The human NT-2 cell line as *in vitro* model system for the excitotoxic cascade during stroke

Vom Fachbereich Biologie der Universität Hannover
zur Erlangung des Grades

Doktor der Naturwissenschaften
Dr. rer. nat.
genehmigte Dissertation

von

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Geboren am 21. Februar 1968 in Kamen

2004

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Tag der Promotion: 7. 11. 2003

Abstract

Cells from the human teratocarcinoma line NTera-2 can be induced to terminally differentiate into postmitotic neurons when treated with retinoic acid. However, this differentiation process is rather time consuming as it takes between 42 and 54 days.

In this study a modified differentiation protocol is introduced which reduces the time needed for differentiation considerably without compromising the quality of the neurons obtained. The introduction of a proliferation step as free floating cell spheres cuts the total time needed to obtain high yields of purified NT-2 neurons to about 24-28 days. The cells obtained show neuronal morphology and migrate to form ganglion-like cell conglomerates. Differentiated cells were characterised using immunocytochemical and histochemical techniques. Among others, the cells express neuronal polarity markers such as the cytoskeleton associated proteins MAP2 and Tau. Moreover, the generation of neurons in sphere cultures induced immunoreactivity to the ELAV-like neuronal RNA-binding proteins HuC and HuD. This finding provides experimental evidence that HuC/D are involved in human neuronal differentiation.

NT-2 neurons were used to establish an *in vitro* assay system, that will allow to test neuroprotective substances during simulated ischaemia. The viability of NT-2 neurons was measured using the Alamar Blue® assay. This assay demonstrated constant viability during several weeks in culture. In experiments of simulated ischaemia the neurons were subjected to anoxia and hypoglycaemia. The viability of NT-2 neuronal cells was significantly reduced by anoxia and further reduced in the presence of glutamate reflecting the cells vulnerability to anoxia/ischaemia and to excitotoxic conditions. The addition of the N-methyl-d-aspartate (NMDA)-receptor antagonist MK-801 reduced glutamate induced neuronal damage.

Fluorescence imaging with Calcium indicator dyes was used to assess the response of NT-2 neurons to glutamate and NMDA. NT-2 neurons showed a strong response to stimulation with glutamate or NMDA, which was abolished in calcium free medium and at low pH values. The NMDA receptor antagonists MK-801, AP 5, and Ketamine reduced the NMDA induced response.

The mitochondrial potential of neurons during anoxia was estimated using the fluorescent dye rhodamine 123. The dye was incorporated in mitochondria of NT-2 cells and was used as an indicator of mitochondrial activity and cell viability. Its fluorescence increased strongly after the onset of anoxia and decreased to background level when the cells died.

These results demonstrate that NT-2 neuronal cells can be used as an *in vitro* test system for the large-scale screening of neuroprotective substances.

Keywords/Schlagworte: Ischaemia, Neuroprotection, Human nerve cells.

Zusammenfassung

Zellen der humanen Teratocarcinoma Zelllinie Ntera-2 (NT-2) können durch Behandlung mit Retinsäure terminal in postmitotische Nervenzellen differenziert werden. Dieser Differenzierungsprozess benötigt zwischen 42 und 54 Tagen.
In dieser Arbeit wird ein modifiziertes Differenzierungsprotokoll vorgestellt, das die zur Differenzierung notwendige Zeit deutlich verkürzt, ohne die Qualität der erhaltenen Nervenzellen zu beeinträchtigen. Die Einführung eines zusätzlichen Proliferationsschrittes, in dem die Zellen als frei flottierende, sphärische Aggregate kultiviert werden, reduziert die benötigte Zeit bis zum Erhalt von gereinigten NT-2 Nervenzellen auf ca. 24 bis 28 Tage. Die so erhaltenen Zellen zeigen eine neuronale Morphologie und bilden ganglionartige Zellcluster. Die differenzierten Zellen wurden durch immuncytochemische und histochemische Methoden charakterisiert. Sie exprimieren u. a. neuronale Polaritätsmarker wie z. B. die Zytoskelett - assozierten Proteine MAP2 und Tau. Außerdem wird durch die neuronale Differenzierung die Immunreaktivität gegenüber den ELAV-ähnlichen, neuronalen RNA-bindenden Proteinen HuC und HuD induziert. Dies ist ein experimenteller Hinweis, dass HuC/D Proteine in der Differenzierung menschlicher Neuronen eine Rolle spielen.
NT-2 Nervenzellen wurden benutzt, um ein *in vitro* Testsystem zu entwickeln, das es erlaubt, mögliche neuroprotektive Substanzen unter Bedingungen einer simulierten Ischämie zu testen. Die Viabilität von NT-2 Nervenzellkulturen wurde mit Hilfe des Alamar Blue® Testsystems bestimmt und war über mehrere Wochen Kultur konstant. In Versuchen zur simulierten Ischämie wurden diese Kulturen anoxischen und hypoglycaemischen Bedingungen ausgesetzt. Die Viabilität von NT-2 Nervenzellkulturen wurde durch die Anoxie signifikant reduziert. Die Zugabe von Glutamat führte zu einer zusätzlichen Reduktion der Viabilität. Dadurch konnte die Empfindlichkeit dieser Zellen gegenüber anoxischen und exzitotoxischen Bedingungen gezeigt werden. Der spezifische Blocker von N-methyl-d-aspartat (NMDA) - Rezeptoren, MK-801, konnte die glutamatinduzierte Schädigung von neuronalen Zellen verringern.
Durch Imagingversuche mit fluoreszierenden Kalziumindikatoren wurde die Reaktion von NT-2 Nervenzellen nach Stimulation mit Glutamat und NMDA bestimmt. NT-2 Nervenzellen zeigten eine signifikante Reaktion auf eine solche Stimulation. Diese ließ sich durch Verwendung von kalziumfreiem Medium und bei niedrigen pH - Werten unterdrücken. Die NMDA-Rezeptor-Antagonisten MK-801, AP 5 und Ketamin konnten die NMDA-induzierte Reaktion reduzieren.
Das mitochondriale Potential von Nervenzellen wurde mit Hilfe des Fluoreszenzfarbstoffs Rhodamin 123 während einer Anoxie bestimmt. Der Farbstoff wurde von den Mitochondrien der NT-2 Nervenzellen aufgenommen und als Indikator für die mitochondriale Aktivität und die Viabilität verwendet. Die Fluoreszenz stieg unmittelbar nach Beginn einer Anoxie steil an und fiel zurück auf das Niveau des Hintergrundes nachdem die Zellen abgestorben waren.
Diese Ergebnisse zeigen, dass es möglich ist NT-2 Nervenzellen für ein *in vitro* Testsystem zu benutzen, mit dem eine große Anzahl von Substanzen auf eine neuroprotektive Wirkung geprüft werden können.

Keywords/Schlagworte: Ischämie, Neuroprotektion, menschliche Nervenzellen.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst habe. Es wurden keine anderen als die angegebenen Quellen und Hilfsmittel verwendet. Diese Dissertation wurde weder als ganzes noch in Teilen für Diplom-, Promotions- oder ähnliche Arbeiten verwendet.

Hannover, den 17. März 2004

François Paquet-Durand

List of used abbreviations

Abbreviation	*Full name*
5-HT	5-hydroxy-tryptamine (serotonin)
AChE	Acetylcholinesterase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoazole-propionic acid
AP	Alkaline phosphatase
AP5	dl-amino-5-phosphono valeric acid
approx.	Approximately
Ara C	1-6-D-arabinofuranosylcytosine
ATP	Adenosine-tri-phosphate
BDNF	Brain derived neurotrophic growth factor
BHA	Butyl-hydroxy-anisole
cGMP	Cyclic guanosine-mono-phosphate
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DCD	Delayed cell death
DMEM	Dulbecco´s minimum essential medium
DMSO	Di-methyl-sulfoxide
EAAT	Excitatory amino acid transporter
EDTA	Ethylene-diamine-tetraacetate
EtOH	Ethanole
F12	Ham´s F12 medium
FBS	Fetal bovine serum
FudR	2'-deoxy-5-fluorouridine
GABA	γ-amino-butyric-acid
GFAP	Glial fibrillary acidic proteine
HEPES	N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid
IBMX	3-isobutyl-1-methylxanthine
LDH	Lactate dehydrogenase
MAP2	Microtubuli associated protein 2
MCAO	Middle cerebral artery occlusion
MeOH	Methanole
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

Abbreviation	*Full name*
NADPH	Nicotine-amide-adenine-dinucleotide-phosphate (reduced)
NBT	Nitro blue tetrazolium
NGF	Neurotrophic growth factor
NMDA	N-methyl-d-aspartate
NO	Nitric oxide
NOS / uNOS	Nitric oxide synthase / universal nitric oxide synthase
NT-2N	NT-2 neuronal cells
NT-3	Neurotrophic factor 3
OGD	Oxygen and glucose deprivation
PBS	Phophate buffered saline
PCP	Phencyclidine
PDE	Phospho-diesterase
pdl	Poly-d-lysine
PFA	Paraformaldehyde
PI	Propidium iodide
PTW	PBS with 0.2 % Tween 20
PTX	PBS with 0.2 % Triton X100
PTX 0.5	PBS with 0.5 % Triton X100
R123	Rhodamine 123
RA	Retinoic Acid
ROI	Region of interest
ROS	Reactive oxygen species
S	Svedberg
sGC	Soluble guanylyl cyclase
SNP	Sodium nitro prusside
SOD	Super-oxide-dismutase
TMRM	Tetra-methyl rhodamine
Urd	1-ß-D-ribofuranosyluracil

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1. Introduction

There is a huge demand in current biomedical research for growing human brain cells in culture systems. Human nerve cells grown *in vitro* could provide valuable insights into the differentiation and function of the nervous system. They could also provide experimental material for investigating cell therapies for disorders of the central nervous system (CNS) and might eventually serve as a source for neural transplantation and brain repair. The generation of human nerve cells on a large scale in the petri-dish would allow for high-throughput screening of neuroactive compounds.

The subject of this study is the human teratocarcinoma cell line NT-2, its differentiation into human nerve cells and its potential uses in basic and applied neurobiological research. Special focus lies on the establishment of an *in vitro* model system for the excitotoxic cascade using differentiated NT-2 neuronal cells. The excitotoxic cascade is triggered by an excessive release of the excitatory neurotransmitter glutamate and eventually leads to the death of neurons which are responsive to glutamate. This process seems to play an important role in stroke and could also be involved in other neurodegenerative diseases of the human brain (Gass, 1997).

1. 1. Stroke, ischaemia and the excitotoxic cascade

Stroke is the third leading cause of death and an important cause of adult disability in industrialised countries with their ageing human populations (Casper et al., 2003). The development of new therapies and new drugs that could at least alleviate the consequences of stroke is therefore an important goal in biomedical research.

The brain has the highest metabolic rate of all organs. It depends predominantly on oxidative metabolism, thus consuming a disproportionately high amount of the body's oxygen and glucose. An interruption of the blood supply caused by an injury, the rupture, or the occlusion of a blood vessel is therefore particularly damaging to the brain.

The main reason for the brains high energy consumption is the maintenance of the membrane potential. This requires a continuous supply of ATP to drive the ion pumps (Na^+/K^+ ATPases) necessary to maintain the membrane potential. Ischaemic neurons deprived of oxygen and glucose are unable to perform oxidative phosphorylation needed to produce ATP. They may be able to perform glycolysis for a limited amount of time using intracellular reserves. However, the ATP generation from glycolysis is relatively small and leads to a build-up of lactic acid, which in turn leads to a drop in intracellular pH. Neurons rapidly deplete their ATP and then depolarise. The disruption of ion homeostasis also alters the osmotic balance leading to an influx of water and a swelling of the affected cell (Voet and Voet, 1995).

The depolarisation of the cell membrane in turn leads to the release of neurotransmitters, in

particular glutamate, which is the most important excitatory neurotransmitter in the central nervous system (CNS) with approximately 90% of all neurons releasing glutamate after excitation (Magistretti et al., 1999). Dying neurons release even more glutamate. Glutamate could additionally be released by astrocytes. Although these are not as vulnerable to anoxia as neurons, their cell membranes also depolarise under anoxic conditions. This may lead to a reversal of the glutamine / glutamate transporter causing the additional release of glutamate (Rossi et al., 2000; Phillis et al., 2000).

The release of glutamate stimulates glutamate receptors which in turn lead to a series of pathological intracellular events.

1. 1. 1. The NMDA receptor

Glutamate binds to different types of receptors, which are divided into 2 main groups, the NMDA and the non-NMDA type receptors. Apart from glutamate, NMDA-type receptors can also be stimulated selectively by NMDA (N-methyl-d-aspartate). It is of particular interest as its activation does not only lead to a membrane depolarisation but also to an influx of extracellular calcium ions and mediates an important part of neuronal cell loss during and after ischaemia (Choi et al., 1985).

The NMDA receptor is connected to a high conductance ion pore, which upon activation allows sodium ions (Na^+) and calcium ions (Ca^{2+}) to enter the cell and potassium ions (K^+) to leave the cell. Opening of the NMDA receptor channel requires extracellular glycine as a cofactor. The channel opening is not only dependent on ligand binding, but also on membrane potential. Inside the ion pore is a magnesium ion (Mg^{2+}) binding site. Mg^{2+} is held in its position by the electrostatic force generated by the cells membrane potential. When the receptor is in its inactive state the passage of ions is blocked by the magnesium ion. In case of a cell membrane depolarisation, the Mg^{2+} block is removed and Na^+ and Ca^{2+} ions enter the cell (Kandel et al., 1996).

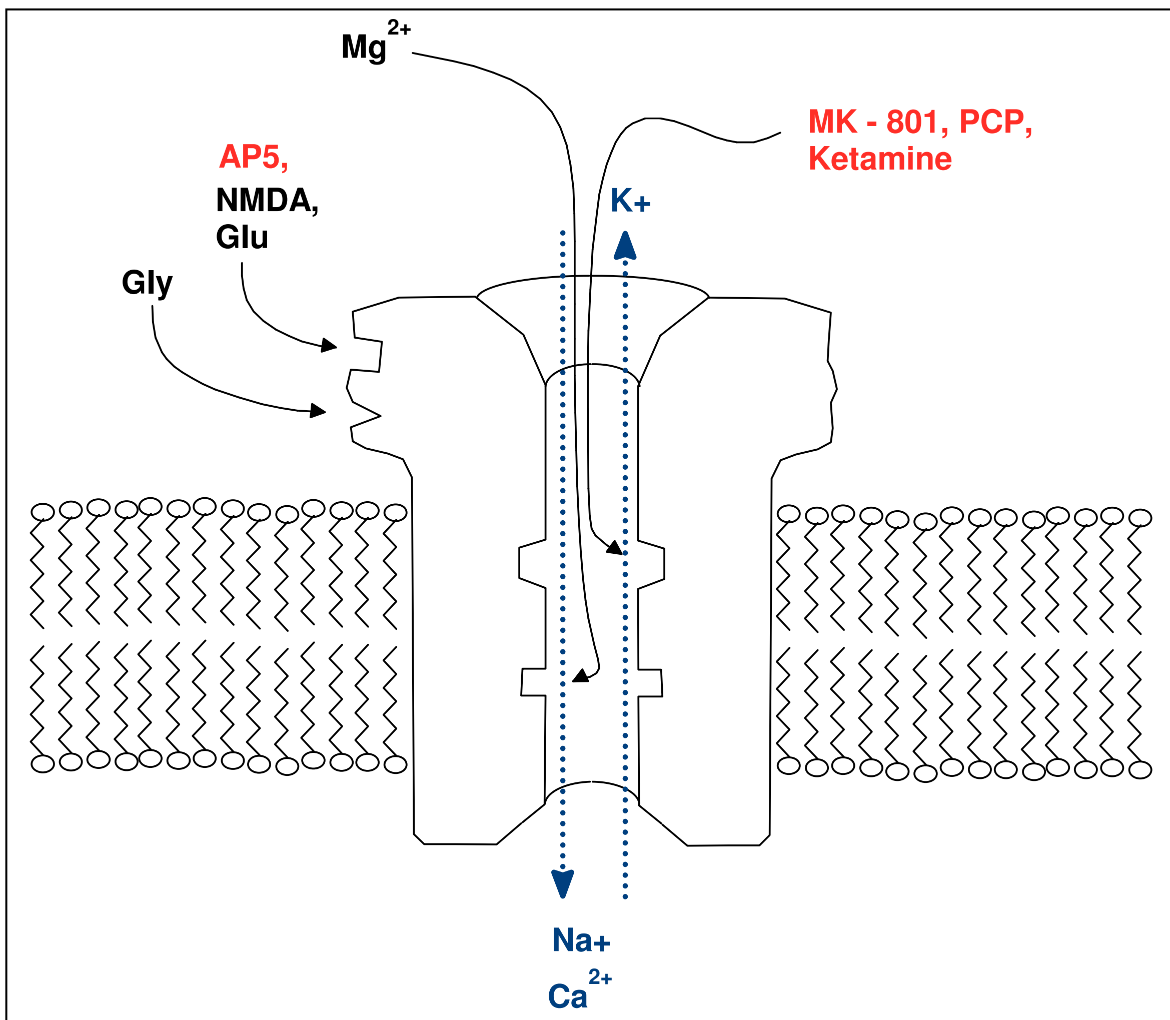


Figure 1: Schematic representation of the NMDA receptor (modified after Kandel et al., 1996). Upon activation with glutamate or NMDA the channel pore becomes permeable for sodium and potassium ions. The action of glutamate is enhanced by a simultaneous binding of glycine. AP5 competitively blocks glutamate activation. Inside the channel pore are binding sites for magnesium, MK-801, or PCP. When these ligands bind to their respective site the passage of ions is blocked. The magnesium block is removed by a depolarisation of the cell. To date it is unclear whether the binding sites for MK-801/PCP are identical with the magnesium binding site or not.

The NMDA receptor is a molecular coincidence detector, as it combines 2 different signals – presence of glutamate and depolarisation of the cell – to an additional input signal, the elevation of intracellular calcium levels. This property of the NMDA receptor is of crucial importance for the formation and strengthening of synapses and has been implied in learning and memory formation (Smirnova et al., 1993; Tang et al., 1999).

Other agonists of the NMDA receptor include aspartate. Glycine increases the receptor´s response to glutamate. There are several NMDA receptor antagonists, some of which are used as drugs in human or veterinary medicine. The most well known of these drugs which block the passage of ions through the receptors ion pore, are PCP (“Angel Dust”), Ketamine, and MK-801.

Their binding site could be identical with the Mg^{2+} binding site (Chazot et al., 1993). Another commonly used drug in neuroscience research – AP5 – competes with glutamate for the binding at its binding site but does not activate the receptor.

1. 1. 2. The excitotoxic cascade

The stimulation of NMDA type glutamate receptors leads to an influx of calcium ions into the cell. This can be damaging to the cell, if the intracellular calcium concentration reaches a critical level (Choi, 1985).

Ca^{2+} is an important secondary messenger in a number of intracellular signal transduction cascades. High concentrations of Ca^{2+} are accumulated in the mitochondria and are known to lead to an increase in the production of reactive oxygen species (ROS) (reviewed in: Kristián and Siesjö, 1997; Nichols and Ward, 2000). An increase of intracellular calcium also stimulates calcium-dependent enzymes such as calcium-dependent proteases, nucleases, and phospholipases, which subsequently trigger the apoptosis (Chakraborti et al., 2002).

The generation of small numbers of ROS and free radicals is a normal by-product of the oxidative phosphorylation. The reduction of O_2 to H_2O in the mitochondria causes their formation even under normal metabolic conditions. These free radicals are destructive to the cell and have to be neutralised by specific radical scavengers (Olanow, 1993). An increase in the activity of oxidative metabolism to levels close to the metabolic maximum drastically increases the amount of free radicals generated. Since neurons depend primarily on oxidative metabolism their excessive stimulation is followed by a rising production of free radicals (Coyle and Puttfarcken, 1993).

ROS generation and Ca^{2+} influx may also be connected to stimulation of AMPA type glutamate receptors. Spinal motor neurons and at least a subset of cortical GABAergic neurons have been shown to be susceptible to AMPA induced Ca^{2+} influx in the mitochondria causing a rise in ROS production levels (Carriedo et al., 2000).

Excessive release and accumulation of extracellular glutamate, stimulates glutamate receptors in neurons, inducing their depolarisation and increases in intracellular calcium levels. The excessive stimulation of the NMDA receptor can cause the death of a neuron. The excessive release of glutamate from one cell causes the death of adjacent cells and even further release of glutamate, creating a kind of feedback loop which increases neuronal damage. This process where the excessive stimulation with an excitatory transmitter eventually causes the death of neuronal cells was first described by Olney (1974) and termed the excitotoxic cascade.

In connection with the excitotoxic cascade, the free radical nitric oxide (NO) plays an important role (Dawson et al., 1993; Iadecola, 1997). As a gas, NO is able to diffuse freely through cell

membranes and may bind to targets in other cells. It is produced by different isoforms of the enzyme NO synthase (NOS). The neuronal isoform, nNOS, is involved in neurotransmission, whereas the endothelial isoform, eNOS, is responsible for the dilatation of blood vessels, thereby regulating blood flow in specific areas of the body (Bredt, 1999). NO has potentially damaging effects on the surrounding tissue if it is released in large amounts. During ischaemia *in vivo,* the endothelium is severely damaged leading to a release of NO from endothelial cells (Xu et al., 2000). This release of NO increases neuronal damage and as a diffusible molecule it could trigger the additional release of glutamate which would then lead to a potentiation of excitotoxic damage. In accordance to this, Dawson et al. (1991, 1993) and Xu et al. (2000) could show that both specific inhibitors of NO synthase and scavengers of NO decreased the vulnerability of primary neurons to oxygen and glucose deprivation (OGD).

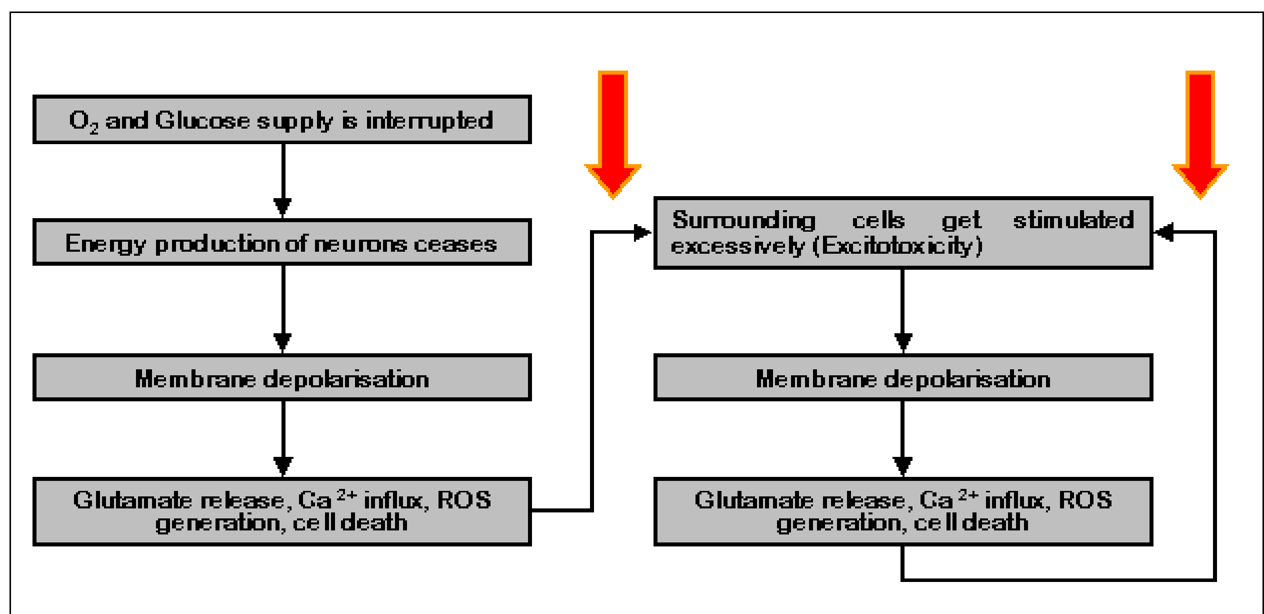


Figure 2: A schematic flow chart of the events during cerebral ischaemia. The exact sequence of events is to date unclear and should therefore only be seen as an indication. For instance, the release of glutamate could occur after depolarisation and/or after cell death. Arrows indicate possible points of therapeutic interventions.

The series of events during cerebral ischaemia can roughly be divided into 2 phases:

In the first, the immediate vicinity of occluded blood vessels is damaged irreversibly through necrotic processes. In this ischaemic core, neuronal death occurs rapidly giving very little or no time for therapeutic interventions.

In the second phase, the excitotoxic cascade comes into play and results in a wave of delayed cell death, which spreads to surrounding tissue, the so called penumbra. This phenomenon results in a drastic increase of total infarct volume (Lee et al., 1999; Dirnagl et al., 1999). In the penumbra, neurons remain viable for many hours, or even days after the primary ischaemic

event, providing a period of time for therapeutic interventions.

1. 1. 3. The excitotoxic cascade and its implications for stroke therapy

In the hours and days after a stroke, the excitotoxic cascade amplifies neuronal damage that would otherwise be restricted to a limited area of the brain (Dirnagl et al., 1999). Within the series of events that take place during the excitotoxic cascade, there are certain points where an interruption seems feasible, providing an opportunity for therapeutic interventions. A successful therapy for the treatment of stroke might include the application of several different neuroprotective substances that are active at the various stages of the excitotoxic cascade.

As calcium influx seems to provoke an important part of ischaemic damage, glutamate receptors which mediate calcium influx are promising targets for such neuroprotective substances. A blockade or reduction in activity of these receptors by specific antagonists could result in increased neuronal survival (Choi et al., 1988; Gidday et al., 1995; Lockhart et al., 2002).

A decrease in ROS generation, or the scavenging of ROS before they can exert their damaging effects on the cell, would increase neuronal survival. The generation of ROS is provoked at later stages of the excitotoxic cascade by high levels of intracellular calcium (Dugan et al., 1995; Kristián and Siesjö, 1997) and could be compensated for by the addition of specific ROS scavengers (O´Neill et al, 1997). A reduction in the generation of ROS could also be possible if neuroprotective substances were targeted directly to the electron transport chain (Dessolin et al., 2002).

The same applies for NO generation. The damaging effects of an excessive NO production (Bredt, 1999) could be relieved by specific inhibitors of NO synthase or inhibitors of the NO receptor guanylyl cyclase (GC).

Still further downstream of the excitotoxic cascade lays the apoptotic cascade. The programmed cell death is triggered by excitotoxic events (Chakraborti et al., 2002). As it is an active, ATP dependent process, it can only be undertaken under conditions of normoxia, in this case after reperfusion. At that point, a number of cells could still be viable and possibly recover if the apoptotic cascade was interrupted before it had reached its final stages. This could lead to a delayed rescue of neurons from cell death.

1. 2. Animal models of ischaemic brain damage

Currently a number of research projects are under way which are trying to identify neuroprotective substances which could intervene during or after an ischaemic event. To investigate the cellular mechanisms occurring in ischaemic brain damage, a variety of rodent

models that mimic the pathogenic environment of nerve cells during stroke have been developed. Two widely employed animal models are the transient global forebrain ischaemia model, in which the entire blood supply to the brain is transiently interrupted reflecting cardiac arrest (De Bow and Colbourne, 2003), and the focal cerebral ischaemia model, in which the middle cerebral artery (MCA) is occluded (Arvidsson et al., 2001). The period of transient ischaemia is followed by reperfusion, then the animal is allowed to recover for some time, then sacrificed, and the brain damage studied in detail. The consequences of stroke cause a high degree of distress in form of pain and anxiety for the living animals (Breder et al., 1999). Since pharmaceutical companies are trying to develop neuroprotective compounds that reduce brain damage after stroke, large numbers of laboratory animals are needed for drug testing.

However, their use implies a number of setbacks: The use of animals is relatively expensive, requiring well trained personnel, both for the animal experiments and the histological evaluation of the outcome of an experiment. Mass screenings (high throughput screening, HTS) using such an animal model are rather costly.

Moreover species specific differences have to be taken into account. Drugs proven to be neuroprotective in a model for transient ischaemia in one species may be ineffective or even have adverse effects on the CNS of another species. For instance the commonly used NMDA channel blocker MK-801 has strong neuroprotective capacities in new-born and adult rats (Gidday et al., 1995; Arvidsson et al., 2001) but it is ineffective in piglets (LeBlanc et al., 1991). Results obtained with laboratory animals may therefore not necessarily be applicable to the human CNS.

1. 3. In vitro models of ischaemia

Cerebral ischaemia is caused by an interruption of blood flow in a specific area of the brain. The interruption is usually caused by the occlusion of a blood vessel by a blood clot. It can also be caused by the rupture of a blood vessel or a trauma. The interruption of blood flow interrupts the supply of oxygen and glucose and the transport of CO_2 and other metabolic waste. This in turn leads to a number of secondary phenomena (i. e. the rise in extracellular pH) which are so far not completely understood.

Such a situation is rather difficult to simulate as it is virtually impossible to generate a complete neuronal tissue in an *in vitro* system. The most important parameters that are altered during ischaemia seem to be the lack of oxygen and glucose in the affected area which is the reason why most *in vitro* systems of simulated ischaemia are focused on these two parameters. Almost all in *vitro* approaches make use of material obtained from test animals which is either cultivated as tissue slices in so called “slice cultures” or as cultures of isolated primary neuronal cells.

1. 3. 1. Brain slice cultures

In many respects, slice cultures are probably as close to the *in vivo* state as one can get outside of a complete organism. Brain slices have almost the same cellular composition as neuronal tissues *in vivo* and many neuronal networks are still intact. They are constantly perfused with an appropriate medium that ensures supply with nutrients and oxygen. Ischaemia in brain slices can be induced relatively easily by stopping perfusion for a certain time. Since the cell density within a brain slice is very high, normal diffusion processes are not sufficient to supply the quantities of oxygen and nutrients needed. Unfortunately there are considerable difficulties connected to the use of brain slices in ischaemia models. No matter how fast the preparation protocol is, during the extraction and the slicing of the brain the blood flow is interrupted and the cells within a brain slice are already subjected to some kind of ischaemia. This strongly influences subsequent ischaemic experiments. On the surface of a brain slice – where it was cut – lay a large number of dead or dying cells which influence the still intact deeper layers in a way which is very difficult to evaluate. Brain slices degrade rapidly and their maximum viability in culture does not exceed 10 days.

1. 3. 2. Neuronal cell cultures

Although cultures of isolated neuronal cells do represent only parts of neuronal tissue, they are easier to handle than are brain slices, provide a much simpler model system and can be kept in culture for several weeks up to a few months. Neuronal cell cultures can be established from certain cell lines or from primary cultures.

Primary neuronal cultures from test animals are widely used in research to address a large number of experimental questions. Ischaemia can be simulated in primary cultures by withdrawing oxygen and/or glucose and such a system can be used to screen for neuroprotective drugs (Bartmann-Lindholm and Carter, 1999). Primary cells isolated from one animal provide experimental material for a large number of assays reducing the total number of test animals needed. However, primary cultures represent a heterogeneous mixture of different cell types which may show contradicting behaviour under specific experimental conditions. Also, their composition can be highly variable from one animal to another.

In a cell culture system with a homogenous, well characterised cell population, the effect of the excitotoxic cascade can be separated from other potentially damaging effects. This allows studying damaging effects in detail and individually, for instance anoxia, glutamate excitotoxicity, and NO toxicity. In addition, several damaging effects can be combined (i.e. by the addition of glutamate, NO or an NO donor such as SNP to an anoxic system).

1. 4. The NT-2 cell line

I focussed on the well characterised human teratocarcinoma cell line Ntera-2 (NT-2) which has been derived from a human testicular cancer. Upon treatment with retinoic acid (RA), a biologically active derivative of vitamin A, the NT-2 cells can be induced to differentiate into cells, that, judged by the expression of many neuronal markers, include postmitotic neurons (NT-2-N cells) (Andrews, 1984; Pleasure et al., 1992). A developmental study suggested that differentiation of NT-2 neurons in cell culture follows a pattern of differential gene expression similar to that of neural precursors during neurogenesis (Simeone et al., 1991). Depending on the cell differentiation protocol, NT-2 cells also have the capacity to develop into astrocytes (Bani-Yaghoub et al., 1999; Sandhu et al., 2002). The neuronal differentiation of NT-2 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). They do express a variety of neurotransmitter phenotypes including GABAergic, catecholaminergic, cholinergic and serotonergic phenotypes (Guillemain et al., 2000; Yoshioka et al., 1997). The cholinergic neurotransmitter phenotype has been shown to be inducible when CNTF is added to the cell culture medium (Zeller and Strauss, 1995).

NT-2 N cells have been shown to possess glutamate channels (Younkin et al., 1993), they respond to glutamate toxicity (Munir et al., 1995) and have also been found to be highly susceptible to oxidative stress (Tamagno et al., 2000). However the formation of free radicals by NT-2 neurons was found to be only of minor importance in hypoxic cell death (Almaas et al., 2002). There is increasing evidence that nerve cells derived from the NT-2 cell line can be used as a model for hypoxic-ischaemic cell injury (Munir et al., 1995). They have been used to study the effects of anoxia and hypoglycaemia *in vitro* (Rootwelt et al., 1998) and to evaluate neuroprotective properties of barbiturates (Almaas et al., 2000) and caspase inhibitors (Almaas et al., 2003) under conditions of simulated ischaemia. They were also used to study the neuroprotective effect of allopregnanolone, in a model of NMDA induced excitotoxicity (Lockhart et al., 2002).

This cell type has also been used in several transplantation studies, which have demonstrated successful engraftment in experimental animals (Ferrari et al., 2000) and humans (Nelson et al., 2002) and improvement of behavioural deficits associated with stroke in human patients (Kondziolka et al., 2001). In culture, NT-2 neuronal cells show neuronal morphology and migrate to form ganglion-like cell conglomerates. Differentiated cells are immunoreactive to neuronal cytoskeletal markers such as MAP2, Tau, and NF-66 and the neuronal membrane bound proteins NCAM, and GAP-43 (Pleasure et al., 1992).

One disadvantage of this cell culture system is the rather lengthy differentiation process. This process requires a 5-6 week treatment with retinoic acid (RA), followed by 2 trypsinisation steps (2 days), a treatment with mitotic inhibitors (7-10 days) and 1-2 more selective trypsinisation steps for neuronal purification. Taken together, the differentiation of NT-2 precursor cells into neurons requires between 44 and 56 days of cell culturing work.

A cell aggregation protocol has been described (Cheung et al., 1999) which shortens the time needed for differentiation to 3 weeks of RA treatment. This method has been successfully used in an expression study of synapsins (Leypoldt et al., 2002). In the procedure, cells were aggregated and treated with RA for 14 days, then plated on poly-d-lysine and laminin coated petri - dishes and treated with mitotic inhibitors for another 7 days. However, the aggregates obtained contain only a low percentage of neuronal cells. My own experiments with this method showed that further purification of neurons was rather difficult. One intention of this study was therefore to optimise and possibly shorten the differentiation process.

1. 5. Simulation of ischaemia in neuronal cell cultures

Cultures of isolated neuronal cells present a number of important differences between the situation *in vivo* and the situation *in vitro*:

In cell cultures, the cell densities are usually considerably lower than in intact tissue. Therefore the consumption of oxygen and glucose is much lower in an *in vivo* environment.

On the other hand, the volume of the surrounding medium is considerably larger than the volume of the interstitial liquid in intact tissue. Thus, *in vitro* only traces of oxygen or glucose, dissolved in the cell culture medium, are sufficient for cell survival. Also, the release of substances from cells within such a culture (i. e. Neurotransmitters, K^+ or CO_2) will have little or no effect on other cells as these substances are heavily diluted in the surrounding medium.

To be able to simulate ischaemia it is therefore imperative to generate complete anoxia by removing even trace amounts of oxygen from the cell culture medium. Low concentrations of glucose are not as problematic as is oxygen, because in the complete absence of oxygen the capacity of glucose to produce ATP is limited and it will therefore be rapidly depleted. If the action of excitatory neurotransmitters such as glutamate is to be studied, these have to be added to the culture medium during the experiment as their release from neurons will probably be too small to induce an excitatory response.

1. 6. Aim of this study

The first intention was to optimise the differentiation process and possibly reduce the time needed to obtain terminally differentiated, postmitotic neurons. To this end, it was tried to combine the amplification of precursors in free floating cell conglomerates (Cheung et al., 1999) with the differentiation and purification procedure described by Pleasure et al. (1992). Using immunocytochemical staining of neuronal markers, it was shown that this new protocol of cell expansion greatly enhances nerve cell differentiation and allows to obtain postmitotic NT-2 neurons in less than half the time needed for the conventional differentiation protocol.

I also wanted to use immunocytochemical methods to follow the neuronal development and to further characterise the neuronal phenotype of the cells obtained.

Using NT-2 neuronal cells it was intended to establish an assay system that would be suitable for the large scale screening of neuroprotective substances, which can be used during or after an ischaemia. Therefore an experimental setup was designed which allows to generate anoxic / hypoglycaemic conditions *in vitro*. The effect of anoxia and high concentrations of glutamate or NMDA on the mitochondrial potential of NT-2 neurons was studied. In order to evaluate the susceptibility of NT-2 neurons to glutamate induced excitotoxicity, the effect of glutamate and NMDA on intracellular calcium concentration was estimated using calcium dependent fluorescent dyes. The development of viability in long term cultures of NT-2 neurons was determined and the effect of oxygen glucose deprivation (OGD) on these cultures was measured using different cell viability assays.

2. Materials and Methods

2. 1. Precursor cell line

NT-2 precursor cells (Passages 52-54) were purchased from Stratagene (La Jolla, CA). The NT-2 cells were treated as indicated in the Stratagene instruction manual. NT-2 precursor cells were differentiated using two different protocols. The first – conventional – differentiation protocol uses layer culture and largely follows the procedure given by Pleasure et al. (1992). The second is a new sphere culture protocol where one additional step greatly reduces the total time needed for neuronal differentiation.

2. 2. Cell culture and expansion

NT-2 cells were cultured in DMEM/F12 medium supplemented with 10 % fetal bovine serum (FBS), 1 % penicilline / streptomycine and 1 % glutamine and kept at 37°C under 5% CO_2 atmosphere. The same medium was used for neuronal cell cultures (NT-2N) where half the medium volume was changed every 2-3 days. Cells were cultured on Falcon T75 or T175 (after 2nd replate) cell culture flasks.

NT-2 precursor cells were split 1:3-1:4 every 3-4 days by applying 2 ml trypsine-EDTA (1X) solution per T75 cell culture flask for 4-5 min. Trypsinisation was stopped by adding 7-8 ml of serum supplemented medium to the flasks. The cells were then counted and seeded for cell expansion on fresh T75 cell culture flasks at a density of 1-2 x 10^6 cells per flask. (6 x 10^6 cells per flask for neuronal differentiation).

In order 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.

2. 3. Coating of cell culture ware

Plastic and glass ware was coated with poly-d-lysine 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 4h. After incubation the liquid was removed and allowed to dry completely at room temperature. This was followed by a matrigel coating. A matrigel matrix solution (approx. 10 mg/ml) was diluted 1:30 in ice cold DMEM/F12 (without serum!). The resulting solution was pipetted up and down 5-10 times to ensure even distribution of matrigel with a fire polished Pasteur pipet. To each well of a 96 well plate 6 drops of this solution were added (approx. 200-250 µl) and incubated for 1h. Afterwards the liquid was removed and the plates dried completely at room temperature. The matrigel coating was repeated at least 2 times. Larger surfaces (such as

glass cover slips) were incubated with a volume of matrigel solution large enough to cover the entire surface. Coated plates were used within 24h after coating.

2. 4. Conventional differentiation protocol

NT-2 precursor cells (Passages 58-72) were seeded in 75 cm^2 tissue culture grade flasks at a density of 6 x 10^6 cells per flask. From the second day on the cell culture medium was supplemented with retinoic acid (RA) to give a final concentration of 10 µM. Every 2-3 days the medium was changed and the cells kept in culture for the next 6 weeks.

Replate 1: After 6 weeks the cells were washed twice with sterile PBS and once with Versen, then trypsinised (Trypsin-EDTA,1X) and seeded on T175 cell culture flasks at a density of approx. 100 x 10^6 cells per flask. Cells were cultured in T175 flasks for 2 days to obtain conditioned medium.

Replate 2: The conditioned medium was collected, centrifuged and sterile filtered. Cells were selectively trypsinised, transferred to T75 flasks (cell density: approx. 30 x 10^6 cells per flask) and supplied with inhibitor medium (Ara C, 1-6-D-arabinofuranosylcytosine: 1 µM; FudR, 2'-deoxy-5-fluorouridine: 10 µM; Urd, 1-ß-D-ribofuranosyluracil: 10 µM).

Replate 3: After 7-10 days neurons were again selectively trypsinised. Then, they were counted and transferred to plates coated with poly-d-lysine and matrigel for further experiments.

2. 4. 1. Modifications of the conventional differentiation protocol

To try and optimise the differentiation protocol the cells were exposed to cell culture medium with different RA concentrations during the 6 week incubation. The concentrations used were 5 µM, 10 µM (as usual), 25 µM, and 50 µM of RA.

NT-2 cells were also subjected to treatment with cell culture medium with 200 µM butyl-hydroxyl-anisole (BHA) together with 1% DMSO instead of RA.

2. 5. Sphere culture differentiation protocol

The difference to the conventional protocol is the introduction of a sphere culture step at the beginning of the differentiation. The following steps are mostly identical to the conventional procedure.

Step1: NT-2 precursor cells (Passages 58 - 72) were seeded in 94 mm, bacteriological grade petri dishes at a density of 4 x 10^6 - 5 x 10^6 cells per dish. On the first day 10 ml of DMEM/F12 medium supplemented with 10 % FBS, 1 % penicilline / streptomycine and 1 % glutamine were 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. This was done by transferring the cell suspension to centrifuge tubes. Remaining cell conglomerates were washed off with a few ml 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. The use of new petri-dishes at this point is very important as the cells adhere to the plastic and do no longer form floating cell conglomerates after a few days in culture.

Step 2: After 7 - 8 days the conglomerates from one petri-dish were seeded in T75 cell culture flasks. After 6 - 8 days in culture the surface of a culture flask was completely covered with aggregates or cells growing as a monolayer.

Step 3: Cells were trypsinized (Trypsin-EDTA,1X) and transferred to T175 cell culture flasks (Replate I). Trypsinisation was done without the use of Versen (according to the Stratagene protocol) which had proven to be of very little use. Cells were cultured in T175 flasks for 2 days to obtain conditioned medium.

Step 4: Cells were selectively trypsinized (Replate II), transferred to T75 flasks and supplied with inhibitor medium (Ara C: 1 µM, FudR: 10 µM, Urd: 10 µM).

Step 5: After 7-10 days neurons were again selectively trypsinized (Replate III). Then, they were counted and transferred to plates coated with poly-d-lysine and Matrigel for further experiments.

Using immunocytochemical stainings for neuronal markers (see below) and nuclear counterstaining with propidium iodide the purity of neuronal cultures was evaluated. Some epitheloid cells were also present in the Petri-dishes, but staining for neuronal markers indicated, that after final plating the cell cultures contained approximately 80 - 95% human nerve cells.

In additional control experiments, RA was omitted from the medium during step 1 of our protocol (see Figure 5). RA treatment started only after cell conglomerates had been seeded in T75 culture flasks.

An important factor that influenced neuronal yield was the number of passages of the commercially supplied NT-2 precursor cells. The passage numbers used in our modified differentiation method were between 58 and 72. In our experience in both the layer culture and the sphere culture protocol, neuronal yield dropped after passage 68.

2. 5. 1. Modifications of sphere culture differentiation protocol

To try and increase neuronal differentiation NT-2 cells were exposed to different concentrations of butyl-hydroxyl-anisole (BHA) together with DMSO during sphere culture.

The concentrations used are given in the table below:

BHA concentration	DMSO concentration	
200 µM	1 %	
100 µM	0.5 %	+ 10 µM RA or without RA
50 µM	0.25 %	
25 µM	0.125 %	

In 2 separate series of experiments, NT-2 cells were exposed to BHA/DMSO alone or together with 10 µM RA.

2. 6. Treatment of NT-2 neurons with neurotrophic factors

The influence of neurotrophic factors on the expression of certain proteins was studied in 2 experiments. The neurotrophic factors used were: recombinant human brain derived neurotrophic factor (rh-BDNF), recombinant human neurotrophic factor 3 (rh-NT-3), mouse 2.5S neurotrophic growth factor (mNGF 2.5S), and mouse 7S neurotrophic growth factor (mNGF 7S). The experiment was begun with the plating of 10.000 NT-2 neuronal cells per well in a 96 well plate coated with poly-d-lysine and Matrigel. After one week in culture the cells were exposed to different concentrations of neurotrophic factors. The concentrations used were: 20 and 40 ng/ml for BDNF, 20 and 40 ng/ml for NT-3, 50 and 100 ng/ml for mNGF 2.5S, and 50 and 100 ng/ml for mNGF 7S.

After 17 days of exposure to neurotrophins the experiment was stopped by the fixation of the cells with 4% PFA (4% PFA + 0.1% glutaraldehyde for GABA staining). For the immunocytochemical and histochemical staining procedure see next chapter (2. 7.) The marker used to assess the reaction of NT-2N cells to neurotrophins were HuC/D, GFAP, tryptophan hydroxylase, 5-HT and GABA. I also used the NADPH-diaphorase staining assay and labelling with the universal NOS (uNOS) antibody, an antibody which recognises a conserved region of all three types of NOS enzymes. The number of cells that labelled positive and negative in the staining procedure was counted. To this end, 5 fields of view at 20 x magnification were counted for each well and the average percentage of positive cells calculated.

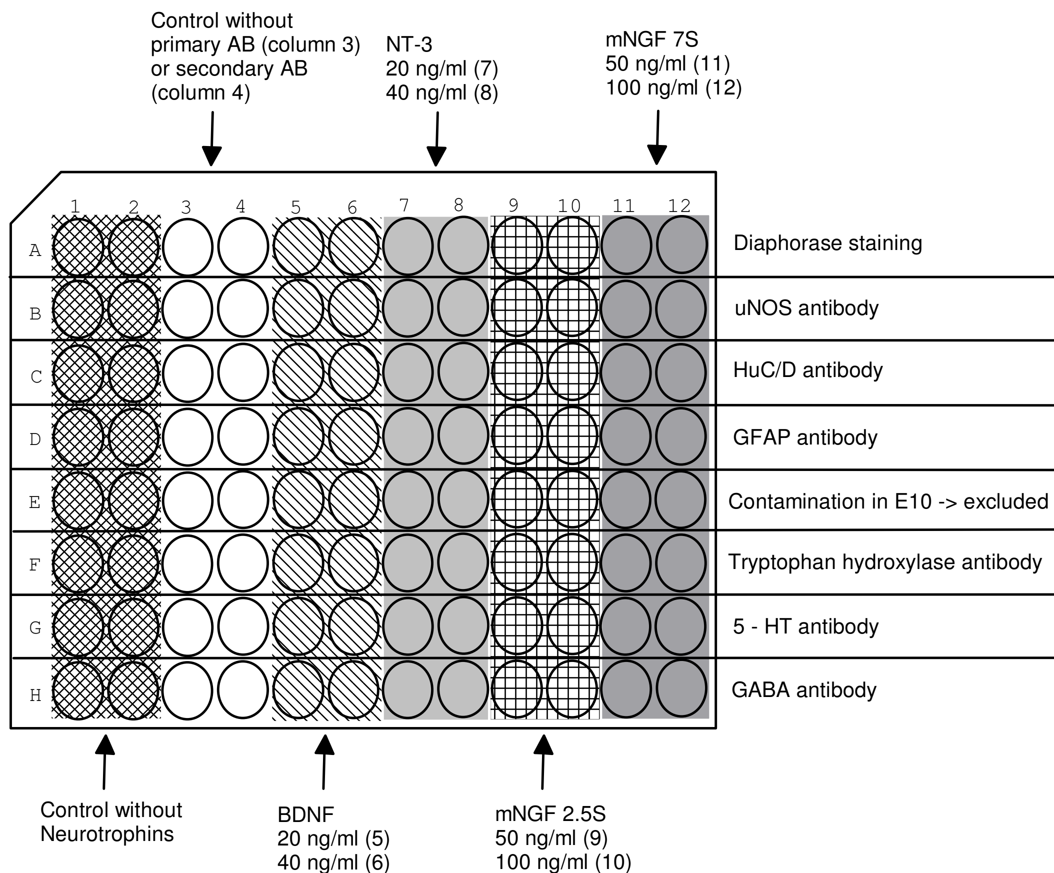


Figure 3: Illustration of the distribution of samples in an experiment with neurotrophic factors. NT-2 neuronal cell cultures were exposed to different concentrations of neurotrophins. After 12 days in culture one well (E10) was contaminated. This well and the surrounding wells (E9 + E11) were sterilized using 1 M NaOH solution, the whole row was excluded from the further experiment. At the end of the experiment, the cells were fixed and stained using immunocytochemical or histochemical staining techniques.

2. 7. Immunocytochemistry

Cells were washed with PBS once and then fixed for 1h in PBS containing 4 % paraformaldehyde (PFA). For the detection of γ-amino-butyric-acid (GABA), 0.1 % glutaraldehyde was added to the fixative. After fixation, cells were washed 3 times for 5 minutes in PBS containing 0.2% Triton X100 (PTX). Blocking solution containing PTX and 5 % normal serum from the host animal, from which the secondary antibody was obtained, was applied for 1 hour. Primary antibodies were diluted in PTX containing 2 % normal serum and applied overnight at 4° C. On the next morning wells were washed 4 times for 5 minutes in PTX and incubated with secondary biotinylated antibody directed against the host animal of the primary antibody (Vector) for one hour at RT. After 3 more washing steps in PTX, cells were washed once in PBS containing 0.2% Tween 20 (PTW). Immunofluorescence was detected by applying streptavidin bound fluorescein, alexafluor 488 or CY3 for 1h. Finally, the cells were washed 3 times in PTW and kept in PTW at 4°C for at least several weeks.

Antibody	*Provider*	*Concentration/ Dilution*
Monoclonal mouse anti-Hu C/D (clone 16A11)	Molecular Probes, Eugene (OR)/USA	5-10 µg/ml
Monoclonal mouse anti-MAP2 (clone AP20)	Sigma, St. Louis (MO)/USA	1:200
Polyclonal rabbit anti-Tau (AB1512)	Chemicon International, Temecula (CA)/USA	1:200
Polyclonal rabbit anti-GFAP	Biotrend, Köln/Germany	1:1000
Polyclonal rabbit anti-GABA	Sigma, St. Louis (MO)/USA	1:500
Polyclonal rabbit anti-5-HT	Sigma, St. Louis (MO)/USA	1:500
Monoclonal mouse anti-PrP (clone 8G8)	kindly provided by A. Stuke, DPZ, Göttingen/Ger.	1:200
Polyclonal sheep anti-tryptophan hydroxylase (AB1541)	Chemicon International, Temecula (CA)/USA	1:1000
Monoclonal mouse anti-uNOS (clone 3F7-B11-B5)	Sigma, St. Louis (MO)/USA	1:200

Table 1: List of the antibodies used for the immunocytochemical characterisation of differentiated human NT-2 neuronal cells. The antibody solution was diluted as indicated.

Cells were incubated for 5-10 min. in PBS containing 500 nM of propidium iodide (PI) as a nuclear counterstain. Controls consisted of omitting the primary antibodies and application of the fluorescence detection system.

Double labelling was done sequentially by applying primary and secondary antibody for the first antigen to be labelled. This was followed by another blocking step with normal serum and then primary and secondary antibody for the second antigen to be labelled. Primary and secondary antibodies were chosen to avoid interference between the first and the second detection step (i. e.: block with horse serum, primary mouse anti-MAP2, secondary horse anti-mouse, block with goat serum, primary rabbit anti-Tau, secondary goat anti-rabbit). Fluorescein was used to label the first antigen, CY 3 to label the second.

2. 8. Histochemistry

Alkaline phosphatase (AP): Cells were fixed and washed (s. a.). A staining solution containing X-phosphate, nitro-blue-tetrazolium (NBT) in TRIS/$MgCl_2$ buffer, pH 9.5 was applied for 2 h and then washed off twice with PTX. Control consisted of staining solution without NBT.

Acetylcholinesterase (AChE): Cells were fixed and washed with PBS, then washed with TRIS-maleate buffer (24.2 g TRIS, 23.2 g maleate, approx. 8.0 g NaOH in 1 l destilled water, pH 5.7). The cells were then incubated for several hours in a staining solution (10 mg actylthiocholine-iodide in 7.5 ml TRIS-maleate buffer + 0.5 ml 0.1 M sodium citrate + 1.0 ml 30 mM $CuSO_4$ + 1.0 ml 5 mM $K_3Fe(CN)_6$). Control consisted of staining solution without actylthiocholine-iodide.

NADPH-diaphorase: Cells were fixed, washed with PBS and permeabilised with PBS containing 0.5 % Triton X100 (PTX 0.5) for 2h. The staining solution consisted of 2.5 mg NBT dissolved in 0.5 ml methanole to which 50 mM TRIS-HCl-buffer was added to give a total volume of 10 ml. To this solution 10 mg NADPH were added. The cells were incubated with staining solution at room temperature in the dark for 20-60 min. Control consisted of staining solution without NBT.

2. 9. Anoxia and excitotoxicity

NT-2 neurons were plated on poly-d-lysine and matrigel coated 96-well plates at a cell density of 20.000 cells per well. In order to limit evaporation from outer wells in long term cultures, cells were not seeded in the rows A and H and the columns 1 and 12. The concentric ring of empty wells around the neuronal cell cultures was filled with sterile PBS. NT-2N cells were cultured in these plates for 1-7 weeks prior to the anoxia / hypoglycaemia experiment. Initially, anoxia experiments were performed in DMEM/F12 medium supplemented with 10 % FBS, 1 % penicilline / streptomycine and 1 % glutamine, in DMEM/F12 without glucose or in PBS. Later experiments were performed in Krebs HEPES Buffer of the following composition:

1 mM NaH_2PO_4, 118.5 mM NaCl, 5.57 mM KCl, 1.25 mM CaCl, 1.2 mM $MgCl_2$, 0.03 mM Na_2EDTA, 0.06 mM Ascorbic Acid, 20 mM HEPES, and pH 7.4

Medium was changed to anoxia medium immediately before the experiment was performed. The volume of anoxia medium was 100 µl in earlier and 30 µl in later experiments. This volume was reduced in order to ensure a rapid deoxygenation with Argon. 1 to 4 mM sodium dithionite were added to the anoxia medium to scavenge oxygen in the medium. Internal positive control was given anoxia medium without sodium dithionite and these wells were closed with airtight caps.

In addition to anoxia, in a number of samples the medium was supplemented with glutamate or NMDA in concentrations ranging from 100 µM to 5000 µM.

pH-dependency of viability was determined in an experiment where samples were exposed to anoxia and pH values of 7.1, 7.7 and 8.1. Some of these samples were also supplemented with 1 mM glutamate and/or with 2 mM dithionite.

The 96-well plates were placed in a sterile, anoxic chamber which was perfused with a mixture of 95% argon and 5% CO_2 during the full length 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 starting point for anoxia was at 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 in the warm water bath. After 1-4 hours the anoxia experiment was stopped with reoxygenation and a viability assay.

To evaluate the effect of sodium dithionite on the viability of NT-2 neurons several experiments were performed in which neurons were subjected to different concentrations of dithionite without being exposed to anoxia.

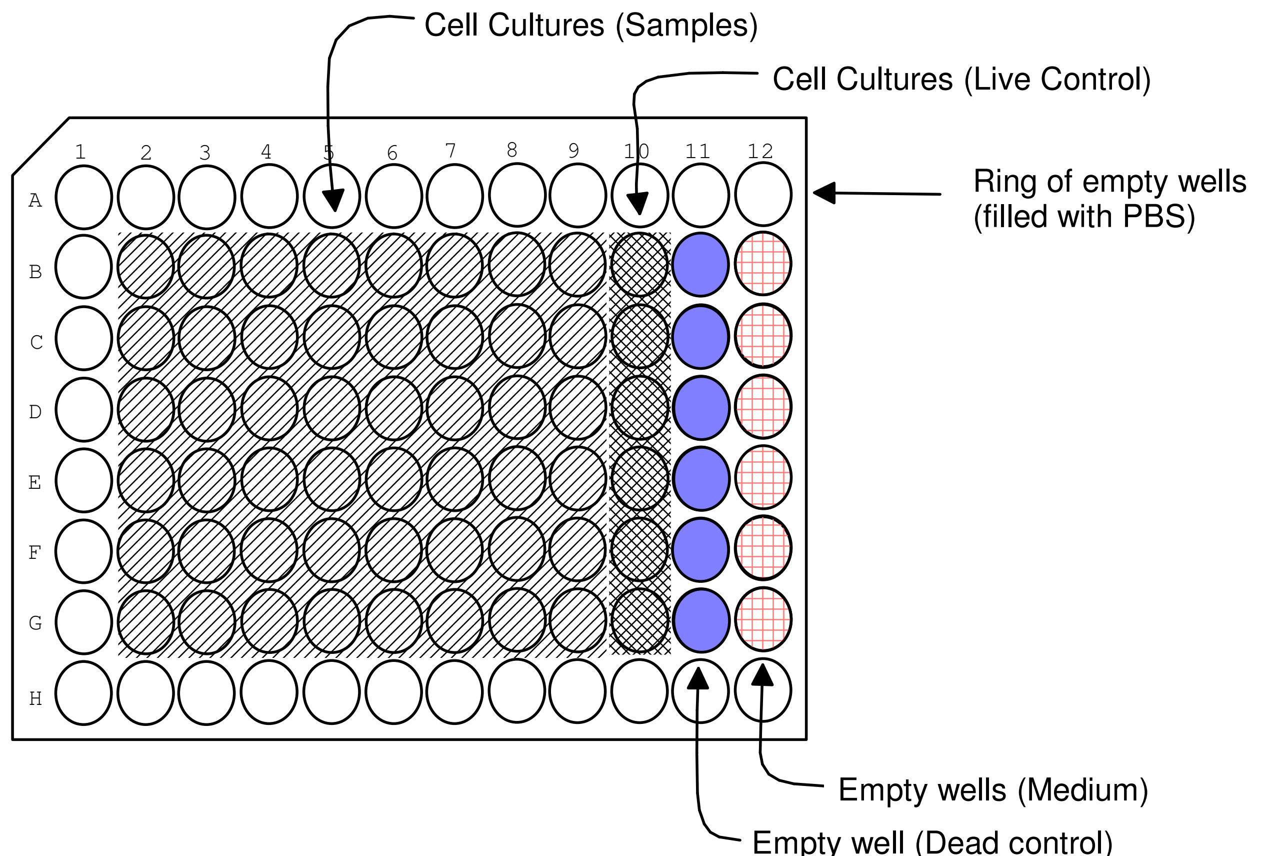


Figure 4: Illustration of the distribution of samples and live control on a 96-well plate. For the Alamar Blue® assay two additional columns were used as blank (empty wells filled with 100 µl cell culture medium) and dead control (empty wells filled with cell culture medium + 3% Alamar Blue solution).

2. 10. Cell viability assay

The development of the viability of NT-2 neuronal cultures used in the anoxia experiments 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 is then detected by measuring the absorbance of the liquid medium in a photometric microplate reader (Tecan Spectra II). 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. During the weeks in culture, before the experiment, the viability was usually measured once per week, there was one measurement in the 24h before the anoxia, and at least 3 measurements at between 4h to 72h after anoxia.

For each measurement the cell culture medium (or Krebs HEPES Buffer) was completely changed and replaced with culture medium to which 3% Alamar Blue® solution was added. Total assay volume in each individual well in a 96 well plate was 100 µl. After 4h incubation at 37°C / 5% CO_2 the plates were read in the microplate reader equipped with 570 nm and 600 nm filters and the medium changed to cell culture medium without Alamar Blue. Each 96-well plate was assayed several times after the anoxia experiment. The viabilities measured in the last measurement before the anoxia were set to 100% for each well in every sample group. Viabilities measured after anoxia, 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.

To compare the absorption values obtained with the Alamar Blue assay to the actual number of living or dead cells, a Live/Dead Assay was performed. In this assay two different dyes are combined that either stain dead cells with ethidium-homo-dimer (EthD-1) in red or living cells with calcein in green. NT-2N cells were incubated 30 min. at 37°C / 5% CO_2 in cell culture medium containing 4 µM EthD-1 and 0.8 µM calcein. After incubation cells were washed 3 times in cell culture medium and viewed under a Zeiss Axiovert 25 microscope. In 10 fields of view neuronal cells were counted for every sample and the average values for living and dead cells were calculated. Samples were compared to live controls which were set as 100% for each measurement.

2. 11. Microscopy and image processing

Optical sections were layered in Adobe Photosphop 6.0 and combined to obtain one single image. Morphological observations, routine light microscopy and fluorescence microscopy were performed on a Zeiss Axiovert 25 microscope equipped with an HBO 25 Halogen lamp and a 450 nm (for R123, alexa fluor 488, fluorescein) or 550 nm (for Cy3, TMRM, PI) filter set.

Cells that were labelled with more than one fluorescent dye were viewed in a Leica TCS 2 laser scanning microscope. Excitation wavelength was 488 nm, emission was measured in 2 or 3 channels ranging from 500 to 525 (fluorescein), 542 to 570 nm (Cy3), and 630 to 700 nm (PI). Confocal images were captured using Leica software. Image overlays and contrast enhancement were done using Adobe Photoshop 6.0.

2. 12. Calcium fluorescence measurement

NT-2 neurons were plated on poly-d-lysine and matrigel coated, round, 25 mm diameter, glass coverslips. Calcium Green 1® was dissolved in a 20% solution of pluronic acid in DMSO and diluted with cell culture medium to give a final concentration of 5 µM. NT-2N cells were incubated with this solution for 30 min. Then cells were washed with Krebs HEPES Buffer, placed in a perfusion chamber which was kept at 37.1°C and mounted onto a Zeiss Axiovert 35 microscope. Calcium fluorescence measurement was performed using a Hamamatsu SIT Camera, Sutter Instrument Lambda 10-2 optical filter changer / shutter and Simple PCI software. Fluorescence excitation was done using a XBO 75W/2 Xenon lamp with a 450 - 490 nm filter set.

The cells were constantly perfused with Krebs HEPES buffer, the volume of the perfusion chamber used was 1 ml, the flow rate was 2.5 to 3 ml/min. Every 2.00-2.30 min. the medium was completely exchanged. Stimulation was done with a 3 sec. application of a solution of 100-1000 µM NMDA or glutamate and 50 - 500 µM glycine in Krebs HEPES buffer. MK-801 (10-500 µM), AP 5 (100-500 µM) and Ketamine (500 µM) were dissolved in Krebs HEPES buffer and administered prior or together with NMDA/glutamate. At the end of a calcium imaging experiment 5 µM of the calcium ionophore Calcimycine (A23187, Molecular Probes) was added to the Krebs HEPES buffer.

Images of the cells were captured every 1.5 to 10 s. Regions of interest (ROI) were defined around the body of the cells and the mean fluorescence of each ROI calculated using Simple PCI software. Background fluorescence (F_B) was subtracted from the fluorescence of the cells (F). For comparison purposes, the value obtained – ΔF – was sometimes divided by the baseline fluorescence of the non-stimulated cells (F_0). The maximum height of glutamate/NMDA induced, calcium dependent fluorescence peaks was compared to baseline fluorescence (F_0) and maximum fluorescence (F_{max}) after application of Calcimycine. To establish whether a distinctive treatment had a significant effect on calcium influx, different samples were compared using Student´s t-test and Vassar Stats (http://faculty.vassar.edu/lowry/VassarStats.html) software.

2. 13. Estimation of mitochondrial potential using rhodamine 123

NT-2 neurons were plated on poly-d-lysine and matrigel coated, round, 25mm diameter, glass coverslips. A stock solution containing 2.5 mg rhodamine 123 (R123) in 1 ml MeOH was diluted 1:400 in cell culture medium. NT-2 N cells were incubated for 30 min at 37°C / 5% CO_2 in the

R123 solution. After incubation the neuronal cells were washed 2 times in Krebs HEPES buffer and transferred to a perfusion chamber which was kept at 37.1°C and mounted onto a Zeiss Axiovert 35 microscope. Rhodamine 123 fluorescence measurement was performed using the equipment already described for calcium fluorescence measurements. Fluorescence excitation was provided by an XBO 75W/2 Xenon lamp with a 450-490 nm filter set.

The cells were perfused constantly with Krebs HEPES buffer. Cells were stimulated by the application of a solution of 500 µM glutamate / 100 µM glycine or 500 µM NMDA / 100 µM glycine. In this experimental setup anoxia was induced with 2 mM sodium dithionite in Krebs HEPES buffer. Images of the cells were captured every 3 to 30 s. ROI were defined around the cells and the mean fluorescence of each ROI calculated using Simple PCI software. Background fluorescence (F_B) was subtracted from the fluorescence of the cells (F) giving a ΔF value.

2. 14. Estimation of mitochondrial potential using tetra-methyl-rhodamine (TMRM)

NT-2 neurons were plated on poly-d-lysine and matrigel coated, round, 25mm diameter, glass coverslips. 1.8 mg tetra-methyl-rhodamine (TMRM) was dissolved in 1440 µl DMEM/F12. This stock solution was diluted 1:250 and 1:1000 in cell culture medium. NT-2 N cells were incubated for 30 min at 37°C / 5% CO_2 in the TMRM solution. After incubation the neuronal cells were washed 2 times in Krebs HEPES buffer and transferred to a perfusion chamber which was kept at 37.1°C and mounted onto a Zeiss Axiovert 35 microscope. TMRM fluorescence measurement was performed using the equipment already described for calcium fluorescence measurements. Fluorescence excitation was provided by an XBO 75W/2 Xenon lamp with a 550 nm filter set. The cells were perfused constantly with Krebs HEPES buffer.

2. 15. SNP stimulation of NT-2 neurons: movement of neurites

NT-2 neurons (Passages 59, 61, 62) were plated on poly-d-lysine and matrigel coated, round, 25mm diameter, glass coverslips. After 1.5-4 days in culture the cells were transferred to a perfusion chamber which was kept at 37.1°C and perfused with Krebs HEPES buffer. The neurites, growth cones and filopodia of NT-2 neurons were viewed under an inverted microscope with 100 X oil immersion objective and phase contrast. For a time lapse sequence, every 30 sec. a picture was taken of the neurites´ movement. The movement of active growth cones and filopodia of NT-2 neurons was observed 30-60 min. prior to the application of the NO donor SNP. The observation was continued for approximately 1 h after application of SNP.

A stock solution of 30 mg SNP in 1 ml Krebs HEPES buffer was allowed to stand for 30 min. before it was diluted 1:100 to a final concentration of 1 mM SNP. This SNP solution was applied

to the cells and their reaction observed.

2. 16. SNP stimulation of NT-2 neurons: effect on cGMP

NT-2 neurons were plated on poly-d-lysine and matrigel coated 96-well plates. The stock solution of 30 mg of the NO donor SNP in 1 ml DMEM/F12 medium was allowed to stand for 30 min before it was diluted to its final concentration. Another stock solution was allowed to stand for at least 24 h to ensure that it was completely degassed before it was diluted.

24 wells of the 96-well plate were divided into 12 groups with 2 wells each. 4 groups were given SNP that had been degassed for only 30 min., while 4 others were given SNP that had been completely degassed (for at least 24 h). SNP was applied at 2 different concentrations (100 µM and 1000 µM). The phosphodiesterase (PDE) inhibitor IBMX was added to the SNP treated cells and to non-treated cells at a concentration of 1 mM according to the scheme below.

Control without 1st Antibody	+ SNP
Control + 1st Antibody	
SNP 100 µM	
SNP 1000 µM	
SNP 100 µM + IBMX 1 mM	
SNP 1000 µM + IBMX 1 mM	
IBMX 1 mM	

Control without 1st Antibody	+ degassed SNP
Control + 1st Antibody	
SNP 100 µM	
SNP 1000 µM	
SNP 100 µM + IBMX 1 mM	
SNP 1000 µM + IBMX 1	
IBMX 1 mM	

After 30 min incubation at 37°C/5%CO_2, the cells were washed with PBS once and fixed in PBS containing 4 % paraformaldehyde (PFA). After fixation cells were washed 3 times for 5 minutes in PBS containing 0.2 % Triton X100 (PTX). Blocking solution containing PTX and 5 % normal horse serum was applied for 1 hour. Cells were double stained with the primary anti-cGMP antibody diluted 1:1000 and the primary anti - MAP2 antibody diluted 1:200. After overnight incubation at 4°C wells were washed 4 times for 5 minutes in PTX and incubated for one hour at RT with secondary antibody labelled with Cy 3 (anti-cGMP) and alexa fluor 488 (anti-MAP2). Propidium iodide was used as a nuclear counterstain.

2. 17. List of chemical substances, biological agents and cell culture material used

A list of the antibodies used for the immunocytochemical characterisation of NT-2 neuronal cells is given in section 3. 7., see page 16.

Item	***Provider***
1-6-D-arabinofuranosylcytosine (Ara C)	Sigma, St. Louis (MO)/USA
1-ß-D-ribofuranosyluracil (Urd)	Sigma, St. Louis (MO)/USA
2'-deoxy-5-fluorouridine (FudR)	Sigma, St. Louis (MO)/USA
actylthiocholine-iodide	Sigma, St. Louis (MO)/USA
Alamar Blue® Assay	Trek Diagnostic Systems, East Grinstead/UK
Alexa fluor 488 (streptavidin bound)	Molecular Probes, Eugene (OR)/USA
Anti-cGMP antibody	kindly provided by Jan de Vente, Univ. Maastricht / NL
AP 5 (dl-amino-5-phophono valeric acid)	Sigma, St. Louis (MO)/USA
Argon/CO_2 gas (95%/5%)	Messer Griesheim, Duisburg/Germany
BDNF	Alomone Labs, Jerusalem/Israel
BHA (butyl-hydroxy-anisole)	Sigma, St. Louis (MO)/USA
$CaCl_2$	Aldrich, Gillingham/UK
Calcimycine (A23187)	Molecular Probes, Eugene (OR)/USA
Calcium Green® 1	Molecular Probes, Eugene (OR)/USA
Cell culture flasks (tissue culture grade)	Falcon, Franklin Lakes (NJ)/USA
$CuSO_4$	Fluka, Buchs/Switzerland
CY3 (streptavidin bound)	Vector, Burlingame (CA)/USA
DMEM/F12 Medium	Gibco, Paisley/UK
DMSO (dimethyl sulfoxide)	Sigma, St. Louis (MO)/USA
Ethanole	Roth, Karlsruhe/Germany
Fetal bovine serum (FBS)	Gibco, Paisley/UK
Fluorescein (streptavidin bound)	Vector, Burlingame (CA)/USA
FM® 1 - 43	Molecular Probes, Eugene (OR)/USA
Glutamate	Merck, Darmstadt/Germany
Glutamine solution	Gibco, Paisley/UK
Glycine	Sigma, St. Louis (MO)/USA
HEPES	ICN Biomedicals, Aurora (OH)/USA
IBMX	Sigma, St. Louis (MO)/USA
$K_3Fe(CN)_6$	Roth, Karlsruhe/Germany

Item	*Provider*
KCl	Sigma, St. Louis (MO)/USA
Ketamine	Sigma, St. Louis (MO)/USA
L - Ascorbic acid	Sigma, St. Louis (MO)/USA
Live/Dead Assay	Molecular Probes, Eugene (OR)/USA
Maleate	Sigma, St. Louis (MO)/USA
Matrigel	Becton dickinson, Franklin Lakes (NJ)/USA
Methanole	Roth, Karlsruhe/Germany
$MgCl_2$	Fluka, Buchs/Switzerland
MK-801 (Dizocilpine)	Sigma/RBI, St. Louis (MO)/USA
mNGF 2.5 S	Alomone Labs, Jerusalem/Israel
mNGF 7S	Alomone Labs, Jerusalem/Israel
Na_2EDTA	Sigma, St. Louis (MO)/USA
NaCl	Sigma, St. Louis (MO)/USA
NADPH	Sigma, St. Louis (MO)/USA
NaH_2PO_4	Merck, Darmstadt/Germany
NaOH	Merck, Darmstadt/Germany
NBT	Sigma, St. Louis (MO)/USA
NMDA (N-methyl-d-aspartic acid)	Sigma, St. Louis (MO)/USA
Normal horse, rabbit and goat sera	Vector, Burlingame (CA)/USA
NT-2 precursor cells	Stratagene Corp., La Jolla (CA)/USA
NT-3	Alomone Labs, Jerusalem/Israel
PBS	Institute of Virology, Vet. School Hannover
Penicilline / streptomycine solution	Gibco, Paisley/UK
Petri dishes (bacteriological culture grade)	Greiner, Hamburg/Germany
PFA	Sigma, St. Louis (MO)/USA
Pluronic acid (F-127, solution in DMSO)	Molecular Probes, Eugene (OR)/USA
Poly-d-lysine (pdl)	Sigma, St. Louis (MO)/USA
Propidium iodide (PI)	Molecular Probes, Eugene (OR)/USA
Retinoic Acid (RA)	Sigma, St. Louis (MO)/USA
Rhodamine 123 (R123)	Sigma, St. Louis (MO)/USA
Secondary biotinylated antibodies	Vector, Burlingame (CA)/USA
SNP (Sodium nitro prusside)	Sigma, St. Louis (MO)/USA
Sodium citrate	Sigma, St. Louis (MO)/USA

Item	*Provider*
Sodium dithionite ($Na_2S_2O_3$)	Merck, Darmstadt/Germany
Tetra-methyl rhodamine (TMRM)	Sigma, St. Louis (MO)/USA
TRIS	Sigma, St. Louis (MO)/USA
Triton X100	Sigma, St. Louis (MO)/USA
Trypsine EDTA solution (1X)	Gibco, Paisley/UK
Tween 20	Sigma, St. Louis (MO)/USA
Versen	Gibco, Paisley/UK

3. Results

3. 1. Neuronal differentiation in sphere cultures

Since neural stem cells have been reported to successfully proliferate on non-adhesive substrates as cell clusters termed neurospheres (Reynolds and Weiss, 1992), a similar approach was used to expand the NT-2 cell line. NT-2 precursors were seeded onto bacteriological grade petri-dishes, on which these cells do not adhere. Under these conditions the cells proliferated as clusters in the shape of free floating spheres (Figure 5) ranging between 50 and 150 µm in diameter. Neuronal differentiation required the simultaneous exposure to RA.

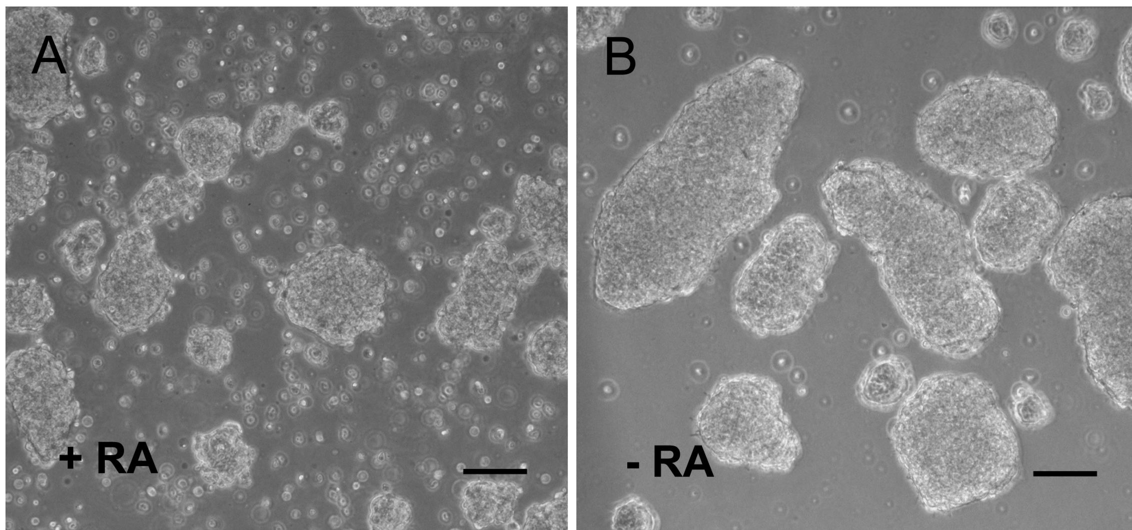


*Figure 5: Expansion of NT-2 cells in sphere culture. Initial expansion for 24h was followed by a treatment with RA for 48h (**A**) and without RA for 48h (**B**). Note the striking differences in the shape of the different aggregates. Scale bar: 100 µm.*

However, in comparison to the layer culture method (Pleasure et al., 1992), cell multiplication in the spherical clusters and concomitant RA treatment shortened the time needed for neuronal differentiation considerably. After the transfer of the spheres to culture flasks, the spherical clusters adhered to the substrate. Within a few days after the transfer, single neurons with rounded, phase-bright cell bodies emerged from the clusters, attached mainly to flattened epithelial-like cells, and began to elaborate neuritic processes (Figure 6A). The appearance of neuron like cells was facilitated if the sphere culture period was prolonged to 10 days or more.

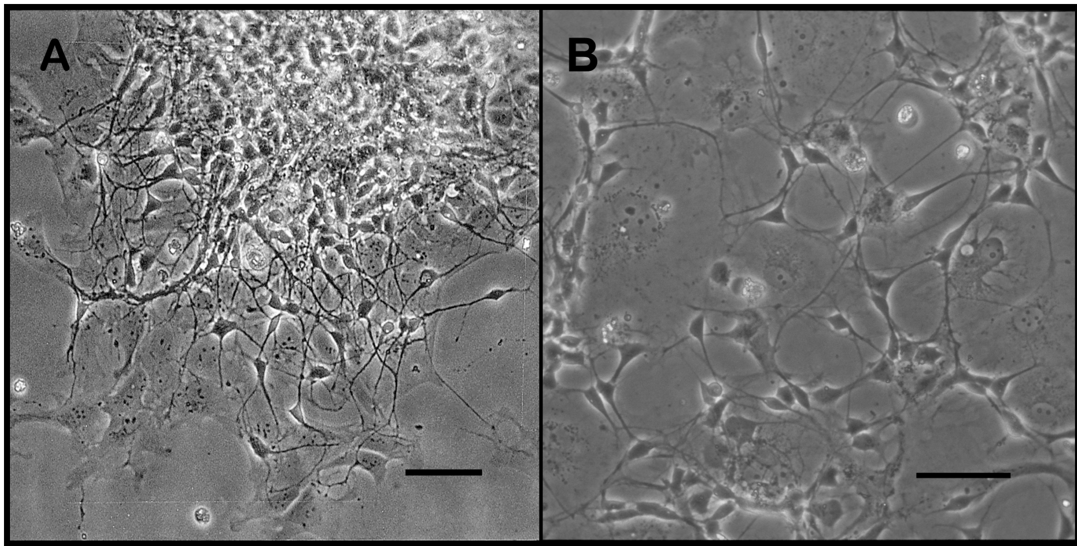


*Figure 6: NT-2 cells during differentiation. (**A**) After 10 days of aggregating treatment with RA, spherical aggregates were plated on adherent substrate and cultured for 3 more days. The surface of the substrate is mostly covered with flattened non-neuronal cells. On top of these cells grow neurons which emerge from the aggregate. (**B**) NT-2 neurons after 2nd replate and 7 days of inhibitor treatment. Scale bars: 50 µm*

Initially, neuronal cell density still proved to be rather low (approx. 0.01%). During the following three replates, neuronal number and purity increased to approximately 80-95%, depending on the selectivity of the final trypsinisation step. After the 2nd replate and 6-10 days of inhibitor treatment large numbers of cells showing neuronal morphology became clearly visible (Figure 6B).

When grown on a poly-d-lysine / Matrigel surface, the differentiated cells aggregated to form ganglion-like cell clusters. Cells within these aggregates showed neuronal morphology and formed numerous processes that connected to adjacent clusters (Figure 9A). We maintained these neurons for up to 3 months in cell culture. Using the sphere culture method, we obtained between 2.0 to 4.6 x 10^5 highly purified (>95%) NT-2 neurons out of 10^6 precursor cells. Eleven experimental trials were run, lasting for 24 days each, yielding an average number of 3.75 x 10^5 neurons per 10^6 undifferentiated precursor cells. We followed the conventional method in 8 trials, obtaining over a time course of 54 days an average yield of 1.2 x 10^6 neurons per 10^6 undifferentiated precursor cells. For a comparison of the two methods, it should be kept in mind that the longer incubation time of the conventional differentiation procedure leads to a higher cell expansion, resulting in the apparently higher yield of neurons.

In control experiments, RA was omitted during the initial sphere expansion step (Figure 5B). RA was applied later on, when the cells had been transferred to tissue culture grade flasks. The cell conglomerates that formed in the absence of RA were considerably larger than those formed

during RA treatment and far more elongated (Figure 5A + B). A following treatment over one week with RA, replating, and inhibitor treatment, did not produce any neurons.
In summary, compared to the layer culture, the sphere culture method appears to be up to 2 times faster than the original procedure (Pleasure et al., 1992) (Figure 7 and table 2).

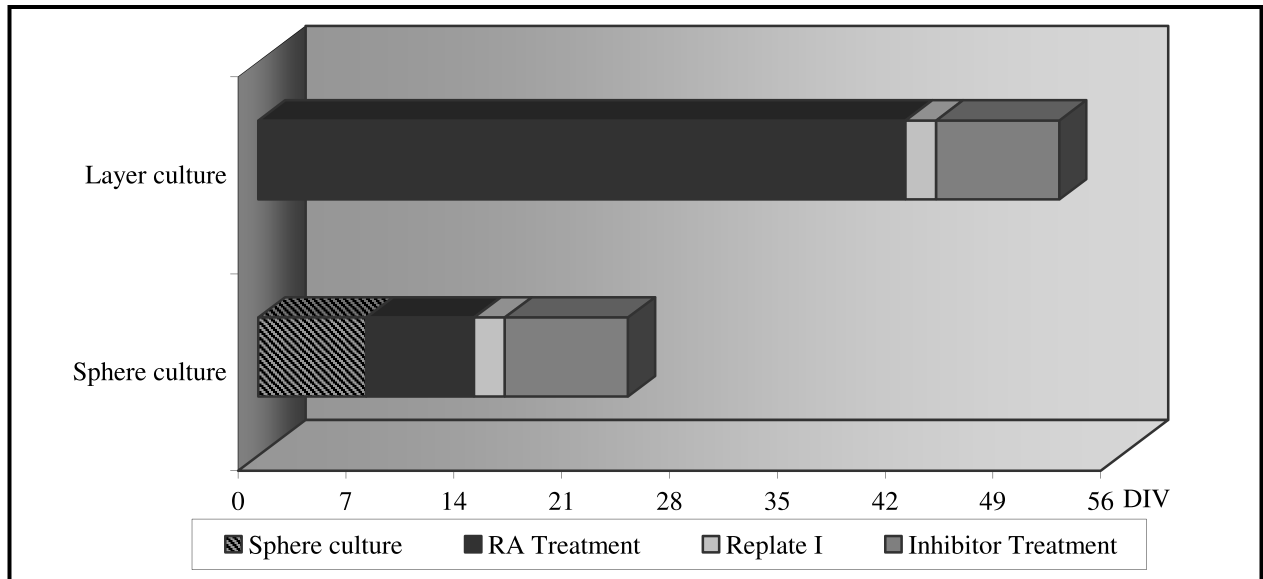


Figure 7: Time needed for neuronal differentiation in the two different differentiation protocols. The additional sphere culture step allows to obtain NT-2 neurons in half the time needed for the conventional differentiation protocol.

Layer culture		***Sphere culture method***	
-	-	7 - 8 days	Cell sphere culture with RA
35 - 42 days	RA treatment	6 - 8 days	RA treatment
2 days	Preparation of conditioned medium (Replate I)	2 days	Preparation of conditioned medium (Replate I)
7 - 10 days	Mitotic inhibitor treatment (Replate II)	7 - 10 days	Mitotic inhibitor treatment (Replate II)
Total: 44 - 54 days	Harvesting of neurons (Replate III)	**Total: 22 - 28 days**	Harvesting of neurons (Replate III)

Table 2: Comparison of the two differentiation protocols. The introduction of an additional sphere culture step at the beginning of neuronal differentiation allows to reduce the following RA treatment in adherent layer cultures from 5-6 weeks to about 1 week. The following treatment is identical to treatment in the differentiation protocol already described (Pleasure et al., 1992). The total time needed for neuronal differentiation is cut to one half in the sphere culture protocol.

3. 1. 1. Modifications of differentiation protocol using different RA concentrations

The presence of RA during culture is crucial for neuronal differentiation in a variety of embryonal carcinoma and neuroblastoma cell lines (Maden, 2001). The amount of neuronal differentiation that occurs is dependent on RA concentration (McBurney et al., 1982). It was therefore tested whether the neuronal differentiation could be increased with RA concentrations higher or lower than previously reported (Pleasure et al., 1992).

An increase of RA concentration to 50 µM during the 6 week treatment resulted in a lower cell density of 30-40 x 10^6 cells per T75 cell culture flask instead of 90-120 x 10^6 in flasks that were treated with 10 µM RA. In samples treated with 25 µM RA the reduction of cell density was not that well marked. After both treatments – 25 and 50 µM RA – no, or very few cells with neuronal morphology could be found and the corresponding experiments were stopped.

A reduction of RA concentration to 5 µM did not result in a lower cell density after RA treatment but did not yield cells with neuronal morphology.

Neither increase nor decrease in RA concentration led to an enhanced neuronal differentiation. Therefore the treatment with 10 µM RA was continued.

3. 1. 2. Modifications of differentiation protocol using BHA/DMSO

Butyl-hydroxy-anisole (BHA) has been suggested to have morphogenic capacities similar to those of RA. It has been implied in neuronal differentiation in particular (Safford et al., 2002). The modifications of both the conventional differentiation protocol and the sphere culture protocol, where BHA was applied alone or together with DMSO and/or RA did not result in an increased neuronal differentiation.

In the experiments where BHA was used at concentrations of 50 µM or above, most of the NT-2 precursor cells had died within 3 days after BHA application and the experiments were stopped at that point. This toxic effect of BHA can not be attributed to the presence of DMSO. NT-2 precursor cell cultures treated with 1% DMSO alone remained viable for at least 10 days.

Experiments where NT-2 precursors in sphere culture had been treated with 25 µM BHA/0.125% DMSO did not yield cells with neuronal morphology. Neuronal cell yield in sphere cultures that were treated with 25 µM BHA/0.125% DMSO/10 µM RA was considerably lower (approx. 50%) than in control cultures that had been treated with 10 µM RA alone.

Since the application of BHA or DMSO did not result in an enhanced neuronal differentiation these experiments were discontinued.

3. 2. Immunocytochemical and histochemical characterisation of NT-2 neuronal cells

The NT-2 neuronal cells obtained using the conventional differentiation protocol have been characterised extensively using immunocytochemical or histochemical methods (Pleasure et al., 1992; Guillemain et al., 2000). The NT-2 neurons obtained from the RA treated sphere cultures were morphologically indistinguishable from NT-2 neurons obtained with the conventional layer culture method (Figure 8A+B). They showed the typical spherical cell bodies and the extension of long neurite-like processes. Yet, whether the neurons obtained with the modified differentiation protocol showed the same expression pattern for certain neuronal markers was still unclear. Also, markers were tested for which the expression in NT-2 neurons had not yet been reported.

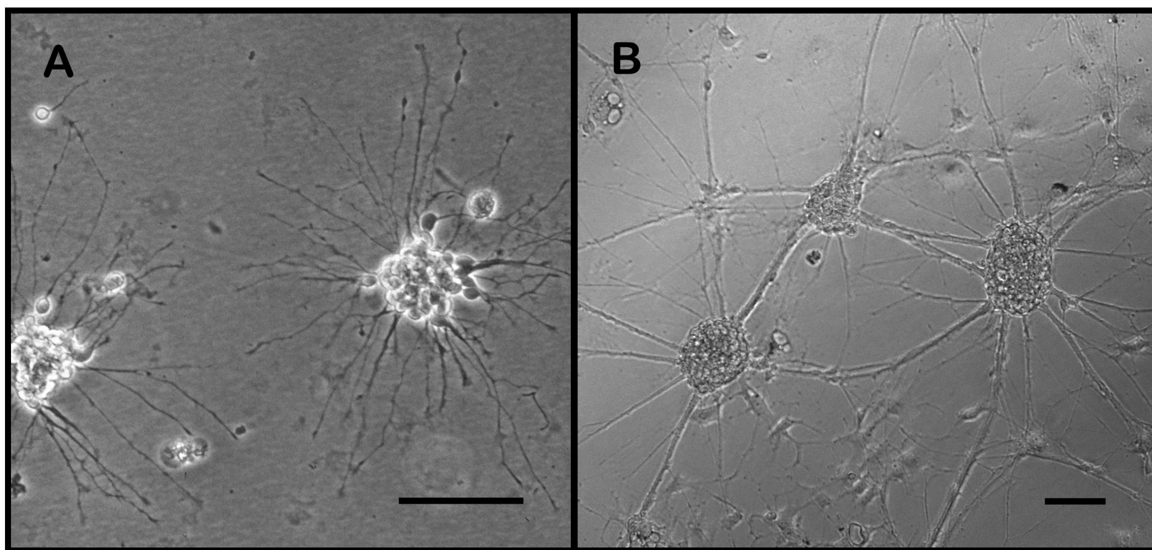


*Figure 8: Differentiated NT-2 neurons in culture. NT-2 neurons have a strong tendency to aggregate and to form clusters. This process continues during culture, leading to a complex network of interconnected ganglion-like clusters of neuronal cells. Neurons in (**A**) are in culture for 7 days, whereas cells in (**B**) have been cultured for 4 weeks. Scale bar: 100 µm*

In order to further characterise the properties of differentiated NT-2 cells, different markers for nerve cells, astrocytes, and neurotransmitters were used. Terminally differentiated NT-2 cells were immunocytochemically stained for the neuronal markers MAP2, Tau, HuC/D, for the neurotransmitters 5-HT, and GABA, for transmitter synthesising enzymes tryptophane hydroxylase, and NOS, for the astrocytic cell marker GFAP, and for prion protein (PrP). They were histochemically stained for the NADPH-diaphorase, acetylcholinesterase (AChE), and for the potential stem cell marker alkaline phosphatase (AP).

Within cultures of differentiated NT-2 cells, at least 3 different types of cell morphologies were easily distinguished. The vast majority of cells (80-95%) had a neuron-like morphology with a small, condensed cell body (approx. 10 µm in diameter) and very long processes that could reach

several hundred micrometer in length. 3-10% of the differentiated cells had a stellate morphology, some with a small cell body (10-20 µm Ø) and long processes, others with a large cell body (up to 50 µm Ø) and fewer processes. The remaining cells belonged to a group which had a very large flattened cell body (up to 100 µm Ø) and very few or no processes. The cell viability assays showed neither increase nor decrease in absolute viability values during long term culture, implicating that none of these cells were dividing (see 3.4., page 38).

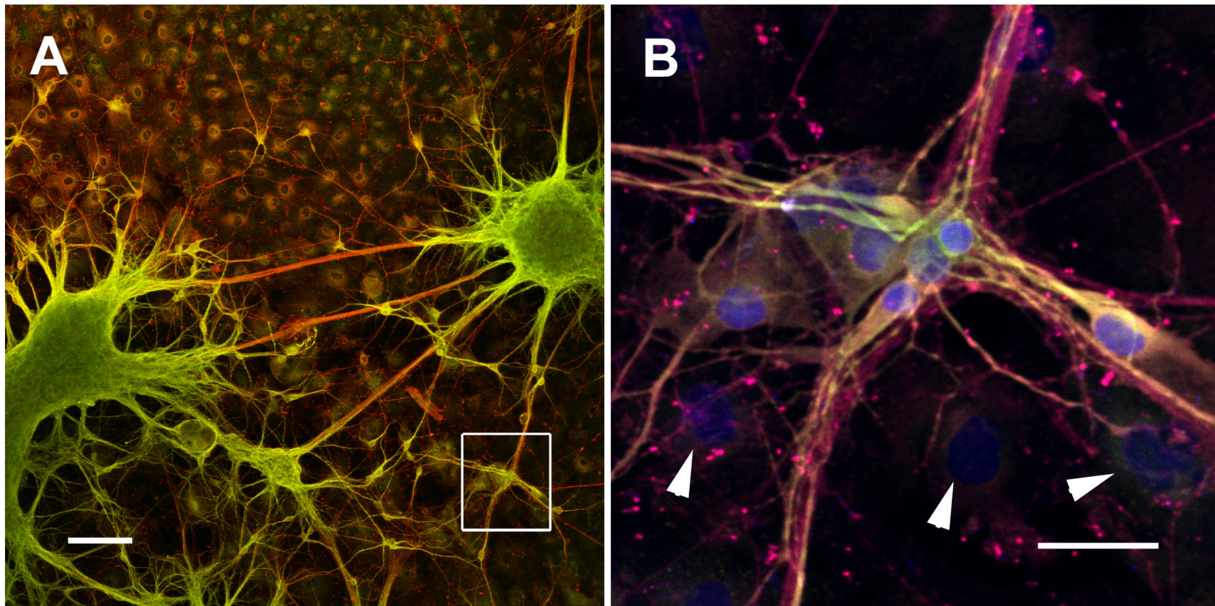


*Figure 9: Clusters of NT-2 neurons after 6 weeks in culture. (**A**) Immunofluorescent double staining for MAP2 (green) and Tau (red). Note that short neurites in the vicinity of neuronal clusters contain predominantly MAP2, whereas longer neurites which connect distant clusters contain predominantly Tau. (**B**) is a magnification of (**A**). PI was used as a nuclear counterstain (blue). Also visible: flattenend non-neuronal cells which do not stain for either MAP2 or Tau (white arrowheads). Scale bar: 100 µm (**A**) and 25 µm (**B**).*

All neuron-like differentiated cells that elaborated processes, were positive for neuronal markers. They expressed neuronal polarity markers such as the cytoskeleton associated proteins MAP2 and Tau (Figure 9). These proteins are commonly used as neuronal markers and are selectively expressed in either the axon – Tau protein – or the dendrites – MAP2. Accordingly, the antibody directed against Tau protein stained predominantly long, elongated neurites which linked distant cell clusters. The soma was not stained. MAP2 immunoreactivity was found only in short neurites close to the cell body and also in parts of the soma which were close to the cells membrane.

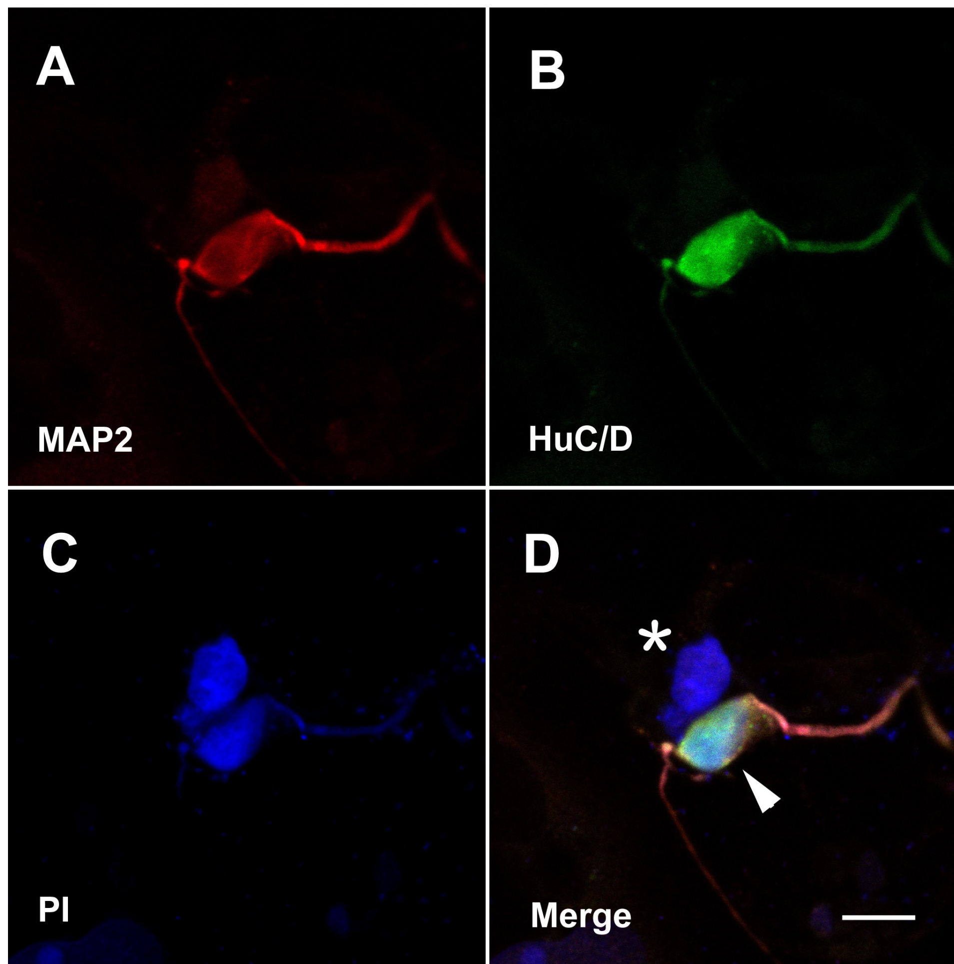


*Figure 10: Immunofluorescence of differentiated NT-2 cells after 5 days in culture. Cells have been stained for MAP2 (**A**, red) and HuC/D (**B**, green). PI (**C**, blue) was used as a nuclear counterstain. The merging of all 3 images (**D**) shows that MAP2 and HuC/D colocalise in a cell with neuronal morphology (arrowhead), whereas a second, non-neuronal cell (asterisk) does not stain with either MAP2 or HuC/D. Scale bar: 10 µm.*

Moreover, the cell bodies were imunoreactive to the ELAV-like neuronal RNA-binding proteins HuC/D, which have been implicated in mechanisms of nerve cell differentiation (Akamatsu et al., 1999; Campos et al., 1987; Wakamatsu and Weston, 1997). The HuC/D antibody stains the soma of neurons; the neurites are only very faintly stained. It was evident in confocal sections that almost all cell bodies in the clusters were HuC/D positive, confirming the successful neuronal differentiation. NT-2 cells that do not show neuronal morphology are not stained. As shown by double labelling in Figure 10, the HuC/D staining of neuronal cell bodies is colocalised with staining against MAP2, confirming that only NT-2 neurons do express HuC/D. To my knowledge, this is the first report that HuC/D is expressed in differentiated NT-2 cells.

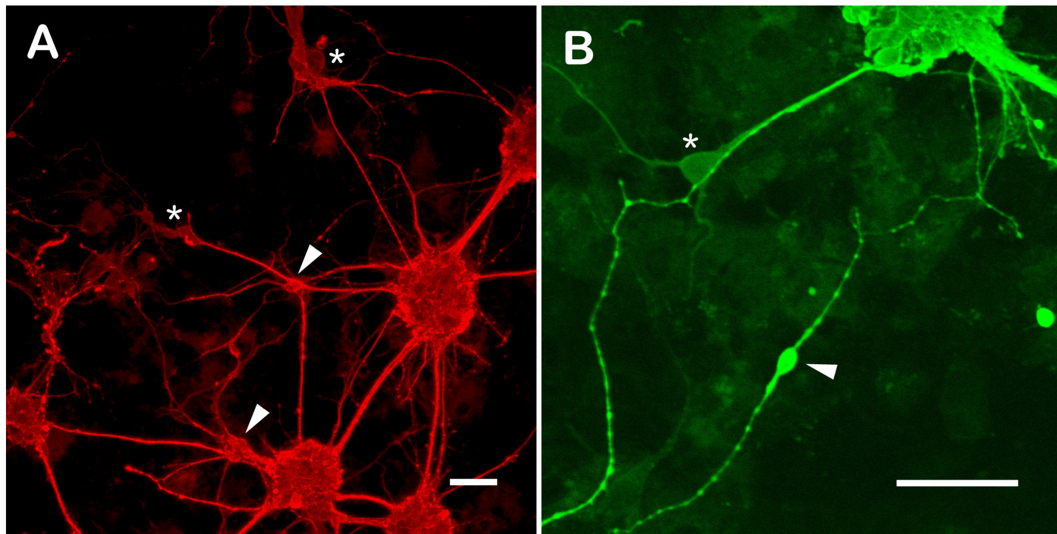


Figure 11: Immunocytochemical staining against γ-amino butyric acid (GABA). GABA immunoreactivity is present in parts of the soma and in the neurites of labelled cells. White arrowheads indicate examples of positively labelled cells, asterisks mark non-labelled cells. Scale bars: 50 µm.

γ-amino butyric acid (GABA) is the main inhibitory neurotransmitter in the CNS. After 5-6 weeks in culture, the antibody directed against GABA stained the somata and the neurites of a large number of neurons (57.5 %), other neurons and cells with glial morphology were only faintly stained, other cell types did not stain (Figure 11). The neurites of GABA positive cells exhibited a large number of regularly spaced varicosities (Figure 11B).

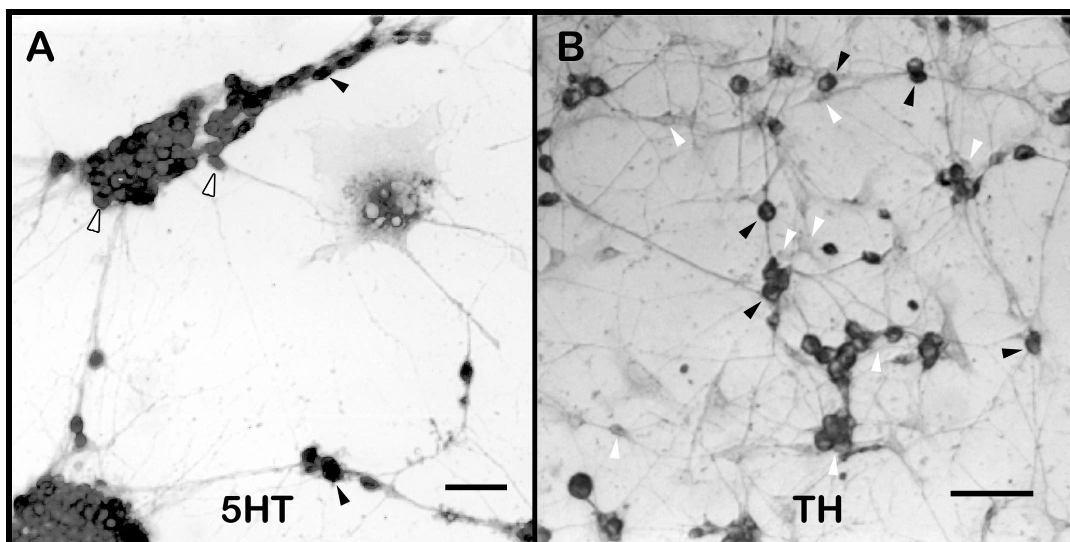


*Figure 12: Immunocytochemical staining against serotonin (5HT) in (**A**) and tryptophan hydroxylase (TH) in (**B**). Antibodies against both 5HT and TH stain the soma of neuronal cells; neurites are only very faintly stained. Examples for labelled cells are indicated by black arrowheads, non-labelled cells are indicated by white arrowheads. Scale bar: 50 µm*

The antibodies against serotonin (5-HT) and its synthesising enzyme tryptophan hydroxylase (TH) did react with some neurons (Figure 12). The antibody directed against 5-HT predominantly stained the somata of 32.5% of all cells in cultures of differentiated NT-2 cells. TH antibody stained the somata of 36% of all cells. Neurites and cells with non-neuronal morphology were only faintly stained.

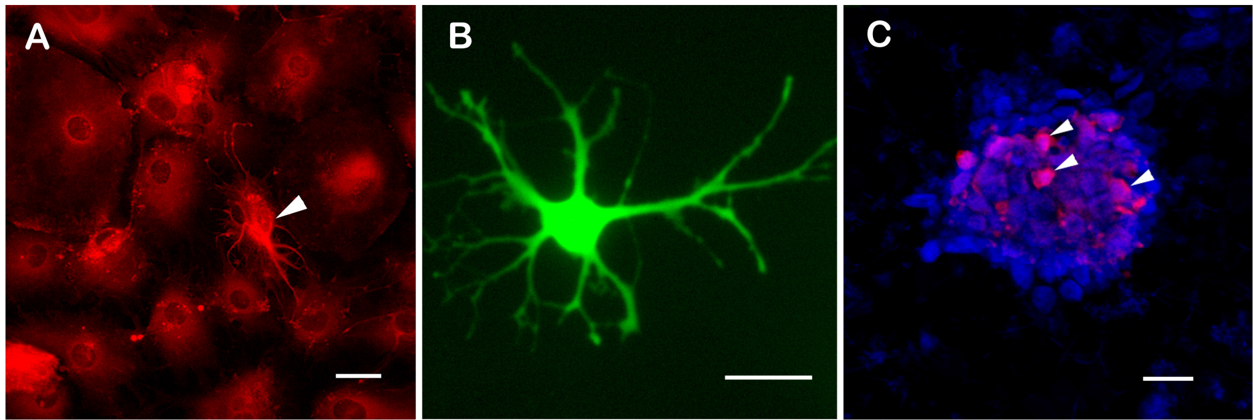


*Figure 13: Immunohistochemical staining of differentiated NT-2 cells with GFAP antibody. Non-neuronal NT-2 cells with large cell bodies are only faintly stained with GFAP antibody. Smaller cells elaborating processes (white arrowhead) are strongly stained (**A**). Many GFAP positive cells show a stellate morphology, typical for astrocytes (**B**). Within clusters of neuronal cells, a small number of cells (white arrowheads) do also react with GFAP antibody (**C**). Nuclei in (**C**) are labelled with PI (blue). Scale bar: 25 µm*

Some residual non-neural cells of epithelial morphology (5-20 % of total cell count), which have already been described by Pleasure et al. (1992), did not react with neuronal markers. In a recent publication (Sandhu et al., 2002) these cells were found to be mostly astrocytes expressing excitatory amino acid transporter proteins (EAAT 1 and 2) in a manner characteristic for primary astrocytes.

Glial fibrilary acidic protein (GFAP) is a protein, that is expressed predominantly in astrocytes of the CNS (Eng et al., 2000). The antibody against GFAP stained predominantly NT-2 cells with an irregular stellate cell body (Figure 13B) and some epithelial-like cells with a large flattened cell body and few processes (Figure 13A). It also stained a small number of cells (3.5%) within clusters of neuronal cells (Figure 13C).

NT-2 cells did not react with an antibody directed against a conserved sequence of the NO synthase enzyme, a sequence that is common to all three types of NOS (uNOS). They were also negative in the NADPH-diaphorase assay, a histochemical assay which is commonly used to identify NOS (Dawson et al., 1991).

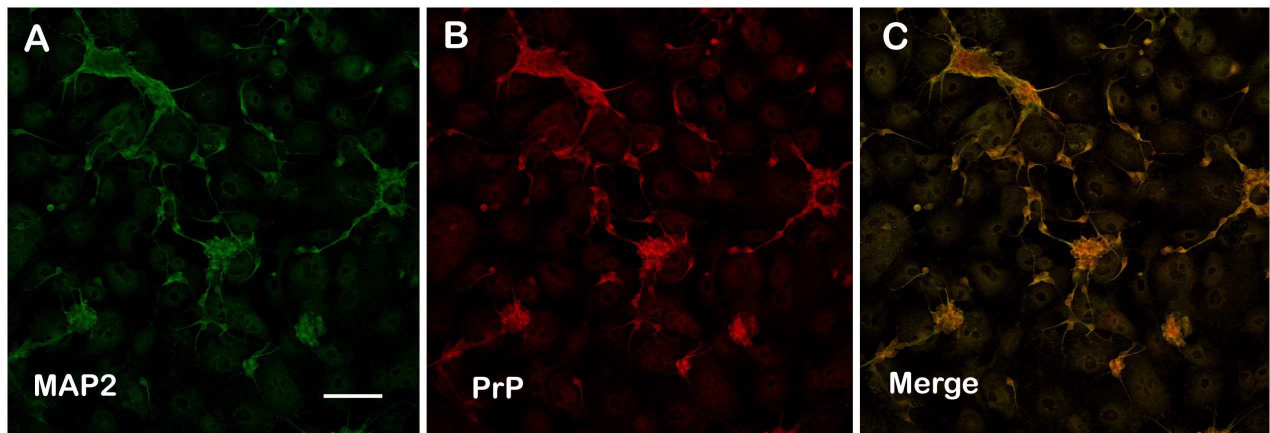


Figure 14: Immunohistochemical staining for MAP2 (green) and PrP (red). MAP2 immunoreactivity is located in the dendrites and in outlying parts of the soma. PrP immunoreactivity is found in the dendrites and in the soma. The merging of both images shows that MAP2 and PrP are colocalised in cells with neuronal morphology. Scale bar: 100 µm.

The antibody against prion protein (PrP) – a protein which is predominantly expressed in the soma and neurites of neurons (Lainé et al., 2001) – stained the soma and the neurites of NT-2 neurons (Figure 14B). Anti-PrP staining was colocalised with MAP2 staining, other cell types did not stain (Figure 14C). This finding confirms that also in differentiated NT-2 cells PrP is mainly expressed in neurons.

The assay for alkaline phosphatase (AP), an enzyme which has been suggested as a marker for embryonic stem cells (Talbot et al., 1993), reveals a small number of positive cells, usually within clusters of neuronal cells. Since these cells were located inside neuronal clusters, it has so far not been possible to establish whether AP positive cells showed neuronal morphology or co expression of neuronal markers.

The acetylcholinesterase assay seemed to react with the matrigel coating, leading to a high background staining. It was therefore not possible to distinguish clearly between labelled cells and the background with this assay. Guillemain et al. (2000) found that up to 49 % of NT-2 neurons are cholinergic as assessed by cholinacetyltransferase (ChAT) immunoreactivity.

The following table summarises the results of immuno- and histochemistry.

Marker	*Cell types*		
	Neuronal morphology	*Stellate morphology*	*Other*
Hu C/D	100 %	-	-
MAP2	100 %	-	-
Tau	100 %	-	-
GFAP	3.5 %	50-60 %	10 %
GABA	57.5 %	-	-
5-HT	32.5%	-	-
TH	36 %	-	-
NOS	-	-	-
NADPH - diaphorase	-	-	-
PrP	100 %	-	-
Alkaline phospatase	<1%	-	-
Acetylcholinesterase	?	?	?

Table 3: Labelling of differentiated NT-2 cells with specific antibodies or histochemical assays. Neurons are cells with a neuron-like morphology, astrocytes show a stellate morphology. (-) indicates no labelling. The question mark in the acetylcholinesterase row indicates labelling of background.

The immunocytochemical and histochemical characterisation of differentiated NT-2 cells confirms their neuronal character. Neuronal cells obtained from either the conventional or the sphere culture differentiation protocols showed no differences in their properties.

3. 3. Treatment of NT-2 neurons with neurotrophic factors

There are indications that the differentiation of neuronal precursor cells is guided by the presence of certain neurotrophins. In neuronal stem cells obtained from rat hippocampus, sequential application of RA and BDNF or NT-3, were able to induce GABAergic, cholinergic and dopaminergic neurotransmitter phenotypes (Takahashi et al., 1998). In NT-2 cells in particular it was shown that ciliary derived neurotrophic factor (CNTF) and leukaemia inhibitory factor (LIF) drive neuronal differentiation towards a cholinergic neurotransmitter phenotype (Zeller and Strauss, 1995). BDNF and NT-3/4 were shown to influence the dendritic complexity of differentiated NT-2 neurons (Piontek et al., 1999). In two experiments the response of NT-2 neurons to the presence of neurotrophins was studied. The parameters checked were the presence of NO synthesising enzymes using the diaphorase stain and an antibody directed against uNOS,

the presence of the neuronal marker HuC/D, the glial marker GFAP, the serotonin synthesising enzyme tryptophan hydroxylase, and the neurotransmitters serotonin (5-HT) and GABA.
When compared with the internal negative control cultures that were not exposed to neurotrophic factors, no significant differences were found (data not shown). Both, the assay for diaphorase and the staining for uNOS were negative as stated before (see 3. 2.). The antibody directed against HuC/D labelled all cells with neuronal morphology.

3. 4. Reaction of NT-2 neurons to anoxia

To characterise insult parameters causing cell death in the NT-2 neuronal cultures, I determined the viability using the Alamar blue assay. The Alamar Blue assay exhibits very low toxicity in general and I could not find any toxic reactions in NT-2 cells at the concentrations used. When compared to other assay systems (such as MTT or LDH assays) this assay has the advantage that it allows repeated measurements of the same cell culture giving additional information of its development over time. I followed the viabilities of neuronal cultures during the weeks before an anoxia experiment to find out whether there were any changes in viability that were unrelated to anoxia.

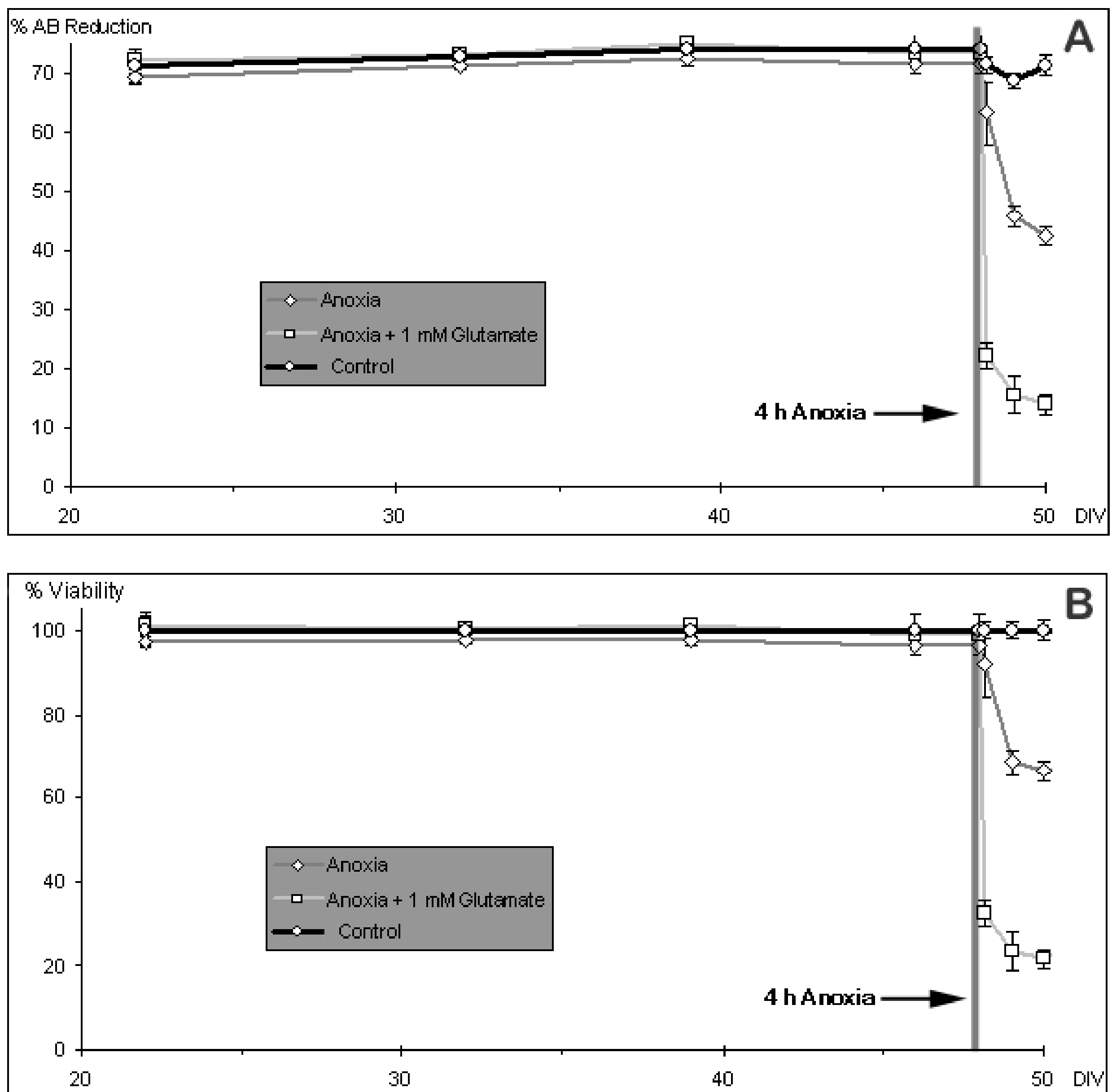


*Figure 15: Viability of NT-2 neurons in long term culture. NT-2 neurons were cultured for 48 days in vitro (DIV) before being subjected to 4h of anoxia. Viabilities of all sample groups remained stable until the onset of anoxia. Anoxia was performed using a gas mixture of 95% Ar / 5% CO_2 and simultaneous application of 1mM Na-dithionite. Within 48h after anoxia the viabilities of the samples that had been exposed to anoxia or anoxia and 1 mM glutamate were drastically decreased. (**A**) shows the measured values of Alamar Blue reduction after 4h incubation with the staining solution. (**B**) shows the relative values in comparison to the live control. Data taken from one typical experiment, mean values for n=4 wells for each measurement, error bars indicate SEM.*

The weekly measurements did not reveal any significant increase nor decrease in the viability of NT-2 neuronal cells during long-term culture. Moreover, the relative viabilities between cultures in the control and in the sample group did not change (Figure 15). To assess the outcome of an experiment, and to be able to compare different experiments with each other, the absolute values

of Alamar Blue® reduction were compared with the internal live control. The internal control was set as 100%. In later experiments, in order to compensate for the differences that occurred between different wells, the values obtained from the last measurement before anoxia were set as 100% for each individual well in all sample groups. These relative values were then compared to the internal live control.

In NT-2 neuronal cultures the induction of anoxia leads to neuronal damage and cell death. The main loss in neuronal cells occurs during anoxia or in the first hours after anoxia. Only a small amount of neuronal cell death occurs between 24h and 48h post anoxia. 48h after anoxia the viabilities of the surviving cells have reached a stable level and remain mostly unchanged afterwards.

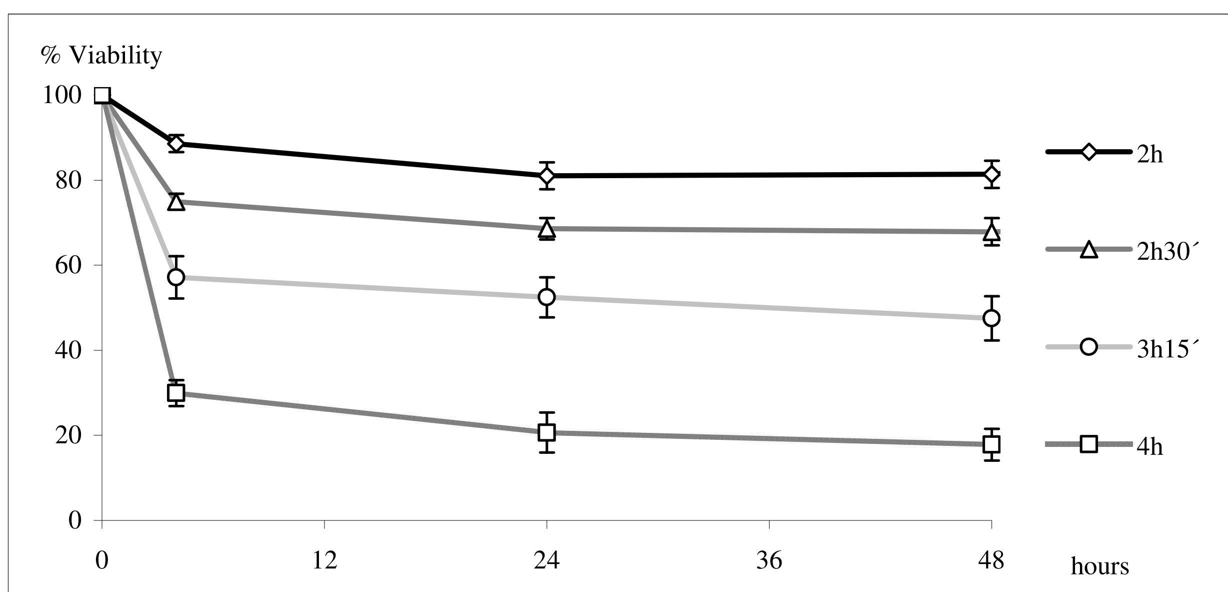


Figure 16: Development of viability of NT-2 neuronal cultures during the 48h after anoxia. Cells were cultured for 32 days before the experiment, and then subjected to anoxia during 2h 00´, 2h 30´, 3h 15´ and 4 hours with simultaneous exposure to 1mM glutamate. Data collected from 4 different experiments, mean values for n=8 samples, error bars indicate SEM.

The duration of anoxia largely influences the survival of neuronal cells (Figure 16). Depending on the exact culture conditions, anoxia times shorter than 2 hours did little or no damage to the NT-2 neurons. After 2 hours of anoxia the neuronal cultures lost approximately 20% of their viabilities when compared to live controls. An increase of anoxia duration resulted in a marked decrease of neuronal viability. After 4 hours of anoxia the viability of the cell cultures was reduced by about 80%. As assessed by immunocytochemical methods, almost none (< 1%) of the surviving cells showed neuronal morphology or stained for neuronal markers. A large number of the surviving cells (50-70%) stained positive for GFAP.

Control experiments with undifferentiated NT-2 precursor cells did not reveal vulnerability to 4h of anoxia.

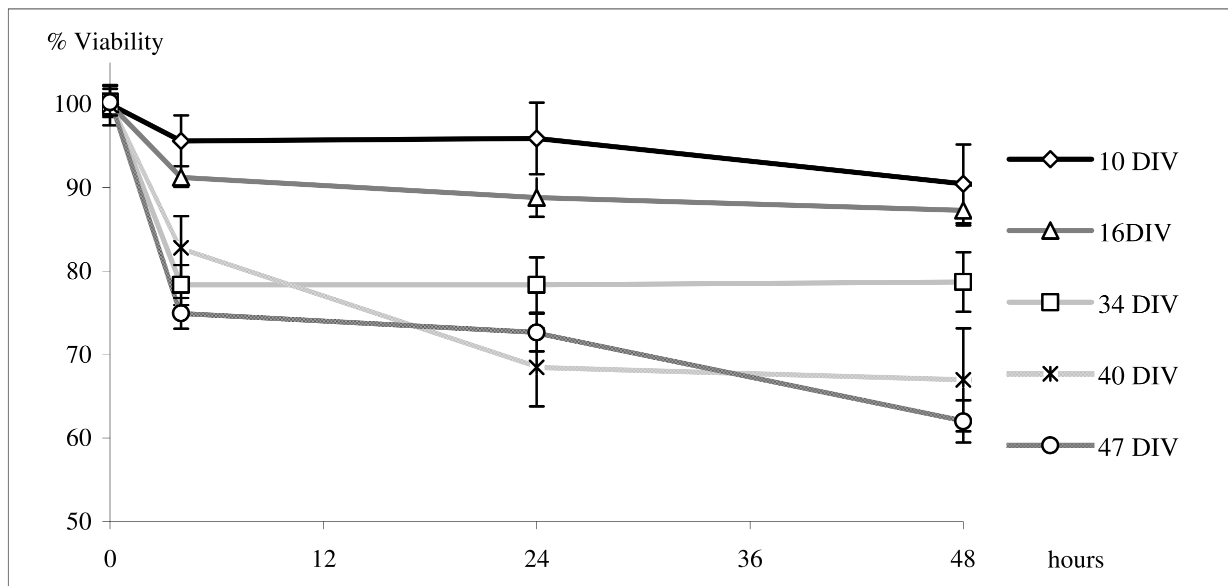


Figure 17: Influence of culture age on vulnerability to anoxic insult. Development of viability of NT-2 neuronal cultures during the 48h after exposure to 2h of anoxia. Cells were cultured for differing number of days in vitro (DIV) before being subjected to anoxia and 1 mM glutamate. Data collected from 5 different experiments, mean values for n=8, error bars indicate SEM.

Neuronal survival after anoxia is strongly influenced by the time NT-2 neuronal cells were cultured before an anoxia (Figure 17). Relatively "young" neuronal cells that were cultured for 10 or less days *in vitro* (DIV) after differentiation, did survive an anoxia of 2 hours mostly unharmed loosing only 5-10% of their original viability. Under the same experimental conditions "older" cell cultures with more than 40 DIV after differentiation responded with a significant decrease in viability loosing approximately 30-35% of their original viabilities. The longer NT-2N cells are cultured, the more vulnerable they are to anoxia.

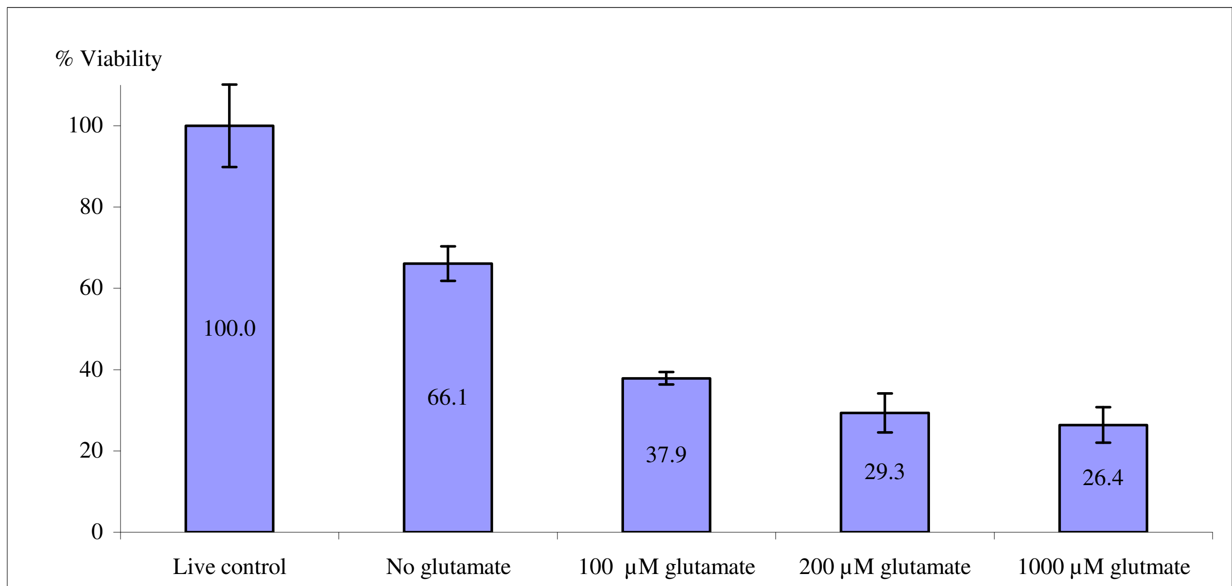


Figure 18: Effect of glutamate on viability of NT-2 neuronal cultures 48h after 4h of anoxia. Cells were cultured for 42 days before being subjected to anoxia. Data collected from one typical experiment, mean values for n=6, Error bars indicate SEM.

Ischaemia in brain tissue is accompanied by an increase in extracellular glutamate which mediates mechanisms of excitotoxic cell death (Gass, 1997). Similarly, anoxic injury in our cell culture system increased with the glutamate concentration in the bath solution (Figure 18). Without glutamate neuronal NT-2 cultures that have been cultured for 42 days, lose about one third of their viability after 4h of anoxia. Under the same experimental conditions the addition of glutamate further decreases their viability in a concentration dependent manner.

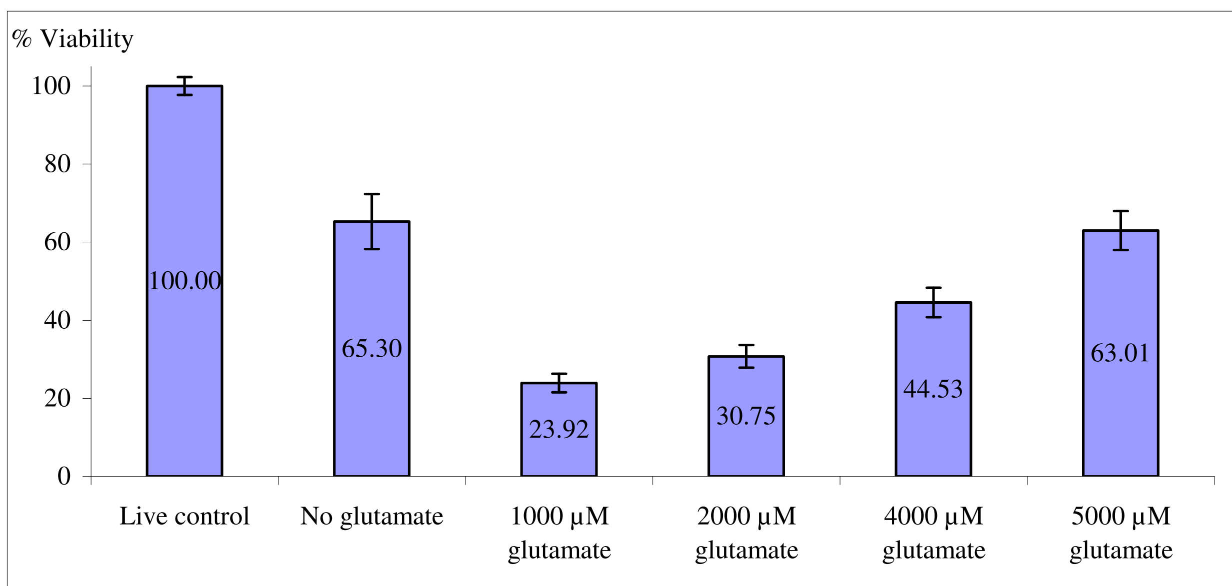


Figure 19: Effect of high concentrations of glutamate on viability of NT-2 neuronal cultures 48h after 4h of anoxia. Cells were cultured for 48 days before being subjected to anoxia. Data from one typical experiment, mean values for n=6, Error bars indicate SEM.

In a different experiment however, very high glutamate concentrations (>3 mM) surprisingly seem to have a protective effect (Figure 19). This effect on viability is unrelated to the pH of the cell culture medium, which was shown to be in the range of 7.2-7.4.

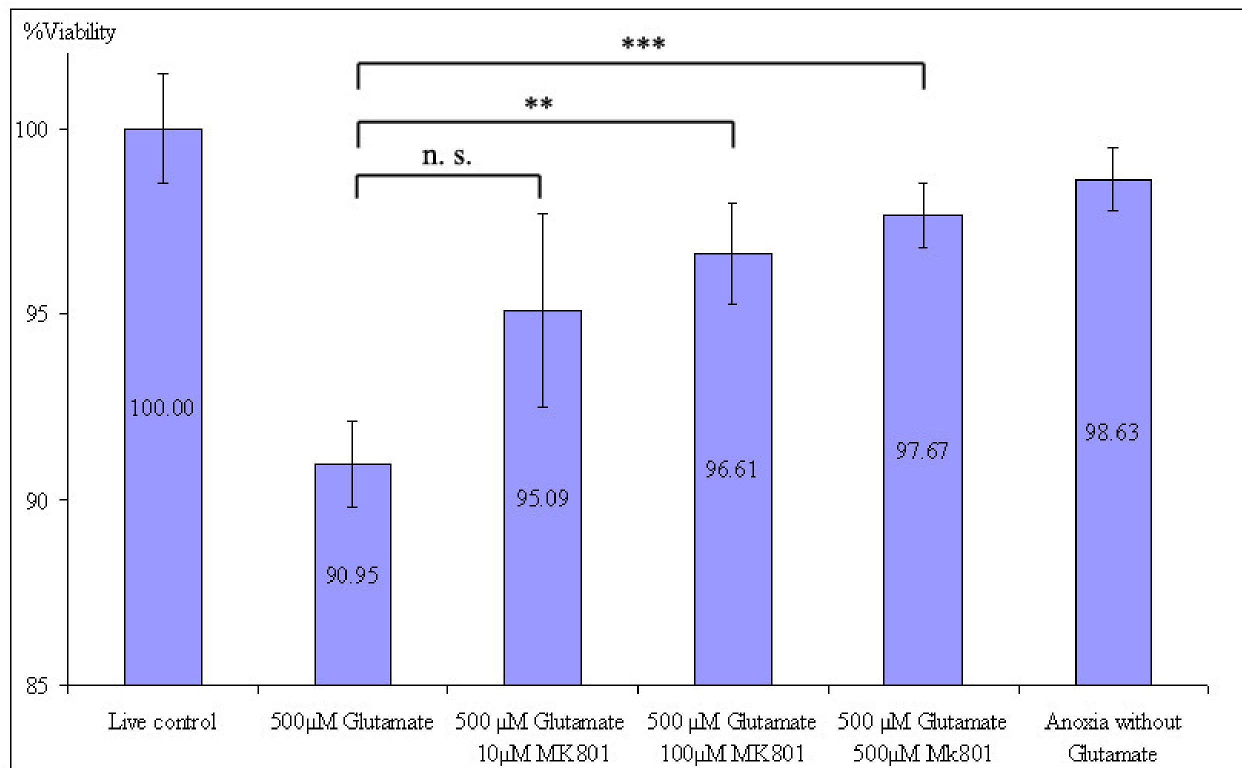


*Figure 20: Effect of NMDA receptor blockade of NT-2 neuronal cultures on viability after 2h of anoxia. Cells were exposed to 500 µM glutamate alone or together with different concentrations of the NMDA channel blocker MK-801. Addition of 500 µM MK-801 protects the neurons from the damage induced by 500 µM glutamate. This effect is significant (** = p<0.005) for 100 µM MK-801 and highly significant (*** = p<0.001) for 500 µM MK-801. 10 µM MK-801 has no significant (n. s.) effect on neuronal viability. Cells were cultured for 16 days before being subjected to anoxia. Data from one typical experiment, mean values for n=10, error bars indicate SEM.*

MK-801, a commonly used NMDA channel blocker, protected NT-2 neurons against relatively mild (1h duration) anoxia. In anoxia lasting 2 hours or more, MK-801 did protect against glutamate induced damage, but it could not restore viabilities to the level of live controls (Figure 20). A statistical comparison of the samples using Student´s t-test showed that low concentrations of 10 µM MK-801 had no significant effect on neuronal viability. 100 µM MK-801 protected the cells significantly ($p<0.005$, n=10). A statistically highly significant level of protection ($p<0.001$, n=10) was achieved with a MK-801 concentration of 500 µM.

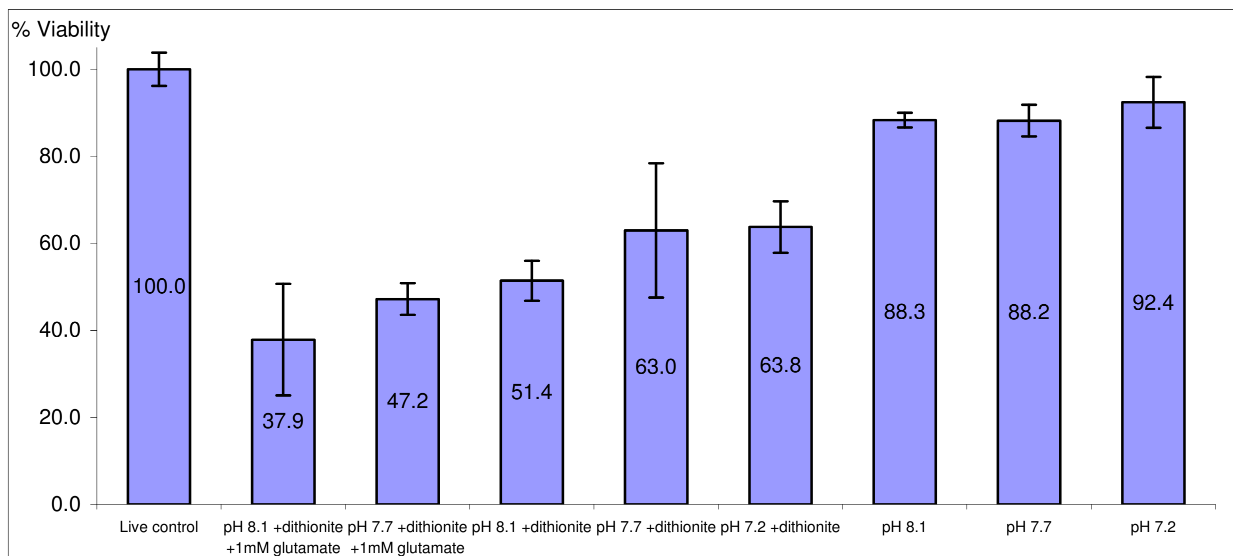


Figure 21: pH dependency of neuronal viability after 3h of anoxia. Samples were exposed to pH values of 7.2, 7.7 and 8.1, with or without sodium 2 mM dithionite, with or without 1 mM glutamate. Data from one experiment only, DIV=35, n=2-4 for samples, n=6 for live control, error bars indicate SEM.

A number of publications suggest that neuronal damage during ischaemia is attenuated by low pH values and exacerbated by high values (Tang et al., 1990; Raley-Sussman and Barnes, 1998; Almaas et al., 2003). To assess the pH dependency of viability during anoxia in NT-2 neurons one experiment was conducted where NT-2 neurons were subjected to 3h of anoxia and the pH of the buffer used set to 7.2, 7.7 and 8.1. Because of a contamination during cell culture, the sample number for individual sample groups was relatively low. Therefore the differences measured between sample groups are not significant and should be regarded as an indication only. Without the use of sodium dithionite – without complete anoxia – neuronal damage was limited to approximately 10 % and there were no significant differences between samples that had been treated with different levels of pH. The use of 2 mM sodium - dithionite during anoxia reduced the viabilities in all samples. Neuronal damage was lower in samples with a pH close to neutral and higher in samples with a basic pH. The addition of 1mM glutamate reduced viabilities even further, with the pH 8.1 samples showing the strongest loss in viability (Figure 21).

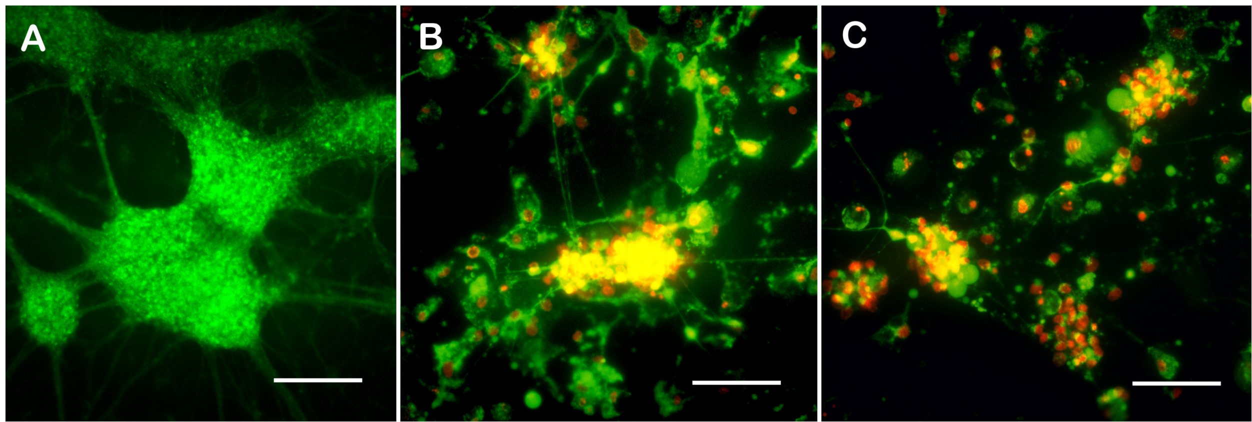


*Figure 22: Live Dead viability assay of NT-2 neurons after anoxia. Living cells are stained in green, whereas dead nuclei are stained red. The internal live control is unaffected at 22h post anoxia (**A**). In samples that were subjected to 3.30h of anoxia a large number of cells have died at 22h after anoxia (**B**). The viability is further reduced at 46h after anoxia (**C**). Scale bar: 100 µm*

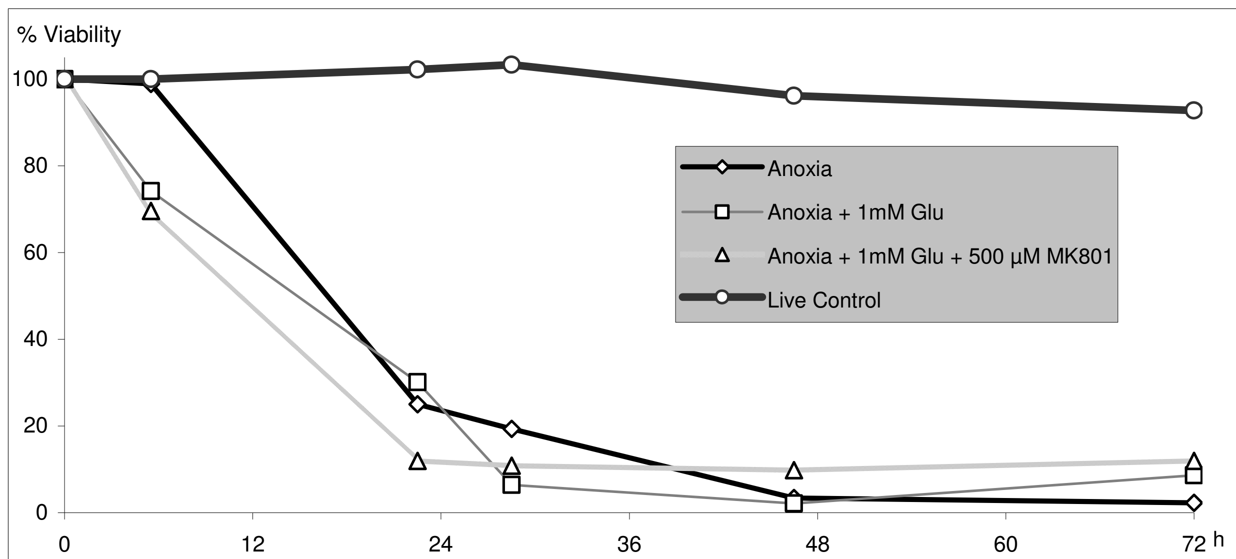


Figure 23: Development of viability in the 72h after anoxia measured with the Live/Dead assay. Cells were exposed to 3h 30´ of anoxia, anoxia with 1mM glutamate (glu), anoxia with 1 mM glutamate and 500 µM MK-801. The last viability count before anoxia was set as 100%, all values are normalized to this reference value. Cells were cultured for 18 days prior to experiment. Data obtained from one typical experiment.

In general, the results obtained from the microscopic evaluation of the Live/Dead® assay (Molecular Probes) after anoxia correlated well with results obtained from the Alamar Blue® assay. Living cells showed intensive green fluorescence, dead cells were stained in red (Figure 22). The anoxia had no effect on the live control. The Live/Dead assay´s toxicity to NT-2N cells is higher than the toxicity of the Alamar Blue assay, but still relatively low. The live control lost approximately 8.5% of their original viability during the 3 days of study (Figure 23). As in the

Alamar Blue assay, the viabilities of the samples that had been subjected to 3h 30´ anoxia and glutamate experienced a sharp drop to about 20% of live controls within the first 24 h post anoxia. Then they dropped to approx. 5-10% in the next 2 days.

The apparent loss of neuronal cells was almost complete in samples subjected to anoxia without glutamate. As the neuronal damage was already at its maximum, the addition of glutamate could not increase neuronal cell death any further and correspondingly there was virtually no protection from MK-801.

3. 5. Measurement of calcium fluorescence in differentiated NT-2 cells

After loading with the indicator dye Calcium Green 1®, NT-2 neurons were constantly perfused with Krebs HEPES buffer and challenged by the addition of solutions containing glutamate receptor ligands. The imaging experiments showed that NT-2-N cells respond to stimulation with glutamate or NMDA with a sharp increase of the cytosolic calcium concentration. After stimulation the fluorescence fell back to baseline level (Figure 24).

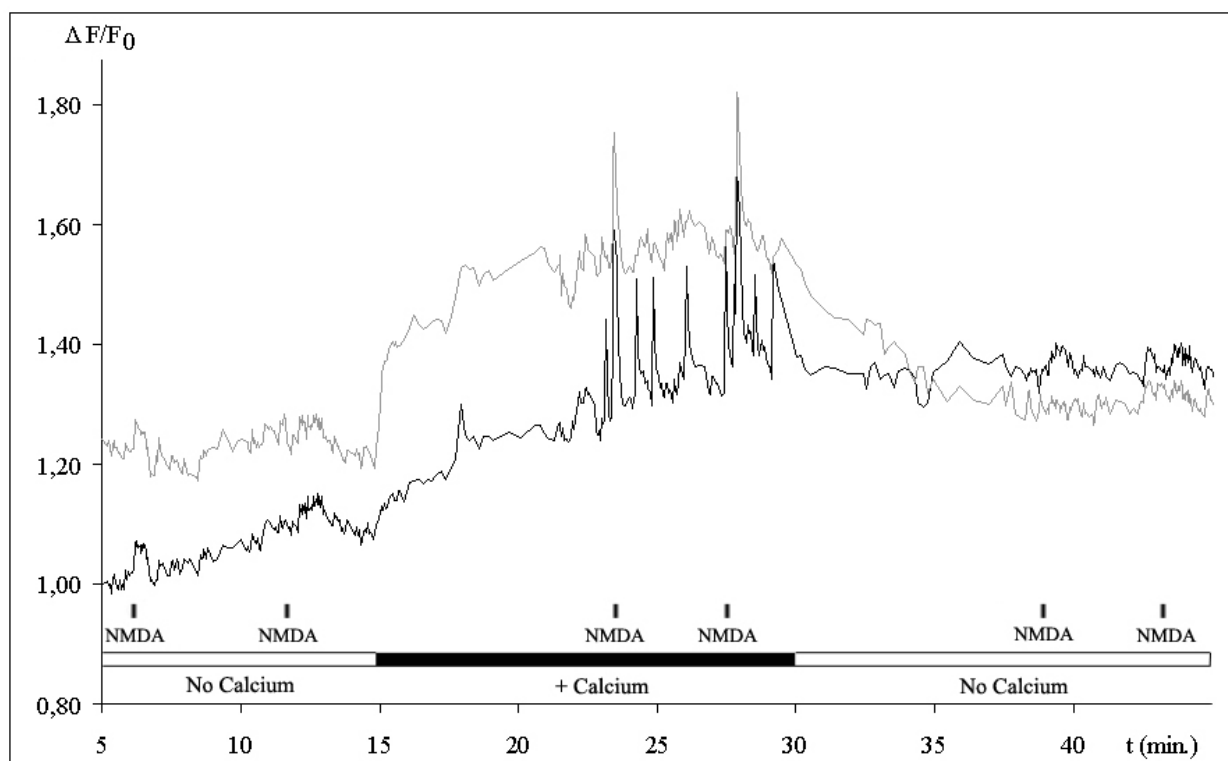


Figure 24: Calcium imaging of NT-2 neurons with the fluorescent dye Calcium Green 1®. NT-2 neurons were first perfused with calcium-free buffer, then with calcium containing buffer, then again with calcium-free buffer. The cells were challenged 6 times with 3s pulses of 50 µM NMDA and 50 µM glycine. The dark curve represents the relative fluorescence intensity of one single neuron showing calcium transients independent of NMDA stimulation. The light curve represents the mean fluorescence intensities of 3 neurons not showing transient fluctuations. In calcium containing medium, exposure to NMDA/glycine evoked a sharp increase in calcium dependent fluorescence, whereas in calcium-free medium such a reaction was not observed. Note the increase in calcium dependent fluorescence after addition of calcium and its decrease after withdrawal of calcium. Data collected from one typical experiment.

I also observed transient fluctuations in intracellular calcium concentration already described by Gao et al. (1998). Both transient fluctuation and glutamate/NMDA stimulation were abolished in calcium free buffer, confirming that the observed fluctuations were due to an influx of extracellular calcium into the cells (Figure 24). In separate experiments, the transient fluctuation and glutamate/NMDA induced response were also abolished when the pH of the Krebs HEPES buffer used during stimulation was lowered to 5.9. This is in line with studies from Tang et al. (1990) who found that an increased external proton [H^+] concentration suppressed NMDA activated currents.

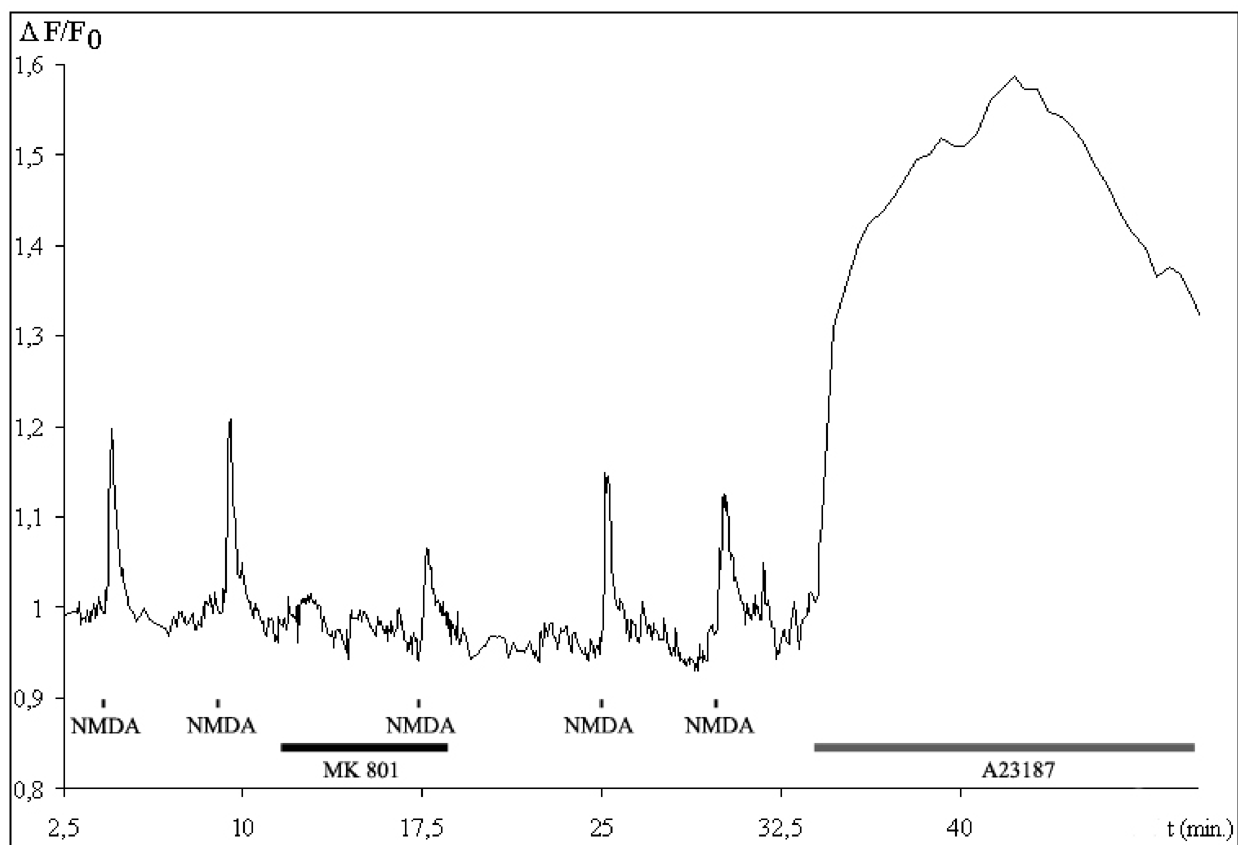


Figure 25: Effect of the NMDA receptor antagonist MK-801 on NMDA evoked calcium influx. NT-2 neurons were constantly perfused with Krebe HEPES buffer and exposed to 3s pulses of 75 µM NMDA and 75 µM glycine. Perfusion with a buffer containing 200 µM MK-801 reduced the NMDA dependent maximum peak height. Finally, 5 µM Calcimycine (A23187) was added to the buffer. The curve illustrates the behaviour of one typical neuron.

In order to test the effect of NMDA receptor antagonists on the NMDA evoked calcium influx, these antagonists were added to the perfusion medium. The application of 200 µM of the NMDA receptor antagonist MK-801 leads to a significant reduction of calcium dependent fluorescence, confirming that most of the calcium influx into the cells is mediated by NMDA receptors.

The application of the calcium ionophore Calcimycine (A23187) completely permeabilises the cell membranes for external calcium and leads to drastically increased calcium dependent fluorescence. The fluorescence values, then reached, indicate the maximum calcium signal in a cell and were taken as a reference (Figure 25). Different inhibitors of NMDA responsive glutamate channels were used to block NMDA receptor mediated calcium influx and their effectiveness was compared. The perfusion with these NMDA channel blockers itself already caused small Ca^{2+} peaks. In order to separate this Ca^{2+} influx not caused by NMDA stimulation from the NMDA response, the NMDA channel inhibitors were added to the perfusion medium 5 minutes before the cells were stimulated with NMDA or glutamate. Depending on the inhibitor concentration, the response to the stimulation was significantly reduced by the inhibitors used. It

was however, not completely abolished (Figure 25).

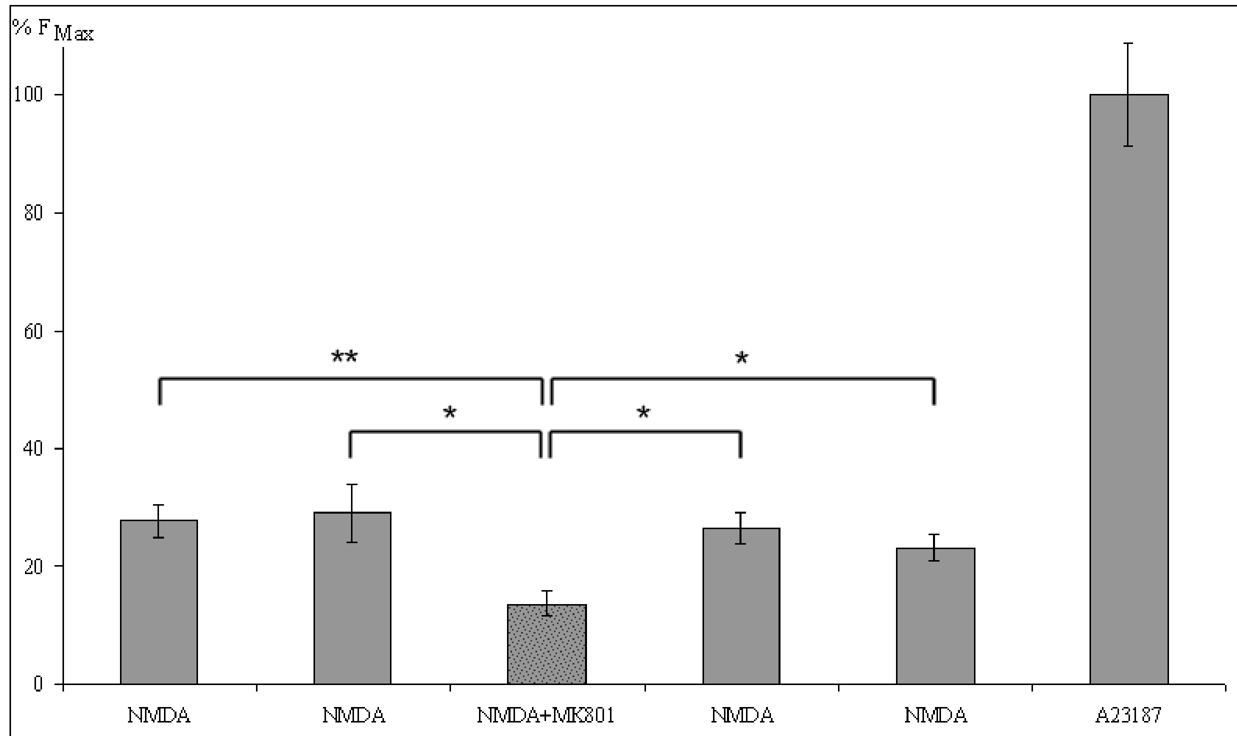


*Figure 26: Maximum peak heights of calcium dependent fluorescence in relation to baseline fluorescence (0%) and maximum fluorescence after Calcimycine (A23187) application (100%). Application of 200 µM MK-801 to the perfusion medium prior and during stimulation with 75 µM NMDA/glycine reduces NMDA induced response by approx. 50%. Example of one typical experiment, values are mean values with n=9 ROI defined around the cell bodies of 9 cells, error bars indicate SEM, * = $p<0.01$, ** = $p<0.005$.*

The baseline fluorescence was set as 0%, the maximum peak height after Calcimycine application was set to 100%. This normalisation was applied to the experiment depicted in Figure 25 leading to the simplified representation in Figure 26. In order to facilitate the comparison of different experiments, the same normalisation was also applied in the following graphs.

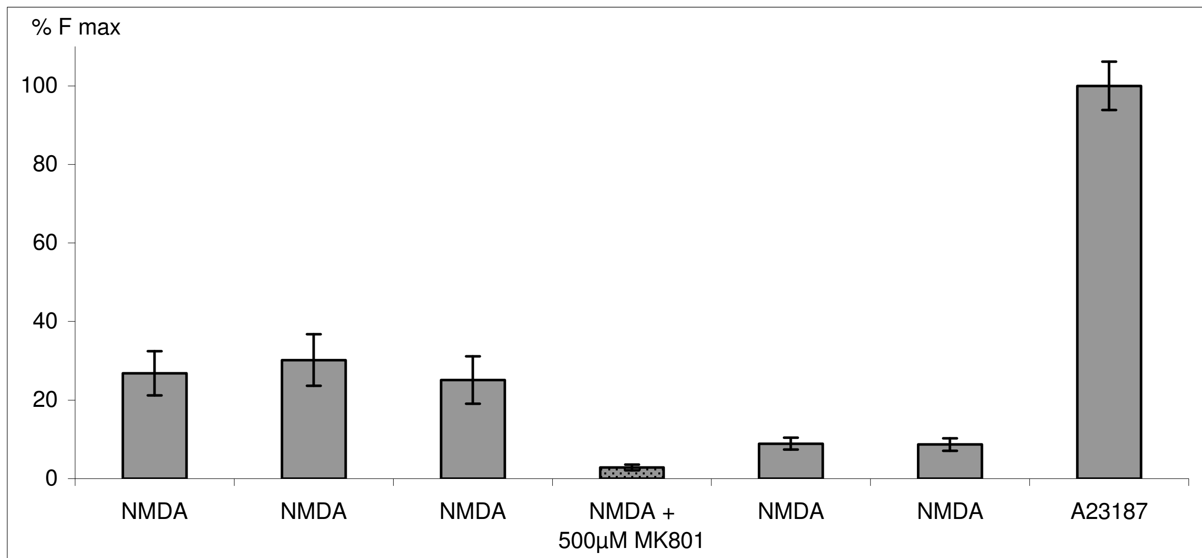


Figure 27: MK-801 reduces calcium influx after NMDA stimulation. The application of 500 µM MK-801 to the perfusion medium reduces response induced by 75 µM NMDA/glycine to approx. 10%. The following stimulations after MK-801 application did not reach their original levels. Example of one typical experiment, n=19 cells, error bars indicate SEM.

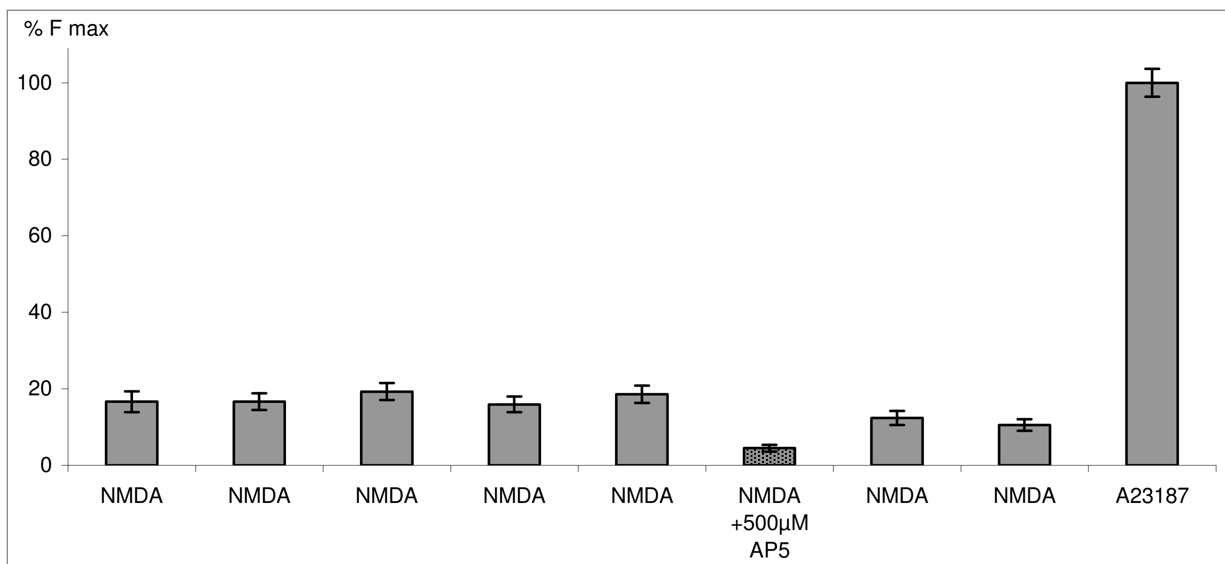


Figure 28: AP5 reduces calcium influx after NMDA stimulation. The application of 500 µM AP5 to the perfusion medium reduces response induced by 50 µM NMDA/glycine to approx. 25%. Example of one typical experiment, n=29 cells, error bars indicate SEM.

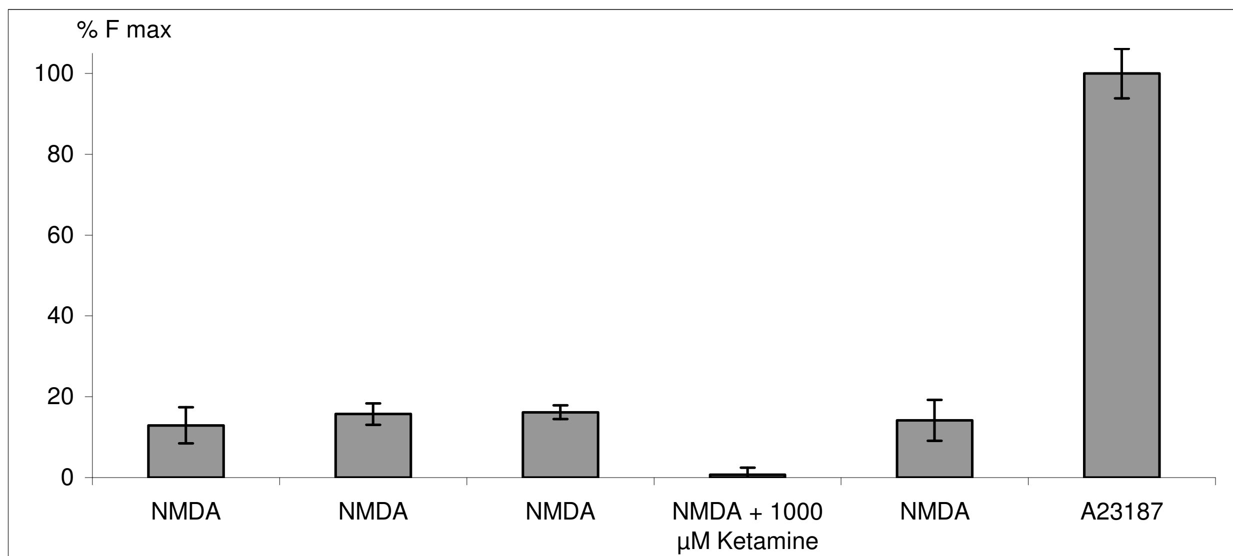


Figure 29: Ketamine reduces calcium influx after NMDA stimulation. The application of 1000 µM Ketamine to the perfusion medium reduces response induced by 75 µM NMDA/glycine to approx. 5%. Example of one typical experiment, n=8 cells, error bars indicate SEM.

The competitive inhibitors AP5 and Ketamine blocked Ca^{2+} ion influx only at high concentrations of 500 or 1000 µM, respectively. These inhibitors were readily washed out by a short period (5 min.) of perfusion with saline (Figure 28 + *Figure 29*29). Stimulation with NMDA after inhibition was as effective as before. The non-competitive NMDA receptor antagonist MK-801 inhibited calcium influx at lower concentrations. 50 µM MK-801 had little effect on calcium influx. 200 µM MK-801 reduced NMDA mediated calcium influx to about 50% of the control level (Figure 26). 500 µM MK-801 reduced this influx to 10 % of control level. After application of 500 µM MK-801, even longer perfusion times of up to 10 min. could not restore NMDA response to the levels before inhibition (Figure 27).

In order to facilitate the comparison of the inhibitory effect of the different NMDA receptor antagonists, the maximum peak height of the first NMDA/glycine stimulation was set as 100%. This was compared to the maximum peak height of later stimulations that were done in the presence of NMDA receptor antagonists. Figure 30 summarises the results of these experiments.

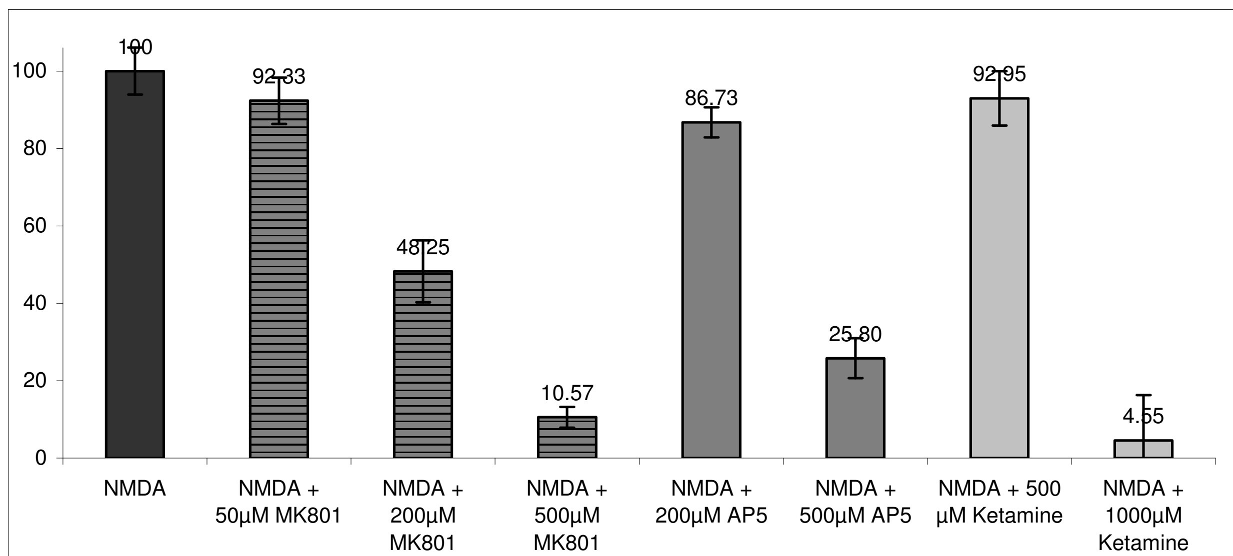


Figure 30: Effect of different NMDA receptor antagonists on the NMDA induced calcium influx. Values are presented as percentage of the response to the first NMDA stimulation. MK-801 has the strongest effect on NMDA/glycine induced response, AP5 and Ketamine are effective only at higher concentrations. Error bars indicate SEM

To address the question whether NMDA receptor mediated Ca^{2+} influx was related to the age of the cells in culture, cultures that were 6 to 23 days old were compared with each other in their response to NMDA stimulation. The maximum height of NMDA induced fluorescence peaks oscillated between +17.34% (SEM=3.06) at 6 DIV, +17.94% (SEM=3.87) at 12 DIV, and +19.72% (SEM=1.85) at 23 DIV. A significant age dependent increase or decrease in NMDA induced response was not detected.

<u>3. 6. Effect of anoxia on mitochondrial potential</u>

Mitochondria play a critical role in cell viability and calcium homeostasis after ischaemic injury (Kristián and Siesjö, 1998). I monitored changes in mitochondrial functioning using the cationic fluorescent dyes rhodamine 123 (R123) and tetra-methyl-rhodamine (TMRM) which accumulate in mitochondria on the basis of their membrane potential. Under UV excitation TMRM fluorescence quickly faded from NT-2 neuronal cells. In addition to this, TMRM seemed to have a toxic effect on NT-2 neurons with the neurons starting to detach from the substrate within 2-3 hours after incubation with TMRM. Rhodamine 123 was retained by NT-2 neurons kept under normal conditions for at least 3 days and did not appear to cause toxic reactions during this time. As a consequence of this, the use of TMRM was discontinued and rhodamine 123 used instead.

R123 incorporates into the mitochondria based upon the strength of their membrane potential. Once the mitochondria are “loaded” with R123, a decrease in mitochondrial potential leads to a “leaking out” of the dye to the cytosol. This increases the apparent fluorescence of the

corresponding cell. When the mitochondrial potential is re-established, the dye is again reincorporated into the mitochondria, decreasing the apparent fluorescence of the cell. The labelling with R123 can thus be taken as an indirect indication of the mitochondrial potential and as an indication of the energetical status of the whole cell. After a complete disruption of mitochondrial potential and the subsequent cell death, R123 leaks out of the cell into the surrounding medium, decreasing the cells fluorescence below baseline values. This final decrease in R123 fluorescence is an efficient and reliable indicator of cell death.

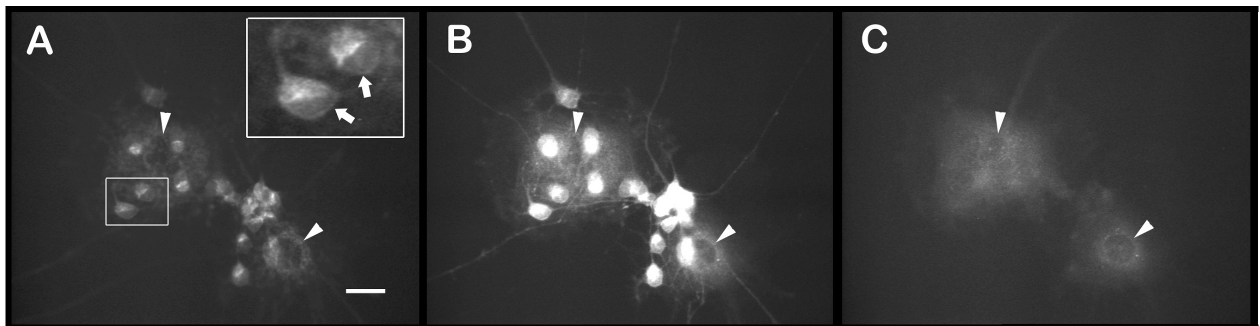
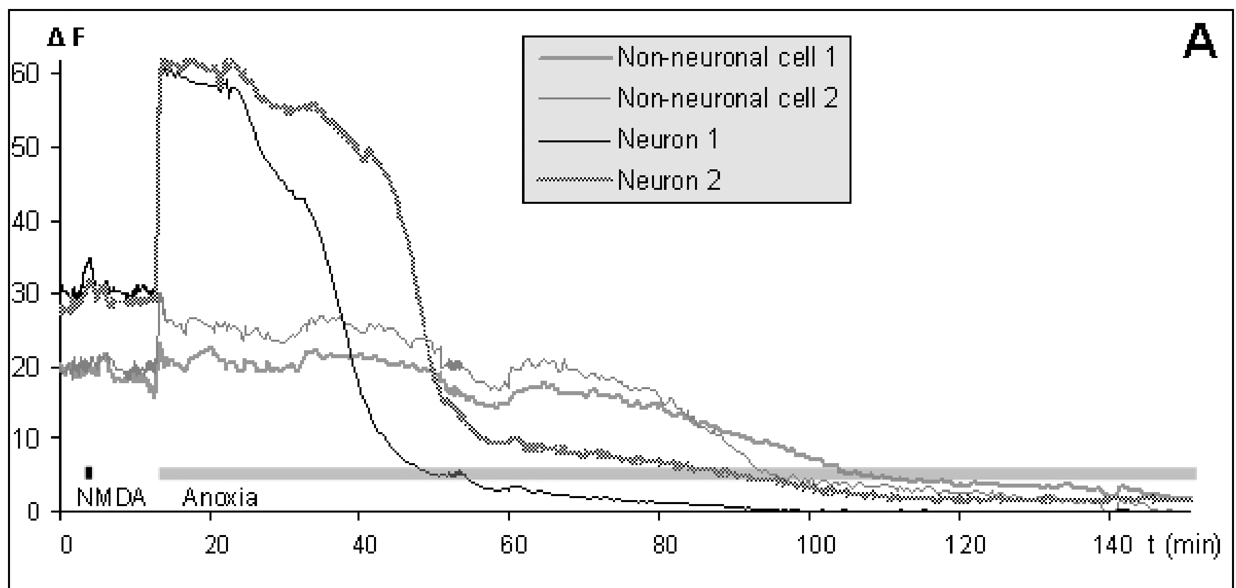


*Figure 31: Imaging of the mitochondrial potential during anoxia with rhodamine 123 (R123). (**A**) NT-2 neurons and non-neuronal cells are stained with R123. The mitochondria are distributed around the nuclei of the cells (small white arrows in the insert). White arrowheads indicate the nuclei of large, flattened non-neuronal cells. (**B**) The generation of anoxia with 2 mM sodium-dithionite leads to a strong increase of R123 fluorescence in neurons. Non-neuronal cells are less affected. (**C**) After 60 min. of anoxia the neurons have died and detached from the substrate. The non-neuronal cells remain viable for another 60 min. Scale bar: 20 µm*

Rhodamine 123 stained predominantly the soma of neuronal and non-neuronal cells (Figure 31). Neurites were faintly stained, the nuclei remained unstained (see insert in Figure 31 A). High resolution images of the large, non-neuronal cells show that the mitochondria are arranged in a grid-like manner, probably connected to parts of the cytoskeleton (image not shown). The total fluorescence of cell bodies was evaluated during anoxic experiments and plotted as a function of time (Figure 32).

When NT-2 neuronal cells are exposed to NMDA/glycine for 1 minute, they show a 12-15% increase in rhodamine 123 fluorescence when compared to baseline fluorescence (Figure 32). The observed reaction to NMDA is, however, much slower than in the calcium imaging experiments. Non-neuronal cells do not exhibit such a reaction to NMDA.



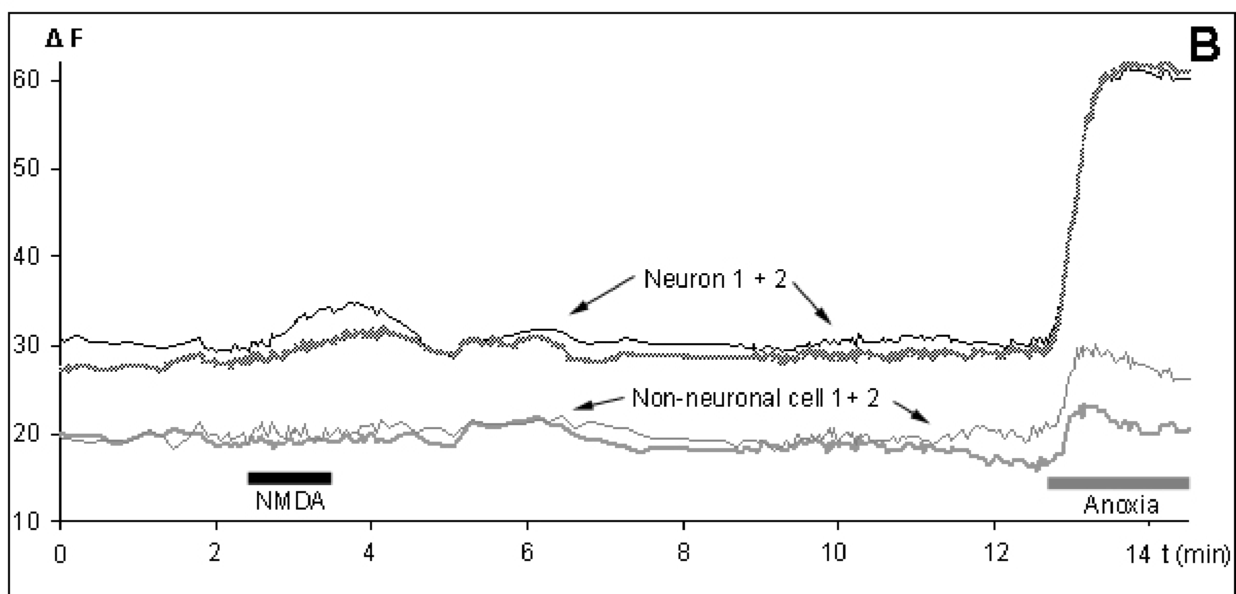


*Figure 32: Rhodamine 123 fluorescence of NT-2 neurons in culture. The culture was constantly perfused with Krebs HEPES buffer (pH 7.4) and maintained at 37.1°C. Neurons and non-neuronal cells were first challenged with 500 µM NMDA / 100 µM glycine (at t=3´25´´) for 1 minute, then exposed to anoxia (at t=12´45´´, with 2 mM sodium - dithionite) until the end of the experiment. (**B**) is a magnification of the first 14 min. of the experiment depicted in (**A**). Note the differences in behaviour of neurons and non-neuronal cells.*

Within 1-2 min after the onset of anoxia the fluorescence of neuronal cells increases sharply by about 100% (Figure 32). Non-neuronal cells show a fluorescence increase that is much less marked (approx. 20%). After a period of 30 min to 1 hour the fluorescence decreases again and finally drops to background level. This final decrease in fluorescence indicates cell death with the corresponding cells detaching from the substrate. In this experimental setting, neuronal cells are the first to die, with non-neuronal cells surviving up to 2 times longer.

It is interesting to note that neuronal cells, which show a strong response to NMDA, die earlier than neurons which show a weak response to NMDA (Figure 32 A+B). The experimental data on this were so far very limited and therefore are not statistically significant.

3. 7. SNP stimulation

The release of nitric oxide (NO) by neuronal cells during ischaemia could play an important part in delayed neuronal cell death (DCD) (Bredt, 1999). There is growing evidence that NO is involved in neuronal development in general, and in the regulation of neuronal growth cone motility in particular (Hess et al., 1993; Van Wagenen and Rehder, 1999). In target cells NO activates soluble guanylyl-cyclase (sGC) which in turn produces cyclic GMP (cGMP). Although NT-2 neurons were shown not to possess nitric-oxide-synthase (NOS) (Munir et al., 1995), a finding which was confirmed by the immunocytochemical and histochemical staining (see 3.2., page 31), it is unclear whether these cells are responsive to NO. Therefore, to test whether NT-2 neurons react to exogenous NO, the reaction of growth cones from NT-2 neurons to the NO donor SNP was observed using time-lapse video microscopy. In addition to this, the intracellular cGMP levels following exposure to SNP were examined in an immunohistochemical assay.

NT-2 N cells do not only tend to actively aggregate and form clusters of neuronal cell bodies but they also rapidly form a complex net of intertwined neurites. In order to be able to identify a SNP specific reaction, only isolated, active growth cones were observed. To find an active growth cone that has no contact to other cell bodies or neurites is getting increasingly difficult the longer the neurons are cultured. Therefore, most of the observed cells had been cultured for 2 days or less (36-40h). In experiments where the cells had been cultured for 3 or 4 days it was rarely possible to find an isolated, active growth cone.

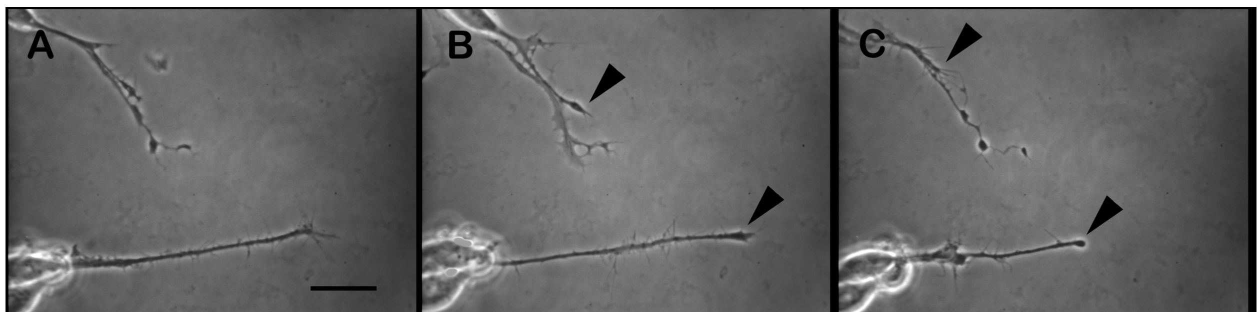


*Figure 33: Reaction of an NT-2 neuron´s growth cone to SNP application. Two growth cones (marked with black triangle) were observed for 1h using time lapse video microscopy with a 100X oil immersion objective (**A**). After 1h 1mM SNP was applied (**B**). The filopodia closest to the growth cone retracted almost immediately. Within the next 30 min. the entire growth cones retracted and the neurite was shortened (**C**). Scale bar: 20 µm.*

Active growth cones seem to be influenced by the application of SNP. In 3 out of 5 experiments

(4 out of 8 observed active growth cones), the filopodia closest to the growth cone retracted after the application of 1 mM SNP. This retraction of filopodia was almost immediate. The filopodia in parts of the neurite closer to the cell body did not retract and were motile during the whole length of the experiment. Within the next 20-30 min. the whole growth cone retracted, and the neurite shortened (Figure 33). However, in one case (out of 8 observed) the growth cone retracted already before the application of SNP and then showed no further reaction to SNP. 3 of the observed growth cones did not show any reaction to SNP.

In a different set of experiments, the effect of SNP application on intracellular cGMP levels was studied. To this end, the concentration of intracellular cGMP was investigated using a specific immunocytochemical assay. These preliminary data seem to indicate that SNP has a stimulating effect on cGMP levels in some but not all NT-2 neurons.

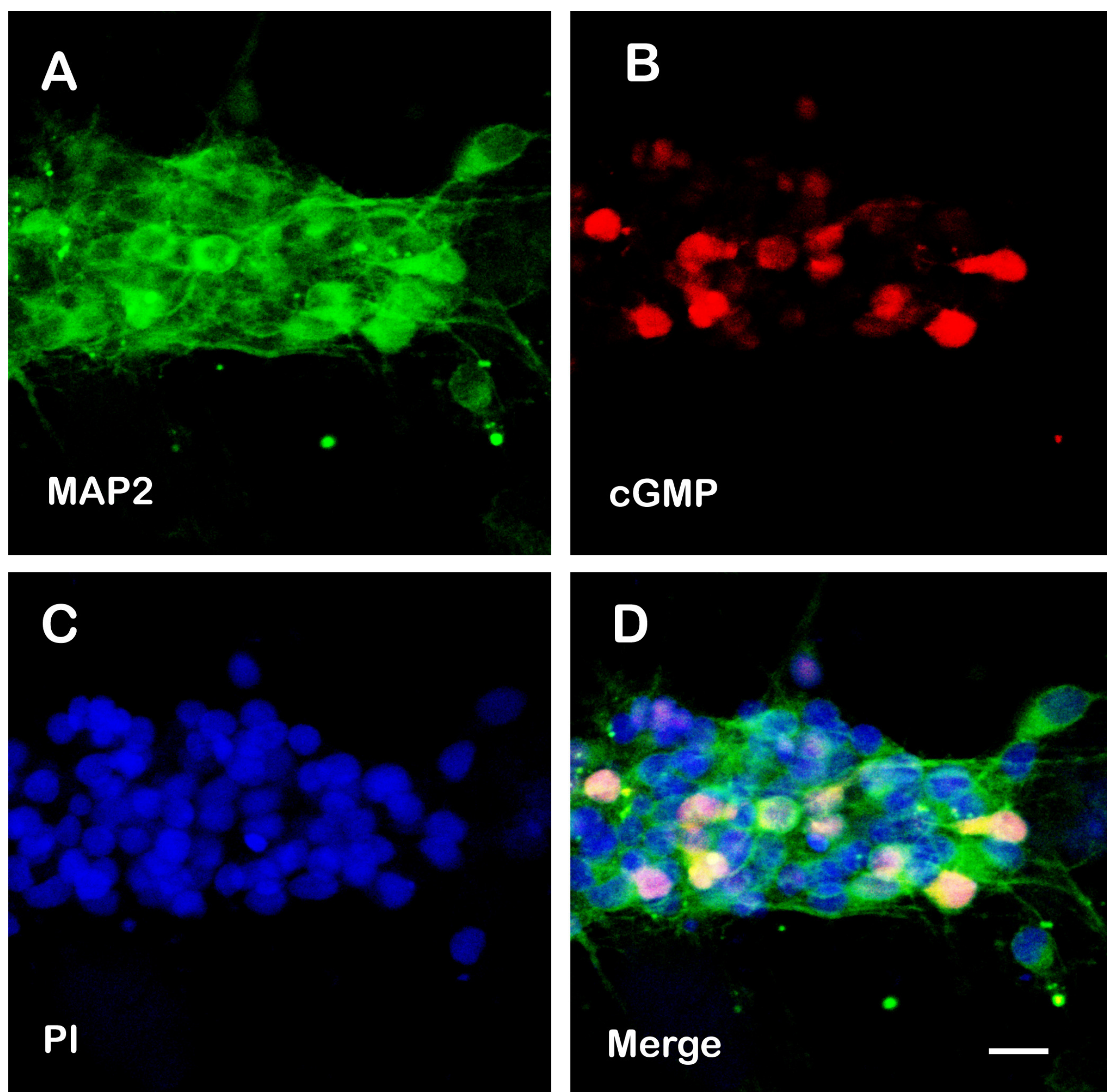


*Figure 34: Reaction of NT-2 neurons to stimulation with SNP. Immunofluorescence staining of NT-2 neurons after 2 weeks in culture and 30 min. exposure to 1 mM of the NO donor SNP and 1 mM of the diesterase inhibitor IBMX. Cells were stained for MAP2 (**A**, green) and cGMP (**B**, red). PI (**C**, blue) was used as a nuclear counterstain. Images were merged in (**D**). Scale bar: 20 µm.*

Double staining against cGMP and the neuronal marker MAP2 confirmed that cGMP positive cells are also positive for MAP2. It also shows that the majority of MAP2 positive cells do not react with increased cGMP synthesis to stimulation with SNP (Figure 34).

The controls, where antibodies for cGMP were applied, but no SNP, were all negative for cGMP. The same is true for the samples where only IBMX – an inhibitor of phosphodiesterase (PDE) activity – and no SNP had been applied. In the samples where SNP had been applied, together with IBMX or not, a number of cGMP positive cells were found. In samples that were incubated with SNP about 15% of the cells counted were positive for cGMP. The number of cGMP positive cells was higher in samples where SNP had been applied together with IBMX. In those samples a maximum of 30 % of counted cells was positive. The samples where SNP, that had been degassed for 24 h, was applied, are mostly negative for cGMP, except for the sample where 100 μM SNP was applied (Table 4).

SNP	*Control*	*0.1 mM SNP*	*1 mM SNP*	*0.1 mM SNP + 1 mM IBMX*	*1 mM SNP + 1mM IBMX*	*1 mM IBMX*
cGMP positive	0	4	4	11	12	0
Total cell count	35	27	24	37	53	25
% positive	0	14.8	16.7	29.7	22.6	0

Degassed SNP	*0.1 mM SNP*	*1 mM SNP*	*0.1 mM SNP + 1 mM IBMX*	*1 mM SNP + 1mM IBMX*
cGMP positive	1	0	0	1
Total cell count	16	32	36	42
% positive	6.3	0	0	2.4

Table 4: Stimulation of NT-2 neuronal cells with SNP. SNP increased cGMP immunoreactivity after 30 min. exposure to a detectable level in approx. 15 - 30% of NT-2 neurons.. SNP that was degassed for 24h had only a small effect on cGMP immunoreactivity. Untreated control and IBMX-only treated cells did not show cGMP immunoreactivity.

4. Discussion

The major aim of this study was to establish a cell culture system for the simulation of ischaemic conditions based on human NT-2 neuronal cells that could be used as a test system for neuroprotective substances.

NT-2 neurons were used in a study on the protective effect of glutamate receptor blockers (Rootwelt et al., 1998) and of barbiturates during ischaemia (Almaas et al., 2000). The model of simulated ischaemia used in these studies, was however, a rather mild one, with only a moderate effect on neuronal viability even after anoxia times of 6-10 hours. Under *in vivo* conditions the duration of experimental ischaemia needed to produce substantial neuronal cell death is approximately 50-100 times shorter. Therefore, it was intended to establish a model for simulated *in vitro* ischaemia that would come closer to the *in vivo* conditions with respect to the duration of anoxia and the culture conditions.

The cell culture systems that are used so far to simulate ischaemic conditions *in vitro* are usually based on primary neuronal cells obtained from rodents. These assay systems are mostly used to address questions of basic research. They also allow the testing of neuroprotective substances *in vitro* (Bartmann-Lindholm and Carter, 1999; Campbell et al., 2000; Milhaud et al., 2000).

The human origin of neurons is a major advantage of the NT-2 based assay system. Since there are considerable differences in the reaction to neuroprotective substances between different species (reviewed in Munir et al., 1995), human cells are obviously the best option to test neuroprotective substances.

The homogenous composition of NT-2 cultures and the possibility to use cocultures of NT-2 neurons and NT-2 astrocytes are additional advantages. Since the lengthy differentiation procedure is a major drawback that limits the use of NT-2 neurons, it was tried to reduce the time needed for neuronal differentiation. A short differentiation protocol was established in this work using a cell aggregation technique.

4. 1. Cell aggregation facilitates neuronal differentiation

Two procedures for expanding neural stem cells in culture have been reported. In the first technique precursor cells are grown on a substrate coated surface, on which the cells expand as a monolayer (Ray et al., 1993). The second technique involves proliferation of precursors in a three-dimensional environment on non-adherent plastic surface as cell clusters termed neurospheres (Reynolds and Weiss, 1992). Motivated by a recent study showing that differentiation of NT-2 cells *in vitro* can also occur in cell aggregates (Cheung et al., 1999), we investigated the expansion of this embryonal carcinoma cell line on a non adhesive substrate. This method of cell proliferation indeed generated cell spheres, that resemble the neurospheres

described in the literature (Figure 5 A). Here, the morphological resemblance should not imply a monoclonal origin of the cell spheres, which most likely derive from several aggregating and proliferating NT-2 progenitor cells.

Our findings suggest that both, cell contact and Retinoic Acid (RA) contribute to a more rapid cell expansion and neuronal differentiation process than in the conventional protocol using cell layer culture (Pleasure et al., 1992). RA has been known for a long time to exhibit morphogenetic properties and to influence cell differentiation during embryogenesis and in cell culture systems (reviewed in Maden, 2001). Presumably, RA exerts its morphogenetic effects via the differential activation of HOX genes which has also been shown for the human NT-2 cell line (Simeone et al., 1991).

The influence of cell contacts on differentiation has been described both for primary cell culture (Reinhardt and Bruinink, 1987) and for cell lines. Cells of the murine P19 line, for instance, will also differentiate to neurons when aggregated and exposed to high concentrations of RA (McBurney et al., 1982). However, the cellular mechanisms, how both RA and cell contact combine to facilitate neuronal differentiation, remain still poorly understood. Moreover, since the differentiation process in floating cell spheres resembles a suspension culture, it is currently difficult to discriminate influences from cell surface contacts in the aggregate from the anchorage-withdrawal effect.

In a recent study on a mouse neuroectodermal progenitor cell line, the influence of RA on cell aggregation and neuronal differentiation has been investigated. Schlett et al. (2000) found that RA treatment promotes the aggregation of cells of the mouse NE-4C cell line when grown on poly-l-lysine surfaces. After 2 days in culture NCAM immunopositive cells started to appear inside of these aggregates. Without RA treatment, neither aggregation nor neuronal differentiation could be observed.

Our finding that cell multiplication in the spheres without simultaneous RA treatment (Figure 5B) does not yield neurons, corresponds to the results of Schlett et al. (2000). In our protocol human NT-2 cells generate cell conglomerates when plated onto non-adhesive substrate even without RA treatment. However, size and shape of the conglomerates cultured without RA differed from the typical cell spheres exposed to RA (Figure 5B). Moreover, no neuronal differentiation could be observed subsequently. This result implies that RA application influences the shape of the cell spheres and proves to be essential for the neuronal differentiation of the NT-2 cell line. It also implies that cell aggregation alone is not sufficient for neuronal differentiation to occur but that both factors must work together to enable the generation of neuronal cells *in vitro*.

In fact, when the conventional layer culture differentiation method described by Pleasure et al.

(1992) was applied, a cell aggregation phenomenon was also observed. After 4-6 weeks in culture and simultaneous exposure to RA the cells did no longer grow as a monolayer, but formed a dense multi-layered structure. In these cultures some areas were denser and a multitude of cell layers grew on top of each other. The overall appearance of such a culture resembled a hilly landscape where areas with a high cell density formed “hills” and areas with a low cell density formed “valleys”. One could speculate that conditions within these “hilly” aggregates are similar to the conditions in the free floating spheres described above. If neuronal differentiation took place primarily inside the cell aggregates, as the data from Cheung et al. (1999) and Schlett et al. (2000) indicate, it would explain why the floating sphere culture protocol allows for a faster neuronal differentiation. When NT-2 cells are grown on an adherent substrate their motility is reduced because they stick to this substrate. The formation of aggregates that facilitate differentiation then takes much longer than in the suspension culture. The faster aggregation in suspension cultures would then be the main reason for the faster neuronal differentiation.

The sphere culture protocol dramatically reduced the time needed for a successful neuronal differentiation to one half when compared with the conventional method (Figure 7). To verify that the neuronal cells generated using the rapid differentiation protocol, were identical to the neurons obtained with the layer culture, we used immunocytochemical labelling techniques.

4. 2. Neurogenesis and expression of neuronal proteins

Neuronal differentiation in our cell sphere protocol was characterised by immunostaining for the neuronal markers MAP2, Tau, and HuC/D (Figure 9, Figure 10). MAP2 is confined to the dendrites of neuronal cells, whereas Tau protein is present only in the axons. Even though the NT-2 neurons were clearly labelled for the cytoskeletal proteins MAP2 and Tau, a selective enrichment of Tau in axonal processes (Piontek et al., 1999) was only evident in the longer neurites connecting distant cell clusters.

The Hu family of proteins have been identified as neuron-specific antigens recognised by autoimmune antiserum from patients with neurodegenerative disorders associated with cancer (Graus et al., 1985). The evolutionary conserved Hu gene family shares homology with the ELAV-gene, which is essential for neuronal development in *Drosophila* embryos (Campos et al., 1987). Gene expression studies have also implicated the Hu/Elav family of RNA - binding proteins into processes of neuronal differentiation in vertebrates (Akamatsu et al., 1999; Wakamatsu and Weston, 1997). The immunocytochemical detection of HuC/D after neuronal cell differentiation in NT-2N cultures suggests a role of these proteins also in human neurogenesis.

Prion protein (PrP) is predominantly expressed in neurons, both in the soma and the neurites

(Lainé et al., 2001; Ford et al., 2002). The detection of cellular PrP in NT-2 neurons (Figure 14) is in line with the work of Shyu et al. (2000), who found that PrP is regularly expressed in NT-2 neurons and that its expression is up-regulated after the induction of a heat shock. So far it is unclear whether such an up-regulation of PrP also takes place after anoxia. The selective expression of PrP in cells with neuronal morphology and colocalisation with the neuronal markers MAP2 and HuC/D further confirms the neuronal nature of differentiated NT-2 cells. This also implicates that NT-2 neurons could be used to investigate the function of PrP in human nerve cells *in vitro*.

Subpopulations of neuronal cells reacted with antibodies directed against the neurotransmitters GABA, serotonin (5-HT), or the serotonin synthesising enzyme tryptophan hydroxylase (Figure 11, Figure 12). This corresponds to the findings of Guillemain et al. (2000) who found that a number of different neurotransmitter phenotypes, including GABA, dopamine, and 5-HT, are expressed in NT-2 neuronal cells. By double-fluorescence immunostaining, it was also found that many NT-2 neurons express more than one neurotransmitter at a time.

The immunocytochemical detection of the main excitatory transmitter glutamate in neurons is rather difficult as it is part of the normal amino acid metabolism present in every cell.

The fact that neither NOS nor NADPH-diaphorase activity were detected in NT-2 neuronal cells corresponds to results obtained by Munir et al. (1995). This finding could be important for further studies of *in vitro* ischaemia and the excitotoxic cascade in this cell line. Since the generation of NO seems to be responsible for an important part of ischaemic damage *in vivo* (Ayata et al., 1997), the use of neurons that do not produce NO themselves would allow to separate the effects of the endogenous release of NO from other damaging effects. This could be achieved for instance via the addition of exogenous NO to neuronal cultures during or after an anoxia/hypoglycaemia. The exact assessment of NO induced neuronal damage in such a model for ischaemia could lead to a redefinition of therapeutic strategies.

The differentiation of NT-2 cells to glial cell types and the corresponding detection of GFAP in NT-2 neuronal cells, particularly in cells with a stellate morphology (Figure 13), was already reported by other groups (Bani-Yaghoub et al., 1999; Sandhu et al., 2002). Since glial cells seem to be important for the establishment of synaptic contact both in primary cultures (Pfrieger and Barres, 1997) and in NT-2N cultures (Hartley et al., 1997), a coculture of NT-2 neurons with NT-2 glia cells could open up new opportunities for the study of synaptic transmission in human NT-2 neurons.

The results obtained from the immunocytochemical staining support the idea that differentiated NT-2 cells are truly neuronal. Neuronal cells differentiated using the faster sphere culture protocol show no significant differences to cells obtained with the conventional layer culture

protocol.

4. 3. Generation of anoxia/hypoxia in vitro

Ischaemia is caused by an interruption of blood flow in a specific area, a situation which is very difficult to simulate in an *in vitro* system. Since the most important parameters that are altered during ischaemia seem to be the lack of oxygen and glucose in the affected area, we focused our investigations on the withdrawal of oxygen and glucose and the subsequent reactions of NT-2 neurons to this.

In cell cultures the cell densities are lower than in intact tissue. Conversely, the volume of the surrounding medium is considerably larger than the extracellular tissue volume. In combination, these two factors lead to the fact that even traces of oxygen are sufficient for the survival of neuronal cell cultures. Thus, an effective *in vitro* simulation of anoxia requires the complete and rapid removal of oxygen from the surrounding liquid medium.

The complete removal of oxygen from a liquid medium is rather difficult and requires thorough exchange with an inert gas. I used argon as an inert gas because it has a lower molecule radius (van der Waals radius) than oxygen or nitrogen (Ar: 1.91 Å, O_2: 2.06 Å, N_2: 2.15 Å) allowing for a faster and more complete displacement of oxygen (Hollemann and Wiberg, 1996). Since oxygen has a high diffusion coefficient in water, even very short exposures of such a degassed solution to ambient air are sufficient for reoxygenation. The relatively long duration of anoxia necessary to produce neuronal cell death in other *in vitro* models that used nitrogen to displace oxygen (i. e. Almaas et al., 2000), could well be due to an incomplete deoxygenation and/or a reoxygenation of the cultures before the transfer to an anoxic chamber. To avoid reoxygenation, the use of an anaerobic work bench is almost imperative.

However, a simple workaround consists in the chemical removal of oxygen from a saline using the reducing agent sodium-dithionite. The addition of sodium-dithionite to the liquid medium reduces oxygen to hydrogen peroxide in a first rapid step. The peroxide is then reduced by a second mole of dithionite to SO_2^-. These reactions scavenge even trace amounts of oxygen, leading to a complete deoxygenation of the liquid medium right after the application of dithionite to the medium. Depending on the concentration of dithionite, the anoxia is maintained for some time.

Although this method of generating anoxia is widely used in cell culture based research (i. e. Cummins et al., 1993), it is unclear whether the generation of peroxides could affect intrinsic membrane properties. In a recent comparison of anoxia generated chemically using dithionite and anoxia generated physically using nitrogen-bubbling, no significant differences in potassium ion currents were observed (Gebhardt and Heinemann, 1999). In order to avoid possible adverse

effects of dithionite on the NT-2 neurons, the concentration of dithionite in the medium was minimised by keeping the exposure time of dithionite deoxygenated solutions to air as short as possible. Cell cultures were immediately placed into an anoxic chamber that was deoxygenated with a mixture of 5 % carbon-dioxide and 95% Argon. In this experimental paradigm, dithionite is only used to achieve a rapid deoxygenation of the cell culture medium in the initial phase of anoxia. In later stages the anoxia is maintained and reoxygenation prevented by the Ar/CO_2 gas mixture.

When compared to other *in vitro* simulations of ischaemia, the deoxygenation with Argon and additional use of dithionite has a stronger negative effect on viability. Almaas et al. (2000) worked with nitrogen-bubbling to deoxygenate the liquid medium. The cell cultures were then treated with this medium and placed into an anoxic chamber. In this experimental setting, the duration of anoxia needed to produce a significant effect on viability of neuronal cell cultures was between 6 and 10 hours.

The complete removal of oxygen from the cell culture medium at the very beginning of the anoxic experiment leads to a strong decrease of neuronal viability. In an *in vivo* experiment durations of ischaemia of 5-15 minutes lead to pronounced cell death in rat hippocampal CA1 neurons (Colbourne et al, 1999). Although my experimental approach is still far from this situation, it might be closer to the *in vivo* setting than other *in vitro* simulations of anoxia.

4. 4. Viability assays

To assess the effect of anoxia on the viability of NT-2 neuronal cultures a number of different viability assays were tested and compared. The measurement of viability of a cell culture is not as simple as it may seem at first sight. In practice, the exact experimental conditions may have a large influence on the outcome of a viability assay. When using a cell viability assay one usually assumes that a certain marker is representative of the number of living or dead cells. There are however limitations to these assumptions.

A number of different assay systems are available for the measurement of viability. Most of them are based on certain enzymatic activities. Still, there is the need to assure that the assay chosen is adequate for the cells used, works reliably under the experimental conditions, and allows to address the experimental questions asked (reviewed in: Lindl, 1999).

For instance, the trypan blue staining – a commonly used assay in cell culture – is based on the fact that the dye accumulates in cells with defective cell membranes, it thus reveals dead cells. However the amount of dead or dying cells can be misestimated because of certain errors that are intrinsic to this method. Cells that have activated apoptosis and are irreversibly committed to die but whose cell membranes are still intact will not be stained by the dye. Also some cells could

already be too fragmented to accumulate the dye or are no longer recognisable as a cell. The actual effect of a specific treatment on viability will therefore be underestimated. The problem is further complicated by the fact that the assay itself may influence viability. The toxicity of trypan blue will increase the number dead cells if the cells are not counted rapidly, the amount of cell death will then be overestimated. To correct for the intrinsic errors of this method is rather difficult as each measurement can only be performed once.

Another commonly used viability assay is the MTT cleavage assay where a soluble tetrazolium salt is reduced by living cells giving an insoluble formazan salt. After a certain incubation time the cells have to be lysed and treated with a detergent to generate a soluble colorant, which can be measured in a photometer. The main disadvantage of this assay is that viability can be measured only once.

The same is true for the LDH viability assay which is widely used in studies of *in vitro* ischaemia. It makes use of the fact that all living cells possess LDH, an enzyme that catalyses the reversible reduction of pyruvate to lactate. Dying cells release this enzyme and because it is relatively stable and retains its enzymatic activity it is easily measurable using a simple biochemical assay.

Depending on the exact experimental conditions the use of the LDH assay should be carefully considered. During anoxia the oxidative metabolism comes to a standstill. The cell can however still generate ATP by using the anaerobic glycolysis. This leads to an increase of intracellular lactate and correspondingly to a drop in pH. It is likely that under conditions of anoxia or hypoxia the increase in glycolytic activity will result in an increase of LDH activity. The reoxygenation / reperfusion following anoxia will again require a high activity of LDH to decrease the pool of lactic acid to normal levels. In the literature there are indications that the activity or the expression level of LDH is indeed up-regulated during and after anoxia/hypoxia. Nowak et al. (1996) found that hypoxia is one of the main factors that lead to an induction of LDH activity in primary cultures of rabbit renal cells. Similarly, Lai et al. (2003) found that chronic hypoxia increases LDH activity between 100% - 370 % in different regions of the rat brain. If viability is assessed using an LDH activity based assay, the rate of cell death will be overestimated in samples that were subjected to anoxia/hypoxia. The corresponding live controls will not be affected by this misestimation. The same is true for certain antioxidant enzymes, like super-oxide-dismutase (SOD), which are also likely to be up-regulated after anoxia / reperfusion. SOD inhibits LDH and leads to a false lower cell death estimation (Almaas et al., 2002). To use the LDH assay for viability measurements in experiments of anoxia/hypoxia is therefore not advisable.

The viability assays mentioned above - Trypan blue, MTT and LDH - do not allow a continuous

measurement of viability. Since there are a number of indications in the literature that the delayed neuronal cell death occurs during several days post ischaemia (i.e. Colbourne et al., 1999), I wanted to study the viability in neuronal cell cultures continuously during and after an anoxic insult. I therefore looked for other viability assays that would allow for a continuous monitoring.

The Alamar Blue® assay is similar to the MTT assay in the way that it also uses the conversion of a dye by living cells. Here, contrary to the MTT assay, the product is still soluble and does not have to be dissolved with the help of detergents. Thus the assay can be repeated several times with the same cell culture. The dye used in the Alamar Blue assay has an oxidising potential close to that of oxygen. It is incorporated into living cells where it serves as an electron acceptor in the oxidative chain of the mitochondria. Thus it can replace oxygen in the oxidative metabolism. This became evident in one of the first experiments with anoxia and the Alamar Blue assay. When Alamar Blue solution was added to the NT-2N cells before they were subjected to anoxia, the consumption of the dye increased dramatically during anoxia and the cells survived the anoxia with no apparent loss in viability. This indicates that in the absence of oxygen NT-2N cells are using Alamar Blue as an oxidant instead. Therefore measurements of viability with this assay are not possible during anoxia and have to be conducted before and after such an experiment. Yet, this assay enabled continuous monitoring of viability under normoxic conditions without measurable toxic effect on the cultures.

The Live/Dead assay uses two different fluorescent dyes that are either incorporated by living cells or intercalate into the DNA of dead cells. Calcein AM is a fluorescent dye that is bound to acetoxy methyl ester (AM). In this form it exhibits low fluorescence and is able to permeate cell walls. Inside a cell the acetoxy methyl ester is cleaved by esterases which are present in every living cell. The cleavage of Calcein AM leads to a large increase in fluorescence and at the same time prevents the dye from leaving the cell. The second fluorescent dye - Ethidium homodimer - is a dye that exhibits a strong fluorescence after intercalation into DNA. As it can not permeate intact cell membranes it intercalates only in the DNA of dead cells, labelling them efficiently.

The Live/Dead assay has the advantage that it can be performed immediately after anoxia at any given point in time whereas the Alamar Blue assay needs a 4h incubation before it can be read out. A disadvantage of the use of the Live/Dead assay with NT-2N cells is that these cells do not grow as a uniform monolayer but form clusters where it is very difficult to do an exact cell count. The bottom of the wells is covered with mostly non-neuronal cells which are less sensitive for anoxic insult but provide for a high background fluorescence. Dead cells quickly detach from the substrate which then leads to an underestimation of cell death. Therefore only living cells were considered in repeated counts before and after anoxia. These factors are severe obstacles

for the use of the Live/Dead assay for the evaluation of viability in NT-2N cells either in an automated readout or in a manual count.

The differences in viability measurements found between Live/Dead assay and Alamar Blue assay - where the viabilities almost never dropped below 20% - can easily be explained by the fact that here only neurons - cells showing neuronal morphology - were counted. The remaining non-neuronal cells which survive the anoxia mostly unharmed, account for the apparently higher viabilities measured with the Alamar Blue assay.

4. 5. Reaction of NT-2 neurons to anoxia and excitotoxic conditions

NT-2 N cells have been shown to possess glutamate channels (Younkin et al, 1993) and respond to glutamate toxicity (Munir et al, 1995). This finding is confirmed by the calcium fluorescence measurements which demonstrate a calcium influx after NMDA or glutamate stimulation (Figure 24). Although Gao et al. (1998) have shown that NT-2 neurons exhibit transient fluctuations of intracellular calcium concentrations, to my knowledge, this is the first time that NT-2 neurons were shown to possess functional, NMDA-type, glutamate receptors.

NT-2 neurons are highly susceptible to oxidative stress (Tamagno et al., 2000), yet, the formation of free radicals in NT-2 neurons was found to be only of minor importance in hypoxic cell death (Almaas et al., 2002). NT-2 neurons have been used to study the effects of anoxia and hypoglycaemia *in vitro* (Rootwelt et al., 1998) and to evaluate neuroprotective properties of barbiturates (Almaas et al., 2000) and caspase inhibitors (Almaas et al., 2003) under conditions of simulated ischaemia. They were also used to study the neuroprotective effect of allopregnanolone, a derivate of progesterone, in a model of NMDA induced excitotoxicity (Lockhart et al., 2002).

In our experimental setup NT-2 neuronal cell cultures react to anoxic conditions with a reduction of their viability (Figure 15). This effect increases with the duration of anoxia and with the culture age (Figure 16, Figure 17). NT-2 N cultures also show a strong reaction to excitotoxic conditions. Primary neuronal cultures respond to anoxic/hypoglycaemic conditions in a way that is very similar to differentiated NT-2 cultures. Bartmann-Lindholm and Carter (1999) found that primary neurons obtained from rat embryos showed extensive cell death (up to 85%) after 2 hours of anoxia. They had used, however, higher cell densities, a factor which may be responsible for the apparent higher cell death rate in these primary cultures. The primary cultures also responded to excitotoxic conditions; maximum glutamate toxicity was observed between 100 and 1000 µM glutamate (Bartmann-Lindholm and Carter, 1999). Thus, in principle it is possible to use the NT-2 cell culture system to simulate ischaemic conditions *in vitro* and the

results obtained in such experiments are comparable to those obtained with primary cultures.
There are however certain limitations to this *in vitro* approach which have to be taken into account. Under certain circumstances, these limitations can even be advantageous
One specific property of this *in vitro* system is that it consists mostly of neurons (> 80%) with a low percentage of glial cells (5-10%) and other so far undefined cell types. This is of course very different to neuronal tissue where glial cell types are much more abundant, and which is composed of endothelium and blood vessels. The results obtained with such an *in vitro* system are therefore limited to neuronal cells.
This limitation can, at the same time, also be an advantage: This cell culture system with a homogenous, well characterised cell population allows to study the effect of simulated ischaemia on human neurons. It allows separating the effects of the excitotoxic cascade from other potentially damaging effects. In this context, the fact that NT-2 neurons do not produce NO (Munir et al., 1995; see also 3.2, page 31) and that the endogenous free radical production after anoxia/reperfusion was found to be only very moderate (Almaas et al., 2002) is particularly advantageous. This situation provides a unique opportunity to study the damaging effects of anoxia, glutamate, NO, and ROS in detail and individually. In addition, several damaging effects can be combined (i.e. by the generation of anoxia, the addition of glutamate, NO or ROS to an anoxic system), and the effects of different combinations of damaging factors can be evaluated. The study of such a system could lead to a reassessment of the factors influencing ischaemic neuronal cell death and indicate new approaches for the therapy of stroke.

4. 5. 1. Delayed neuronal cell death (DCD) in NT-2 neuronal cultures

The main loss in viability seems to occur during the first 12 hours after anoxia (Figure 15, Figure 16). Between 24h and 48h post anoxia the loss in viability is rather small or even neglible. This is in contrast to findings *in vivo* where an important part of neuronal loss seems to occur between 1-3 days after ischaemia (Colbourne et al., 1999). The reasons for this delayed neuronal cell death (DCD) *in vivo* are not yet well understood. They have been attributed to glutamate excitotoxicity, NO toxicity, ROS generation during reperfusion, or to apoptotic processes (Coyle and Puttfarken, 1993; Bredt, 1999; Jin et al., 2002).
The fact that there is a small number of DCD even in the *in vitro* model can not be explained with the release of glutamate from dying neurons because this is quickly diluted in the surrounding cell culture medium and the medium was changed several times after anoxia. The production of NO can also not be responsible for DCD because NT-2 N cells do not possess NO synthase (Munir et al., 1995) and in the corresponding experiments there was no addition of exogenous NO. ROS generation has been shown to be of minor importance in post ischaemic

cell death (Almaas et al., 2002). The observed small amount of DCD between 24 h and 48 h post anoxia could therefore be due to either ROS generation or to apoptotic processes.

The mentioned differences between the hypothetical *in vivo* situation and the *in vitro* model explain the observed differences in behaviour at least in part. This also allows to establish more realistic conditions, that resemble *in vivo* conditions closer, by the addition of exogenous of glutamate, NO, or ROS to the cell culture medium after the anoxia, during the subsequent reoxygenation. Such an addition of these factors is very likely to lead to another increase in DCD after anoxia.

4. 5. 2. Effect of glutamate and involvement of NMDA receptors

In combination with anoxia NT-2 neuronal cultures show a strong reaction to glutamate with their viabilities being decreased by approximately one third when compared to anoxia without glutamate (Figure 18). This effect of glutamate on viability can be reversed by the addition of MK-801, a commonly used NMDA-channel blocker (Figure 20). To completely protect NT-2 neurons from glutamate induced damage during anoxia I had to use MK-801 concentrations considerably higher than previously reported. In a model of relatively mild hypoxia, Rootwelt et al. (1998) and Almaas et al. (2002) report that MK-801 at a concentration of 10 µM offers almost complete protection. To obtain the same level of protection I had to use MK-801 concentrations of at least 100 µM. This is in line with results obtained by Lockhart et al. (2002) who in a model of NMDA induced cell death found reliable compensation of NMDA related effects at an MK-801 concentration of 100 µM.

The finding that low concentrations of MK-801 did not protect NT-2 neurons against glutamate toxicity is supported by the calcium imaging experiments. MK-801 concentrations of 10 or 100 µM had no significant effect on calcium ion influx after stimulation with NMDA. A significant reduction of calcium influx was only achieved with MK-801 concentrations of 200 µM or above (Figure 26). A similar effect was also achieved with high concentrations of the NMDA-receptor antagonists AP 5 (APV, 500 µM) or Ketamine (1000 µM) (Figure 28, Figure 29). This finding implies that MK-801 could be less effective in human NT-2 neurons than it is in other species.

Surprisingly, high concentrations of glutamate – above 3 mM – seem to have a positive effect on viability. This could be due to an effect on the solutions osmolarity. Another explanation for this phenomenon would be that glutamate is incorporated into the cells and metabolised in the Krebs cycle to generate ATP. This could alleviate the severe “energy crisis” the neurons are facing during anoxia and explain their survival.

4. 5. 3. Effect of NMDA and anoxia on mitochondrial potential

The fact that NT-2 neurons are indeed facing a severe energy crisis during anoxia is confirmed by the imaging of the mitochondrial potential using the cationic fluorescent dye rhodamine 123 (R123).

Mitochondria produce ATP, the main "energy currency" of every living cell. The mitochondrial potential is of prime importance for the survival of a cell. Its disruption quickly leads to cell death, either via necrotic or apoptotic mechanisms. ATP consumption and the subsequent ATP generation in the mitochondria decrease in the mitochondrial potential.

R123 fluorescence increased reversibly after stimulation of NT-2 neurons with NMDA (Figure 32B). This apparent increase in fluorescence was caused by a release of R123 by the mitochondria into the neurons´ cytosol. This R123 release was caused by the decrease of mitochondrial potential which in turn was caused by a high demand of ATP needed for the repolarisation of the cell membrane. After the end of the NMDA challenge, the mitochondrial potential returned to its initial value, leading to a reincorporation of the fluorescent dye into the mitochondria.

The response of NT-2 cells to a challenge with NMDA is similar to the behaviour of primary cortical cell cultures obtained from embryonic mice. Melzian et al., (1999) found that these cultures respond to a stimulation with 300 µM glutamate with a slow and reversible increase in R123 fluorescence.

The R123 fluorescence of NT-2 neurons showed a marked increase after the onset of oxygen and glucose deprivation (OGD) (Figure 32). In the first 20-30 minutes the fluorescence decreased only slowly, a phenomenon which is probably due to a leaking out of the dye from the cytosol to the surrounding medium. After this initial phase, the fluorescence drops much faster and reaches background levels within the next 30-40 minutes, indicating the disruption of the membrane integrity and the cells´ death. In fact, many of the neurons observed detached from the substrate and disappeared in this phase. The response to OGD in NT-2 neurons is almost identical to the responses observed in primary cultures from embryonic mice (Melzian et al., 1999). In these cultures OGD leads to drastic increase of R123 fluorescence after the onset of anoxia, a slow decrease of fluorescence after the peak value has been reached, and fast decrease of fluorescence below baseline values after approx. 60 min of anoxia.

The imaging of the mitochondrial potential points to the fact, that the energetical status of NT-2 neurons during NMDA challenge or anoxia is significantly altered. It confirms the vulnerability of NT-2 neurons to anoxia and allows to monitor the reaction of every individual cell during the whole length of the experiment.

4. 5. 4. Effect of age on neuronal vulnerability

The finding that older NT-2 neuronal cultures are more vulnerable to an ischaemic insult than younger ones could be explained by an ongoing differentiation of NT-2 cells in culture. This idea is supported by the work of Guillemain et al. (2000) who found that the number of NT-2 neurons with a peptidergic phenotype increases considerably between 2 and 4 weeks in culture. This process could somehow lead to an increased sensitivity of NT-2 neurons against neurotransmitters and thus to an increased vulnerability to excitotoxicity. One could for instance assume, that the number of neurotransmitter receptors present on the cell surface continues to increase for some time. In this case, the NMDA induced response of neurons should increase with age. So far, in the calcium imaging experiments such an age dependent increase in response was not found. Also, signal transduction cascades might continue to develop during the first 6 weeks in culture leading to an increased response to stimulation that lies downstream of the intracellular calcium signal. This is a possibility that can not be ruled out so far.

Another explanation takes into account the fact that NT-2 neurons in culture have a strong tendency to form large, ganglion-like aggregates. Inside these aggregates, conditions are likely to be very different from the conditions that isolated cells in young neuronal cultures are facing. Within aggregates, cells are densely packed and directly exposed to substances released from neighbouring cells. Because of the consumption by surrounding cells, the concentration of oxygen and glucose will reach critical levels inside an aggregate much earlier than outside (Pardo and Honegger, 1999). This setting is probably much closer to *in vivo* conditions and might contribute to the increased vulnerability of older NT-2N cultures.

5. Conclusion

In recent years the NT-2 cell culture system has proven to be a valuable tool for the study of ischaemic brain injury on human neurons *in vitro* (Munir et al., 1995; Rootwelt et al., 1998; Lockhart et al., 2002; Almaas et al., 2003). However, the widespread use of NT-2N cultures for the screening of neuroprotective compounds for stroke therapy was countered by the fact that the differentiation of these cells was rather time-consuming and that the generation of anoxic / hypoxic conditions required sophisticated and expensive technical equipment.

The modified differentiation protocol established in this work allows for a faster and more efficient generation of NT-2 derived terminally differentiated neurons. This will greatly facilitate all the studies involving the NT-2 cell culture system and open up new opportunities to study the mechanisms of human neuronal cell differentiation and pathophysiology.

The NT-2 neurons present many of the characteristics of primary cultures of nerve cells with the advantage of the homogeneous cellular composition of clonally derived neurons. The immunocytochemical labellings against neuron specific markers confirm the neuronal status of these cells. This is further confirmed by the functional expression of NMDA-type glutamate receptors, and their significant vulnerability to anoxic insult. These properties make them an excellent subject for large drug scale screening.

The investigations conducted have shown that in principle it is possible to use NT-2 neurons for the *in vitro* simulation of ischaemia. The procedure is reasonably simple to perform and requires only standard laboratory equipment. The viability of NT-2 neuronal cultures is significantly reduced by experimental anoxia, an effect which is increased by the addition of glutamate. Since known neuroprotective substances, such as the glutamate channel blocker MK-801, do protect NT-2 neurons against anoxia, the NT-2 neurons can be used as a model system for ischaemic brain damage suitable to screen for neuroprotective substances. This procedure may be a useful paradigm that could facilitate the discovery and development of drugs that could be used during or after a stroke.

The NT-2 model system has the potential of replacing the use of laboratory animals in basic investigation of the excitotoxic cascade *in vitro*. It has the additional capacity to reduce the number of experimental animals needed in initial stages of drug screening and routine testing. In addition, genetic differences between human cells and animal cells can be ruled out. Thus, it might be easier to relate the experimental results directly to human diseases. Although biomedical research often depends on animal models, there is now a practical alternative available to work directly with human neurons avoiding ethical problems linked to animal experimentation.

6. Future prospects

The differentiation of NT-2 cells to mature, functional, postmitotic neurons in culture provides a unique opportunity to study the development of human neuronal cells *in vitro*. Using recently developed chip technology even subtle changes in the expression pattern of non-neuronal precursors to fully functional neurons will be discovered and can be studied in detail. This approach could give new insights in the delicate processes of neuronal differentiation and could eventually lead to the discovery of new genes involved with neuronal development (Satoh and Kuroda, 2000; Leypoldt, et al., 2001; Bani-Yaghoub et al., 2001).

A number of questions concerning the use of NT-2 neuronal cells in models of simulated ischaemia are still open. The reaction of NT-2 neurons to basic or acidic pH-values and to high concentrations of glutamate needs to be studied in more detail. Whether NT-2 neurons after anoxia die via a necrotic or an apoptotic pathway is not yet well known. Although it seems that apoptosis plays a minor role (Almaas et al., 2003), the questions which pathway is taken at different time points during and after anoxia and how this can be influenced by experimental conditions, are still open. In particular, it could be interesting to find out whether the observed small amount of delayed neuronal cell death (see 3.4. page 38) can be reduced by inhibitors of apoptose.

From the preliminary data collected on the effect of NO, it is likely that NT-2 neurons or subpopulations of NT-2 neurons are responsive to NO. The effect of NO on the viability of neuronal cultures during anoxia, alone or in combination with glutamate, could be worth further investigations. The same is true for the effect of ROS which have already been shown to produce an impact on NT-2N cells (Tamagno et al., 2000). Various combinations of these 4 factors (anoxia, glutamate, NO, ROS) or all of them together in NT-2 cells could lead to a new understanding of ischaemic processes.

The NT-2 cell culture system presents very promising characteristics that could allow to do mass screenings of potentially neuroprotective substances. The initial cell expansion and the neuronal differentiation are reasonably simple to be performed with a minimal amount of personnel. The following processes, from cultivation of neurons, to testing of compounds, to readout and evaluation of results could all be performed in a highly automated system. This could greatly facilitate the search for and discovery of new lead substances to be used in therapies of CNS diseases. This is especially true for neuroprotective compounds that could be employed in stroke therapy, yet the NT-2N cell culture system seems to be versatile enough to adapt the screening process to the search for other kinds of therapeutics.

Having capabilities similar to those of neuronal stem cells, NT-2 cells could be suitable for a cell replacement therapy. This kind of therapy could be adopted for diseases of the brain that are connected to a neuronal cell loss in focal points, like stroke or Parkinson. NT-2N have already proven that they can be successfully engrafted into a human CNS (Nelson et al., 2002) and there are indications that the engraftment restores neuronal functions. (Kondziolka et al., 2001). Even though it is not yet known how engrafted NT-2 neurons exert their beneficial effect, restorative brain surgery based on differentiated NT-2 cells could be available in the not too distant future.

7. Acknowlegments/Danksagung

This research project was supported by the 3R foundation (Project No.: 69-99), Münsingen, Switzerland.

The PrP antibody was kindly provided by Andreas Stuke from Deutsches Primatenzentrum (DPZ), Göttingen/Germany. The cGMP antibody was kindly provided by Jan de Vente from Universiteit Maastricht/The Netherlands.

Danksagung

Ich bedanke mich bei Professor Gerd Bicker für die Überlassung des Themas, die ständige Diskussionsbereitschaft und die hervorragende Betreuung.

Ich bedanke mich sehr herzlich bei Saime Tan die mich in die Zellkulturtechniken eingeführt hat, und mir viele wertvolle Tipps zu deren Handhabung gegeben hat.

Bei Thorsten Heins bedanke ich mich für die Hilfe bei Computerproblemen aller Art und beim einscannen von Photos und Dias.

Dr. Michael Stern, Mario Naujock und Christian Brueser-Pieper danke ich für das Korrekturlesen dieser Arbeit. Ohne diese Mithilfe wären noch viel mehr Fehler darin enthalten….

Bei allen aktuellen und ehemaligen Mitarbeitern (und auch zeitweiligen Gästen) der AG Bicker möchte ich mich ganz herzlich für das hervorragende Arbeitsklima und die vielen netten und anregenden Gespräche in den Kaffeerunden bedanken. Es hat vielleicht nicht immer danach ausgesehen, aber auch daraus haben sich eine Reihe von wertvollen Anstößen für diese Doktorarbeit ergeben.

8. Publications

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

- Bicker, G., and Paquet-Durand, F. (2002): “Vom Teratocarcinom zum Neuron: Menschliche Nervenzellen in der Petrischale”, TiHo Forschungsmagazin, 31-34

- Paquet-Durand, F., Tan, S., and Bicker, G. (2003): “Simulation of stroke related damage in cultured human nerve cells.”, 3R Info Bulletin, Vol. 23, www.forschung3r.ch/de/publications/bu23.html

- Paquet-Durand, F., Tan, S., and Bicker, G. (2003): “Turning teratocarcinoma cells into neurons: rapid differentiation of NT-2 cells in floating spheres”, Developmental Brain Research, Vol. 142, 161 – 167

- Paquet-Durand, F., and Bicker, G. (2004): “Hypoxic/ischaemic cell damage in cultured human NT-2 neurons”, Brain Research, submitted

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Curriculum vitae

21. 2. 1968 Born in Kamen/Germany,

1974 – 1978 Attended primary school in Albersloh (Germany)

1978 – 1983 Attended secondary school in Münster/Westf. (Germany)

1983 – 1984 Stayed 1½ year in Paraguay, attended Colegio San Blas in Obligado (Paraguay)

1984 – 1988 Return to Germany, finished secondary school with „Abitur“ in Münster/Westf. (Germany) in June 1988

1988 – 1989 August 1988 – August 1989: military service in the French army

1989 – 1998 Biochemistry studies at the university of Hannover (beginning in winter term 1989)

1993 First exam („Vordiplom“) in April 1993

1993 – 1994 June 1993 – October 1994: Studies of tropical and marine biology at the Universidad Nacional in Heredia (Costa Rica) with the IAS programme of Hannover university.

1. *Work placement (6 months) in pesticide analysis laboratory (LAREP) of the school for environmental research: „Implementation of analysis methods for the pesticides Quinclorac, 2-4D and Propanil in water samples using HPLC.“*
2. *Work placement (6 weeks) at the wildcat rehabilitation center „Profelis“ in Las Flores de Upala / Costa Rica: „Pre – release behavioural studies on Felis wiedii (Margay)“*

1995 - 1996 Practical training in different laboratories (6 – 10 weeks each) on the following subjects:

1. *Isolation of toxic substances from the brazilian fungus Ramaria flavobrunescens, chemical institute of Hannover Veterinary school*
2. *Effects of stimulating and inhibiting agents on the in vitro release of Growth Hormone (GH) and Prolactin (PRL): Example of VIP and somatostatin agonists, INSERM, Unité 159, Paris, France*
3. *Molecular biology of the Kat1 potassium channel of Arabidopsis thaliana and its*

expression in E. Coli, institute for biophysics of Hannover University

4. *Analysis of enzyme activities in Zea mays, Botanical institute of Hannover Veterinary school*
5. *Optimisation in the synthesis of Cephalostatinanaloga, Institute for Organic chemistry of Hannover University*

1997 *Passed the final exams in Biochemistry in December 1997, beginning of the 6 months diploma thesis: „Effects of Salt Stress on enzyme activities in Zea mays"*

1998 Finished biochemistry studies with the diploma in June 1998

1998 - 2003 Doctoral thesis on the subject: „The human NT-2 cell line as *in vitro* model system for the excitotoxic cascade during stroke" (beginning in November 1998)

2003 End of doctoral thesis

Hannover, 7. Juli 2003

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