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CELLULAR BASIS OF STEROID NEUROPROTECTION IN THE WOBBLER MOUSE, A GENETIC MODEL OF MOTONEURON DISEASE

María C. González Deniselle, Susana L. González and Alejandro F. De Nicola [1]

Laboratory of Neuroendocrine Biochemistry, Instituto de Biología y Medicina Experimental; Dept. of Human Biochemistry, Faculty of Medicine, University of Buenos Aires; and Instituto Universitario de Ciencias de la Salud, Fundación Barceló, Argentina. [1] Instituto de Biología y Medicina Experimental. Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina. E-mail: denicola@proteus.dna.uba.ar.

Abstract

The Wobbler mouse suffers an autosomal recessive mutation producing severe motoneuron degeneration and astrogliosis in the spinal cord. It has been considered a suitable model of human motoneuron disease, including the sporadic form of amyotrophic lateral sclerosis (ALS). Evidences exist demonstrating increased oxidative stress in the spinal cord of Wobbler mice, whereas antioxidant therapy delayed neurodegeneration and improved muscle trophism. 21-Aminosteroids are glucocorticoid-derived hydrophobic compounds with antioxidant potency 3 times higher than vitamin E and 10 times higher than methylprednisolone. They do not bind to intracellular receptors, and prevent lipid peroxidation by insertion into membrane lipid bilayers. In common with the spinal cord of ALS patients, Wobbler mice present astrocytosis with hyperexpression of glial fibrillary acidic protein (GFAP), and increased expression of nitric oxide synthase (NOS) and growth-associated protein (GAP-43) in motoneurons. Here, we review our studies on the effects of a 21-aminosteroid on GFAP, NOS and GAP-43. First, we showed that 21-aminosteroid treatment further increased GFAP-expressing astrocytes in gray matter of the Wobbler spinal cord. This effect may provide neuroprotection if one considers a trophic and beneficial function of astrocytes during the course of degeneration. Other neuroprotectans used in Wobbler mice (T-588) also increased preexisting astrocytosis. Second, histochemical determination of NADPH-diaphorase, a parameter indicative of neuronal NOS activity, showed that the 21-aminosteroid down-regulated the high activity of this enzyme in ventral horn motoneurons. Therefore, suppression of nitric oxide by decreasing NADPH-diaphorase (NOS) activity, may provide neuroprotection considering that excess NO is highly toxic to motoneurons. Finally, 21-aminosteroid treatment significantly attenuated the aberrant expression of both GAP-43 protein and mRNA in Wobbler motoneurons. Hyperexpression of GAP-43 possibly indicated abnormal synaptogenesis, denervation and muscle atrophy, parameters which may return to normal following antioxidant steroid treatment. Besides 21-aminosteroids, other steroids also behave as neuroprotectans. In this regard, degenerative diseases may constitute potential targets of these hormones, based on the fact that the spinal cord expresses in a regional and cell-specific fashion, receptors for androgens, progesterone, adrenal steroids and estrogens.

Key words: Wobbler mouse; amyotrophic lateral sclerosis; spinal cord; neurodegeneration; astrogliosis; 21-aminosteroids.

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Resumen

El ratón Wobbler padece de una mutación autosómica recesiva que le produce una severa degeneración de motoneuronas y astrogliosis de la médula espinal. Se lo ha considerado un modelo apropiado de las enfermedades humanas de motoneurona, incluida la forma esporádica de la esclerosis lateral amiotrófica (ELA). Existen evidencias que demuestran aumento del estrés oxidativo en la médula espinal del ratón Wobbler, mientras que las terapias antioxidantes retardan la neurodegeneración y mejoran el trofismo muscular. Los 21-aminoesteroides son compuestos hidrofóbicos derivados de los glucocorticoides cuya potencia antioxidante es 3 veces mayor a la vitamina E y 10 veces mayor que la metilprednisolona. No se unen a los receptores intracelulares y previenen la peroxidación lipídica por inserción en la bicapa lipídica de las membranas. En común con la médula espinal de pacientes con ELA, los ratones Wobbler presentan astrocitosis con hiperexpresión de la proteína ácida fibrillar de la glia (GFAP), aumentada expresión de la óxido nítrico sintetasa y de la proteína asociada al crecimiento (GAP-43) en motoneuronas. En este trabajo nosotros resumimos nuestros estudios sobre los efectos de los 21-aminoesteroides sobre la GFAP, NOS y GAP-43. Primero, mostramos que el tratamiento con el 21-aminoesteroide aumentó aún más la alta expresión de la GFAP por astrocitos de la sustancia gris de la médula espinal de ratones Wobbler. Este efecto posiblemente sea neuroprotector si se considera la función trófica y beneficiosa de los astrocitos en el curso de la neurodegeneración. Otros neuroprotectores empleados en el ratón Wobbler como el T-588 también aumentan la astrocitosis. Segundo, la determinación histoquímica de la NADPH-diaforasa, un parámetro indicador de la actividad de NOS, mostró que el 21-aminoesteroide redujo la alta actividad de esta enzima en las motoneuronas del asta ventral de los Wobbler. De esta manera, la supresión del óxido nítrico al disminuir la enzima que lo produce, también sería un efecto neuroprotector considerando que el exceso de óxido nítrico es tóxico para las motoneuronas. Finalmente, el tratamiento con el 21-aminoesteroide significativamente atenuó la expresión aberrante del ARNm y proteína para la GAP-43, una acción que atenuaría las anormalidades de la sinaptogénesis, desnervación y atrofia muscular. Además de los 21-aminoesteroides, otros esteroides también se comportan como neuroprotectores. En este sentido, las enfermedades neurodegenerativas pueden constituirse en un blanco terapéutico preferencial para estas hormonas, dado que la médula espinal presenta una alta regionalización y especificidad celular en la expresión de receptores para los andrógenos, progesterona, esteroides adrenales y estrógenos.

Palabras clave: ratón Wobbler; esclerosis lateral amiotrófica; médula espinal; neurodegeneración; astrogliosis; 21-aminoesteroides.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive devastating disease affecting motoneurons that innervate voluntary muscles. It belongs to a family of diseases characterized by motoneuron degeneration. Following an anatomical criteria, these diseases are classified into three groups [Cudkowicz et al, 1998]: 1) Motoneuron diseases affecting lower motoneurons of the brain stem and spinal cord and upper motoneurons of the corticospinal and corticobulbar systems; 2) Diseases affecting lower motoneurons only; 3) Diseases comprising exclusively upper motoneurons. ALS is a typical example of the first group. The drama imposed by ALS is due to its fatal outcome, and the minor effectiveness of available treatments. At the late stages of the disease, patients show increased ambulatory difficulties, muscle weakness, inability to swallow, and risk respiratory paralysis in the light of a relative mental awareness [Bradley, 1996]. According to the NIH Publication # 1984 [Amyotrophic Lateral Sclerosis, 1984] 95% of ALS patients do not report a family history of the disease, for which reason they belong to the "sporadic form", while the re-

maining 5% present the "familial form", transmitted by a dominant gene. Other available data indicate that familial ALS cases account for 15-20%. In both forms, however, surviving time is limited to about 5 years [Cudkowicz et al, 1998].

Examples of the group of motoneuron diseases affecting lower motoneurons only are the types I to IV forms of spinal muscular atrophy, characterized by progressive motoneuron degeneration transmitted by an autosomic recessive gene. To this group also belongs the spinobulbar muscular atrophy linked to the X chromosome, which presents a defect in the spinal cord androgen receptor, and the gangliosidosis caused by GM2 accumulation. Lastly, a typical example of upper motoneuron disease is the familial spastic paraplegia, which shows an autosomic dominant transmittance.

Animal models of motoneuron disease

Biomedical research is the only hope to cure or delay motoneuron disease. In this sense, animal models become useful tools to develop new pharmacological treatments based on experimental findings *in vivo* and *in vitro*. Rodents are the most common animal models of genetic or induced motoneuron disease. An exemption is the Brittany Spaniel dog characterized by a profuse filament deposition in motoneurons [Amyotrophic lateral sclerosis, 1984] but unfortunately, little has been done in these dogs regarding treatment responses. Following the reports of Cudkowicz et al [1998] and Price [1994], murine models may be classified into four categories:

- The Wobbler mouse, a model for ALS and infantile spinal muscular atrophy.
- The progressive motor neuropathy mouse (pmn).
- The "wasted" (wst), "tumbler" (tb) and three varieties of mice with motoneuron disease known as mnd1, mnd2 and mnd3.
- Transgenic animals expressing a mutant form of the enzyme superoxide dismutase type I (SOD1) and transgenics with hyperexpression of light (NF-L) and heavy (NF-H) neurofilament subunits .

Animal models of familial ALS: The Gurney mouse

For FALS, the best-known and widely used model is the mouse developed by Gurney et al [1994] at Upjohn-Pharmacia laboratories in Kalamazoo, MI. The discovery was prompted by the observation that in about 20% of patients with FALS, a primary defect exists in the cytosolic Cu,Zn-superoxide dismutase type I (SOD1) [Rosen et al, 1993]. At the present time, more than 50 mutations of this enzyme have been reported. Fiszman et al [1999] considered that the activity of SOD1 progressively decreases with advancing age in red blood cells of FALS patients. Thus, functional modifications in SOD1 occur both in humans with FALS and experimental models of this disease.

The enzyme SOD1 homodimerizes and its primary function is to detoxify superoxide anion, by conversion into H_2O_2. The last product is detoxified to H_2O by the enzymes catalase and glutathion peroxidase. If present in excess, superoxide anion interacts with nitric oxide (NO) forming peroxinitrite ($ONOO^-$), a highly toxic product causing protein nitrosylation and generation of hydroxyl radicals (OH^-) [Cohen and Author, 1982]. In patients with FALS, the reduction in the activity of SOD1 amounts to 25-50% in brain, suggesting that this disease can originate in abnormalities in free radical homeostasis, with a subsequent increase in oxidative stress.

However, a problem exists since diseases with loss of function of a specific protein are recessive in nature, while FALS is inherited in a dominant fashion. Thus, it is likely that mutant SOD1 acquired a gain of function or other properties. In the transgenic mice, three out of four mutants of the human SOD1 introduced caused motoneuron degeneration: glycine 93 → alanine, glycine 85 → arginine and glycine 37 → arginine. Starting at 3-4 months of age, transgenics bearing these mutations developed motoneuron disease characterized by limb weakness, posterior limb paralysis, tremor, death due to inability to chew, loss of the enzyme acetyl-cholinesterase, motoneuron death, accumulation of neurofibrillary material, loss of axons in

ventral roots, dendrite microvacuolization, hiperexpression of SOD1, with aggregates of the enzyme in neurons and astrocytes, increased oxidative stress in the spinal cord with decreased levels of vitamine E, and increased susceptibility to glutamate neurotoxicity [Gurney et al 1994; Li et al2000; Spooren and Hengerer, 2000].

To explain these abnormalities, several hypothesis were advanced, such as: a) a lower affinity for superoxide anion by the mutant SOD1, the accumulation of which increased oxidative stress; b) high affinity for peroxinitrite, with elevated levels of nitronium ions causing protein nitrosylation; c) enzyme precipitation forming aggregates which kill motoneurons. The last possibility gained considerable attention, as enzyme deposits may cause neuronal death. Recently, Li et al [2000] demonstrated in SOD1 transgenics that neuronal death occurs by apoptosis due to activation of executioner caspases. These group also showed that a caspase 3 inhibitor was able to reverse apoptosis and prolong life span in the transgenic mice. Therefore, the Gurney mouse resulted a valuable tool not only for basic neurochemical research but also for testing new pharmacological treatments which may benefit humans suffering from FALS.

The Wobbler mouse: genetics, clinical outcome and neuropathology

The Wobbler mouse mutant contains a mutation of autosomic recessive expression (wr) producing early degeneration of motoneurons of the spinal cord and brain stem [Duchen and Strick, 1968] (Figure 1). Although the wr gene has not been identified, it maps to chromosome 11 close to the glutamine synthetase gene [Kaupmann et al, 1992]. Due to the clinical symptomatology and neuropathological findings, Wobblers become useful models of motoneuron disease including sporadic ALS and infantile spinal muscular atrophy (Werdenig-Hoffman disease).

In homozygous animals (wr \ wr) the first manifestations of the disease are observed at 3-4 weeks of age, consisting in progressive weakness of front limbs, tremor and ambulatory difficulty, followed by muscle atrophy, small body size, front limb flexion and extension of rear limbs [Duchen et al, 1965]. Heterocygote mice (wr \ +) are clinically normal. Yung et al [1982] have established different stages of the disease, taking into consideration the condition of the limbs (0 = normal; 1 = atrophy; 2 = curled digits; 3 = curled wrists; 4 = complete thoracic flexion of front limbs) and deambulation (0 = normal; 1 = tremor with normal deambulation; 2 = wobbling; 3 = curled paw walking; 4 = jaw walking).

Histologically, perikaryal vacuolar degeneration with cromatolisis and cellular edema occurs in lower medium and large size motoneurons of Wobbler mouse spinal cord. Typically, an eccentric nucleus can be observed. Additionally, dense astrocytosis (Figure 2) with increased levels of glial fibrillary acidic protein (GFAP) and axonal degenera-

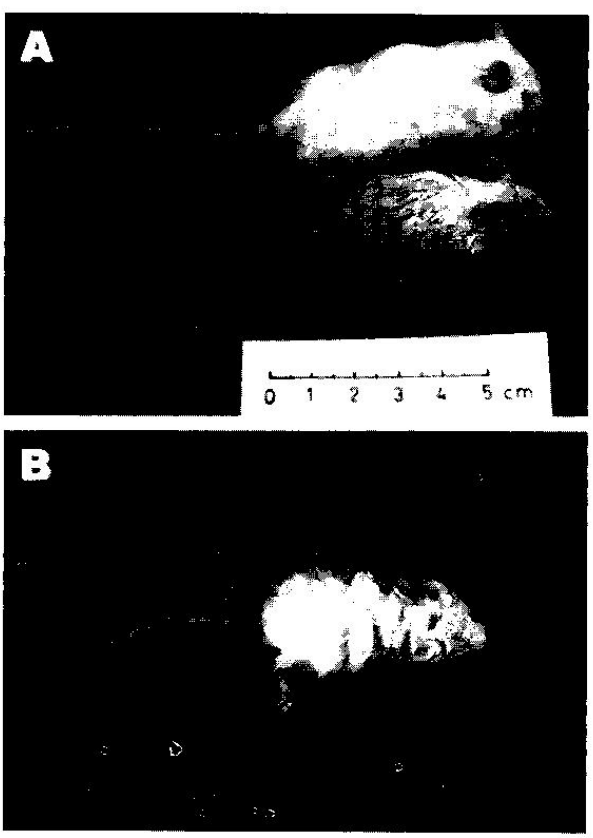


Fig. 1. Representative photomicrographs of control and Wobbler mice. A) Control (top) and Wobbler (bottom) mice, 1 month old. B) Wobbler mouse, 3 months old.

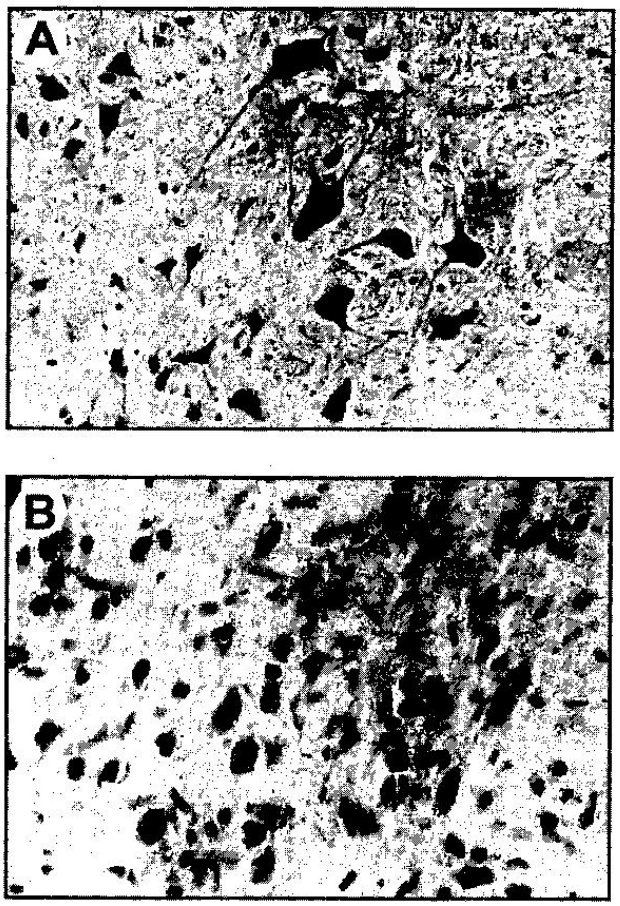


Fig. 2. Photomicrographs representing cresyl violet staining of control (A) and Wobbler (B) mice cervical spinal cord. Magnification: 400 X.

tion characterize the Wobbler mouse cervical spinal cord. According to some authors the astrogliosis represent a primary event [Laage et al, 1988], whereas others consider it a response to neurodegeneration.

Mitsumoto and Gambetti [1986] demonstrated that the slow axonal transport is impaired in Wobbler mice, an event explained by the reduction of axonal number or diameter. Also, myelinated fibers undergo axonal degeneration. In this respect, the number of larger fibers diminishes in an advance stage of the disease while smaller fibers are not affected. Therefore, neuronal degeneration involves a motoneurons similar to humans with motoneuron disease. Interestingly, axonal pathology in Wobblers coincides with the appearance of clinical symptoms [Mitsumoto and Bradley, 1982].

Over the years, several pathological abnormalities found in Wobbler mice closely resembling those observed in the spinal cord of patients with ALS, encouraging authors to use Wobblers as models for human motonenuron disease. These are listed in Table 1.

Molecular pathology of Wobbler mice spinal cord

During the course of degeneration, the spinal cord of Wobbler mice shows increased expression of some genes involved in neurotrophism or in differentiation during embryonic life. Some of the trophic factors produced by degenerating neurons could also influence astrogliosis.

The most relevant hyperexpressed genes are those coding for brain-derived neurotrophic factor (BDNF) and its associated receptors trkB and p75 NTR, c-Jun, transforming growth factor alpha (TGFα) and the growth-associated protein known as GAP-43. On the other hand, both choline acetyl-transferase (ChAT) mRNA and protein are reduced, whereas expression of NGF, neurotrophin 3 and its receptor trkC are unchanged [Junier et al, 1994; Junier et al, 1998; Popper et al, 1997].

Motoneurons expressing high levels of BDNF and its high affinity and low affinity related receptors, may do so in an attempt to delay degeneration, considering that this factor promotes neuronal survival after axotomy and differentiation of embryonic neurons [Henderson, 1986; Enfors et al, 1989]. However, the high levels of BDNF cannot avoid the decline in ChAT, the enzyme synthesizing acetylcholine, a main neurotransmitter released by motoneurons. It is also possible that the high expression of trophic factors and their receptors are a manifestation of cellular stress, or typify a reversal of the gene pattern of mature motoneurons to an embryonic profile. Another factor hyperexpressed by degenerating motoneurons is TGFα, which according to Junier et al [1994] is the main cause of astrogliosis. She and her group demonstrated that after TGFα is produced and released by motoneurons, it binds to astrocyte receptors, inducing their hy-

Table 1. Histopathological abnormalities found in the spinal cord of patients with ALS and Wobbler mice

- ➢ Degeneration of medium and large size motoneurons, including loss of Nissl substance.
- ➢ Axonal changes identical to Wallerian degeneration.
- ➢ Neufibrillary hyperplasia with impaired axonal transport.
- ➢ Lack of lipofuscin grains in motoneurons.
- ➢ Astrogliosis with strong expression of GFAP.
- ➢ Increase expression of the growth associated protein (GAP-43).
- ➢ Higher expression of nitric oxide synthase.

pertrophy and proliferation.

An important contribution to Wobbler mice spinal cord pathology was carried out by Ma and Vacca-Galloway [1994]. They found a pronounced degeneration of α motoneurons in ventral horn, accompanied by plastic changes in γ motoneurons. There is also intense axonal sprouting in presynaptic neurons, accompanied by high immunoreactive levels of the neuropeptides substance P, TRH, endorphin and ACTH receptors [Deng et al, 1996; Smith and Hughes, 1994]. These changes in presynaptic neurons follow α-motoneuron degeneration.

Astrocyte pathology in Wobbler mice

Wobbler mice present in common with ALS patients astrogliosis in gray and white matters of the spinal cord, primary motor cortex and subpial regions [Murayama et al, 1991]. The reactive astrocytes found in Wobbler and ALS spinal cord express high levels of GFAP [Laage et al, 1988]. The cause of astrocytosis in ALS is unknown, and some abnormalities in the function of these cells have been reported. Some authors consider this event a secondary response to neuronal illness, in which case TGF α could act as the neuronal inducer of astrogliosis. However, Wobbler astrocytes in primary culture also present some abnormalities. In this respect, primary Wobbler astrocytes exhibited abnormal cell-cell contacts, defects in glutamate transport and intercellular networks in culture in comparison to control astrocytes [Hantaz-Ambroise et al, 1994; 1995; González Deniselle et al, 1999c]. Furthermore, direct addition of Wobbler astrocyte conditioned medium or cocultures with Wobbler astrocytes led to a decrease in neuron number in primary mixed neuronal cultures [Ait-Ikhlef et al, 2000].

Besides, previous work from our laboratory demonstrated: 1) low (^{3}H)-thymidine incorporation into astrocytes derived from the spinal cord of Wobbler mice in contrast to control astrocytes, 2) unresponsiveness of Wobbler astrocytes to corticosterone and interleuquin 1 in doses that readily stimulated proliferation of control astrocytes, 3) selective stimulation of [^{3}H] thymidine incorporation by TGF β1 in Wobbler mice astrocytes to levels similar to control cells. In this sense, TGF β1 could have a role in the development of astrogliosis. Thus, available evidences suggest several abnormalities exist in number, morphology, and function of Wobbler mouse astrocytes, indicating that in addition to neurons, glial cells are profoundly affected in the course of the Wobbler disease and possibly in ALS.

Oxidative stress in Wobbler mouse spinal cord

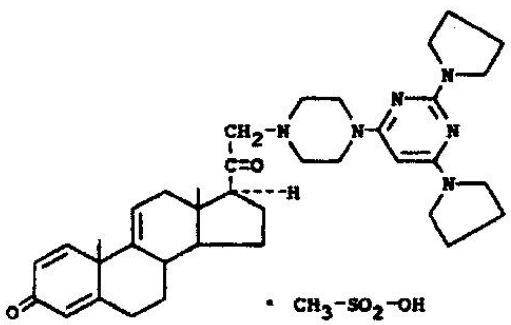
In contrast to studies carried out with the SOD1 transgenics, participation of oxidative stress in Wobbler mice spinal cord neuropathology rests mainly on indirect evidences. For example, Ikeda et al [1995] suggestion that a defective SOD1 may play a role

in the Wobbler neurodegeneration, was supported by replacement of the abnormal enzyme with a lecithinized derivative. This treatment delayed neurodegeneration and muscle atrophy. In other studies, inhibition of oxygen free radicals with n-acetylcysteine [Henderson et al, 1996] or attenuation of excess NO by administration of the NOS inhibitor nitroindazol [Ikeda et al, 1998] resulted to some extent in delayed neurodegeneration and attenuation of motor dysfunction. The latter result is consistent with Clowry and McHanwell [1996] who showed very high levels of NOS activity in the spinal cord motoneurons of the mutant animals.

Effects of an antioxidant 21-aminosteroid on astrogliosis, NOS and GAP-43 expression in the Wobbler mouse spinal cord

The above mentioned observations suggested that antioxidant steroid therapy may be a suitable treatment for neurodegeneration. In this sense, most steroid types provide neuroprotection, including estrogens, androgens, glucocorticoids, 21-aminosteroids and progesterone [Jones et al, 1997; McEwen, 1999; Hall, 1992; Schumacher et al, 2000]. 21-Aminosteroids, also called "Lazaroids" are glucocorticoid-derived synthetic compounds

U-74389F (16-desmethyl tirilizad)



INHIBITION OF IRON CATALYZED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATES

COMPOUND	IC 50
VITAMIN E	28μM
U-74006F (tirilazad)	8μM
METHYLPREDNISOLONE	>100μM

Fig. 3. Chemical structure of a 21-aminosteroid (top). Inhibition of iron dependent lipid peroxidation by vitamin E, a 21-aminosteroid and the glucocorticoid methylprednisolone (bottom). [References from Hall et al, 1991, and McCall et al, 1989].

showing strong antioxidant activity (Figure 3). These molecules do not bind to intracellular glucocorticoid receptors, and they are devoid of classical glucocorticoid properties, including the unwanted secondary effects found in chronic glucocorticoid therapy [Hall et al, 1993; De Nicola, 2000].

21-Aminosteroids are highly hydrophobic compounds inserting in the lipid phase of cell membranes [McCall et al, 1996]. Thus, they are ideally suited to inhibit lipid peroxidation, because they scavenge free oxygen radicals and stabilize membranes [Braughler et al, 1984; Hall et al, 1993]. Based on previous studies showing that oxygen-derived free radicals produced neuronal death in cases of CNS injury, stroke and neurodegenerative diseases, pharmacological trials employing 21-aminosteroids revealed strong neuroprotection of these substances in models of experimental CNS injury, hypoxia and ischemia [Hall et al, 1993; Beck and Bielenberg, 1991]. In our laboratory, we administered the 21-aminosteroid U-74389F to Wobbler mice, aiming to investigate the potential of this compound to change existing spinal cord neuropathology. Our work was focused in three molecules abnormally expressed by the spinal cord of ALS patients and Wobbler mice. These include neuronal NOS, neuronal GAP-43 and astrocyte GFAP.

Effects of a 21-aminosteroid on GFAP-expressing astrocytes

GFAP is an intermediate filament protein which participates in growth of astrocyte processes [Eng, 1985: Wilkin et al, 1990], regulation of motility, cell shape and relation of the astrocytes with surrounding neurons [Laping et al, 1994]. GFAP has also become a useful marker for astrocytes in immunocytochemical studies. Our experiments demonstrated that sc implantation of a 50 mg pellet of U-74389F for 4 days to Wobbler mice increased the size and\or number of glial cells showing GFAP immunoreactivity in the spinal cord and internal capsule. Quantitation of the changes using computerized densitometry reinforced the view that U-74389F effects on GFAP-positive astrocytes occurred in spite of a preexisting astrocytosis (Figure 4). Significant up-regulation of GFAP under U-74389F therapy was observed in cells localized in the ventral and dorsal horns (Figure

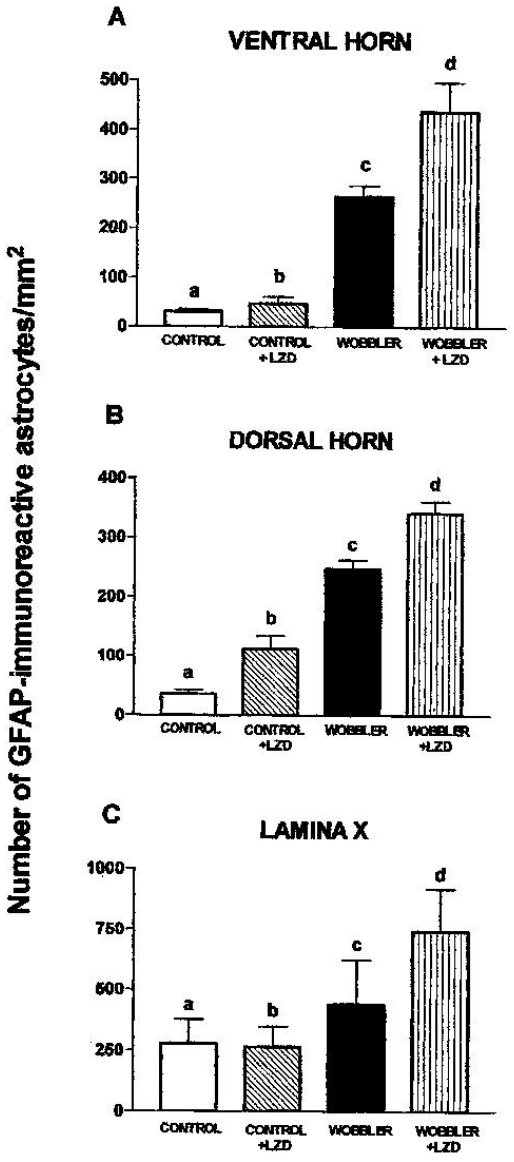


Fig. 4. Number of GFAP immunoreactive astrocytes/mm^2 in gray matter areas of the spinal cord of control mice (open columns), controls receiving the 21-aminosteroid Lazaroid U-74389F (LZD; cross hatched columns), Wobbler mice (black column), and Wobblers receiving U-74389F (dotted column). (A) Ventral horn; (B) dorsal horn; (C) central canal or lamina X. Results represent the mean ± S.E. Statistical significance: (A) c vs a, $p<0.01$; c vs d, $p < 0.01$; (B) a vs b, $p < 0.05$; a vs c, $p < 0.01$; c vs d, $p < 0.01$; (C) a vs b, NS; c vs d, NS (ANOVA and Duncan test).

4) without changes in white matter astrocytes found in the ventrolateral funiculus and corticospinal tract of the spinal cord [González Deniselle et al, 1996].

Although the biological significance of this finding and its relation to neuronal sickness of Wobbler mice may be open to discussion, we interpret it in terms of the known beneficial role of astrocytes on neuronal function. Thus, neuronal damage caused by ischemia, toxins, excitotoxicity, injury and neurodegeneration is first followed by astrocyte hypertrophy and then hyperplasia with GFAP hyperexpression [Norenberg, 1994]. In this way, stimulated astrocytes could be better suited to provide neuroprotection. For a number of years, it has been realized that among other properties, astrocytes take up excess glutamate and potassium released during neurotransmission, provide neurons with trophic factors, glucose and lactate used for energy purposes and even play a role in myelination of axons [Magistretti and Pellerin, 1999: Liedtke et al, 1996]. Thus, stimulation of the preexisting astrocytosis would be a positive effect, in contrast to traditional concepts which associate astrocytosis with increased pathology and inhibition of neuronal regeneration [McKeon et al, 1991]. Recently, Ikeda et al [2000] treated Wobbler mice with T-588, a non-steroidal antioxidant. By the time T-588 enhanced motor function, muscle parameters and survival time of the affected animals, it also stimulated astrocytosis. These data, in conjunction with ours, support that some antioxidant neuroprotectants also up-regulate astrocyte number and\or function.

Effects of a 21-aminosteroid on NOS (NADPH-diaphorase)

We have already discussed existing reports which postulate that free radicals play a substantial role in Wobbler mice neurodegeneration. Thus, increased activity of NOS was noted from determination of NADPH-diaphorase histochemistry [Clowry and McHanwell, 1996]. Also, treatment of Wobbler mice with the antioxidants OPC-14117 [Abe et al, 1997], n-acetylcysteine [Henderson et al, 1996], lecythinized superoxide dismutase [Ikeda et al, 1995] T-588 [Ikeda et al, 2000] or the NOS inhibitor nitroindazol [Ikeda et al, 1998], corrected the motor dysfunction of the animals. Possibly, this beneficial action resulted from blockage of free radical damage to motoneurons.

In confirmation with the report of Clowry and McHanwell [1996], we also found that Wobbler mouse spinal cord showed a sizable amount of neurons giving strong histochemical staining for NADPH-diaphorase [González Deniselle et al, 1999a]. In certain sections, positive motoneurons were surrounded by a complex network of fibres and processes strongly stained for NADPH-diaphorase. Treatment of Wobblers with the 21-aminosteroid U-74389F during 4 days, significantly reduced NADPH-diaphorase activity in motoneurons of the ventral horn (Figure 5). NO is a powerful neurotoxic agent, contributing to pathogenesis of neuronal death [Resink, 1996]. In our case, increased production of NO may contribute to neurodegeneration in the Wobbler spinal cord, considering that after coupling with superoxide anion, NO forms peroxinitrites. Lipids, proteins and DNA are preferred targets of the oxidative cascade originated by the excess NO. In the Wobbler spinal cord, this cycle may be suppressed by

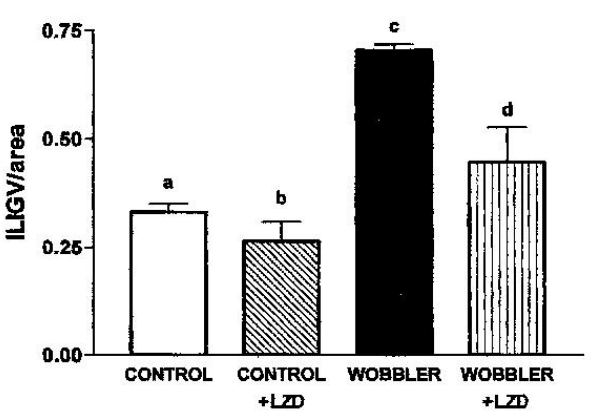


Fig. 5. Quanitative analysis of NADPH-diaphorase histochemistry in ventral horn motoneurons. Results represent the reaction intensity (ILIGV/area) in control mice (CONTROL), controls receiving U-74389F (CONTROL + LZD), Wobbler mice (WOBBLER) and Wobblers receiving U-74389F (WOBBLER + LZD). Significance: a vs c: $p < 0.01$; c vs d: $p < 0.01$.

antioxidants such as U-74389F, because this compound reduces the activity of NADPH-diaphorase (NOS), the enzyme responsible for NO overproduction.

Effects of a 21-aminosteroid on GAP-43

A third protein with abnormal expression in Wobbler spinal cord (as well as in patients with ALS) is the growth-associated protein or GAP-43. Physiologically, this phosphoprotein is found in high concentrations in embryonic life, during synaptogenesis and in axons regenerating after injury [Benowitz et al, 1997]. Usually, the high expression of GAP-43 subsides when growing axons reach their target or myelinize [Oestreicher et al, 1997]. However, in the normal adult spinal cord, moderate levels of GAP-43 are found in axon terminals of the dorsal horn, in the corticospinal tract and in the area surrounding the central canal Lamina X but is absent from motoneurons [Curtis et al, 1993a]. However, GAP-43 is up-regulated in motoneurons after experimental spinal cord trauma [Curtis et al, 1993b]. In humans, ventral horn motoneurons are devoid of GAP-43 but high mRNA levels appeared in ALS patients [Parhad et al, 1992]. In cases of Alzheimer´s disease, aberrant expression of GAP-43 was found in astrocytes [De la Monte et al, 1995], whereas abnormal expression also occurred in the hippocampus and gyrus cinguli of schizophrenic patients [Blennow et al, 1999].

In Wobbler mouse spinal cord, an intense expression of protein and mRNA corresponding to GAP-43 was found in ventral horn neurons (Figures 6 and 7) [González Deniselle et al, 1999a; 1999b]. In the mRNA studies, a synthetic ^{35}S-labeled oligonucleotide complementary to mouse GAP-43 mRNA [Clowry and McHanwell, 1996] was hybridized to spinal cord sections. Computer-assisted image analysis was used to quantitate the number of grains per cell, a measurement proportional to the amount of cytoplasmic mRNA [Mitsumoto and Gambetti, 1986]. As observed in Figure 7, GAP-43 mRNA was almost undetectable in control mice motoneurons, in contrast with the intense expression in Wobbler motoneurons. 21-Aminosteroid treatment significantly depressed the abnormal levels of GAP-43 mRNA. In the same direction, the 21-aminosteroid also reduced the high expression of GAP-43 protein [González Deniselle et al, 1999a]. These effects were not exclusive of the antioxidant steroid, since corticosterone treatment led to identical changes. We believe that in order to down-regulate GAP-43 mRNA and protein, corticosterone employed a non-genomic mechanism for two reasons: first, the GAP-43 gene lacks a steroid-responsive element [Chao et al, 1998]. Second, antioxidant activity is not exclusive of 21-aminosteroids, but of glucocorticoids as well [Hall, 1993].

As was the case with NOS, it is not unreasonable to hypothesize that down-regulation of GAP-43 mRNA and protein may be closely related to steroid neuroprotection. A priory, increased accumulation of GAP-43 protein may be caused by retardation of its transport from perikaryon to the axon growth cone [Benowitz et al, 1993]. However, the severe muscle atrophy typical of Wobbler mice suggests, as an alternative mechanism, that muscle denervation could originate the GAP-43 hyperexpression. Degenerating motoneurons would react to denervation and synaptic loss by compensatory but useless strengthening of the remaining synapsis. Hyperexpression of GAP-43 may be of little help, considering that abnormal synaptic contacts including collateral synaptogenesis and hypomyelination are common companions of degeneration in Wobbler mice and ALS patients [Benowitz et al, 1997; Kapfhammer et al, 1994; Parhad et al, 1992]. Actually, in these cases as well as in other neurophatologies (Alzheimer' s disease and schizophrenia) GAP-43 may be a marker for neurodegeneration.

Therefore, down-regulation of GAP-43 mRNA and protein expression with the 21-aminosteroid could indicate a slow down of the exaggerated response to muscle degeneration. This assumption is supported by the strong myogenic activity displayed by 21-aminosteroids [Metzinger et al, 1992] and by data of Oestreicher et al [1997] who showed that the level of motoneuronal GAP-43 is highly dependent on muscle activity. Therefore, enhancement of muscle trophism could

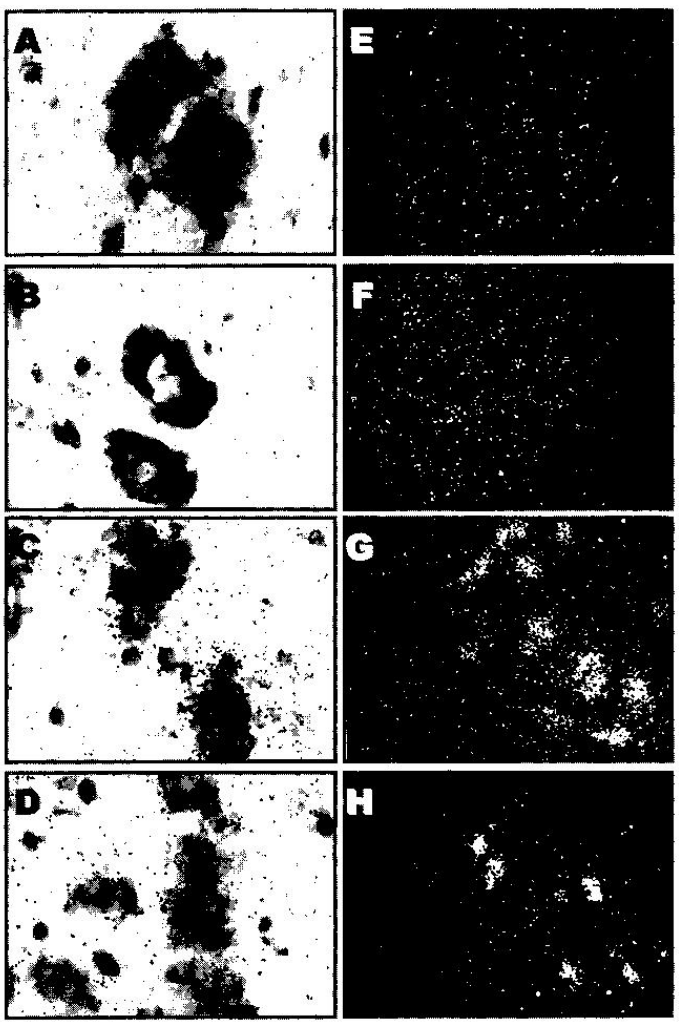


Fig. 6. Photomicrographs representing in situ hybridization for GAP-43 mRNA in the spinal cord ventral horn from control mouse (A, bright field; E, dark field); control mouse treated with the lazaroid U-74389F (B, bright field; F, dark field); Wobbler mouse (C, bright field; G, dark field); Wobbler mouse treated with the lazaroid U-74389F (D, bright field; H, dark field). Modified from González Deniselle [1999b].

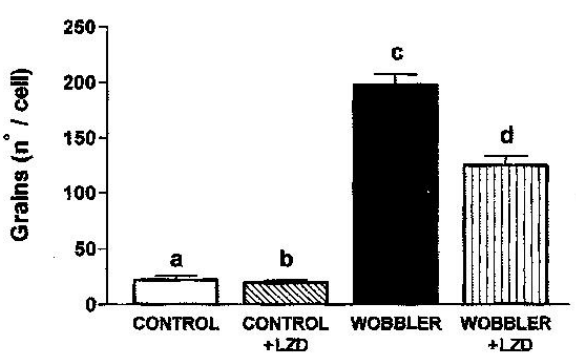


Fig. 7. Results of in situ hybridization for GAP-43 mRNA in ventral horn motoneurons from control mice (open column), controls receiving U-74389F (cross-hatched column), Wobbler mice (dark column) and Wobblers receiving U-74389F (vertical line column). Significant differences using ANOVA and Newman Keuls post hoc test were demonstrated for c vs a ($p<0.001$) and c vs d ($p < 0.001$).

counteract GAP-43 hyperexpression. Eventually, this hypothesis does not preclude direct effects of the 21-aminosteroid on the motoneuron to stop or delayed neurodegeneration based on its antioxidant activity.

Other effective treatments for Wobbler mice

Many literature reports testify the beneficial effects of trophic factors and neurotrophins to delay disease progression in Wobbler mice. In this sense, brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) enhanced muscle trophism, increase the number of myelin fibres and as a whole improved the clinical status of the animals [Mitsumoto et al, 1994], whereas insulin growth factor – 1 (IGF-1) increased muscle fiber diameter, muscle strength and histochemical parameters of muscle trophism [Hantai et al, 1991]. Basic fibroblast growth factor (bFGF) was able to increase muscle strenght and reduce limb spasticity [Ikeda et al, 1991]. Finally, interleukin 6 (IL-6) prevented denervation and motoneuron degeneration [Ikeda et al, 1996].

These effects of trophic factors resemble the beneficial activity of antioxidants, including OPC-14117, lecithinized SOD1, n-acetylcysteine, 7-nitroindazol and T-588 [Abe et al, 1997; Ikeda et al, 1995, 1998; 2000; Henderson et al, 1996]. An intriguing possibility would be that trophic factor neuroprotection enhances natural antioxidant defenses. If true, common intracellular signaling pathways could be modulated by trophic factors and antioxidants, resulting in powerful neuroprotection in this mouse model of ALS.

Conclusions

Together with SOD1 transgenics, Wobbler mice constitute valuable models for the study of familial and sporadic forms of ALS, respectively. In the case of Wobbler mice, changes of spinal cord neurochemistry and muscle electrophysiology in conjunction with clinical data, demonstrated the usefulness of several pharmaceutical compounds to delay neurodegeneration. Among them, antioxidant steroids are molecules changing spinal cord neurochemistry in a positive way, besides being devoid of major secondary effects. Their actual value for human studies needs to be further investigated. In experimental animals, further studies need to be employed at the final and at the presymptomatic stages of neurodegeneration. Furthermore, considering that other types of steroid hormones also bring neuroprotection, trials in this and other animal models of motoneuron disease are worth attempting. In this connection, it should considered that the spinal cord constitutes a target of steroid hormones, expressing in a regional and cell-specific fashion receptors for androgens, progesterone, adrenal steroids and estrogens [De Nicola, 1993]. These receptors, as well as other recognized steroid non-genomic mechanisms, may provide the cellular basis of steroid neuroprotection in future investigations employing animal models of motoneuron disease.

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