TOWARDS GENE THERAPY IN

AMYOTROPHIC LATERAL SCLEROSIS

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Towards gene therapy in amyotrophic lateral sclerosis

ISBN: 90-393-3826-4

Druk: Febodruk, Enschede

Omslag: Rat-1 fibroblasten getransfecteerd met catalase op een achtergrond van motorische neuronen. Ontworpen door Freya Wisman.

TOWARDS GENE THERAPY IN AMYOTROPHIC LATERAL SCLEROSIS

Ontwikkeling van gentherapie voor amyotrofische laterale sclerose
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
dinsdag 26 oktober 2004 des middags te 2.30 uur

door

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geboren op 14 juli 1976 te Zeist

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The study presented in this thesis was financially supported by the "Prinses Beatrix Fonds".
Financial support for the publication of this thesis was provided by Dr. Ir. Van de Laar Stichting and J.E. Jurriaanse Stichting.

Contents

List of abbreviations

AAV	adeno-associated viral vector(s)
ANOVA	analysis of variance
ALS	amyotrophic lateral sclerosis
BBB	blood brain barrier
BHK	baby hamster kidney
BSA	bovine serum albumin
CMV	cytomegalovirus
CNS	central nervous system
CNTF	ciliary neurotrophic factor
COX-2	cyclooxygenase-2
CSF	cerebrospinal fluid
Ct	cycle treshold
DHK	dihydrokainic acid
EAAT	excitatory amino acid transporter
FALS	familial amyotrophic lateral sclerosis
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
HEK	human embryonic kidney
HIV	human immunodeficiency virus
IGF-1	insulin growth factor-1
iNOS	inducible nitric oxide synthase
LVV	lentiviral vector(s)
mSOD1	mutated Cu,Zn-superoxide dismutase
NAC	N-acetyl-L-cysteine
NeuN	neuronal nuclei
NF-κB	nuclear factor-κB

NSC-34	neuroblastoma-spinal cord hybrid cells
PaGE	paw grip endurance
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pmn	progressive motoneuropathy
qPCR	quantitative polymerase chain reaction
ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction
RVG	rabies virus glycoprotein
SALS	sporadic amyotrophic lateral sclerosis
SOD1	Cu,Zn-superoxide dismutase
TU	transducing units
VEGF	vascular endothelial growth factor
VSV-G	vesicular stomatitis virus glycoprotein

CHAPTER 1

GENERAL INTRODUCTION

Contents of the introduction

1. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common motoneuron disease in human adults, described as early as 1869 by a French neurobiologist and physician, Jean-Martin Charcot (Charcot and Joffroy, 1869). ALS is a relentlessly progressive, fatal disorder of the nervous system that results from the degeneration of upper and lower motoneurons. At this moment there is no effective treatment available for this condition.

1.1 Epidemiology and classification

The incidence of ALS is 1-3 per 100,000 persons. In most cases the clinical onset manifests itself in the forth or fifth decade of life. Denervation of the respiratory muscles and diaphragm, with consequent respiratory insufficiency is generally the main fatal event that occurs within 2-5 years after onset (Rowland and Shneider, 2001; Strong, 2003). Gender probably plays a role in ALS, as the number of men developing the disease is 1.5 times higher than that of women. This difference declines after the menopause, suggesting a role for estrogens in the pathogenesis of ALS (Norris et al., 1993; Haverkamp et al., 1995).

Two distinct forms of ALS have been identified, a familial form (FALS) and a sporadic form (SALS). FALS accounts for about 10% of all cases of ALS, in the remaining 90% no genetic linkage has been implicated and these are therefore characterised as sporadic. In 1993 mutations in the gene coding for Cu,Zn-superoxide dismutase (SOD1), a superoxide radical scavenging enzyme, were found in FALS patients (Rosen et al., 1993). To date, 15-20% of FALS patients can be linked to mutations in the SOD1 gene (Cudkowicz et al., 1997), and more than 100 mutations have been identified, spanning all exons of the SOD1 gene (Cleveland, 1999; Valentine and Hart, 2003).

1.2 Clinical and neuropathological features

Initially, the site of onset determines the clinical features. Limb onset is found in 65-80% of all ALS cases, and results in weakness of arms and legs. Onset associated with bulbar dysfunction, i.e. initial swallowing and speech difficulties, is found in the remaining 20-25% of the cases. As the disease progresses the clinical expression becomes quite

uniform, with a progressive decline of muscular function resulting in paralysis, speech and swallowing disabilities, and ultimately, respiratory failure causing death 2-5 years after disease onset. Over the last years it has been recognised that also alterations in cognition are a common factor in ALS patients (Wilson et al., 2001). Thus, frontal and temporal lobe atrophy can be demonstrated in a subset of ALS patients by CT-scanning (Kato et al., 1993). However, early manifestations of frontotemporal lobe dysfunction can only be visualised with more sensitive dynamic neuroimaging techniques (Strong et al., 1999).

The major neuropathological features correlate with the principal clinical manifestation, namely a degeneration of motoneurons in the spinal cord, brainstem, and motor cortex (figure 1). Loss of motoneurons is associated with intracellular inclusions such as aggregates that are immunoreactive for neurofilaments, Bunina bodies immunoreactive for cystatin C, and Lewy body-like inclusions. Another feature of ALS pathology is the accumulation and activation of both astrocytes and microglial cells indicative for a neuroinflammatory response in affected areas (McGeer and McGeer, 2002; Turner et al., 2004). Also myelin pallor in the corticospinal tracts can be found as a consequence of axonal loss in this region. Remarkably, motoneurons of the Onuf's nucleus in the spinal cord, and motoneurons of the cranial nerve nuclei III, IV, and VI are usually spared (Mannen et al., 1977; Schroder and Reske-Nielsen, 1984; DePaul et al., 1988). This corresponds with the clinical finding that pelvic floor muscle control and oculomotor functioning seem to be normal in ALS patients.

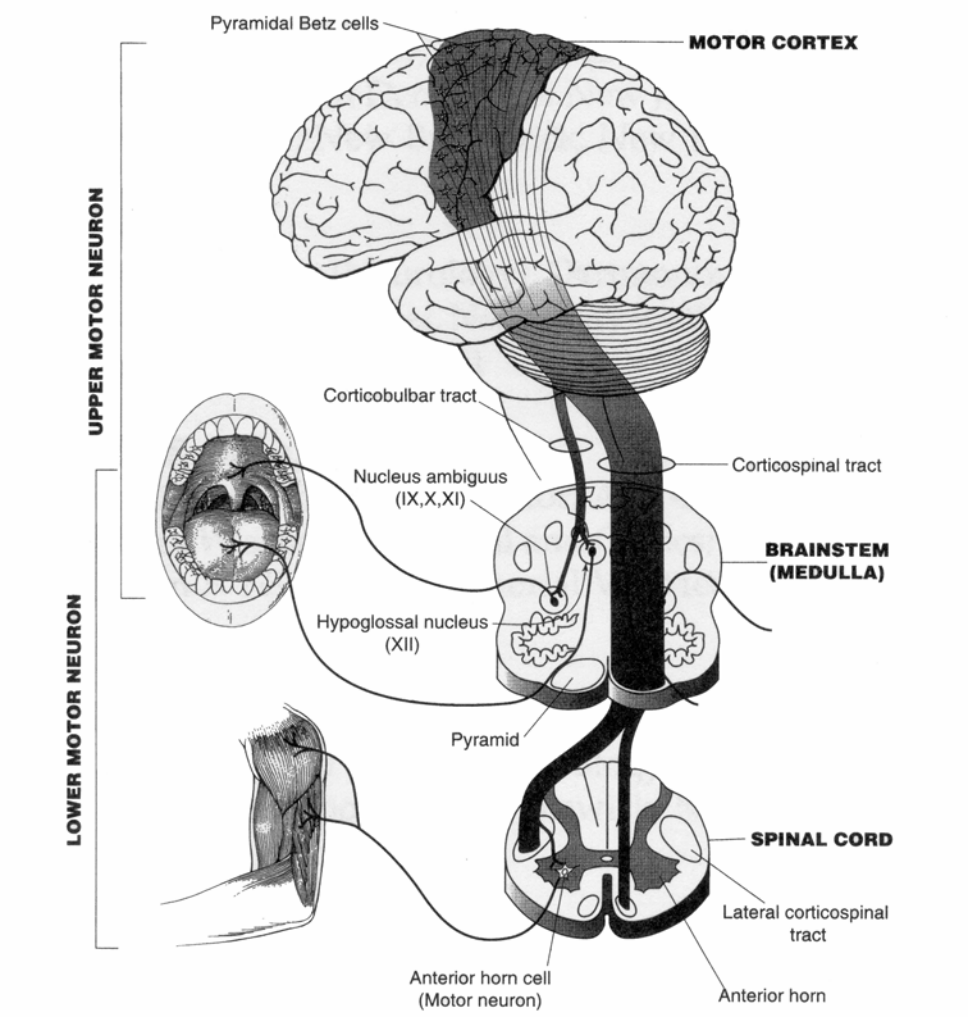

Figure 1. *ALS is associated with a preferential loss of both upper (motor cortex, also known as Betz cells)- and lower (brain stem and spinal cord) motoneurons. As indicated, the site of onset determines the first clinical signs (from Belsh and Schiffman, 1996).*

1.3 Mutated SOD1 based animal models for ALS

Transgenic mice and rats overexpressing FALS associated forms of mutated human SOD1 develop a paralytic syndrome that closely replicates the clinical and pathological hallmarks of ALS (Gurney et al., 1994; Wong et al., 1995; Bruijn et al., 1997; Howland et al., 2002). The first clinical sign in these mice is a fine tremor in at least one limb, which develops into weakness and atrophy of proximal muscles. At end-stage of the disease the animals are severely paralysed and unable to eat and drink. The initial neuropathological hallmarks are vacuolar degeneration of motoneurons with undetectable neuronal loss or gliosis. As the disease progresses, however, α-motoneurons in the ventral horn decrease in number, their rough endoplasmatic reticulum undergoes vacuolar dilatation, and mitochondrial changes take place. At end-stage of disease there is a dramatic loss of motoneurons, and Lewy body-like intracellular inclusions can be found. This is accompanied by marked gliosis and accumulation of activated microglial cells (Dal Canto and Gurney, 1995). Other features of the disease that can be observed in SALS patients are also reproduced in these ALS animal models, including a loss of the glutamate transporter GLT-1, signs of neuroinflammation, and oxidative stress (Dunlop et al., 2003; Howland et al., 2002; Bendotti et al., 2001; Bruijn et al., 1997; McGeer and McGeer, 2002; Tu et al., 1997).

The most commonly used mutated SOD1 (mSOD1) animal model in ALS research is a transgenic mouse overexpressing human SOD1 containing a substitution of glycine to alanine at position 93 (G93A-hSOD1) (Gurney et al., 1994). The amount of incorporated copies of the G93A-hSOD1 gene determines the severity of disease in the ALS mice. G93A-hSOD1 mice that express high amounts of the mSOD1 gene are called high-copy mice and die at approximately 4-5 months of age, while the low-copy mice generally die within 8 months (Dal Canto and Gurney, 1995).

2. Disease mechanisms in ALS

ALS is a multi-factorial disease, and many pathological mechanisms have been suggested to play a role in the aetiology of the disease (Strong, 2003; Bruijn et al., 2004; Veldink et

al., 2004). In this thesis I will focus on oxidative stress and excitotoxicity as two, highly interrelated, contributing factors to the selective degeneration of motoneurons.

2.1 Oxidative stress in ALS

Oxidative stress is a consequence of a misbalance between the production of oxidants and the ability of a cell or tissue to defend itself against them. Potential oxidants are free radicals, such as the superoxide and hydroxyl radicals, as well as molecules with a strong oxidative potential, such as hydrogen peroxide, nitric oxide, and peroxynitrite. All these oxidants, often referred to as reactive oxygen species (ROS) and reactive nitrogen species, can lead to a wide range of intracellular- and extracellular effects, including damage to DNA, proteins and lipids.

Markers indicative for oxidative damage to proteins, lipids, and DNA have been found in tissue derived from motor cortex and spinal cord of SALS patients (Ferrante et al., 1997a; Beal et al., 1997). Also, in peripheral cells of ALS pateints, such as fibroblasts (Aguirre et al., 1998) and blood cells (Bonnefont-Rousselot et al., 2000), an increased sensitivity for oxidative stress was observed. Furthermore, oxidative stress markers have also been found in FALS patients and ALS animal models identifying lipid modifications (Ferrante et al., 1997b), DNA damage (Ferrante et al., 1997a; Warita et al., 2001), and protein modifications (Beal et al., 1997; Ferrante et al., 1997b).

The finding of mutations in the SOD1 gene in FALS patients, together with the neuropathological features observed in the mSOD1 transgenic mice, have provided strong impetus for the oxidative stress hypothesis in ALS. Under physiological conditions, SOD1 catalyses the conversion of superoxide into hydrogen peroxide, which is then further metabolised into water by glutathion peroxidase or catalase. Since a knock-out mouse for SOD1 does not develop a motoneuron disease (Reaume et al., 1996) and some FALS mutations do not cause a decrease in enzymatic SOD1 activity (Ripps et al., 1995; Wong et al., 1995), it is generally accepted that the toxic effect of mSOD1 is due to a gain of function rather than a loss of enzymatic SOD1 activity per se (Julien, 2001). Several hypotheses on the mechanism of this toxic property of mSOD1 have been put forward (figure 2). One proposal is that misfolding of mSOD1 allows abnormal substrates, such as peroxynitrite (Beckman et al., 1993) and hydrogen peroxide (Wiedau-Pazos et al., 1996),

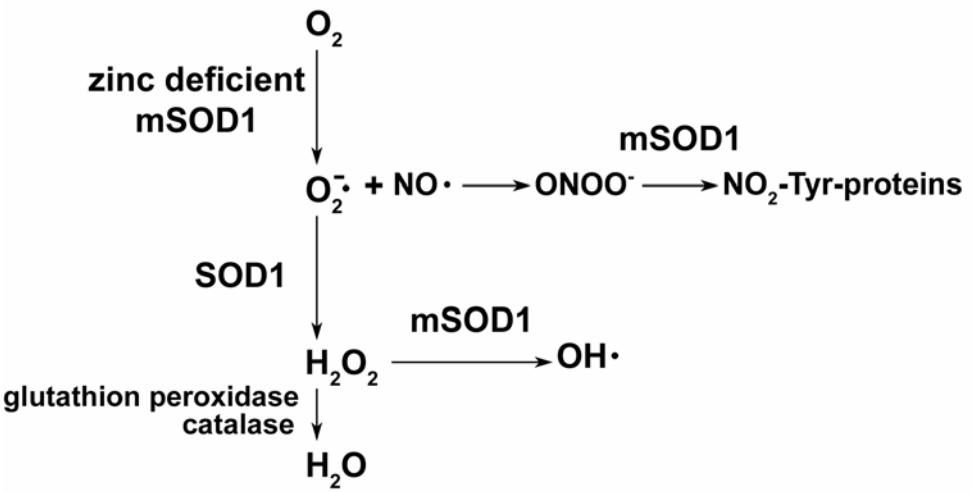

Figure 2. *Proposed enzymatic mechanisms to explain the toxic gain of function of mutant SOD1. Normally, SOD1 catalyses the conversion of superoxide into hydrogen peroxide, which is further metabolised into water by catalase and glutathion peroxide. One proposed mechanism for mSOD1 toxicity is that mutated SOD1 is misfolded, allowing abnormal substrates, such as peroxynitrate and hydrogen peroxide, to react with its catalytic site leading to the production of reactive oxygen/nitrogen species, such as peroxynitrite and hydroxyl radicals. Another mechanism could be the inability of mSOD1 to properly bind zinc. Zinc-deficient mSOD1 may catalase the conversion of molecular oxygen into superoxide radicals.*

to react at the copper atom in its catalytic site. The reaction of peroxynitrite catalysed by mSOD1 leads to the nitration of tyrosine residues and a consequent loss of function of critical proteins (Beckman et al., 1993). Hydrogen peroxide, in its turn, may be used as a substrate by mSOD1 to generate hydroxyl radicals, which can damage DNA, proteins, and lipid membranes, ultimately leading to cell death (Wiedau-Pazos et al., 1996). Another mechanism by which mSOD1 could exert its toxic gain of function is its inability to properly bind zinc, leading to a reduction to the Cu^{+}-form of mSOD1. This reduced form of the enzyme may catalyse a reaction in which oxygen is converted to superoxide radicals. These superoxide radicals reacts fast with nitric oxide to produce the reactive peroxynitrite (Estevez et al., 1999), which is a strong oxidant, as well as an aberrant substrate for mSOD1, as described above. Another hypothesis is that the mSOD1 aggregates to form cytoplasmic and intramitochondrial inclusions (Pasinelli et al., 2004). Aggregates immunoreactive for SOD1 are found in the mSOD1 ALS mouse model as well as in post-mortem tissue obtained from human ALS cases linked to mSOD1 (Bruijn

et al., 1998). These aggregates could be toxic by disrupting axonal transport or by co-aggregating with essential cellular proteins. For example, it has been demonstrated that mSOD1 can aggregate with heat shock proteins, making them unavailable to exert their anti-apoptotic function (Okado-Matsumoto and Fridovich, 2002).

Besides mSOD1 related changes, also additional causes of oxidative stress are known to play a role in the pathogenesis of FALS and SALS. One of the factors that is known to be implicated is mitochondrial dysfunction. The onset of disease in mSOD1 mice is associated with an increase in the number of dilated mitochondria, suggesting that the toxicity of mSOD1 may be mediated by damage to mitochondria (Kong and Xu, 1998). Similar ultrastructural changes in mitochondrial morphology are found in post-mortem CNS tissue from ALS patients (Sasaki and Iwata, 1996). In addition, increases in complex I and II/III activity (Bowling et al., 1993), and decreases in complex IV activity (Fujita et al., 1996) have been found in spinal cord of ALS patients. Chronic mitochondrial inhibition by the complex II inhibitor malonate in organotypic spinal cord cultures showed that motoneurons are specifically vulnerable to mitochondrial dysfunction (Kaal et al., 2000). Dysfunction of mitochondria is associated with altered Ca^{2+}-homeostasis, the production of excess amounts of ROS, and can cause the release of the apoptotic signal cytochrome c.

The accumulation and activation of microglial cells and astrocytes in spinal cord and motor cortex is a prominent feature of ALS (Troost et al., 1990; Lampson et al., 1990; Kawamata et al., 1992; Turner et al., 2004). Neuroinflammation is also thought to contribute to oxidative stress. For example, activated microglia can release superoxide free radicals via the respiratory burst system, which in turn can contribute to motoneuron degeneration (Colton and Gilbert, 1987; McGeer and McGeer, 2002). Moreover, similar to astrocytes, activated microglia express inducible nitric oxide synthase (iNOS) and release nitric oxide into the extracellular milieu. Finally, high levels of cyclooxygenase-2 (COX-2), expressed by activated microglia (Bauer et al., 1997), have been reported in spinal cord of ALS patients (Yasojima et al., 2001). COX-2 catalyses the conversion of arachidonic acid into prostaglandins, an enzymatic process associated with the production of superoxide radicals. Another source of ROS can be attributed to glutamate-induced excitotoxicity. An imbalance in calcium homeostasis induced by glutamate toxicity can

cause the formation of ROS and an activation of intracellular death inducing pathways (Rothstein, 1996; Shaw and Ince, 1997). This phenomenon will be discussed in more detail below.

2.2 Excitotoxicity

Before reviewing the evidence that a dysfunction of the glutamate neurotransmission system may contribute to motor neuron injury in ALS, the basic physiology of this process and the concept of excitotoxicity will be discussed briefly.

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system (CNS). In normal glutamatergic neurotransmission (figure 3), glutamate is released from synaptic vesicles in the presynaptic terminal and diffuses into the synaptic cleft. At the postsynaptic neuron glutamate activates either ionotropic (e.g. NMDA, AMPA, or kainate) or metabotropic (G-protein coupled) glutamate receptors. Ionotropic receptors are directly linked to ion channels, and activation of these receptors increases transmembrane calcium and sodium fluxes. Metabotropic receptors do not have ion-channels as part of their structure. Instead, they affect channels by activation of G-proteins and evoke a variety of functions by mediating intracellular signal transduction. The excitatory signal is terminated by active removal of glutamate from the synaptic cleft by excitatory amino acid transporters (EAATs) (Danbolt, 2001). This family has five members, designated EAAT1-5, each having specific physiological properties and distribution throughout the CNS. EAAT2 is mainly localised on astrocytes and is called GLT-1 in rodents. It has an important role in extracellular glutamate clearance in the CNS, as GLT-1 knock-out mice retained less than 10% of total glutamate transport (Tanaka et al., 1997). In astrocytes glutamate can be converted to α-ketoglutarate, which may be further metabolised in the tricarboxylic acid cycle. Alternatively, as part of the so-called glutamate-glutamine shuttle, glutamate can also be converted to glutamine by glutamine synthase (Hertz et al., 1999). Glutamine, in turn, is transported back to the neuronal terminal where it is reconverted to glutamate by glutaminase and stored again into synaptic vesicles (Shigeri et al., 2004).

Under pathophysiological conditions, however, the excitatory action of glutamate can turn into a toxic event resulting in neuronal injury and death, due to overactivation of either

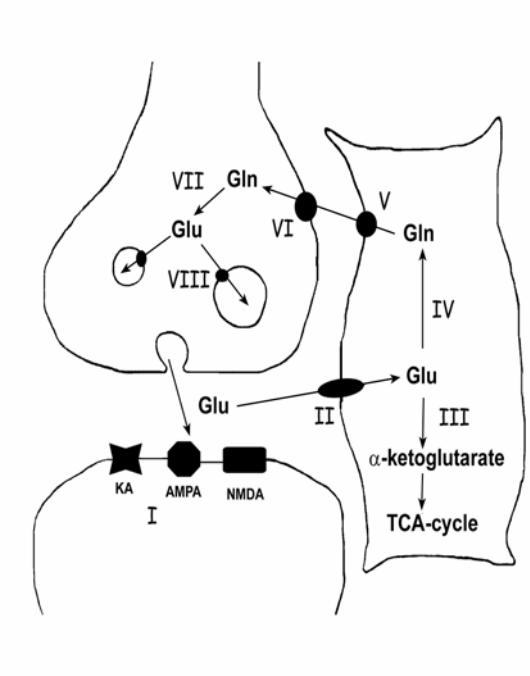

Figure 3. *Glutamatergic neurotransmission. Glutamate released from synaptic vesicles in the presynaptic neuron activates glutamate receptors (I), such as NMDA, AMPA, or kainate, on the postsynaptic membrane. The excitatory signal is terminated by active removal from the synaptic cleft mainly by the glutamate transporter EAAT2 (II). Glutamate is converted to α-ketoglutarate by glutamate dehydrogenase (III) or is converted to glutamine by glutamine synthetase (IV) in the so-called glutamate-glutamine shuttle. Glutamine is transported back to the motoneuron by glutamine transporters (V), and is reconverted to glutamate by glutaminase (VI), and stored again in presynaptic vesicles by vesicular glutamate transporters (VIII).*

NMDA- or calcium-permeable AMPA receptors resulting in an excess influx of calcium (Heath and Shaw, 2002; Arundine and Tymianski, 2003). Under normal circumstances intracellular calcium concentrations are tightly controlled at a level below 100 nM. An increased level of intracellular free calcium can activate multiple calcium-dependent enzymes, such as calpain, protein kinase C, phospholipases, endonucleases, proteases, xanthine oxidase, and nitric oxide synthase. The activation of these enzymes can directly damage the neuron and sometimes induce the formation of free radicals. In addition, an increased level of free intracellular calcium will trigger mitochondrial Ca^{2+}-loading in an attempt to buffer the excess influx of calcium. This can lead to a loss in mitochondrial membrane potential, with a consequent decrease in ATP production and an increase in ROS production. All these processes lead to cell death.

Excitotoxicity can also be a secondary process triggered by a compromised energy status of the motoneuron. This will lead to a loss of the normal resting membrane potential and a consequent loss of the voltage-dependent Mg^{2+}-block of NMDA receptors. Now, even normal levels of glutamate can cause an excess influx of calcium and thereby activate the cascade of toxic processes.

2.2.1 Excitotoxicity in ALS

The first indication that alterations in glutamate homeostasis and consequent excitotoxicity might be implicated in the pathogenesis of ALS was the finding of increased glutamate levels in the cerebrospinal fluid (CSF) of ALS patients (Rothstein et al., 1990; Shaw et al., 1995). Although not all studies could confirm this result (Perry et al., 1990), results from a recent study, in which a more sensitive HPLC method was used to measure glutamate levels, showed that in a subpopulation of the ALS patients elevated glutamate levels could be found in the CSF (Spreux-Varoquaux et al., 2002). The finding of decreased EAAT2 protein levels indicated a potential mechanism that can explain the elevated glutamate levels in ALS patients. In 60-70% of the SALS patients, a 30-90% loss of EAAT2 protein in both motor cortex and spinal cord was found (Rothstein et al., 1995). In addition, a decrease in functional sodium-dependent glutamate uptake was observed in synaptosomes from the spinal cord and motor cortex of SALS patients (Rothstein et al., 1992).

2.2.2 Loss of EAAT2

The important role of EAAT2 in motoneuron degeneration is illustrated, inter alia, by anti-sense GLT-1 oligonucleotide treatment in rats, which resulted in a progressive motoneuron syndrome, showing hind limb paralysis within 7 days of treatment (Rothstein et al., 1996). In theory, several mechanisms may explain for the loss in EAAT2 functioning in ALS. First, truncated mRNA species were found in motor cortex from ALS patients indicative for an abnormal splicing of EAAT2 mRNA and consequent production of functional defective EAAT2 mRNA (Lin et al., 1998). However, while this first study indicated that these altered EAAT2 splice products were specific for ALS affected regions in the CNS, later studies identified these splice variants also in control tissue (Meyer et al., 1999; Flowers et al., 2001). A second explanation may be given by the fact that EAAT2 protein levels are regulated by a EAAT2 promoter. In recent years it has become evident that microglial cell activation is a prominent feature in ALS pathogenesis (McGeer and McGeer, 2002). Activated microglial cells can release several factors that can damage motoneurons, including glutamate and TNFα (McGeer and McGeer, 2002). In this context, it is worth mentioning that with the identification of the human EAAT2 promoter

it was found that TNFα can inhibit EAAT2 transcription (Su et al., 2003). Third, glutamate uptake by EAATs is driven by a sodium-gradient. The sodium gradient across the membrane is maintained by the Na^+-K^+-pump, which is dependent on the hydrolysis of ATP. Mitochondrial dysfunction and consequent ATP depletion can therefore result in a decrease in glutamate uptake with, a consequential accumulation of extracellular glutamate (Rossi et al., 2000). Although this phenomenon is not directly examined in ALS, it can not be excluded that it could also play a role in the pathogenesis, as a global loss of Na^+-K^+-ATPase activity is found in the G93A-hSOD1 ALS mouse model (Ellis et al., 2003). The final, and most compelling factor that can explain transporter loss, is the disruption of glutamate transporters by oxidants leading to a reduction in glutamate uptake capacity (Volterra et al., 1994; Trotti et al., 1996). Accordingly, oxidative modifications to EAAT2 have been reported in both ALS patients and the mSOD1 mouse model (Pedersen et al., 1998; Deitch et al., 2002).

2.3 Selective motoneuron vulnerability and neuronal death in ALS

Given the widespread distribution of glutamate receptors throughout the CNS, the question arises why motoneurons are selectively vulnerable to excitotoxicity and oxidative stress. This could be due to a combination of factors. First, in the context of excitotoxicity it is noteworthy that motoneurons express Ca^{2+}-permeable AMPA receptors (Heath and Shaw, 2002; Weiss and Sensi, 2000). AMPA receptors are composed of heteromeric complexes of various combinations of four specific receptor subunits, called GluR1 to 4. The Ca^{2+}-permeability of the AMPA receptor is determined by the presence of the GluR2 subunit. Thus, while GluR2 prevents Ca^{2+}-influx, GluR2-lacking AMPA receptors have a high permeability for Ca^{2+} (Hollmann et al., 1991). Interestingly, AMPA receptors expressed by motoneurons seem to be relatively deficient in GluR2 compared to dorsal horn neurons, which are less sensitive to an excitotoxic insult (Van Damme et al., 2002; Shaw et al., 1999). When GluR2 is present in the AMPA receptor complex, a posttranscriptional substitution of a glutamine residue (Q) to an arginine residue (R) at the so-called Q/R-site determines the Ca^{2+}-permeability of the receptor. AMPA receptors containing the unedited form of GluR2 (Q) have high calcium permeability. Normally, this editing process occurs with virtually 100% efficiency from the embryonic stage

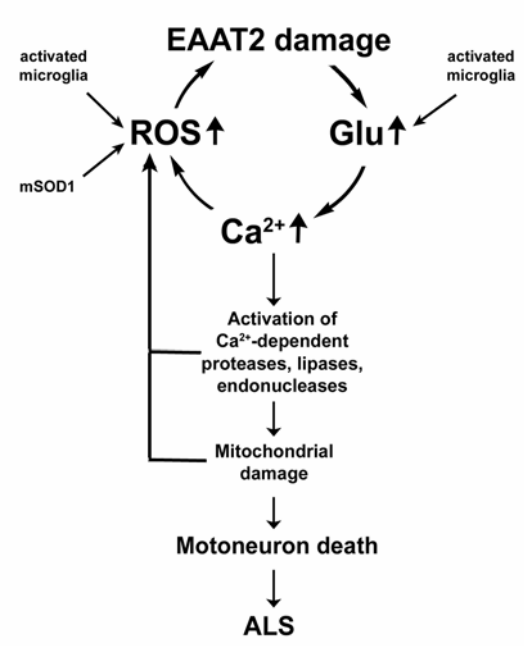

Figure 4. *Schematic overview of the impact of oxidative stress and excitotoxicity in ALS pathogenesis. Oxidative stress and excitotoxicity are highly interrelated pathogenic mechanisms, which can result in a vicious feed-forward cycle. As indicated, ROS can damage the glutamate transporter EAAT2, leading to elevated levels in glutamate, increased influx of calcium, mitochondrial damage, and excitotoxic death of motoneurons. In turn, excitotoxicity can increase ROS, which can further damage EAAT2. The impact of the mutated SOD1 and the neuroinflammatory response in ALS is illustrated by the fact that mSDO1 and activated microglial cells are known to cause the release of large amounts of ROS, glutamate, or a combination thereof.*

onwards. In a recent study, using laser microdissection to analyse single motoneurons, it was shown that the editing efficiency in approximately 56% of the motoneurons from ALS patients was incomplete, while 100% editing efficiency was found in controls (Kawahara et al., 2004). Incomplete editing, such as found in ALS, will result in Ca^{2+}-permeable AMPA receptors, leading to increased Ca^{2+}-influxes, and consequent excitotoxicity.

Second, and in relation to the above phenomenon, it is noteworthy that motoneurons have a low calcium buffering capacity (Ince et al., 1993). Apart from active Ca^{2+}-pumps, also Ca^{2+}-binding proteins, including parvalbumin and calbindin D28k, are implicated in the control the intracellular calcium levels. Motoneurons seem to express lower levels of these calcium-binding proteins compared to motoneurons that are relatively spared in ALS (Ince et al., 1993). As described above, increased intracellular levels of calcium will lead to the activation of Ca^{2+}-dependent enzymes and to mitochondrial dysfunction. This can be specifically injurious to cells with high metabolic rates, such as motoneurons.

These features could, at least in part, explain the selective degeneration of motoneurons in ALS. In this context it is important to note that excitotoxicity and oxidative stress are considered to be highly interrelated mechanisms in ALS pathogenesis (Rao and Weiss, 2004). Thus, progressive motoneuron death can be explained by a feed-forward cycle in which ROS, either or not generated by excitotoxic processes in motoneurons, damage the glutamate transporter and lower glutamate uptake capacity of the surrounding astrocytes. As illiustrated in figure 4, this could converge into a self-propagating process driven by a further increase in the synaptic glutamate concentration, excitotoxicity, and consequent production of ROS. This vicious feed-forward cycle can explain the progressive nature of ALS and emphasises the potential use of therapeutic interventions aimed to target both oxidative stress and excitotoxicity.

3. Therapeutic strategies in ALS

As stated above, several pathogenic mechanisms may lead to motoneuron death in ALS. Consequently, many different therapeutic approaches have been evaluated for possible treatment of ALS patients (Morrison, 2002; Bruijn, 2002; Strong, 2003). In this section I will discuss therapeutic strategies aimed to limit oxidative stress and excitotoxicity in ALS disease models and ALS patients.

3.1 Therapy targeted at oxidative stress

Several anti-oxidants have been tested on their neuroprotective efficacy in ALS. One of these is vitamin E (α-tocopherol), a lipophilic agent that is present in membranes and acts as a chainbreaker, thus protecting lipids from peroxidation. In 1939, Lou Gehrig, a famous baseball player in the USA, was diagnosed with ALS. At that time, there was a great interest in vitamin E as treatment for nervous system disorders. Gehrig was one of the first ALS patients treated with vitamin E (Reider and Paulson, 1997), the course of his disease, however, appeared to be unaltered by vitamin E treatment. The therapeutic potential of vitamin E was rekindled when new interest in the role of oxidative stress emerged (Rosen et al., 1993). In high-copy mSOD1 ALS mice vitamin E treatment delayed disease onset and progression but did not prolong survival (Gurney et al., 1996). A placebo-controlled

trial in 289 ALS patients showed that, although biochemical markers of oxidative stress were decreased in treated patients, vitamin E treatment did not affect their motor function and survival (Desnuelle et al., 2001).

Another anti-oxidant tested in both the mSOD1 ALS mouse model and in ALS patients is N-acetyl-L-cysteine (NAC). NAC can act as a free radical scavenger and can increase glutathion levels by increasing the L-cysteine level, which is the rate-limiting precursor for glutathion synthesis (Holdiness, 1991; Burgunder et al., 1989). In high-copy mSOD1 ALS mice, NAC-treatment delayed onset with approximately one week, while survival was prolonged by approximately 10 days (Andreassen et al., 2000). However, a randomised, double-blind, placebo-controlled clinical trial in 110 ALS patients failed to detect any effect of treatment with NAC on either disease progression or survival (Louwerse et al., 1995).

The last anti-oxidant discussed here is catalase. Catalase can convert hydrogen peroxide and inhibits the formation of nitric oxide, a precursor of peroxynitrate (Mittal, 1993). In primary motoneuron cultures, injections of catalase cDNA or catalase itself significantly decreased the vulnerability of these cells for hydrogen peroxide-induced cell death (Herpers et al., 1999). In high-copy mSOD1 ALS mice, the synthetic SOD/catalase mimetic EUK-134 reduced oxidative stress and prolonged survival in ALS mice (Jung et al., 2001). Interestingly, the efficacy of EUK-134 has been attributed mainly to its catalase activity (Baker et al., 1998). Putrescine-modified catalase has also been tested in mSOD1 mice. This modified derivative of catalase, that has an increased blood-brain-barrier permeability (Reinholz et al., 1999a), significantly delayed disease onset in high copy ALS mice (Reinholz et al., 1999b). In low-copy mSOD1 ALS mice, continuous infusion of putrescine-catalase showed a therapeutic effect on both onset and survival (Poduslo et al., 2000). So far, catalase or catalase-based drugs have not been tested as anti-oxidant treatment in ALS patients, which is mainly due to biostability and delivery issues inherent to catalase therapy in the CNS (Pong, 2003). Intriguingly, since gene therapy could circumvent these issues, it is proposed that delivery of catalase by gene therapy would be an interesting therapeutic option that warrants evaluation on its neuroprotective potential in ALS.

3.2 Therapy targeted at excitotoxicity

The only FDA-approved agent registered for causative treatment of ALS patients is riluzole. It is thought to reduce excitotoxicity by inhibiting glutamate release, blocking postsynaptic NMDA receptors, and blocking presynaptic voltage-dependent sodium channels (Doble, 1996). The exact mechanism responsible for the therapeutic effects in ALS is still not known. In high-copy mSOD1 ALS mice riluzole treatment significantly prolonged survival, albeit without changing the time of onset (Gurney et al., 1996). In a first placebo-controlled trial with 155 ALS patients, a 3-month difference in mean survival was observed in the riluzole-treated group compared to the control group (Bensimon et al., 1994). In a second trial of 959 patients the positive result of riluzole treatment on survival was confirmed (Lacomblez et al., 1996).

Other compounds that can diminish excitotoxicity in experimental in vitro- and/or in vivo-models have not shown to be beneficial in ALS patients. One of these compounds is gabapentin, an anti-convulsant which decreases the pool of releasable glutamate in the axon terminal (Taylor, 1997). Although this drug increased the survival of high-copy mSOD1 ALS mice with approximately 10 days (Gurney et al., 1996), it was not effective in a clinical trial ALS patients (Miller et al., 1996; Miller et al., 2001). Similarly, also branched-chain amino acids, which are proposed to act by activating glutamate dehydrogenase (Tandan et al., 1996; The Italian ALS Study Group, 1993), L-threonine that can increase the concentration of inhibitory transmitter glycine (Blin et al., 1992), and dextromethorphan (Gredal et al., 1997), a NMDA receptor antagonist, did not show any beneficial effects in ALS patients.

As discussed earlier, a decrease in EAAT2 protein expression is considered an important contributing factor to excitotoxicity in ALS. Therefore, it has been suggested that increasing the glutamate uptake capacity in the surroundings of motoneurons would protect them from excitotoxicity (Morrison, 2002; Maragakis and Rothstein, 2004; Herrera-Marschitz, 2004). Since most of the glutamate uptake is accounted for by the glial transporter EAAT2 (Tanaka et al., 1997), from the therapeutical point of view it seems reasonable to try and increase the EAAT2 protein level. The neuroprotective potential of EAAT2 is underscored by a recent in vivo study showing that increased expression of EAAT2 modulates excitotoxicity and delays the onset of disease in EAAT2/mSOD1

double transgenic ALS mice (Guo et al., 2003). Although this cross-breeding study in transgenic mice demonstrates the neuroprotective efficacy of EAAT2 overexpression, it is obvious that such a strategy can not be employed for clinical use. Theoretically, expression of EAAT2 in astrocytes can be induced by physiological modulators such as EGF, TGF-α (Zelenaia et al., 2000) and cAMP analogs (Swanson et al., 1997) that are known to interact with the promoter region of the EAAT2 gene. However, the clinical use of these agents is greatly hampered by the fact that, due to their pleiotropic nature, they interfere with multiple cellular pathways other than EAAT2 regulation. Taking into account the above considerations, it is proposed that gene therapy might be a promising approach to locally increase EAAT2 expression and to attenuate excitotoxicity in ALS.

4. Gene therapy in neurodegenerative diseases

As described above, delivery of anti-oxidant proteins (i.c. catalase) or antiglutamatergic proteins (i.c. EAAT2) in the vicinity of affected motoneurons can be hypothesised to be of therapeutic value in ALS. However, to exert their neuroprotective effect appropriate delivery of these proteins to the CNS is essential. Systemic delivery of proteins to the CNS is only possible if specific uptake sites in the blood brain barrier (BBB) are present, or if BBB permeabilisers are linked to the parent protein. A disadvantage of systemic delivery of therapeutic proteins is the occurrence of severe systemic side effects and their rapid degradation. Direct delivery of therapeutic proteins into the CNS is likely to circumvent these limitations. Therefore, direct delivery techniques have been developed including gene therapy, which allows long-term, localised, regulatable expression of therapeutic genes. At present, to introduce therapeutic genes into the CNS, both ex vivo gene therapy and in vivo gene therapy can be exploited.

4.1 Ex vivo gene therapy

Ex vivo gene therapy comprises the delivery of therapeutic proteins via genetically engineered cells overexpressing the therapeutic gene of interest. The manipulated cells can be transplanted into the body in three different manners, i.e. as naked cells, or encapsulated in micro- or macrocapsules, respectively. When cells are transplanted as

naked cells several critical biosafety aspects come into play. When allogeneic, xenogeneic cells, or cell lines are used, one important limitation is the possibility to trigger an immune response, and consequent rejection of the foreign cells. Moreover, even when immunosuppression is used to delay possible rejection of the graft, these implants may develop into tumors (Jaeger, 1985; Bing et al., 1988). Although the use of autologous cells is expected to circumvent part of these problems, it entails that for each individual a specific cell bank has to be produced, which might not be clinical feasible in all cases. Another major problem associated with the transplantation of naked cells relates to the impossibility of retrieving the transplanted cells, e.g. by operative means, in case adverse effects take place.

To overcome these problems, alternative strategies have been developed in which the genetically engineered cells are encapsulated by a permeable membrane. Encapsulation of the cells will shield them from the immune system, but still enables these cells to release the therapeutic protein and to take up small molecules, including nutrients or disease related pathogenic factors. Immunoisolation of the genetically engineered cells from the host even allows the use of xenogeneic cells. Two types of encapsulation techniques exist, designated as micro-encapsulation or macro-encapsulation.

The encapsulation of small clusters of cells by a thin, semi-permeable membrane made of poly-electrolytes is called the micro-encapsulation technique. This technique, in combination with dopamine-secreting PC12 cells, has shown to be effective in reducing behavioural deficits in animal models for Parkinson's disease (Winn et al., 1991). However, the use of poly-electrolytes makes the capsule vulnerable for chemical and mechanical damage and, hence, of no value for use in humans (Aebischer and Ridet, 2001).

The biosafety issues described above can be circumvented by the use of the macro-encapsulation technique using hollow, cylindrical tubes with semi-permeable polymer membranes to encapsulate the genetically engineered cells. Moreover, when necessary, anchorage of the tubes to a catheter allows the retrieval of the cells by a simple surgical procedure. The usefulness of macrocapules was proven in animal models for different neurodegenerative disorders. For example, fibroblasts overexpressing adenosine were successful in suppressing seizures in the rat kindling model of epilepsy (Huber et al.,

2001), baby hamster kidney-cells (BHK) genetically engineered to overexpress ciliary neurotrophic factor (CNTF) delayed disease progression in progressive motoneuropathy (pmn) mice (Sagot et al., 1995), and L-DOPA and dopamine-producing PC12 cells improved motor behaviour in rodent and primate models for Parkinson's disease (Lindner and Emerich, 1998).

The clinical feasibility of macrocapsules in humans was first assessed in a safety study, performed by the group of Aebischer and colleagues, in which ALS patients received a macrocapsule containing CNTF-producing BHK-cells (Aebischer et al., 1996). Delivery of CNTF to the CSF was obtained through placement of the capsule in the CSF among the nerve roots of the cauda equina. Increased amounts of CNTF were measured in the CSF of these patients up to 20 weeks after the initial implant, but not in blood (Zurn et al., 2000). After removal of the capsule, histochemical and biochemical analyses confirmed the survival and functional activity of the encapsulated cells. Moreover, no immune reaction to the capsule could be observed after retrieval, despite the fact that no immunosupression was used during the post-transplantation period (Aebischer et al., 1996; Zurn et al., 2000). Unfortunately, no disease modifying effect was found in these patients. Importantly, however, adverse effects such as found upon systemic CNTF administration were not observed. This study established the use of the macro-encapsulation technique as a safe way to deliver neuroprotective proteins in a chronic and local manner (Aebischer et al., 1996). This form of gene therapy is especially useful for secreted therapeutic proteins. However, it can also be used for genetically engineered cells that express intracellular proteins that can counteract specific molecular pathological mechanisms e.g. oxidative stress and excitotoxicity. Consequently, and in the context of ALS, genetically engineered cells constructed to overexpress either the anti-oxidant enzyme catalase or the glutamate uptake carrier EAAT2 warrant evaluation for use in combination with ex vivo gene therapy.

4.2 In vivo gene therapy

The feasibility of in vivo gene therapy as a means to directly transfer genes into the CNS has gained strong impetus by recent developments towards the biosafety of the technique and the viral vectors used (Davidson and Breakefield, 2003). Genes encoding therapeutic

proteins can be delivered by viral- and non-viral vectors. As non-viral vectors are associated with a low transduction efficiency in vivo, in this chapter only in vivo gene therapeutic strategies based on viral vectors will be discussed. The most promising, and most widely used, viral vectors include the adeno-associated viral vectors and lentiviral vectors. Both vectors have the ability to infect dividing and non-dividing cells and show long-term gene expression both in vitro and in vivo. Therefore, both vectors are extensively studied for their use in the treatment of neurodegenerative diseases (Buchschacher, Jr. and Wong-Staal, 2000; Davidson and Breakefield, 2003).

4.2.1 Adeno-associated virus vectors

Adeno-associated viral vectors (AAV) are non-pathogenic human parvoviruses. Thus, while the viral genes are completely deleted, injections of AAV particles do not lead to an acute inflammatory response or toxic side effects. AAV can enter the cell via its interaction with membrane heparin sulfate proteoglycans. It can transduce the gene of interest through both episomal transgene expression and by random chromosomal integration (Hermens and Verhaagen, 1998). AAV-based in vivo gene therapy has been used in many animal models for neurodegenerative diseases, since it can transduce both neurons and astrocytes. For example, while intrastriatally delivered AAV- GDNF (glial cell line-derived neurotrophic factor) showed structural and functional neuroprotection in a rat model of Huntington's disease (McBride et al., 2003), intrastriatal transduction of GDNF mediated by AAV showed functional recovery in a rat model for Parkinson (Kirik et al., 2000). Besides injections into cortical regions, direct intraspinal injections of AAV have also been investigated in the mSOD1 mouse model for ALS. For example, it has been reported that Bcl-2, an anti-apoptotic protein, delivered by AAV to motoneurons innervating the hind limbs, delayed disease onset but did not prolong survival in ALS mice. Interestingly, AAV mainly transduced neurons in the spinal cord, whereas transduction of astrocytes was only scarcely observed (Azzouz et al., 2000).

When injected into the muscle, AAV binds to and enters neuronal terminal and can be retrogradely transported by neurons. This property makes AAV extremely suitable to target motoneurons (Kaspar et al., 2002; Boulis et al., 2003; Davidson et al., 2000). Kaspar and co-workers used the retrograde transport ability of AAV to deliver insulin

growth factor-1 (IGF-1) to motoneurons in high-copy mSOD1 ALS mice by injections into the respiratory- and motor limb-muscles. IGF-1 treatment started at 60 days of age delayed onset by 31 days and the median survival was increased by 37 days compared to control mice. Interestingly, when AAV-IGF-1 was applied at the time of onset (i.e. injections at 90 days of age) the life-span of IGF-1 treated animals was still increased by 22 days compared to the control group (Kaspar et al., 2003). In another study in which AAV injections into the gastronemicus and triceps brachii muscles were used to deliver GDNF to the spinal cord, treatment starting at 9 weeks of age delayed onset and survival of ALS mice. The delivery of GDNF to the motoneurons is thought to be mediated by retrograde transport of the GDNF protein itself rather than retrograde transport of AAV-GDNF, as injection of AAV-β-galactosidase retrograde transport did not lead to β-galactosidase expression in motoneurons (Wang et al., 2002).

Unfortunately, however, AAV also have some major disadvantages that make them less suitable for use in gene therapy. AAV only has a small packaging capacity, limiting the transgene to a size of < 5 kb, and a limited diffusion capacity in tissues in vivo. Moreover, indirect delivery of AAV to the spinal cord by intramuscular injections with consequent axonal retrograde transport will only target motoneurons (Kaspar et al., 2003). Although this is ideally for a neuroprotective protein such as catalase, it would not be effective for astrocytic proteins, such as EAAT2. In addition even when AAV is directly injected into the spinal cord, transduction of astrocytes is only scarcely observed (Azzouz et al., 2000). Therefore, AAV is not a suitable vector to deliver neuroprotective proteins to spinal cord astrocytes.

4.2.2 Lentiviral vectors

Lentiviral vectors (LVV) belong to the family of retrovirus and are in fact derived from the human immunodeficiency virus (HIV) (Buchschacher, Jr. and Wong-Staal, 2000). Since LVV gene transduction relays on active transport of the pre-integration complex through the nucleopore by the nuclear import machinery, these vectors are capable to infect both dividing and non-dividing cells. The past few years, major advances have been made towards the biosafety and efficiency of these vectors (Kay et al., 2001; Davidson and Breakefield, 2003). These include the deletion of all viral genes, thus preventing virus

replication and recombination, and the generation of viral particles with a vesicular stomatitis virus G-protein (VSV-G) envelope. When LVV are pseudotyped by the VSV-G envelope, the tropism is increased resulting in a greater stability of the virus particles and making it a more efficient vector for gene therapy. LVV pseudotyped with a VSV-G envelope showed long-term expression in rodents (Zala et al., 2004; Naldini et al., 1996a) and non-human primates (Kordower et al., 1999). In some brain regions, such as the striatum and hippocampus, direct injections of LVV lead to a preferential neuronal transduction (Naldini et al., 1996a), while in other anatomical structures, such as the midbrain, transduction of astrocytes was also observed (Rosenblad et al., 2000). This latter aspect would make LVV potentially useful as tool to transfer astrocytic proteins. So far however, experiments using direct injections of LVV into the spinal cord of experimental animal models have not been performed.

A major disadvantage of LVV pseudotyped with a VSV-G envelope is their inability to be retrogradely transported in neurons in case one wants to specifically motoneurons. This problem can be circumvented, however, by pseudotyping the LVV with a rabies virus glycoprotein (RVG) envelop (Mazarakis et al., 2001). The therapeutic potential of LVV-RVG is supported by the finding that injections of LVV-vascular endothelial growth factor (VEGF) into hind limb gastronemicus, diaphragm, intercostal, facial, and tongue muscles of high-copy mSOD1 mice resulted in a marked delay in onset of 28 days. Also, the lifespan was dramatically extended by an average of 38 days. Furthermore, the therapeutic potential of LVV-VEGF was tested when started at time of onset. LVV-VEGF treatment started at 90 days of age slowed down the loss of motor performance and delayed the survival with 19 days (Azzouz et al., 2004).

Although LVV have a modest packaging capacity of about 8-9 kb and a limited diffusion capacity in tissue in vivo, these properties are more favourable than those observed in AAV vectors. So far, direct LVV injections into the spinal cord have not been investigated in ALS. However, given the potential of LVV to transduce astrocytes, it is proposed that in vivo gene therapy with LVV-EAAT2 is an interesting option to deliver EAAT2 to astrocytes in the spinal cord.

5. Aim of the thesis

The research described in this thesis focuses on the use of ex vivo and in vivo gene therapy as therapeutic options in ALS. As outlined in the preceding paragraphs, oxidative stress and excitotoxicity are two important pathogenic mechanisms operative in ALS. The anti-oxidant enzyme catalase and the glutamate transporter EAAT2 might be of therapeutic value to combat these pathogenic mechanisms. However, the neuroprotective efficacy of these proteins greatly depends on the mode of delivery to the CNS. Therefore, the general aim of this thesis is to explore the use of gene therapy as a tool to deliver either catalase or EAAT2 in experimental models for ALS.

This aim can be divided in two parts:

Ex vivo gene therapy implies the construction of genetically engineered cell lines overexpressing the gene of interest. It is a clinical safe method to deliver neuroprotective proteins to the CNS. One of the aims of this thesis is to investigate whether cell lines overexpressing either catalase or EAAT2 can be constructed that show neuroprotective efficacy in in vitro models for ALS.

LVV have become an interesting tool to deliver neuroprotective genes to the CNS. In vivo gene therapy implicates the use of viral vectors to deliver neuroprotective genes to the CNS. In this study we will explore the use of LVVs as a tool to deliver transgenes of interest (i.c. EAAT2) to astrocytes. For this purpose both mouse organotypic spinal cord cultures as well as primary human astrocytes were used. Furthermore, the neuroprotective efficacy of LVV-EAAT2 was tested in a behavioural phenotypic study in high-copy mSOD1 ALS mice.

CHAPTER 2

CELLS OVEREXPRESSING CATALASE DO NOT PROTECT MOTONEURONS FROM OXIDATIVE STRESS IN VITRO: IMPLICATIONS FOR EX VIVO GENE THERAPY

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Abstract

Amyotrophic lateral sclerosis is a fatal neurodegenerative disease characterised by a loss of motoneurons. Oxidative stress plays a critical role in the pathogenesis of ALS. Hence, it is hypothesised that ex vivo gene therapy with encapsulated genetically engineered cells overexpressing anti-oxidant proteins might be a way to protect motoneurons. Therefore, in the present study, genetically engineered Rat-1 fibroblasts overexpressing catalase were generated and tested for their neuroprotective efficacy in Rat-1 fibroblast/motoneuron cocultures. Although catalase overexpressing cells were found to be more resistant to oxidative stress themselves, in coculture they were unable to rescue either primary motoneurons or neuroblastoma-spinal cord (NSC-34) hybrid cells respectively from hydrogen peroxide induced oxidative cell death. Given the outcome of this study it is concluded that the use of ex vivo gene therapy with catalase overexpressing cells might not be sufficient to effectively protect affected motoneurons.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disease characterised by a loss of upper and lower motoneurons. Ten percent of all ALS cases are hereditary and in about 20% of these patients a mutation in the Cu,Zn-superoxide dismutase (SOD1) gene is identified (Rosen et al., 1993). Rodents carrying a mutated form of the human SOD1 gene develop ALS (Gurney et al., 1994; Howland et al., 2002). At this moment approximately 100 different mutations in SOD1 have been identified, throughout the entire gene (Cleveland, 1999; Valentine and Hart, 2003). Since these mutations do not cause a decrease in SOD1 enzymatic activity (Wong et al., 1995; Ripps et al., 1995) and SOD1 knock-out mice do not develop ALS like symptoms (Reaume et al., 1996), it is hypothesised that the cellular toxicity of mutated SOD1 (mSOD1) involves a pro-oxidative gain of function.

Besides the propensity to form intracellular protein aggregates (Bruijn et al., 1998), it has been reported that mSOD1 generates oxidative stress by two distinct but not mutually exclusive mechanisms. First, point mutations in SOD1 lead to aberrant protein folding allowing abnormal substrates, such as peroxynitrate (Beckman et al., 1993) and hydrogen peroxide (Wiedau-Pazos et al., 1996), to reach and react at the catalytic site of the enzyme. As reported, these reactions lead to serious tissue damage e.g. via nitration of critical proteins and the release of toxic hydroxyl radicals. According to the second mechanism, mSOD1 is unable to properly bind zinc atoms. Having lost zinc mutated SOD1 will catalyse the release of superoxide, which in the presence of nitric oxide, gives rise to production the neurotoxin peroxynitrate (Estevez et al., 1999). Other factors that might contribute to oxidative stress in ALS include glutamate-induced excitotoxicity (Rao and Weiss, 2004), mitochondrial dysfunction (Beal, 1998), AGE modified SOD aggregates (Takamiya et al., 2003; Shibata et al., 2002; Shibata et al., 2002), and the activation of microglial respiratory burst and NO production (McGeer and McGeer, 2002).

Accordingly, there is considerable evidence indicating that elevated oxidative stress and elevated oxidative damage are present in the tissues of ALS transgenic mice and ALS patients. Enhanced oxygen radical production has been reported in mutant SOD1 mice (Liu et al., 1998), and several markers indicative for elevated protein oxidation, protein

nitration, lipid peroxidation, and DNA oxidative damage have been demonstrated in peripheral (Bonnefont-Rousselot et al., 2000) and central nervous system tissue of sporadic and familial ALS patients (Ferrante et al., 1997a; Beal et al., 1997; Beal et al., 1997).

Given the above findings and the critical role of oxidative stress (caused mainly by H_2O_2, NO) in motoneuron survival (Kaal et al., 1998), it is tempting to speculate that limiting oxidative stress might be neuroprotective in ALS. There are many enzymes involved in the cellular defence mechanism against oxidative stress. Of these, the anti-oxidant enzyme catalase is of particular interest as it not only detoxifies hydrogen peroxide but also inhibits the formation of nitric oxide, a precursor of peroxynitrate (Mittal, 1993).

Previously we reported that motoneurons in vitro are protected against an H_2O_2 challenge when they are micro-injected with catalase cDNA or the enzyme itself (Herpers et al., 1999). However, in the in vivo situation cDNA micro-injection of single motoneurons would be rather inefficient, if not impossible. Moreover, direct delivery of extra catalase to the CNS is greatly hampered by the blood brain barrier (BBB) (Pong, 2003; Reinholz et al., 1999a; Reinholz et al., 1999a). In theory, using an ex vivo gene therapy approach with encapsulated catalase overexpressing cells, it might be possible to by-pass the BBB and to increase catalase activity in the vicinity of threatened motoneurons (Aebischer et al., 1996).

In this study we used Rat-1 fibroblasts to construct genetically engineered cells overexpressing human catalase. To evaluate their use for ex vivo gene therapy, besides testing their capacity to detoxify H_2O_2, the neuroprotective efficacy of these cells was tested in Rat-1 fibroblasts/motoneuron coculture models.

Materials and methods

Rat-1 fibroblasts

Rat-1 fibroblasts, originally described by Prasad and colleagues (Prasad et al., 1976), were transfected with a pBK-CMV vector encoding human catalase (kindly provided by Dr. H. van Remmen, University of Texas Health Science Center at San Antonio, USA) using the calcium-phosphate technique (Wisman et al., 2003). After one week in culture, transfected

cells were selected by adding 400 µg/ml of the antibiotic G418. From the resistant cells, stable monoclonal cell lines were created by limiting dilution. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere at 37ºC and 5% CO_2. For application in Rat-1 fibroblast/motoneuron coculture experiments, wild-type or catalase Rat-1 fibroblasts were grown on 12 mm diameter inserts (Millipore) with a poly-L-lysine (20 µg/ml) coating.

Immunocytochemical characterisation of catalase expressing Rat-1 fibroblasts

Rat-1 fibroblasts were fixed using 4%-paraformaldehyde for 20 min by 4ºC. Cells were washed with 0.1 M phosphate buffer (PB) and incubated in 200 mM glycine in PB for 20 min. Upon blocking of aspecific binding sites by incubation with PB with 0.5% bovine serum albumin (BSA) and 0.2% gelatine (PBG) containing 2% normal goat serum for 30 min, cells were incubated at 4ºC for 16 hours with a polyclonal rabbit antibody against human catalase diluted 1:100 (Calbiochem) in PBG with 0.05% Triton X-100. Thereafter, cells were washed with PB and incubated with biotinylated goat-anti-rabbit antibody for 1 hour at room temperature. Finally, after washing with PB, cells were labelled with streptavidin-FITC and embedded in 0.1 M Tris/HCl (pH=8.5) containing 25% glycerol, 10% DABCO (Sigma-Aldrich), and 10% Mowiol 4-88 (Sigma-Aldrich). Catalase immunoreactivity was visualised under a Leitz Orthoplan fluorescence microscope.

NSC-34 motoneuron cell line

NSC-34, a hybrid cell line of motoneuron enriched embryonic mouse spinal cord cells with mouse neuroblastoma (courtesy Dr. N.R. Cashman, Centre for Research in Neurodegenerative Diseases, University of Toronto, Canada) (Cashman et al., 1992), were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere at 37°C and 5% CO_2. For application in Rat-1 fibroblast/motoneuron coculture NSC-34 cells were seeded at a density of 15,000 cells/cm^2 in a 24-wells plate one day in advance.

Primary motoneuron culture

Primary motoneurons were isolated from the ventral part of the spinal cord of embryonic day 15 Wistar rats by a combination of enzymatic and mechanical dispersion as previously described (Kaal et al., 1998; Wisman et al., 2003; Wisman et al., 2003). Motoneurons were purified by metrizamide density centrifugation, collected from the interphase, and seeded at low density in a 24-wells culture plate (1360 cells/cm^2) on an poly-L-ornithin (1.5 µg/ml) and laminin (1.5 mg/well) coating. The motoneuron cultures were grown at 6% O_2/ 5%CO_2 at 37ºC for 4 days before application in Rat-1 fibroblast/motoneuron coculture.

H_2O_2-challenge test in Rat-fibroblasts

Wild-type or catalase expressing Rat-1 fibroblasts were plated onto a 96-wells plate at a density of 12500 cells/cm^2. After 24 hours, Rat-1 fibroblasts were challenged by replacing the entire medium with freshly prepared medium containing different concentrations of H_2O_2. After 20 hours, the cells were stained with Hoechst 53388 and fixed with 4%-paraformaldehyde.

Using Leica Quantimet software, the survival was determined by counting the number of cell nuclei in 4 automatically selected fields covering 4.4 mm^2 of each well, and was expressed as a percentage of untreated control.

H_2O_2-challenge in Rat-1 fibroblast/motoneuron cocultures

To evaluate the neuroprotective efficacy of the genetically engineered catalase expressing cells, Rat-1 fibroblast/motoneuron cocultures were treated with H_2O_2. To this end, Rat-1 fibroblasts inserts were placed on top of NSC-34 or primary motoneurons, respectively, 30 min before exposure to different concentrations of H_2O_2 (0-900 µM). After 20 hours, the inserts were removed and motoneuron survival was determined. For NSC-34, cells were incubated with Alamar Blue (Biosource) in a final concentration of 10% (v/v) for two hours before the amount of reduced, fluorescent “resazurin” Alamar Blue, as measure of survival was determined on a Fluostar optima reader (BMG) with excitation at 544 nm and emission at 590 nm (Nociari et al., 1998). For primary motoneurons, cell survival was determined under phase-contrast microscopy as previously described (Wisman et al., 2003).

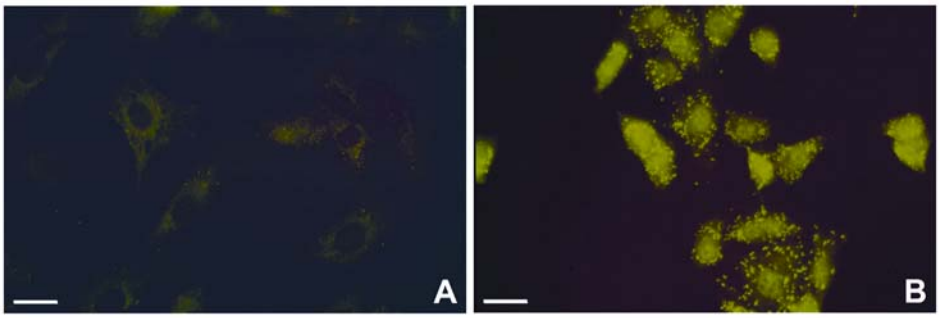

Figure 1. *Cellular expression of catalase in wild-type Rat-1 fibroblasts (A) and in Rat-1 fibroblasts overexpressing human catalase (B). Catalase is localised in peroxisomes and in the cytoplasm of the cells. Scale bar is 20 μm.*

Statistics

All data are given as mean ± SEM. Statistical analysis was performed with the SPSS 9.0 for Windows software. After testing for homogeneity of variance and for normality of residuals, an analysis of variance (ANOVA) was performed for multiple comparison between groups followed by Bonferoni's post hoc test to compare group means. p values <0.05 were considered significant.

Results

After calcium phosphate transfection and selection with G418, several stable catalase expressing cell lines were generated. In wild-type Rat-1 fibroblasts catalase immunoreactivity was predominantly present in the peroxisomes (figure 1). When stable cell lines were evaluated for the cellular expression of catalase marked differences in the intensity of immunoreactivity were found. In line with catalase being a peroxisomal marker protein, besides moderate cytosolic staining, a punctated staining pattern was observed (figure 1). Whereas in six cell lines catalase immunoreactivity was not more abundant than in the wild-type Rat-1 fibroblasts, in ten cell lines the catalase immunostaining was dramatically increased. Of these, one cell line was selected for further experiments (figure 1).

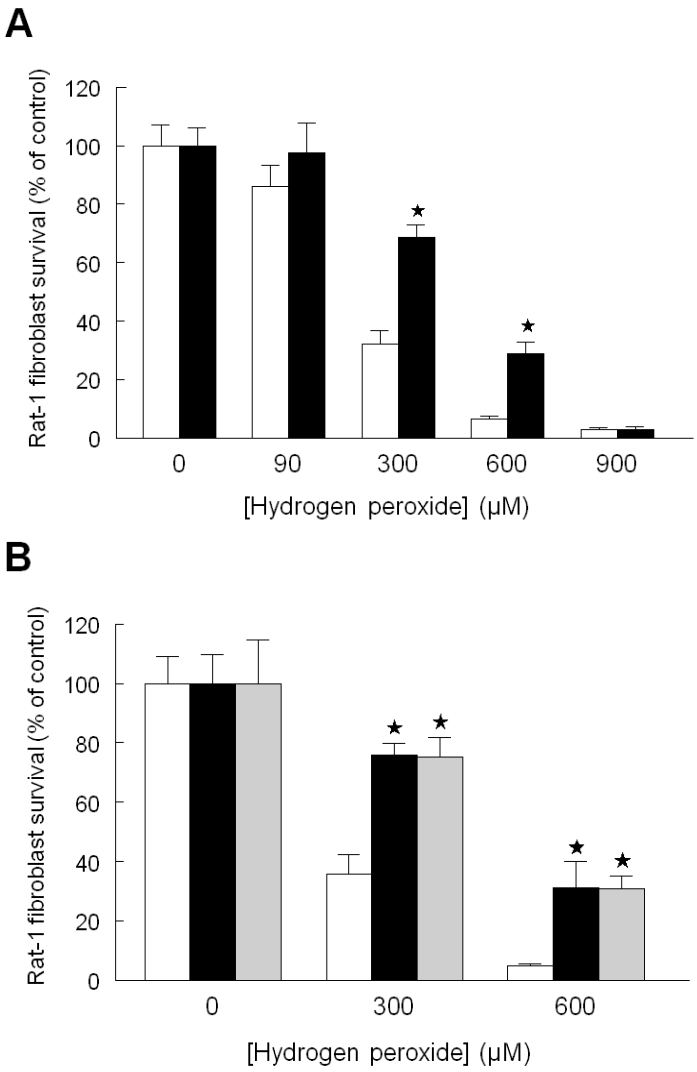

Figure 2. *Catalase overexpressing Rat-1 fibroblasts are able to protect themselves against an H_2O_2-challenge. A) Wild-type (open bars) or catalase overexpressing (solid bars) Rat-1 fibroblasts were exposed to H_2O_2 for 20 hours. Survival was determined by Hoechst staining and expressed as percentage of untreated controls. B) catalase overexpressing cells from passage number 74 (black bars) are equally capable of protecting themselves from H_2O_2 as cells from passage number 83 (grey bars). Data are mean ± SEM of three independent experiments, each consisting of four observations per group. * $p<0.05$ versus wild-type cells.*

First, as indication for enhanced functional expression of catalase, the cell line was challenged with different concentrations of H_2O_2. As illustrated in figure 2A, catalase expressing Rat-1 fibroblasts were less vulnerable to H_2O_2 induced toxicity. At 300 and 600 μM H_2O_2, survival amounted to 68.7% ± 4.3 and 28.7% ± 4.2 (mean ± SEM), respectively, which is significantly higher than the 32.1% ± 4.7 and 6.4% ± 1.0 observed in the presence of wild-type Rat-1 fibroblasts. To verify whether the expression of functional catalase was stable over time, the H_2O_2-challenge test was performed after different passages in culture. As illustrated in figure 2B the percentage of surviving Rat-1 fibroblasts, when challenged with H_2O_2, did not significantly change from passage no. 74 to passage no. 83, which spans approximately 2 months in culture.

Secondly, to evaluate the neuroprotective properties of the genetically engineered cells overexpressing catalase, Rat-1 fibroblasts/motoneuron cocultures were established. When NSC-34 cells in coculture were exposed to H_2O_2, a dose-dependent decrease in cell survival could be observed. In NSC-34/Rat-1 catalase fibroblast cocultures, a small though statistically significant neuroprotective effect of the catalase overexpressing cells could only be observed at a concentration of 900 μM H_2O_2 (figure 3A). Similarly, while a dose-dependent decrease in survival was observed in H_2O_2 treated primary motoneuron/Rat-1 fibroblast cocultures, no difference was found between the survival of motoneurons grown either in the presence of catalase-expressing or wild-type cells (figure 3B).

Discussion

Aiming at an anti-oxidant therapy for ALS, we have constructed a genetically engineered Rat-1 fibroblast stably over-expressing the H_2O_2- and NO- detoxifying enzyme catalase. Immunocytochemical analysis revealed a dramatic increase in catalase expression in transfected cells. Similar to naive cells, and in keeping with its normal localisation, catalase protein was mainly found in the peroxisomes and cytoplasm (Tolbert and Essner, 1981; Singh, 1996; Singh, 1996). The functionality of stably expressed catalase was demonstrated by the fact that these cells were less vulnerable to oxidative stress inflicted by H_2O_2.

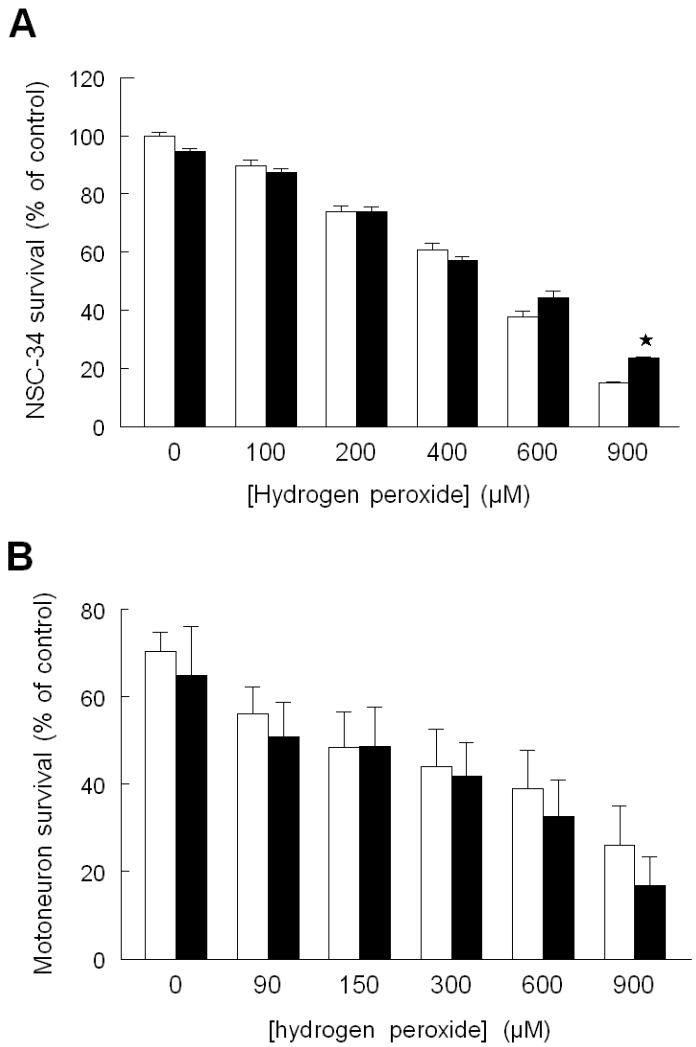

Figure 3. *Catalase overexpressing Rat-1 fibroblasts do not protect motonurons from oxidative stress in vitro. NSC-34 cells (panel A) or pimary motoneurons (panel B) were exposed to vehicle or H_2O_2, at the indicated concentrations, either in the presence of wild-type (open bars) or catalase overexpressing (solid bars) Rat-1 fibroblasts, respectively.*

*Panel A: NSC-34 survival was assessed after 20 hours with the Alamar Blue assay. Data are mean ± SEM, each consisting of 4 observations per group. * $p<0.05$ versus wild-type cells. Panel B: Motoneuron survival was assessed under phase-contrast microscopy after 20 hours. Data are mean ± SEM of two independent experiments, each consisting of 4 observations per group.*

In earlier experiments, catalase has shown neuroprotective efficacy in in vitro and in vivo ALS models. While catalase cDNA or protein could protect primary motoneurons in vitro (Herpers et al., 1999), the synthetic SOD/catalase mimetic EUK-134 was shown to reduce oxidative stress and to prolong survival of low-copy mutant G93A-hSOD1 ALS mice (Jung et al., 2001). Interestingly, the efficacy of EUK-134 has been attributed mainly to its catalase activity (Baker et al., 1998).

In other studies, in which catalase was modified by the addition of putrescine to increase the blood-brain-barrier permeability, catalase treatment was tested in high- and low copy G93A-hSOD1 mice. While in high copy mice a delay in onset was observed (Reinholz et al., 1999b), in low copy mice continuous infusion putrescine-catalase showed a therapeutic effect on both onset and survival (Poduslo et al., 2000). The above studies emphasise the important role of oxidative stress in the pathogenesis of ALS, and, in addition, indicate that catalase could be used as a neuroprotective treatment. Unfortunately, however, so far efforts to clinically develop catalase treatment have met serious pitfalls due to biostability and delivery issues (Pong, 2003).

As underscored by ex vivo gene transfer of catalase in a rat arthritis model (Dai et al., 2003) and CNTF delivery studies in ALS patients (Aebischer et al., 1996), we hypothesise that ex vivo gene therapy with catalase overexpressing cells could be an effective and clinically feasible approach to chronically enhance catalase activity in the vicinity of affected motoneurons. To this end, we created a stable catalase expressing cell line, and tested these cells on their ability to protect motoneurons from oxidative stress. The outcome of the current in vitro study showed that, although the catalase expressing Rat-1 fibroblasts themselves were more resistant to H_2O_2-induced cell death, they were not capable to rescue NSC-34 or primary motoneurons from H_2O_2-induced oxidative damage in coculture. There may be several reasons to explain the inefficiency of these cells in our in vitro models. The choice to deliver catalase in the vicinity of motoneurons via ex vivo gene therapy, entails introducing catalase in a foreign cell, in this case the Rat-1 fibroblasts. Since catalase is a non-secreted enzyme, this implicates that introducing these cells does not directly cause an increase of catalase activity in the extracellular milieu. Rather, it is speculated that the catalase overexpressing cells work as an H_2O_2-sink, thereby indirectly lowering oxidative stress in the vicinity of motoneurons. Thus, H_2O_2 generated in the extracellular milieu, or even worse, within motoneurons, has to diffuse

over several membranes before it can be detoxified by the catalase overexpressing Rat-1 fibroblasts. Although it is known that H_2O_2 can diffuse over membranes (Carampin et al., 2003), the relatively large diffusion distance together with the high intrinsic reactivity of H_2O_2, can explain the inability of the overexpressing catalase cells to confer neuroprotection. Secondly, and in relation to this, the catalase activity in the overexpressing cells might simply be too low to efficiently work as a sink. Hence, to effectively protect cells, it appears that catalase should be introduced preferably at the same place of H_2O_2 production, i.e. intracellularly (Herpers et al., 1999). Finally, very recent data suggests that in order to adequately battle oxidative stress a combined overexpression of catalase, glutathion peroxidase and SOD isoforms might be required (Baud et al., 2004; Lortz and Tiedge, 2003; Lortz and Tiedge, 2003).

In summery, although catalase treatment seems to be promising to limit oxidative stress and to combat ALS pathogenesis, at least from our in vitro study, we conclude that ex vivo gene therapy with catalase expressing cells is not likely to be effective in vivo. Rather, given the neuroprotective efficacy of intracellular delivery of catalase cDNA on motoneurons in vitro (Herpers et al., 1999) and the recent advances in AAV-mediated retrograde gene transfer to motoneurons (Kaspar et al., 2003), we recommend in vivo gene therapy of catalase to be evaluated as experimental therapy in ALS.

Acknowledgement

This work was supported by the Prinses Beatrix Fonds. The authors wish to thank Eva de Jong, Rogier van Gent, and Esther Slootjes for their excellent technical support.

CHAPTER 3

CELLS OVEREXPRESSING EAAT2 PROTECT MOTONEURONS FROM EXCITOTOXIC DEATH IN VITRO

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Neuroreport, 2003, 14(15):1967-70

Abstract

Amyotrophic lateral sclerosis is an incurable disease in which cerebral and spinal motoneurons degenerate, causing paralysis and death within 2-5 years. One of the pathogenic factors of motoneuron death is a chronic excess of glutamate, which exceeds its removal by astrocytes, i.e. excitotoxicity. Extra glutamate uptake in the spinal cord may slow down or prevent motoneuron death. We have engineered cells overexpressing the main glutamate transporter and tested their potential to rescue motoneurons exposed to high levels of glutamate in vitro. The engineered cells protected motoneurons in a motoneuron-astrocyte coculture at glutamate concentrations when astrocytes were no longer capable of removing glutamate. This suggests that engineered cells, introduced into the spinal column, can help remove glutamate, thereby preventing motoneuron death.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterised by a selective loss of motoneurons in the motor cortex, brain stem and spinal cord. The precise mechanism of motoneuron death is still largely unknown, but the finding of increased glutamate levels in the cerebrospinal fluid of ALS patients (Plaitakis and Caroscio, 1987; Spreux-Varoquaux et al., 2002; Spreux-Varoquaux et al., 2002; Plaitakis and Caroscio, 1987; Spreux-Varoquaux et al., 2002) has induced the hypothesis that a disturbed glutamate homeostasis is implicated in the pathogenesis of ALS. Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). Glutamate, released from glutamatergic neurons can activate several types of pre- and post-synaptic glutamate receptors. Excessive glutamate induces excitotoxic neuronal death of motoneurons through activation of calcium-permeable receptors leading to increased cellular calcium influx (Carriedo et al., 1996). The subsequent rise in the intracellular calcium concentration causes mitochondrial dysfunction, the generation of reactive oxygen species, and the activation of proteases, phospholipases, and endonucleases, and ultimately cell death (Shaw and Ince, 1997).

The primary mechanism for the inactivation of glutamate signalling is its removal from the extracellular space by a sodium-dependent glutamate transport system, comprised of so-called excitatory amino acid transporters (EAATs). So far, five human transporter subtypes have been characterised, designated EAAT1 to EAAT5 (Gegelashvili and Schousboe, 1997). EAAT2, known as GLT-1 in rodents, is expressed mainly by astrocytes and accounts for the major part of glutamate uptake in the CNS. When different subtypes of the glutamate transporter were examined in sporadic ALS patients, a marked decrease in the protein level of the glial glutamate transporter EAAT2 was found in the spinal cord and motor cortex, while levels of other glutamate transporter subtypes were not changed (Rothstein et al., 1995). In addition, a decrease in high affinity uptake of glutamate was found in synaptosomes of ALS patients, but not in controls (Rothstein et al., 1992; Rothstein et al., 1992).

A recent study (Rothstein et al., 1996), investigating the neuropathological effect of chronic treatment with EAAT2 antisense oligonucleotides in rats, has indicated that a decrease in EAAT2 protein, and a consequent rise in glutamate levels, is likely to

contribute to selective motoneuron death. This EAAT2 antisense treatment produced a progressive motoneuron syndrome, with animals showing a slowing of hindlimb movements followed by paresis of the hindlimbs within seven days.

If a decrease in EAAT2 protein level does indeed contribute to motoneuron death, it seems valid to postulate that local overexpression of the EAAT2 gene can protect motoneurons from glutamate excitotoxicity. From a clinical viewpoint, to date the safest method of overexpressing a putative neuroprotective gene *in vivo* is the *ex-vivo* gene therapy approach (Aebischer et al., 1996) using encapsulated, genetically engineered cell lines, thereby avoiding immune rejection and tumour formation. In the present study, genetically engineered cell lines that overexpress EAAT2 were constructed and tested for their ability to protect motoneurons against excitotoxicity *in vitro*.

Materials en Methods

Generation of stable EAAT2 expressing cell lines

Human Embryonic Kidney (HEK)-293 cells were cultured in Dulbecco's Modified Eagle Medium with 25 mM HEPES (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere at 37ºC and 5% CO_2.

A 2 kb cDNA for the human glial glutamate transporter EAAT2 (GenBank: U01824) (kindly provided by Dr. D. Trotti, Department of Neurology, Cecil B. Day Laboratory for Neuromuscular Research, Harvard Medical School) was subcloned into a pCI-neo mammalian expression vector using the EcoRI site. HEK-293 cells were transfected using the calcium-phosphate technique.

After one week in culture, transfected cells were selected by adding 400 µg/ml of the antibiotic G418. From the resistant cells, monoclonal cell lines were created by limiting dilution. For application in the motoneuron coculture experiments, wild-type- or EAAT2-HEK cells were grown on 12 mm diameter inserts (Millipore) with a poly-L-lysine (20 µg/ml; Sigma) coating.

Western blot analysis

The presence of EAAT2 protein in HEK cells was determined by using Western blot analysis. Cell protein samples were prepared by homogenisation in lysis buffer containing 0.5% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride. Protein content was determined according to Lowry (Lowry et al., 1951).

Protein samples, prestained molecular weight standards (Gibco) and rat cortex homogenate as a positive EAAT2 control were separated on 10% SDS-polyacrylamide gels. Separated proteins were transferred to a polyvinylidene fluoride membrane (Roche) by electroblotting (100 V, 60 min) in Towbin buffer containing 12.4 mM Tris, 95.5 mM glycin and 20% methanol.

After blocking non-specific binding by overnight incubation at 4ºC in phosphate buffered saline containing 0.1% Tween (PBS-Tw) and 3% bovine serum albumin, the blots were incubated with the EAAT2-reactive guinea-pig-anti-glutamate transporter GLT-1 antibody (1:2,500; Chemicon) in PBS-Tw for 3.5 hours. Next, membranes were washed with PBS-Tw and incubated with biotinylated goat-anti-guinea-pig antibody (1:500; Vector) in PBS-Tw for 1 hour. After washing, the membranes were incubated with avidin-biotin-conjugated peroxidase reagent (ABC-HRP; Vectastain) 1:100 diluted in PBS-Tw for 1 hour. Finally, membranes were washed and immunoreactive proteins were visualised using ECL substrate (Roche).

Glutamate uptake assay

The functionality of EAAT2 in the transfected HEK cells was estimated with the glutamate uptake assay according to Debler and Lajtha with minor adjustments (Debler and Lajtha, 1987). Briefly, HEK cells were plated into a 24-wells culture plate at a density of 350,000 cells per well and grown for 16-24 hours. Thereafter, medium of HEK cells was replaced by transport buffer (10 mM HEPES, 5 mM Tris, 10 mM D(+)glucose, 3.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 1.2 mM K_2HPO_4) containing either 140 mM NaCl or 140 mM cholineCl. The cells were kept in this buffer for 10 min at 37ºC. Glutamate uptake was started by the addition of 1 μM glutamate containing 9.52 pM [^{3}H]-glutamate (specific activity 42 Ci/mmol) (ICN Pharmaceuticals). After incubation for 30

min at 37ºC, glutamate uptake was stopped by adding ice-cold transport buffer containing 1 mM glutamate. Cells were resuspended in transport buffer with 1mM glutamate and harvested on a filter with a Beckman cell harvester. The filters were rinsed three times with ice-cold transport buffer with 1 mM glutamate. Radioactivity on the filters was quantified by liquid scintillation counting. Na^+-dependent uptake, expressed as the amount of glutamate taken up within 30 min, was assessed by subtracting the choline values from the sodium values and by correcting for protein content.

The non-selective EAAT inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid (K_i values 5-10 μM for EAAT2,4 and 5, and 50-100 μM for EAAT1 and 3) and the EAAT2 selective inhibitor dihydrokainic acid (K_i value 15-60 μM for EAAT2), were added 5 min before and during the incubation with [^{3}H]-glutamate.

Primary motoneuron culture

Primary motoneurons were prepared from the ventral part of the spinal cord of embryonic day 15 Wistar rats by a combination of enzymatic and mechanical dispersion as previously described (Kaal et al., 1998). Motoneurons were purified by metrizamide density centrifugation, collected from the interphase, and seeded at low density in a 24-wells culture plate (1360 cells/cm^2) on a 3-week-old astroglial feeder layer previously prepared from the pellet fraction of the metrizamide density centrifugation. To obtain pure astrocytes, the pellet fraction was plated in 25 cm^2 culture flasks and grown to confluence. In the third week of culture non-astrocytic contaminating cells were shaken off on a rotary shaker (Dijkstra et al., 1999). The motoneuron-astrocyte cocultures were grown at 6% O_2/5% CO_2 at 37ºC for 4 days. To evaluate for the neuroprotective efficacy of genetically engineered HEK cells, inserts containing HEK cells were placed on top of 4-day-old cocultures 30 min before exposure to glutamate (250 or 600 μM) or vehicle. To monitor the survival of motoneurons over time, a subpopulation of motoneurons (±30 neurons/well) was identified under phase contrast microscopy and mapped using the Leica DM IRBE microscope with the Leica Quantimet images analysis system software. After 20 hours, the viability of mapped motoneurons was assessed under phase contrast using well-established criteria (Kaal et al., 1998).

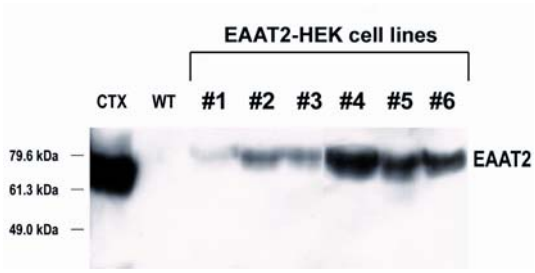

Figure 1. *Western Blot analysis for EAAT2 protein expression in different genetically engineered cell lines. Lane 1, CTX: rat cortex homogenate. Lane 2, WT: wild-type-HEK cells. Lane 3-8: EAAT2-HEK cell lines #1 to #6, respectively.*

Statistical evaluation

All data are given as means ± SEM. Statistical analysis was performed using the SPSS 9.0 for Windows software. After testing for homogeneity of variance and for normality of residuals, an analysis of variance (ANOVA) was performed for multiple comparison between groups followed by Bonferoni's post hoc test to compare group means. *p* values <0.05 were considered significant.

Results

After transfection of the HEK cells with the construct encoding human EAAT2, and the selection of stable cell lines with G418, several EAAT2 expressing cell lines were obtained. They were all examined for the presence of EAAT2 protein with Western blot analysis. While wild-type cells showed no immunoreactivity for EAAT2, 6 out of 45 stable cell lines expressed EAAT2 at a detectable levels. Figure 1 shows that the expression level of EAAT2 differed considerably between cell lines. The EAAT2-HEK cell lines express EAAT2 protein with a molecular weight of appr. 79 kDa, which is slightly higher than the molecular weight of EAAT2 in rat cortex homogenate.

Next, using the [^{3}H]-glutamate uptake assay, we tested whether the EAAT2 in the HEK cell lines could functionally participate in glutamate uptake. To differentiate between sodium-dependent glutamate uptake and aspecific glutamate binding, the assay was performed in the presence or absence of sodium. The uptake for wild-type- and EAAT2-HEK cells is still linear at 60 min (data not shown), we measured at 30 min. In both wild-type-HEK cells and the stable EAAT2-HEK cell lines glutamate uptake in the absence of sodium was very low, indicating that the uptake is sodium-dependent. Wild-type cells also showed some sodium-dependent glutamate uptake (± 0.46 nmol/mg protein/30 min),

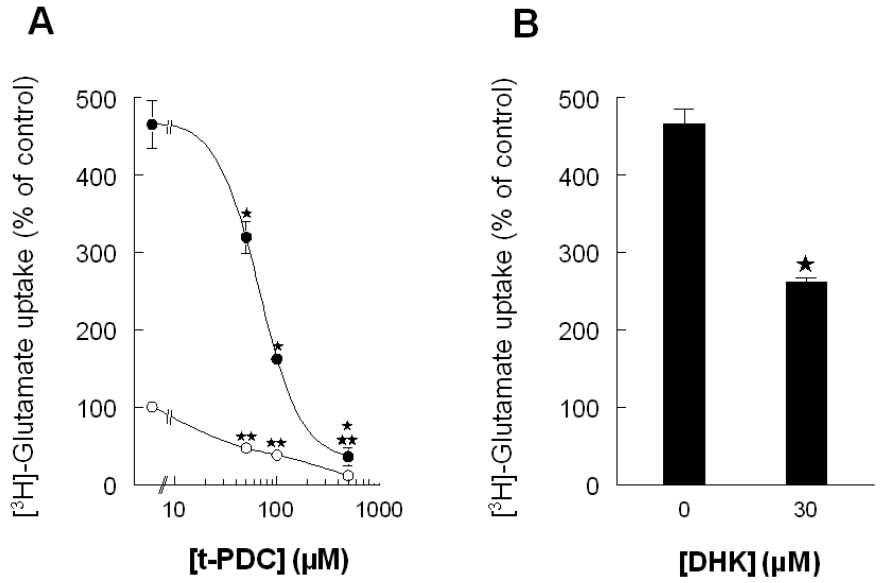

Figure 2. *Pharmacological characterisation of [^{3}H]-glutamate uptake in the EAAT2-HEK cell line. [^{3}H]-Glutamate uptake in EAAT2-HEK cells (●, solid bars) is determined in the presence of L-trans-pyrrolidine-2,4-dicarboxylic acid (panel A) and dihydrokainic acid (panel B). [^{3}H]-Glutamate uptake values are expressed as percentage of control; i.e. the uptake of glutamate in untreated wild-type-HEK cells (○). Uptake in wild-type cells amounted to 0.40 ± 0.05 nmol/mg protein/30 min. *,** p<0.05 versus respective controls, i.e. uptake of glutamate in the absence of inhibitor.*

which can be contributed to the expression of the EAAT3 subtype (Toki et al., 1998), but this value is considerably lower than that observed in EAAT2-HEK cells. Glutamate uptake in the EAAT2-HEK cell lines varied from 0.6-2.1 nmol/mg protein/30 min, with cell line 5 showing the highest level. Thus, cell line #5 was selected for further experiments.

With specific glutamate uptake inhibitors we first examined the transport characteristics. The non-selective transporter inhibitor L-trans-pyrrolidine-2,4-dicarboxylic acid completely inhibited glutamate uptake in a concentration-dependent manner, both in wild-type- and EAAT2-HEK cells (fig 2). To further differentiate between transporter subtypes, the specific EAAT2 inhibitor dihydrokainic acid was used at 30 µM, which is in the range of its reported K_i value. While dihydrokainic acid had no effect on the glutamate

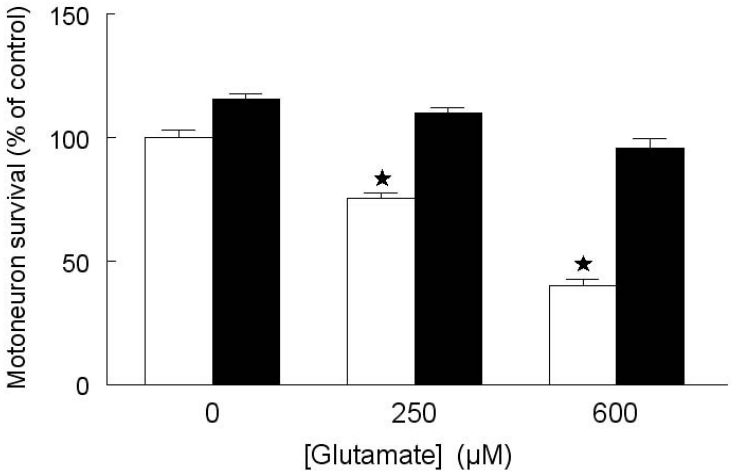

Figure 3. *EAAT2 overexpressing HEK cells protect motoneurons from excitotoxic death in vitro. Motoneuron-astrocyte cocultures were exposed to vehicle or glutamate, either in the presence of wild-type- (open bars) or EAAT2-HEK cells (solid bars). Motoneuron survival was assessed after 20 hours of glutamate exposure. Data are mean ± SEM of two independent experiments, each consisting of 4 observations per group. * $p<0.05$ versus control, i.e. the survival of untreated cultures in the presence of wild-type-HEK cells.*

uptake in wild-type cells, uptake in the EAAT2-HEK cells was inhibited by appr. 50% (fig. 2).

Finally, having shown specific and functional glutamate uptake, we tested if the EAAT2-HEK cell line could protect motoneurons on a astrocyte feeder layer from glutamate toxicity *in vitro*. Astrocytes in these cultures also express EAAT2, their uptake being appr. 1.5-fold higher than that of EAAT2 cells (data not shown). When motoneurons were exposed to glutamate in the presence of wild-type-HEK cells, significant dose-dependent cell death was observed (fig. 3). In contrast, glutamate-induced toxicity was virtually absent when motoneuron were exposed to glutamate in the presence of the EAAT2-HEK cells. Motoneuron survival remained unaffected even when the cultures were exposed to glutamate at a dose as high as 600 μM (fig. 3).

Discussion

One of the most important hypotheses for the pathogenesis of amyotrophic lateral sclerosis is glutamate induced motoneuron cell death. While high glutamate levels generally cause toxicity in neurons, for motoneurons even small increases in glutamate levels can be lethal as these cells are more sensitive for glutamate than other neurons (Ludolph et al., 2000). Motoneurons express glutamate receptors lacking the GluR2 subunit, which makes these receptors more permeable for calcium (Shaw et al., 1999; Vandenberghe et al., 2000; Vandenberghe et al., 2000; Vandenberghe et al., 2000). Also, motoneurons express less calcium binding proteins such as calbindin D28K and parvalbumin (Ince et al., 1993). To prevent excitotoxicity, it is of great importance that the glutamate concentration in the synaptic cleft is kept low. It is known that protein levels (Rothstein et al., 1995) and the high-affinity uptake (Rothstein et al., 1992) of the glutamate transporter EAAT2 are decreased in the spinal cord and motor cortex of appr. 65% of sporadic ALS patients. Hence, induction of EAAT2 expression and consequent lowering of spinal glutamate levels might be of therapeutic value in amyotrophic lateral sclerosis. Theoretically, expression of EAAT2 in glia can be induced by physiological modulators such as EGF, TGF-α (Zelenaia et al., 2000) and cAMP analogs (Swanson et al., 1997). However, the clinical use of these agents is greatly hampered by the fact that, due to their pleiotropic nature, they interfere with multiple cellular pathways other than EAAT2 regulation. Therefore, notwithstanding the importance of the recent characterisation of the EAAT2 promoter and future pharmacotherapeutic value thereof (Su et al., 2003), at this moment the most promising and clinically feasible approach to increase the expression of the glutamate transporter EAAT2 is the use of gene therapy (Aebischer et al., 1996). It is our hypothesis that with EAAT2 gene therapy it might be possible to chronically lower the local spinal glutamate concentration and, hence, to prevent excitotoxic motoneuron death.

Therefore, we have decided to construct EAAT2 overexpressing cell lines suitable for *ex vivo* gene therapy. Creation of such cells was successful and EAAT2 protein was stably expressed in 6 different HEK cell lines. Although the EAAT2 in these genetically engineered cells has a somewhat higher molecular weight compared to that in rat cortex homogenate, probably due to altered posttranslational modification, EAAT2-HEK cells

were able to actively take up considerable amounts of glutamate in a sodium-dependent manner. Pharmacological characterisation revealed that, while the modest uptake in wild-type-HEK cells was mediated by non-EAAT2 transporters, the increase in uptake capacity observed in the genetically engineered cells was for the greater part mediated by EAAT2. Dihydrokainic acid inhibited glutamate uptake in EAAT2-HEK cell line by appr. 50%, in line whit its K_i-value. To test the neuroprotective potential of EAAT2-HEK cells, we exposed astroglial-motoneuron cocultures to glutamate at concentrations previously shown to be sufficient to trigger cellular calcium-influx, mitochondrial dysfunction, and ROS generation in cultured rodent motoneurons (Rao et al., 2003). Importantly, the genetically engineered cell line that was selected for its high uptake capacity, was capable to fully protect motoneurons from glutamate exposure *in vitro*. Thus, whereas 60% of motoneurons died upon glutamate exposure in the presence of wild-type-HEK cells, in the presence of EAAT2-HEK cells virtually no excitotoxicity was observed, even when motoneurons were exposed to a dose as high as 600 µM. Although not explicitly investigated, the neuroprotective effect observed in this model at least indicates that the EAAT2-HEK cells are capable to efficiently scavenge and/or metabolise glutamate in a non-toxic manner. The clinical safety of ex-vivo gene therapy, using encapsulated cells, has previously been demonstrated in ALS patients (Aebischer et al., 1996). Hence, on the basis of the shown neuroprotective potency of EAAT2 expressing cell lines *in vitro*, it seems worthwhile to investigate whether or not ex vivo gene therapy for EAAT2 might be an efficacious and feasible approach to combat degeneration of motoneurons in amyotrophic lateral sclerosis.

Conclusion

The stable cell lines created in this study expressed functional EAAT2 protein. Overexpression of EAAT2 resulted in a profuse increase in glutamate uptake compared to wild-type cells. Furthermore, these genetically engineered cells were able to protect motoneurons from glutamate exposure *in vitro*. As glutamate excitotoxicity is thought to be important in motoneuron death *in vivo*, it is anticipated that encapsulated EAAT2-overexpressing cells can be of therapeutic value in ALS.

CHAPTER 4

INTRASPINAL LENTIVIRAL VECTOR-MEDIATED DELIVERY OF EAAT2 IN A TRANSGENIC ALS MOUSE MODEL

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal paralytic disease characterised by specific degeneration of motoneurons. Although the exact pathogenesis of ALS is still not clear, glutamate excitotoxicity seems to play an important role in motoneuron cell death. By overexpressing the glutamate transporter EAAT2, and thereby lowering the glutamate concentration in the vicinity of motoneurons, we aim to confer neuroprotection in the G93A-hSOD1 ALS mouse model. Gene therapy with the use of lentiviral vectors (LVVs) is an effective approach to deliver therapeutic proteins to the central nervous system. LVVs encoding EAAT2 or green fluorescent protein (GFP) were constructed to examine both the cellular pattern of transduction and the neuroprotective efficacy of intraspinally delivered LVV-EAAT2. In organotypic spinal cord cultures it was found that LVV-GFP was mainly transduced in astrocytes leaving the neuronal cells virtually unaffected. Likewise, when LVV-GFP was introduced intraspinally into young adult mice at the level of vertebra L1, immunohistochemical analysis again showed preferential transduction of the transgene into spinal astrocytes at the site of injection with no evidence for neuronal transduction.

Next, using motor performance and body weight as clinical parameters, the neuroprotective efficacy of LVV-EAAT2 was evaluated in G93A-hSOD1 mice. While EAAT2 was effectively transduced in the spinal cord, disease onset and survival in LVV-EAAT2 treated mice did not significantly differ from either LVV-GFP-treated or naive control mice. It is concluded that, although LVV-EAAT2 did not effect the clinical outcome of ALS mice, as such LVVs can be successfully used to deliver therapeutic genes into spinal astrocytes.

Introduction

Amyotrophic lateral sclerosis (ALS) is a paralytic neurodegenerative disorder, leading to death approximately 3-5 years after diagnosis. ALS is characterised by a preferential loss of upper- and/or lower-motoneurons (Haverkamp et al., 1995; Strong, 2003; Haverkamp et al., 1995). Although the exact pathogenesis of ALS is largely enigmatic, it is known that glutamate plays an important role in motoneuron cell death. Glutamate is one of the major neurotransmitters in the central nervous system. Relatively high levels of glutamate can lead to neurodegenerative processes via overstimulation of Ca^{2+}-permeable glutamate receptors, a process known as excitotoxicity (Shaw and Ince, 1997). The consequent rise in intracellular calcium concentration causes a cascade of toxic processes including mitochondrial dysfunction, the activation of Ca^{2+}-dependent proteases, phospholipases, and endonucleases, and the generation of reactive oxygen species (ROS), resulting ultimately in cell death (Arundine and Tymianski, 2003). When compared to other neurons, motoneurons are particularly vulnerable for AMPA/kainate receptor mediated excitotoxicity (Carriedo et al., 1996; Weiss and Sensi, 2000; Weiss and Sensi, 2000). In ALS, this vulnerability might be explained by the fact that, due to a defect in the editing of the mRNA encoding the GluR2 AMPA receptor subunit, motoneurons express a specific subset of Ca^{2+}-permeable AMPA receptors (Kawahara et al., 2004). In addition, motoneurons are less capable to handle large amounts of intracellular Ca^{2+}, since the expression of the Ca^{2+}-binding proteins such as calbindin D28K and parvalbumin is relatively low in motoneurons as compared to other neurons (Ince et al., 1993).

Under physiological conditions, glutamate activates ionotropic and metabotropic glutamate receptors on the postsynaptic neuronal membrane. To inactivate this signal, glutamate is quickly cleared from the synaptic cleft by sodium-dependent glutamate transporters, so-called excitatory amino acid transporters (EAATs) (Gegelashvili and Schousboe, 1997). The glutamate transporter subtype EAAT2, which is predominantly expressed by astrocytes, accomplishes the majority of glutamate uptake under physiological conditions (Tanaka et al., 1997). In ALS patients, as well as in ALS animal models, supporting evidence for a disturbed glutamate homeostasis has been found. Thus, while an increased level of glutamate is observed in the cerebrospinal fluid of ALS patients (Spreux-Varoquaux et al., 2002), both EAAT2 protein level (Rothstein et al.,

1995) and the functional glutamate uptake capacity in brain and spinal cord tissue are decreased (Rothstein et al., 1992).

As recently described by Rao and Weiss (Rao and Weiss, 2004), in ALS progressive motoneuron death can be explained by a feedforward cycle in which ROS, generated by excitotoxic processes in motoneurons, damage the glutamate transporter and lower glutamate uptake capacity of the surrounding astrocytes. This could converge into a self-propagating process driven by a further increase in synaptic glutamate concentration and consequent excitotoxicity. In this context, and in relation to the marked inflammatory response in ALS, also excess glutamate release from activated microglia is noteworthy (McGeer and McGeer, 1998; McGeer and McGeer, 2002; McGeer and McGeer, 2002). Accordingly, it can be hypothesised that by increasing astrocytic glutamate uptake capacity, e.g. via overexpression of EAAT2, it might be possible to halt this glutamate driven cascade and to protect the motoneurons from excitotoxicity.

While, in a previous study, we showed that primary motoneurons could effectively be protected against excitotoxicity by the presence of EAAT2-overexpressing HEK cells in coculture (Wisman et al., 2003), the neuroprotective potential of EAAT2 is underscored further by an in vivo study showing that increased expression of the glial glutamate transporter EAAT2 modulates excitotoxicity and delays the onset of disease in an EAAT2/G93A-hSOD1 double transgenic ALS mouse model (Guo et al., 2003).

In a first attempt to translate the above concept into a clinically feasible approach, we examined whether in vivo gene therapy can be exploited to locally increase EAAT2 expression in the spinal cord and to improve the clinical phenotype of ALS mice. Therefore, lentiviral vectors (LVVs) have been constructed encoding EAAT2 or the marker gene green fluorescent protein (GFP), under the transcriptional control of the cytomegalovirus (CMV) promoter. Upon characterisation of these LVVs in vitro and in vivo, the clinical efficacy of intraspinal delivery of LVV-EAAT2 has been investigated in the G93A-hSOD1 ALS mouse model.

Materials and methods

Cloning and production of lentiviral vectors

To generate lentiviral vectors, a three plasmid expression system was used. A 2 kb cDNA encoding the human glial glutamate transporter EAAT2 (GenBank: U01824; kindly provided by Dr. D. Trotti, Department of Neurology, Cecil B. Day Laboratory for Neuromuscular Research, Massachusetts General Hospital, Harvard Medical School) was subcloned into the transfer vector (pHR2). As a control, GFP cDNA was subcloned into the same vector. While the pCMVΔR-8.74 plasmid was used as minimal packaging construct, the pMD.G plasmid, containing the vesicular stomatitis virus G protein (VSV-G) envelope, was used as third plasmid. Replication-defective lentiviral vectors were generated by transient cotransfection of 293T human embryonic kidney (HEK) cells with the three-plasmid combination (Naldini et al., 1996a). High-titer stocks were obtained by centrifugation at 20,000 rpm with a SW28 rotor for at least 140 minutes at 16°C. The supernatant was carefully discarded and the pellets of GFP- or EAAT2-expressing LVVs were resuspended in phosphate-buffered saline (PBS) containing 0.5% IgG free and low endotoxin bovine serum albumin (BSA).
Particle content was measured by a p24 antigen ELISA (Perkin Elmer Life Sciences, MA, USA) and correlated to a previous determined GFP-LVV concentration. The latter concentration was assayed for transduction efficiency on growing 293T cells by counting GFP positive cells two days after infection.

Characterisation of LVV-EAAT2 in vitro

Transduction of 293T-HEK cells

293T HEK cells were cultured in IMDM (Sigma, Zwijndrecht, The Netherlands) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were plated in a 24-wells plate at a density of $1x10^5$ cells/well. After 24 hours, cells were transduced with EAAT2- or GFP-LVV at a concentration of $1x10^6$ or $1x10^7$ transducing units (TU), respectively. The next day the medium was replaced by fresh medium and the cells were grown for an additional two

days before EAAT2 expression and EAAT2 function were characterised by Western blot analysis or a glutamate uptake assay, respectively.

Western blot analysis

EAAT2 protein expression was determined by Western blot analysis (Wisman et al., 2003). Protein samples were prepared by homogenisation in 50 mM Tris-HCl lysis buffer containing 0.5% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, and 1 mM PMSF (pH=7.4). Protein content was determined according to Lowry using BSA as a standard (Lowry et al., 1951).

Protein samples, prestained molecular weight standards (Gibco, Invitrogen, Breda, The Netherlands), and rat cortex homogenate as a positive EAAT2 control were separated on 10% SDS-polyacrylamide gels. Separated proteins were transferred to a polyvinylidene fluoride membrane (Roche, Basel, Switzerland) by electroblotting (100 V, 60 min) in Towbin buffer containing 12.4 mM Tris, 95.5 mM glycin, and 20% methanol.

After blocking non-specific binding by overnight incubation at 4ºC in PBS containing 0.1% Tween (PBS-Tw) and 3% BSA, blots were incubated with the EAAT2 reactive guinea-pig-anti-glutamate transporter GLT-1 antibody (1:2,500; Chemicon, Temecula, CA, USA) in PBS-Tw for 3.5 hours at room temperature. As a loading control, blots were also stained for mouse anti-β-actin (1:40,000; Sigma, Zwijndrecht, The Netherlands). Next, membranes were washed with PBS-Tw and incubated with biotinylated goat-anti-guinea-pig antibody or biotinylated horse-anti-mouse (1:500; Vector Laboratories, Burlingame, CA, USA) in PBS-Tw for 1 hour. After washing, the membranes were incubated with avidin-biotin-conjugated peroxidase reagent (ABC-HRP; Vector Laboratories, Burlingame, CA, USA) 1:100 diluted in PBS-Tw for 1 hour. Finally, membranes were washed and immunoreactive proteins were visualised using ECL (Roche, Basel, Switzerland).

Glutamate uptake assay

The functionality of EAAT2 was estimated with a glutamate uptake assay according to Debler and Lajtha with minor adjustments (Debler and Lajtha, 1987; Wisman et al., 2003; Wisman et al., 2003). Briefly, after transduction of the 293T cells with LVVs, medium

was replaced by transport buffer (10 mM HEPES, 5 mM Tris, 10 mM D(+)glucose, 3.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 1.2 mM K_2HPO_4; pH=7.4) containing either 140 mM NaCl or 140 mM cholineCl. The cells were kept in this buffer for 10 min at 37ºC before glutamate uptake was started by the addition of 1 µM glutamate containing 9.52 pM [^{3}H]-glutamate (specific activity 42 Ci/mmol; Valeant Pharmaceuticals International, Costa Mesa, CA, USA). After incubation for 20 min at 37ºC, glutamate uptake was stopped by adding an equal volume of ice-cold transport buffer containing 1 mM glutamate. Following resuspension of the cells in the stop buffer, cells were harvested and washed on a Beckman cell harvester. Radioactivity on the filters was quantified by liquid scintillation counting. Na^+-dependent uptake, expressed as the amount of glutamate taken up per mg protein/min, was assessed by subtracting the choline values from the sodium values and by correcting for protein content.

Cellular localisation of LVV-transduced proteins in vitro and in vivo

LVV-treated organotypic spinal cord cultures

Organotypic spinal cord cultures were prepared from the spinal cord of 8 day-old mouse pups as described previously (Kaal et al., 2000; Rothstein et al., 1993; Rothstein et al., 1993). In brief, lumbar spinal cords were dissected and sliced into 350 µm thick sections. The slices were cultured on inserts in medium containing 25% Hank's balanced salt solution (Gibco, Invitrogen, Breda, The Netherlands), 25% horse serum, 50% modified essential medium (Gibco, Invitrogen, Breda, The Netherlands), 35 mM glucose, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. On the day of preparation, the slices were transduced with $1x10^6$ TU LVV-GFP per slice. After 10 days in culture, the slices were fixed with 4% paraformaldehyde for 1 hour. The slices were then impregnated in 25% sucrose in PBS for at least 30 minutes. A block of 4% agar is frozen on a tissue holder with M-1 embedding matrix (Shandon, Pittsburgh, PA, USA) and the slices are quickly frozen on the agar with cooled isopentane. Serial transverse cryosections of 6 µm were mounted on chrome-alum gelatine coated coverglasses and used for immunohistochemistry.

LVV-treated wild-type B6SJLF1/J mice

At 50 days of age, female B6SJLF1/J mice (The Jackson Laboratories, Bar Harbour, Maine, USA) were subjected to intraspinal surgery. Briefly, mice were anaesthetised by an injection of pentobarbital (96 mg/kg, ip). Animals were placed in a stereotactic apparatus and their spinal cords were immobilised with the use of a, so-called, spinal adaptor (Azzouz et al., 2000). Following a laminectomy at the level of vertebra L1, 0.5 µl LVVs ($1x10^9$ TU/ml) were injected into the ventral spinal cord (-0.7 mm ventral of the dura) through a 5 µl Hamilton syringe fitted with a blunt 33 gauge needle. Injections were controlled by an infusion pump at a rate of 0.2 µl/min. Following injection, the needle was left in place for 5 min before being retrieved.

For localisation studies with LVV-GFP, mice were injected bilaterally at one site (i.e., two injections placed on opposite sites of the midline of the spinal cord). The mice were sacrificed by a lethal dose of pentobarbital (180 mg/kg, i.p.) and perfused transcardially with saline containing 20 IU/ml heparin, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). The spinal cord was dissected free from the vertebral column and postfixed in the same fixative at 4ºC overnight. Subsequently, the tissue was cryoprotected in 25% sucrose in PBS (pH=7.4). Hereafter, tissue containing the injection site was frozen in cooled isopentane, and stored at –70ºC until further use. Transverse cryosections (10 µm) were mounted on chrome-alum gelatine coated coverslips.

Immunohistochemistry

After air-drying for at least 1 hour, sections of either organotypic spinal cord cultures or perfused mouse tissue were permeabilised by incubation with aceton for 5 min. Thereafter, the sections were incubated with primary antibodies diluted in PBS containing 0.2% BSA and 0.1% sodium azide (PBS-BSA) for 16 hours at RT. For the identification of neuronal and astroglial cells, respectively, mouse anti-neuronal nuclei (NeuN 1:400; Chemicon, Temecula, CA, USA) and rabbit anti-glial fibrillary acidic protein (GFAP 1:4000; Dako, Glostrup, Denmark) were used. Thereafter, sections were rinsed 3 times in PBS, and incubated with horse-anti-mouse- or goat-anti-rabbit-biotinylated secondary antibodies (1:220; Vector Laboratories, Burlingame, CA, USA) in PBS-BSA. Next, after washing three times in PBS, sections were incubated with streptavidin-Cy3 in PBS-BSA

for 1 hour. After rinsing 3 times in PBS, sections were mounted in veronal buffered saline (pH=8.6) containing 90% glycerol (v/v), and 10% DABCO, and evaluated by immunofluorescence microscopy. To visualise microglial cells sections are processed for tomato-lectin (*Lycopersicon esculentum*) histochemistry as described previously (Acarin et al., 1994).

LVV-treated G93A-hSOD1 ALS mice

Animals

All animal protocols were approved by the animal care committee of the University Medical Center Utrecht. Transgenic mice carrying G93A human SOD1 (B6SJL-TgN (SOD1-G93A) 1Gur/J) were purchased from Jackson Laboratories (Bar Harbour, Maine, USA). The colony was maintained in a B6SJLF1/J background. Genotyping of transgenic mice was assessed by standard PCR amplification of DNA extracted from tail tissue with specific primers for hSOD1. The mice were housed in isolated cages in an infection unit with a 12-12 hour light-dark cycle. All animals had free access to water and food.
To study the neuroprotective efficacy of LVV-EAAT2, at 50 days of age G93A-hSOD1 mice were randomised into three groups. LVV-EAAT2 treated mice (n=9) and LVV-GFP treated mice (n=9) were subjected to stereotactic surgery as described above albeit that these mice were injected bilaterally at two sites, separated by 2 mm in the rostral-caudal direction (Azzouz et al., 2000). An additional group of mice (n=6) remained untreated and served as naive controls. Since onset of disease and mortality differ among the sexes, experimental groups existed of female mice only (Veldink et al., 2003).

Clinical phenotyping

Starting from one week after the surgical procedure onwards, to monitor disease onset and progression mice were evaluated for signs of motor deficits using the paw-grip-endurance-test (PaGE) and the beam-balance test, respectively (Veldink et al., 2003; Weydt et al., 2003; Veldink et al., 2003). In the PaGE test each mice was placed on the wire-lid of a conventional housing cage. The lid was gently shaken to prompt the mouse to hold onto the grid before it was swiftly turned upside down. The latency until the mouse let go with at least both hind limbs of the grid was timed, with an arbitrary

maximum of 90 sec (Weydt et al., 2003). The time point at which mice performed under 90 sec. was used as an event to establish an Kaplan-Meier curve. For the beam balance test, mice were placed on a 15 cm raised platform connected to a dark box via a beam of 1.5 cm in width and 27 cm in length. The time needed to reach the dark box was assessed as a measure for disease progression. The time point at which mice were not able to reach the dark platform within two times their average performance, i.e. the time needed to reach the dark box assessed before onset occurred, was used as event (test failure) to establish a Kaplan-Meier curve (Trieu and Uckun, 1999; Veldink et al., 2003; Trieu and Uckun, 1999). These tests were performed twice a week and were stopped when the mice were unable to hold on to the grid or beam for at least three consecutive attempts.

To monitor disease progression and to determine the clinical endpoint, body weight was monitored twice a week using an electronic balance. For ethical reasons, the artificial endpoint for death was defined as the time point at which mice had lost over 20% of the adult body weight (i.e. mean body weight as determined at week 9-11), or were unable to right themselves when placed on their side within 30 sec (Veldink et al., 2003). After sacrificing these animals, tissue from the lumbar- and cervical spinal cord, the cortex, the liver, and the adductor magnus muscle was collected for PCR analysis of EAAT2 expression.

EAAT2 expression in LVV-EAAT2 treated mice

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was purified from tissues by means of a RNAzol based extraction method according to the manufacturer's instructions (Invitrogen, Breda, The Netherlands). After the integrity and the amount of total RNA was analysed by electrophoresis, cDNA was made by reverse transcription-polymerase chain reaction (RT-PCR). In brief, after heating the RNA for 5 min at 60°C in the presence of random hexamer [pd(N)6] primers and RNAsin, and after subsequent incubation on ice for 3 min, 10 µl samples of total RNA were reverse transcribed to cDNA for 120 min at 42°C in a reaction mixture (total volume 50 µl) containing Promega M-MLV RT reaction buffer (50 mM Tris-HCl (pH = 8.3), 3 mM $MgCl_2$, 75 mM KCl, 10 mM DDT), RNase/DNase-free BSA, 2.25 µg random hexamer primers, 0.6 U/µl RNaSIN, 20 U/µl Moloney Murine Leukemia Virus reverse

transcriptase (M-MLV-RT), and 1 mM of each of the deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP). cDNA samples were stored at -20°C prior to use.

Polymerase chain reaction for EAAT2 and mouse GAPDH

For PCR amplification, 1 µl of cDNA was used. The amplification was carried out in 20 µl 50 mM Tris-HCl (pH=8.0) containing 50 mM KCl, 1.5 mM $MgCl_2$, 20 pmol of each of the respective forward- and reverse primer, 2.5 mM dNTP, and 0.75 U Platinum Pfx DNA polymerase. The primers used are listed in table 1. As a negative control, PCR mixture with no template was included. PCR amplification was performed in 40 three-step cycles as follows: 1 min at 94°C to denaturate, 1 min at 55°C to anneal, and 1 min at 68°C to extend.

Statistical analysis

All statistical analyses were performed with the SPSS 9.0 for Windows software. For the comparison of glutamate uptake values of the transduced HEK cells, after testing for homogeneity of variance and for normality of residuals, an analysis of variance (ANOVA) was performed for multiple comparison between groups followed by Bonferoni's post hoc test to compare group means. Statistical analysis of the disease onset and survival was performed using the Kaplan-Meier method with a log rank test. p values <0.05 were considered statistically significant.

Table 1. *Primer sequenches used to amplify EAAT2 and GAPDH*

Gene	Genbank #	primers	Amplicon size (bp)
EAAT2	U01824	Fw 5'-CAA CGC AAC CAG CGC TGT TG-3' Rv 5'-GCA ATG ATC TTT CCA CAG ATC AG-3'	300
GAPDH	BC020407	Fw 5'-TTA GCA CCC CTG GCC AAG G-3' Rv 5'-CTT ACT CCT TGG AGG CCA TG-3'	80

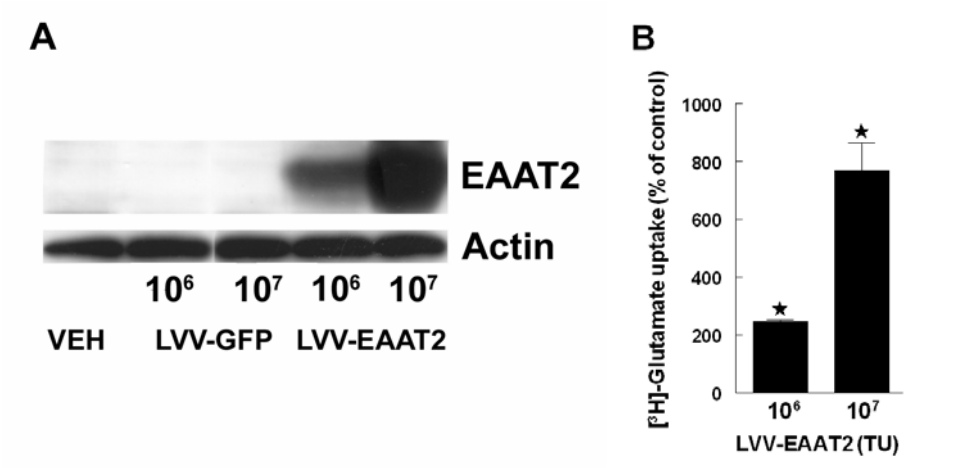

Figure 1. *Functional characterisation of LVV-EAAT2 in vitro. HEK cells were exposed to LVV-GFP or LVV-EAAT2, at a dosage of 1x10^6 or 1x10^7 TU, before EAAT2 protein expression (panel A) and [^{3}H]-Glutamate uptake capacity (panel B) were determined. Panel A: Western blot analysis of HEK cells either treated with vehicle (lane 1), LVV-GFP (lanes 2 and 3), or LVV-EAAT2 (lanes 4 and 5), at the indicated concentrations (TU). Panel B: [^{3}H]-Glutamate uptake in LVV-EAAT2 transduced cells. Sodium-dependent glutamate uptake is expressed as percentage of control, i.e. the uptake observed in cells treated with an equivalent amount of TU of LVV-GFP. * p<0.05 versus respective control.*

Results

Characterisation of LVV-EAAT2 in vitro

Upon validation of the engineered LVV-EAAT2 construct by sequencing and controlled digestion (data not shown), the expression and functionality of the LVV-EAAT2 construct protein was evaluated in HEK cells. First, transduced cells were examined for the presence of EAAT2 protein by Western Blot analysis. In contrast to naive, or LVV-GFP treated cells, respectively, in cell lysates from 1x10^6 TU LVV-EAAT2-treated HEK cells a clear protein band with a molecular weight of 79 kDa, corresponding to EAAT2 was found. As shown in Figure 1A, the amount of transduced EAAT2 protein increases profoundly when cells are treated with an increased amount of LVV-EAAT2, i.e. 1x10^7 TU. Next, to analyse the functionality of the transduced protein, a [^{3}H]-glutamate uptake assay was performed on EAAT2- or GFP-transduced HEK cells. While the Na^+-dependent

[^{3}H]-glutamate uptake was approximately 2.5 times higher in LVV-EAAT2 treated cells as compared to GFP transduced cells when $1x10^6$ TU of LVVs were used, in case of $1x10^7$ TU, the observed increase in uptake in EAAT2 transduced cells was even more pronounced and amounted to 800% (figure 1B).

Cellular localisation of LVV-transduced proteins in organotypic spinal cord cultures

Organotypic mouse spinal cord cultures were transduced with LVV-GFP and fixed 10 days after infection. From control experiments it appeared that the day of infection is of critical importance for successful transduction. Thus, where the transduction efficiency differed considerably between DIV 0 and DIV 3, starting the infection at DIV 0 invariably showed the highest transduction efficiency (data not shown). The cellular localisation of LVV-transduced proteins was studied using immunohistochemical (double) staining with antibodies raised against neuronal and glial cell markers, i.e. NeuN and GFAP, respectively. As illustrated in figure 2 (A-F), in organotypic spinal cord cultures GFP was found to be expressed predominately by astrocytes, leaving the neuronal cells virtually unaffected.

Cellular localisation of LVV-transduced proteins in mouse spinal cord

Mice were given a bilateral intraspinal injection of LVV-GFP and were sacrificed for immunohistochemical analysis two weeks thereafter. As illustrated in figure 2 (G-L), GFP expression is mainly colocalised with GFAP, a marker for astrocytes. Although some GFP expression could be found in NeuN positive cells, the majority of neurons lacked GFP expression. Interestingly, as shown in figure 2I, neuronal cells are intimately surrounded by GFP transduced glia cells and their processes. By analysing serial sections around the site of injection, it appeared that LVV-induced transfer of GFP could be observed approximately 1.5-2 mm from the injection site, spreading caudally and rostrally into the spinal cord. Moreover, histological evaluation of the lumbar spinal cord revealed that intraspinal LVV delivery as such was not associated with astrocytic proliferation and/or microglial activation.

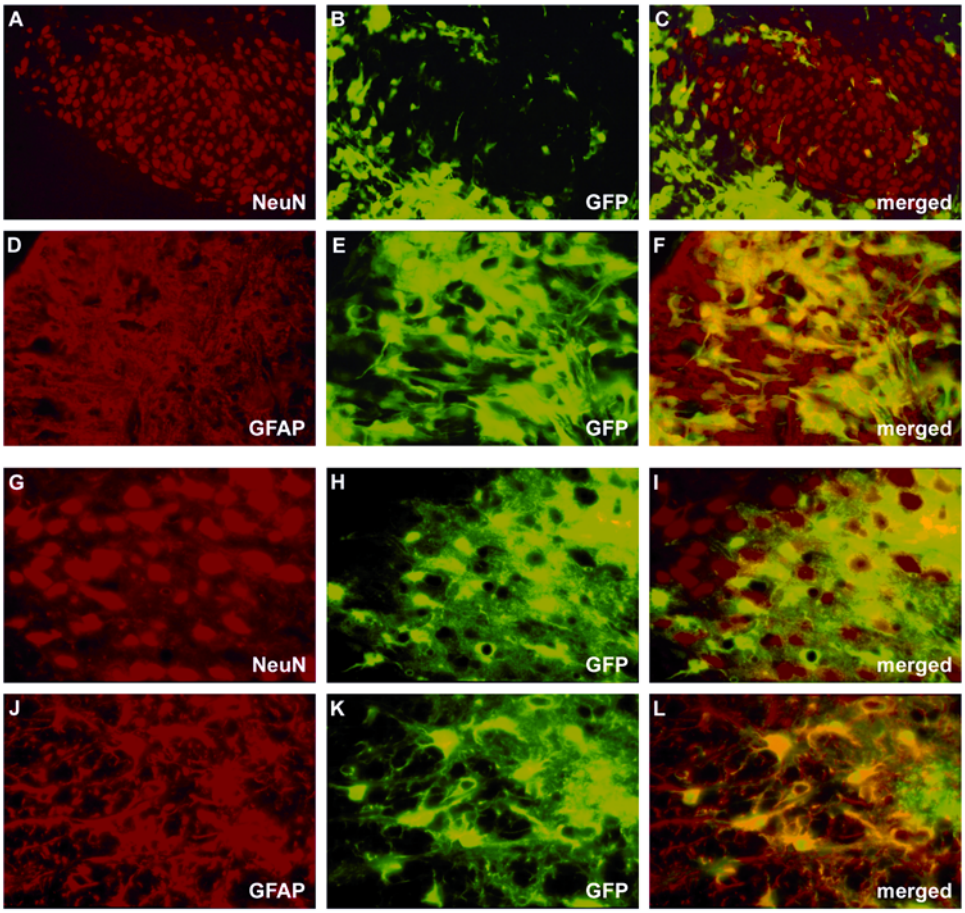

Figure 2. *Cellular localisation of LVV-transduced proteins in vitro and in vivo. Panel A-H: Immunohistochemical analysis of LVV-GFP treated organotypic spinal cord cultures. Cultured spinal cord slices were treated with LVV-GFP (green) at DIV 0 and processed for immunohistochemistry using NeuN (A-C, red) or GFAP (D-F, red) as markers for neuronal or glial cells, respectively. In merged pictures double stained cells appear as yellow. Panel G-H: Immunohistochemical analysis of LVV-GFP injected mice. Mice were injected with LVV-GFP (green) at the level of vertebra L1 and sacrificed two weeks later. Immunohistochemistry for the neuronal markers NeuN (G-I, red) and the astroglial marker GFAP (J-L, red) show a predominant colocalisation for the transgene GFP with astrocytes. Overall, GFP expression was found to be expressed predominately by astrocytes, leaving the neuronal cells virtually unaffected.*

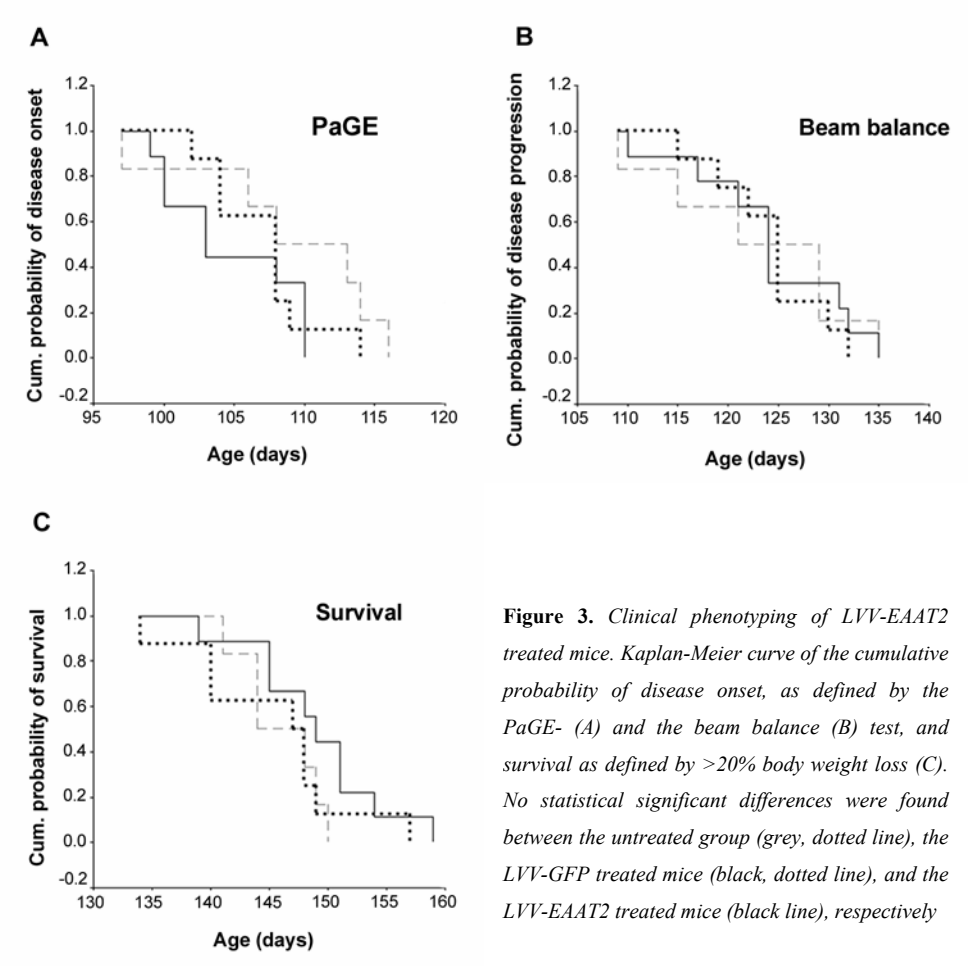

Figure 3. *Clinical phenotyping of LVV-EAAT2 treated mice. Kaplan-Meier curve of the cumulative probability of disease onset, as defined by the PaGE- (A) and the beam balance (B) test, and survival as defined by >20% body weight loss (C). No statistical significant differences were found between the untreated group (grey, dotted line), the LVV-GFP treated mice (black, dotted line), and the LVV-EAAT2 treated mice (black line), respectively*

Clinical phenotyping of LVV-EAAT2 treated G93A-hSOD1 mice

To determine the neuroprotective efficacy of LVV-EAAT2, two groups of mice were injected bilaterally at two separate sites either with LVV-GFP or LVV-EAAT2. An additional group of mice remained untreated. Although, in the LVV-GFP-treated group one animal died as a consequence of the surgical procedure, no adverse affects were found in any of the other operated animals. Thus, although some mice showed a transient drop in the gain of body weight, all mice exhibited normal motor behaviour as soon as one day postoperative. As shown in figure 3A, the time of onset of the disease, as defined by a first

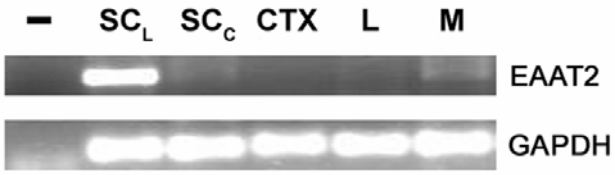

Figure 4. *PCR analysis of EAAT2 mRNA expression in LVV-EAAT2 treated mice. Mice used to test the clinical efficacy of LVV-EAAT2 were sacrificed at end stage of the disease. Tissue from lumbar- (SC_L), and cervical spinal cord (SC_C), cortex (CTX), liver (L), and adductor magnus muscle (M), respectively, was taken out for PCR analysis. In LVV-EAAT2 treated mice, an amplicon band at the anticipated size of 300 bp was found in tissue from the injection site (SC_L), but not in any of the other tissues analysed. GAPDH was used as an internal control (bottom panel).*

decline in latency time (<90 sec) in the PaGE test, did not differ between the untreated, LVV-GFP- or LVV-EAAT2-treated animals showing a mean time of onset of 109.0 ± 2.58, 107.13 ± 1.33, and 105.5 ± 1.33 (days ± SD), respectively (log rank ρ = 0.23). In addition, no difference in disease progression was observed. Thus, while no differences were observed in the ALS associated loss of body weight (data not shown), the performance in the beam balance test was equal for all groups (log rank ρ = 0.94) showing first test failure at 123.0 ± 4.0, 124.13 ± 1.95, and 124.22 ± 2.6 (days ± SD) for untreated, LVV-GFP- and LVV-EAAT2-treated groups, respectively (figure 3B).

Finally, the survival of the mice was determined by using a greater than 20% loss of body weight as an artificial clinical endpoint. As illustrated in figure 3C, no statistically significant differences in survival were observed between the experimental groups. A mean survival of 146.0 ± 1.44, 145.38 ± 2.51, and 149.0 ± 1.92 (days ± SD) was found for the untreated-, LVV-GFP-, and LVV-EAAT2-treated mice, respectively (log rank ρ = 0.30).

After sacrificing the mice at end-stage of disease, tissue from lumbar- (injection site), and cervical-spinal cord, brain, liver and adductor magnus muscle was collected for EAAT2 expression analysis. PCR analysis using selective primers for EAAT2 and the housekeeping gene GAPDH revealed that EAAT2 was successfully transduced in all of the LVV-EAAT2-treated mice. As illustrated in figure 4 for one of the LVV-EAAT2-treated animals, an amplicon band of the anticipated size (300 bp) was found in tissue taken from the injection site, but not in any of the other regions examined.

Discussion

In the present study, we examined whether in vivo gene therapy LVV-EAAT2 can be exploited to increase EAAT2 expression in the spinal cord and tested the therapeutic effect of LVV-EAAT2 in an ALS mouse model.

For this purpose, an LVV construct encoding EAAT2 was engineered and characterised using both in vitro and in vivo models. In vitro studies using HEK cells showed a clear induction of EAAT2 protein expression in LVV-EAAT2 treated cells, while at the same time the glutamate uptake capacity of these transduced cells was dramatically increased. Thus, an increase in the sodium-dependent glutamate uptake up to 800% was observed in LVV-EAAT2 treated cells as compared to GFP-transduced control cells. Hence, it is concluded that LVV-EAAT2 is an effective tool to achieve functional EAAT2 gene transfer.

The LVVs used in this study are pseudotyped with a VSV-G envelope and a CMV promoter, which enables these LVVs to transduce a broad range of cell types including both neurons and astrocytes. To investigate the cellular expression pattern of transgenes carried by these VSV-G/CMV-LVVs, organotypic mouse spinal cord cultures were transduced with LVV-GFP. Furthermore, under stereotactic guidance mice were intraspinally injected with LVV-GFP at the level of vertebra L1. Strikingly, in both studies it was found that the transgene GFP is almost exclusively expressed by astrocytes with no evidence for neuronal transduction. Similar studies for LVV-mediated EAAT2 expression in mouse were inconclusive due to the fact that specific antibodies that can distinguish between endogenous mouse GLT-1 and human EAAT2 are not yet available (data not shown). However, based on the GFP data, it is anticipated that in mouse spinal cord EAAT2 is similarly transduced in astrocytes. While previous studies with LVVs often showed a transgene expression in neurons (Blomer et al., 1997; Naldini et al., 1996b; Naldini et al., 1996a; Naldini et al., 1996a; Naldini et al., 1996b), it has now become evident that the cellular preference of transgene delivery and/or expression by LVV is not uniform in the CNS and can also be found in astrocytes (Rosenblad et al., 2000). Accordingly, regional differences in the cellular expression of the VSV-G receptor and/or the activity of the promoters used, may explain the preferential expression of the transgene in spinal astrocytes such as observed in our in vitro and in vivo studies. Thus, while retrograde AAV-based gene therapy approaches can be used to target neuronal cell

bodies (Kaspar et al., 2003), from our studies it appears that VSV-G/CMV-LVVs are an effective means to accomplish gene transfer into spinal astrocytes. This property makes these vectors especially useful when one wants to introduce potential neuroprotective molecules that are normally expressed by astrocytes, including EAAT2 or glial derived neurotrophic factors.

To test the clinical efficacy of in vivo gene therapy for EAAT2, in the present study G93A-hSOD1 transgenic ALS mice have been treated with LVV-EAAT2 using an approach previously described by Azzouz and colleagues (Azzouz et al., 2000). In the G93A-hSOD1 mouse model the first signs of clinical onset are usually visible in the hind limbs reflecting motoneuron loss in the lumbar spinal cord (Gurney et al., 1994). Hence, to protect spinal motoneurons and to delay disease onset, LVV has been delivered intraspinally using a spinal adaptor positioned at the level of vertebra L1. To monitor the development of motoneuron disease, and to detect early deficits in hind limb motor function, we decided to use the PaGE test. This basic test of motor function, that requires balance and motor strength rather than good motor coordination, has recently been reported to be among the most sensitive methods to detect early motor signs in G93A-hSOD1 ALS mice (Weydt et al., 2003). In addition, to further differentiate between experimental groups, the beam balance performance and body weight were used as clinical parameters (Veldink et al., 2003). Notwithstanding the fact that in all experimental animals the transcription of EAAT2 was confirmed by RT-PCR analysis of spinal cord tissue obtained at end stage, it was found that transduction of EAAT2 did not show a neuroprotective effect on disease onset and disease progression. Moreover, similar to that observed in double EAAT2/G93A-hSOD1 transgenic mice (Guo et al., 2003), overexpression of EAAT2 by in vivo gene therapy did not prolong the lifespan of G93A-hSOD1 mice.

There are several factors that may explain the lack of clinical efficacy of LVV-EAAT2 in our mouse model. For example, it can be argued that the transduction of LVV-EAAT2 was not persistent over time. However, in line with previous data showing long-term expression of LVV-transduced marker genes in the CNS (Blomer et al., 1997; Naldini et al., 1996b; Blomer et al., 1997), and given our observation that EAAT2 mRNA was still present in end-stage animals, this option seems unlikely. It is still possible, however, that the dosage and/or the location of LVV injections used were inadequate. That is, ALS is a

widespread disease characterised by a degeneration of motoneurons in the motor cortex, brainstem, and spinal cord. Consequently, transduction of EAAT2 into a selective subpopulation of astrocytes located in the vicinity of spinal motoneurons only, such as accomplished in the present study, might not be sufficient to successfully combat disease progression in G93A-hSOD1 ALS transgenic mice.

Notwithstanding the fact that in our studies LVV-EAAT2 therapy was started at an early presymptomatic stage, it may be that transduction of EAAT2 at this time point was too late to effectively inhibit the glutamate-driven pathological cascade leading to excitotoxicity and progressive motoneuron loss. Finally, given its vulnerability for oxidative damage (Trotti et al., 1996; Trotti et al., 1999; Trotti et al., 1999), it can not be ruled out that newly delivered EAAT2 is subjected to oxidative stress caused by e.g. mitochondrial dysfunction, excitotoxic processes, neuroinflammation, and/or the respiratory burst of activated microglial cells (McGeer and McGeer, 2002). Although the aforementioned factors are likely to be operative in (sporadic) ALS patients also, it is noteworthy that in G93A-hSOD1 transgenic ALS animal models, mutant SOD1 constitutes an additional source of oxidative stress that cannot be neutralised by EAAT2 overexpression per se (Julien, 2001). While the presence of mutant SOD1 might provide an other explanation for the lack of any therapeutic effect of LVV-EAAT2 treatment, at the same time the above issues argue for the use of adjuvant therapy using e.g. a combination of antioxidants, anti-inflammatory drugs and/or the glutamate release inhibitor Rilutek, to possibly improve the clinical efficacy of in vivo gene therapy with LVV-EAAT2 (Kriz et al., 2003; Weiss et al., 2004).

Acknowledgement

This work was supported by the Prinses Beatrix fonds. The authors wish to thank Jan. H. Brakkee and Jacqueline A. Sluijs, from the Rudolf Magnus Institute of Neuroscience and the Netherlands Institute for Brain Research, respectively, for their excellent technical support.

CHAPTER 5

EFFICIENT LENTIVIRAL VECTOR-MEDIATED GENE TRANSFER OF THE GLUTAMATE TRANSPORTER EAAT2 TO PRIMARY ADULT HUMAN ASTROCYTES IN VITRO

L.A.B. Wisman, R. Veerhuis, D.S. Counotte, P.R. Bär, E.M. Hol, F.L. van Muiswinkel

Abstract

Glutamate excitotoxicity plays a critical role in the pathogenesis of ALS, a paralytic disease characterised by a progressive loss of motoneurons. The dominate glutamate transporter EAAT2 accounts for the majority of glutamate transport in the brain, and is decreased in ALS patients. It is hypothesised, therefore, that delivery of EAAT2 to astrocytes in the surroundings of affected motoneurons could be an effective means to protect these cells from excitotoxicity. In the present study, we demonstrated that LVV-EAAT2 is an effective tool to deliver EAAT2 to adult human astrocytes in vitro.

A series of human cortical- and spinal cord primary astrocyte cultures were exposed to LVV-EAAT2 before the functional expression of EAAT2 was evaluated by qPCR, Western blot analysis, and a glutamate uptake assay. In all cultures tested an increase in functional glutamate uptake was observed as compared to LVV-GFP treated controls. This increase was accompanied by an increase in EAAT2 mRNA and EAAT2 protein levels, respectively. In addition, it was found that functional expression of EAAT2 in LVV-EAAT2 treated cells was even further enhanced in the presence of TNF-α. It is concluded that LVV-EAAT2 is an effective means to increase functional glutamate uptake capacity in adult cortical and spinal human astrocytes.

Introduction

Amyotrophic lateral sclerosis (ALS) is a severe paralytic disease characterised a preferential loss of motoneurons and by progressive muscle atrophy. Glutamate excitotoxicity is thought to play an important role in the pathogenesis of ALS. Under normal conditions, glutamate released from the presynaptic neuron acts as a neurotransmitter by activating glutamate receptors on postsynaptic terminals. The glutamate signal is terminated by active uptake of glutamate by sodium-dependent glutamate carriers, the so-called excitatory amino acid transporters (EAATs). The dominate glutamate transporter subtype EAAT2 (GLT-1 in rodents) is expressed mainly by astrocytes (Chaudhry et al., 1995) and accounts for the majority of the glutamate transport (Tanaka et al., 1997).

Under pathological conditions, relatively high levels of glutamate can lead to neurodegenerative processes triggered by over-stimulation of Ca^{2+}-permeable glutamate receptors. The subsequent rise in the intracellular calcium concentration causes the activation of Ca^{2+}-dependent enzymes such as proteases, phospholipases, and endonucleases. In addition, the elevated calcium levels will lead to mitochondrial dysfunction and the generation of reactive oxygen species. This multifactorial proces can ultimately end in cell death and is known as excitotoxicity (Heath and Shaw, 2002; Arundine and Tymianski, 2003).

The cerebrospinal fluid (CSF) of ALS patients contains elevated levels of glutamate (Rothstein et al., 1990; Spreux-Varoquaux et al., 2002). One potential explanation for these increased levels may be the finding that in 60-70% of the sporadic ALS patients a marked loss of EAAT2 is observed in the motor cortex and the spinal cord (Rothstein et al., 1995). Similarly, in mutated SOD1 (mSOD1) rodent models for ALS a reduction in GLT-1 protein is also observed (Bruijn et al., 1997; Howland et al., 2002). By using anti-sense oligonucleotides to knock-down GLT-1 in rats, it has been demonstrated that a loss in EAAT2 protein can lead to an ALS-like syndrome. Thus, knock-down of GLT-1 resulted in a reduction of GLT-1 protein and led to motoneuron degeneration with hind limb paralysis within seven days (Rothstein et al., 1996).

Given the pivotal role of EAAT2 in glutamate clearance, we hypothesise that overexpression of EAAT2 in affected areas, such as the motor cortex and spinal cord,

might be an effective means to protect motoneurons from excitotoxicity. This view appears to be underscored, at least in part, by a recent in vivo study showing that overexpression of GLT-1 attenuates excitotoxicity and delays disease onset in mSOD1/EAAT2 compound transgenic mice (Guo et al., 2003). In ALS patients, in vivo gene therapy using lentiviral vectors (LVV) encoding EAAT2 appears to be a reasonable, clinical feasible, option to consider. Therefore, in the present study an EAAT2-expressing LVV was developed and tested for its ability to transduce human adult cortical and spinal cord astrocytes in culture. Moreover, given the recently identified relationship between TNF-α and EAAT2 transcription (Su et al., 2003) and taking into account the marked increase in TNF-α levels in ALS (Poloni et al., 2000; Hensley et al., 2002), also the impact of TNF-α on the glutamate uptake capacity of transduced adult human astrocytes was investigated.

Materials and methods

Cloning and production of lentiviral vectors

To generate lentiviral vectors, a three-plasmid expression system was used. A 2 kb cDNA encoding the human glial glutamate transporter EAAT2 (GenBank: U01824; kindly provided by Dr. D. Trotti, Department of Neurology, Cecil B. Day Laboratory for Neuromuscular Research, Massachusetts General Hospital, Harvard Medical School) was subcloned into the transfer vector (pHR2). As a control, GFP cDNA was subcloned into the same vector. While the pCMVΔR-8.74 plasmid was used as minimal packaging construct, the pMD.G plasmid, containing the vesicular stomatitis virus G protein (VSV-G) envelope, was used as third plasmid. Replication-defective lentiviral vectors were generated by transient co-transfection of 293T human embryonic kidney (HEK) cells with the three-plasmid combination (Naldini et al., 1996a). High-titer stocks were obtained by centrifugation at 20,000 rpm for at least 140 minutes at 16°C. The supernatant was carefully discarded and the pellets of GFP- or EAAT2-expressing LVVs were resuspended in phosphate-buffered saline (PBS) containing 0.5% IgG free and low endotoxin bovine serum albumin (BSA).

Particle content was measured by a p24 antigen ELISA (Perkin Elmer Life Sciences, MA, USA) and correlated to a previous determined LVV-GFP concentration. The latter concentration was assayed for transduction efficiency on growing 293T cells by counting GFP positive cells two days after infection.

Adult primary human astrocytes

Human spinal cord or cortical brain tissue was obtained by rapid autopsy according to standardized procedures under the management of the Netherlands Brain Bank (Amsterdam, The Netherlands; coordinator Dr. R. Ravid). Medical records were obtained for all patients prior to autopsy, and pathological evaluation was performed to confirm the clinical diagnosis. Primary human astrocyte cultures were isolated as described previously (De Groot et al., 1997) and were cultured in Dulbecco's modified Eagle medium with an equal amount of Ham-F10 suplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere at 37°C and 5% CO_2.

Transduction of primary adult human astrocytes

Primary adult human astrocytes were plated in a 24-wells plate at a density of $1.25x10^4$ cells/cm^2 in a volume of 500 μl. After three days in culture, cells were transduced with EAAT2- or GFP-LVV at a dosage of $1x10^6$ transducing units (TU). The next day the medium was replaced by fresh medium and the cells were grown for an additional three days before EAAT2 expression and EAAT2 function were characterised by quantitative PCR, Western blot analysis, and a glutamate uptake assay, respectively. When indicated, TNF-α (100 ng/ml) treatment was started after removal of the LVV-containing medium, i.e. 48 hours prior to the Western blot analysis or glutamate uptake assay.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was purified of (transduced) human astrocytes by means of a RNAzol based extraction method according to the manufacturer's instructions (Invitrogen, Breda, The Netherlands). After the integrity and the amount of total RNA was analysed by electrophoresis, cDNA was made by reverse transcription-polymerase chain reaction (RT-PCR). In brief, after heating the RNA for 5 min at 60°C in the presence of random

hexamer [pd(N)6] primers and RNAsin, and after subsequent incubation on ice for 3 min, 10 µl samples of total RNA were reverse transcribed to cDNA for 120 min at 42°C in a reaction mixture (total volume 50 µl) containing Promega M-MLV RT reaction buffer (50 mM Tris-HCl (pH = 8.3), 3 mM $MgCl_2$, 75 mM KCl, 10 mM DDT), RNase/DNase-free BSA, 2.25 µg random hexamer primers, 0.6 U/µl RNaSIN, 20 U/µl Moloney Murine Leukemia Virus reverse transcriptase (M-MLV-RT), and 1 mM of each of the deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP). cDNA samples were stored at -20°C prior to use.

Quantitative PCR

Quantitative PCR (qPCR) was carried out using the SYBR Green PCR kit (Applied biosystems, Foster City, CA, USA). Amplification was performed in a total volume of 20 µl in MicroAmp Optical 96 well plates (Applied biosystems) on an ABI 5700 sequence detection system (Applied biosystems). Reaction mixture was composed of 10 µl SYBR Green mix, 3 µl primers (2 pmol of each primer), 1.5 µl cDNA, and 5.5 µl H_2O. Reaction conditions were: 10 min 95°C, followed by 40 cycles of 15 sec at 95°C, and 1 min at 60°C. The following primer sets were used to detect EAAT2 (Genebank: AY066021): Forward 5'-CTG GGC ACC GCT TCC A-3', Reverse 5'-ACG AAT CTA GTC ACA GGC TTA TCA A-3'.

The data, fluorescence intensity and cycle thresholds, were acquired and processed automatically by Sequence Detection Software (Applied Biosystems). The amount of EAAT2 is calculated by a modified ΔΔCt method (Wu et al. 2003), by raising the primer efficiency of EAAT2 to the power of - cycle threshold. This is normalised and divided by the average of the two normalised housekeeping gene expressions, human elongation factor EF-1-α and E2 ubiquitin (Warrington et al. 2000). The efficiency of each primer pair used was determined with the LinREG program (Ramakers et al. 2003). The EAAT2 expression values of LVV-EAAT2 transduced human astrocytes were normalised to the expression values of naive cells, which was set to 1.

Western blot analysis

EAAT2 protein expression was determined by Western blot analysis (Wisman et al., 2003). Protein samples were prepared by homogenisation in 50 mM Tris-HCl lysis buffer containing 0.5% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, and 1 mM PMSF (pH=7.4). Protein content was determined according to Lowry using BSA as a standard (Lowry et al., 1951).

Protein samples and prestained molecular weight standards (Gibco, Invitrogen, Breda, the Netherlands) were separated on 10% SDS-polyacrylamide gels. Separated proteins were transferred to a polyvinylidene fluoride membrane (Roche, Basel, Switzerland) by electroblotting (100 V, 60 min) in Towbin buffer containing 12.4 mM Tris, 95.5 mM glycin, and 20% methanol.

After blocking non-specific binding by overnight incubation at 4ºC in PBS containing 0.1% Tween (PBS-Tw) and 3% BSA, blots were incubated with the EAAT2 reactive guinea-pig-anti-glutamate transporter GLT-1 antibody (1:2,500; Chemicon, Temecula, CA, USA) in PBS-Tw for 3.5 hours at room temperature. Next, membranes were washed with PBS-Tw and incubated with biotinylated goat-anti-guinea-pig antibody (1:500; Vector Laboratories, Burlingame, CA, USA) in PBS-Tw for 1 hour. After washing the membranes were incubated with avidin-biotin-conjugated peroxidase reagent (ABC-HRP; 1:100, Vector Laboratories, Burlingame, CA, USA) diluted in PBS-Tw for 1 hour. Finally, membranes were washed and immunoreactive proteins were visualised using ECL (Roche, Basel, Switzerland). To visualise β-actin as a loading control on the same blot, the so-called Rainbow Western method was used (Krajewski et al., 1996). Therefore, blots were first incubated with 3,3'-diaminobenzidine/0.01% H_2O_2 to block and visualise EAAT2-immunoreactivity. Next, the blots were stained as described above with mouse anti-β-actin (1:40,000; Sigma, Zwijndrecht, The Netherlands) as primary antibody, and biotinylated horse-anti-mouse (1:500; Vector Laboratories, Burlingame, CA, USA) as secondary antibody, respectively. After visualisation of β-actin immunoreactivity by ECL, β-actin bands were coloured also by incubation with 3-amino-9-ethylcarbazole/0.01% H_2O_2.

Glutamate uptake assay

The activity of transduced EAAT2 was estimated with a glutamate uptake assay (Debler and Lajtha, 1987; Wisman et al., 2003). Briefly, medium on LVV-transduced cells was replaced by transport buffer (10 mM HEPES, 5 mM Tris, 10 mM D(+)glucose, 3.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 1.2 mM K_2HPO_4; pH=7.4) containing either 140 mM NaCl or 140 mM cholineCl. The cells were kept in this buffer for 10 min at 37ºC before glutamate uptake was started by the addition of 0.5-200 μM glutamate containing 9.52 pM [^{3}H]-glutamate (specific activity 42 Ci/mmol; Valeant Pharmaceuticals International, Costa Mesa, CA, USA). After incubation for 20 min at 37ºC, glutamate uptake was stopped by adding an equal volume of ice-cold transport buffer containing 1 mM glutamate. Cells were washed five times with this buffer before the cells were lysed with 0.3 M NaOH. Radioactivity was quantified by liquid scintillation counting. Na^+-dependent uptake, expressed as the amount of glutamate taken up per mg protein/min, was assessed by subtracting the choline values from the sodium values and by correcting for protein content. When indicated, the EAAT2 selective inhibitor dihydrokainic acid (DHK) (K_i value 15-60 μM for EAAT2), was added 10 min before and during the incubation with [^{3}H]-glutamate.

Statistical evaluation

All data are given as means ± SD. Statistical analysis was performed using the SPSS 9.0 for Windows software. After testing for homogeneity of variance and for normality of residuals, an analysis of variance (ANOVA) was performed for multiple comparison between groups followed by Bonferoni's post hoc test to compare group means. *p* values <0.05 were considered significant.

Results

In the present study, a series of primary adult human astrocyte cultures derived from cortical- or spinal cord-autopsy material was used to investigate the use of LVV as a tool to deliver EAAT2. All cultures were >98% positive for the astrocytic marker GFAP. Details of the different primary human astrocyte cultures are given in Table 1.

Table 1. ***Clinical autopsy and experimental data of the primary adult human astrocyte cultures used in this study.***

Astrocyte culture	Origin	Sex	Age	Clinical diagnosis	Glutamate uptake (μM glutamate/mg protein/min)		%
					LVV-GFP	LVV-EAAT2	
Case 1	CTX	M	73	VD	4.85±0.81	14.6±4.43*	302
Case 2	CTX	F	85	AD	5.11±0.51	11.3±0.51*	221
Case 3	CTX	F	62	AD	8.14±0.43	13.6±1.96*	167
Case 4	CTX	M	92	C	2.40±0.84	6.75±1.81*	281
Case 5	CTX	F	82	AD	4.70±0.85	8.80±0.80*	187
Case 6	CTX	F	58	AD	13.6±0.56	38.8±3.58*	286
Case 7	CTX	M	56	C	1.83±0.48	6.09±0.64*	333
Case 8	SC	M	61	MS	6.35±0.85	10.6±1.38*	150
Case 9	SC	F	85	C	1.99±0.76	25.4±3.52*	1277
Case 10	SC	F	86	AD	2.33±1.49	14.4±3.36*	620

*The glutamate uptake capacity of LVV-GFP- and LVV-EAAT2-treated astrocytes, expressed as μM glutamate/mg protein/min, and the increase in glutamate uptake in LVV-EAAT2 treated astrocytes as percentage of LVV-GFP treated astrocytes (%), are shown. Data shown are given as means ± SD (n=4 cultures per experimental group). * $p<0.05$ versus respective control, i.e. LVV-GFP treated cells. CTX: cortex, SC: spinal cord, AD: Alzheimer's disease, VD: vascular dementia, MS: multiple sclerosis, C: control.*

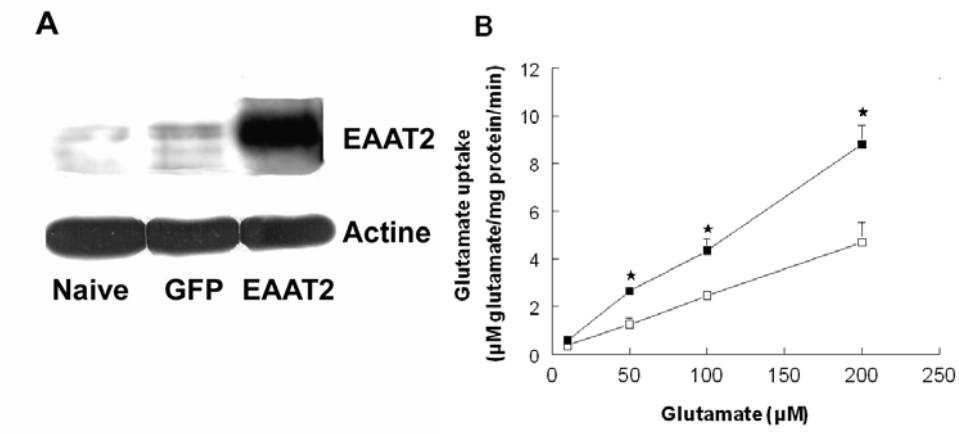

Figure 1. *Functional characterisation of LVV-EAAT2 in adult primary human astrocytes. Human astrocytes (case 5) were exposed to 1x106 TU LVV-GFP or LVV-EAAT2 for 24 hours before EAAT2 protein expression (panel A) and glutamate uptake capacity (panel B) were determined 48 hours thereafter.*
*LVV-GFP- (open squares) and LVV-EAAT2 treated astrocytes (filled squares) were exposed to increasing concentration of glutamate for 20 min at 37ºC. Sodium-dependent glutamate uptake is expressed as µM glutamate/mg protein/min. Data shown are means ± SD (n=4), * p<0.05 versus respective control, i.e. LVV-GFP treated cells.*

EAAT2 expression of LVV-treated human astrocytes

EAAT2 expression in human astrocyte cultures, either or not treated with $1x10^6$ TU LVV-EAAT2, was analysed with qPCR- and Western blot-analysis, respectively. As illustrated for cultures prepared from case 5, an increase in EAAT2 mRNA was observed in LVV-EAAT2 treated cells compared to naive human astrocytes. The increase amounted to 5700%. Accordingly, while endogenous EAAT2 protein could be demonstrated in LVV-GFP transduced- or naive cells, Western blot analysis revealed a marked increase in EAAT2 protein in LVV-EAAT2 treated human astrocytes (figure 1A).

Functional glutamate uptake in LVV-treated human astrocytes

Glutamate uptake assays were performed to determine whether LVV-EAAT2 mediated gene transfer resulted in functional EAAT2 expression. Cultured human astrocytes were exposed to different concentrations of glutamate, which resulted in a linear, dose-

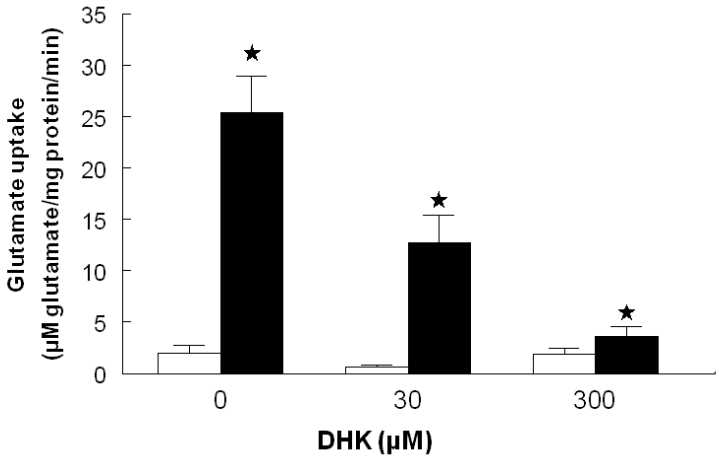

Figure 2. *Pharmacological characterisation of glutamate uptake in LVV-GFP- (open bars) and LVV-EAAT2-treated (black bars) human adult spinal cord astrocytes (case 9). Glutamate uptake is determined in the absence or presence of the selective EAAT2 inhibitor dihydrokainic acid and is expressed as µM glutamate/mg protein/min. Data shown are means ± SD (n=4), * p<0.05 versus the uptake of glutamate in vehicle treated LVV-EAAT2 treated astrocytes.*

dependent increase in glutamate uptake in LVV-GFP treated, and LVV-EAAT2 treated-human astrocytes (figure 1B). The glutamate uptake in human astrocytes appeared to be linear up to 30 minutes (data not shown). As anticipated, the glutamate uptake in LVV-EAAT2 treated human astrocytes was markedly increased as compared to naive- or LVV-GFP treated-cells. In subsequent experiments, aimed to analyse a series of different primary astrocyte cultures prepared from cortical and spinal cord tissue the sodium-dependent glutamate uptake is measured after incubation with a fixed concentration of 200 µM glutamate for 20 minutes. In this study, the uptake capacity of LVV-EAAT2 treated human astrocytes was compared to LVV-GFP treated control cells. As illustrated in table 1, in all cultures tested, LVV-EAAT2 transduction invariably led to a profound increase in the sodium-dependent glutamate uptake, increases ranging from approximately 150% to 1250% compared to LVV-GFP transduced control cells. While in three astrocyte cultures (case 1, 2, and 5), LVV-GFP transduction resulted in a small, decrease of approximately 20% in glutamate uptake compared to naive human astrocytes, in seven out of ten cultures no significant differences were observed between naive- and LVV-GFP treated-cultures.

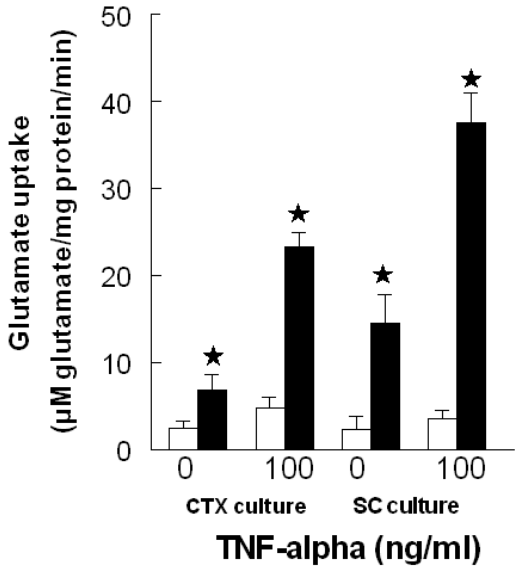

Figure 3. *Effect of TNF-α on glutamate uptake capacity of human adult astrocytes, treated either with LVV-GFP (open bars) or LVV-EAAT2 (black bars). Human astrocytes either prepared from cortical (case 4) or spinal cord (case 10) tissue were exposed to 100 ng/ml TNF-α 48 hours prior to the glutamate uptake assay. Glutamate uptake is expressed as µM glutamate/mg protein/min. Data shown are means ± SD (n=4), * p<0.05 versus respective controls, i.e. uptake of glutamate in LVV-GFP treated astrocytes.*

Pharmacological characterisation of glutamate uptake

To investigate whether the glutamate transporter subtype EAAT2 was indeed responsible for the increase in glutamate uptake capacity such as observed in the LVV-EAAT2 treated cells, the EAAT2-specific glutamate transporter inhibitor DHK was used for pharmacological characterisation. As shown in figure 2, while the LVV-EAAT2 mediated increase amounted to 1250%, the presence of DHK resulted in a dose-dependent decrease in glutamate uptake in LVV-EAAT2 treated human spinal cord astrocytes. In LVV-EAAT2 treated cells a 50% reduction in glutamate uptake was observed in the presence of 30 µM DHK. Similar pharmacological effects were found in LVV-EAAT2 treated cortical astrocyte cultures (data not shown).

Effect of TNF-α on the glutamate uptake capacity of human astrocytes

Finally, the effect of TNF-α treatment on glutamate uptake was investigated in adult primary human astrocytes derived from cortical or spinal cord tissue. As shown in figure 3, treatment with TNF-α resulted in an increased sodium-dependent glutamate uptake in

naive-, LVV-GFP treated and LVV-EAAT2 treated-human astrocytes. While in the LVV-EAAT2 treated cortical culture a twofold induction in glutamate uptake was observed, upon treatment with 100 ng/ml TNF-α for 48 hours the uptake was dramatically enhanced and amounted to 970% as compared to vehicle treated LVV-GFP cultures. In spinal cord astrocyte cultures LVV-EAAT2 transduction resulted in an increase of 620%, which was further enhanced to 1610% by TNF-α treatment.

Discussion

In this study we investigated whether LVVs can be considered as a tool for functional EAAT2 gene delivery to astrocytes, e.g. in the surrounding of affected spinal and/or cortical motoneurons in ALS. In recent years, major advances have been made to optimise the cloning capacity and biosafety of LVVs. When pseudotyped with a vesicular stomatitis virus G-protein (VSV-G) envelope, LVVs are able to transduce a broad range of cells, including astrocytes (Buchschacher, Jr. and Wong-Staal, 2000; Vigna and Naldini, 2000; Rosenblad et al., 2000). As shown in chapter 4, LVV pseudotyped with a VSV-G envelope showed a preferential astrocytic expression both in organotypic spinal cord cultures and mouse spinal cord. However, the ability of LVVs to transduce human adult astrocytes has never been investigated. Primary human astrocytic cultures were used to investigate whether LVVs can be employed to increase functional EAAT2 protein expression and, hence, to boost the glutamate uptake capacity of these cells. Treatment with LVV-EAAT2 resulted in a profound increase in glutamate uptake in both cortical and spinal cord adult primary human astrocytes. This corresponded with an increase in EAAT2 mRNA and protein levels in LVV-EAAT2-treated astrocytes as demonstrated by qPCR and Western blot analysis, respectively. As shown by the glutamate transporter inhibitor DHK, the increase in glutamate uptake in LVV-EAAT2-treated astrocytes could be completely attributed to EAAT2. The IC_{50} value of DHK in LVV-EAAT2-treated cells amounted to approximately 30 μM, which is similar to that described for DHK in other systems (Arriza et al., 1994). On the basis of our in vitro study, it is concluded that LVV-EAAT2 is a suitable tool to overexpress EAAT2 in adult human astrocytes and, hence, can be considered as a experimental therapeutic option to combat neurodegenerative excitotoxic processes known to be operative in ALS (Heath and Shaw, 2002) and other

neurodegenerative diseases including Alzheimer's disease (Hynd et al., 2004), and Parkinson's disease (Sherer et al., 2001).

ALS is a multifactorial disease, in which besides excitotoxicity also other neurodegenerative processes play a role (McGeer and McGeer, 2002). In line with the prominent role of neuroinflammation in ALS, it is noteworthy that ALS pathogenesis is associated with the accumulation of microglial cells (Turner et al., 2004) and increased levels of the inflammatory cytokine TNF-α (Poloni et al., 2000). Recently, it has been reported that TNF-α can down-regulate EAAT2 protein expression via an interaction with the EAAT2 promoter region (Su et al., 2003), a phenomenon that may also explain the loss of EAAT2 protein such as observed in ALS patients and rodent models for ALS (Rothstein et al., 1995; Howland et al., 2002; Bendotti et al., 2001).

Expression of LVV-EAAT2 is driven by the CMV-promoter, which can be strongly activated by TNF-α, via the induction of the transcription factor nuclear factor-κB (NF-κB) (Prosch et al., 1995; Laegreid et al., 1995). Given the above association between ALS pathogenesis, TNF-α, and endogenous EAAT2 gene transcription, it was anticipated that, in contrast to endogenous EAAT2, the LVV-mediated EAAT2 production would not be inhibited by the high levels of TNF-α such known to be present in the ALS affected brain. We have tested the influence of TNF-α on (LVV-EAAT2-transduced) astrocytes, and found that TNF-α treatment indeed resulted in a profound increase in the glutamate uptake capacity of LVV-EAAT2-treated human astrocytes. This phenomenon was invariably observed both in cortical and spinal cord cultures. Although on the basis of the present study we can not exclude possible effects of TNF-α on glutamate metabolism, glutamate retention (Kazazoglou et al., 1996; Szymocha et al., 2000a; Szymocha et al., 2000b) or the post-transcriptional modification (Lin et al., 1998), increased turnover, or altered trafficking of EAAT2 transporters, it is tempting to conclude that the TNF-α induced increase in LVV-mediated EAAT2 uptake capacity is attributable to a TNF-α mediated induction of NF-κB.

Interestingly, and in contrast to our expectations which were mainly based on studies in astrocyte cultures prepared from human fetal brain tissue (Fine et al., 1996; Wang et al., 2003), we found that TNF-α treatment also slightly enhanced glutamate uptake in naive- and LVV-GFP treated-human astrocytes, indicating that in contrast to fetal human astrocytes the function of endogenous EAAT2 can be increased by TNF-α in adult human

astrocytes. As yet, the underlying biochemical or molecular processes responsible for the TNF-α-induced increase in glutamate uptake in naive- and LVV-EAAT2 treated-human astrocytes remain elusive.

In conclusion, LVV-EAAT2 has been shown to be an effective tool to overexpress functional EAAT2 in adult human cortical and spinal cord astrocytes. Moreover, the fact that TNF-α appears to boost the functional uptake capacity of LVV-EAAT2 treated astrocytes, underlines our thesis that in vivo gene therapy with LVV-EAAT2 warrants evaluation on its neuroprotective potential in ALS.

CHAPTER 6

SUMMARY AND DISCUSSION

Summary

In this thesis I have explored the possibilities to use gene therapy to deliver neuroprotective proteins to the CNS and, hence, to combat ALS.

Chapter 1 provides a background on ALS, in which I focussed on oxidative stress and excitotoxicity as important contributing factors in the pathogenesis of this disease. The anti-oxidant enzyme catalase and the glutamate transporter EAAT2 were introduced as potential neuroprotective proteins to tackle oxidative stress and excitotoxicity, respectively. In addition, an introduction is given on various strategies for gene therapy, notably ex vivo and in vivo gene therapy.

In **chapter 2** we aimed to protect motoneurons from oxidative stress by overexpressing the anti-oxidant enzyme catalase via ex vivo gene therapy. To this end, genetically engineered Rat-1 fibroblasts overexpressing catalase were generated. Although the engineered cells themselves were more resistant to oxidative stress, in coculture they were unable to rescue either primary motoneurons or NSC-34 cells from oxidative cell death induced by hydrogen peroxide. These data suggest that ex vivo gene therapy using catalase-overexpressing cells might not be sufficient to protect motoneurons from oxidative stress in vivo, probably due to the relative large distance between the site of radical formation and the genetically engineered cells.

In **chapter 3** we aimed to protect motoneurons from excitotoxicity by overexpressing the glial glutamate transporter EAAT2 via ex vivo gene therapy. Overexpression of EAAT2 in HEK cells resulted in a profuse increase in glutamate uptake compared to wild-type cells. Furthermore, the engineered cells protected motoneurons in a motoneuron-astrocyte coculture from glutamate-induced toxicity. Hence, it seemed worthwhile to investigate whether ex vivo gene therapy for EAAT2 might be of therapeutic value in amyotrophic lateral sclerosis.

In the second part of this thesis, the possibilities of in vivo gene therapy with lentiviral vectors were explored.

The aim of the study described in **chapter 4** was twofold. The first aim was to investigate the cellular pattern of LVV-mediated gene transduction in mouse spinal cord both in vitro and in vivo. Therefore, organotypic mouse spinal cord cultures were treated with LVV-GFP in vitro and wild-type mice were intraspinally injected with LVV-GFP at the level of vertebra L1 under stereotactic guidance. In both situations, GFP expression was found to be expressed predominantly by astrocytes, while leaving the neuronal cells virtually unaffected. The second aim was to examine the neuroprotective efficacy of intraspinally delivered LVV-EAAT2 on the course of the disease in G93A-hSOD1 ALS mice by using motor performance and body weight as clinical parameters. We found that EAAT2 was effectively transduced in the spinal cord, but that disease onset and survival in the treated mice did not significantly differ from either LVV-GFP-treated or naive control mice. We concluded that, although LVV-EAAT2 did not effect the clinical outcome of ALS mice, as such LVVs can be successfully used to deliver therapeutic genes into spinal astrocytes.

The aim of the study described in **chapter 5** was to investigate whether LVV-EAAT2 could be used as a tool to deliver EAAT2 to primary adult human astrocytes derived from either cortical or spinal post-mortem brain tissue. Concomitant with a marked increase in EAAT2 mRNA and EAAT2 protein in LVV-EAAT2-treated cells, a significant increase in functional glutamate uptake was observed in all astrocytic cultures tested as compared to LVV-GFP-treated controls. Given the recently identified relationship between TNF-α and EAAT2 transcription (Su et al., 2003) and taking into account the marked increase in TNF-α levels in ALS (Poloni et al., 2000; Hensley et al., 2002), the effect of TNF-α treatment on the glutamate uptake capacity was also investigated. It was found that functional expression of EAAT2 in LVV-EAAT2 treated cells was even further enhanced in the presence of TNF-α. We conclude that LVV-EAAT2 is an effective means to deliver EAAT2 to primary human astrocytes and that the functional expression of LVV-EAAT2 is enhanced by TNF-α, a cytokine known to be present at high levels in ALS tissue.

Discussion

In this thesis I explored the use of gene therapy to deliver therapeutic proteins to the CNS, in order to be able to alleviate the damage induced by ALS. Because gene therapy provides localised, sustained delivery of proteins to the CNS, it circumvents problems associated with systemic delivery of therapeutic agents, such as the inability of some proteins to cross the BBB, the instability and degradation of proteins, and their systemic side effects (Aebischer and Ridet, 2001). In the introduction two forms of gene therapy were introduced, namely ex vivo gene therapy with the use of macrocapsules containing the manipulated cells, and in vivo gene therapy using lentiviral vectors (LVV). Both forms of gene therapy can result in a long-term expression of the transgene (Aebischer et al., 1996; Zurn et al., 2000; Kordower et al., 2000; Naldini, 1998). However, the delivery of the therapeutic protein to the CNS differs between both approaches.

Ex vivo gene therapy

Ex vivo gene therapy is especially tailored for the delivery of secreted proteins (Aebischer and Ridet, 2001), since the genetically engineered cells overexpressing the therapeutic protein of interest are delivered to the CNS inside macrocapsules that are placed intrathecal. In this thesis we wanted to deliver the non-secreted proteins catalase and EAAT2, and investigated whether cells overexpressing these proteins might work as a "sink" for hydrogen peroxide and glutamate, thus diminishing oxidative stress and excitotoxicity, respectively.

We found that catalase-overexpressing cells were not able to protect motoneurons from hydrogen peroxide. Previously it was demonstrated that overexpression of catalase within the motoneuron itself could protect the motoneuron from oxidative stress (Herpers et al., 1999). The relatively large diffusion distance between the genetically engineered cells and the locus where radicals are formed, i.e. in the extracellular milieu or within the motoneuron itself, together with the high intrinsic reactivity of H_2O_2, may explain the inability of the cells overexpressing catalase to confer neuroprotection. It is concluded that, in order to effectively protect motoneurons from oxidative stress, catalase needs to be expressed directly in the motoneuron itself and should therefore be delivered to the CNS in a different manner than via ex vivo gene therapy.

As shown in chapter 3, cells overexpressing the glutamate transporter EAAT2 did protect motoneurons in a motoneuron/astrocyte coculture from glutamate-induced death. However, in this co-culture model the distance between the motoneuron and the EAAT2-overexpressing cells was relatively short and separated only by a few millimetres of culture medium, while in ALS patients this distance is expected to be much larger. In addition, to be effectively cleared, glutamate, present in the direct vicinity of motoneurons has to diffuse through the spinal cord parenchyma before reaching the CSF where the capsule with the genetically engineered cells is localised. Hence, it remains to be seen whether EAAT2 overexpressing cells will have the same neuroprotective effect in vivo. Unfortunately, however, we were unable to test the neuroprotective efficacy of the engineered EAAT2 overexpressing cells in an ALS mouse model due to the fact that the currently available capsules are too large to be implanted into the mouse spinal cord. Since the clinical safety of ex-vivo gene therapy using encapsulated cells has previously been demonstrated in ALS patients (Aebischer et al., 1996), it seems worthwhile to investigate whether or not ex vivo gene therapy for EAAT2 might be an efficacious and feasible approach to combat degeneration of motoneurons in amyotrophic lateral sclerosis patients.

In vivo gene therapy

In contrast to ex vivo gene therapy, by using in vivo gene therapy with viral vectors it is possible to deliver neuroprotective genes to specific areas in the brain. This means that in the case of ALS therapeutic genes can be targeted to motoneurons themselves or to their surrounding astrocytes (Davidson and Breakefield, 2003). Taking into account that EAAT2 is the dominant glial glutamate transporter, it is anticipated that EAAT2 should be expressed predominantly by astrocytes in order to be able to function properly. As discussed in chapter 4, it appears that LVV-mediated gene transduction is mainly directed to astrocytes as shown by transduction of mouse organotypic spinal cord cultures and by direct intraspinal injections in mice. In addition, LVV encoding EAAT2 was able to deliver functional EAAT2 to primary adult human astrocytes (chapter 5).

Direct delivery of LVV-EAAT2 into spinal cord did not lead to a delay in disease onset or survival in the G93A-hSOD1 mouse model. There are several factors that may explain the lack of clinical efficacy of LVV-EAAT2 in our mouse model. The two most important

factors, in my opinion, will be discussed shortly. First, local delivery of LVV by injecting the mouse spinal cord at one section might be inadequate. Considering that ALS is a widespread disease characterised by a degeneration of motoneurons in the motor cortex, brainstem, and spinal cord. Consequently, transduction of EAAT2 into a selective subpopulation of astrocytes located in the vicinity of spinal motoneurons only, such as accomplished in the present study, might not be sufficient to successfully delay disease onset or survival. Second, since excitotoxicity and oxidative stress are two highly interrelated pathogenic mechanisms in ALS, and given the vulnerability of EAAT2 for oxidative damage (Trotti et al., 1996; Rao and Weiss, 2004), it can not be excluded that the newly delivered EAAT2 is damaged by oxidants. This means that, in order for EAAT2 to effectively exert its anti-glutamatergic action, concomitant anti-oxidant treatment to protect the glutamate transporter from oxidants is necessary.

Implications for ALS therapy

Lentiviral vectors have improved considerably over the last years (Buchschacher, Jr. and Wong-Staal, 2000). The first clinical trials using LVV to transduce autologous CD4+ T-cells to treat HIV infection are currently under way (MacGregor, 2001). For use as tool to deliver therapeutic proteins to the CNS, several factors of LVV can be further optimised. These include the specificity of LVV for a specific cell population, regulation of the delivered transgene, and the necessity to reach large areas.

By manipulating the envelope protein and promoter used it is possible to direct the transgene expression to a specific cell population. From our studies it appears that LVV is a suitable tool to transduce astrocytes when it is directly injected into the mouse spinal cord. The LVV used in these studies are pseudotyped with a VSV-G envelope and driven by a CMV promoter. This combination has shown previously to transduce mainly neurons when tested in other brain regions (Blomer et al., 1997; Kordower et al., 1999). For example, using the Ross River Virus glycoprotein instead of VSV-G as an envelope, changes the tropism of LVV towards a glial preference in the striatum (Kang et al., 2002). Similarly, changing the promoter from CMV- to a GFAP-promoter of LVVs pseudotyped with a VSV-G envelope directs transgene expression towards a preferential glial expression instead of a neuronal expression in the striatum (Jakobsson et al., 2003). Recently, targeting motoneurons to deliver genes of interest has been shown to be

relatively simple, as both AAV (Kaspar et al., 2003) and LVV (Azzouz et al., 2004) can be retrogradely transported from the neuromuscular synapse to the cell body, where it is incorporated into the genome. In this way, motoneurons that innervate muscles such as the gastrocnemius, diaphragm, intercostal, facial, and tongue muscles, are accessible by an intramuscular injection into the respective muscles, followed by axonal retrograde transport. This approach is well suited for a protein such as catalase, since catalase most likely needs to be expressed within the motoneuron itself to properly protect the motoneuron from oxidative stress (chapter 2).

Since ALS is a disease that ultimately affects lower- and upper-motoneurons, it is expected that for in vivo gene therapy to be efficacious, LVV-mediated gene transduction will be required in a large area, preferentially the whole spinal cord and motor cortex. By using a retrograde delivery technique as described above it is possible to reach many motoneurons by injecting multiple muscles. Using direct injections of LVV, to deliver for example EAAT2 to astrocytes, has as disadvantage that it restricts the number of transduced cells, as the volume that can be injected is limited to a maximum of 1-3 µl for rodents, and the diffusion of LVV in tissue is limited to a few millimetres. In case one wants to deliver viral vectors to a large area in the brain a systemic route can be considered. Injecting viral vectors into the muscles can lead to transduction of motoneurons via retrograde axonal transport of these viral vectors, as described above. However, this approach is only suitable for the delivery of neuronal proteins. For astrocytic proteins such as EAAT2 other modes of delivery have to be considered to reach global transduction. To date, injecting viral vectors into the ventricles in order to deliver LVV globally via the CSF seems to be the most promising option. However, so far, this has resulted only in a periventricular delivery (Ghodsi et al., 1999), so that at this moment multiple injections are still necessary.

Another important aspect to take into consideration for gene therapeutic applications is the ability to regulate transgene expression, as sustained gene expression can lead to unwanted side-effects. Several systems have been generated to control expression of a transgene by an exogenous drug, both at the transcriptional (Gossen et al., 1995; Wang et al., 1994; No et al., 1996; Rivera et al., 1996; Pollock et al., 2000) and at the translational (Werstuck and Green, 1998) level. Among them, the tetracycline-dependent system has been best characterised. LVV with an incorporated tetracycline system showed an

efficient regulation of transgene expression both in vitro and in vivo upon treatment with doxycycline (Kafri et al., 2000; Vigna et al., 2002).

The question remains whether the anti-oxidant enzyme catalase and the glutamate transporter EAAT2 are able to halt neurodegenerative processes in ALS even when optimally delivered to the CNS. Previous studies in ALS mouse models showed that both catalase and EAAT2 are able to delay onset (Guo et al., 2003; Poduslo et al., 2000). However, as described in the general introduction, multiple pathways lead to motoneuron death (Bruijn et al., 2004). The most promising therapeutic approach is probably by using a combination of proteins that are targeted at all or most pathophysiological components: oxidative stress, excitotoxicity and neuroinflammation.

Concluding remarks

Although both catalase and EAAT2 have great neuroprotective potential, their efficacy greatly depends on the mode of delivery to the CNS. The recent advances in the development of viral vectors shows great promise for viral vectors in therapeutic applications. However, it is important that for each specific application the best viral vector is chosen. LVV seems a suitable option to deliver EAAT2 to astrocytes, since direct injections of LVV into the spinal cord of mice mainly transduced astrocytes. Using viral vectors that can be retrogradely transported from neuromuscular synapse to the cell body of motoneurons, e.g. LVV pseudotyped with the rabies virus glycoprotein, seems to be an interesting option as means to deliver catalase to affected motoneurons. The effect of combined gene therapy with catalase and EAAT2 should be the focus of further research.

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Samenvatting

Amyotrofische laterale sclerose (ALS) is een zeer ernstige ziekte waarbij motorische neuronen afsterven. Het verlies van motorische neuronen leidt tot een toenemende zwakte van spieren in het aangezicht, de armen, de benen, de buik en de rug. Daarnaast treedt er ook zwakte op van de spieren die betrokken zijn bij het spreken, slikken en ademhalen. Na het stellen van de diagnose is de gemiddelde levensduur gemiddeld 2-5 jaar. De belangrijkste doodsoorzaak is respiratoire insufficiëntie. Tot op heden is er nog maar één medicijn voor ALS geregistreerd, Riluzole, dat de levensduur van ALS patiënten met gemiddeld drie maanden verlengt. Hoewel het onbekend is hoe de ziekte exact ontstaat, wordt gedacht dat glutamaat excitotoxiciteit en oxidatieve stress een grote rol spelen bij het afsterven van de motorische neuronen. Glutamaat is een van de belangrijkste neurotransmitters in de hersenen. Verhoogde concentraties van glutamaat kunnen schadelijk zijn voor de (motorische) neuronen, een proces dat bekend staat als glutamaat excitotoxiciteit.

Oxidanten zijn zeer reactieve moleculen die kunnen ontstaan tijdens tal van fysiologische en pathofysiologische processen in de cel. Indien er sprake is van een wanverhouding tussen de geproduceerde oxidanten en het verdedigingsmechanisme daartegen in een cel, kan er een teveel aan oxidanten ontstaan, en spreekt men van een situatie van oxidatieve stress. Een overmaat aan oxidanten leidt tot oxidatieve schade aan belangrijke onderdelen van een cel zoals eiwitten, DNA en membranen, en resulteert uiteindelijk in celdood.

Oxidatieve stress en glutamaat excitotoxiciteit zijn twee sterk gerelateerde processen in ALS. Uit de literatuur is het bekend dat glutamaat excitotoxiciteit leidt tot verhoogde mate van oxidatieve stress. Bovendien kan oxidatieve stress de glutamaat opnamecapaciteit van astrocyten verkleinen door de “excitatory amino acid transporter”-2 (EAAT2) te beschadigen (zie onder). Hierdoor ontstaat er een vicieuze cirkel, waarbij de schadelijke processen oxidatieve stress en glutamaat excitotoxiciteit elkaar meer en meer verergeren.

Het doel van deze studie is om de motorische neuronen te beschermen tegen deze schadelijke processen. Hiertoe worden het anti-oxidant eiwit catalase of het membraan gebonden EAAT2 verhoogd tot expressie gebracht met behulp van gentherapie. Catalase is een sleutelenzym in het beschermingsmechanisme van de cel tegen oxidatieve stress.

Het zet waterstofperoxide, een sterke oxidant, om in water. Door catalase tot overexpressie te brengen hopen wij de oxidatieve stress gerelateerde schade aan motorische neuronen te verhinderen. EAAT2 is het belangrijkste membraaneiwit in de hersenen voor de opname van glutamaat, en wordt voornamelijk gevonden in astrocyten. Deze astrocyten, die rondom de (motorische) neuronen liggen, zorgen mede voor het in stand houden van het extracellulaire milieu. Het membraan gebonden eiwit EAAT2, dat zorgt voor de opname van glutamaat in de astrocyt, speelt een belangrijke rol bij de regulatie van de extracellulaire glutamaat concentratie en voorkomt dat concentraties bereikt worden die schadelijk zijn voor de motorische neuronen. In ALS patiënten blijkt dat het EAAT2-eiwit verlaagd is. Door EAAT2 verhoogd tot expressie te brengen hopen wij de glutamaat excitotoxiciteit te verhinderen.

Zoals eerder genoemd zal het verhoogd tot expressie brengen van deze twee eiwitten met behulp van gentherapie worden bewerkstelligd. In grote lijnen bestaan er twee strategieën om gentherapie uit te voeren. Ten eerste kan gebruikt worden gemaakt van cellen die buiten het lichaam genetisch gemanipuleerd worden om op die manier een beschermende factor verhoogd tot expressie te brengen. Deze gemanipuleerde cellen kunnen vervolgens in het lichaam geplaatst worden, al dan niet verpakt in een beschermende capsule. Deze benadering wordt ex vivo gentherapie genoemd. Ten tweede is het mogelijk om gebruik te maken van in vivo gentherapie. Hiertoe wordt gebruikt gemaakt van een virus dat het gen van interesse direct in de cellen van het aangedane weefsel kan brengen, in ons geval het ruggenmerg. Virussen zijn van nature gespecialiseerd in het infecteren van een cel om daarna hun eigen genen in de celkern ‘aan het werk te zetten’. Virussen die worden gebruikt bij gentherapie zijn zó veranderd dat ze geen ziekte kunnen veroorzaken en zich niet meer kunnen vermenigvuldigen, maar nog wel de cellen kunnen bereiken die genetisch gemanipuleerd moeten worden. Om EAAT2 tot overexpressie te brengen maken we in dit onderzoek gebruik van vectoren afkomstig van het lentivirus.

Ex vivo gentherapie

In het eerste deel van het onderzoek hebben we de mogelijkheden van ex vivo gentherapie in het kader van ALS nader onderzocht. Om de motorische neuronen te beschermen tegen oxidatieve stress is een cellijn gecreëerd die catalase tot overexpressie brengt. Daarvoor zijn Rat-1 fibroblasten getransfecteerd met een plasmide met het gen coderend voor

catalase. De getransfecteerde cellijn die op deze manier ontstond, is gecontroleerd op de aanwezigheid van catalase door middel van immunohistochemie. Gevonden werd dat de getransfecteerde cellen meer catalase bevatte, wat voornamelijk werd gevonden in de peroxisomen. Daarna is onderzocht of het catalase-eiwit in de catalase-cellijn ook functioneel actief was. Dit werd getest door de getransfecteerde cellen bloot te stellen aan waterstofperoxide, het reactieve substraat voor catalase. Het bleek uit onze experimenten dat de catalase-cellijn zichzelf beter kon beschermen tegen waterstofperoxide schade dan ongetransfecteerde Rat-1 fibroblasten. Daarna hebben we gekeken of deze catalase-cellen in staat waren om motorische neuronen te beschermen tegen waterstofperoxide geïnduceerde celdood. Hiervoor werden motorische neuronen samen in kweek gebracht met de catalase-cellen en vervolgens blootgesteld aan waterstofperoxide. In dit co-culture model bleek dat de motorische neuronen niet werden beschermd tegen waterstofperoxide door de aanwezigheid van de catalase-cellen.
Deze resultaten suggereren dat ex vivo gentherapie met behulp van cellen die catalase verhoogd tot expressie brengen waarschijnlijk niet voldoende effectief is om de motorische neuronen tegen oxidatieve stress te beschermen. Dit kan verklaard worden door de relatief grote afstand tussen de plaats waar oxidanten gevormd worden, i.c. in het motorische neuron zelf, en de plaats waar de overmaat van catalase zich bevindt, dus in de genetische gemanipuleerde cellen die relatief ver van het motorische neuron gelegen zijn.

Om motorische neuronen tegen glutamaat excitotoxiciteit te beschermen werd een cellijn gecreëerd die de glutamaat transporter EAAT2 tot overexpressie brengt. Daarvoor zijn humane embryonale nier (HEK) cellen getransfecteerd met een plasmide coderend voor EAAT2. De cellijnen die op deze wijze ontstonden zijn gecontroleerd op de aanwezigheid van EAAT2-eiwit door middel van Western blotting. Met de cellijnen die een duidelijke verhoging van EAAT2-eiwit lieten zien werd verder onderzoek verricht. Eerst hebben we met behulp van een glutamaatopname test gecontroleerd of het tot expressie gebrachte EAAT2 ook functioneel was. De verschillende EAAT2-cellijnen lieten allemaal een verhoogde glutamaat opnamecapaciteit zien. De cellijn die de grootste glutamaat opnamecapaciteit liet zien, hebben we vervolgens gebruikt om te onderzoeken of EAAT2-cellen in staat zijn om motorische neuronen te beschermen tegen glutamaat. Hiervoor werden motorische neuronen samen in kweek gebracht met HEK cellen die EAAT2 tot

overexpressie brachten en behandeld met glutamaat. De EAAT2-cellen waren in staat de motorische neuronen te beschermen tegen glutamaat excitotoxiciteit. De bovenstaande bevinding was voor ons mede de aanleiding om verder onderzoek te verrichten naar de therapeutische waarde van het verhogen van de glutamaat opnamecapaciteit in een diermodel voor ALS.

In vivo gentherapie

In het tweede gedeelte van het onderzoek hebben we de mogelijkheden van in vivo gentherapie voor EAAT2 nader onderzocht met behulp van lentivirale vectoren. Om te bepalen welk celtype voornamelijk geïnfecteerd wordt door lentivirale vectoren, hebben we eerst gebruikt gemaakt van een organotypisch kweekmodel van ruggenmerg afkomstig van muizen. Deze ruggenmerg kweken zijn geïnfecteerd met lentivirale vectoren coderend voor een groen fluorescerend eiwit (GFP) en na 10 dagen werd er door middel van immunohistochemie met markers voor verschillende celtypen gecontroleerd welke cellen getransduceerd waren. De cellen die GFP tot expressie brengen, bleken voornamelijk astrocyten te zijn. Dit zijn de cellen rondom de motorische neuronen waarin EAAT2 normaal ook tot expressie komt. Om dit ook in het levende dier te onderzoeken, zijn gewone muizen intraspinaal geïnjecteerd met lentivirale vectoren coderend voor GFP. Hiertoe wordt gebruikt gemaakt van een stereotactische operatie op het niveau van ruggenmergsegment L1. Ook nu werd gevonden dat het transgen GFP voornamelijk tot expressie komt in astrocyten.

Vervolgens hebben we onderzocht of lentivirale vectoren coderend voor de glutamaat transporter EAAT2 (LVV-EAAT2) in staat zijn om EAAT2 verhoogd tot expressie te brengen in humane astrocyten. Voor dit doel is gebruikt gemaakt van astrocyten verkregen uit post-mortem weefsel van cortex- en ruggenmergweefsel van mensen. Na behandeling met LVV-EAAT2 werd een verhoging gevonden in zowel mRNA- als eiwitgehalte van EAAT2. Bovendien werd gevonden dat de functionele glutamaat opnamecapaciteit in alle geteste cultures verhoogd was na behandeling met LVV-EAAT2. We kunnen dan ook concluderen dat LVV-EAAT2 een effectief middel is om EAAT2 verhoogd tot expressie te brengen in humane astrocyten afkomstig van cortex en ruggenmerg.

Ten slotte hebben we onderzocht wat het effect is van tumor necrosis factor-α (TNF-α) op de glutamaat opnamecapaciteit van LVV-EAAT2 behandelde humane astrocyten. TNF-α is een cytokine dat verhoogd aanwezig is in ALS weefsel en dat volgens de literatuur een direct remmend effect kan hebben op de EAAT2 promotor. In onze studie werd echter gevonden dat de opnamecapaciteit van astrocyten behandeld met LVV-EAAT2 juist sterk verhoogd was in de aanwezigheid van TNF-α. Een mogelijke verklaring is het feit dat de expressie van LVV-EAAT2 wordt aangestuurd door een CMV-promotor. Deze promotor kan sterk geactiveerd worden door TNF-α via de inductie van transcriptie factor nucleair factor-κB (NF-κB). Het lijkt waarschijnlijk dat, bij een eventuele toepassing van LVV-EAAT2 als gentherapie bij ALS patiënten, het effect van extra glutamaat opnamecapaciteit mogelijk nog wordt versterkt door de lokale aanwezigheid van hoge TNF-α spiegels.

In het laatste gedeelte van dit onderzoek hebben we de beschermende werking van extra EAAT2 in transgene muizen die een op ALS gelijkend ziektebeeld ontwikkelen, onderzocht. Om een behandelingseffect van intraspinale injecties van LVV-EAAT2 vast te stellen, hebben we de aanvang van de ziekte vastgesteld en het ziekteverloop gevolgd door middel van motorische gedragstesten. Ook is de overleving bepaald door gebruik te maken van een artificieel eindpunt. Om ethische redenen is gekozen voor een maximum verlies (>20%) van lichaamsgewicht. Ondanks het feit dat de transductie van EAAT2 succesvol was, werden er geen significante verschillen waargenomen in ziekteaanvang, beloop en overleving tussen de muizen die waren behandeld met LVV-EAAT2 en de verschillende controle groepen. Een verklaring hiervoor kan zijn dat het tot verhoogd expressie brengen van EAAT2 op het niveau van ruggenmergsegment L1 alleen niet voldoende is om de ziekte te beïnvloeden. Mogelijk is het zo dat EAAT2 in de gehele hersenen en ruggenmerg verhoogd tot expressie moet komen om beschermend te kunnen werken. Ondanks het feit dat een lokale injectie van LVV-EAAT2 geen effect heeft op het verloop van de ziekte in dit muismodel, kunnen we uit deze studie wel concluderen dat de lentivirale vectoren beschouwd kunnen worden als succesvol middel om beschermende genen verhoogd tot expressie te brengen in astrocyten gelegen in het ruggenmerg.

Algemene conclusie

Over het geheel genomen kunnen we concluderen dat de wijze waarop en de plaats alwaar beschermende factoren tot expressie worden gebracht in de hersenen van essentieel belang is voor deze factoren om hun beschermende werking optimaal uit te kunnen voeren. Zo lijkt het van groot belang dat catalase in de motorische neuronen zelf tot expressie gebracht wordt, wil het deze cellen optimaal kunnen beschermen tegen oxidatieve stress. Door recente ontwikkelingen in het optimaliseren van virussen voor gentherapie is het transduceren van motorische neuronen inmiddels een reële mogelijkheid geworden. Er zijn namelijk virussen ontwikkeld, die na inspuiten in een spier, opgenomen kunnen worden door het uiteinde van een axon om daarna retrograad getransporteerd te worden naar het cellichaam van een motorisch neuron. Hierdoor bestaat de mogelijkheid om specifiek de motorische neuronen te transduceren met bv. catalase. Om glia specifieke eiwitten, zoals EAAT2, verhoogd tot expressie te brengen, zijn lentivirale vectoren een uitstekend middel aangezien de lentivirale vectoren voornamelijk astrocyten transduceren.
Zoals eerder beschreven is, zijn oxidatieve stress en glutamaat excitotoxiciteit twee sterk gerelateerde schadelijke processen. Immers oxidatieve stress zorgt voor een verlaging van de glutamaat opnamecapaciteit door EAAT2 te beschadigen. Dit kan leiden tot verhoogde glutamaat spiegels, wat weer kan leiden tot glutamaat excitotoxiciteit met als gevolg verhoogde oxidatieve stress. Voor toekomstig onderzoek is het dan ook interessant te bekijken wat het effect is van gecombineerde gentherapie waarbij zowel catalase en EAAT2 verhoogd tot expressie worden gebracht, zodat oxidatieve stress en glutamaat excitotoxiciteit gelijktijdig aangepakt kunnen worden.

Dankwoord

Na vier jaar onderzoek is mijn proefschrift af. Hieraan hebben veel mensen op hun eigen manier aan bijgedragen, vakinhoudelijk of juist helemaal niet. Graag wil ik iedereen daarvoor bedanken. Een aantal mensen wil ik daarvoor speciaal mijn dank betuigen.

Tijdens mijn hoofdstage werd ik door Loes Schrama gevraagd voor een AIO-project bij de vakgroep Experimentele Neurologie. Hierover heb ik niet lang hoeven na te denken. Het enthousiasme van Loes was altijd zeer inspirerend. Het is daarom moeilijk te bevatten dat zij er niet meer is. Toch zou ik haar graag willen bedanken voor de tijd dat ze mij heeft begeleid.

Mijn promotor Dop Bär. Beste Dop, je was de afgelopen vier jaar de constante factor in mijn onderzoek. Jouw input op bepaalde momenten is essentieel geweest voor mij om door te gaan met het onderzoek. Graag wil ik je dan ook bedanken voor alle hulp, adviezen en enthousiasme.

Mijn co-promotoren Freek van Muiswinkel en Elly Hol. Beste Freek, door jou enthousiasme en vele suggesties voor mijn onderzoek vond ik het altijd prettig samenwerken. Het snelle en vooral grondige nakijken van mijn manuscripten was vooral op het einde van mijn onderzoek zeer belangrijk. Je verhalen en humor zorgden altijd voor een goede sfeer op het lab. Beste Elly, jouw hulp en suggesties waren zeer belangrijk voor de moleculair biologische kant van mijn werk. Doordat je altijd zeer snel reageerde op al mijn mail was de afstand tussen Utrecht en Amsterdam voor mij geen probleem. En als het nodig was, kon ik ook zonder enige moeite terecht op je lab.

Ook wil ik Bert Joosten bedanken voor de begeleiding tijdens het eerste jaar.

Henk Veldman en Peter Sodaar ben ik dankbaar voor de hulp bij veel experimenten. Henk, zonder jouw hulp bij de intraspinale injecties van de muizen waren de operaties niet zo soepel verlopen. Verder kon ik jou altijd alles vragen op het lab en wist je op alles een antwoord. Peter, jouw hulp bij het kweken van de motorneuronen en de

ruggenmergplakken was zeer belangrijk. Daarnaast heb je veel studenten die ik begeleidde wegwijs gemaakt op het lab. Marjolein Vliem wil ik bedanken voor de gezelligheid op het lab.

Tijdens de afgelopen vier jaar heb ik met veel plezier vele studenten mogen begeleiden. Bij deze wil ik ze allemaal bedanken voor hun inzet. Eva de Jong, Rogier van Gent, Sandra Coenen, Marie-Jose Claessen, Michael van Es, Wouter van Wingerden, Danielle Counotte en Esther Slootjes bedankt voor alle hulp!

Alle medebewoners van de AIO-kamer wil ik bedanken voor de afgelopen periode. Tijdens de beginperiode van mijn AIO-periode was er het altijd gezellige AIO-overleg. Sipke, Mariëtte, Evert, Diane en Ildiko, bedankt voor alle gezellige etentjes. Later zorgden Paul van Vught, Hanneke Bunte, Sanne Piepers, Geert-Jan Groeneveld en Jan Veldink voor de nodige afleiding op de AIO-kamer. Als experimenten of het schrijven niet wilden lukken, kon ik altijd even klagen. Paul, Sanne en Hanneke wil ik veel succes wensen met het afronden van jullie eigen promotieonderzoek. Ook wil ik alle AIO's, en in het bijzonder Lars van der Heide, van de vakgroep farmacologie en anatomie bedanken voor alle gezelligheid tijdens summerschools en in New Orleans.

Pierre de Graan wil ik bedanken voor de hulp bij de glutamaat opname testen. Ook Henk Spierenburg wil ik bedanken voor alle hulp op het RA-lab. Bij Ineke Bos kon ik altijd terecht met vragen over glutamaat transporters, bedankt.

Voordat ik alle GGO-vergunningen had aangevraagd om in Utrecht met de lentivirale vectoren te mogen werken, kon ik op het NIH in Amsterdam alvast beginnen. Hierdoor was een vliegende start van het tweede gedeelte van mijn project mogelijk. Ik wil daarom ook graag Joost Verhaagen bedanken voor deze mogelijkheid. Daarnaast wil ik iedereen van het lab van Elly Hol en Joost Verhaagen bedanken voor de hulp en gezelligheid tijdens mijn periode in Amsterdam. In het bijzonder wil ik Jacqueline Sluijs bedanken voor het wegwijs maken van vele nieuwe technieken.

I am very grateful to professor Aebischer to give me the change to learn the surgical procedure for the intraspinal injections in mice at his laboratory. I would also like to thank Sandrine Guillot for her effort and time to demonstrate the surgical procedure.

Jan Brakkee wil ik bedanken voor alle hulp bij het opzetten van de intraspinale injecties in het ruggenmerg van muizen. Toen ik begon met het opzetten van deze operatie heb ik vaak gedacht dat we het nooit voor elkaar zouden krijgen. Maar mede door jouw enthousiaste inzet is het toch gelukt. Bij deze wil ik ook Pieter Looijmans bedanken voor alle hulp bij het opzetten van de operatie.

Ik ben mijn paranimfen Suzanne Bouwman en Laurette Goedhard dankbaar dat ze mij willen begeleiden op deze voor mij belangrijke dag. Onze afspraken en gesprekken zorgden altijd voor de nodige ontspanning en afleiding.

Mijn ouders wil ik bedanken voor hun ondersteuning gedurende de afgelopen jaren. Mijn zus(je) Freya wil ik bedanken voor haar creatieve inbreng in de omslag van mijn proefschrift.

Lieve Jeroen, zonder jouw steun en begrip was het niet gelukt.

Curriculum Vitae

Liselijn Agatha Barendina werd geboren op 14 juli 1976 te Zeist. Ze behaalde het VWO diploma in juni 1994 aan de katholieke scholengemeenschap De Breul. In september van datzelfde jaar begon ze met een studie Medische Biologie aan de Universiteit Utrecht. In het kader van deze studie liep ze twee onderzoeksstages. Haar bijvakstage voerde zij uit bij de vakgroep Endocrinologie onder begeleiding van dr. F.M. Verheijen en Prof. dr. M.A. Blankenstein. In dit onderzoek werd de relatie tussen de progesteron receptor en meningioma's onderzocht. Haar hoofdvakstage werd gedaan bij de vakgroep Farmacologie en Anatomie van het Rudolf Magnus Instituut voor Neurowetenschappen onder begeleiding van dr. L.H. Schrama. Hier werd de functie van het eiwit B-50 nader onderzocht. In 2000 legde ze het doctoraal examen Medische Biologie af. In mei van dat jaar begon ze als assistent in opleiding bij de vakgroep Neurologie van het Rudolf Magnus Instituut voor Neurowetenschappen aan een onderzoek naar de ontwikkeling voor gentherapeutische strategieën voor de behandeling van amyotrofische laterale sclerosis onder begeleiding van Prof.dr. P.R. Bär, dr. F.L. van Muiswinkel en dr. E.M. Hol. De resultaten van het onderzoek dat in deze periode werd verricht, staan beschreven in dit proefschrift.

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