

Delayed administration of VEGF rescues spinal motor neurons from death with a short effective time frame in excitotoxic experimental models *in vivo*

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ABSTRACT

VEGF (vascular endothelial growth factor) prevents neuronal death in different models of ALS (amyotrophic lateral sclerosis), but few studies have addressed the efficacy of VEGF to protect motor neurons after the onset of symptoms, a critical point when considering VEGF as a potential therapeutic target for ALS. We studied the capability of VEGF to protect motor neurons after an excitotoxic challenge in two models of spinal neurodegeneration in rats induced by AMPA (α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid) administered either chronically with osmotic minipumps or acutely by microdialysis. VEGF was administered through osmotic minipumps in the chronic model or injected intracerebroventricularly in the acute model, and its effects were assessed by immunohistochemical and histological analyses and motor performance tests. In the chronic model, VEGF stopped the progression of the paralysis and protected motor neurons when administered after AMPA before the onset of the motor symptoms, whereas no protection was observed when administered after the onset. VEGF was also protective in the acute model, but with a short time window, since the protection was effective when administered 1 h but not 2 h after AMPA. Our results indicate that while VEGF has an indubitable neuroprotective effect, its therapeutic potential for halting or delaying the progression of motor neuron loss in ALS would likely have a short effective time frame.

Key words: amyotrophic lateral sclerosis, neurodegeneration, neuroprotection, spinal cord, rat.

INTRODUCTION

ALS (amyotrophic lateral sclerosis) is the most common type of motor neuron disease and is characterized by the selective and progressive loss of motor neurons in the spinal cord, brainstem and motor cortex, with the first symptoms occurring in mid-adult life. Depending on its origin ALS is categorized into two types, FALS (familial ALS) or SALS (sporadic ALS), the latter accounting for ~90% of the cases. ALS is a multi-factorial and non-cell autonomous disease (see Ilieva et al., 2009, for a review). The molecular and cellular processes that cause the loss of motor neurons still need to be clarified, but, regardless of the mechanism of neuronal death, VEGF (vascular endothelial growth factor) has been consistently shown to be an effective protector against motor neuron loss under different experimental paradigms, both *in vitro* (Li et al., 2003; Van Den Bosch et al., 2004; Tolosa et al., 2008) and more importantly, *in vivo*. Several studies have proven that VEGF protects motor neurons from mutant SOD1 (superoxide dismutase 1)-induced neuronal loss (Azzouz et al., 2004; Zheng et al., 2004; Storkebaum et al., 2005; Lunn et al., 2009), and we have demonstrated that VEGF is also effective against motor neuron degeneration induced by excitotoxicity (Tovar-y-Romo et al., 2007) through a mechanism that involves the direct activation of VEGFR2 (VEGF receptor 2) expressed in these neurons (Tovar-y-Romo and Tapia, 2010). Interestingly, the expression of this receptor is diminished in ALS surviving motor neurons (Brockington et al., 2006; Lunn et al., 2009).

In most of the studies with experimental paradigms *in vivo* VEGF has been administered prior to any noticeable motor deficits. Nevertheless, in the majority of SALS cases and given

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Abbreviations: ALS, amyotrophic lateral sclerosis; AMPA, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid; BDNF, brain-derived neurotrophic factor; ChAT, choline acetyltransferase; FALS, familial ALS; GFAP, glial fibrillary acidic protein; i.c.v., intracerebroventricular; IGF-1, insulin-like growth factor 1; PB, phosphate buffer; PGE, paw grip endurance; SALS, sporadic ALS; SOD1, superoxide dismutase 1; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

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the lack of reliable biomarkers (Pradat and Dib, 2009) is unfeasible to predict who will develop the disease at a later stage in life, so that an early preventive treatment starting before the onset would be practically futile. Therefore, it is indispensable to assay the efficacy of VEGF or any other intended therapeutic agent, under experimental conditions in which the motor neuron death process start before the administration of the neuroprotective agent, in order to assess its effectiveness for stopping such a neurodegenerative process.

In this work, we administered VEGF to adult rats after they were infused with the glutamate receptor agonist AMPA (α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid) in the spinal cord, either chronically or acutely, in two experimental models of excitotoxic spinal neurodegeneration that we previously reported to cause hind limb paralysis due to the loss of lumbar motor neurons (Corona and Tapia, 2004; Tovar-y-Romo et al., 2007). While the controversy on the role that excitotoxicity plays in the actual human disease still goes on (Corona et al., 2007; Tovar-y-Romo et al., 2009b; Bogaert et al., 2010), this mechanism provides a means by which we can assay the neuroprotective properties of VEGF *in vivo* and correlate the motor performance with the histopathology of the spinal cord.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (270–290 g) were used in all the experiments. Procedures were performed in accordance with the Rules for Research in Health Matters (Mexico), with the approval of the local Animal Care Committee (Office of Laboratory Animal Welfare ID number A5281-01). Animals were housed in a laboratory environment with a 12 h light/12 h dark cycle and with food and water *ad libitum*. All surgical procedures were performed under anaesthesia, and all efforts were made to minimize suffering during the experimental procedures.

Surgical implantation of the osmotic minipumps

The implantation of osmotic minipumps was carried out as previously described (Tovar-y-Romo and Tapia, 2010), slightly modified to allow the connection of a second osmotic minipump to the same infusion cannula at a later time point. Briefly, rats were anaesthetized with 5% isoflurane in a 95% O₂/5% CO₂ mixture and placed in a stereotaxic spinal unit. Anaesthesia was lowered and maintained at 1–2% isoflurane during surgery. A longitudinal incision of the back skin was made at the lumbar region and muscles surrounding lumbar vertebrae were cut and retracted. On the second lumbar vertebra, the spinous process was removed and an ~1-mm-diameter hole was drilled on the

left side of the lamina in which a stainless-steel screw (1 mm diameter; 3.7 mm long) was inserted to anchor the implant. An ~2 mm² laminectomy was made in the right side of the lamina of the same vertebra and a small cut of the meninges was made to insert into the dorsal parenchyma (~1 mm deep) the tip of a double concentric cannula (CMA/7 Stockholm, Sweden) from which the dialysis membrane was removed by cutting the end of the shaft. Thus, this modified infusion cannula has two inlet tubes, and one of them was sealed at the moment of implantation. Dental cement was poured over the screw and the plastic support of the cannula and the open inlet was connected to an osmotic minipump (Alzet, model 2004, approximate capacity 200 μ l, flow rate 0.25 μ l/h; Durec) containing a solution of 7.5 mM AMPA (Tocris) in 0.1 M PB (phosphate buffer), pH 7.4. This concentration of AMPA has been shown before to be the most convenient for the purpose of the experiment (Tovar-y-Romo et al., 2007). The pump was implanted subcutaneously in the back of the rat, the skin incision was closed with surgical stainless-steel clips and rats received an intramuscular dose of penicillin.

The second minipump was connected at a later time point (see the Results section), but not less than 24 h after the first implant surgery. For this, rats were anaesthetized with isoflurane and placed in a stereotaxic frame as described above. The surgical clips were removed, the previous incision was gently pulled apart to expose the implanted cannula, and the tip of its free sealed end was cut and connected to a minipump containing recombinant rat VEGF₁₆₄ (Sigma) dissolved in 0.1 M PB at a concentration calculated to deliver 24 ng/day (Tovar-y-Romo et al., 2007). The pump was placed subcutaneously and the skin incision was again closed with surgical stainless-steel clips.

Pumps were filled at least 40 h before the implantation and incubated in sterile saline at 37°C for stabilization.

Spinal cord microdialysis

Microdialysis administration of AMPA in the rat spinal cord was performed as previously described (Corona and Tapia, 2004). Briefly, rats were anaesthetized with isoflurane and placed in a stereotaxic spinal unit as described above. Animals were maintained under low anaesthesia (1–2% isoflurane) throughout the experiment. Lumbar vertebrae were exposed as described above and a ~2 mm hole was drilled on the right side of the lamina on L2. Then, after carefully cutting the meninges an intact microdialysis probe (CMA/7) was lowered down into the right dorsal horn of the spinal cord. The probes were continuously perfused with a Krebs–Ringer solution (118 mM NaCl, 4.5 mM KCl, 2.5 mM MgSO₄, 4.0 mM NaH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃ and 10 mM glucose, pH 7.4), at a flux rate of 2 μ l/min. AMPA was administered by perfusing for 25 min a 6 mM solution prepared in Krebs–Ringer medium in which osmolarity was maintained by reducing the NaCl concentration proportionally. This AMPA concentration was chosen on the basis of previous results (Corona and Tapia, 2004). At the end of the

experiment, the microdialysis probe was gently removed and the skin incision was closed with surgical clips.

i.c.v. (intracerebroventricular) administration of growth factors

In the acute model of AMPA by microdialysis, VEGF or recombinant human BDNF (brain-derived neurotrophic factor; Sigma) were microinjected intracerebroventricularly 30 min before or at 1 or 2 h after AMPA perfusion, using the following stereotaxic co-ordinates: AP -0.5 mm from Bregma, L $+1.5$ mm from mid-line, V -4 mm from skull surface. Control rats received a $5 \mu\text{l}$ volume of Krebs–Ringer medium. The experimental groups were injected with 50 ng of recombinant VEGF in a $5 \mu\text{l}$ volume or 30 ng of BDNF in a $3 \mu\text{l}$ volume, with a flux rate of $1 \mu\text{l}/\text{min}$ (concentrations of VEGF and BDNF are closely equimolar).

Assessment of motor function in the chronic model of spinal neurodegeneration

Motor performance was evaluated as described previously (Tovar-y-Romo et al., 2007). Rats were trained for 2 days prior to the surgery on two motor tests: a variation of the PGE (paw grip endurance) task and the rotarod (Columbus Instruments). Animals were evaluated in each test routinely until fixation. For the PGE test, rats were placed individually on a horizontally placed grid (30×19 cm) attached to a mechanical rotator. The grid was gently turned (3 rev./min) until reaching a vertical position. The time taken by the rats for climbing to the top of the grid and reaching a stable position or the latency to fall from the grid when they were unable to climb was scored with a cut-off of 40 s. For the rotarod test, rats walked individually on an accelerating (0.2 rev./min per s) rod, starting from 10 rev./min with a cut-off of 120 s, and the time on the rod was scored. In addition to the motor tests, the overall stride pattern of the hind footprints was qualitatively analysed; for this purpose, the hindpaws of treated rats were inked with non-toxic Chinese ink before animals walked along a paper runway.

Histology and immunohistochemistry

For histological and immunohistochemical analyses, the rats implanted with osmotic minipumps were fixed when they reached the lowest scores on the motor tests or at 20 days after implantation when they did not score low, and the rats subjected to spinal cord microdialysis were fixed 24 h after AMPA perfusion. For fixation, animals were anaesthetized with barbiturate and perfused transcardially with 250 ml of ice-cold 0.9% saline, followed by 250 ml of ice-cold 4% paraformaldehyde in PB, pH 7.4. Spinal cords were removed, postfixed at 4°C and successively transferred to sucrose solutions (up to 30%). Transverse $40 \mu\text{m}$ sections of the lumbar region, where the infusion or microdialysis cannula was implanted, were obtained in a cryostat. Alternate sections

were stained with Cresyl Violet or immunostained for ChAT (choline acetyltransferase) and GFAP (glial fibrillary acidic protein). Free-floating sections were blocked with 5% BSA in PBS–Triton X-100 (0.3%) for 2 h, and then incubated with goat polyclonal anti-ChAT (1:200, Chemicon) and rabbit anti-GFAP antibodies (1:1000; Sigma), for 48 h at 4°C . Sections were washed 3×10 min in PBS–Triton X-100 and incubated with biotin-conjugated mouse anti-goat IgG (1:200, Vector) for 1 h. After three washes, sections were incubated for 2 h with avidin–Texas Red conjugate (1:200, pH 8.2, Vector) and fluorescein-conjugated anti-rabbit antibody (1:250 Zymed) for 2 h. Finally, sections were washed and mounted on silane (γ -methacryloxypropyltrimethoxysilane; Sigma)-covered slides and coverslipped with fluorescent mounting medium (DAKO). Sections were visualized under confocal microscopy (Olympus IX81); merged images are the overlay of two laser sections in the Z-plane, using the Olympus Fluoview 1.6 Viewer. Morphologically undamaged motor neurons in the Nissl preparations (with a soma diameter $>25 \mu\text{m}$ and a distinguishable nucleus) were counted in a $\times 10$ microscopic field. The number of cells was determined in sections where the trace of the cannula (infusion or microdialysis) was evident; five sections per rat were analysed and the values were averaged.

Statistical analysis

Comparisons regarding number of motor neurons were made using ANOVA followed by a Fisher's *post-hoc* test. $P < 0.05$ was considered statistically significant.

RESULTS

Chronic administration of VEGF after AMPA but before the onset of symptoms rescues motor neurons and prevents the progression of paralysis

Chronic delivery of AMPA in the rat lumbar spinal cord, by means of osmotic minipumps, causes a progressive paralysis of the rear limbs (Tovar-y-Romo et al., 2007). The first symptoms of motor impairment are characterized mainly as an ipsilateral limping that becomes evident after 24 h of surgery. The limping progresses to a state of rigid flexion of the ipsilateral limb by days 2–3 after surgery and at this time motor deficits begin to appear in the contralateral limb. The motor dysfunction progresses to a state of complete bilateral paralysis over a period of 20 days. However, by day 10 the paralysis, although not complete, is clearly evident, as rats perform poorly in the rotarod and even though they are still able to climb on the PGE test, the scores are significantly lower than baseline. In previous work, we have shown that

control rats infused only with vehicles (Krebs-Ringer medium or DMSO) or VEGF alone do not develop any motor alteration at any time (Tovar-y-Romo et al., 2007; Tovar-y-Romo and Tapia, 2010).

We administered 24 ng/day of recombinant VEGF via a second osmotic minipump, to rats previously implanted with an AMPA-filled pump, before and after the time they showed a clear ipsilateral limp. This time was variable, occurring between days 2 and 4 after the implantation of AMPA minipump, so that the pre-symptom administration of VEGF through the second minipump started 24–36 h afterwards and the post-symptom administration started at 2–4 days. None of the rats of the post-symptom administration group ($n=8$) was protected and all animals showed irreversible and progressive motor impairments reaching bilateral paralysis, as described above for animals receiving only AMPA. This was reflected by a continuous decrease in rotarod and PGE scores (Figure 1). In contrast, all the animals receiving VEGF before the appearance of the ipsilateral limping ($n=6$) displayed only a transitory decrease in both rotarod and PGE scores that did not progress to a permanent paralytic state. Even more, all the rats recovered a normal motor performance by day 7 after AMPA administration (6 days of VEGF treatment) (Figure 1). In this group, the ipsilateral limp remained but did not progress to the rigid flexion seen in the non-protected rats (see footprints in Figure 1).

As expected, the rats treated with VEGF after the onset of symptoms showed a remarkable loss of motor neurons, which correlated with their paralytic state, as well as an intense

astrocytic inflammatory response detected by GFAP immunolabelling (Figure 2), similar to the changes observed after the infusion of AMPA alone (Tovar-y-Romo et al., 2007). In contrast, the early administration of VEGF before the symptoms clearly prevented neuronal death and astrogliosis. As shown in Figure 2, in these rats most of the motor neurons are preserved in the ipsilateral side of the spinal cord and no neurodegeneration whatsoever occurred in the contralateral side that shows the same baseline number of motor neurons as in rats treated with Krebs-Ringer medium, with DMSO or with VEGF alone (results not shown; see Tovar-y-Romo et al., 2007; Tovar-y-Romo and Tapia, 2010). Notably, the number of neurons protected by VEGF, both in the ipsilateral and contralateral sides, is remarkably similar to the number of healthy motor neurons preserved when VEGF is co-administered together with AMPA (Tovar-y-Romo et al., 2007).

i.c.v. injection of VEGF protects motor neurons against AMPA-induced acute excitotoxicity

In order to test whether a systemic (i.e. non-local) administration of VEGF could also be protective, a single dose of VEGF (50 ng) was injected in the lateral cerebral ventricle in the acute model of spinal neurodegeneration. Microdialysis perfusion of AMPA in the lumbar spinal cord, but no Krebs-Ringer, L-2,4-*trans*-pyrrolidine dicarboxylate, leupeptin or NMDA perfusion, causes a rapid, irreversible and complete paralysis of the ipsilateral limb that develops over the course of 6–12 h, due to an almost complete progressive

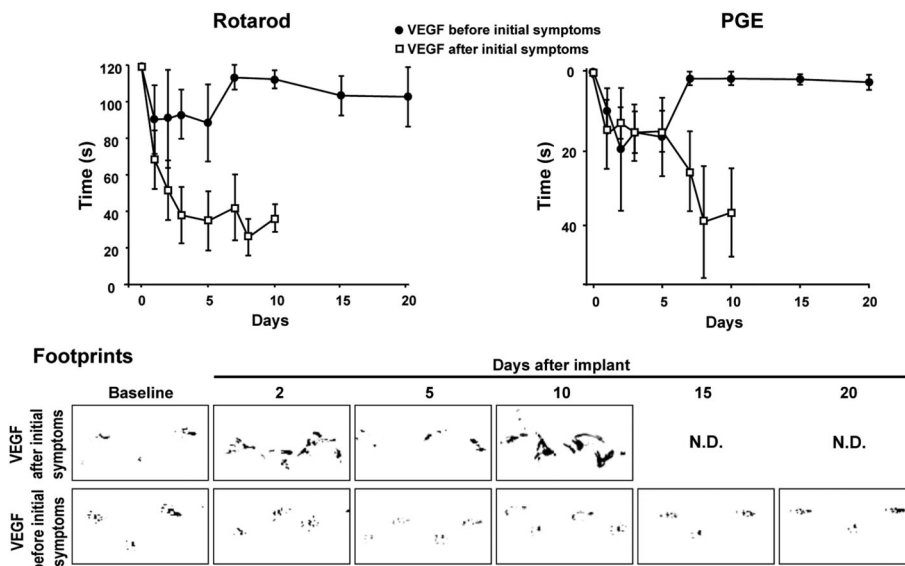


Figure 1 Chronic administration of VEGF after AMPA but before the onset of symptoms prevents the progression of paralysis
 Top panels: time course of rotarod and PGE performances of rats infused with AMPA that received VEGF before (closed circles, $n=6$) or after (open squares, $n=8$) the initial symptoms of motor impairment. Each point in the graphs is the means \pm S.E.M. of the best score out of three trials obtained by each rat at the time points tested. Bottom panel: representative footprints of rats treated with AMPA followed by VEGF before and after the initial motor disturbances. Notice the progression of paralysis in the rat treated after the initial symptoms, observed by a continuous mark left by the dragging of hindlimbs, whereas the rat treated before the symptoms presented only an ipsilateral limp depicted as a shrunken footprint (right footprint on top of the lane). N.D., not determined.

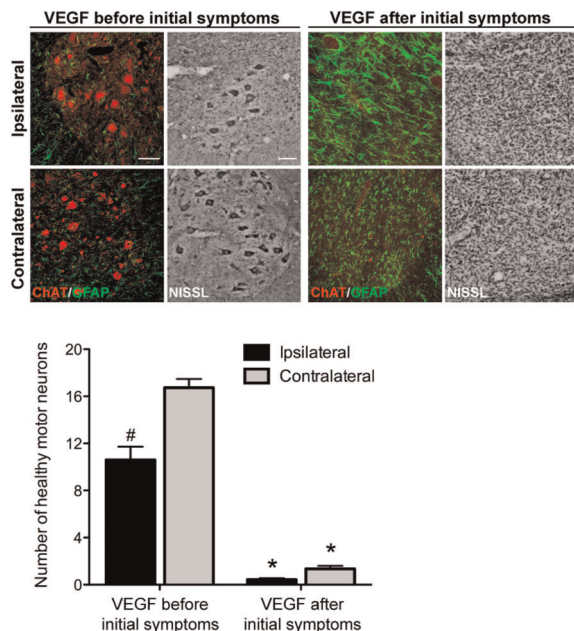


Figure 2 Chronic administration of VEGF after AMPA but before the onset of symptoms rescues motor neurons from death

Representative micrographs of the ventral horns immunostained for ChAT (red) and GFAP (green) in the lumbar section of spinal cord of rats treated with AMPA followed by VEGF before or after the initial symptoms of paralysis. Note the remarkable loss of motor neurons and the intense glial reaction induced by AMPA in both sides of the spinal cord in the rat treated with VEGF after the symptoms onset and the protection exerted by VEGF when infused after AMPA but before the onset. Scale bar=100 μ m. The graph shows the number of healthy motor neurons counted on the Nissl staining. Values are means \pm S.E.M. (five slices per rat, $n=6$ in the group treated with VEGF before and $n=8$ in the group treated after the initial symptoms). * $P<0.0001$ as compared with corresponding side in the opposite group. # $P<0.0001$ as compared with the contralateral side in the same group (ANOVA followed by Fisher's *post-hoc* test).

loss of spinal motor neurons (Corona and Tapia, 2004, 2007, 2008). We found that control rats that received Krebs–Ringer medium i.c.v. 30 min before AMPA infusion displayed the previously observed progression of motor deficits, reaching complete paralysis of the ipsilateral limb at approx. 12 h, and showed a near 90% motor neuron loss in the perfused side of the spinal cord 24 h after surgery ($n=4$) (Figure 3). In contrast, the rats that received VEGF i.c.v. 30 min before AMPA were remarkably protected from paralysis, as no motor impairments occurred during the following 24 h and, accordingly, neuronal death was almost totally prevented ($n=8$) (Figure 3).

To test if this protection was specifically mediated by VEGF, we assayed the administration of a closely equimolar dose of BDNF (brain-derived neurotrophic factor; 30 ng) under the same experimental conditions. BDNF 30 min before AMPA did not exert any protective effect on motor neuron survival. These animals also showed progressive motor deficits reaching complete paralysis of the ipsilateral hindlimb and most motor neurons were lost at 24 h, similar to control rats receiving i.c.v. Krebs–Ringer medium ($n=8$) (Figure 3).

Then, we explored whether a delayed i.c.v. administration of VEGF could still be protective. When VEGF was injected 1 h after the spinal perfusion of AMPA there was a remarkable preservation of the motor neurons in the perfused side of the spinal cord. Although there was a significant decrease of $\sim 25\%$ in the number of motor neurons as compared with the group treated with VEGF 30 min before AMPA ($P=0.022$), the healthy motor neurons remaining at 24 h were sufficient to maintain normal motor function, since no overt motor deficits were observed in any of the rats and the ipsilateral hindlimb paralysis did not occur ($n=8$). However, when VEGF was injected i.c.v. 2 h after AMPA, in spite of a slightly significant protection regarding the number of healthy motor neurons as compared with the control group treated with Krebs medium ($P=0.038$), there was a $\sim 75\%$ motor neuron loss after 24 h ($n=9$, Figure 3) and all the rats developed hindlimb palsy, similar to the i.c.v. Krebs–Ringer control rats.

DISCUSSION

In the present work, we demonstrate that the intraspinal or i.c.v. administration of VEGF after the initiation of a neurodegenerative process generated by a chronic or acute excitotoxic stimulus prevents the degeneration and death of spinal motor neurons, although within a brief time window. This indicates that the potential of VEGF for protection rapidly decreases once the neurodegeneration process has surpassed a threshold of spinal motor neuron damage.

Despite the accumulating studies on the molecular and cellular mechanisms that cause motor neuron death in ALS, the precise processes underlying the disease in humans are still not fully elucidated. There is a considerable amount of evidence indicating that toxic Ca^{2+} -dependent processes such as excitotoxicity, mitochondrial dysfunction, endoplasmic reticulum stress and oxidative stress, as well as other processes not necessarily dependent on Ca^{2+} deregulation, like protein misfolding and aggregation, axonal transport impairment and inflammation, may play a role in the pathogenesis (Grosskreutz et al., 2010). From the studies in the transgenic models of FALS that harbour mutant forms of SOD1 we know that all these alterations may occur in motor neurons and their neighbour cells, and the fact that VEGF delays the onset of symptoms, retards the paralysis progression and therefore extends the survival in these animals, indicates that VEGF can significantly hinder at least some of the toxicity resulting from these events. Nonetheless, the mutant SOD1 transgenic experimental models only reproduce the mechanism of death that happens in less than 3% of ALS cases (Tovar- γ -Romo et al., 2009a).

We previously demonstrated that when administered together with AMPA, VEGF completely prevents the spinal neurodegeneration elicited by AMPA-induced excitotoxicity (Tovar- γ -Romo et al., 2007) and that this protection is

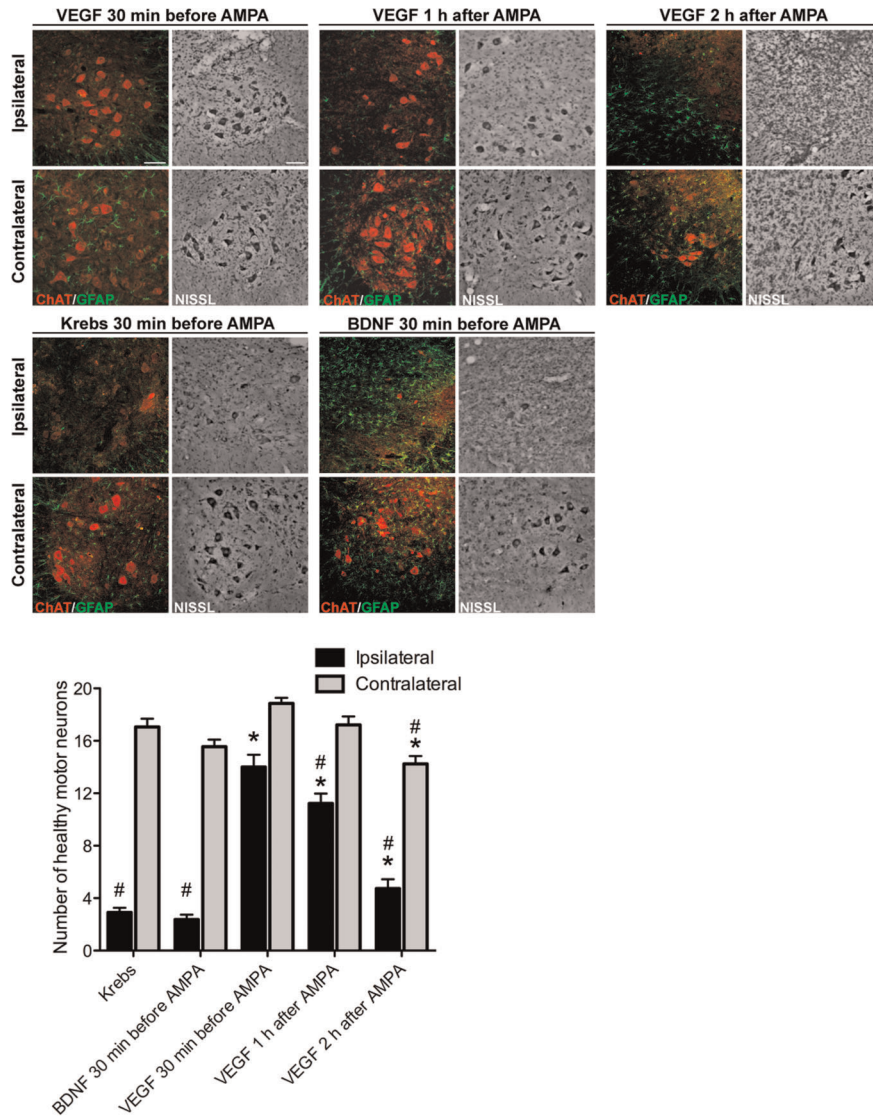


Figure 3 i.c.v. injection of VEGF shortly after AMPA microdialysis perfusion protects motor neurons against death and prevents paralysis Representative micrographs of the ventral horns immunostained for ChAT (red) and GFAP (green) in the lumbar section of spinal cord of rats perfused with AMPA by means of microdialysis and injected i.c.v. with Krebs–Ringer medium 30 min before AMPA ($n=4$), BDNF 30 min before AMPA ($n=8$) and VEGF 30 min before AMPA ($n=8$) or at 1 h ($n=9$) or 2 h ($n=9$) after AMPA. Note the loss of motor neurons in the ipsilateral side of the control groups treated with Krebs or with BDNF and the remarkable protection induced by VEGF injected 30 min before AMPA. At 1 h this protection is still present, although significantly reduced, while at 2 h it is almost completely lost. Scale bar=100 μ m. The graph shows the number of healthy motor neurons counted on the Nissl staining. Values are means \pm S.E.M. (five slices per rat). * $P<0.0001$ as compared with the corresponding side in the group treated with Krebs. # $P<0.0005$ as compared with the corresponding side in the group treated with VEGF 30 min before AMPA (ANOVA followed by Fisher's *post-hoc* test).

achieved by the activation of VEGFR2 and the downstream activation of the survival signalling pathway PI3K (phosphoinositide 3-kinase) and the inhibition of p38 (Tovar-γ-Romo and Tapia, 2010). This indicates that the trophic support of VEGF is protective in spite of the toxic processes to which motor neurons are subjected, even when they are not due to mutations in SOD1 or any other altered gene. In fact, this trophic factor is required for the normal function of motor neurons, as demonstrated by the paralysis that mice with a deleted hypoxia response element in the promoter of the *Vegf*

gene develop due to the loss of spinal motor neurons (Oosthuysen et al., 2001).

The experimental models that were used in this study are based on the toxicity elicited by the over-activation of AMPA receptors, in accordance to one of the main hypotheses on the neuronal death in ALS (Corona and Tapia, 2007). As mentioned before, these models offer the advantage of evaluating the protective effects of VEGF on motor neurons that do not bear genetic alterations, which is the most common scenario in the actual human SALS disease. The

exact molecular and cellular processes that lead to motor neuron death in the genetic rodent models of ALS, specifically those with mutations in SOD1, are not fully understood, and while excitotoxicity has been considered to be involved, there is not a definitive demonstration that this actually occurs (Tovar-y-Romo and Tapia, 2006; Tovar-y-Romo et al., 2009a). Nonetheless, the fact that VEGF rescues motor neurons from death elicited either by genetic mutations or by excitotoxicity may predict the existence of intersecting points in these two types of neuronal injury. Targeting these common pathways may be a useful approach to understand the basic biological alterations that occur in motor neurons.

As we demonstrate here, while VEGF can clearly protect motor neurons from AMPA-mediated excitotoxicity, it has a short-time frame for protection once the noxious process is triggered in the neurons. In the chronic model, the beginning of the neuronal death process does not result in any obvious symptom of paralysis, such as limping, but it can be measured with the aid of motor tests, as shown by the decline in both rotarod and PGE scores of the rats treated with VEGF after AMPA but before the onset of symptoms. This decline, however, is not due to the loss of motor neurons, as shown by the histological analysis of the protected rats. In this sense, it is noteworthy that the transgenic models of FALS, bearing human (Dal Canto and Gurney, 1995; Bruijn et al., 1997) or murine (Morrison et al., 1998) mutant SOD1 do not present a significant loss of motor neurons prior to the onset of symptoms, and the time course of neuronal loss occurs at a very fast rate over a period of 10 days (Morrison et al., 1998). Notably, in human histological studies of spinal cord there is a large variability between the degree of motor neuron loss and muscle weakness (Stephens et al., 2006). This could mean that a very early intervention with growth factors could still be effective. In fact, in the FALS models the administration of VEGF (Azzouz et al., 2004; Storkebaum et al., 2005) or IGF-1 (insulin-like growth factor 1; Kaspar et al., 2003; Dodge et al., 2008) at the onset of symptoms delays the progression and increases lifespan, although the administration of these growth factors well before the onset confers a significant better protection. This difference possibly means that VEGF is helpful at preventing the accumulating toxicity that arises from neurodegenerative processes that begin before motor neuron death or symptom onset (Dal Canto and Gurney, 1995; Bendotti et al., 2001).

In the acute model, we previously found that although the neuronal death occurs fairly quickly, over the course of 6–12 h, the first histological alterations appear as soon as 3 h after microdialysis delivery of AMPA (Corona and Tapia, 2008). In the present work, we found that when the i.c.v. administration of VEGF is delayed 1 h after AMPA, VEGF is still capable of rescuing the majority of motor neurons and therefore prevent the ipsilateral paralysis. However, this protective effect is significantly hampered when VEGF is given 2 h after AMPA. We have consistently found in our experimental settings that there is a threshold that motor neuron death must overpass in order to be reflected as

motor impairments and paralysis. Experimental data from our laboratory show that energetic metabolites or antioxidant agents that rescue at least half the motor neurons in the lumbar spinal cord from AMPA-induced excitotoxic death also prevent the ipsilateral hind limb paralysis that follows AMPA administration, whereas drugs that rescue less than half motor neurons do not prevent motor impairments (Corona and Tapia, 2007, 2008; LD Santa-Cruz and R Tapia, unpublished data).

The i.c.v. administration of VEGF has also been proven efficient in the rat transgenic model of FALS (Storkebaum et al., 2005). Opposite to the local administration in the spinal cord achieved by the continuous infusion of VEGF in the chronic model, the i.c.v. administration allows the diffusion of the growth factor throughout the entire spinal cord, although this administration way must probably create a concentration gradient (Storkebaum et al., 2005). This is of special interest when considering that, in the actual human disease, cellular alterations take place along the entire spinal cord, which might be a target particularly difficult to reach. Therefore, assessing different ways to deliver trophic factors is worth trying. The continuous perfusion of trophic factors in the spinal cord by intrathecal infusions or into the brain by i.c.v. injections overcome the blockade that the blood–brain barrier represents to the delivery of these molecules. Intrathecal injections in fact have been tried in ALS patients for the delivery of IGF-1, with modest results (Nagano et al., 2005).

Our present results also point out at the specificity of VEGF to protect motor neurons, since BDNF failed to do so in this experimental model. This result is in line with previous reports showing that BDNF was not able to protect motor neurons from excitotoxicity (Fryer et al., 2000). Even more, BDNF may also increase the toxicity for neurons through the activation of NADPH oxidase (Kim et al., 2002), an enzyme that has been shown to be involved in motor neuron pathology by damaging the survival pathways activated by trophic factors (Wu et al., 2006). All this could account for the failure of BDNF in human clinical trials (The BDNF Study Group, 1999).

Because of the therapeutic success in animal models, currently there is little doubt about the potential for protection that VEGF has on motor neurons. However, the majority of treatments that have been found to be effective in experimental models do not correlate with a success in clinical trials (Corona et al., 2007). Trophic factors are not the exception: clinical trials assaying the protection conferred by ciliary neurotrophic factor (ALS CNTF Treatment Study Group, 1996) and IGF-1 (Lai et al., 1997; Borasio et al., 1998) failed to improve the condition in ALS patients even when they were found to be protective in the transgenic rodent model (Kaspar et al., 2003; Dodge et al., 2008). Particularly concerning growth factor therapies, special attention should be given to growth factor stability, sustained delivery and exposure, doses, their ability to cross blood–brain barrier, the mid-life of proteins and the unwanted side effects in non-targeted cells (Suzuki and Svendsen, 2008). However, it is also

important to take into account that the moment at which these proteins are administered can result crucial for their therapeutic success. Unfortunately, due to the many parameters that have to be met in order to get a correct diagnosis of ALS, this process is rather slow (Shook and Pioro, 2009; Bedlack, 2010) and the earliest intervention with VEGF once a patient is diagnosed may already be too late. Clinical trials for VEGF are now under way to assess the safety and tolerability of VEGF₁₆₅ (Siciliano et al., 2010), and the working time frame for VEGF therapy is an important factor that needs to be considered regarding any clinical therapeutic success.

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