Epidermal growth factor and growth hormone-releasing peptide-6: Combined therapeutic approach in experimental stroke

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Abstract.

Purpose: Stroke is the second cause of mortality worldwide, with a high incidence of disability in survivors. Promising candidate drugs have failed in stroke trials. Combined therapies are attractive strategies that simultaneously target different points of stroke pathophysiology. The aim of this work is to determine whether the combined effects of Epidermal Growth Factor (EGF) and Growth Hormone-Releasing Peptide-6 (GHRP6) can attenuate clinical signs and pathology in an experimental stroke model.

Methods: Brain global ischemia was generated in Mongolian gerbils by 15 minutes of carotid occlusion. After reperfusion, EGF, GHRP6 or EGF+GHRP6 were intraperitoneally administered. Clinical manifestations were monitored daily. Three days after reperfusion, animals were anesthetized and perfused with an ink solution. The anatomy of the Circle of Willis was characterized. Infarct volume and neuronal density were analyzed.

Results: EGF+GHRP6 co-administration reduced clinical manifestations and infarct volume and preserved neuronal density. No correlation was observed between the grade of anastomosis of the Circle of Willis and clinical manifestations in the animals receiving EGF+GHRP6, as opposed to the vehicle-treated gerbils.

Conclusions: Co-treatment with EGF and GHRP6 affects both the clinical and pathological outcomes in a global brain ischemia model, suggesting a suitable therapeutic approach for the acute management of stroke.

Keywords: Stroke, combined therapy, EGF, GHRP6, global brain ischemia, neuroprotection, brain protection

1. Introduction

Stroke is the world's second leading cause of mortality, with a high incidence of severe morbidity in surviving victims (Woodruff et al., 2011). Many strategies have been implemented in an attempt to provide neuroprotection from cerebral ischemia. However, to date, the efforts to successfully translate the results from pre-clinical studies into an effective therapeutic strategy have been disappointing (Ginsberg, 2008; Traystman, 2010).

After stroke, brain cells die as a result of a range of processes including excitotoxicity, oxidative stress, neuroinflammation and programmed cell death (Lo et al., 2003; Macrez et al., 2011). The interrelatedness

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between various pathways leading to cell death may explain why targeting single points from individual pathways has not been successful (Lo, 2008; Traystman, 2010). A more complete approach that targets different pathways within the neurovascular unit is indicated (Lo, 2008; Ovbiagele et al., 2003; Traystman, 2010).

Epidermal Growth Factor (EGF) and Growth Hormone Releasing-Peptide-6 (GHRP6) have demonstrated to be beneficial in both experimental and clinical paradigms. These effects relate to the recruitment of cell survival mechanisms, providing protection against a broad range of pathologic processes (Berlanga et al., 1998, 2002; Caballero et al., 2001; Cibrian et al., 2006). EGF and GHRP6 target a range of processes within the pathophysiological cascade of ischemic damage. These molecules share anti-apoptotic (Delgado-Rubin et al., 2009; Niidome et al., 2006; Peng et al., 1998) and anti-excitotoxic (Casper & Blum, 1995; Delgado-Rubin de Celix A. et al., 2006) effects, while EGF promotes neurogenesis (Baldauf & Reymann, 2005) and remyelination (Aguirre et al., 2007) and GHRP6 induces endogenous neuroprotective factors (Frago & Chowen, 2005; Guan, 2008) as exclusive effects. Thus, the combined administration of EGF and GHRP6 is likely to have beneficial consequences in stroke.

Here we show that the combined administration of EGF and GHRP6 has neuroprotective effects in global brain ischemia experiments that can be demonstrated at the clinical and pathological levels.

2. Materials and methods

2.1. Animals and experiments

All the experimental protocols were approved by the ethical committee of animal facilities of the Center for Genetic Engineering and Biotechnology (La Habana, Cuba).

Male Mongolian gerbils (*Meriones unguiculatus*), (CENPALAB, Cuba) 25 to 30 weeks old and weighing 70 to 90 g were used in the experiments. Animals were maintained on a 12-hour light/dark cycle with unlimited access to food and water. Prior to surgery the animals were randomly assigned to experimental groups using the software LabTools 1.1 (P. Skládal, Brno, Czech Republic).

2.2. Surgical procedures

The animals were anesthetized with chloral hydrate (400 mg/kg). A ventral midline cervical incision was made; the carotid arteries were exposed and clamped with micro-aneurysm clips. After a 15-minute occlusion, the clips were removed and renewal of blood flow was confirmed (Butterfield et al., 1978). The animals were sutured and allowed to recover. Sham-operated animals ($n = 10$) were treated in the same manner except that the carotid arteries were not occluded. During the surgical procedure and until the animals had completely recovered from anesthesia, rectal temperature was recorded using a digital thermometer (Digi-Sense, Cole-Parmer, Barnant Company, USA). The rectal temperature was kept close to physiological values ($37 \pm 0.5°C$) with a feedback-controlled heating system.

2.3. Experimental design

Four experimental groups were formed according to the treatment after reperfusion: EGF (200 μg/kg) ($n = 20$), GHRP6 (660 μg/kg) ($n = 20$), EGF+GHRP6 (200 μg/kg and 660 μg/kg, respectively) ($n = 20$), and saline (vehicle) ($n = 43$).

The administration of potential neuroprotectants (or vehicle control) took place immediately after blood flow was restored and subsequently 30 minutes, 2 hours and 4 hours later. Treatment was maintained during three days, injecting every 12 hours on days 2 and 3 after reperfusion.

2.4. Neurological grade assessment

To reveal clinical signs of infarction, animals were examined 24, 48 and 72 hours after reperfusion using modified Lawner criteria (Lawner et al., 1979) (Table 1). Examination was conducted by trained observers, blinded with respect to the treatment applied to each animal. The score assigned to each animal was called neurological grade and included the evaluation of palpebral ptosis, grip strength and flexor reflex, body posture, gait pattern (including speed and circling). The general activity was registered, with behavioral patterns ranging from hypoactivity, in which the animal walks slowly or stays at the same point (prostration) once it is placed in the open field arena, to hiperactivity, in which the animal walks uninterruptedly instead of stopping to explore the environment. The occurrence

Table 1
Parameters for neurological grade assessment

Clinical Signs	Score	Criteria
Bristling	1	pilo-erection in the head or spine
	2	Hyperactive
General activity	2	Hypoactive
	6	Extreme prostration
Hypotonia	2	Presented in one foreleg
Decreased grip strength	4	Presented in both forelegs
Hyporreflexia	2	Presented in one rear paw
Decreased flexor reflex to stretching	4	Presented un both rear paws
Body posture	3	Head cocked; body with a "C" posture
	2	Slow
Walking	3	Concentric turns in the same place or circling
	4	Null
	1	Shake ears
	2	Shake ears with no walking
	3	Tonic-clonic movements of the head, neck and ears
Seizures	4	Generalized tonic-clonic movements
	5	Generalized tonic-clonic movements with falls and flips
	6	Generalized tonic-clonic seizures with death

of seizures during manipulation was also registered (Loskota et al., 1974).

The normal condition was assigned a score of 0. When the neurological grade involved continuous seizures or extreme prostration the animals were euthanized in order to avoid unnecessary suffering according to institutional requirements.

2.5. Examination of the Circle of Willis

Following the last day of clinical observation, animals were deeply anesthetized with diethyl ether and transcardially perfused with phosphate-buffered saline. Thereafter, 0.5 mL of 3% bromophenol blue solution in an equal volume of 7.0% gelatin were transcardially injected. In order to evaluate the posterior vasculature, the brains were removed and examined using a stereomicroscope (Zeiss, Stemi 2000, Germany). The patency of the posterior communicating arteries (Fig. 1A–C) was registered, photographed and classified according to the following scale: 0 = no posterior communicating arteries; 1 = thin unilateral posterior communicating artery; 2 = thick unilateral posterior communicating artery; 3 = thin bilateral posterior communicating arteries and 4 = thick bilateral

posterior communicating arteries. The encephala were collected immediately after the examination of the Circle of Willis and cut on a brain matrix to obtain 1-mm serial coronal slides which were incubated into 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) solution during 20 minutes at 37°C. Stained slides were photographed using a stereomicroscope ($10\times$ magnification) in order to calculate the infarct volume. All the brains were subjected to histological processing.

2.6. Histological and morphometric analysis

The coronal sections were immersed in paraffin, cut and stained with hematoxilin-eosin. The qualitative evaluation included a general assessment of the cerebral cortex, hippocampus, thalamus and caudate-putamen nucleus. The quantitative analysis was based on the neuronal density of the structures mentioned above (Fig. 1D). Every lamina was digitalized (40x magnification). The total area of each image was 141 158 μm^2. Viable neurons were counted using tools from the Image J program (Rasband, 2006) (National Institutes of Health, Bethesda, Maryland, USA). Viability was assessed according to the following criteria: sharply delineated nucleus with ellipsoid or round shape; clearly distinguishable nucleolus located centrally within the nucleus; nucleus slightly darker than surrounding neuropil; neuronal cytoplasm clearly demarcated from surrounding neuropil and less than one third of the neuron surrounded by confluent vacuolization (Stummer et al., 1994). Cell counts were performed blindly, and the data from both hemispheres of each animal were averaged for final evaluation. Neuronal density was calculated for every region (viable neurons/mm^2).

Infarct area was measured in each serial coronal slide using tools from the *Image J* program. Infarct volume was calculated by using the following formula: Infarct volume = infarct area * slide thickness.

2.7. Statistical analysis

Survival analyses were made using a log-rank test. Differences in neurological grade, neuronal density, and infarct volume were determined using the Kruskal-Wallis test followed by the Dunn's multiple comparison test. Statistical correlations were studied using the Spearman's correlation coefficient. Differences were considered significant with a two-tailed α-error probability of less than 5%. All the statistical
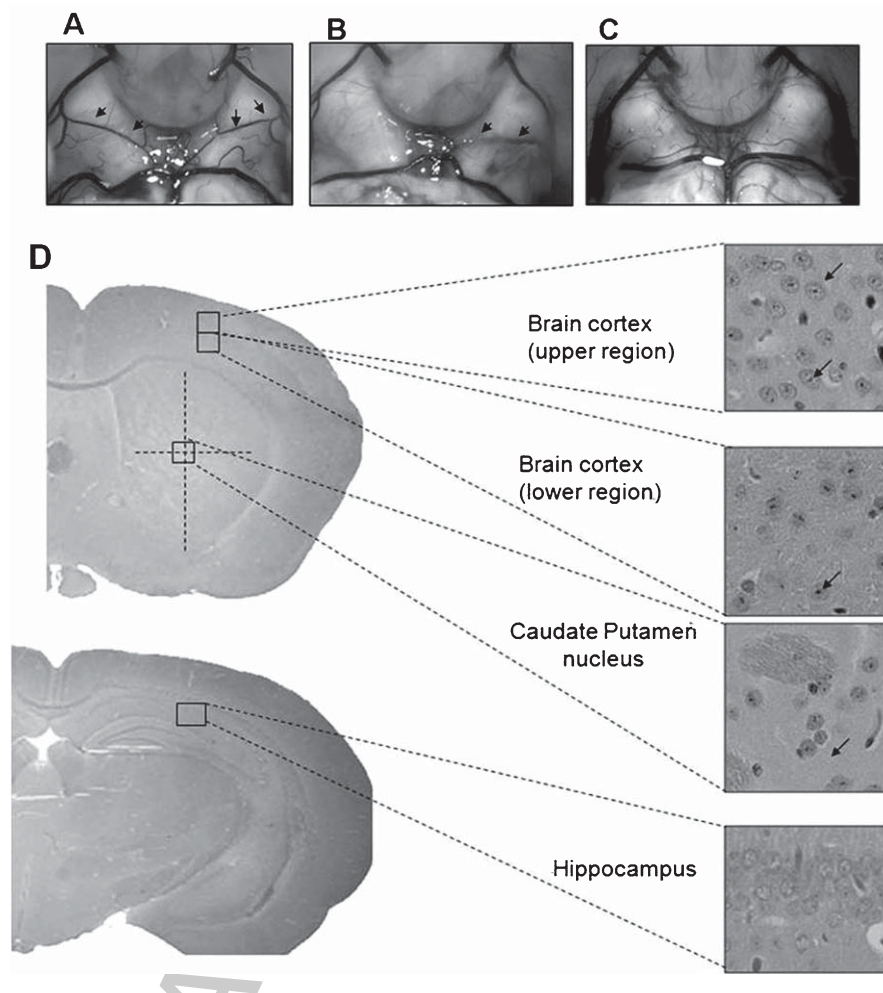
Fig. 1. Variations of posterior cerebral vasculature in surviving Mongolian gerbils. (A) Bilateral posterior communicating arteries; (B) Unilateral posterior communicating artery and (C) No communicating artery. Scale bar: 1 mm. (D) Schematic representation of the procedure to analyze neuronal density in these regions. Photomicrographs of typical coronal sections (7 μm) taken between+0.7 mm_0 mm and −1.3 mm_ −2 mm from Bregma in the gerbil brain. Black arrows indicate viable neurons.

evaluations were made using the Graph PadPrism Software (Graph-Pad Software Inc., San Diego, CA, USA).

3. Results

Forty percent of the animals receiving vehicle or GHRP6 died. On the contrary, there was 100% survival in the groups treated with EGF+GHRP6 or EGF alone (Fig. 2A).

The main neurological manifestations observed in this study included hypotonia, hyperactivity and gait disorders. The neurological grade score in the EGF+GHRP6-treated group was the lowest at

24 and 48 hours after reperfusion (Fig. 2B). The EGF+GHRP6-treated group exhibited a better clinical evolution and survival.

A correlation analysis showed that the degree of arterial anastomosis was negatively correlated with the neurological grade in the vehicle- and EGF-treated groups. The GHRP6-treated group exhibited a non-significant negative correlation. On the contrary, in the EGF+GHRP6-treated group this inverse correlation was prevented (Fig. 2C).

Animals receiving vehicle or treated with EGF or GHRP6 alone had infarcts located in the cortex, caudate-putamen and hippocampus. The animals treated with EGF+GHRP6 had no infarcts in the
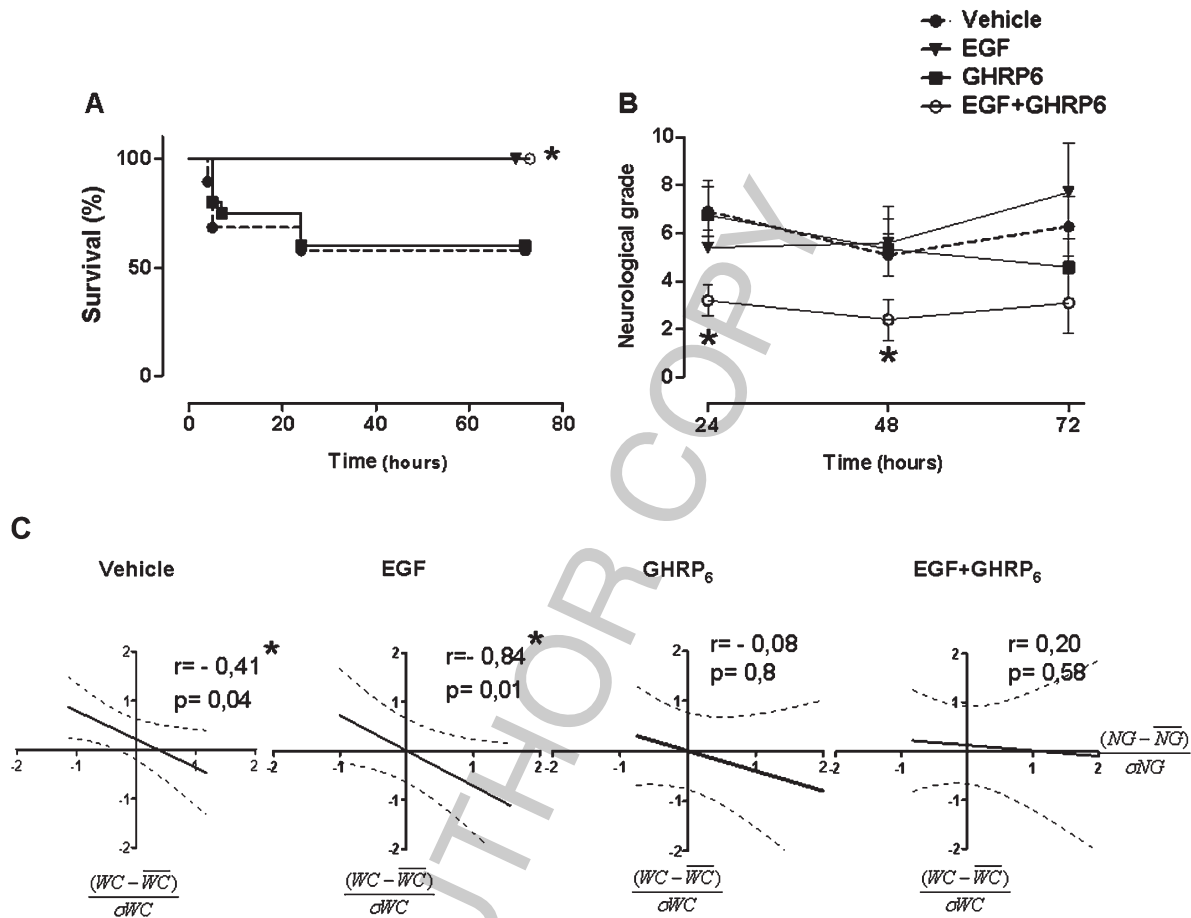
Fig. 2. Neurological grade and pathology of the global brain ischemia experiment. **A:** Survival curves. Log-rank test. **B:** Clinical evolution. Kruskal-Wallis and Dunn tests. **C**: Correlation between neurological grade (NG) and number of communicating arteries in the Willis's Circle (WC). Spearman's correlation test. r = Spearman's correlation coefficient. Asterisks indicate significant differences.

cerebral cortex or in the hippocampus, and had only small infarcts in the caudate-putamen. The infarct volume calculated for the entire brain was significantly lower in the EGF+GHRP6-treated group than in the group that received vehicle (Fig. 3A, B). Accordingly, 96% of the animals in the vehicle-treated group had cerebral infarction, as compared with only 50% of the animals treated with the combination. Neuronal density was also preserved in brain cortex, caudate-putamen and hippocampus in the EGF+GHRP6-treated group (Figs. 4 and 5).

4. Discussion

Despite the fact that rat and mouse models of brain ischemic lesions are more frequently used than the Mongolian gerbil in stroke research, the latter species has a group of advantages. These advantages include the reliability and simplicity of the surgical procedures when 15 minutes of carotid occlusion are set and the resemblance of the grade of anastomosis of the Circle of Willis to that of humans (Manninen et al., 2009; Yang et al., 2003).

Until few years ago it was universally accepted that Mongolian gerbils had an incomplete Circle of Willis. That imperfect anatomical structure facilitated the induction of cerebral injury, by occluding both common carotid arteries. A five minute-long carotid occlusion has commonly been used to restrict histopathological changes to the CA1 region of the dorsal hippocampus (Clark et al., 2003; Plahta et al., 2004). However, the finding by D.T. Ladley (Laidley et al., 2005) and J.B. Seal (Seal et al., 2006) that the grade
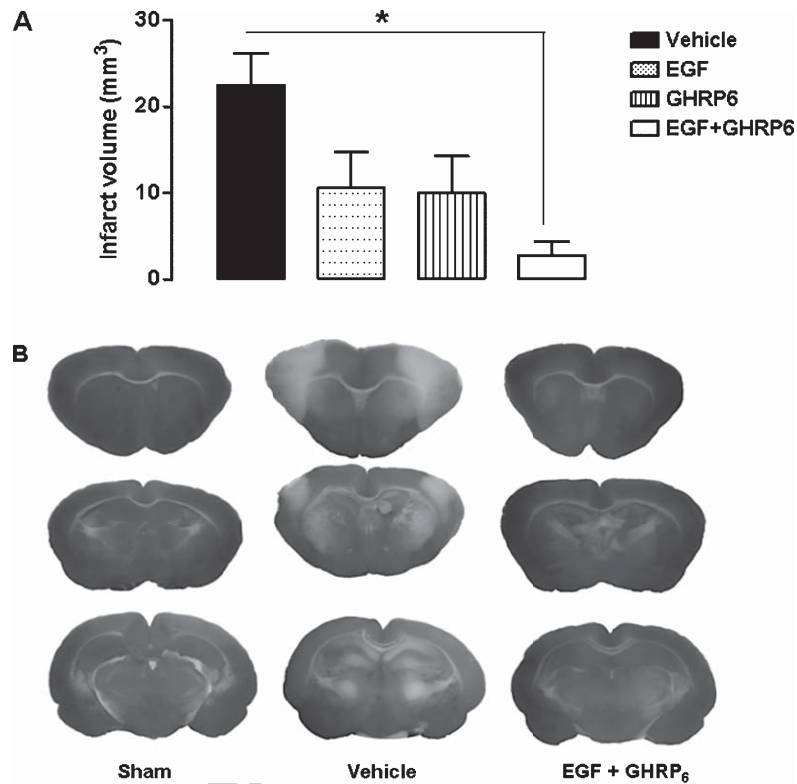
Fig. 3. **A**: Infarct volume comparison between experimental groups. Kruskal-Wallis and Dunn tests. Asterisks indicate significant differences. **B**: Representative photographs of TTC-stained brain slides from false-operated, vehicle-treated and EGF+GHRP$_6$-treated animals.

of anastomosis of the vertebrobasilar and the carotid arterial systems differ among gerbils, explains why the global cerebral ischemia induced in Mongolian gerbils is not considered a reliable model by many authors (Laidley et al., 2005). Compared to other experiments using gerbils where carotid occlusion lasted 5 or 10 minutes, the 15-minute period of ischemia produced more damage at different cerebral regions, predominantly in both hemispheres. Since the reliability of this model strongly correlates with the variability of the posterior communicating arteries in the Circle of Willis (Laidley et al., 2005; Seal et al., 2006), we used the grade of anastomosis of the Circle of Willis as a covariate for a more accurate interpretation of the clinical and pathological results. By doing so, we can guarantee that the time of carotid occlusion determines the degree of tissue injury, even in the presence of thick posterior communicating arteries: 96% of the animals from the vehicle-treated group exhibited ischemic damage. Thus we confirmed the hypothesis of a negative correlation between the neurological grade and the grade of anastomosis of the Circle of Willis. We

also showed that this negative correlation is prevented by the therapeutic intervention with EGF+GHRP6 or GHRP6 (Fig. 2C). This result, together with the clinical and pathological evidences, suggests that the effect of the combination EGF+GHRP6 is potent enough to abolish the contribution of insufficient communicating arteries to the severity of the ischemic insult.

The neuroprotective effect of EGF+GHRP6 was observed in every cerebral region, in terms of neuronal density and infarct volume (Figs. 3 and 4). The high preservation of the neuronal density in the hippocampus of EGF+GHRP6-treated animals (Figs. 4 and 5) is an important result since neuroprotection experiments focused on hippocampus integrity have explored carotid occlusion for only 5 and 10 minutes (Calapai et al., 2000; Plahta et al., 2004). The fact that EGF+GHRP6 protected hippocampal neurons after 15 minutes of ischemia is remarkable and constitutes a robust proof of principle of the neuroprotective effect of the combined therapy with EGF+GHRP6.

The high mortality rate observed in the vehicle-treated group was similar to that previously reported
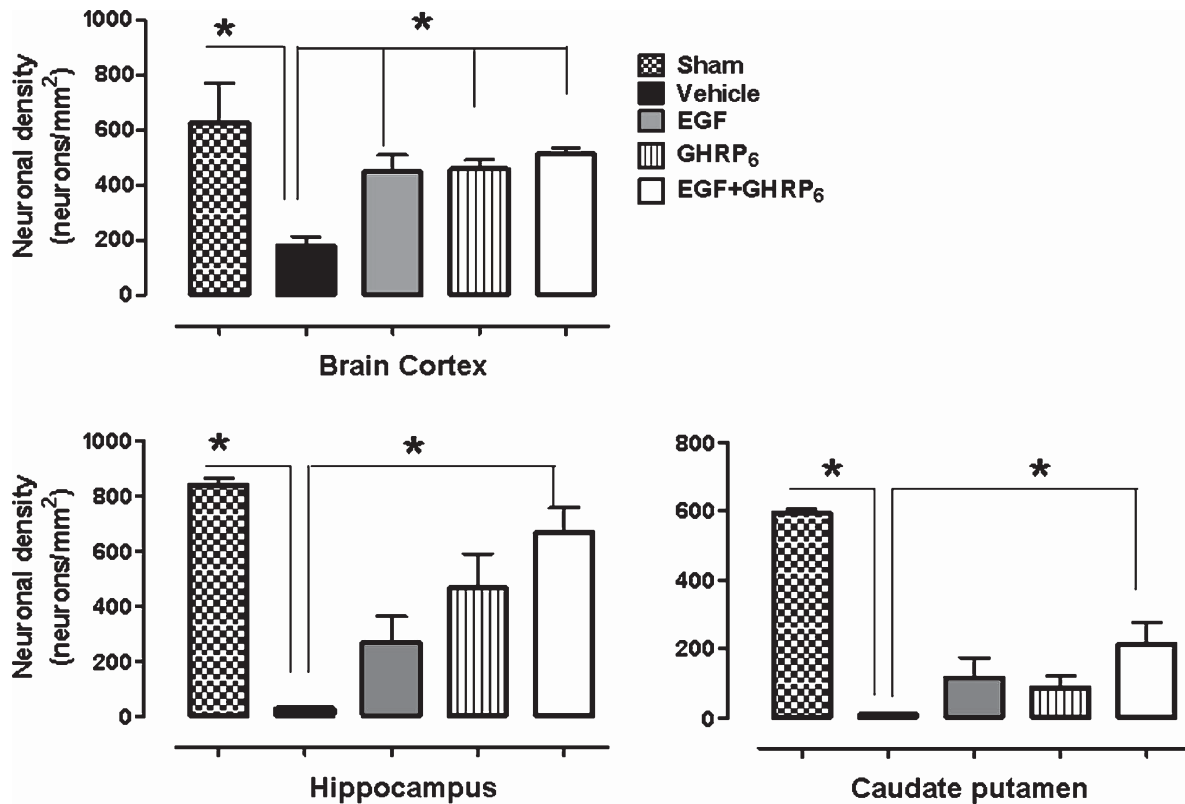
Fig. 4. Neuronal density analysis in brain cortex, caudate, and hippocampus of the global ischemia experiment. Kruskal Wallis and Dunn tests. Asterisks indicate significant differences.

by W. Stummer (Stummer et al., 1994) and is a consequence of the severity of the damage. Thus, the impact of EGF+GHRP6 on the survival rate is relevant and consistent with its effects on neurological grade and pathology.

The direct association between clinical and pathological results demonstrated in this work is in agreement with the recommendations of the stroke therapy academic industry roundtable (STAIR) for the preclinical evaluation of candidate drugs (Fisher et al., 2009; Stroke Therapy Academic Industry Roundtable (STAIR), 1999), and provides a strong proof of principle in support of the neuroprotective role of EGF+GHRP6 co-administration.

4.1. Benefits of combined therapy strategies in neuroprotection

Considering the multiple pathophysiologic mechanisms elicited after an ischemic insult (Shuaib & Hussain, 2008; Yakovlev & Faden, 2004), the use of

a combined therapy is more likely to maximize neuroprotection. Such strategy would allow a concerted blocking of key points of the ischemic cascade.

The plethora of failed clinical trials using neuroprotective drugs for acute ischemic stroke has led to therapeutic nihilism. However, a retrospective view of the design of these failed trials reveals that single mechanisms of cell death were targeted (Lo et al., 2005).

After an ischemic insult, oxygen and glucose deprivation is accompanied by endothelium, astrocyte and oligodendrocyte damage and microglia activation (Lo, 2008). It is well known that neuronal viability depends on the homeostatic relationship between neurons, glial cells, and endothelium (Lo & Rosenberg, 2009). The high preservation rate of neuronal density observed in this work strongly suggests that the other cells from the neurovascular unit are also likely to be protected by the effects of EGF+GHRP6. The therapeutic approach using EGF+GHRP6 is in agreement with the principle that successful protection of the central nervous system after an ischemic event requires that all the components
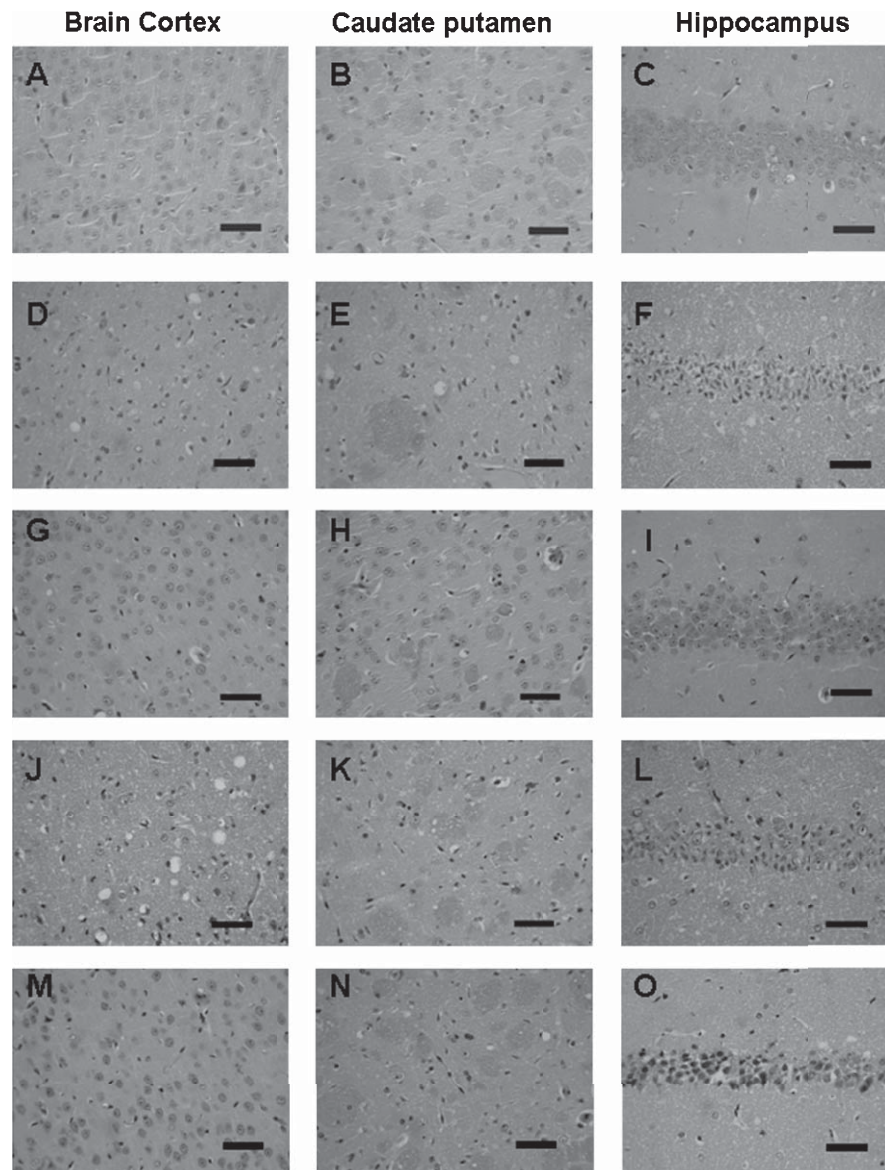
Fig. 5. Representative photograph of brain cortex, caudate putamen and hippocampus from animals that were false-operated (A, B and C), or treated with vehicle (D, E and F), EGF+GHRP$_6$ (G, H, and I), EGF (J, K, and L) or GHRP6 (M, N and O). Barr = 50 μm.

of the neuroaxis be protected (Guo & Lo, 2009; Lo, 2008).

4.2. EGF and GHRP6 interventional targets

Both EGF and GHRP6 have general cytoprotective properties, leading to the marked effects described in our experiments (Brywe et al., 2005; Yu et al., 2009). The more relevant cytoprotective effects of these compounds are directed against oxidative stress-induced damage (Berlanga et al., 2007; Peng et al., 1998), mitochondrial dysfunction (Gibson et al., 2002; Sheng et al., 2007) and glutamate-induced excitotoxicity (Delgado-Rubin de Celix A. et al., 2006; Hicks et al., 1998).

Immediately after ischemic reperfusion there is an increase in the concentration of free radicals and lipoperoxidated products, which are responsible of

both oedema and neuronal damage (Lee et al., 2004). The properties of EGF of protecting against oxidative damage and preventing the accumulation of lipid peroxidation derivatives may help explain the cytoprotective effect of this protein on ischemia-induced neuronal death (Peng et al., 1998). Additionally, GHRP6 has a remarkable effect on both reducing reactive oxygen species, and preserving natural antioxidant systems, specifically the superoxide dismutase enzyme (Berlanga et al., 2007).

A pivotal effect of the action of EGF on mitochondrial integrity is the inhibition of Bax expression and the upregulation of Bcl-2 expression (Ge et al., 2009). GHRP6, in turn, inhibits caspases and interrupts apoptosis (Paneda et al., 2003). Therefore, the co-administration of EGF+GHRP6 is likely to control apoptosis at two different levels.

In the context of brain ischemia, the insulin-like growth factor (IGF-1) induced by GHRP6 (Frago et al., 2005) could share synergistic effects with EGF in controlling apoptosis in a similar fashion to what was reported by K.H. Limedand et al. (Limesand et al., 2003). Furthermore, the biological effects of IGF-1 and EGF have been previously demonstrated on neural precursors in terms of survival and proliferation (Gago et al., 2003). The neuroprotective effects of EGF+GHRP6 after ischemia and reperfusion could be associated with the transduction and activation of survival signals (Gibson, 2004; Zheng et al., 2000). The EGF receptor (EGFR) is one of the most relevant receptors regulating cell survival. The transduction signals activated by EGF involve PI3K/AKT, Ras/MAPK and JAK/STAT, all of which target cell survival mechanisms (Gibson, 2004). GHRP6, in turn, induces IGF-1 (Frago et al., 2002) which then switches on the PI3K pathway (Zheng et al., 2000). Thus, the activation of PI3K, mediated directly by EGF and indirectly by GHRP6, may explain why the therapeutic effect of the combination EGF+GHRP6 is better than the effect of EGF or GHRP6 administered independently. This was previously confirmed in experiments by our group in which diseased animals treated with EGF+GHRP6 had a better clinical outcome associated to an increase of IGF-1 brain transcript (Del Barco et al., 2011).

Ischemic tolerance in response to repeated transient ischemic insult is one of the more relevant examples of endogenous neuroprotection (Davis & Patel, 2003) and the endogenous expression of both EGF and IGF-1 has been demonstrated to be among the molecular events that sustain the effects of ischemic preconditioning (Naylor et al., 2005). Furthermore, the improvement of clinical signs and the reduction of infarct volume mediated by the transplantation of mesenchymal stem cells after brain ischemic insult have been associated to the induction of both IGF-1 and EGF (Wakabayashi et al., 2010).

Other actions of EGF and GHRP6 simulate endogenous mechanisms of neuroprotection such as neurogenesis (Aberg, 2010; Baldauf & Reymann, 2005), activation of the ontogenic neuroprotective pathway (Brodie et al., 1991; Ge et al., 2009) and induction of neuroprotective factors like growth hormone and IGF-1(Frago et al., 2005, 2002). The imitation of brain endogenous protective mechanisms may be the key to future successful approaches to neuroprotection (Ehrenreich & Sirén, 2001).

Although the aim of this work was to assess the effect of the co-administration of EGF+GHRP6 in the acute phase of stroke, additional experiments are in progress in order to evaluate a possible long-lasting effect of this therapy during the recovery phase and the existence of a therapeutic window, wide enough to allow the translatability of this approach to clinic.

In summary, our evidences suggest that this combined therapy is particularly advantageous because its components could be simultaneously directed to multiple targets. Although further work is required, our data suggest that future clinical trials should seek to combine compounds with multiple effects for maximum neuroprotection.

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