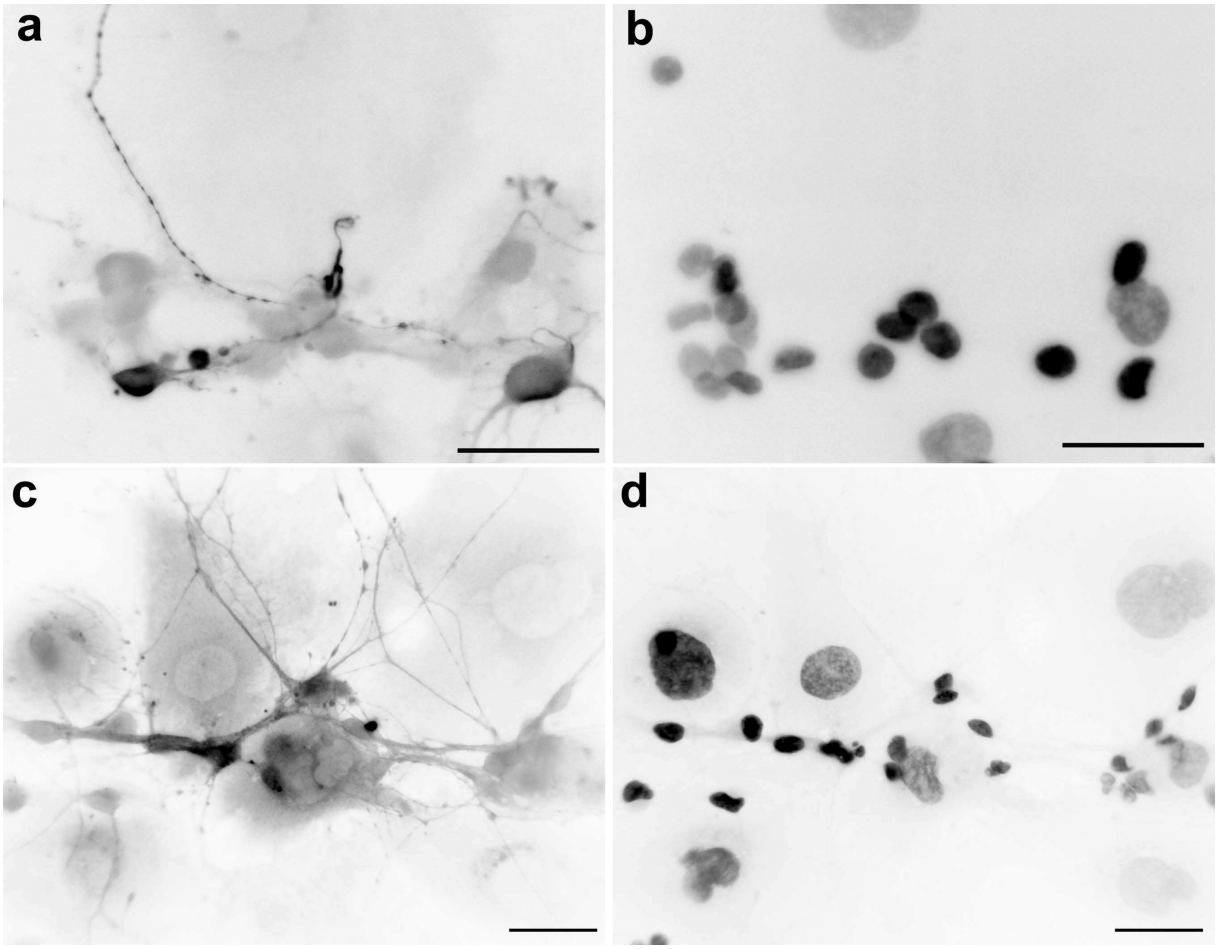


Fig. 7 *Inverted immunofluorescence images of NT2-neurons stained for the neurotransmitters GABA (**a**) and acetylcholine (**c**) and the corresponding DAPI labeled nuclei (**b, d**). **a** A subset of NT2-neurons is strongly immunoreactive for GABA; **c** A large population of neurons are immunopositive for the cholinergic marker choline acetyl transferase. Bars 50µm*

Previous reports showed differentiation into dopaminergic NT2 neurons by exposure either to lithium (Zigova et al. 1999, 2000) or to a mix of growth factors, dopamine, and high intracellular cAMP levels by the application of forskolin and IBMX (Iacovitti et al. 2001). In each case, with or without these treatments, no indicators of a dopaminergic phenotype could be observed. Two different primary antibodies against tyrosine hydroxylase, antityrosine hydroxylase MAB318 (**Fig. 8a**) and anti-tyrosine hydroxylase AB152 (**Fig. 8c**) were used. Both of which stained appropriate control tissue (mouse brain slice, see inset in **Fig. 8a**; human tissue microarray, see inset in **Fig. 8c**). Anti-dopamine immunostaining was absent (**Fig. 8e**) unless the cells were preincubated for 15 min in 1 mM dopamine prior to fixation (see inset in **Fig. 8e**). This confirmed the reliability of the staining procedure.

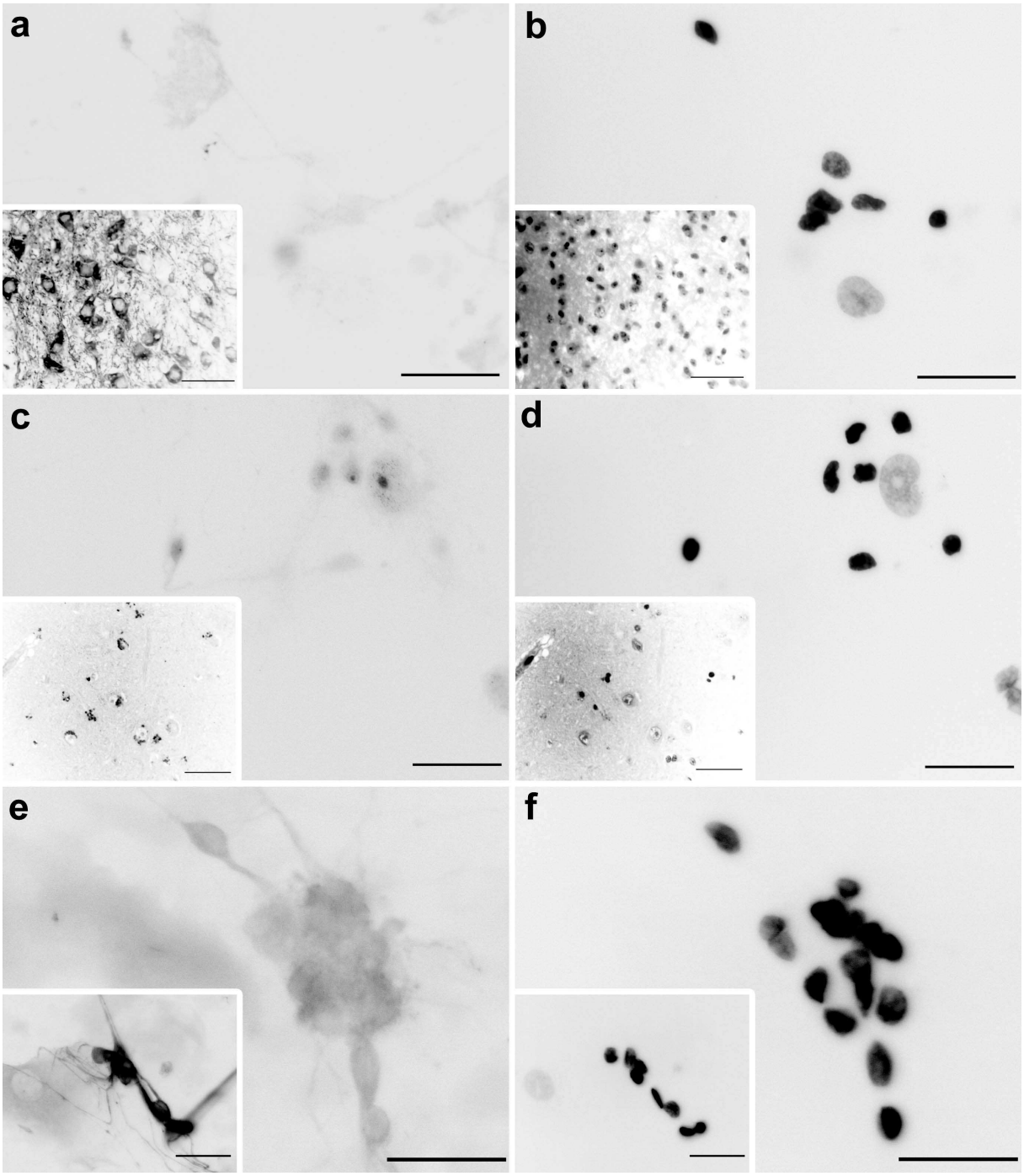


Fig. 8 *Inverted immunofluorescence images of NT2 neurons cells stained for tyrosine hydroxylase and dopamine; DAPI was used as a nuclear counterstain (**b, d, f**). Several primary antibodies were used all of which delivered negative results. **a** Anti-tyrosine hydroxylase MAB318. **c** Anti-tyrosine hydroxylase AB152. **e** Anti-dopamine AHP847. Insets: Positive controls, mouse brain slice (**a**), human brain tissue microarray (**c**), and NT2 neurons pretreated with 1 mM dopamine for 15 min before fixation (**e**). Bars 50 μm*

3.2 Anoxia in NT2 neuronal cell cultures

3.2.1. Response of NT2 neuronal cultures to anoxic conditions

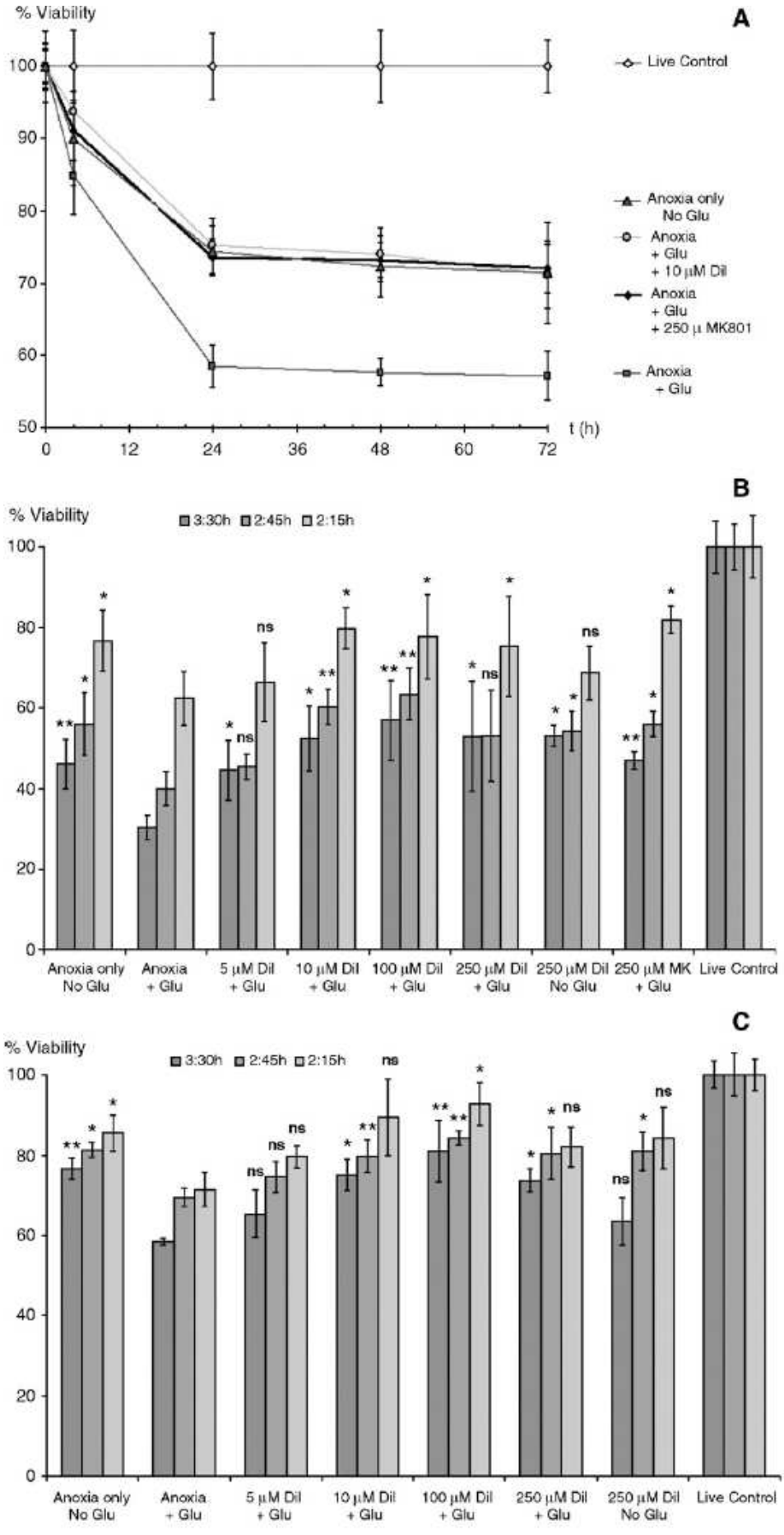
Exposure to anoxic conditions caused neuronal damage and cell death in differentiated NT2 neuron cultures as assessed by the decrease in their viabilities. The main loss in neuronal cells occurred either during anoxia or in the first 24 h after anoxia. Only a small amount of cell death occurred between 24 h and 72 h post anoxia (**Fig. 9a**). For this reason and for the sake of clarity, only the data from the 48 h post anoxia time point are shown in the following figures. The extent of damage was dependent on the duration of anoxia, with an anoxia of 3.30 h showing the strongest loss of viability (dark bars in **Fig. 9b, c**). Both anoxia with and without the addition of glutamate reduced the viability of the neuronal cultures. The decline in viability was, however, far more pronounced when glutamate was added to the medium (**Fig. 9**).

3.2.2. Neuroprotective effect of diltiazem on NT2 neuronal cultures

It has been reported that MK801, a commonly used NMDA channel blocker, protects NT2 neurons in the model of simulated ischemia against glutamate-induced damage (Paquet-Durand and Bicker, 2004). In this study, the neuroprotective capacities of diltiazem and MK801 after an in vitro simulation of ischemia were compared. Like MK801, diltiazem was able to reduce the excitotoxic effect of glutamate on viability of NT2 neuronal cell cultures. In experiments with durations of anoxia ranging from 2.15 h to 3.30 h, the damage caused by the addition of glutamate was reduced by both drugs to the level of samples that were not exposed to glutamate. However, the diltiazem concentrations necessary to achieve this effect were considerably lower than MK801 concentrations (**Fig. 9b**). Both MK801 and diltiazem were unable to reduce the percentage of cell death caused by the anoxic treatment alone. At a concentration of 10 μM, diltiazem already exhibited a significant protection of NT2 neuronal cultures from excitotoxic injury. The optimal levels of neuroprotection were seen at 10 and 100 μM (**Fig. 9b**). At 250 μM, diltiazem did not increase the viability of NT2 neuronal cell cultures any further. Whereas diltiazem was effective under conditions simulating ischemia, it showed no significant neuroprotective action against anoxia without glutamate in the medium.

3.2.3. Neuroprotective effect of diltiazem on mouse primary cortical neuronal cultures

To confirm the protective effect of diltiazem in a primary culture system, additional experiments were performed using cortical neuron cultures obtained from neonatal mice. The impact of simulated ischemia on the viability of the murine cultures was less pronounced than in the NT2 neuronal cultures. While the NT2 neuronal cultures lost approximately 70 % of their original viabilities after 3.30 h of anoxia, the murine cultures lost only about 40 % of their viabilities in the same period (**Fig. 9b, c**). However, at a concentration of 100 μM, diltiazem also demonstrated a significant neuroprotective effect for all three durations of anoxia. The use of 250 μM diltiazem led to a slight reduction of the viability when compared to the 100 μM diltiazem values or the samples without glutamate, pointing towards a possible adverse effect of diltiazem at higher doses (**Fig. 9c**).


A
% Viability
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12
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+ Glu
100 μM Dil
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250 μM Dil
+ Glu
250 μM Dil
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250 μM MK
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5 μM Dil
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+ Glu
250 μM Dil
No Glu
Live Control

Fig. 9 *Protective effects of diltiazem and MK801 on viabilities of NT2 neuronal cell cultures and mouse primary cortical neuron cultures. (**A**) NT2 neurons were cultured for 28 days in vitro (DIV) and then exposed to 2.45 h of anoxia and 500 μM glutamate/glycine (Glu). The time course of viability in the cell cultures was followed during 72 h post anoxia. (**B**) Comparison of the neuroprotective effects of diltiazem (Dil) and MK801 (MK) 48 h after anoxia. After 20 DIV, the NT2 neurons were subjected to differing lengths of anoxia (2.15 h, 2.45 h, 3.30 h). Samples treated with glutamate and diltiazem or MK801 are compared to samples that were treated with glutamate only. Diltiazem was added to the samples in concentrations ranging from 5–250 μM. At 10 μM, diltiazem compensates the glutamate-induced neuronal damage achieving a similar level of protection as 250 μM MK801. The bars show mean values with n=3–7 independent experiments with 5–6 individual measurements in each. (**C**) Comparison of the neuroprotective effect of different concentrations of diltiazem 48 h after anoxia on mouse primary cortical neuron cultures. After 5 DIV, the neuronal cultures were subjected to differing lengths of anoxia (2.15 h, 2.45 h, 3.30 h). The effect of anoxia on the viability of the primary neuron cultures was less pronounced when compared with NT2 neuron cultures. Still, at 100 μM, diltiazem completely protected the primary neurons against glutamate-induced damage. The bars show mean values taken fromn=2–3 independent experiments with 6 individual measurements in each. Levels of significance (**B, C**) were calculated by comparing Dil+Glu values with the corresponding Anoxia+Glu value (i.e. the second group of columns in the histogram from the left); error bars represent SEM; significance levels are: *p <0.05; **p <0.01; ns=not significant.*

3.3 Migration of NT2 neurospheres

3.3.1 Observation of migration

When one-week retinoic acid treated NT2 neurospheres were seeded on an adherent surface cells started to migrate out of these spheres. To visualize how cells migrate out, the cells were observed under a Zeiss Axiovert 200 light microscope and time lapse video microscopy were performed. The cells do not migrate in the observation chamber, when time lapse video microscopy were started one hour after seeding. For that reason, the cells were incubated for 24 h at 37 °C and 5 % CO_2 before starting the observation. The resulting time lapse video visualized the migration process in an impressive way. The picture sequence taken at 4 hours time interval of **Fig. 10** provides a representation of the migration process.

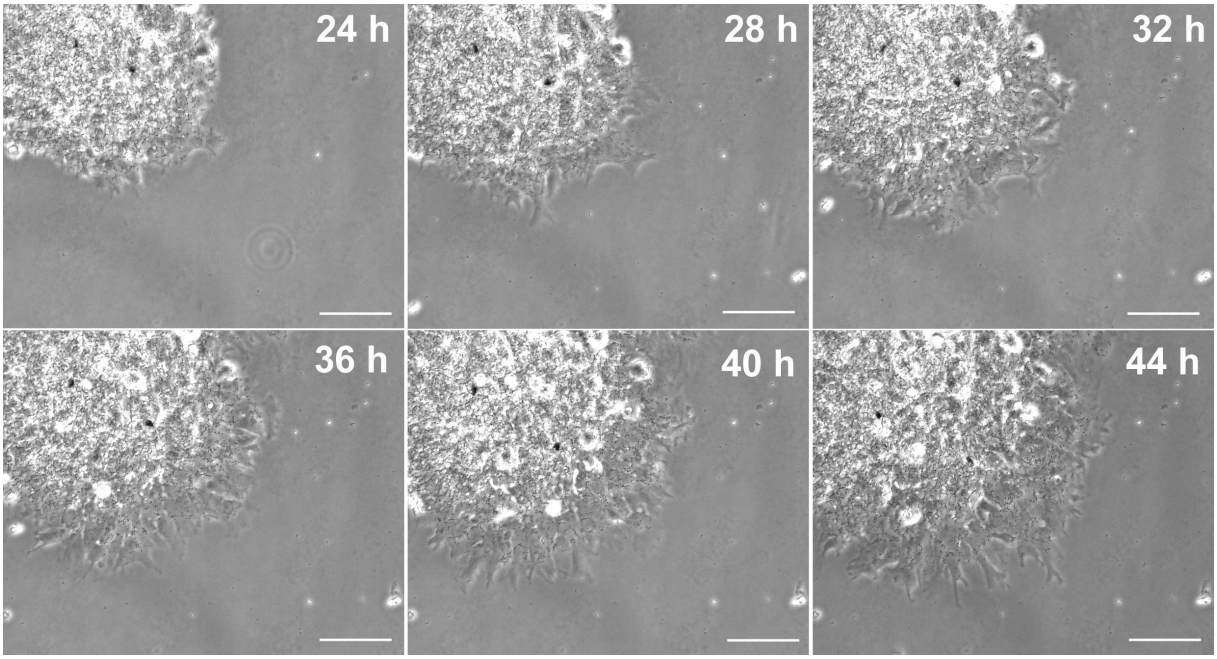

Fig. 10 *Migration of NT2 neurospheres after 24, 28, 32, 36, 40 and 44 hours. Bars 100 µm*

In a further experiment, the migration of NT2 neurospheres was observed over one week and every day up to every third day a picture were taken and analysed. The cells migrated at constant speed. These demonstrated that this cell migration is a linear process without any slow down of motility (**Fig. 11**).

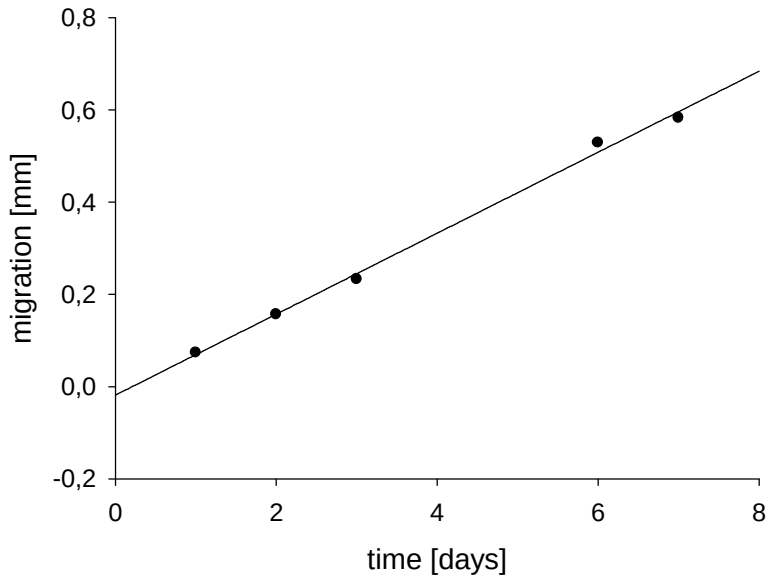

Fig. 11 *Migration of NT2 cells over a period of one week demonstrate the linear process of the cell migration.*

3.3.2 Immunocytochemical characterisation of migrating NT2 neurospheres

To characterise the NT2 neurospheres and the migrating cells after 24 and 48 hours migration several immunocytochemical stainings of cytoskeletal markers were performed. For 24 h migrated neurospheres show similar results as 48 h migrated cells. The intermediate filament nestin which is typical for neuronal precursor cells is expressed in the neurosphere as well as in the migrating cells (**Fig. 12a**). Type III ß-tubulin, which is a marker for neuronal cells is expressed in only a few cells and is located in the neurosphere (**Fig. 12b**). A staining with phalloidin visualised the actin cytoskeletal structure of the neurosphere and the migrating cells (**Fig. 12c**).

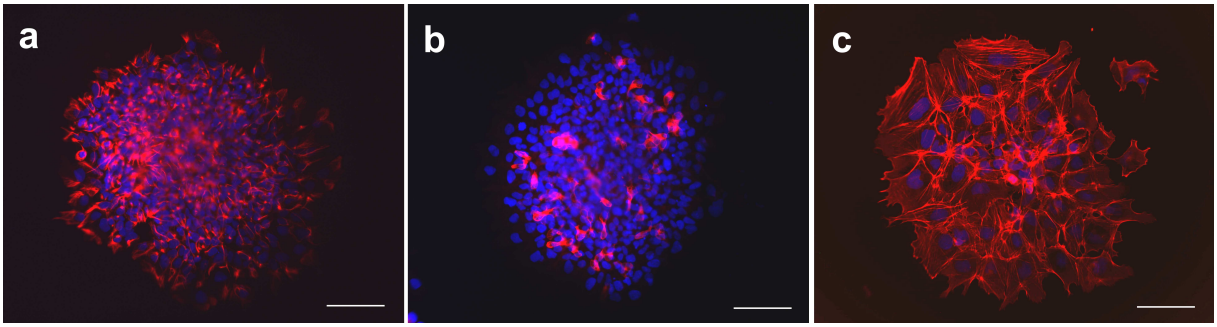

Fig. 12 *Immunocytochemical labelling of cytoskeletal markers (red) and corresponding DAPI-labeled nuclei (blue) of 24 h migrated NT2 neurospheres.* ***a*** *Nestin is expressed in the neurosphere as well as in the migrating cells,* ***b*** *Type III ß-tubulin is expressed in only a few cells and is located in the neurosphere and* ***c*** *phalloidin visualised the actin cytoskeletal structure. Bars 100 µm*

For the detection of cell proliferation, cells were exposed to BrdU for 2 hours and labelled for incorporated BrdU with an anti-BrdU antibody (**Fig. 13**). BrdU was incorporated into 24.2 ± 2.4 % cells after 24 h migration and into 14.8 ± 0.7 % after 48 h migration (n=4 spheres counted). This decrease of cell proliferation after a longer migration period is probably caused by application of retinoic acid which is a known inhibiter of cell proliferation (Kikuchi et al., 1984).

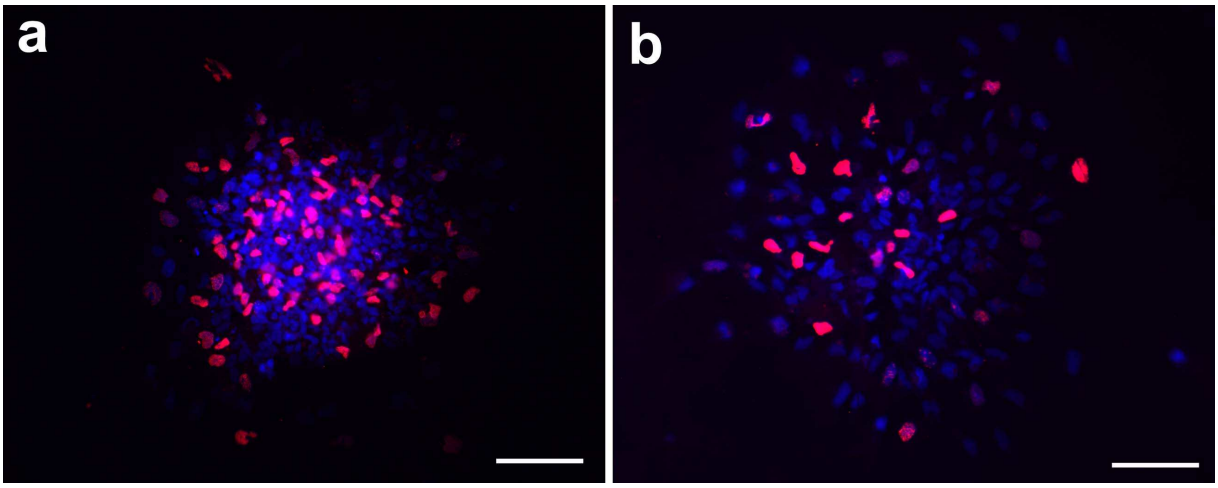

Fig. 13 *Cell proliferation of NT2 neurospheres. Cells labelled for incorporated BrdU (red), nuclear counterstaining (DAPI) appears blue. After 24 h migration* ***(a)*** *24.2 ± 2.4 % cells were labelled and after 48 h migration* ***(b)*** *14.8 ± 0.7 %. Bars 100 µm*

3.3.3 Migration assays in present of test compounds

To investigate the effects of several test compounds on migration of NT2 neurospheres a principle of evaluation has been established according to the method described in Moors et al. (2007) (**Fig. 14**). After 24 h and 48 h migration the distance between the edge of the sphere and the furthest migrated cells have been measured in treated and untreated cells at four distinct positions per neurosphere (n=32 distances per sample were measured). Dose/effect-profiles and resultant EC_{50} values (= compound concentration with 50 % effect) in relation to the untreated positiv control were calculated and compared with acute toxicity values determined by an Alamar Blue ® viability assay. Each experiment was performed two or three times.

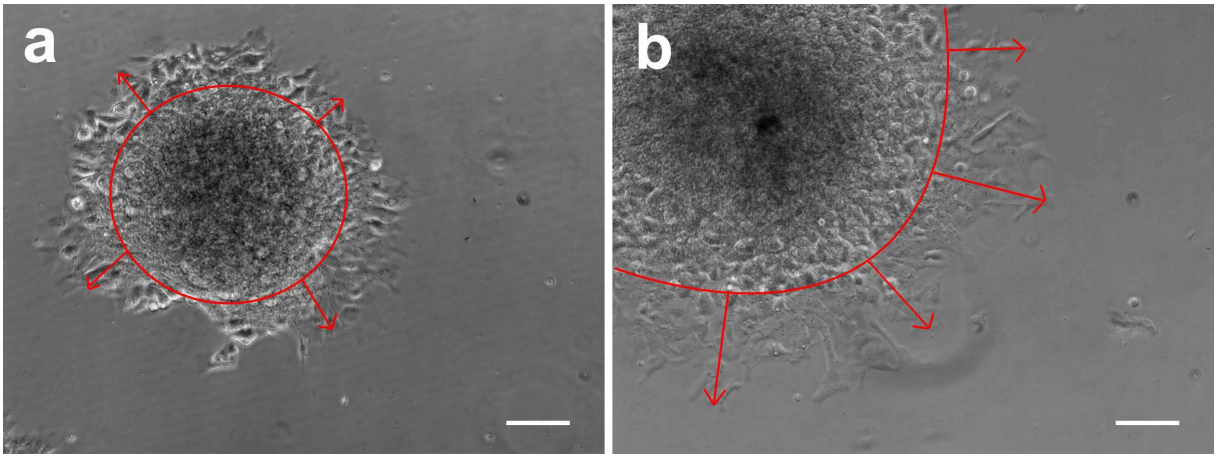

Fig. 14 *Principle of evaluation illustrated on untreated migrating NT2 neurospheres. The distance (red arrows) between the edge of the sphere (red circle) and the furthest migrated cells were measured after* ***a*** *24 h and* ***b*** *48 h migration at four distinct position per neurosphere. Bars 100 µm*

In some cases problems with this evaluation method occurred. The migration of cells was disturbed when the matrigel coating was inhomogeneous (**Fig. 15a**). When neurospheres were to small and no edge was visible (**Fig. 15b**) or when too many spheres were seeded (**Fig. 15c**), it was not possible to evaluate these spheres.

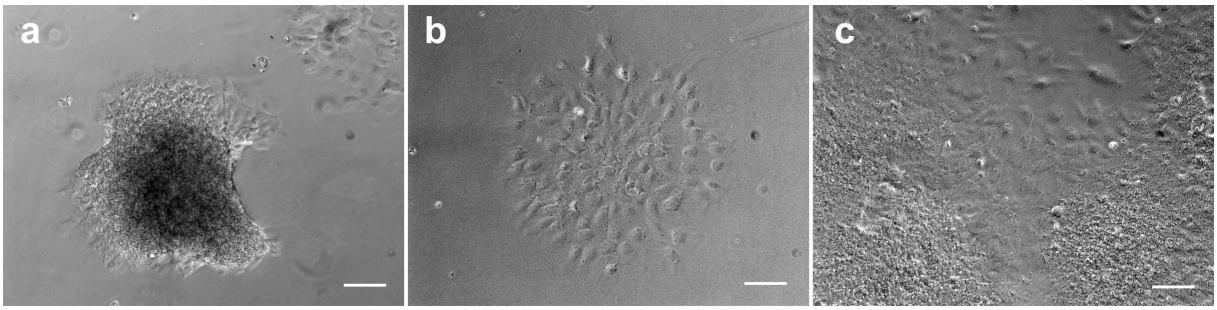

Fig. 15 *Problems of evaluation:* ***a*** *Inhomogeneous Matrigel coating,* ***b*** *no edge in the sphere and* ***c*** *too many cells. Bars 100 µm*

To establish this migration assay, two compounds with known effects on neuronal migration were tested. On the one hand ethanole (EtOH) was tested which is a known developmental neurotoxic agent as seen in the fetal alcohol syndrome (Guerri, 1998). On the other hand, I used Phorbol-12-myristate-13-acetate (PMA) which is an activator of protein kinase C (PKC) signaling (Isakov et al., 1985) and stimulates migration of neural cells *in vivo* (Sears and Ciment, 1988) and *in vitro* (Moors et al., 2007). After exposure to 2 % EtOH the migration out of NT2 neurospheres was decreased to 59 ± 3.2% after 24 h and to 34.9±1.9 % after 48 h

when compared to untreated controls. In contrast, exposure to 10 nM PMA resulted in a stimulation of migration to 302 ± 10.6 % of the controls after 24 h and to 251.6 ± 10.1 % after 48 h (**Fig. 16a, b**). These changes in cell migration differ from acute viability values (**Fig. 16c, d**). EC_{50} values calculated for the inhibition via Ethanol demonstrated the difference between the migration and cytotoxicity effect (**Table 2**) and that changes in migration are not caused by changes in viability. These results obtained in the migration assays indicate that the selected chemicals impair neural migration *in vitro*.

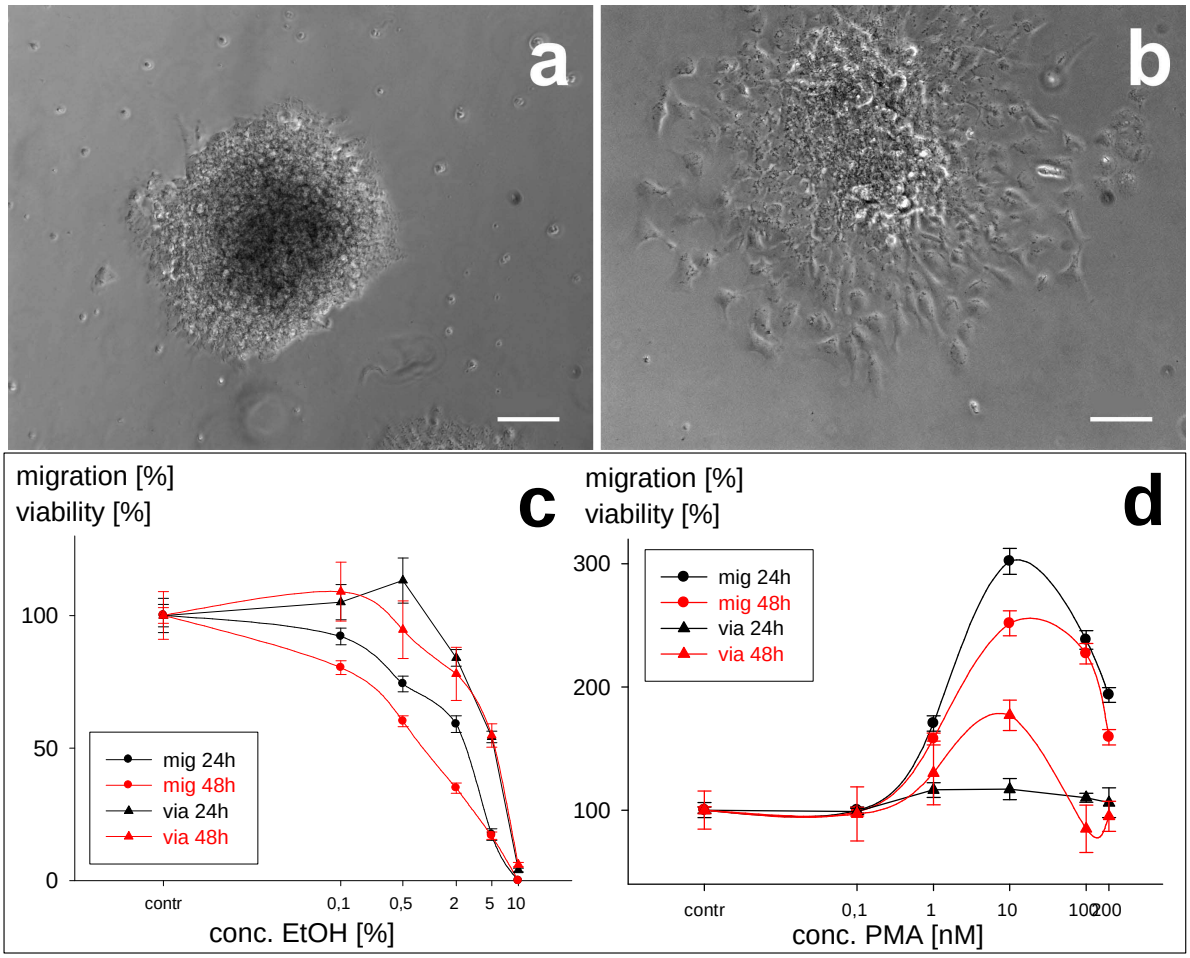

Fig. 16 a *EtOH [2 %] treated and* ***b*** *PMA [10 nM] treated NT2 neurospheres after 24 h migration. With EtOH treatment there is a clear decrease of migration and with PMA treatment a clear increase of migration becomes visible. Bars 100 µm. Migration (circles) and viability values (triangles) of* ***c*** *EtOH treated and* ***d*** *PMA treated NT-2 neurospheres after 24 h (black) and 48 h (red) migration validate this effect. The changes in cell migration differ from acute viability values. Data taken from one typical experiment, mean values for n=32 measurements, error bars indicate ± SEM.*

EtOH [%]	EC_{50} Migration	EC_{50} Viability	Difference
24 h	1.3 ± 0.7	5.1 ± 0.7	yes
48 h	1.0 ± 0.5	4.2 ± 0.5	yes

Table 2 *EC_{50} values calculated for migration and viability of the inhibition via Ethanol demonstrated the difference between the migration and cytotoxicity effect. Each value represents the mean ± SEM of two or three separate experiments*

In the context of the BMBF (Federal Ministry for Education and Research) project: „Development of predictive *in vitro* tests as replacement of animal experiments for testing developmentally specific neurotoxicity“ potential developmental neurotoxic compounds [methylazoxy methanol acetate (MAM), methyl mercury (MeHgCl), valproic acid (VPA), lead-acetate (Pb-ac)] and non developmental neurotoxic compounds [glutamate and acetaminophen] (**Table 3**) were analyzed in the neurosphere migration assays. The potential developmental neurotoxic compounds all have known neurotoxic properties. MeHgCl is a compound which disturbs cell migration *in vivo* (Castoldi et al., 2001; Clarkson, 2002) as well as MAM, which also inhibits neuronal migration *in vivo* (Baraban and Schwartzkroin, 1995; Colacitti et al., 1998). Pb is a potent neurotoxic agent, especially during nervous system development (White, 2007). The anti-epileptic drug VPA acts as tumor suppressor *in vivo* and *in vitro*, but also as teratogen causing various malformations including neural tube defects (Gotfryd et al., 2007) and inhibits neural cell motility *in vitro* (Fuller et al., 2002). The neurotransmitter glutamate and the analgesic drug acetaminophen, also known as paracetamol, supposedly do not have any developmental neurotoxic properties and serve as negative controls.

A

Substance	CAS-No.	Provider	Catalog-No.	Lot-No.	Solvent
MAM (Methylazoxy methanole acetate)	592-62-1	NCI	NCI #F0040, MRI #213	ET-11-109-1 Batch 3	PBS
MeHgCl (Methylmercury (II) chloride)	115-09-3	Sigma	442534	08409KH	PBS
VPA (Valproic acid sodium salt)	1069-66-5	Sigma	P4543	036K0731	Medium
Lead(II) acetate trihydrate (Pb-ac)	6080-56-4	Sigma	467863	07523HD	H_2O dest.

B

Substance	CAS-No.	Provider	Catalog-No.	Lot-No.	Solvent
L-Glutamic acid	142-47-2	Sigma	G1626	096K0111	Medium
Acetaminophen	103-90-2	Sigma	A5000	096K0072	DMSO

Table 3 *BMBF project test substances for* ***A*** *developmental neurotoxicity and for* ***B*** *cytotoxicicity, but not developmental neurotoxicity.*

The results of the migration assays performed with the BMBF project test substances indicate that methyl mercury is the most toxic compound and glutamate the fewest toxic (**Fig. 17**). The 24 h and 48 h results of migrated, treated NT2 neurospheres show different effects. The EC_{50} values for migration differ from viability after 24 h for the test substances VPA, MAM and methyl mercury, whereas EC_{50} values for the non developmental neurotoxin glutamate but also for lead-acetate did not differ from acute toxicity values (**Table 4**). In contrast after 48 h the developmental neurotoxin lead-acetate inhibited specifically migration, whereas for the non developmental neurotoxin glutamate but also for VPA, MAM and methyl mercury did not. These results indicate that it is important to measure at several timepoints to see all effects on migration. The EC_{50} values for acetaminophen could not be determined (N.D.) because it was not possible to solve adequate high concentrations.

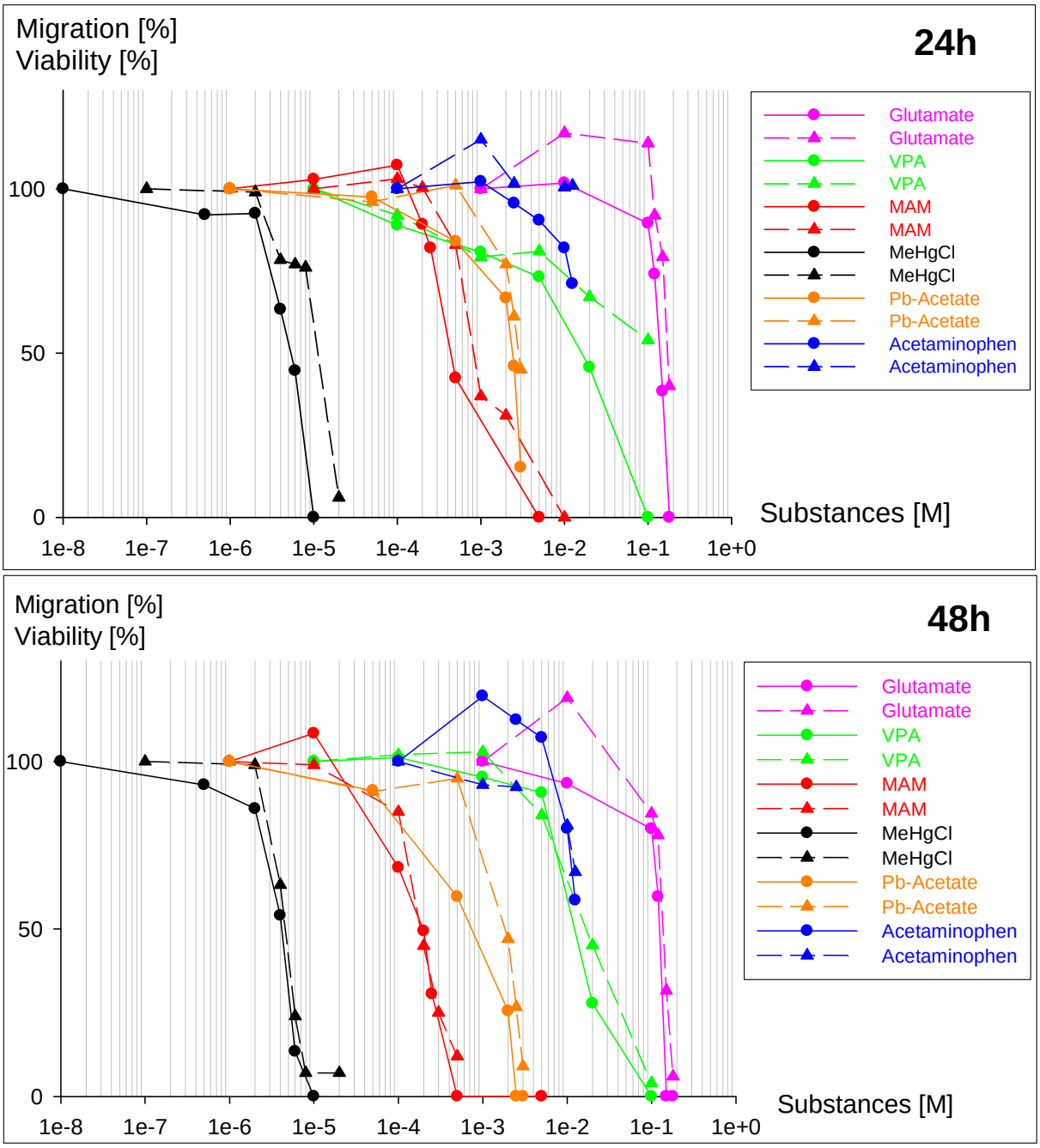

Fig. 17 *Migration (circles) and viability values (triangles) of the tested BMBF compounds after 24 h and 48 h migration. After 24 h migration values differ from viability values for the test substances VPA, MAM and methyl mercury; after 48 h lead-acetate inhibited specifically migration. Data taken from one typical experiment, mean values for n=32 measurements, error bars indicate ± SEM.*

24 h	Substance	EC_{50} Migration	EC_{50} Viability	Difference
	VPA [mM]	15.8 ± 2.2	91.6 ± 28.3	yes
	MAM [µM]	291.8 ± 83.5	1038.2 ± 226.6	yes
	MeHgCl [µM]	4.7 ± 0.5	9.6 ± 1.7	yes
	Pb-ac [mM]	2.3 ± 0.1	2.6 ± 0.2	no
	Glutamate [mM]	129.7 ± 9.3	182.5 ± 14.6	no
	Acetaminophen	N.D.	N.D.	

48 h	Substance	EC_{50} Migration	EC_{50} Viability	Difference
	VPA [mM]	11.0 ± 2.9	11.4 ± 7.0	no
	MAM [µM]	180.5 ± 36.6	208,8 ± 58,7	no
	MeHgCl [µM]	4.0 ± 0.3	4.1 ± 0.5	no
	Pb-ac [mM]	0.6 ± 0.1	2.1 ± 0.4	yes
	Glutamate [mM]	126.3 ± 6.6	143.8 ± 11.4	no
	Acetaminophen	N.D.	N.D.	

Table 4 *EC_{50} values calculated for migration and viability for the tested BMBF compounds after 24 h and 48 h migration. The values demonstrated the difference between migration and viability and the associated developmental neurotoxic effect of these test compounds. The EC_{50} values for acetaminophen were not determinable (N.D.) Each value represents the mean ± SEM of two or three separate experiments*

In migration assays with VPA treatment the cells show a modified cellular geometry (**Fig. 18a**). The ratio between length and width of migrated cells increased by treatment with VPA. This effect is highly significant (*** = $p<0.001$) for 5mM VPA treated cells after 48 h migration (**Fig. 18b**). Such an effect did not appear with any other treatment and seems to be characteristic for VPA treatment.

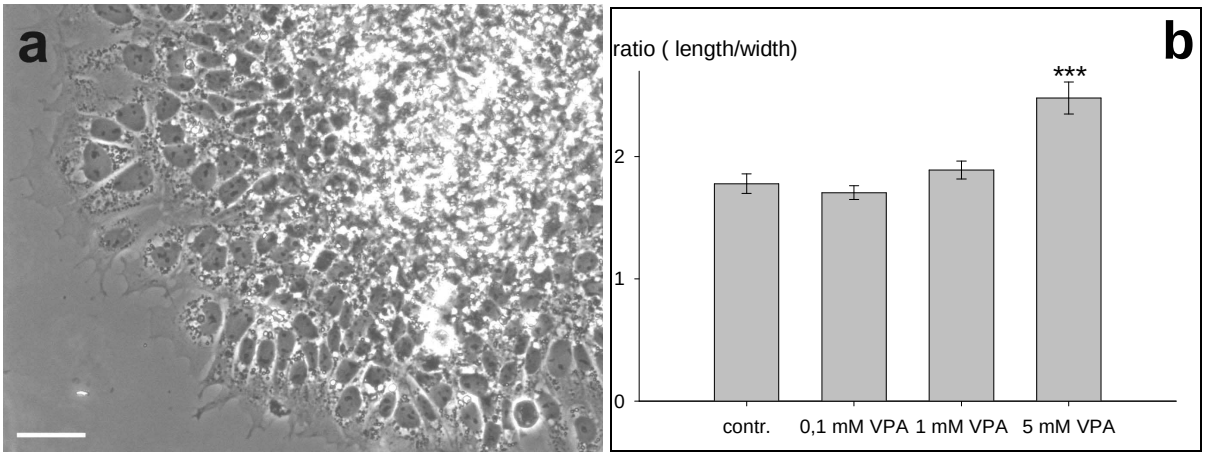

Fig. 18 *Cellular geometry of VPA treated cells after 48 h migration.* ***a*** *Migrated, 5mM VPA treated, cells show a changed cellular geometry. Bar 50 µm.* ***b*** *The ratio between length and width of migrated cells increased by treatment with VPA. This effect is highly significant (*** = p<0.001) for 5mM VPA treated cells, mean values for n=40, error bars indicate ± SEM.*

4. Discussion

4.1 NT2 neurons: Neuronal phenotype, synaptic marker and neurotransmitters

Morphological analysis and staining for the major cytoskeletal markers have confirmed that the improved differentiation protocol (Paquet-Durand et al., 2003) results in the development of differentiated human neurons within less than 1 month. The cultures contain pure neurons with less than 5 % contamination by undifferentiated, but postmitotic, cells. In one week and two week cultured neurons a punctate staining for the presynaptic marker synapsin could be observed. The number of synaptic punctae increased over time. Whether these sites of local concentration of synaptic vesicle- associated protein represent functional synapses remains to be analyzed. On the postsynaptic side, there is evidence for functional NMDA receptors in NT2 neurons as derived both from the standard differentiation protocol (Sandhu et al., 2003) and from the protocol involving floating spheres (Paquet-Durand and Bicker, 2004). Podrygajlo et al. (2010) detected spontaneous postsynaptic currents in human NT2 neurons differentiated in aggregate culture using the outside-out patch-clamp configuration. Moreover it has been shown that these human model neurons display synaptic vesicle recycling (Tegenge et al., 2009).

Conventionally generated NT2 neurons have been shown to display a broad variety of neurotransmitter phenotypes (Guillemain et al., 2000), including cholinergic, GABAergic, catecholaminergic, serotonergic, and peptidergic phenotypes. The percentage of cholinergic neurons generated under the rapid differentiation protocol (35 %) is in the same range as that reported for the adherent culture differentiation protocol (47 %, Guillemain et al., 2000). In contrast the observed number of GABAergic neurons is much lower (only 15 %), as opposed to 62 % (Guillemain et al., 2000). For the dopaminergic phenotype, the difference is even more striking, since I was unable to observe any markers for a dopaminergic phenotype, compared with 50 % TH positive cells observed by Guillemain et al. (2000). Even all attempts to induce this phenotype via lithium application (Zigova et al., 1999, 2000) or the application of a mix of FGF1 and various co-activators (Iacovitti et al., 2001) were not successful. At present, it is unclear whether these variations in the number of GABAergic and dopaminergic cells are attributable to differences in the neuronal differentiation protocol alone, or whether other factors, e.g., media composition, passage number of precursors, or their genetic instability, also play a role here.

The NT2 neurons develop in the presence of high concentrations of RA, which is known to induce caudal positional identity in developing nervous tissue (Maden, 2002). This caudalizing effect of RA alone might be sufficient to induce a high number of cholinergic neurons from NT2 precursors. Thus, NT2 neurons might provide a promising tool for transplantation based therapeutical strategies in motor neuron diseases such as ALS, where they have been successfully used in animal models (Garbuzova-Davis et al., 2002; Saporta et al., 2002).

4.2 NT2 neurons in a model of simulated ischemia

Screening for neuroprotective substances in vivo involves mainly rodent (Li et al., 1999; Colbourne et al., 1999) or pig (Foster et al., 2001) animal models. Similarly, the cell culture systems established to simulate ischemic conditions in vitro, are also largely based on primary neuronal cells and slice preparations obtained from rodents (Bartmann-Lindholm and Carter, 1999; Campbell et al., 2000; Melzian et al., 1999; Schurr et al., 1995a,b). Since there are considerable differences in the response to neuroprotective substances between different species (reviewed in Munir et al., 1995), human nerve cells are especially desirable in screening assays for protective drugs. NT2 neurons integrate, survive and promote functional recovery in rodent brains (Kleppner et al., 1995; Borlongan et al., 2000; Watson et al., 2003). Their successful transplantation into human stroke patients (Kondziolka et al., 2001) shows that NT2 neurons are indeed very similar to primary neurons, underlining their value for the screening of drugs that are intended for human use. Consequently, in vitro systems of ischemic human NT2 neurons have been used to evaluate the protective effects of glutamate receptor blockers (Rootwelt et al., 1998), barbiturates (Almaas et al., 2000) and of Coenzyme Q10 (Sandhu et al., 2003). The application of simple technical equipment for the simulation of hypoxic/ischemic conditions in postmitotic NT2 neurons (Paquet-Durand and Bicker, 2004) further improved the usefulness of these human neurons for basic research in the pathophysiology of ischemic injury. Excitotoxicity caused by an over stimulation with the neurotransmitter glutamate is thought to be a major factor of neuronal cell damage in the pathology of cerebral stroke (Olney, 1974; Kristián and Siesjö, 1998). Even though the cellular mechanisms accounting for glutamate excitotoxicity are not well understood, a major cause seems to be the excessive cellular influx of Ca^{2+}. Using Ca^{2+} imaging, Gao et al. (1998) have shown that NT2 neurons exhibit transient fluctuations of intracellular Ca^{2+} concentrations. These spontaneous Ca^{2+} transients were also observed in the NT2 culture

system and are due to an influx of extracellular Ca^{2+}, since they do not occur in Ca^{2+} free saline (Paquet-Durand and Bicker, 2004). Moreover, the NT2 neurons generated by the rapid cell differentiation protocol show NMDA- and glutamate- stimulated Ca^{2+} influx, offering the possibility to investigate excitotoxic cell death. The functional expression of NMDA-type glutamate receptors and the vulnerability to anoxic insult provide further confirmation for the neuronal status of rapidly differentiated NT2 cultures. Unfortunately, NMDA receptor antagonists that appear to be neuroprotective *in vitro* and in animal models, have so far failed in human clinical trials (Hoyte et al., 2004a). Combined with anoxia, glutamate application reduced cellular viability in the NT2 cultures effectively, thus mimicking ischemic neuronal damage. The main loss in viability seemed to occur during the first 24 h after anoxia. This effect of glutamate on viability could be prevented by the addition of relatively high concentrations of MK-801, a commonly used NMDA-channel blocker (McDonald et al., 1987). In models of relatively mild hypoxia/hypoglycemia (Rootwelt et al., 1998; Almaas et al., 2000; Sandhu et al., 2003), 10 μM MK801 was found to completely protect NT2 neurons from glutamate- induced cell damage. In the NT2 model of strong anoxia (Paquet-Durand and Bicker, 2004) and in a model of NMDAinduced excitotoxicity (Lockhart et al., 2002), however, concentrations of at least 100 μM MK-801 were needed to effectively protect NT2 neurons. In this work, optimal neuroprotection was achieved with MK801 concentrations of 250 μM and above. Here, it has been shown that far lower concentrations of diltiazem completely protected the NT2 neurons against glutamate-induced cell damage in the model of simulated ischemia (10 μM diltiazem versus 250 μM MK-801). The additional experiments performed using cortical neuron cultures obtained from neonatal mice were able to confirm the protective effect of diltiazem in a primary culture system. However, the effect of simulated ischemia on the viability of the murine cultures was less pronounced than in the NT2 neuronal cultures.

D-cisdiltiazem is an L-type voltage gated calcium channel blocker that was introduced as a drug for human use in the 1970s. Since then, ample experience has been acquired concerning the treatment of human heart arrhythmias, high blood pressure and angina pectoris with diltiazem (Chaffman and Brogden, 1985; Weir, 1995; O'Connor et al., 1999). Ono et al. (1991) demonstrated that diltiazem had a positive effect on neurologic recovery score after global brain ischemia in dogs. Diltiazem was also able to partly rescue rod photoreceptor cells in a mouse model of retinal degeneration (Frasson et al., 1999, Vallazza-Deschamps et al., 2005). Furthermore, diltiazem and MK-801 have been shown to decrease neuronal damage in *in vitro* and *in vivo* rat models of cerebral ischemia (Schurr et al., 1995a,b; Li et al., 1999).

Using electrophysiological investigations, Schurr et al. (1995a,b) could show that both diltiazem and MK-801 are able to protect hippocampal slices against hypoxic damage alone. In the NT2 cell culture model, diltiazem did not protect neurons after anoxia treatment without added glutamate. However, a direct comparison of the anoxic period, lasting for more than 2 h in dissociated neuronal cell culture to the hypoxic conditions in tissue slices, lasting from 12 to 20 min (Schurr et al., 1995a,b) is rather difficult to draw. In extensive studies on the use of calcium antagonists for the treatment of hypertension (Hansson et al., 2000), the incidence of stroke in human patients treated with diltiazem was found to be relatively low, indicating that diltiazem might have an additional neuroprotective function (reviewed in Grossman and Messerli, 2004). To my knowledge, it has, however, not been tried on human neurons in ischemia related models. Moreover, I am not aware of any studies using diltiazem in the therapy of stroke patients.

Interestingly, neither diltiazem nor MK-801 was able to completely protect neuronal cells since they were unable to restore viabilities beyond the level of cultures subjected to anoxia without glutamate. This implies that in the NT2 experimental system the viabilities of neuronal cultures are governed by both glutamate-dependent and independent forms of cell death.

Based on its neuroprotective properties in the cultures of human model neurons the antihypertensive drug diltiazem, or structurally related compounds, are promising candidates for the treatment of ischemic brain injury during the acute phase of a stroke. Moreover, it will be essential to develop efficient strategies for delivering these compounds to the damaged brain tissue.

4.3 NT2 neurospheres in migration assays

The developing human brain is highly vulnerable to injury caused by neurotoxic compounds, much more than the adult brain. Only few industrial chemicals (lead, methylmercury, polychlorinated biphenyls [PCBs], arsenic and toluene) are proven to cause human brain injury by exposure to these chemicals during early fetal development (Grandjean and Landrigan, 2006). These few chemicals might be only the tip of the iceberg in consideration of the several thousands of known but untested chemicals (**Fig. 19**). About thousand of chemicals known to be toxic in experiments, only 200 chemicals are known to cause neurotoxic effects in adult human beings. According to the absence of systematic testing of

chemicals there is a great need to generate new approaches which are able to give clear evidences about the potential of chemicals to disturb human brain development.

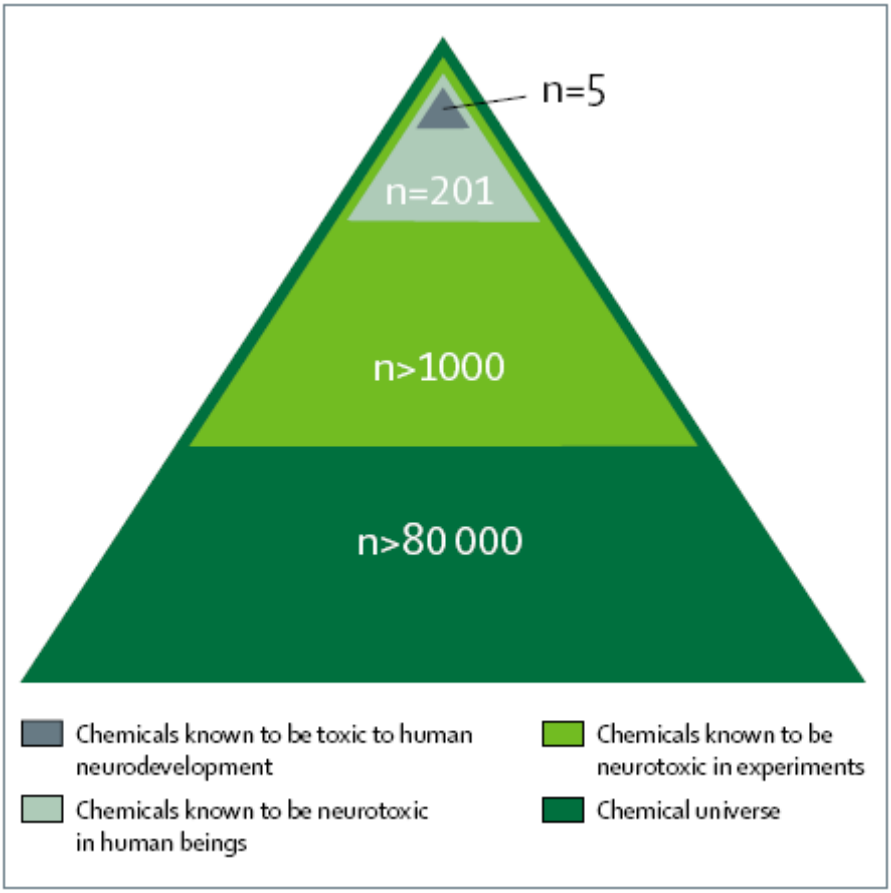

Fig. 19 *Diagram of the extent of knowledge of neurotoxic chemicals. Of the thousands of known chemicals, only a small fraction have been proven to cause developmental neurotoxicity in humans. Figure from Grandjean and Landrigan (2006)*

For discovering potential developmental neurotoxic chemicals and the mechanism that control neuronal migration animal models have often been employed (Fishman and Hatten, 1993, Sass et al., 2001, Haase and Bicker, 2003). Neurosphere-based animal cultures have been used to study cell proliferation, differentiation and migration (Leone et al. 2005; Mizuno et al. 2005). To replace animal models normal human neural progenitor (NHNP) cells (Moors et al., 2007) or human NT2 cells (Hill et al., 2008) are a suitable alternative. Tegenge and Bicker (2009) showed that aggregates of human NT2 precursor cells are a useful tool to study regulatory mechanism of cell motility. According to this, the use of one-week 10 µM RA treated NT2 neurospheres show great promise to create a human model system for developmental neurotoxicity. Aggregated NT2 precursor cells started to migrate out after seeding on an adherent surface. The microscopic observation of the migration demonstrated

the linearity of this process and that no slow down of migration takes place over a period of one week after seeding. The proliferation of NT2 neurosphere cells decreased after a migration period of 48 hours caused by RA treatment which is a known inhibitor of cell proliferation (Kikuchi et al., 1984). Most of the cells in the neurosphere and the migrating cells were nestin-positive. Only a few cells in the neurosphere displayed type III ß-tubulin staining. This indicates that the migrating cells mainly consisted of neuronal precursor cells and that the NT2 aggregate culture system is a suitable human model for developmental neurotoxicity drug screening.

To evaluate the distances the cells migrate relatively to a certain time intervall several images were acquired after 24 h and 48 h migration according to the method described in Moors et al. (2007). To obtain statistical reliability 32 distances for every sample were measured. In some early experiments, an inhomogeneous matrigel coating disturbed the migration. Therefore, the coating is a critical step for the successfully performed experiment. An alternative for matrigel coating would be using a laminin coating, which is also a known adhesive surface for neuronal cells (Pleasure et al. 1992). In this work, matrigel was preferred because of lower costs. The dose/effect-profiles and resultant EC_{50} values calculated from each drug treated migration experiment were compared with EC_{50} values of acute toxicity determined by an Alamar Blue ® viability assay to ensure whether migratory effects differ from toxic effects. Every experiment was repeated at least one or two times to intensify the results. The Alamar Blue ® viability assay was already established in the experiments of simulated ischaemia with NT2 neuronal cells. It has the advantage in comparison to commonly used tests (like MTT, Trypan blue and LDH) that cells survive the staining and can be monitored continuously. First migration assays were performed with exposure to model substances with known effects on migration like ethanole and PMA (Moors et al., 2007). The expected inhibitory effect of ethanole and the stimulatory effect of PMA on migration have been clearly verified after 24 h as well as after 48 h migration. The concentrations needed to obtain migratory effects differ from those of toxic effects and indicate that it is possible to impair neural migration *in vitro* in both directions - inhibitory and stimulatory.

In the context of the BMBF project: „Development of predictive *in vitro* tests as replacement of animal experiments for testing developmentally specific neurotoxicity“ potential developmental neurotoxic compounds (MAM, MeHgCl, VPA and Pb-ac) and non developmental neurotoxic compounds (glutamate and acetaminophen) were analyzed by performing the established migration assays with NT2 neurospheres. MeHgCl is a compound which disturbs cell migration *in vivo* (Castoldi et al., 2001; Clarkson, 2002). This inhibitory

effect on migration could be confirmed by exposure NT2 neurospheres to MeHgCl. However, this effect on migration only differs after 24 h migration from the toxic effect in contrast to the 48h values that do not differ. The results of migration assays performed with MAM, which is also a known inhibiter of neuronal migration *in vivo* (Baraban and Schwartzkroin, 1995; Colacitti et al., 1998), were similar to MeHgCl results. Only the 24 h migration values displayed a clear inhibition of migration which is not caused by toxicity. The anti-epileptic drug VPA is known as an tumor suppressor *in vivo* and *in vitro*, but also as teratogen causing various malformations including neural tube defects (Gotfryd et al., 2007) and inhibits neural cell motility *in vitro* (Fuller et al., 2002). In this work the inhibition of migration via VPA exposure of NT2 neurospheres was reflected after 24 h but not after 48 h. Interestingly, it was also observed that VPA treated cells displayed an altered morphology after migration. The ratio between length and width of migrated cells increased significantly in contrast to untreated cells. These changes in cellular geometry might accompany with a changed composition of the actin cytoskeleton properties by VPA exposure published by Walmod et al. (1998 and 1999). Also modifications of dendritic arborization in VPA treated motor cortex neurons are known (Snow et al., 2008). These findings indicate that VPA affects the cell motility by changing the cell morphology. The neurotoxic agent Pb, which affects especially during nervous system development (White, 2007), displayed a clear inhibition of migration out of NT2 neurospheres which is not caused by cytotoxicity. In contrast to the other tested compounds the migration were affected only after 48 h and not after 24 h. As expected for the neurotransmitter glutamate which is known to not have any developmental neurotoxic properties, there were no effects on migration of NT2 neurospheres detectable. The EC_{50} values for the analgesic drug acetamniophen, which also to not have any developmental neurotoxic properties, could not be determined because it was not possible to solve adequate high concentrations.

The different results of 24 h and 48 h migrated cells after drug treatment indicate that measuring at several timepoints is a critical step for a successfully performed experiment. Also the number of repetitions of every experiment is essential for statistical significance. Since the evaluation method is a rather time-consuming process, in future projects an automatical evaluation method for higher throughput would be useful. A first step in this direction would be to design software programs, which are able to automatically perform the image uptake or to automatically measure the distances of cell migration.

In summary, the results demonstrate that it is possible to perform with the human NT2 cell line *in vitro* tests for developmental neurotoxicity which are predictive for the effects of known and suspected developmental neurotoxins.

5. Publications

Part of this thesis has been published in the following research articles:

- **Bicker, G., Gierse, A., Tan, S., Paquet-Durand, F.** (2007): "Simulation of Stroke-related Damage in Cultured Human Nerve Cells" ALTEX 24, Special Issue, 16-18

- **Paquet-Durand, F., Gierse, A., Bicker, G.** (2006) „Diltiazem protects human NT-2 neurons against excitotoxic damage in a model of simulated ischemia" Brain Research, 1124, 45–54

- **Podrygajlo, G., Tegenge, M.A., Gierse, A., Paquet-Durand, F., Tan, S., Bicker, G., Stern, M.** (2009) "Cellular phenotypes of human model neurons (NT2) after differentiation in aggregate culture" Cell and Tissue Research 336, 439-452

6. Literature

1. **Almaas**, R., Saugstad, O.D., Pleasure, D., Rootwelt, T. (2000): “Effect of barbiturates on hydroxyl radicals, lipid peroxidation, and hypoxic cell death in human NT2-N neurons”, Anesthesiology 92, 764–774
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