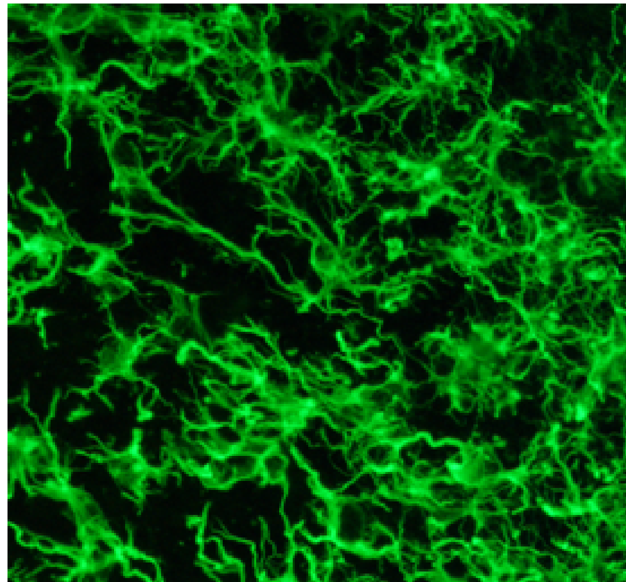
Neuroprotective gene therapy strategies applied to the acutely damaged immature rat brain

PhD Thesis



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A Dami

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CERTIFICAN:

Que el trabajo de investigación titulado "Neuroprotective gene therapy strategies applied to the acutely damaged immature rat brain", presentado por Hugo Peluffo Zavala para optar al grado de Doctor en Biología por la Universidad Autónoma de Barcelona, ha sido realizada bajo su dirección y cumple con todos los requisitos para ser defendido públicamente.

Y para que así conste, firma la presente en Bellaterra a 24 de marzo de 2006.

Dra. Laia Acarin — Dr. Bernardo Castellano

"The mental features discoursed of as the analytical, are, in themselves, but little susceptible of analysis. We appreciate them only in their effects. We know of them, among other things, that they are always to their possessor, when inordinately possessed, a source of the liveliest enjoyment. As the strong man exults in his physical ability, delighting in such exercises as call his muscles into action, so glories the analyst in that moral activity which disentangles. He derives pleasure from even the most trivial occupations bringing his talents into play. He is fond of enigmas, of conundrums, of hieroglyphics; exhibiting in his solutions of each a degree of acumen which appears to the ordinary apprehension preternatural. His results, brought about by the very soul and essence of method, have, in truth, the whole air of intuition".

The Murders in the Rue Morgue
Edgar Allan Poe

"Las características de la inteligencia denominadas analíticas son en sí mismas poco susceptibles de análisis. Las apreciamos sólo en sus efectos. Sabemos de ellas, entre otras cosas, que son, para los que las poseen en alto grado, fuente del mayor goce. Así como el hombre fuerte se complace en su destreza física, deleitándose con ejercicios que pongan en acción sus músculos, así goza el analista en la actividad espiritual que significa *desenredar*. Se complace aun en las ocupaciones más triviales que ponen en juego su talento. Le gustan los enigmas, los acertijos, los jeroglíficos y al solucionarlos demuestra un grado de perspicacia que, para el resto de las mentes, parece sobrenatural. En realidad, sus resultados, obtenidos a través del alma y la esencia del método, tienen todo el aire de la intuición."

Los asesinatos de la calle Morgue
Edgar Allan Poe

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OVERVIEW

This Thesis intends to contribute to the knowledge of the neurodegenerative mechanisms of the acutely damaged immature brain, in particular those related with oxidative stress and inflammation. Moreover, it undertakes the development of neuroprotective strategies involving antioxidant genes by using non-viral modular recombinant non-pro-inflammatory gene therapy vectors.

Acute central nervous system (CNS) damage consists of a multitude of inter-related and complex events determining in the case of focal ischemia two overall lesion areas as a function of time and space: one or several early lesion cores very difficult to rescue and a late lesion periphery. Excitotoxicity, oxidative stress and inflammation play an important role in the initial and secondary injury. In particular, the immature brain displays several characteristics that make it special in its reactions against an acute injury. Several differences in the three mentioned lesion mechanisms will contribute to the differences in lesion outcome. In this context, this Thesis contributes to the basic understanding of the oxidative stress and inflammation occurring after an NMDA-mediated excitotoxic lesion to the immature brain, and to the development of neuroprotective gene therapy strategies for reducing immature brain damage by inhibiting oxidative stress.

This Thesis consists of five complementary studies. In particular, the first study (**Article 1**, page 39) shows the *in vivo* expression and cell localization of one of the most important antioxidant proteins, Cu/Zn superoxide dismutase (SOD), whose expression was observed mainly in neuronal cells and nervous system boundaries in the normal immature brain. However, after an excitotoxic lesion, the expression of this enzyme rapidly disappeared (at 4 hours) from the acutely affected neurons before they undergo cell death. One day after, expression of Cu/Zn SOD begun to increase in reactive astrocytes, present in the lesion for up to 7 days, the last time studied. In the second study (**Article 2**, page 41), the presence of nitrotyrosine, an important oxidative protein product affecting several cellular mechanisms and capable of triggering cell death was analyzed. In the normal brain nitrotyrosine was observed in neurons and scattered astrocytes, however, after the excitotoxic lesion it was early increased in neurons of the lesion core, and after at 1 day post-lesion it increased also in astrocytes. Interestingly, the nitrated hypertrophied astrocytes from 3 days post-lesion onward represented a separated population of cells sharing several markers such as vimentin, metallothionein, Cu/Zn SOD, and high GFAP content and hypertrophy, being always phenotypically the most reactive astrocytes. In addition, although being heavily nitrated and showing activated caspase 3 in their nuclei, nitrated astrocytes did not display any morphological sign of cell death nor TUNEL staining at any time-point studied.

In this lesion context, the next step of this Thesis was to explore the putative beneficial effects of the overexpression of Cu/Zn SOD after the NMDA excitotoxic lesion. To accomplish this, the third study (**Article 3**, page 43) focused on the development of a new gene therapy

strategy based on a non-viral modular recombinant protein vector. This vector had previously been described for *in vitro* settings, and was shown to maintain the beneficial properties of viral attachment and internalization processes while avoiding most of the inconveniences associated with potentially infective material. The study showed that these vectors could deliver a transgene to the whole excitotoxically lesioned zone of the immature brain when injected 2 or 4 hours after the lesion. Moreover, it could transfect neurons, astrocytes and microglial cells, without generating additional inflammation. With these promising results, in the fourth study (**Article 4**, page 45), one of these protein vectors was used for overexpressing Cu/Zn SOD 2 hours after the excitotoxic lesion. Cu/Zn SOD overexpressing animals displayed decreased nitrotyrosine formation, reduced lesion volume, increased neuronal survival, and a complete functional recovery after 3 days in relation to NMDA+saline injected animals. However, surprisingly control lesioned animals injected with the protein vector overexpressing the transgene for the green fluorescent protein (GFP) or with the naked protein vector without any DNA, showed also a reduced lesion volume, suggesting that the vector itself plays a neuroprotective role. To explore this possibility, we designed the last study (**Article 5**, page 47), which showed that the neuroprotective potential of the protein vectors was mediated by the vectors integrin-interacting Arg-Gly-Asp (RGD) motif, as a cyclic RGD peptide was sufficient to induce this neuroprotection. Accordingly, both the protein vector and the cyclic RGD peptide were neuroprotective against a NMDA mediated injury to mixed cortical cultures. However, none of these molecules were neuroprotective under the same treatment conditions in cortical neuron purified cultures, suggesting that the neuroprotective mechanisms include triggering of a glial derived neurotrophic phenotype.

This Thesis concludes that oxidative stress, and in particular the O_2^-/$ONOO^-$ pathway is a mayor contributor to lesion expansion in the acutely injured immature brain, and that the overexpression of Cu/Zn SOD is an interesting neuroprotective strategy. In addition, it shows that modular recombinant protein vectors are efficient gene therapy vectors that can be applied to therapeutic interventions to the acutely lesioned immature brain.

INTRODUCTION

The nervous system displays several unique characteristics like need of high energy supply, activity-dependent modulation of local blood flow, strict control of blood-brain barrier permeability and the composition of the narrow extracellular space, as well as complex neurotrophic interactions to maintain neuronal survival and modulate synaptic plasticity. When damage occurs, wide range of molecular cascades trigger a coordinated response. In particular, acute damage to the CNS as a consequence of hypoxia/ischemia due to stroke or cardiac arrest, sustained seizures, trauma, axotomy, or profound hypoglycemia, consists of a multitude of inter-related and complex events ranging from altered homeostasis, anatomical breakdown and energy failure, to ischemia/hypoxia, oxidative stress, inflammation and glial activation, and cell death.

In the case of focal hypoxia/ischemia, the pathological mechanisms determine two overall lesion areas as a function of time and space: an early degenerating lesion core and a delayed damaged lesion periphery immediately adjacent or at some distance from the core. The initial core consists of rapidly dying cells by necrosis, due to the direct trauma and/or the hypoxic/ischemic environment. Under this circumstance, a critical cell death mechanism is excitotoxicity, which consists of glutamate-mediated over excitation of neurons, triggering necrotic or apoptotic cell death depending on the intensity of the insult. In fact, the induction of excitotoxic injuries by excitatory amino acids analogs is a well-known model for ischemia or epilepsy. The lesion core is a zone that is very difficult to rescue, however the secondary, gradually progressing adjacent lesion also called the penumbra, is composed of less seriously injured tissue that undergoes a series of changes leading to delayed cell death mainly by apoptosis, and where cell death and functional recovery can be modulated. Accordingly, most of the therapies are directed against the neuropathological events occurring in this zone. However, despite that perinatal brain injury is a serious problem and that the immature brain displays several interesting particularities, most neurodegeneration studies have been done in adult rats.

An emerging therapeutic approach is the overexpression of transgenes in the lesioned brain. A wide variety of vectors capable of transferring the desired DNA to brain cells are being generated, both for stable expression of transgenes for the use in the correction of inherited diseases, and for transient expression, more suitable for the treatment of acute damages. The main vectors used due to their natural potential are viral-derived vectors, which have shown important success in some experimental settings and clinical trials. However, they present several drawbacks, especially when considering the therapeutic intervention in acute brain injuries, as they can induce inflammation/immune system activation or cell transformation. In this Thesis, we intend to contribute to the development of effective vectors for gene delivery to acute CNS injuries, which do not induce additional inflammation or immune system activation. Non-viral vectors can fulfill these conditions, but need further development.

In the Introduction of this Thesis we will first analyze the main underlying mechanisms of cell death and its amplification, like excitotoxicity, oxidative stress and glial-mediated inflammation. Then, the particularities of the immature brain and of its injury will be introduced, and finally the gene therapy strategies available will be presented.

1. Acute lesions to the CNS

1.1. Excitotoxicity

The process of glutamate-mediated neuronal death has been called excitotoxicity and was discovered more than three decades ago by Olney and co-workers (Olney 1969). Neurons in culture can be killed by exposure to glutamate or aspartate, but also by natural excitotoxins as kainic acid, or synthetic agonists of the diverse glutamate receptors as α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) or N-methyl-D-aspartate (NMDA) (Bonfoco et al. 1995). These agonists, as well as antagonists of glutamate receptors, were then used to demonstrate the central role of the over activation of glutamate receptors in various models of acute CNS injury including stroke (Dirnagl et al. 1999), traumatic brain and spinal cord injury (Hayes et al. 1992; Obrenovitch and Urenjak 1997), perinatal hypoxic/ischemic injury (Johnston et al. 2002), and neurodegenerative disorders including Alzheimer's disease (Cotman 1998), Parkinson's disease (Sonsalla et al. 1998), Huntington's disease (Petersen et al. 1999), and Amyotrophic Lateral Sclerosis (Ludolph et al. 2000). For instance, glutamate antagonists for ionotropic receptors have been shown to possess a strong neuroprotective effect against hypoxic/ischemic brain damage both in adult (Kochhar et al. 1988) and neonatal animals (Andine et al. 1988)(reviewed in Berger and Garnier 1999). Thus, the induction of an excitotoxic damage by direct *in vitro* or *in vivo* injection of glutamate receptor agonists, including NMDA or kainate, are well established models for different acute CNS injuries, specially for hypoxic/ischemic-like brain injuries in both adult and immature brain (Acarin et al. 1996; Doble 1999; Mattson 2003; McDonald and Johnston 1990).

The complex cascade of events triggered by glutamate receptor activation involves changes in different subcelullar compartments including the cytosol, mitochondria, endoplasmic reticulum, and nucleus (Figure 1). The Ca^{2+} entrance is key event that activates several processes and molecules like cysteine proteases called calpains that cleaves a variety of cellular substrates as cytoskeletal proteins, membrane receptors, metabolic enzymes, and caspases (Bano et al. 2005; Caba et al. 2002; Guttmann et al. 2002). Moreover, the excess of Ca^{2+} triggers apoptosis (Ankarcrona 1998), probably by induction/activation of pro-apoptotic proteins, leading to mitochondrial membrane permeability changes, release of cytochrome C, caspase activation, and liberation of apoptosis inducing factor (AIF) (Culmsee et al. 2001; Dargusch et al. 2001; Duan et al. 1999). It is not clear however to what extent excitotoxic cell death depends on caspases,

especially caspase 3, or on calpains and AIF (Kroemer and Martin 2005; Volbracht et al. 2005). In fact, other non-cell death related functions of caspases have been recently described, and their complex role is a currently very active research field.

One of the main deleterious effects of Ca^{2+} is the induction of oxidative stress through several mechanisms including activation of oxygenases (Goodman et al. 1994), perturbation of mitochondrial Ca^{2+} and energy metabolism (Sengpiel et al. 1998), and induction of membrane lipid peroxidation (Goodman et al. 1996). The reactive oxygen species produced in response to glutamate-induced Ca^{2+} influx include superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and peroxynitrite ($ONOO^-$) (Culcasi et al. 1994; Mattson et al. 1995; Sengpiel et al. 1998).

Accordingly, cultured hippocampal, cortical, and cerebellar neurons exhibit increased levels of $O_2^{\cdot-}$ and H_2O_2 when exposed to excitotoxic concentrations of glutamate or NMDA (Gunasekar et al. 1995; Lafon-Cazal et al. 1993; Mattson et al. 1995). Interestingly, the production of both $O_2^{\cdot-}$ and $\cdot NO$ seems to be necessary for excitotoxic neuronal death in various models (Dawson et al. 1993; Lipton et al. 1993), suggesting a central role for ONOO- produced by the combination of $O_2^{\cdot-}$ and $\cdot NO$. Moreover, while oxidative stress contributes to the excitotoxic patophysiology downstream of glutamate receptor activation, it can also render neurons more vulnerable to excitotoxicity. For example, lipid peroxidation resulting from exposure to Fe^{2+} or β-amyloid peptide impairs the activities of the plasma membrane Na^+/K^+-ATPase, Ca^{2+}-ATPase, and glucose transporter (Mark et al. 1995; Mark et al. 1997a; Mark et al. 1997b), potentiating the Ca^{2+} overload and cell depolarization. In fact, the pathobiology of all acute injuries to the CNS includes production of reactive oxygen and nitrogen species that rapidly induce oxidative stress (Halliwell 2001). In particular, $O_2^{\cdot-}$ has been identified as an important mediator of oxidative injury during ischemia-reperfusion and many other neurological injuries (Chan 1996). Accordingly, cerebral ischemia (Armstead et al. 1988; Fabian et al. 1995) and traumatic brain injury (Kontos and Povlishock 1986) cause a rapid and sustained increase in the formation of $O_2^{\cdot-}$, which is accelerated during mitochondrial dysfunction, and may also result from increased activity of several cytosolic enzymes as phospholipase A_2 (Chan and Fishman 1980) or cyclooxygenase 2 (COX_2) (Armstead et al. 1988).

Besides neuronal cells, glial cells also show glutamate receptors, but their susceptibility to excitotoxicity differs from each cell type. While astrocytes and microglial cells are resistant (Mattson and Rychlik 1990), oligodendrocytes are highly susceptible (Martinez-Palma et al. 2003; Matute et al. 2006). The involvement of glial cells in the excitotoxic processes and inflammation will be discussed further on in this Thesis.

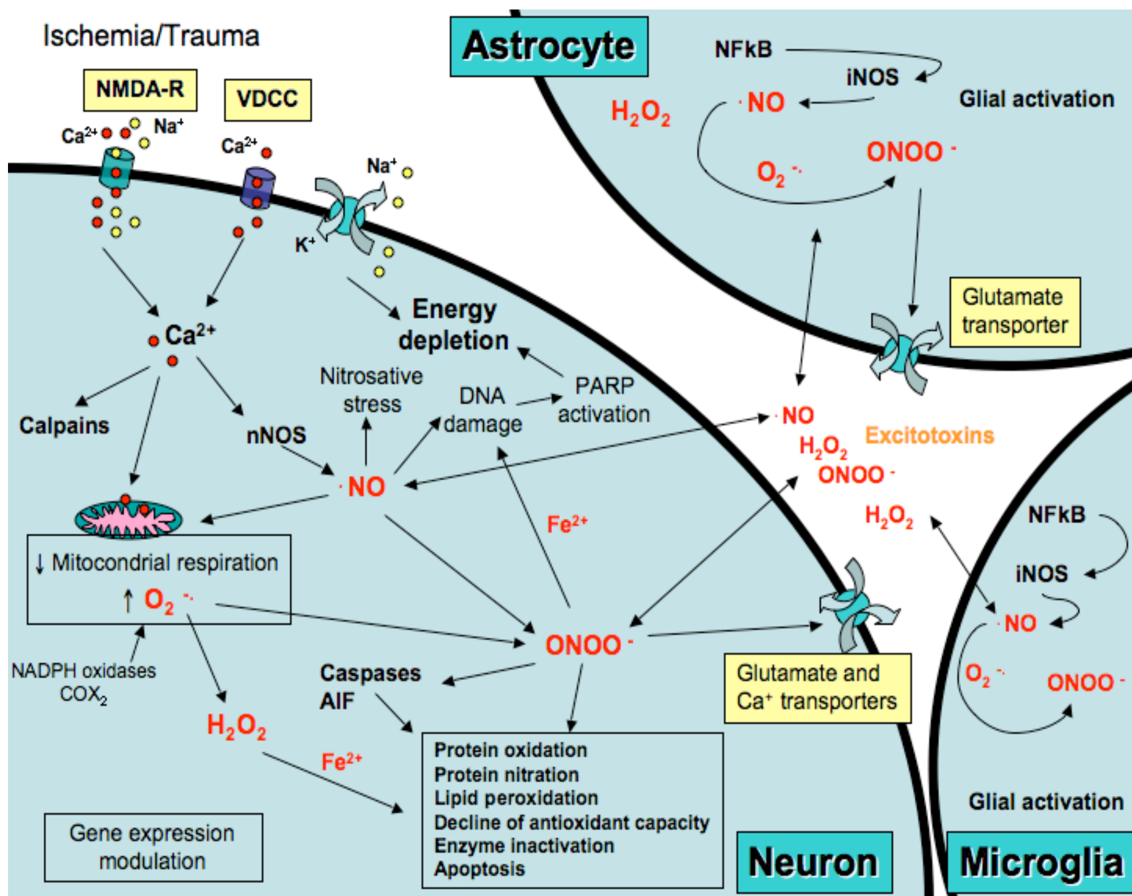

Figure 1. The excitotoxic process. When an excitotoxic process occurs, the overactivation of NMDA glutamate receptors (NMDA-R) induces an elevation of intracellular Ca^{2+} and Na^+ levels, which will have several important effects: i) activation of voltage-dependent Ca^{2+} channels (VDCC) that will further increase cytosolic Ca^{2+}; ii) endoplasmic reticulum release of Ca^{2+} due to metabotropic glutamate receptor activation; iii) activation of calpains; iv) alter mitochondrial respiration inducing the production of oxygen free radicals as superoxide ($O_2^{·-}$); v) activate the neuronal isoform of nitric oxide synthase (nNOS) increasing the nitric oxide (·NO) levels, which will react with $O_2^{·-}$ to produce the potent oxidant and nitrating specie peroxynitrite ($ONOO^-$); and vi) modulate gene expression. The oxidative stress in turn, will have several important effects: i) induction of protein nitrosylation and nitration; ii) inhibition of glutamate and Ca^{2+} transporters; iii) DNA damage and poly (ADP-ribose) polymerase (PARP) activation; iv) activation of caspases; v) liberation of apoptosis inducing factor (AIF) and cytochrome c; and vi) further amplificates the damage by inducing additional oxidative stress in neighboring cells. These cell death mechanisms will depend on the level of stimulation, inducing rapid necrosis or delayed apoptosis. Finally, oxidative stress and cell death will induce inflammatory phenotypes in neighboring glial cells by different mechanisms including the activation of nuclear factor κ B (NFκB).

1.2. Oxidative stress

Oxidative stress occurs when the production of reactive nitrogen and oxygen species surpasses the antioxidant defenses. As it has been commented for excitotoxicity, it is a very important mechanism of cell injury, but also a trigger of inflammatory processes and auto-toxic loops.

1.2.1. Reactive nitrogen and oxygen species

Nitric oxide is a free radical synthesized in various cell types by one of the three isoforms of the nitric oxide synthase (NOS) enzyme. Two of them are constitutive: neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3); and one inducible (iNOS or NOS2) (Leone et al. 1991)(Figure 2). Among the physiological functions described is the relaxation of smooth muscle, the inhibition of platelet aggregation, or the cytotoxicity mediated by immune cells (reviewed in Ignarro 2000). In the SNC, ·NO has been implicated in neurotransmission modulation, synaptic plasticity, and learning. It is a gas that diffuses across cell membranes and thus has been postulated to diffuse in living tissues up to 300 μM, though it is limited by blood vessels being highly reactive with heme proteins (Wood and Garthwaite 1994). Neurons (Garthwaite et al. 1988), as well as microglial cells (Boje and Arora 1992; Chao et al. 1992), astrocytes (Galea et al. 1994; Murphy et al. 1990), and endothelial cells (Ignarro 1989) can act as sources of ·NO. This free radical participates in numerous chemical reactions in the cell, being the interaction with heme groups one of the main mechanisms by which it mediates its biological functions (Figure 2), including the activation of the guanylate cyclase by nitrosilating its heme group (Murad 1994).

This free radical has also been implicated in the physiology and patophysiology of several systems where it exerts dual roles. A protective role has sometimes been described, as inhibiting inflammation by attenuating platelet and neutrophil vascular attachment (Hickey et al. 1997; Yan et al. 1996), inhibiting the NMDA receptor by direct nitrosylation (Lipton et al. 1993), and inactivating caspases (Melino et al. 1997). These neuroprotective effects seem to depend on low steady state fluxes of ·NO as those produced generally by eNOS or nNOS (Kilbourn et al. 1997). However, ·NO has been linked as a toxic agent in a wide range of pathological processes when produced at elevated steady state fluxes, generally mediated by iNOS, but also by nNOS under some circumstances (Dawson and Dawson 1998; Gross and Wolin 1995). In the CNS it can mediate toxic effects by directly inhibit axonal transport (Redford et al. 1997), or the mitochondrial electron transport (Brown and Cooper 1994; Cassina and Radi 1996; Cleeter et al. 1994). Moreover, it has been postulated that many of the important toxic effects of ·NO are mediated by its reaction with $O_2^{-\cdot}$ forming the potent oxidant and nitrating agent $ONOO^-$ (Beckman et al. 1990; Radi et al. 1991) (Figure 2). This reaction between ·NO and $O_2^{-\cdot}$ occurs at a diffusion limiting rate, being ·NO one of the few known molecules capable of competing with the superoxide dismutase (SOD) enzyme for the $O_2^{-\cdot}$ (Huie and Padmaja 1993).

Among all the reactive oxygen species one of the most important molecules is $O_2^{-\cdot}$, which is produced as a consequence of the oxidative metabolism that leads to an increase of the univalent reduction of oxygen in the mitochondria, and also by some enzymes as NADPH oxidase and xanthine oxidase (Freeman and Crapo 1982; Turrens and Boveris 1980) (Figure 2). It can be transformed into a potent oxidant when reacting with transition metals as iron or copper forming hydroxyl radical (·OH), or with ·NO to form $ONOO^-$ as already described. Compared with ·OH,

which is highly reactive, $ONOO^-$ or $\cdot NO$ are less reactive, and thus these compounds can target specific molecular domains. Interestingly, $ONOO^-$ was shown to diffuse across cell membranes (Denicola et al. 1998), and thus, unlike $O_2^{\cdot-}$, can act in cells near the one where it was generated.

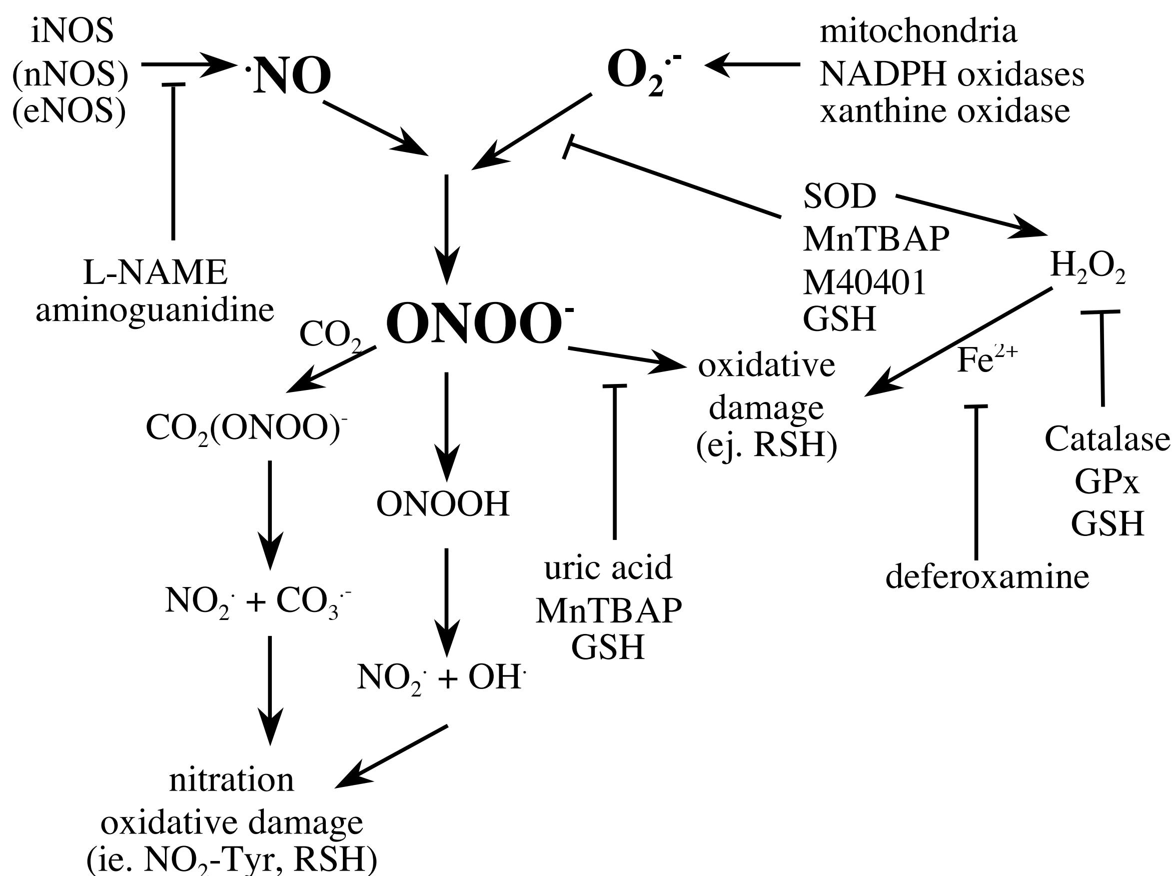

Figure 2. Formation and reactivity of the main oxygen and nitrogen reactive species and their endogenous or pharmacological scavengers/inhibitors. This figure depicts the formation of reactive species and their main reactions. Several strategies have been used to limit the oxidative damage, as inhibition of nitric oxide synthases (NOS) with aminoguanidine or L-nitro-arginine-methyl-esther (L-NAME), overexpression of the antioxidant enzymes superoxide dismutases (SOD), catalase or glutathione peroxidase (GPx), pharmacological SOD mimetics (MnTBAP, M40401), increase of the major non-enzymatic antioxidant defense molecule glutathione (GSH), or administration of iron chelators (deferoxamine). Cysteine groups of proteins (RSH) can be oxidated and nitrosilated, and tyrosine residues of proteins can be nitrated (NO2-Tyr).

Endogenous or exogenously added $ONOO^-$ can induce apoptosis by a variety of mechanisms in different systems including primary cortical neuron cultures (Bonfoco et al. 1995), and motor neuron cultures (Estevez et al. 1998). Among the suggested mechanisms involved in the proapoptotic effects of $ONOO^-$ are: i) general cell oxidative damage to membranes, DNA and fundamental molecules, inactivating important metabolic enzymes as aconitase (Castro et al. 1998), succinate dehydrogenase (Radi et al. 1994), or fumarate reductase (Rubbo et al. 1994), and other important molecules like iron responsive elements (IREs)(Soum and Drapier 2003), Ca^{2+}-

ATPase (Viner et al. 1996), manganese superoxide dismutase (Mn SOD) (MacMillan-Crow et al. 1996), and tyrosine hydroxylase (Ara et al. 1998); ii) induction of the mitochondrial transition permeability pore and liberation of cytochrome c to the cytosol (Packer and Murphy 1994); iii) activation of caspase 3 (Lin et al. 1998); and iv) nitration of protein bound or free tyrosines (Beckman et al. 1992; Radi 2004), which contributes to the neuropathology of both acute and progressive neurological disorders (Ischiropoulos and Beckman 2003; Sarchielli et al. 2003). For example, free nitrotyrosine at biologically relevant concentrations is neurotoxic to cultured motor neurons (Peluffo et al. 2004). In fact, nitration can inhibit essential signal transduction cascades by several mechanisms including nitration of tyrosines competing with its phosphorilation (Kong et al. 1996; Martin et al. 1990) or inactivating zinc finger proteins (Crow et al. 1995). Moreover, the nitration of cytoskeletal proteins as neurofilaments, actin or tubulin can alter the intracellular transport mechanisms (Aslan et al. 2003; Crow et al. 1997; Eiserich et al. 1999; Peluffo et al. 2004). It should be noted that an $ONOO^-$ independent mechanism of nitration has been described involving the neutrophil enzyme myeloperoxidase (Eiserich et al. 1998), however these cells are not normally present or only transitory present in the CNS, and thus nitrotyrosine has been used as a footprint of $ONOO^-$ formation (Radi 2004).

1.2.2. Antioxidant defenses in the CNS

The CNS displays high levels of antioxidant defenses (Adlard et al. 1973; Makar et al. 1994; Slivka et al. 1987), but several evidences have shown that neurons are vulnerable to oxidative stress (Bonfoco et al. 1995; Estevez et al. 1998; Pechan et al. 1992; Whittemore et al. 1994), whereas astrocytes display higher resistance (Bolanos et al. 1995) (Langeveld et al. 1995; Pechan et al. 1992). For instance, in culture, astrocytes can be treated with elevated concentrations of $ONOO^-$ without generating cell death nor decreasing their mitochondrial enzyme activities or glutathione levels, while all these problems are seen in isolated neuron cultures treated with four times less $ONOO^-$ (Bolanos et al. 1995). This elevated resistance of astrocytes is mediated by their high levels of two of the main endogenous non-enzymatic antioxidants: vitamin E and glutathione (Barker et al. 1996; Makar et al. 1994). Accordingly, astrocytes unlike neurons, are capable of synthesizing cysteine from cystine, the rate-limiting step in the synthesis of glutathione (Sagara et al. 1993a). In this manner, astrocytes are not only capable of synthesizing their own glutathione, but also of releasing cysteine which can in turn be used by neurons to maintain their glutathione, which could explain why neurons are more resistant to oxidative stress when co-cultured with astrocytes (Langeveld et al. 1995). Moreover, trophic factors, which are fundamental molecules determining neuronal survival, have been shown to increase the antioxidant enzyme levels in neurons (Mattson et al. 1995; Pan and Perez-Polo 1993) and to decrease their nNOS expression, inhibiting cell death (Estevez et al. 1998; Novikov et al. 1995), also pointing to antioxidant defenses as critical pro-survival molecules. Moreover, while

·NO inhibits the respiratory mitochondrial electron chain in astrocytes in culture, it does no trigger their death as it does with neurons, possibly due to a metabolic shift, where the astrocytes increase the glycolytic rate and lactate release (Bolanos et al. 1994). Finally, astrocytes react to injury by elevating their antioxidant enzyme levels including Cu/Zn SOD (Liu et al. 1993) and Mn SOD (Komori et al. 1998). Thus, after a lesion, astroglial cells can act as a buffer of oxidative stress, not only by having elevated endogenous antioxidant capabilities, but also by increasing them, and in addition increasing the trophic and metabolic support for neurons.

1.2.3. Superoxide dismutase

In the last decade many studies have focused on the antioxidant proteins dealing with oxidative stress in physiological conditions and after injury. Superoxide dismutases are among the most important cellular mechanisms coping with oxidative stress. Cellular levels of $O_2^{-\cdot}$ are normally low due to the action of cytosolic Cu/Zn SOD and mitochondrial Mn SOD. These key enzymes catalyze the dismutation of $O_2^{-\cdot}$ to oxygen and H_2O_2 (McCord and Fridovich 1969)(Figure 2). Hydrogen peroxide is then further detoxified by catalase and glutathione peroxidase, the final step in the detoxification chain of reactive oxygen species.

In the adult CNS, Cu/Zn SOD is widely expressed by different neuronal populations. Regarding glial cells, it is generally assumed that microglial cells and oligodendrocytes do not show expression of Cu/Zn SOD, but the astroglial expression of this enzyme is controversial. Astrocytes have been described as the main and most intense cells immunoreactive for Cu/Zn SOD in few studies, but most works have failed to detect this astroglial expression, or have reported expression only in some scattered astrocytes. On the other hand, expression of Cu/Zn SOD has been well documented in reactive astrocytes several days after different types of adult brain injury, like transient cerebral ischemia (Liu et al. 1993), kainate treatment (Kim et al. 2000), quinolinic treatment (Noack et al. 1998), or Alzheimer's and Down's Syndrome (Furuta et al. 1995). Surprisingly, there are no studies showing the cell-specific expression of Cu/Zn SOD in the immature brain.

In agreement with the antioxidant role of this enzyme, its over-expression in adult transgenic animals shows pronounced neuroprotection in most acute CNS injury models (Chan et al. 1991; Mikawa et al. 1996; Yang et al. 1994). Accordingly, conjugation of the enzyme to a fusion protein is neuroprotective against a transient global ischemic insult (Eum et al. 2004), and synthetic SOD mimetics as $O_2^{-\cdot}$ dismuting metalloporphyrins protect against transient ischemic injuries (Mackensen et al. 2001; Mollace et al. 2003). In addition, the targeted deletion of the Cu/Zn SOD gene or extracellular SOD gene worsens the outcome after focal ischemia in the adult brain (Kondo et al. 1997; Sheng et al. 1999). Importantly, though endogenous Cu/Zn SOD is normally expressed in neurons, it is rapidly downregulated after several types of adult acute brain insults (DeKosky et al. 2004; Kim et al. 2000; Liu et al. 1994; Liu et al. 1993) rendering the brain more

susceptible to oxidative stress. It is not known whether this phenomenon occurs in immature brain, where overall findings suggest a differential scenario for oxygen and nitrogen reactive species in the evolution of a brain injury (Ferriero 2004) (see below).

1.3. Glial inflammatory response against injury

Damage to the CNS triggers a response that depending on the severity of the insult can involve several cell types, including neurons, glial cells, endothelial cells in blood vessels, and several blood-derived cells. Whereas mild or indirect trauma like axotomy only shows entrance to the CNS of some T lymphocytes, when neuronal cell death occurs enhanced number of T lymphocytes are recruited and found in close vicinity of activated phagocytic microglial cells (Raivich et al. 1998). On the other hand, direct mechanical injury (Carlson et al. 1998), ischemia (Clark et al. 1995) and excitotoxicity (Acarin et al. 2002b; Bolton and Perry 1998) lead to a massive influx of leukocytes, specially monocytes and neutrophils, being the latter the ones showing the most rapid recruitment and also being directly linked with exacerbation of the damage. Finally, the recognition of specific antigens in neural tissue, as in autoimmune diseases or infection, triggers a different scenario, where not only antigen specific lymphocytes enter the nervous tissue, but also macrophages, eosinophils, natural killer cells and non-specific activated cytotoxic T-lymphocytes (Hemmer et al. 2004; Meisel et al. 2005). In all these situations a coordinated response with glial cells occurs, which is generally referred as glial reactivity.

Considering all forms of brain pathology, glial cells develop an evolutionary conserved, graded and apparently stereotyped response, which is thought to play an important role in the protection and repair of the nervous system, protection against pathogens, but also in degeneration (reviewed in Raivich et al. 1999 and Lucas et al. 2006). A mild injury to neurons, as happens after axonal injury or in the initial phase of several neurodegenerative diseases induce a mild response of astroglial and microglial cells, whereas after severe injuries a robust glial response occurs.

1.3.1. Astroglial reactivity

Astroglial cells, the predominant cell type of the nervous system, undergo changes when a lesion occurs, which depend on the external trauma. Both white matter fibrillary highly GFAP positive astrocytes, and grey matter protoplasmic astrocytes poorly positive for GFAP or with undetectable levels of this protein, undergo rapid non-apparent intracellular changes including the complex activation of several pro-inflammatory transcription factors such as nuclear factor κ B (NFκB) or signal transducers and activators of transcription (STATs) (Acarin et al. 2000b; Brambilla et al. 2005; Justicia et al. 2000) and of cellular anti-inflammatory cascades as those mediated by SHP1 phosphatase (Horvat et al. 2001) or the suppressor of cytokine signaling (SOCS) proteins (Campbell 2005). Within 24 hours after injury, there is an increase in the

content of GFAP: in the case of a mild injury like axotomy the appearance of small GFAP processes, and in the case of an intense injury like ischemia the appearance of velate GFAP-positive plaque-like reactive astrocytes (Raivich et al. 1999). However, in both cases after 2-3 days the astrocytes transform into hypertrophic cells with increased size of GFAP positive soma and proximal processes (Eddleston and Mucke 1993). Finally, these cells form a glial scar that isolates injured individual cells (also called synaptic stripping) or entire lesioned tissue areas from the healthy functional ones. While this process can be reversed when it is localized and restricted around individual neurons, it is not reversible when it occurs along large areas separating zones of unstructured neural parenchyma and functional tissue.

As already commented, the phenotype of glial cells has an important effect on neuronal survival, both under physiological conditions and after injury. In the normal brain, astrocytes provide neurons with a suitable structural support, synthesize essential extracellular matrix molecules (Noble et al. 1984; Selak et al. 1985a), produce and secrete precursors for the antioxidant support of neurons (Sagara et al. 1993b) and a wide range of trophic factors including neurotrophins (Carroll et al. 1993; Condorelli et al. 1994; Moretto et al. 1996; Neveu et al. 1994), constitute a metabolic bridge and functional coupling between neurons and blood vessels (Selak et al. 1985b; Takano et al. 2006), and maintain the extracellular medium ionic and neurotransmitter concentrations for correct neurotransmission. When damage occurs, reactive glial cells acquire new phenotypes that tend to cope with the insult, as induction or increase in the expression of trophic factors (Pechan et al. 1992), antioxidant enzymes, extracellular matrix molecules, and anti-inflammatory mediators (Pinteaux et al. 2005), products that have been the target of neuroprotective mechanisms. For example Riluzole, the only treatment approved for Amyotrophic Lateral Sclerosis, was shown to be one of the pharmacological compounds that can act on astrocytes, inducing astrocyte-derived neurotrophic activity for motor neurons (Peluffo et al. 1997). Another compound, ONO-2506, that decreases astrocytic proinflammatory activation and increases GLT1 and GLAST glutamate transporters protects the CNS from MPTP-induced Parkinson's disease (Kato et al. 2003) and focal cerebral ischemia (Tateishi et al. 2002). Interestingly, a closer look to these works reveal that although ONO-2506 decreases the number of heavily reactive hypertrophic astrocytes at 7 days after the injection of MPTP, it actually enhances the rapid appearance of slightly reactive astrocytes at 3 days after the injection, correlating with the recent suggestion that spatially or temporally different subsets of reactive astrocytes could play either toxic or beneficial roles.

Accordingly, the study of the integrative molecular-morphological-functional phenotype of the glial cells in the non-injured brain has lead to the accumulation of evidence suggesting that there are several cellular phenotypes coexisting in the brain. For example, under physiological conditions, recent data suggest that there are at least three functionally and structurally different populations of GFAP expressing astrocytes in the grey matter of the hippocampus (Matthias et al.

2003). One of these phenotypes included cells with a net of short thin processes, showing very low levels of GFAP, AMPA glutamate receptors, while a second group resembling the classical protoplasmic astrocytes, displayed irregularly shaped soma bearing expanded branched nets of processes, high levels of GFAP expression, and glutamate transporters. The third subpopulation expressed low levels of GFAP and no glutamate receptors nor transporters. Thus, the first astrocytic population would be excited by extracellular glutamate, the second would contribute to diminish the extracellular glutamate, and the third would be insensitive to glutamate. The integration of this view in reactive astrocyte biology will probably contribute to a better understanding of the properties, functions, and therapeutic opportunities for the interaction with these cells. For instance, the activation of astrocytes with different pro-inflammatory mediators induces different phenotypes of these cells. Activation of cultured astrocytes by innate immune mediators like bacterial lipopolysaccharide LPS (through activation of Toll receptors 3 and 4) showed interleukin 6 (IL-6), tumor necrosis factor α (TNFα), interferon α_4 (INFα_4), interferon β (INFβ) cytokine production, as well as induction of iNOS and several chemokines and adhesion molecules. On the other hand, when stimulated with interferon γ (INFγ) and TNFα adaptive immune cytokines, astrocytes only upregulated the same chemokines and adhesion molecules, but in addition they expressed MHC II, showing the functional ability to activate $CD4^+$ T lymphocytes (Carpentier et al. 2005). However, it is not known if these two phenotypes can coexist in the lesioned brain, or if they represent different stages of reactivity in time.

1.3.2. Microglial reactivity

It has for long been recognized that the post-lesion transformation of both grey and white matter resting microglia towards different activated states imply their increase in cell body size, thickening of proximal processes and decrease of distal ramifications, which can develop in an amoeboid morphology without cell processes or finally in a round macrophagic state (reviewed in Kreutzberg 1996 and Raivich et al. 1999). As already commented for astrocytes, different microglial phenotypes are possible both in normal and lesioned brain. For instance, resting parenchymal microglia and perivascular macrophages seem to constitute two separate populations of cells, not only by its location but also by their molecular profile (Graeber et al. 1989). Very recently, in vitro models for microglial activation have been developed to elucidate this differential activation, where microglial cells are not only activated with the classical bacterial endotoxin (LPS) treatment paradigm, but instead with conditioned medium from lesioned neurons. Interestingly, in these types of experiments, two different microglial activation states at the molecular level were achieved. Furthermore, reactive microglia has been separated in two groups as showing a phagocytic-innate phenotype or an antigen presenting-adaptive phenotype (Town et al. 2005). In fact, resident activated microglia can phagocyte discrete neuronal populations that die by apoptosis during the development without generating inflammation. Finally, a third group of

activated microglia should also be considered, including the mixed phenotype between the two described phenotypes when microglial cells are challenged with pathogen derived products, when they display enhanced phagocytosis, pro-inflammatory cytokine production, as well as adaptive activation of T lymphocytes.

One of the differential mediators making possible that microglial cells react to cell damage are the high energy purine nucleotides ATP, UDP and UTP, as it has been shown very recently by Davalos and co-workers in a very elegant work (Davalos et al. 2005). They combined mice overexpressing enhanced green fluorescent protein (EGFP) only in microglial cells (under the promoter of fractalkine receptor, CX3CR1) with *in vivo* trans-cranial two-photon laser-scanning microscopy, being able to show the effects of a localized laser lesion to the cortex and the microglial reactions. However, the molecular characterization of the different activation stages of microglial cells is still not well defined.

In summary, the balance between these activated microglial and astroglial phenotypic states seems to depend on several factors: i) whether glial cells are exposed to pro-inflammatory TH1 cytokines as INFγ, TNFα or IL-6 or to anti-inflammatory TH2 cytokines as interleukin 4 (IL-4), interleukin 10 (IL-10) and perhaps transforming growth factor β1 (TGFβ1); ii) whether pathogen-derived molecules are present; iii) in the case of microglia, whether co-stimulatory molecules like CD40 are involved or also if other directly stimulatory or inhibitory receptors are present on microglial cell membranes (like TREM2, Takahashi et al. 2005, or CD200R, Broderick et al. 2002) and are engaged by their ligands.

1.3.3. Glial reactivity and oxidative stress

In addition to disrupting neuronal homeostasis, oxidative stress has important effects on glial cells by activating these cells to display pro-inflammatory properties. For instance, astrocyte cultures treated with $ONOO^-$ undergo morphological and functional phenotypic changes, which includes ·NO-dependent neurotoxic properties towards co-cultured motor neurons (Cassina et al. 2002), while if this occurs *in vivo* is not clear. Moreover, the prototypical pro-inflammatory transcription factor NFκB was described very early to become directly activated by oxidative stress and to regulate main changes in glial cell gene expression (Schreck R et al. 1991; Storz and Toker 2003; Takada et al. 2003). Microglial cells which overexpress Cu/Zn SOD, when activated by LPS, showed less ·NO, TNFα and IL1β production than wild type microglia, suggesting that endogenous $O_2^{-\cdot}$ also contributes to microglial activation (Chang et al. 2001). On the other hand, activated glial cells can also induce oxidative stress by several mechanisms. *In vitro*, proinflammatory activation of astroglial cells induces iNOS expression (Bolanos et al. 1994; Galea et al. 1994; Lee et al. 1993) and mediates neuronal cell death by ·NO overproduction (Cassina et al. 2002; Chao et al. 1996; Dawson et al. 1994; Hewett et al. 1994; Hu et al. 1997; Skaper et al. 1995; Stewart et al. 2000). *In vivo*, diverse neuropathologies show iNOS positive astrocytes and

increased nitrotyrosine immunoreactivity, including Amyotrophic Lateral Sclerosis (Almer et al. 1999; Cha et al. 1998), Multiple Sclerosis (Bagasra et al. 1995; Bo et al. 1994), Parkinson's disease (Liberatore et al. 1999), Progressive Supranuclear Palsy (Komori et al. 1998), Cerebral Ischemia (Endoh et al. 1994), and HIV cerebral pathology (Boven et al. 1999). Interestingly, oxidative stress, including that mediated by $ONOO^-$, can inhibit neuronal and astrocytic glutamate transporters (Trotti et al. 1996; Volterra et al. 1994), potentiating the excitotoxic process.

It is now well established that astrocytes interact by various and complex mechanisms with microglial cells and perivascular macrophages (Raivich et al. 1999; Streit et al. 1989). Activated microglia can secrete proinflammatory cytokines, $\cdot NO$, $O_2^{\cdot-}$ (with the consecutive formation of $ONOO^-$), and excitotoxins which mediate important effects on the astroglial phenotype (Giulian and Baker 1985) and induce neurotoxicity *in vitro* (Boje and Arora 1992; Chao et al. 1992) and *in vivo* (Liberatore et al. 1999). In turn, reactive astrocytes are involved in the microglial activation by secretion of similar diffusible factors (Malipiero et al. 1990; Thery et al. 1992). By these mechanisms, both astrocytes and microglial cells can potentiate their inter-activation and the resultant neurotoxicity.

2. The immature brain

Perinatal brain injury is the major contributor to perinatal morbidity and mortality. It is usually the consequence of cerebral ischemia, cerebral hemorrhage or intrauterine infection (Perlman et al. 1996; Wu and Colford 2000). For example, after brain damage from perinatal hypoxic/ischemic insults, a considerable number of children will develop cerebral palsy, severe learning disabilities, and other disorders. Several lines of evidence suggest that the immature brain responds in a particular fashion to brain injuries (Ferriero 2004; Vannucci and Hagberg 2004), at least in part due to the fact that many of the adult gene expression patterns, neural circuits organization, cell differentiation or myelination have not yet been achieved (Dobbing and Sands 1979; Romijn et al. 1991). For instance, in white matter, a very important proliferation of oligodendrocyte progenitors occurs which contributes to the ongoing myelination (Jacobson 1963). In the cortex, there is still ongoing axonal projection as well as formation and maturation of synapses, which constitutes the major final steps in corticogenesis (Bourgeois et al. 1994; Bourgeois and Rakic 1993). In fact, around the perinatal period and for some time after there is a burst in synapse formation, a process responsible for naming the period as the "plasticity window", and that ranges in the human neocortex around 470 days (E120-P310) (Huttenlocher and Dabholkar 1997; Rakic et al. 1994), and from P2 to P16 in rats (Bourgeois 1997).

2.1. Oxidative stress in the immature brain

Oxidative stress is known to play a fundamental role in brain damage as a result of a perinatal hypoxic/ischemic-like insult (Berger and Garnier 1999; Ferriero 2004). However, several differences between immature and adult animals in regards to oxidative stress and antioxidant defenses have been described. In regards to oxidant deleterious mechanisms, it is now known that in the immature brain free iron accumulates more rapidly, within 4 hours after transient cerebral ischemia, stimulating Fenton reactions (Kondo et al. 1995; Palmer et al. 1999) and that immature brain has a peak of nNOS, iNOS, and nitrotyrosine levels (Fernandez et al. 2003) which contributes to the neurotoxicity after hypoxic/ischemic injury (Ferriero et al. 1996; Palmer et al. 1994; Tsuji et al. 2000). Moreover, the postnatal brain is more sensitive than the adult brain to the neurotoxic actions of NMDA (McDonald et al. 1988), which will lead to increased $O_2^{-\cdot}$ generation (Dugan et al. 1995; Lafon-Cazal et al. 1993), that may contribute, together with a slower clearance of glutamate (Diamond 2005) due to its developmental increase (Furuta et al. 1997; Ullensvang et al. 1997), to an increased susceptibility to excitotoxicity. Finally, it is well known that total brain levels of antioxidant enzymes, including those of Cu/Zn SOD, vary throughout life, and reach the adult pattern only after the first month of life (Acarin et al. 1999; Khan and Black 2003; Nishimura et al. 1992).

However, contradictory results have been reported in regard to the role of Cu/Zn SOD after hypoxic/ischemic injury to the immature CNS. While a slightly worsened neuropathological outcome was observed in postnatal transgenic mice over-expressing Cu/Zn SOD (Ditelberg et al. 1996), several antioxidant molecules including SOD mimetics like $O_2^{-\cdot}$ dismuting metalloporphyrins (Shimizu et al. 2003), or the iron chelator and nitration inhibitor desferrioxamine (Palmer et al. 1994) have been shown to be neuroprotective. Consequently, oxidative mechanisms in the acutely injured immature brain, in particular those related with $O_2^{-\cdot}$ and SOD, need further study. As this Thesis provides some new data on the field of oxidative stress in the immature brain, this will be further discussed in the Discussion section.

2.2. Glia and inflammation in the immature brain

Inflammation in the immature brain also shows several characteristics that make it different from the adult brain, like those regarding glial reactivity and regeneration. In fact, the differences observed regarding regeneration in very young and old mammals seem to be at least in part due to the changes in astroglial reaction to injury. For instance, hippocampal neurons cultured on nitrocellulose filters that had previously been implanted in the cortex of young or mature animals and thus have "scar-on-a-dish" characteristics showed that neurites grew better on the young implant (Rudge and Silver 1990). In accordance, adult cultured reactive astrocytes from the optic nerve inhibited projection outgrowth of both mature and immature retinal ganglion

neurons, while immature reactive astrocytes from the same origin induced outgrowth from both mature and immature retinal ganglion neurons (Bahr et al. 1995). Furthermore, immature but not mature reactive astrocytes transplanted into adult brain on nitrocellulose filters (Smith and Silver 1988), or in cell suspension (Smith and Miller 1991), could inhibit scar formation modifying the response of adult astrocytes. Thus, in spite of astroglial reactivity and scar formation, the immature brain has a greater potential for regeneration than adult and old animals, in part due to the different astroglial biology, a fact that is probably related to the physiological "plasticity window" which is ongoing in the postnatal brain as previously commented.

Another important difference is observed in the recruitment of leukocytes and the phenotype of microglial cells. For example, the reaction towards proinflammatory mediators is developmentally regulated, since injection of LPS or IL-1β into the brain parenchyma of immature rodents elicits a more florid response than that seen in adults (Anthony et al. 1997; Lawson and Perry 1995), with kinetics and characteristics typical of non-neural tissues. In the immature animals, larger numbers of neutrophils were recruited and there was more conspicuous breakdown of the blood brain barrier (BBB) (Anthony et al. 1997). Moreover, during the first two weeks of postnatal life in the rat two different types of microglial cells coexist: in the white matter tracts there are amoeboid microglial cells, which appear during prenatal life deriving from monocytes or primitive/fetal macrophages (Boya et al. 1987; Dalmau et al. 1997; Ling and Wong 1993), and maintain several macrophage characteristics such the expression of major histocompatibility complexes (MHC) (Ling et al. 1991); in the gray matter there are primitive ramified microglial cells (Dalmau et al. 1998), a transition form towards the formation of adult ramified microglial cells found in the mature CNS and derived from the progressive ramification of amoeboid cells. This will have consequences for the outcome of brain injury to the immature brain. Though the main cell types recruited are normally monocyte/macrophages and neutrophils, other cell types as mastocytes are also involved in lesion outcome. The normal immature brain shows a peak of entry of mastocytes (Lambracht-Hall et al. 1990), which under some circumstances can exacerbate excitotoxic lesions to the immature brain (Patkai et al. 2001). Thus, the study of the inflammatory responses in the immature brain, and their degree of influence on lesion outcome need further study.

Our group has been studying the role of glial cells in the neuropathology of acute immature brain injury (reviewed in Acarin et al. 2001), mainly using a well-characterized excitotoxic model for hypoxic/ischemic injury to the postnatal brain based on the intracortical stereotaxic injection of NMDA (Ikonomidou et al. 1989; Olney 1990). We described several rapid and delayed changes that occur in transcription factors, cytokine levels, and glial reactivity (summarized in Table 1).

Table 1. Inflammatory events in excitotoxically injured immature brain				
Cell type	*Early phase (0-12h)*	*Acute phase (12h-3d)*	*Late phase (3d-14d)*	*References*
Neurons	Necrotic cell death Activation of NFκB Expression of IL-6 and COX_2	Apoptotic cell death Expression of TNFα, COX_2, and some iNOS	Apoptotic cell death (until day 5)	(Acarin et al. 2000a; Acarin et al. 2000b; Acarin et al. 2002b)
Microglia	Very little and restricted activation of STAT3 and NFκB Expression of IL-1β, HSP32, and COX_2	Massive increase in number and TL binding Expression of IL-1β, MHC1, MHC11, MT1-11, vimentin, HSP32, and COX_2	Expression of HSP32, IL-1β, and TGF-β	(Acarin et al. 2000a; Acarin et al. 2000b; Acarin et al. 1996; Acarin et al. 1997; Acarin et al. 1999; Acarin et al. 2002a; Acarin et al. 2002b)
Astroglia	Activation of STAT3 and NFκB Expression of iNOS Expression of HSP47 and some HSP27	Activation of STAT3 and NFκB Expression of GFAP, TNFα, MT1-11, vimentin, IL-6, and iNOS Expression of HSP47 and some HSP27	Activation of STAT3 and NFκB Expression of GFAP, vimentin, IL-1β, IL-6, TGF-β, and TNFα Expression of HSP47 and some HSP27 Scar formation	(Acarin et al. 2000a; Acarin et al. 2000b; Acarin et al. 1999; Acarin et al. 2002a; Acarin et al. 2002b)
Various	Blood brain barrier break-down begins Neutrophil infiltration, some of them expressing iNOS	Massive monocyte infiltration Neutrophil infiltration, some of them expressing iNOS	Glial scar formation	(Acarin et al. 1996; Acarin et al. 1997; Acarin et al. 1999; Acarin et al. 2002b)

3. Gene therapy in the CNS

The impressive advances in molecular and cellular biology have made possible the modification and utilization of virus, natural vectors for the delivery of genes, for the transfer of DNA or RNA in a wide variety of situations (Kirik and Bjorklund 2003; Somia and Verma 2000). Moreover, other non-viral methods for the delivery of genes have been designed and show a promising future (Aris and Villaverde 2004; Li and Ma 2001; Mastrobattista et al. 2006; Roth and Sundaram 2004). The field of application of gene therapy strategies is broad and has been used for the correction of genetic diseases, treatment of acquired diseases, prophylactic vaccination, generation of models for these diseases, or the easier development of transgenic animals or knockouts.

An important concept that has arisen from these studies is that there is not a single vector suitable for most applications of gene therapy, and on the contrary, there is a specific type of vector appropriate for each experimental or pathologic condition. For instance, some genetic diseases need the overexpression or the downregulation of one or more specific proteins during

the lifetime of the organism. On the other hand, the treatment of various acute diseases needs the overexpression or downregulation of specific proteins during a short period. Moreover, under some situations a specific cell type must be targeted, and in other cases reaching the highest possible number of cells is needed. In addition, desired levels of transgene expression can also vary.

The therapeutic approach in space and time is also determinant of which type of vector can be successful. In some cases an *ex vivo* strategy can be suitable, as in the case of the pioneer work of Marina Cavazzana-Calvo, Alain Fisher and their colleagues on children with Severe Combined Immuno Deficiency (SCID) (Cavazzana-Calvo et al. 2005). In most cases an *ex vivo* gene therapy is not possible, and thus direct injection of the vectors is needed. Some organs can be targeted using specific vectors, however a direct injection into the organ or in a clamped local vascular setting must be performed in many cases. Consideration of the therapeutic time frame for the application of gene therapy strategies to acute injuries is also critical, especially when considering acute injuries to the CNS. Most vectors need at least 4-6 hours to induce the production of a transgene, which in addition needs to reach therapeutic-relevant concentrations.

Though the design and construction of vectors is ongoing a burst, there are still several limitations that include the loss of transgene expression, induction of an immunologic response against the vector or the transgenic protein, limitation in size of the insert of DNA or RNA, easy and reproducible production at high scale, controlled insertion into the cellular genome, and biosafety. For these reasons the continuous improvement and design of new vectors are needed (Branca 2005).

The multifactorial approach to the pathologies, designing combined treatments targeting several critical mechanisms of injury and repair is emerging as the necessary step for an important advance in the pathological outcome. Gene therapy offers the opportunity of a combined treatment with several therapeutic transgenes, as for example several enzymes forming a sequential cascade, like for the production of dopamine in Parkinson's disease (Azzouz et al. 2002), or acting on different independent therapeutic targets (Sapolsky 2003).

3.1. Viral vectors

Retroviral vectors based on the murine leukemia virus (MuLV) have been widely used in part due to historical reasons, as it was the first vector established and used for a clinical trial around 1990. The *Retroviridae* family is characterized by possessing a linear RNA genome that is converted to DNA inside the cell. As with other viral vectors, most viral genes can be substituted by the therapeutic genes and with the aid of a helper cell line that produces the viral capsid proteins *in trans,* a chimeric replicative deficient viral vector can be generated. These viral vectors are only capable of a dead-end infection, as they lack most of the essential components for viral propagation, which in addition reduces the risk of the appearance of recombination-induced

infectious virus. The main advantage of these types of vectors, shared with the adeno-associated viral vectors, is the long lasting expression of the transgene as they are integrative. However, this can also generate drawbacks as its integration can dysregulate endogenous gene expression which can generate tumors as in the case of the *ex vivo* SCID treatment. In this sense, efforts are being made to regulate the site of insertion of the transgenes (Dildine et al. 1998; Maguire-Zeiss and Federoff 2004). Another disadvantage of the retroviral vectors is that they have a limited capacity of transgene size that can be accommodated into its genome (around 8,3Kb for MuLV), and their inability to infect non-dividing cells. Thus they do not effectively infect neurons, muscle, liver or lungs. However, this characteristic can constitute an advantage in the case of suicidal gene therapy directed against tumors, where the dividing cells should be targeted. It must be noted that within this same family, the lentiviral-derived vectors are capable of transducing non-dividing cells, as for example the Human Immunodeficiency Virus (HIV), which in addition has been modified to be non-integrative (Yanez-Munoz et al. 2006).

Adeno-associated based viral (AAV) vectors are also being developed. They are small, non-pathogenic, single stranded DNA viruses of the *Parvoviridae* family, and have turned out to be in general an efficient and useful gene delivery vehicle (Carter and Samulski 2000), in particular for the CNS (Peel and Klein 2000). As already commented, sustained expression of transgenes can be obtained in several species and tissues using these vectors, however it is not clear what proportion of expression originates from integrated or extra-chromosomal DNA (Miao et al. 1998). Two of the main problems of these vectors are the difficulties to scale up the production and the restricted packaging capacity (around 4,5 Kb). Moreover, though they are less immunogenic than other viral vectors, the long-lasting expression of a therapeutic transgene in human clinical trials is still hampered by the host immune response (Manno et al. 2006).

The *Adenoviridae* viral (AdV) family consists of double chain DNA viruses that cause benign respiratory and eye infections in humans. After infection their genome remains episomal in the nucleus, and can transduce both dividing and non-dividing cells, including the CNS (Bajocchi et al. 1993). Replicative defective recombinant virus and the so termed "gutless" AdV can be generated in high titers with commercial-grade (Alba et al. 2005; Kochanek et al. 1996). When injected *in vivo*, these vectors can generate high levels of transgene expression, and their expression only lasts for 2 to 4 weeks in the majority of organs, although it can last for longer periods in some organs as the brain (Di Polo et al. 1998), or when modifications are made to diminish their immunogenic potential (Michou et al. 1997). One of the major problems of these vectors is the immunologic response against them (Michou et al. 1997), which can however constitute an advantage in the case of gene therapy against tumors where an exacerbation of the immunologic response is desired to eliminate the cells.

Other virus that have been used for generation of vectors are the herpes simplex virus (HSV) (Fink and Glorioso 1997) and the alphavirus (Xiong et al. 1989). The HSV has been

specially used for transduction of CNS due to their effectiveness in infecting neuronal cells where they can develop a latent state without integration into the host genome (During et al. 1994).

The CNS was for a long time considered to be immune privileged. More recently, the use of viral vectors has contributed to the demonstration that this is not the case, as both innate and adaptive arms of the immune system can be activated following viral gene therapy (Maguire-Zeiss and Federoff 2004). In fact, one of the biggest challenges that the viral vector faces is the immune response of the host, which is capable of eliminating transgene expression (Somia and Verma 2000). Cellular immunity eliminates the transduced cells, whereas humoral immunity precludes the repeated administration of the vector because of the subsequent antibody response that will be boosted by memory cells (Dai et al. 1995; Kafri et al. 1998). These responses are observed mostly against adenoviral vectors. In fact, retroviral, lentiviral and AAV vectors do not seem to suffer from cytotoxic-T cell responses. Studies examining the immune and inflammatory responses to viral vectors have begun to decipher the molecular mechanisms involved. A systematic evaluation of both the innate and adaptive responses of the immune system would enhance our understanding and aid in the development of safer vectors.

3.2. Non-viral vectors

Though viral vectors are very efficient gene therapy vectors, they show several limitations, in particular for the treatment of acute disorders. As mentioned, they can show some toxicity, immunogenicity, possibility of insertional mutagenesis, inadequate cell-targeting, limited packaging capacity, elevated cost of production, and risk of generation of infectious virus by recombination (Branca 2005; Maguire-Zeiss and Federoff 2004). In this context, the development of alternative non-viral vectors have experienced a boost, as they are normally easy to use and to produce in high scale, and they display a low or absent specific immunologic response (Aris and Villaverde 2004; Li and Ma 2001; Roth and Sundaram 2004). Efficient vectors need to be able to transfer the DNA to the cell nucleus sorting several critical steps: condensation and protection of the DNA from nucleases, internalization into the cell, releasing into the cytosol escaping in some cases from the endosomes, nuclear entry, and liberation of the DNA in the nucleus (Figure 3).

The simplest approach is the use of naked plasmids, which have shown an important degree of success under specific therapeutic and experimental paradigms. In 1990, Wolff and colleagues reported that intramuscularly injected naked plasmid DNA could be efficiently expressed in myofibers (Wolff et al. 1990). This technique has lead to several interesting developments like for example "DNA vaccination" (Danko and Wolff 1994), overexpression of erythropoietin (Fattori et al. 2005), anti-cancer therapy (He et al. 1998), treatment of Duchenne muscular dystrophy (Romero et al. 2004), and therapeutic angiogenesis for clinical limb or myocardial ischemia (Isner 2000; Vincent et al. 2000). Several further technical improvements have

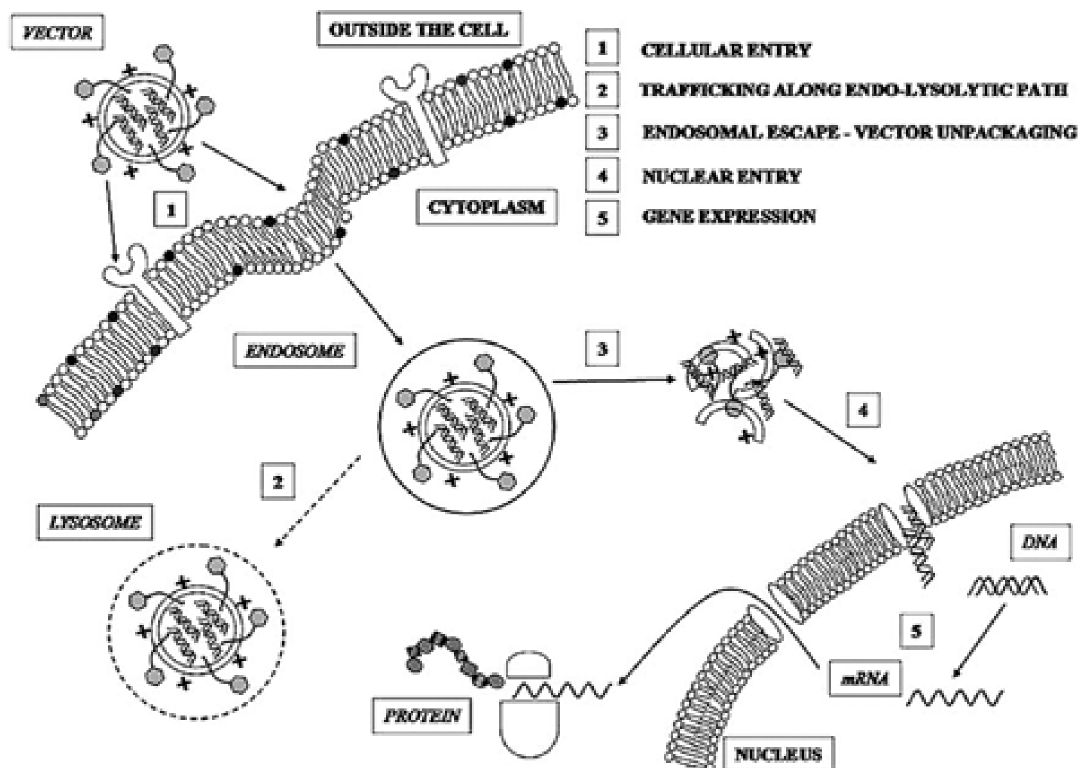

Figure 3. Critical steps for efficient transgene delivery (taken from Roth and Sundaram 2004)

increased the utility of naked plasmid injection like intravascular injection under high hydrostatic pressure (Budker et al. 1998), electroporation (which is being explored in CNS with some success, see Li and Ma 2001), and interaction with polymers (Mumper et al. 1996).

A further development in transfection efficiency was obtained by the addition to the DNA of several lipids like liposomes, micelles, emulsions, and other organized structures of lipids. Neutral and anionic liposomes encapsulate the DNA inside vesicles, while cationic liposomes suffer an extensive lipid rearrangement when mixed with DNA and are also called lipoplexes. In this manner, these molecules are thought to enter the cell by endocytosis or by fusing with cell membranes, and can transfect both dividing and non-dividing cells. These vectors possess some limitations, as although neutral or anionic liposomes are less toxic and more compatible with biological fluids than cationic liposomes, they have difficulties in entrapping the DNA, show insufficient interaction with cells, and lack of endosome disruption mechanisms. While some new lipids are more efficient than first generation cationic lipids, the goal of finding a "magic" lipid has been elusive. However, they have been used *in vivo* with some degree of success in selective settings, as tumor treatments, airway administration, intraperitoneal disease treatments, or direct intravenous administration (Li and Ma 2001), including for transfection of normal and acutely lesioned CNS (Iwamoto et al. 1996; Yang et al. 1997)(see Table 2). In fact, several clinical trials are ongoing and have shown some success in the treatment of cystic fibrosis (Alton et al. 1999),

melanomas (Nabel et al. 1993), or ovarian cancer (Hui et al. 1997). Liposomes have been combined with several molecules to increase transfection efficiency. For example, polycations as poly-L-lysine, which are capable of condensing the DNA protecting it from nucleases, were combined with DNA and cationic liposomes, improving the transfection efficiency (Gao and Huang 1996). In particular, these types of complexes can deliver a transgene to the CNS by direct intracranial injection and have been used in clinical trials (Leone et al. 2000), and can in addition induce transgene expression in many organs by intravenous injection (Li and Huang 1997). Finally, the combination of liposomes with antibodies can direct tissue-specific expression of transgenes, as is the case of antibodies anti-transferrin receptor coupled to liposomes, which can direct the expression of a transgene to the CNS after intravenous injection (Shi and Pardridge 2000).

3.3. Modular recombinant protein vectors

These vectors consist of a chimeric recombinant protein produced from a unique DNA sequence, that enables its purified production in scalable settings. The original gene of the scaffold protein is engineered to express a chimeric protein with several functional domains that enable the resultant protein to act as a gene therapy vector (reviewed in Aris and Villaverde 2004). The selection of the scaffold protein and the functional motifs, and an equilibrated combination and spatial distribution of such partner elements is essential for the development of an efficient vector for use in a particular situation. As introduced earlier, these gene delivery devices must perform relevant functions that mimic those of viruses; namely, nucleic acid condensation, targeted cell attachment and internalization, endosomal escape and nuclear transfer. Promising prototypes, able to deliver expressible DNA to tissue culture but also to specific cell-types in whole organisms are being generated.

In living cells, DNA is usually associated with cationic proteins, such as protamine and histones, as thermodynamically stable, tight complexes. In the gene therapy context, the condensed state of such interactions is essential for nuclease protection and stabilization in the extracellular media (Tagawa et al. 2002), and for efficient translocation to the cell nucleus and further transgene expression (Liu et al. 2003). Consequently, cationic peptide sequences, complete proteins or protein domains have often been incorporated into non-viral vehicles for DNA condensation. Among them, polylysine segments, either included as part of recombinant proteins (Aris et al. 2000), as synthetic free peptides (Harbottle et al. 1998) or coupled to viral proteins (Wagner et al. 1992), are probably the most widely used DNA ligands.

In addition to DNA attaching/condensing domains, the incorporation on vectors of powerful cell-binding domains for receptor-mediated uptake has been used, not only for strict cell or tissue targeting but also to promote robust cell attachment. Although selective receptor recognition would be more convenient in systemic delivery, promiscuous ligands could dramatically improve cell binding and internalization in local administration. Arg-Gly-Asp (RGD)-

containing integrin-binding domains are used by adenoviruses (Wickham et al. 1993a) and some picornaviruses (Evans and Almond 1998) for cell infection and have been exploited as efficient ligands in (i) whole viral capsid proteins (Carlisle et al. 2001), (ii) short peptides accommodated in multifunctional proteins (Aris et al. 2000; Aris and Villaverde 2003), (iii) synthetic proteins as tandem repeats (Hosseinkhani and Tabata 2003) or (iv) as part of DNA-condensing synthetic constructs (Harbottle et al. 1998). RGD-interacting integrins, are widely distributed, and thus represent attractive receptors in cases where high selectivity is not required. However, it has been also shown that RGD peptide tags, incorporated into intravenously administered bacteriophages, provide them with important tumor tropism (Pasqualini et al. 1997). Moreover, another factor that should also be carefully considered is the charge of the whole particle, and not only the specificity and presentation of the cell-binding domain, as it dramatically influences the efficiency of cell attachment (Schaffer and Lauffenburger 1998).

Receptor-mediated endocytosis generally results in the entrance of the ligands into the lysosomal pathway for their destruction; however, several gene therapy vectors are capable of enhancing gene delivery by escaping from this pathway. In fact, there are some specific endosome escaping motifs that are used among others by viral vectors (Wagner et al. 1992) (Medina-Kauwe et al. 2001) that can be engineered into modular protein vectors enhancing their transfection efficiency.

The nuclear membrane is an efficient barrier to naked DNA, being the nuclear pore complex in charge of nuclear transport by the recognition of short peptide sequences acting as nuclear localization signals (NLSs). Therefore, associated proteins would act as enhancers of nuclear transfer, provided they display such targeting signals. Several viral protein segments, like the SV40 NLS peptides from the T antigen (Fritz et al. 1996) or from the VP1 capsid protein (Navarro-Quiroga et al. 2002), and complete viral proteins like the adenovirus hexon protein (Carlisle 2002) enhance the transgene expression levels when incorporated into vectors.

Another important fact is that the synthesis of therapeutic amounts of recombinant proteins have been developed and extensively studied, in contrast to the problems encountered for the large-scale production of viral vectors (Wu and Ataai 2000) (Kamen and Henry 2004). Production bioprocesses are well established for bacteria, yeast and filamentous fungi, from which many GRAS (**g**enerally **r**ecognized **a**s **s**afe) species, used as recombinant hosts, would minimize the eventual presence of undesirable host by-products, such as pyrogenic compounds. Though some proteins are largely insoluble or toxic for the hosts systems, significant bottlenecks in the production would not be expected. Moreover, single step purification has been shown to be possible by several methods when including, as additional modules, standard purification signals for affinity chromatography, like the histidine tag (Uherek et al. 1998) or enzyme activities in the modular vectors. Although, in general, the efficiency of gene expression offered by non-viral vehicles has been lower than that achieved with viral vectors, the potential to improve their

efficiency (through further engineering), their biosafety, and the facilities for large-scale bioproduction prompt careful consideration of chimeric, multifunctional proteins as appealing alternatives to viral vectors. Also, for specific purposes, such as expression of growth factors, low levels of transgene expression would be more desirable, in particular in the CNS where no strategy of this kind has been applied. An important step would be to assess the potential neuroprotective efficacy of these vectors in different therapeutically relevant experimental settings, including acute neurological injuries.

The contribution of Antoni Villaverde, Anna Arís and colleagues has been critical for the development of these types of vectors. They used as scaffold protein the β-galactosidase of *E. Coli* for several reasons: it had been extensively studied and thus its three-dimensional structure is well known (Jacobson et al. 1994), it enables an efficient recombinant production in bacteria, it can be purified by activity columns, and it has an endogenous nuclear localization motif (McInnis et al. 1995). To enable cell attachment and internalization of the enzyme, they introduced an integrin interacting motif composed of the GH motif of the foot and mouth disease virus (23 amino acids from 134-156 of VP1 protein, serotype C, clone C-S8c1), which displays a prototypical RGD integrin-interacting motif (Mason et al. 1994; Villaverde et al. 1996). Finally, they also introduced to this modular protein a polylysine tail, which as has already been commented, permits condensation and attachment of the DNA to the vector. The recombinant modular vector so generated was termed 249AL and showed efficient *in vitro* gene delivery (Aris et al. 2000; Aris and Villaverde 2000). This Thesis took advantage of this interesting gene therapy approach for the delivery of transgenes, and evaluated their potential for neuroprotective gene therapy after immature CNS acute injury.

3.4. Gene therapy for acute neurological injuries

It is now well known that acute injuries to the CNS like hypoxia-ischemia due to stroke or cardiac arrest, sustained seizures, trauma, axotomy, and profound hypoglycemia alter gene expression and that some of the induced or downregulated genes may determine whether injured neurons will survive or will die. Among the many genes that have been identified to participate in these pathologies, those possessing neuroprotective properties would be good candidates for gene therapy (Dumas and Sapolsky 2001; Sapolsky 2003), and it has been shown that gene transfer using viral vectors or liposomes in experimental stroke and other acute injuries is feasible and that different degrees of neuroprotection can be achieved (Table 2). However, it is currently limited by the vector administration routes or by the regions or cell types that the vectors can transfect.

Noteworthy, functional recovery has generally not been addressed in these neuroprotection studies. This is an important point since the decrease on the number of apoptotic neurons or even the decrease on the lesion volume does not necessarily imply that a

functional recovery occurs (Dumas and Sapolsky 2001; Phillips et al. 2001). Some transfected genes act on very early steps as hyperexcitability (glutamate transporter downregulation or potassium channel overexpression), energy failure (glucose transporter Glut1 overexpression),

Table 2. Neuroprotective gene therapy after acute injuries to the CNS

Lesion model/age	*Therapeutic gene*	*Vector*	*Methodology*	*Effect*	*Reference*
Ischemia/adult	Bcl-2	Liposomes	Single immediate intrathecal injection 12µg DNA/anim.	30% decrease in lesion volume and apoptotic cells at 48 hrs.	(Cao et al. 2002)
Ischemia/adult	Bcl-2	AAV	Single intracerebral injection 1 hr. after ischemia	35% decrease in apoptotic cells in CA1 at 48 hrs.	(Shimazaki et al. 2000)
Ischemia/adult	Bcl-2	HSV	Single intracerebral injection 1,5 hs. after ischemia	60% decrease in neuronal degeneration (only transfected neurons) at 48 hrs.	(Lawrence et al. 1997)
Ischemia/	GDNF	AAV	Single immediate intracerebral injection	35% decrease in lesion volume at 72 hrs.	(Tsai et al. 2000)
Ischemia/adult	Midkine	AdV	Single intracerebral injection 90 min. after ischemia	40% decrease in lesion volume and apoptotic cells at 48 hrs.	(Takada et al. 2005)
Ischemia/adult	Hsp72	HSV	Single intracerebral injection 2 hrs. after ischemia	15% decrease in neuronal degeneration (only transfected neurons) at 48 hrs.	(Hoehn et al. 2001)
Ischemia/adult	GPx	HSV	Single intracerebral injection 5 hrs. after ischemia	25% decrease in neuronal degeneration (only transfected neurons) at 48 hrs.	(Hoehn et al. 2003)
Ischemia/adult	HGF	Liposomes	Single immediate injection in the cisterna magna	60% increase in survival of hippocampal neurons at 7 d.	(Hayashi et al. 2001)
TBI/adult	NGF	Liposomes	Single intracerebral injection of 12µg DNA/anim. 1 day after TBI	Prevents degeneration of septal cholinergic neurons at 14 d.	(Zou et al. 1999)
Excitotoxicity/adult	NGF	Tf-lipoplexes	Single immediate intracerebral injection of 5µg DNA/anim.	60% decrease in lesion volume 48 hrs. after and no lesion observed at 7 d.	(da Cruz et al. 2005)
Excitotoxicity/adult	Calbindin D28K	HSV	Single immediate intracerebral injection	40% decrease in lesioned area at 72 hrs.	(Phillips et al. 1999)
Excitotoxicity/adult	SK2 or Kv1.1	HSV	Single immediate intracerebral injection	70 and 30% resp. decrease in lesioned area at 72 hrs. and reduced seizures	(Lee et al. 2003)
Excitotoxicity/adult	Glut1	HSV	Single intracerebral injection 1 h. after	30% decrease in lesioned area at 48 hrs.	(Lawrence et al. 1995)
Excitotoxicity/adult	Glut1	HSV	Single immediate intracerebral injection	Hippocampal function improvement	(McLaughlin et al. 2000)
Nerve crush injury/adult	GDNF	AdV	Single immediate injection at the nerve crush site	Functional and histological improvements at 4 weeks	(Araki et al. 2006)
Spinal cord hemisection/adult	Bcl-2	Lipofecta-mine	Single immediate injection at the lesioned nucleus of 16.7µg DNA/anim.	Prevents neuronal death and decreases atrophy at 2 months	(Takahashi et al. 1999)
6-hydroxydopamine injection/adult	TH	HSV	Single intracerebral injection after lesion	Increased striatal levels of TH and dopamine, and 65% recovery in rotation behavior at 1 year	(During et al. 1994)
6-hydroxydopamine injection/adult	TH	Pegylated TfR-immunolip.	Single intravenous injection 4 weeks after of 10µg DNA/anim.	Normalization of striatal TH levels and 80% reduction in rotation behavior at 3 d.	(Zhang et al. 2004)

Only studies injecting the vector after the lesion were considered. Abbreviations: calcium-activated potassium channel (SK2), voltage-gated potassium channel (Kv1.1), tyrosine hydroxylase (TH), transferrin (Tf), transferrin receptor (TfR), nerve growth factor (NGF), glial derived growth factor (GDNF,) brain derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), B-cell leukemia/lymphoma 2 (Bcl-2), glucose transporter 1 (Glut1), glutathione peroxidase (GPx), traumatic brain injury (TBI), adeno-associated viral vector (AAV), adenoviral vector (AdV), herpex simplex viral vector (HSV).

calcium excess (calbindin D28K overexpression), or oxidative stress (glutathione peroxidase overexpression). Other genes target later events as protein damage/miss-folding (heat shock protein Hsp72 overexpression), or the apoptotic machinery (overexpression of antiapoptotic genes like Bcl-2 or NAIP)(see Table 2). Finally, another useful strategy is targeting of inflammatory cascades that amplify secondary damage (like overexpression of interleukin 1 receptor antagonist, IL1-Ra). Importantly, some of these transgenes maintain the neuronal body, but do not have an effect on its neurite arborizations or neurotransmission machinery, while others are able to maintain the functionality of the neuron integrated in its circuit, therefore inducing different degrees of functional recovery (McLaughlin et al. 2000).

Another important factor for gene therapy is that overexpression of therapeutic proteins acting in the extracellular compartment, like trophic factors or cytokines, will be able to diffuse in the lesioned brain parenchyma, and thus will have increased probabilities of success. In addition, these type of molecules act normally at very low concentrations. In particular, neuroprotection from excitotoxicity to the immature brain can be induced by lentiviral-mediated overexpression of diffusible molecules as BDNF (Bemelmans et al. 2006) or adenoviral overexpression of IL1-Ra (Hagan et al. 1996).

Thus, significant advances have been made regarding gene therapy for acute CNS injuries, but there is still need for flexible, safe, and more effective vectors, that in addition show functional recovery. Moreover, the development of these vectors offers wide opportunities for basic research models and experimental interventions. When I begun with this Thesis work, some non-viral vectors had been analyzed for *in vivo* gene delivery to the CNS, but to our knowledge no study had been published showing gene delivery to the brain using modular recombinant vectors, nor neuroprotective gene transfer by means of this type of vectors. In fact, out of the CNS, just a few examples of functional improvements with recombinant vectors had been shown (Ferkol et al. 1993; Sato et al. 2000).

General aim

This Thesis intends to contribute to the knowledge of the neurodegenerative mechanisms of the acutely damaged immature brain, in particular those related with oxidative stress and inflammation. Moreover, it undertakes the development of neuroprotective strategies involving antioxidant genes by using non-viral modular recombinant non-pro-inflammatory gene therapy vectors.

Specific aims

1) Evaluate the time course and the cell specific expression of Cu/Zn SOD after acute excitotoxic immature brain injury.
2) Study the possible production of nitrotyrosine and its location after an acute excitotoxic injury to the immature brain.
3) Evaluate if the overexpression of Cu/Zn SOD after an excitotoxic lesion to the immature brain is neuroprotective.
4) Assess whether non-viral modular recombinant protein vectors are suitable for gene therapy to the excitotoxically injured immature brain.

Article number 1:

Cu/Zn Superoxide Dismutase Expression in the Postnatal Rat Brain Following an Excitotoxic Injury

HUGO PELUFFO, LAIA ACARIN, MARYAM FAIZ, BERNARDO CASTELLANO, and BERTA GONZÁLEZ

Overview

As oxidative stress is a main contributor to cell death and inflammation, this Thesis characterized the temporal and cell-specific expression of the Cu/Zn SOD after excitotoxic damage to the immature brain, which had not been described before. This study was also necessary for the subsequent design of a gene therapy strategy based on the overexpression of Cu/Zn SOD. We found that Cu/Zn SOD was very rapidly downregulated in the lesion core after 4 hours of excitotoxic injury, and increased from 24 hours after, being upregulated mainly in reactive astrocytes until 7 days after, the last time analyzed. Thus, these results show that when most of the reactive oxygen species are formed, Cu/Zn SOD is degraded, and thus there is a temporally window when Cu/Zn SOD overexpression could be neuroprotective after an excitotoxic injury. In addition, we provided a careful description of which cell types express this enzyme in the immature brain, when the antioxidant enzymes levels are still being changing to reach adult levels.

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Cu/Zn superoxide dismutase expression in the postnatal rat brain following an excitotoxic injury

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Abstract

Background: In the nervous system, as in other organs, Cu/Zn superoxide dismutase (Cu/Zn SOD) is a key antioxidant enzyme involved in superoxide detoxification in normal cellular metabolism and after cell injury. Although it has been suggested that immature brain has a different susceptibility to oxidative damage than adult brain, the distribution and cell-specific expression of this enzyme in immature brain and after postnatal brain damage has not been documented.

Methods: In this study, we used immunohistochemistry and western blot to analyze the expression of Cu/Zn SOD in intact immature rat brain and in immature rat brain after an NMDA-induced excitotoxic cortical injury performed at postnatal day 9. Double immunofluorescence labelling was used to identify Cu/Zn SOD-expressing cell populations.

Results: In intact immature brain, Cu/Zn SOD enzyme was widely expressed at high levels in neurons mainly located in cortical layers II, III and V, in the sub-plate, in the pyriform cortex, in the hippocampus, and in the hypothalamus. Glial fibrillary acidic protein-positive cells only showed Cu/Zn SOD expression in the glia limitans and in scattered cells of the ventricle walls. No expression was detected in interfascicular oligodendroglia, microglia or endothelial cells. Following excitotoxic damage, neuronal Cu/Zn SOD was rapidly downregulated (over 2–4 hours) at the injection site before neurodegeneration signals and TUNEL staining were observed. Later, from 1 day post-lesion onward, an upregulation of Cu/Zn SOD was found due to increased expression in astroglia. A further increase was observed at 3, 5 and 7 days that corresponded to extensive induction of Cu/Zn SOD in highly reactive astrocytes and in the astroglial scar.

Conclusion: We show here that, in the intact immature brain, the expression of Cu/Zn SOD was mainly found in neurons. When damage occurs, a strong and very rapid downregulation of this enzyme precedes neuronal degeneration, and is followed by an upregulation of Cu/Zn SOD in astroglial cells.

Background

It has been shown that ~2–5% of the electron flow in isolated brain mitochondria produces superoxide anion radicals ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) [1]. Cellular levels of $O_2^{-\cdot}$ are normally low due to the action of cytosolic copper zinc superoxide dismutase (Cu/Zn SOD) and mitochondrial manganese superoxide dismutase (Mn SOD). These key enzymes catalyze the dismutation of $O_2^{-\cdot}$ to oxygen and H_2O_2 [2]. Increased production of superoxide and its derivatives can induce injury by diverse mechanisms including initiation of lipid peroxidation, inactivation of enzymes, damage to DNA, and protein sulfhydryl oxidation. In particular, in the presence of nitric oxide (NO·), $O_2^{-\cdot}$ and NO· rapidly and spontaneously react to form the potent oxidant peroxynitrite ($ONOO^-$), which is capable of nitrating tyrosine [3,4] contributing to the neuropathological process [5]. In this sense, superoxide radicals have been identified as important mediators of oxidative injury during ischemia-reperfusion and many other neurological injuries [6]. Cerebral ischemia [7-9] and traumatic brain injury [10] cause a rapid and sustained increase in the formation of $O_2^{-\cdot}$, which is accelerated during mitochondrial dysfunction, and may also result from increased activity of several cytosolic enzymes as phospholipase A_2 [11] or cycloxygenase 2 (COX2) [7].

In the adult central nervous system (CNS), Cu/Zn SOD is widely expressed in different neuronal populations: hippocampal CA pyramidal neurons and granule neurons of the dentate gyrus, cortical neurons, especially pyramidal cells, neurons of the substantia nigra, and at very high levels in motor neurons of the spinal cord [12-17]. In regards to glial cells, it is generally assumed that microglial cells and oligodendrocytes do not show expression of Cu/Zn SOD [12,16-19], but the astroglial expression of this enzyme is controversial. Some studies have found Cu/Zn SOD expression primarily and most intensely in astrocytes [18,19], but a number of works have failed to detect this expression in astrocytes [13-15,17], or have reported expression only in some scattered astrocytes [12,16]. On the other hand, expression of Cu/Zn SOD has been well documented in reactive astrocytes several days after different types of adult brain injury, like transient cerebral ischemia [13], kainate treatment [14], quinolinic treatment [18], or Alzheimer's and Down's Syndrome [16].

According with its antioxidant role, in most adult CNS injury models the over-expression of Cu/Zn SOD is thought to be neuroprotective [20-22]. In agreement, synthetic $O_2^{-\cdot}$ dismuting metalloporphyrins protect against transient middle cerebral artery occlusion [23], and targeted deletion of the Cu/Zn SOD or extracellular SOD genes worsens outcome after focal ischemia [24,25]. However, contradictory results have been reported regarding the toxicity of $O_2^{-\cdot}$ after hypoxic/ischemic injury to the immature brain. Whereas several antioxidant molecules including SOD mimetics (as $O_2^{-\cdot}$ dismuting metalloporphyrins) have been shown to be neuroprotective [26], a slightly worsened neuropathological outcome is observed in transgenic mice over-expressing Cu/Zn SOD [27]. Several other differences in regards to oxidative stress and antioxidant defences have been reported in the immature versus the mature brain. For example, and in comparison to the adult brain, in the immature damaged brain glutathione peroxidase is not upregulated after trauma [28]; free iron accumulates more rapidly within 4 hours after transient cerebral ischemia stimulating Fenton reactions [29,30]; and metallothioneins, potent antioxidant enzymes that bind transition metals as Zn, are less concentrated [31,32]. Finally, the postnatal brain is more sensitive than the adult brain to the neurotoxic actions of N-methyl-D-aspartate (NMDA) [33], which will lead to increased $O_2^{-\cdot}$ generation [34,35]. Interestingly, it has been reported that Cu/Zn SOD is rapidly downregulated after several types of injury in the mature CNS [13,14,36,37], but no data is available regarding this phenomenon in immature brain, where overall findings suggest a differential scenario for oxygen and nitrogen reactive species in the evolution of a brain injury [38].

In this context, the aim of our study was to evaluate the temporal and spatial dynamics, and the identity of Cu/Zn SOD expressing-cells, in the intact immature rat brain, and following an excitotoxic injury.

Methods

Excitotoxic lesions

Nine-day-old Long-Evans black-hooded rat pups of both sexes were placed in a stereotaxic frame adapted for newborns (Kopf) under isofluorane anaesthesia. The skull was opened using a surgical blade, and 0.15 μl of saline solution (0.9% NaCl, pH 7.4) containing 18,5 nmol of N-methyl-D-aspartate (NMDA) (Sigma, M-3262, Germany) were injected into the right sensorimotor cortex at the level of the coronal suture (2 mm lateral from bregma and at 0.5 mm of depth). Control animals received an injection of 0.15 μl of vehicle saline solution. After suture, pups were placed in a thermal pad and maintained at normothermia for 2 hours before being returned to their mothers. Experimental animal work was conducted according to Spanish regulations, in agreement with European Union directives. This experimental procedure was approved by the ethical commission of the Autonomous University of Barcelona. All efforts were made to minimize animal suffering in every step.

Immunocytochemical study

Intact immature brains from P9, P10, P12, and P16 rats of both sexes where used for the analysis of Cu/Zn SOD

expression under physiological conditions (2–4 animals per time). Moreover, at 2, 4, and 10 hours, and 1, 3, 5 and 7 days after NMDA (3–4 animals per time) or saline injection (2 animals per time), rats were anaesthetized and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed in the same fixative for 2 hours and sunk in a 30% sucrose solution before being frozen with dry CO_2. Coronal sections (30-μm-thick) were obtained using a Leitz cryostat. Free-floating parallel sections were treated with 10% foetal calf serum in Tris-buffered saline (TBS: 0.05 M Trizma base containing 150 mM of NaCl, pH 7.4) +1% triton X-100 for 1 hour, and incubated overnight at 4°C with sheep polyclonal anti-Cu/Zn SOD (574597, Calbiochem, Darmstad, Germany) (1:300) in the same solution. Afterwards, sections were rinsed and incubated for 1 hour at room temperature with Cy3-conjugated anti-sheep secondary antibody (AP147C, Chemicon, California, USA) (1:150). As negative controls, sections were incubated in media lacking the primary antibody.

Double labelling with specific cell markers

Double labelling was carried out in order to identify Cu/Zn SOD-expressing cells in sections previously immunoreacted for Cu/Zn SOD as reported above.

For neuronal identification, sections were further incubated with a monoclonal anti-NeuN antibody (MAB377, Chemicon, California, USA) (1:1000) and Cy2-conjugated anti-mouse secondary antibody (PA-42002, Amersham Pharmacia Biotech, England) (1:1000).

For astroglial labelling, sections were incubated with a polyclonal anti-GFAP antibody (Z-0334, Dakopatts, Denmark) (1:1800), and immunostaining was visualized with Cy2-conjugated anti-rabbit secondary antibody (PA-42004, Amersham Pharmacia Biotech, England) (1:1000).

As a microglial marker, sections were processed for double staining with tomato lectin histochemistry by incubating with the biotinylated lectin from Lycopersicon esculentum (tomato) (Sigma, L-9389, Germany) diluted to 6 μg/ml, followed by Cy2-conjugated streptavidin (PA-42000, Amersham Pharmacia Biotech, England) (1:1000).

Selected sections of all double labelling techniques were incubated for 5 min with a 0.00125 μg/ml solution of 4, 6-diamino-2-phenylindole (DAPI) in TBS. Double-stained sections were analyzed using a Nikon Eclipse E600 epifluorescence microscope and a LEICA TCS SP2 AOBS confocal microscope.

TUNEL labelling

Terminal dUTP Nick End Labelling (TUNEL) staining for detection of DNA fragmentation was performed on parallel sections mounted on slides. Tissue sections were rinsed in Tris buffer (10 mM, pH 8) and EDTA (5 mM) and then incubated in the same buffer plus Proteinase K (20 μg/ml) for 15 min. at room temperature. After several washes with EDTA (5 mM), sections were incubated for 10 min. in TdT buffer (Tris 30 mM, 140 mM Sodium Cacodilate, 1 mM Cobalt chloride, pH 7.7). Sections were then incubated in TdT buffer plus 0.161 Units/ μl TdT enzyme (Terminal Transferase, 3333566 Roche, Manheim, Germany) and 0.0161 nmol/ μl of biotin-16-dUTP (1093070, Roche, Manheim, Germany) for 30 min. at 37°C. The reaction was stopped by washing the sections in citrate buffer (300 mM sodium chloride, 30 mM sodium citrate, 5 mM EDTA). After several washes with TBS, sections were incubated with avidin-peroxidase (P-0364, Dakopatts, Denmark) (1:400) for another hour at room temperature. Finally, the peroxidase reaction product was visualised in 100 ml of Tris buffer containing 50 mg of 3'-diaminobenzidine and 0.01% of hydrogen peroxide.

Western blotting and densitometry

Three or four NMDA-injected and two intact and saline-injected animals for each survival time were decapitated, and the complete injected cortex quickly extracted, chopped and frozen in liquid nitrogen. Samples were resuspended in Tris/HCl (50 mM, pH7.8), EDTA (1 mM), DTT (1 mM), PMSF (100 μg/ml), pepstatin A (2 μg/ml), leupeptin (2 μg/ml), trypsin inhibitor (10 μg/ml), benzamidine (0.2 mM) and submitted to mechanical dissociation. Total protein concentration was measured by the bicinchoninic acid method and equal quantities of protein were run on a 15% SDS-polyacrilamide gel electrophoresis (SDS-PAGE). Electrotransfered protein samples to polyvinylidene fluoride (PVDF) membranes were incubated overnight at 4°C with TBS+0.3% Tween 20 and 5% non-fat milk, and for 2 hours at room temperature with sheep polyclonal anti-Cu/Zn SOD (574597, Calbiochem, Darmstad, Germany)(1:1000). Membranes were rinsed and incubated in biotinylated anti-sheep antibody (1:1000) (RPN-1025, Amersham Pharmacia Biotech, England), avidin-peroxidase (1:2000) (P0364, Dakopatts, Denmark) and finally in the chemiluminiscent substrate SuperSignal West Pico (PIERCE) combined with exposure on Hyperfilm ECL (Amersham).

Semi-quantitative estimation of western blot protein signals were performed by measuring band integral intensity with analySIS® software after high resolution scanning.

Statistical analysis

All results are expressed as mean ± standard error mean (SEM). ANOVA followed by Fisher's PLSD post-hoc test

was used to determine significant differences ($p<0.05$) after western blot densitometry.

Results

Cu/Zn superoxide dismutase expression in the immature rat brain

As there is no available description of Cu/Zn SOD cell-specific expression in the immature brain, we began our study with this general description. No changes in the distribution pattern and cellular identity of Cu/Zn-expressing cells were found between P9 to P16 and thus will be described in general. The immature rat brain showed widespread immunoreactivity for Cu/Zn SOD, mainly located in neurons. The most intense staining was observed in pyramidal neurons located in cortical layers V, III and II (Fig. 1A, B), in the pyriform cortex, and in the pyramidal neurons of the olfactory tubercle. Sub-plate neurons, a transient cell population located beneath the cortical plate [39] were also among the most intense cells (Fig 4A). In addition, hippocampal CA pyramidal neurons and some inter neurons (Fig. 1C), as well as dentate gyrus granular and sub-granular neurons, medial septum neurons, and hypothalamic neurons also displayed intense staining. There was also more diffuse staining in many other neuronal populations as for example in various thalamic nuclei, substantia nigra (Fig. 1D), and striatum. Most of the immunoreactivity was mainly observed in neuronal soma although less intense staining was also present in the nuclei, sparing the nucleolus.

No specific Cu/Zn SOD immunoreactivity was detected in parenchymal grey matter GFAP-positive astrocytes (Fig 2A). However, co-expression of Cu/Zn SOD and GFAP was observed in scattered tanycytes of the third ventricle wall (Fig 2B), in *corpus callosum*, and in astrocytes forming the glia limitans (Fig 2C). Most ciliated ependymal cells expressed Cu/Zn SOD (Fig. 2B). Moreover, besides the ependymal cells described above, co-localization confocal studies with tomato lectin, which specifically detects microglial cells as well as endothelium [40], did not show overlap with Cu/Zn SOD staining (Fig 2D). No consistently Cu/Zn SOD-labelled cells were observed in white matter tracts, which indicated that oligodendrocytes at this location do not seem to express this enzyme.

Finally, western blots showed a significant developmental increase in the total amount of brain Cu/Zn SOD between P9 and P16 (Fig. 3B), evidenced as a single band of 16 KDa corresponding to the expected molecular mass of the enzyme monomer.

Control intracortical saline injection caused a local and transient increase in Cu/Zn SOD immunoreactivity mainly in cortical neurons (data not shown) 10 hours later surrounding the injection site. A trend towards a rapid and transient increase could also be observed by western blots (Fig 3B).

Cu/Zn superoxide dismutase expression after excitotoxic injury to the immature brain

As shown before, intracortical NMDA administration is a model of excitotoxic damage that triggers rapid neuronal death and tissue injury, which expands rostro-caudally and includes part of the cortex, *corpus callosum*, dorsal striatum, septum and rostral hippocampus [33,41].

In contrast to saline injected animals, as observed both by western blot tendencies (Fig. 3) and immunohistochemistry (Fig. 4, 5) at 2–4 hours after NMDA injection, the lesioned neural parenchyma showed a drastic downregulation of total Cu/Zn SOD immunoreactivity in neuronal cells, which returned to basal levels 24 hours after the lesion and increased significantly thereafter due to expression in astroglial cells. Interestingly, the early decrease in neuronal Cu/Zn SOD immunoreactivity at 2–4 hours (Fig. 4A–B) was accompanied by a slight condensation of nuclear chromatin (Fig. 4C–D) and changes in NeuN neuronal marker (Fig. 4F–G), in the absence of apparent signs of neuronal degeneration or TUNEL staining (Fig. 4H). At later time points, from 10 hours, degenerating neurons still showed downregulated Cu/Zn SOD immunoreactivity, but displayed condensed nuclei, nuclear blebbing (Fig. 4E) and also DNA fragmentation observed by TUNEL staining (Fig. 4I). Downregulation of Cu/Zn SOD and signs of neuronal degeneration were observed from 10 hours in primary cortical degenerating areas and from 1 day post-lesion in secondary degenerating regions such as the cortical, striatal and hippocampal penumbra (Fig. 5A). However, the total Cu/Zn SOD enzyme level increased from 1 day post-lesion, reaching a maximum induction after 3–7 days (Fig 3B and 5B), due to astroglial upregulation of the enzyme. Cu/Zn SOD was induced in the soma and proximal projections of the most hypertrophied and most intensely GFAP-immunopositive astroglial cells of the whole degenerative area including the white matter (Fig. 5C), but not in slightly activated astrocytes with lower GFAP immunoreactivity. This elevated expression continued at 5 and 7 days post-lesion when most astrocytes of the degenerative area were strongly hypertrophic and formed the glial scar (Fig. 5D). Only scattered reactive microglial cells expressed Cu/Zn SOD at 3 days after lesion, but most reactive microglial cells of the lesioned parenchyma remained negative.

Discussion

In the last decade, progress has been made regarding the complex evolution of damage in the developing brain [38] and how it differs with adult brain injury. In this sense, one of the divergences proposed is that immature brain deals poorly with oxidative stress. In this study, we

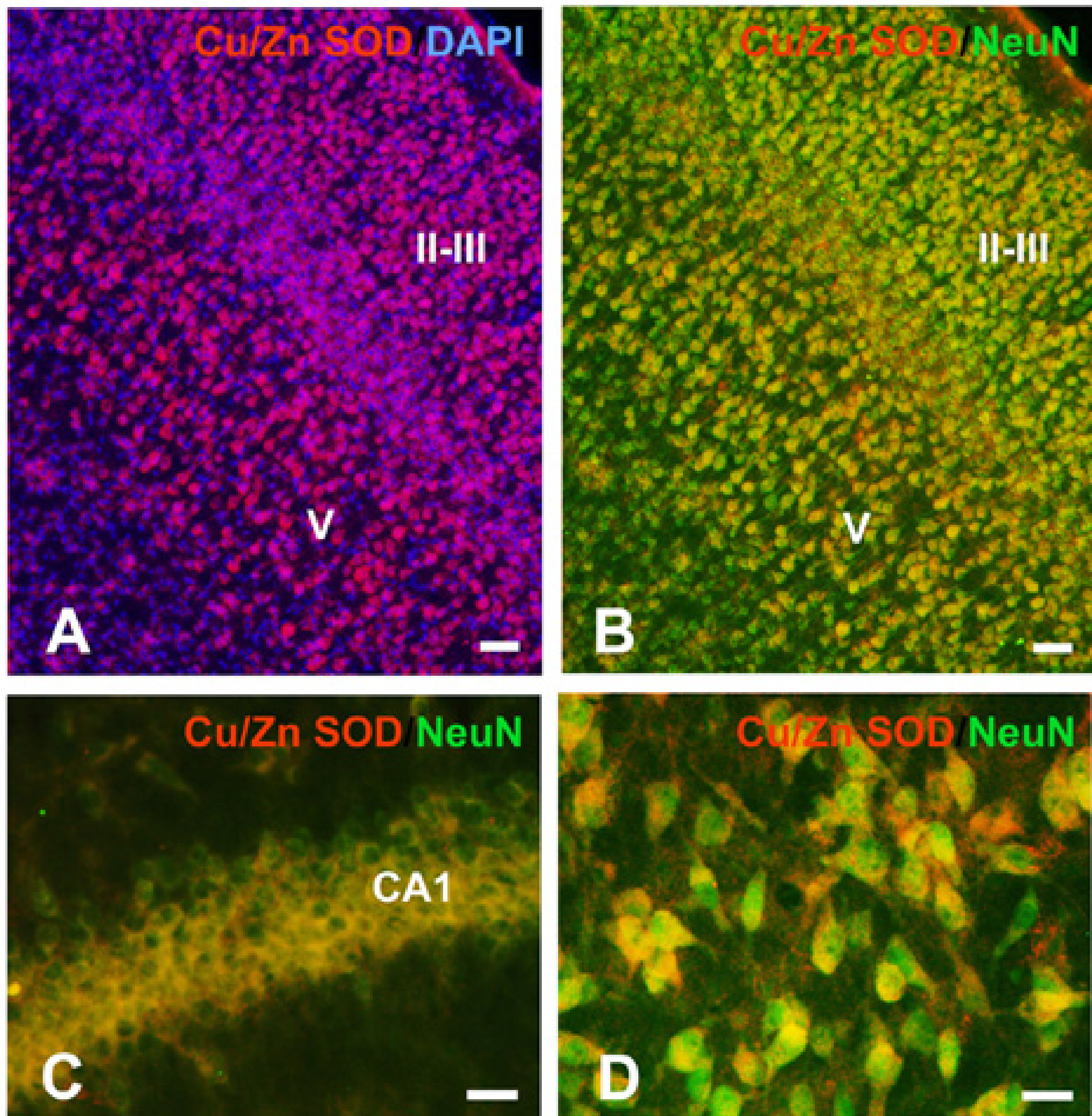

Figure 1

Neuronal Cu/Zn SOD immunoreactivity in intact immature brain. Widespread immunoreactivity for Cu/Zn SOD was observed mainly in neurons, both in the cytoplasm and nucleus, sparing the nucleolus. In the cortex, the highest expression was observed in cortical pyramidal neurons of layers V and III and II (**A**, **B**: Parietal cortex). In addition, the hippocampal pyramidal layer (CA1; **C**), and of substantia nigra (**D**) also displayed immunoreactivity. Scale bars: 50 μm in A and B; 20 μm in C and D.

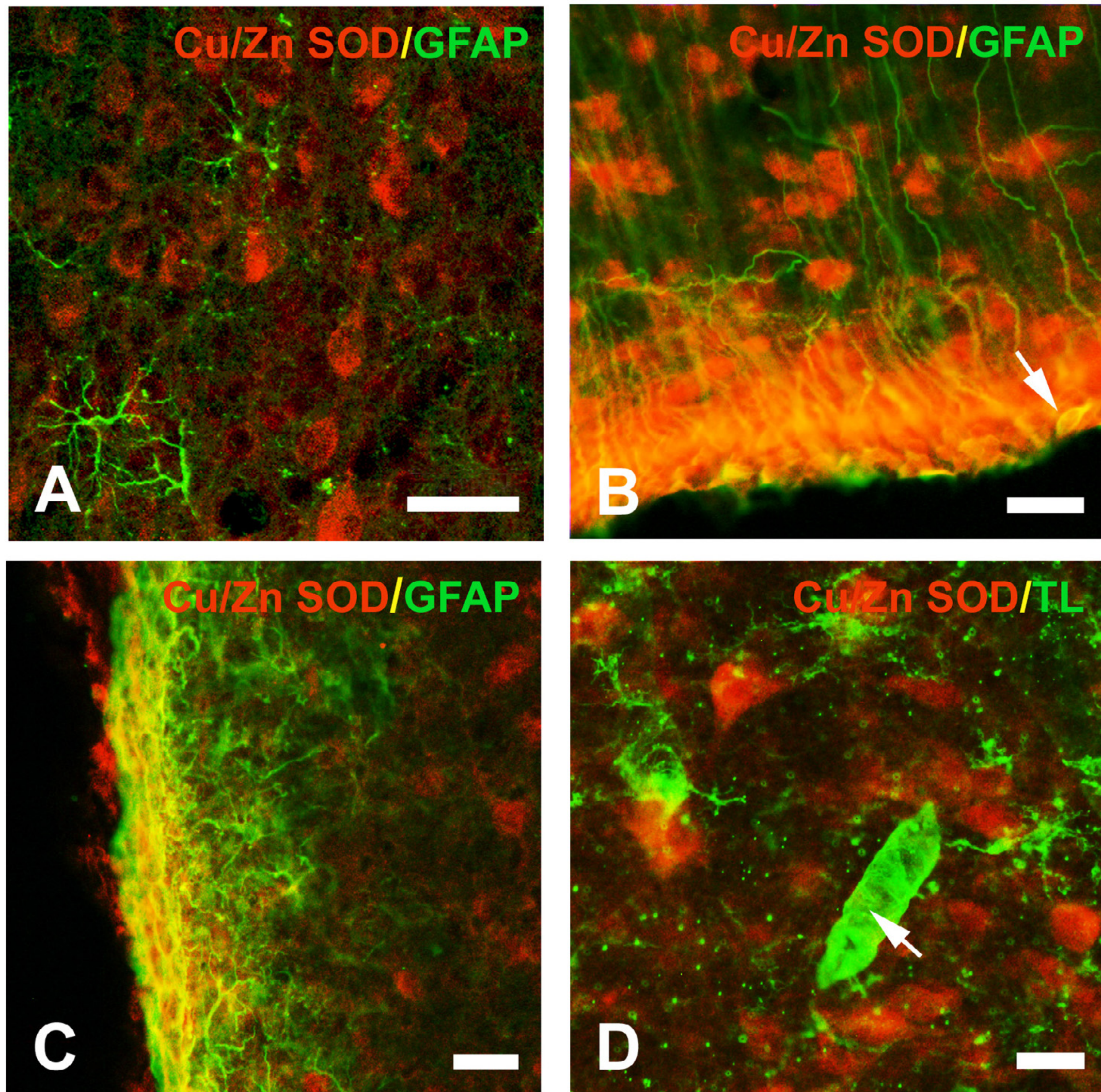

Figure 2

Glial distribution of Cu/Zn SOD immunoreactivity in intact immature brain. Cu/Zn SOD was not observed in the GFAP-expressing astrocyte population of the brain parenchyma (**A**: cortex, confocal image). However, GFAP-expressing astrocytes showed co-localization with Cu/Zn SOD in the glia limitans (**C,** confocal image) and in the ventricle walls (**B**: third ventricle; arrow: tanycyte). Microglial cells and endothelium (arrow), identified with tomato lectin histochemistry, were negative for Cu/Zn SOD (**D**: cortex; confocal image). Scale bars in A: 40 μm; in B, C and D: 20 μm.

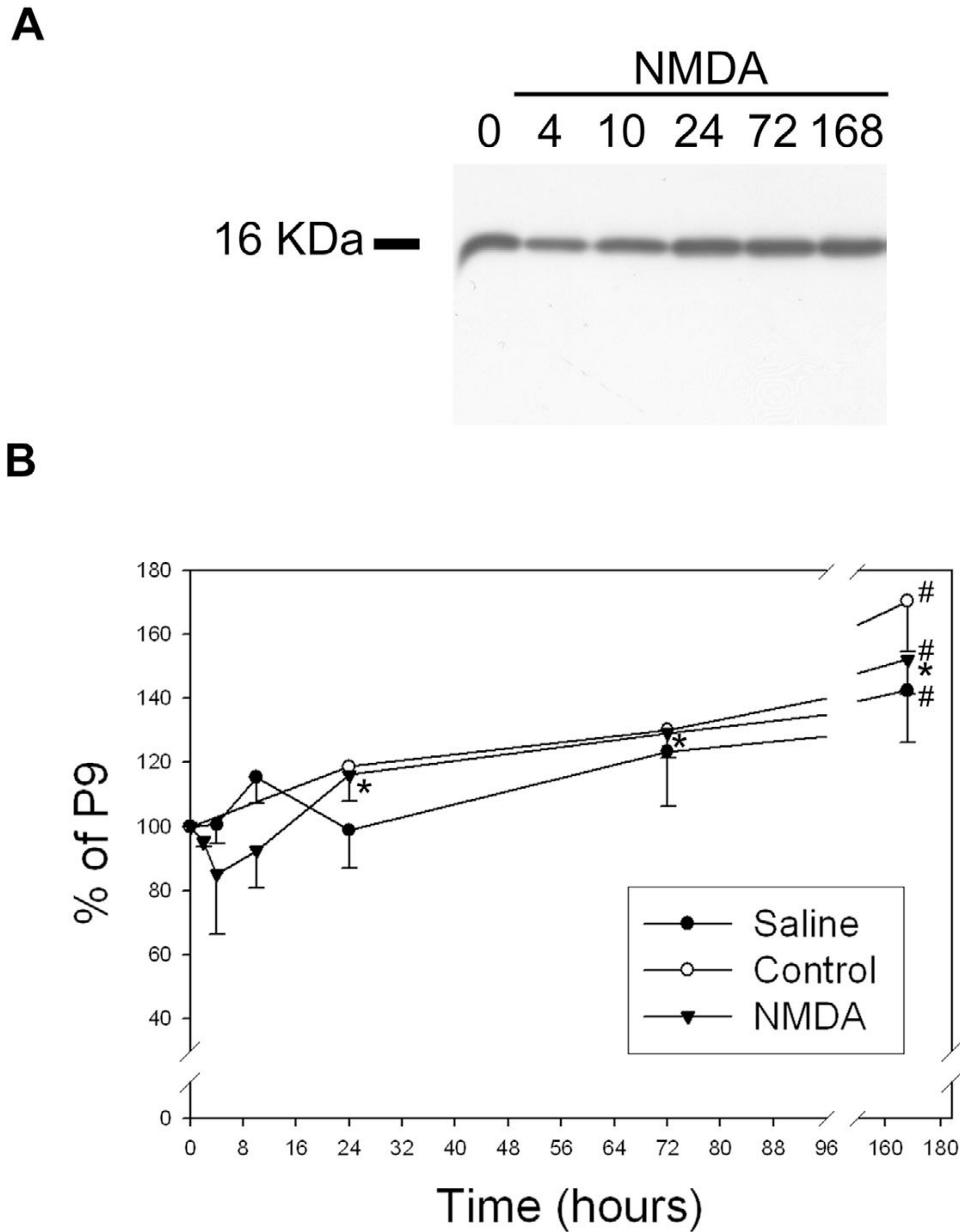

Figure 3
Total Cu/Zn SOD expression in control, and saline or NMDA injected postnatal cortex. Western blot (**A**) and semi-quantification of total levels of Cu/Zn SOD protein from intact, saline-injected, or NMDA-injected brain cortical extracts are represented as percentage of Cu/Zn SOD level in P9 (**B**). A single band of the expected molecular weight of 16 KDa of the Cu/Zn SOD monomer was observed in all samples (**A**). The intracortical injection of saline solution induced a trend towards the transient upregulation of Cu/Zn SOD which showed a peak at 10 hours post-injection (**B**: solid circles). In contrast, after NMDA injection, total cortical Cu/Zn SOD expression showed a trend towards rapidly diminishing (**A**, **B**: triangles). This trend of Cu/Zn SOD reduction at 4 hours after NMDA injection in total cortical extracts is comparable to the % of lesioned cortex which was around 20% (not shown). However, at later time points, Cu/Zn SOD was significantly upregulated peaking at 7 days (168 hours) post-lesion. In intact control brain (**B**: opened circles), total enzyme level significantly increased from postnatal day 9 to postnatal day 16. (# $p < 0.05$ in relation to P9 control animals and * $p < 0.05$ in relation to NMDA 4 hours).

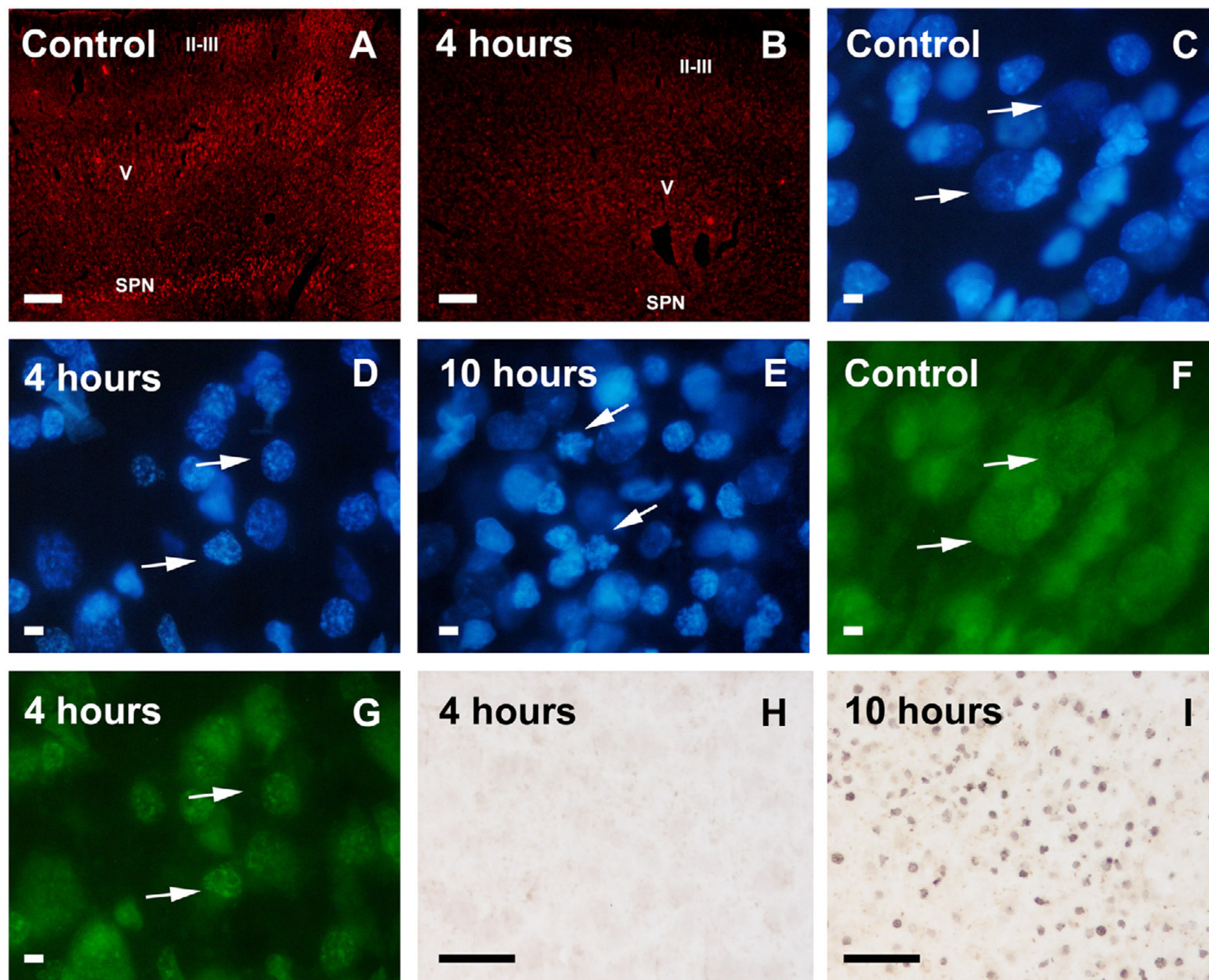

Figure 4

Cell-specific Cu/Zn SOD expression in the early excitotoxicaly lesioned immature brain. In the injection site, Cu/Zn SOD immunoreactivity decreased from 2–4 hours after the NMDA injection, and continued to be downregulated until 24 hours after injection (**A-B**,**SPN**: sub-plate neurons, **V** and **II-III**: cortical layers). Neurons from the injected cortex after 4 hours showed slightly condensed chromatin (compare **C** and **D**: arrows), and a shift of NeuN staining from the soma and nucleus towards the nucleus (compare **F** and **G**: arrows) in the absence of TUNEL staining (**H**). From 10 hours after lesion onward, neuronal nuclei showed clear apoptotic signals like condensation and blebbing (**E**: arrows) and TUNEL staining (**I**). Scale bars in A-B: 200 μm; in C-G 5 μm; and in I-H: 40 μm.

have shown that the expression of the key antioxidant enzyme Cu/Zn SOD is mainly found in neuronal cells. When damage occurs, a strong neuronal downregulation of this enzyme precedes both neuronal cell death and the subsequent Cu/Zn SOD upregulation in astroglial cells.

Cu/Zn SOD in the intact immature brain

It is well known that total brain levels of antioxidant enzymes vary throughout life [32,41,42]. Accordingly, brain glutathione peroxidase and Mn SOD were reported to increase during the first month of postnatal rat life, and to continue increasing slightly during adulthood and aging [28,43-46]. Catalase has been reported to peak around the first week of life and then decline to reach a plateau by the first month [43,44]. Regarding Cu/Zn SOD, it is known that its levels increase rapidly after birth, peaking around the second postnatal week, in agreement with our western blot studies. Later on, Cu/Zn SOD decreases

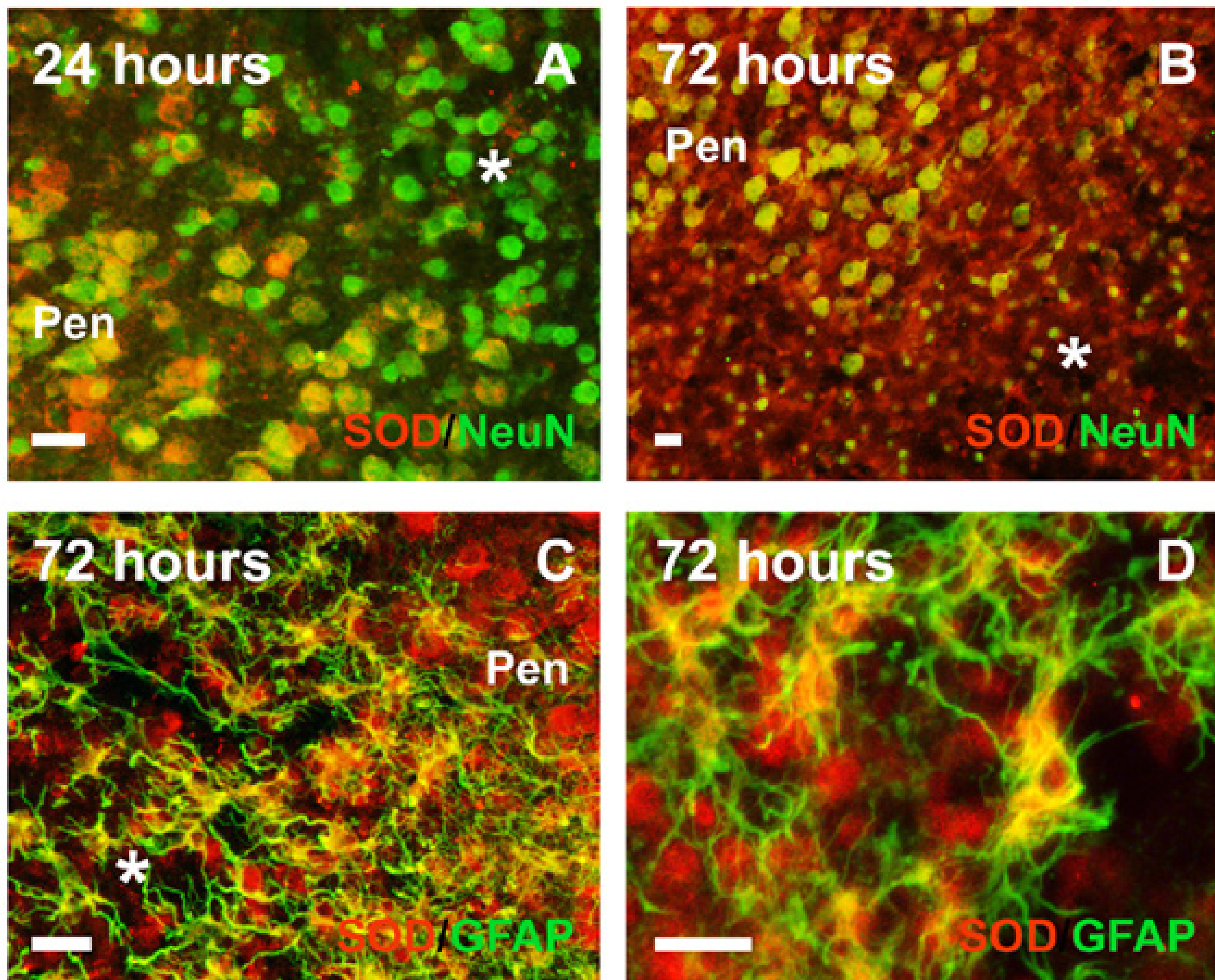

Figure 5
Cell-specific Cu/Zn SOD expression in the late excitotoxicaly lesioned immature brain. One day after the lesion (**A**), neuronal bodies were condensed in the lesioned zone (*) when compared to the penumbra zone (**Pen**). Moreover, the lesion core (*) showed very low or no expression of Cu/Zn SOD. In contrast, 3 days after injection, cortical expression of Cu/Zn SOD increased in the whole lesion (**B**, *). Neurons in the penumbra (**B**, **Pen**) and reactive hypertrophic astrocytes (**C**) within the degenerative core (*) expressed Cu/Zn SOD after 3 days. These GFAP-positive hypertrophic astrocytes were the main cell type expressing Cu/Zn SOD in the lesion core (**D**). This pattern of expression was maintained until 7 days post-lesion (the last time analyzed), when the glial scar was evident. Scale bars in A-D: 20 μm.

slightly to reach adult levels, but increases slightly again in aging [42,44,45]. Thus, it seems clear that immature rat brain has a different balance of antioxidant enzymes, which reach the adult overall pattern only after the first month of life.

We have shown that in the postnatal brain the majority of Cu/Zn SOD immunoreactivity is observed in neuronal somas with less intense staining present in the nuclei, sparing the nucleoli, as has been reported earlier for adult animals [12-17,47]. Accordingly, even though changes in Cu/Zn SOD levels occur in postnatal development, the cell type distribution of this enzyme does not change. Neurons are the brain cells with the highest oxygen consumption, constantly submitted to oxidative stress as it is suggested by $O_2^{\cdot-}$ mediated oxidation of hydroethidine

[9], or by the presence of nitrotyrosine [48,49]. Accordingly, it is not surprising that high levels of Cu/Zn SOD are observed in a wide number of neuronal populations, specifically in the large neurons with high energetic requirements, like pyramidal neurons, or catecholaminergic neurons which are submitted to elevated oxidative stress due to catecholamine metabolism [50]. Very recently, a site for the pro-inflammatory transcription factor NFκB has been reported at the human Cu/Zn SOD promoter, which can induce its expression [51]. Accordingly, in the CNS, neurons are the main cells that display constitutively activated NFκB [52,53], and its activity is required for their survival [54]. In addition, the other important cellular superoxide dismutase, the Mn SOD, is also enriched in subsets of neurons [55], although different from those enriched in Cu/Zn SOD.

No immunoreactivity for Cu/Zn SOD was observed in the GFAP-expressing astrocyte population of the neural parenchyma in the immature brain, as has been previously reported for adult brain [13-15,17,47], and despite the fact that immature astrocytes differ from adult astrocytes. This lack of Cu/Zn SOD expression contrasts with the higher tolerance of astrocytes to oxidative stress [48,56]. Accordingly, and in comparison with neuronal cells, astrocytes in culture have been shown to have higher levels of glutathione and the lipophilic antioxidant vitamin E [57], and they are capable of synthesizing their own glutathione from cysteine [58], mechanisms that probably confer upon astrocytes an elevated antioxidant status and increased resistance towards oxidative stress, despite the absence of detectable levels of Cu/Zn SOD. Noteworthy, Cu/Zn SOD immunoreactivity is only found in glia limitans astrocytes and in GFAP-positive tanycytes, which could be attributed to the contact of these astrocytes with non-CNS molecules, which are also known to induce the expression of several other activation markers observed previously in these regions, like GFAP overexpression, vimentin or metallothioneins [41]. Regarding microglial cells, neither resting ramified microglia nor amoeboid microglial cells found in the immature brain showed Cu/Zn SOD immunoreactivity, as has been shown for adult resting microglial cells [12,17,47]. In addition, we were unable to find consistent immunoreactivity for Cu/Zn SOD in white matter oligodendrocytes of immature brain, as has been described in the adult [12,17,47]. The lack of this enzyme could help explain the well known high sensitivity of oligodendrocytes to excitotoxicity and oxidative stress [59], especially in the immature brain where white matter injury is thought to be the underlying mechanism of brain damage [38].

Cu/Zn SOD in the injured immature brain

After an excitotoxic injury to the postnatal brain, we have observed a dramatic and rapid neuronal downregulation of Cu/Zn SOD in the NMDA injection site, which is early evident 2–4 hours after injection in neurons that only show slight and very early signs of degeneration and are negative for TUNEL staining. In fact we have previously shown that these neurons display, 10 hours after NMDA injection, NFκB activation and COX2 upregulation, suggesting that they are still active and functional [53,60]. The Cu/Zn SOD downregulation also coincides with neuronal nitration [48] suggesting endogenous $O_2^{-\cdot}$/peroxynitrite formation at these very early time points in compromised neurons. Although to our knowledge this is the first study describing the expression of Cu/Zn SOD after immature brain damage, studies on adult brain injury have also shown, as early as 4 hours after transient cerebral ischemia, Cu/Zn SOD immunoreactivity downregulation in striatum and cortex [37] and in neurons of the hippocampal CA region [13]. Moreover, 24 hours after injection of kainate to adult rat hippocampus, Cu/Zn SOD immunoreactivity is also downregulated in neurons of CA, despite an absence of apparent neuronal degeneration at that time-point [14]. Accordingly, it is known that superoxide scavengers protect from injury at these early time points [23,26,61]. Although the mechanism whereby this rapid downregulation occurs is not clear, it appears that it could be mediated by oxidative stress, as PC12 cells treated with H_2O_2 rapidly (after 4 hours) downregulate Cu/Zn SOD [51]. Interestingly, hypothermia, the most powerful neuroprotective strategy known, not only inhibits the rapid downregulation of Cu/Zn SOD after a traumatic brain injury but also induces its overexpression [36]. Most surprisingly, this effect is specific for Cu/Zn SOD, and in fact hypothermia induces a less significant upregulation of other antioxidant enzymes such as catalase and glutathione peroxidase in comparison with non-hypothermic brain.

Contradictory results have been reported regarding the toxicity of $O_2^{-\cdot}$ after hypoxic/ischemic injury to the immature brain. Whereas slightly worsened neuropathological outcome was observed in transgenic mice overexpressing Cu/Zn SOD and submitted to severe hypoxia/ischemia [27], several antioxidant molecules including SOD mimetics as $O_2^{-\cdot}$ dismuting metalloporphyrins were shown to be neuroprotective [26]. One hypothesis is that Cu/Zn SOD transgenic mice produce excess H_2O_2 that in the immature brain is not cleared by the upregulation of the glutathione peroxidase as has been reported for adult animals [28,62]. However, an additional explanation would be that as the postnatal brain express increased amounts of the NMDA receptor [63], whose activation leads to increased $O_2^{-\cdot}$ generation [34,35], an initial enhanced oxidative stress would occur. Taken together, this data suggest that in the immature brain subjected to ischemia/excitotoxicity, neurons at the lesion zone are very early submitted to an elevated oxidative stress, and

therefore Cu/Zn SOD downregulation contributes to the further amplification of cell damage and neuronal cell death.

Although Cu/Zn SOD expression remains very low in compromised neurons, a return to normal expression levels is seen by 24 hours after lesion, and an increase in total enzyme level is observed later on. This secondary Cu/Zn SOD induction is due to upregulation in reactive hypertrophic astrocytes within the lesion site. We have shown in previous studies that these reactive astrocytes display activated NFκB from 10 hours after the excitotoxic lesion [53], which could contribute to the induction of the Cu/Zn SOD observed here. We have also shown that the hypertrophic Cu/Zn SOD-overexpressing astrocytes are heavily nitrated, and also display metallothionein I-II expression, suggesting an elevated rate of oxidative stress in this particular group of cells [48]. Our findings in immature brain are in accordance with studies of adult brain damage that have shown induction of Cu/Zn SOD and Mn SOD in astrocytes several days after focal ischemia [37] excitotoxicity [14,18], or in Alzheimer's disease and Down's Syndrome [16]. In addition to the upregulated enzymatic antioxidant defences, astrocytes have been shown to produce their own glutathione and also provide neurons with cysteine, a rate-limiting precursor in neuronal glutathione synthesis [58]. Thus, astrocytes seem to be the main cell type increasing the total antioxidant capabilities in the nervous tissue after a lesion, which can in addition explain their elevated resistance to cell death after an injury. In this sense, previous studies showed that Cu/Zn SOD-overexpressing astrocytes have increased resistance to oxidative damage [64] and attenuated oxidative inhibition of glutamate uptake [65], allowing for a maintenance of their physiological functions after a lesion.

Regarding microglial cells, it is somehow surprising that only a reduced number of reactive ameboid microglial cells express Cu/Zn SOD, and that this occurs very transiently, as in some circumstances activated microglia produce large amounts of oxygen radicals after a lesion including $O_2^{-\cdot}$ [66].

We believe that the results presented here highlight the importance of *in vivo* cell-localization studies for antioxidants, as some of the reactive species like $O_2^{-\cdot}$ do not diffuse across cell membranes and thus will most probably react within the cell where it is formed.

Conclusion

In conclusion, we show that in the intact immature rat brain during the plasticity window, Cu/Zn SOD is mainly expressed in neurons, though it is also expressed at the central nervous system boundaries like the glia limitans or the ependymal cells. Moreover, we show that brain Cu/Zn SOD expression levels vary after an excitotoxic injury: it is rapidly downregulated in neurons, rendering the affected neurons even more susceptible to oxidative damage, and later on it is upregulated in highly hypertrophic astrocytes. Therefore, as no changes in cell specificity are found in the immature versus the adult brain, further studies would be needed to elucidate the mechanisms underlying the different susceptibility to oxidative damage in these two lesion paradigms.

List of abbreviations

SOD: superoxide dismutase; $O_2^{-\cdot}$: superoxide; H_2O_2: hydrogen peroxide; NO·: nitric oxide; COX: cycloxygenase; CNS: central nervous system; NFκB: nuclear factor kappa B; NMDA: N-methyl-D-aspartate; TUNEL: terminal dUTP nick end labelling; DAPI: 4, 6-diamino-2-phenylindole; GFAP: glial fibrilary acidic protein.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

HP carried out part of the brain lesions and animal work, performed most of the immunohistochemical and western blot studies, conceived the study and drafted the manuscript. LA carried out part of the brain lesions and animal work, participated in the design of the study and helped to draft the manuscript. MF carried out part of the immunohistochemistry and helped to draft the manuscript. BC and BG coordinated and supervised the development of the study, were responsible for the project giving economical support and helped in the last version of the manuscript. All authors read and approved the final manuscript.

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Astroglial Nitration after Postnatal Excitotoxic Damage: Correlation with Nitric Oxide Sources, Cytoskeletal, Apoptotic and Antioxidant Proteins

LAIA ACARIN, HUGO PELUFFO, LUIS BARBEITO, BERNARDO CASTELLANO, and BERTA GONZÁLEZ

Overview

The very fruitful interaction between the hypothesis of the Uruguayan laboratory directed by Luis Barbeito where I worked before, and the Catalonian laboratory where I made this Thesis work made this article possible. This study was conceived merging two main ideas: i) astrocyte activation is selectively induced by peroxynitrite, which can in turn trigger a neurotoxic phenotype of these cells towards motor neurons in culture, that we had shown before with the Uruguayan laboratory (Cassina et al. 2002), and ii) the know-how of *in vivo* astrocyte activation following acute lesions involving cytokines, antioxidant enzymes, and transcription factors as NFκB and STAT3, that the group of Barcelona had previously published (summarized in Table 1). In the present study, we observed that *in vivo*, as we had seen *in vitro*, nitrated astrocytes (presumably exposed to peroxynitrite) conform a subpopulation of astrocytes that show several special characteristics: they were the most hypertrophied astrocytes, the ones that expressed the highest levels of GFAP, the only ones that expressed metallothionein I-II, and the ones that showed *de novo* expression of vimentin and Cu/Zn SOD. Moreover, in the early stages of the excitotoxic lesion, they also expressed iNOS, and thus contributed to the lesion progression, as they do *in vitro*. These data support the notion that *in vivo*, reactive oxygen species and in particular peroxynitrite, are directly pro-inflammatory. In addition, a very surprising finding was that nitrated astrocytes showed immunoreactivity to activated caspase 3, while not showing any sign of cell death along different times after the lesion. These latter findings have now been confirmed by our group in adult and old rats and also by other groups, but its biological significance is still elusive.

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Astroglial Nitration after Postnatal Excitotoxic Damage: Correlation with Nitric Oxide Sources, Cytoskeletal, Apoptotic and Antioxidant Proteins

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ABSTRACT

Oxygen free radicals and nitric oxide (NO) participate in the pathogenesis of acute central nervous system (CNS) injury by forming peroxynitrite, which promotes oxidative damage and tyrosine nitration. Neuronal nitration is associated with cell death, but little is known of the characteristics and cell fate of nitrated astrocytes. In this study, we have used a postnatal excitotoxic lesion model (intracortical NMDA injection) and our aims were (i) to evaluate the temporal and spatial pattern of astroglial nitration in correlation with the neuropathological process and the sources of NO; and (ii) to establish, if any, the correlation among astrocyte nitration and other events such as expression of cytoskeletal proteins, antioxidant enzymes, and cell death markers to cope with nitration and/or undergo cell death. Our results show that after postnatal excitotoxic damage two distinct waves of nitration were observed in relation to astrocytes. At 24 h post-lesion, early-nitrated astrocytes were found within the neurodegenerating area, coinciding with the time of maximal cell death. These early-nitrated astrocytes are highly ramified protoplasmic cells, showing diffuse glial fibrillary acidic protein (GFAP) content and expressing inducible NOS. At later time-points, when astrogliosis is morphologically evident, nitrated hypertrophied reactive astrocytes are observed in the penumbra and the neurodegenerated area, displaying increased expression of GFAP and vimentin cytoskeletal proteins and of metallothionein I–II and Cu/Zn superoxide dismutase antioxidant proteins. Moreover, despite revealing activated caspase-3, they do not show TUNEL labeling. In summary, we show that nitrated astrocytes in vivo constitute a subpopulation of highly reactive astrocytes which display high resistance towards oxidative stress induced cell death.

Key words: caspase-3; Cu/Zn SOD; metallothionein; nitrotyrosine; oxidative stress; TUNEL; vimentin

INTRODUCTION

There is increasing evidence that oxygen free radicals and nitric oxide (NO) play an important role in the pathogenesis of acute central nervous system (CNS) injury. The main neurotoxic properties of these elements involve the reaction of NO with superoxide anion, leading to the formation of the potent oxidant peroxynitrite ($ONOO^-$) (Beckman et al., 1990; Radi et al., 1991) which promotes oxidative damage, inducing lipid perox-

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idation, mitochondrial dysfunction, cell energy depletion, DNA damage and protein modification by the nitration of tyrosine residues (Beckman et al., 1992).

By using the detection of nitrotyrosine as a footprint for the demonstration of peroxynitrite formation, in the last few years different studies have shown the presence of tyrosine nitration under several neuropathological conditions, including ischemia (Tanaka et al., 1997; Coeroli et al., 1998; Hirabayashi et al., 2000), traumatic injury (Bidmon et al., 1998; Scott et al., 1999; Liu et al., 2000), multiple sclerosis (Oleszak et al., 1998), Alzheimer's disease (Smith et al., 1997) and amyotrophic lateral sclerosis (ALS) (Abe et al., 1995). In all these CNS pathologies, peroxynitrite is thought to play a key role in neuronal oxidative damage and cell death, as neuronal cell nitration by peroxynitrite formation causes cell dysfunction, mitochondrial damage and apoptosis (Bonfoco et al., 1995; Estévez et al., 1998; Ischiropoulos and Beckman, 2003).

Neuronal cell death is always accompanied by reactive astrogliosis, characterized by astroglial cell hypertrophy, cytoskeletal changes and production of both neurotrophic and cytotoxic molecules (Ridet et al., 1997). In this sense, reactive astroglial cells have been shown to express the inducible form of nitric oxide synthase (iNOS) and become nitrated in different neuropathological conditions (Endoh et al., 1994; Wallace and Bisland, 1994; Schmidt et al., 1995; Loihl and Murphy, 1998; Almer et al., 1999), pointing to reactive astrocytes as possible producers of detrimental peroxynitrite. In agreement, *in vitro* evidence has demonstrated that astrocytes are more resistant to peroxynitrite-induced cell damage (Bolaños et al., 1995; Cassina et al., 2002) and that this reactive species induces phenotypical changes in cultured astrocytes including upregulation of glial fibrillary acidic protein (GFAP), transition towards a fibrous morphology, expression of iNOS and inhibition of high-affinity glutamate transporters, causing cytotoxicity for cocultured neurons (Trotti et al., 1996; Cassina et al., 2002). However, the characteristics and cell fate of nitrated peroxynitrite-exposed astrocytes after *in vivo* damage are unknown.

In this study we have used a well-characterized *in vivo* postnatal excitotoxic lesion model (Acarin et al., 1999a,b, 2000, 2002) to evaluate the temporal and spatial pattern of astroglial nitration in correlation with the neuropathological process and the sources of NO. As nitration is commonly found in cytoskeletal proteins, we have analyzed the correlation of major astroglial cytoskeletal proteins with nitration. Furthermore, we have analyzed whether nitrated astrocytes increase expression of antioxidant mechanisms to cope with nitration and/or undergo cell death.

MATERIALS AND METHODS

Excitotoxic Lesions

Nine-day-old Long-Evans black-hooded rat pups of both sexes were placed in a stereotaxic frame adapted for newborns (Kopf) under isofluorane anaesthesia. The skull was opened using a surgical blade, and 0.15 μL of saline solution (0.9% NaCl, pH 7.4) containing 37 nmols of *N*-methyl-D-aspartate (NMDA) (Sigma, M-3262, Germany) were injected into the right sensorimotor cortex using a 0.5-μL Hamilton microsyringe. Control animals received an injection of 0.15-μL of the vehicle saline solution. After suture, pups were placed in a thermal pad and maintained at normothermia for 2 h before being returned to their mothers. Experimental animal work was conducted according to Spanish regulations, in agreement with European Union directives. This experimental procedure was approved by the ethical commission of the Autonomous University of Barcelona. All efforts were made to minimize animal suffering in every step.

Immunocytochemical Study of Nitrotyrosine

At 2, 4 and 10 h, and 1, 3, 5 and 7 days after NMDA or saline injection, rats were anesthetized by ether inhalation and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). A minimum of four NMDA-injected animals and two saline-injected controls were used for each survival time. Brains were postfixed in the same fixative for 2 h and sunk in a 30% sucrose solution before being frozen with dry CO_2. Coronal sections (30-μm-thick) were obtained using a Leitz cryostat.

After endogenous peroxidase blocking with 2% H_2O_2 in 70% methanol, free-floating parallel sections were treated with 10% fetal calf serum in tris buffered saline (TBS) + 1% triton X-100 for 30 min, and incubated overnight at 4°C with either rabbit anti-nitrotyrosine polyclonal antibody (06-284, Upstate Biotechnology, Lake Placid, NY) (1:100) or rabbit anti-nitrotyrosine polyclonal antibody (4709, kindly provided by A. Estevez and J.S. Beckman). Afterwards, sections were rinsed and incubated for 1 h at room temperature with biotinylated anti-rabbit secondary antibody (RPN-1004, Amersham Pharmacia Biotech, England) (1:200). After the incubation, sections were rinsed in buffer and immersed in avidin-peroxidase (P-0364, Dakopatts, Denmark) (1:400) for another hour at room temperature. Finally, the peroxidase reaction product was visualized in 100 mL of tris buffer containing 50 mg of 3′-diaminobenzidine and 33 μL of hydrogen peroxide. As negative controls for immunohistochemistry, sections

were incubated in media lacking primary antibodies. For control of nitrotyrosine labeling, some sections were incubated in nitrotyrosine primary antibody solution containing free aminoacid nitrotyrosine (Sigma, N-7389) at 40 mM in TBS + 10% FCS (pH 7.4).

Double Immunohistochemistry for Nitrotyrosine and NOS Isoforms

In order to correlate astroglial nitration with the sources of NO, we performed double immunocytochemical detection of nitrotyrosine and iNOS or nNOS, isoforms responsible for the production of the largest amount of NO after CNS damage. Sections were immunoreacted for nitrotyrosine as reported above, but using Cy3-conjugated anti-rabbit secondary antibody (PA-43004, Amersham Pharmacia Biotech, Buckinghamshire, UK) (1:1000). On the same sections, iNOS and nNOS immunohistochemistry was performed by using rabbit anti-iNOS (NOS-2) polyclonal antibody (AB5382, Chemicon, CA) (1:6000) or rabbit anti-nNOS (NOS-1) polyclonal antibody (N53130, Transduction Laboratories, Lexington, KY) (1:3000) and Cy2-conjugated anti-rabbit secondary antibody (PA-42004, Amersham Pharmacia Biotech) (1:1000). Double-stained sections were analysed using a LEICA TCS 4D confocal microscope.

Double Immunohistochemistry for Nitrotyrosine and Cell Markers/Cytoskeletal Proteins

We used double-staining techniques for the identification of nitrotyrosine-positive cells by using specific cellular markers. Sections were immunoreacted for nitrotyrosine as reported above, but using Cy3-conjugated anti-rabbit secondary antibody (PA-43004, Amersham Pharmacia Biotech) (1:1000). Sections were then further processed by using one of the following primary antibodies: (i) polyclonal anti-GFAP (Z-0334, Dakopatts, Glostrup, Denmark) (1:1000) and (ii) monoclonal anti-vimentin (M725, Dakopatts, Denmark) (1:1000) for astroglial labeling; (iii) monoclonal anti-NeuN (MAB377, Chemicon, CA) (1:1000) for neuronal labeling; (iv) polyclonal anti-myeloperoxidase (MPO) (A0398, Dakopatts, Denmark) (1:400) for labeling of neutrophils. Afterwards, Cy2-conjugated anti-rabbit secondary antibody (PA-42004, Amersham Pharmacia Biotech) (1:1000), or Cy2-conjugated anti-mouse secondary antibody (PA-42002, Amersham Pharmacia Biotech) (1:1000) were used to visualize labeling.

Moreover, additional nitrotyrosine-immunoreacted sections were processed for double staining with tomato lectin histochemistry for the demonstration of microglial cells. Sections were incubated with the biotinylated lectin from *Lycopersicon esculentum* (tomato) (Sigma, L-9389, Germany) diluted to 6 μg/mL and with Cy2-conjugated streptavidin (PA-42000, Amersham Pharmacia Biotech) (1:1000). Double-stained sections were analyzed using a LEICA TCS 4D confocal microscope.

Double Immunohistochemistry for Nitrotyrosine and Antioxidant or Apoptotic Markers

To evaluate whether additional antioxidant mechanisms were found in nitrated astrocytes, we correlated nitrotyrosine immunolabeling with the expression of the antioxidant proteins heat shock protein 27kDa (HSP27), metallothionein I–II and Cu/Zn superoxide dismutase (Cu/Zn SOD) by performing double immunohistochemistry using the following primary antibodies: (i) polyclonal anti-HSP27 (SPA-801, Stressgene, Victoria, Canada; 1:2500); (ii) monoclonal anti-metallothionein I–II (kindly provided by J. Hidalgo, Autonomous University of Barcelona; 1:200); (iii) polyclonal anti Cu/Zn SOD (574597, Calbiochem, Darmstad, Germany; 1:500).

Finally, we evaluated the activation of apoptotic or anti-apoptotic cell death markers in nitrated astrocytes, by analyzing the double immunolabeling for nitrotyrosine and the pro-apoptotic protein active caspase-3 using polyclonal anti-cleaved caspase-3 (9661, Cell Signaling Technology, Beverly, MA; 1:200); and the anti-apoptotic protein bcl-2 using monoclonal anti-bcl-2 (M0887, Dakopatts, Denmark; 1:100).

In addition, double labeling with nitrotyrosine and terminal dUTP nick end labeling (TUNEL) staining was also performed. Tissue sections previously processed for nitrotyrosine Cy3-immunolabeling were rinsed in Tris buffer (10 mM, pH 8) and EDTA (5 mM) and then incubated in the same buffer plus Proteinase K (20 μg/mL) for 15 min at room temperature. After several washes with EDTA (5 mM), sections were incubated for 10 min in TdT buffer (Tris 30 mM, 140 mM sodium cacodilate, 1 mM cobalt chloride, pH 7.7). Sections were then incubated in TdT buffer plus 0.161 Units/μL TdT enzyme (terminal transferase, 3333566 Roche, Manheim, Germany) and 0.0161 nmol/μL of biotin-16-dUTP (1093070, Roche, Manheim, Germany) for 30 min at 37°C. The reaction was stopped by washing the sections in citrate buffer (300 mM sodium chloride, 30 mM sodium citrate, 5 mM EDTA). After several washes with TBS, sections were incubated with Cy2-conjugated streptavidin (PA-42000, Amersham Pharmacia Biotech; 1:1000).

Selected sections were incubated for 5 min with a 0.00125 μg/mL solution of 4,6-diamino-2-phenylindole (DAPI) in TBS. Double-stained sections were analyzed using a LEICA TCS 4D confocal microscope.

RESULTS

Injection of NMDA into the right sensorimotor neocortex of 9-day-old rats caused a lesion involving neuronal loss and glial response across the entire thickness of the cortex and the dorsal striatum at the level of the injection site, as we have previously described in detail (Acarin et al., 1999a,b, 2000). However, injection of saline solution caused no neuronal degeneration and only a very focal and transient glial response up to 3 days post-injection restricted to the needle track.

In regards to nitrotyrosine immunohistochemistry, results have been summarized in Figure 1. As similar results were obtained with the two different nitrotyrosine antibodies used, only results using the antibody from Upstate are shown in the figures. In saline-injected controls, only a faint nitrotyrosine labeling was observed in some neuronal cells of neocortical layers, thalamus and hippocampus, both in the ipsilateral and contralateral brain hemispheres (Fig. 1). Moreover, very faint nitrotyrosine-positive astroglial cells were observed in the cingulum of the corpus callosum (Fig. 2B). This constitutive nitrotyrosine labeling was markedly reduced in saline-injected control animals from 5 days post-injection (postnatal day 14).

In contrast, in lesioned animals, nitrotyrosine immunoreactivity was found in some neuronal cells and infiltrated neutrophils besides astrocytes, the main scope of the study (results are summarized in Fig. 1). Noteworthy, microglia/macrophages were never positive for nitrotyrosine at any of the survival times studied.

Nitrotyrosine in Astrocytes: Time course, Distribution and Characterization

According to the time of appearance, specific location, morphology and immunohistochemical labeling for NOS, antioxidant and apoptotic proteins, we distinguish two waves of nitrated astrocytes (Fig. 1 and Table 1): (i) early-activated highly ramified protoplasmic astrocytes, and (ii) late-activated reactive hypertrophied astrocytes.

Early-nitrated highly ramified protoplasmic astrocytes showed low and diffuse GFAP content and no signs of cell hypertrophy (Figs. 2A, 3A), resembling the early activated astroglial phenotype previously described by Raivich et al. (1999) in the damaged gray matter. These highly ramified protoplasmic activated astrocytes were only observed at 24 h post-lesion evenly distributed

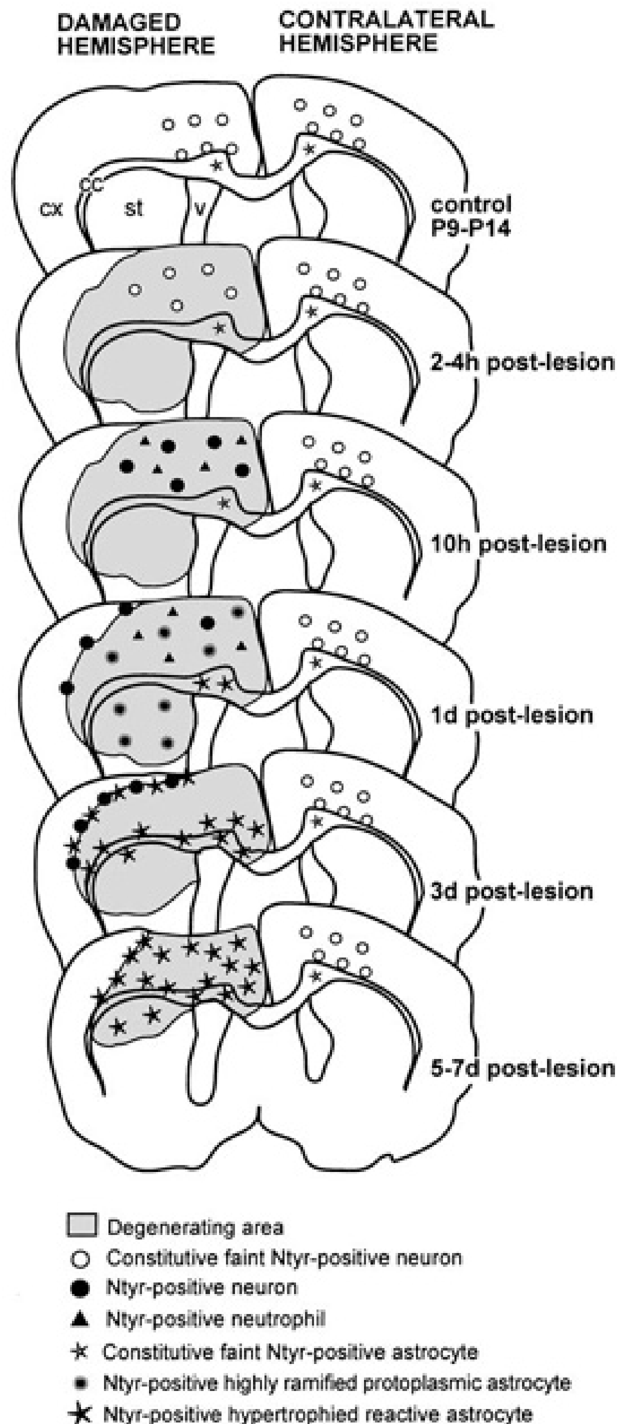

FIG. 1. Temporal and spatial distribution of nitrotyrosine-positive cells in the normal postnatal brain and at different survival times following a cortical excitotoxic lesion. The neurodegenerating area is shaded in the ipsilateral hemisphere. Nitrotyrosine-positive cells are identified as neurons (circles), neutrophils (triangles), and two morphologically distinct astrocytes (asterisks and stars). Nitrated astrocytes show a highly ramified protoplasmic morphology at 24 h post-lesion (asterisks) and are hypertrophied reactive cells at longer survival times (stars). Cx, cortex; cc, corpus callosum; st, striatum; v, ventricle.

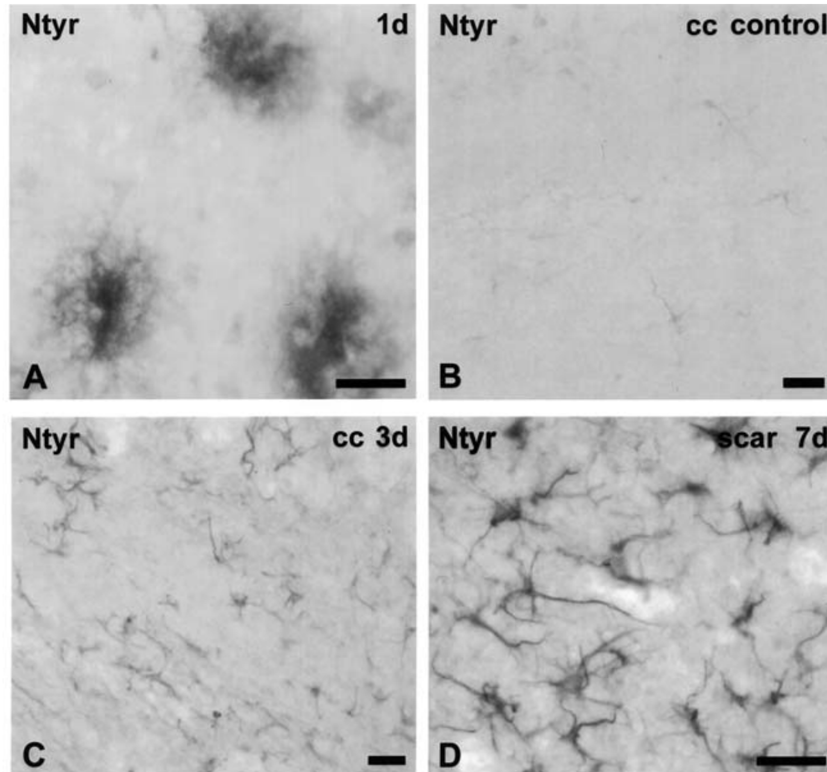

FIG. 2. Distribution of nitrotyrosine-positive (Ntyr) astrocytes. Early-nitrated astrocytes are observed at 1 day post-lesion (**A**) as highly ramified protoplasmic cells. In comparison to control animals (**B**), at 3 days post-lesion, nitrated reactive astrocytes are found in the corpus callosum (cc) (**C**). At 5–7 days post-lesion, nitrated highly reactive hypertrophied astrocytes cover the neurodegenerating area and form the glial scar (**D**). Bar = 30 μm.

throughout the neurodegenerating areas in the cortex and striatum.

Late-nitrated reactive astrocytes were observed in the, 3 days post-lesion. These cells were characteristically hypertrophied and showed increased GFAP labeling with defined and thick GFAP-positive filament bundles (Figs. 2D, 3E). At 3 days post-lesion, these nitrated reactive astrocytes were observed in the borders of the lesion, contacting penumbra neurons with their cell bodies or cytoplasmic processes (Fig. 3D), as well as in the adjacent corpus callosum (Fig. 2C), extending their projections towards the degenerating areas in the cortex. At 5–7 days post-lesion, they covered the degenerating area (Fig. 2D) and accumulated in the lateral margins of the lesion and the upper cortical layers, forming the glial scar. The phenotypical characterization of the two types of nitrated astrocytes using double labeling techniques are summarized in Table 1 and detailed below.

Nitrated Astrocytes and Sources of NO

As previously described (Acarin et al., 2002), iNOS expression in lesioned animals was strongly induced at 10–24 hours post-lesion, in highly ramified protoplasmic activated astrocytes found throughout the neurodegenerating area. Noteworthy, at 1 day post-lesion but not at earlier timepoints, iNOS expression in highly ramified protoplasmic activated astrocytes co-localized with nitrotyrosine labeling (Fig. 3B), although not all iNOS-expressing astrocytes were nitrated. In contrast, nitrated late reactive hypertrophied astrocytes seen from 3 days post-lesion, including those at the glial scar, did not display iNOS expression. No co-localization between either types of nitrated astroglia and nNOS was observed at any time, although nNOS positive neurons were always found surrounding the area occupied by nitrated astrocytes (Fig. 3C).

Nitrated Astrocytes and Cytoskeletal Proteins

Whereas nitrated early highly ramified protoplasmic astrocytes at 24 h showed low and diffuse GFAP content (Fig. 3A) and no vimentin expression, nitrated late reactive hypertrophied astrocytes seen from 3 days post-lesion showed strong GFAP labeling (Fig. 3E) and *de novo* expression of vimentin (Fig. 3F). Notably, most, but not all GFAP-positive reactive astrocytes showed nitrotyro-

Table 1. Characterization of Nitrated Astrocytes Following Excitotoxic Damage to the Immature Rat Brain

		NO sources		Cytoskeletal protein		Antioxidant proteins		Apoptotic/anti-apoptotic markers		
Nitrated astrocytes	*Time post-lesion*	*nNOS*	*iNOS*	*GFAP*	*Vimentin*	*Cu/Zn SOD*	*MT I-II*	*Bcl-2*	*Active Caspase-3*	*TUNEL*
Highly ramified protoplasmic astrocyte	24 h	−	++	−/+ diffuse	−	−	−	−	−	−
Reactive hypertrophied astrocyte	3–7 days	−	−	++	++	+	+	−	+	−

Cu/Zn SOD, copper/zinc superoxide dismutase; GFAP, glial fibrillary acidic protein; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; TUNEL, terminal UTP nick end labeling. Grading of immunoreactivity: −, no labeling; −/+, faint labeling; +, labeling; ++, strong labeling.

sine labeling, and nitrated astrocytes were always the more hypertrophied and showing the strongest GFAP overexpression (Fig. 3E). At the cellular level, nitrotyrosine was found in thick GFAP-positive cell projections, but thinnest and distal GFAP-positive processes often lacked nitrotyrosine (Fig. 3E). In regards to vimentin, only nitrated hypertrophied astrocytes expressed vimentin, and when observed at high magnification, nitrotyrosine labeling strongly co-localized with the cellular location of this intermediate filament (Fig. 3F).

Nitrated Astrocytes and Antioxidant Proteins

Nitrated early highly ramified protoplasmic astrocytes found at 24 h post-lesion lacked expression of HSP27, metallothionein I–II, or Cu/Zn SOD (Table 1). However, the majority of nitrated late reactive hypertrophied astrocytes presented *de novo* expression of metallothionein I–II (Fig. 3G) and Cu/Zn SOD (Fig. 3H) in their soma and proximal projections. No correlation was found between nitration and HSP27 immunoreactivity (data not shown), as HSP27 induction was only observed in some nitrated astrocytes, and HSP27-positive non-nitrated astroglial cells were also found.

Nitrated Astrocytes and Apoptosis-Related Markers

Nitrated highly ramified protoplasmic astrocytes found at 24 h post-lesion neither showed activation of caspase-3 nor TUNEL staining (Table 1). In contrast, most nitrated reactive hypertrophied astrocytes found from 3 days post-lesion and, until 7 days, the last survival time analyzed, showed immunoreactivity for active caspase-3 in their nuclei (Fig. 3I,J). However, caspase-3–positive nitrated astrocytes did not show any signs of nuclear condensation using DAPI nuclear staining (Fig. 3J) and were not positive by the TUNEL staining at any of the survival times studied (Fig. 3K,L). Finally, nitrated astrocytes did not show expression of the anti-apoptotic protein bcl-2 at any time (data not shown).

DISCUSSION

In this study, we have shown that after postnatal excitotoxic damage two distinct waves of nitrated astrocytes are observed. Around 24 h post-lesion, nitrated astrocytes are highly ramified protoplasmic cells, showing diffuse GFAP content and expressing iNOS. At later time-points, when astrogliosis is more evident, nitrated astrocytes are hypertrophied and display increased expression of the cytoskeletal proteins GFAP and vimentin, expression of the antioxidant proteins metallothionein I–II and Cu/Zn SOD, as well as activation of caspase-3 in the absence of nuclear fragmentation. These late-nitrated reactive astrocytes do not express iNOS or nNOS.

Early-Nitrated Highly Ramified Protoplasmic Astrocytes Express iNOS

Although earlier survival times of 2, 4 and 10 h post-lesion were analysed, nitration of astrocytes was not found until 24 h post-lesion, in highly ramified protoplasmic astrocytes, resembling the early-activated reactive velate astroglial phenotype that has been previously described by Raivich et al. (1999) following ischemia and trauma and recently by Campos (2003) in transgenic mice expressing *Lmo1-lacZ*. These nitrated astrocytes show iNOS expression, although not all iNOS-expressing astrocytes were nitrated. Noteworthy, iNOS expression is already seen in these types of astroglial cells at 10 h post-lesion (Acarin

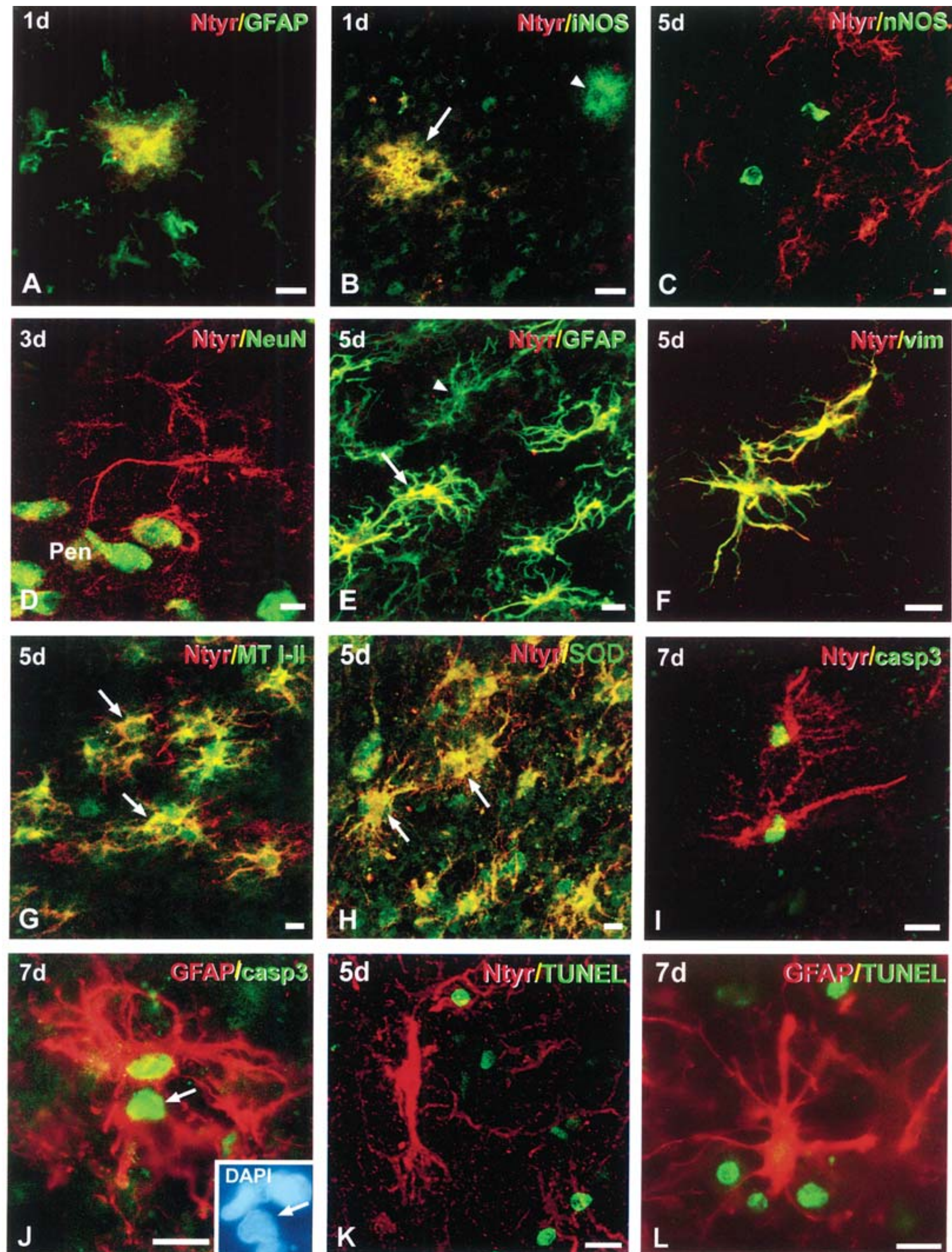

FIG. 3. Co-localization of nitrated astrocytes with NO sources and cytoskeletal, antioxidant, and apoptotic proteins. Nitrotyrosine (**A–I,K**) or GFAP (**J,L**) labeling are shown in red and either GFAP (A,E), iNOS (B), nNOS (C), NeuN (D), vimentin (vim) (F), metallothionein I-II (MT I–II) (G), Cu/Zn superoxide dismutase (SOD) (H), caspase-3 (casp3) (I–J) or TUNEL (K–L) are shown in green. Yellow shows co-localization of markers. At 24 h post-injury, nitrated hairy astrocytes show mild GFAP (A) and iNOS (arrow in B) labeling, and not all iNOS-positive astrocytes are nitrated (arrowhead in B). No co-localization between nitrated astrocytes and nNOS staining is seen at any time (C). At 3 days post-injury, some nitrated astrocytes are found surrounding NeuN-positive neurons in the penumbra region (Pen) (D). Nitrated astrocytes are reactive hypertrophied and the most strongly GFAP-positive (arrow in E), although not all GFAP-positive astrocytes are nitrated (arrowhead in E). Only nitrated astrocytes show vimentin staining (F) and nitrotyrosine labeling co-localizes with the cellular location of vimentin filaments (F). All nitrated astrocytes express MT I-II (e.g., see arrows in G) and SOD (e.g., see arrows in H). Note that mainly astrocytes express MT I-II, but SOD is expressed by nitrated astrocytes and by all neurons. Nitrated reactive astrocytes show caspase-3 positive nuclei (I and J); however, neither showed nuclear condensation by DAPI staining (arrow in inset in J) nor DNA cleavage by TUNEL labeling (K, L). Bar = 10 μm.

et al., 2002), more than 12 h prior to nitration. In this sense, it is reasonable to think that tyrosine nitration in these astrocytes may represent peroxynitrite formed within the cell, by NO derived from endogenous iNOS in combination with superoxide anion, which is largely produced in excitotoxicity (Lafon-Cazal et al., 1993). In addition, a tyrosine nitration mechanism involving neutrophil myeloperoxidase has also been described (Eiserich et al., 1999), although no close association between neutrophils and nitrated astrocytes was found.

iNOS is the enzyme isoform associated with the production of large amounts of NO and its cytotoxic actions (Iadecola et al., 1995; Yun et al., 1996). Accordingly, iNOS is supposed to be responsible for the production of NO to form peroxynitrite after CNS damage, as nitrotyrosine formation is strongly reduced after treatment with iNOS inhibitors (Takizawa et al., 1999; Ikeno et al., 2000) and in iNOS knockout mice (Liberatore et al., 1999; Hirabayashi et al., 2000). In these models, iNOS inhibition, which may imply decreased peroxynitrite formation, is associated with neuroprotection (Iadecola et al., 1995, 1997; Lecanu et al., 1998; Liberatore et al., 1999; Tsuji et al., 2000). Therefore, in our excitotoxic model, early iNOS-expressing nitrated astrocytes, whose appearance correlates with the time of maximal neuronal death in this model (Acarin et al., 1999a), could contribute to neuronal damage, either by NO/peroxynitrite production or by toxic 3-nitrotyrosine release (Mihm et al., 2001; Cassina et al., 2002; Peluffo et al., 2004). However, it should be noted that different cell types besides astrocytes, mainly infiltrating neutrophils, become nitrated and express iNOS (Iadecola et al., 1996; Bidmon et al., 1998; Coeroli et al., 1998; Grzybicki et al., 1998; Loihl et al., 1999; Acarin et al., 2002). In this sense, the putative neurotoxic role of astroglial-derived NO and peroxynitrite, which has been demonstrated using astroglial cultures (Dawson et al., 1994; Stewart et al., 2000; Cassina et al., 2002), would be *in vivo* potentiated by neutrophils.

Nitration of Reactive Hypertrophied Astrocytes

Later on, when reactive astroglial cells cover the neurodegenerates area and form the glial scar, nitrotyrosine labeling is found in hypertrophied reactive astrocytes. These nitrated astrocytes do not show iNOS/nNOS expression, which suggests that they do not produce NO themselves, at least when nitration is occurring. Another possibility could involve the astroglial production of superoxide anion, but difficult to demonstrate *in vivo*. Reactive astrocytes in several neurodegenerative conditions are known to activate cytosolic phospholipase A2, producing arachidonic acid (Stephenson et al., 1999), an inducer of superoxide formation in astroglial cultures (Chan et al., 1988). Superoxide anion can form peroxynitrite by combining with NO, which may reach astroglial cells by diffusion from surrounding nNOS neuronal cells, as neuronal nNOS has been previously shown to play an important role in cell nitration after NMDA-induced excitotoxicity (Ayata et al., 1997).

Nevertheless, we cannot rule out that reactive astrocytes could be nitrated by incorporating 3-nitrotyrosine from the extracellular media, released by early-nitrated astrocytes, neutrophils and neurons in the degenerating area. However, the short half-life of parenchymal nitrotyrosine after ischemia (little more than 2 h) (Takizawa et al., 1999) does not support this mechanism. In the same way, incorporation of exogenous nitrotyrosine into astroglial cells would result in the nitration of tubulin by the activity of tyrosine-tubulin ligase (Eiserich et al., 1999; Peluffo et al., 2004), and in this lesion model no nitrotyrosine-positive band around 55 KDa, the molecular weight of dissociated alfa-tubulin and other cytoskeletal proteins, is observed in western blots (H. Peluffo, unpublished observations).

In order to further characterize nitrated astrocytes and evaluate the putative nitration of other cytoskeletal proteins, we correlated nitrotyrosine labeling with the expression of the intermediate filament proteins, GFAP and vimentin. Vimentin is found in astrocytes during brain development but is lost when the brain reaches maturity and GFAP becomes the major astroglial intermediate filament protein (Dahl, 1981). However, astroglial cells show de novo expression of vimentin in response to several types of brain damage (Takamiya et al., 1988; Acarin et al., 1999b; Eliasson et al., 1999). Here we show that, although nitrotyrosine labeling is not found in all GFAP-overexpressing astrocytes and nitration is not observed in all GFAP-containing cell processes, nitrotyrosine co-localizes with vimentin-positive filaments. Noteworthy, all vimentin-positive astrocytes are nitrated, and all vimentin-containing cell processes show nitrotyrosine labeling. Astroglial vimentin expression has been linked to motility and proliferation (Takamiya et al., 1988; Janeczko, 1993; Lepekhin et al., 2001), intracellular transport mechanisms (Pixley and De Vellis, 1984; Hutchins and Casagrande, 1989), and as support for protein kinases (Ciesielski-Treska et al., 1995). Therefore, it is likely that proteins associated with vimentin-intermediate filaments, more than the vimentin protein itself, could become nitrated as a result of peroxynitrite formation in astrocytes.

Expression of Apoptotic and Antioxidant Proteins in Nitrated Astrocytes

Although it is known that neuronal nitration induces cellular damage and activation of apoptotic mechanisms

(Bonfoco et al., 1995; Endres et al., 1998; Estévez et al., 1998; Oka et al., 2000), the effect of nitration in astrocytes is largely unknown. Besides modifications in microtubule assembly, tyrosine nitration can disrupt the phosphorylation/dephosphorylation dynamics of tyrosine kinase residues involved in cell signalling (Kong et al., 1996; Knapp et al., 2001). In spite of this, Bolaños and coworkers have demonstrated that cultured astrocytes are more resistant to peroxynitrite-mediated mitochondrial damage due to a rise in glutathione levels and a switch towards glycolytic metabolism (Bolaños et al., 1995; Bolaños and Almeida, 1999), which may also account for the absence of astroglial death after peroxynitrite treatment *in vitro* (Cassina et al., 2002).

In this report we have observed that early-nitrated highly ramified protoplasmic astrocytes do not show activation of the apoptotic protein caspase-3, whereas late-nitrated reactive hypertrophied astrocytes do. Caspase-3 is a proteolytically activated enzyme which is considered one of the major executioners of apoptosis (Nicholson, 1999; Springer et al., 2001) and is induced by peroxynitrite in different cell types *in vitro* (Estévez et al., 1998; Lin et al., 1998; Cassina et al., 2002). Nevertheless, it should be noted that nitrated reactive astrocytes showing caspase-3–positive nuclei did not show signs of nuclear fragmentation and were TUNEL-negative, even at the last survival time studied, suggesting the existence of a time frame between caspase-3 activation and the execution of apoptotic death (Brecht et al., 2001) or pointing to an activated state of caspase-3 positive cells in the absence of cell death, as has been reported in a model of ischemic preconditioning (McLaughlin et al., 2003) and in status epilepticus (Narkilahti et al., 2003).

Finally, it is thought that one of the mechanisms by which astroglial cells compensate for oxidative stress and avoid cell death is by increasing different antioxidant mechanisms. Accordingly, in this study we have shown that, whereas no correlation between nitrated astrocytes and expression of HSP27 was observed, reactive nitrated astrocytes present de novo expression of Cu/Zn SOD and metallothionein I–II. Cu/Zn SOD is largely recognised as one of the main free radical scavenger in ischemia and excitotoxicity (Noack et al., 1998; Kim et al., 2000) and its over-expression provides astrocytes an increased resistance to oxidative damage (Chen et al., 2001). Metallothionein I–II is a metal-binding protein upregulated in response to stress and inflammation, playing a key role in the detoxification of heavy metals and scavenging of free radicals (Hidalgo et al., 1994). Interestingly, transgenic over-expression of metallothionein I in astrocytes and microglial cells dramatically decreases inflammation, reduces astroglial and microglial iNOS expression and diminishes lipid peroxidation and nitrotyrosine labeling, decreasing neuronal apoptosis (Giralt et al., 2002; Penkowa et al., 2002). Accordingly, both Cu/Zn SOD and metallothionein could serve nitrated reactive hypertrophied astrocytes as an additional antioxidant mechanism to cope with oxidative stress and cell death.

In conclusion, we report that an excitotoxic lesion to the postnatal brain induces the nitration of a subpopulation of astrocytes in two waves that differ in cell characteristics and temporal location. An early wave included a rapid iNOS induction in nitrated astrocytes, coinciding with the time of maximal cell death. A second and later astrocytic nitration wave was characterized by cell hypertrophy, increased GFAP expression, and induction of vimentin and the antioxidant proteins metallothionein and Cu/Zn SOD. These nitrated astrocytes, despite displaying caspase-3 activation, seemed to be highly resistant towards oxidative stress induced cell death, probably by the overexpression of antioxidant enzymes.

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Article number 3:

Nonviral Gene Delivery to the Central Nervous System Based on a Novel Integrin-Targeting Multifunctional Protein

HUGO PELUFFO, ANNA ARÍS, LAIA ACARIN, BERTA GONZÁLEZ, ANTONI VILLAVERDE, and BERNARDO CASTELLANO

Overview

This work is the fruit of a very intense collaboration of our laboratory with Anna Arís and Antoni Villaverde from the Institute of Biotechnology and Biomedicine of our University. To pursue the aim of developing new strategies for reducing CNS damage after an acute excitotoxic lesion, a gene therapy approach was needed that would not induce more inflammation, as many of the well-characterized viral vectors do. Arís and Villaverde had developed a very elegant strategy for delivering DNA to cells. It was based on the combination of functional motifs in a recombinant scaffold protein, which could then mimic the viral infection of cells. They used the β-galactosidase of *E. Coli* as scaffold protein because its structure is very well known and because it presents a natural nuclear localization motif. They introduced two additional functional motifs to this protein: a poly-lysine tail which binds and condense DNA, and an integrin targeting domain that enables the molecule, termed 249AL, to attach to the cell and is then endocytosed. They had shown that it was effective in *in vitro* settings, and we ought to analyze if it could also be effective in an *in vivo* CNS acute injury paradigm. We found that the 249AL vector could transfer the green fluorescent protein (GFP) transgene to the whole lesioned brain area, and that it did not induce a significant inflammatory reaction after 6 days of direct intraparenchymal injection. Thus, this non-viral modular protein vector could be suitable for the neuroprotective gene therapy strategy pursued in this Thesis.

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Nonviral Gene Delivery to the Central Nervous System Based on a Novel Integrin-Targeting Multifunctional Protein

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ABSTRACT

Successful introduction of therapeutic genes into the central nervous system (CNS) requires the further development of efficient transfer vehicles that avoid viral vector-dependent adverse reactions while maintaining high transfection efficiency. The multifunctional protein 249AL was recently constructed for *in vitro* gene delivery. Here, we explore the capability of this vector for *in vivo* gene delivery to the postnatal rat CNS. Significant transgene expression was observed both in the excitotoxically injured and noninjured brain after intracortical injection of the DNA-contaning–249AL vector. In the injured brain, a widespread expression occurred in the entire lesioned area and retrograde transport of the vector toward distant thalamic nuclei and transgene expression were observed. Neurons, astrocytes, microglia, and endothelial cells expressed the transgene. No recruitment of leukocytes, demyelination, interleukin-1β expression, or increase in astrocyte/microglial activation was observed at 6 days postinjection. In conclusion, the 249AL vector shows promising properties for gene therapy intervention in the CNS, including the targeting of different cell populations.

OVERVIEW SUMMARY

The introduction of therapeutic genes for the treatment of central nervous system (CNS) pathologies requires the development of flexible vehicles devoid of inflammatory and immune reactions that are many times triggered by viral vectors. 249AL, a multifunctional protein vector, was recently generated based on *Escherichia coli* β-galactosidase. Taking advantage of the intrinsic nuclear localization motifs located in this protein, two additional functional domains were introduced: an Arg-Gly-Asp (RGD) integrin-binding and cell internalization peptide and a polylysine tail with DNA condensing properties. The resulting protein construct efficiently delivered expressible DNA to cultured cells. Here we show the competent *in vivo* 249AL-mediated transgene delivery to intact and injured postnatal rat CNS in the absence of an inflammatory reaction or immune response at 6 days postinjection. After an excitotoxic insult, transgene expression was clearly enhanced, was more widespread, and was observed in neurons, astrocytes, microglia, and endothelium. In summary, the 249AL vector displays promising properties for future gene therapy intervention in the CNS, especially in tissue-damaging conditions, and furthermore, offers a flexible modular design adaptable to other specific therapeutic requirements.

INTRODUCTION

CONSIDERING THE CURRENT sequencing and expected solving of the complete human genome, gene therapy approaches are likely to acquire an extremely high importance in the improvement of human health. The transitory or stable introduction of therapeutic genes in target cells requires appropriate vehicles for DNA delivery and nuclear transfer. In the last few years, adenoviruses, parvoviruses, herpesviruses, and retroviruses have been engineered to generate DNA transfer vehicles, which exploit relevant viral properties such as cell attachment, internalization, nuclear transport, and stable DNA expression. These efforts have resulted in prototypes that showed an important degree of success under experimental conditions (Sapolsky and Steinberg, 1999; Costantini *et al.*, 2000; Mountain, 2000; Brooks *et al.*, 2002). However, a set of viral-dependent adverse reactions have been observed in clinical trials (Jane *et al.*, 1998; NIH Report 2002) and are accompanied by an increasing concern about the possible risks associated with the release of manipulated infectious agents and their scale-up production difficulties. For these reasons, development of safer, stable, and eventually more efficient nonviral vehicles for gene transfer is necessary (Jane *et al.*, 1998; Navarro *et al.*, 1998). Cationic lipids (Mountain, 2000; Li and Ma, 2001) and synthetic peptides (Sparrow *et al.*, 1998) have been explored as

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coating devices for expressible DNA, and multifunctional proteins for cell-targeted DNA delivery have been constructed by combining bioactive protein domains from different origins (Fominaya and Wels, 1996; Paul *et al.*, 1997; Arís *et al.*, 2000). These independent elements can supply DNA-condensing, cell-binding, cell-internalization, endosome-disrupting, and nuclear-targeting activities to the resulting vehicles without most of the inconveniences of potentially infective material (Navarro *et al.*, 1998). The intrinsic flexibility of these vectors offers wider perspectives for the generation of promising vehicle prototypes for specific therapeutic needs.

For the central nervous system (CNS) these constructs are of special relevance because DNA delivery by different viral species has been achieved (Dewey *et al.*, 1999; Sapolsky and Steinberg, 1999; Costantini *et al.*, 2000; Brooks *et al.*, 2002) but accompanied by unacceptable toxicity (Nilaver *et al.*, 1995), persistent inflammation (Dewey *et al.*, 1999), immune activation (Byrnes *et al.*, 1996b; Wood *et al.*, 1996), and demyelination (Byrnes *et al.*, 1996a; Nilaver *et al.*, 1995). In addition, viral delivery has been shown to potentially break tolerance to endogenous proteins (Zinkernagel *et al.*, 1990). Likewise, the peripheral readministration of viral vectors to animals previously injected intracerebrally with the same vectors induces a delayed-type hypersensitivity reaction in the neural parenchyma (Byrnes *et al.*, 1996a).

Alternative gene delivery strategies for the CNS, such as intracerebral injection of polyethylenimine/DNA (Bousiff *et al.*, 1995) or lipid/DNA complexes (Shi and Pardridge, 2000; Hecker *et al.*, 2001) have been explored but need further improvement (Li and Ma, 2001). We have previously reported the construction of 249AL, a nonviral vector based on an engineered β-galactosidase protein (Arís *et al.*, 2000). Taking advantage of β-galactosidase nuclear localization motifs (McInnis *et al.*, 1995), two additional functional domains were introduced into this bacterial protein: an Arg-Gly-Asp (RGD) integrin-binding and cell-internalization peptide and a polylysine tail with DNA condensing properties, rendering this multifunctional protein an efficient vector for gene delivery to cultured cells (Arís *et al.*, 2000). In this study, we have characterized 249AL as a vector for *in vivo* gene delivery to the intact and damaged postnatal rat CNS.

Brain damage as a result of perinatal hypoxic-ischemic insult is a serious clinical problem with severe neurologic consequences such as spastic paresis, choreoatheosis, ataxia, disorders of sensomotor coordination, or impairment of intellectual ability (Berger and Garnier, 1999). In this regard, because glutamate-mediated excitotoxicity is implicated in this insult, NMDA receptor activation has been largely used as a model for hypoxic-ischemic injury to the postnatal brain (Ikonomidou *et al.*, 1989; Olney, 1990).

In the context of our approach, we have focused on the evaluation of the transfection efficiency of 249AL in neurons and glia, the extent of transgene expression, and the exploration of the putative induction of an inflammatory immune response.

MATERIALS AND METHODS

Protein, DNA, and formation of protein-DNA complexes

Protein 249AL is an engineered form of *Escherichia coli* β-galactosidase that displays a 27-mer peptide with an integrin-targeted RGD-based motif. This segment, inserted between residues 249 and 250 of the bacterial enzyme, reproduces the cell-attachment region of the VP1 capsid protein of the foot-and-mouth disease virus (Arís and Villaverde, 2000). The additional presence of a decca-lysine tail, attached to the amino

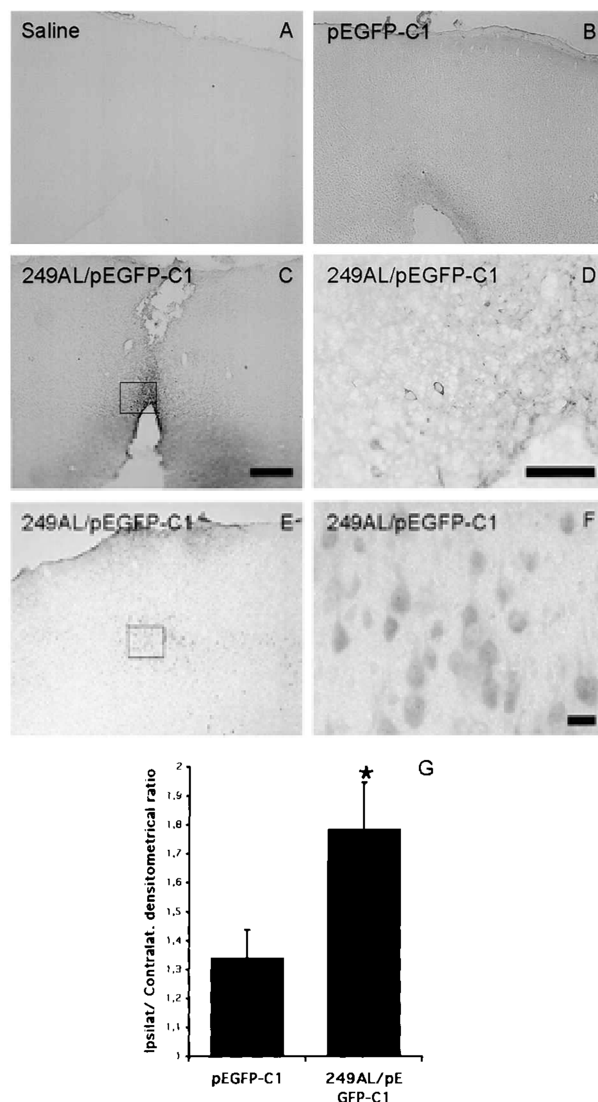

FIG. 1. 249AL transgene delivery. Green fluorescent protein (GFP) immunoreactivity (Abcam antibody) at different survival times after the intracerebral injection of saline solution (**A**), pEGFP-C1 DNA (**B**), or 249AL/pEGFP-C1 (**C–F**). Saline injected brains do not show any immunoreactivity (A). One day after injection with pEGFP-C1 DNA, only a mild immunoreactivity is observed in the vicinity of the injection site (B). Brains injected with 249AL/pEGFP-C1 show GFP immunoreactivity in the parenchyma along the needle track and subjacent corpus callosum at 1 day postinjection (B, C) and in sparse neurons in the ipsilateral cortex (E, F) at 7 days postinjection. D and F: Magnifications from squares in (C) and (E), respectively. Densitometric analysis for GFP immunoreactivity 1 day after injection show a significant (*$p < 0.05$) increase in sections from animals injected with 249AL/pEGFP-C1 compared to naked pEGFP-C1 DNA injected animals (**G**). Scale bars: (A–C), (E) = 300 μm; (D) = 50 μm; (F) = 20 μm.

terminus of the construct, and a still unidentified enzyme segment with nuclear targeting properties (McInnis *et al.*, 1995) enables 249AL to promote DNA delivery. The 249AL protein was produced in bacteria and purified from crude cell extracts as described previously (Arís *et al.*, 2000). A red-shift variant of jellyfish Aequorea Victoria green fluorescent protein (GFP) gene encoded into plasmid pEGFP-C1 (Clontech, Palo Alto, CA) under the control of the cytomegalovirus promoter and the SV40 enhancer element was used as a reporter gene to monitor the efficiency of DNA delivery. All protein and DNA complexes were formed by incubation in 0.9% NaCl at room temperature for 1 hr using 0.04 μg DNA per microgram of 249AL protein. Details of complex formation can be found elsewhere (Arís and Villaverde, 2000).

Experimental animals

Experimental animal work was conducted according to Spanish regulations in agreement with European Union directives. A total of forty-three 9-day old Long-Evans black-hooded nonlesioned or excitotoxically prelesioned rat pups (15–20 g, both genders; Janvier, Le Genest Saint Isle, France) were used. Experimental procedures were approved by the ethical commission of the Autonomous University of Barcelona. All efforts were made to minimize animal suffering in every step.

Injection of 249AL into nonlesioned cortex

In two independent experiments the 249AL protein/pEGFP-C1 DNA complexes or naked pEGFP-C1 DNA were injected intracerebrally and animals were sacrificed 24 hr later. Intracerebral injections were made into the right sensorimotor cortex at the level of the coronal suture (2 mm lateral of bregma and 0.5 mm depth) using a stereotaxic frame adapted for newborns (Kopf Instruments, Tujunga, CA) under gas anesthesia. The skull was opened with a surgical blade and injections of either 1 μl of pEGFP-C1 DNA (0.032 μg/μl in NaCl 0.9%) or 249AL protein/pEGFP-C1 complexes (0.032 μg/μl pEGFP-C1 in NaCl 0.9%) were made using an automatic injector at 0.33 μl/min. The needle was left in place for an additional 3 min to allow for diffusion. In a separate experiment to evaluate possible toxicity associated with 249AL, only the purified 249AL protein (0.8 μg/μl) or saline were injected intracerebrally as described above and animals were sacrificed 6 days later. Inflammation was assessed by immunohistochemical analysis of sections for: (1) astroglial demonstration using antibodies to the astrocyte glial fibrillary acidic protein (GFAP), (2) microglial/macrophage visualization using histochemistry for tomato lectin, (3) the proinflammatory cytokine interleukin 1β (IL-1β) expression, (4) the presence of infiltrating CD8$^+$ or CD4$^+$ T lymphocytes, and (5) the expression of myelin marker myelin basic protein (MBP).

Injection of 249AL into prelesioned cortex

Excitotoxic lesions were done as previously described (Acarin *et al.*, 1999). 37 nmol of *N*-methyl-D-aspartate (NMDA) (Sigma, St. Louis, MO) diluted in 0.15 μl of saline solution (0.9% NaCl) or control saline solution was injected at a rate of

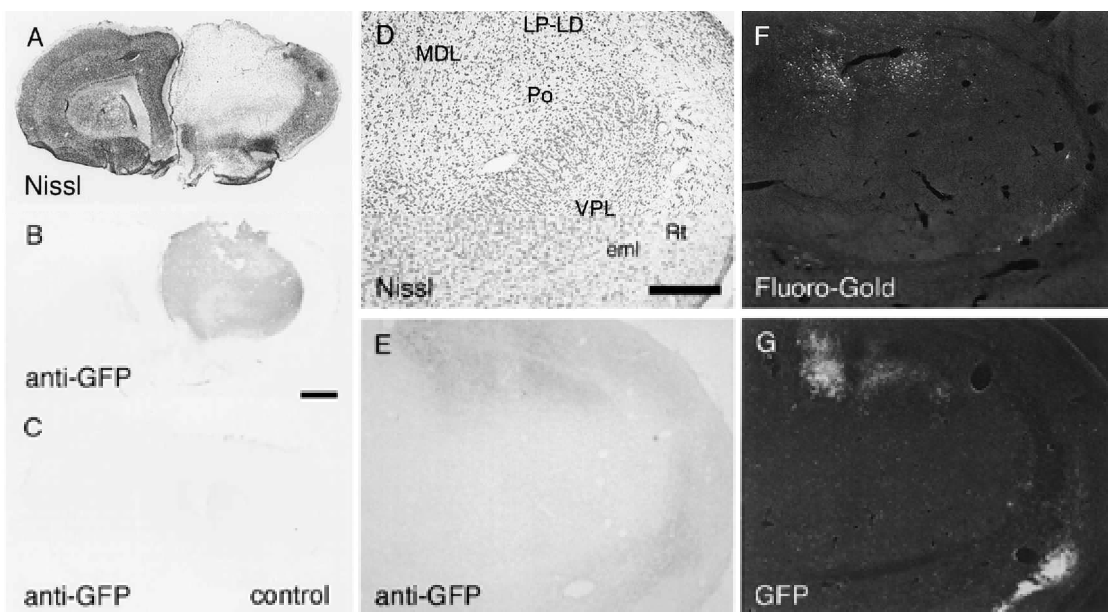

FIG. 2. Widespread transgene delivery after an excitotoxic lesion. Green fluorescent protein (GFP) immunoreactivity in coronal sections at the level of striatum (B–C) and thalamus (D–G) of excitotoxically lesioned animals postinjected with 249AL/pEGFP-C1 at 1 (**A**–**C**) and 7 days postinjection (**D**–**G**). At 1 day, the neurodegenerative area, clearly visualized with Nissl stain (A), is entirely covered by GFP immunoreactivity (B). No immunopositive signal is observed in *N*-methyl-D-aspartate (NMDA)-lesioned animals injected only with saline solution (C). In the retrogradely connected thalamic nuclei, as identified in Nissl-stained sections (D), diffuse GFP immunoreactivity is found in the mediodorsal lateral nucleus (MDL), posterior nuclear group (Po), reticular nucleus (Rt), and lateral nuclear group (LP-LD) (E). Nonlesioned animals injected intracortically with the retrograde tracer Fluorogold (F) show fluorescence in MDL, Po, and ventral posterolateral nucleus (VPL). Similar findings are observed by GFP epifluorescence in thalamus (G), although GFP expression is observed in Rt nucleus located on the other side of the external medullary lamina (eml). Scale bars: A–C = 1 mm; D–G = 400 μm.

0.05 μl/min at the same coordinates used for the 249AL injections. After suturing, pups were placed on a thermal pad for 2 hr at 36°C to maintain normothermia. Excitotoxically lesioned animals were injected with 249AL/pEGFP-C1 or pEGFP-C1 6 hr later, and sacrificed at 24 hr postlesion for immunohistochemical or Western blot analysis. One additional group was injected with 249AL/pEGFP-C1 and sacrificed at 7 days post-injection for immunohistochemical analysis.

Injection of Fluorogold

In order to comparatively assess retrograde transport from sensorimotor cortex toward connected thalamic nuclei, the retrograde tracer Fluorogold was injected (0.15 μl, 2%) in nonlesioned rat pups as was done for injection of the 249AL vector. Rats were sacrificed 5 days postinjection.

Fixation and histology

Animals were anesthetized and perfused intracardially for 7 minutes with 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4). The brains were removed immediately and postfixed for 4 hr in the same fixative, cryoprotected with 30% sucrose solution, and quickly frozen with dry CO_2. Alternate cryostat sections were mounted on gelatin-coated slides (15 μm thick) or stored free-floating in antifreeze buffer (30 μm thick).

Immunohistochemistry

Sections were processed for endogenous peroxidase inactivation and blocked for 1 hr in Tris-buffered saline (TBS, pH 7.4), 10% fetal calf serum. Sections were incubated overnight at 4°C in the same blocking solution with primary antibodies against either GFP (1:1500 Abcam ab290 rabbit polyclonal, or 1:100 Clontech JL-8 mouse monoclonal), GFAP (1:1800 Dakopatts Z-0334 rabbit polyclonal), IL-1β (1:200 Santa Cruz sc-1252 goat polyclonal), CD8 (hybridoma supernatant) (Malissen *et al.*, 1982), CD4 (hybridoma supernatant) (Carrera *et al.*, 1987), or MBP (1:300 Dakopatts A623 rabbit polyclonal). After several washes they were incubated for 1 hr with biotinylated anti-rabbit (1:200, Amersham RPN-1004) or biotinylated anti-mouse (1:200, Amersham RPN-1001). Specific staining was evidenced by incubation with avidin-peroxidase (1:400 Dakopatts P0364) for 1 hr and subsequent 3,3′-diaminobenzidine (DAB)-hydrogen peroxide developing procedure. Only for GFP immunohistochemistry the biotin-streptavidin LSAB2 System (Dakopatts K0675) was used, followed by a developing solution of 0.1 *M* acetate buffer pH 6 containing 0.027 g/L glucose oxidase, 0.4 g/L ammonium chloride, 24 g/L ammonium nickel sulfate, 2 g/L glucose, and 0.2 g/L DAB.

Microglia/macrophages were demonstrated by histochemistry for tomato lectin *Lycopersicon esculentum*, performed by incubation for 1 hr at room temperature in TBS with the biotinylated lectin (6 μg/ml, Sigma L-9389) followed by avidin-peroxidase incubation and DAB-hydrogen peroxide developing procedure as described previously (Acarin *et al.*, 1994).

For double-staining procedures, sections were first processed as described above for GFAP immunolabeling or tomato lectin histochemistry and developed with DAB-hydrogen peroxide. These sections were then incubated as described above with primary antibody to GFP (Abcam ab290), amplified with the avidin-biotin complex and incubated in developing buffer consisting of phosphate 10 m*M* pH 7.4, 390 μM filtered benzidine, 840 μM sodium nitroprusside dehydrate, and 0.008% hydrogen peroxide. Control sections were processed simultaneously for detection of GFAP or tomato lectin followed by GFP detection but omitting the GFP primary antibody.

Western blotting

Western blots for GFP were performed in order to comparatively assess transgene expression between 249AL/pEGFP-C1 (n = 5) and pEGFP-C1 (n = 4) injected lesioned animals. After decapitation, the brains were quickly removed and the hemispheres were separated, chopped and frozen in liquid nitrogen. Samples were resuspended in sodium dodecyl sulfate (SDS) 1%, aprotinin (1 μg/ml), phenylmethanesulfonylfluoride (PMSF) (1 m*M*), pep-

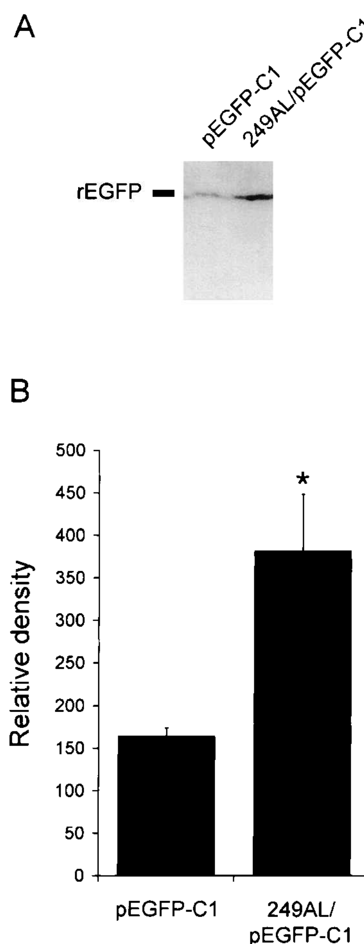

FIG. 3. 249AL increase transgene expression in *N*-methyl-D-aspartate (NMDA)-lesioned brain. Twenty-four hours after injection, protein samples from lesioned brain hemispheres of 249AL/pEGFP-C1 or naked pEGFP-C1 DNA injected animals were extracted and submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot for GFP (**A**). Only one immunopositive band per lane is observed, which comigrates with recombinant enhanced green fluorescent protein (rEGFP). Densitometric analysis of the immunopositive bands shows a significant (*$p < 0.05$) increase in transgene expression in 249AL/pEGFP-C1 injected animals in comparison with naked pEGFP-C1–injected animals (**B**).

statin A (1 μM), leupeptin (100 μM), and disrupted with a Braun Labsonic U sonicator (20 KHz, 50 W). Total protein concentration was determined using the bicinchoninic acid method. Equal amounts of protein were used for 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrotransfered protein samples to polyvinylidene fluoride (PVDF) membranes were incubated in blocking buffer (TBS plus 5% nonfat milk and 0.05% Tween 20) overnight at 4°C and then incubated in primary GFP antibody (1:1000 Clontech JL-8) in blocking buffer for 2 hr at room temperature. The membranes were then incubated in secondary biotinylated anti-mouse antibody (1:800, Amersham RPN-1001), avidin-peroxidase (1:3000 Dakopatts P0364), and finally in chemiluminescent substrate SuperSignal West Pico (Pierce Biotechnology, Rockford, IL) combined with exposure on Hyperfilm ECL (Amersham, Piscataway, NJ).

Densitometric measurements

GFP immunohistochemically processed sections were digitized with a Nikon DXM1200 digital camera attached to an E600 Nikon microscope and further densitometrical measurements were performed using NIH Image 1.52 software as previously described (Acarin *et al.*, 1997). Briefly, images from two areas (0.6 mm^2 each) of the cortical injection site and one from contralateral cortex were digitized from three parallel sections of each animal (249AL/pEGFP-C1, $n = 6$; pEGFP-C1, $n = 5$). Data are expressed as the mean densitometric ratio of each section (ipsilateral/contralateral). Western blot protein signals were estimated by measuring band areas with one-dimensional Quantity One (BioRad, Hercules, CA) software after high-resolution scanning. All results are reported as mean values ± standard error of the mean (SEM). Differences between two groups were evaluated for equal variance (*F* test) followed by unpaired Student's *t* test, and probabilities < 0.05 were considered statistically significant.

RESULTS

249AL mediated delivery of the transgene in the damaged and intact brain

249AL vector-mediated gene delivery to the postnatal CNS was evaluated after stereotaxic intracortical injection in intact and excitotoxically damaged animals. Injection of the 249AL vector complexed with pEGFP-C1 DNA (249AL/pEGFP-C1) or naked pEGFP-C1 DNA into intact brains, showed a local expression of the GFP reporter gene in the ipsilateral neocortex along the needle track and the subjacent corpus callosum at 1 day postinjection. This expression was observed immunohistochemically with two different GFP antibodies (Fig. 1C and 1D) and confirmed by epifluorescence for GFP (not shown). The GFP protein was still present in neurons of the injected cortex 7 days postinjection (Fig. 1E and 1F), and in sparse neurons of the contralateral cortex. In contrast, animals injected with naked pEGFP-C1 DNA only showed mild immunoreactivity for GFP at the injection site (Fig. 1B). Densitometric analysis of GFP immunohistochemically processed sections showed a significant ($p < 0.05$) increase in cortical transgene expression in animals injected with 249AL/pEGFP-C1 compared to animals injected with naked pEGFP-C1 DNA (Fig. 1G).

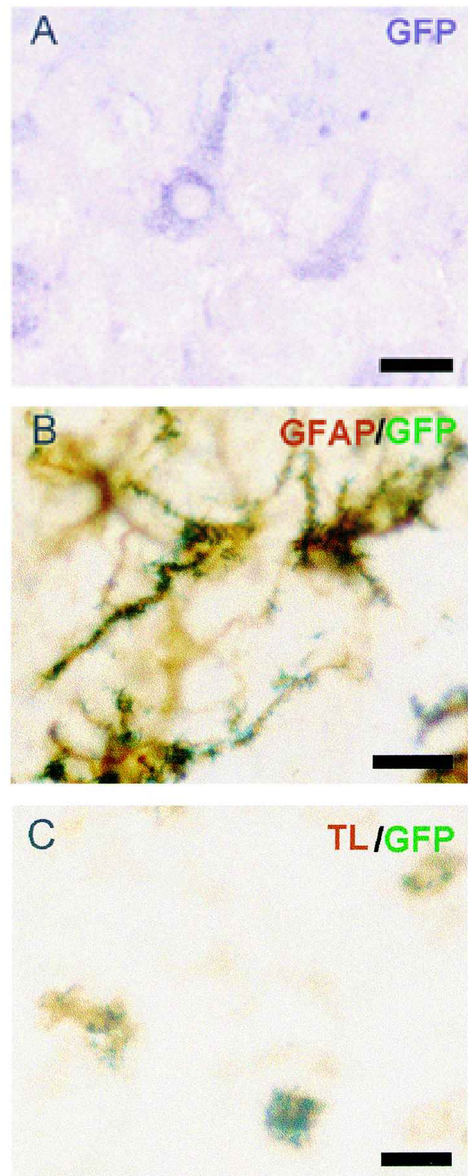

FIG. 4. 249AL-mediated transgene expression in different cell types. Green fluorescent protein (GFP) immunoreactivity show transgene expression in neurons (**A**) as well as in glial cells (**B–C**) at 1 day postinjection. Double immunohistochemical staining indicate that both astrocytes (B) and microglia (**C**) express the transgene as shown by colocalization of glial fibrillary acidic protein (GFAP; brown in B) and GFP (green in B), or tomato lectin (TL, brown in C) and GFP (green in C), respectively. Scale bars: (A) = 10 μm; (B), (C) = 20 μm.

The vector 249AL also increased GFP transgene expression in NMDA-lesioned brains. NMDA administration is a well-known model of excitotoxic damage that triggers rapid neuronal death and tissue injury, which expands rostro-caudally and includes part of the cortex, corpus callosum, dorsal striatum, and septum (Fig. 2A, Acarin *et al.*, 1999). When 249AL/pEGFP-C1 was injected into the damaged brain, there was widespread GFP transgene expression, which covered the en-

tire lesioned area at 24 hr postinjection (Fig. 2B) and that was still observed 7 days later. Interestingly, at 7 days postlesion, these animals also showed GFP green fluorescence (Fig. 2G) as well as GFP immunoreactivity (Fig. 2E) in several thalamic nuclei, which are anatomically connected to the injected cortex (Sherwood and Timiras, 1970; Faull and Mehler, 1985). This GFP expression profile correlated with the staining pattern of thalamo-cortical projecting nuclei, labeled after intracortical injection of the Fluorogold retrograde tracer into the cortex of intact animals (Fig. 2F).

In addition to immunohistochemical techniques, 249AL transgene delivery efficiency in damaged brains was also assessed by comparative Western blot densitometrical analysis of 27-kd bands that comigrated with recombinant GFP. A significant ($p < 0.05$) 132% $\pm$ 17% increase in GFP expression was observed in animals injected with 249AL/pEGFP-C1 versus animals injected with naked pEGFP-C1 DNA (Fig. 3).

In both damaged and intact brains, GFP was efficiently expressed in neurons, scattered endothelial cells and glia. Double labeling with GFAP astroglial marker or tomato lectin microglial/macrophage marker showed enhanced GFP expression in these cells after the lesion (Fig. 4).

Finally, appropriate controls were performed and none of them showed GFP immunoreactivity. These included: (1) sections from intact saline injected brains (Fig. 1A); (2) sections from damaged saline-injected brains (Fig. 2C); (3) sections that were not incubated with either primary antibody; (4) sections processed with primary antibody preincubated with recombinant enhanced green fluorescent protein (EGFP) peptide (0.1 mg/ml, not shown).

Absence of histopathological alterations after 249AL intracerebral injection

To evaluate possible changes of several immune/inflammatory parameters elicited by 249AL, we injected intracerebrally the 249AL vector alone, devoid of DNA, in order to exclude putative transgenic protein-derived effects. Six days after the vector injection, sections stained with toluidine blue showed a well-organized brain parenchyma lacking inflammatory infiltrates or neuronal affectation (Fig. 5A and 5B). The immunohistochemical staining for GFAP showed local astroglial hypertrophy at the needle track and subjacent corpus callosum (Fig. 5C), which was indistinguishable from that observed in saline injected controls. Similarly, sections processed for tomato lectin to demonstrate microglia/macrophages did not show any significant reactivity (Fig. 5D). Only a slight increase in macrophage number was observed in the needle track, whereas normal ramified resting microglia were observed in the neighboring parenchyma (Fig. 5E). No significant infiltration of CD8- or CD4-positive T lymphocytes was observed (Fig. 5F). Regarding the expression of the proinflammatory cytokine IL-1β, 249AL injected animals presented similar results to saline-injected controls, that is to say only some immunopositive endothelial cells (not shown). Finally, MBP immunohistochemistry only showed a slight demyelination in the corpus callosum at the injection site in both saline and 249AL injected animals, which was most likely associated with the injection procedure itself (Fig. 5G).

DISCUSSION

In this study, we propose a multifunctional vector prototype (249AL) as an alternative to viral vectors for *in vivo* CNS gene delivery, which maintains the beneficial properties of viral attachment and internalization processes while avoiding most of the inconveniences associated with potentially infective material.

249AL efficiently delivered the GFP transgene

After an intracerebral injection into the intact or damaged postnatal rat brain, the 249AL vector efficiently delivered the GFP transgene. The 249AL vector used in the present work displays an RGD motif, which interacts with $\alpha_V\beta_3$, $\alpha_V\beta_5$ and at least some other α_V integrins (Wickham *et al.*, 1993). In this sense, the high transfection efficiency observed is probably because of an enhanced cell internalization of the DNA, mediated by interactions between the RGD motif and the plasma membrane α_V integrins that are expressed in the brain (Akiyama *et al.*, 1991; Ellison *et al.*, 1998; Pinkstaff *et al.*, 1999; Masumura *et al.*, 2001). In fact, cell internalization via receptor-dependent endocytosis of many viruses, including most adenovirus serotypes and foot-and-mouth disease virus, is specifically mediated by interactions of a viral RGD peptide with cellular α_V integrins (Wickham *et al.*, 1993). In addition, 249AL polylysine-DNA condensation properties have been shown to protect DNA from nuclease activity and increase cell internalization (Fominaya and Wels, 1996), thus contributing further to the enhanced transfection efficiency. Interestingly, an additional increase in transgene expression was observed when DNA complexed to the 249AL vector was injected into damaged brain. A possible explanation could be the lesion-triggered upregulation of $\alpha_V\beta_3$ integrin expression that has been reported in hypertrophic astrocytes, microglia, and microvessels after ischemia (Okada *et al.*, 1996; Ellison *et al.*, 1998; Masumura *et al.*, 2001), Alzheimer (Akiyama *et al.*, 1991), or experimental autoimmune encephalomyelitis (Previtali *et al.*, 1997). Increased glial transgene expression after the lesion reported here further supports this idea. Noteworthy, the 249AL vector was capable of transfecting cells of the entire excitotoxically lesioned area, from the most rostral to the most caudal areas, including different brain regions. This fulfills one of the most important requirements for successful CNS gene therapy (i.e., whole-lesion widespread gene delivery and expression [Sapolsky and Steinberg, 1999]). Therefore, 249AL could be an appropriate vector to preferentially induce transgene expression in injured areas, sparing most of the normal parenchyma.

249AL does not induce an immune/inflammatory response

One of the biggest challenges facing vectors is the host immune response. Whereas at the cellular level, recruitment of cytotoxic T lymphocytes eliminates vector-targeted cells and triggers tissue injury (Yang *et al.*, 1994; Byrnes *et al.*, 1996b), at the humoral level, antibodies against the vector preclude the repeated administration (Kajiwara *et al.*, 2000). In this regard, viruses have been shown to potentially break tolerance to endogenous previously non-immunogenic proteins, acting as po-

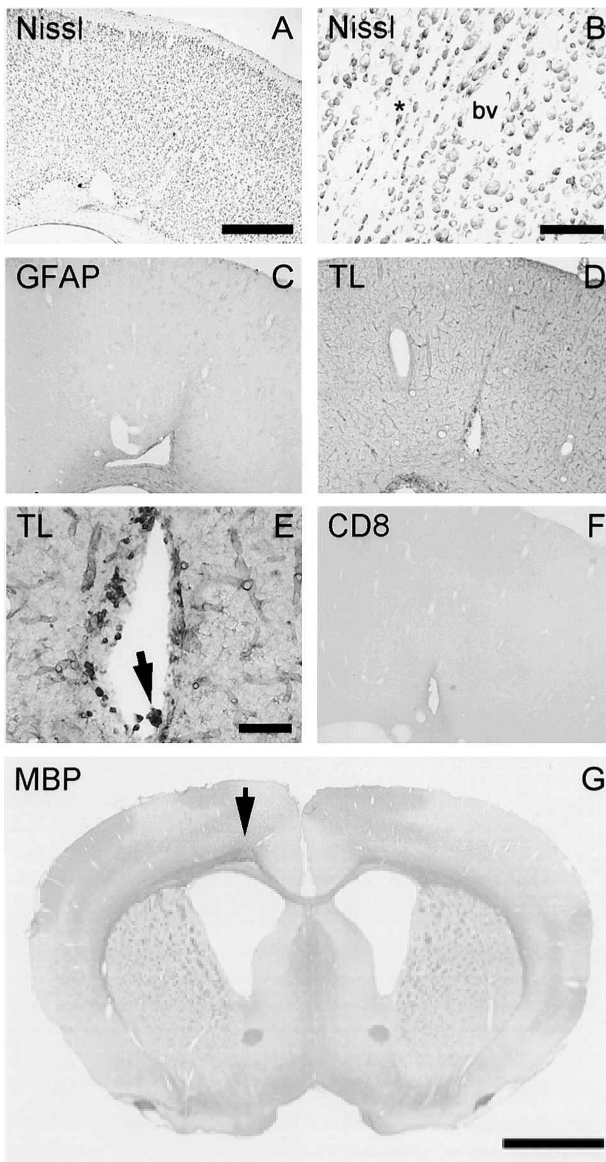

FIG. 5. Absence of histopathologic alterations after 249AL injection. Nissl staining (**A**, **B**), several markers of inflammation and immune activation (**C**–**F**) and myelin staining (**G**) in 249AL-injected brains after 6 days' survival. Nissl staining shows no degenerating neurons, perivascular leukocyte infiltrates (blood vessel, bv) or general alterations in parenchymal structure except for the needle track (*) (A, B). Glial fibrillary acidic protein (GFAP) immunostaining shows no differences in astrocytosis (C). Microglial marker tomato lectin shows normal ramified resting microglia (D, E) and scattered macrophages only at the needle track (arrow in E). No further differences in immunoreactivity of CD8 lymphocyte marker (F), or myelin protein MBP (G) are observed in comparison to saline injected animals. Scale bars: (A), (C), (D), (F) = 500 μm; (B) = 30 μm; (E) = 50 μm; (G) = 2 mm.

tent adjuvants in the generation of immune responses (Zinkernagel *et al.*, 1990). Particularly in the brain, unacceptable persistence of inflammation has been observed on injection of different viral vectors (Dewey *et al.*, 1999) as well as immune activation (Wood *et al.*, 1996) and demyelination (Nilaver *et al.*, 1995; Byrnes *et al.*, 1996a). Accordingly, the intracerebral injection of adenoviral vectors induces a rapid increase of IL-1β (Cartmell *et al.*, 1999), followed by activation of microglia/macrophages and astroglia (Wood *et al.*, 1996), increased expression of major histocompatibility complex class I (Wood *et al.*, 1996) and a rise in anti-adenovirus antibodies in serum at the first 48 hours (Kajiwara *et al.*, 2000). Moreover, accumulation of activated CD8$^+$ and CD4$^+$ lymphocytes occurs in the neural parenchyma by the first week (Byrnes *et al.*, 1996b). In contrast, our results show that none of these histopathological changes occurred 6 days after 249AL injection, suggesting that this vector does not trigger an inflammatory reaction or an immune response in the host in a timeframe where viral vectors do.

249AL transport and transgene expression in distal brain areas

Several viruses including adenoviruses (Akli *et al.*, 1993) are internalized at axon terminals and retrogradely transported to the neuronal soma, thus making possible the selective transfection of a population of distant neurons by a noninvasive approach. It is believed that terminal internalization of viruses into neurons is mediated by interactions of α_V integrins with RGD motifs of viral capsid proteins, as described in other cell types (Wickham *et al.*, 1993). Interestingly, in this study transgene expression was observed in sparse neuronal soma in the contralateral cortex, and in several thalamic nuclei, which are known to have afferent/efferent cortical projections (Faull and Mehler, 1985). It is therefore conceivable that when injected cortically, the 249AL vector could be internalized by somatosensory thalamo-cortical axonal terminals and by retrograde transport, transfect distant neuronal soma. The presence of the Fluorogold retrograde tracer in contralateral cortical neurons and in the MDL and posterior nuclear group (Po) thalamic nuclei further supports the idea of efficient 249AL retrograde transport. A similar spatial pattern of transgene expression was reported after adenoviral intracortical injection into the ischemic brain (Abe *et al.*, 1997). These results suggest that the 249AL vector retains the retrograde transfecting property of several viruses, and hence could be used for transgene delivery by a noninvasive administration route to target specific distal neuronal populations in the CNS.

Nonviral gene delivery using the 249AL vector offers promising insights of future therapies for acute CNS injuries, because it preserves most of the relevant viral properties while avoids the inflammatory, immune-activating, infectious, and scale-up production derived difficulties of viral vectors. The intrinsic flexibility of this modular vector provides wider perspectives for the generation of specific prototypes for different therapeutic needs.

Gene therapy-mediated neuroprotection after acute neurologica insults such as hypoxia-ischemia, hypoglycemia, seizures, or traumatic brain injury has been achieved in different animal models (Sapolsky and Steinberg, 1999; Costantini *et al.*, 2000) including postnatal rats (Hagan *et al.*, 1996). These strategies involve the transgenic overexpression of neuron-survival promoters including: calcium-binding proteins, glucose trans-

porters, hyperpolarizing ion channels, anti-inflammatory proteins, neurotrophic factors, or antiapoptotic genes (reviewed in Sapolsky, 2003). It should be noted that several of the neuroprotective effects of these molecules are based on a transient postinjury overexpression and not on a constitutively increased expression. For example, it has been shown that constitutive overexpression of calbindin disrupts neuronal plasticity (Chard *et al*., 1995). In this context, the rapid and transient expression of the transgene after an acute injury, as observed with the 249AL vector in the postnatal brain, is likely to be a successful clinical approach.

Finally, it should be noted that upregulation of α_V integrins in cerebral microvessels after ischemia (Okada *et al*., 1996) or in tumor microvasculature (Pasqualini *et al*., 1997) makes possible an intravenous route for vector administration targeting these specific areas of interest. Future studies must be undertaken to establish therapeutically relevant transgene expression levels achievable with the 249AL vector under different experimental paradigms.

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Neuroprotection from NMDA Excitotoxic Lesion by Cu/Zn Superoxide Dismutase Gene Delivery to the Postnatal Rat Brain by a Modular Protein Vector

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Overview

In the previous work we showed that the 249AL vector was capable of delivering a transgene to the lesioned immature brain. However, we here asked whether this recombinant vectors could induce the expression of biologically-relevant concentrations of transgenic protein. We decided to overexpress, in the same immature injury model, the gene of the Cu/Zn SOD antioxidant enzyme as: i) it is highly neuroprotective in transgenic adult rats, and ii) it is rapidly downregulated or degraded in the immature brain as we showed previously in Article 1. Moreover, Arís and Villaverde had developed a new version of the 249AL vector termed NLSCt, which had an additional nuclear localization functional domain from SV40 virus, and displayed increased transfection efficiency *in vitro*. So we used this improved vector for *in vivo* overexpression of Cu/Zn SOD two hours after the excitotoxic injury. This treatment was neuroprotective when analyzed 3 days after, not only at the tissue level but also at the functional level, although part of the neuroprotection observed was directly mediated by the vector itself.

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NEUROPROTECTION FROM NMDA EXCITOTOXIC LESION BY Cu/Zn SUPEROXIDE DISMUTASE GENE DELIVERY TO THE POSTNATAL RAT BRAIN BY A MODULAR PROTEIN VECTOR

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Abstract

Background

We have analyzed the neuroprotective efficacy of the transient overexpression of antioxidant enzyme Cu/Zn Superoxide dismutase (SOD) after excitotoxic injury to the immature rat brain by using a recently constructed modular protein for non-viral gene delivery termed NLSCt.

Results

Injection of the NLSCt vector carrying the Cu/Zn SOD transgene 2 hours after intracortical N-methyl-D-aspartate administration showed improved functional outcome and a reduced lesion volume at 3 days post lesion. In secondary degenerative areas, increased neuronal survival as well as decreased numbers of degenerating neurons and nitrotyrosine immunoreactivity was seen. Interestingly, injection of the NLSCt vector carrying the control GFP transgene also displayed a significant neuroprotective effect but less pronounced.

Conclusions

When the appropriate levels of Cu/Zn SOD are expressed transiently after injury using the non-viral modular protein NLSCt a neuroprotective effect is seen. Thus recombinant modular protein vectors may be suitable for in vivo gene therapy.

Background

The pathobiology of acute damage to the CNS includes production of the superoxide anion ($O_2^{-\cdot}$) and other reactive oxygen species that rapidly induce oxidative injury by lipid peroxidation, DNA damage and protein nitration (Halliwell 2001) Many studies in the last decade have focused on the study of antioxidant proteins dealing with oxidative stress in physiological conditions and after injury.

Superoxide dismutases are among the most important cellular mechanisms that cope with oxidative stress. In normal conditions, cytosolic copper zinc superoxide dismutase (Cu/Zn SOD) and mitochondrial manganese superoxide dismutase (Mn SOD) are responsible for maintaining low levels of intracellular $O_2^{-\cdot}$ by catalyzing its dismutation to oxygen and H_2O_2 (McCord and Fridovich 1969). In neuronal cells, endogenous Cu/Zn SOD is normally expressed but is rapidly downregulated after several types of acute brain insults (DeKosky et al. 2004; Kim et al. 2000; Liu et al. 1993; Peluffo et al. 2005) rendering the brain more susceptible to oxidative stress. In agreement with its antioxidant role, over-expression of Cu/Zn SOD in adult transgenic rats show pronounced neuroprotection in most acute CNS injury models (Chan et al. 1991; Mikawa et al. 1996; Yang et al. 1994) and targeted deletions of the Cu/Zn SOD gene or extracellular SOD genes worsens the outcome after focal ischemia in the adult brain (Kondo et al. 1997; Sheng et al. 1999).

After hypoxic/ischemic injury to the immature CNS, contradictory results have been reported in regards to the role of Cu/Zn SOD. Brain damage as a result of perinatal hypoxic-ischemic insult is a serious clinical problem with severe neurological consequences, where oxidative stress is known to play a fundamental role (Berger and Garnier 1999; Ferriero 2004). In previous studies using N-methyl-D-aspartate (NMDA)-mediated excitotoxicity, a model for hypoxic-ischemic injury to the postnatal brain (Ikonomidou et al. 1989; Olney 1990), we have shown an upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Acarin et al. 2002) and increased levels of the oxidative marker nitrotyrosine (Acarin et al. 2005). However, while a slightly worsened neuropathological outcome was observed in postnatal transgenic mice over-expressing Cu/Zn SOD (Ditelberg et al. 1996; Fullerton et al. 1998), several antioxidant molecules including SOD mimetics like $O_2^{-\cdot}$ dismuting metalloporphyrins have been shown to be neuroprotective (Shimizu et al. 2003). In this sense, several differences between immature and adult animals in terms of oxidative stress and antioxidant defenses have been described including: upregulation of gluthathione peroxidase in the damaged adult brain but not in the damaged immature brain after trauma (Fan et al. 2003), the rapid free iron accumulation within 4 hours after transient cerebral ischemia stimulating Fenton reactions in the immature brain (Kondo et al. 1995; Palmer et al. 1999), the lesser concentration of metallothioneins, potent metal-binding antioxidant enzymes in the immature brain (Ebadi 1986; Nishimura et al. 1992). Finally, the postnatal brain is more sensitive than the adult brain to the neurotoxic actions of NMDA (McDonald et al. 1988) which will lead to increased $O_2^{-\cdot}$ generation (Dugan et al. 1995; Lafon-Cazal et al. 1993). In view of these contradictory findings of Cu/Zn SOD

expression, we induced a transient post-injury overexpression of Cu/Zn SOD after excitotoxic damage to the immature rat brain using a novel non-viral gene therapy approach.

The design of non-viral vectors showing transgene expression in the brain has recently gained interest (Peluffo et al. 2003; Shi and Pardridge 2000; Wang et al. 2002; Xiang et al. 2003) due to the limitations imposed by viral vectors (Bessis et al. 2004; Maguire-Zeiss and Federoff 2004). Non-viral modular approaches for gene therapy vectors based on the combination of several functional domains in a single polypeptide chain are of particular interest because of recombinant DNA methodologies that allow tailored designed vectors. The production of such protein vectors also permits a convenient scaling up and the vehicles exhibit high stability suitable for therapeutic uses. We have previously reported the construction of a recombinant modular protein vector that combines different functional domains displayed by E. coli β-galactosidase then engineered to conveniently accommodate a poly-lysine tail with DNA condensing/attaching properties and an integrin targeting RGD motif with cell attachment and internalization properties (Aris et al. 2000; Aris and Villaverde 2000). The resulting vector was capable of transferring a transgene to the intact and lesioned brain without any detectable acute inflammatory reaction or immune activation (Peluffo et al. 2003). We have further improved the transfection efficiency of this modular vector by introducing the nuclear localization motif of the SV40 virus, generating the vector NLSCt (Aris and Villaverde 2003).

The aim of this study was to assess the potential efficacy of the recombinant modular protein vector NLSCt complexed to the Cu/Zn SOD gene to improve neurological outcome after an acute excitotoxic injury to the immature brain.

Results

Lesion volume and neuronal cell death

The injection of NMDA into the sensorimotor cortex of the postnatal brain induces a well-characterized excitotoxic lesion (Acarin et al. 1996) which includes the sensorimotor cortex, the dorso-medial striatum, and the rostral hippocampus.

Rat brains injected two hours after the lesion with the NLSCt vector carrying the antioxidant enzyme Cu/Zn SOD transgene (NMDA+Cu/Zn SOD group) showed a pronounced reduction in the neurodegenerative area when compared to both the NMDA only group and the NMDA+saline group. The percentage of lesioned hemisphere volume was reduced by 42.6% in comparison with NMDA+saline animals (Figure 1A). The distribution of the lesion along the antero-posterior axis showed that the reduction in the neurodegenerative area extended rostrocaudally to all affected regions including cortex, striatum and hippocampus (Figure 1B, 2A). Both primary lesioned areas such as the sensorimotor cortex, and secondary lesioned areas like the caudal sub-plate neuronal layer, striatum and hippocampus showed a reduction in the extent of neurodegeneration. Surprisingly, the negative control, with the NLSCt vector carrying the GFP transgene (NMDA+GFP group), showed a less pronounced but significant reduction in the neurodegenerative area. The NMDA+GFP group was only significantly different from the NMDA+saline group and not the NMDA only group (the % of lesioned hemisphere volume was reduced by 29.4% with respect to the NMDA+saline group, Figure 1A, B, 2A).

To confirm the reduction in the extent of neurodegeneration, remaining neurons (by Nissl staining) and degenerating neurons (by FluoroJade B staining) present in various secondary degenerating regions were quantified. In NMDA+Cu/Zn SOD animals, the number of Nissl stained neurons in secondary degenerating regions such as, the lateral sensorimotor cortex where higher than those observed in NMDA+GFP animals. NMDA+Cu/Zn SOD animals also showed increased neuroprotection at the caudal region of hippocampal CA1 layer (Figure 2A, B). Accordingly, in NMDA+Cu/Zn SOD animals, Fluoro-Jade B stained degenerating neuron cell counts showed a significant reduction in the number of stained neurons at the caudal region of hippocampal CA1 layer whereas a not significant reduction was seen in the temporal cortex (caudal border of lesion) (Figure 2C, D). In contrast, in the NMDA+GFP group similar numbers of Nissl stained neurons in the sensorimotor cortex were seen when compared to NMDA+saline animals. Moreover, the number of Nissl-stained neurons at the CA1 layer of the hippocampus was only significantly higher at a greater distance from the lesion site in NMDA+GFP animals when compared to NMDA+saline animals, showing less pronounced neuroprotection than the NMDA+Cu/Zn SOD animals (Figure 2B). Although NMDA+GFP animals displayed reduced numbers of FluoroJade B degenerating neurons in the hippocampal CA1 region in relation to the NMDA+saline group, the reduction occurred only to a limited extent when compared to NMDA+Cu/Zn SOD animals (Figure 2C, D).

To determine if the reduction in the neurodegenerative area observed after the treatment with the NLSCt vector carrying the control GFP transgene was due to the DNA molecule or intrinsic to the NLSCt vector, we injected lesioned rats with the naked NLSCt vector. Interestingly, the NLSCt vector alone induced a significant 31.2% reduction of lesion volume (data not shown), reproducing the results obtained after the treatment with the NLSCt vector carrying the GFP transgene.

Tyrosine nitration

Protein tyrosine nitration is a footprint of peroxynitrite and other reactive species formation (Radi 2004). As we have previously described in detail (Acarin et al. 2005), excitotoxic damage induces nitration in astrocytes and neuronal cells within the neurodegenerating area during the first 24 hours post-lesion and in the border of the lesion at longer survival times (Figure 3). In NMDA+Cu/Zn SOD animals, densitometrical measurements in a degenerating area at the border of the lesion such as the caudal region of the CA1 hippocampal layer showed a significant reduction in tyrosine nitration (immunoreactivity grade of 1.9±0.3) in relation to NMDA+saline animals (immunoreactivity grade of 3.8±0.7). Densitometry in the NMDA+GFP animals (2.4±0.4) was not significantly different from the NMDA+saline group (Figure 3).

Functional outcome

Interestingly, neurological tests carried out showed that only the Cu/Zn SOD overexpressing animals recovered significantly from the injury. In the inclinated grid climbing test, where general motor coordination is evaluated, only NMDA+Cu/Zn SOD animals showed significant and near complete recuperation of the time spent in the inclinated grid at 3 days, statistically indistinguishable from the non-lesioned saline injected animals (Figure 4A). Both NMDA+saline and NMDA+GFP injected animals showed a reduced performance in this task until 3 days, the last time analyzed. In addition to the improvement of the NMDA+SOD injected animals observed in the grid climbing test, the spontaneous turning behaviour, a neurological sign of un-balanced striatal neurotransmission, also showed a similar profile (Figure 4B). NMDA+saline and NMDA+GFP animals showed a significant spontaneous net turning toward the ipsilateral side at 1 day when compared with non-lesioned saline injected animals. In contrast, NMDA+Cu/Zn SOD animals did not show any bias on their net turning behaviour, not differing significantly from the non-lesioned saline injected rats. All groups performed equally well on an open field motor task (Figure 4C), demonstrating similar motor activity, which could not account for the differences observed in both of the neurological tests performed. In addition, a general improvement of the Cu/Zn SOD overexpressing rats could also be observed in the development-mediated daily weight increase (Figure 4D) where again, only the Cu/Zn SOD over-expressing animals were statistically indistinguishable from the non-lesioned saline injected controls.

Discussion

This study shows for the first time that consistent functional and neuropathological recovery from acute immature brain damage can be achieved by post-lesion overexpression of the Cu/Zn SOD antioxidant enzyme delivered through a modular multifunctional protein vector. This treatment protected against excitotoxic damage, thought to be an important mechanism underlying neuronal death after hypoxic/ischemic injury to the neonate (Ferriero 2004; Johnston et al. 2002), but also in acute adult brain neurodegenerative conditions such as stroke (Dirnagl et al. 1999) and traumatic brain injury (Obrenovitch and Urenjak 1997), as well as in chronic ones as Alzheimer's (Cotman 1998), Parkinson's (Jenner and Olanow 1998) and Huntington's (Petersen et al. 1999) disease.

Cu/Zn SOD as a therapeutic transgene

In this study, the Cu/Zn SOD transgene was expressed under the control of the cytomegalovirus immediate early promoter and as such, expressed in any cell type. We have previously shown that after postnatal excitotoxic injury, neurons, astrocytes and microglial cells are the main cell types showing transgene expression mediated by NLSCt-type vectors (Peluffo et al. 2003). Under physiological conditions, Cu/Zn SOD is mainly expressed in neurons (Pardo et al. 1995; Peluffo et al. 2005), however, in our experimental conditions several glial cell types are also transfected (Peluffo et al. 2003) and could indirectly mediate the neuroprotective effect. Noteworthy, $O_2^{-\cdot}$ produced after damage in both neurons and glial cells can be dismutated to H_2O_2 by Cu/Zn SOD activity, hindering the formation of the potent oxidant and protein nitrating agent peroxynitrite (Beckman et al. 1990; Radi et al. 1991). The reduction in the levels of nitrotyrosine reported here support this mechanism of Cu/Zn SOD neuroprotection. Interestingly, there is a great deal of evidence that suggests that $O_2^{-\cdot}$/peroxinitrite species toxicity is higher than that of H_2O_2. A study of Cu/Zn SOD overexpressing astrocytes exposed to $O_2^{-\cdot}$ found that they had higher survival rates than control astrocytes even when glutathione peroxidase and catalase activities were blocked and GSH levels depleted (Chen et al. 2001). Cu/Zn SOD overexpressing astrocytes also survived better that control astrocytes after oxygen glucose deprivation, in the absence of glutathione peroxidase upregulation and with a lower catalase upregulation in comparison to control astrocytes (Wang et al. 2005). These Cu/Zn SOD overexpressing astrocytes, unlike controls, also maintained elevated GSH concentration. Peroxinitrite but not H_2O_2 has also been shown to trigger an in vitro reactive phenotype of astrocytes that is toxic for co-cultured motor neurons (Cassina et al. 2002). These data suggest that overproduction of H_2O_2 is not a major factor in astrocytic injury. On the other hand, specific scavenging of $O_2^{-\cdot}$ can also increase neuronal survival under some pathologically relevant conditions. For example, motor neurons can be rescued from trophic factor withdrawal by liposome-mediated Cu/Zn SOD protein delivery (Estevez et al. 2000) and synthetic SOD mimetics (Estevez et al. 1998; Peluffo et al. 2004). Neuronal cultures can also be protected from excitotoxicity by SOD mimetics (Vergun et al. 2001), adenovirally mediated overexpression of Cu/Zn SOD (Barkats et al. 1996), or

transgenically overexpressed Cu/Zn SOD (Chan et al. 1990). However, in some particular cases, Cu/Zn SOD overexpression can reduce neuronal survival during direct extracellular exposure to superoxide generators by a mechanism involving excess H_2O_2 accumulation (Ying et al. 2000). Finally, some research suggests that $O_2^{-\cdot}$ is more toxic than H_2O_2 by reacting with ·NO to form peroxinitrite (Beckman et al. 1990; Radi et al. 1991) as been shown by inhibition of ·NO production in neuronal cultures submitted to an excitotoxic damage. This treatment is sufficient for inducing neuroprotection (Dawson et al. 1993; Gunasekar et al. 1995; Lipton et al. 1993), while $O_2^{-\cdot}$ and H_2O_2 are still being formed but will not be so toxic. Thus, the increase in Cu/Zn SOD expression in neurons and astrocytes most likely contributes to the neuroprotection observed in vivo.

Regarding lesion volume, we show a significant decrease in all NLSCt treated animals when compared to NMDA+saline injected animals, but no difference between these NLSCt treated groups. However, only the Cu/Zn SOD treated animals showed a significant reduction in lesion volume compared to NMDA alone lesioned animals, which is in fact the real control of the therapeutic gene therapy approach. It is important to consider the functional recovery induced by Cu/Zn SOD therapy in both of the neurological evaluation tests that could reflect increased preservation of functional synaptic contacts and white matter tracts. The reduction in the developmental increase in daily body weight observed in lesioned animals and GFP treated animals was not observed in Cu/Zn SOD treated animals, which also supports the general improvement of these animals.

In accordance with our findings, SOD mimetics (such as $O_2^{-\cdot}$ dismuting metalloporphyrins) were shown to be neuroprotective after ischemia in the immature brain (Shimizu et al. 2003). However, our results contrast with the previously reported exacerbation of hypoxic/ischemic injury occurring in immature transgenic mice over expressing Cu/Zn SOD (Ditelberg et al. 1996). Though the reason for this difference is not clear, several reasons besides species specificity and lesion model could contribute to its explanation. In our experimental conditions, the NLSCt vector induced a transient and lower level of Cu/Zn SOD transgene expression compared to the higher and permanent expression found in transgenic mice. It has previously been shown that very high levels of Cu/Zn SOD, as those observed in transgenic animals, can produce alterations such as, an increase in basal lipid peroxidation (Ceballos-Picot et al. 1992), mitochondrial vaquolation (Dal Canto and Gurney 1995; Jaarsma et al. 2000), abnormalities in neuromuscular junctions (Avraham et al. 1988), or deficits in long-term potentiation (LTP) and spatial memory (Gahtan et al. 1998). Furthermore, after life-long overexpression of Cu/Zn SOD, compensatory changes in the basal levels or induction of other antioxidant enzymes like Mn SOD (Ceballos-Picot et al. 1992), heme oxygenase (Weinzierl et al. 2003), or glutathione peroxidase (Sheldon et al. 2004) have been documented that provide an altered redox balance in transgenic animals.

In regards to expression levels, it has been reported that polyethylene glycol-conjugated Cu/Zn SOD treatment after focal ischemia showed a U-shaped dose-response curve, implying that the effective neuroprotective dose of this enzyme may be in fact concentration restricted (He et al. 1993).

Therefore, several parameters like protein levels, time-course and cell population of Cu/Zn SOD expression could affect the overall outcome after the lesion and underlie the differences between gene therapy and transgenic mice approaches.

NLSCt vector for neuroprotective gene therapy

Studies showing phenotypic or functional effects derived from transgene expression in the CNS are generally absent (Dumas and Sapolsky 2001). Regarding non-viral vectors, only one flexible liposome/antibody-conjugated non-viral vector has been reported to induce functional recovery, reversing motor abnormalities after a 6-hydroxydopamine striatal lesion (Zhang et al. 2004).

Several multifunctional protein vectors have been developed by combining functional modules from different origins, driving the four main steps for successful DNA transfer to the cell nucleus: DNA ligation-condensation, cell attachment-internalization, endosome disruption-escaping, and nuclear import. Although many of these prototypes can transfect cells in culture, their efficiency in vivo is very limited (reviewed in (Aris and Villaverde 2004)). We have previously shown that an earlier version of the NLSCt modular vector had a restricted capacity for transgene delivery to the intact brain. However, it was very efficient in conducting widespread transgene expression after an excitotoxic lesion (Peluffo et al. 2003), probably due to the disruption of the extracellular matrix and the small size of the vector/DNA complexes (20-40nm diameter) (Aris and Villaverde 2000). In this study, we show that NLSCt had a very high transfection efficiency, only 24ng of NLSCt-coated Cu/Zn SOD plasmid was able to reduce oxidative stress and rescue neurons from cell death in different areas of the lesion border, that considerably reduced the total lesion volume and increased functional outcome. This transfection efficiency could be due to integrin mediated endocytosis and enhanced transit towards the nuclear compartment mediated by the SV40 viral nuclear localization sequence of NLSCt (Aris and Villaverde 2003). It could also be due to massive neuronal endocytosis that reaches the nuclear compartment, a process previously described in neuronal cells within a few hours after injection of toxic and sub-toxic doses of NMDA or kainate (Borsello et al. 2003a; Borsello et al. 2003b).

Transient Cu/Zn SOD expression induced by NLSCt-mediated gene therapy seems to be an important parameter in conferring neuroprotection. In the same experimental model as the one used here, and also using the transgene regulated by the CMV promoter, the expression of the GFP transgene was transient, almost disappearing at 7 days postinjury (Peluffo et al. 2003). Whereas a prolonged stable delivery of a transgene would be desirable for long-term improvement of pathologies such as inherited monogenic defects, transient transgene delivery may be more beneficial than a constitutive expression for therapeutic intervention after acute CNS damage.

Interestingly, both the NLSCt vector carrying a control transgene and the nude vector showed a significant grade of neuroprotection, indicating that the effect was intrinsic to the NLSCt vector. One of the bioactive motifs of NLSCt is the foot-and-mouth disease virus integrin-interacting RGD peptide, which interacts preferentially with different α_V integrins (Duque and Baxt 2003). This may suggest that

our results with the vector show a neuroprotective effect mediated by the interaction between the RGD motif of NLSCt and specific integrins through an unknown mechanism. This is supported by a recent study showing that blocking αD/β2 integrins is strongly neuroprotective after spinal cord injury (Gris et al. 2004). In addition, although the NLSCt vector inhibits the interaction of RGD dependent integrins with their natural extracellular matrix ligands, it could be directly activating integrin outside-in signaling events (Qin et al. 2004).

Conclusions

We show that transient overexpression of Cu/Zn SOD after an excitotoxic injury to the immature rat brain using a modular protein for non-viral gene delivery can be neuroprotective and improve functional outcome; signalling this vector as a promising tool for in vivo gene therapy strategies for acute CNS lesions.

Methods

Protein, DNA and protein-DNA complexes

Protein NLSCt is an engineered form of Escherichia coli beta-galactosidase that displays an integrin-targeted RGD motif. This segment, inserted between residues 249 and 250 of the bacterial enzyme, reproduces the cell-attachment region of the VP1 capsid protein of foot-and-mouth disease virus (Aris and Villaverde 2000) that binds host cells preferentially by integrin $\alpha v\beta 3$ but also by integrins $\alpha v\beta 6$, $\alpha v\beta 5$, and $\alpha v\beta 8$ (Duque and Baxt 2003). The additional presence of a deca-lysine tail joined to the amino terminus of the construct and a still unidentified enzyme segment with nuclear targeting properties and the SV40 NLS at carboxi terminus of the recombinant protein (McInnis et al. 1995) enables NLSCt to promote efficient DNA delivery. The NLSCt protein was produced in bacteria and purified from crude cell extracts as described previously (Aris et al. 2000). The human Cu/Zn SOD gene cloned into the plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) under the control of a cytomegalovirus immediate early promoter was used (generously provided by AG Estévez and JS Beckman). A red-shift variant of jellyfish Aequorea Victoria green fluorescent protein (GFP) gene encoded into plasmid pEGFP-C1 (Clontech, Palo Alto, CA, USA) under the control of the same cytomegalovirus promoter was used as a control. Protein and DNA complexes were formed by incubation in 0.9 % NaCl at room temperature for 1 hour at ratios of 0.03 μg DNA per μg of NLSCt protein. Details of complex formation are provided elsewhere (Aris et al. 2000).

Excitotoxic injury and treatment paradigm

Nine-day old Long-Evans black-hooded rat pups (15-20 gr., both sexes; Janvier, France) were used. All intracerebral injections were made into the right sensorimotor cortex at the level of the coronal suture (2 mm lateral of bregma and 0.5 mm depth) using a stereotaxic frame adapted for new-borns (Kopf Instruments) under isoflurane (Baxter International Inc.) anesthesia. Excitotoxic lesions were performed as previously described (Acarin et al. 1999), by injecting 18.5 nmol of N-methyl-D-aspartate (NMDA) (Sigma-Aldrich, St. Louis, MO, USA) diluted in 0.15 μl of saline solution (0.9 % NaCl) at a rate of 0.05 μl/min using an automatic injector. One microliter of either the NLSCt vector (0.8 μg/μl) carrying the control EGFP plasmid or the Cu/Zn SOD plasmid, the NLSCt vector alone (0.8 μg/μl), or the vehicle (NaCl 0.9 %) was injected 2 hours after the excitotoxic lesion at the same coordinates at 0.2 μl/min. After suture, pups were placed on a thermal pad for 2 hours at 36 ºC to maintain normothermia. Experimental animal work was conducted according to Spanish regulations in agreement with European Union directives. Experimental procedures were approved by the ethical commission of the Autonomous University of Barcelona. All efforts were made to minimize animal suffering.

Behavioural and neurological testing.

Quantitative methods for the evaluation of adult motor performance (Marshall and Ungerstedt 1977) have been adapted here for postnatal pups (P9-P12). These methods of scoring gave consistent values for different observers. From day 1 until day 3 post-lesion, all rats were weighed and tested once a day in each of the following tasks: general motor activity, net turns and inclinated grid climbing. For the evaluation of general motor activity and net turns, rats were placed in an open field (70cm x 70cm) immediately after the separation from the mother and their spontaneous activity was recorded as number of new squares (10x10 cm) visited. Simultaneously, the total number of spontaneous complete turns (ipsilateral=positive and contralateral=negative) were recorded. As previously shown elsewhere, un-lesioned animals as well as saline injected animals did not show turning bias towards either side. In this test, lesioned rats show spontaneous turning bias towards the ipsilateral side while the lesion over-stimulates the ipsilateral parenchyma. After one day it disappears due to the complete destruction of neurons and then turning can only be observed by injection of amphetamine. The inclinated grid climbing test was performed by allowing the rats to climb an inclined grid (metal bars of 3.5 mm in diameter and separated by 7.5 mm) at an angle of 45°. Climbing the grid is a spontaneous response. However, in the few cases when a rat stayed still on the grid, it was removed and placed again on the grid. The total time climbing on the grid until five consecutive falls occurred was recorded each day for every rat. The task was interrupted after 5 falls or after 360 seconds on the grid. Unlesioned animals and saline injected animals consistently climbed for longer time periods than lesioned animals.

Histology and lesion volume measurement

Three days post-lesion, rats were anaesthetized and perfused intracardially with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were post-fixed in the same fixative for 2 hours and sunk in a 30% sucrose solution before being frozen with dry CO_2. Coronal sections of the entire brain (30-μm thick) were obtained using a Leitz cryostat. Parallel sections of the entire brain every 240 μm were collected directly on a slide, stained for Nissl and used for the measurement of the total lesioned area and total hemisphere area after high resolution digitizing. Using the analySIS® software, the pale Nissl stained lesioned area was quantified by parallel observation of the slides on a microscope. To avoid misinterpretations due to possible tissue edema or postmortem alterations during cutting or mounting, data are presented as percentage of the ipsilateral hemisphere.

Immunohistochemistry for nitrotyrosine and densitometrical analysis

Nitrotyrosine labeling, a marker of peroxynitrite and other reactive oxygen species formation, was used to evaluate the level of oxidative stress. Sections were processed for endogenous peroxidase inactivation and blocked for 1 hour in Tris-buffered saline (TBS, pH 7.4), 10 % fetal calf serum plus 1% Triton X-100. Sections were incubated overnight at 4°C in the same blocking solution with a primary

antibody against nitrotyrosine (1:60)(06-284, Upstate Biotechnology, Lake Placid, NY, USA). After several washes they were incubated for 1 hour with biotinylated anti-rabbit (1:200, Amersham RPN-1004). Specific labeling was evidenced by incubation with avidin-peroxidase (1:400 Dakopatts P0364) for 1 hour and subsequent 3,3´-diaminobenzidine (DAB)-hydrogen peroxide developing procedure. For densitometrical measurements, digitalized images from ipsilateral lesion border (penumbra) and contralateral hippocampal CA1 regions were analyzed with the analySIS® software for total grey intensity. Data was expressed as the ratio between immunolabeling intensity in the injured and contralateral hemispheres.

Fluoro-Jade B staining

Neuronal degeneration was detected as previously described (Schmued and Hopkins 2000). Briefly, free-floating sections were mounted and air dried overnight. After dehydration in ethanol (30%, 50%, 70%, 96% and 100%) and rehydration (etanol 96% and 70%), sections were rinsed with distilled water and oxidized with MnO4K (0.06% in water, 15 min.). Then sections were rinsed with distilled water, incubated with Fluoro Jade B (Histo-Chem, Inc. Jefferson, USA) (0.0004% in water plus 1% acetic acid glacial, 20 min.), washed with distilled water, air dried and mounted.

Statistical analysis

All results are expressed as mean ± standard error mean (SEM). Six rat litters containing at least 3 different experimental groups were used. A total of 8 saline injected animals and 51 NMDA injected animals were used (NMDA only, n=4; NMDA+saline, n=14; NMDA+NLSCt/pSOD, n=13; NMDA+NLSCt/pEGFP, n=16; and NMDA+NLSCt, n=4). ANOVA followed by Fisher`s PLSD post-hoc test was used to determine significant differences ($p<0.05$) in lesion volume, cell counts, nitrotyrosine densitometry and number of squares visited measurements. Repeated measures ANOVA followed by Fisher`s PLSD post-hoc test were used to evaluate significant differences ($p<0.05$) between groups in weight increase and in inclinated grid walking. Analysis of significant differences between groups in the measurements of the number of net turns was performed by ANOVA followed by Fisher`s PLSD post-hoc test after ranking of the data.

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Figures

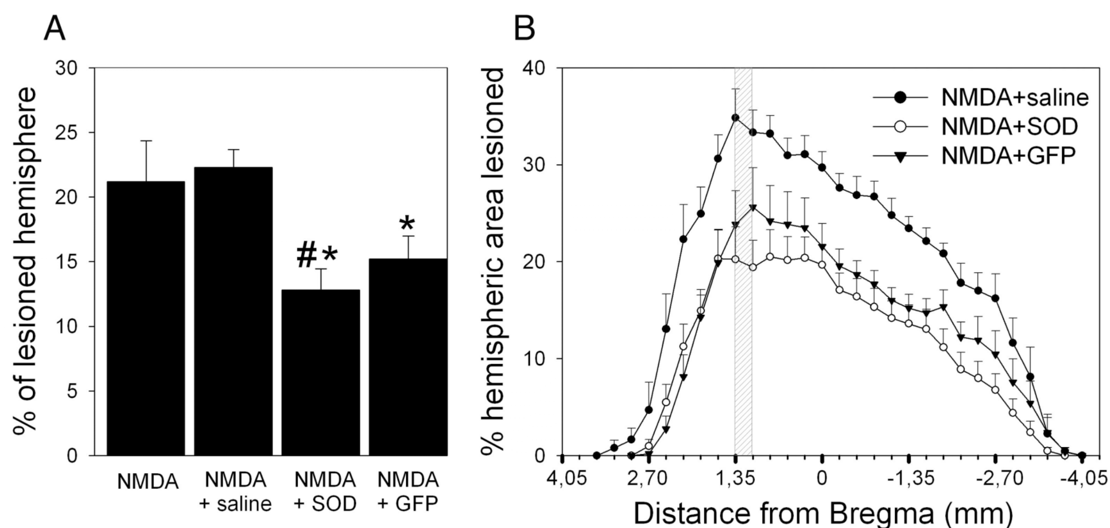

Figure 1 - Post-lesion over-expression of Cu/Zn SOD is neuroprotective

NMDA lesioned animals were not re-injected or re-injected 2 hours after with either saline solution, the NLSCt vector carrying the transgene for Cu/Zn SOD or the NLSCt vector carrying the transgene for EGFP (NMDA, NMDA+saline, NMDA+SOD and NMDA+GFP respectively). The percentage of total lesioned hemisphere observed in A show that animals injected with the transgene for Cu/Zn SOD displayed a significant reduction in lesion volume in comparison with NMDA+saline (*$p<0.05$) or in comparison to NMDA alone (#$p<0.05$). The lesion volume in NMDA+saline injected animals was 36.2mm^3. Noticeably, animals injected with the GFP transgene also displayed a significant reduction, though only when compared to NMDA+saline injected animals; a reduction that was less pronounced than those found in Cu/Zn SOD injected animals. The rostro-caudal percent of lesioned hemispheric area is shown in B. The overall lesion was reduced at all levels of the brain of Cu/Zn SOD and GFP injected animals. The injection site is highlighted in grey.

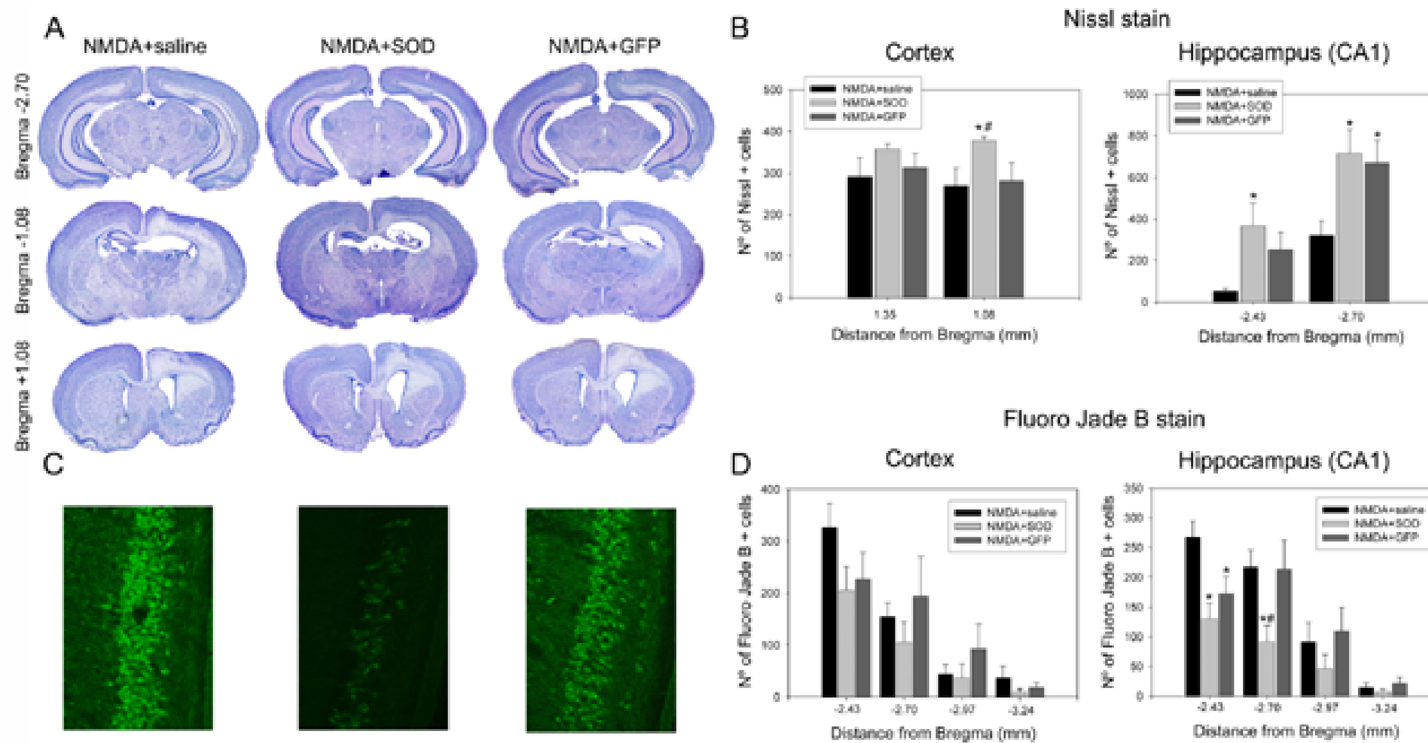

Figure 2 - Post-lesion over-expression of Cu/Zn SOD increases neuronal survival

Nissl stained sections showing the lesion distribution in the animals post-injected with saline (NMDA+saline), NLSCt+Cu/Zn SOD (NMDA+SOD), or NLSCt+GFP (NMDA+GFP)(A). Quantification of Nissl stained neurons in the sensorimotor cortex and hippocampal CA1 penumbra showed increased cell survival in Cu/Zn SOD overexpressing animals (B). GFP overexpressing animals only showed an increase in neuronal number in hippocampal CA1 at a greater distance from the lesion point (B). Degenerating neurons were stained with Fluoro-Jade B staining (C), and quantitative analysis showed a significant reduction of degenerating neurons in Cu/Zn SOD overexpressing animals (D). (*$p<0.05$ in relation to NMDA+saline injected animals and #$p<0.05$ in relation to NMDA+GFP injected animals).

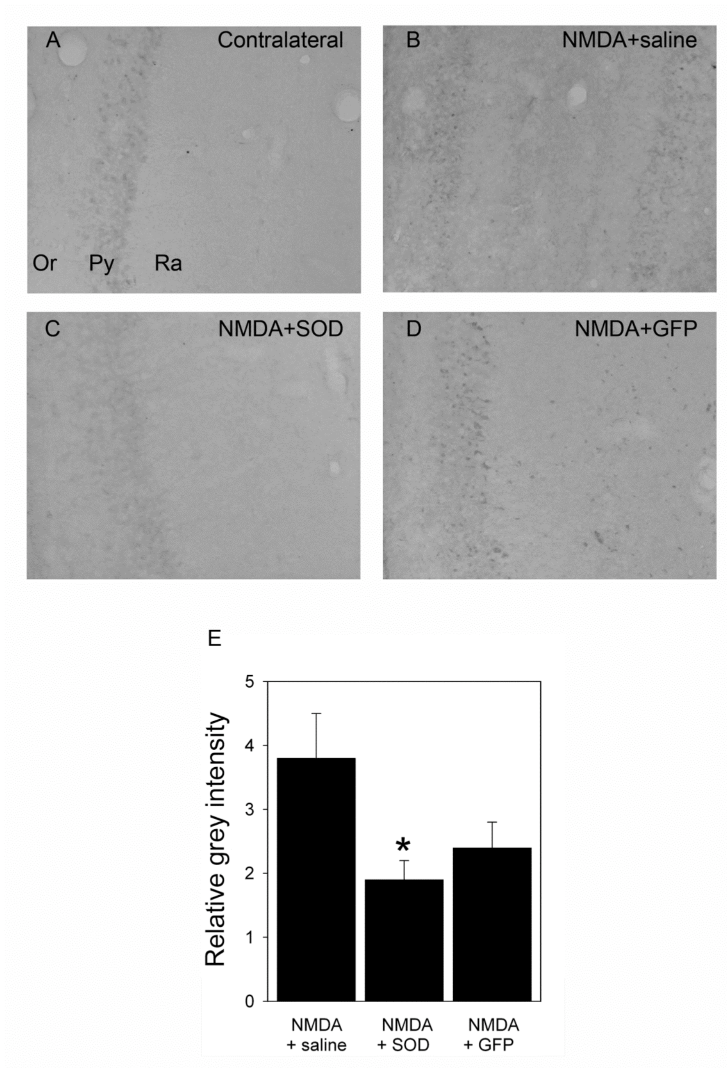

Figure 3 - Post-lesion overexpression of Cu/Zn SOD decreases nitrotyrosine immunoreactivity

Sections from the caudal region of the CA1 hippocampal layer (bregma -2.43) of the contralateral side showed a basal nitrotyrosine immunoreactivity (A, Or: stratum oriens; Py; stratum pyramidalis; Ra: stratum radiatum). After NMDA injection, an increased immunorectivity was detected (B). However, animals overexpressing Cu/Zn SOD showed reduction in nitrotyrosine immunoreactivity (C), while animals overexpressing GFP showed a less pronounced reduction (D). Quantitative analysis confirmed these observations whereby, only animals overexpressing Cu/Zn SOD showed a significant ($p<0.05$) decrease in nitrotyrosine immunoreactivity (E).

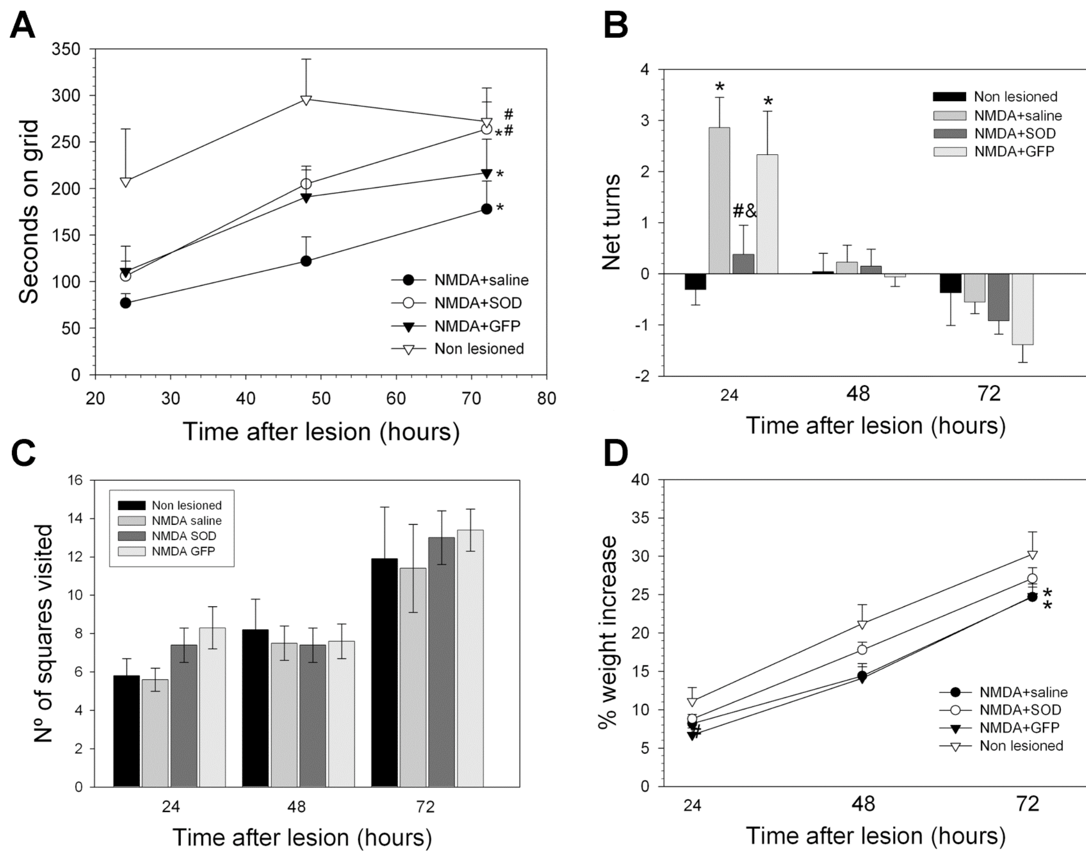

Figure 4 - Functional evaluation of NLSCt-Cu/Zn SOD injected animals

At 24, 48 and 72 hours post-lesion, animals were subjected to several neurological tests including: the estimation of coordination skills by measuring the total climbing time until falling when placed on an inclinated grid (A); spontaneous turning behaviour in an open field (total turns recorded in 1.5 min.)(B); and spontaneous motor activity (C). Percentage of body weight increase was also followed (D). Lesioned animals injected with saline (NMDA+saline) showed a significant decrease in the time spent climbing on the inclinated grid in comparison to non-lesioned saline injected animals (Non lesioned)(A). Interestingly, only animals injected with the NLSCt vector carrying the Cu/Zn SOD transgene (NMDA+SOD) displayed a significant recovery in the time spent on the inclinated grid when compared to NMDA+saline animals (#p<0.05). Animals injected with NMDA+saline or NMDA plus NLSCt vector carrying the EGFP transgene (NMDA+GFP) showed a significant increase in net turns compared to saline injected animals (*p<0.05). In addition, only animals overexpressing Cu/Zn SOD showed a turning behaviour indistinguishable from non-lesioned control saline injected rats and significantly different from NMDA+saline (#p<0.05) or NMDA+GFP injected animals (&p<0,05)(B). There were no differences in the open field general motor activity of all experimental groups (C). Body weight of both NMDA+saline and NMDA+GFP injected animals showed a significant decrease in the developmentally physiological body weight increase in relation to saline injected animals (*p<0.05). Overexpression of Cu/Zn SOD hindered this decrease and these animals showed an increase in body weight indistinguishable from control non-lesioned saline injected rats.

Article number 5:

RGD Integrin Interacting Domains are Neuroprotective after an Excitotoxic Lesion to the Immature Brain by a Glial Dependent Mechanism

HUGO PELUFFO, PAU GONZÁLEZ, ANNA ARÍS, LAIA ACARIN, ANTONI VILLAVERDE, BERNARDO CASTELLANO, and BERTA GONZÁLEZ

Overview

In the previous work, we were surprised to observe that the NLSCt vector displayed endogenous neuroprotective capacity. To understand this finding and to explore how the vector could interact with the neuroprotective gene delivery strategy that we pursued in this Thesis, we designed several *in vivo* and *in vitro* experiments. Based on previous literature, our main hypothesis was that the integrin-targeting domain of NLSCt could be at least in part responsible for this effect. Thus, we compared the effect of the NLSCt vector with those of an RGD containing peptide in our acute excitotoxic injury model. Both the NLSCt protein vector and the RGD containing peptide showed a similar and significant neuroprotection and a modulation of the microglial response after an *in vivo* excitotoxic injury. Interestingly, the neuroprotective effect was dependent on glial cells, as glial cells were necessary in cortical cultures for neuroprotection from NMDA treatment.

Manuscript.

RGD INTEGRIN INTERACTING DOMAINS ARE NEUROPROTECTIVE AFTER AN EXCITOTOXIC LESION TO THE IMMATURE BRAIN BY A GLIAL DEPENDENT MECHANISM

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Abstract

Integrin binding to extracellular matrix ligands, including those presenting the prototypical RGD motif, modulate diverse cellular responses such as proliferation, migration, survival, differentiation, synaptogenesis and neuritic outgrowth. Within the brain, many molecules like vitronectin, osteopontin, and fibronectin, which are induced after an acute lesion, present RGD motifs. Previous work from our group showed that a recombinant gene therapy vector termed NLSCt, which displayed an RGD motif, was neuroprotective after an excitotoxic injury to the immature rat brain. In this study, we have analyzed whether this neuroprotection was mediated by the RGD motif by injecting the RGD-containing synthetic peptide GPenGRGDSPCA (GPen) two hours after an N-Methyl-D-Aspartate (NMDA)-mediated excitotoxic lesion to the P9 rat brain and analysing the extent of brain damage and the glial and inflammatory response 3 days later. The animals treated with the GPen peptide showed a significant lesion volume reduction up to 28%, which was dose dependent in the 0.007-1mM range, and a decrease in the number of FluorojadeB positive degenerating neurons in the hippocampal lesion periphery, when compared to lesioned animals treated with saline. Additionally, NMDA-injected animals treated with either NLSCt or GPen at the neuroprotective doses showed a significant increase in microglial reactivity and microglia/macrophage cell number, but no differences in neutrophil infiltration and the astroglial response. Finally, in order to determine whether the neuroprotective effect was dependent on glial cells, we performed in vitro studies using neuron-purified primary cultures and neuron-enriched cultures containing glial cells, where we have evaluated the neuroprotective effect of NLSCt and GPen after NMDA-induced cell death. Interestingly, whereas no neuroprotection was seen after 3 days in neuron-purified cultures, a significant and complete neuroprotection accompanied by activation of the ERK kinase intracellular signalling cascade was seen in neuron-enriched cultures containing glial cells. These overall results suggest that RGD containing molecules are neuroprotective after an excitotoxic injury by indirect glial-dependent mechanisms.

Introduction

Brain damage as a consequence of perinatal cerebral hypoxia/ischemia is a major cause of acute mortality and severe chronic disabilities. Several lines of evidence suggest that the immature brain responds in a particular fashion to brain injuries (Ferriero 2004; Vannucci and Hagberg 2004), partly due to the fact that many of adult gene expression patterns, neural circuits organization, cell differentiation and myelination have not yet been achieved (Bourgeois and Rakic 1993; Jacobson 1963). In the last years, we have characterized the neurodegenerative process and the associated glial, oxidative and inflammatory response following excitotoxic brain damage to the postnatal brain (Acarin et al. 2000a; Acarin et al. 2000b; Acarin et al. 1999; Acarin et al. 2005; Acarin et al. 2002b), and have developed several pharmacological and gene therapy strategies for neuroprotection (Acarin et al. 2001; Acarin et al. 2002a; Peluffo et al. 2006). In this sense, as non-viral gene therapy using recombinant protein vectors constitutes a promising tool for the introduction of therapeutic genes (Aris and Villaverde 2004), we have been using a recombinant multifunctional vector termed NLSCt for gene delivery to the damaged postnatal CNS (Peluffo et al. 2003) (Peluffo et al. 2006). This vector uses β-galactosidase as scaffold protein (Aris et al. 2000; Aris and Villaverde 2000), holds a polylysine tail for DNA attachment, displays an integrin interacting motif composed of the GH region of the foot and mouth disease virus (FMDV) presenting a prototypical three amino acid Arg-Gly-Asp (RGD) integrin-interacting motif (Villaverde et al. 1996) and bears a short peptide sequence acting as nuclear localization signal for nuclear transport (Aris and Villaverde 2003). Surprisingly, in a recent work studying the putative neuroprotective effect of antioxidant enzyme gene delivery, we found that NLSCt carrying a control DNA was neuroprotective when injected after an excitotoxic lesion to the immature brain (Peluffo et al. 2006). As integrin interaction has been implicated in proliferation, migration, survival and differentiation in a variety of cell types, we have hypothesized that the neuroprotective effect of NLSCt may be mediated by the interaction of the RGD domain of this vector with cell integrins present in neurons and/or glial cells.

Integrins are a very complex family of molecules as they can form more than 20 different glycoprotein receptors composed of one α and one β subunit, they show some degree of overlapping ligands (Miranti and Brugge 2002), they transduce both outside-in and also inside-out signals (Qin et al. 2004), and a single integrin can recognize two different ligands and transduce different signals in response to each of them (Butler et al. 2003). In particular, in the CNS, integrins are widely but differentially expressed in distinct neuronal populations (Pinkstaff et al. 1999; Schmid and Anton 2003), wherein they are mainly found in the cell membrane of growth cones and synaptic terminals (Bahr and Lynch 1992; Bahr et al. 1991; Einheber et al. 1996), and have been implicated in neuronal migration, survival, differentiation, synaptogenesis and neuritic outgrowth (Clegg et al. 2003). In addition to neurons, glial cells also express a wide variety of integrins (Schmid and Anton 2003), and integrin-mediated adhesion to extracellular matrix and other cell types can lead to signaling events affecting cell activation, motility, proliferation and apoptosis (Berton and Lowell 1999; Clegg et al. 2003). The finding

that RGD, only a three amino acid sequence, was an essential cell adhesion motif (Pierschbacher and Ruoslahti 1984), and that a family of integrins could recognize RGD motifs in different extracellular matrix proteins, gave this motif a central role in cell adhesion biology as the prototype adhesion signal (Ruoslahti 1996). Within the CNS, extracellular matrix proteins like vitronectin, osteopontin, fibronectin, and L1 neurite-promoting molecule present RGD domains (Clegg et al. 2003). Among the integrins who bind the RGD motif are $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha v\beta 8$, $\alpha 5\beta 1$, $\alpha 8\beta 1$ and $\alpha IIb\beta 3$, integrins which are present in the cell membrane of neurons, astrocytes, microglia, oligodendrocytes, endothelium or immune inflammatory cells (Clegg et al. 2003).

In this context, the aim of this study was to determine whether the neuroprotective effect of the NLSCt vector was mediated by the integrin-interacting motif RGD by injecting the cyclic RGD peptide GPen (GPenGRGDSPCA) following excitotoxic damage to the postnatal brain. Moreover, as integrin interactions are thought to play an important role in glial cell activation and inflammatory cell recruitment, we have examined whether NLSCt and GPen modulate the astroglial and microglial response to injury, and influence neutrophil recruitment in vivo. Finally, in order to determine whether the neuroprotective effect is dependent on glial cells, we have performed in vitro studies using neuron-enriched primary cultures containing glial cells and neuron-purified cultures, where we have evaluated the putative neuroprotective effect of NLSCt and GPen and the activation or inhibition of integrin intracellular cascades by analyzing the phosphorylation of p42/44 MAPK (ERK1/2), a downstream effector of integrin signaling.

Materials and Methods

In vivo studies

Excitotoxic injury and RGD treatment paradigm. The experimental animal work was conducted according to Spanish regulations in agreement with European Union directives. Experimental procedures were approved by the ethical commission of the Autonomous University of Barcelona. Nine-day old Long-Evans black-hooded rat pups (15-20gr., both sexes; Janvier, France) were used. Intracerebral injections were made into the right sensorimotor cortex at the level of the coronal suture (2mm lateral of bregma and 0.5mm depth) using a stereotaxic frame adapted for new-borns (Kopf Instruments) under isoflurane (Baxter International Inc.) anesthesia. Excitotoxic lesions were performed as previously described (Acarin et al. 1999), by injecting 18.5nmol of N-methyl-D-aspartate (NMDA) (Sigma-Aldrich, St. Louis, MO, USA) diluted in 0.15μl of saline solution (0.9% NaCl) at a rate of 0.05μl/min using an automatic injector. The needle was left in place for 10 additional minutes to allow for diffusion. The nude NLSCt protein vector (herein nNLSCt) or NLSCt vector mixed with pEGFP plasmid (herein NLSCt) were prepared for *in vivo* injection as previously described in detail (Peluffo et al. 2003). One μl of either NLSCt vector (7μM), nNLSCt (7μM), GPenGRGDSPCA (GPen, 7-1000μM, Gibco BRL) or vehicle (NaCl 0.9%) was injected 2 hours after the excitotoxic lesion at the same coordinates at 0.2μl/min. The needle was left in place for 10 additional minutes to allow for diffusion. After suture, pups were placed on a thermal pad for 2 hours at 36°C to maintain normothermia.

In vivo studies were designed into two independent series of experiments involving several litters and mixing experimental groups in each litter. For the first series, a total of 43 NMDA injected animals were used (n=18 NMDA+saline, 3 day survival; n=21 NMDA+NLSCt 7μM, 3 day survival; and n=4 NMDA+nNLSCt 7μM, 3 day survival). For the second experimental series, 43 NMDA injected animals were also used (n=5 were used for each treatment for the 12 hour survival time; n=12 NMDA+saline, 3 day survival; n=9 NMDA+GPen 7μM, 3 days; n=4 NMDA+GPen 100μM, 3 day survival; and n=8 NMDA+GPen 1mM, 3 day survival).

Histology and lesion volume measurement. Twelve hours and three days after the lesion, rats were anaesthetized and perfused intracardially with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were post-fixed in the same fixative for 2 hours and sunk in a 30% sucrose solution before being frozen with dry CO_2. Coronal sections of the entire brain (30-μm thick) were obtained using a Leitz cryostat. Parallel coronal sections of the whole brain separated by 240 μm (every eighth section) were mounted directly on a slide, stained for Nissl, and used for the measurement of lesion volume and total hemisphere volume after high resolution digitalization with a slide scanner. Using analySIS® software and simultaneous microscope observation, the lesioned area identified by its pale Nissl staining was quantified in each section. To avoid miss-interpretations related to possible tissue edema or shrinkage, data are presented as the percentage of total ipsilateral hemisphere volume.

Immunohistochemistry and histochemistry. Sections were processed for endogenous peroxidase inactivation and blocked for 1 hour in Tris-buffered saline (TBS, pH 7.4), 10% fetal calf serum and 1% triton X-100. Sections were incubated overnight at 4ºC in the same blocking solution with primary antibodies against either GFAP (1:1800, Dakopatts Z-0334 Denmark, rabbit polyclonal) for astroglial cell labeling, or myeloperoxidase (MPO) (1:400, Dakopatts A0398 Denmark, rabbit polyclonal) for neutrophil labeling. After several washes they were incubated for 1 hour with anti-rabbit or anti mouse horseradish peroxidase labeled secondary antibodies (1:200, Amersham Biosciences NA934V, or 1:200, Amersham Biosciences NA931V respectively), and 3,3´-diaminobenzidine (DAB)-hydrogen peroxide was used as developing procedure.

Microglia/macrophages were demonstrated by histochemistry for Lycopersicon esculentum (tomato) lectin, performed by incubation for 2 hours at 37°C in TBS with the biotinylated lectin (6μg/ml, Sigma L-9389) followed by avidin-peroxidase incubation and DAB-hydrogen peroxide developing procedure as previously described (Acarin et al. 1994).

Fluoro-Jade B staining. Neuronal degeneration was detected as previously described (Schmued and Hopkins 2000). Briefly, free-floating sections were mounted and air dried overnight. After defatting in increasing ethanol and subsequent rehydration, sections were oxidized with MnO_4K (0.06% in water, 15 min.). Sections were then rinsed in distilled water, incubated with Fluoro Jade B (Histo-Chem, Inc. Jefferson, USA) (0.0004% in water + 1% glacial acetic acid, 20 min.), washed with distilled water, air-dried and coverslipped..

Labelling densitometry and cell number quantification. For densitometrical measurements, images taken at 20x from the lesion core and the contralateral control cortex conformed the region of interest (ROI) and were analyzed with analySIS® software. As previously described (Acarin et al. 1997; Peluffo et al. 2006), the mean grey value labelling density of ROI, and the area occupied by immunoreactive cells within ROI were obtained for each section. Microglia/macrophage cell number was counted in 20x micrographs from the cortical lesion core in three consecutive averaged sections for each animal. MPO positive cell number was counted in 20x micrographs from the cortex (including lesion core and periphery) and the striatum (lesion periphery), also in three consecutive averaged sections for each animal. Fluoro Jade B positive cells were counted in 20x micrographs from two different aeas of the ipsilateral hippocampal CA1 region (lesion periphery). In all cell counts, three consecutive sections were counted for each animal and averaged.

In vitro studies

Cell cultures. Primary cortical cultures were prepared from E17 OFA rat embryos by dissecting the cortices free of meninges, and after dissociation, cells were plated at a density of 1.8×10^6 cells/ml in BME medium (Pan Biotech, 04-25050) supplemented with 5% fetal horse serum (Invitrogen) and 5%

fetal calf serum (Invitrogen), glutamine 2mM, 0.6% glucose, 50U/ml penicillin and 50μg/ml streptomycin on 10μg/ml polylysine coated wells. At 7 days in vitro, enriched cortical neuron cultures were prepared by taking away the 5% fetal calf serum and adding a total of 10% fetal horse serum and by adding cytosine arabinoside (AraC) 10μM. Under these conditions, at 10-12 days in vitro when cultures were used, they approximately contained 65% of neuronal cells, 22% astrocytes and 13% microglial cells. Purified cortical neuron cultures were prepared in the same manner but after dissociation, cells were plated at the same density but in Neurobasal medium (Invitrogen) supplemented with 2% B27 supplement (Invitrogen), 0.5mM GlutaMaxI (Invitrogen), 100U/ml penicillin and 100μg/ml streptomycin. In this case, AraC 3μM was added at 4 days in vitro. Under these conditions, at 10-12 days in vitro when cultures were used they approximately contained 97% of neuronal cells, and less than 3% glia.

Enriched cortical neuron cultures and purified cortical neuron cultures were treated with 300μM NMDA in the culture medium for 15 min. and 10 min. respectively. After several washes, cells were incubated with different concentrations of nNLSCt, GPen, or K10lacZ (control vector without the RGD motif) in 200μl OPTIMEM medium (Invitrogen), and after 1 hour 800μl of the culture medium described above for each neuron culture type were added to each well, and incubated for 72 hours.

Neuronal cell viability. Purified neuron cortical cultures were evaluated for neuronal viability using the spectrophotometric 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) assay by incubating cultures for 1 hour at 37°C with 5% CO_2 and 0,2mg/ml MTT. Cells were then lysed in DMSO and the amount of MTT product (coloured formazan) was determined by measuring absorbance at 570nm and 630nm, and the 570-630 subtracted data were used. in enriched neuron cortical cultures, which also contained glial cells, the evaluation of neuronal cell viability was achieved by neuronal cell immunocytochemical labelling with microtubule associated protein 2 (MAP2, MAB 3418, 1:1500, Chemicon Intl.) and anti-mouse HRP-linked (1:200, Amersham Bioscience, NA931V) as secondary antibody. Immunoreactive cells were counted in eight 40x fields in each well.

Western blotting. Cell cultures were lysed in distilled water with SDS 1 %, aprotinin (1 μg/ml), PMSF (1 mM), pepstatin A (1 μM), leupeptin (100 μM), and orthovanadate 1mM, and disrupted with a Braun Labsonic U sonicator (20 KHz, 50 W). Total protein concentration was determined using the bicinchoninic acid (BCA) assay and equal amounts of protein were used for 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrotransfered protein samples to nitrocellulose membranes were incubated in blocking buffer (PBS + 5 % non-fat milk + 0.05 % Tween 20) overnight at 4 °C and then incubated in primary anti-phosphoryated Erk1/2 antibody (1:1000, 9101 Cell Signaling Technology, Inc.) in blocking buffer for 2 hours at room temperature. The membranes were then incubated in secondary peroxidase conjugated anti-rabbit antibody (1:2000, 170-6515 BioRad), and finally in chemiluminiscent substrate SuperSignal West Pico (Pierce) combined with exposure on Hyperfilm ECL (Amersham).

Data processing and statistical analysis. All results are expressed as mean ± standard error mean (SEM). ANOVA followed by Fisher`s PLSD post-hoc test was used to determine significant differences ($p<0.05$) in lesion volume, cell counts, and densitometry.

Results

Neuroprotective effect of RGD-containing molecules after excitotoxic damage to the immature brain

The NMDA injection into the sensorimotor cortex of the postnatal day 9 immature rat brains induced a well-characterized excitotoxic lesion that included the sensorimotor cortex, the dorsomedial striatum, and the hippocampus. As previously described (Peluffo et al. 2006), when rats were injected two hours after the excitotoxic lesion, at the same location, with 7µm of the gene therapy NLSCt vector combined with the control transgene Green Fluorescent Protein (GFP), a pronounced neuroprotection was observed 3 days after the lesion. Interestingly, when the nude NLSCt vector (nNLSCt) was injected under the same circumstances, it also induced a significant neuroprotection. The overall % of lesioned hemispheric volume was reduced by 28±6% with NLSCt and 28±5% with nNLSCt (Figure 1A). When the GPen peptide was injected at different doses 2 hours after the excitotoxic lesion, the neuroprotective effect measured by the reduction in the % of lesioned hemispheric volume was clearly dose-dependent (7µM: 12±10%; 100µM: 20±5%; 1000µM: 25±8% reduction)(Figure 1B). However, the GPen peptide needed a three orders of magnitude higher concentration than NLSCt to show a significant lesion volume decrease. The distribution of the lesion along the antero-posterior axis showed that the reduction in the neurodegenerative area extended rostrocaudally to all affected regions including cortex, striatum and hippocampus. Both primary lesioned areas such as the sensorimotor cortex, and secondary lesioned areas like the caudal sub-plate neuronal layer, striatum and hippocampus showed a reduction in the extent of neurodegeneration.

To further characterize the grade of neuroprotection, degenerating Fluoro Jade B stained neurons were quantified in the secondary degenerating hippocampus. As previously described (Peluffo et al. 2006), lesioned animals treated with NLSCt vector showed a significant decrease in the number of Fluoro Jade B degenerating neurons at two different caudal levels of the CA1 hippocampal layer (Figure 2). Accordingly, lesioned animals treated with GPen at the neuroprotective dose, also showed a significant reduction in the number of degenerating neurons in the same hippocampal area (Figure 2).

Neuroprotection mediated by RGD-containing molecules is associated with a modulation of the microglial but not the astroglial response

Animals injected with NLSCt 7µm or GPen 1mM two hours post-lesion did not show a significant difference regarding astroglial reactivity at 12 hours or 3 days post-lesion when compared to NMDA+saline injected animals. Neither the morphology (Compare Figures 3A, B and C), GFAP immunolabelling densitometry (Figure 3D), nor the area of a given ROI occupied by GFAP overexpressing astrocytes (Figure 3E) were different within these groups.

In contrast, NMDA-injected animals treated with either NLSCt or GPen at the same neuroprotective doses showed significant differences in the microglial/macrophage response at 12 hours post-lesion when compared to control NMDA+saline animals (Figure 4A, B and C). At this time,

tomato lectin densitometry (not shown), the area of ROI occupied by reactive microglial/macrophage cells (not shown), and the microglia/macrophage cell number (Figure 4G), were higher in NLSCt and GPen treated animals. At 3 days post-lesion, when the characteristic NMDA-induced increase in microglial/macrophage reactivity at the lesion core is observed (Figure 4D and G), a significant increase in microglial/macrophage cell number was found in NLSCt but not GPen treated animals, in comparison with control NMDA+saline injected animals (Figure 4E, F and G).

Neuroprotection mediated by RGD-containing molecules is not correlated with changes in the number of infiltrated neutrophils

Quantification of MPO immunohistochemistry at 12 hours post-lesion, when maximal presence of neutrophils is observed in postnatal excitotoxic lesions (Acarin et al. 2002b), showed no significant differences in the number of MPO positive cells between lesioned animals injected with saline or GPen, neither in the cortical lesion core and surrounding cortex (NMDA+saline = 66±15 MPO+cells; NMDA+GPen = 95±20 MPO+cells) or in the striatum (NMDA+saline = 20±4 MPO+cells; NMDA+GPen = 23±4 MPO+cells).

Neuroprotective effect of RGD-containing molecules after excitotoxicity in vitro

Enriched cortical neuron cultures composed of approximately 65% cortical neurons, 22% astrocytes and 13% microglial cells, were treated with either nNLSCt or GPen both under basal conditions and after NMDA-induced neurodegeneration. The addition of nNLSCt vector to these neuron-enriched cultures under basal conditions did not affect neuronal survival (Figure 5A), but at 72 hours after NMDA treatment, the low concentration of 0,01μM of nNLSCt prevented completely excitotoxic neuronal death, but surprisingly did not show any effect at higher concentrations (Figure 5B). The control vector without the RGD motif (K10lacZ) did not show significant neuroprotection (Figure 5B). Like nNLSCt, the GPen peptide did not affect neuronal survival under basal conditions (Figure 5A), but showed complete protection from excitotoxicity when added after NMDA treatment (Figure 5B). However, in comparison with NLSCt, 1.000 to 10.000 times more concentrated GPen still provided neuroprotection. In order to confirm whether the neuroprotective effect was mediated directly by the RGD domains on neuronal cells we repeated the experiments on purified cortical neuron cultures containing less than 3% of total glial cells. Neither nNLSCt nor GPen induced significant effects when added for 3 days on neuron-purified cultures under basal conditions (data not shown). Importantly, both nNLSCt and GPen failed to show neuroprotection when these neuron-purified cultures were previously treated with NMDA (Figure 5C).

RGD-containing molecules induce ERK phosphorylation in vitro

Enriched cortical neuron cultures showed a rapid and transient phosphorylation of ERK1/2 peaking at 15 minutes after exposure to nNLSCt or GPen, which returned to basal levels 1 hour later (Figure 6). Surprisingly, though 0,01μM nNLSCt was sufficient to induce Erk1/2 phosphorylation, higher

concentrations as 1μM nNLSCt did not induce its phosphorylation (not shown). Again, the concentrations of nNLSCt needed to induce ERK1/2 phosphorylation were 100 times smaller than that of GPen, and in addition, high concentrations of GPen still induced Erk1-2 phosphorylation. A control cyclic RGE peptide did not induce ERK1/2 phosphorylation (not shown).

Discussion

Taken together, this work shows that small cyclic RGD peptides, like GPen, are neuroprotective against excitotoxic damage *in vivo* and *in vitro*. Interestingly, the neuroprotective effect of RGD domain induced an activation of microglial reactivity *in vivo*, and was only observed in the presence of glial cells *in vitro*, suggesting an important glia-dependent effect of the neuroprotection elicited by this domain. RGD domains may exert their effects not only by inhibiting the interaction of integrins with their natural ligands as previously thought, but also directly activating outside-in integrin signaling cascades, like ERK1/2 activation.

The GPen peptide, a cyclic form of RGDS (Pierschbacher and Ruoslahti 1987) which shows a higher specificity and a lower IC50 than linear RGD, is a very efficient inhibitor of αVβ3 and αVβ5-mediated cell attachment to vitronectin but shows nearly no activity towards attachment on fibronectin by α5β1 (Pierschbacher and Ruoslahti 1987). Accordingly, GPen may be, at least in part, exerting its neuroprotective effect by binding to αVβ3 and αVβ5, either inhibiting its binding to the extracellular matrix or activating intracellular mechanisms. In particular, in monocytes and macrophages, αvβ3 integrin expression is involved in movement, trans-endothelial migration, chemotaxis, production of the free radical superoxide after the induction of the respiratory burst (Berton and Lowell 1999), and contributes to the macrophage/microglial phagocytosis of affected neurons, a process that can be partly inhibited with RGDS peptides (Witting et al. 2000). Moreover, αVβ3 is known to be upregulated in astroglial cells after focal stroke in adult rats, whereas its main ligand, osteopontin, is expressed by surrounding microglial cells at early phases of the degenerative process (Ellison et al. 1998), suggesting that αVβ3-osteopontin binding may play a role in glial cell activation, migration and tissue remodeling after brain ischemia. Furthermore, a recent study has shown that osteopontin treatment is neuroprotective after oxygen glucose deprivation in cortical neuron cultures and when administered intracerebroventricularly before and after transient middle cerebral artery occlusion (Meller et al. 2005). In fact, neuroprotection of osteopontin in these cortical neuronal cultures was dependent on the RGD motif of osteopontin and mediated by activation of phosphatidyl inositol-3 kinase/Akt (PI3K/Akt) and ERK1/2 signalling pathways (Meller et al. 2005), which together with focal adhesion kinase (FAK), and nuclear factor kappa B (NFκB) mediate integrin outside-in intracellular signalling (Berton and Lowell 1999). In agreement, we here show that both NLSCt and GPen activated ERK1/2 in neuron-enriched cultures, but only at the neuroprotective doses.

Additionally, GPen has also been reported to completely inhibit the adhesion of denatured collagen or fibrinogen to immobilized platelet αIIbβ3 integrin, implying that GPen could also interact with αIIbβ3 integrin (Mogford et al. 1996). In fact, αIIbβ3 acts as a functional receptor for fibrinogen, but also for vitronectin, fibronectin, and vascular adhesion molecules like von Willebrand factor and ICAM-1, and has been shown to support platelet aggregation and thrombus formation, being therefore a target for antithrombotic therapy, like in a study where αIIbβ3 antagonist intravenous injection has shown beneficial effects after ischemic damage (Quinn et al. 2003).

Significantly, although both RGD domains were neuroprotective, the effects of NLSCt protein and GPen peptide administration were not identical. The different neuroprotective profiles of GPen and NLSCt could be explained by several reasons; first, it should be noted that the effective concentration of the two molecules was different, being necessary to use a thousand times higher concentration of GPen than that of NLSCt to exert neuroprotection after an excitotoxic injury. In agreement, it has recently been demonstrated that cyclic RGD peptides in the context of larger proteins show a thousand fold increase in their $\alpha v\beta 3$ integrin binding affinity (Schraa et al. 2002), a fact that could contribute to explain this differences. In fact, linear peptides inhibit FMDV infection in the milimolar range whereas longer peptides, like several GH loop peptides as that in NLSCt, do the same but at an IC50 of 0,8μM (see (Mateu et al. 1996) for review), a similar concentration at which we observed the neuroprotective effects of NLSCt. Second, conformational effects could also explain the slight differences observed with these two molecules, as the RGD domain in the GH loop of NLSCt holds a higher degree of flexibility. Third, the slightly different integrin specificity between NLSCt and GPen, could also account for those differences. The RGD motif in the NLSCt protein mediates FMDV attachment to the cell membrane to mediate viral infection (Fox et al. 1989) by interacting with integrins $\alpha v\beta 3$ (Berinstein et al. 1995), $\alpha v\beta 6$ (Jackson et al. 2000b), $\alpha v\beta 1$ (Jackson et al. 2002), and $\alpha v\beta 8$ (Jackson et al. 2004), which have been shown to act as receptors for the FMDV, and the infection of cells expressing these integrins can be inhibited by RGD peptides or anti-αv antibodies. Noteworthy, $\alpha v\beta 5$ is the only αv integrin consistently not involved in FMDV attachment (Berinstein et al. 1995; Duque and Baxt 2003). Additionally, although it is controversial whether $\alpha 5\beta 1$ integrin acts as a receptor for FMDV (Berinstein et al. 1995; Jackson et al. 2000a; Jackson et al. 2002), the GH loop of FMDV inserted into another macromolecule including β-galactosidase do bind this integrin (Chambers et al. 1996; Villaverde et al. 1996), and therefore we can not discard that NLSCt also binds to $\alpha 5\beta 1$. Therefore, we can not rule out that in addition to $\alpha V\beta 3$, other integrins could also contribute to the neuroprotective effect observed. In fact, the GPen interaction with other αV integrins besides $\alpha V\beta 3$ has not been published, and thus considering that NLSCt also recognizes $\alpha V\beta 1$, $\alpha V\beta 6$, and $\alpha V\beta 8$, it is possible that these integrins are also involved and account for the increased neuroprotection observed with NLSCt in comparison to GPen. And finally, it must be taken into account when trying to comprehend the consequence of integrin binding to its ligands that the resulting effect is highly dependent on the ligand. For example, although $\alpha v\beta 3$ binds to vitronectin and fibronectin, engagement of $\alpha v\beta 3$ to vitronectin (but not fibronectin) induces PI3K/Akt signalling activation and association of PI3K to $\beta 3$ integrin in a phosphorylation dependent manner (Butler et al. 2003). Moreover, inside-out signaling can also alter ligand specificity of one integrin. For instance, cells that under basal conditions attach to vitronectin and fibronectin via $\alpha v\beta 3$ will only attach to vitronectin when activated (Butler et al. 2003).

Another important point is to evaluate whether RGD motifs are interacting with neurons, glia or inflammatory cells. On one hand, the RGD peptides could be acting directly on neuronal cells, as vitronectin has been shown to induce neuronal differentiation (Wechsler-Reya 2001), and RGD

peptides can induce upregulation of neurotrophins like BDNF, NT3 and NGF and expression of their receptors TrkB and TrkC in hippocampal slices (Gall et al. 2003). In this sense, interaction of β1 integrins with laminin in neuronal cultures activates the pro-survival pathway PI3K/Akt, and blockade of these interactions renders neurons more susceptible to excitotoxicity (Gary and Mattson 2001). On the other hand, it should be noted that whereas no neuroprotection to excitotoxicity was observed after either NLSCt or GPen treatment in neuron-purified cortical cultures with minor glial cell presence, a neuroprotective effect was achieved with both RGD containing molecules after excitotoxicity in cortical neuron-enriched cultures with around 35% of glial cells. This finding leads to the conclusion that RGD domains mediate integrin signaling in glial cells, which could be inducing a neurotrophic phenotype, as integrins can modulate growth factor receptor signaling (Miranti and Brugge 2002), and therefore provide neuroprotection by a glial-dependent mechanism, as occurs in other neuronal culture systems (Peluffo et al. 1997). Interestingly, the most striking effect of both RGD-containing molecules in glial cells was the increase in microglial/macrophage reactivity and cell numbers after the excitotoxic lesion, a phenomenon which has classically been linked to exacerbation of damage but its role is being revisited in the last years. Although the effects of αv blocking on endogenous microglial cells are not known, αvβ3 integrins are important to monocytic transendothelial migration under inflammatory conditions, and αv antibodies or deficiency in β3 subunit have been shown to reduce monocyte transendothelial migration (Weerasinghe et al. 1998), further implying endogenous microglial cells in the neuroprotective effect. In regards to the infiltration of inflammatory cells, especially neutrophils, which are associated with tissue damage exacerbation, it has been described that the interaction of this cell type with extracellular matrix can be potently inhibited by antibodies against β3 in epithelial cell cultures (Bruyninckx et al. 2001). However, in our in vivo excitotoxic damage model, RGD motifs do not reduce the entrance of neutrophils after the lesion, and thus this mechanism cannot account for the neuroprotection observed.

In conclusion, this study shows a neuroprotective action of RGD domains, which is mediated by glial cells and is accompanied by an increased reactivity of microglial cells, suggesting that the activation and/or inhibition of particular integrins could modulate the glial and inflammatory response after nervous system lesions. However, the exact mechanisms underlying this neuroprotection as well as the possible participation of other cell types need further study.

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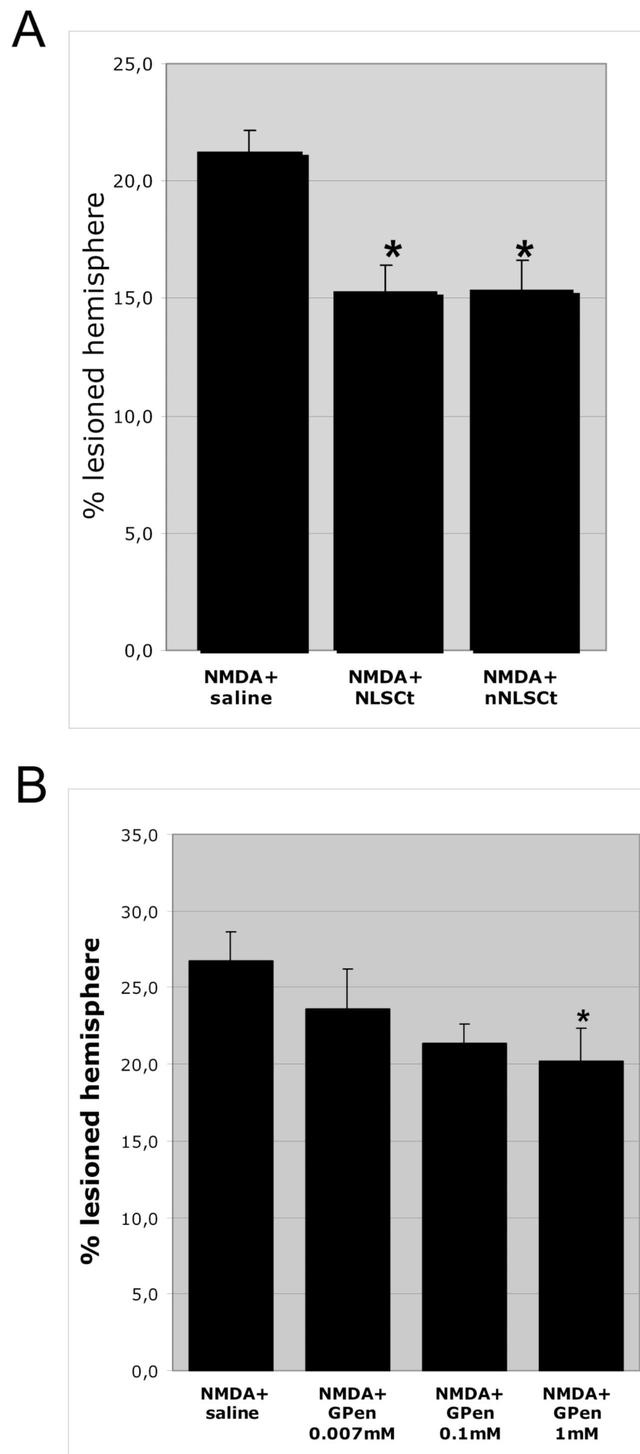


Figure 1. RGD containing molecules are neuroprotective after in vivo excitotoxic injury. Administration of the NLSCt vector or the nude vector (nNLSCt) 2 hours post-lesion showed, after 3 days, a similar significant (*p<0.05) reduction in the % of lesioned hemisphere when compared to NMDA+saline injected animals (A). Interestingly, in a separate series of experiments, when lesioned animals were injected with the RGD peptide GPen under the same conditions, a dose-dependent decrease in the % of lesioned hemisphere was observed when compared to NMDA+saline injected animals (*p<0.05).

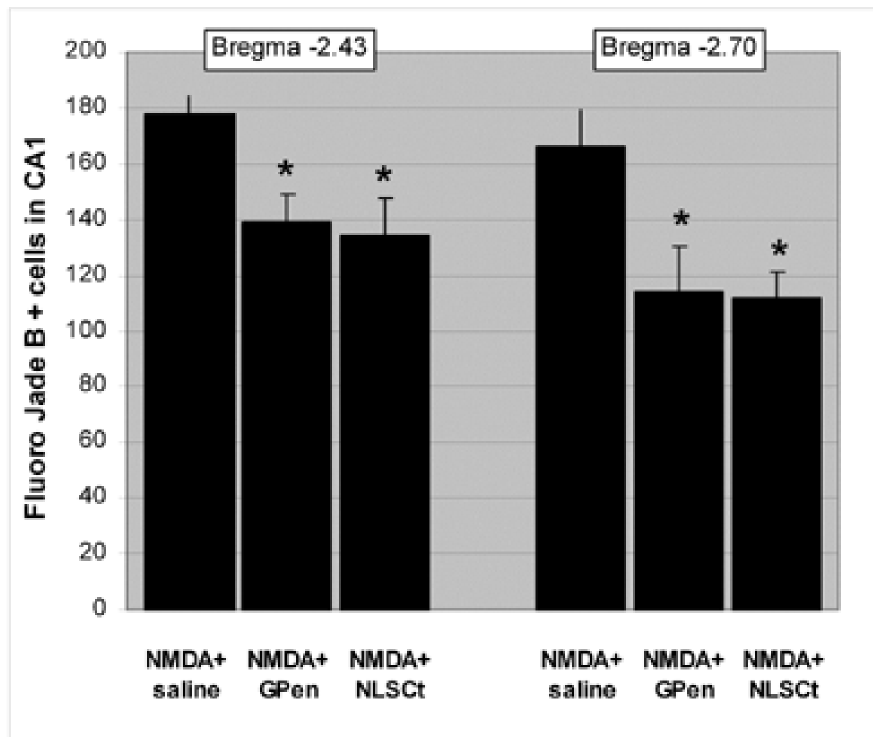


Figure 2. RGD containing molecules show neuroprotection at CA1. Two hours post-lesion administration of the RGD peptide GPen (1mM) or the NLSCt vector (7μM) showed, after 3 days, a significant reduction (*$p<0.05$ when compared to NMDA+saline injected animals) in the number of degenerating neurons stained with Fluoro Jade B in the CA1 layer of the hippocampus. This reduction was observed at two different zones of the caudal CA1 layer at two rostro-caudal levels from the lesion core.

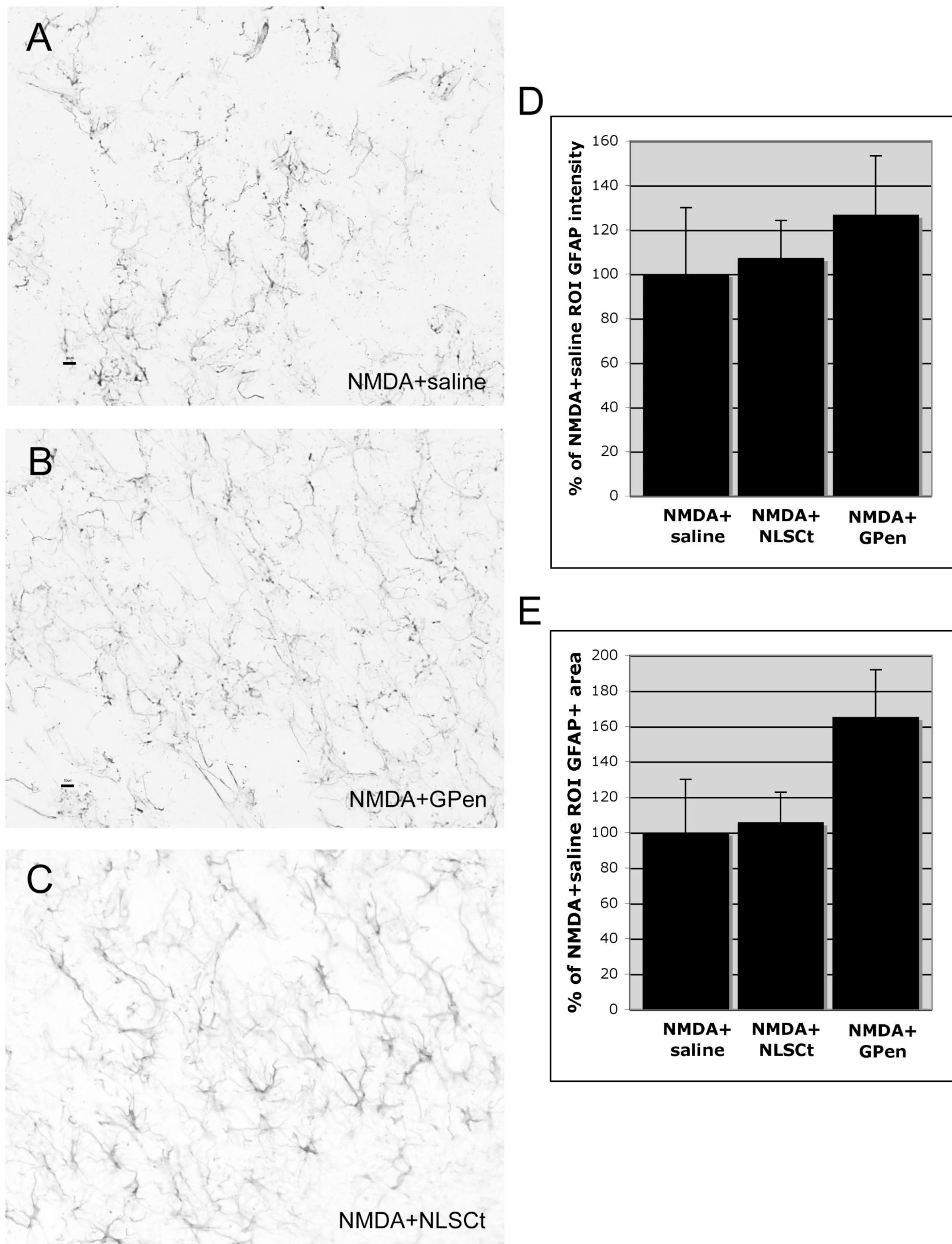


Figure 3. RGD containing molecules do not modify GFAP immunoreactivity after in vivo excitotoxic injury. Administration of the NLSCt vector (7μM) or GPen (1mM) showed no significant variation in GFAP immunoreactivity (compare A-C) by densitometrical measurement after 3 days (D, expressed as percentage of control NMDA+saline). Moreover, no significant variation was observed in the total area of GFAP immunoreactivity after 3 days (E, expressed as percentage of area occupied by immunoreactive cells in ROI of control NMDA+saline).

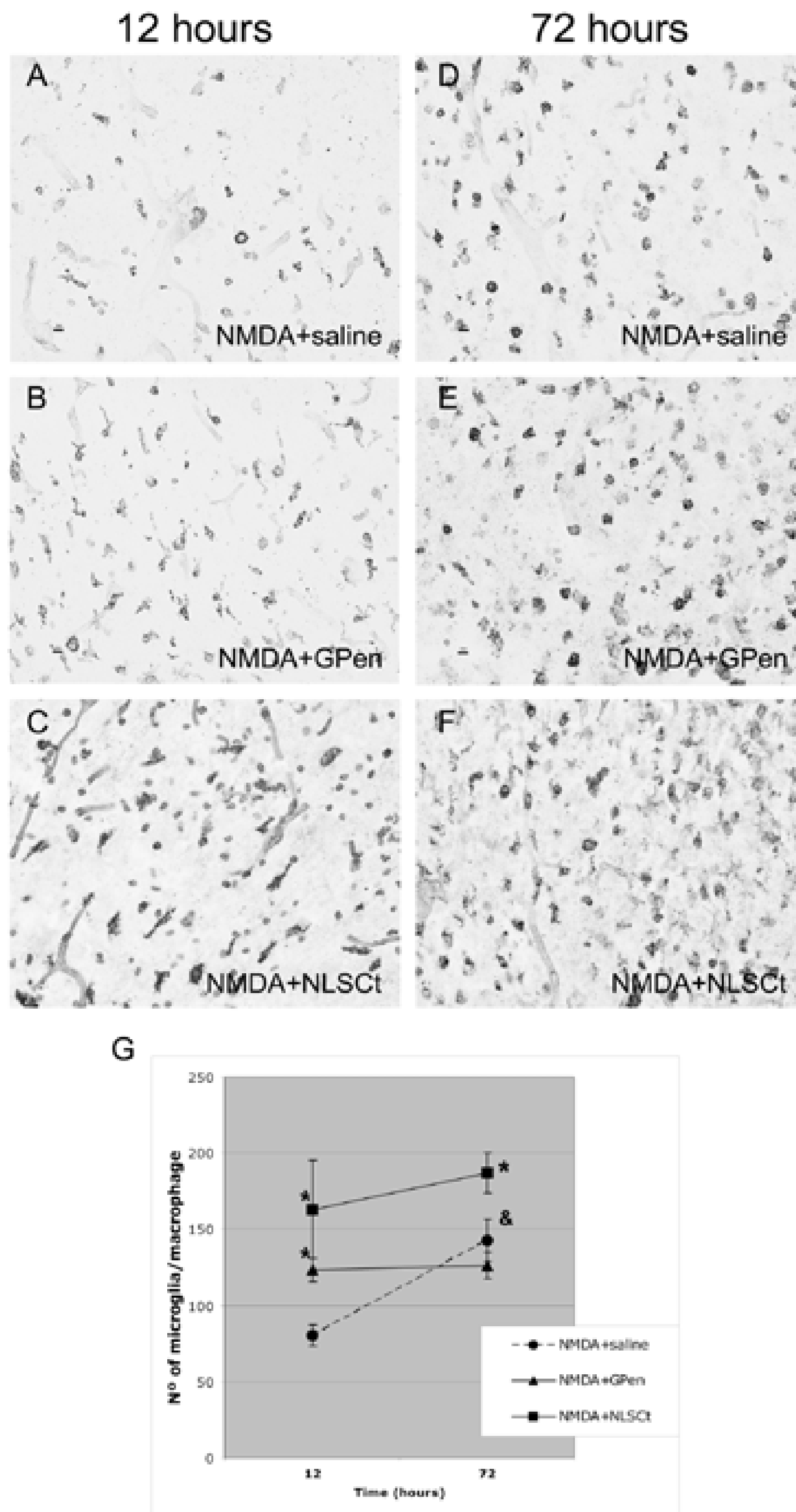


Figure 4. RGD containing molecules modify microglial/macrophage reactivity after excitotoxic injury. Administration of GPen (1mM, B) or NLSCt vector (7μM, C) showed a significant variation in microglial/macrophage reactivity observed in the lesion core at 12 hours in comparison with NMDA+saline animals (A), which was not so evident at 72 hours (D, E, F). Quantification of the number of tomato lectin microglia/macrophages (G), showed an increase at 12 hours post-lesion in both treatments (*p<0.05 compared to control NMDA+saline at the same time point, and &p<0.05 compared to NMDA+saline at 12 hours).

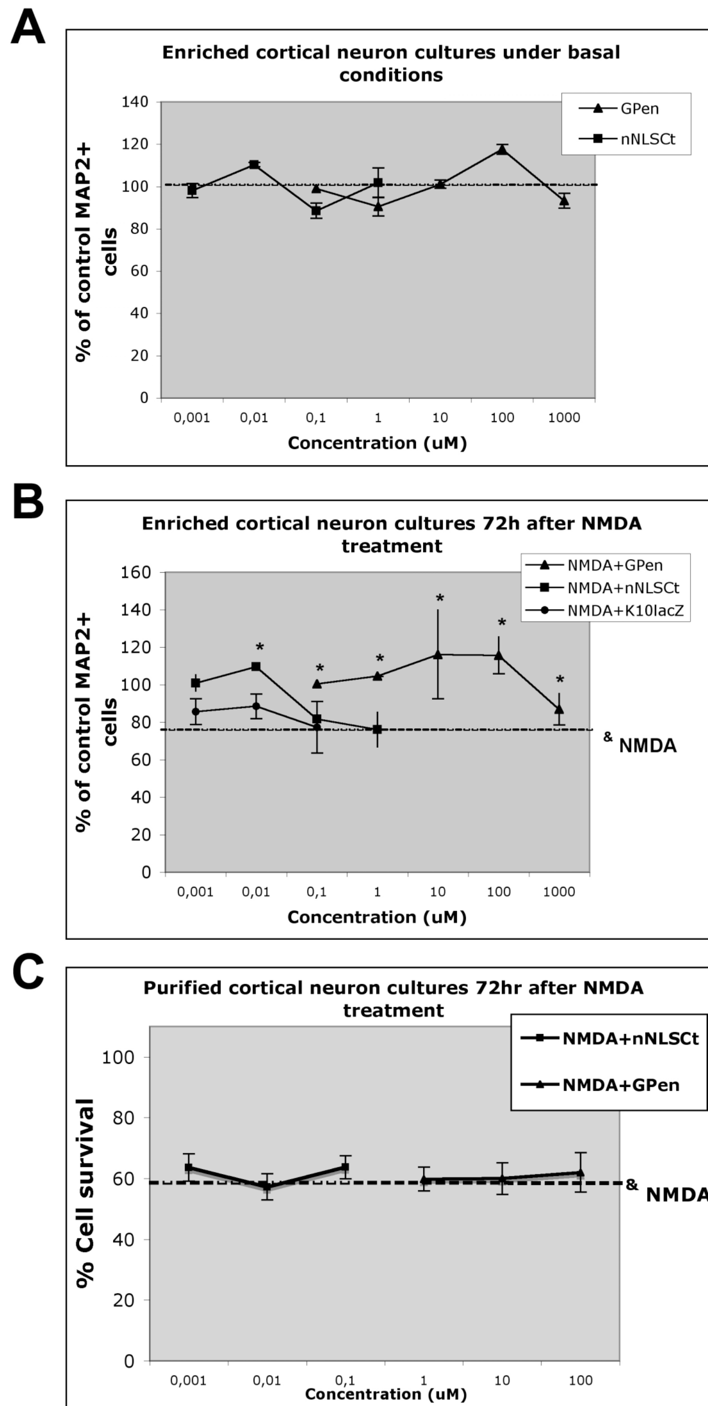
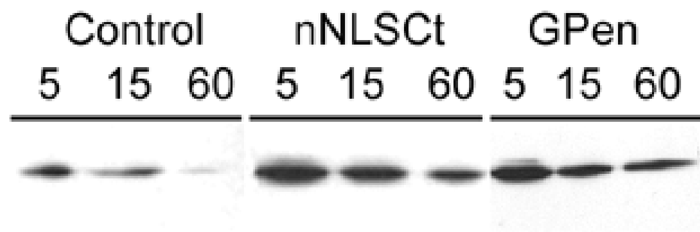


Figure 5. RGD containing molecules are neuroprotective after in vitro excitotoxic injury. Enriched cortical neuron cultures were exposed to saline (A) or NMDA (B), and then incubated at the indicated concentrations of NLSCt, GPen or saline. NMDA caused a significant decrease in neuronal viability after 72 hours (&$p<0.05$, dotted line in B and C). Although no effect was observed with NLSCt or GPen under basal conditions (A), a significant increase in MAP2 positive neurons was observed in NMDA treated cultures incubated with NLSCt or GPen (B). However, when purified cortical neuron cultures were exposed to saline, NLSCt or GPen after NMDA treatment, no significant increase in neuron viability was observed by MTT measurement (C).

A



B

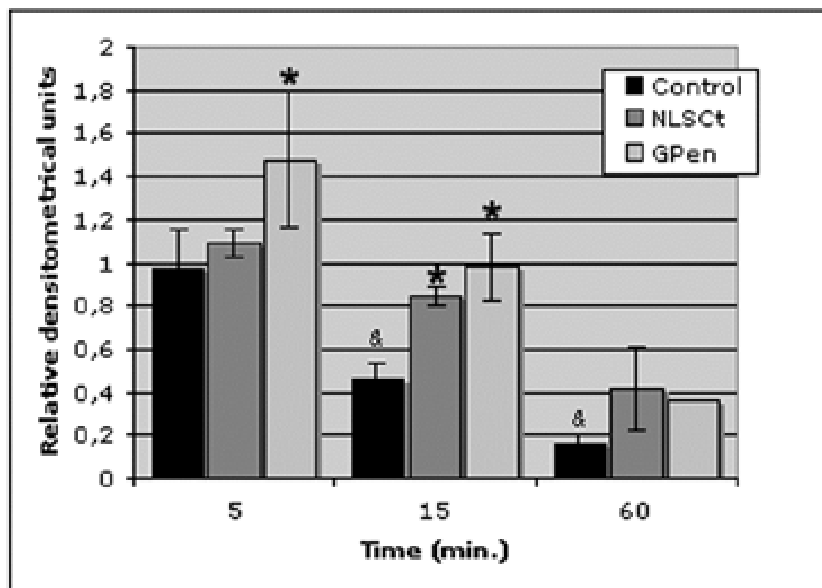


Figure 6. RGD containing molecules activate Erk1/2 pathway. Control enriched cortical neuron cultures incubated in OPTIMEM medium showed a significant decrease with time in the density of bands immunostained for phospho Erk1/2 (A and B: & $p<0,05$ compared to control al 5 minutes). However, treatment of enriched cortical neuron cultures with nNLSCt (0,01μM) or GPen (1μM) induced a rapid (15 minutes) significant increase in Erk1/2 phosphorylation (A and B: * $p<0,05$ compared to each corresponding control time).

DISCUSSION

Oxidative stress in the acutely lesioned immature brain

In the last decade, progress has been made regarding the complex evolution of damage in the developing brain (Ferriero 2004; Johnston et al. 2002) and how it differs from adult brain injury. One of the main divergences proposed is that immature brain deals poorly with oxidative stress. In this sense, the question that arises is: which are then the known differences regarding oxidative stress between acute brain insults in the adult and the immature brain?

First, the **production of reactive oxygen species** after an acute lesion has also been analyzed in mature and immature brain. In both cases, $O_2^{-\cdot}$ and H_2O_2 are produced, but it is difficult to evaluate the total levels produced and their relative quantities. However, immature brain possesses an increased sensitivity to glutamate due to the overexpression of NMDA receptors (McDonald and Johnston 1990), which will generate $O_2^{-\cdot}$ upon activation (Lafon-Cazal et al. 1993), and also by the slower clearance of glutamate (Diamond 2005), due to the ongoing developmental increase in glial glutamate transporter expression (Furuta et al. 1997; Ullensvang et al. 1997). Thus it would be expected that the immature brain under excitotoxic conditions would produce increased quantities of $O_2^{-\cdot}$ in comparison to adult brain, which could lead to the subsequent increased production of H_2O_2 and other reactive species. In fact, after an ischemic brain lesion, while the adult H_2O_2 brain levels decrease, the immature levels remain stable (D. Ferriero personal communication). Moreover, in the immature brain free iron accumulates more rapidly, within 4 hours after transient cerebral ischemia (Kondo et al. 1995; Palmer et al. 1999), a fact that would increase the oxidative stress due to reactions with $ONOO^-$ or the H_2O_2-mediated Fenton reactions.

Second, regarding the production and **toxicity of reactive nitrogen species in combination with reactive oxygen species**, there are also some similarities and differences between adult and immature CNS. For example, after different acute neurological injuries in both mature and immature brain there is an increased ·NO production, which is neurotoxic at elevated fluxes when produced by nNOS and iNOS, as the inhibition of these enzymes is neuroprotective (Ferriero et al. 1996; Iadecola et al. 1997; Iadecola et al. 1995; Lecanu et al. 1998; Liberatore et al. 1999; Samdani et al. 1997; Tsuji et al. 2000). However, in addition to the post-lesion induction of ·NO production, there is a peak in nNOS and iNOS enzyme levels and activity in normal rat brain at P5-P7, which decreases rapidly thereafter to adult levels in the case of nNOS and to very low levels in the case of iNOS (Fernandez et al. 2003). We have also observed iNOS expression in normal P9 rat parenchyma and its induction post-excitotoxic lesion (Acarin et al. 2002b). It is thus expectable that these basal increased ·NO production in normal immature rat brain will predispose the immature brain to neurotoxicity. The time frame of iNOS induction in immature rat brain after an acute excitotoxic or hypoxic/ischemic lesion is much faster, showing a peak at

10-48 hours post-lesion and decreasing thereafter (Acarin et al. 2002b; Coeroli et al. 1998; Ikeno et al. 2000), when compared to the one observed in adult brain after similar lesions, where the peak is at 2-3 days (Bidmon et al. 1998; Endoh et al. 1994; Iadecola et al. 1996; Loihl et al. 1999; Nogawa et al. 1998; Schmidt et al. 1995). This correlates with the slower progression of neurodegeneration in adult brain. Moreover, like nNOS and iNOS, nitrotyrosine also shows a peak in P5-P7 in neuronal cells of normal rat brain, the same cell-type showing the NOS enzymes upregulation (Fernandez et al. 2003). Interestingly, there was a specific regulation of nitrated proteins, suggesting that nitration during the postnatal development is a specific and physiological mechanism that may contribute to the definitive cortical conformation and also to the lesion progression (Fernandez et al. 2003). Accordingly, this Thesis also shows nitrotyrosine in some neurons of the neocortex, thalamus, and hippocampus, as well as in some astrocytes at *cingulum* of the *corpus callosum* of normal P9 rat brain (Article 2). This strengthens the hypothesis of increased basal oxidative stress in immature brain. In addition, increased levels of nitrotyrosine are formed after an acute excitotoxic injury to the immature brain (Article 2), as reported for the adult and immature CNS after different types of acute injuries (Bidmon et al. 1998; Coeroli et al. 1998; Fernandez et al. 2003; Hirabayashi et al. 2000; Scott et al. 1999; Takizawa et al. 1999; Tanaka et al. 1997).

Third, it has been observed that **nitrative and oxidative injury to developing oligodendrocytes** plays a major role in immature brain lesions (Haynes et al. 2003). The age at which premature infants are at greatest risk for periventricular leukomalacia (24 to 32 gestational weeks), a white matter injury which is one of the leading causes of cerebral palsy, coincides with the time interval when premyelinating (O4 and O1) oligodendrocytes, preferentially populate the telencephalic white matter (Back et al. 2001). Thus, it has been postulated that these cells in particular are susceptible to free radical injury and death (Levison et al. 2001; Volpe 2001). During this early myelination, a great synthesis of lipid-rich myelin sheaths occur, which imposes high metabolic demands to these early oligodendrocytes, which will in turn generate increased oxidative stress, specifically after an injury. This susceptibility has been demonstrated in rat oligodendrocyte cultures, in which premyelinating but not mature myelin basic protein-positive oligodendrocytes are particularly vulnerable to glutathione deprivation (Back et al. 1998). Interestingly, samples of developing white matter from premature infants show normal levels of catalase and glutathione peroxidase, while Cu/Zn SOD and Mn SOD are decreased, and will only increase after birth, a fact that will contribute to the high sensitivity of these infants to brain injury (Folkerth et al. 2004).

Fourth, it is well known that total brain **levels of antioxidant enzymes** vary throughout life (Acarin et al. 1999; Folkerth et al. 2004; Khan and Black 2003; Nishimura et al. 1992). Accordingly, brain glutathione peroxidase and Mn SOD levels were reported to increase during the first month of postnatal rat life, and to continue increasing slightly during adulthood and

aging (Aspberg and Tottmar 1992; Buard et al. 1992; Ceballos-Picot et al. 1992; Fan et al. 2003; Hamilton et al. 2001). Catalase has been reported to peak around the first week of life and then declines to reach a plateau by the first month (Aspberg and Tottmar 1992; Buard et al. 1992). Metallothioneins, potent metal-binding antioxidant enzymes, are less concentrated in the immature brain (Ebadi 1986; Nishimura et al. 1992). Regarding Cu/Zn SOD, it is known that its levels increase rapidly after birth, peaking around the second postnatal week. Later on, Cu/Zn SOD decreases slightly to reach adult levels, but increases slightly again with aging (Aspberg and Tottmar 1992; Ceballos-Picot et al. 1992; Folkerth et al. 2004; Khan and Black 2003). Accordingly, we observed in this Thesis an increase in the total amount of Cu/Zn SOD from P9 to P16 (Article 1). We also describe the general cell-type expression of this enzyme in immature brain both before and after an excitotoxic lesion, which does not vary with respect to the reported for adult patterns. Taken together, these reports show that immature rat brain has a different balance of antioxidant enzymes, which reach the adult overall pattern only after the first month of life.

Fifth, the **regulation of the antioxidant enzyme levels** after acute lesions is also an important factor. For instance, though adult animals subjected to traumatic brain injury upregulate glutathione peroxidase, immature animals do not (Fan et al. 2003). Moreover, it has been reported that after acute injuries to the adult brain, Cu/Zn SOD is very rapidly downregulated (Kim et al. 2000; Liu et al. 1994; Liu et al. 1993). Accordingly, after an excitotoxic injury to the postnatal brain, we have observed a dramatic and rapid neuronal downregulation of Cu/Zn SOD in the NMDA injection site, which is early evident from 2-4 hours after injection in neurons which only show slight and very early signs of degeneration and are negative for TUNEL staining (Article 1). In fact we have previously described that these neurons display, 10 hours after NMDA injection, NFκB activation and COX_2 upregulation, suggesting that they are still active and functional (Acarin et al. 2000b; Acarin et al. 2002b). The Cu/Zn SOD downregulation also coincides with neuronal nitration (Article 2) suggesting endogenous $O_2^{-\cdot}$/$ONOO^-$ formation at these very early time points in compromised neurons. Though the mechanism where-by this rapid downregulation occurs is not clear, it appears that it could be mediated by oxidative stress, as PC12 cells treated with H_2O_2 rapidly (after 4 hours) downregulated Cu/Zn SOD (Rojo et al. 2004), and Cu/Zn SOD fragmentation *in vitro* can rapidly be induced by H_2O_2 (Choi et al. 1999) or by peroxyl radicals ($ROO^\cdot$) (Kwon et al. 2000). To our knowledge this Thesis is the first study describing the expression of Cu/Zn SOD after immature brain damage.

Contradictory results have been reported regarding the toxicity of $O_2^{-\cdot}$ and the role of Cu/Zn SOD after hypoxic/ischemic injury to the immature brain. Whereas slightly worsened neuropathological outcome was observed in transgenic mice over-expressing Cu/Zn SOD and submitted to severe hypoxia/ischemia (Ditelberg et al. 1996), SOD mimetics like $O_2^{-\cdot}$ dismuting metalloporphyrins protected from hypoxia/ischemia (Shimizu et al. 2003) and we show in this Thesis that gene therapy mediated overexpression of this enzyme after an excitotoxic lesion is

neuroprotective (Article 4). Though the basis of these differences are not clear, several reasons besides species specificity and lesion model could contribute to its explanation. In our experimental conditions, the NLSCt vector induced a transient and lower level of Cu/Zn SOD transgene expression compared to the higher and permanent expression found in transgenic mice. In fact, it has previously been shown that very high levels of Cu/Zn SOD observed in transgenic animals, can produce alterations (discussed in Article 4). Furthermore, compensatory changes in the basal levels or induction of other antioxidant enzymes like Mn SOD (Ceballos-Picot et al. 1992), heme oxygenase (Weinzierl et al. 2003), glutathione peroxidase (Sheldon et al. 2004), or catalase after life-long overexpression of Cu/Zn SOD have been documented in transgenic animals, providing an altered redox balance in these animals. Although one wonders if in this case the data from transgenic animals is physiologically valid, it is also true that when ischemia is performed in these same transgenic animals at the adult stage, the lesion is reduced, indicating that something critical is ongoing in immature animals. Another important finding comes from the direct intravenous administration of polyethylene glycol-conjugated Cu/Zn SOD after adult focal brain ischemia, where an U-shaped dose-response curve was observed (He et al. 1993), implying that the effective neuroprotective dose of this enzyme may be in fact concentration restricted, and that too much Cu/Zn SOD can be deleterious (McCord 2004). A similar U-shaped dose-response curve for Mn SOD was observed for heart reperfusion, where administration of high doses of SOD where toxic (Nelson et al. 1994). Finally, an additional factor which could contribute to the observed differences is that whereas transgenic animals will only overexpress the Cu/Zn SOD in neurons, in our experimental paradigm of Cu/Zn SOD overexpression (Article 3) and also with the treatment with SOD mimetics, several cell-types besides neurons will be targeted. Another hypothesis suggests that Cu/Zn SOD transgenic mice produce excess H_2O_2 after an acute lesion, which in the immature brain would not be cleared by the upregulation of the glutathione peroxidase, as would be the case in adult animals (Fan et al. 2003; Fullerton et al. 1998). Accordingly, the Cu/Zn SOD transgenic immature animals suffered from increased H_2O_2 accumulation after injury (Fullerton et al. 1998). However, there are several facts that contradict this hypothesis: i) catalase levels are very high in immature brain (Aspberg and Tottmar 1992; Buard et al. 1992); ii) Cu/Zn SOD transgenic animals show increased baseline expression of glutathione peroxidase (Sheldon et al. 2004); and iii) if the Cu/Zn SOD transgenic immature animals suffer from increased H_2O_2 accumulation after injury, they would also show increased levels of oxidative stress like lipoperoxidation, which does not occur (Fullerton et al. 1998). Therefore, several parameters like antioxidant protein levels, and the time-course and the cell population that express Cu/Zn SOD could affect the overall outcome after the lesion and underlie the differences between gene therapy or SOD mimetics and transgenic mice approaches.

Interestingly, several evidences suggest that the toxicity of $O_2^{\cdot-}$/$ONOO^-$ species is higher than that of H_2O_2. A study found that H_2O_2 is not highly toxic for astrocytes, as Cu/Zn SOD

overexpressing astrocytes exposed to $O_2^{-\cdot}$ had higher survival rates than control astrocytes even when glutathione peroxidase and catalase activities were blocked and glutathione levels depleted (Chen et al. 2001). Moreover, Cu/Zn SOD overexpressing astrocytes also survived better than control astrocytes after oxygen glucose deprivation, and this occurred in the absence of glutathione peroxidase upregulation, and with a delayed a lower catalase upregulation in comparison to control astrocytes, suggesting again that the H_2O_2 produced was not toxic (Wang et al. 2005). In fact, these Cu/Zn SOD overexpressing astrocytes maintained elevated glutathione concentration unlike the control ones. Moreover, $ONOO^-$ but not H_2O_2 could trigger an in vitro reactive phenotype of astrocytes that was toxic for co-cultured motor neurons (Cassina et al. 2002). These data suggests that overproduction of H_2O_2 is not a major factor in the astrocytic injury. Additional evidences suggest that for neuronal cells the toxicity of $O_2^{-\cdot}$/$ONOO^-$ species is higher than that of H_2O_2. Specific scavenging of $O_2^{-\cdot}$ can increase neuronal survival under some pathologically relevant conditions. For example motor neurons can be rescued from trophic factor withdrawal by liposome-mediated Cu/Zn SOD protein delivery (Estevez et al. 2000), or by synthetic SOD mimetics (Estevez et al. 1998; Peluffo et al. 2004). Neuronal cultures can also be protected from excitotoxicity by SOD mimetics (Vergun et al. 2001), adenovirally mediated overexpression of Cu/Zn SOD (Barkats et al. 1996), or transgenically overexpressed Cu/Zn SOD (Chan et al. 1990). However, in some particular cases, Cu/Zn SOD overexpression can reduce neuronal survival during direct extracellular exposure to superoxide generators by a mechanism involving excess H_2O_2 accumulation (Ying et al. 2000). Finally, an additional group of evidences suggesting that $O_2^{-\cdot}$ is more toxic than H_2O_2 by its reaction with $\cdot NO$ to form $ONOO^-$ (Beckman et al. 1990; Radi et al. 1991)(see Introduction section) comes from the inhibition of $\cdot NO$ production in neuronal cultures submitted to an excitotoxic damage. This treatment is sufficient for inducing neuroprotection (Dawson et al. 1993; Gunasekar et al. 1995; Lipton et al. 1993), while $O_2^{-\cdot}$ and H_2O_2 are still being formed but will not be so toxic. Thus, the increase in Cu/Zn SOD expression in neurons and astrocytes most likely contributes to the neuroprotection observed *in vivo*.

Taken together, all the available data on acute damage and oxidative stress suggest that in the immature brain subjected to hypoxia/ischemia-excitotoxicity, neurons at the lesion zone are very early submitted to enhanced excitotoxicity due to increased NMDA receptor expression and decreased glutamate clearance. Moreover, a further potentiation of the damage would occur by an increased generation of oxidative stress mediators as $O_2^{-\cdot}$, H_2O_2, and $ONOO^-$/nitrotyrosine due to the increased excitotoxicity and NOS enzyme levels, and also by less availability of antioxidant enzymes by having still not reached the adult levels or by decreased post-insult levels.

Contributions of astrocytes to the oxidant and antioxidant status

As already commented, oxidative stress is an important trigger of inflammation, and the oxidative/antioxidant molecules are also important components of the inflammatory responses. The balance between the triggering mechanisms and inflammatory responses determines if the lesion will be auto-limited or in contrast will fall into an auto-toxic amplifying cascade.

After an acute CNS injury, a delayed upregulation of antioxidant mechanisms is observed both in mature and immature animals. This Thesis shows that although Cu/Zn SOD expression remains very low in compromised neurons after the excitotoxic lesion, a return to normal expression levels is seen by 24 hours after, and an increase in total enzyme level is observed later on. This secondary Cu/Zn SOD induction is due to upregulation in reactive hypertrophic astrocytes within the lesion site. It has been shown in previous studies that these reactive astrocytes display activated NFκB from 10 hours after the excitotoxic lesion (Acarin et al. 2000b), a transcription factor that is activated by oxidative stress (Schreck R et al. 1991; Storz and Toker 2003; Takada et al. 2003), and which could contribute to the induction of the Cu/Zn SOD observed here (Rojo et al. 2004). This Thesis also shows that the hypertrophic Cu/Zn SOD over-expressing astrocytes are heavily nitrated, and also display metallothionein I-II expression, suggesting an elevated grade of oxidative stress in this particular group of cells (Article 2). Our findings in the immature brain are in accordance with studies in adult brain damage where induction of Cu/Zn SOD and Mn SOD in astrocytes several days after focal ischemia (Liu et al. 1994) excitotoxicity (Kim et al. 2000; Noack et al. 1998), or in Alzheimer's disease and Down's Syndrome (Furuta et al. 1995) have been shown. In addition to the enzymatic antioxidant defenses upregulated in astrocytes, they produce their own glutathione and also provide neurons with cysteine, a rate-limiting precursor in neuronal glutathione synthesis (Sagara et al. 1993b). Thus, astrocytes seem to be the main cell type increasing the total antioxidant capabilities in the nervous tissue after a lesion, which can in addition explain their elevated resistance to cell death after an injury. Due to this high antioxidant capacity of astrocytes they have often been referred to as a "sink" of reactive oxygen and nitrogen species. In this sense, previous studies showed that increased Cu/Zn SOD overexpression in astrocytes mediates their increased resistance to oxidative damage (Chen et al. 2001), to oxygen glucose deprivation (Wang et al. 2005), and attenuated oxidative inhibition of glutamate uptake (Chen et al. 2000), allowing for a better maintenance of their physiological functions after a lesion.

In contrast, astrocytes can also be producers of oxygen and nitrogen reactive species. As commented in detail in the introduction section, astrocytes can produce neurotoxic fluxes of ·NO. Moreover, this Thesis shows evidence of oxidative stress in astrocytes (Article 2), where nitration was found 24 hours post-lesion in highly ramified protoplasmic astrocytes, resembling the early-activated reactive velate astroglial phenotype that has been previously described by Raivich and coauthors following ischemia and trauma (Raivich et al. 1999) and recently by Campos in a

transgenic mice expressing Lmo1-lacZ (Scotti Campos 2003). These nitrated astrocytes showed iNOS expression, although not all iNOS-expressing astrocytes were nitrated. Noteworthy, iNOS expression is already seen in these type of astroglial cells at 10 hours post-lesion (Acarin et al. 2002b), more than 12 hours prior to nitration. In this sense, it is reasonable to think that tyrosine nitration in these astrocytes may represent $ONOO^-$ formed within the cell by $\cdot NO$ derived from endogenous iNOS in combination with $O_2^{\cdot-}$, which is largely produced in excitotoxicity (Lafon-Cazal et al. 1993) and by some reactive astrocytes (Chan et al. 1988; Stephenson et al. 1999). Therefore, in our excitotoxic model, early iNOS-expressing nitrated astrocytes, whose appearance correlates with the time of maximal neuronal death in this model (Acarin et al. 1999), could contribute to neuronal damage, either by $\cdot NO/ONOO^-$ production or by toxic nitrotyrosine release (Cassina et al. 2002; Mihm et al. 2001; Peluffo et al. 2004). However, it should be noted that different cell types besides astrocytes, mainly infiltrated neutrophils, become nitrated and express iNOS (Acarin et al. 2002b; Bidmon et al. 1998; Coeroli et al. 1998; Grzybicki et al. 1998; Iadecola et al. 1996; Loihl et al. 1999). In this sense, the putative neurotoxic role of astroglial-derived $\cdot NO$ and $ONOO^-$, which has been demonstrated using astroglial cultures (Cassina et al. 2002; Dawson et al. 1994; Stewart et al. 2000), would be *in vivo* potentiated by neutrophils.

Regarding microglial cells, it is somehow surprising that only scattered reactive amoeboid microglial cells express Cu/Zn SOD, and that it occurs very transiently, as in some circumstances activated microglia produce large amounts of oxygen radicals after a lesion including $O_2^{\cdot-}$ (Colton and Gilbert 1993). However, it has been shown that microglial cells possess elevated levels of other antioxidant defense mechanisms, specially glutathione, glutathione peroxidase, and catalase, which increase after several types of lesions (Dringen 2005).

We believe that the overall results presented here highlight the importance of *in vivo* cell-localization studies for antioxidants, as: i) some of the reactive species like $O_2^{\cdot-}$ do not diffuse across cell membranes and thus will most probably react within the cell where they are formed, and ii) due to the high oxygen tension needed for cell survival in culture, the study of the expression of antioxidants in vitro is misleading, tending to overestimate the endogenous antioxidant potential of the cells, and being most probably a better indicator of antioxidant defenses in stressed cells. A typical example occurs with astrocytes, that express Cu/Zn SOD *in vitro* but not *in vivo* (Article 1)(Kim et al. 2000; Liu et al. 1993; Moreno et al. 1997; Thaete et al. 1986; Viggiano et al. 2003).

Do nitrated astrocytes constitute a selective population of reactive astrocytes?

As commented in the introduction section, the closer study of the integrative molecular-morphological-functional phenotype of the astroglial cells in the non-injured brain has lead to the accumulation or evidence suggesting that there are several cellular phenotypes coexisting in the brain. This concept is beginning to be applied also to reactive astrocytes. This Thesis shows that

when reactive astroglial cells cover the neurodegenerating area and form the glial scar, nitrotyrosine labeling is found in a selective population of hypertrophied reactive astrocytes. In order to further characterize nitrated astrocytes and evaluate the putative nitration of other cytoskeletal proteins, we correlated nitrotyrosine labeling with the expression of the intermediate filament proteins, GFAP and vimentin. Although nitrotyrosine labeling is not found in all GFAP-overexpressing astrocytes, and nitration is not observed in all GFAP-containing cell processes, nitrotyrosine co-localizes with vimentin-positive filaments. Noteworthy, all vimentin-positive astrocytes are nitrated and all nitrated astrocytes express vimentin. In fact all vimentin-containing cell processes show nitrotyrosine labeling. It is likely that proteins associated to vimentin-intermediate filaments, more than the vimentin protein itself, could become nitrated as a result of peroxynitrite formation in astrocytes, though thus needs further study. In addition, the hypertrophic, with very high GFAP content, nitrated, vimentin expressing astrocytes also show de novo expression of Cu/Zn SOD and metallothionein-I-II. Both Cu/Zn SOD and metallothionein could serve nitrated reactive hypertrophied astrocytes as an additional antioxidant mechanism to cope with oxidative stress and cell death. Further studies are needed for dissecting the possible selective nitration targets, and how this could influence the lesion progression and astrocytic phenotype.

Although it is known that neuronal nitration induces cellular damage and activation of apoptotic mechanisms (Bonfoco et al. 1995; Endres et al. 1998; Estevez et al. 1998), the effect of nitration in astrocytes is largely unknown. As already pointed in the Introduction section, it is well established that astrocytes are more resistant to oxidative and nitrative stress than neurons. This Thesis shows that early-nitrated highly ramified protoplasmic astrocytes do not show activation of the apoptotic protein caspase-3, whereas all late-nitrated reactive hypertrophied astrocytes do. Caspase-3 is a proteolytically activated enzyme which is considered one of the major executioners of apoptosis (Nicholson 1999; Springer et al. 2001) and is induced by peroxynitrite in different cell types *in vitro* (Cassina et al. 2002; Estevez et al. 1998; Lin et al. 1998). Nevertheless, it should be noted that nitrated reactive astrocytes showing caspase-3 positive nuclei did not show signs of nuclear fragmentation and were TUNEL-negative, even at 7 days post lesion, suggesting either the existence of a time frame between caspase-3 activation and the execution of apoptotic death (Brecht et al. 2001), or pointing to an activated state of caspase-3 positive cells in the absence of cell death. In fact, the presence of activated caspase-3 in non-dying cells is a very active and recent research field, and this observation had not been made before I begun with this Thesis. Interestingly, the first caspases described were the interleukin converting enzymes (ICEs)(Jacobson and Evan 1994), which early suggested a relation between these types of enzymes and inflammation besides of their pro-apoptotic potential. However, most of the efforts were directed to elucidate the pro-apoptotic properties of the caspases. Very recently, it was reported that in a model of ischemic preconditioning (McLaughlin et al. 2003) and in status epilepticus (Narkilahti et

al. 2003), caspase 3 was activated but did not cause cell death. It was also observed in normal tissues in the absence of cell death, like in neurons and some of their projections in the brain (Shimohama et al. 2001; Yan et al. 2001), in granular neurons of the cerebellar cortex (Oomman et al. 2004), and it is essential for synaptic plasticity (Bravarenko et al. 2006; Dash et al. 2000). In addition, it was observed in a subpopulation of astrocytes expressing GLAST glutamate transporter from several CNS locations (Noyan-Ashraf et al. 2005). Moreover, it was proposed that it could be involved cell differentiation of different cell types as Bergmann glia (Noyan-Ashraf et al. 2005; Oomman et al. 2005), erythroid cells (Zermati et al. 2001), skeletal muscle (Fernando et al. 2002), neural stem cells (Fernando et al. 2005), and osteoblasts (Mogi and Togari 2003). Moreover, caspase 3 activation is also important in inflammatory as well as neuropatic pain (Joseph and Levine 2004), and it is also cleaved during T lymphocyte activation (Wilhelm et al. 1998), and thus could contribute to the intracellular signaling cascades of inflammation. The unique localization of activated caspase 3 in the nucleus of nitrated astrocytes (Article 2) and other cells (Oomman et al. 2005), suggests the possible ways by which it could influence differentiation or phenotypic adjustments, possibly acting on transcription factors (Charvet et al. 2003), proteins involved in the cell cycle and DNA synthesis/repair enzymes, which have been shown to be targets of this enzyme. Moreover, this localization may hinder the activation of the pro-apoptotic machinery. Also alternative mechanisms, as expression of survivin that blocks cleaved caspase 3, have been postulated to mediate astrocyte survival in spite of displaying activated caspase 3 (Johnson et al. 2005).

These data overall indicate that nitrated astrocytes conform a subpopulation of astrocytes in two waves that differ in cell characteristics and temporal location. An early wave included a rapid iNOS induction in nitrated astrocytes, coinciding with the time of maximal cell death. A second and later astrocytic nitration wave was characterized by cell hypertrophy, increased GFAP expression, and induction of vimentin and the antioxidant proteins metallothionein and Cu/Zn SOD. These nitrated astrocytes, despite displaying caspase-3 activation, seemed to be highly resistant towards oxidative stress induced cell death, probably by the overexpression of antioxidant enzymes. Treatment of astrocytes with $ONOO^-$ *in vitro* also nitrated astrocytes and induced a similar phenotype (Cassina et al. 2002). Additional studies are needed to evaluate the *in vivo* mechanisms of induction of this phenotype, and to analyze if the nitrated astrocytes observed in several pathologies also conform a discrete cell population with specific gene expression patterns.

249AL and NLSCt as efficient tools for acute CNS lesion gene therapy

Several multifunctional protein vectors have been developed by combining functional modules from different origins. However, although many of these prototypes can transfect cells in culture, their efficiency *in vivo* is very limited (reviewed in Aris and Villaverde 2004). This Thesis

shows that 249AL, a modular recombinant protein vector displayed a restricted capacity for transgene delivery to the intact brain, but it was very efficient in conducting widespread transgene expression after an excitotoxic lesion (Article 3). The 249AL vector used displays an RGD motif, which interacts with $\alpha v\beta 3$, but also $\alpha v\beta 1$, $\alpha v\beta 5$, $\alpha v\beta 8$, $\alpha B\beta 1$ and $\alpha 5\beta 1$ integrins (Chambers et al. 1996; Villaverde et al. 1996). In this sense, the high transfection efficiency observed is probably due to an enhanced cell internalization of the DNA, mediated by interactions between the RGD motif and the plasma membrane αv integrins which are expressed in the brain (Akiyama et al. 1991; Ellison et al. 1998; Masumura et al. 2001; Pinkstaff et al. 1999). In fact, cell internalization via receptor-dependent endocytosis of many viruses, including most adenovirus serotypes and foot-and-mouth disease virus, is specifically mediated by interactions of a viral RGD peptide with cellular αv integrins (Wickham et al. 1993b). In addition, 249AL polylysine-DNA condensation properties have been shown to protect DNA from nuclease activity and increase cell internalization (Fominaya and Wels 1996), thus contributing further to the enhanced transfection efficiency. Interestingly, an additional increase in transgene expression was observed when DNA complexed to the 249AL vector was injected into damaged brain. Some experimental facts suggest that transfection efficiency can vary in different conditions. For instance *in vivo* transfection can be better than *in vitro* transfection, as when the same amount of naked DNA injected directly into muscle transfects double the number of cells compared with in vitro transfection with lipofectamine, or much more compared with naked DNA only (Budker et al. 2000). The transfection conditions of transferrin-lipoplexes based vectors have also been shown to differ between neuronal cultures and direct intracerebral injection (da Cruz et al. 2005). Thus, in vitro and *in vivo* transfection efficiency and mechanisms can vary, and there may be some uptake system that enhances the *in vivo* transfection efficiency. In the case of the 249AL and NLSCt vectors, the enhanced transgene delivery after the lesion is probably due to the disruption of extracellular matrix and the small size of the vector/DNA complexes (20-40nm diameter) (Aris and Villaverde 2000). This transfection efficiency could also be due to increased integrin-mediated endocytosis, as lesion-triggered upregulation of $\alpha V\beta 3$ integrin expression has been reported in hypertrophic astrocytes, microglia and microvessels after ischemia (Ellison et al. 1998; Masumura et al. 2001; Okada et al. 1996), Alzheimer (Akiyama et al. 1991) or experimental autoimmune encephalomyelitis (Previtali et al. 1997). Increased glial transgene expression after the lesion reported here further supports this idea. Moreover, increased transfection efficiency can be due to massive neuronal endocytosis, reaching the nuclear compartment, a process previously described in neuronal cells within a few hours after injection of toxic and sub-toxic doses of NMDA or kainate (Borsello et al. 2003a; Borsello et al. 2003b). Finally, the introduction of the SV40 viral nuclear localization sequence to 249AL vector enhanced the transit towards the nuclear compartment and increased transfection efficiency in vitro of the resultant NLSCt vector (Aris and Villaverde 2003). In fact, NLSCt provided very high transfection efficiency in vivo, as only 24ng of

NLSCt-coated Cu/Zn SOD plasmid was able to reduce oxidative stress and rescue neurons from cell death in different areas of the lesion border, considerably increasing functional performance of the animals (Article 4, Figure 4). Noteworthy, the 249AL and NLSCt vectors used in this Thesis were capable of transfecting cells of the entire excitotoxically lesioned area, from the most rostral to the most caudal areas, including different brain regions (Article 3). This fulfills one of the most important requirements for successful CNS gene therapy, i.e. whole-lesion widespread gene delivery and expression (Sapolsky and Steinberg 1999).

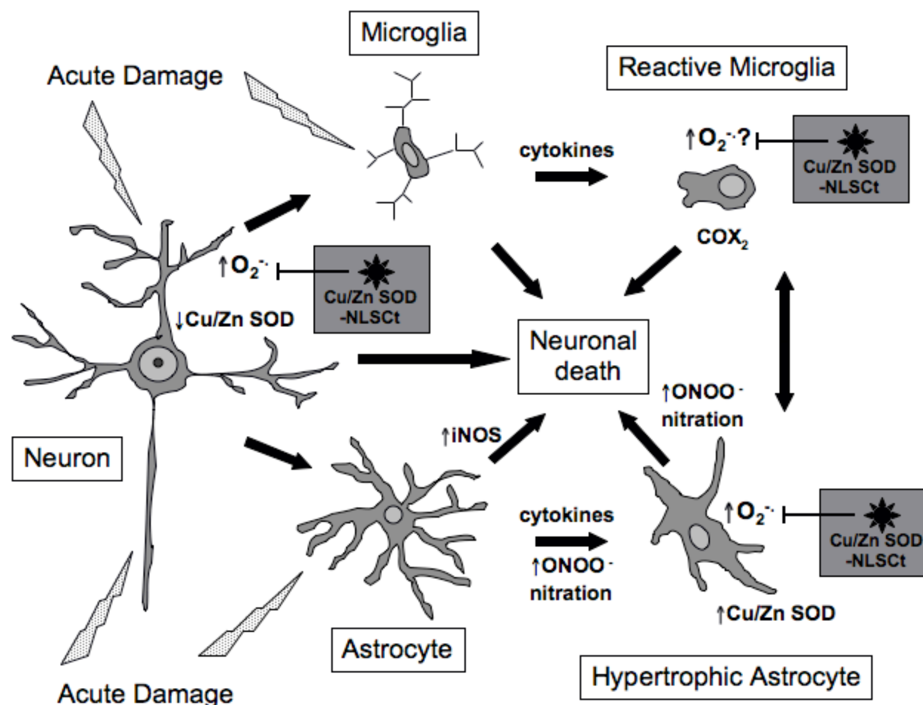


Figure 4. Representation of the main findings of this Thesis and the mechanism of neuroprotection by Cu/Zn SOD overexpression by means of the NLSCt modular recombinant protein vector.

As commented in the Introduction section, one of the biggest challenges facing vectors is the host immune response. Particularly in the brain, unacceptable persistence of inflammation has been observed upon injection of different viral vectors (Dewey et al. 1999) as well as immune activation (Wood et al. 1996) and demyelination (Byrnes et al. 1996a; Nilaver et al. 1995). Accordingly, the intracerebral injection of adenoviral vectors induces a rapid increase of IL-1β (Cartmell et al. 1999), followed by activation of microglia/macrophages and astroglia (Wood et al. 1996), increased expression of major histocompatibility complex class I (Wood et al. 1996) and a rise in anti-adenovirus antibodies in serum at the first 48 hours (Kajiwara et al. 2000). Moreover, accumulation of activated CD8+ and CD4+ lymphocytes occurs in the neural parenchyma by the first week (Byrnes et al. 1996b). In contrast, our results show that none of these histopathologic changes occurred 6 days after 249AL injection to the immature brain, suggesting that this vector does not trigger an inflammatory reaction or an immune response in the host in a timeframe

where viral vectors do (Article 3).

Production of biologically relevant quantities of transgenic protein

Although the benefits of administering exogenous neurotrophic factors for neuronal recovery are well known, the limitations imposed by protein degradation and dilution, and inefficient systemic delivery makes gene transfer systems more favorable for in situ expression. For example, the transfer and expression of hepatocyte growth factor (HGF) was sufficient to reduce delayed neuronal death, and this approach was more effective than administration of recombinant HGF (Hayashi et al. 2001). Although it is accepted that gene delivery by non-viral vectors has little safety risks, their frequently observed low transfection efficiency constitutes a major concern. Nevertheless, if we consider that trophic factors are potent molecules and that they are secreted, a low transgene expression and/or a small number of transfected cells is sufficient to drive significant biological effects, and the transfection efficiency issue will be of minor significance. Our gene therapy strategy using the NLSCt vector showed neuroprotective effects overexpressing an antioxidant enzyme that will only act inside the cells where it is produced, and that needs a higher concentration than trophic factors for showing a neuroprotective effect (Article 4). In agreement, we have observed that the overexpression of interleukin 10 after an excitotoxic lesion to the immature brain by means of the NLSCt vector can induce biological effects (Pau González and Hugo Peluffo unpublished results). In addition, in other ongoing experiments in the laboratory we have observed that overexpression of the NFκB interacting protein A20 in excitotoxically lesioned immature brain by means of NLSCt can induce significant increase in lesion volume, and also that the NLSCt vector can direct neuroprotective gene expression in excitotoxically injured adult striatum. These data show that the NLSCt vector can produce biological relevant concentration of a transgenic protein, and thus could also be used in the immature as well as mature acute CNS injuries for overexpression of molecules acting intracellularly and also extracellularly. In fact, semi-quantitative western blot analysis of transgene level production in our model suggested that the 249AL vector was capable of producing 1ng of transgenic protein in the brain (unpublished results). If we consider that the transgenic protein is distributed in the whole lesion as observed (Article 3), it would reach an effective concentration of around 10ng/ml. This concentration is in the order of magnitude of the active concentration of neurotrophic factors and cytokines. The use of our non-viral vector would thus mediate neuroprotective effects without life-long overexpression of this trophic factor in the brain. Moreover, though not confirmed *in vivo*, the NLSCt is capable of producing 30 times enhanced transgenic protein levels compared to 249AL *in vitro* (Aris and Villaverde 2003), and thus the *in vivo* concentrations of the transgenic protein would be conceivably greater. These data overall support the hypothesis that the modular recombinant vectors, and in particular NLSCt, can be suitable for different neuroprotective gene therapy strategies to the acutely injured brain.

Therapeutic considerations for gene therapy against acute CNS injuries

There is increasing evidences showing that gene therapy strategies can be applied to the acutely injured CNS (see Table 2), but these findings raise several questions. Firstly, it is important to understand the underlying mechanisms by which overexpression of a transgene has a particular effect. This is critical if we consider the multifactorial/multitarget approach that is emerging as necessary for a successful therapy. In our experimental model, the mechanism of neuroprotection by overexpression of Cu/Zn SOD is the increase in the total $O_2^{-\cdot}$ dismuting activity of neurons where the endogenous enzyme is normally expressed, but also in glial cells which do not have detectable levels of Cu/Zn SOD until 24-48 hours after the lesion (Article 1). Importantly, the transgenic Cu/Zn SOD also replaces the endogenous one which dimishes within 2-4 hours in the lesion core (Article 1).

Secondly, another important question is whether or not rescuing of neurons from death also spares them from dysfunction (Dumas and Sapolsky 2001). One can imagine scenarios in which the prolonged presence of non-functional neurons in neuronal networks could be deleterious. For example, the sparing of retinal photoreceptors from light induced degeneration by fibroblast growth factor 2 overexpression does not restore function as assessed by electroretinograms (Spencer et al. 2001). Moreover, the introduction of the Glut1 transgene (an early intervention) or the Bcl-2 transgene (a late intervention) generates comparable reduction in neurotoxicity, however Glut1 was more effective in sparing synaptic plasticity and cognition (Dumas et al. 2000; McLaughlin et al. 2000). Thus targeting early critical events in the neurotoxicity cascade seem to induce more robust neuroprotection than targeting later events as apoptotic/necrotic mechanisms. In agreement, we show here that targeting the early generation of $O_2^{-\cdot}$ by overexpressing the Cu/Zn SOD can induce important neuroprotection, not only at the level of neuron preservation but also at the level of functional improvement (Article 4).

Thirdly, is it possible to target the earliest steps of neurotoxicity following insult? For example, to target the early event of hyperexcitability efficiently, potassium channels must be overexpressed within thirty minutes post-insult. However, there is a 2-4 hours window for the delivery of Glut1 or calbindin (Phillips et al. 2001), and a 6-8 hours window for the delivery of Bcl-2 (McLaughlin et al. 2000). For the decomposition of $O_2^{-\cdot}$ there seems to be a therapeutic window, which includes sparing of function, of up to 6 hours after focal ischemia as observed with synthetic SOD mimetics (Mackensen et al. 2001). We delivered the Cu/Zn SOD 2 hours after the insult, but did not perform studies with larger delay times. However, the facts that the SOD mimetics act very rapidly and that the transgene expression *in vivo* needs 2-4 hours to be initiated suggests that gene therapy with Cu/Zn SOD will not be effective if administered at longer times after the lesion. Several additional evidences suggest that Cu/Zn SOD and other antioxidant enzymes are very interesting candidates for gene therapy. First, it is known that superoxide

scavengers protect from injury at early time points both in mature and immature animals (Ikeda et al. 2002; Mackensen et al. 2001; Mollace et al. 2003; Shimizu et al. 2003). Second, trophic factors that are very powerful neuroprotective agents, show as one of their neuroprotective mechanism, the upregulation of antioxidants and antioxidant enzymes (Jackson et al. 1994; Pan and Perez-Polo 1993), including SOD (Mattson et al. 1995; Nistico et al. 1992). Third, and particularly important regarding Cu/Zn SOD, is the observation that hypothermia, the most powerful neuroprotective strategy known, not only inhibits the rapid downregulation of Cu/Zn SOD that normally occurs after a traumatic brain injury but also induces its over-expression (DeKosky et al. 2004). Most surprisingly, this effect is specific for Cu/Zn SOD, and in fact hypothermia induces a less significant upregulation of other antioxidant enzymes such as catalase and glutathione peroxidase in comparison with non-hypothermic brain.

Another limiting factor for efficient gene therapy is that both intraventricular and direct intracerebral injections are invasive surgical procedures and may inflict additional trauma and injury to the brain. Though these direct injections have been performed in patients with success, other approaches as direct intrathecal injection into the cisterna magna, offers easy access to the intracranial CSF space and is usually carried out in a fully conscious and non-anesthetized patient. Thus, this procedure seems to be less invasive and easier to perform, and offers a more global access of vector to the CNS. Several studies have shown that gene transfer to the brain by intrathecal injection of vectors may be efficient and may take part in different compartments (Kramm et al. 1996; Meuli-Simmen et al. 1999; Ooboshi et al. 1995). In this Thesis we injected normal animals with the 249AL vector intravenously, but were unable to recover any transgene expression in the brain, though it was found in other organs (not shown). An interesting experiment would be to inject the modular vectors intravenously but after an acute brain lesion, where a blood-brain barrier breakdown is known to occur, and thus some transgene expression may also occur.

An additional possible barrier to gene therapy interventions is the downregulation of protein synthesis after an ischemic insult, which has been reported to delay transgenic protein expression (Lawrence et al. 1997). However, in the case of the immature brain this phenomenon is more transient than in mature brain, with complete recuperation of protein synthesis levels by 12 hours after transient ischemia while still partly inhibited in adult brain by 24 hours after (Berger et al. 1996). Thus the immature brain constitutes an interesting model for the development of gene therapy approaches, and here gene therapy approaches are more likely to be effective.

Finally, it should be noted that there are very few works showing neuroprotection after an acute injury to the CNS by means of non-viral vectors (Cao et al. 2002; da Cruz et al. 2005; Takahashi et al. 1999; Zhang et al. 2004). The first article showing neuroprotection using a non-viral gene therapy strategy used direct injection of liposome-mediated overexpression of Bcl-2 after spinal cord hemisection into the axotomized Clark Nucleus (Takahashi et al. 1999). They

observed an important reduction in cell death in this nucleus and a partial protection from neuronal atrophy, which lasted at least two months. However, no functional evaluation was performed. After I begun with this Thesis work, this same strategy was applied after occlusion of the middle cerebral artery-mediated transient ischemic lesion to the adult brain (Cao et al. 2002). Here, the overexpression of the Bcl-2 transgene under the control of the strong CMV promoter, but not under the control of a hypoxia-inducible human vascular endothelial growth factor promoter, induced a 30% reduction in lesion volume. Furthermore, this Thesis shows for the first time that consistent functional and neuropathological recovery from an acute immature brain damage can be achieved by post-lesion overexpression of the Cu/Zn SOD antioxidant enzyme by means of a modular recombinant protein vector. In summary, these works suggests that modular recombinant protein vectors can also be useful neuroprotective gene therapy vectors for acute CNS injuries, and that this rapidly expanding field will surely contribute to the improvement of the therapeutic intervention. Moreover, the intrinsic flexibility of the modular recombinant protein vectors used in this Thesis provides wider perspectives for the generation of specific prototypes for different therapeutic needs.

Neuroprotective of integrin-interacting RGD domains

Surprisingly, both the NLSCt vector carrying a control transgene and the nude vector showed a significant grade of neuroprotection from the *in vivo* excitotoxic damage, indicating that this effect was intrinsic to the NLSCt vector. One of the bioactive motifs of NLSCt is the foot-and-mouth disease virus integrin-interacting RGD peptide. Interestingly, the injection of the cyclic RGD peptide GPen after the excitotoxic injury showed a similar neuroprotection than NLSCt (Article 5), confirming that the RGD domain of NLSCt mediates the neuroprotection. In fact, a previous study showed that blocking αD/β2 integrins is strongly neuroprotective after spinal cord injury (Gris et al. 2004). In addition, although the NLSCt vector inhibits the interaction of RGD dependent integrins with their natural extracellular matrix ligands, it could also be directly activating integrin outside-in signaling events (Qin et al. 2004). In fact, both NLSCt and GPen were able to directly activate Erk1/2 and NFκB signaling. This Thesis also shows that both NLSCt and GPen modulated microglial reactivity in vivo after an excitotoxic lesion, and importantly it also suggests that they modulate glial neurotrophic activity (Article 5), in agreement with a study showing that in hippocampal slices linear RGD peptides induce upregulation of BDNF, NT3 and NGF neurotrophins and also of the neurotrophin receptors TrkB and TrkC (Gall et al. 2003). Though this study suggests that this upregulation occurred mainly in pyramidal and granular neurons cell layers, as no cell specific labeling was performed it is not known if it occurred only in neuronal cells or also in glial cells. Alternatively, in addition to modulating neurotrophic factor receptor expression, integrins can also modulate growth factor receptor signaling (Miranti and Brugge 2002), and thus the RGD containing molecules could be modulating the activity of glial

derived growth factors already present in the medium. We can not rule out however that other mechanisms are contributing to the neuroprotection observed by NLSCt and GPen, like partial inhibition of microglial phagocytosis of degenerating neurons (Witting et al. 2000), or inhibition of cells attachment/migration. Further studies are being conducted in our laboratory to further dissect the mechanisms of neuroprotection like glial cell type involved, neurotrophic factors production, and phagocytic activity.

Future directions

This Thesis opens several questions in different related fields, and also leaves several complementary experiments to be done to reduce its limitations.

An interesting issue would be to continue with the characterization of the nitrated astrocyte subpopulation of reactive astrocytes and their influence on lesion progression. In particular, further insights into the functional properties of these cells would be critical. Moreover, the evaluation of the role of the activated caspase 3 in these cells, specially regarding its role on astrocyte activation and is influence on neuronal survival would be of high interest. Finally, considering that nitrated astrocytes have been described in several pathologies, knowing whether the observations of this Thesis can be generalized to other acute and progressive CNS pathologies would be desirable.

Further research on the selective immature CNS lesion mechanisms and diagnosis is also needed. The elucidation of the mechanisms of immature brain damage will aid in the development of selective therapies, and the improvement in diagnosis will dictate when and how to intervene, and will also diminish the important lag-time between the insult and the treatment. In this sense, additional experiments evaluating the therapeutic window for the NLSCt-mediated gene therapy approach are needed.

Regarding the gene therapy strategy used in this Thesis, it would be interesting to explore alternative administration routes of the NLSCt vector, like intravenous administration after a lesion, where the blood brain barrier becomes disrupted. In addition, upregulation of αv integrins in cerebral microvessels after ischemia (Okada et al. 1996) or in tumor microvasculature (Pasqualini et al. 1997) makes possible an intravenous route for vector administration targeting these specific areas of interest. Studies analyzing the long-term effects of treatment with the NLSCt vectors should also be conducted. Moreover, as these vectors can be retrogradely transported by axonal terminals they could be useful for the treatment of spinal motor neuron diseases by a non-invasive intramuscular injection approach. Finally, further enhancement of the *in vivo* transfection efficiency of the NLSCt vector would be important. This could be explored by introducing an endosome escaping domain to the vector. In parallel, modification of targeting specificity by introducing functional domains like more RGDs, transferrin domains, or mannose, could be useful for the transfection of specific cell types.

The experience so far confirms that in many cases a multifactorial scope is required to understand a pathological event, and thus the design of an effective therapy would require this same approach. Therefore, an effective therapeutic strategy would then consist of the combination of several compounds, cells or gene therapy approaches distributed along the time of the neuropathological process. Within the gene therapy approach, the combinatorial gene therapy to increase neuroprotection could include the overexpression of Cu/Zn SOD along with other antioxidant enzymes as glutathione peroxidase, or these enzymes along with anti-inflammatory genes and antiapoptotic genes. Undoubtedly further research on combined therapies will induce a twist on future therapeutic interventions.

Main conclusions

i) In the immature rat brain the Cu/Zn SOD enzyme is mainly expressed in neurons and glia limitans and ventricular walls. This expression pattern does not differ substantially from the reported for the adult rat brain.

ii) In the immature rat brain neuronal of Cu/Zn SOD showed a rapid and drastic reduction after a NMDA excitotoxic injury, which could contribute to explain the major vulnerability of neurons to oxidative stress.

iii) In the immature rat brain, after an excitotoxic injury, nitrotyrosine was found not only in the primary and secondary inured neurons but also in relation to a subpopulation of highly reactive astrocytes.

iv) These heavily nitrated astrocytes are resistant to cell death in spite of displaying activated caspase 3 in their nuclei, which suggest new functions for this protein.

v) Modular recombinant protein vectors can be used for in vivo gene delivery to the excitotoxically injured immature brain without exacerbating neurotoxic inflammation.

vi) Modular recombinant protein vectors induce biological and therapeutic relevant concentrations of transgenic protein when administered 2-4 hours after an excitotoxic lesion.

vii) The overexpression of Cu/Zn SOD in the excitotoxically lesioned immature rat brain is neuroprotective and may have a putative therapeutic application.

viii) Integrin interacting RGD motifs are neuroprotective after an acute excitotoxic injury to the immature brain by a still unknown glial dependent mechanism.

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APPENDIX

Presentation of the Thesis work in scientific meetings

1) Oral presentation: "Gene therapy and neuroprotection: inhibition of NFkB glial pathway in an excitotoxic injury model". H. Peluffo, L. Acarin, A. Aris, A. Villaverde, B. González and B. Castellano. Second Meeting of the Spanish Glial Network, 25-27 May 2001, Miraflores, Madrid.

2) Oral presentation: "Non-viral vectors as tools for the design of gene therapy strategies for central nervous system". H. Peluffo, L. Acarin, A. Aris, A. Villaverde, B. González and B. Castellano. First Meeting of the Nervous System Regeneration and Reparation Network, 25-26 October 2001, University of Barcelona, Spain.

3) Presentation of two posters: "Phenotypic characterization of nitrated astrocytes following postnatal excitotoxic damage", L. Acarin, H. Peluffo, B. González and B. Castellano; and "In vivo gene delivery to the CNS using a novel integrin targeting multifunctional protein", ". H. Peluffo, L. Acarin, A. Aris, A. Villaverde, B. González and B. Castellano, at the 5th European Meeting on Glial Cell Function in Health and Disease, Rome, Italy, May 2002.

4) Presentation of two posters: "Phenotypic characterization of nitrated astrocytes following postnatal excitotoxic damage", L. Acarin, H. Peluffo, B. González and B. Castellano; and "In vivo gene delivery to the CNS using a novel integrin targeting multifunctional protein", ". H. Peluffo, L. Acarin, A. Aris, A. Villaverde, B. González and B. Castellano, in the 3rd Forum of European Neuroscience, Paris, France, July 2002.

5) Oral presentation: "Inhibition of NF-kB mediated by gene therapy or drugs", Hugo Peluffo, Laia Acarin, Berta Gonzalez and Bernardo Castellano, in the 2nd Meeting of the Network for Experimental Models for Central Nervous System Pathologies, in the Center of Genome Regulation, Barcelona, Spain, December 2002.

6) Poster presentation: "Expression of Cu/Zn superoxide dismutase in immature rat brain after excitotoxic damage", Hugo Peluffo, Laia Acarin, Berta Gonzalez and Bernardo Castellano, in the 6th European Meeting on Glial Cell Function in Health and Disease, Berlin, Germany, September 2003.

7) Poster presentation: "Non-viral gene delivery to the CNS based on a novel integrin targeting multifunctional protein ", H. Peluffo, L. Acarin, A. Aris, A. Villaverde, B. González y B. Castellano, in: Towards clinical gene therapy: pre-clinical assessment of gene transfer, organized by the European Program of GENETHON – VectEuroTrain, dedicated to the promotion of the cooperation and the training in gene therapy techniques in Europe. Universidad Autónoma de Barcelona, Bellaterra, Spain, February 2004.

8) Poster presentation: "In vivo gene transfer by non-viral, multifunctional proteins", A. Aris, U. Pérez, H. Peluffo, L. Acarin, B. González, B. Castellano and A. Villaverde, en el congress: Towards clinical gene therapy: pre-clinical assessment of gene transfer, organized by the European Program of GENETHON – VectEuroTrain, dedicated to the promotion of the cooperation and the training in gene therapy techniques in Europe. Universidad Autónoma de Barcelona, Bellaterra, Spain, February 2004.

9) Poster presentation: "Neuroprotective non-viral gene delivery to the postnatal CNS based on novel integrin targeting multifunctional proteins". H. Peluffo, L. Acarin, A. Aris, B. González,

A. Villaverde, y B. Castellano, in the 4th Forum of European Neuroscience, Lisbon, Portugal, Julio 2004.

10) Poster presentation: "Neuroprotective superoxide dismutase gene delivery to the postnatal rat brain based on a novel non-viral multifunctional protein vector". **H. Peluffo**, L. Acarin, A. Arís, B. González, A. Villaverde and B. Castellano. In the 34th Annual meeting of the Society for Neuroscience, San Diego, USA, 2004.

11) Poster presentation: "Neuroprotective mechanisms of αv integrins interacting peptides after an excitotoxic lesion to the immature brain". **H. Peluffo**, P. Gonzalez, A. Aris, L. Acarin, B. Castellano, A. Villaverde and B. Gonzalez. In the 35th Annual meeting of the Society for Neuroscience, Washington DC, USA, 2005.

12) Oral presentation: "Neuroprotection from excitotoxic damage by Cu/Zn superoxide dismutase gene delivery to the postnatal rat brain using a modular non-viral protein vector". H. Peluffo, L. Acarin, A. Aris, P. González, B. Castellano, A. Villaverde and B. Gonzalez. In the 4th Annual Meeting of Mouse Models in Neuroscience Research Network, Center for Genomic Regulation, Spain, December, 2005.

Additional published scientific work by the author not being part of this Thesis

1) V. Brovia, A. Costa, G. Bedó, **H. Peluffo**, E. Manta and L. Barbeito. N-Acetylaspartylglutamate Acetoxymethyl ester (NAAG-AM) as a tool to permeabilise the neuropeptide NAAG and Succinimidyl-NAAG into intact cells: effects on ·H-Dopamine exocitosis. Brazilian Journal of Medical and Biological Research, 29:249-258 (1996)

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