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# Broad therapeutic treatment window of [Nle$^4$, D-Phe$^7$]α-melanocyte-stimulating hormone for long-lasting protection against ischemic stroke, in Mongolian gerbils

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**Abstract**

Melanocortin peptides have been shown to produce neuroprotection in experimental ischemic stroke. The aim of the present investigation was to identify the therapeutic treatment window of melanocortins, and to determine whether these neuropeptides chronically protect against damage consequent to brain ischemia. A 10-min period of global cerebral ischemia in gerbils, induced by occluding both common carotid arteries, caused impairment in spatial learning and memory (Morris test: four sessions from 4 to 67 days after the ischemic episode), associated with neuronal death in the hippocampus. Treatment with a nanomolar dose (340 μg/kg i.p., every 12 h for 11 days) of the melanocortin analog [Nle$^4$, D-Phe$^7$]α-melanocyte-stimulating hormone (NDP-α-MSH), starting 3–18 h after the ischemic episode, reduced hippocampal damage with improvement in subsequent functional recovery. The protective effect was long-lasting (67 days, at least) with all schedules of NDP-α-MSH treatment; however, in the latest treated (18 h) gerbils, some spatial memory deficits were detected. Pharmacological blockade of melanocortin $MC_4$ receptors prevented the protective effects of NDP-α-MSH. Our findings indicate that, in conditions of brain ischemia, melanocortins can provide strong and long-lasting protection with a broad therapeutic treatment window, and with involvement of melanocortin $MC_4$ receptors, 18 h being the approximately time-limit for stroke late treatment to be effective.


*Keywords:* Ischemic stroke; Learning and memory; Hippocampal damage; Melanocortins; Therapeutic window; (Mongolian gerbil)

## 1. Introduction

Following a cerebrovascular accident, brain cell damage may occur within minutes to days and through several, perhaps parallel, mechanisms including excitotoxicity, inflammatory response and apoptosis (Choi, 1996; Dirnagl et al., 1999; Leker and Shohami, 2002). The excitatory amino acids glutamate and aspartate are released in uncontrolled manner in ischemic areas, and excitotoxicity directly and/or indirectly generates large amounts of radical species (Choi, 1996; Leker and Shohami, 2002). Neuronal and inducible nitric oxide (NO) synthases are up-regulated, and the overproduced NO reacts with oxygen species to produce highly reactive radicals, including peroxynitrite, deleterious for neuronal survival (Leker and Shohami, 2002). Proinflammatory mediators produced by the neurochemical cascade triggered by ischemia include interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), adhesion molecules and tissue metalloproteinases (Leker and Shohami, 2002). Apoptosis may be responsible for up to 50% of cellular

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deaths in cerebral ischemia. The mechanisms leading to inflammatory response and apoptotic death in ischemic brain injury involve several possible pathways including a mitogen-activated protein kinase (MAPK)-dependent pathway (Beyaert et al., 1996; Herlaar and Brown, 1999; Sugino et al., 2000), a nuclear factor-kB-dependent pathway (Clemens et al., 1997), and the activation of inducible proapoptotic members of the Bcl-2 family (Choi, 1996; Matsushita et al., 1998). Besides an abundant production of proinflammatory cytokines, these pathways lead to the activation of caspases, also involved in inflammation (Schulz et al., 1999). The caspase pathway culminates in the formation of effector caspases, which in turn activate DNA breaking enzymes and energy consuming DNA repair enzymes, leading to breakdown of DNA and cell death (Choi, 1996; Ni et al., 1998; Schulz et al., 1999).

Several innovative neuroprotective approaches have been shown to reduce brain lesions in animal models of stroke (Amemiya et al., 2005; Borsello et al., 2003; Brott and Bogousslavsky, 2000; Endres et al., 2004; Leker and Shohami, 2002; Ottani et al., 2003; Sun et al., 2003; Wise et al., 2005). However, clinical trials failed to confirm animal data so far. The reasons for these disappointing results could be: presence of toxic side effects, short therapeutic treatment window and a single-mechanism neuronal damage blockade (Gladstone et al., 2002; Leker and Shohami, 2002; Wise et al., 2005).

Melanocortins are endogenous peptides of the adrenocorticotropin/melanocyte-stimulating hormone (ACTH/MSH) group. Besides a few reports on the protective effects of α-MSH in conditions of experimental brain ischemia (Huang and Tatro, 2002; Huh et al., 1997) — but not of $\gamma_2$-MSH and the ACTH-(4-9) analog ORG 2766 (Herz et al., 1996, 1998) — it has been recently provided (Giuliani et al., 2006) the first clear evidence that [Nle$^4$, D-Phe$^7$] α-MSH (NDP-α-MSH), which activates melanocortin $MC_1$, $MC_3$, $MC_4$ and $MC_5$ receptor subtypes, causes a strong protection, with a therapeutic treatment window of at least 9 h, against inflammatory, apoptotic, histopathological and behavioral consequences of brain ischemia, through the activation of central nervous system (CNS) melanocortin $MC_4$ receptors.

From a practical point of view, effective protection against ischemic stroke should be definitive, and it requires an as much as possible broad therapeutic treatment window. The aim of the present study, therefore, was to precisely identify the therapeutic treatment window of melanocortins, and to determine whether these neuropeptides chronically protect against damage consequent to transient global brain ischemia.

## 2. Methods

### 2.1. Transient global brain ischemia in gerbils

Male Mongolian gerbils (Charles River Breeding Laboratories, Calco, Como, Italy), weighing 70–80 g, were used. They were kept in air-conditioned colony rooms (temperature 21 ± 1 °C, humidity 60%) on a natural light/dark cycle, with food in pellets and tap water available ad libitum. Housing conditions and experimental procedures were in strict accordance with the European Community regulations on the use and care of animals for scientific purposes (CEE Council 89/609; Italian D.L. 22-1-92 No. 116) and were approved by the Committee on Animal Health and Care of Modena and Reggio Emilia University. The animals were acclimatized to our housing conditions for at least 1 week before use. Transient global brain ischemia was induced, under general anesthesia with chloral hydrate (400 mg/kg i.p.; Sigma, St. Louis, MO, USA), by occluding with atraumatic clips both common carotid arteries for 10 min (Giuliani et al., 2006; Wiard et al., 1995). This experimental model represents human stroke conditions due, for example, to atherosclerotic involvement of the common carotid arteries, respiratory arrest, cardiac arrest (Adams et al., 1993; Fisher, 1982). Rectal and cranial (left temporalis muscle) temperatures were monitored with temperature probes, from the induction of anesthesia and for 11 days, and maintained close to 37 °C by means of heating lamps. This procedure has been adopted to rule out a role of a possible melanocortin-induced hypothermia in neuroprotection (Ren et al., 2004; Sinha et al., 2004; Spulber et al., 2005). Animals were allowed to recover from surgery for 4 days before starting behavioral studies. Sham ischemic gerbils received the same surgical procedure except that the carotid arteries were not occluded.

### 2.2. Drug and treatment schedules

The synthetic melanocortin analog NDP-α-MSH (kindly provided by Prof. Paolo Grieco, Department of Pharmaceutical and Toxicological Chemistry, University of Naples Federico II, Naples, Italy) and HS024 (Neosystem, Strasbourg, France), were dissolved in saline (1 ml/kg) and administered i.p. Control animals (ischemic or sham ischemic) received equal volume of saline by the same route. NDP-α-MSH was administered every 12 h (for 11 days) starting 3, 9, 12 or 18 h after the ischemic episode. Pretreatment with HS024 (cyclic MSH analog, potent and highly selective melanocortin $MC_4$ receptor antagonist) (Kask et al., 1998) or saline, when done, was performed i.p. 20 min before each administration of NDP-α-MSH or saline. The doses of NDP-α-MSH (340 μg/kg) and HS024 (130 μg/kg) were chosen because maximally effective in affording neuroprotection and blockade of melanocortin $MC_4$ receptors, respectively, in the same experimental model of ischemic stroke (Giuliani et al., 2006).

### 2.3. Assessment of spatial learning and memory

We used the modified Morris water-maze test (Giuliani et al., 2006; Morris, 1984; Ottani et al., 2003, 2004; Wiard et al., 1995) in a double-blind manner. This test measures gerbil's ability to learn, remember and go to a place in space defined only by its position relative to distal extramaze cues. The apparatus consisted of a circular white pool (80 cm in diameter and 55 cm in height) filled to a depth of 15 cm with water (27 °C) rendered opaque with milk powder. Gerbils were trained to find the spatial location of a platform of clear perspex hidden by arranging for its top surface (7 cm in diameter) to be 1 cm below the water level; the platform occupied a fixed position at 20 cm from the pool wall. Four points on the apparatus wall (North, South, East,

West) were defined by means of different geometrical figures. Conspicuous cues (wall plates, door, the observer himself) were placed in a fixed position around the pool. On the day before starting training, each gerbil was given 60 s of adaptation to the pool (i.e., the gerbil was placed in the pool — without platform — and allowed to swim freely with no opportunity for escape). During training, a trial began when the gerbil, held facing the side wall, was immersed in the water. Latency to escape onto the hidden platform was recorded. If the gerbil failed to locate the platform within 60 s, it was placed on it. The gerbil remained on the platform for 15 s, then it was removed. Each gerbil received three daily trials with a 5-min intertrial interval starting each time from a different cardinal point in a random succession. Gerbils were subjected to a 5-day training sequence (to assay learning) starting 4 days after the ischemic episode, then, 2 days after the end of learning assay (that is, 11 days after ischemia), to a 1-day training (to assay memory). Sixty days after the ischemic episode, gerbils were subjected to a third session (5-day training sequence); finally, 2 days after the end of such session (that is, 67 days after ischemia), to a fourth session (1-day training). Tests were performed between 10:00 a.m. and 3:00 p.m. in a sound-proof room. The pool was drained and cleaned each day at the end of testing.

### 2.4. Histology

A the end of behavioral studies, in 128 gerbils the brains were removed (under deep general anesthesia) and processed as previously described (Giuliani et al., 2006; Ottani et al., 2003, 2004). Hippocampus morphology was studied on hematoxylin–eosin stained sections (7 μm-thick). Glial fibrillary acidic protein and antiapoptotic activity of cells were analyzed on slides immunocytochemically treated with monoclonal anti-GFAP (Zymed Laboratories, St. Francisco, CA, USA) and monoclonal anti-Bcl-2 (Dako, Glostrup, Denmark), respectively. The slides were incubated overnight at 4 °C with the antibodies, in a moist and darkened chamber. The slides were then incubated with 1:200 streptavidin biotinylated complex (Dako) for 60 min and developed in diaminobenzidine (Fluka, Buchs, Switzerland), and counterstained in Harris hematoxylin. Morphological analyses were performed using an Axiophot photomicroscope (Carl Zeiss, Jena, Germany). Histometrical analyses were performed at the magnification factor on TV screen of ×50 (length) and ×800 (thickness and cell number) using an image system (Vidas-Zeiss). Thickness of the pyramidal cell layer, ischemic extent (percentage of the linear size of the hippocampus CA1–CA4 subfields containing, after hematoxylin–eosin stain, neurons having red cytoplasm and picnotic or shrunk nuclei), viable neurons (neurons having granular cytoplasm and euchromatic nucleus with large nucleoli; hematoxylin–eosin stain), number of astrocytes (cells positive to glial fibrillary acidic protein) and of cells positive to Bcl-2 were evaluated on 3 different slides of serial sections for each hippocampal sample. The density of neurons, astrocytes and cells positive to Bcl-2 was estimated in a 100 μm-thick band overlapping the pyramidal cell layer.

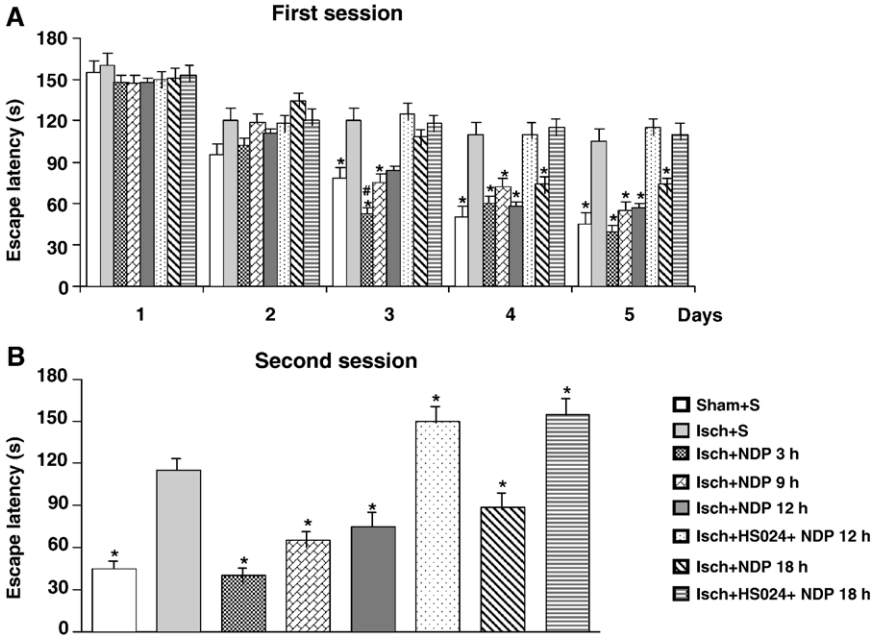


Fig. 1. NDP-α-MSH improves, with a broad therapeutic treatment window, learning and memory in gerbils subjected to transient global brain ischemia. Histograms' height indicates latency to escape onto the hidden platform (Morris water-maze test; mean values ± S.E.M.; $n = 18–20$ gerbils per group). The first session (A) started 4 days after the ischemic episode; the second session (B) took place 2 days after the end of the first session. The effect of NDP-α-MSH (340 μg/kg i.p., twice daily for 11 days following brain ischemia) was prevented by gerbil pretreatment with the melanocortin $MC_4$ receptor antagonist HS024 (130 μg/kg i.p., before each administration of NDP-α-MSH). Pretreatment with saline did not affect the outcomes of NDP-α-MSH or saline treatment in ischemic gerbils (not shown for the sake of clarity). Sham = sham ischemic; Isch = ischemic; S = saline; NDP = NDP-α-MSH; 3, 9, 12 and 18 h = first treatment at 3, 9, 12 and 18 h after injury, respectively. $^{\star}P < 0.05$, at least, versus the corresponding value of ischemic gerbils treated with saline; $^{\#}P < 0.05$ versus the corresponding value of sham ischemic gerbils.

### 2.5. Statistical analysis

All data were analyzed by means of one-way analysis of variance followed by Student–Newman–Keuls' test. A value of $P<0.05$ was considered significant.

## 3. Results

### 3.1. Learning and memory performance

The Mongolian gerbil is an useful laboratory animal for studying the consequences of cerebral ischemia, including the effects on learning and memory, as well as for evaluating neuroprotective drugs in ischemic stroke (Katsuta et al., 2003; Kirino, 1982; Simon et al., 1984; Wiard et al., 1995). We investigated, therefore, the ability of gerbils subjected to a 10-min period of global cerebral ischemia to learn, remember and go to the platform of Morris apparatus (Giuliani et al., 2006; Morris, 1984; Ottani et al., 2003, 2004). In control gerbils (treated with saline), such period of ischemia caused a significant impairment (as compared with sham ischemic) in place finding both during the first training session (assay of learning) and during the second session (assay of memory) (Fig. 1A,B). On the other hand, in gerbils i.p. treated (starting 3, 9, 12 or 18 h after the ischemic episode) with the melanocortin NDP-α-MSH (340 μg/kg every 12 h for 11 days) there was a significant improvement in learning (first session) and memory (second session) performance, if compared with ischemic control animals (Fig. 1A,B). However, when treatment started 12 or 18 h after ischemia, there was a 1-day delay in learning, if compared with earlier started treatments (Fig. 1A,B). Interestingly, when treatment started 3 h after ischemia, NDP-α-MSH-treated gerbils learned more rapidly than sham ischemic ones (Fig. 1A).

In the third session (a 5-day training sequence), started 60 days after ischemia, NDP-α-MSH-treated gerbils maintained the previously showed better ability (compared with ischemic control animals) to find the spatial location of the platform, but, in the latest treated (18 h) ones, some spatial memory deficits were detected (Fig. 2A). However, a significantly better performance was observed also in the fourth session, that is 67 days after ischemia, with all schedules of NDP-α-MSH-treatment (Fig. 2B).

In all four sessions of Morris test, according to our previous data (Giuliani et al., 2006), the protective effect on learning and memory of the most delayed treatments (12 and 18 h) with NDP-α-MSH was completely prevented by a pretreatment with the selective melanocortin $MC_4$ receptor antagonist HS024 (Figs. 1 and 2). Moreover, HS024 worsened memory, as detected in the second session (Fig. 1B).

### 3.2. Hippocampus histological damage

The hippocampus — particularly the CA1 subfield — is an area of the brain that plays a critical role in learning and memory. A brief, transient period of global cerebral ischemia causes selective loss of CA1 pyramidal cells 2–3 days after the ischemic episode (delayed neuronal death), and a more prolonged period extends the damage to CA2–CA4 subfields

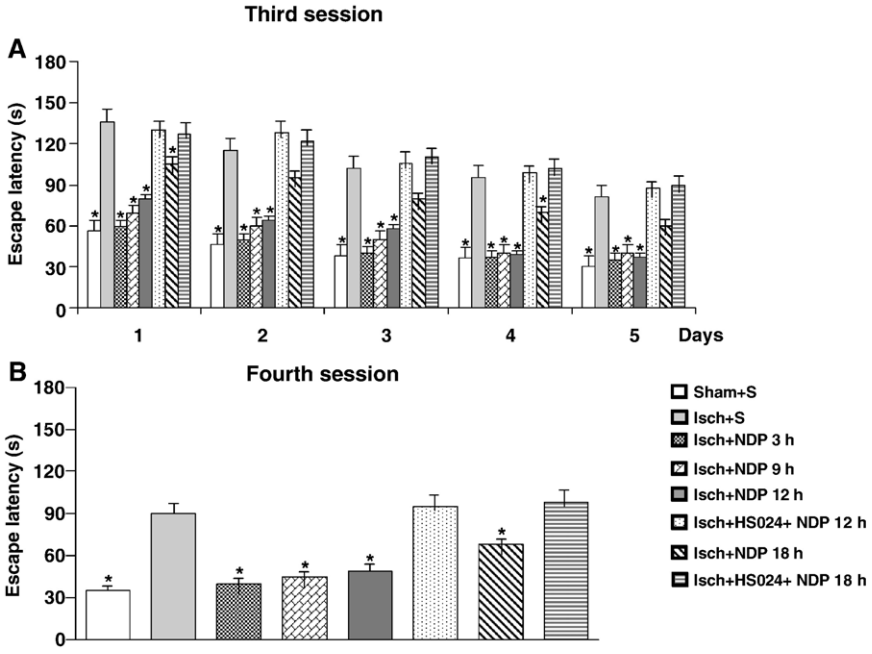


Fig. 2. Long-lasting improvement in learning and memory by NDP-α-MSH in gerbils subjected to transient global brain ischemia. Histograms' height indicates latency to escape onto the hidden platform (Morris water-maze test; mean values ± S.E.M.; $n=10–12$ gerbils per group). The third session (A) started 60 days after the ischemic episode; the fourth session (B) took place 2 days after the end of the third session. The effect of NDP-α-MSH (340 μg/kg i.p., twice daily for 11 days following brain ischemia) was prevented by gerbil pretreatment with the melanocortin $MC_4$ receptor antagonist HS024 (130 μg/kg i.p., before each administration of NDP-α-MSH). Pretreatment with saline did not affect the outcomes of NDP-α-MSH or saline treatment in ischemic gerbils (not shown for the sake of clarity). Sham = sham ischemic; Isch = ischemic; S = saline; NDP = NDP-α-MSH; 3, 9, 12 and 18 h = first treatment at 3, 9, 12 and 18 h after injury, respectively. $^{\star}P<0.05$, at least, versus the corresponding value of ischemic gerbils treated with saline.

(Katsuta et al., 2003; Kirino, 1982; Simon et al., 1984; Wiard et al., 1995). At the end of both the second and fourth sessions of behavioral study, therefore, we processed the hippocampus for histology and histometry. After the second session, in saline-treated gerbils subjected to transient brain ischemia we observed loss of hippocampal neurons mostly inside CA1 (followed by CA2) subfield, partially replaced by glial cell hyperplasia (astrocytes) as indicated by glial fibrillary acidic protein positivity (Fig. 3B,D,F). Moreover, we found a great number of dead neurons showing pyknosis, nuclear dust, swollen perikaryon, cellular shrinkage and absence of Nissl substance; accordingly, we detected a scanty expression of antiapoptotic activity (anti-Bcl-2 reaction, Fig. 3E). The hippocampus of gerbils treated with NDP-α-MSH (340 μg/kg, i.p., every 12 h for 11 days, starting 3, 9, 12 or 18 h after the ischemic episode) resulted to have an ischemic extent quite similar to that of saline-treated ones, but with a significantly larger thickness of the pyramidal cell layer in the CA1 (and CA2, not shown) subfield (Fig. 3A,C). In these subfields we also recorded a significantly higher number of viable neurons, if compared with the corresponding areas of saline-treated gerbils (Fig. 3D,F). Astrocyte immunoreaction was quite similar to that observed in saline-treated gerbils (Fig. 3B), whereas the antiapoptotic activity was more expressed (Fig. 3E).

After the fourth session of behavioral studies (67 days after ischemia), in saline-treated ischemic rats we found a histological picture of the hippocampus characterized again by neuronal degeneration, cellular debris and reactive astrocytes, but it resulted improved, if compared with that found after the second session in the corresponding control animals: in fact, a larger thickness of the pyramidal cell layer, and a higher number of viable neurons were detected [likely due to hypoxia-induced neurogenesis (Arvidsson et al., 2002; Sharp et al., 2002)] (Fig. 4A,B). Albeit a similar thickness of the pyramidal cell layer was found in all groups (Fig. 4A), a significantly lesser degree of morphological damage was again detected in NDP-α-

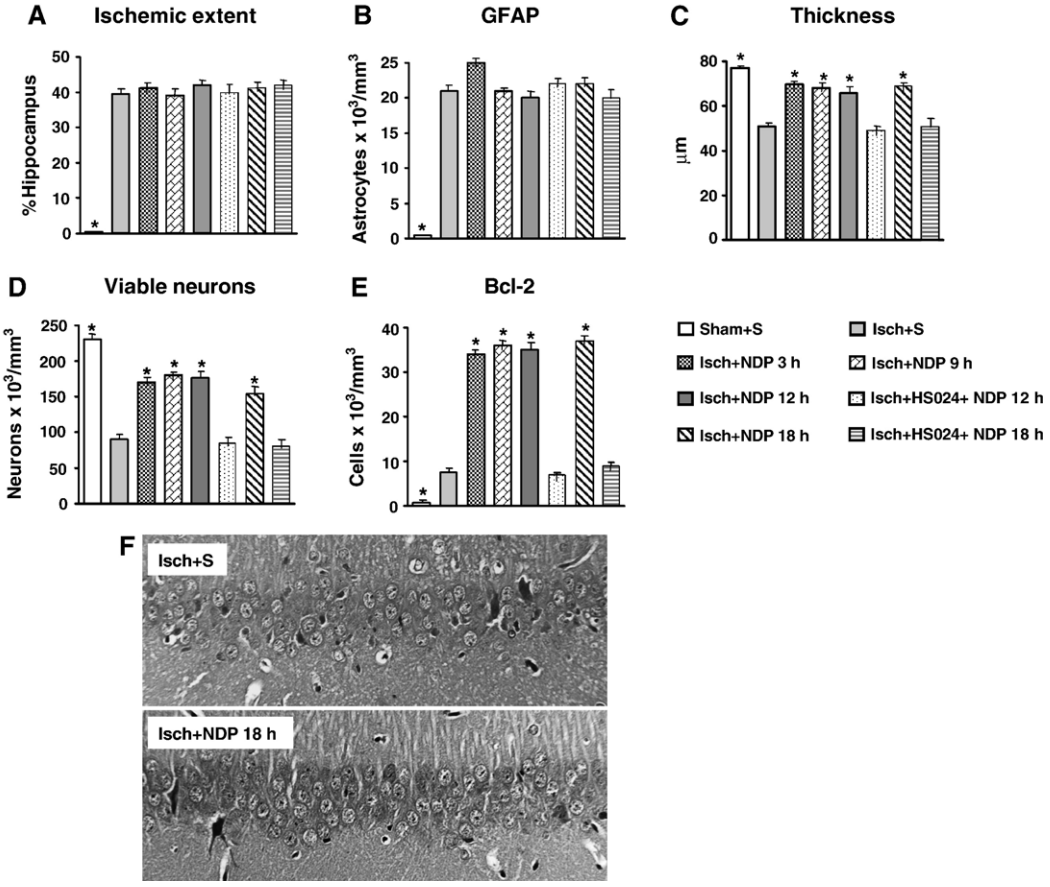


Fig. 3. NDP-α-MSH protects, with a broad therapeutic treatment window, against histological damage and neuronal death in the hippocampus CA1 subfield of gerbils subjected to transient global brain ischemia. Histograms' height indicates mean values ± S.E.M. ($n = 8$ gerbils per group) obtained after the second session of Morris test (11 days after ischemia). The amount of ischemic areas (A) and glial cell hyperplasia (B: number of astrocytes in the CA1 subfield) of the pyramidal layer resulted quite similar in all groups. In the CA1 subfield of NDP-α-MSH-treated gerbils (340 μg/kg i.p., twice daily for 11 days following brain ischemia) the thickness of the pyramidal cell layer was larger (C), and the number of viable neurons (D) and cells reactive to anti-Bcl-2 (E) was greater. (F) Note the relative greater number of viable neurons in the NDP-α-MSH-treated gerbil; hematoxylin-eosin stain. The protective effects of NDP-α-MSH were prevented by gerbil preatreatment with the melanocortin $MC_4$ receptor antagonist HS024 (130 μg/kg i.p., before each administration of NDP-α-MSH). Pretreatment with saline did not affect the outcomes of NDP-α-MSH or saline treatment in ischemic gerbils (not shown for the sake of clarity).Sham = sham ischemic; Isch = ischemic; S = saline; NDP = NDP-α-MSH; 3, 9, 12 and 18 h = first treatment at 3, 9, 12 and 18 h after injury, respectively; GFAP = astrocyte immunoreaction to anti-glial fibrillary acidic protein; $^{\star}P < 0.001$ versus the corresponding value of ischemic gerbils treated with saline. Field width: $F = 408$ μm.

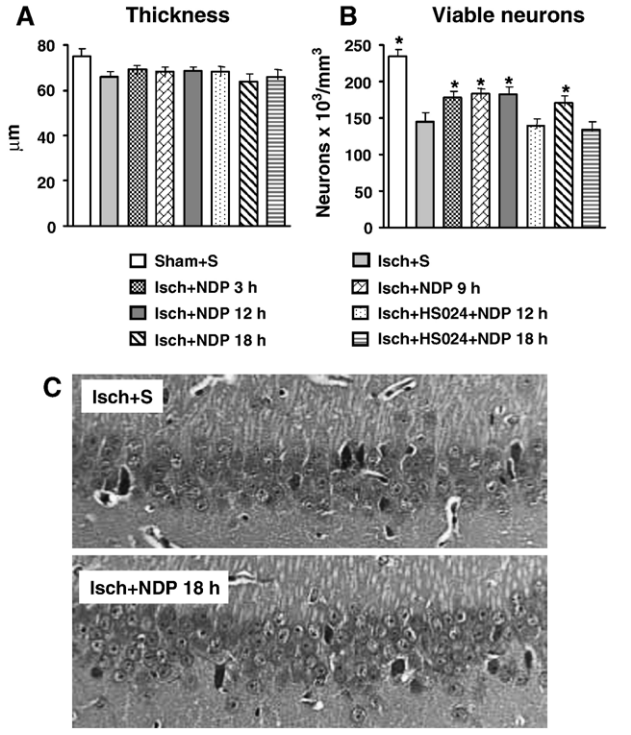


Fig. 4. NDP-α-MSH chronically protects against histological damage and neuronal death in the hippocampus CA1 subfield of gerbils subjected to transient global brain ischemia. Histograms' height indicates mean values±S.E.M. ($n$=8 gerbils per group) obtained after the fourth session of Morris test (67 days after ischemia). The thickness of the pyramidal cell layer in the CA1 subfield (A) was similar in all groups. In the CA1 subfield of NDP-α-MSH-treated gerbils (340 μg/kg i.p., twice daily for 11 days following brain ischemia) the number of viable neurons (B) was greater than that of saline-treated ones. (C) Note the relative greater number of viable neurons in the NDP-α-MSH-treated gerbil; hematoxylin–eosin stain. The protective effects of NDP-α-MSH were prevented by gerbil pretreatment with the melanocortin $MC_4$ receptor antagonist HS024 (130 μg/kg i.p., before each administration of NDP-α-MSH). Pretreatment with saline did not affect the outcomes of NDP-α-MSH or saline treatment in ischemic gerbils (not shown for the sake of clarity).Sham=sham ischemic; Isch=ischemic; S=saline; NDP=NDP-α-MSH; 3, 9, 12 and 18 h=first treatment at 3, 9, 12 and 18 h after injury, respectively; $^{\star}P<0.05$ versus the corresponding value of ischemic gerbils treated with saline. Field width: $C$=408 μm.

MSH-treated ones, with a number of viable neurons significantly higher than that of saline-treated ischemic gerbils, also in the latest (18 h) treated animals (Fig. 4B,C).

After the second session of behavioral studies, the effect of the most delayed treatments (12 and 18 h) with NDP-α-MSH on thickness of the pyramidal cell layer, number of viable neurons, and Bcl-2 immunoreaction, resulted which was prevented by pretreatment of gerbils with the selective melanocortin $MC_4$ receptor antagonist HS024 (Fig. 3). Sixty-seven days after ischemia, the favourable effect of NDP-α-MSH on the number of viable neurons resulted which was counteracted by melanocortin $MC_4$ receptor blockade (Fig. 4).

## 4. Discussion

The only approved therapy for ischemic stroke is early (within 3 h) thrombolysis with alteplase (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). Because of several concomitant factors, including a narrow treatment window, no innovative drugs have been proven successful in advanced clinical trials (Gladstone et al., 2002). A recent meta-analysis suggests the time window of alteplase might be extended up to 4–5 h after stroke onset (Hacke et al., 2004), and a phase II study of a new intravenous thrombolytic drug, desmoteplase, suggests their time window might be extended up to 9 h, but only in selected patients (Hacke et al., 2005). Recently, a significant degree of neuroprotection has been reported with new compounds administered up to 6–10 h after experimental brain ischemia, and such late treatment also reduced the behavioral consequences of the ischemic episode for at least 14 days (Borsello et al., 2003; Williams et al., 2004).

Several melanocortins such as ACTH-(1-24), α-MSH, shorter fragments and synthetic analogs, like NDP-α-MSH, have a life-saving effect in animal and human conditions of circulatory shock (Bertolini et al., 1986a,b,c; Guarini et al., 2004; Ludbrook and Ventura, 1995; Noera et al., 2001; Squadrito et al., 1999), as well as in other severe hypoxic conditions, including prolonged respiratory arrest (Guarini et al., 1997) and myocardial ischemia (Bazzani et al., 2001, 2002; Guarini et al., 2002; Vecsernyes et al., 2003). Recently (Giuliani et al., 2006), we have demonstrated that the melanocortin peptide NDP-α-MSH significantly protects also against impairment in learning and memory caused by transient global brain ischemia in gerbils. This neuroprotective effect occurs also when treatment starts up to 9 h after ischemia and is associated with a modulation of the inflammatory response and of the apoptotic process in the hippocampus, with consequent reduction of the morphological damage and increase of viable neurons (Giuliani et al., 2006).

Here we show that NDP-α-MSH protects against impairment in learning and memory, following transient global brain ischemia in gerbils, also when treatment starts 18 h after the ischemic episode. Since gerbils perform better than ischemic control animals 67 days after a stroke, this protection seems to be long-lasting and appears definitive. Moreover, this protective effect is associated with a reduction of the morphological damage in the hippocampus, including a reduction of neuronal death, a larger thickness of the pyramidal cell layer and an overexpression of Bcl-2 immunoreactivity. However, with the latest treatment (18 h) the behavior improvement is unstable, this indicates that 18 h are the approximate time-limit for stroke late treatment with melanocortins to be effective.

The broad therapeutic window of melanocortins could be the consequence of their influence on the following mechanisms. (i) The inflammatory reaction to brain ischemia is a complex and multi-step delayed process which includes marked increase in macrophage infiltration into the ischemic area from 24 to 72 h after injury. Attenuation of this process, therefore, could produce an extension of the therapeutic time window (Williams et al., 2004). Accordingly, melanocortins have a peculiar anti-inflammatory activity (Catania et al., 2004; Getting, 2002; Guarini et al., 2004; Huang and Tatro, 2002; Wikberg et al., 2000); indeed, they modulate the inflammatory response, by decreasing TNF-α and IL-6 levels, also in our gerbil model of transient global brain ischemia (Giuliani et al., 2006). (ii)

Neuronal death in brain ischemia is largely due to excitotoxic mechanisms which activate the MAPK members C-jun N-terminal kinases (JNKs). JNKs (expecially JNK3) seem to be involved in mediating neuronal death (Borsello et al., 2003; Kuan et al., 2003); in fact, recent data indicate that, despite early activation of JNKs, continuous JNKs activation for several hours is required for efficient neuronal death (Borsello et al., 2003). Accordingly, in our experimental model of a brain ischemia, NDP-α-MSH blunts JNKs activation including JNK3 (Giuliani et al., 2006). (iii) In brain ischemia, the therapeutic treatment window for neuroprotection can be prolonged through a delay of caspase activation (Fink et al., 1998). Accordingly, as previously reported (Giuliani et al., 2006), NDP-α-MSH inhibits activation of the downstream executioner caspase-3 in the same gerbil model of brain ischemia.

Obviously, the long-lasting protection by melanocortins could be the result of definitive, direct blockade of these pathological, and likely parallel, mechanisms. However, nor can we rule out indirect effects mediated by melanocortins, such as diminished death signals from non-neuronal cells, e.g., astrocytes (Newman, 2003). Besides neuroprotective actions, targeting ischemic tissue and aimed at limiting brain damage, melanocortins might promote functional recovery after a stroke through the stimulation of repair mechanisms, including neurogenesis. Accordingly, it has been reported that melanocortins beneficially affect neural growth during development, improve functional recovery in rats subjected to diencephalic hemisection and promote nerve regeneration in several experimental models of nerve injury and neuropathies (Benelli et al., 1988; de Wied, 1999; Starowicz and Przewlocka, 2003).

Melanocortin $MC_3$ and $MC_4$ receptors are the predominant subtypes expressed in the CNS (Catania et al., 2004; Getting, 2002; Wikberg et al., 2000). The mechanisms by which systemically administered NDP-α-MSH protects against damage following transient global brain ischemia seem to involve direct activation of brain melanocortin $MC_4$ receptors. In fact, pretreatment with the selective melanocortin $MC_4$ receptor antagonist HS024 prevents the protective effect of NDP-α-MSH, both when treatment starts early (Giuliani et al., 2006) and when starts late (present data). Interestingly, NDP-α-MSH-treated gerbils learn more rapidly than sham ischemic ones (present data; Giuliani et al., 2006); consistently, after the blockade of melanocortin $MC_4$ receptors there is a more serious post-stroke evolution of the behavioral injury (memory), and an increase in DNA fragmentation in the hippocampus, compared with ischemic control animals (present data; Giuliani et al., 2006). Again, melanocortins increase production of the antiinflammatory cytokine IL-10 in modulating the inflammatory cascade (for reviews see: Catania et al., 2004; Wikberg et al., 2000); on the other hand, low plasma concentrations of IL-10 are associated with early worsening of neurological symptoms in patients with acute ischemic stroke (Vila et al., 2003). Finally, α-MSH plasma levels are decreased in patients with acute traumatic brain injury, and patients with the lowest circulating levels have an unfavourable outcome (Catania et al., 2004). Taken together, these data suggest that, in conditions of brain ischemia, melanocortins might be physiologically involved in neuroprotection.

In conclusion, in our opinion, further investigations by using other animal species and other animal models of brain ischemia, such as focal ischemia, should be encouraged: the confirmation of the full efficacy of these neuropeptides could take into account the possibility of such therapeutic intervention in humans. In fact, in view of the broad time window for successful drug treatment and the long-lasting protection (present data), the activity against several ischemia-related mechanisms of damage (Giuliani et al., 2006) and the lack of appreciable toxicity (Catania et al., 2004), melanocortin peptides could provide the potential to develop a new and more physiological approach to treatment of human ischemic stroke. This is also the "state of the art" drawn by Tatro (2006) in the Endocrinology journal editorial that has been dedicated to our previous paper (Giuliani et al., 2006).

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