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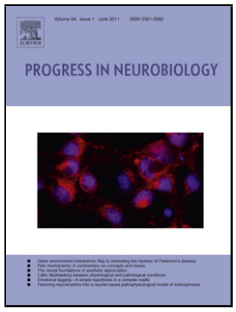

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Multiple beneficial effects of melanocortin MC_4 receptor agonists in experimental neurodegenerative disorders: therapeutic perspectives

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Highlights

- Melanocortins induce neuroprotection and neurogenesis in neurodegenerative disorders
- Melanocortins improve synaptic activity and neurological performance
- These effects are mediated by melanocortin MC4 receptors

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ABSTRACT

Melanocortin peptides induce neuroprotection in acute and chronic experimental neurodegenerative conditions. Melanocortins likewise counteract systemic responses to brain injuries. Furthermore, they promote neurogenesis by activating critical signaling pathways. Melanocortin-induced long-lasting improvement in synaptic activity and neurological performance, including learning and memory, sensory-motor orientation and coordinated limb use, has been consistently observed in experimental models of acute and chronic neurodegeneration. Evidence indicates that the neuroprotective and neurogenic effects of melanocortins, as well as the protection against systemic responses to a brain injury, are mediated by brain melanocortin 4 (MC_4) receptors, through an involvement of the vagus nerve. Here we discuss the targets and mechanisms underlying the multiple beneficial effects recently observed in animal models of neurodegeneration. We comment on the potential clinical usefulness of melanocortin MC_4 receptor agonists as neuroprotective and neuroregenerative agents in ischemic stroke, subarachnoid hemorrhage, traumatic brain injury, spinal cord injury, and Alzheimer's disease.

*Abbreviations***:** Aβ, β-amyloid; ACTH, adrenocorticotropic hormone; AD, Alzheimer's disease; BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2'-deoxyuridine;

CNS, central nervous system; DG, dentate gyrus; ERK, extracellular signal-regulated kinases; HMGB-1, High Mobility Group Box-1; JNK, c-jun N-terminal kinases; MSH, melanocyte-stimulating hormone; NDP-α-MSH, [Nle^4,D-Phe^7]α-melanocyte-stimulating hormone; SAH, subarachnoid hemorrhage; SCI, spinal cord injury; SGZ, subgranular zone; Shh, Sonic hedgehog; IL, interleukin; SVZ, subventricular zone; TBI, traumatic brain injury; TNF-α, tumor necrosis factor-α.

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Keywords: Neurodegenerative diseases, Pathophysiological mechanisms, Melanocortin receptor agonists, Neuroprotection, Neurogenesis, Functional recovery

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1. Introduction

Acute neurodegenerative conditions including stroke (ischemic or hemorrhagic), traumatic brain injury (TBI), spinal cord injury (SCI), and chronic neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease, amyotrophic lateral sclerosis, and more, are responsible for high mortality and disability. Unfortunately, acute and chronic neurodegenerative disorders have no effective treatment options, as current therapies only relieve symptoms (with varying effectiveness, depending on the condition of individual patients), without counteracting the degenerative process progression (Adams et al., 2007; Antel et al., 2012; Banerjee et al.; 2010; Bragge et al., 2012; Galimberti et al., 2013; Iqbal and Grundke-Iqbal, 2011; Laskowitz and Kolls, 2010; Lazarov et al., 2010; Loane and Faden, 2010; Tayeb et al., 2012; Van der Walt et al., 2010; Zhang and Chopp, 2009). Thus, these diseases bear a very high cost in both economic terms and life quality deterioration. A recent study by the European Brain Council estimated the total cost (direct and indirect) of brain disorders in Europe was about 800 billion euros in 2010, and neurodegenerative diseases account for a very large proportion (Gustavsson et al., 2011).

Preclinical investigations aimed at developing novel therapeutics for neurodegenerative disorders indicate that melanocortins could be promising drug candidates. The melanocortin family encompasses adrenocorticotropic hormone (ACTH), α-, β- and γ-melanocyte-stimulating hormones (α-, β- and γ-MSH) and shorter fragments: all are products of the pro-opiomelanocortin gene, and all melanocortins share a core sequence of four amino acids, His-Phe-Arg-Trp, which are the residues (6-9) in ACTH and α-MSH (Brzoska et al., 2008; Caruso et al., 2014; Catania et al., 2004; Getting, 2006; Giuliani et al., 2012; Schiöth, 2001; Versteeg et al., 1998; Wikberg and Mutulis, 2008). Intensive preclinical research by several independent groups started in the fifties of the last century, as well as more recent clinical investigations, showed many extra-hormonal effects of melanocortins: in this research field, the studies by the pharmacological schools of Modena (Italy) and Utrecht (The Netherlands) have given an important

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propulsion. Indeed, a growing body of evidence indicates that melanocortin peptides and synthetic analogs affect many central and peripheral body functions, such as food intake, sexual behavior, pain sensitivity, fever control, pigmentation, learning and memory (Bertolini et al., 1969, 1986; Brzoska et al., 2008; Catania et al., 2004; de Wied, 1969; de Wied and Bohus, 1966; Diano, 2011; Ferrari, 1958; Ferrari et al., 1963; Getting, 2006; Gispen et al., 1970; O'Donohue and Dorsa, 1982; Schiöth, 2001; Tatro and Sinha, 2003; Vergoni and Bertolini, 2000; Wikberg and Mutulis, 2008). Of note, melanocortins also play a protective and life-saving role in experimental hypoxic and degenerative conditions (Altavilla et al., 1998; Bazzani et al., 2001, 2002; Bertolini, 1995; Bertolini et al., 1989; Giuliani et al., 2007a, 2010, 2012; Guarini et al., 1990, 1996, 1997, 2004; Jochem, 2004; Lonati et al., 2012; Minutoli et al., 2011a, 2011b; Mioni et al., 2003; Ottani et al., 2013, 2014;Versteeg et al., 1998), including acute and chronic neurodegenerative disorders (Catania, 2008; Corander et al., 2009; Gatti et al., 2012; Giuliani et al., 2006a, 2006b, 2007b, 2009, 2011, 2012, 2014a, 2014b, 2015; Holloway et al., 2011; Lasaga et al., 2008).

In the present paper we review the protective actions of melanocortins in the experimental neurodegenerative conditions thus far investigated, including mechanisms of action, and discuss the possibility to extend the use of melanocortin agonists for treatment of other neurodegenerative disorders.

2. Neurodegeneration, endogenous compensation and brain repair

Impairment in cognitive, behavioral, motor and basic vital functions are common in patients with neurodegenerative disease. The broad variety of neurodegenerative phenotypes is consistent with differences in the initial disease triggers and pathological hallmark biomarkers of the disease. However, despite differences in origins, many disease-related pathways that cause neuronal damage and death are common to most acute and

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chronic neurodegenerative disorders (Antel et al., 2012; Banerjee et al., 2010; Blennow, 2010; Bragge et al., 2012; Drouin-Ouellet and Cicchetti, 2012; Dumont et al., 2001; Friedlander, 2003; Gao et al., 2012; Glass et al., 2010; Haass, 2010; Heneka et al., 2015; Iqbal and Grundke-Iqbal, 2011; Karbowski and Neutzner, 2012; Kumar and Loane, 2012; Leker and Shoami, 2002; Leuner et al., 2012; Lo, 2010; Loane and Faden, 2010; Mehta et al., 2013; Moskowitz et al., 2010; Walsh et al., 2014; Yuan and Yankner, 2000; Zipp and Aktas, 2006). It has been hypothesized that the spread of neurodegeneration occurs not only by proximity, but also transneuronally through propagation of toxic molecules along network connections (Guo and Lee, 2014; Raj et al., 2012; Zhou et al., 2012). Therefore, over the last decades, significant progress has been made in the understanding of pathophysiological mechanisms of neurodegeneration. In particular, the discovery of the important role played by excitotoxicity, oxidative stress, mitochondrial dysfunction, inflammatory response, defective autophagy and apoptosis provided potential targets for novel neuroprotective drugs. There is evidence that also astrocyte dysfunction can play a pivotal role in neurological disorders (Sofroniew, 2015). Epigenetic mechanisms ─ that is, various types of reversible DNA aberrant methylation and histone modifications ─ have been recently considered to be involved in neurodegeneration. Consistently, methyl donors and histone deacetylase inhibitors are under investigation in treatment of neurodegenerative disorders (Adwan and Zawia, 2013; Jakovcevski and Abkarian, 2012; Lardenoije et al., 2015).

Notably, neurodegenerative stimuli also induce endogenous compensation and brain repair, through mechanisms that could likewise be implemented as novel drugs. Such endogenous mechanisms include triggering of neuroprotective pathways, activation of latent or parallel neuronal circuits, synaptic plasticity and remodelling of discrete networks (Banerjee et al., 2010; Drouin-Ouellet and Cicchetti, 2012; Giuliani et al., 2010, 2012; Iqbal and Grundke-Iqbal, 2011; Lo, 2010; Moskowitz et al., 2010; Walsh et al., 2014; Zhang et al., 2011; Zipp and Aktas, 2006).

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Intense basic and clinical investigations are aimed at developing effective neuroprotective strategies (pharmacological and not pharmacological), mainly targeting the pathophysiological pathways that lead to neurodegeneration. However, no novel drug so far, despite promising preclinical data, has revealed successful in large clinical trials, mainly because of toxic side effects, narrow therapeutic treatment window (for acute disorders) and blockade of single molecular pathways responsible for neuronal damage (Adams et al., 2007; Baldwin et al 2010; Banerjee et al., 2010; Blennow, 2010; Bragge et al., 2012; Ehrenreich et al., 2009; Galimberti et al., 2013; Gladstone et al., 2002; Iqbal and Grundke-Iqbal, 2011; Kumar and Loane, 2012; Laskowitz and Kolls, 2010; Leker and Shoami, 2002; Lo, 2010; Loane and Faden, 2010; Moskowitz et al., 2010; O'Collins et al., 2006; Rogalewski et al., 2006; Schiöth et al., 2012; Schumacher et al., 2014; Spence and Voskuhl, 2012; Stetler et al., 2014; Tayeb et al., 2012; Van der Walt et al., 2010; Yepes et al., 2009; Zigmond and Smeyne, 2014; Zipp and Aktas, 2006).

Neurogenesis, a component of brain plasticity, is a significant endogenous mechanisms of compensation and repair . It is now recognized that neural stem cells occur in the central nervous system (CNS) of all adult mammals, including humans. New neural progenitors are mainly produced in two brain regions, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). Additional neuronal progenitors have been found in the forebrain parenchyma, posterior periventricular region surrounding the hippocampus and spinal cord (Benarroch, 2013; Duan et al., 2008; Gage, 2000; Iqbal and Grundke-Iqbal, 2011; Lichtenwalner and Parent, 2006; Lie et al., 2004; Lo, 2010; Suh et al., 2009; Wiltrout et al., 2007). Interestingly, a recent study on the food chain (plants - herbivorous animals - humans), based on the principle that atmosphere ^{14}C is incorporated into DNA during cell division, indicates that about 1400 DG cells were born daily in the human brain, and approximately 80% of human DG neurons undergo renewal in adulthood (Spalding et al., 2013). Conversely in mice such a renewal only occurs in about 10% of DG neurons (Imayoshi et al., 2008). Growing evidence indicates that generation of new neurons can increase under physiological

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stimuli (e.g., physical exercise, environmental enrichment, etc.), as well as in pathological conditions including acute and chronic neurodegenerative diseases such as ischemic and hemorrhagic stroke, TBI, SCI, AD to compensate neuronal loss (Arvidsson et al., 2002; Deng et al., 2010; Duan et al., 2008; Gage, 2000; Kazanis, 2009; Lazarov et al., 2010; Lichtenwalner and Parent, 2006; Lie et al., 2004; Liu et al., 1998; Ohira, 2011; Suh et al., 2009; Vessal et al., 2007; Zhang and Chopp, 2009). Of note, brain insults stimulate endogenous neural progenitor migration from the germinal zones to damaged areas, where they can form mature neurons and glial cells, with potential functional integration if the microenvironment is favorable.

Therapeutic strategies, designed to improve functional recovery in neurodegenerative conditions, are under investigations. These strategies mainly rely on pharmacologically-induced proliferation of endogenous progenitors through an action on different cell targets (Chohan et al., 2011; Giuliani et al., 2011; Irwin and Brinton, 2014; Lichtenwalner and Parent, 2006; Lie et al., 2004; Sun et al., 2003; Suzuki et al., 2007; Wang et al., 2004). A potential target is represented by primary cilia. Indeed, it is well established that primary cilia – microtubule-based organelles – regulate neuronal function in the developing and adult CNS and primary cilia dysfunction causes complex human diseases (Louvi and Grove, 2011). Primary cilia are involved in modulation of signaling pathways, including the canonical Wnt-3A/β-catenin and Sonic hedgehog (Shh) pathways, whose persistent expression in adult mammals play a key role in regulating neural stem/progenitor cell proliferation and migration (Alvarez-Medina et al., 2009; Breunig et al., 2008; Inestrosa and Arenas, 2010; Kuwabara et al., 2009; Lee and Gleeson, 2010; Zhang et al., 2013). Many ion channels and receptors, including melanocortin receptors, are expressed in the membrane of primary cilia (Lee and Gleeson, 2010; Louvi and Grove, 2011; Siljee-Wong et al., 2010):As stated above, primary cilia are involved in the neurogenic process, therefore, could be significant targets. Another therapeutic strategy presently under investigation is based on intracerebral transplantation or systemic injection of a variety of cell types of both

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human and non-human origin, including neural stem/progenitor cells from embryonic and fetal tissue, gene-modified cells, immortalized neural cell lines, multipotent cells such as hematopoietic/endothelial progenitors and stromal cells from bone marrow, umbilical cord blood, and adipose tissue (Burns et al., 2009; Glat and Offen, 2013; Lindvall and Kokaia, 2010; Rodrigues et al., 2012; Vaquero and Zurita, 2011; Zhang and Chopp, 2009). Albeit of great interest, cell-based therapy appears very problematic (Hayes and Zavazava, 2013).

3. Melanocortins and their receptors

Melanocortins are endogenous peptides that occur in several peripheral tissues and within the CNS. The melanocortin system exerts modulation and homeostasis functions. These peptides act through activation of five melanocortin G protein-linked, seven transmembrane receptors (MC_1 to MC_5), all positively coupled with adenylyl cyclase (Table 1) (Brzoska et al., 2008; Caruso et al., 2014; Catania, 2007, 2008; Catania et al., 2004; Corander et al., 2009; Diano, 2011; Getting, 2006; Giuliani et al., 2012; Lasaga et al., 2008; Mountjoy, 2010; Mountjoy et al., 1992; Patel et al., 2010; Schioth, 2001; Schioth et al., 2005; Tatro, 1990, 1996; Tatro and Entwistle, 1994, Versteeg et al., 1998; Wikberg et al., 2000; Wikberg and Mutulis, 2008). The core sequence (6-9) shared by natural melanocortins is required for binding to all MC receptors (Catania et al., 2004; Oosterom et al., 1999; Getting, 2006; Schiöth, 2001). Although the transmembrane signaling primarily involves activation of a cAMP-dependent pathway, melanocortin signaling is also conveyed through additional, cAMP-independent pathways.

MC receptors are widely distributed within the CNS and in peripheral tissues and, similar to other G protein-linked receptors, they form dimeric or oligomeric complexes. The MC_1 receptor is expressed in melanocytes and other skin cells, hair follicles, testis, kidney, periaqueductal gray of the midbrain, endothelial cells, immune/inflammatory cells, and liver. MC_2 is the ACTH receptor and is mainly expressed in the adrenal glands,

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although it has also been detected in adipocytes, skin, testis and fetus brain. MC_3 is expressed in the CNS, gut, kidney, heart, immune system, testis, and skeletal muscle. MC_4 has been mainly found in various brain areas, although low expression levels are also found in the periphery. The MC_5 receptor is ubiquitous in peripheral organs even if it has also been detected in the telencephalon of mammal embryos. Despite their possible expression outside the brain, MC_3 and MC_4 are the predominant receptor subtypes in the CNS. MC_4 expression is clearly broader than that of MC_3, and recent evidence indicates that MC_4 receptors play a key protective role against neuronal injury (Catania, 2008; Giuliani et al., 2010, 2012; Lasaga et al., 2008; Mountjoy, 2010).

Melanocortins, modulate pathophysiological processes including excitotoxicity, oxidative stress, inflammation and apoptosis and contribute to protect the host from damage caused by excessive reactions. An interesting and potentially relevant feature is that melanocortins reduce, but do not abolish, local and systemic inflammatory responses. Therefore they do not impair the defence mechanisms in which a certain degree of inflammation is beneficial (Catania, 2007, 2008; Patel et al., 2010).

In view of their potential therapeutic use, synthesis of melanocortin-related peptides and peptidomimetics as selective agonists (Table 1) and antagonists at MC receptors is in progress. It has been consistently reported that most of melanocortins and their synthetic analogs reach the CNS in pharmacologically-relevant concentrations after systemic injection, and experimental evidence also supports this idea (Banks and Kastin, 1995, Benoit et al., 2000; Catania, 2008; Corander et al., 2009; Giuliani et al., 2006a, 2006b; Guarini et al., 1999, 2004; Holloway et al., 2011; Mioni et al., 2005; Spaccapelo et al., 2011; Wikberg and Mutulis, 2008). Furthermore, permeability of the blood-brain barrier significantly increases in pathological conditions that cause brain damage (Krizbai et al., 2005; Michalski et al., 2010; Sharma et al., 2012), thus facilitating achievement of adequate brain concentrations of substances.

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4. Melanocortins and ischemic stroke

4.1. Neuroprotective effects

Ischemic stroke is the leading cause of adult disability and the second main cause of death in the United States and Europe (Lloyd-Jones et al., 2009). Transient or prolonged severe decrease in cerebral blood flow can eventually lead to delayed neuronal death. Activation of multiple pathological pathways within minutes to days following the cerebrovascular accident are responsible for the brain damage. Indeed, damage is caused by several mechanisms, including excitotoxicity, inflammatory response, defective autophagy, and apoptosis (Baldwin et al., 2010, Banerjee et al., 2010; Dotson and Offner, 2017; Gladstone et al., 2002, Leker and Shoami, 2002; Moskowitz et al., 2010, Zipp and Aktas, 2006). So far, no novel drugs have established effectiveness in neuroprotection, and the only approved therapy is thrombolysis within 3-4.5 h of symptom onset (Adams et al., 2007; Baldwin et al., 2010; Ramee and White 2014; Yepes et al., 2009).

Neuroprotective properties of melanocortin agonists have been demonstrated in several models of brain ischemia (Table 2). α-MSH administration improved the recovery of auditory-evoked potentials in a dog model of transient brain stem ischemia (Huh et al., 1997) and reduced brain inflammation in transient global and focal cerebral ischemia in mice (Huang and Tatro, 2002). Recently, we provided the first evidence that short-term treatment (up to 11 days) with nanomolar amounts of [Nle^{4},D-Phe^{7}]α-MSH (NDP-α-MSH) and its analog RO27-3225 induce a strong neuroprotection against damage consequent to global or focal cerebral ischemia in gerbils and rats. Of particular interest, the neuroprotective effect occurred also when treatment was started several hours (up to 12-18) after ischemia (Giuliani et al., 2006a, 2006b, 2007b, 2009; Ottani et al., 2009; Spaccapelo et al., 2011). Forslin Aronsson and coworkers (2006) and Chen and coworkers (2008) confirmed the neuroprotective effect of α-MSH in

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other models of global and focal cerebral ischemia in rats. The consistently proved melanocortin-induced functional recovery after stroke including improvement in learning, memory, sensory-motor orientation, and limb coordination was accompanied by a treatment-associated reduction in morphological damage with a greater number of viable neurons (Chen et al., 2008; Forslin Aronsson et al., 2006; Giuliani et al., 2006a, 2006b, 2007b, 2009; Savos et al., 2011; Spaccapelo et al., 2011).

A growing body of evidence indicates that the neuroprotective effects of melanocortins occur through antagonism of excitotoxic, inflammatory and apoptotic responses that are the main ischemia-related mechanisms of damage (Table 2 and Fig. 1). Indeed, α-MSH rescued neurons in a rat model of kainic acid-induced excitotoxicity (Forslin-Aronsson et al., 2007). Further, short-term treatment of stroke animals with the melanocortins α-MSH, NDP-α-MSH, and RO27-3225 reduced the brain level/activity of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), extracellular signal-regulated kinases (ERK), c-jun N-terminal kinases (JNK), p38 mitogen-activated protein kinases (p38) and caspase-3, and increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL, with consequent decrease in DNA fragmentation, which is the ultimate feature of apoptosis (Huang and Tatro, 2002; Giuliani et al.,2006b; Ottani et al., 2009; Spaccapelo et al., 2011).

The broad time-window of melanocortin treatment for a successful neurological recovery, and the activity against the main ischemia-related mechanisms of brain damage, originate a potential, innovative neuroprotective approach.

4.2. Neurogenic effects

Data from our laboratory indicate that melanocortin treatment causes long-lasting - or even definitive- functional recovery from ischemic stroke associated with overexpression of the synaptic activity-regulated gene Zif268 in the hippocampus (Giuliani et al., 2006b, 2009). Zif268 is an early

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growth response gene involved in injury repair. Together with other early growth response gene family members it is rapidly induced as transcription factor by a variety of physiological and pathological stimuli. The essential role of Zif268 in synaptic plasticity, as well as in long-term survival, maturation, and functional integration of newborn neurons, has been clearly demonstrated (Beckmann and Wilce, 1997; Giuliani et al., 2009, 2011, 2014a; Veyrac et al., 2013). Melanocortins have established neurotrophic effects, improve neural plasticity and are involved in fetal development including CNS development (Catania, 2008; Gispen et al., 1986; Simamura et al., 2011; Starowicz and Przewłocka, 2003). Through stimulation of repair mechanisms, including neurogenesis, melanocortins might likewise promote functional recovery after stroke.

Consistently, in a long-term study on stroke in gerbils we recently found (Giuliani et al., 2011) that administration of NDP-α-MSH for 11 days markedly increased the number of hippocampus cells labeled with 5-bromo-2'-deoxyuridine (BrdU). In these experiments, BrdU, a thymidine analog that is incorporated into DNA during cell division was injected intraperitoneally in gerbils for 11 days to label proliferating cells . Confocal microscopy examination on day 50 after stroke, showed that most of BrdU immunoreactive cells were localized within the DG of NDP-α-MSH-treated animals. In these experiments, almost all BrdU positive cells expressed the mature neuronal marker NeuN, but not the glial fibrillary acidic protein (marker of astrocytes), indicating that melanocortin treatment causes a shift toward the neuronal phenotype. Furthermore, in NDP-α-MSH-treated stroke gerbils almost all of BrdU-NeuN immunoreactive cells colocalized with the early functional gene Zif268 (Table 2) (Giuliani et al., 2011). This gene is primarily expressed after synaptic activation and is used as an indicator of functionally integrated neurons (Becker et al., 2007; Jessberger and Kempermann, 2003; Tashiro et al., 2007).

With regard to the molecular mechanisms underlying melanocortin-induced neurogenesis, recent findings from our laboratory indicate that treatment of stroke gerbils with NDP-α-MSH produces DG over-expression of the principal regulators of neural stem cell proliferation and fate

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determination (Table 2 and Fig. 2), namely Wnt-3A/β-catenin and Sonic hedgehog (Shh), as well as that of the early and immature neuronal marker doublecortin (Giuliani et al., 2011; Spaccapelo et al., 2013). This effect was observed over the early stage of neural stem/progenitor cell development (days 1-10 after the ischemic insult). Consistent with an essential role played by the Wnt-3A/β-catenin and Shh pathways in the melanocortin-induced neurogenesis, gerbil pretreatment with the Wnt-3A antagonist Dickkopf-1, or the Shh antagonist cyclopamine, reduced the capacity of NDP-α-MSH to induce neural stem/progenitor cell proliferation. Furthermore, DG up-regulated expression of Zif268 was detected during the early stage of neurogenesis. Notably, this gene plays an essential role in synaptic plasticity, selection, maturation, and functional integration of newborn neurons (Veyrac et al., 2013). Finally, high levels of IL-10 were also detected in the DG, particularly in brain vessels, thus suggesting a distant origin (Spaccapelo et al., 2013); indeed, melanocortins induce production of the anti-inflammatory cytokine IL-10 in peripheral blood monocytes and cultured monocytes (Catania et al., 2004; Giuliani et al., 2012), and IL-10 is known to provide a favourable microenvironment for neurogenesis after ischemic stroke (Morita et al., 2007). Primary cilia activity could be a target of melanocortins in the stimulation process of neurogenesis. Primary cilia, in fact, modulate the Wnt-3A/β-catenin and Shh signaling pathways (Alvarez-Medina et al., 2009; Breunig et al., 2008; Inestrosa and Arenas, 2010; Kuwabara et al., 2009; Lee and Gleeson, 2010), and melanocortin receptors are expressed on the membrane of neuron primary cilia (Siljee-Wong et al., 2010); however, an involvement of these organelles in the melanocortin-induced neurogenesis has not yet been investigated.

Induction of neuroprotection and neurogenesis with a broad time-window, together with development of mature and functional neuron properties by the newly generated cells, suggest an interesting pharmacological profile of melanocortins.

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5. Melanocortins and hemorrhagic stroke

5.1. Neuroprotective effects

Subarachnoid hemorrhage (SAH) is a type of hemorrhagic stroke generally caused by the rupture of an aneurysm in a cerebral artery that produces discharge of blood into the basal cisterns (Citerio et al., 2007; Laskowitz and Kolls, 2010; Macdonald et al., 2007). The most common and severe complication of SAH is a late vasospasm. Approximately 50% of patients with symptomatic vasospasm develop infarction, and 20% to 50% of these patients experience a disabling stroke or die from progressive ischemia (Lanterna et al., 2005; Laskowitz and Kolls, 2010; Macdonald et al., 2007). Intracerebral hemorrhage is another type of hemorrhagic stroke characterized by intraparenchimal hemorrhage. It is can be caused by a vessel rupture or it can occur as a complication of thrombolytic treatment after ischemic stroke (Aronowski and Hall, 2005; Xi et al., 2006). Conventional drugs are used to maintain vital functions, and various agents are being tested for prevention or reduction of vasospasm, including magnesium sulfate, statins and intra-arterial infusion of vasodilators. However, no consistently efficacious therapies have been implemented in clinical practice (Moretti et al., 2015; Naggara and Nataf, 2013). Oxidative stress, excitotoxicity, inflammatory response and apoptosis play a crucial role in the pathogenesis of cerebral vasospasm after SAH. Data indicate that the initial event after SAH is activation of genes involved in angiogenesis, inflammation, and extracellular matrix remodeling in cerebral arteries, that promote arterial contractility (Cahill et al., 2006; Gatti et al., 2012; Maddahi et al., 2011; Vikman et al., 2006). It appears, therefore, that correction of key steps of this pathophysiological process may prevent vasoconstriction and brain damage. Oxidative stress, excitotoxicity, inflammation and apoptosis play a fundamental pathogenetic role also in intraparenchimal hemorrhage (Aronowski and Hall, 2005; Lee et al., 2010).

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The potential therapeutic effect of melanocortins in SAH has been recently investigated in a rat model of the disorder (Table 2) through evaluation of early gene expression profiling (at 4 h) and delayed vasospasm (at day 5) in the basilar artery (Gatti et al., 2012). In this study, NDP-α-MSH prevented SAH-induced alterations of gene expression profile in the basilar artery. In particular, the peptide inhibited induction of genes involved in inflammation, stress response, apoptosis and vascular remodelling, whereas it enhanced expression of genes with a salutary role. Further, NDP-α-MSH reduced vasospasm on day 5. The modulatory effect of melanocortins on several detrimental pathways could account for their potential beneficial effects in these neurodegenerative disorders.

A limited compensatory neurogenesis occurs after hemorrhagic stroke, and the newly proliferating progenitors tend to migrate from SVZ and SGZ to the site of injury (Masuda et al., 2007; Shen et al., 2008). However, there are no data on a potential melanocortin-induced amplification of neurogenesis in these conditions.

6. Melanocortins and traumatic injury of the CNS

6.1. Neuroprotective effects

Traumatic brain injury (TBI) and spinal cord injury (SCI) are major and complex CNS disorders, frequently associated wih a poor prognosis and lifelong neurological deficits (Bragge et al., 2012; Dumont et al., 2001; Gupta et al., 2010; Kumar and Loane, 2012; Leker and Shoami, 2002; Loane and Faden, 2010; Maegele et al., 2007) In TBI, caused by direct head impact, injury is mostly focal and results in cortical damage, vascular injury, and hemorrhage accompanied by ischemia; delayed diffuse brain injury also includes axonal damage. Both the primary events and the delayed secondary alterations contribute to neurological deficits. Similar to stroke, traumatic Injury triggers an excitotoxic and inflammatory response, and

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activation of an apoptotic pathway. SCI primarily produces axonal disruption, vascular and metabolic changes, followed by secondary extensive damage due to excitotoxicity, inflammation and apoptosis. Furthermore, TBI is an established risk factor for Alzheimer's disease (Sivanandam and Thakur, 2012). Methylprednisolone is the most prescribed agent in SCI, and anticonvulsant drugs are generally recommended in TBI, also because some of these products are thought to be neuroprotective. Unfortunately, there is no clinically proven neuroprotective therapy. Similar to stroke, the proposed new therapeutic approaches for TBI and SCI failed for comparable reasons (Bragge et al., 2012; Kabadi and Faden, 2014; Kumar and Loane, 2012; Loane and Faden, 2010; Losiniecki and Shutter, 2010; Silva et al., 2014; Xiong et al., 2009).

We recently found that delayed (up to 6 hours) and short-term (up to 7 days) treatment of TBI rats with the melanocortin analog NDP-α-MSH produces marked protective effects (Table 2), including modulation of the inflammatory response and inhibition of the apoptotic cascade (Bitto et al., 2012). Remarkable observations were reduced brain level/activity of nitrites, TNF-α, ERK, JNK, BAX and caspase-3, and the increased expression of Bcl-2. Serum concentrations of High Mobility Group Box-1 (HMGB-1) and IL-6 were decreased, whereas those of the anti-inflammatory cytokine IL-10 were augmented. These changes resulted in a lesser degree of brain damage in melanocortin-treated TBI animals with greater number of viable neurons in the cortical region and in the hippocampus and reduced axonal degeneration in the corpus callosum. In these studies, biomolecular and morphological improvement was distinctly correlated with functional recovery, in particular with sensory-motor orientation and limb use coordination, learning and memory (Bitto et al., 2012). The therapeutic treatment window of melanocortins in TBI was sufficiently broad, although shorter than that observed in stroke conditions (Giuliani et al., 2006a, 2006b). Of note, the weight-drop model used by Bitto et al. (2012) strengthens the value of this study, because it allows to assess the therapeutic potential in TBI conditions ranging from focal to diffuse brain injuries that accurately reproduce potential clinical damage.

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Neuroprotective effects of some low molecular weight non-peptide compounds, with affinity and activity at melanocortin receptors, have been documented in a rat model of focal SCI induced by incision into a right dorsal horn (Table 2) (Sharma et al., 2006). Topical application of ME10501 to the spinal cord 5 min after injury, markedly reduced cell damage, cell loss, sponginess and edema at 5 h after injury. Further, in a mouse model of SCI induced by compression of the T10-T12 spinal tract α-MSH reduced histological damage and improved hind limb motor function as assessed at day 14 after injury (Bharne et al., 2011).

Evidence indicates that reactive neurogenesis also occurs after TBI and SCI, and newborn cells tend to migrate to the site of injury (Bye et al., 2011; Vessal et al., 2007; Wiltrout et al., 2007; Xiong et al., 2012; Zheng et al., 2013). Thus far, a possible melanocortin-induced amplification of neurogenesis in these neurodegenerative conditions has not been studied.

7. Melanocortins and Alzheimer's disease

7.1. Neuroprotective effects

AD is a chronic neurodegenerative disorder marked by severe cognitive and behavioral deficits. Sporadic AD is the most common cause of dementia in the elderly, whereas the less prevalent form of Alzheimer (familial or genetic AD) can occur in younger subjects (Galimberti et al., 2013; Iqbal and Grundke-Iqbal, 2010, 2011; Tayeb et al., 2012). Typical features of AD brains are accumulation of extra-cellular β-amyloid (Aβ) fibrillar deposits (Aβ plaques), intra-neuronal tau neurofibrillary tangles (composed of hyperphosphorylated tau protein), and extensive neuronal loss in vulnerable regions as the cortex and hippocampus (Blennow, 2010; Iqbal and Grundke-Iqbal, 2010, 2011; Ittner and Götz, 2011; O'Bryant et al., 2016; Sivanandam and Thakur, 2012; Sperling et al., 2013; Tayeb et al., 2012). Evidence indicates that β-amyloid accumulation and tau

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hyperphosphorylation trigger excitotoxic and inflammatory responses that cause neurodegeneration and apoptotic cell death. Glutamate, free radicals, cytokines, mitogen-activated protein kinases and caspases play a significant role in these detrimental pathways (Bagheri et al., 2011; Friedlander, 2003; Giuliani et al., 2013; Heneka et al., 2015; Tayeb et al., 2012; Sivanandam and Thakur, 2012; Yuan and Yankner, 2000; Zhang et al., 2011; Zipp and Aktas, 2006). Impaired cholinergic transmission and excessive glutamate activity within the brain are hallmarks of the disorder. Consequently, currently approved therapies for AD are mostly based on cholinergic and anti-glutamatergic strategies, but this approach generally induces transient and modest cognitive improvement (Galimberti et al., 2013; Tayeb et al., 2012). In a scenario in which there are no effective therapeutic solutions, new effective pharmacological approaches are needed to at least retard disease progression. Because disturbances occurring in AD patients are multifaceted, cardiologists, ophthalmologists, and other specialists should be involved in the therapeutic approach together with neurologists (Cermakova et al., 2015; Ho et al., 2012; Schiöth et al., 2012; Sivak, 2013).

Beacause of their potential beneficial effects, melanocortins have been the subject of several AD studies. A clinical investigation reported low levels of ACTH/α-MSH in the cerebrospinal fluid/brain of patients with AD-related dementia (Arai et al., 1986; Facchinetti et al., 1984; Rainero et al., 1988). In an *in vitro* model of AD, α-MSH reduced production of inflammatory mediators by cultured murine microglial cells stimulated with Aβ (Galimberti et al., 1999). Recent results from our laboratory showed that melanocortins protect against progression of experimental AD (Giuliani et al., 2014a). Indeed, in a triple-transgenic mouse model of AD, which closely mimics human AD features (these mice harbor human transgenes APP_{Swe}, $PS1_{M146V}$ and tau_{P301L}), daily treatment with nanomolar doses of NDP-α-MSH (started at the age of 12 weeks and carried on until 30 weeks) significantly reduced cerebral cortex/hippocampus concentration/phosphorylation levels of amyloid precursor protein, presenilin-1, Aβ deposits, tau (phosphorylated at sites Ser 202, Ser 396 and Thr 181), malondialdeyde, nitrites, inflammatory and apoptotic mediators. Relative to

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untreated controls, NDP-MSH-treated animals also showed reduced neuronal loss, with over-expression of the synaptic activity-dependent gene Zif268 and improvement in cognitive functions. In another experimental model of AD transgenic mice (APP_{Swe} mice; Tg2576), NDP-α-MSH treatment started at the age of 24 weeks and administered for 7 weeks reduced cerebral cortex/hippocampus level of Aβ deposit, decreased neuronal loss, induced up-regulation of the activity-dependent gene Zif268, and improved cognitive performance (Giuliani et al., 2014b). Further, in TgCRND8 AD mice, α-MSH treatment for 28 days (started at the age of 12 weeks) prevented GABAergic neuronal loss and anxiety alterations, and improved spatial memory (Ma and McLaurin, 2014). Collectively, these findings indicate that melanocortins counteract the progression of experimental AD by targeting pathophysiological pathways up- and down-stream of Aβ and tau, and through an improvement of synaptic plasticity.

7.2. Neurogenic effects

A very limited, if any, compensatory neurogenesis occurs in AD, depending on age and disease severity (Becker et al., 2007; Ben Menachem-Zidon et al., 2014; Iqbal and Grundke-Iqbal, 2011; Lilja et al., 2013; Lo, 2010; Marlatt and Lucassen, 2010; Mu and Gage, 2011).

Encouraged by the impressive results obtained in stroke gerbils (Giuliani et al., 2011), we recently investigated effects of melanocortins in AD neurogenesis (Giuliani et al., 2015). We found that a 50-day NDP-α-MSH treatment of Tg2576 mice with moderate AD, not only preserved cerebral cortex and hippocampus morphology and counteracted learning and memory decline, but also induced an intense hippocampus DG proliferation of neural stem/progenitor cells. Almost all BrdU positive cells counted on day 50 of the study were also NeuN immunoreactive (but not GFAP positive) and expressed Zif268, thus confirming that melanocortin treatment shifts cells toward a neuronal phenotype and promotes functional integration of newborn neurons.

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The mechanism underlying the neurogenic effect of melanocortins has not been investigated in AD, but it is reasonable to hypothesize that the same molecular mechanisms identified in stroke could lie behind the neurogenic effect of melanocortins in AD animals. In particular, a melanocortin-induced up-regulation of Wnt-3A/β-catenin, Shh, IL-10 and Zif268 (Giuliani et al,2009, 2011, 2014a, 2014b) associated with down-regulation of IL-1β signaling expression could have an eminent role. (Giuliani et al., 2014a). Indeed, these changes could promote over-expression of neurogenesis facilitating factors, including brain-derived neurotrophic factor (BDNF) (Caruso et al, 2012, 2014). Both neurogenic and neuroprotective effects depict an interesting pharmacological profile of melanocortins also in AD.

8. Involvement of MC_4 receptors

As melanocortin MC_4 receptors are the predominant melanocortin receptor subtype expressed in the CNS (Catania, 2008; Getting, 2006; Giuliani et al., 2012; Mountjoy, 2010; Wikberg and Mutulis, 2008), these receptors have been investigated in neurodegenerative disorders (Table 2). Several investigations determined effects of MC_4 blockade on melanocortin effects. Results showed that pretreatment with selective melanocortin MC_4 receptor antagonists prevent the neurogenic and/or neuroprotective effect(s) induced by NDP-α-MSH (agonist at MC_1, MC_3, MC_4 and MC_5 receptors) and RO27-3225 (selective agonist at MC_4 receptors) in ischemic stroke, TBI and AD (Bitto et al., 2012; Giuliani et al., 2006a, 2006b, 2007b, 2009, 2011, 2014a, 2014b, 2015; Spaccapelo et al., 2011, 2013). Furthermore, ME10501, a compound that exerts a potent neuroprotective action in SCI, has high affinity and activity at MC_4 receptors (Sharma et al., 2006). Treatment of cerebral ischemia with the ACTH-(4-7) fragment linked to its C terminal to the Pro-Gly-Pro sequence likewise been induces neuroprotection (Dmitrieva et al., 2010; Stavchansky et al., 2011), although an involvement of MC receptors in this effect has not been reported. As a matter of fact, this compound does not bear the common core

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sequence (6-9) of MSH, necessary for binding to all MC receptor and for agonist activity (Brzoska et al., 2008; Catania et al., 2004; Getting, 2006; Oosterom et al., 1999; Schiöth, 2001; Versteeg et al., 1998); thus it may be that the mechanism of action of this molecule differs from that of melanocortin peptides. Finally, the MC_4 receptor agonist RY767 (a small non peptide molecule) did not induce neuroprotection in a rat model of ischemic stroke (Regan et al., 2009); however, this research only assessed indirect infarct volume at day 3 after stroke, but pathophysiological pathways, neuron viability and functional recovery were not investigated.

It can be hypothesized that MC_4 receptor-mediated signal transduction of melanocortins inhibits neurodegeneration-related key pathophysiological pathways. Consequently, repair mechanisms could be favorably modulated by a physiologically based self-defense machinery. Consistent with a significant physiological role played by MC_4 receptors in neurodegeneration, enhanced expression of MC_4 receptors has been reported in brains of rats subjected to ischemic stroke (Mountjoy et al., 1999). Further, electrophysiological, immunocytochemical and biomolecular studies have recently given evidence that MC_4 receptors play a crucial role in regulation of hippocampal synaptic plasticity (Shen et al., 2013).

9. Melanocortins and associated peripheral effects of brain injury

Systemic responses following brain injury have been characterized, and there is evidence that modulation of systemic responses could have cerebral protective effects (Fig.1) (Anthony et al., 2012; Catania et al., 2009; Mravec, 2010; Ottani et al., 2009). Further, a bidirectional communication between the injured brain and the peripheral immune system has been clearly documented (An et al., 2014).

In recent research on a model of focal brain ischemia in rats, the melanocortin peptide NDP-α-MSH suppressed the inflammatory and apoptotic cascades and the excitotoxic reaction, not only in the brain but also in peripheral tissues (Table 2) (Ottani et al., 2009). Indeed, NDP-α-MSH

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inhibited JNK, ERK and caspase-3 activation, as well as DNA fragmentation and TNF-α overexpression, in the striatum, liver ,and blood. These findings are consistent with the established ability of melanocortins to reduce systemic inflammatory responses in several conditions including circulatory shock (Giuliani et al., 2010; Guarini et al., 2004). In TBI rats, increases in circulating IL-6 and HMGB-1 (Bitto et al., 2012) could promote systemic responses that, in turn, are detrimental to other organs and their inhibition could, therefore, be beneficial. In this regard, it is of note that NDP-α-MSH blunted the TBI-induced systemic release of HMGB-1 (Table 2) (Bitto et al., 2012), as this cytokine is a key mediator of systemic inflammation, and a necessary and sufficient mediator for lethal inflammation (Tracey, 2007).

Overproduction of inflammatory mediators in chronic neurodegenerative disorders is associated with microglia activation and chronic systemic inflammation may, in turn, accelerate the disease progression (Cunningham et al., 2013). There is no direct information on influences of melanocortins on peripheral alterations in other neurodegenerative conditions, although it seems reasonable to hypothesize that the beneficial effects exerted in ischemic stroke and TBI could likewise occur in hemorrhagic stroke, SCI and possibly other, acute and chronic, neurodegenerative disorders.

10. Melanocortins and the vagus nerve against neurodegeneration

Data suggest that the "cholinergic anti-inflammatory pathway" could be involved in the protective effect of melanocortins against neurodegeneration. The cholinergic anti-inflammatory pathway is the effector arm of the "inflammatory reflex", a self-defense mechanism identified through studies on the local and systemic inflammatory responses that follow circulatory shock, myocardial ischemia and other diseases (Borovikova et al., 2000; Giuliani et al., 2010, 2012; Guarini et al, 2003, 2004; Mioni et al, 2005; Tracey, 2002, 2007). The beneficial effects

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induced by activation of this pathway are mediated by the efferent vagal fibers through acetylcholine – the main vagus nerve neurotransmitter – and peripheral α7 subunit-containing nicotinic acetylcholine receptors. Effects consist of reduced expression of several pro-inflammatory cytokines, including IL-6, TNF-α and the late mediator of severe inflammation HMGB-1 (Giuliani et al., 2010, 2012; Guarini et al., 2003; Rosas-Ballina et al. 2011; Tracey, 2002, 2007). Further, it appears that the vagus nerve exerts an immunomodulatory function in the molecular communication between the injured brain and the peripheral immune system (An et al., 2014).

Recent investigations found that bilateral cervical vagotomy and nicotinic receptor blockade with chlorisondamine prevent the protective effects of melanocortins against brain and systemic consequences of ischemic stroke (Ottani et al., 2009). Peripheral inflammation associated with ischemic stroke, a significant component in both acute and long-term outcome, seems to be favourably modulated by the vagus nerve. Reduction of peripheral inflammation could consequently attenuate development of systemic inflammation-induced neuroinflammation (Mravec, 2010; Ottani et al., 2009). Accordingly, in experimental intracerebral hemorrhage, activation of the cholinergic anti-inflammatory pathway through intracerebroventricular injection of muscarinic receptor agonists reduced the inflammatory responses in peripheral tissues and in the brain, with improvement in functional recovery (Lee et al., 2010). A modulatory activity by the vagus nerve has been likewise assumed in the melanocortin-induced decrease in circulating IL-6 and HMGB-1 and in functional recovery in TBI rats (Bitto et al., 2012). Similarly, electrical stimulation of the intact vagus nerve reduced infarct size in rat focal cerebral ischemia, presumably through activation of the cholinergic anti-inflammatory pathway, and via an afferent vagal fiber-mediated modulation of brain noradrenergic neuronal activity (Ay et al., 2009; Mravec, 2010). Interestingly, electrical stimulation of the intact vagus nerve increased hippocampal progenitor cell proliferation in adults rats, likely through a norepinephrine/serotonin-dependent mechanism (Revesz et al., 2008). The effect of vagus stimulation on cognition and memory remains

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controversial. However, in pilot studies, stimulation of the intact vagus nerve improved cognitive functions in AD patients, likely as a consequence of synaptic activation at multiple brain sites; therefore, a potential therapeutic use is under investigation (Iturri Clavero et al., 2010; Vonck et al., 2014). Finally, α7 nicotinic receptor agonists have been reported to promote neuronal differentiation of neural stem/progenitor cells in mice (Narla et al., 2013).

These findings indicate that vagal afferent and efferent pathways could be involved in the modulation of peripheral and central inflammation, neuronal plasticity and perhaps compensatory neurogenesis in neurodegenerative conditions. Notably, seminal observations on the significant role played by the vagus nerve and acetylcholine in the anti-shock effects of melanocortins were reported about thirty years ago (Guarini et al. 1986). In subsequent research on experimental hemorrhagic shock and myocardial ischemia, melanocortins were found to stimulate the motor arm of the inflammatory reflex. Namely, the vagus nerve-mediated cholinergic anti-inflammatory pathway was activated through stimulation of brain MC_3/MC_4 receptors located in the vagus dorsal motor nucleus and/or nucleus ambiguus (Giuliani et al., 2010, 2012; Guarini et al., 2004; Mioni et al., 2005). The effect of melanocortins on the sensory arm (afferent vagal fibers) has not been directly investigated; however, pretreatment with capsaicin ─ which induces desensitization of certain sub-populations of primary afferent nerve fibers, including vagal fibers ─ prevented the anti-shock effect of ACTH-(1-24) in the rat (Guarini et al., 1992). Collectively, these findings suggest that endogenous melanocortin neuropeptides could be physiologically involved in neuroprotection and in neurogenesis, and in protection against the systemic effects of a brain injury. This effect could occur not only via a direct protective effect within the CNS but also through activation of the vagus nerve (Fig. 1). Of note, the dorsal vagal complex, encompassing the sensory nuclei of the solitary tract, area postrema and dorsal motor nucleus of the vagus nerve lacks the blood-brain barrier (Tracey, 2002), thus this brain region can be easily reached by systemically administered molecules including melanocortin peptides.

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11. Potential therapeutic use

Evidence from prolonged preclinical studies, with observation times up to two months after brain injury, suggests that both neuroprotection and amplification of reactive neurogenesis could contribute to a long-lasting melanocortin-induced functional recovery in different types of acute brain insults, including focal and global cerebral ischemia, SAH, TBI and SCI (Bitto et al., 2012; Chen et al., 2008; Forslin Aronsson et al., 2006; Gatti et al., 2012; Giuliani et al., 2006a, 2006b, 2007b, 2011; Savos et al., 2011; Sharma et al., 2006) (Fig. 3). Based on experimental evidence in these conditions, the potential therapeutic use of melanocortins could be conceptually extended to include intracerebral hemorrhage. Additional disorders, such as those characterized by brain injury caused by carotid damage, could likewise benefit from melanocortin treatment. However, appropriate studies using specific experimental models (Guarini, 1996) should be carried out. The broad therapeutic treatment window of melanocortins is remarkable and represents a key component together with the low doses required (nanomolar amounts/daily) and the short treatment duration (approximatel up to 1-2 weeks) required for a successful approach to acute neurodegenerative conditions. Moreover, recent data suggest that chronic treatment (up to 7- 18 weeks) with nanomolar doses of melanocortins could counteract AD progression (Giuliani et al., 2014a, 2014b, 2015) in the absence of harmful side effects. Indeed, the absence of toxicity is a consistent melanocortin characteristic that has been dependably pointed out also in protracted treatments and in different pathological conditions (Brzoska et al., 2008; Catania et al., 2004; Giuliani et al., 2012; Minutoli et al., 2011a; Wikberg and Mutulis, 2008). Therefore, when considering the potential therapeutic use of melanocortins, in AD, a chronic disorder marked by neurological symptoms associated with impairment of several other organs and systems, this favourable feature should be included.

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Endogenous melanocortins exert modulatory functions, and several data support the idea that these peptides may play a physiological, protective role against different types of brain injuries, including ischemic stroke, SAH, TBI, SCI and AD. Indeed, clinical investigations reported that α-MSH plasma levels are decreased in patients with ischemic stroke, SAH and TBI with an unfavourable outcome (Magnoni et al., 2003; Zierath et al., 2011). In addition, low ACTH/α-MSH levels were found, in sporadic studies, in the cerebrospinal fluid and certain brain areas of AD-type dementia patients (Arai et al., 1986; Facchinetti et al., 1984;), and in the cerebrospinal fluid of patients with late onset of AD-type dementia (Rainero et al., 1988). Further, melanocortins have established ability to induce neurotrophic effects on central cholinergic neurons, and for this reason many years ago it was hypothesized that α-MSH deficiency could be cause of AD (Anderson, 1986). It is relevant in this regard the observation that melanocortins increase serum levels of the anti-inflammatory cytokine IL-10 in TBI animals (Bitto et al., 2012). Such effect could indeed contribute to reduction of the inflammatory responses in such conditions, as low plasma concentrations of IL-10 are associated with early worsening of neurological symptoms in patients with acute stroke (Vila et al., 2003). Further, evidence for a role of IL-10 in the regulation of the systemic inflammatory response following trauma is well documented (Schneider et al., 2004). It appears that IL-10 is required in $CD4^+$ T cells-mediated neuroprotection (Xin et al., 2011). Finally, the established ability of melanocortins to activate the vagus nerve could play a key protective role not only in acute, but also in chronic neurodegenerative disorders, such as AD, in which vagus nerve stimulation is under investigation as a potential therapeutic strategy (Iturri Clavero et al., 2010).

Therefore, the proven ability of melanocortins to act at brain MC_4 receptors to prevent injury by targeting multiple pathways in acute and chronic experimental neurodegeneration strongly needs well-constructed randomized controlled trial/blinded studies to cross from pre-clinical into clinical

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arena. Indeed, the capacity of MC_4 activation to promote tissue repair and functional recovery within the brain, suggests encouraging perspectives on the clinical use MC4 agonists.

12. Potential developments to treat other neurodegenerative disorders

Triggers and pathological biomarkers of neurodegenerative diseases are quite different. This wide spectrum includes ischemia, trauma, subarachnoid and intracerebral hemorrhage, plaques and tangles, Lewy bodies, motoneuron death, nerve demyelination, huntingtin protein accumulation, and more. Nevertheless, as stated above, multiple convergent pathophysiological pathways leading to neuronal death are activated in both acute and chronic neurodegenerative diseases (Adwan and Zawia, 2013; Antel et al., 2012; Banerjee et al., 2010; Drouin-Ouellet and Cicchetti, 2012; Dumont et al., 2001; Kumar and Loane, 2012; Friedlander, 2003; Gao et al., 2012; Glass et al., 2010; Haass, 2010; Heneka et al., 2015; Iqbal and Grundke-Iqbal, 2010,2011; Jakovcevski and Abkarian, 2012; Karbowski and Neutzner, 2012; Leker and Shoami, 2002; Leuner et al., 2012; Lo, 2010; Loane and Faden, 2010; Moskowitz et al., 2010; Sofroniew, 2015; Walsh et al., 2014; Yuan and Yankner, 2000; Zhang et al., 2011; Zipp and Aktas, 2006). Treatments targeting these common pathways could have a broader use in neurodegenerative diseases. Melanocortins meet this criterion and, therefore, they deserve further investigation.

As stated above, melanocortins may play a physiological protective role in several acute and chronic neurodegenerative disorders (Fig. 3), and melanocortin treatment could induce not only neuroprotection but also neurogenesis. For example, acute episodes of severe hypoxia, which are among the most common stressors in newborn children, can lead to neurodegeneration (Florio et al., 2010). Melanocortins could provide neuroprotection in these neonates. Consistent with this idea, hypoxia increases plasma levels of ACTH and ACTH precursors in the near-term ovine

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fetus, and shifts towards an ACTH-dependent physiological response in the neonatal rat (Bruder et al., 2008; Myers et al., 2005). Brain damage can also occur in acute carbon monoxide poisoning (Busl and Greer, 2010), and reactive increases in circulating ACTH were observed in patients with acute carbon monoxide poisoning (Raff et al., 1985), thus supporting a possible neuroprotective effect of melanocortins. Increased cerebrospinal levels of melanocortins have been found in patients with Parkinson's disease; such increase has been interpreted as a neuromelanin-mediated compensatory mechanism for protection of neural components of the substantia nigra (Halabe Bucay, 2008). Conversely, low plasma concentrations of α-MSH were found during exacerbation phases of multiple sclerosis (Sandyk and Awerbuch, 1992). It is well known that astrocyte products contribute to neuroprotection. This is particularly interesting as MC_4 receptor activation induces expression of BDNF in rat astrocytes (Caruso et al., 2012). This factor was found to be neuroprotective in experimental models of AD, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (Nagahara and Tuszynsky, 2011). Multiple sclerosis patients have decreased plasma concentrations of BDNF relative to healthy controls, but levels increased after clinical recovery from a relapsing phase (Frota et al., 2009); this observation is consistent with a neuroprotective role for this neurotrophin also in this disease.

Width of treatment time window is crucial in acute disorders such as stroke or TBI, and evidence indicates that the protective effects of melanocortins occur also when treatment is started several hours after the insult (Bitto et al., 2012; Giuliani et al., 2006a, 2006b, 2009; Spaccapelo et al., 2011). It is reasonable to hypothesize that timeliness is also essential for treatment of chronic neurodegenerative diseases, such as Alzheimer, Parkinson, amyotrophic lateral sclerosis, or multiple sclerosis. It appears obvious that neuronal dysfunction is more easily controlled by pharmacological stimulation of endogenous mechanisms of compensation, remodeling and repair in the initial phases of the disorder, when patients are still asymptomatic. As disease severity advances, stimulation of endogenous protective mechanisms, both neuroprotective and neurogenic,

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would likely be less effective. It is worth noting that the majority of studies on experimental chronic neurodegenerative diseases are based on early disease stage, whereas patients usually start therapy when the disease has already reached advanced severity. Therefore, investigations in aged animals and in a late stage of chronic neurodegenerative disorders should be encouraged to assess the potential beneficial effects of melanocortins.

13. Conclusions

The proved ability of melanocortins to modulate, with a broad time window, central and peripheral pathophysiological responses to different types of brain injury establishes a strong pharmacological framework for a potentially successful use of these molecules in treatment of neurodegenerative disorders. Up-regulation of the Zif268 gene, which is essential for synaptic transmission and plasticity and is involved in many processes related to injury repair, induction of new cells that develop properties of mature and functional neurons in the injured brain areas, and improvement of neurocognitive and motor performance, support this idea.

Interestingly, melanocortins induce phenotype changes both in the heart and in the brain that resemble ischemic preconditioning (Catania et al., 2010; Gatti et al., 2012). Therefore, the preconditioning-like effect could further contribute to neuroprotection in brain injury. Consistently, preconditioning and postconditioning as innovative strategies against ischemia/reperfusion injury of several organs including the brain ─ and against other CNS insults ─ are under investigation, with encouraging results (Granfeldt et al., 2009; Stetler et al., 2014; Veighey and Macallister, 2012). Moreover, the antipyretic action of melanocortins is well established, and melanocortin-induced hypothermia can likewise contribute to neuroprotection against acute neurodegeneration (Holloway et al., 2011; Spulber et al., 2005; Tatro, 2006; Tatro and Sinha, 2003). Experimental and clinical studies provide increasing evidence that therapeutic hypothermia could be useful in several brain injuries (Marion and Bullock, 2009;

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Maybhate et al., 2012; Moore et al., 2011; Salerian and Saleri, 2008; Yenari and Han, 2012). Indeed, hypothermia decreases blood flow and metabolism, and preserves blood-brain barrier. Further, it blunts the cascade of detrimental processes that occur after a brain injury, such as excitotoxicity, free radical discharge, inflammation and apoptosis. It has been suggested that chronic cooler core body temperatures may prolong longevity and retard neurodegeneration. Hypothermia has been also reported to enhance neurogenesis and angiogenesis.

The melanocortin system is thought to be activated in inflammatory or stress conditions where it likely contributes to resolution of inflammation. It may be that chronic inflammation results from ineffective engagement of these physiological peptides including their receptors (Ahmed et al., 2014). It is worth considering that melanocortins are modulators of several pathophysiological processes that contribute to protect the host from damage caused by excessive reactions: indeed melanocortins reduce, but not abolish, local and systemic inflammatory responses, and therefore they do not impair the defence mechanisms (Catania, 2007, 2008; Catania et al., 2004, 2006; Patel et al., 2010). Further, these peptides do not reduce microbial killing activity of neutrophils but they rather enhance it, and exert direct broad-spectrum antimicrobial effect (Catania et al., 2009; Grieco et al., 2013). This peculiar characteristic could be of great relevance for treating neurodegenerative conditions in immunocompromised patients, e.g., patients with ischemic stroke, TBI, SAH (where infections are a prominent cause of death), and in AD (where old age-induced vulnerability to infectious agents may contribute to the onset and progression of the disease) (Catania et al., 2009; Kourbeti et al., 2012; Sy et al., 2011; Westendorp et al., 2011).

Finally, it is important to underscore that all the melanocortin effects described in this review are independent from adrenal steroid production: indeed, the melanocortin peptides used in the cited experiments (α-MSH, NDP-α-MSH, RO27-3225, ME10501) do not bind MC_2 receptors, expressed in adrenal cortex and required for glucocorticoid release and, therefore, are devoid of corticotropic activity (Benoit et al., 2000; Catania et

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al., 2004; Corander et al., 2009; Giuliani et al., 2010, 2012; Patel et al., 2010; Wikberg and Mutulis, 2008). Thus, adverse effects associated with glucocorticoid increases would be avoided. Notably, certain melanocortin compounds have already been tested in clinical trials for treatment of weight disorders, sexual dysfunction and skin diseases, with no toxicity problem (for reviews see: Brzoska et al., 2008; Caruso et al., 2014; Corander et al., 2009; Wikberg and Mutulis, 2008). However, in normal animals, some melanocortins (e.g., γ_1-MSH, γ_2-MSH) increase blood pressure and heart rate after intravenous administration, whereas ACTH-(1-24), α-MSH and analogs lacking the C-terminal Arg-Phe sequence do not cause blood pressure increase in normal animals; interestingly, ACTH-(1-24) is able to decrease arterial blood pressure and to increase heart rate (Mioni et al., 2003). This solid clinical experience should be very helpful for implementation of therapeutic use of melanocortins in neurodegeneration.

Clinical advantages of melanocortin treatment after a brain injury include the approach easiness, not only for neuroprotection but also for brain regeneration. This would be a significant difference relative to the novel and controversial, stem cell-based approaches presently under investigation. Indeed, such therapies not only require demonstration of efficacy and cell survival, but they also need a strategy to control cell proliferation and safety (Burns et al., 2009; Hayes and Zavazava, 2013; Vaquero and Zurita, 2011). Therefore, scientists, clinicians, regulators and ethicists should deeply collaborate for a responsible clinical translation of such approaches. A potential limitation linked to treatment of neurodegenerative conditions with natural melanocortins could be the fast breaking down of these peptides in the body fluids although several of the generated fragments are biologically active (Brzoska et al., 2008; Catania et al., 2004). Another potential problem of the natural peptides is the lack of selectivity at melanocortin receptors , and, consequently, a broad activity on central and peripheral tissues and functions (Brzoska et al., 2008; Catania et al., 2004; Corander et al., 2009; Getting, 2006; Schiöth, 2001; Vergoni and Bertolini, 2000; Wikberg and Mutulis, 2008). Therefore,

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stable and highly selective agonists at MC_4 receptor are needed to cure neurodegenerative disorders. Finally, it should be considered that the neurogenic effect of melanocortins has been investigated and proved so far only in an experimental model of ischemic stroke and AD; thus the observation needs to be extended to other conditions.

In conclusion, the exciting results so far obtained encourage melanocortin studies in various experimental models that reflect the other human neurodegenerative diseases. These investigations could constitute an important challenge for the development of innovative drugs, with both neuroprotective and neurogenic properties, against acute and chronic neurodegenerative disorders. Parallel efforts should be devoted to design of synthetic peptides with potentially improved pharmacological profile.

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Figure Captions

Fig. 1. Schematic representation of pathways involved in the neuroprotective effects of melanocortins in neurodegenerative diseases. Peripheral beneficial effects are also highlighted. Melanocortins induce both direct and indirect neuroprotection. Direct effects (reduction of neuropathological hallmark lesions, excitotoxicity, inflammation and apoptosis, and increase in synaptic plasticity) occur through the stimulation of MC_4 receptors located in the central nervous system (CNS) injured area. Indirect neuroprotection is consequent to the activation of the vagus nerve-mediated cholinergic anti-inflammatory pathway, through the stimulation of MC_4 receptors seemingly located in the vagus dorsal motor nucleus (DMN) and/or nucleus ambiguous (NA): this activation promotes acetylcholine release from the efferent vagal fibers in organs of the reticuloendothelial system (heart, liver, etc.). Acetylcholine released interacts with α7 subunit-containing nicotinic acetylcholine receptors on tissue macrophages and other immune cells, and counteracts damage mediator production, with a consequent inhibition of excitotoxic, inflammatory and apoptotic responses at peripheral level. Reduction of such peripheral pathological responses may result in cerebral protective signals and, consequently, reduced development of similar responses in the CNS. These protective signals from the periphery towards the CNS are likely conveyed , at least in part, via the afferent vagal fibers, which are widely distributed throughout the CNS.

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Giuliani et al., Fig. 1

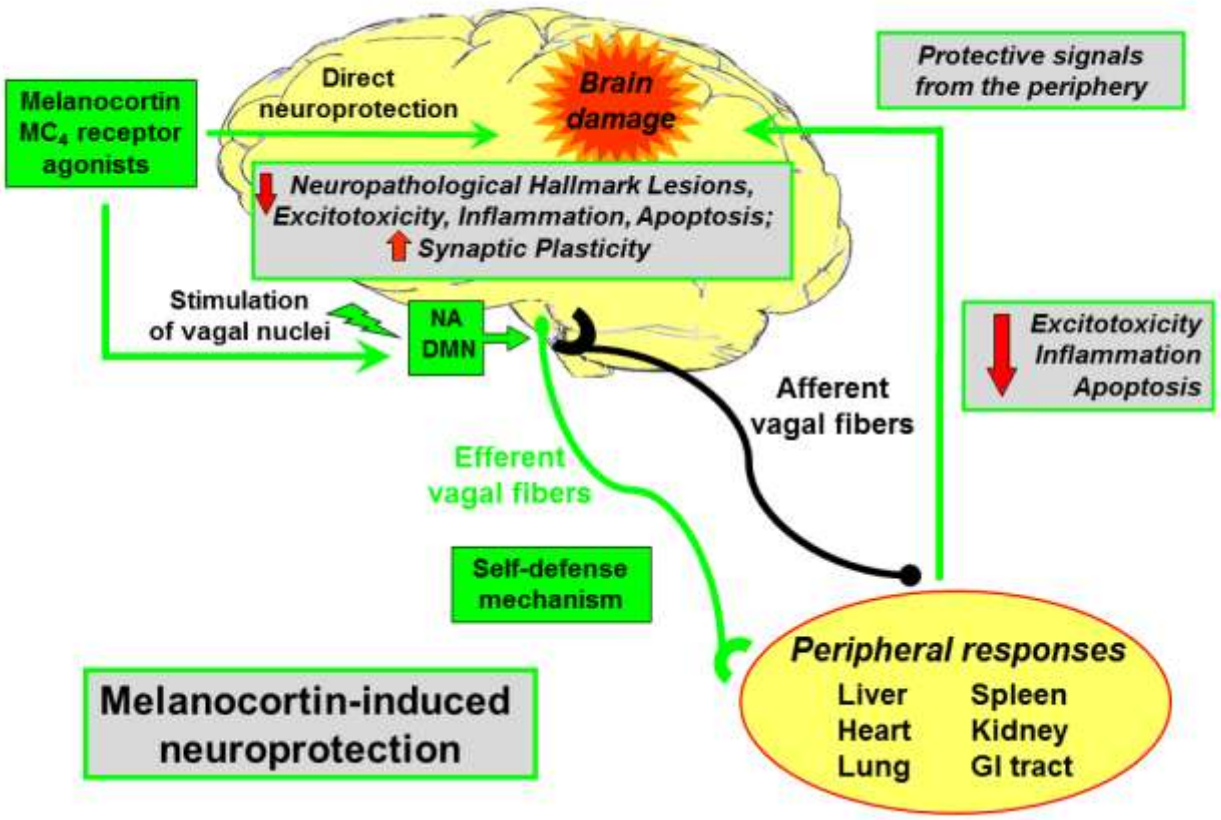

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Fig. 2. In neurodegenerative disorders melanocortins induce brain generation of new cells that develop properties of mature and functionally integrated neurons. Schematic representation of neurogenesis steps, and main mediators (Wnt-3A, β-catenin, Shh, Zif268, DCX, NeuN) whose expression in neural stem/progenitor cells and newborn cells is up-regulated by MC_4 receptor agonists; IL-1β (secreted by microglia) and BDNF (secreted by microglia and other brain mature cells such as neurons and astrocytes) are down-regulated and up-regulated, respectively, by MC_4 receptor agonists; IL-10 (secreted by inflammatory cells, and up-regulated by MC_4 receptor agonists) likely has a distant origin. Stimulation of the intact vagus nerve (not highlighted) also seems to induce an increase in neural progenitor cell proliferation in adults mammals. SGZ, subgranular zone; SVZ, subventricular zone; Shh, Sonic hedgehog; IL-10, interleukin-10; IL-1β, interleukin 1β; DCX, doublecortin; BDNF, brain-derived neurotrophic factor.

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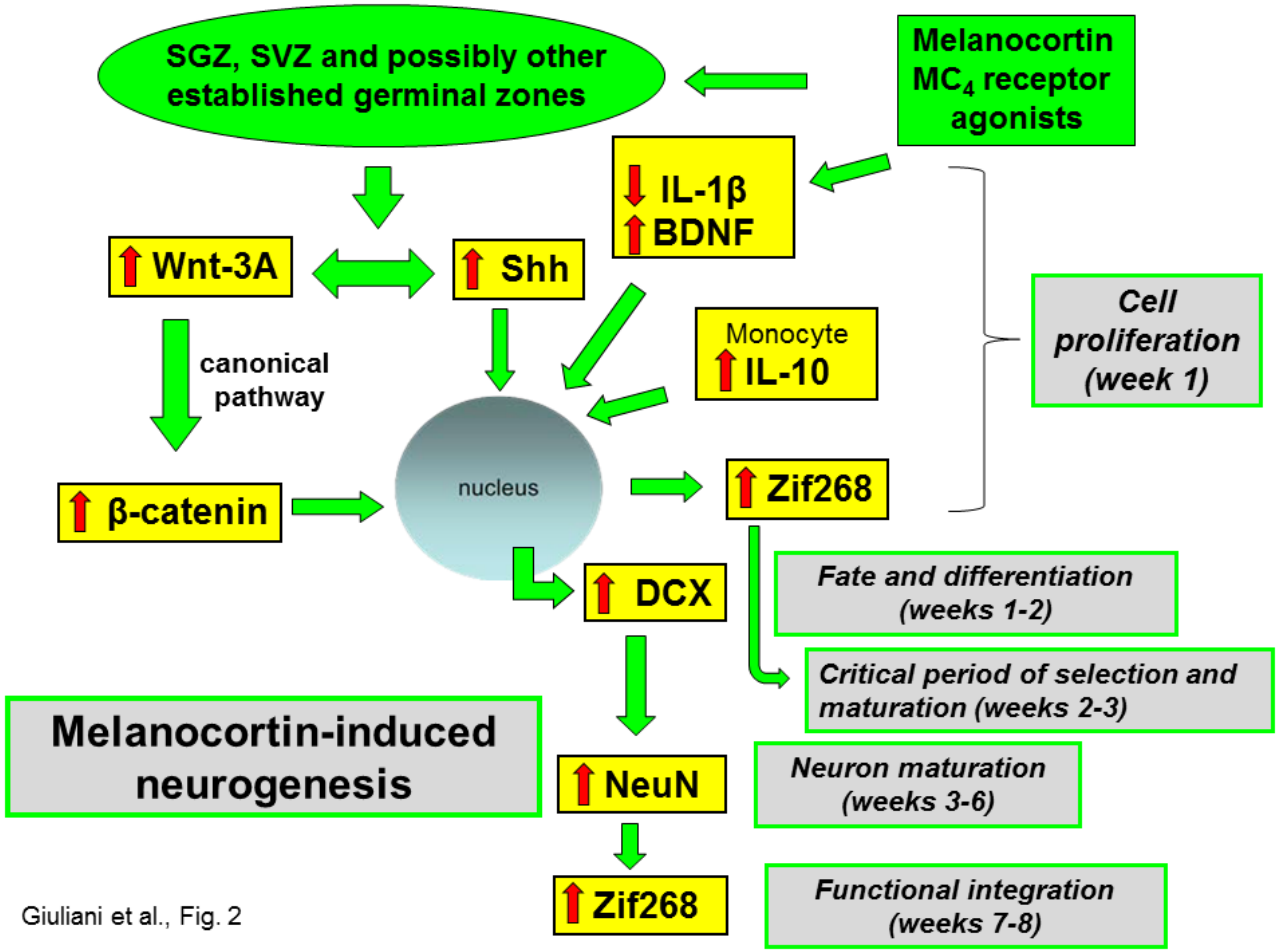

Giuliani et al., Fig. 2

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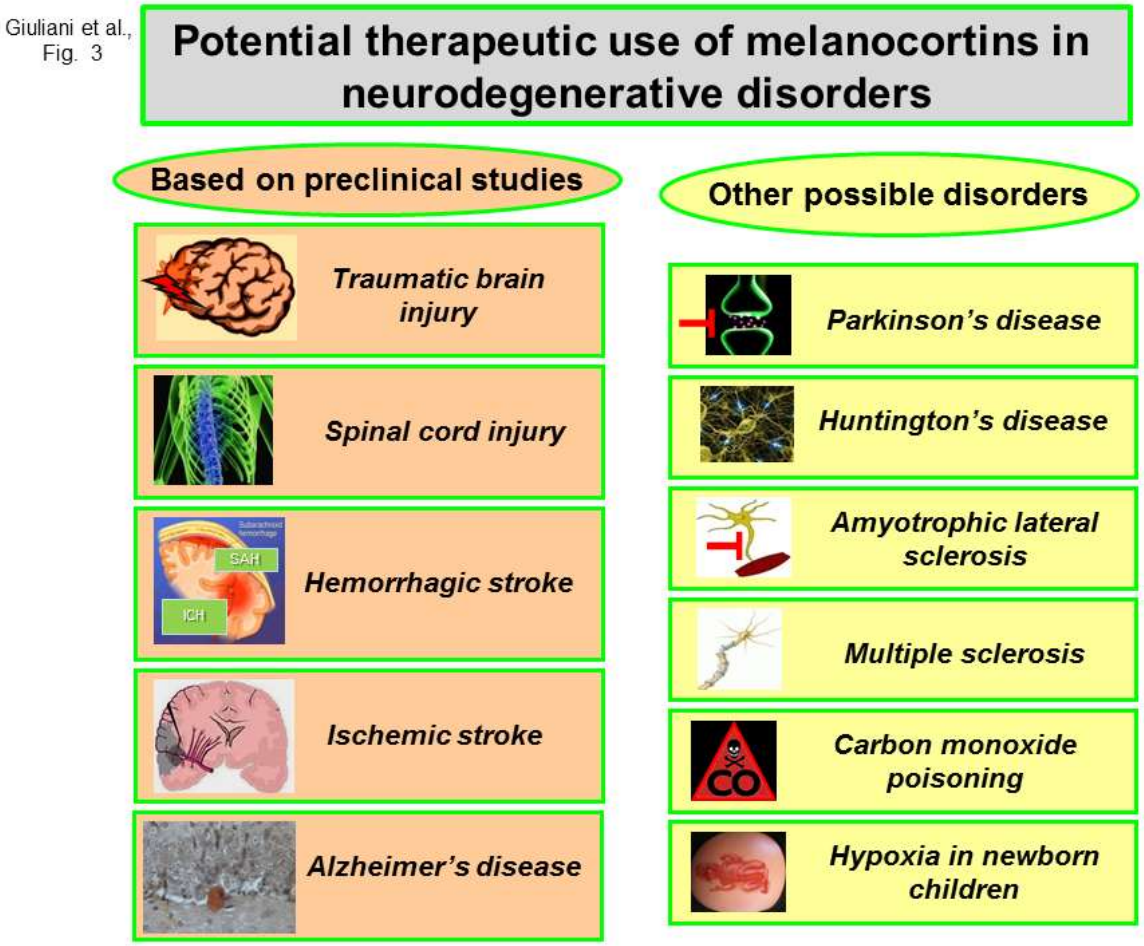
Fig. 3. Potential therapeutic uses of melanocortins in neurodegenerative diseases. Left: suggestion based on results in preclinical studies; right: proposal based on conceptual extension to other neurodegenerative disorders. SAH: subarachnoid hemorrhage; ICH: intracerebral hemorrhage.



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Tables

Table 1 Melanocortin receptor (MC) subtypes, prevalent distribution and affinity of MC agonists

Receptor subtype	Agonist affinity	Prevalent distribution of receptor subtypes
MC_1	MTII > NDP-α-MSH > α-MSH = ACTH-(1-24) ≥ ACTH-(1-39) > β-MSH >> γ_1-MSH ≥ γ_2-MSH > RO27-3225	Melanocytes, hair follicles, glial cells, periaqueductal gray of the midbrain, immune cells, endothelial cells, liver
MC_2	ACTH-(1-39) = ACTH-(1-24)	Adrenal cortex; also detected in adipocytes, skin, testis and fetus brain
MC_3	MTII > NDP-α-MSH > [D-Trp^8]γ_2-MSH > γ_1-MSH ≥ γ_2-MSH = β-MSH = ACTH-(1-39) = ACTH-(1-24) > α-MSH	Brain, heart, kidney, gastrointestinal tract, immune cells
MC_4	RO27-3225 > PG-931 > ME10501 > MTII ≅ NDP-α-MSH >> α-MSH = ACTH-(1-39) = ACTH-(1-24) > β-MSH >> γ_1-MSH ≥ γ_2-MSH	Brain; expressed at low levels in some peripheral organs/tissues
MC_5	PG-911 > α-MSH = NDP-α-MSH = MTII ≥ ACTH-(1-39) = ACTH-(1-24) = β-MSH >> γ_1-MSH ≥ γ_2-MSH	Widespread in many peripheral organs/tissues; detected in the telencephalon of mammal embryo

The receptor affinity of the main MC agonists is shown.

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Table 2 . Preclinical studies on the protective effects of melanocortins in neurodegenerative disorders

Preclinical condition	Treatment	Observed effects	References
Transient global cerebral ischemia by bilateral common carotid artery occlusion (gerbil, C57 BL/6 mouse)	α-MSH, NDP-α-MSH, RO27-3225	↓ Hippocampal and cortical damage, neuronal death, excitotoxic, inflammatory and apoptotic mediators; ↑ Zif268, functional recovery, neurogenesis, Wnt-3A, β-catenin, Shh, doublecortin; broad time window; effects reversed by MC_4 receptor blockade	Giuliani et al., 2006a, 2006b, 2009, 2011; Huang and Tatro, 2002; Spaccapelo et al., 2011, 2013
Focal cerebral ischemia by intrastriatal microinjection of endothelin-1 (rat)	NDP-α-MSH	↓ Striatal damage, neuronal death, systemic damage, excitotoxic, inflammatory and apoptotic mediators; ↑ functional recovery; broad time window; effects reversed by MC_4 receptor blockade and vagotomy	Giuliani et al., 2007b; Ottani et al., 2009
Transient global cerebral ischemia by four vessel occlusion (rat)	α-MSH	↓ Astrocyte proliferation; ↑ hippocampus viable neurons	Forslin Aronsson et al., 2006
Kainic acid-induced brain damage (rat)	α-MSH	↓ Excitotoxicity; ↑ hippocampus viable neurons	Forslin Aronsson et al., 2007
Transient focal cerebral ischemia by unilateral middle cerebral artery occlusion (mouse, rat)	α-MSH	↓ Infarct size, cortical TNF-α and IL-1β, TH1 immune response; ↑ sensory-motor orientation and limb coordination	Chen et al., 2008; Huang and Tatro, 2002; Savos et al., 2011
Transient brain stem ischemia by ventraspinal and vertebral artery occlusion (dog)	α-MSH	↑ Recovery of auditory evoked potentials	Huh et al., 1997
Subarachnoid hemorrhage by injection of blood into the cisterna magna (rat)	NDP-α-MSH	Down-regulation of genes involved in inflammation, stress response, apoptosis, vascular remodelling; ↓ ERK 1/2, IkBα, vasospasm	Gatti et al., 2012
Impact-acceleration model of traumatic brain injury (rat)	NDP-α-MSH	↓ Hippocampal and cortical damage, neuronal death, inflammatory and apoptotic mediators; ↓ serum levels of IL-6 and HMGB-1; ↑ functional recovery, broad time window; effects reversed by MC_4 receptor blockade	Bitto et al., 2012
Spinal cord injury by incision into the right dorsal horn (rat)	ME10501	↓ Cell damage, cell loss, sponginess, edema; effect mediated by MC_4 receptors	Sharma et al., 2006
Spinal cord injury by compression of the T10-T12 tract (mouse)	α-MSH	↓ Histological damage; ↑ hind limb motor function	Bharne et al., 2011

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Alzheimer's disease ($APP_{Swe}/PS1_{M146V}/tau_{P301L}$ mouse)	NDP-α-MSH	↓ Hippocampal and cortical amyloid plaques, proteins of amyloid/tau cascade, excitotoxic, inflammatory and apoptotic mediators, neuronal death; ↑ Zif268 and cognitive performance; effects reversed by MC_4 receptor blockade	Giuliani et al., 2014a
Alzheimer's disease (Tg2576 mouse)	NDP-α-MSH	↓ Hippocampal and cortical amyloid plaques, neuronal death; ↑ Zif268, cognitive performance, neurogenesis; effects reversed by MC_4 receptor blockade	Giuliani et al., 2014b, 2015

ERK 1/2: extracellular signal-regulated kinases 1/2; HMGB-1: High Mobility Group Box-1; IkBα: kBα inhibitor; IL: interleukin; α-MSH: α-melanocyte-stimulating hormone; NDP-α-MSH: [Nle^4,D-Phe^7]α-melanocyte-stimulating hormone; Shh: Sonic hedgehog; TH1: T-helper 1 cells; TNF-α: tumor necrosis factor-α. IL-1β: interleukin-1β; IL-6: interleukin-6;