Experimental Autoimmune Encephalomyelitis: A Neurological Challenge for Gonadotropin-Releasing Hormone (GnRH)

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Abstract: Experimental Autoimmune Encephalomyelitis (EAE), a model of Multiple Sclerosis (MS), is a disorder characterized by the infiltration of lymphocytes and monocytes, the activation of the microglia, demyelination and an axonal loss. Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide with a role as a primary regulator of gonadal function. However, it has been established that GnRH is expressed in many non-hypothalamic tissues. This neurohormone presents a variety of actions including neurotrophic properties. The administration of GnRH to animals with EAE reduces the severity of the disease. GnRH produces a significant recovery in the clinical signs of locomotion, an increase in both the level of the proteins involved in the process of neuroregeneration and in the axonal diameter of spinal cord neurons. This fact could lead to the generation of novel therapies against MS.

Keywords: GnRH, neurotrophic factor, multiple sclerosis, experimental autoimmune encephalomyelitis, neurofilaments, neuroregeneration.

MULTIPLE SCLEROSIS - EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Introduction

The central nervous system (CNS) was considered for many years as an immunologically privileged site, as a region of the body that does not generate a normal inflammatory immune response to strange antigens. However, now it is clear that inflammatory immune responses may occur in it and although they differ from classic immune inflammatory responses in the periphery, many of the same cells are involved [1]. Multiple sclerosis (MS) is an autoimmune inflammatory disease affecting more than one million people worldwide, women two times more than men [2]. It occurs in approximately 0.1 % of Caucasians in North and Central Europe, and it is the most common demyelinating disease in young adults. It has a range from 10 to 59 years old [3], with the majority of cases between 20 and 40 years old. In rare cases, the onset of illness may occur before 10 years of age or after 59 years old [4].

To begin with, MS normally occurs as a recidivist-remittent disease with recurrent attacks producing a set of neurological deficits that include loss of sensations, blindness, lack of motor coordination or autonomous disorders of intestine and bladder [5]. Symptoms may disappear completely between attacks but often persist as the disease progresses [1].

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Neurological Characteristics

MS is a disorder characterized by the infiltration of lymphocytes and monocytes, the activation of the microglia, demyelination and by an axonal loss [2]. Neurodegeneration in MS is a consequence of demyelination, which generates plaques in the white substance, which are features of this pathology. Specifically, a plaque is an area of white matter in which the inflammatory response has stripped it of its myelin coating, and then non-neuronal cells in the brain or spinal cord have caused cicatrization, including the microglia [1]. In spite of inflammation, infiltration of immune cells and demyelination in the brain and spinal cord are the main characteristics of injuries caused in MS [6]. The patients with neurological alterations correlate best with axonal degeneration [7].

Immunologic Characteristics

It is believed that MS occurs in individuals genetically biased and exposed to a environmental trigger that induces the activation of specific T cells against myelin, as it occurs in a viral infection [8] and this fact allows T cells to cross the blood-brain barrier [6]. However, it is important to recognize that such genetic variants associated with the disease may not necessarily cause it, but are perhaps markers for it [7].

Immune responses in the CNS involve not only the activation of resident cells including the microglia, astrocytes and endothelial cells, but also the infiltration of circulating immune cells (monocytes, neutrophils and T cells). As in the periphery, both activated resident cells as well as infiltrated cells express, release and/or respond to pro and anti-inflammatory cytokines in the immune regulation in the CNS, as well as the glial function [1].

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This point of view, in which we consider MS a T lymphocyte- mediated autoimmune disease, is derived mainly from studies on a single animal model, the experimental autoimmune encephalomyelitis (EAE) [9].

Physiopathology

In the early stages of MS, a decrease in density and opening of the channels of Na^+ in the internodes occurs, which leads to inflammation and results in the release of immune products such as cytokines, adhesion products and nitric oxide from the cell [10,11]. This fact reduces the velocity of nervous impulse conduction through the axons and to generate the symptoms of the disease [10]. Another process involved in the physiopathology of MS is protein carbonylation (non-enzymatic addition of aldehydes or ketones) to specific aminoacids. Likely, the oxidative stress conditions during the course of the disease are sufficient to cause protein carbonylation and generate proteolysis of cytoskeletal proteins [12].

Pathogenesis

The most currently accepted hypothesis is that MS is the result of a particular genetic predisposition and an unknown environmental factor that would cause T cells to be autoreactive, which after a period of latency -10 to 20 years- would be activated by a systemic or local factor [7,13]. This would lead to an autoimmune reaction in which the blood-brain barrier would be crossed by the autoreactive T cells, which could cause an inflammatory reaction and subsequent demyelination [6,10].

To start inflammation in the CNS, specific T cells against myelin must be activated on the periphery, gain access to the CNS and then be reactivated by the antigen presenter cells. Reactivation of T cells induces the production of soluble mediators by many cell types that recruit other inflammatory cells. While the CNS is protected from cell infiltration by the blood-brain barrier and by the barrier of cerebrospinal fluid, memory and activated T cells can lower its defense mechanism because they express adhesion molecules, chemokines and integrins enabling them to cross these barriers [6].

The cytokines are intimately involved in the pathology of MS and the EAE [2]. Previously it was believed that Th1 cells directed by IL-12 were pathogenic in MS and EAE [14], because T cells as IL-12 directed differentiation of reactive Th1 cell myelin and probably facilitate entry in the CNS, weakening the blood-brain barrier. In addition, two very marked effects of IL-12 in the physiopathology of MS is the increase in inflammation of the CNS and activation of the glia. This fact could be due to IL-12 mediating these effects through the production of IFN-gamma and nitric oxide [11]. However, recent data have established that T CD4+ cells that produce IL-17 targeted by IL-23, called Th17 cells, play an essential role in the pathogenesis of EAE. These results were obtained through experiments in which the signaling of IL-12 routes and their subunits is blocked (IL-12 is a heterodimeric cytokine composed of p35 and p40 subunits), in which a decline in EAE was expected to be observed. However, when working with p35 subunit deficient mice, these were susceptible to EAE; while deficient mice in the p40 subunit were resistant [14]. It has also been shown that dendritic cells of patients with MS secrete large amounts of IL-23 and express high levels of IL-23p19 mRNA [15]. Therefore, the axis IL-23/Th17 is important for the development of EAE, rather than IL-12/Th1, still important not only for animal models but also in chronic inflammatory diseases [14].

However, the adoptive transfer cells Th1 and Th17 may induce EAE and clinical paralysis in mice, but the induced pathology by Th17 cells differs from that induced by Th1 cells. Therefore, it is unlikely that Th17 cells could be unique participants in the development of damage to tissues in this classic model of autoimmunity [16].

There are many cytokines that have influence on the pathogenesis of MS, some of which trigger an inflammatory response in the white substance of the CNS, as is the case for IL-6, IL-17, TGF-β and IFN-γ. Moreover, all cytokines involved are important in the processes of cell differentiation towards Th1 and Th17 profiles. Th1 and Th17 differentiated cells express other proinflammatory cytokines; for example, Th1 cells secrete tumor necrosis factor (TNF-α) which is also part of the development of EAE [16] producing autoimmune inflammation in the brainstem and spinal cord exacerbating the clinical symptoms of the disease [2].

EAE as MS Model

The origins of the EAE date from the 1920s, when Koritschoner and Schweinburg led inflammation in the spinal cord of rabbits by inoculation with human spinal cord [9]. In the 1930s, some researchers tried to reproduce the brain complications associated with the vaccination of rabies by repetitive immunization with tissue of the CNS in rhesus monkeys [17]. Since then, EAE has been developed in different species, including rodents and primates, and from these studies it has become clear that EAE can reproduce many clinical, neuropathological and immunological aspects of MS [18-20].

EAE can be induced in sensitive animals by immunization with brain homogenized and purified neural antigens, such as: myelin basic protein (MBP), proteolipidic protein (PLP), myelin oligodendrocyte glycoprotein (MOG), or by adoptive transfer of specific T-cells to neural antigens [21], and includes the breakdown of the blood-brain barrier, nervous system infiltration by TCD4+ cells and macrophages, the activation of endogenous glial cells (microglia and astrocytes) and the demyelination due to loss of oligodendrocytes [22].

GONADOTROPIN-RELEASING HORMONE

Introduction

The gonadotropin-releasing hormone (GnRH) is a decapeptid (Glu-His-Try-Ser-Tyr-Gly-Leu-Arg-Pro-Gly) of 1.2 kDa of molecular weight, which is synthesized mainly in hypothalamic neurons from medial preoptic area. The effect that this hypothalamic hormone exerts on pituitary cell gonadotropes is well demonstrated, which is to increase the synthesis and secretion of gonadotropic hormones: follicle-stimulating hormone (FSH) and luteinizing hormone (LH); which in turn induce both the synthesis and secretion of sex hormones, as well as the generation of gametes (ova and sperm) [23].

Nerve cells that synthesize and release GnRH (GnRHergic neurons) have their embryonic origin in the olfactory placodes. Since embryonic origin, cells migrate and colonize the basal forebrain, around the preoptic area and the median basal hypothalamus. In mammals, the median eminence contains a large amount of GnRH, so it is the area in which the peptide is stored in the neural terminals before their release to the hypothalamic-hypophysial portal vessels system [24].

Molecular Synthesis

GnRH is synthesized as a pro-hormone, containing 92 aminoacids. The peptide signal is located in the first 23 amino, followed by the decapeptid of GnRH, with an excision of 3 aminoacids and a 56-aminoacid product site as an associated peptide to GnRH (GAP). Post-transcriptional GnRH processing involves breakdown by a pro-hormone convertase, elimination of basic aminoacids by a carboxypeptidase, modification of the terminal amino by action of glutaminyl cyclase and modification of the terminal carboxy by peptidylglycine α-amido mono-oxygenase. This enzyme processing produces mature GnRH [25].

Secretion

After the synthesis, GnRH is packaged in secretory granules and transported to the median eminence where the peptide is released from GnRHergic terminals within the perivascular system space [26]. This is due to the fact that in the hypothalamus, two types of glial cells -astrocytes and ependimocytes- which constitute the latero-ventral portion of the third ventricle, regulate the secretory neuroendocrin neurons activity. These cells regulate the GnRH secretion in two ways: 1) involving the production of growth factors through receptors with tyrosin-kinase activity and 2) involving plastic adjustments of the adhesion between GnRHergic neurons and the glia. The GnRHergic axons reach the median eminence -at least in part- guided by basic fibroblast growth factor [27].

In adult rodents, GnRH is released from median eminence with a frequency of one pulse each 30 min; while in primates a frequency of release of GnRH at intervals of approximately 50-60 min has been observed [26].

GnRH Outside the Hypothalamo-Pituitary-Reproductive Axis

In the last decades, it has been established that GnRH is expressed in many non-hypothalamic tissues, including ovarian, uterus, placenta, testes, breast, immune system, pancreas, adrenal glands, tooth and prostate, as well as in a large amount of cell lines and tumors. A considerable number of studies showed that GnRH has a variety of actions, including the facilitation of steroidogenesis, cell proliferation, apoptosis, fertilization, embryo implantation, adhesion to extracellular matrix and cell migration. An important role of GnRH and its receptor has been identified in the regulation of growth tumor in ovarian cancer, breast tumors, endometrial carcinoma and prostate cancer as an autocrine/paracrine mechanism [28,29].

The GnRH Receptor

GnRH actions are mediated by its receptor in the pituitary gonadotropes. To date, only a conventional GnRH receptor subtype (GnRH-R type I) with the loss of terminal carboxy has been found in humans [23]. GnRH receptors are classic receptors coupled to protein-$G_{\alpha q}$-11-phospholipase C with seven transmembrane domains connected by intracellular and extracellular loops [30]. However, it has been described recently that the signal pathway may be different from protein-$G_{\alpha i}$ in extrapituitary tissues such as ovarian, prostate and tumoral cells, implying the activation of mitogen-activated protein kinase (MAPK) phosphatidyl-inositol-3-kinase (PI3K) and nuclear factor kappa B (NF-k B) [31].

GnRH receptors have also been found in different areas of the brain such as the hippocampus, lateral septal nucleus, amygdala, lateral band of Broca and lateral cortex of the cingula. In addition, these receptors have functional characteristics similar to the pituitary gonadotropes [32].

Recently in our laboratory, we have found the presence of the receptor to GnRH in cerebral cortex neurons and spinal cord neurons of embryo and of adult rat, using immunohistochemistry, RT-PCR and electrophoresis techniques. Likewise the activation of this receptor by its natural agonist (GnRH) induces a decrease in the expression of the mRNA to its own receptor as a down regulation mechanism [33,34].

GnRH isoforms

To date, about 20 isoforms have been characterized in species of mammals and non-mammals [28]. However, only two forms of GnRH have been identified in the human genome [35]: GnRHI and GnRHII [23], whose genes are found in chromosomes 8 and 20 respectively [25].

The first form of GnRH (GnRHI) was isolated in mammals by Guillermin and Schally in 1967 [25] and the second sub-type of GnRH (GnRHII) was originally identified in chicken hypothalamus and has been found in humans [23].

GnRHI shares 60 % of identity among mammals and the tunicata, while GnRHII is highly conserved (in 100 %) between birds and mammals [25].

The second form differs from GnRHI by three aminoacids in positions 5, 7 and 8 (His $^{5\,7}$ Trp^{7} Tyr ^{8} GnRHI) [23]. In contrast to GnRHI, GnRHII expression is significantly elevated outside the brain and is particularly abundant in the kidney, bone marrow and prostate. Although GnRHII can stimulate gonadotropin secretion, its efficiency is much lower than that of GnRHI (only about 2%), which suggests that this is not its main role, acting in fact as neuromodulator. The exact functions of GnRHII are still unknown. However, some are known extra-pituitary actions in human, such as tumoral proliferation suppression, despite the transcript from the GnRHII receptor has not been identified in any human tissue or cell type [23]. GnRHI has a very short half-life (less than 4 minutes), which suggests that GnRHI acts locally in an autocrine and/or paracrine way [28].

EAE AND GnRH

Neurotrophic Factors

In MS or EAE, the immune system causes a degenerative process via autoimmune inflammatory mechanisms. Thus, axon damage or neural loss occurring during a late phase of the disease play a crucial role and are determinant in the destabilization of the nervous system. However, members of the neurotrophins such as the Brain Derived Neurotrophic

Factor (BDNF), neurotrophins NT-3 and NT-4 are important regulators of neuronal function and survival. Their role during neuronal growth processes is well established, the regulation of synaptic plasticity and capacity to protect neurons against several pathogens [36-38].

BDNF in particular, presents an important protection against neuronal degeneration and further promotes axonal growth and myelin repair, acting as well as a modulator in the CNS with very important therapeutic prospects [39]. The increased levels of neurotrophins in late stages of EAE may reflect a similarity to neurogenesis initiated by EAE and other brain alterations. They also exert an anti-apoptotic effect which is mediated in part by stimulation in the production of proteins of the Bc1-2 family, which inhibit the destruction of caspases. However, limitations to clinical use of these factors are present because they do not cross the blood-brain barrier. One alternative is the introduction of transformed cells (producers of neurotrophins) [39]. Also, it has been recently found that the Ciliary Neurotrophic Factor (CNTF) promotes myelogenesis and reduces inflammation in animals with EAE [40].

Neurofilaments

On the other hand, we find very close links between elements of the neuronal cytoskeleton when neurodegenerative processes occur, where the most representative proteins are the neurofilaments (NFs) [41]. The NFs are the largest type of intermediate filaments in neurons. They maintain neuronal size and shape and axonal caliber. NFs increase with development; this increase stabilizes the cytoarchitecture and neuronal size. NFs consist of three subunits: light weight 68 kDa (NF-L), medium of 160 kDa (NF-M) and heavy of 200 kDa (NF-H) [42].

Recently NFs and glial fibrillary acidic protein (GFAP) have been used as markers for axonal and glial damage. An increase in both NF-L and GFAP was particularly noted in cerebrospinal fluid of patients with MS or the EAE model [12,43]. Both humans and experimental models histological cuts of spinal cord reveal a clear decrease in axonal diameter evaluated by immunohistochemistry against neurofilaments in EAE [44].

GnRH as Neurotrophic Factor

The primary function of GnRH is to stimulate the synthesis and release of FSH and LH from pituitary gonadotropes of vertebrates. Its action is mediated by specific receptors located on the external cell membrane surface. However, as described above, both GnRH and its receptor are located in other extrahypothalamic and extrapituitary tissues respectively. There are evidences on the possible role of GnRH in other tissues outside the reproductive system.

In experiments in our laboratory, we have found that neurons of cerebral cortex and spinal cord of rat, from the embryonic stage until the adult stage, expressed the receptor to GnRH and that it is sensitive to GnRH. The latter was found when these neurons were incubated with GnRH applied the pulsatile way and the level of expression of the mRNA for the receptor decreased significantly. This regulation of the expression of the receptor can be as it occurs in the pituitary gonadotropes [33,34]. In histological analysis in cuts of spinal cord, we found GnRH receptor presence in the gray substance, particularly in the motoneurons [34].

Considering the possible role of GnRH with neurotrophic properties, in another series of experiments using cultured cerebral neurons of rat embryos, we have found that GnRH induces an increase in both growth and number of neurites, as well as an increase in the expression of neurofilaments, particularly NF-M and NF-H [45]. These results give the possibility that GnRH can act as a neurotrophic factor as it occurs in the hippocampus, where treatment with GnRH induces changes in the density of dendritic spines [46] and envelopes the synaptic transmission mediated by the receptor to glutamate [47].

This background strongly suggests the possibility that GnRH can act as a neurotrophic factor also in spinal cord neurons. This consideration is based on the presence of its receptors to GnRH in the motoneurons [34,48]. In the case that some neurological disorder could affect the spinal cord, where the axons were compromised, either in their diameter as well as in its NF content, GnRH treatment could be effective for the formation of new neurites, and both the increase of axonal diameter as well as the expression of NFs. This possibility is a viable alternative for treatment against neurodegenerative diseases or neuronal regeneration.

GnRH Treatment in Animals with EAE

In accordance with these possibilities, we decided to perform experiments on rats with EAE where neurological degeneration occurs.

Our results indicate that treatment with GnRH modifies the severity of the disease. We have specifically noted that in male rats with EAE (castrated to avoid the steroid sex hormones influence), the treatment with low doses in the physiological range of GnRH [49] produced a significant recovery in the clinical signs of locomotion [50].

Furthermore, we find that the axonal diameter of neurons of the spinal cord is increased significantly in animals with EAE treated with GnRH, compared with animals without treatment. Very similar results were obtained in the normal control animals without EAE. In the electrophoretical analysis of spinal cord of male animals with EAE treated with GnRH, the levels of expression of NFs of 68, 160 and 200 kDa were higher than those without treatment and the values were similar to normal animals [50].

These facts indicate that the administration of GnRH to animals with EAE significantly reduces the severity of the disease, both in locomotor signs as well as in the expression of proteins involved in the process. The increase in the expression of NFs is correlated directly with axonic diameter, which suggests a better response in the locomotion of animals with EAE. There is a strong hypothesis on the participation of mitochondria in axonal spinal cord damage during MS [51]. However, it would be difficult to associate GnRH with mitochondrial stability due to the routes of intracellular signaling.

The mechanism by which GnRH exerts its effect on morphological and biochemical recovery of spinal cord neurons in EAE is unknown. According to the background mentioned above, some options may be considered: a) that the signaling path is through G-protein$_{\alpha q}$-11-phospholipase C or b) pro-

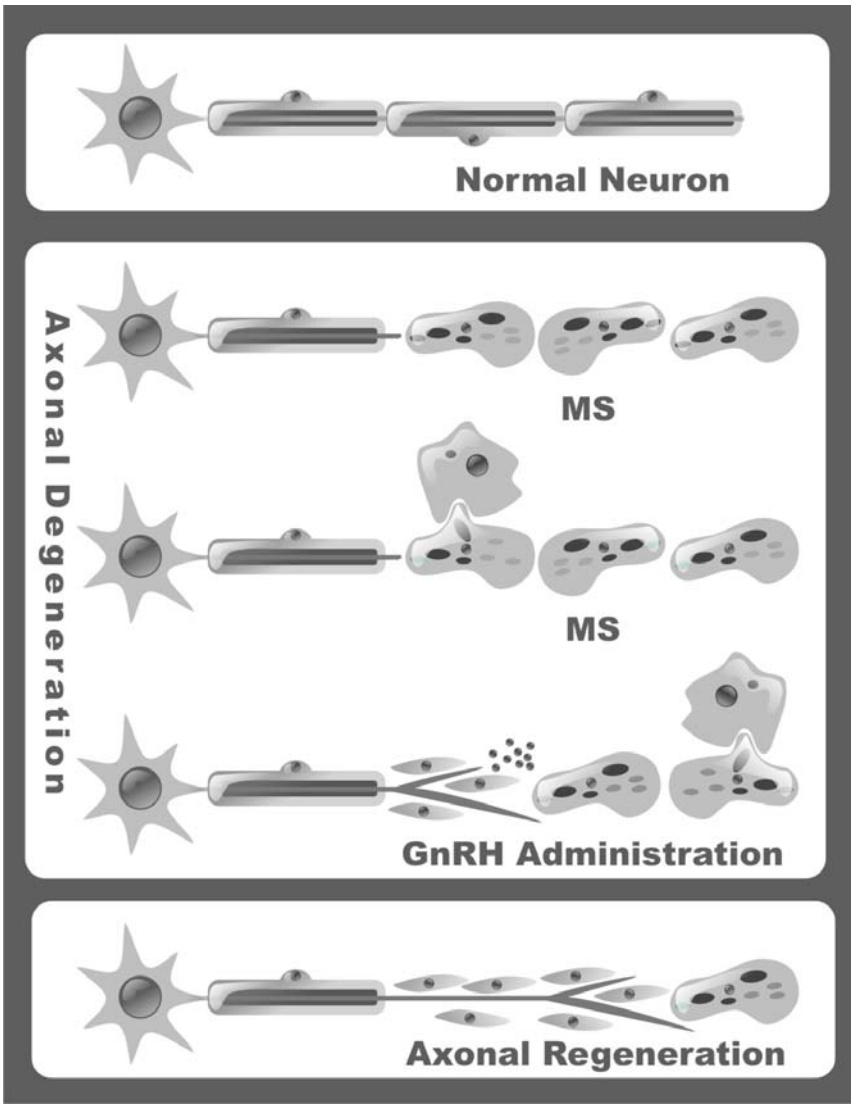

Fig. (1). Possible effect of GnRH on axonal regeneration in multiple sclerosis (MS). During MS the myelin is destroyed by immune system cells which may generate axonal degeneration. GnRH administration induces axonal regeneration.

tein-$G_{\alpha i}$ that would involve the activation of MAPK, PI3K and NF-κ B. In both cases, signaling would get to the cell nucleus and would lead gene expression for the synthesis of proteins such as NFs and thus, their neurites.

Although the administration of GnRH reduces neurological damage caused by EAE, we still do not have evidence that this neurohormone can exert some effect on the level of autoimmune response against its own nervous system. Administering immunosuppressive agents in animals with EAE could have a synergic impact with the GnRH treatment; i.e. to reduce the level of attack (immunosuppression) and to induce the regeneration (GnRH) of neurons damaged by disease (Fig. **1**).

Immunoreactive material to GnRH has been found in spinal cord neurons of sheep [48]. This fact could mean the possible participation of GnRH as neurotransmitter or neuromodulator in the physiological processes of spinal cord. However, as an endogenous therapeutic mechanism is not possible, because the fact that recovery from MS or EAE does not occur naturally.

CONCLUSION

This overview shows that MS or EAE is a disorder characterized by the infiltration of lymphocytes and monocytes, the activation of the microglia, demyelination and by an axonal loss. GnRH modulates a variety of functions outside of reproductive axis. Particularly, that GnRH could be used as a neurotrophic factor. The administration of GnRH to animals with EAE significantly reduces the severity of the disease, both in locomotor signs as well as in the expression of proteins involved in the process of neuroregeneration. This fact could lead to the generation of novel therapies against MS.

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