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Gene Therapy and Cell Reprogramming for the Aging Brain: Achievements and Promise

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Abstract: 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. Spontaneous or environmental neurotoxin-mediated 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 are of clinical interest. Among them, Insulin-like Growth Factor I and Glial cell line-Derived Neurotrophic Factor are emerging as powerful neuroprotective molecules. This article discusses the experimental evidence supporting the neuroprotective relevance of these and related factors in the aging brain. The availability of induced pluripotent stem cells offers a new promise for the treatment of pathologies associated with the loss of specific cell types as for instance, nigral DA neurons (in PD) or basal forebrain cholinergic neurons (BFCN) in the early stages of AD. Recent studies documenting the use of cell reprogramming for the generation of multipotent neuronal precursors as well as functional BFCN and DA neurons are reviewed.

Keywords: Aging, alzheimer, gene therapy, cell reprogramming, Parkinson, neurodegeneration, neurotropic factors, transdifferentiation.

IMPACT OF AGING ON COGNITION AND BRAIN DOPAMINERGIC ACTIVITY

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 and environmental insults. 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 in the substantia nigra (SN), affects 0.1-0.3% of the population and 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.

Although age is considered to be the most important risk factor for the development of neurodegenerative diseases, it should be pointed out that in recent years a growing number of epidemiological studies have shown an association between the exposure to environmental toxins, such as pesticides, and a rising incidence of AD and PD in younger individuals [8-10]. In this context, treatments that can prevent or delay the decline in cognitive function and central DA activity during normal aging, or after long-term exposure to envi-

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ronmental toxins, may prove to be effective interventions to prevent or delay the progress of AD and PD.

Neuroprotective Gene Therapy

Gene therapy has undergone a remarkable development in the last 20 years. Particularly important advances have been made in the improvement of gene vehicles [11] 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 poses 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 [12].

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. 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 achieved only 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 harbor. 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 accommodate or a wide variety of cells they can transduce or an extended duration of transgene expression [13].

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 (ALS), stroke and some brain insults [13].

INSULIN-LIKE GROWTH FACTOR I (IGF-I)

Biochemistry and Endocrinology of IGF-I

Structurally, IGF-I is a small peptide with a molecular weight of 7649 Da and like insulin, has an A and B chain connected by disulphide bonds [14]. It binds to at least two cell surface receptors: the IGF-I receptor (IGFIR), and the insulin receptor. The IGFIR is a heterotetramer composed of two extracellular α subunits and two transmembrane β subunits. The former have binding sites for IGF-I and are linked by disulphide bonds, whereas the β subunits have a short extracellular domain, a transmembrane domain, and an intracellular domain. The latter contains a tyrosine kinase domain, which constitutes the signal transduction mechanism. Similar to the insulin receptor, the IGF-I receptor undergoes ligand induced autophosphorylation [15]. The activated IGF-I receptor is capable of phosphorylating other tyrosine containing substrates, such as insulin receptor substrate 1 (IRS-1), and continues a cascade of enzyme activations via phosphatidyl-inositol-3 kinase (PI3-kinase), Grb2 (growth factor receptor bound protein 2), Syp (a phophotyrosine phosphatase), Nck (an oncogenic protein), and Shc (src homology domain protein), which associated to Grb2, activates Raf, leading to a cascade of protein kinases including Raf, mitogen activated protein (MAP) kinase, 5 G kinase, and others [16].

Circulating IGF-I is the mediator of the anabolic and mitogenic activity of GH. Although IGF-I is produced by a number of tissues where it acts locally as a paracrine hormone [17], most of the circulating peptide is secreted by the liver in a GH-regulated fashion [18]. Historically, the clinical significance of IGF-I has been related to severe primary IGF-I deficiency, whose classic form is the Laron syndrome [19]. Due to its mitogenic activity, oversecretion of IGF-I has a pathogenic role for certain types of cancer.

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, [20] cortical injury [21, 22] and spinal cord lesions [23]. 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 [24]. 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 [25]. *In vitro* studies have shown that IGF-I increases cell survival in primary hypothalamic cell cultures [26] and stimulates differentiation of rat mesencephalic DA neurons [27]. A protective effect of IGF-I has been reported in immortalized hypothalamic cells exposed to reduced glutathione-depleting agents [28], in human DA cell cultures exposed to the toxin salsolinol [29] and in human and rodent neuronal cultures exposed to toxic doses of DA [30].

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 (6-OHDA)-lesioned rats suggest that IGF-I mediates the neuroprotective effect of estrogen on nigral DA neurons [31]. In a rat model of cerebellar ataxia (induced by 3-acetylpyridine (AC)), subcutaneous (sc) or intracerebroventricular (icv) administration of IGF-I restored motor coordination and partially rescued inferior olive neurons from the toxic effect of AC [25]. 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 [32]. Furthermore, IGF-I has been reported to protect hippocampal neurons from the toxic effects of amyloid peptides [33]. In-

terestingly, IGF-I treatment of mice overexpressing a mutant Aβ amyloid peptide markedly reduced their brain burden of Aβ amyloid [34].

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-OHDA, 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 [35].

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 [36].

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 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 PRL 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 [37] (Fig. **1**). 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 [38]. 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) [39]. 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 [38]. RAd-IGF-I-injected rats but not RAd-GFP-injected controls, showed

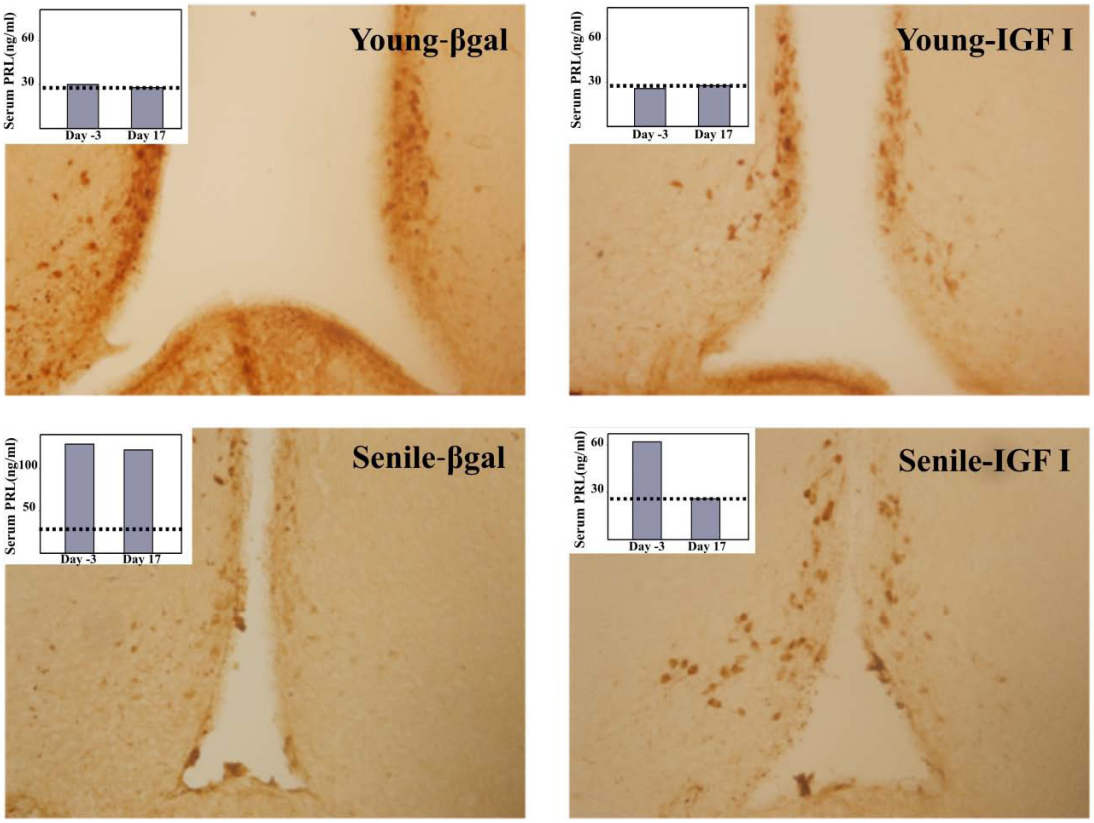

Fig. (1). Effect of IGF-I gene therapy on the DA neurons of the ARC-PeV hypothalamic region in young and senile females.- The representative coronal hypothalamic sections shown pass through the medial hypothalamus and were immunolabeled with a monoclonal anti-rat TH antibody. Animals were sacrificed 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. Obj. X 20. Insets show serum PRL levels before and 17 days after vector injection. From ref. 37 with permission.

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 modest but significant amelioration in motor performance in aged but not in young animals.

In rats, there is substantial evidence that age-related ovarian failure is preceded by abnormal responsiveness of the neuroendocrine axis to estrogen positive feedback [40]. 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 [41]. A bicistronic recombinant 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 (Fig. **2**). These results suggest that overexpression of IGF-I in the MBH prolongs normal ovarian function in M-A female rats [41].

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 rodent model of 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 [42].

GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR (GDNF)

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 DA neurons in disso-

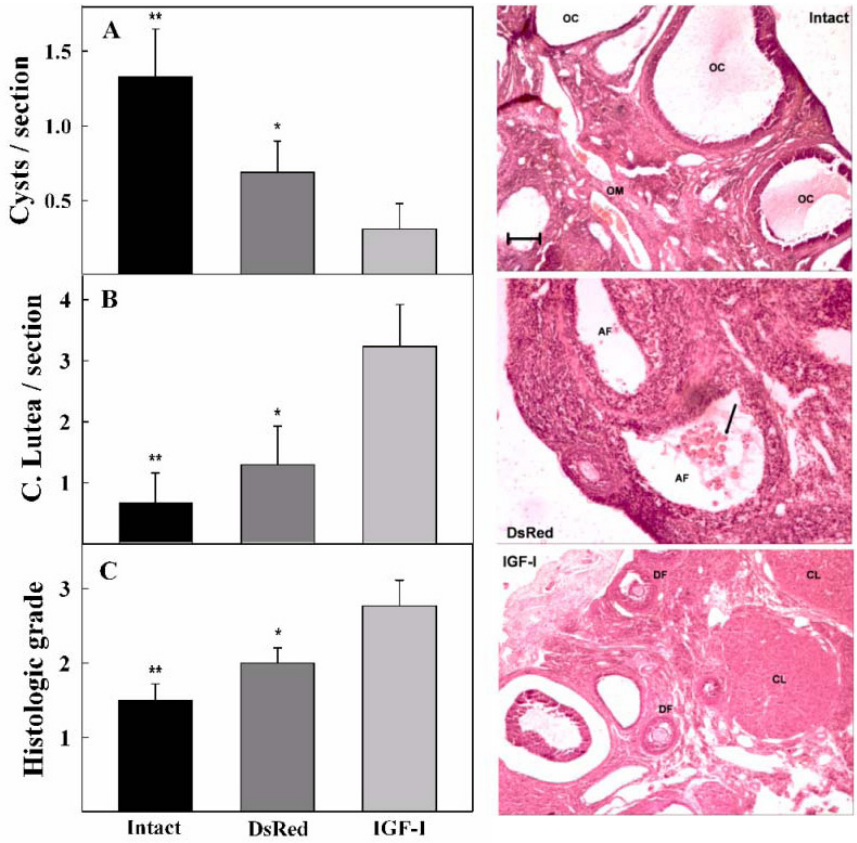

Fig. (2). Right panels- Histology of representative ovaries from control and experimental 49.5 weeks months old middle-aged (M-A) rats submitted to long-term (3 mo) IGF-I gene therapy in the MBH. Ovarian section from an intact animal (upper panel). It was assigned grade 1 and shows large ovarian cysts (OC) but no corpora lutea (CL) or mature follicles (MF). **Ovarian section from a DsRed rat (middle panel).** It was assigned grade 2 and shows numerous atretic follicles (AF) but no CL or MF. Apoptotic cells can be observed in the follicular space (arrow). Interstitial connective tissue is abundant and highly cellular. **Ovarian section from an animal submitted to IGF-I gene therapy (bottom panel).** It was assigned grade 3 and shows developing follicles (DF) and CL of normal aspect as well as some AF. Scale bar, 300 µm; OM, ovarian medulla. **Left panels- Histomorphometric assessment of the ovaries of control and RAd-IGF-I-treated M-A rats.** The number of follicular cysts (A), and corpora lutea (B) per section as well as the histologic grade (C) was assessed in the Intact (N=6), DsRed (N=13) and IGF-I (N=13) groups. Asterisks refer to differences versus the IGF-I group (ANOVA followed by the Tukey´s test), ** ($p<0.01$), *($p<0.05$). From ref. 41 with permission.

ciated rat mesencephalic cultures, and to increase their neurite length and cell size as well as their high affinity dopamine uptake; non-DA neurons or glial cells are not significantly affected by GDNF [43, 44]. GDNF is the founding member of the GDNF family of neurotrophic factors, which additionally includes three other structurally related members: neurturin (NTN) [45], persephin [46], and artemin [47]. The members of the GDNF family belong to the transforming growth factor (TGFβ) 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 [43]. GDNF and NTN have proved to enhance the survival of DA neurons in rodent and primate models of PD, previously treated with DA neurotoxins [48-50].

Infusion of the GDNF peptide into the lateral ventricle, striatum, or SN in rodent or primate models of PD has been reported to protect DA neurons in the SN, increase dopamine levels in the striatum and SN, and ameliorate behavioral deficits [49,51-53]. GDNF is active in protecting not only DA 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 [54].

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, administration in the striatum or in the SN of adenoviral [55-57], herpes simplex virus (HSV)-derived [58], lentiviral (LV) [59-61] or rAAV vectors [62-64] for GDNF has been shown to protect nigral DA neurons from the toxic action of DA toxins. In this context, rAAV and LV 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, icv 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 [65, 66]. In another study, 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 [67]. Although this study 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 [66,68,69]. 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 [70]. In order to improve safety, GDNF was replaced by NTN, as this member of GDNF family did not develop neutralizing anti-NTN antibodies or cerebellar degeneration [68,71,72].

Therapeutic Potential of GDNF for Non Parkinsonian Neurodegenerative Models

Therapeutic approaches with GDNF have extended to other neurodegenerative models, e.g. motor neuron degeneration [73,74], cerebral ischemia [75], limbic seizure [76], spinal cord motoneuron degeneration [73, 77, 78], noradrenergic neuron degeneration of the locus coeruleus [79], cerebellar Purkinje cell degeneration [80], degeneration of cholinergic neurons of the basal forebrain [81], as well as peripheral sensory and autonomic neuron degeneration [82].

There is also evidence that GDNF is active in the hypothalamus as GDNF gene delivery in the MBH induces body weight loss and ameliorates age-related obesity in rats [83]. 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 DA neuron number [84]. At the cognitive level, GDNF was shown to significantly improve spatial learning in aged-impaired Fisher 344 rats after icv administration of the peptide [85]. Furthermore, lentiviral mediated-GDNF gene therapy in the CA1 dorsal hippocampus improved neurotransmitter secretion and reversed cognitive deficit in aged Fisher 344 rats [86].

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 [72,45]. 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 primate models of PD as well as in aged rats and aged monkeys [72,87,88].

Phase I trial 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 [89]. 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. Since these clinical trials enrolled patients with advanced PD and presumably significant DA neuron loss, it could be expected that neuroprotective therapies be more effective in earlier stage patients [90].

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) [91]. 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 [92].

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 [93].

MISCELLANEOUS FACTORS ASSESSED FOR GENE THERAPY OF AD

Nerve growth factor (NGF), an important factor in the cholinergic basal forebrain for promoting and maintaining synaptic contact between the neurons of hippocampus and cortex, has been assessed for the treatment of AD. Thus, a phase 1 clinical trial of NGF gene therapy for AD was carried out in eight patients with early-stage probable AD. NGF was administered to the brain using ex vivo gene delivery. Autologous fibroblasts obtained from small skin biopsies in each subject were genetically modified to produce and secrete human NGF using retroviral vectors [94]. NGF production was measured, and cells were stereotaxically injected into the cholinergic basal forebrain in one surgical session. Cognition was assessed in six subjects safely completing NGF delivery. Mean Mini-Mental Status Examination (MMSE) scores before treatment showed that overall decline was reduced 51% compared to preoperative rate for the mean 22-month period after treatment. Positron emission tomography (PET) scan findings indicate that NGF stimulation indeed broadly increases cortical glucose uptake [94]. Taken together, these results suggest that NGF ex vivo gene therapy may be vastly superior to current medications for AD that only provide a 5% improvement in cognitive scores [95]. However, the ex-vivo gene transfer approach is limited by a decline in NGF protein expression from the cells over 18 months post-implantation, while manufacturing complexities and costs make this approach impractical for application to larger number of patients. Therefore, CEREGENE constructed CERE-110, a replication defective AAV-2 that contains the full-length human β-nerve growth factor (NGF) cDNA with which a series of pre-clinical experiments were performed to provide support for a phase I clinical study [96]. Interim data from a Phase 1 clinical trial of CERE-110 for six patients with mild to moderate AD revealed that after nearly three years of follow-up, the administration of CERE-110 was well tolerated [97-99]. All subjects underwent stereotactic neurosurgery that delivered CERE-110 into Nucleus Basalis of Meynert (NBM). Cognitive testing data suggest that a reduction in the rate of cognitive decline may have been achieved. PET scans were also obtained from the patients' brains at baseline, six and 12 months, and increases in brain metabolism were observed in several cortical regions at six months. Further increases in metabolism were measured at 12 months, representing a potential reversal of patterns typically observed in AD [97-99]. In September 2009, Ceregene initiated a double-blind, placebo-controlled (sham surgery), randomized, multicenter study evaluating CERE-110 gene delivery in subjects with mild to moderate AD. Estimated completion date is in December 2014 [100].

Brain-derived neurotrophic factor (BDNF) is another important neurotrophin which plays a key role in synaptic plasticity and memory formation in the hippocampus. It has been assessed in monkeys for the treatment of AD. Aged monkeys (24.5 ± 1.2 years) characterized on a visuospatial discrimination task sensitive to temporal lobe function received bilateral injections of lentiviral vectors expressing either BDNF or GFP, into four locations distributed over the rostral to caudal extent of the medial temporal cortex, targeting entorhinal cortex neurons projecting to the hippocampus. BDNF gene-treated monkeys showed a significant improvement in performance compared to control aged monkeys, by the tenth postoperative session. Analysis of brain sections showed accurate targeting of the lentiviral vectors to the entorhinal cortex and elevated BDNF protein distribution in the hippocampus, suggesting anterograde transport. Furthermore, mean entorhinal neuronal size significantly increased in the BDNF gene-treated monkeys compared to monkeys receiving lenti-GFP [101]. Taken together, these results establish a rationale for BDNF delivery to the entorhinal cortex as a means for treating entorhinal and hippocampal degeneration in AD.

Proteolytic degradation has emerged as a key pathway involved in controlling levels of the AD-associated amyloid-β (Aβ) peptide in the brain. The endopeptidase neprilysine (NEP), a major Aβ degrading enzyme in mice and humans [102], was initially used for implementing short- and medium-term gene therapy for overexpressing NEP in mutant amyliod precursor protein (APP) transgenic mice. This approach resulted in a reduction of Aβ accumulation, neurodegeneration and behavioral deficit [103-105]. More recently, brain long-term (6 months) NEP gene therapy using a LV vector not only lowered the amyloid plaque load and the levels of intracellular Aβ immunoreactivity but also improved behavioral performance in the water maze tests and reduced synaptic pathology in APP transgenic mice [106].

Another approach to reduce APP levels in the brain involved the construction of a rAAV encoding the cDNA for the light and heavy chains of a monoclonal antibody against the Aβ peptide [107]. A single i.m. injection of the vector into C57BL/6 mice generated serum levels of anti-Aβ antibody in excess of 0.1 mg /ml for up 64 weeks. When the vector was i.m. administered to transgenic mice overexpressing APP, their brain Aβ load was significantly lowered [107].

THE PROMISE OF CELL REPROGRAMMING FOR THE AGING BRAIN

Reprogramming of Fibroblasts into Neurons and Neural Stem Cells

In 2006, Takahashi and Yamanaka [108] reported that the transfer of the four pluripotency genes Oct4, Sox2, cMyc and Klf4 (known as the Yamanaka genes), to somatic cells can reprogram them, taking the cells to a stage in which they

behave as embryonic stem cells. The possibility of generating this type of cells, known as induced pluripotent stem cells (iPSC), has opened a horizon of hitherto unimagined possibilities for the development of personalized therapeutic strategies [109,110]. Cell reprogramming is also a powerful methodology to transdifferentiate a somatic cell type into a different somatic cell lineage. In particular, there is a keen interest in transdifferentiating fibroblasts and other somatic cell types to either mature neurons or neural precursors (NP) which can later be used for implementing cell therapy for neurodegenerative pathologies like AD and PD. It was initially shown that transfer of the genes for three neural-lineage–specific transcription factors namely, *Ascl1*, *Brn2* (also called *Pou3f2*) and *Mit1l,* was able to convert mouse embryonic and postnatal fibroblasts into functional neurons *in vitro*. These induced neurons (iN) express multiple neuron-specific proteins, generate action potentials and form functional synapses [111]. The genes for the same three factors can generate functional neurons from human pluripotent stem cells as early as 6 days after transduction with a Tet-On lentiviral vector expressing the above genes. When combined with the gene for the basic helix-loop-helix transcription factor NeuroD1, these factors could also convert fetal and postnatal human fibroblasts into iN displaying typical neuronal morphologies and expressing multiple neuronal markers. These iN were able to generate action potentials and could establish synaptic contacts when co-cultured within primary mouse cortical neurons [112].

These results were followed by efforts to transdifferentiate fibroblasts into neural stem cells (NSC) or neural progenitor cells (NPC). NSC are self-renewing, tripotent cells that are capable of producing the three major cell types of the the CNS i.e., neurons, astrocytes and oligodendrocytes [113]. NSCs give rise to unipotent or multipotent NPC with limited self-renewal capacity. There are two main strategies to reprogram fibroblats to NSC or NPC. The first one was initially used by *Kim et al.* [114] and is based on the use of the Yamanaka genes, which are transiently (3 to 6 days) expressed under the control of a DOX-inducible promoter in genetically modified mouse fibroblasts in order to make the cells epigenetically unstable and therefore, amenable to be induced to transdifferentiate. When such epigenetically unstable mouse fibroblasts are grown in a medium that contains the neurogenic molecules, fibroblast growth factor 2 (FGF2), FGF4 and epidermal growth factor (EGF), colonies of cells showing many of the features of NPC or NSC are generated after additional 8-9 days in culture [114]. In a conceptually similar approach the Yamanaka genes were expressed in mouse fibroblasts for 5 days and then the expression of Oct4 was selectively repressed (keeping the other three Yamanaka genes overexpressed). In this way tripotent induced NSC were generated. These cells showed an extensive self-renewal capacity compared with the limited passaging ability of the bipotent NSC/NPC generated by Kim *et al.* [114].

In all of the above studies the reprogramming strategy involved integrative gene transfer with the well-known risk of insertional mutagenesis or gene silencing. We suggest that adenoviral vectors offer a safer reprogramming alternative. We have designed a regulatable helper-dependent adenovector expressing the four Yamanaka genes and the humanized green fluorescent protein (hGFP) gene which should allow implementing the strategy of Kim *et al.* [114] in a non integrative fashion (Fig. **3**). In order to overcome the lower transduction efficiency of adenoviral as compared with retroviral vectors, we propose to use the magnetofection technique [115], during cell reprogramming. Magnetofection markedly increases the transduction efficiency of HD-adenovectors in cell culture (Fig. **4**).

The second reprogramming strategy, which was separately developed by Lujan *et al.* [116] and Han *et al.* [117]**,** started with a list of 11 candidate genes for transciption factors which were transferred to mouse fibroblasts by means of retroviral vectors. This resulted in the appearance of induced NSC or NPC. Systematic elimination reduced the list of effective factors to a small number. In one study [116] it was found that *SOX2* and *FoxG1* overexpressed in embyonic fibroblasts are capable of generating self-renewing bipotent induced NPC that give rise to astrocytes and functional neurons. When the gene for the *Brn2* transcription factor was added to *SOX2* and *FoxG1*, tripotent NPC were generated which could be differentiated into neurons, astrocytes and oligodendrocytes [116]. In the other study [117]**,** *Sox2, cMyc, Klf4* and *Brn4* were pinpointed as active factors sufficient to induce direct transdifferentiation of mouse fibroblasts into induced NSC. This was a gradual process in which the fibroblast transcriptional program was silenced over time. The induced NSC so generated exhibit the cell morphology, gene expression, epigenetic features, differentiation potential and self-renewing capacity of natural NSC. They also show *in vitro* and *in vivo* functionality similar to those of wild-type NSC [117].

The Potential of Cell Reprogramming for the Treatment of PD and AD

Cell reprogramming offers a major advantage over embryonic stem cell (ESC)-based therapeutic approaches for PD and AD. In effect, iPSC as well as induced NSC and NPC can be derived from easily accessible somatic cells from the patient (skin fibroblasts, for instance). The induced

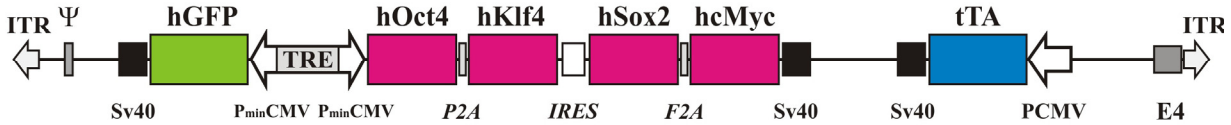

Fig. (3). Diagrammatic representation of the genome of an HD-adenovector suitable for non integrative cell reprogramming. The vector can express the Yamanaka genes and hGFP (as a reporter) for the desired period after which expression of the pluripotency genes and hGFP can be readily inhibited by doxycycline addition to the culture media. TRE, tetracycline-responsive element; PminCMV, human cytomegalovirus minimal promoter; PCMV, CMV full promoter; tTA, tetracycline-responsive transcriptional activator; hGFP, Reporter cDNA; SV40pA, simian virus 40 polyadenylation signal; ψ, packaging signal; ITR, inverted terminal repeats; P2A, F2A, CHYSEL self-processing sequences; IRES, internal ribosome entry site, hOct4, hKlf4, hSox2, hcMyc, Yamanaka pluripotency genes.

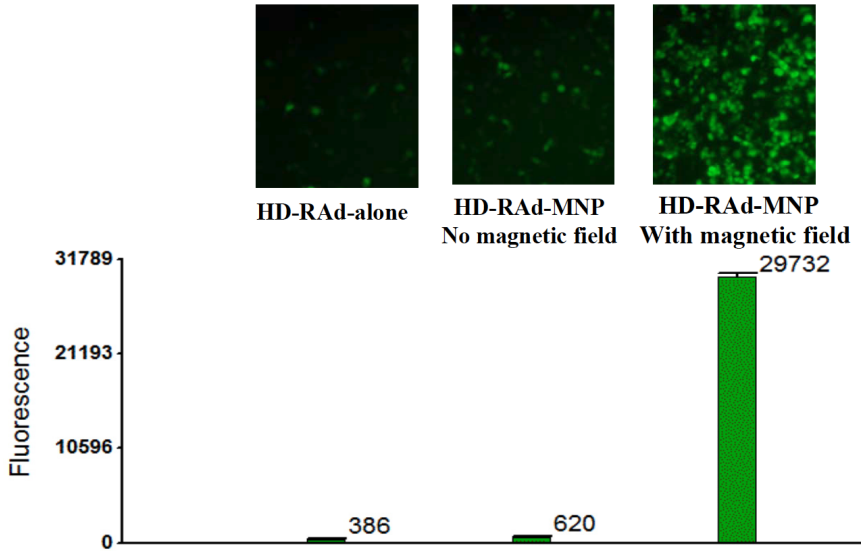

Fig. (4). Magnetofection in N2a neuronal cells. Using a HD-RAd harboring the gene for GFP, transgene expression was quantitated measuring fluorescence in the corresponding cell lysates 4 days after the vector was added to the cultures. **Left column**, vector alone was added (2 X10^9 viral particles/ml); **Center column**, the same concentration of vector was complexed with the magnetic nanoparticle PEI-Mag2 (2.5 fg Fe /physical viral particles) and then added to the cultures without applying a magnetic field; **Right column**, the same vector complex was added to cells and a magnetic field was applied for 30 min. When the magnetic field was applied, GPF fluorescence (arbitrary units) was over 50 times higher than in the lysates from cells exposed to the naked vector. Inset images above represent GFP expression in the corresponding cell cultures. Obj. 20X.

neurons derived from the patient's own cells will be autologous for him/her and therefore will not induce immunologic rejection when implanted.

The use of ESC as a source of DA neurons for the treatment of PD is a topic of growing interest and there is evidence that DA neurons derived from mouse ESC survive for extended periods (over 37 weeks) in rodent models of PD [118]. Furthermore, mouse ESC can spontaneously differentiate into DA neurons when implanted in the striatum of rats whose nigral DA neurons have been pharmacologically lesioned [119]. The newly generated DA neurons cause a gradual and sustained behavioral restoration of DA-mediated motor asymmetry. Like ESC, iPSC can be induced to differentiate into DA neurons and can be subsequently implanted into the brain of rat models of PD where they are able to improve behavior [120].

The initial stages of AD are characterized by an early substantial loss of basal forebrain cholinergic neurons (BFCN) which leads to deficits in spatial learning and memory. Consequently, cholinergic neuron replacement constitutes a relevant therapeutic goal and, interestingly, it has been recently shown that BFCN can be consistently derived from human ESC [121]. It has been also demonstrated that functional neurons can be generated by reprogramming skin fibroblasts from normal individuals and familiar AD patients [122]. It seems therefore plausible to hypothesize that in the not-too-distant future a mature cell reprogramming technology will make it possible to implement personalized regenerative medicine for neurodegenerative diseases.

CONCLUDING REMARKS

The increase of the elderly population is an almost worldwide phenomenon. Consequently, the incidence of age-related neurological (and other) pathologies like PD and AD is becoming a problem of significant medical and economic impact which is further exacerbated by exposure of the general population to increasing levels of environmental pollutants. In this context, research and development of novel therapeutic tools for neurodegenerative diseases, like gene therapy, may open 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.

In recent years, cell reprogramming has emerged as a powerful technology that promises to make it possible to implement personalized regenerative medicine for neurodegenerative diseases.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

Aβ	=	Amyloid-β
ALS	=	Amyotrophic lateral sclerosis
APP	=	Amyloid precursor peptide
AR-JP	=	Autosomal recessive juvenile parkinsonism
BFCN	=	Basal forebrain cholinergic neurons
BDNF	=	Brain-derived neurotrophic factor
ESC	=	Embryonic stem cell
NSC	=	Neural stem cell
NPC	=	Neural progenitor cell
iN	=	Induced neuron
PRL	=	Prolactin
FGF2 and 4	=	Fibroblast growth factor 2and 4
EGF	=	Epidermal growth factor
GDNF	=	Glial cell line-derived neurotrophic factor
hNPC	=	Human neural progenitor cells
IGF-I	=	Insulin like-grrowth factor I
MBH	=	Medial basal hypothalamus
NGF	=	Nerve growth factor
NTN	=	Neurturin
NBM	=	Nucleus Basalis of Meynert
TIDA	=	Tuberoinfundibular dopaminergic
TGFβ	=	Transforming growth factor β

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