Astrocytes: New Targets of Melanocortin 4 Receptor Actions

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Abstract

Astrocytes exert a wide variety of functions with paramount importance in brain physiology. After injury or infection, astrocytes become reactive and they respond by producing a variety of inflammatory mediators that help maintain brain homeostasis. Loss of astrocyte functions as well as their excessive activation can contribute to disease processes thus it is important to modulate reactive astrocyte response. Melanocortins are peptides with well-recognized anti-inflammatory and neuroprotective activity. Although melanocortin efficacy was shown in systemic models of inflammatory disease, mechanisms involved in their effects have not yet been fully elucidated. Central anti-inflammatory effects of melanocortins and their mechanisms are even less well known and in particular the effects of melanocortins on glial cells are poorly understood. Of the five known melanocortin receptors (MCR) only subtype 4 is present in astrocytes. MC4R has been shown to mediate melanocortin effects on energy homeostasis, reproduction, inflammation and neuroprotection and, recently, to modulate astrocyte functions. In this review we will describe MC4R involvement in anti-inflammatory, anorexigenic, and anti-apoptotic effects of melanocortins on the brain. We will highlight MC4R action in astrocytes and discuss their possible mechanisms of action. Melanocortin effects on astrocytes provide a new means of treating inflammation, obesity, and neurodegeneration, making them attractive targets for therapeutic interventions in the central nervous system.

INTRODUCTION

Melanocortins are conserved regulatory peptides with anti-inflammatory, anti-pyretic and neuroprotective effects (Catania 2008; Catania, et al. 2004). Astrocytes are the most abundant cell type in the central nervous system (CNS), regarded for a long time merely as support cells for neurons. In recent decades, a growing body of evidence has demonstrated that astrocytes are fundamental pieces in the maintenance of brain homeostasis. Although melanocortin action in astrocytes was reported as early as 1984, only recently were melanocortin receptors identified in these cells. The effects of melanocortins in astrocytes are only beginning to be understood. In this review, we will discuss astrocytes as targets of melanocortin action in the brain.

1. Astrocytes

Astrocytes are organized in a non overlapping manner in the brain and have been classified as protoplasmic or fibrous depending on their morphology and localization. Protoplasmic astrocytes are found in gray matter and have several fine branches with uniform distribution whereas fibrous astrocytes are present in white matter and have few but longer processes. Nevertheless, the diversity of astroglial cells seems to be wider. The human cerebral cortex has several subtypes of astrocytes not found in rodents, and human astrocytes are larger, more diverse, and more complex than rodent astrocytes (Oberheim, et al. 2009). Morphologic studies showed that astrocytes have processes closely contacting blood vessels known as vascular end-feet which enable astrocytes to interact directly with endothelial cells and to contribute to maintain and regulate the blood brain barrier (Abbott, et al. 2010). Astrocytes also contact neuronal synapses with their neuronal end-feet (Grosche, et al. 2002; Grosche, et al. 1999; Ventura and Harris 1999) and thereby they can modulate

neuronal activity. Astrocytes also connect with each other through gap junctions that allow calcium signaling and metabolic coupling between them.

Astrocytes are positioned between blood vessels and neurons allowing them to rapidly respond to changes in the extracellular space. They uptake K^+ that is accumulated in the synaptic space as a consequence of neuronal activity through K^+-channels present in astrocytes (Kofuji and Newman 2004). Also, astrocyte membranes have Na^+/H^+ exchangers and bicarbonate transporters to regulate proton shuttling (Obara, et al. 2008). One of the most important functions of astrocytes is the removal of glutamate from synaptic space through glutamate transporters present in their plasma membrane (Anderson and Swanson 2000), this being the main mechanism by which astrocytes modulate synaptic transmission (Kang, et al. 1998). Excessive glutamate release induces excitotoxicity, which may cause neuron death. Activated astrocytes have increased protein levels of glutamate transporters (Krum, et al. 2002) that enable them to eliminate excess glutamate in the extracellular space. Within the cytoplasm, glutamate is converted to glutamine by glutamine synthetase. Glutamine is then released by astrocytes and can be taken up by neurons and used to renew glutamate stores.

The regulated release of molecules stored in vesicles in glial cells is known as gliotransmission. Astrocytes can release several gliotransmitters such as glutamate, γ-aminobutyric acid, D-serine, neuropeptides and ATP in a calcium-dependent manner (Parpura and Zorec 2010). Astrocytes release D-serine at glutamatergic synapses where it then acts as a co-agonist for NMDA receptors (Henneberger, et al. 2010). ATP secreted by astrocytes into the extracellular space contributes to regulate postsynaptic efficiency at glutamatergic synapses (Gordon, et al. 2005). Also, astrocytes can produce neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor in response to damage, disease or cytokines (Albrecht, et al. 2002; Marz, et al. 1999; Rudge, et al. 1995; Schwartz and Nishiyama 1994) .

Astrocytes and the inflammatory response

Inflammation is a physiological response to pathogens, injury or damage but when it is exacerbated or becomes chronic it can contribute to the onset of neurodegenerative disorders. There are several mediators of this response such as cytokines, chemokines, nitric oxide (NO) and prostaglandins (PGs). In the brain, astrocytes and microglia are immune effector cells that recognize pathogenic antigens, become reactive or activated, and elicit an inflammatory response. They recruit immune cells contributing to the induction of pathogen specific immune adaptive responses (Iwasaki and Medzhitov 2004). Activation of glial cells leads to signal transduction pathways that activate nuclear factor-κB (NF-κB), a transcription factor that regulates the production of inflammatory mediators. Bacterial lipopolysaccharide (LPS) has been used extensively to produce systemic and brain inflammation. LPS activates its receptor TLR4 (toll-like receptor 4) resulting in NF-κB activation. After systemic LPS administration TNF-α, IL-1β and IL-6 are increased in the brain (Laye, et al. 1994). Although all brain cells can synthesize NO, astrocytes and microglia can produce high amounts of NO in response to LPS or pro-inflammatory cytokines by increasing expression of inducible NO synthase (iNOS) whereas the other two NOS isoforms are constitutively active and produce discrete amounts of this molecule. Similar to NOS, cyclo-oxygenase 1 (COX-1) is constitutively expressed in the brain whereas COX-2 expression can be induced by pro-inflammatory stimuli such as LPS leading to PG synthesis (Caruso, et al. 2004). Although neurons can also produce PGs, astrocytes synthesize higher levels of PGs than neurons (Luo, et al. 1998).

Reactive astrogliosis involves cellular hypertrophy, proliferation, increased production of intermediate filaments such as vimentin and glial fibrillary acidic protein (GFAP). In severe injuries, astrogliosis leads to the formation of the glial scar by proliferating astrocytes which can prevent axon growth in the damaged area, but

also limits damage to a specific area and thus protecting the surrounding tissue (Buffo, et al. 2010). Reactive astrocytes can exacerbate damage by releasing pro-inflammatory cytokines, NO and reactive oxygen species. In sites distant from the damage or when it is minor, astrocytes grow in size and increase their production of antioxidants such as glutathione that protect cells from oxidative stress (Wilson 1997), and growth factors that increase neuron survival (Schwartz and Nishiyama 1994). This mild reactive astrogliosis is associated with better recovery from damage. In fact, deletion of reactive astrocytes in a model of spinal cord injury causes failure in blood-brain barrier repair, leukocyte infiltration, severe demyelination and death of oligodendrocytes and neurons (Faulkner, et al. 2004). GFAP knock-out mice develop more severe experimental autoimmune encephalomyelitis (EAE) than wild type mice, which argues in favor of a protective role for astrocytes in this model (Liedtke, et al. 1998). On the other hand, over-expression of TNF-α in astrocytes results in neurodegeneration, gliosis, and the development of chronic encephalopathy (Stalder, et al. 1998). iNOS expression in astrocytes was observed in Alzheimer's disease (AD) (Luth, et al. 2002), multiple sclerosis (MS) (Bo, et al. 1994), EAE (Tran, et al. 1997), and ischemia (Zhu, et al. 2003). When NF-κB expression was impaired only in astrocytes, animals were normal and showed a better recovery from spinal cord injury (Brambilla, et al. 2005). Inflammatory substances released by astrocytes can have harmful effects and even cause death of brain cells contributing to neurodegeneration. However, other factors also released from these glial cells can promote cell survival, thus astrocyte activation cannot be regarded simply as beneficial or detrimental. The net result of their activation depends on several factors such as brain environment, type of injury, and time of exposition to injury. Attenuation of pro-inflammatory mediator release without abolishing the release of beneficial factors constitutes a balanced strategy for the treatment of neuroinflammatory diseases.

Astrocytes and energy homeostasis

The brain has a high energy requirement and its energy supply is also regulated by astrocytes. Glucose enters the brain via endothelial cells and astrocyte end-feet processes. Astrocytes may convert glucose into lactate by performing glycolysis; it may alternatively be used to synthesize glycogen. In fact, glycogen in astrocytes is considered as storage for lactate rather than glucose (Dringen, et al. 1993). Lactate is transported to extracellular space via specific transporters where it can be taken up by neurons. Then, neurons convert lactate into pyruvate which can be used to obtain energy, a mechanism known as the astrocyte-neuron lactate shuttle (Pellerin, et al. 2007). The astrocyte network actually mediates diffusion of glucose and lactate from vasculature to neurons, especially in sites with high demand of neuron energy (Gandhi, et al. 2009; Rouach, et al. 2008). Lactate was also shown to be neuroprotective after cerebral ischemia in mice (Berthet, et al. 2009).

Fatty acids can also be processed as energy substrates. High levels of fatty acids are found in obesity, metabolic syndrome and high-fat diet (HFD), and it is known that fatty acids can cross the blood-brain barrier (Dhopeshwarkar and Mead 1973). Obesity-induced inflammation is a local inflammatory response, maintained in a chronic state, induced by nutrients involving metabolic cells interfering with normal metabolism and disrupting insulin and leptin signaling (Gregor and Hotamisligil 2011). Increased expression of pro-inflammatory cytokines in hypothalamus is observed in HFD-treated rats compared to lean controls (De Souza, et al. 2005). Also, TNF-α knockout improved insulin sensitivity and lowered circulating free fatty acids in mice fed a HFD (Uysal, et al. 1997). Since cytokines are targets of NF-κB, this factor is thought to be critically involved in obesity. Indeed, overnutrition induces hypothalamic activation of NF-κB in HFD animals (Zhang, et al. 2008).

Given that obesity influences brain functions a potential role for hypothalamic astrocytes in these effects is postulated (Garcia-Caceres, et al. 2013; Yi and Tschop 2012). In fact, GFAP-immunoreactive astrocytes are increased in obese Zucker rats (Tomassoni, et al. 2013) and exposure of mice to HFD induces an increase in GFAP mRNA levels as well as in astrocyte numbers and/or processes (Horvath, et al. 2010). Interestingly, recent work shows that saturated but not unsaturated fatty acids induce TNF-α and IL-6 release from astrocytes via TLR4 activation (Gupta, et al. 2012), implicating these cells in obesity-induced inflammation. Moreover, HFD is considered a risk factor for the onset of AD and palmitic acid-treated astrocytes were shown to induce amyloid processing leading to toxic fragment accumulation (Patil, et al. 2006). Hypothalamic astrocytes were reported to express adiponectin receptor 1 which recognizes adiponectin, an adipose tissue secreted hormone involved in the control of energy homeostasis (Guillod-Maximin, et al. 2009). Leptin modulates synaptic inputs in hypothalamus and induces anorexic signaling in neurons whereas in obesity increased leptin levels are found together with leptin resistance (Schwartz 2006). Astrocytes express leptin receptor in hypothalamus and obesity induced by HFD increases leptin receptor expression in hypothalamic astrocytes (Hsuchou, et al. 2009). Also, both overnutrition and chronic leptin treatment of rats increased GFAP and vimentin expression in hypothalamus (Garcia-Caceres, et al. 2011). A recent report shows that neonatal overnutrition increased body weight and leptin, affecting glial cells since GFAP, glucose and glutamate transporter expression were increased in hypothalamus, further suggesting that physiological changes in metabolic state can modulate astrocyte functions (Fuente-Martin, et al. 2012). Moreover, astrocyte leptin-receptor knockout mice showed less severe obesity than wild type mice (Jayaram, et al. 2013). While much remains to be understood about the astrocyte role in energy homeostasis, it seems clear that these cells are key players in the CNS response to obesity.

2. Melanocortin system

The melanocortin system consists of melanocortins, five melanocortin receptors (MCRs), and two endogenous antagonists. Melanocortins include α-, β-, and γ-melanocyte stimulating homones (MSH), and adrenocorticotropin (ACTH), and are generated by proteolytic cleavage of the precursor peptide pro-opiomelanocortin (POMC) by pro-hormone convertases (PCs). Both PC1 and PC2 are needed to produce α-MSH (Benjannet, et al. 1991), after which this peptide suffers additional modifications to become mature α-MSH (Wilkinson 2006). The main source of α-MSH is the pars intermedia of the pituitary gland (Usategui, et al. 1976) although it is also synthesized in several other peripheral tissues. α-MSH is synthesized in the arcuate nucleus of the hypothalamus (O'Donohue and Dorsa 1982), and in the nucleus of the solitary tract in the brain stem (Bronstein, et al. 1992); from there POMC neurons project throughout the brain (Bagnol, et al. 1999). This system has also two endogenous antagonists: Agouti and Agouti-related peptide (AGRP). Agouti is produced in the skin (Blanchard, et al. 1995) where it regulates pigmentation. AGRP is present in the brain only in neurons of the arcuate nucleus (Dinulescu and Cone 2000) where it acts as a competitive antagonist of MC3R and MC4R.

Melanocortin receptors (MCRs)

Five MCRs have been described to date, products of five different genes. All MCRs belong to the family A of G protein-coupled receptors with seven transmembrane domains. MCRs activate adenylate cyclase and induce cAMP production. MC2R is activated only by ACTH. γ-MSH is a selective MC3R agonist (Roselli-Rehfuss, et al. 1993) whereas α-MSH, β-MSH and ACTH are agonists of all other MCRs (Schioth, et al. 1996). **MC1R** was the first MCR to be cloned from melanocytes (Chhajlani and Wikberg 1992; Mountjoy, et al. 1992), and it is

expressed in the skin where its activation by α-MSH induces melanogenesis. MC1R is also found in immune cells where it mediates the anti-inflammatory action of α-MSH in leukocytes (Catania 2007). Adrenal gland **MC2R** activation by ACTH results in production of steroids (Mountjoy et al. 1992). MC2R is also present in rodent adipocytes (Boston and Cone 1996), in human keratinocytes (Slominski, et al. 1996), and in bone cells (Isales, et al. 2010). **MC3R** is widely distributed within the brain (Roselli-Rehfuss et al. 1993) and is also present in several peripheral organs (Gantz, et al. 1993a). MC3R knock-out mice are obese and hyperphagic (Chen, et al. 2000a) and MC3R is thought to function as an autoreceptor in POMC neurons (Cowley, et al. 2001). It also has protective effects in rat heart ischemia (Guarini, et al. 2002) and is involved in the anti-inflammatory effects of melanocortins in macrophages (Getting, et al. 2006). **MC4R** is expressed predominantly in the brain (Mountjoy, et al. 1994) although it was also detected in adipose tissue (Chhajlani 1996), in human skin melanocytes (Spencer and Schallreuter 2009), and in rat heart, lung, kidney and testis (Mountjoy, et al. 2003). **MC5R** is widely found in peripheral tissue (Gantz, et al. 1994; Labbe, et al. 1994) and has also been detected in some areas of the CNS (Griffon, et al. 1994). Data from MC5R knock-out mice show that this receptor regulates secretion of both lachrymal and sebaceous glands (Chen, et al. 1997b).

MC4R

MC4R is an intronless gene that encodes a protein of 332 amino acids with four potential glycosylation sites and two potential palmitoylation sites. It has high levels of homology with the other MCRs. This receptor is also very similar between species. Mouse and rat MC4R have 99% identity whereas rat and human MC4R have 93% identity and their conformation is very similar (Figure 1). Signaling pathway for MC4R involves G protein-mediated activation of adenylate cyclase and increased cAMP production (Gantz, et al. 1993b). It was shown that α-MSH activates the cAMP responsive element binding protein (CREB) in neurons of the hypothalamic paraventricular nucleus (Sarkar, et al. 2002), the solitary nucleus (Sutton, et al. 2005), and in hypothalamic cultured neurons (Caruso, et al. 2010). We recently reported that MC4R activation in astrocytes also involves cAMP- protein kinase A (PKA) -CREB activation (Caruso, et al. 2012). In addition, MC4R stimulation activates the mitogen-activated protein kinase (MAPK) extracellular regulated-kinase (ERK)-1/2 *in vivo* (Daniels, et al. 2003; Sutton et al. 2005) and *in vitro* (Chai, et al. 2006; Daniels et al. 2003; Patten, et al. 2007; Vongs, et al. 2004), an effect that may involve phosphoinositol-3 kinase activation (Vongs et al. 2004). An increase in intracellular Ca^{2+} levels was also detected after MC4R stimulation (Mountjoy, et al. 2001). There is also interaction between signaling pathways since MC4R activation enhances insulin-stimulated mTOR signaling (Chai, et al. 2010) and potentiates leptin signaling (Zhang, et al. 2009). Apart from G protein other proteins may interact with MC4R. Melanocortin 2 receptor accessory protein (MRAP) and MRAP-2 reduce cAMP accumulation induced by melanocortins and are therefore negative regulators of all MCRs except for MC2R (Chan, et al. 2009). Also, it was reported that mahoganoid protein reduces MC4R coupling to cAMP (Perez-Oliva, et al. 2009). The proteoglycan syndecan-3 enhances AGRP antagonism at MC4R (Reizes, et al. 2003). However, further study is needed to fully understand the role of accessory proteins in MC4R functions.

All melanocortins activate MC4R with the exception of γ-MSH. Some synthetic molecules also act as selective MC4R compounds. (Nle^4, $D\text{-}Phe^7$)-α-MSH (NDP-MSH) is the most potent linear analogue of α-MSH (Sawyer, et al. 1980) with high affinity for all MCRs. Melanotan II (MTII) is a non selective agonist of all MCRs except MC2R. Ro27-3225 is a selective agonist for MC4R (Benoit, et al. 2000) and was shown to protect against haemorrhagic shock (Giuliani, et al. 2007). THIQ is a MC4R agonist that reduced food intake in rats (Muceniece, et al. 2007). D-Tyr MTII is another selective MC4R agonist recently proved to stimulate MC4R in hippocampal neurons (Shen, et al. 2013). Also, another highly selective MC4R agonist, BIM-22493, proved to be effective centrally

(Kievit, et al. 2013). Of all the antagonists, SHU9119 is a widely used potent antagonist of both MC3R and MC4R (Schioth, et al. 1999). HS014 was the first selective MC4R antagonist designed since it has about 20-fold higher affinity for MC4R over MC3R (Schioth et al. 1999). HS024 antagonizes MC4R (Kask, et al. 1998) with 100 times more affinity for MC4R than for MC3R, although it antagonizes all MCRs except MC2R.

3. MC4R-mediated actions

Melanocortins exert a variety of brain effects that have been reviewed in detail elsewhere (Bertolini, et al. 2009). Several and diverse melanocortin effects in the brain involve MC4R activation (for review see (Tao 2010)). Some examples of these effects are shown in Table I. Melanocortins through MC4R activation influence energy homeostasis acting within the hypothalamus and promoting weight loss. The anorexigenic effect of α-MSH is mediated by MC4R (Marsh, et al. 1999). In fact, targeted disruption of MC4R gene causes obesity-diabetes syndrome (Huszar, et al. 1997), and mutations in MC4R gene are associated with severe early-onset obesity (Yeo, et al. 1998). MC4R activation was shown to regulate food intake by inducing the release of BDNF in the hypothalamus (Xu, et al. 2003) and a great amount of current research on MC4R is conducted on this field. MC4R activation was also shown to increase sexual and reproductive function (Schioth and Watanobe 2002; Van der Ploeg, et al. 2002) and to augments pain sensitivity (Bertorelli, et al. 2005; Starowicz, et al. 2002). Antagonists of this receptor are also being evaluated as a treatment for cachexia (DeBoer 2010).

MC4R and inflammation

Melanocortins have a well documented role as potent anti-inflammatory agents in several models of inflammation in peripheral organs (reviewed in (Catania et al. 2004)). The anti-inflammatory action of α-MSH reduces secretion of mediators such as cytokines, NO and PGs, and impairs leukocyte activation and infiltration into damaged tissues. Different MCRs may be responsible for the anti-inflammatory properties of melanocortins depending on the tissue or cell type involved. More recent research has provided knowledge on the central action of melanocortins in inflammation. Systemically administered α-MSH reduces cytokine expression in cerebral ischemia (Huang and Tatro 2002), and in brain inflammation induced by LPS (Rajora, et al. 1997). α-MSH was shown to inhibit PGE_2 release induced by LPS or IL-1β from hippocampal fragments (Weidenfeld, et al. 1995), but not from hypothalamic fragments (Mirtella, et al. 1995). However, we reported that melanocortins inhibit the production of NO and PGs induced by IL-1β in rat hypothalamus (Cragnolini, et al. 2006). α-MSH, β-MSH and γ-MSH were found to exert an anti-inflammatory action in a model of neuroinflammation in mice by reducing LPS-induced NO production (Muceniece, et al. 2004). Since MC3R and MC4R expression in the CNS is high they are more likely responsible for central melanocortin actions. MC4R involvement in anti-inflammatory actions of melanocortins in the brain has been suggested (Lasaga, et al. 2008). Central administration of α-MSH markedly reduces induction of hypothalamic iNOS and COX-2 gene expression in rats injected with LPS, an effect prevented by central administration of selective MC4R antagonist HS024 (Caruso et al. 2004), indicating for the first time a role for MC4R in inflammation. We also showed that α-MSH attenuates TNF-α expression induced by LPS and interferon-γ (INF-γ) in hypothalamic cultured neurons that express MC4R (Caruso et al. 2010). Although a role for MC3R in these

effects cannot be completely ruled out, evidence suggests that MC4R is involved in the anti-inflammatory effects of melanocortins in the brain.

Effects of melanocortins on astrocytes have been known since 1984 when α-MSH was shown to induce cAMP accumulation in astroglial cultures (Evans, et al. 1984). Proliferative effects of α-MSH were reported in 7-day-old cultured astrocytes, an effect no longer observed at later times (Zohar and Salomon 1992), suggesting that melanocortins might have a developmental role in these cells. α-MSH was reported to inhibit TNF-α release induced by LPS in human astrocytoma cells (Wong, et al. 1997), although it had no effect on basal or IL-1β-induced PGE_2 levels in astrocytes (Katsuura, et al. 1989). More recently, cloning of the MCRs led to the identification of the subtypes present in astrocytes. Selkirk et al. demonstrated that only MC4R mRNA is expressed in rat astrocytes (Selkirk, et al. 2007). Considering that they are central cells in the initiation and maintenance of the inflammatory response and that we detected MC4R expression at both mRNA and protein levels in rat astrocytes (Caruso, et al. 2007), we hypothesized that astrocytes might be targets of MC4R action. In fact, α-MSH attenuates LPS+IFN-γ-induced inflammatory response in astrocytes since α-MSH treatment decreased iNOS and COX-2 expression and consequently NO and PGE_2 release and HS024 also prevented these effects (Caruso et al. 2007).

Fever is a host-defense response to inflammation mediated mainly by cytokines (IL-1β, TNF-α, and IL-6) and PGE_2. Recently, the RANKL/RANK system was described as another important mediator of fever caused by LPS or cytokines in mouse brain (Hanada, et al. 2009). Since levels of α-MSH increase in the brain during fever (Bell and Lipton 1987) and circulating levels of α-MSH increase in response to endotoxin administration in humans (Catania, et al. 1995), a physiological role for melanocortins in fever has been suggested. Melanocortins are also considered endogenous antipyretics whose effect on fever has been known for some time (Tatro 2000). Central administration of α-MSH reduces fever caused by LPS (Huang, et al. 1997), IL-1β (Daynes, et al. 1987) and TNF-α (Martin, et al. 1991). Although the mechanisms involved in the antipyretic action of α-MSH remain unknown, the decrease of pro-inflammatory cytokines and PGs production in the brain can contribute to fever reduction. Indeed, ip administration of α-MSH was shown to inhibit fever by activating central MCRs (Huang, et al. 1998), and the antipyretic effect of central administered α-MSH was also blocked by HS014, a selective MC4R antagonist, thereby highlighting MC4R involvement in α-MSH effect on LPS-induced fever (Sinha, et al. 2004). Astrocytes also participate in fever since they produce the inflammatory mediators that cause it, but surprisingly this issue has been scantily investigated. One recent report showed that astrocytes are involved in fever induced by RANKL and cytokines. Hanada et al. (2009) showed that inactivation of the RANK receptor in neuronal progenitor cells as well as inactivation of this receptor only in astrocytes abolished fever in response to RANKL, IL-1β and TNF-α (Hanada et al. 2009), indicating that astrocytes are major contributors to inflammation-induced fever. Thus, MC4R activation in astrocytes could help reduce fever by inhibiting release of mediators such as cytokines and PGs.

MC4R and energy homeostasis

Melanocortin system in the arcuate nucleus (ARC) of hypothalamus plays a central role in energy homeostasis. POMC neurons in the ARC release α-MSH in response to peripheral signals such as leptin (Cowley et al. 2001) or insulin (Benoit, et al. 2002), after which α-MSH induces an anorexigenic effect by activating MC4R in target neurons. As a result, food intake decreases and metabolic rate increases, promoting weight loss. Leptin and

insulin are considered to act as adiposity signals since their blood levels increase in proportion to body fat mass and access the brain where these hormones promote negative energy balance. In addition, in the ARC AGRP neurons induce the opposite effect when activated, since they have orexigenic effects and also inhibit POMC neurons. AGRP neurons are inhibited by leptin and insulin. Neuronal targets of POMC and AGRP neurons involve the paraventricular nucleus (PVN) and lateral hypothalamic area (LHA). Neurons in the PVN produce peptides that decrease food intake and increase metabolic rate such as oxytocin, corticotrophin-releasing hormone and thyrotropin-releasing hormone (Schwartz 2006). On the contrary, neurons of the LHA stimulate food intake and promote weight gain by releasing orexins and melanin concentrated hormone. MC4R is expressed in both PVN and LHA neurons where it has a prominent role in energy homeostasis. MC4R knockout mice are hyperphagic and obese with a decreased energy expenditure (Huszar et al. 1997). Recently, treatment with a selective MC4R agonist (BIM-22493) was shown to induce transient decreases in food intake and weight loss over 8 weeks of treatment in diet-induced obese rhesus macaques, which also showed decreased adiposity and improved glucose tolerance (Kievit et al. 2013). Mutations in MC4R gene are associated with severe early-onset obesity (Yeo et al. 1998). Variation of nucleotide sequence in one allele of human MC4R can cause obesity by disrupting MC4R signaling (Ho and MacKenzie 1999). In obesity, increased circulating leptin levels are not correlated with increased MC4R activation since there is also leptin resistance. Astrocytes as well as POMC neurons express adipokine receptors, including leptin receptors, and in response to HFD they showed increased expression of leptin receptors (Hsuchou et al. 2009). In obesity, reactive astrocytes with enlarged ensheathments impede POMC neuron ability to sense leptin in blood, which was proposed to contribute to leptin resistance (Yi and Tschop 2012). However, a study by Horvath et al. (Horvath et al. 2010) found that gliosis in HFD might not be the cause of leptin resistance since POMC neuron firing in these mice was as expected in response to a strong leptin input. Since melanocortins reduce astrocyte activation (Forslin Aronsson, et al. 2007; Forslin Aronsson, et al. 2006) and production of inflammatory mediators (Caruso et al. 2007), they could be beneficial for reducing obesity-induced inflammation. Moreover, since we proved that melanocortins induce BDNF expression in astrocytes (Caruso et al. 2012)and BDNF is a mediator of MC4R effects on energy balance (Xu et al. 2003), BDNF released by astrocytes may possibly contribute to anorexigenic effects of MC4R.

Cell response to decreased substrate availability or excess of nutrients is triggered by AMP-activated protein kinase (AMPK), thereby acting as a sensor and regulator of cellular energy levels. In hypothalamus, activation of AMPK regulates the entire body's energy balance by reducing energy expenditure and enhancing food intake (Minokoshi, et al. 2004). Indeed, MC4R stimulation by α-MSH induces inhibition of AMPK in GT1-7 hypothalamic cells (Damm, et al. 2012). In another study, Escartin et al. showed that in ciliary neurotrophic factor-activated astrocytes in the striatum AMPK is activated and these cells were more resistant to glycolysis inhibition and less affected by palmitate toxicity (Escartin, et al. 2007). Also, AMPK was detected in spinal astrocytes and was activated by ADP treatment resulting in ATP production in these cells (Cui, et al. 2011). Therefore, we may speculate that MC4R activation in astrocytes can modulate AMPK, which might in turn influence hypothalamic response to energy levels.

MC4R and neuroprotection

Melanocortins participate in the development and regeneration of the CNS. α-MSH can act as a neurotrophic factor during development as well as in the adult brain (Strand, et al. 1991). A recent study showed that NDP-MSH induces neurogenesis in the hippocampus of gerbils after global ischemia and that this effect is mediated

by MC4R (Giuliani, et al. 2011). This treatment also improved the animals' memory and learning. α-MSH through MC4R was also shown to reverse amnesia (Gonzalez, et al. 2009), as well as memory reconsolidation impairment (Machado, et al. 2010), induced by IL-1β administration in the hippocampus of male rats. Melanocortins also exert neuroregenerative actions such as re-growth stimulation of injured axons in rat adult spinal cord (Joosten, et al. 1999). α-MSH-induced neurite-like outgrowth was blocked with a specific MC4R antagonist (Adan, et al. 1996) and by a selective MC4R antagonist in dorsal root ganglia neurons (Tanabe, et al. 2007). Also, topical application of a selective MC4R agonist was found to be neuroprotective in spinal cord injury (Sharma, et al. 2006). Therefore, MC4R is involved in the neuroregenerative effects of melanocortins.

Melanocortin treatment has proven to be neuroprotective through MC4R activation in brain injury. In a model of focal cerebral ischemia in gerbils delayed treatment with α-MSH (Giuliani et al. 2007) or treatment with NDP-MSH but not with the MC3R agonist γ-MSH (Giuliani, et al. 2006) reduced neuron death. Also, NDP-MSH reduced neuron death after kainate-induced excitotoxicity (Forslin Aronsson et al. 2007). NDP-MSH was shown to protect a hypothalamic cell line, which expresses MC4R, from serum deprived-induced apoptosis (Chai et al. 2006). In a rat model of traumatic brain injury NDP-MSH increased the number of viable neurons in the cortex and the hippocampus (Bitto, et al. 2012). This protection correlated with decreased TNF-α and NO production, and decreased expression of pro-apoptotic Bax and caspase-3 activation, and also with increased serum levels of IL-10 and Bcl-2 expression induced by NDP-MSH. All these effects were blocked by HS024 (Bitto et al. 2012). In cerebral ischemia, neuroprotection by NDP-MSH also involves activation of MC4R and Bcl-2 up-regulation (Giuliani et al. 2006). Moreover, melanocortins reduce hippocampal damage and improve learning and memory as long as 50 days after ischemia (Giuliani, et al. 2009).

Apoptotic characteristics such as DNA fragmentation were shown to occur in astrocytes adjacent to cerebral ischemia (Chen, et al. 1997a; Li, et al. 1995) as well as increments in Bax and active caspase-3 levels (Benjelloun, et al. 2003). Inflammatory stimuli such as LPS (Suk, et al. 2001), cytokines (Ehrlich, et al. 1999; Saas, et al. 1999), and NO (Durand, et al. 2010; Kim, et al. 2001) can induce apoptosis of astrocytes. Astrocyte apoptosis can have beneficial as well as detrimental effects on neurons. In neuron-astrocyte co-cultures, the presence of astrocytes diminishes neuron death induced by oxidative stress (Blanc, et al. 1998), and blocking astrocyte gap junctions induces neuron death in response to glutamate (Ozog, et al. 2002). Indeed, cultured spinal cord astrocytes exposed to peroxinitrites for 24 h promote activation of caspase-3 and apoptosis of motor neurons that grow on top of them (Cassina, et al. 2002). In contrast, astrogliosis is observed in AD, Huntington's disease, and Parkinson's disease. Moreover, in MS, Huntington's disease, ischemia and brain injury, astrocytes die by apoptosis (Maragakis and Rothstein 2006; Takuma, et al. 2004). Therefore, reduction of the essential functions performed by astrocytes as well as their activation can directly contribute to neurodegeneration. In models of neuron death induced by cerebral ischemia or by excitotoxicity, systemic administration of α-MSH decreased neuron death and astrocyte activation by decreasing the number of GFAP-positive cells (Forslin Aronsson et al. 2007; Forslin Aronsson et al. 2006). However, nothing was known about melanocortin action on astrocyte death. We demonstrated that MC4R activation by α-MSH protects astrocytes from apoptosis induced by LPS+IFN-γ (Caruso et al. 2007). Melanocortins prevent astrocyte death by decreasing caspase-3 activity and the expression of Bax induced by LPS+IFN-γ and by increasing the expression of Bcl-2. Since melanocortins increase astrocyte survival this can contribute to their neuroprotective effects.

Astrocytes are able to produce neurotrophic factors in response to damage, disease or cytokines that can promote neuron survival (Albrecht et al. 2002; Rudge et al. 1995; Schwartz and Nishiyama 1994). ACTH was observed to down-regulate ciliary neurotrophic factor mRNA levels without modifying other neurotrophins in

cultured astrocytes (Kokubo, et al. 2002). In contrast, an analogue of ACTH increased BDNF mRNA levels in rat glial cell cultures (Shadrina, et al. 2001) and after cerebral ischemia (Dmitrieva, et al. 2010). Concordantly, we showed that MC4R activation induces expression of BDNF in cultured rat astrocytes (Caruso et al. 2012) suggesting that neuroprotection by MC4R can involve neurotrophic factor release.

4. Mechanisms of MC4R-mediated effects

The broad effects exerted by melanocortins can be explained by the fact that α-MSH inhibits NF-κB, a transcription factor that regulates the inflammatory response, by activating transcription of inflammatory mediators (Li and Verma 2002). α-MSH was shown to reduce the activation of NF-κB *in vitro* (Manna and Aggarwal 1998) and *in vivo* in the brain (Ichiyama, et al. 1999a). However, the scenario seems to be different for astrocytes. α-MSH in A172 human glioma cells reduced (Ichiyama, et al. 1999b), whereas in H4 glioma cells did not modify (Sarkar, et al. 2003), NF-κB activation. We also showed that NF-κB activation was not modified in rat astrocytes (Caruso et al. 2012). Thus in addition to NF-κB inhibition an alternative mechanism of action may exist for melanocortins in astrocytes. It was shown that NF-κB activity can also be inhibited by the anti-inflammatory cytokine IL-10 in monocytes (Wang, et al. 1995). Indeed, SHU9119, a MC3R/4R antagonist, reduces *per se* IL-10 serum release induced by LPS (Vulliemoz, et al. 2006), and IL-10 is released from human peripheral mononuclear cells (Yamaoka-Tojo, et al. 2006), indicating that melanocortins can also be physiological modulators of IL-10. Indeed, we recently reported that NDP-MSH via MC4R activation did not modify IL-10 released from astrocytes whereas it did increase IL-10 release from microglial cultured cells (Carniglia, et al. 2013). Apart from NF-κB and IL-10 modulation, melanocortins activate CREB transcription factor which is involved in neuron proliferation and survival, learning and memory as well as in neuroprotection (Lonze and Ginty 2002). CREB is activated by α-MSH in hypothalamic neurons and although α-MSH decreased TNF-α expression, it did not affect NF-κB activation in these cells (Caruso et al. 2010). In astrocytes, α-MSH increases cAMP intracellular levels and also induces CREB activation (Caruso et al. 2012). Congruently, we blocked BDNF expression induced by MC4R activation in astrocytes with adenylate cyclase and PKA inhibitors (Caruso et al. 2012) confirming that cAMP-PKA-CREB pathway is activated in astrocytes by MC4R stimulation.

Mechanisms of neuroprotection by melanocortins involve modulation of MAPK activation and expression of proteins from the Bcl-2 family. In ischemia models melanocortins were reported to reduce MAPKs activation (p38, JNK and ERK1/2) and to increase Bcl-2 expression promoting survival of brain cells (Giuliani et al. 2006). In a rat model of traumatic brain injury, NDP-MSH also decreased JNK and ERK1/2 activation as it increased serum levels of IL-10 and Bcl-2 expression (Bitto et al. 2012). However, ERK activation was induced by melanocortins in rat hypothalamus (Daniels et al. 2003) and in solitary nucleus of the rat (Sutton et al. 2005). ERK1/2 was also activated in GT1-7 hypothalamic cells in response to α-MSH (Damm et al. 2012). Our very recent data also indicate that α-MSH abolishes the reduction in ERK2 phosphorylation induced by IL-1β in the hippocampus (Gonzalez, et al. 2013) and that ERK1/2 is activated by NDP-MSH in astrocytes (Caruso, et al. 2013). Moreover, ERK1/2 activation is known to have protective effects, which is also true for MC4R-mediated ERK1/2 activation since ERK1/2 inhibitor decreases the anti-apoptotic effect of MC4R activation in GT1-1 cells (Chai et al. 2006). Hence, ERK activation and modulation of Bcl-2 expression by melanocortins seem to be important mechanisms underlying neuron and astrocyte survival by melanocortins.

Since BDNF has proved to be protective in neurodegenerative diseases such as AD, MS and PD (Nagahara and Tuszynski 2011) and its expression is increased in response to melanocortins in the hypothalamus and also in astrocytes, it is another possible mediator of melanocortin actions. BDNF could have protective effects on neurons and on astrocytes themselves, the latter being an issue that has not been thoroughly investigated. BDNF stimulates S100β expression in mouse astrocytes (Djalali, et al. 2005), and it increases intracellular calcium levels of rat astrocytes (Climent, et al. 2000) but much study is still needed to fully understand the effects of BDNF on astroglial cells.

Transforming growth factor-β (TGF-β) is another cytokine that modulates inflammatory responses and CNS homeostasis (Aigner and Bogdahn 2008). This cytokine inhibits the LPS-induced expression of TNF-α in astrocytes and microglia (Benveniste, et al. 1995; Lodge and Sriram 1996). TGF-β can also have neuroprotective effects that are mediated by glial cells (Qian, et al. 2008). We recently showed that NDP-MSH increases TGF-β release from astrocytes and thus it is also a possible mediator of melanocortin actions.

A growing body of evidence has implicated peroxisome proliferator-activated receptors (PPARs) in the regulation of inflammatory processes in the CNS (Bright, et al. 2008). In glial cells, PPARs (α, β, and γ) modulate the production of pro-inflammatory mediators (Aleshin, et al. 2009; Lovett-Racke, et al. 2004). PPAR-γ agonists were found to inhibit the release of pro-inflammatory cytokines by microglial cells and astrocytes (Storer, et al. 2005). Anti-inflammatory action of PPAR-β agonists has also been demonstrated in these cells (Polak, et al. 2005) and in a model of focal cerebral ischemia (Arsenijevic, et al. 2006). Our recent findings show for the first time that MC4R activation modulates PPAR expression in glial cells. NDP-MSH increases PPAR-γ protein levels whereas it decreases PPAR-β protein levels in astrocytes (Carniglia et al. 2013), an effect that has also been described for LPS (Jana and Pahan 2012). In addition, PPARs anti-inflammatory effects led to the use of their agonists in *in vitro* and *in vivo* models of neurodegenerative diseases with successful results especially in AD (Heneka and Landreth 2007). Thus, PPARs are also strong candidates to mediate MC4R action in the brain.

All together these data suggest that MC4R activation in astrocytes modulates BDNF and PPAR expression, activates CREB, and induces TGF-β release. The role of these factors in MC4R-mediated effects remains to be determined and their actions to be explored further to prove their therapeutic value.

5. Conclusions

MC4R mediates anti-inflammatory, anorexigenic and neuroprotective effects of melanocortins within the brain. Since astrocytes play a major role in inflammation, the control of their response and the induction of anti-inflammatory and neuroprotective factors by MC4R activation (Figure 2) may restore astrocyte functionality and thereby lead to ameliorate inflammatory disorders and neurodegenerative diseases. Also, recent evidence shows that astrocytes might be involved in the regulation of energy homeostasis (Figure 3). However, in view of the variety of effects produced by MC4R activation, development of more selective and potent agonists and antagonists is needed. Knowledge about the MC4R mechanism of action is of great importance in order for MC4R agonists to become effective therapeutically. Although some progress has been made in this direction more studies are needed to validate glial MC4R as a potential new therapeutic target.

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DECLARATION OF INTEREST

The Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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Figure Legends

Figure 1- Differences between human, mouse and rat MC4R proteins.

Sequence alignment of human, mouse and rat MC4R protein. Amino acids that differ from the human MC4R sequence are highlighted in grey. Mouse and rat MC4R share 93% of the amino acid sequence with human MC4R and being very similar and share the same structure. The N-terminal portion is extracellular whereas the C-terminal portion is intracellular. TM: Transmembrane domain, IL: Intracellular loop, EL: Extracellular loop.

Figure 2- MC4R activation in astrocytes.

Activation of MC4R by α-MSH induces production of cAMP which leads to CREB activation. This pathway is most likely involved in the anti-inflammatory and anti-apoptotic effects of melanocortins in astrocytes. Although NF-κB is involved in the anti-inflammatory effects of melanocortins, this remains a controversial fact for astrocytes. Instead, MC4R activation induces release of the anti-inflammatory agents PPAR-γ and TGF-β probably through cAMP-CREB pathway. BDNF released after MC4R activation occurs through cAMP-PKA-CREB in astrocytes and this neurotrophin through TrkB receptor can have direct effects on neurons promoting their

survival. MC4R activation inhibits apoptosis since it increases Bcl-2 protein levels and reduces Bax protein levels thus promoting cell survival against apoptotic stimuli in astrocytes as well as in neurons.

Figure 3- Astrocytes and MC4R in obesity.

In response to a HFD diet the circulation of saturated fatty acids and cytokines is increased. Astrocytes can be activated by both, saturated fatty acids and cytokines, and thus they respond by increasing GFAP and vimentin expression and by activating NF-κB which in turn induces cytokines expression. Both cytokines (inflammation) and saturated fatty acids (HFD) promote diet-induced obesity. Stimulation of MC4R by α-MSH reduces astrocyte activation and pro-inflammatory mediators, so it could also be possible that these cells help to induce decrease energy balance and thus this may decrease diet-induced obesity susceptibility.

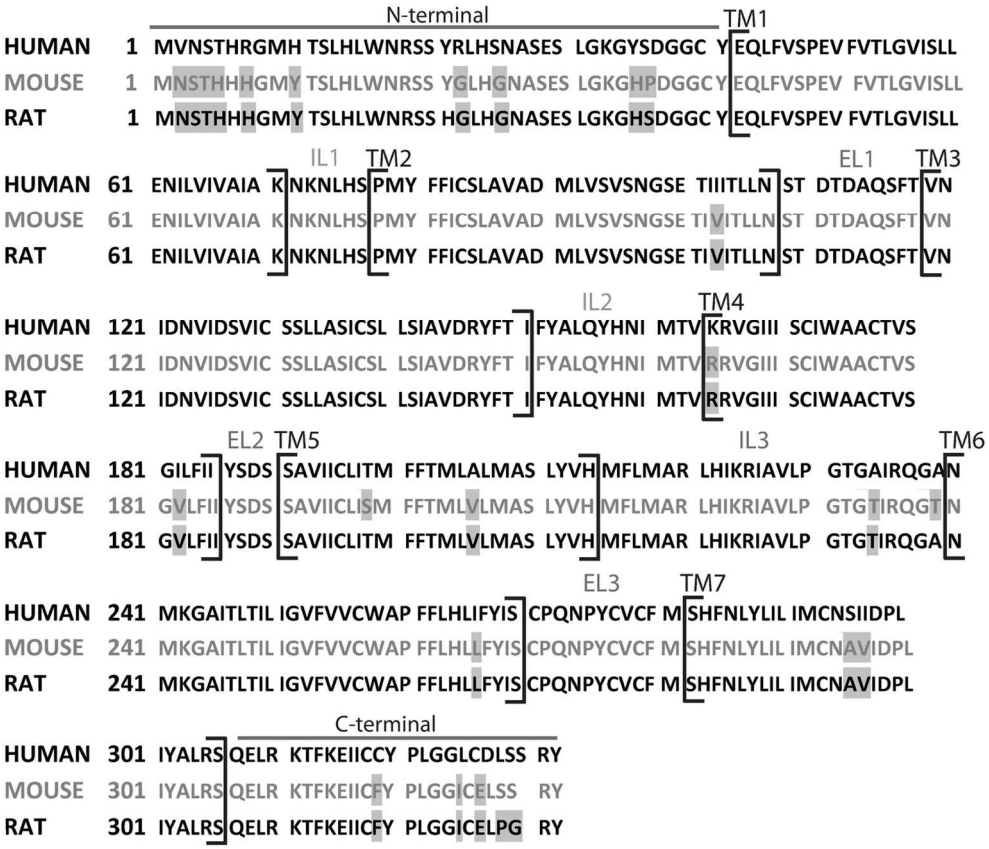
ABBREVIATIONS

Central nervous system (CNS)
Brain-derived neurotrophic factor (BDNF)
Nitric oxide (NO)
Prostaglandins (PGs)
Nuclear factor-κB (NF-κB)
Lipopolysaccharide (LPS)
TLR4 (toll-like receptor 4)
Inducible NO synthase (iNOS)
Cyclo-oxygenase 1 (COX-1)
Glial fibrillary acidic protein (GFAP)
Experimental autoimmune encephalomyelitis (EAE)
Alzheimer's disease (AD)
Multiple sclerosis (MS)
High-fat diet (HFD)
Melanocyte stimulating hormone (MSH)

Adrenocorticotropin (ACTH)
Pro-opiomelanocortin (POMC)
Pro-hormone convertases (PCs)
Agouti-related peptide (AGRP)
cAMP responsive element binding protein (CREB)
Protein kinase A (PKA)
Mitogen-activated protein kinase (MAPK)
Extracellular regulated-kinase (ERK)
Melanocortin 2 receptor accessory protein (MRAP)
(Nle^4, $D\text{-}Phe^7$)-α-MSH (NDP-MSH)
Melanotan II (MTII)
Interferon-γ (INF-γ)
Paraventricular nucleus (PVN)
Arcuate nucleus (ARC)
Lateral hypothalamic area (LHA)
AMP-activated protein kinase (AMPK)
Transforming growth factor-β (TGF-β)
Peroxisome proliferator-activated receptors (PPARs)

Table I- Central MC4R-mediated effects

Effect on	MC4R agonist or antagonist action	References
Energy homeostasis	α-MSH and β-MSH decrease food intake	(Abbott, et al. 2000)
	MTII agonist reduces food intake and increases metabolic rate	(Chen, et al. 2000b)
	AGRP antagonist increases food intake	(Rossi, et al. 1998)
Sexual function	THIQ agonist increases erectile activity and enhances copulatory behavior	(Van der Ploeg et al. 2002)
	MTII induces and SHU9119 completely blocks penile erection	(Wessells, et al. 2003)
Reproduction	MTII agonist increases luteinizing hormone and prolactin secretion in female fasted rats	(Schioth, et al. 2001)
	AGRP reduces luteinizing hormone and prolactin surge in female fasted rats	(Watanobe, et al. 2001)
	AGRP induces luteinizing hormone and follicular stimulating hormone release in male rats	(Stanley, et al. 1999)
	MC4R function restores pubertal onset, fertility and lactation in mice	(Israel, et al. 2012)
Cachexia	AGRP attenuates cardiac cachexia in heart failure and in mice-bearing tumor	(Scarlett, et al. 2010) (Joppa, et al. 2007)
	Small-melanocortin inhibitors attenuate cachexia	(DeBoer 2010)
Pain	MTII increases and antagonist SHU9119 decreases sensitivity to pain.	(Starowicz et al. 2002) (Bertorelli et al. 2005)
	AGRP reduces mechanical allodynia in a model of chronic pain in rats	
Neuroprotection	HS024 selective MC4R antagonist blocks NDP-MSH protective effect on cerebral ischemia	(Giuliani et al. 2006) (Giuliani et al. 2009)
	MC4R antagonist prevents the increase in neurite outgrowth induced by α-MSH in Neuro2A cells	(Adan et al. 1996)
Memory	HS014 blocks α-MSH-induced recovery from memory impairment produced by IL-1β in rats	(Gonzalez et al. 2009) (Machado et al. 2010)
	NDP-MSH improves memory and learning of gerbils	(Giuliani et al. 2011)
Inflammation	HS024 blocks α-MSH-induced reduction of iNOS and COX-2 expression induced by LPS in rat hypothalamus and by LPS+IFN-γ in astrocytes	(Caruso et al. 2004) (Caruso et al. 2007)
Fever	Selective MC4R agonist MRLOB-001 suppresses LPS-induced fever	(Sinha, et al. 2003)
	HS014 blocks α-MSH anti-pyretic effect	(Sinha et al. 2004)



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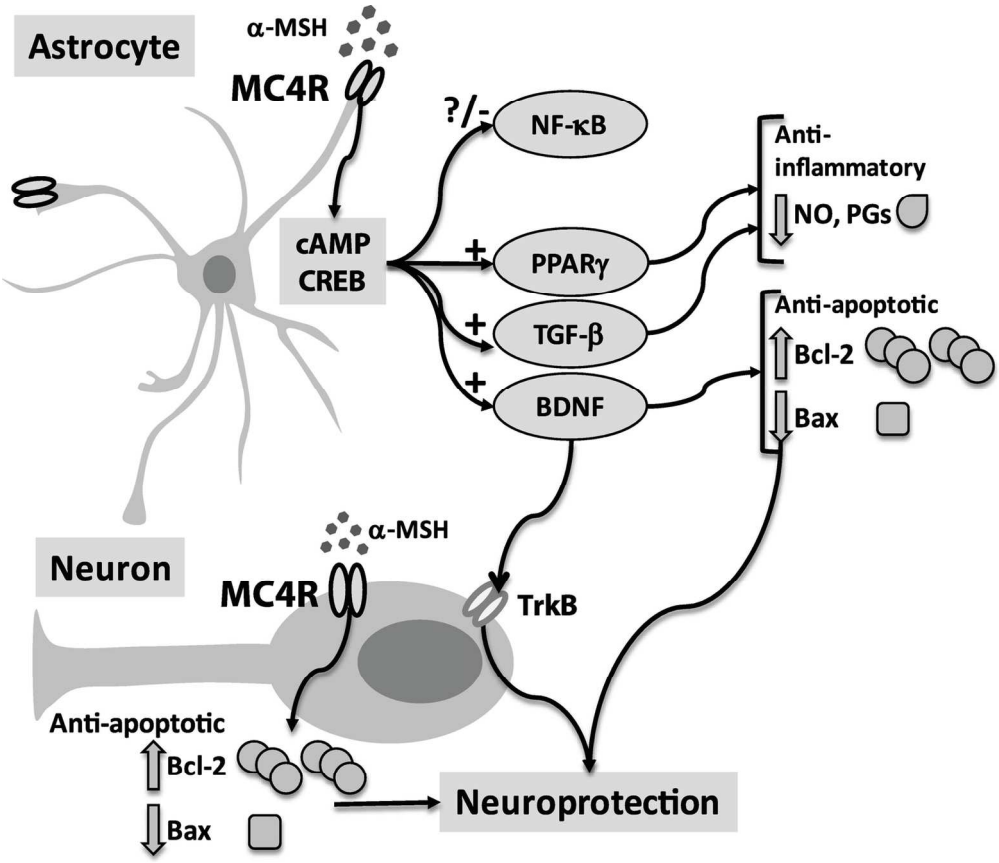

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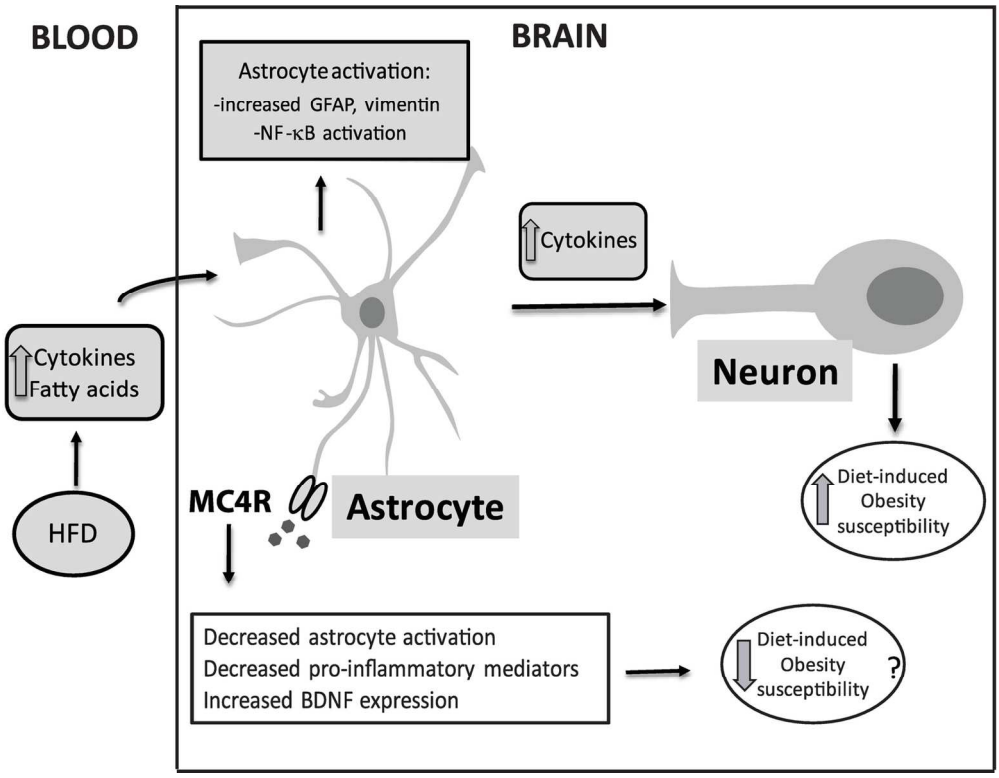

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