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ORIGINAL ARTICLE

Restorative effect of insulin-like growth factor-I gene therapy in the hypothalamus of senile rats with dopaminergic dysfunction

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Insulin-like growth factor-I (IGF-I) is emerging as a powerful neuroprotective molecule that is strongly induced in the central nervous system after different insults. We constructed a recombinant adenoviral vector (RAd-IGFI) harboring the gene for rat IGF-I and used it to implement IGF-I gene therapy in the hypothalamus of senile female rats, which display hypothalamic dopaminergic (DA) neurodegeneration and as a consequence, chronic hyperprolactinemia. Restorative IGF-I gene therapy was implemented in young (5 months) and senile (28 months) female rats, which received a single intrahypothalamic injection of 3×10^9 plaque-forming units of RAd-βgal (a control adenoviral vector expressing β-galactosidase) or RAd-IGFI and were killed 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-positive cells in the hypothalamus of experimental as compared with control senile animals (5874 ± 486 and 3390 ± 498, respectively). Our results indicate that IGF-I gene therapy in senile female rats is highly effective for restoring their hypothalamic DA dysfunction and thus reversing their chronic hyperprolactinemia.
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Keywords: *aging; DA neurodegeneration; TIDA neurons; hyperprolactinemia; IGF-I*

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), dopaminergic (DA) neurons are among the cells most susceptible to the deleterious effects of age. Thus in humans, Parkinson's disease, a degeneration of nigrostriatal DA neurons, which affects 0.1–0.3% of the population, is the most conspicuous reflection of the vulnerability of DA neurons to age.[1] In rats, aging brings about a progressive dysfunction 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 lactotrophic cell proliferation in the adenohypophysis.[2] Degeneration and loss of TIDA neurons during normal aging are associated, in the female rat, with progressive hyperprolactinemia[3] and the development of pituitary prolactinomas.[4] This neuroendocrine pathology of aging female rats provides a convenient animal model to assess the efficacy of therapeutic strategies aimed at protecting central DA neurons. Interestingly, Parkinsonian patients usually reveal functional alterations in the hypothalamo-PRL axis.[5]

Neurotrophic factors that prevent the degeneration and enhance the recovery of remaining DA neurons are of clinical interest. Among them, insulin-like growth factor-I (IGF-I) is emerging as a powerful neuroprotective molecule, which is strongly induced in the CNS after different insults such as ischemia,[6] cortical injury[7,8] and injury of the spinal cord.[9] In situations involving cytotoxic damage in the hippocampus, the microglia of this region dramatically increase the production of IGF-I- and IGF-I-binding protein 2, which suggests a neuroprotective role of these molecules in the CNS.[10] Furthermore, IGF-I has been reported to protect hippocampal neurons from the toxic effects of amyloid peptides.[11] Interestingly, IGF-I treatment of mice overexpressing a mutant Aβ amyloid peptide markedly reduced their brain burden of Aβ amyloid.[12] *In vitro* studies have shown that IGF-I increases cell survival in primary hypothalamic cell cultures[13] and stimulates differentiation of rat mescencephalic DA neurons.[14] A protective effect of IGF-I has been reported in immortalized hypothalamic cells exposed to reduced glutathione-depleting agents,[15] in human DA cell cultures exposed to the toxin salsolinol[16] and in human and rodent neuronal cultures exposed to toxic doses of DA.[17]

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Prompted by the above evidence, we constructed an adenoviral vector harboring the gene for rat IGF-I and used it to implement IGF-I gene therapy in the hypothalamus of hyperprolactinemic senile female rats. The present report documents the effectiveness of this therapeutic strategy in reversing hyperprolactinemia and increasing DA function in the hypothalamus of the senescent animals.

Results

In vitro *IGF-I gene transfer*

Recombinant adenoviral vector harboring the gene for rat IGF-I (RAd-IGFI) induced a significant overexpression of IGF-I in both B92 glial and N2a neuronal cells as compared with their corresponding counterparts incubated with RAd-βgal (Figure 1). In the glial cells, RAd-IGFI induced a marked increase in the release of IGF-I. Both cell types produced comparable levels of IGF-I when incubated for 3 days with either RAd-βgal or medium alone (data not shown). No cytopathic effects were detected in either B92 or N2a cells at the vector concentrations used.

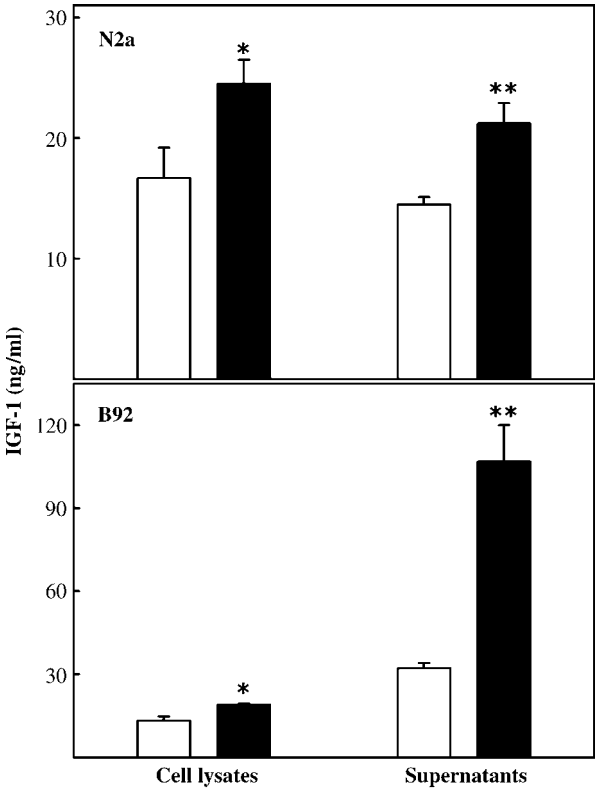

Figure 1 Intracellular and secreted levels of IGF-I in RAd-IGFI-transduced neuronal and glial cells. N2a mouse neuroblastoma cells (upper panel) and B92 rat glial cells (lower panel) were incubated for 3 days with either RAd-βgal (open columns) or RAd-IGFI (solid columns). Supernatants were collected, cells scraped from the well's bottom and resuspended in medium (see Materials and methods section for further details). Total IGF-I was assayed in supernatants and lysates and the peptide concentration referred to the original volume of medium per well. Bars on columns represent s.e.m. ($n=5$). *$P<0.05$; **$P<0.01$.

In vivo *hypothalamic IGF-I gene transfer*

Intrahypothalamic injection of RAd-βgal or RAd-IGFI in young (5 months) female rats followed by killing of the animals at different times post-injection, showed that RAd-IGFI-injected animals overexpressed IGF-I in the hypothalamus for about 50 days. At post-injection day 70, hypothalamic IGF-I content was not significantly different between RAd-βgal- and RAd-IGFI-injected rats (Figure 2).

IGF-I gene therapy in the hypothalamus of senile female rats

Seventeen-day IGF-I gene therapy was implemented according to the experimental design described in Materials and methods (Figure 3).

Enzymohistochemical assessment of the hypothalamus from young and senescent female rats, 17 days after RAd-βgal injection, revealed a widespread distribution of X-gal-positive cells in both the arcuate-periventricular (ARC-PeV) and the paraventricular (PaV) regions (Figure 4). At the end of the treatment, total IGF-I content in the medial basal hypothalamus (MBH) of senescent animals was higher in the RAd-IGFI- than in the RAd-βgal-injected group (Figure 4, lower inset).

In the young animals, serum PRL profiles were not affected by hypothalamic injection of either RAd-βgal or RAd-IGFI (Figure 5, lower panel). In contrast, treatment with RAd-IGFI but not with RAd-βgal induced a marked fall of serum PRL levels in the senescent female rats, thus reversing the chronic hyperprolactinemia typically present in aged female rats (Figure 5, upper panel). As expected, there was a slight age-related increase in serum estradiol, but the RAd-IGFI treatment did not affect the circulating levels of this steroid (Figure 5, inset).

Quantitative immunohistochemical assessment revealed that in the senile but not in the young female rats, IGF-I gene therapy induced an increase in the number of hypothalamic tyrosine hydroxylase positive (TH+)

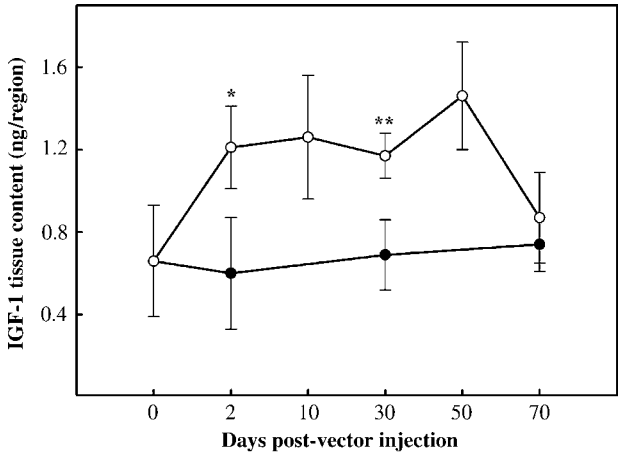

Figure 2 Assessment of the length of IGF-I expression in the Arc-PeV region of young rats intrahypothalamically injected with RAd-IGFI. On experimental day 0, animals received a bilateral stereotaxic injection of either RAd-βgal (solid symbols) or RAd-IGFI (open symbols) in the ARC-PeV hypothalamic region. At the indicated times, groups of 3–4 rats were killed, the ARC-PeV-median eminence region removed by micropunch and assayed for IGF-I. Bars on columns represent s.e.m. *$P<0.05$; **$P<0.01$

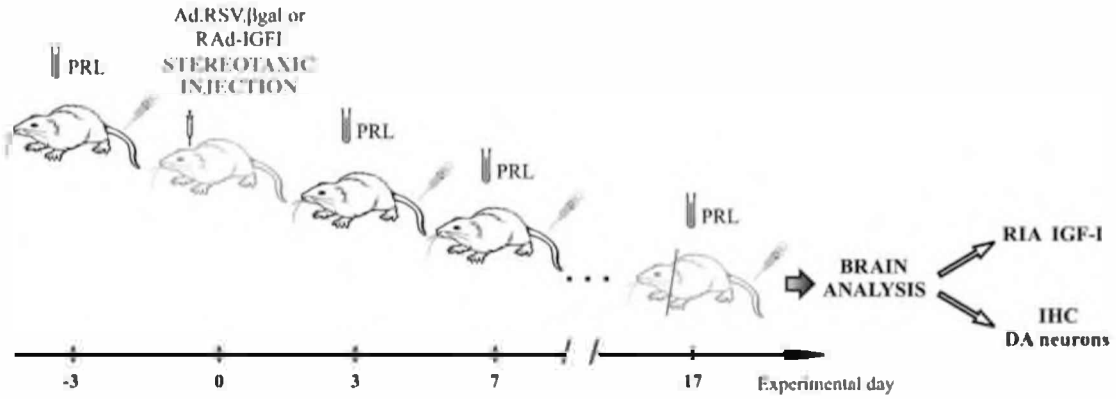

Figure 3 Experimental design for IGF-I gene therapy in the hypothalamus of female rats. Two identical but independent experiments were performed using this design. Animals were bled at experimental days −3, 3, 7, 10, 14 and 17, taking 0.3–0.4 ml blood from the tail veins in each sampling. RAd-βgal (controls) or RAd-IGFI (experimental) were stereotaxically injected in the hypothalamus of young and senile female rats on experimental day 0. See Results for further details.

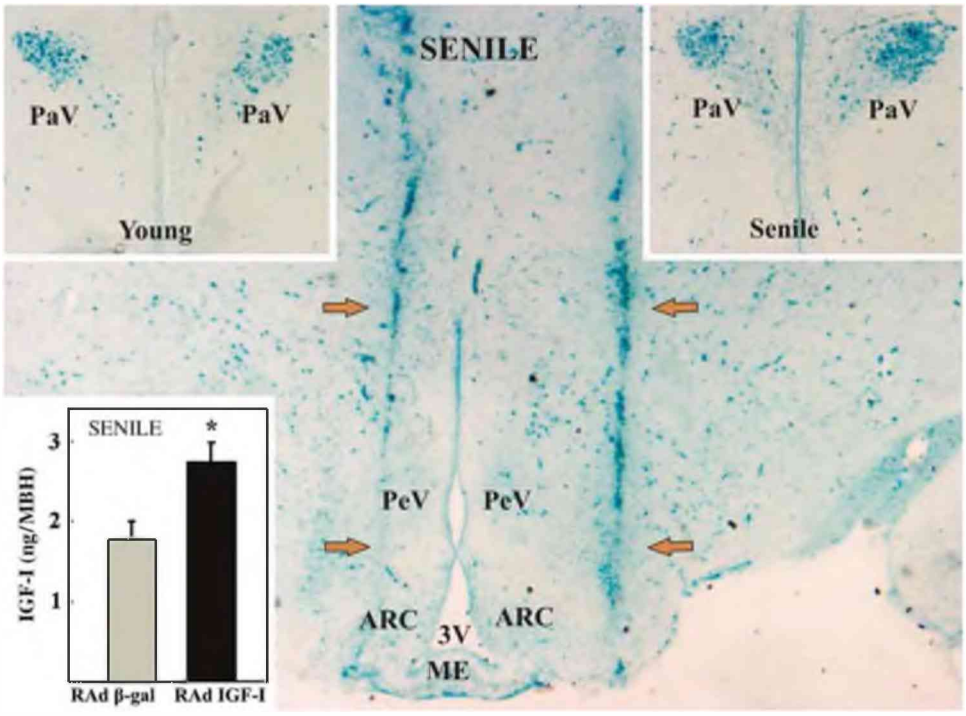

Figure 4 Expression of β-gal in the hypothalamus of young and senile female rats 17 days after RAd-βgal injection. Control rats were killed 17 days after receiving a single bilateral intrahypothalamic injection of RAd-βgal. Expression of β-gal in hypothalamic sections was detected by the X-gal technique (positive cells stained in blue). The main panel shows β-gal expression in the medial hypothalamus of a representative senile female rat. High frequency of transduced cells is observed along the tracks left by the needle during vector injection (arrows). Left and right upper insets show β-gal expression in the PaV region of a young and senile control animal, respectively. The lower inset on the left shows IGF-I content in the MBH of RAd-βgal- and RAd-IGFI-treated senile rats, 17 days after the corresponding vector injection. *A significant ($P<0.05$) difference between the two groups (N per group = 4). For further technical details see Materials and methods. ARC: arcuate hypothalamic nucleus; PeV: periventricular hypothalamic nucleus; PaV: paraventricular hypothalamic nucleus; 3V: third ventricle. Objectives ×1.5 and ×10 for main panel and insets, respectively.

neurons as compared with the RAd-βgal-treated counterparts (Figure 6). This change was significant in the PaV nucleus ($P<0.05$) and near significant in the ARC-PeV region ($P=0.07$). Morphometric analysis of the effect of IGF-I gene therapy on other morphologic characteristics of hypothalamic DA neurons including cell perimeter, cell area and roundness revealed that the treatment induced no significant changes in the young rats and only minor changes in the senile animals (data not shown). Although the number of DA hypothalamic neurons was comparable between young and senile control rats, TH immunostaining was generally fainter in the latter. This was quantitatively assessed by morphometrically estimating the average optical density per TH cell in the different experimental groups (data not shown). This loss of immunoreactivity in the senile

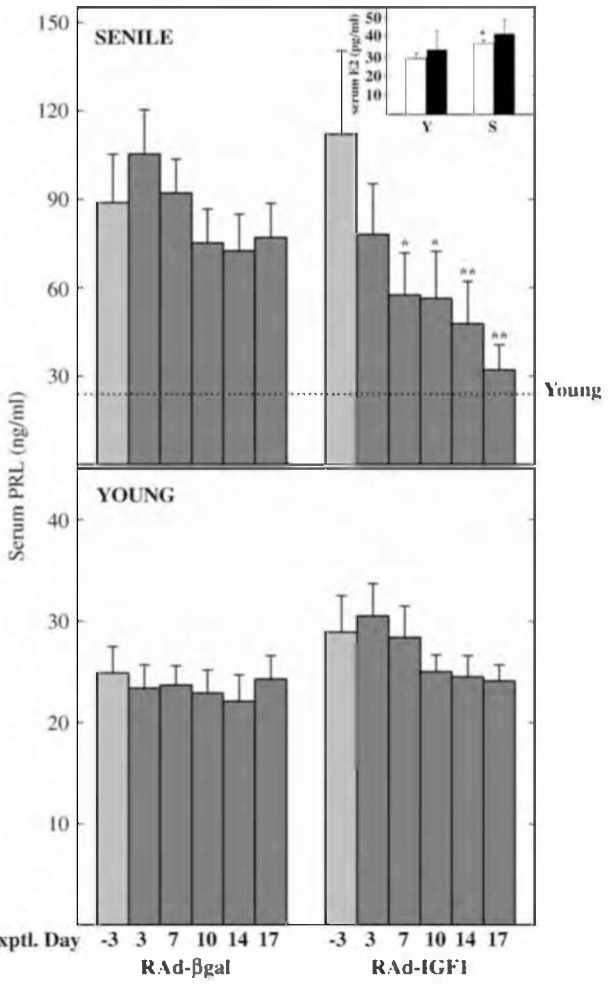

Figure 5 Effect of IGF-I gene therapy on serum PRL levels in young and senile female rats. The panels show the effect of intrahypothalamic injection of RAd-βgal or RAd-IGFI on serum PRL profiles during a 17-day-long post-injection period. The lower and upper panels correspond to young (5 months) and senile (28 months) animals, respectively. The columns in light gray (experimental day -3) indicate pre-injection PRL levels. Notice that at pre-injection time all senile female rats were hyperprolactinemic as compared to reference values from intact young female rats (dotted line). The number of animals in the young control, young experimental, senile control and senile experimental groups were 10, 11, 9 and 12, respectively. Bars over columns represent s.e.m. values. The significance of differences between post-injection versus the corresponding pre-injection PRL value is indicated by * ($P<0.05$) or ** ($P<0.01$) over columns. Inset shows 17β-estradiol levels in the serum of RAd-βgal- (open columns) and RAd-IGFI-treated rats of both age groups at experimental day 17. Y: young; S: senile. Asterisk (*) on column indicates a significant difference of senile controls versus young controls. *N* values for estradiol data are 4, 5, 5 and 4, for Y-βgal, Y-IGFI, S-βgal and S-IGFI, respectively.

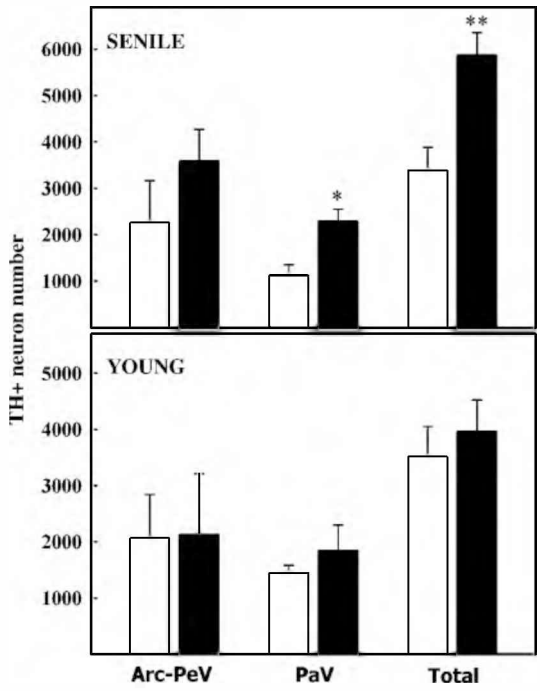

Figure 6 Effect of IGF-I gene therapy on DA neuron number in the hypothalamus of young and senile rats. The graphs show the quantitation of TH+ neurons in the ARC-PeV region, and in the PaV nucleus as well as the total TH+ neuron number in the hypothalamus. Animals were killed 17 days after the corresponding vector injection in the hypothalamus. Open and solid columns correspond to animals injected with RAd-βgal or RAd-IGFI, respectively. TH+ neuron counting was performed both manually and automatically using an appropriate image analysis software (see Materials and methods for further details). Bars over columns represent s.e.m. values ($N=4$). Significant differences between the RAd-βgal- and RAd-IGFI-injected groups for each pair of columns are indicated by asterisks (*) over the solid column. *$P<0.05$; **$P<0.01$.

animals was partly reversed by IGF-I gene therapy (Figure 7).

Discussion

A number of *in vivo* models have been developed for the study of the pathophysiology of Parkinson's disease as well as for the assessment of new therapeutic strategies for this devastating disease. They include the use of neurotoxins to lesion nigral DA neurons in primates and rodents and the generation of transgenic models overexpressing α-synuclein.[18] Although each of these paradigms has provided useful information for the understanding of Parkinson's disease, they share a significant limitation namely, that the neurological lesions they study are caused by experimental manipulations rather than by aging, the only unequivocal risk factor for Parkinson's disease.[19,20] In this context, the aging female rat emerges as a unique model of spontaneous and progressive age-related DA dysfunction. Besides, the functional status of TIDA neurons can be readily monitored in the animals by measuring circulating PRL levels. The advantages of the neuroendocrine system for the evaluation of gene therapy strategies in the CNS has been already demonstrated in the Brattleboro rat, a mutant lacking arginine-vasopressin (AVP), which is used as a model of diabetes insipidus.[21] When an adenoviral vector encoding the rat AVP cDNA was stereotaxically injected into the supraoptic nucleus of Brattleboro rats, a substantial expression of AVP in magnocellular cells as well as the presence of immunohistochemically detectable AVP in their axons projecting to the posterior pituitary was detected. Measurement of urine output and urine osmolality showed that the symptoms of diabetes insipidus in the Brattleboro rats

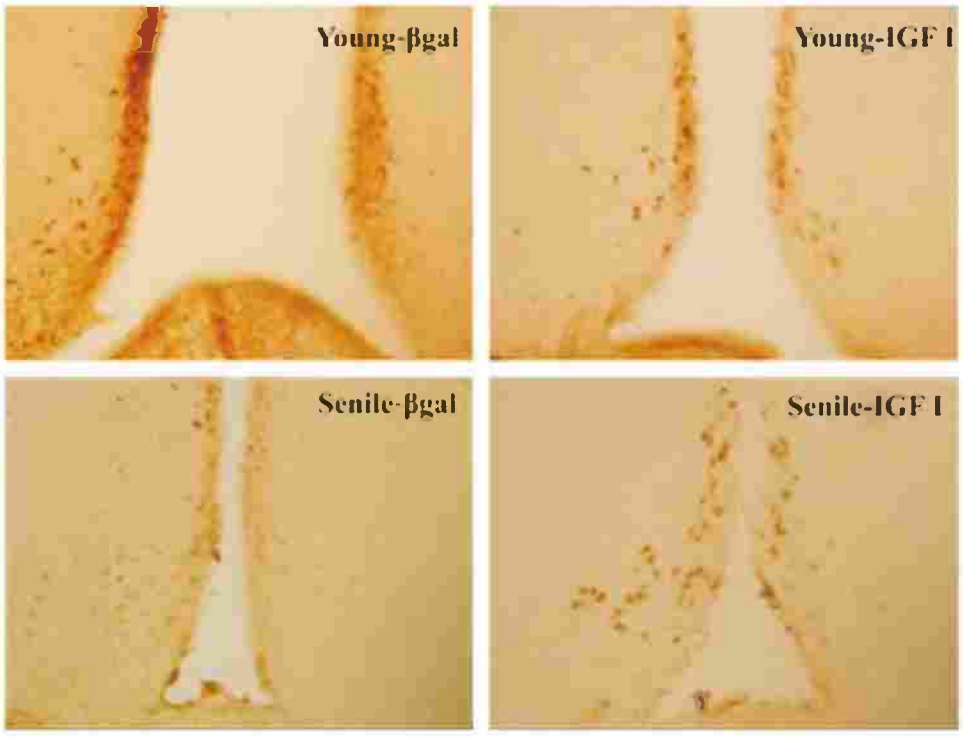

Figure 7 Effect of IGF-I gene therapy on the DA neurons of the ARC-PeV hypothalamic region in young and senile female rats. The representative coronal hypothalamic sections shown pass through the medial hypothalamus and were immunolabeled with a monoclonal anti-rat TH antibody. Animals were killed 17 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. Objective $\times 20$.

were significantly reduced for up to 4 months after injection of the viral vector.[22]

The present study is, to our knowledge, the first attempt to use IGF-I gene therapy for the restoration of DA neuron function in aged rats. The RAd-IGFI vector constructed for this purpose proved to be effective in inducing IGF-I overexpression in glial and neuronal cell lines as well as in the hypothalamus of young and old rats. The length of expression (50 days) of transgenic IGF-I achieved *in vivo* with our adenoviral vector was shorter than that reported for adenovirally delivered AVP (4 months) in the above-mentioned studies in the hypothalamus of Brattleboro rats.[22] This may be due to the fact that the two transgenes were driven by different promoters. Morphologic assessment of RAd-βgal-injected rats 17 days post-surgery revealed a widespread distribution of transduced cells in the medial hypothalamus, which suggests that transgenic IGF-I also reached the PaV and ARC-PeV regions. RAd-βgal has not the same backbone as RAd-IGFI, but we chose to use it despite this fact because its performance in the hypothalamus is well characterized in our laboratory.

The DA neurons of the rat hypothalamus are grouped into two main areas, A_{12} and A_{14},[23,24] with the DA perikarya of the A_{12} area being located in the ARC nucleus and in the periarcuate region.[25] The A_{14} DA neurons are mainly located within the PaV and PeV nuclei, with a few scattered DA neurons in the anterior ventromedial hypothalamic area.[25,26] The A_{12} area and its corresponding axon terminals constitute the TIDA system, whereas the A_{14} area and its fibers are known as the periventricular dopaminergic system. Both systems regulate PRL secretion by exerting a tonic inhibitory control on both PRL secretion and lactotroph proliferation.[27] In early studies, TIDA neuron function was reported to decline during aging in rats, with a marked reduction in hypothalamic, median eminence and neurointermediate lobe DA content in old (24–26 months) as compared with young (4 months) rats.[28] More significant, the rate of DA secretion into the hypophysial portal blood of aged (20–26 months) male and female rats was found to decline drastically when compared with young (2–4 months) counterparts.[29,30] Although the above age-related alterations in hypothalamic DA secretion were ascribed to a functional decline of TIDA neurons rather than to TIDA neuron loss,[31] more recent work in very old female rats (32 months) showed that at extreme ages, DA neuron loss occurs in the rat hypothalamus.[32] In the present study, comparison of total hypothalamic DA neuron number in 5- versus 28-month-old control female rats (3516 versus 3390, respectively) shows a slight nonsignificant decline, which is in acceptable agreement with the above reports. The marked hyperprolactinemia of our senile animals in the face of minimal TIDA neuron loss, supports the hypothesis proposed by others namely, that in the aging female rat most TIDA neurons survive but become progressively dysfunctional.[31] In line with this idea, the poor TH immunoreactivity of hypothalamic sections from senile control rats suggests low levels of neuronal TH. In this context, the marked reversion of chronic hyperprolactinemia effected by RAd-IGFI but not RAd-βgal treatment in the senile female rats, strongly suggests that overexpression of IGF-I in the hypothalamus of the

aged animals restored DA neuron function and/or number (see below).

Although we are unaware of studies on the protective activity of IGF-I on adult hypothalamic DA neurons, our results are not unexpected in view of the well-established neuroprotective activity of IGF-I in other brain regions. In a rat model of cerebellar ataxia (induced by 3-acetylpyridine (AC)), subcutaneous or intracerebroventricular administration of IGF-I restored motor coordination and partially rescued inferior olive neurons from the toxic effect of AC.[33] Two- and 4-week 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.[34] 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.[35]

Our morphometric data show an increase in TH+ neurons in the hypothalamus of senile rats treated with RAd-IGFI. This increase could be accounted for by a trophic effect of transgenic IGF-I on pre-existing dysfunctional DA neurons expressing low levels of TH (putative TH− DA neurons), which would be rendered TH+ by the treatment. Nevertheless, our findings are also consistent with a neurogenic effect of IGF-I in the hypothalamus of the senile animals. Although the assessment of neurogenesis was beyond the scope of the present study, this possibility should be mentioned. In the hippocampus of adult rats, IGF-I has been reported to selectively induce neurogenesis.[36] Furthermore, the exercise-induced increase in the number of hippocampal neurons has been shown to be mediated by circulating IGF-I.[37] Interestingly, it has been recently reported that neural progenitor cells exist in the ependymal layer of the adult rat's third ventricle and that they may migrate and differentiate into hypothalamic neurons.[38]

The present study describes the implementation of restorative gene therapy in an unexplored animal model of age-related central DA neuron degeneration. Using this paradigm, we demonstrate that IGF-I gene therapy in the hypothalamus of hyperprolactinemic senile female rats is highly effective in reversing TIDA dysfunction. Future studies exploring the restorative ability of IGF-I in the substantia nigra of Parkinson's disease models as well as the possible neurogenic activity of IGF-I in the hypothalamus and substantia nigra of aged rats may prove to be highly rewarding.

Materials and methods

Adenoviral vectors

RAd-IGFI. A RAd vector harboring the rat *IGF-I gene* (kindly donated by Dr Peter Rotwein, Oregon Health Sciences University) was constructed by a variant of the two-plasmid method[39] employing the AdMax plasmid kit (Microbix, ON, Canada). Briefly, the cDNA coding for rat *IGF-I gene* (obtained from the mRNA for the IGF-Ib precursor form)[40] was excised from plasmid pBluescript KS, subcloned in pCA14 and inserted in the multiple cloning site (MCS) of shuttle pDC515 (one of the plasmids of the kit), which contains an expression cassette consisting of the mouse cytomegalovirus promoter and the simian virus 40 polyadenylation signal, immediately upstream and downstream to the MCS, respectively. Downstream this cassette, pDC515 also contains an frt recognition site for the yeast FLP recombinase. The second plasmid of the kit, the genomic plasmid pBHGfrt(del)E1,3 FLP, consists of the entire genome of adenovirus 5 (Ad5), containing deletions in the regions E1 and E3. Upstream to the E1 deletion, pBHGfrt(del)E1,3 FLP contains an expression cassette for the gene for yeast FLP recombinase and immediately downstream to the E1 deletion, there is an frt recognition site. Both plasmids were co-transfected in HEK293 cells, a line stably transfected with a portion of the Ad5 E1 genomic region. In co-transfected HEK293 cells, FLP recombinase is readily expressed and efficiently catalyzes the site-directed recombination of the expression cassette of pDC515 into pBHGfrt(del)E1,3 FLP, thus generating the genome of the desired recombinant adenoviral vector, RAd-IGFI. The newly generated RAd was rescued from HEK293 cell lysates and plaque purified. It was further purified by ultracentrifugation in a CsCl gradient. Final virus stocks were titrated by a serial dilution plaque assay.

RAd-βgal. This RAd was kindly provided by Dr Michel Perricaudet, Institut Gustave Roussy, CNRS, Paris. In this vector, the E1 genomic region has been replaced by an expression cassette containing the *Escherichia coli lac Z reporter gene* under the control of the Rous sarcoma virus long terminal repeat. The vector was expanded in 293 cells and purified and titrated as indicated for RAd-IGFI.

In vitro *studies*

Cell cultures. The B92 rat glial cell line and the N2a mouse neuroblastoma cell line were used to test the effectiveness of RAd-IGFI *in vitro*. Cells were grown in Eagle's minimum essential medium (MEM), 16.8 mM Hepes buffer (pH 7.0), 2 mM glutamine, 0.1 mM non-essential amino acids, 20 mg/l penicillin/streptomycin, 3.3 mg/l amphotericin B, 2.2 mg/l $NaHCO_3$ and 10% (v/v) fetal bovine serum. They were grown at 37°C in a humidified atmosphere of 95% air–5% CO_2. Cells were fed every 3–4 days and split when confluent.

Cell transduction protocol. Cells were plated on six-well plates. When a density of $1.5–2.0 \times 10^5$ cells/well was reached, the medium was replaced by 1 ml fresh medium containing 2×10^8 plaque-forming units (p.f.u./ml) RAd-βgal or RAd-IGFI. After 3 days, cell supernatants were collected by gentle aspiration, 1 ml MEM per well was added to cells and they were scrapped off. Cell suspensions were freeze-thawed three times, centrifuged at 1000 *g* for 10 min and lysates collected. Total IGF-I was measured in both supernatants and lysates.

Animals

Young (Y, 5 months) and senescent (S, 28–31 months) female Sprague–Dawley rats, raised in our gerontological rat colony, were used. Animals were housed in a temperature-controlled room (22±2°C) on a 14:10 h light/dark cycle. Food and water were available *ad libitum*. In our gerontological rat colony, the average 50%

survival time for female rats, studied in groups of 50–60 animals, is 33 months (range 32–34 months). All experiments with animals were performed according to the Guidelines on the Use of Animals in Neuroscience Research (the Society of Neuroscience) using Institutional Animal Care and Use Committee (IACUC) approved procedures (approval date 6 January 2004) and Animal Welfare Guidelines of NIH (INIBIOLP's Animal Welfare Assurance No. A5647-01).

Experimental design for in vivo *IGF-I gene therapy*

Young and senescent female rats were allotted to a control or experimental group, thus forming four groups: young control (Y-βgal), young experimental (Y-IGFI), senescent control (S-βgal) and senescent experimental (S-IGFI). Beginning at experimental day −3, a small blood sample (0.3–0.4 ml) was taken from the tail veins of all rats at the indicated times (Figure 3). Serum was obtained and kept at −20°C for hormone assay.

On Experimental day 0, control and experimental animals received bilateral 1.5-μl intrahypothalamic injections containing 3×10^9 p.f.u. RAd-βgal or RAd-IGFI, respectively. For this purpose, rats were anesthetized by injection of ketamine hydrochloride (40 mg/kg, intraperitoneal) and xylazine (8 mg/kg, intramuscular), and placed in a stereotaxic frame. In order to access the ARC-PeV region, the tip of a 26 G needle fitted to a 10 μl syringe was brought to the following coordinates relative to the bregma: 3.0 mm posterior, 10.0 mm ventral and 0.6 mm right and left.[41]

On experimental day 17, part of the rats from the four groups were killed by rapid decapitation, the brain was rapidly removed from the cranium, placed on a dry ice block and the MBH dissected and homogenized for determination of IGF-I content (in some animals, the ARC-PeV-median eminence region was obtained by micropunch). The remaining animals were placed under deep anesthesia and perfused with phosphate-buffered formaldehyde 4% (pH 7.4) fixative. Each brain was removed and trimmed down to a block containing the whole hypothalamus. The block was then serially cut into coronal sections 40 μm thick on a freezing microtome.

Immunohistochemistry

In each block, one every six serial sections was selected in order to obtain a set of non-contiguous serial sections spanning the whole hypothalamus. Typically, a whole hypothalamus comprised about 48 coronal sections, thus yielding six sets of eight non-contiguous serial sections. For counting purposes, each set was considered as representative of the whole hypothalamus. For each animal, one set of sections was immunohistochemically processed using an anti-TH monoclonal antibody (Calbiochem Inc., La Jolla, CA, USA). For detection, the Vectastain Universal ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) employing 3,3-diamino benzidine tretrahydrochloride as chromogen was used. Sections were dehydrated, mounted and used for image analysis.

Image analysis

Stereological quantitation of hypothalamic TH neurons was performed as previously described, with minor modifications.[32] Briefly, images of the DA areas of hypothalamic sections were captured using an Olympus DP70 digital camera attached to an Olympus BX51 microscope (Tokyo, Japan). Digital images were analyzed using the ImagePro Plus (IPP) v5.1 image analysis software (Media Cybernetics, Silver Spring, MA, USA). In order to avoid double counting errors, only TH neurons showing clearly shaped nuclei were counted. The total number (N) of TH neurons per nucleus was estimated using the following equation:

$$N = \frac{d}{n.s} \sum_{i=1}^{n} x$$

Where, d = length (mm) of the rostrocaudal axis of the hypothalamus being assessed; n = number of slices assessed per hypothalamus; s = thickness of sections (40 μm); x = number of TH neurons counted per slice assessed.

Enzymohistochemistry

In the animals injected with RAd-βgal, the hypothalamic sections were submitted to enzymohistochemistry for *E. coli* β-galactosidase. Sections were incubated overnight in 0.01% 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal; Calbiochem, San Diego, CA, USA), 5 mM $K_3Fe(CN)_6$-$K_4Fe(CN)_6$, 2 mM $MgCl_2$ solution, dehydrated and mounted for light microscopy assessment.

Hormone assays

For all hormones, serum or brain samples were assayed in duplicate. Serum PRL was measured by a specific radioimmunoassay (RIA) following a protocol previously described[4] and using the rat materials provided by Dr AF Parlow, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA, USA. Serum PRL was expressed in terms of NHPP rPRL RP-3.

For IGF-I RIA, cell culture samples and hypothalamic specimens were subjected to acid–ethanol cryoprecipitation as described by Breier *et al*.[42] For B92 and N2a cells, 150 μl lysates or supernatants prepared as indicated above, were submitted to acid–ethanol extraction. The MBH and ARC-PeV-median eminence regions were dissected and homogenized in 150 μl Tris-HCl (60 mM), ethylenediaminetetraacetic acid (1 mM) with protease inhibitors, pH 6.8. Homogenates were centrifuged at 800 g, and 90 μl supernatants were subjected to acid–ethanol extraction. IGF-I was determined as previously described[43] using antibody (UB2-495) provided by Drs L Underwood and JJ Van Wyk, and distributed by the Hormone Distribution Program of the NIDDK and Dr AF Parlow. Recombinant human IGF-I (Chiron Corp., Emeryville, CA, USA) was used as radioligand and unlabeled ligand.

Serum 17β-estradiol was measured by RIA using a commercial solid phase kit (Coat-A-Count, DPC, Los Angeles, CA, USA) and following the manufacturer's instructions.

Statistical analysis

The t-test or the analysis of variance was used, as appropriate, to evaluate group differences. Tukey's method was chosen as a *post hoc* test. Significant differences between rat groups for each hypothalamic area were defined as those with a $P<0.05$. Highly significant differences were defined as those with a P-value <0.01.

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