

Centre for Systems Neuroscience
University of Veterinary Medicine Hannover

The role of synaptic transmission in the
pathophysiology and therapy of neurodegenerative
disease, amyotrophic lateral sclerosis

Dissertation

Submitted in partial fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY
-Ph.D.-

At the centre for Systems Neuroscience Hannover
Awarded by the University of Veterinary Medicine Hannover

by

Daniela Ragancokova
Kosice, Slovakia

Hannover 2009

Department of Neurology and Neurophysiology
Medical University Hannover

Supervisor: Prof. Dr. med. Susanne Petri
PD Dr. med. Klaus Krampfl

1. referee: Prof. Dr. med. Susanne Petri
PD Dr. med. Klaus Krampfl

2. referee: Prof. Dr. med. Claudia Grothe

3. referee: Prof. Dr. med. Christoph Fahlke

External referee: PD Dr. Sibylle Jablonka

Date of final exam October 24th 2009

Funding This work was supported by a Georg-Christoph-Lichtenberg scholarship of the state of Lower Saxony and the Center for Systems Neuroscience.

Publications:

Analysis of Neuroprotective Effects of Valproic Acid on Primary Motor Neurons in Monoculture or Co-cultures with Astrocytes or Schwann Cells. Cell Mol Neurobiol., 2009

Ragancokova, D., Jahn, K., Kotsiari, A., Schlesinger, F., Haastert, K., Stangel, M., Petri, S. and Krampfl, K.

Modulation of synaptic transmission and analysis of neuroprotective effects of valproic acid derivatives in motoneuron.

Cell Mol Neurobiol (submitted)

D. Ragancokova, Y. Song, H. Nau, R. Dengler, K. Krampfl and S. Petri

pH- induced Blocking Effect on Recombined Glycine Receptor Channels (submitted)

Y. Song, F. Schlesinger, D. Ragancokova, R. Dengler and K. Krampfl

Characterization and applications of SV40 large T antigen-immortalized rat ventral mesencephalic neuronal stem cells (submitted)

André Nobre, Ieva Kalve, Konstantin Cesnulevicius, Daniela Rangancokova, Nina Halfer, Andreas Ratzka, Maike Wesemann, Klaus Krampfl, Peter Claus, Claudia Grothe

Influence of an IFN- β therapy on oligodendrocyte proliferation and differentiation by sera from untreated and treated MS patients (submitted)

A. Kotsiari, E. Voss, R. Pul, T. Skripuletz, D. Ragancokova and M. Stangel

Posters:

“Modulation of synaptic transmission and analysis of neuroprotective effects of valproic acid derivatives in rat embryonic motoneuron cultures”

D. Ragancokova, Y. Song, H. Nau, R. Dengler, S. Petri and K. Krampfl

7th European ALS Congress, Research workshop and young investigators meeting, May 2009, Turin, Italy

“Is valproic acid neuroprotective in primary motor neuron culture? “

D. Ragancokova, K. Jahn, A. Kotsiari, L.J. Jin, F. Schlessinger, S. Petri and K. Krampfl

6th International Symposium on Experimental and Clinical Neurobiology, September 2008, Kosice, Slovakia,

“Analysis of neuroprotective effects of valproic acid and the analogues in motor neurons“

D. Ragancokova, F. Schlesinger, K. Jahn, , S. Petri, R. Dengler and K. Krampfl

6th FENS Forum of European Neuroscience, July 2008, Geneva, Switzerland

Oral Presentations:

“Electrophysiological studies and toxicity test of primary motoneuron cultures”

D. Ragancokova, K. Jahn, A. Kotsiari, S. Petri, R. Dengler and K. Krampfl

6th European ALS Congress, Research workshop and young investigators meeting, June 2008, Lisbon, Portugal

Dedicated in love to my dearest family,

Contents

ABBREVIATIONS

CHAPTER I INTRODUCTION **1**

AMYOTROPHIC LATERAL SCLEROSIS (ALS)	1
EXCITOTOXICITY	2
VALPROIC ACID AND ITS DERIVATES	5
MOTOR NEURONS AND GLIAL CELLS	7

CHAPTER II ANALYSIS OF NEUROPROTECTIVE EFFECTS OF VALPROIC ACID ON PRIMARY MOTOR NEURONS IN MONOCULTURE OR CO-CULTURES WITH ASTROCYTES OR SCHWANN CELLS. **8**

ABSTRACT	8
INTRODUCTION	9
MATERIALS AND METHODS	11
RESULTS	14
DISCUSSION	21

CHAPTER III MODULATION OF SYNAPTIC TRANSMISSION AND ANALYSIS OF NEUROPROTECTIVE EFFECTS OF VALPROIC ACID DERIVATES MOTONEURON. **23**

ABSTRACT	23
INTRODUCTION	24
MATERIALS AND METHODS	27
RESULTS	31
DISCUSSION	40

CHAPTER IV GENERAL DISCUSSION **44**

REFERENCE LIST **49**

SUMMARY **59**

ZUSAMMENFASSUNG **61**

ACKNOWLEDGMENTS **63**

ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
SOD1	Superoxid-dismutase 1
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
EPSCs	Excitatory postsynaptic currents
NMDA	N-methyl D-aspartate
MK-801	Cyclohepten-5,10-imine maleate
VPA	Valproic acid
DNA	Desoxy-Ribonucleic Acid
HDAC	Histone deacetylase
3-PHA	3-propylheptanoic acid
R-PE-4-yn	R (+) -2-n-pentyl-4-pentynoic acid
S-PE-4-yn	S (-) -2-n-pentyl-4-pentynoic acid
Gsk-3b	Glycogen synthasekinase-3-b
GABA	Gamma- amino- butyric acid
rHuCNTF	human recombinant ciliary neurotrophic factor
rHuBDNF	human recombinant brain derived growth factor
EPSC	Excitatory Postsynaptic current
IPSC	Inhibitory Postsynaptic current
DIV	Day in vitro
EAAT2	Excitatory amino acid transporter
FCS	Fetal Calf Serum
DAPI	4', 6-diamidino-2-phenylindole
InsP ₃	Inositol-1,4,5-triphosphate
NCAM-PSA	Neuronal Cell Adhesion Molecules-Polysialic Acid
TSA	Trichostanin

CHAPTER I INTRODUCTION

Amyotrophic lateral sclerosis (ALS)

ALS is a late-onset neurodegenerative disorder characterized by a loss of motor neurons in the motor cortex, brainstem, and spinal cord. This results in progressive muscle weakness and wasting and death, usually from respiratory failure, within 2-5 years. It has been recognized that motor neurons might be particularly susceptible to degeneration induced by toxicity because of their size, a somatic diameter of 50-60 μ m with a long axonal process, and the high energy requirements of such a large cell (Heath and Shaw, 2002).

This is a common disease, with an annual incidence rate of 0.4 to 1.76 per 100,000 population. Men are affected somewhat more frequently than women (1.4 to 1). Most patients are more than 50 years old at the onset of symptoms and the incidence increases with each decade of life. The majority of ALS patients has no affected family members and is considered to have sporadic ALS. Familial ALS occurs 5-10% of cases and predominantly has an autosomal dominant inheritance. In 20% of familial cases (which represents only 1-2% of all cases), there are mutations in superoxid dismutase-1 (SOD1) gene on chromosome 21q.

Clinically, the selective motor neuron loss results in progressive paresis of bulbar, respiratory and limb muscles, while sensory and cognitive functions are preserved in most cases. Neurological examination reveals lower motor neuron signs (paresis, muscle atrophy, fasciculations) and upper motor neuron signs (paresis, hyperflexia, Hoffman and Babinski signs, increase muscle tone). At the ultrastructural level several abnormalities have been described in motor neurons from ALS patients: eosinophilic Bunina bodies, ubiquitinated inclusions and neurofilament inclusions. These abnormalities can be present

in both familial and sporadic cases, highlighting the similarity between both forms of the disease. The exact pathogenic mechanism underlying the selective motor neuron death in ALS is not yet elucidated, although a number of possible mechanisms in sporadic ALS and SOD1-linked ALS have been proposed. These include oxidative stress, axonal strangulation from neurofilamentous accumulations, toxicity from intracellular protein aggregates, mitochondrial dysfunction, inflammation, decreased availability of growth factors and excitotoxic death of motor neurons (Van Damme, et al., 2005).

There is no specific treatment for any of motor neuron diseases, and only supportive measures can be utilized. The antiglutamate agent, riluzole appears to slow the progression of ALS, and improve survival in patients with disease of bulbar onset, adding, at best, 3 months to life expectancy (Principle of Neurology, R.D.Adams). Presynaptically it prevents propagation of action potentials via inhibition of sodium channels. Therefore it inhibits also GABAergic but preferentially glutamatergic neurotransmission.

Excitotoxicity

Excitotoxicity is maybe not the newest and most spectacular hypothesis in the ALS field, but it is undoubtedly one of the most robust pathogenic mechanisms. It is not only supported by an impressive amount of indirect evidence, the therapeutic efficacy of riluzole is most likely due to its anti-excitotoxic properties. Excitotoxicity is neuronal degeneration induced by overstimulation of glutamate receptors and this mechanism is thought to contribute to the neuronal damage in stroke, neurotrauma, epilepsy, and many neurodegenerative disorders (Van Damme, et al., 2005). Excitotoxicity can occur when the extracellular glutamate concentration increases or when the postsynaptic neuron becomes

vulnerable to normal glutamate levels (**Fig.1**). In the central nervous system the extracellular glutamate concentration is kept low (approximately 0.6 μM) in spite of a high intracellular concentration of approximately 10 mM and frequent release of glutamate at glutamatergic synapses. Elevation of the extracellular glutamate concentration to 2–5 μM is considered sufficient to cause degeneration of neurons through excessive stimulation of glutamate receptors (Rosenberg, et al., 1992). Elevated extracellular glutamate concentrations can occur when the release from presynaptic terminals is augmented or when the re-uptake from the synaptic cleft is insufficient. In addition, lethal injury to neurons, astrocytes or microglia can lead to the release of the intracellular glutamate content and result in excitotoxic death of surrounding neurons. Acute elevations of glutamate are thought to increase neuronal damage in conditions such as stroke, status epilepticus and neurotrauma, while more chronic and milder elevations of glutamate are believed to induce excitotoxicity in affected neuronal populations in neurodegenerative diseases. Glutamate released from presynaptic terminals acts on post- and presynaptic glutamate receptors. Glutamate receptors are divided into ionotropic glutamate receptors, which are ligand-gated cation channels, and metabotropic glutamate receptors, which are G-protein-coupled receptors that control second messenger systems. The ionotropic receptors can further be subdivided into three classes according to their preferred synthetic agonist (Hollmann and Heinemann, 1994, Seeburg, 1993): AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), NMDA (N-methyl- *D* -aspartate) and KA (kainate) receptors. KA has a high affinity for KA receptors resulting in rapidly desensitizing currents, but is mostly used as an agonist of AMPA receptors since it induces non-desensitizing AMPA receptor currents. AMPA receptors are permeable to Na^+ , K^+ and in a variable degree to Ca^{2+} . Functionally, AMPA receptors are the most important glutamate receptors to mediate fast excitatory transmission. NMDA receptors mediate the late component of excitatory transmission and play a key role in the induction

of synaptic plasticity. The role of KA receptors in physiological and pathological conditions is less clear (Van Den Bosch, et al., 2006).

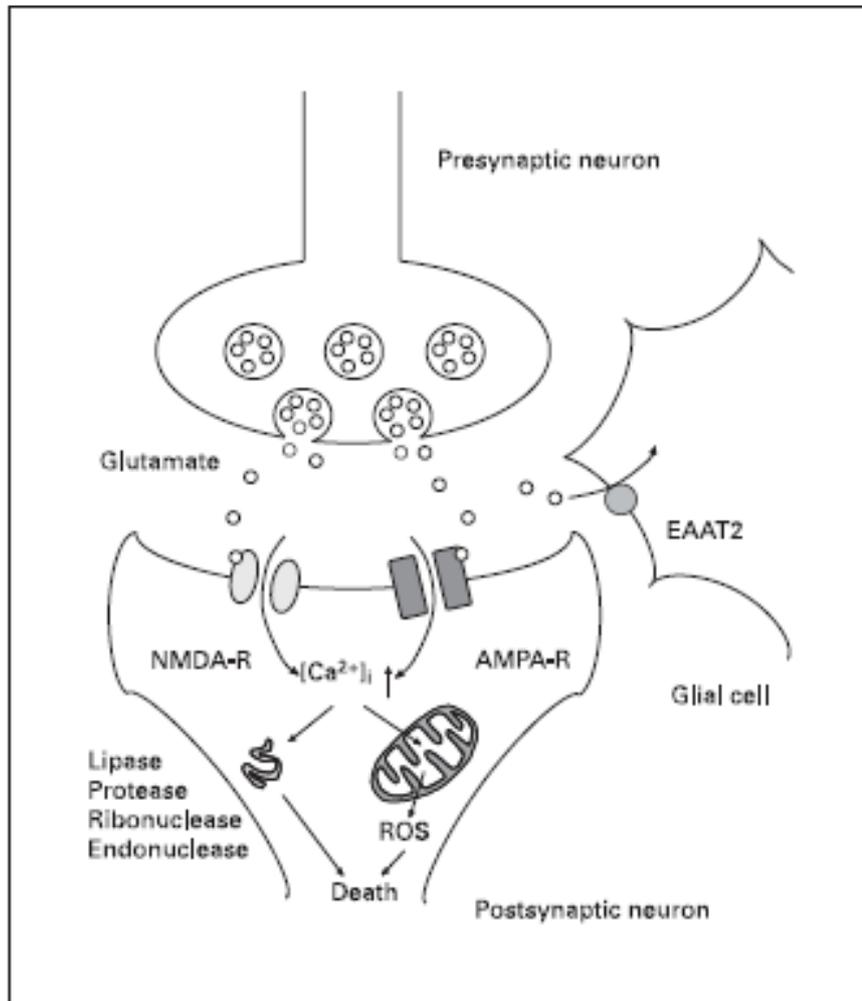


Fig.1. Mechanisms of excitotoxicity. Elevation of the extracellular glutamate concentration, induced by increased release or deficient re-uptake of glutamate gives rise to excessive glutamate receptor stimulation. Changes in properties of glutamate receptors and an increased vulnerability of energetically compromised neurons can also result in excitotoxic damage. Ca^{2+} influx through glutamate receptors (NMDA receptors or AMPA receptors) induces activation of toxic cellular enzymes and mitochondrial dysfunction with the formation of reactive oxygen species (ROS), which results in the death of the postsynaptic neuron (Van Damme, et al., 2005).

Valproic acid and its derivatives

Valproic acid (sodium valproate, VPA) is the most commonly used antiepileptic drug today in the treatment of epilepsy and recently used as a mood stabilizer. In spite of its wide use for many years, the mechanism of VPA action is still not fully understood. Its simple structure of a short-branched fatty acid differs to a great extent from the substituted heterocyclic ring structures characterizing the traditionally used antiepileptic drugs. The antiepileptic action of VPA is probably due to a combination of several effects in the CNS because of its wide spectrum of activity against different types of seizures and status epilepticus. During recent years VPA has also shown its effectiveness in several other neurological and psychiatric disorders. Attention has also been focused on the modification of the main inhibitory neurotransmitter in the brain, (-aminobutyric acid (GABA) by VPA. Impairment of GABAergic inhibitory neurotransmission can lead to convulsions, whereas potentiation of GABAergic activity results in anticonvulsant effects (Loscher, 1989). Favoring the GABAergic hypothesis for VPA, is inhibition of the onset of seizures induced by GABA antagonists (Frey and Loscher, 1978). VPA selectively enhances GABAergic inhibition in the cerebral cortex (Baldino and Geller, 1981). Antiepileptic properties of VPA are also linked with the inhibition of sodium channels and possibly T-type calcium channels (Trojnar, et al., 2004).

During the past years, it has become evident that VPA is also associated with anti-cancer activity. VPA not only suppresses tumor growth and metastasis, but also induces tumor differentiation in vitro and in vivo. Several modes of action might be relevant for the biological activity of VPA:

- (1) VPA increases the DNA binding of activating protein-1 (AP-1) transcription factor, and the expression of genes regulated by the extracellular-regulated kinase (ERK)-AP-1 pathway;
- (2) VPA downregulates protein kinase C (PKC) activity;
- (3) VPA inhibits glycogen synthase kinase-3b (GSK-3b), a negative regulator of the Wnt signaling pathway;
- (4) VPA activates the peroxisome proliferator-activated receptors PPAR α and δ ;
- (5) VPA blocks HDAC (histone deacetylase), causing hyperacetylation.

The findings elucidate an important role of VPA for cancer therapy. VPA might also be useful as low toxicity agent given over long time periods for chemoprevention and/or for control of residual minimal disease (Blaheta and Cinatl, 2002).

Despite VPA's wide spectrum of action, in some cases its use is limited due to specific pharmacokinetics and dangerous adverse effects. These include hepatotoxicity and teratogenicity. Such limitations account for intensive research that has been carried out in order to develop new analogues or derivatives of VPA.

In our studies, we focus on three out of number of synthesized VPA derivatives that have lately been under investigation: 3-propyl-heptanoic acid (3-PHA) and 2 pentyl-4-yn enantiomers (R- and S- PE-4-yn). 3PHA, the non-teratogenic analogue of VPA was identified as a very potent inducer of differentiation without evidence of HDAC-inhibition (Deubzer, et al., 2006). 2 pentyl-4-yn enantiomers possess enantiospecific effects. Thus, the S-PE-4-yn has a high teratogenic potency, whereas the R-PE-4-yn is a moderate teratogen. Moreover, R- but not S-PE-4-yn has been reported to improve spatial learning, whereas only the S-PE-4-yn has been demonstrated to induce depletion of inositol-1,4,5-triphosphate (InsP3) and up-regulation of cyclinD3, thereby inducing growth-cone enlargement and cell cycle arrest, respectively (Eickholt, et al., 2005, Gotfryd, et al., 2007, O'Loinsigh, et al., 2004).

Motor neurons and glial cells

Glial cells constitute 90% of cells in the human nervous system, but comparatively little is known about many of their functions about the beneficial or detrimental role of neuron-glia interactions (Ullian, et al., 2004). Two glial cell types, astrocytes and Schwann cells, ensheath CNS and PNS synapses, respectively. Synaptic glia clear extracellular ions and neurotransmitters from the synaptic cleft and recent studies suggest that astrocytes actively regulate synapse formation, function, and stability. For example, astrocytes in culture sense and respond to neuronal activity by increasing their internal Ca^{2+} concentration, which in turn triggers them to release their own chemical transmitters that regulate neuronal activity (Araque, et al., 1999). Perisynaptic Schwann cells also detect synaptic activity by increasing intracellular Ca^{2+} and respond to this activity by regulating neurotransmitter release (Reist and Smith, 1992, Rochon, et al., 2001). These studies strongly suggest that these two perisynaptic glial cells, astrocytes and Schwann cells are intimately involved in the process of synaptic function.

Strong evidence for the hypothesis that motor neuron degeneration in ALS is non-cell-autonomous comes from experiments with chimeric SOD1 and from in vitro studies in co-cultures of wild type or transgenic rodent embryonic stem cell-derived motor neurons and glial cells: it could be shown that normal motor neurons degenerated in presence of mutated non-neuronal cells, and that vice versa wild type non-neuronal cells were neuroprotective (Clement, et al., 2003, Di Giorgio, et al., 2007, Nagai, et al., 2007)

Chapter II Analysis of neuroprotective effects of valproic acid on primary motor neurons in monoculture or co-cultures with astrocytes or Schwann cells.

Abstract

Chronic dysregulation of the intracellular Ca^{2+} homeostasis (excitotoxicity) is thought to contribute to the development of motor neuron diseases. Valproic acid (VPA) is widely used as an antiepileptic drug and acts mainly by inhibition of sodium channels and by enhancing the level of the inhibitory neurotransmitter γ -aminobutyric acid (GABA). Neuroprotective capacities of VPA are supposed to arise also from the inhibition of histone deacetylases. We investigated the viability of highly purified rat embryonic motor neurons cultured on glial feeder layers, composed of either astrocytes or Schwann cells, or in the absence of glia, monoculture in presence of VPA and/ or kainate (KA) using immunocytochemistry and calcium imaging. A significant effect of the culture- and co-culture conditions on the viability of motor neurons in our in vitro model of excitotoxicity was detected. The neuroprotective effect of VPA on primary embryonic motor neuron cultures was not proven. A functional interaction between VPA and KA occurred during the first ten days in culture.

Introduction

The etiopathogenesis of motor neuron disorders such as amyotrophic lateral sclerosis (ALS) still remains largely elusive. Chronic excitotoxicity is believed to play a major pathogenic role. It refers to the abnormal activation of glutamate receptors which leads to a final common pathway of neuronal death (Heath and Shaw, 2002). Several lines of evidence indicate that α -amino-3-hydroxy-5-methyl-4-isoxasole propionic acid (AMPA) and kainate (KA) receptors may be of pathogenic importance in ALS. Besides the antiglutamatergic agent Riluzole with marginal neuroprotective effects, no therapeutic options have been developed to halt the rapidly progressive degeneration of motor neurons in ALS.

Valproic acid (2-propylpentanoic acid, VPA), a widely used antiepileptic and mood-stabilizing drug has recently been considered to provide neuroprotective effects (Coyle and Duman, 2003). Although the mode of action of VPA as a mood stabilizer has not been fully elucidated, inhibition of glycogen synthase kinase 3-b (GSK-3b) and upregulation of Bcl-2, an antiapoptotic protein, appear to be involved (Eldar-Finkelman, 2002, Leng, et al., 2008). Antiepileptic properties of VPA could be related to its ability to enhance γ -aminobutyric acid (GABA) transmission (Deckers, et al., 2000, Loscher, 2002). VPA has further been shown to be an inhibitor of histone deacetylases (HDACs) and could lead to up-regulation of antioxidant and antiapoptotic genes as it has already been shown for other HDAC inhibitors such as sodium phenylbutyrate in transgenic ALS mice (Petri, et al., 2006, Ryu, et al., 2005). VPA and other HDAC inhibitory drugs have also been considered for treatment of another motor neuron disease, as spinal muscular atrophy (Hahnen, et al., 2006). In order to address a potential neuroprotective role of VPA in motor neurons, we first studied the interactions of highly purified populations of developing neurons under different culture conditions. Glial cells constitute 90% of cells

in the human nervous system, but comparatively little is known about many of their functions about the beneficial or detrimental role of neuron-glia interactions (Ullian, et al., 2004). Strong evidence for the hypothesis that motor neuron degeneration in ALS is non-cell-autonomous comes from experiments with chimeric SOD-1 and from in vitro studies in co-cultures of wild type or transgenic rodent embryonic stem cell-derived motor neurons and glial cells: it could be shown that normal motor neurons degenerated in presence of mutated non-neuronal cells, and that vice versa wild type non-neuronal cells were neuroprotective (Clement, et al., 2003, Di Giorgio, et al., 2007, Nagai, et al., 2007)

In a second attempt, we investigated the effects of co-application of VPA together with KA in our different co-culture systems. Excitotoxicity was induced by KA which is known to trigger a selective rise in cytoplasmic Ca^{2+} concentration due to the opening of AMPA/KA-gated glutamate receptor channels (Hollmann, et al., 1991) and subsequently activates mechanisms such as reactive oxygen species-formation and mitochondrial Ca^{2+} overload in motor neurons (Arundine and Tymianski, 2003, Carriedo, et al., 2000, Vandenberghe, et al., 2000). Long- and short-term incubation of the cells with VPA as well as dose-dependency of VPA- and KA- effects was tested under the hypothesis that VPA could be a suitable neuroprotective drug for the prevention of chronic motor neuron death.

Materials and Methods

Primary culture

Purification of motor neurons was performed as previously described (Haastert, et al., 2005). For harvesting of cells, a minimal number of rats were housed and sacrificed according to German law on animal care. Lumbar ventral spinal cords were dissected from Sprague-Dawley rat embryos (gestational age: E14/15). After tissue dissociation the motoneurons were enriched by gradient density centrifugation using OptiPrep (Sigma-Aldrich, Steinheim, Germany). Culture medium (Neurobasal medium, Gibco Invitrogen, Germany; with 2% (v/v) horse serum; 2% (v/v) B27-supplement, Gibco Invitrogen, Germany; 0.5mM l-glutamax; 5 ng/ml rHuBDNF Peprtech, Germany and 5 ng/ml rHuCNTF Peprtech, Germany) was added to the resulting pellet. Highly enriched motor neurons were seeded on glass coverslips either pre-incubated first with polyornithin (diluted 1:1000, Sigma) and then with laminin1 (conc. 2, 5µg/ml, Invitrogen) or pre-incubated first with poly-L-lysine (diluted 1:1000, Sigma) and then plated with primary astrocyte prepared from newborn Sprague-Dawley rat cerebra as previously described (McCarthy and de Vellis, 1980, Stangel and Bernard, 2003) or Schwann cell cultures prepared from newborn Sprague-Dawley rat sciatic nerves as previously described (Haastert, et al., 2005). Purified motor neurons were added after 24 hours and cultures were fed every second day with culture medium for motor neurons.

Immunocytochemistry

Cells were fixed with 4% *para*-formaldehyde (PFA). Glial feeder layers were stained with antibodies against S100 (1:200, Dako, Denmark) as a marker for Schwann cells, microtubule-associated protein 2 (1:1000, MAP2) as neuronal marker (Chemicon, Germany) and glial fibrillary acidic protein (1: 400, GFAP) to identify astrocytes (Sigma, Germany).

Motor neuron survival was quantified by double-staining with antibodies against β III-tubulin (1:140, Upstate, USA) which detects all neurons, and SMI 32 (1:1500, Covance, USA) which is specific for motor neurons from embryonic spinal cord preparations. The nuclei of all cultured cells were stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma). Cell counts were done in 12 wells of 4-6 different preparations per each condition. Counting was performed using an ocular counting grid to quantify all cells on a horizontal stripe crossing the complete well on a fluorescence microscope BX-70.

Toxicity experiments

Motor neuron-rich cell fractions were prepared as described above and seeded on laminin or on glial feeder layers of neonatal rat astrocytes or Schwann cells at an average density of 3×10^4 cells/ cm^2 . After 10 day in vitro (DIV 10), cell-cultures were incubated for 48 hours with KA and VPA in different concentrations (30 μ M, 100 μ M, 300 μ M and 1 μ M, 10 μ M, 100 μ M, respectively) to test for dose-dependent effects on motor neuron survival. Furthermore, we performed neurotoxicity experiments adding 10 μ M VPA in combination with 30 μ M KA. After 48 hrs of KA/VPA exposure, cells were fixed with PFA and motor neuron survival was assessed as described above.

Calcium Imaging

For calcium imaging studies, all cultures were incubated for 20 minutes at room temperature with the membrane permeable ester form of the high-affinity ratio-metric calcium dye FURA 2 AM (4 μ M, Sigma, Germany). Cells chosen for measurements had to exceed at minimum a diameter of 20 μ m to be considered as motor neurons. Fluorescent images were obtained at temporal and spatial resolution (5Hz, TILL Vision Imaging System by Photonics, Munich, Germany). Standard extracellular solution contained

HEPES 11.6 mM, Na⁺ 129.1 mM, Cl⁻ 143.8 mM, K⁺ 5.9 mM, Mg²⁺ 1.2 mM, Ca²⁺ 3.2mM and glucose 10.0 mM at pH 7.3 (NaOH). Imaging experiments were conducted at room temperature and after obtaining baseline values, local perfusion through the applicator was started using either short 5 s pulses of 30 μ M KA or 100s of 10 μ M VPA. For the analysis of Ca²⁺ transients, background subtraction was used and subcellular regions of interest were defined over the cytosol, nucleus and neurite

Statistical analysis

All results were expressed as mean \pm S.E.M. GraphPad Prism3.0 software (www.graphpad.com) was used for statistical evaluation. Comparisons between different conditions were performed using one-factor analysis of variance (ANOVA) followed by Kruskal-Wallis test.

Results

We first investigated the ability of central and peripheral glial cell-feeder layers to improve the survival of developing motor neurons as compared to the monoculture condition. Motor neurons developed increasingly large somata with an extensive dendritic arborisation and generally one longer axon-like process. All SMI32 positive motor neurons in 10-days-old (DIV 10) cultures had cell bodies larger than 20 μ m and a distinct morphology, fulfilling the criteria used by Carriedo, Yin et al. (**Fig.1 A, B, D, F**). In co-cultures, there were larger neuronal networks as compared to monocultures. Astrocytes were determined by anti-GFAP-immunostaining (Vandenberghe, et al., 1998) and appeared as irregular cells with thick and short processes (**Fig.1 C**). An antibody against the calcium-binding protein S100 which is detected in the cytoplasm of Schwann cells (Schumacher, et al., 1993) was used for labelling of the motor neuron-Schwann cell co-cultures (**Fig.1 E**).

Motor neuron survival in the three different culture conditions was examined by counting the proportion of SMI 32-positive motor neurons following double-immunostaining for β III-tubulin. The quantitative analysis in control groups showed a significant increase of the number of motor neurons in both co-culture systems. On the Schwann-cell feeder layer, survival of motor neurons was highest (40.5 \pm 4.8 % of relative number of motor neuron survival, $p < 0.001$, 10 different counts per experiment, $n=6$) as compared to the motor neuron-astrocyte co-culture and monoculture (35.5 \pm 3.9 %, $p < 0.001$ and 20.99 \pm 2.7 %, respectively).

Dose-dependent effects of VPA on developing spinal motor neurons under the three different culture conditions were assessed using three different concentrations of VPA (1, 10, 100 μ M) starting from DIV 10 for 48 hours. There was no considerable positive effect of VPA on the relative number of motor neurons with 1 or 10 μ M VPA as shown in **Fig.2 A**. At a VPA concentration of 100 μ M, there was a reduction in the motor neuron proportion

that reached significance in case of Schwann cell co-cultures and monocultures ($p < 0.01$; $10.6 \pm 5.22\%$ and $p < 0.05$; $7.3 \pm 2.6\%$ difference between motor neuron survival in control and $100\ \mu\text{M}$ VPA conditions, respectively) while in the astrocyte co-culture system, there was just a trend towards lower relative motor neuron numbers (not significant; $4.0 \pm 3.8\%$ difference between control and $100\ \mu\text{M}$ VPA groups).

To induce excitotoxicity, 10-day-old cultures were exposed to KA in concentrations ranging from $30 - 300\ \mu\text{M}$ for 48 h. (**Fig.2 B**) and a dose-dependency was observed, as previously described. Interestingly, after exposure to $30\ \mu\text{M}$ KA, relative numbers of motor neurons on astrocyte feeder-layers were significantly higher than on Schwann cells ($11.1 \pm 3.6\%$ reduction of the relative number of motor neurons cultured on astrocytes, $p < 0.05$, as compared to $14.6 \pm 3.4\%$ reduction of motor neurons seeded on Schwann cells, $p < 0.001$). Monocultures showed just $6.1 \pm 2.4\%$ ($p < 0.05$) reduction of the motor neurons exposed to $30\ \mu\text{M}$ KA compared to the untreated control group.

The results suggest that astrocytes in our culture system provided a better neuroprotection under KA exposure even though the Schwann cell co-culture condition led to the highest absolute motor neuron viability (**Fig.2 B**).

In a next step, $30\ \mu\text{M}$ KA was applied together with $10\ \mu\text{M}$ VPA for 48 hours to test for its possible neuroprotective effect. There was no significant difference between the relative numbers of motor neurons after exposure to $30\ \mu\text{M}$ KA alone as compared to the simultaneous addition of VPA (**Fig.3**). In the monoculture system, co-application of KA/VPA did not show higher motor neuron survival as compared to KA-exposure alone ($15.08 \pm 2.2\%$ relative number of motor neurons exposed to KA/VPA compared to $14.9 \pm 2.1\%$ motor neurons survival applying KA). In the two co-culture conditions, there was a slight but not significant protective effect of VPA between motor neurons survival under KA/VPA incubation to KA incubation alone in both coculture systems. Thus, in presence of a glial cell feeder layer VPA develops a marginal neuroprotective effect against KA-

induced neurotoxicity which is not seen in the absence of glial cells in the monoculture condition (**Fig.4**).

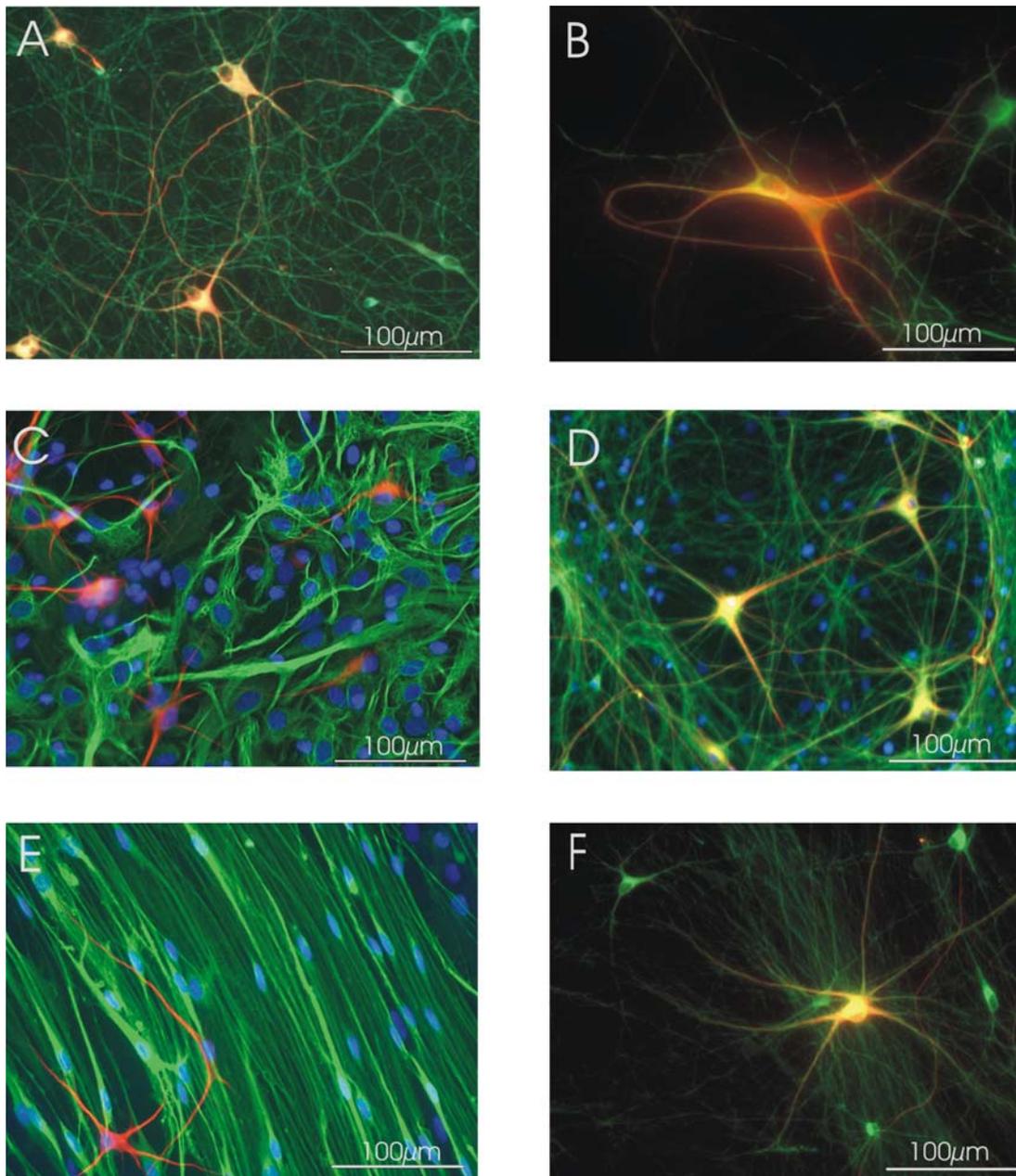


Fig. 1 Immunocytochemical analysis of the neuronal/glial cell populations. Photographs show immunolabelled representative cultures at DIV 10 on glial free feeder layer, monoculture (**A,B**), on astrocytes layer (**C,D**) and on Schwann cells layer (**E,F**). Neuronal cells were double-stained using antibodies against β III-tubulin (green fluorescence) and SMI-32, non-phosphorylated neurofilament H/M (red fluorescence) (**A, B, D, F**). (**C**) Astrocytes demonstrating GFAP-immunoreactivity (green) neighbouring neurons stained by MAP2 (red). (**E**) Schwann cells immunopositive for S100 (green) and MAP2 antibody-labelled neurons. Stained nuclei of cultured cells appear in blue (DAPI). Scale bars =100µm.

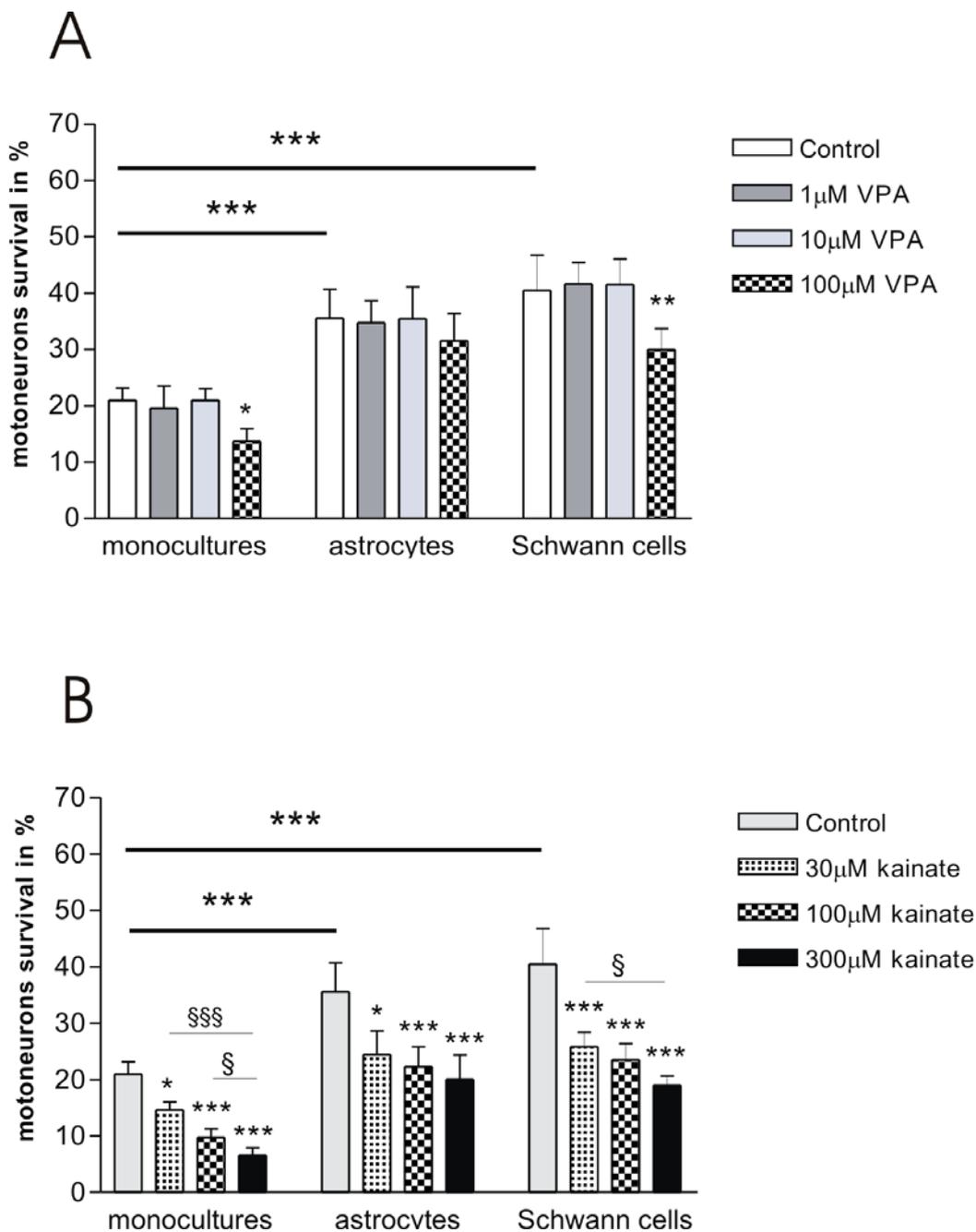


Fig. 2 Quantification of cultured motor neurons. Immunocytochemical analysis of monocultures (n=4) or neuronal-glia co-cultures (n=6) on astrocytes or Schwann cells at DIV 12 revealed the percentage of motor neurons (immunopositive for SMI32) in the β III-tubulin-immunoreactive total neuronal cell fraction. In all conditions Schwann cells and astrocytes increased survival of motor neurons. **(A)** 48 hours application of VPA in different concentrations showed dose-dependency of motor neuron survival, 100µM VPA has toxic effect for developing motor neurons. **(B)** The neurotoxic effect of KA is AMPA/KA mediated and dose-dependent. Astrocyte feeding layers show mild neuroprotective effect to neurons in case of application of KA. Values represent mean \pm S.E.M., *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

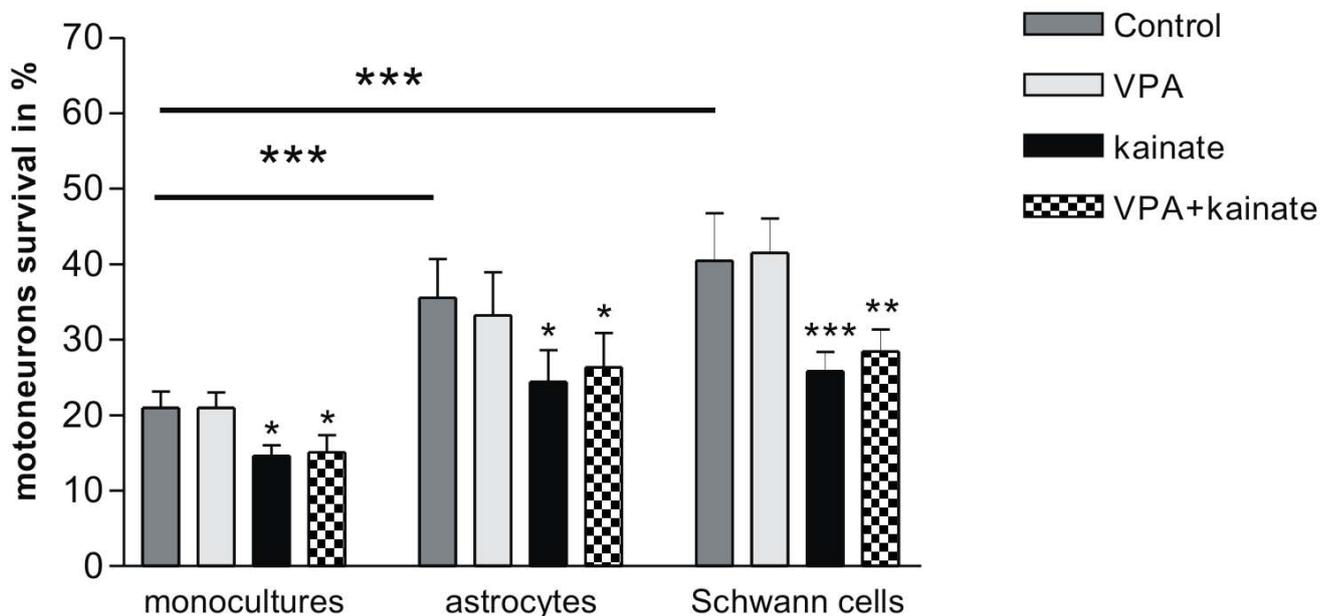


Fig. 3 Test for a neuroprotective effect of VPA in presence of KA. Incubation of motor neuron/glia cultures for 48 hours with 10 μ M VPA and 30 μ M KA. The motor neuron specific viability in presence of KA was significantly reduced in monocultures as well as in co-cultures. However, co-application of VPA leads to a slight increase of neuronal survival in co-cultures. Values represent mean \pm S.E.M., *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

To address the question how motor neurons react upon short-term exposure to KA in presence or absence of VPA at the physiological level, we performed calcium imaging experiments (**Fig.4**). Cultures <DIV 10 were observed to be non-spontaneously active, i.e. they did not show occurrence of spontaneous intracellular Ca^{2+} transients but Ca^{2+} transients could be elicited by a pulse-wise application of KA. Starting at DIV 10, large cells (diameter > 20 μM) which were considered to be motor neurons, were spontaneously active and showed quite regular Ca^{2+} transients occurring at a frequency around 0.02 to 0.07 Hz (97% of 42 observed events in 65 cells). In non-spontaneously active cultures we applied short 5 s pulses of 30 μM KA for three times. Upon that Ca^{2+} transients were observed in all three culture conditions (monoculture, astrocytes, Schwann cells). Before the 3rd KA pulse, 10 μM VPA was applied for 100s (**Fig.4 A, B, C**). Non-spontaneously active motor neurons in absence of glial cells showed a higher vulnerability to Ca^{2+} transients during the application of VPA over the cytosol, nucleus and neurite. 75% of the evaluated cells showed an intracellular Ca^{2+} overload after the 3rd KA pulse followed by no reaction of the Ca^{2+} level, which could indicate neuronal cell death. In 25% of the surviving motor neurons the intracellular Ca^{2+} never returned to the baseline (**Fig.4 A**).

Non-spontaneously active motor neurons on glial feeder layers under VPA incubation showed higher intracellular Ca^{2+} transients with a rising phase of around 1 s and a slower decay after the 3rd KA exposure (**Fig.4 B, C**). However, cytosolic Ca^{2+} concentration levels after KA application were 2 to 4 times higher in motor neurons cultured on Schwann cell feeder layers than on astrocytes, while the intracellular Ca^{2+} concentration always returned to the baseline in both co-culture systems. VPA application did not alter the spontaneous Ca^{2+} transients regarding frequency and shape (**Fig.4 D, E, F**).

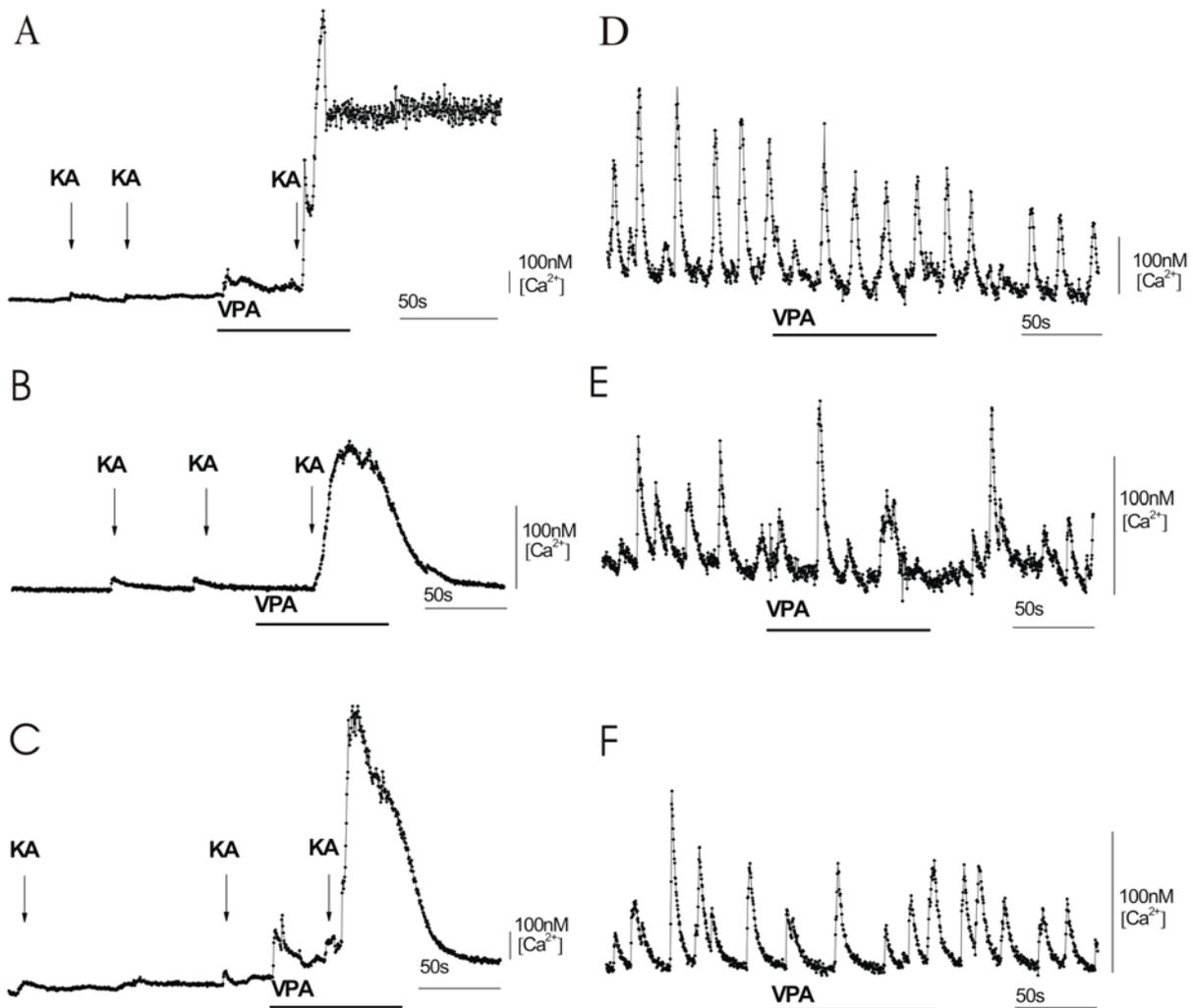
Non-spontaneously activeSpontaneously active

Fig. 4 Microfluorometric Ca^{2+} measurements monitoring cytosolic calcium transients. Non-spontaneously active cultures (< DIV 10, **A**, **B**, **C**) were exposed to two 5s pulses of $30\mu\text{M}$ KA followed by application of $10\mu\text{M}$ VPA (100s) during which a third pulse of KA was applied. Spontaneously active cultures (> DIV 10, **D**, **E**, **F**) were exposed to $10\mu\text{M}$ VPA for 100s. **(A)** Motor neuron cultures in absence of glial cells showed calcium influx upon application of KA. A successive short stimulation with KA in presence of VPA led to an excessive rise in intracellular Ca^{2+} levels and motor neuron cell death. **(B)** Motor neurons seeded on astrocytes and **(C)** on Schwann cells. Motor neurons cultured on astrocytes showed lower Ca^{2+} influx comparing to motor neurons in presence of Schwann cells. On glial feeding layers the intracellular Ca^{2+} concentration in motor neurons always returned to the baseline. In spontaneously active cells no direct effect of the VPA application on calcium transients was visible (**D**, **E**, **F**).

Discussion

By studying the viability of highly purified populations of rat embryonic spinal motor neurons in the absence or presence of glial cells (astrocytes vs. Schwann cells) in vitro, we could show that glial feeding layers significantly enhance the number and survival motor neurons with a trend towards a more pronounced effect of Schwann cells as compared to astrocytes. These findings are in line with the previously established observation that Schwann cells and astrocytes in co-cultures provide a trophic source for motor neurons and allow physiological cell-cell interactions during neurite-outgrowth in vitro (Mirsky, et al., 2002, Pennica, et al., 1996). In both the monoculture and the two different co-culture conditions, brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) were added to the media to prevent degeneration of motor neurons as previously described (Sendtner, et al., 1992, Sendtner, et al., 1992). The differences in cell viability can therefore not be attributed to a lack of growth factors in the media but rather to additional neuroprotective effects provided by the different glial feeder layers.

Selective vulnerability of motor neurons towards excitotoxic stress has been proposed as a central pathomechanism in motor neuron diseases such as ALS (Rao and Weiss, 2004, Van Den Bosch, et al., 2006). We showed dose dependent neurotoxic effects of the glutamate receptor agonist KA in our three different motor neuron culture systems which validate them as a proper tool to study excitotoxicity in vitro and to screen potentially neuroprotective compounds. In contrast to the data from Vandenberghe and colleagues where kainate-induced neuronal death in a motor neuron-enriched co-culture system was potentiated by astrocytic glial cells, we observed a protective effect of astrocyte feeder layers against KA excitotoxicity (Vandenberghe, et al., 1998). Most probably, the addition of growth factors to our culture medium contributes to the contradictory results.

Addressing the presumed neuroprotective capacities of VPA in our in vitro system, we observed that lower VPA doses showed neither protective nor toxic capacities. Higher concentrations ($>100\mu\text{M}$), were even considerably toxic for the cells. This confirms the data of Sugai and colleagues, where cultured tissues became thin and fragile after exposure to high concentrations of VPA (Sugai, et al., 2004). Supporting the observation of rather deleterious than protective VPA effects, the HDAC inhibitory capacities of VPA have been suggested to lead to induction of embryonic malformations (Eikel, et al., 2006). We demonstrated that VPA did not exert significant protective effects against KA-excitotoxicity as a major cause of neuronal death via exaggerated Ca^{2+} -influx through AMPA receptor channels (Grosskreutz, et al., 2007, Kawahara, et al., 2003).

To further assess this mechanism in our culture system, we performed calcium imaging experiments with and without KA and/or VPA exposure. Motor neurons in general display a significantly higher occurrence of spontaneous Ca^{2+} transients than non-motor neurons suggestive of increased AMPAergic synaptic input (Jahn, et al., 2006). This correlates to the fact that motor neurons appear to have AMPA receptors with higher relative Ca^{2+} -permeability than other neuronal populations. In our cultures, this spontaneous activity of motor neurons occurred after DIV 10 and was not influenced by short term application of VPA. In cells younger than DIV 10, we used KA to elicit Ca^{2+} influx. Our observation of highest KA sensitivity of motor neurons in monoculture further supports the notion that surrounding glial cells can protect motor neurons against excitotoxic stimuli. The application of VPA to the co-cultures in this experimental paradigm was again not protective against KA exposure but even increased motor neuron vulnerability. Altogether these data show that primary rodent motor neurons can not be protected by VPA against KA-induced toxicity. Our calcium imaging data indicate that the observed toxic effects of VPA on motor neurons can at least in part be attributed to an increased Ca^{2+} influx triggering excitotoxicity.

Chapter III Modulation of synaptic transmission and analysis of neuroprotective effects of valproic acid derivatives motoneuron.

Abstract

ALS is a devastating neurodegenerative disorder for which no effective treatment exists. There is some evidence for neuroprotective effects of valproic acid (VPA), an established drug in the long-term therapy of epilepsy, due to its influence on a variety of intracellular signalling pathways. The beneficial effects, however, are limited due to the adverse effects of VPA. To overcome this problem, a number of VPA derivatives with fewer side effects have been synthesized. In the present study, we investigated the viability of highly purified embryonic motoneurons cultured on glial feeder layers, composed of either astrocytes or Schwann cells, or in monoculture, in presence of VPA and its three derivatives 3-propyl-heptanoic acid (3-PHA), PE-4-yn enantiomers (R- and S- PE-4-yn). An excitotoxic stimulus, kainate (KA), was added at day in vitro 9 (DIV9) and the neuroprotective effect of either simultaneous incubation (DIV9) or pre-incubation (DIV1) of VPA and its derivatives was tested. The survival of motoneurons under simultaneous application of KA and VPA derivatives was not remarkably increased. Pre-incubation with VPA and even more with the derivatives before the addition of KA, however, significantly reduced their vulnerability against the KA-induced neurotoxic effect. These data suggest that the neuroprotective capacities of VPA and its three derivatives tested here drastically increase when they are added several days before the excitotoxic stimulus. The PE-4-yn enantiomers could be identified as particularly promising neuroprotective agents with a possibly more favourable side-effect profile than VPA. Using whole cell patch clamp recordings, their protective capacity could be in part attributed to an increase of inhibitory postsynaptic transmission and was further dependent on the co-culture conditions.

Introduction

Amyotrophic lateral sclerosis (ALS) is a multifactorial disease, in which oxidative stress, mitochondrial damage, excitotoxicity, protein aggregation, and impairment of axonal transport play a role, and a multisystem disease, in which damage to motoneurons in the ventral horn of the spinal cord, brain stem and primary motor cortex is linked to damage to nonneuronal cells (astrocytes, microglia, muscle cells) (Bendotti and Carri, 2009). Besides the antiglutamatergic agent Riluzole with marginal neuroprotective effects, no therapeutic options have been developed to halt the rapidly progressive degeneration of motoneurons in ALS. Valproic acid (sodium valproate, VPA), one of the most frequently used antiepileptic drugs, has also been established in the treatment of bipolar disorders, neuropathic pain, and as a migraine prophylaxis. Antiepileptic properties of VPA are related to an increase of the level of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), with acute administration causing a 15-45% increase of GABA (Johannessen, 2000). Although the underlying mechanisms are unclear, a growing body of evidence suggests that VPA has neuroprotective and neurotrophic capacities (Chen, et al., 2006, Sugai, et al., 2004). VPA has also been shown to possess tumor suppressor activity which is in part mediated by inhibition of histone-deacetylases (HDACs) (Blaheta, et al., 2005) and could lead to up-regulation of antioxidant and antiapoptotic genes as it has already been shown for other HDAC inhibitors such as sodium phenylbutyrate in transgenic ALS mice (Petri, et al., 2006, Ryu, et al., 2005). Despite its wide spectrum of action, the clinical use of VPA in some cases is limited due to specific pharmacokinetics and dangerous adverse effects. These include hepatotoxicity and teratogenicity. Such limitations have induced intensive research in order to develop novel less toxic analogues or derivatives of VPA. In the present study, we focus on three out of a number of

synthesized VPA derivatives that have lately been under investigation: 3-propyl-heptanoic acid (3-PHA) and PE-4-yn enantiomers (R- and S- PE-4-yn). 3PHA, a non-teratogenic analogue of VPA was identified as a very potent inducer of cell-differentiation without evidence of HDAC-inhibition (Deubzer, et al., 2006). 2 pentyl-4-yn enantiomers possess enantiospecific effects: S-PE-4-yn is highly teratogenic, whereas R-PE-4-yn is a moderate teratogene. Moreover, R- but not S-PE-4-yn has been reported to improve spatial learning, whereas only S-PE-4-yn has been demonstrated to induce depletion of inositol-1,4,5-triphosphate (InsP3) and up-regulation of cyclin D3, thereby inducing growth-cone enlargement and cell cycle arrest, respectively (Eickholt, et al., 2005, Gotfryd, et al., 2007, O'Loinsigh, et al., 2004).

In the present study, we intended to address the neuroprotective effects of VPA and these derivatives and to test for a potential correlation with their pharmacological effects on synaptic activity. We performed simultaneous incubation of rat primary motoneurons together with a neurotoxic agent (kainate, KA) or pre-incubation of the cells with the tested substances before the addition of KA in three different culture conditions (motoneurons in monoculture or co-culture with astrocytes or Schwann cells). Selective vulnerability of motoneurons towards excitotoxic stress is believed to play a major pathogenic role in motoneuron diseases such as ALS (Rao and Weiss, 2004, Van Den Bosch, et al., 2006). KA induces mild excitotoxicity which is known to trigger a selective rise in cytoplasmic Ca^{2+} concentration due to the opening of AMPA/KA-gated glutamate receptor channels (Hollmann, et al., 1991). Strong evidence for the hypothesis that motoneuron degeneration in ALS is non-cell-autonomous comes from experiments showing that normal motor neurons degenerated in presence of mutated non-neuronal cells, and that vice versa wild type non-neuronal cells were neuroprotective (Clement, et al., 2003, Di Giorgio, et al., 2007, Nagai, et al., 2007). In lien with these findings, we have recently shown that astrocyte- and Schwann cell-feeder layers led to the higher absolute survival rates of

motoneurons and that co-culture of motoneurons with astrocytes was protective against the neurotoxic effect of KA (Ragancokova, et al., 2009). In the present study, we first observed the survival of highly purified populations of developing neurons following application of the four tested substances simultaneously with KA. In a second attempt we pre-incubated motoneurons with the tested substances before adding KA. To further characterize the mechanism underlying the positive effects of a pre-incubation with VPA-derivates, we performed whole-cell patch-clamp recordings of motoneurons in all 3 culture conditions and analyzed changes in spontaneous synaptic transmission.

Materials and Methods

Primary culture

Purification of motoneurons was performed as previously described (Haastert, et al., 2005). For harvesting of cells, a minimal number of rats were housed and sacrificed according to German law on animal care. Lumbar ventral spinal cords were dissected from Sprague-Dawley rat embryos (gestational age: E14/15). After tissue dissociation the motoneurons were enriched by gradient density centrifugation using OptiPrep (Sigma-Aldrich, Steinheim, Germany). Culture medium (Neurobasal medium, Gibco Invitrogen, Germany; with 2% (v/v) horse serum; 2% (v/v) B27-supplement, Gibco Invitrogen, Germany; 0.5mM l-glutamax; 5 ng/ml rHuBDNF Peprtech, Germany and 5 ng/ml rHuCNTF Peprtech, Germany) was added to the resulting pellet. Highly enriched motoneurons were seeded on glass coverslips either pre-incubated first with polyornithin (diluted 1:1000, Sigma) and then with laminin1 (conc. 2, 5µg/ml, Invitrogen) or pre-incubated first with poly-L-lysine (diluted 1:1000, Sigma) and then plated with primary astrocyte prepared from newborn Sprague-Dawley rat cerebra as previously described (McCarthy and de Vellis, 1980) or Schwann cell cultures prepared from newborn Sprague-Dawley rat sciatic nerves as previously described (Haastert, et al., 2005). Purified motoneurons were added after 24 hours and cultures were fed every second day with culture medium for motoneurons.

Neurotoxicity experiments

Motor neuron-rich cell fractions were prepared as described above and seeded on laminin or on glial feeder layers of neonatal rat astrocytes or Schwann cells at an average density

of 3×10^4 cells/ cm^2 . Simultaneous incubation of DIV9 cell cultures was performed together with $30 \mu\text{M}$ KA and with $10 \mu\text{M}$ of VPA (a concentration which had previously shown the best neuroprotective effect) (Ragancokova, et al., 2009) or with $10 \mu\text{M}$ of the derivatives: 3-propyl-heptanoic acid (3-PHA), R(+)- and S(-)-2-n-pentyl-4-pentynoic acid (R- and S-PE-4-yn). In a second set of experiments, cells were pre-incubated from DIV1 with VPA, 3-PHA; R- and S- PE-4-yn. At DIV9, $30 \mu\text{M}$ KA was added to the cells for 48 hours. Following incubation, cells were fixed with 4% *para*-formaldehyde (PFA) at DIV11 and motoneuron survival was assessed by immunocytochemical analysis: Anti- β III-tubulin (1:140, Upstate, USA) was used to detect all neurons, an antibody against SMI32 (1:1500, Covance, USA) revealed the percentage of motoneurons. Glial feeder layers were stained with antibodies against S100 (1:200, Dako, Denmark) as a marker for Schwann cells and glial fibrillary acidic protein (1: 400, GFAP) to identify astrocytes (Sigma, Germany). The nuclei of all cultured cells were stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma, Germany). Cell counts were done in 12 wells of 3-5 different preparations per each condition. Counting was performed using an ocular counting grid to quantify all cells on a horizontal stripe crossing the complete well on a fluorescence microscope BX-70.

Electrophysiology, whole-cell patch clamp recordings

Whole- cell recordings were made from motoneurons exceeding at minimum a diameter of $20 \mu\text{m}$ (Jahn, et al., 2006). Pipettes were placed on the soma with a tip resistance of 8-10 $\text{M}\Omega$ filled with intracellular solution containing (in mM): 140 KCl, 11 EGTA, 10 Hepes, 10 glucose, 2 MgCl_2 , 1 CaCl_2 . The osmolarity was adjusted to 340mosm/L with mannitol. For the experiments the culture medium was replaced by pseudoextracellular solution containing (in mM) 162 NaCl, 5.3 KCl, 2 CaCl_2 , 0.85 NaHPO_4 , 0.22 KH_2PO_4 , 15 Hepes and 6.11 glucose. The pH of both solutions was initially adjusted to 7.4. Cells were

perfused with extracellular solution with 2 ml/min at room temperature which was prepared freshly before each experiment. After formation of a high-resistance seal ($>1\text{G}\Omega$) and break-in, only neurons with input resistance of $>100\text{M}\Omega$ were used for subsequent analysis. Whole-cell voltage signals were clamped and held at -70mV using Axopatch 200B patch clamp amplifier (Axon Instruments, Foster city, CA, USA). Ensemble currents were sampled with 20 kHz using a Digidata 1200 Interface and the pCLAMP6 software suit on a PC (Axon Instruments, Foster City, CA, USA). For further analysis data were filtered at 5 kHz. For any tested condition at least three independent recordings from three different preparations were performed. The GluR subtypes mediating EPSCs were identified by application of specific blocking compounds CNQX (6-cyano-7-nitroquinoxaline-2, 3- dione; $10\mu\text{M}$) and MK-801 (Dizocilpine; $100\mu\text{M}$) specific for AMPARs (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate-receptors) and NMDARs (N-methyl-D-aspartic acid-receptors), respectively. GABA receptor-mediated channels were specifically blocked by Bicuculline ($10\mu\text{M}$). All blockers were purchased from Sigma-Aldrich, Germany. Baseline noise ranged from 2.5 to 5.0 pA peak to peak, and just synaptic events with an amplitude $>10\text{pA}$ were analyzed. Synaptic currents were characterized by the following parameters: peak current amplitude, frequency of current transients and the parameters of the current decay phase as derived from the fitting procedure. The time constant of current decay was determined by fitting the synaptic current decay from 90% to 10% peak amplitude with a single exponential and is given in milliseconds throughout the text.

All results were given as mean \pm S.E.M. GraphPad Prism5.02 software (www.graphpad.com) was used for statistical evaluation of comparisons between simultaneous incubation and pre-incubation experiments using one-way analysis of variance ANOVA followed by Tukey post-test. The mean for each parameter for all synaptic events (see Table 1.) recorded from individual neurons was obtained using Origin7G (OriginLab Corporation, Northampton, MA, USA). The time constant of decay was analyzed by best monoexponential fit (Clampfit, Axon Instruments). Moreover, we performed a Gaussian fit and it showed up that we had two main populations of events. The population of spikes with a time decay $\tau < 20\text{ms}$ which was also proven to arise from glutamatergic currents by specific blockers (EPSCs) and the population with $\tau > 20\text{ms}$ proven to arise from GABAergic currents by specific blockers (IPSCs) as described above. Parameters of functional cell properties and spikes characteristics were determined using Student's unpaired *t* test.

Results

We investigated the neuroprotective effects of VPA and three different VPA-derivates on primary motoneurons, each compound in the three different co-culture conditions. In a previous study we had shown the ability of the glial cell-feeder layers to improve the survival of developing motoneurons as compared to the monoculture condition. We had further shown a dose-dependency of the effect of VPA on motoneuron survival and a marginal neuroprotective effect against KA-induced neurotoxicity in presence of glial cells (Ragancokova, et al., 2009). In this study we now examined the effects of the VPA derivates 3-propyl-heptanoic acid (3-PHA), R (+) - and S(-)-2-n-pentyl-4-pentynoic acid (R- and S-PE-4-yn) on motoneuron survival (**Fig.1**).

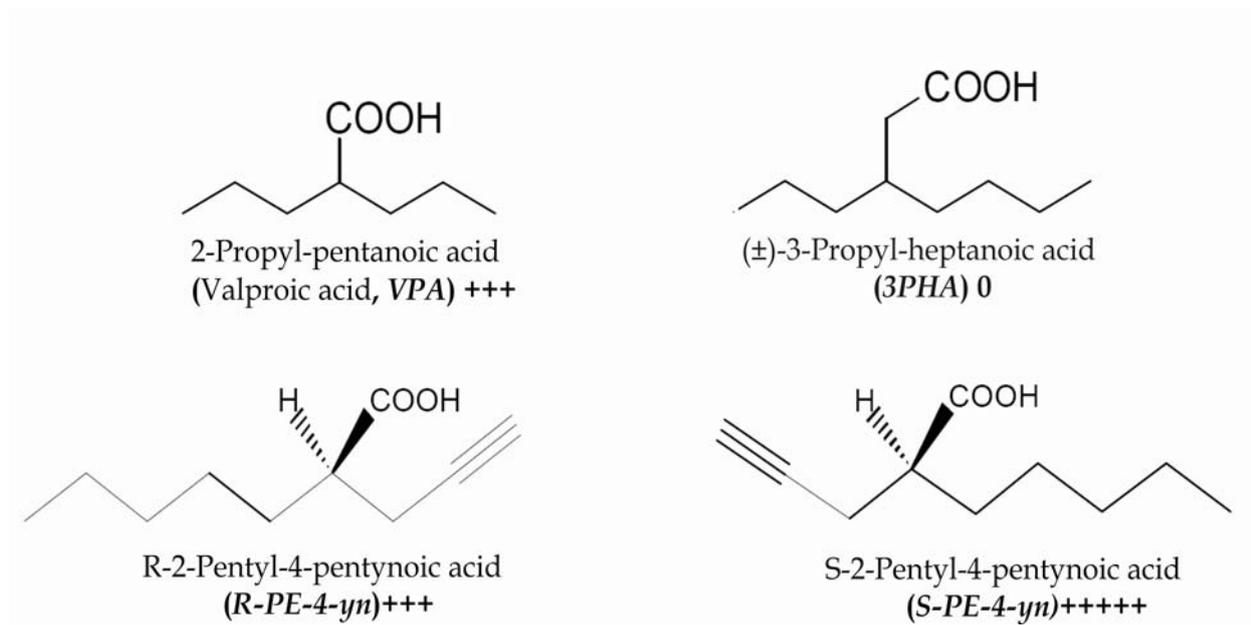


Fig. 1 The chemical structure of the VPA and its congeners investigated in this study. Their respective teratogenic potency are on the arbitrary scale from 0 (no detectable teratogenic potential) to +++++ (very high teratogenic potential).

To test the neuroprotective effects of these derivatives, we induced excitotoxicity by applying 30 μ M KA at DIV9. Tested substances were applied either simultaneously with KA or added 8 days before, at DIV1. In a first step, motoneurons (**Fig.2**) in either monoculture or co-culture with astrocytes or Schwann cells were simultaneously incubated in 10 μ M of VPA or its derivatives together with 30 μ M KA starting from DIV9 for 48h. In the monoculture system (**Fig.2 A**), co-application of VPA, 3PHA or R-PE-4-yn with KA did not increase motoneuron survival as compared to KA-exposure alone. S-PE-4-yn/KA showed a trend towards higher motoneuron survival. In the two co-culture conditions (**Fig.2 B and 2C**), there was a slight but not significant protective effect of VPA, 3PHA and S-PE-4-yn. R-PE-4-yn, however, considerably reduced motoneuron vulnerability to KA-toxicity in motoneurons seeded on astrocytes and even more so on Schwann cells (26.6 \pm 3.9% of motoneurons survived when exposed to KA alone as compared to 35.3 \pm 4.5% under co-application of R-PE-4-yn/KA, $p < 0.05$). We then wanted to evaluate whether pre-incubation of motoneurons (**Fig.3**) with 10 μ M VPA, 3-PHA, R- and S-PE-4-yn starting at DIV1 had better anti-excitotoxic effects. At DIV9, 30 μ M KA was added to the cells for 48 h. In this paradigm, the vulnerability of motoneurons in presence of KA was reduced in monoculture as well as in co-cultures with astrocytes and with Schwann cells. The effects of pre-incubation of motoneurons with the tested compounds was again dependent on the culture condition: motoneurons in monoculture pre-incubated with VPA and 3PHA before KA-addition showed just a trend towards higher cell survival, R-PE-4-yn/KA and S-PE-4-yn/KA compared to KA alone led to a significant increase ($p < 0.001$, 5.8 \pm 0.8% and 4.4 \pm 0.7%, respectively) (**Fig.3 A**). In the astrocyte co-culture system, pre-incubation with all 4 substances improved cell survival, with best effects for VPA ($p < 0.05$, 5.3 \pm 0.8% difference to KA alone) and S-PE-4-yn ($p < 0.01$, 6.5 \pm 0.8%) (**Fig.3 B**). When motoneurons were grown on Schwann cell feeder layers, pre-incubation with all tested substances was significantly protective against KA-toxicity. The difference between VPA/KA and S-PE-4-

yn/KA as compared to KA alone was $3.9 \pm 0.9\%$ and $7.3 \pm 6\%$, respectively ($p < 0.001$)

(Fig.3 C).

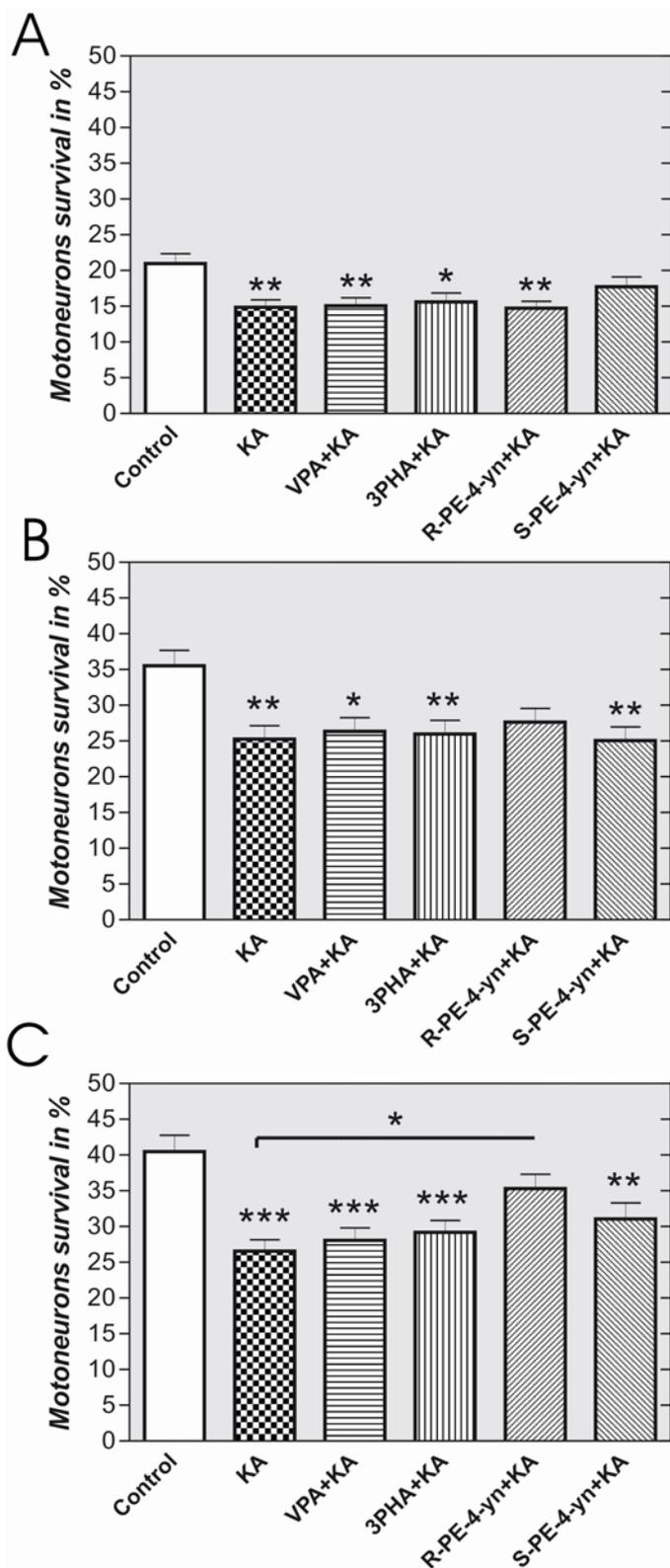


Fig. 2 Simultaneous incubation of $10\mu\text{M}$ VPA and derivatives (3-PHA, R- and S- PE-4-yn) and $30\mu\text{M}$ KA at DIV 9 for 48 hours on monocultures ($n=4$, **A**) or neuronal-glia co-cultures ($n=6$); astrocytes (**B**) or Schwann cells (**C**). The motor neuron specific viability in presence of KA was significantly reduced in monocultures as well as in co-cultures. However, co-application of VPA/KA and 3-PHA/KA leads just to trend of increasing neuronal survival in co-cultures. The derivate S-PE-4-yn shows reasonable neuroprotection against potentiate kainate-induced neuronal death on glial feeder layers, whereas R-PE-4-yn significantly enhanced basal survival of motoneurons in monoculture. Values represent mean \pm S.E.M., *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

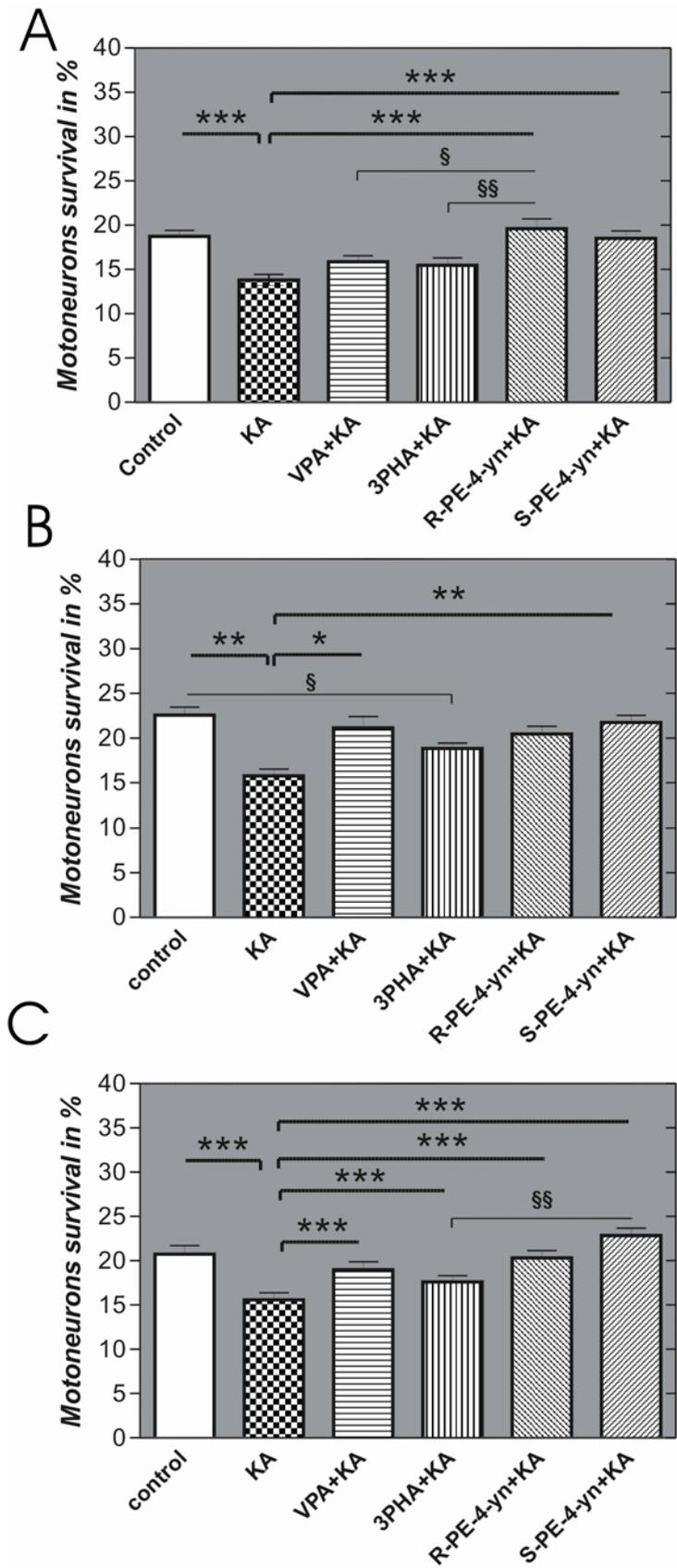


Fig. 3 Preincubation of 10 μ M neuroprotective substances (VPA, 3-PHA, R- and S-PE-4-yn) from DIV1 on monocultures (n=4, **A**) or neuronal-glia co-cultures (n=6); astrocytes (**B**) or Schwann cells (**C**). In DIV9 neurotoxic stimulus, 30 μ M KA was added for 48hours. The motoneuron viability in preincubation of tested substances in presence of KA was significantly increased in all co-culture conditions. However, R-PE-4-yn and S-PE-4-yn show significantly reduced vulnerability against potentiate KA-induced neuronal death. Values represent mean \pm S.E.M., *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

To summarize, pre-incubation with 3PHA had minor anti-excitotoxic effects. VPA pre-

incubation was significantly neuroprotective in the astrocyte- and Schwann cell co-culture. R-PE-4-yn in monoculture and S-PE-4-yn in all three culture systems significantly enhanced motoneuron survival.

To better understand the mechanism underlying the observed protective effects, we performed whole-cell patch-clamp recordings from motoneurons. For these experiments, cells were incubated with the substance which had shown the best neuroprotective effects in the respective co-culture system, i.e. R-PE-4-yn in the monoculture and S-PE-4-yn in the astrocyte- and in the Schwann cell co-culture condition. (Fig.3). Changes in spontaneous synaptic transmission of the cells incubated with the test substance were compared to controls incubated in medium only. The average diameter of measured motoneurons was $26 \pm 3 \mu\text{m}$ in monocultures and $27 \pm 4 \mu\text{m}$ in co-cultures. The patch pipette was positioned on the cell soma in all three conditions (**Fig.4 A**). We analyzed cell properties (average diameter and resting membrane potentials). A significant difference in the resting potential was seen in the astrocyte co-culture system: incubation with S-PE-4-yn resulted in an average potential of $-72 \pm 2.8 \text{mV}$ as compared to $-69 \pm 1.1 \text{mV}$ (see Table 1). We further compared the number of events as well as peak current amplitude and frequency of current transients of spontaneously activate motoneurons. In monoculture, the peak current of amplitude was significantly higher in control cells ($-35 \pm 1.6 \text{pA}$) than in motoneurons incubated with R-PE-4-yn ($-27 \pm 1.6 \text{pA}$). Incubation of motoneurons grown on glial feeder layers did not significantly modify the peak current of amplitude. Surprisingly, motoneurons in co-culture both with astrocytes and Schwann cells showed a significant difference in the frequency of current transient when incubated with S-PE-4-yn as compared to control (see Table 1). The spontaneous excitatory postsynaptic currents (EPSCs) were abolished by the specific blocker of kainate- and AMPA-type GluR channels CNQX ($10 \mu\text{M}$) and NMDA-channels by MK- 801 ($100 \mu\text{M}$). Few minutes after washout the occurrence of spontaneous inward currents resumed (**Fig. 4 B**). It was therefore

concluded that the spontaneous currents measured in motoneurons correspond to spontaneous AMPA- and NMDA-ergic excitatory postsynaptic currents. The spontaneous inhibitory postsynaptic currents (IPSCs) could be blocked by Bicuculline (10 μ M) and must therefore represent GABA-ergic inhibitory postsynaptic currents (**Fig.4 C**).

It has previously been shown that VPA can potentiate postsynaptic responses by reducing the frequency of events but not the amplitude of both spontaneous EPSC and IPSC (Cunningham, Woodhall et al. 2003) We therefore measured the synaptic transmission of motoneurons in whole cell configuration at a holding potential of -70mV in all three co-culture conditions. The synaptic transmission was analyzed by a Gaussian fit. Two main populations of events could be distinguished, the population of spikes with a time decay $\tau < 20$ ms which was also proven to arise from glutamatergic currents by specific blockers (EPSCs) and the population with $\tau > 20$ ms proven to arise from GABAergic currents by specific blockers (IPSCs) (see methods). Motoneurons in monoculture had significantly more EPSCs when pre-treated with R-PE-4-yn (**Fig.5 A, a**). On the contrary, motoneurons in co-culture with astrocytes showed a significant increase of IPSCs when pre-treated with S-PE-4-yn (**Fig.5 B, b**). Motoneurons cultured on Schwann cells and incubated with S-PE-4-yn showed just a trend towards enhancing of IPSCs (**Fig.5 C, c**).

To sum up, in the paradigm of simultaneous application together with KA, significant neuroprotective effects were only observed for one out of 4 compounds, R-PE-4-yn, and only in the two co-culture systems. More relevant anti-excitotoxic effects were seen when motoneurons were pre-incubated before KA-addition, with most robust results for the VPA-derivative S-PE-4-yn. Our patch-clamp experiments revealed that S-PE-4-yn reduces the frequency of postsynaptic currents and enhances events with slow decay time constant (IPSCs) in motoneurons cultured on glial feeder layers.

Chapter III

Table 1 Motoneuron properties

	MN on laminin		MN on Astrocytes		MN on Schwann cells	
	Control	R-PE-4-yn	Control	S-PE-4-yn	Control	S-PE-4-yn
Cell properties (number of cells)	10	8	9	7	6	6
Soma diameter, μm	26 \pm 2	26 \pm 3	27 \pm 4	27 \pm 3	27 \pm 5	27 \pm 4
Resting membrane potential, mV	-73 \pm 1.3	-77 \pm 3.7	-72 \pm 2.8	*** -69 \pm 1.1	-71 \pm 0.2	-73 \pm 2.0
<u>Spike characteristics (number of events)</u>	150	150	100	100	150	150
Peak current amplitude, pA	-35 \pm 1.6	*** -27 \pm 1.6	-24 \pm 1.6	-20 \pm 1.1	-19 \pm 0.6	-20 \pm 1.0
Frequency of current transient, Hz	0.7 \pm 0.04	0.7 \pm 0.04	1.2 \pm 0.09	*** 0.7 \pm 0.08	0.8 \pm 0.04	** 0.7 \pm 0.05

The comparison of motoneurons properties between controls and substance P tested cultures seeded on laminin, astrocytes or Schwann cells.

The values are expressed as a mean \pm S.E.M. and statistical differences were tested using unpaired Student's *t* Test.

Significance is indicated by * ($P < 0.05$) and *** ($P < 0.001$). The holding potential was held at -70 mV.

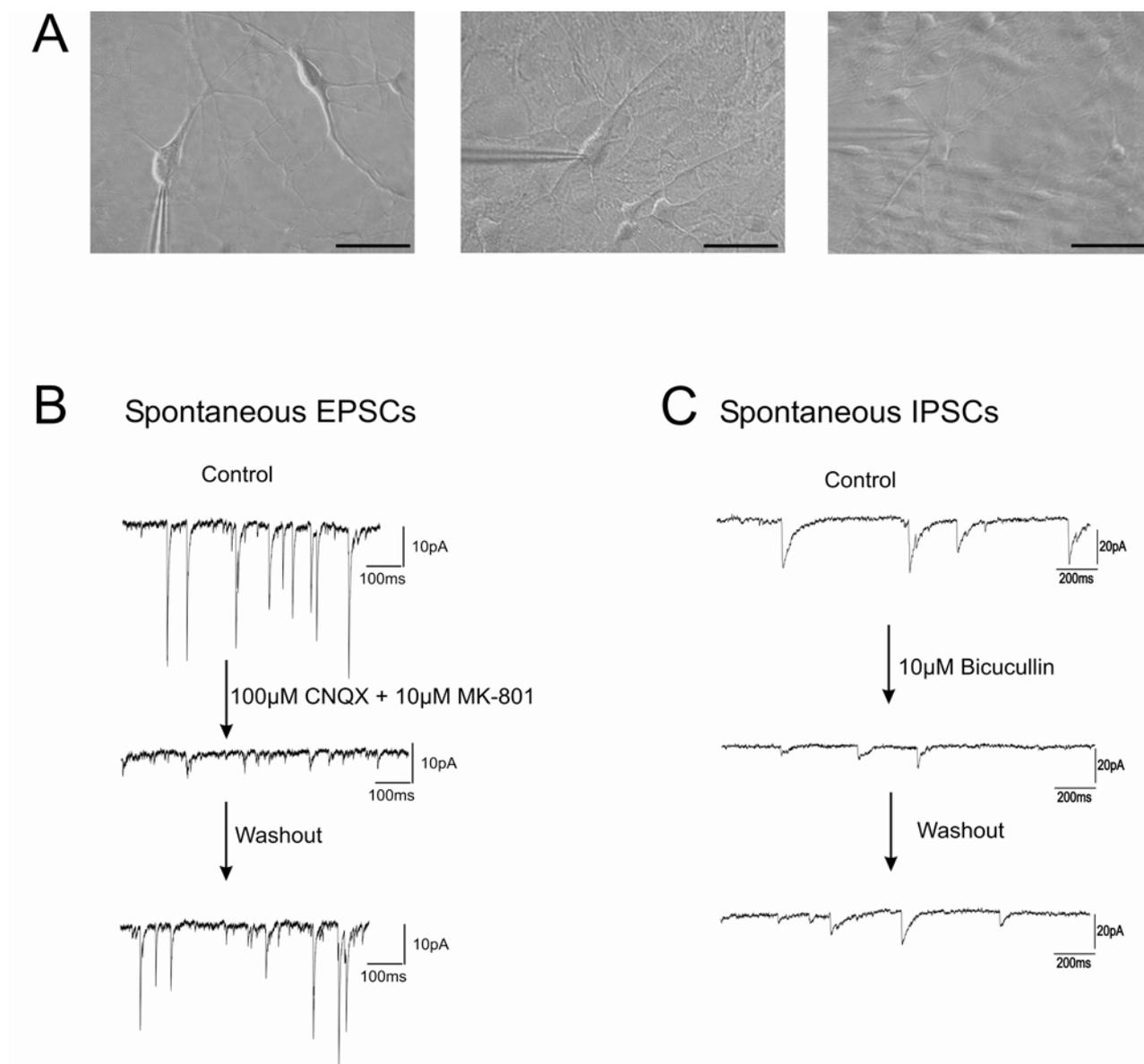


Fig. 4 Representative patch-clamp recordings from spontaneously active motoneurons in *in vitro* cultures. (A) Patched motoneuron in a whole cell configuration of embryonic ventral spinal cords cultured alone (left), motor neurons layered on astrocytes (middle) and on Schwann cells (right). The patch pipette is positioned on the cell soma shown by phase-contrast micrograph. Length of scale bar is 100μm. (B) Spontaneous excitatory postsynaptic currents (EPSC) of motoneurons before (upper trace), during and after (lower trace) incubation of CNQX and MK-801. (C) Spontaneous inhibitory postsynaptic currents which were abolished by addition of Bicucullin and after washed out. The holding potential was kept at -70mV.

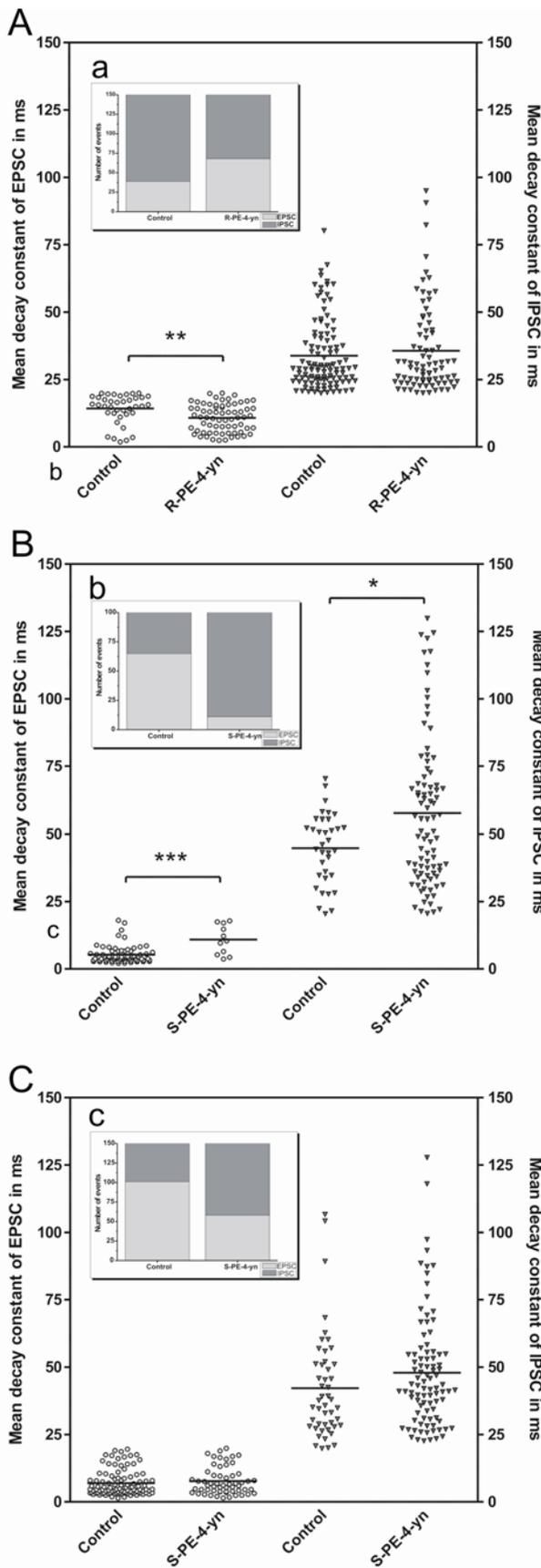


Fig. 5 Modulation of synaptic transmission by *preincubation* with 10µM R-PE-4-yn on monocultures and S-PE-4-yn on co-cultures from DIV1 compared to pre-incubation with control medium. Derivates of VPA increase the number of events with a slow decay time in motoneurons on glial-feeder layers. Measurements were done between DIV 9-15. Spontaneous spikes were divided by the mean decay constant into fast postsynaptic currents, EPSC ($\tau < 20\text{ms}$) and slow postsynaptic currents, IPSC ($\tau > 20\text{ms}$). The number of events was analyzed at least from > 6 cells, 3 different preparations (see Table 1). Insets (a, b, c) show comparisons of EPSCs and IPSCs between controls and VPA-derivate treated cells. Student's unpaired *t* test used to compare controls and tested conditions. Values represent mean \pm S.E.M., *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. (Aa) Purified motoneurons demonstrate evidential more events of EPSCs in cells treated by R-PE-4-yn. Contrary, (Bb) motoneurons cultured on astrocytes show a significant increase of IPSCs. (Cc) Motoneurons cultured on Schwann cells show just trend to enhance IPSCs.

Discussion

VPA is a drug used for the treatment of an increasing number of diseases and conditions including epilepsy, migraine, and bipolar disorder. It can also inhibit tumor growth, metastasis and angiogenesis, and may therefore also be utilized in the treatment of cancer (Blaheta, et al., 2005, Johannessen, 2000). Recently VPA was reported to reduce neurodegeneration, showing neuroprotective effects in *in vivo* and *in vitro* models (Dou, et al., 2003, Sugai, et al., 2004). VPA therefore was considered a promising novel therapy for a variety of neurodegenerative diseases such as ALS. In the ALS transgenic mouse model, long-term dietary administration of VPA did not affect lifespan but significantly delayed motoneuron death (Crochemore, et al., 2009, Rouaux, et al., 2007). In a mouse model of spinal muscular atrophy (SMA), low doses of VPA appeared to be a well tolerable and effective treatment (Tsai, et al., 2007).

Addressing the presumed neuroprotective capacities of VPA in our *in vitro* system, we had previously observed that lower VPA doses possess protective capacities. Higher concentrations (>100 μ M), however, were considerably toxic for primary motoneurons. We had further shown dose dependent neurotoxic effects of the glutamate receptor agonist KA in three different motor neuron culture systems which validate them as a proper tool to study excitotoxicity *in vitro* and to screen potentially neuroprotective compounds (Ragancokova, et al., 2009).

To overcome the problem of adverse effects of VPA administered in higher dosages, a number of chemical derivatives of VPA have been synthesized. Even slight changes in the molecular structure of VPA derivatives (e.g. enantiomers) can completely prevent teratogenicity without substantially influencing the pharmacokinetic properties (Hauck and Nau, 1992). In the present study, we now compared neuroprotective properties of VPA and three potentially more potent and less toxic derivatives in our *in vitro* model of

excitotoxic motoneuron death. We did not detect significant neuroprotective effects of the non-teratogenic VPA-derivative 3PHA, which was identified as a very potent inducer of differentiation without evidence of HDAC-inhibition (Deubzer, et al., 2006). However, we observed significant increases in motoneuron survival following pre-incubation in R-PE-4-yn and S-PE-4-yn before the addition of the excitotoxic stimulus. R-PE-4-yn was even protective when administered simultaneously with KA in the astrocyte- and Schwann cell co-culture systems.

These results correspond to a previous study showing that the administration of PE-4-yn enantiomers significantly enhances rodent water maze learning and presents a favourable toxicological profile, with no overt organ toxicity (Murphy, et al., 2001). In vitro, PE-4-yn enantiomers were demonstrated to induce neuritogenesis in the neuroblastoma cell line, where S-PE-4-yn induced longer neurites than R-PE-4-yn (O'Loinsigh, et al., 2004). PE-4-yn enantiomers can induce expression of the neuroplastic marker NCAM-PSA both in vivo and in vitro in a dose dependent manner. NCAM-PSA is down-regulated in the brain during ageing (Ni Dhuill, et al., 1999), and increased expression of this neuroplastic substrate is directly associated with both neuritogenesis in vitro (Gallagher, et al., 2000) and synapse remodelling in vivo (Murphy and Regan, 1998). PE-4-yn enantiomers therefore seem to have all the neuroplastic qualities of the neurotrophins but be without their disadvantage of impermeability to the blood brain barrier (Pardridge, et al., 1994). Moreover, the ability of PE-4-yn enantiomers to attenuate the age-related decrease in NCAM polysialylation state suggests that it may have a significant advantage in slowing onset of degenerative deficits by enhancing neuroplasticity. It was found that the expression of PSA-NCAM was increased in a concentration dependent manner by both R- and S-PE-4-yn, an observation consistent with the fact, that the racemic mixture of PE-4-yn enantiomers induces the expression of one of the enzymes, PST1, responsible for attaching PSA to NCAM (Lampen, et al., 2005). This indicates that R- and S-PE-4-yn have

largely similar effects *in vitro*. However, in line with our findings, S-PE-4-yn has consistently been reported to be more potent than R-PE-4-yn. Furthermore, S-PE-4-yn has higher anti-apoptotic potency than the R-enantiomer (Gotfryd, et al., 2007). The available *in vivo* data suggest that PE-4-yn enantiomers are generally safe drugs that may be of potential use in the clinical treatment of neurodegenerative diseases (Murphy, et al., 2001) even though there is some concern that these drugs may have similar teratogenic effects as the parent compound VPA. Although the molecular mechanisms underlying this *in vivo* teratogenicity are unknown, it is clear that they directly relate to the antiproliferative/pro-differentiative potency which can be detected *in vitro* in various cell types (Martin and Regan, 1991).

To further assess the mechanism of action of these VPA derivatives, we performed whole-cell patch-clamp recordings to assess whether they could alter the sum of excitatory and inhibitory postsynaptic currents in motoneurons. If they acted as antagonists of ligand-gated ion channels, one would expect significant alterations of the physiological state of the cells. Cunningham et al. showed that VPA modifies excitation and inhibition at cortical synapses and suggested that VPA can potentiate postsynaptic responses, possibly by interaction with benzodiazepine regulatory site of the GABA α receptor (Cunningham, et al., 2003). The addition of specific AMPA- and NMDA-receptor antagonists (CNQX, MK-801) or the GABA α receptor antagonist bicuculline would significantly alter the physiological state of the pharmacological interventions of a tested substance (Buldakova, et al., 2005). By whole-cell recordings, we could indeed confirm an enhancement of inhibitory transmission following incubation of motoneurons with S-PE-4-yn when they were plated on glial feeder layers. The frequency of both excitatory and inhibitory postsynaptic currents was decreased only when motoneurons were co-cultured with glial cells. This observation underlines the crucial role of glial cells in function and survival of motoneurons. Glial cells can exert protective effects on motoneuron via several

mechanisms of actions; they are an important source of neurotrophic factors (Arce, et al., 1998, Van Den Bosch and Robberecht, 2008) and induce neuronal differentiation (Haastert, et al., 2005, Vandenberghe, et al., 1998). Astrocytes in culture sense and respond to neuronal activity by increasing their internal Ca^{2+} concentration, which in turn triggers them to release their own chemical transmitters that regulate neuronal activity (Araque, et al., 1999). Schwann cells also detect synaptic activity by increasing intracellular Ca^{2+} and respond to this activity by regulating neurotransmitter release (Rochon, et al., 2001). Our patch clamp studies revealed pre-incubation with tested VPA derivate increased inhibitory currents and a decreased the frequency of synaptic transmission only when motoneurons were cultured on glial cells. These results further underline that astrocytes and Schwann cells are intimately in the process of synaptic function and can unfold neuroprotective capacities via an enhancement of antiexcitotoxic mechanism

To conclude, our data show that glial cells can potentiate the effects of antiexcitotoxic compound in motoneurons in general and that pre-incubation of the cells with the tested agents consistently led to more robust effects than simultaneous addition together with the neurotoxic stimulus. The PE-4-yn VPA derivates, particular S-PE-4-yn, were identified as promising neuroprotective agents against motoneuron death. By electrophysiological techniques, we could show that similar to VPA their effect can be -at least partially- attributed to an increase in inhibitory synaptic transmission. Their higher potency and more favourable side-effect profile in comparison to VPA makes them interesting candidates for further preclinical and clinical evaluation in motoneuron diseases.

CHAPTER IV General Discussion

It is important to understand how neurons can be protected from excitotoxicity which is thought to play a crucial role in the pathogenesis of chronic neurodegeneration. Especially with respect to the missing success of translation of preclinical neuroprotection achieved by a great variety of compounds during the last decade to the patients suffering from progressive neurodegenerative diseases like amyotrophic lateral sclerosis, the pharmacological research has to be addressed back to the molecular mechanisms of action being relevant for neuroprotection, and exact drug profiles have to be studied and compared to biological effects. Only a systematic research approach seems to be promising for the development of molecular drug design in this field of research.

Valproic acid (VPA) has long been used as an antiepileptic drug and recently as a mood stabilizer, and evidence is increasing that VPA exerts neuroprotective effects through changes in a variety of intracellular signalling pathways including upregulation of Bcl-2 protein with an antiapoptotic property and inhibiting glycogen synthase kinase 3- β , which is considered to promote cell survival. Although the neuroprotective effects of VPA have been demonstrated in a murine model of human immunodeficiency virus-1 encephalitis, there have been just some reports on the effect of VPA in chronic progressing neurodegenerative disease models including amyotrophic lateral sclerosis (ALS). Recently it was reported that VPA reduce neurodegeneration, showing neuroprotective effects in *in vivo* and *in vitro* models (Dou, et al., 2003, Sugai, et al., 2004). VPA therefore was considered a promising novel therapy for a variety of neurodegenerative diseases such as ALS. In the ALS transgenic mouse model, long-term dietary of VPA did not affect lifespan but significantly delayed motoneuron death (Crochemore, et al., 2009, Rouaux, et al., 2007). In a mouse model of spinal muscular atrophy (SMA), low doses of VPA appeared to be a well tolerable and effective treatment (Tsai, et al., 2008).

By studying the viability of highly purified populations of rat spinal motor neurons in the absence or presence of glia (astrocytes vs. Schwann cells) in vitro, we could show that glial feeding layers profoundly enhance the number and survival of cells immunostained by SMI32, a marker for non-phosphorylated neurofilament, identifying motor neurons (Carriedo, et al., 1996). Motor neurons in monocultures showed a reduced survival, similar to the results of Pennica et al. These findings are therefore in line with the previously established observation that Schwann cells and astrocytes in co-cultures provide a trophic source for motor neurons and allow physiological cell-cell interactions during neurite-outgrowth in vitro (Mirsky, et al., 2002). There is well known that astrocytes are pivotal to maintain the low extra-neuronal concentrations of glutamate by scavenging this neurotransmitter from the synaptic cleft. By far the most important glutamate transporter expressed in astrocytes is EAAT2/GLT-1 and it was found that astrocytes induce neuronal differentiation and potentiate the sensitivity of motor neurons to AMPA-receptor mediated glutamate stimulation (Van Den Bosch and Robberecht, 2008). Perisynaptic Schwann cells also detect synaptic activity by increasing intracellular Ca^{2+} and respond to this activity by regulating neurotransmitter release (Rochon, et al., 2001). These studies strongly suggest that these two perisynaptic glial cells, astrocytes and Schwann cells are intimately involved in the process of synaptic function.

Selective vulnerability of motor neurons towards excitotoxic stress has been proposed as a central pathomechanism in the motor neuron diseases such as ALS (Rao and Weiss, 2004, Van Den Bosch, et al., 2006). We showed dose dependent neurotoxic effects of the glutamate receptor agonist KA in our 3 motor neuron culture systems which validate them as a tool to study excitotoxicity in vitro and to screen for potentially neuroprotective compounds. To further assess the presumed neuroprotective capacities of VPA, we therefore analyzed the effect of different doses of VPA on primary motor neurons. While lower VPA doses showed neither protective nor toxic capacities, higher

concentrations ($>100\mu\text{M}$) started to be considerably toxic for the cells which confirms the data of Sugai et al, where cultured tissues became thin and fragile after exposure to high concentration of VPA. The neuroprotective effect of VPA on primary embryonic motor neuron cultures was not significant under the conditions tested by simultaneous incubation.

In a search of new drugs with selective anticonvulsant activities and less toxicity, numerous derivatives and various metabolites of VPA have been investigated and found anticonvulsant activity. Even slight changes in the molecular structure of VPA derivatives (e.g. enantiomers) can completely prevent teratogenicity without substantially influencing the pharmacokinetic properties (Hauck and Nau, 1992).

Acetylation and deacetylation of histones play significant roles in the regulation of gene transcription in many cells. There are two classes of enzymes involved in the acetylation state of histones, histone acetyl transferases, and histone deacetylases (HDACs). It was shown that VPA inhibits HDAC activity and that peroxisome proliferator-activated receptor (PPAR(α)) is depressed by HDAC inhibition (Gottlicher, et al., 2001, Phiel, et al., 2001). Trichostatin (TSA) is an anticancer compound and a well characterized HDAC inhibitor able to induce differentiation. It is interesting that TSA is also teratogenic compound that induces neural tube defects very similar to those induced by VPA (Phiel, et al., 2001). Therefore, HDAC inhibition seems to be involved in the teratogenic mechanism.

We focused on three out of a number of synthesized VPA derivatives that have lately been under investigation: 3-propyl-heptanoic acid (3-PHA) and PE-4-yn enantiomers (R- and S- PE-4-yn). 3PHA, a non-teratogenic analogue of VPA was identified as a very potent inducer of cell-differentiation without evidence of HDAC-inhibition (Deubzer, et al., 2006). 2 pentyl-4-yn enantiomers possess enantiospecific effects: S-PE-4-yn is highly teratogenic, whereas R-PE-4-yn is a moderate teratogene. Our results show that pre-

incubation with 3PHA had minor neuroprotective effect against KA-induced neurotoxicity. VPA preincubation was significantly neuroprotective in the astrocyte- and Schwann cell co-culture. R-PE-4-yn in monocultures and S-PE-4-yn in both co-cultures significantly enhance motoneuron survival. However, simultaneous incubation of motor neurons with VPA and its derivatives did not show such neuroprotective potential as pre-incubation.

To further assess this mechanism in our culture system, we performed calcium imaging experiments and whole-cell recording. Motor neurons in general display a significantly higher occurrence of spontaneous Ca^{2+} transients than non-motor neurons suggestive of increased AMPAergic synaptic input (Jahn, et al., 2006). This correlates to the fact that motor neurons appear to have AMPA receptors with higher relative Ca^{2+} -permeability than other neuronal populations. In our cultures, this spontaneous activity of motor neurons occurred after DIV 10 and was not influenced by short term application of VPA. In cells younger than DIV 10, we used KA to elicit Ca^{2+} influx. Our observation of highest KA sensitivity of motor neurons in monoculture further supports the notion that surrounding glial cells can protect motor neurons against excitotoxic stimuli. The application of VPA to the co-cultures in this experimental paradigm was again not protective against KA exposure but even increased motor neuron vulnerability. Altogether these data show that primary rodent motor neurons can not be protected by VPA against KA-induced toxicity. Our calcium imaging data indicate that the observed toxic effects of VPA on motor neurons can at least in part be attributed to an increased Ca^{2+} influx triggering excitotoxicity.

Moreover, we performed the whole-cell patch-clamp recordings to assess whether they could alter the sum of excitatory and inhibitory postsynaptic currents in motor neurons of pre-incubated motor neurons with tested substances. For these experiments, cells were incubated with the substance which had shown the best neuroprotective effects in the respective co-culture system, i.e. R-PE-4-yn in the monoculture and S-PE-4-yn in the

astrocyte- and in the Schwann cell co-culture condition. Cunningham et al. showed that VPA modifies excitation and inhibition at cortical synapses and suggested that VPA can potentiate postsynaptic responses, possibly by interaction with benzodiazepine regulatory site of the GABA α receptor (Cunningham, et al., 2003). By whole-cell recordings, we could indeed confirm an enhancement of inhibitory transmission following incubation of motor neurons with derivate of VPA, S-PE-4-yn on glial feeder layers. The frequency of both excitatory and inhibitory postsynaptic currents was decreased only when motor neurons were co-cultured with glial cells that play a crucial role in the function and survival of motor neurons.

Summarizing our data, we could show that glial feeder layers can increase viability of motor neurons as compared to motor neuron monoculture. We confirmed the previously described dose-dependent neurotoxicity of KA and VPA which was neurotoxic at a concentration of 100 μ M in primary cell cultures. Our data suggest that the neuroprotective capacities of VPA and its three derivatives tested here drastically increase when they are added several days before the excitotoxic stimulus. The importance of this effect might be due to an increase of inhibitory postsynaptic transmission and was further dependent on the co-culture conditions.

Up to now there is no curative therapy for the treatment of ALS. The only substance with a proven beneficial effect is Riluzole. It prolongs survival of ALS patients for about three months. The recent research indicates that therapeutic options do not have to focus on motor neurons alone, as ALS seems to be a non-cell-autonomous disease. In conclusion, a combined pharmacological interference with the many faces of excitotoxicity both at the motor neurons and at the surrounding cells will be most likely essential to extend survival of ALS patients.

REFERENCE LIST

1. Araque, A., Sanzgiri, R. P., Parpura, V., and Haydon, P. G., 1999. Astrocyte-induced modulation of synaptic transmission. *Can J Physiol Pharmacol* 77, 699-706.
2. Arce, V., Pollock, R. A., Philippe, J. M., Pennica, D., Henderson, C. E., and deLapeyriere, O., 1998. Synergistic effects of schwann- and muscle-derived factors on motoneuron survival involve GDNF and cardiotrophin-1 (CT-1). *J Neurosci* 18, 1440-1448.
3. Arundine, M., and Tymianski, M., 2003. Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell Calcium* 34, 325-337.
4. Baldino, F., Jr., and Geller, H. M., 1981. Effect of sodium valproate on hypothalamic neurons in vivo and in vitro. *Brain Res* 219, 231-237.
5. Bendotti, C., and Carri, M. T., 2009. Amyotrophic Lateral Sclerosis: Mechanisms and Countermeasures. *Antioxid Redox Signal*.
6. Blaheta, R. A., and Cinatl, J., Jr., 2002. Anti-tumor mechanisms of valproate: a novel role for an old drug. *Med Res Rev* 22, 492-511.
7. Blaheta, R. A., Michaelis, M., Driever, P. H., and Cinatl, J., Jr., 2005. Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. *Med Res Rev* 25, 383-397.
8. Buldakova, S. L., Tikhonov, D. B., and Magazanik, L. G., 2005. Analysis of the excitatory and inhibitory components of postsynaptic currents recorded in pyramidal neurons and interneurons in the rat hippocampus. *Neurosci Behav Physiol* 35, 835-843.

9. Carriedo, S. G., Sensi, S. L., Yin, H. Z., and Weiss, J. H., 2000. AMPA exposures induce mitochondrial Ca²⁺ overload and ROS generation in spinal motor neurons in vitro. *J Neurosci* 20, 240-250.
10. Carriedo, S. G., Yin, H. Z., and Weiss, J. H., 1996. Motor neurons are selectively vulnerable to AMPA/kainate receptor-mediated injury in vitro. *J Neurosci* 16, 4069-4079.
11. Chen, C. L., Sung, J., Cohen, M., Chowdhury, W. H., Sachs, M. D., Li, Y., Lakshmanan, Y., Yung, B. Y., Lupold, S. E., and Rodriguez, R., 2006. Valproic acid inhibits invasiveness in bladder cancer but not in prostate cancer cells. *J Pharmacol Exp Ther* 319, 533-542.
12. Clement, A. M., Nguyen, M. D., Roberts, E. A., Garcia, M. L., Boillee, S., Rule, M., McMahon, A. P., Doucette, W., Siwek, D., Ferrante, R. J., Brown, R. H., Jr., Julien, J. P., Goldstein, L. S., and Cleveland, D. W., 2003. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science* 302, 113-117.
13. Coyle, J. T., and Duman, R. S., 2003. Finding the intracellular signaling pathways affected by mood disorder treatments. *Neuron* 38, 157-160.
14. Crochemore, C., Virgili, M., Bonamassa, B., Canistro, D., Pena-Altamira, E., Paolini, M., and Contestabile, A., 2009. Long-term dietary administration of valproic acid does not affect, while retinoic acid decreases, the lifespan of G93A mice, a model for amyotrophic lateral sclerosis. *Muscle Nerve* 39, 548-552.
15. Cunningham, M. O., Woodhall, G. L., and Jones, R. S., 2003. Valproate modifies spontaneous excitation and inhibition at cortical synapses in vitro. *Neuropharmacology* 45, 907-917.

16. Deckers, C. L., Czuczwar, S. J., Hekster, Y. A., Keyser, A., Kubova, H., Meinardi, H., Patsalos, P. N., Renier, W. O., and Van Rijn, C. M., 2000. Selection of antiepileptic drug polytherapy based on mechanisms of action: the evidence reviewed. *Epilepsia* 41, 1364-1374.
17. Deubzer, H., Busche, B., Ronndahl, G., Eikel, D., Michaelis, M., Cinatl, J., Schulze, S., Nau, H., and Witt, O., 2006. Novel valproic acid derivatives with potent differentiation-inducing activity in myeloid leukemia cells. *Leuk Res* 30, 1167-1175.
18. Di Giorgio, F. P., Carrasco, M. A., Siao, M. C., Maniatis, T., and Egan, K., 2007. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat Neurosci* 10, 608-614.
19. Dou, H., Birusingh, K., Faraci, J., Gorantla, S., Poluektova, L. Y., Maggirwar, S. B., Dewhurst, S., Gelbard, H. A., and Gendelman, H. E., 2003. Neuroprotective activities of sodium valproate in a murine model of human immunodeficiency virus-1 encephalitis. *J Neurosci* 23, 9162-9170.
20. Eickholt, B. J., Towers, G. J., Ryves, W. J., Eikel, D., Adley, K., Ylinen, L. M., Chadborn, N. H., Harwood, A. J., Nau, H., and Williams, R. S., 2005. Effects of valproic acid derivatives on inositol trisphosphate depletion, teratogenicity, glycogen synthase kinase-3 β inhibition, and viral replication: a screening approach for new bipolar disorder drugs derived from the valproic acid core structure. *Mol Pharmacol* 67, 1426-1433.
21. Eikel, D., Hoffmann, K., Zoll, K., Lampen, A., and Nau, H., 2006. S-2-pentyl-4-pentynoic hydroxamic acid and its metabolite s-2-pentyl-4-pentynoic acid in the NMRI-exencephaly-mouse model: pharmacokinetic profiles, teratogenic effects, and histone deacetylase inhibition abilities of further valproic acid hydroxamates and amides. *Drug Metab Dispos* 34, 612-620.

22. Eldar-Finkelman, H., 2002. Glycogen synthase kinase 3: an emerging therapeutic target. *Trends Mol Med* 8, 126-132.
23. Frey, H. H., and Loscher, W., 1978. Distribution of valproate across the interface between blood and cerebrospinal fluid. *Neuropharmacology* 17, 637-642.
24. Gallagher, H. C., Odumeru, O. A., and Regan, C. M., 2000. Regulation of neural cell adhesion molecule polysialylation state by cell-cell contact and protein kinase C delta. *J Neurosci Res* 61, 636-645.
25. Gotfryd, K., Owczarek, S., Hoffmann, K., Klementiev, B., Nau, H., Berezin, V., Bock, E., and Walmod, P. S., 2007. Multiple effects of pentyl-4-yn-VPA enantiomers: from toxicity to short-term memory enhancement. *Neuropharmacology* 52, 764-778.
26. Gottlicher, M., Minucci, S., Zhu, P., Kramer, O. H., Schimpf, A., Giavara, S., Sleeman, J. P., Lo Coco, F., Nervi, C., Pelicci, P. G., and Heinzl, T., 2001. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *Embo J* 20, 6969-6978.
27. Grosskreutz, J., Haastert, K., Dewil, M., Van Damme, P., Callewaert, G., Robberecht, W., Dengler, R., and Van Den Bosch, L., 2007. Role of mitochondria in kainate-induced fast Ca²⁺ transients in cultured spinal motor neurons. *Cell Calcium* 42, 59-69.
28. Haastert, K., Grosskreutz, J., Jaeckel, M., Laderer, C., Bufler, J., Grothe, C., and Claus, P., 2005. Rat embryonic motoneurons in long-term co-culture with Schwann cells--a system to investigate motoneuron diseases on a cellular level in vitro. *J Neurosci Methods* 142, 275-284.
29. Haastert, K., Grosskreutz, J., Jaeckel, M., Laderer, C., Bufler, J., Grothe, C., and Claus, P., 2005. Rat embryonic motoneurons in long-term co-culture with

- Schwann cells-a system to investigate motoneuron diseases on a cellular level in vitro. *J Neurosci Methods* 142, 275-284.
30. Hahnen, E., Eyupoglu, I. Y., Brichta, L., Haastert, K., Trankle, C., Siebzehnruhl, F. A., Riessland, M., Holker, I., Claus, P., Romstock, J., Buslei, R., Wirth, B., and Blumcke, I., 2006. In vitro and ex vivo evaluation of second-generation histone deacetylase inhibitors for the treatment of spinal muscular atrophy. *J Neurochem* 98, 193-202.
 31. Hauck, R. S., and Nau, H., 1992. The enantiomers of the valproic acid analogue 2-n-propyl-4-pentynoic acid (4-yn-VPA): asymmetric synthesis and highly stereoselective teratogenicity in mice. *Pharm Res* 9, 850-855.
 32. Heath, P. R., and Shaw, P. J., 2002. Update on the glutamatergic neurotransmitter system and the role of excitotoxicity in amyotrophic lateral sclerosis. *Muscle Nerve* 26, 438-458.
 33. Hollmann, M., Hartley, M., and Heinemann, S., 1991. Ca²⁺ permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* 252, 851-853.
 34. Hollmann, M., and Heinemann, S., 1994. Cloned glutamate receptors. *Annu Rev Neurosci* 17, 31-108.
 35. Jahn, K., Grosskreutz, J., Haastert, K., Ziegler, E., Schlesinger, F., Grothe, C., Dengler, R., and Bufler, J., 2006. Temporospatial coupling of networked synaptic activation of AMPA-type glutamate receptor channels and calcium transients in cultured motoneurons. *Neuroscience* 142, 1019-1029.
 36. Johannessen, C. U., 2000. Mechanisms of action of valproate: a commentary. *Neurochem Int* 37, 103-110.
 37. Kawahara, Y., Kwak, S., Sun, H., Ito, K., Hashida, H., Aizawa, H., Jeong, S. Y., and Kanazawa, I., 2003. Human spinal motoneurons express low relative

- abundance of GluR2 mRNA: an implication for excitotoxicity in ALS. *J Neurochem* 85, 680-689.
38. Lampen, A., Grimaldi, P. A., and Nau, H., 2005. Modulation of peroxisome proliferator-activated receptor delta activity affects neural cell adhesion molecule and polysialyltransferase ST8SiaIV induction by teratogenic valproic acid analogs in F9 cell differentiation. *Mol Pharmacol* 68, 193-203.
 39. Leng, Y., Liang, M. H., Ren, M., Marinova, Z., Leeds, P., and Chuang, D. M., 2008. Synergistic neuroprotective effects of lithium and valproic acid or other histone deacetylase inhibitors in neurons: roles of glycogen synthase kinase-3 inhibition. *J Neurosci* 28, 2576-2588.
 40. Loscher, W., 1989. Valproate enhances GABA turnover in the substantia nigra. *Brain Res* 501, 198-203.
 41. Loscher, W., 2002. Basic pharmacology of valproate: a review after 35 years of clinical use for the treatment of epilepsy. *CNS Drugs* 16, 669-694.
 42. Martin, M. L., and Regan, C. M., 1991. The anticonvulsant valproate teratogen restricts the glial cell cycle at a defined point in the mid-G1 phase. *Brain Res* 554, 223-228.
 43. McCarthy, K. D., and de Vellis, J., 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 85, 890-902.
 44. Mirsky, R., Jessen, K. R., Brennan, A., Parkinson, D., Dong, Z., Meier, C., Parmantier, E., and Lawson, D., 2002. Schwann cells as regulators of nerve development. *J Physiol Paris* 96, 17-24.
 45. Murphy, K. J., Fox, G. B., Foley, A. G., Gallagher, H. C., O'Connell, A., Griffin, A. M., Nau, H., and Regan, C. M., 2001. Pentyl-4-yn-valproic acid enhances both spatial and avoidance learning, and attenuates age-related NCAM-

- mediated neuroplastic decline within the rat medial temporal lobe. *J Neurochem* 78, 704-714.
46. Murphy, K. J., and Regan, C. M., 1998. Contributions of cell adhesion molecules to altered synaptic weightings during memory consolidation. *Neurobiol Learn Mem* 70, 73-81.
 47. Nagai, M., Re, D. B., Nagata, T., Chalazonitis, A., Jessell, T. M., Wichterle, H., and Przedborski, S., 2007. Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat Neurosci* 10, 615-622.
 48. Ni Dhuill, C. M., Fox, G. B., Pittock, S. J., O'Connell, A. W., Murphy, K. J., and Regan, C. M., 1999. Polysialylated neural cell adhesion molecule expression in the dentate gyrus of the human hippocampal formation from infancy to old age. *J Neurosci Res* 55, 99-106.
 49. O'Loinsigh, E. D., Gherardini, L. M., Gallagher, H. C., Foley, A. G., Murphy, K. J., and Regan, C. M., 2004. Differential enantioselective effects of pentyl-4-yn-valproate on spatial learning in the rat, and neurite outgrowth and cyclin D3 expression in vitro. *J Neurochem* 88, 370-379.
 50. Pardridge, W. M., Yoshikawa, T., Kang, Y. S., and Miller, L. P., 1994. Blood-brain barrier transport and brain metabolism of adenosine and adenosine analogs. *J Pharmacol Exp Ther* 268, 14-18.
 51. Pennica, D., Arce, V., Swanson, T. A., Vejsada, R., Pollock, R. A., Armanini, M., Dudley, K., Phillips, H. S., Rosenthal, A., Kato, A. C., and Henderson, C. E., 1996. Cardiotrophin-1, a cytokine present in embryonic muscle, supports long-term survival of spinal motoneurons. *Neuron* 17, 63-74.
 52. Petri, S., Kiaei, M., Kipiani, K., Chen, J., Calingasan, N. Y., Crow, J. P., and Beal, M. F., 2006. Additive neuroprotective effects of a histone deacetylase

- inhibitor and a catalytic antioxidant in a transgenic mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis* 22, 40-49.
53. Phiel, C. J., Zhang, F., Huang, E. Y., Guenther, M. G., Lazar, M. A., and Klein, P. S., 2001. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem* 276, 36734-36741.
 54. Ragancokova, D., Jahn, K., Kotsiari, A., Schlesinger, F., Haastert, K., Stangel, M., Petri, S., and Krampfl, K., 2009. Analysis of Neuroprotective Effects of Valproic Acid on Primary Motor Neurons in Monoculture or Co-cultures with Astrocytes or Schwann Cells. *Cell Mol Neurobiol*.
 55. Rao, S. D., and Weiss, J. H., 2004. Excitotoxic and oxidative cross-talk between motor neurons and glia in ALS pathogenesis. *Trends Neurosci* 27, 17-23.
 56. Reist, N. E., and Smith, S. J., 1992. Neurally evoked calcium transients in terminal Schwann cells at the neuromuscular junction. *Proc Natl Acad Sci U S A* 89, 7625-7629.
 57. Rochon, D., Rouse, I., and Robitaille, R., 2001. Synapse-glia interactions at the mammalian neuromuscular junction. *J Neurosci* 21, 3819-3829.
 58. Rosenberg, P. A., Amin, S., and Leitner, M., 1992. Glutamate uptake disguises neurotoxic potency of glutamate agonists in cerebral cortex in dissociated cell culture. *J Neurosci* 12, 56-61.
 59. Rouaux, C., Panteleeva, I., Rene, F., Gonzalez de Aguilar, J. L., Echaniz-Laguna, A., Dupuis, L., Menger, Y., Boutillier, A. L., and Loeffler, J. P., 2007. Sodium valproate exerts neuroprotective effects in vivo through CREB-binding protein-dependent mechanisms but does not improve survival in an amyotrophic lateral sclerosis mouse model. *J Neurosci* 27, 5535-5545.

60. Ryu, H., Smith, K., Camelo, S. I., Carreras, I., Lee, J., Iglesias, A. H., Dangond, F., Cormier, K. A., Cudkowicz, M. E., Brown, R. H., Jr., and Ferrante, R. J., 2005. Sodium phenylbutyrate prolongs survival and regulates expression of anti-apoptotic genes in transgenic amyotrophic lateral sclerosis mice. *J Neurochem* 93, 1087-1098.
61. Schumacher, M., Jung-Testas, I., Robel, P., and Baulieu, E. E., 1993. Insulin-like growth factor I: a mitogen for rat Schwann cells in the presence of elevated levels of cyclic AMP. *Glia* 8, 232-240.
62. Seeburg, P. H., 1993. The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. *Trends Neurosci* 16, 359-365.
63. Sendtner, M., Holtmann, B., Kolbeck, R., Thoenen, H., and Barde, Y. A., 1992. Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. *Nature* 360, 757-759.
64. Sendtner, M., Schmalbruch, H., Stockli, K. A., Carroll, P., Kreutzberg, G. W., and Thoenen, H., 1992. Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. *Nature* 358, 502-504.
65. Stangel, M., and Bernard, D., 2003. Polyclonal IgM influence oligodendrocyte precursor cells in mixed glial cell cultures: implications for remyelination. *J Neuroimmunol* 138, 25-30.
66. Sugai, F., Yamamoto, Y., Miyaguchi, K., Zhou, Z., Sumi, H., Hamasaki, T., Goto, M., and Sakoda, S., 2004. Benefit of valproic acid in suppressing disease progression of ALS model mice. *Eur J Neurosci* 20, 3179-3183.
67. Trojnar, M. K., Wierzchowska-Cioch, E., Krzyzanowski, M., Jargiello, M., and Czuczwar, S. J., 2004. New generation of valproic acid. *Pol J Pharmacol* 56, 283-288.

68. Tsai, L. K., Tsai, M. S., Ting, C. H., and Li, H., 2008. Multiple therapeutic effects of valproic acid in spinal muscular atrophy model mice. *J Mol Med* 86, 1243-1254.
69. Tsai, L. K., Yang, C. C., Hwu, W. L., and Li, H., 2007. Valproic acid treatment in six patients with spinal muscular atrophy. *Eur J Neurol* 14, e8-9.
70. Ullian, E. M., Harris, B. T., Wu, A., Chan, J. R., and Barres, B. A., 2004. Schwann cells and astrocytes induce synapse formation by spinal motor neurons in culture. *Mol Cell Neurosci* 25, 241-251.
71. Van Damme, P., Dewil, M., Robberecht, W., and Van Den Bosch, L., 2005. Excitotoxicity and amyotrophic lateral sclerosis. *Neurodegener Dis* 2, 147-159.
72. Van Den Bosch, L., and Robberecht, W., 2008. Crosstalk between astrocytes and motor neurons: what is the message? *Exp Neurol* 211, 1-6.
73. Van Den Bosch, L., Van Damme, P., Bogaert, E., and Robberecht, W., 2006. The role of excitotoxicity in the pathogenesis of amyotrophic lateral sclerosis. *Biochim Biophys Acta* 1762, 1068-1082.
74. Vandenberghe, W., Robberecht, W., and Brorson, J. R., 2000. AMPA receptor calcium permeability, GluR2 expression, and selective motoneuron vulnerability. *J Neurosci* 20, 123-132.
75. Vandenberghe, W., Van Den Bosch, L., and Robberecht, W., 1998. Glial cells potentiate kainate-induced neuronal death in a motoneuron-enriched spinal coculture system. *Brain Res* 807, 1-10.

SUMMARY

In summary our results indicate that VPA might be applicable to neurodegenerative diseases such as ALS. As multiple mechanisms have been shown to be involved in ALS pathogenesis, a combination of several neuroprotective compounds with additive effects appears might be a promising strategy for future clinical trials.

In our studies we proved that high concentrations of VPA, however, as well as KA application alone, led to dose-dependent loss of motor neurons as was shown by immunocytochemistry. In order to address a potential neuroprotective role of VPA in motor neurons, we first studied the interactions of highly purified populations of developing neurons under different culture conditions regarding cell survival and its dependency on glial feeding co-cultures, i.e. astrocytes or Schwann cells. Glial feeder layers significantly enhanced basal survival of motor neurons, and slightly protected them against KA neurotoxicity in presence of VPA.

Calcium imaging experiments revealed no effect of VPA on calcium transients once motor neurons were spontaneously active (>10 days in culture). In non-spontaneously active motor neurons (<10 days in culture), however, KA induced intracellular calcium transients were significantly increased in presence of VPA.

To overcome the problem of adverse effects of VPA administered in higher dosages, a number of chemical derivatives of VPA have been synthesized. We focus on three out of a number of synthesized VPA derivatives that have lately been under investigation: 3-propyl-heptanoic acid (3-PHA) and PE-4-yn enantiomers (R- and S-PE-4-yn).

The survival of motor neurons under simultaneous application of KA and VPA derivatives was not significantly increased. Pre-incubation of the cells with VPA and

even more with the derivatives before the addition of KA, however, significantly reduced their vulnerability against the KA-induced neurotoxic effect. The highest increase in motoneuron survival was seen after pre-incubation with S-PE-4-yn. We additionally tested the hypothesis of a correlation of the modulation of synaptic activity and a neuroprotective effect on motoneurons using whole cell patch clamp technique. The analysis of spontaneous synaptic activity in untreated cells and in presence of the test-compounds on glial feeder layers revealed a shift towards increased transsynaptic inhibition. VPA derivative, S-PE-4-yn reduced the frequency but not the amplitude of both spontaneous excitatory (EPSCs) and inhibitory (IPSCs) postsynaptic currents in motoneurons cultured on glial feeder layers.

Clearly, further studies are necessary to identify the exact mechanism of action of VPA and its derivatives that are involved in beneficial action of potential neuroprotective effect.

ZUSAMMENFASSUNG

Unsere Ergebnisse zeigen, dass Vaproat (VPA) eine therapeutische Option bei neurodegenerativen Erkrankungen wie der Amyotrophen Lateralsklerose (ALS) darstellt. Da multiple Pathomechanismen zur Entstehung der ALS beitragen, erscheint eine Kombinationstherapie aus unterschiedlichen neuroprotektiven Substanzen mit additiven Effekten als vielversprechende Strategie für zukünftige klinische Studien

In unseren Untersuchungen konnten wir mittels Immunzytochemie zeigen, dass VPA in hohen Konzentration zu vergleichbaren toxischen Effekten (dosisabhängiger Motoneuronverlust) führt wie die Applikation der exzitotoxischen Substanz Kainat. Um potenzielle neuroprotektive Effekte von VPA zu charakterisieren, untersuchten wir zunächst die Interaktion hochaufgereinigter embryonaler Motoneurone in unterschiedlichen Zellkulturbedingungen im Hinblick auf Zellüberleben in Abhängigkeit von der Präsenz ko-kultivierter Schwann-Zellen oder Astrozyten. Die basale Überlebensrate von Motoneuronen nahm signifikant zu, wenn diese auf glialen Zellen kultiviert wurden, zudem erbrachte die Ko-Kultur mit glialen Zellen zusammen mit der VPA-Applikation auch gewisse protektive Effekte gegen die neurotoxische Wirkung von Kainat.

Calcium imaging-Experimente zeigten keinen Einfluss von VPA auf die Kalziumtransienten von Motoneuronen, die bereits das Stadium der Spontanaktivität erreicht hatten (>10 Tage in Kultur). In den ersten 10 Tagen, in denen noch keine Spontanaktivität nachweisbar war, wurden die Kainat-induzierten spontanen Kalziumtransienten in Anwesenheit von VPA jedoch signifikant gesteigert.

Um das Problem toxischer Nebenwirkungen von hochdosiertem VPA zu umgehen, wurden in den letzten Jahren eine Anzahl chemischer VPA-Derivate

synthetisiert. Wir untersuchten drei vor kurzem entwickelte Substanzen: 3-propylheptanoic acid (3-PHA) und die PE-4-yn-Enantiomere (R- and S- PE-4-yn) bezüglich ihrer neuroprotektiven Effekte.

Bei simultaner Inkubation von Motoneuronen mit Kainat und VPA oder den untersuchten VPA-Derivaten ergaben sich keine signifikanten Effekte auf das Zellüberleben. Präinkubation der Zellen mit VPA und den Derivativen vor der Kainat-Applikation führte jedoch zu signifikanter Reduktion der Zellvulnerabilität. Die höchste Steigerung des Zellüberlebens wurde nach Präinkubation mit S-PE-4-yn beobachtet. Mittels der Patch Clamp-Technik untersuchten wir die Hypothese einer Korrelation zwischen neuroprotektivem Effekt und einer Modulation der synaptischen Aktivität. Die Analyse spontaner synaptischer Aktivität in unbehandelten im Vergleich zu mit den Testsubstanzen inkubierten Motoneuronen in Ko-Kultur mit glialen Zellen ergab eine Veränderung in Richtung vermehrter transsynaptischer Inhibition. Das VPA-Derivat S-PE-4-yn reduzierte die Frequenz, aber nicht die Amplitude von sowohl exzitatorischen als auch inhibitorischen postsynaptischen Potenzialen, wiederum jedoch nur, wenn die Motoneurone auf glialen Zellen kultiviert wurden.

Weitere Studien sind erforderlich, um die exakten Wirkmechanismen von VPA und den hier untersuchten Derivaten zu identifizieren, die den beobachteten neuroprotektiven Effekten zugrunde liegen.

ACKNOWLEDGMENTS

I would like to thank:

Prof. Dr.med.Susanne Petri and PD Dr.med.Klaus Krampfl for giving me a chance to run this project and for their unrestricted support, enthusiastic motivation, academic advice and confidence throughout every phase of my Ph.D. study.

Prof. Dr. med. Claudia Grothe and Prof. Dr. med.Christoph Fahlke for their interest in my work. Many thanks for your helpful comments on my experiments and for your excellent advice.

Prof. Reinhard Dengler for his inspiration and providing a highly professional scientific environment.

Dr.med. vet. Kirsten Haastert and Maike Wesseman from the Institute of Neuroanatomy, Hannover and PD Dr. Sibylle Jablonka from Institute of Neurobiology, Würzburg for their help in my beginnings with establishing cell culture.

Christiane Hotopp-Herrgesell, Carola Kassebaum for their perfect technical assistance and Frau Dreier from Animal House, MHH for the hundreds of animals she mated for me. They were definitely the best!

Andreas Niesel for his excellent technical and mental support, particularly on the way to kiosk.

I kindly thank my colleagues Friedrich Schlesinger, Kirsten Jahn, Elke Ziegler and Katja Kollwe of our department for constructive discussions.

Many thanks to my current colleagues Junping Song, Sarah Knippenberg, Alexander Sarlette, Jaenne de la Roche, Vanesa Buchholz and Nadine Thau for really making me look forward to coming to the lab (almost) every morning! I really enjoyed the atmosphere in lab and definitely appreciated this base station and also the lunch break gossip!

Great acknowledge to the organizers of The Center for Systems Neuroscience (ZSN, Hannover, Germany) for well-organized programme and providing facilities for interdisciplinary team works, which gave us the opportunity to take advantage of this interactive programme during the study.

I should never forget my closest friend Alexandra Kotsiari, who made my life easier here and meaningful.

Finally I thank to all my family for their support and affection through the distance, especially my mum EVA, grandmu KATARINA, MONIKA and Rudy. Dakujem.