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5. Neuroprotective gene therapy in the aging brain

Joaquín Pardo*, Gustavo Ramón Morel, Andrea Soledad Pereyra, Micaela López-León, Oscar Alfredo Brown, Maria José Bellini and Rodolfo Gustavo Goya

Institute for Biochemical Research (INIBIOLP)-Cathedra of Histology B, Cathedra of Pathology B, School of Medicine, National University of La Plata La Plata, Argentina

Abstract. Aging is associated with a progressive increase in the incidence of neurodegenerative diseases in both laboratory animals and humans. In the central nervous system, cholinergic and dopaminergic (DA) neurons are among the cells most susceptible to the deleterious effects of age. Thus, the basal forebrain cholinergic system is known to undergo moderate neurodegenerative changes during normal aging as well as severe atrophy in Alzheimer's disease (AD). Parkinson's disease (PD), a degeneration of nigro-striatal DA neurons is the most conspicuous reflection of the vulnerability of DA neurons to age. Overall, there is growing evidence that a progressive decline in cognitive function and central DA activity represents basic features of normal aging both in humans and laboratory rodents. Exacerbation of these processes contributes to the symptoms of AD and PD, respectively. In this context, neurotrophic factors that can prevent or delay the decline in cognitive function and central DA activity during normal

Correspondence/Reprint request: Dr. Rodolfo G. Goya, INIBIOLP, School of Medicine, UNLP, CC 455, 1900 La Plata, Argentina. E-mail: goya@isis.unlp.edu.ar; *E-mail: joaquin_p88@hotmail.com

aging may reveal new therapeutic avenues for treatment of AD and PD and are therefore of clinical interest. Among the peptide factors, Insulin-like Growth Factor I (IGF-I) and Glial cell line-Derived Neurotrophic Factor are emerging as powerful neuroprotective molecules for the treatment of neurodegenerative pathologies. Among the neurosteroids, estrogens are recognized as neuroprotective and seem to act synergistically with IGF-I and possibly other neurotrophic peptides. This chapter will discuss the evidence supporting the neuroprotective relevance of the above factors.

1. Introduction

Aging is associated with a progressive increase in the incidence of neurodegenerative diseases in both laboratory animals and humans. In the central nervous system (CNS), cholinergic and dopaminergic (DA) neurons are amongst the cells most susceptible to the deleterious effects of age. Thus, the basal forebrain cholinergic system is known to undergo moderate neurodegenerative changes during normal aging as well as severe atrophy in Alzheimer´s Disease (AD). In fact, the cholinergic degeneration in AD seems to occur against a background of age-related atrophy and the exacerbated atrophy in AD can be detected at very early stages of cognitive impairment [1]. In rats, aging is associated with degenerative and/or atrophic changes in the forebrain cholinergic system and these morphologic changes are paralleled by a decline in spatial learning ability [2].

Parkinson's disease (PD), a motor disorder characterized by progressive loss of DA neurons of the substantia nigra (SN), affects 0.1-0.3% of the population, is the most conspicuous reflection of the vulnerability of DA neurons to age. In rats, aging brings about a progressive degeneration and loss of another group of central DA neurons namely, the hypothalamic tuberoinfundibular dopaminergic (TIDA) neurons, which are involved in the tonic inhibitory control of prolactin (PRL) secretion and lactotropic cell proliferation in the adenohypophysis [3]. Progressive dysfunction and loss of TIDA neurons during normal aging is associated in the female rat, with chronic hyperprolactinemia [4] and the development of pituitary prolactinomas [5]. Although aging rats do not develop parkinsonian symptoms, even at 32 months of age, they lose 35-40% nigral DA neurons and show a marked decline in motor performance [6]. In humans, normal aging is also associated with a decline in motor performance and a progressive loss of nigral DA neurons [7]. Therefore, progressive decline in cognitive function and central DA activity seems to represent basic features of normal aging both in humans and laboratory rodents. Exacerbation of these processes would lead to AD and PD, respectively.

In this context, treatments that can prevent or delay the decline in cognitive function and central DA activity during normal aging may prove to be effective interventions to prevent or delay the progress of AD and PD.

2. Neuroprotective gene therapy

Gene therapy, the transfer of genetic material for therapeutic purposes, has undergone a remarkable development in the last 20 years. Particularly important advances have been made in the improvement of gene transfer and expression technology, with current efforts focusing on the design of safer and longer-expression gene vectors as well as systems possessing cell-type specificity for transgene delivery and regulatability of its expression by small molecules.

Gene transfer to the CNS possesses significant challenges due to both the relative inaccessibility of the brain and spinal cord and the extraordinary complexity of CNS structures. An important hurdle is the blood-brain-barrier (BBB), which prevents gene vectors from reaching their therapeutic targets within the CNS. There are some approaches to overcome this problem, e.g. using osmotic disruption of the BBB, a technique to increase the delivery of suspensions to the brain that has been used clinically for more than 10 years. Typically, mannitol solutions disrupt the BBB transiently and reversibly by shrinking endothelial cells and opening the tight junctions [8].

On the other hand, gene therapy offers unique advantages for the long-term delivery of neurotrophic factors to specific CNS regions affected by neurodegenerative processes. Up to now, a number of vectors to deliver therapeutic genes have been designed in order to accomplish appropriate delivery to the host. Non-viral gene delivery vehicles, such as naked DNA, RNA, liposomes and nanoparticles, which are able to harbor large cargo and possess lower costs than viral systems have so far only achieved low levels of therapeutic gene expression during short periods. On the other hand, a large number of genetically modified viruses have proved to efficiently transduce host cells with the therapeutic gene they carry. Viral vectors commonly used are, helper-dependent adenoviral, adeno-associated, retroviral and herpes-derived vectors, which have specific advantages such as a large size of the gene insert they can accomodate, a wide variety of cells they can transduce or an extended duration of transgene expression [9].

There is a growing number of endogenous molecules now recognized as neuroprotective, some of which have been used or are amenable to be used for implementing neuroprotective gene therapy in neurodegenerative processes. They have been tested *in vitro* and in animal models of neurological disorders like PD, AD, epilepsy, amyotrophic lateral sclerosis, stroke and some brain insults.

3. Insulin-like growth factor I (IGF-I)

3.1. IGF-I as a physiologic neuroprotective molecule

There is clear evidence that insulin-like growth factor-I (IGF-I) plays a physiologic role in neuroprotection. Thus, IGF-I is strongly induced in the CNS after different insults such as ischemia, [10] cortical injury [11-12] and spinal cord lesions [13]. In situations involving cytotoxic damage in the hippocampus, the microglia of this region dramatically increases the production of IGF-I and IGF-I binding protein 2, which suggests a neuroprotective role of these molecules in the CNS [14]. Also, the neuroprotective effect of physical exercise in rodent models of ataxia, domoic acid-mediated hippocampal damage and inherited Purkinje cell degeneration (pcd mouse model) was reported to be mediated by circulating IGF-I [15]. *In vitro* studies have shown that IGF-I increases cell survival in primary hypothalamic cell cultures [16] and stimulates differentiation of rat mesencephalic DA neurons [17]. A protective effect of IGF-I has been reported in immortalized hypothalamic cells exposed to reduced glutathione-depleting agents [18], in human DA cell cultures exposed to the toxin salsolinol [19] and in human and rodent neuronal cultures exposed to toxic doses of DA [20].

3.2. Therapeutic potential of IGF-1 for neuroprotection

Direct IGF-I infusion has been used to protect different brain regions. For instance, studies in 6-hydroxydopamine-lesioned rats suggest that IGF-I mediates the neuroprotective effect of estrogen on nigral DA neurons [21]. In a rat model of cerebellar ataxia (induced by 3-acetylpyridine (AC)), subcutaneous (sc) or intracerebro-ventricular (icv) administration of IGF-I restored motor coordination and partially rescued inferior olive neurons from the toxic effect of AC [15]. Continuous infusion of IGF-I in the lateral ventricle, partially restored reference and working memory in 32- as compared to 4-month old male rats [22]. Furthermore, IGF-I has been reported to protect hippocampal neurons from the toxic effects of amyloid peptides [23]. Interestingly, IGF-I treatment of mice overexpressing a mutant Aβ amyloid peptide markedly reduced their brain burden of Aβ amyloid [24]. A novel approach for IGF-I delivery involves the use of IGF-I-producing human neural progenitor cells (hNPC) which were transplanted into a rat model of PD generated by nigral injection of 6-hydroxydopamine (6-OHDA), a dopaminergic toxin, 7 days prior to the hNPC treatment. The results

showed that the treatment reduced asymmetry rotation and DA neuron loss and increased overall survival of hNPC [25].

A recent study showed that subcutaneous injection of IGF-I coupled to polyethylene glycol in *pmn* mutant mice, a model with typical dying-back motoneuron degeneration, prolonged survival, protected against late stage weight loss and significantly maintained muscle force and motor coordination [26].

3.3. Gene Therapy for IGF-I

Gene therapy for IGF-I has shown promising results in the brain of aging rats. Thus, a recombinant adenoviral vector (RAd-IGFI) harboring the gene for rat IGF-I was used to implement IGF-I gene therapy in the hypothalamus of senile female rats, which display TIDA neurodegeneration and, as a consequence, chronic hyperprolactinemia. Restorative IGF-I gene therapy was implemented in young (5 mo.) and senile (28 mo.) female rats, which received a single intrahypothalamic injection of RAd-βgal (a control adenoviral vector expressing β-galactosidase) or RAd-IGFI and were sacrificed 17 days post-injection. In the young animals, neither vector modified serum prolactin levels but in the RAd-IGFI-injected senile rats a nearly full reversion of their hyperprolactinemic status was recorded. Morphometric analysis revealed a significant increase in the total number of tyrosine hydroxylase (TH) positive cells in the hypothalamus of experimental as compared with control senile animals (Fig. 1) [27]. These results suggest, although do not prove, that IGF-I may have a neurogenic action on the hypothalamic DA neuron population in senile animals. Interestingly, IGF-I gene therapy did not affect DA neuron population in the hypothalamus of young rats.

In the same animal model, icv IGF-I gene therapy ameliorated the reduced motor performance of the senile animals [28]. This was achieved taking advantage of the fact that the ependimal route for adenovirally-mediated gene delivery is an effective strategy to increase IGF-I levels in the cerebrospinal fluid (CSF) [29]. Thus, in very old rats (30-31 mo.), which show severe motor deterioration, icv IGF-I gene therapy showed a beneficial impact on motor performance. In this study, RAds expressing either green fluorescent protein (GFP) or rat IGF-I were injected into the lateral ventricle which led to high transgene expression in the ependymal cell layer in the brain and cervical spinal cord [28]. RAd-IGF-I-injected rats but not RAd-GFP-injected controls, showed significantly increased levels of CSF IGF-I. Motor tests showed the expected age-related decline in aged rats. Seventeen-day of IGF-I gene therapy induced a significant amelioration in motor performance in aged but not in young animals.

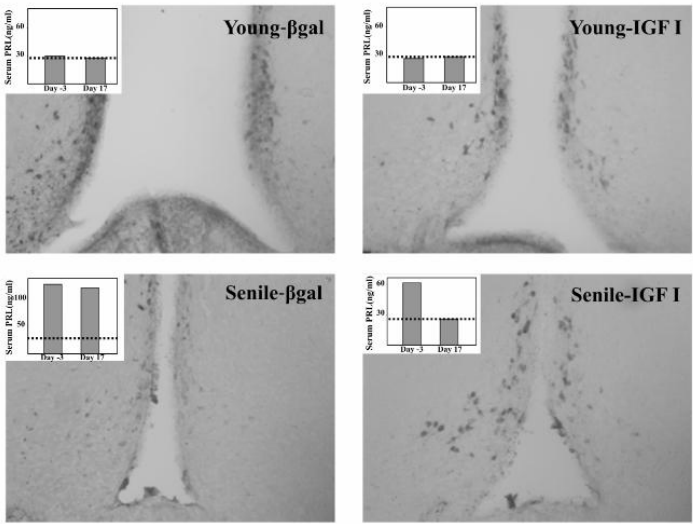

Figure 1. Effect of IGF-I gene therapy on the DA neurons of the ARC-PeV hypothalamic region in young and senile females.- The representative coronal hypothalamic sections shown pass through the medial hypothalamus and were immunolabeled with a monoclonal anti-rat TH antibody. Animals were sacrificed 17 days after the corresponding vector injection in the hypothalamus. The upper panels correspond to young animals injected with either RAd-βgal (left) or RAd-IGFI (right). The lower panels show the corresponding senile counterparts. Insets represent serum PRL levels for the corresponding animal, on experimental day -3 and 17. The dotted line indicates normal values for serum PRL in young female rats. Obj. X 20

In rats, there is substantial evidence that age-related ovarian failure is preceded by abnormal responsiveness of the neuroendocrine axis to estrogen positive feedback [30]. Since IGF-I seems to act as a permissive factor for proper gonadotropin-releasing hormone (GnRH) neuronal response to estrogen positive feedback and taking into consideration that the hypothalamic content of this peptide declines in middle-aged (M-A) rats, the effectiveness of long-term IGF-I gene therapy in the medial basal hypothalamus (MBH) of M-A female rats to extend regular cyclicity and preserve ovarian structure was assessed [31]. A bicistronic adeno-associated vector (rAAV) harboring the genes for IGF-I and the red fluorescent protein DsRed2 was used. Most of the M-A rats injected with the IGF-I rAAV had, on the average, well-preserved estrous cyclicity as well as a generally normal ovarian histology, whereas the control groups

showed a high percentage of acyclic rats at the end of the study and ovaries with numerous enlarged cysts and scarce corpora lutea. These results suggest that overexpression of IGF-I in the MBH prolongs normal ovarian function in M-A female rats [31].

Evidence for the potential of IGF-I as a survival factor for motor neurons comes from a study where high levels of IGF-I expression in the cervical spinal cord of $hSOD1^{G93A}$ rats, a murine model of familial ALS, were induced by means of intraspinal cord injection of a rAAV harboring the gene for IGF-I. This approach reduced the extent of motor neuron loss in the treated segments of the spinal cord in males but, not in females [32].

4. Glial cell line-derived neurotrophic factor (GDNF)

4.1. GDNF and neuroprotection

Glial cell line-derived neurotrophic factor (GDNF) was identified and purified from the B49 glioma cell line based on its ability to promote the survival of dopaminergic neurons in dissociated rat mesencephalic cultures, and to increase their neurite length and cell size as well as their high affinity dopamine uptake; non-dopaminergic neurons or glial cells are not significantly affected by GDNF [33-34]. GDNF is the founding member of the GDNF family of neurotrophic factors, which additionally includes three other structurally related members: neurturin (NTN) [35], persephin [36], and artemin [37]. The members of the GDNF family belong to the transforming growth factor (TGF)-β superfamily. GDNF is active as glycosylated disulfide-bonded homodimer, which triggers a multicomponent receptor complex comprising the transmembrane Ret tyrosine kinase and GFRα, a member of a family of glycosylphosphatidylinositol (GPI)-anchored cell surface proteins [33]. GDNF and NTN have proved to enhance the survival of dopaminergic neurons in rodent and primate models of PD, previously treated with dopaminergic neurotoxins [38-40].

Infusion of the GDNF peptide into the LV, striatum, or SN in rodent or primate models of PD has been reported to protect dopaminergic neurons in the SN, increase dopamine levels in the striatum and SN, and ameliorate behavioral deficits [39, 41-43].

GDNF is active in protecting not only dopaminergic neurons, but also motor, cholinergic and other types of neurons, which makes this molecule a promising candidate for the treatment of several neurodegenerative pathologies, such as PD, AD and ALS [44].

4.2. GDNF peptide and gene therapy for PD

The neuroprotective properties of GDNF mentioned above have generated a great deal of interest in the development of GDNF-based gene therapy strategies to treat PD. In rats and non-human primates, the administration in the striatum or in the SN of adenoviral [45-47], herpes simplex virus (HSV)-derived [48], lentiviral [49-51] or adeno-associated viral (AAV) vectors [52-54] for GDNF has been shown to protect nigral DA neurons from the toxic action of dopaminergic toxins. In this context, AAV and lentiviral vectors are emerging as the most promising tools for long-term high-level transgene expression in the brain.

The successful results in animal models led to the implementation of GDNF peptide therapy in PD patients. However, intraventricular injection of GDNF peptide did not improve the motor scores of PD patients and caused many side effects such as nausea, anorexia, vomiting, weight loss, hyponatremia and paresthesias, including Lehrmitte signs and psychotic manifestations [55-56]. In other studies, the direct administration of GDNF peptide into the putamen of five PD patients improved the patients' United Parkinson's Disease Rating Scale (UPDRS) score in the off -medication state after one year with no serious side effects [57]. Although these studies demonstrated effectiveness, intraputaminal delivery of the GDNF protein was not completely successful. Therefore GDNF clinical trials have been halted largely due to the failure of the neurotrophin to reach a large enough target area within the human striatum and, more importantly, because of the presence of neutralizing anti-GDNF antibodies in a subset of treated patients [58,56,59]. In addition, further review of earlier non-human primate data revealed the presence of cerebellar Purkinje cell degeneration, suggesting that there was some GDNF peptide leakage outside the injection site [60]. In order to improve safety, GDNF was replaced by neurturin (NTN), as this member of GDNF family did not develop neutralizing anti-NTN antibodies or cerebellar degeneration [58,61-62].

4.3. Therapeutic potential of GDNF for non parkinsonian neurodegenerative models

Therapeutic approaches with GDNF have extended to other neurodegenerative models, e.g. motor neuron degeneration [63-64], cerebral ischemia [65], limbic seizure [66], spinal cord motoneuron degeneration [63, 67-68], noradrenergic neuron degeneration of the locus coeruleus [69], cerebellar Purkinje cell degeneration [70], degeneration of cholinergic neurons of the basal forebrain [71], as well as peripheral sensory and autonomic neuron degeneration [72].

Furthermore, there is evidence that GDNF is active in the hypothalamus as it induces body weight loss and ameliorates age-related obesity in rats [73-74]. Short-term GDNF gene therapy in the hypothalamus of senile female rats restored DA neuron function partially and consequently reversed chronic hyperprolactinemia without restoration of dopaminergic neuron number [75]. Another line of evidence suggests that GDNF exerts inhibition of kainic acid-induced seizure activity and prevents the associated neuronal cell loss in hippocampal, thalamic and amygdaloid regions [76]. To our knlowledge, the potential of GDNF against age-related cognitive deterioration has not been fully explored. Interestingly, heterozygous mice for targeted deletion of the GDNF gene demonstrated a significant and selective impairment of performance in the spatial version of the Morris water maze which suggests a role for GDNF in cognition [77]. In another study, when GFRα2-knockout mice were submitted to different learning and memory tests they displayed reduced contextual memory and context discrimination in the fear-conditioning paradigm, impaired behavioral flexibility in spatial learning and decreased retention of conditioned taste aversion [78].

In a pioneer study, GDNF was shown to significantly improve spatial learning in 344 aged impaired Fisher rats after icv administration of the peptide [79]. In gerbils, a single icv administration of Sendai virus harboring the GDNF gene, effectively prevented or delayed hippocampal CA1 neuron death induced by occlusion of both carotid arteries [80]. Lentiviral mediated-GDNF gene therapy in the CA1 dorsal hippocampus improved neurotransmitter secretion and reversed cognitive deficit in aged Fisher 344 rats [81].

5. Neurturin gene therapy

Phase I and II gene therapy trials were completed by Ceregene, Inc. and involved intraputaminal injections of CERE-120, a rAAV2-NTN vector [62, 35]. Previously, this CERE-120 had displayed a consistent pattern of bioactivity and efficacy following CERE-120 administrations, demonstrating that the NTN protein expressed is robustly and consistently bioactive, protecting and/or restoring DA neuron function in rat and non-human primatemodels of PD, as well as in aged rats and aged monkeys [62, 82-83]. Phase I performed in 58 patients with advanced bilateral idiopathic PD demonstrated safety and tolerability as well as an improvement in the off-medication motor subscore of the UPDRS, however, other measures of motor function were not significantly improved [84]. Disappointingly, Phase II trial

did not demonstrate any significant differences in the protocol defined primary by endpoint of UPDRS-motor off score at 12 months between patients treated with CERE-120 and control subjects. In addition, 30 patients were clinically followed in a double-blind fashion for an additional 18 months at which time Ceregene officials reported a modest but statistically significant effect on the UPDRS-motor off score as well as on several secondary measures of motor function. Overall, the Ceregene studies showed that AAV2-NTN treatment had a positive minor restorative effect in PD patients. The small magnitude of the effect was enigmatic since preclinical studies had indicated robust NTN expression from this vector. In conclusion, these clinical trials enrolled patients with advanced PD and presumably significant dopamine neuron loss. Neuroprotective therapies may be more effective in earlier stage patients [85].

6. Parkin

Parkin, a protein encoded by the gene PARK2, is an E3 ubiquitin-protein ligase, whose mutations lead to autosomal recessive juvenile parkinsonism (AR-JP) [86].

Elevating parkin expression in cells reduces markers of oxidative stress while blockade of parkin expression increases oxidative stress. In parkin gene knock down mouse and fly models, mitochondrial function is deficient. Although Parkin is neuroprotective against a variety of toxic insults, it remains unclear which of the above properties of parkin may mediate its protective actions. Some models suggest a putative interaction with α-synuclein and Tau proteins. Parkin interaction with the protein α-synuclein, which aggregates to form parkinsonian Lewy bodies, has been suggested by studies showing that they co-immunoprecipitate. Furthermore, ubiquitinated α–synuclein is present in α-synucleinopathy.

Parkin gene therapy performed in animal models revealed that injection in the SN of a rAAV expressing parkin mitigates α–synuclein toxicity in a rat model of α–synucleinopathy induced by prior nigral injection of a rAAV-expressing α–synuclein [87].

The microtubule-associated protein tau is another protein that self-aggregates in certain neurodegenerative diseases that also involve loss of DA neurons, like frontotemporal dementia with parkinsonism linked to chromosome 17. It has been shown that gene therapy with rAAV vectors expressing Parkin can prevent loss of DA neurons in the SN of rats overexpressing tau [88].

7. Estrogen

7.1. Estrogen and cognition in animal models

Estrogen is a potent steroid of both gonadal and neuronal origin that exerts profound and enduring effects on cognitive function during development, adulthood and aging.

Estrogen, which is one of the neurosteroids synthesized in the brain, is capable of enhancing synaptic plasticity, neurite growth, hippocampal neurogenesis, and long-term potentiation. It also protects against neuron apoptosis and neural injury in a variety of experimental settings, including toxicity-induced by excitatory neurotransmitters, β-amyloid, oxidative stress, and ischemia **[89].** Most data derive from studies in females, but there is evidence that estrogen plays important roles in the male brain, where it can be generated from circulating testosterone by local aromatase or synthesized de novo by neurons and glia **[90].**

Many estrogen actions are potentially relevant to cognitive changes occurring after menopause, but for most, the clinical implications are yet unclear **[91-92].** However, it is important to remark that estradiol does not enhance all aspects of cognition.

7.2. Estrogen therapy and cognitive function- points and counterpoints in clinical trials

Results from studies conducted in animals suggest that the initiation of estrogen therapy at the time of menopause, or soon after ovariectomy, provides an opportunity to prevent memory loss in females, whereas administration of the hormone with a considerable delay has little or even no valuable effects [for review see 93-94]. In ovariectomized rats, estrogen therapy improved synapse formation in hippocampi, blood flow, glucose metabolism, as well as reduced deposition of β-amyloid and prevention of mitochondrial damage were reported after estrogen therapy in ovariectomized rats [see review of the original articles in 95]. Although encouraging, it should be pointed out that the results in animal models need to be confirmed in clinical protocols. Many observational studies and controlled clinical trials have been performed to date. They were done in postmenopausal women in order to clarify whether estrogens augment cognitive functions, prevent cognitive decline or in fact have the opposite effects. Unfortunately, little certainty and much controversy have resulted mainly due to important methodological heterogeneity that makes outcomes not always comparable. Below, a summary of the current points and counterpoints is presented

regarding the role of Hormone Replacement Therapy (HRT) on some cognitive domains such as verbal memory, verbal fluency, visual memory and concentration.

One of the most recent data compilations was performed by Hogervorst and Bandelow in 2010 [96] where over 40 randomized controlled trials that measure cognitive functions in postmenopausal women were reviewed in a meta-analysis. Both, strict and lenient criteria were used in order to include a larger number of studies than those admitted in previously published meta-analysis [**96].** The data revealed that there are several outcome-determinants worth noticing. Some of them rely on participant´s characteristics such as menopausal status (natural or surgical), presence of symptoms, current age and age of menopause. Other factors affecting the outcome are the type of cognitive function, individual tests used, prior training and expertise of testers; results can be also modified by treatment characteristics such as type of estrogen, association with progestagens, route of administration, duration of treatment and time elapsed since menopause onset [96]. The latter issues are probably the most addressed and controversial. In 2004, the Women's Health Initiative Study on Cognitive Aging (WHISCA) revealed an increased risk of cognitive impairment or dementia when estrogens +/- progestins were used in the late postmenopausal stage [97]. Later on in 2006, an ancillary study to the WHI regarding memory (WHIMS), showed that a combination of conjugated equine estrogen with medroxyprogesterone (CEE+MPA) in postmenopausal women had a negative impact on verbal memory and a trend to a positive impact on figural memory over time when compared with placebo [98]. No effect above chance was detected on verbal fluency, concentration, visual memory, or visuospatial tests.

One of the main critiques made to the majority of the existing experimental designs is the age of the women enrolled. They all were 65 years or older at the time of randomization and therefore over a decade beyond menopause onset in most cases. The advocates of the “Timing Hypothesis” claim that the time elapsed before HRT is initiated -in years since menopause- determines the treatment outcome on cognitive functions. Supporting evidence from different studies is reviewed by Rocca et al [95]; the authors show that women with ovarian preservation had a reduced long-term risk of cognitive decline or dementia as compared to women who underwent bilateral oophorectomy before menopause. Treatment with estrogen in the early postmenopausal stage (most commonly at ages 50–60 years) was associated with a reduced long-term risk of cognitive decline or dementia while initiation of estrogen treatment in the late postmenopausal stage (ages 65–79 years) was associated with an increased risk of cognitive impairment or dementia. A theoretical framework for this assumption

emerged from cell culture experiments and is termed “The Healthy Cell Bias Theory”. It proposes that neurons undergoing pathological changes, but not healthy ones, accelerate their pathway towards programmed cell death when exposed to an estrogen-rich environment. This may be more likely to occur in older cells and may be implicated in dementia and accelerated cognitive decline. It might also explain why estrogens were found to have negative effects in older, but not in middle-aged women [99].

As mentioned earlier, other major determinants of HRT and cognitive function seem to be the regimes, route of administration and duration of the treatments. Most of the studies using CEE +MPA showed negative outcomes compared to Estradiol (E2) alone. The retrieved data on mode of administration showed a trend effect for bolus injections to be only associated with positive outcomes while transdermal applications were never associated with negative outcomes. Oral treatment was more likely to show negative effects on verbal memory, while other tests showed no differences in outcomes between modes of administration. Regarding duration, from all studies that had a positive outcome, 79 % had been performed for 12 weeks or less whereas all negative effects were found only in longer duration studies **[96]**. The Cochrane Revision of 2012 found a significant increase in the risk of venous thrombo-embolism, fatal or nonfatal heart attack, stroke and dementia when HRT (both combined and estrogen alone) was used for more than 12 months in women over 65 years [100].

At this time, evidence seems to support the idea that HRT, if recommended at all for neuroprotection, should be done only for a short period of time, using E2 alone and at early postmenopause.

7.3. Mechanisms of action

The effects of estradiol could be mediated by different mechanisms: (1) a genomic Estrogen Receptor (ER) mediated mechanism, (2) a nongenomic mechanism involving mitogen-activated protein kinase (MAPK) and/or phosphotidylinositol-3-kinase (PI3K) signaling, and (3) a receptor-independent antioxidant free radical scavenging mechanism. The first two mechanisms, genomic and nongenomic signalling, can be observed at low physiological doses of 17β-E2. In contrast, the third mechanism (antioxidant free radical scavenging) is only observed at high nonphysiological doses of 17β-E2 [101-102]. ERs are found in both the hippocampus and the frontal lobes which subserve verbal memory, working memory, and retrieval. Estradiol is responsible for mediating two types of changes in memory, those which are both delayed in onset and sustained (genomically mediated) as well as those that are rapid and short-lived (membrane mediated). The changes

observed include rapid increases in spine density, in the brain areas mentioned above. Ultimately, estradiol regulates vital neuronal and glial functions by various mechanisms of action such as modulation of inhibitory and excitatory neurotransmitter receptors and also regulates the expression of genes involved in apoptosis and of genes coding for growth factors and their receptors.

Considering the overall evidence, it is reasonable to hypothesize that this hormone might play an important protective role against the deterioration in cognitive function that occur with normal aging [103-104].

As we mentioned early in this section, estradiol exerts its actions via different mechanisms, one of which involves interacting with different neurotrophic factor receptors. Among them is IGF-I, which is a potent neuroprotectant [105].

There is a substantial body of evidence for a close interdependence between the actions of IGF-I and estradiol in the brain [106]. Both factors have in common the duality of being hormones, as well as locally produced neuromodulators, and they exert similar pleiotropic actions in the developing and adult brain. There are several potential points of convergence between estradiol and IGF-I receptor (IGF-IR) signaling in the brain. Estrogen activates the mitogen-activated protein kinase (MAPK) pathway and has a synergistic effect with IGF-I on the activation of Akt, a kinase downstream phosphoinositol-3 kinase. In addition, IGF-IR is necessary for the estradiol induced expression of the anti-apoptotic molecule Bcl-2 in hypothalamic neurons. The interplay of ERs and IGF-IR in the brain may depend on interactions between neural cells expressing ERs with neural cells expressing IGF-IR, or on direct interactions of the signaling pathways of ERs and IGF-IR in the same cell, since most neurons expressing IGF-IR also express at least one of the ER subtypes [107-109].

It is also known that IGF-I regulates ER transcriptional activity and, as we mentioned above, estradiol regulates IGF-I receptor signalling in neural cells and some details on the molecular mechanisms involved in this cross-talk have been established. Future directions may include the assessment of the interaction of ERs and IGF-I receptors with other signalling systems [109].

7.4. Possible therapies with estradiol

As we have already discussed, there are numerous neuroprotective actions of estrogens that have direct relevance to neurodegenerative diseases and menopause-associated symptoms. Despite these actions, the promise of estrogen-based therapies for reducing the risk for neurodegeneration remains

to be fulfilled. On the other hand, the administration of systemic estradiol presents the greatest risk regarding cancer. Therefore, as ongoing research continues to address these crucial and immediate concerns, an emerging area of investigation is the development of natural and synthetic hormone mimetics that will preferentially activate estrogen neuroprotective mechanisms while minimizing adverse effects in other tissues.

One promising strategy could be to implement gene therapy to over-express the enzyme aromatase. Aromatase, is the enzyme that synthesizes estrogens from androgen precursors and is expressed in the brain. Some studies suggest that modifications in brain aromatase activity may impact on cognitive function [for review see 90]. We have constructed a recombinant adenoviral vector that overexpresses the enzyme (RAd-P450aro; **Fig**.2), and propose that overexpression of this brain aromatase is likely to play an important role in the protection of neural tissue by increasing local estrogen levels in specific areas of the brain where it could exert its actions without affecting other regions. We found that RAd-P450aro, efficiently transduces

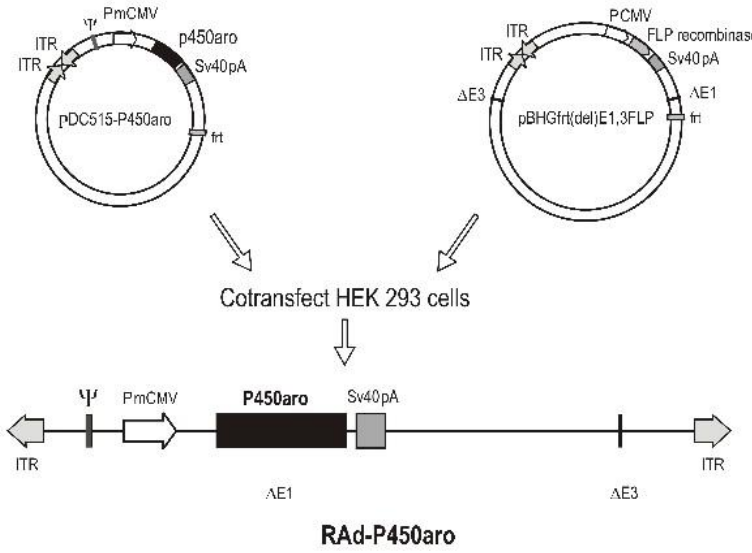

Figure 2. Scheme of construction of RAd-P450aro: mCMV promoter (PmCMV); SV40 polyadenylation signal SV40pA); encapsidation signal, ψ; shuttle vector (pDC515-P450aro);genomic plasmid (pBHGfrt(del)E1,3 FLP); deletion regions (ΔE1 and ΔE3); permissive HEK 293 cells; recognition element for the yeast FLP recombinase (frt); inverted terminal repeats (ITR).

primary cultures of astrocytes or neurospheres, and that it also overexpresses the cDNA for aromatase in astrocytes from the cerebral cortex of mice transduced with the viral vector. Estrogen levels in the supernatant of cells transduced with the virus was higher than in non-transduced cells. Our findings suggest that RAd-P450-Aro may constitute a suitable tool for the study of aromatase function [110]. Brain gene therapy with aromatase also emerges as a potential therapeutic approach for the treatment of cognitive dysfunction in older women.

8. Concluding remarks

The increase of the elderly population is an almost worldwide phenomenon. Consequently, the incidence of age-related neurological (and other) pathologies like Parkinson´s and Alzheimer´s disease is becoming a problem of significant medical and economic impact. In this context, research and development of novel therapeutic tools for neurodegenerative diseases, like gene therapy, explore new avenues for the treatment of these devastating pathologies. At present, only a small number of neuroprotective genes have been tested, mostly in animal models. However, it is likely that assessement of new neuroprotective genes will follow in the near future. Multiple neuroprotective factor-gene therapy is also a plausible alternative for potentiating neuroprotection in age-associated neurodegenerative pathologies.

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