# Endocrinology

# HYPOTHALAMIC INSULIN-LIKE GROWTH FACTOR-I GENE THERAPY PROLONGS ESTRAL CYCLICITY AND PROTECTS OVARIAN STRUCTURE IN MIDDLE-AGED FEMALE RATS

--Manuscript Draft--

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| **Manuscript Number:** | EN-13-1069R2 |
| **Full Title:** | HYPOTHALAMIC INSULIN-LIKE GROWTH FACTOR-I GENE THERAPY PROLONGS ESTRAL CYCLICITY AND PROTECTS OVARIAN STRUCTURE IN MIDDLE-AGED FEMALE RATS |
| **Short Title:** | Hypothalamic IGF-I and ovarian aging |
| **Article Type:** | Original Article |
| **Section/Category:** | Reproduction - Development |
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| **Order of Authors Secondary Information:** | |
| **Abstract:** | There is substantial evidence that age-related ovarian failure in rats is preceded by abnormal responsiveness of the neuroendocrine axis to estrogen positive feedback. Since insulin-like growth factor I (IGF-I) seems to act as a permissive factor for proper GnRH neuronal response to estrogen positive feedback and considering that the hypothalamic content of IGF-I declines in middle-aged (M-A) rats, we assessed 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. We used three groups of M-A rats: one group of intact animals and two groups injected, at 36.2 weeks of age, in the MBH with either a bicistronic adeno-associated vector (rAAV) harboring the genes for IGF-I and the red fluorescent protein DsRed2, or a control rAAV expressing only DsRed2. Daily vaginal smears were taken throughout the study which ended at 49.5 weeks of age. We measured serum levels of reproductive hormones and assessed ovarian histology at the end of the study. While most of the rats injected with the IGF-I rAAV had, on the average, well-preserved estrous cyclicity as well as a generally normal ovarian histology, the intact and control rAAV groups showed a high percentage of acyclic rats at the end of the study and ovaries with numerous enlarged cysts and scarce corpora lutea. Serum LH was higher and hyperprolactinemia lower in the treated animals. These results suggest that overexpression of IGF-I in the MBH prolongs normal ovarian function in M-A female |

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| | rats. |
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29 March, 2013

Ref: EN-13-1069-revision 2

Dr. Andrea C. Gore,
Editor-in –Chief,
Endorcrinology,

We are submitting the re-revised version of the manuscript entitled, **Hypothalamic insulin-like growth factor-I gene therapy prolongs estral cyclicity and protects ovarian structure in middle-aged female rats,** by SS Rodriguez, JI Schwerdt, CG Barbeito, AM Flamini, Y Han, MC Bohn and RG Goya, to be considered for publication in Endocrinology.

Sincerely,

Rodolfo Goya,
Senior Scientist

# RESPONSES TO REVIEWERS' COMMENTS

**Reviewer 1** #1:

**1) If the cysts do not have unovulated oocytes and hence reflect annovulatory events the authors should explain what the ovarian cysts represent.**

We performed a review of some of the ovarian micrographs from CE M-A rats previously published (refs 41, 42, 45 of the MS) and failed to see ovarian cysts with oocytes. In these papers, the authors do not state whether or not they observed oocytes in the cysts. Therefore, our obsevation that ovarian cysts lack an oocyte seems to be a confirmatory result. We cannot rule out the possibility that, occasionally, cysts that contain an oocyte may be observed.

As to what the ovarian cysts represent, we concur with the hypothesis proposed by others (ref 42, 44, 45, for instance) that the follicular cysts begin as primary follicles that start to develop, but due to the inappropriate hormonal environment present in M-A rats, undergo degenerative changes and do not ovulate. The original oocyte probably degenerates and is lost during the process. The landmark evidence that there has been no ovulation is the lack (or scarcity) of corpora lutea in these ovaries. Summing up, follicles that develop normally, release their oocyte and undergo luteinization whereas follicles that fail to ovulate in M-A females, keep growing as large cysts.

**2) LH levels reported are especially low and not consistent with those that might be expected on proestrus (see R. Pineda, Endocrinology 151(2):722-30. The authors should address this. Is it possible that the samples were not collected at the appropriate time/during proestrus?**

See below.

**3) The LH surge is delayed in middle-aged females (see Downs and Wise, Mol Cell Endocrinology 299(1):32-8). It does not appear that the experimental model used accounts for this fact. This is quite relevant and makes the relevance of the LH data difficult to interpret.**

Considering the inconsistencies pointed out by the reviewer, we decided to remove Fig. 1 and all text mentioning LH and E2 measurements in intact young, M-A and senescent rats. The text that we left, succintly descibes the chronology of changes in estrous cycle profiles during the the transition from regular cyclicity to CE in our characterization study.

**4) The authors do not provide a convincing argument that the trauma associated with the vector may have caused IGF-1 release that subsequently rescued ovarian physiology. How would trauma rescue ovarian physiology without improving the hormonal profile? This point should be removed from the manuscript.**

As recommended, we removed the relevant paragraph from the manuscript as well as the associated references.

**5) There are still too many supplemental figures. The authors should reorganize their figures so that they are more efficient with the use of space.**

Taking into account the reviewer's comment, we decided to remove supplemental Fig. 1. In the text, we refer to the data of this figure as data not shown.

We have also removed suppl. Fig. 4 (see aditional explanation below) leaving only two supplemental figures in the paper.

**6) A reference should be provided for the "In rats, reproductive senescence occurs in midlife, whereas in some nonhuman primates, such as rhesus monkeys, this process occurs much later in life." This point is not true for all nonhuman primates therefore the word some should be added before nonhuman as provided above.**
A reference is now provided (ref. 54) and the word "some" was added as recommended.

**Reviewer# 2 #**
**Reviewer 2 response to authors: "I believe the authors can use chi-squared or Fisher's to analyze the data as it is now without replicating the whole gene therapy study. Without a statistical analysis, the figure is rendered meaningless."**
Considering the difficulty we found to perform appropriate statistics that render relevant differences statistically significant, we opted for deleting these results from the text as well as Suppl. Fig. 4. This action served a second purpose namely, to reduce the number of supplemental figures, therefore complying with one of reviewer's 1 requests. We also deleted Table 1 which reported length of time in estrus of young, old and senescent rats and served as reference for designing Suppl. Fig. 4.

*Manuscript (MUST INCLUDE TITLE PAGE AND ABSTRACT)
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**EN-13-1069-Revision 2**

# HYPOTHALAMIC INSULIN-LIKE GROWTH FACTOR-I GENE THERAPY PROLONGS ESTRAL CYCLICITY AND PROTECTS OVARIAN STRUCTURE IN MIDDLE-AGED FEMALE RATS

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**Running title:** Hypothalamic IGF-I and ovarian aging

**Key words:** Aging- hypothalamus- GnRH neurons- ovary- IGF-I- gene therapy

**Word count: 3,750**

**Financial Support:** This work was supported in part by grant # R01AG029798-3 (from the National Institute on Aging and the Fogarty International Center, USA), ANPCYT grant #PICT08-369 and CONICET grant PIP2378 to RGG.

**Disclosure summary:** None of the authors has anything to disclose.

[*] These two authors contributed equally to this work.

34 **ABSTRACT**

35 There is substantial evidence that age-related ovarian failure in rats is preceded by abnormal
36 responsiveness of the neuroendocrine axis to estrogen positive feedback. Since insulin-like
37 growth factor I (IGF-I) seems to act as a permissive factor for proper GnRH neuronal
38 response to estrogen positive feedback and considering that the hypothalamic content of IGF-I
39 declines in middle-aged (M-A) rats, we assessed the effectiveness of long-term IGF-I gene
40 therapy in the medial basal hypothalamus (MBH) of M-A female rats to extend regular
41 cyclicity and preserve ovarian structure. We used three groups of M-A rats: one group of
42 intact animals and two groups injected, at 36.2 weeks of age, in the MBH with either a
43 bicistronic adeno-associated vector (rAAV) harboring the genes for IGF-I and the red
44 fluorescent protein DsRed2, or a control rAAV expressing only DsRed2. Daily vaginal
45 smears were taken throughout the study which ended at 49.5 weeks of age. We measured
46 serum levels of reproductive hormones and assessed ovarian histology at the end of the study.
47 While most of the rats injected with the IGF-I rAAV had, on the average, well-preserved
48 estrous cyclicity as well as a generally normal ovarian histology, the intact and control rAAV
49 groups showed a high percentage of acyclic rats at the end of the study and ovaries with
50 numerous enlarged cysts and scarce corpora lutea. Serum LH was higher and
51 hyperprolactinemia lower in the treated animals. These results suggest that overexpression of
52 IGF-I in the MBH prolongs normal ovarian function in M-A female rats.

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59 **INTRODUCTION**

60 There is substantial evidence that age-related ovarian failure in rats is preceded by abnormal
61 responsiveness of the neuroendocrine system to estrogen positive feedback. Thus, middle-
62 aged (M-A), eugonadal rats demonstrate increased FSH serum levels as well as delayed and
63 attenuated LH surges despite normal estrous cycle lengths **(1).** Changes in the positive
64 feedback effects of estradiol on the LH surge in regularly cycling, M-A rats are not due to
65 primary pituitary dysfunction **(2, 3),** or reduced estrogen receptor α (ESR1) or progestin
66 receptor expression and/or binding in the hypothalamus **(4-6)** or the pituitary **(7, 8)**. Instead,
67 available data overwhelmingly point to an impaired hypothalamic response to estradiol
68 positive, but not negative **(9, 10)**, feedback as the etiology of the alterations in LH release
69 typically observed in reproductively aging rodents **(11).** At the hypothalamic level, the age-
70 related dysfunction of the LH surge is attributable neither to reduced numbers or abnormal
71 morphology of GnRH neurons **(12, 13-15)** nor to reduced GnRH peptide content which
72 remains unchanged or even increases with age **(3).** On the other hand, push-pull perfusion
73 measurements of *in vivo* GnRH output from the mediobasal hypothalamus (MBH) of M-A
74 rats suggest that GnRH peptide release is attenuated under estradiol positive feedback
75 conditions **(16)**. Moreover, GnRH mRNA may decrease **(17)**, and fewer GnRH neurons
76 express cFos **(12, 18)**, a marker for GnRH neuron activation, in M-A compared to young rats
77 **(12, 18)**. Taken together, the available data suggest that decreased preovulatory GnRH release
78 most likely reflects impaired GnRH neuronal activation under estrogen positive feedback
79 conditions **(19).** There is solid evidence that M-A rats do not respond to estradiol positive
80 feedback with appropriate modulation of excitatory and inhibitory hypothalamic
81 neurotransmitter release **(2, 18, 20-23)** which in turn could cause reduced activation of GnRH
82 neurons, reduced GnRH release and an abnormal LH surge **(18, 23).**
83 The initial deterioration of the modulation of excitatory and inhibitory hypothalamic

84 neurotransmitter release in M-A female rats may stem from a reduction in the activity of
85 permissive factors like insulin-like growth factor-I (IGF-I), whose content is reduced in the
86 brains of M-A rats **(17)**. Interestingly, it has been reported that intracerebroventricular (icv)
87 infusion of IGF-I partially rescues the LH surge in M-A rats **(3)**. IGF-I signaling, presumably
88 in the hypothalamus, is necessary for estradiol positive feedback and may modulate the
89 synthesis or release of kisspeptin or vasointestinal peptide (VIP) and/or the expression of
90 glutamate receptors **(24, 25)**. Thus, it is possible that reduced hypothalamic IGF-I indirectly
91 affects GnRH neuron activity by disrupting excitatory inputs mediated by glutamate and
92 kisspeptin **(26-28).**
93 Besides its permissive role in the modulation of the GnRH system, IGF-I is known to be a
94 powerful neurotrophic molecule which appears to be part of the physiologic self-repair
95 mechanisms of the adult brain **(29)**. Furthermore, gene therapy for IGF-I has shown
96 promising results in the brain of aging rats. Thus, IGF-I gene therapy in the MBH of senile
97 female rats was highly effective to restore hypothalamic dopaminergic (DA) neuron number
98 and correct the chronic hyperprolactinemia associated with depressed tuberoinfundibular DA
99 (TIDA) neuron function in old female rats **(30)**. In the same animal model, icv IGF-I gene
100 therapy ameliorated the reduced motor performance of the senile animals **(31, 32).**
101 In the present study we assessed the effectiveness of long-term IGF-I gene therapy in the
102 MBH of cycling adult female rats to extend regular cyclicity and preserve ovarian structure
103 when the animals progress through middle age.
104

105 **MATERIALS AND METHODS**

106 **AAV Vectors.**

107 The recombinant adeno-associated virus (rAAV) shuttle plasmids, pAAV-CMV-IGF-1b-ires-
108 DsRed and pAAV-CMV-ires-DsRed, were made by cloning an expression cassette between

109 the two inverted terminal repeats (ITR) of an AAV2 shuttle plasmid using standard
110 techniques. Both plasmids contain an expression cassette driven by a cytomegalovirus
111 (CMV) promoter containing a ß-globin intron enhancer. The 934 bp rat IGF-Ib sequence was
112 obtained from Peter Rotwein. Both plasmids also contain an internal ribosome entry site
113 (ires) upstream of the cellular reporter gene DsRed2. The expression cassettes were confirmed
114 by DNA sequencing and expression of DsRed2 in transfected HeLa cells prior to preparing
115 high titer, helper-free recombinant rAAV. Both vectors were packaged as rAAV2/2 by the
116 Children's Memorial Viral Vector Core following the protocol of Zolotukhin et al. **(33)** with
117 minor modifications. In brief, a shuttle plasmid and the pDG packaging plasmid **(34,**
118 generously provided by Jürgen Kleinschmidt) were used at a ratio of 1:3, respectively, for
119 $CaCl_2$ transfection into 293T cells. Cells were lysed three days after transfection by freeze-
120 thawing in order to collect virus and cellular debris was removed by centrifugation. The
121 supernatant was treated with octyl-β-D-glucopyranoside and benzonase and then applied to a
122 15-60% iodixanol discontinuous gradient. The 40% layer was further purified using a
123 Mustang Q ion exchange membrane. A Centriplus 100,000 MS cut off membrane was used
124 to concentrate virus, which was stored in phosphate buffered saline (PBS), pH 7.4 containing
125 5% sorbitol and 0.001% PF-68. qRT-PCR was used to determine viral titers. Viral titers
126 were: rAAV-IGF-I-ires-DsRed, 2.0 x $10^{12}$ vector genomes (vg)/ml; rAAV-ires-DsRed, 3.9 x
127 $10^{12}$ vg/ml.
128
129 ***In vitro* Studies**
130 **Cell cultures.** The HEK293 human embryo kidney cell line was used to test the performance
131 of rAAV-IGF-I-ires-DsRed *in vitro*. Cells were grown in Eagle's minimum essential medium
132 (MEM), 16.8 mM Hepes buffer (pH 7.0), 2 mM glutamine, 0.1 mM nonessential amino acids,
133 20 mg/l penicillin/streptomycin, 3.3 mg/l amphotericin B, 2.2 mg/l $NaHCO_3$ and 10% (v/v)

134 fetal bovine serum. They were grown at 37 ºC in a humidified atmosphere of 95% air-5%
135 $CO_2$. Cells were fed every 3-4 days and split when confluent.

136 **Cell transduction protocol.** Cells were plated on 12-well plates. When 70-80% confluence
137 was reached, the medium was replaced with fresh medium containing 4.8 X $10^9$ vg/ml rAAV-
138 IGF-I-ires-DsRed or 2.5 X $10^9$ vg/ml rAAV-ires-DsRed. At appropriate times cell
139 supernatants were collected by gentle aspiration, 1 ml 0.1% Triton X100 in PBS per well was
140 added to cells and they were scrapped off. Cell suspensions were freeze-thawed 3 times,
141 centrifuged at 1,000 g for 10 min and lysates collected for fluorescence determination by
142 spectrofluorometry. Total IGF-I was measured in supernatants.

143

144 **Animals and *in vivo* procedures**

145 Female Sprague-Dawley rats aged 3, 8, 10 and 26 months were used. The animals were raised
146 in our institution (INIBIOLP) and housed in a temperature-controlled room (22 ± 2ºC) on a
147 12:12 h light/dark cycle (lights on from 7 to 19 o'clock). Food and water were available *ad*
148 *libitum.* All experiments with animals were performed according to the Animal Welfare
149 Guidelines of NIH (INIBIOLP's Animal Welfare Assurance No A5647-01).

150 **Stereotaxic injections.** Rats were anesthetized with ketamine hydrochloride (40 mg/kg; ip)
151 plus xylazine (8 mg/kg; im) and placed on a stereotaxic apparatus. In order to access the
152 MBH, the tip of a 26G needle fitted to a 10µl syringe was brought to the following
153 coordinates relative to the bregma: 3.0 mm posterior, 8.0 mm ventral and 0.6 mm right and
154 left **(35)**.

155 **Vaginal smears.** Vaginal secretion was collected daily, between 11 and 13 o'clock, with a
156 plastic pipette filled with 20 µl normal saline (NaCl 0.9%) by inserting the tip into the rat
157 vagina, but not deeply. A drop of vaginal fluid was smeared on a glass slide and the unstained
158 material was observed under a light microscope, with a 40X phase-contrast objective. Three

159 types of cells can be recognized: round and nucleated ones are epithelial cells; irregular ones
160 without nucleus are cornified cells; and little round ones are leukocytes. The proportion
161 among them was used for determination of the estrous cycle phases **(36. 37),** which are
162 indicated as follows, P, proestrus; E, estrus, M, metestrus; D, diestrus; Pe, proestrus entering
163 estrus; Dp, Diestrus entering proestrus.
164 In M-A rats spending several days in a row in constant estrus (CE), a CE cycle was defined,
165 for quantitation purposes, as a period of 5 consecutive days of vaginal smears showing only
166 cornified cells. For instance, if an animal spent 13 days in a row in CE they were counted as
167 13/5=2.6 CE cycles.
168

169 **Experimental design for long-term IGF-I gene therapy in cycling females**

170 Eight-month old (34 wk) cycling females were allotted to a control or experimental group,
171 thus forming 3 groups: Intact control (**Intact**), rAAV-DsRed-injected control (**DsRed**) and
172 rAAV-IGF-I-ires-DsRed-injected experimental (**IGF-I**). Beginning at age 35.5 wk, a small
173 blood sample (0.3-0.4 ml) was taken (between 11 and 13 o'clock) from the tail veins of all
174 rats at appropriate intervals throughout the experiment. Serum was obtained and kept at -20
175 $^{o}$C for hormone assay. Vaginal smears were assessed daily from the beginning to the end of
176 the study. At 36.2 wk of age the DsRed and IGF-I groups received bilateral 2.0-µl
177 intrahypothalamic (MBH) injections containing 4 x $10^{9}$ vg rAAV-DsRed or rAAV-IGF-I-ires-
178 DsRed, respectively. The experiment was ended when the animals reached age 49.5 wk.
179

180 **Brain processing for fluorescence microscopy**

181 Five DsRed and 5 IGF-I animals were placed under deep anesthesia and perfused with
182 phosphate buffered formaldehyde 4%, (pH 7.4) fixative. Each brain was removed and serially
183 cut into coronal sections 40 µm thick on a vibratome. Sections were placed on regular slides,

184 mounted with Fluoromount G (Electron Microscopy Sciences, Hatfield, PA) and observed
185 under an Olympus BX51 fluorescence microscope (Tokyo, Japan). Digital images were
186 captured with an Olympus DP70 digital camera.
187
188 **Histologic and Histomorphometric assessment of ovaries**
189 Ovaries were removed, fixed in 4% formaldehyde, dehydrated and embedded in paraffin.
190 Four µm-thick serial sections, cut following the organ's major axis, were stained with H&E.
191 Micrographs of ovarian sections were taken with a digital camera CANON MC30 attached to
192 an Olympus CX31 microscope. The number of mature and developing follicles, corpora lutea
193 (CL) and cysts per ovarian section was determined using 6 images per gonad (which were at
194 least 100 µm apart) taken with a 4X objective. The assessment of the ovaries was done by two
195 blind observers (CGB, MAF). The criteria for histologically grading the ovaries were based
196 on previous studies in young rats **(38, 39)** and adapted for the ovarian features of M-A rats.
197 The grades are as follows:
198 **Grade 1,** corresponds to ovaries with an average of one or more large cysts, less than one CL
199 and less than one mature or growing follicle per section.
200 **Grade 2,** corresponds to ovaries showing small or medium-sized but not large, cysts; between
201 one and less than 2.5 CL and between one and 1.5 mature or growing follicles per section.
202 **Grade 3,** corresponds to ovaries without medium-sized or large cysts and with an average of
203 2.5 or more CL as well as more than 1.5 mature or growing follicles.
204
205 **Hormone Assays**
206 **IGF-I assay.** IGF-I was extracted from serum by acid-ethanol cryoprecipitation and was
207 radioimmunoassayed as previously described **(30)** using antibody UB2-495 distributed by AF.
208 Parlow, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance
209 CA. Recombinant human IGF-I rhIGF-I (Cell Sciences Inc., Canton, MA) was used as tracer

210 and unlabeled ligand. Cell supernatants were homogenized in PBS, and IGF-I was extracted
211 by acid-ethanol cryoprecipitation and quantified by radioimmunoassay (RIA). The sensitivity
212 and intra-assay CV for IGF-I was 2.4 ng/ml and 11%, respectively.

213 **Pituitary hormone assays.** Serum PRL and LH were measured by specific RIA using the rat
214 materials provided by Dr. A. F. Parlow. Serum PRL and LH were expressed in terms of
215 NHPP rPRL RP-3, and rLH-RP-2, respectively. The sensitivity and intra-assay CV for LH
216 and PRL was 0.6 ng/ml, 14% and 2.3 ng/ml, 13%, respectively.

217

218 **Steroid hormone assays.** Serum progesterone ($P_4$) levels were measured by solid phase RIA
219 using a commercial kit (Coat-A-Count, DPC, Diagnostic Products Corporation, Los Angeles,
220 CA). Serum β-estradiol ($E_2$) was measured using a liquid phase commercial RIA kit (Estradiol
221 Ultrasensitive DSL 4800, Webster, TX). The sensitivity and intra-assay CV for $E_2$ and $P_4$
222 were 4.1 pg/ml, 8% and 27 ng/ml, 5%, respectively.

223

224 **Statistical Analysis**

225 Data are expressed as mean ± SEM, unless otherwise indicated. For multiple experimental
226 groups statistical comparisons were performed by one-way ANOVA followed by the Tukey
227 pos hoc test when the ANOVA was significant. For comparisons between pairs of means the
228 Student's t-test was used when the SD´s did not differ significantly. Otherwise, the Welch's
229 approximate t estimator was used.

230

231 **RESULTS**

232

233 **Characterization of estrous cyclicity of female rats at different ages**

234 **Estrous cycle patterns throughout the lifespan-** Preliminary characterization in our female
235 rat colony of estrous cycle patterns from youth through very old age showed that transition
236 from regular to irregular cyclicity takes place at around 9 months of age and is followed by a

237 prevalence of CE status from age 10 to 18-20 months. Thus, 3 month old females typically
238 show 4-5-day estrous cycles characterized by one day or less of P (in some cycles P was so
239 short that when vaginal smear was performed the cell proportion was consistent with
240 proestrus entering estrus or sometimes just estrus), one day of E and 2-3 days of M and/or D
241 (data not shown). Ten-month old females typically show a prevalence of lengthy CE periods
242 with interspersed irregular cycles (data not shown).
243

244 ***In vitro* DsRed and IGF-I gene transfer**

245 Both rAAV-DsRed and rAAV-IGF-I-ires-DsRed induced strong red fluorescence in HEK293
246 cell cultures (data not shown). rAAV-IGF-I-ires-DsRed showed a strong overexpression of
247 IGF-I when compared with non treated cells or cells incubated with rAAV-DsRed **(Suppl.**
248 **Fig. 1, graph**). IGF-I concentration in the supernatants peaked on day 2 after vector addition
249 and remained steady afterwards. No cytopathic effect was detected in either control or
250 experimental cells at the vector concentrations used.
251

252 **IGF-I gene therapy in the MBH**

253 **Expression of transgenic DsRed in the MBH.** Both rAAV-DsRed and rAAV-IGF-I-ires-
254 DsRed induced strong red fluorescence in the MBH of M-A females from post-vector
255 injection day 3 until the end of the treatment at post-vector injection day 93 **(Suppl. Fig. 1,**
256 **images).** As expected, most of the transduced cells were neurons and in most of the animals
257 were distributed radiating from the needle track within the MBH. Vector diffusion did not
258 extend beyond this region. Visual comparison of DsRed expression in different animals
259 revealed some inter-animal variability concerning the location where the vector was injected
260 within the MBH but around each needle track the number of fluorescent neurons appeared
261 comparable between sides and among animals.

262

263 **Effect of long-term hypothalamic IGF-I gene therapy on estrous cycles in M-A rats**
264 Injection of rAAV-IGF-I-ires-DsRed in the MBH of 36.2 weeks old females had a favorable
265 impact, when compared to rAAV-DsRed-injected or intact animals, on the average regularity
266 of the estrous cycle patterns assessed during 13.3 weeks post-vector injection **(Supplemental**
267 **Fig. 2).** As expected, stereotaxic surgery induced a slight loss in body weight that lasted for
268 around 10 days in both DsRed and IGF-I rats. Subsequently, animals in the three groups
269 gained weight at the same rate.
270 In quantitative terms, the average number of regular cycles per week before and after IGF-I
271 vector injection was not significantly different. In contrast, intact rats or those receiving the
272 DsRed control vector showed significantly lower numbers of regular cycles per week during
273 the post-treatment period as compared to the pre-treatment period (**Fig. 1**). The frequency of
274 irregular cycles in the three groups was comparable before and after treatment whereas the
275 number of CE cycles per week in the intact and DsRed, but not in the IGF-I animals, was
276 significantly higher after than before treatment **(Fig. 1).**
277

278 **Effect of long-term IGF-I gene therapy on ovarian histology in M-A rats**
279 At the end of the study, when rats were aged 49.5 weeks, animals were sacrificed and gonads
280 histologically assessed. The ovaries of intact and DsRed M-A rats weighed significantly
281 ($P<0.01$) less than those of intact young (3 mo.) counterparts. Interestingly, the weight of the
282 ovaries from IGF-I-treated animals did not differ significantly from those of intact young rats
283 (data not shown). The ovaries of both intact and DsRed, M-A females showed, on the
284 average, clear structural alterations, the most conspicuous of which was the presence of large
285 follicular cysts, (**Fig. 2, upper and middle images, respectively**) although the extent of these
286 alterations varied substantially among animals even within the same group. In the females
287 from the IGF-I group the ovaries had, on the average, substantially milder structural

288 alterations and most of them showed follicles in all developmental stages as well as normal
289 CL **(Fig. 2, bottom image).** Histomorphometric analysis revealed a significantly lower
290 incidence of follicular cysts in the ovaries of the IGF-I group **(Fig. 2, A)**. The number of CL
291 was significantly lower in the Intact and DsRed groups than in the IGF-I animals **(Fig. 2, B),**
292 while the number of atretic, growing and mature follicles was not significantly different
293 among groups (data not shown). The histologic grade, an index of the structural integrity of
294 the ovaries, was significantly higher in the IGF-I group than in the Intact and DsRed rats **(Fig.**
295 **2, C)**. The rats with histologic grade 3 had a significantly better preserved regularity of
296 estrous cycles than the animals with histologic grade 1 (data not shown).
297
298 **Effect of MBH IGF-I gene therapy on serum hormone levels in M-A rats**
299 Pituitary weight was comparable in the three experimental groups at 49 weeks of age (data
300 not shown). Hormones were measured before treatment (weeks 35-36) and at the end of the
301 study (weeks 48-49). Serum PRL increased significantly in the post treatment period only in
302 the Intact and DsRed groups. The IGF-I group showed a trend towards an increase but it did
303 not attain significance (**Fig 3, middle panel).** Serum LH increased with age and after vector
304 injection but this change was significant only in the DsRed and IGF-I groups (**Fig 3, bottom**
305 **panel).** Serum $E_2$ was affected neither by age nor by the treatment **(Fig 3, upper panel).**
306 Serum $P_4$ levels were measured at the end of the study only and no significant differences
307 were detected among the three experimental groups (data not shown).
308
309 **DISCUSSION**
310 Although the sequence of changes that take place during reproductive aging in female rats is
311 qualitatively similar in most strains, the timing is likely to differ among strains and in
312 different laboratory environments. This is why we considered it necessary to characterize the
313 chronology of reproductive changes in our female rat colony before attempting to implement

314 long-term protective gene therapy in cycling M-A animals. In qualitative terms, the age
315 changes in vaginal cytology observed in our Sprague-Dawley females are in agreement with
316 early reports in Long-Evans rats. Thus, in Long-Evans females the vaginal smears show
317 regular 4-5 day cycles from 2 to 10 months of age, transitioning to irregular cycles and
318 persistent vaginal cornification (CE) during the following two months **(40, 41).** In our M-A
319 females this transition takes place earlier. Based on the above results, we started hypothalamic
320 IGF-I gene therapy at 36.2 weeks of age, when a significant proportion of our rats were still
321 cycling regularly. The bicistronic vector used allowed us to visually monitor transgene
322 expression in the hypothalamus at different times after virus injection.
323 The average number of regular and CE cycles per week was taken as an index of reproductive
324 capacity, the former being high in rats with regular ovulatory activity and the latter being high
325 in anovulatory rats **(42)**. The numerous medium and large ovarian follicular cysts and scarce
326 CL shown by the intact and DsRed rats at the end of the study constitute a morphology
327 consistent with the sustained secretion of FSH, estradiol and estrone and the low $P_4$ serum
328 concentrations known to exist in anovulatory M-A rats **(41, 43-45)**. In contrast, the rats
329 submitted to IGF-I gene therapy showed substantially better preserved ovaries.
330 Taken together, the well-preserved histologic features generally observed in the ovaries from
331 the rats submitted to IGF-I gene therapy and the vaginal cytology data from the same animals,
332 strongly suggest that the above intervention delayed the onset of anovulation in M-A females.
333 Additionally, the hormone data indicate that the treatment increased serum LH levels and
334 attenuated the progressive hyperprolactinemia that develops during aging in female rats **(46)**.
335 We conclude that long-term overexpression of IGF-I in the MBH of female rats, started
336 before they transition to the CE stage, prolongs normal hypothalamo-ovarian function.
337 Although the mechanism by which this was achieved remains unknown, some of the studies
338 reviewed above point to IGF-I as a permissive factor necessary for a proper functioning of the

339 positive feedback of $E_2$ on GnRH release from GnRHergic terminals into the ME portal
340 system. This idea is consistent with the firm evidence that IGF-I and estrogens act
341 synergistically in the brain both on reproductive function and in neuroprotection. In effect, an
342 extensive colocalization has been documented for estrogen and IGF-I receptors both in
343 neurons and astrocytes **(47, 48)**. This colocalization suggests a cooperative cross-talk between
344 the two receptors **(49).**
345 Since IGF-I content is reduced in the hypothalamus of M-A rats (**17**), it could be hypothesized
346 that by preventing the reduction of IGF-I levels in the MBH of our M-A rats we preserved the
347 positive feedback of $E_2$ on GnRH release. This in turn may not only have maintained normal
348 ovulatory cycles, but it may also have prevented or at least delayed the progressive disruption
349 that typically occurs in ovarian steroid secretion in M-A rats **(45, 50).** The progressive delay
350 and amplitude reduction in proestrus LH surges (and the increased FSH secretion) in M-A
351 female rats are thought to lead to persistently sustained ovarian $E_2$ secretion which over time
352 exerts an inhibitory action on the steroid positive feedback mechanism on hypothalamic
353 GnRH release **(51, 52),** thus further inhibiting the preovulatory LH surge**.** In any case, IGF-I
354 is probably one of many permissive molecules contributing to a proper functioning of the
355 positive steroid feedback mechanism on GnRH. Thus, hypothalamic IGF-I overexpression by
356 itself should not be expected to indefinitely prolong regular cyclicity in M-A rats. As time
357 passes, the hypothalamic expression of other permissive factors may also drop below critical
358 levels and the positive steroid feedback mechanism on GnRH driving preovulatory LH surges
359 will eventually fail even in rats undergoing hpothalamic IGF-I gene therapy. Since the
360 pituitary gland and the ovaries of old female rats transplanted into young hypophysectomized
361 /ovariectomized recipients can sustain vaginal cyclicity **(53),** it appears that the hypothalamus
362 is the critically age-sensitive component of the hypothalamo-pituitary-ovarian axis in rats.
363 Therefore, the implementation of hypothalamic multi-gene therapy for an appropriate set of

364 permissive factors may allow extending normal ovarian function of M-A female rats well into
365 old age.
366 In rats, reproductive senescence occurs in midlife, whereas in some nonhuman primates, such
367 as rhesus monkeys, this process occurs much later in life (54). Despite this and other inter-
368 species differences in reproductive aging, understanding the basic mechanisms that trigger
369 age changes in the rodent reproductive hypothalamus is likely to shed light on hypothalamic
370 aging in primates, including humans.
371

372 **ACKNOWLEDGEMENTS**

373 CGB and RGG are CONICET career researchers. The authors are grateful to Ms. Yolanda E.
374 Sosa for technical assistance and to Dr. Allison Ebert for pilot data confirming that the AAV-
375 IGFI-ires-DsRed shuttle plasmid expresses IGF-I peptide.
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389 **REFERENCES**

390 1. Wise PM 1982 Alterations in proestrus LH, FSH, and prolactin surges in middle-aged
391 rats. Proc Soc Exp Biol Med 169:348–354.
392
393 2. **Neal-Perry G, Lebesgue D, Lederman M, Shu J, Zeevalk GD, Etgen AM** 2009
394 The excitatory peptide kisspeptin restores the luteinizing hormone surge and
395 modulates amino acid neurotransmission in the medial preoptic area of middle-aged
396 rats. Endocrinology 150:3699–3708.
397
398 3. **Todd BJ, Merhi ZO, Shu J, Etgen AM, Neal-Perry GS** 2010 Hypothalamic Insulin-
399 Like Growth Factor-I Receptors Are Necessary for Hormone-Dependent Luteinizing
400 Hormone Surges: Implications for Female Reproductive Aging. Endocrinology 151:
401 1356-1366.
402
403 4. **Chakraborty TR, Hof PR, Ng L, Gore AC** 2003 Stereologic analysis of estrogen
404 receptor alpha (ER alpha) expression in rat hypothalamus and its regulation by aging
405 and estrogen. J Comp Neurol 466:409–421.
406
407 5. **Funabashi T, Kimura F** 1994 Effects of estrogen and estrogen receptor messenger
408 RNA levels in young and middle-aged female rats: comparison of medial preoptic area
409 and mediobasal hypothalamus Acta Biol Hung 45:223–231.

410 6. **Funabashi T, Kleopoulos SP, Brooks PJ, Kimura F, Pfaff DW, Shinohara K,**
411 **Mobbs CV** 2000 Changes in estrogenic regulation of estrogen receptor alpha mRNA
412 and progesterone receptor mRNA in the female rat hypothalamus during aging: an in
413 situ hybridization study. Neurosci Res 38:85–92

414 7. **Rubin BS, Fox TO, Bridges RS** 1986 Estrogen binding in nuclear and cytosolic
415 extracts from brain and pituitary of middle-aged female rats. Brain Res 383:60–67.
416
417 8. **Zheng W, Jimenez-Linan M, Rubin BS, Halvorson LM** 2007 Anterior pituitary
418 gene expression with reproductive aging in the female rat. Biol Reprod 76:1091–1102.
419
420 9. **Neal-Perry GS, Zeevalk GD, Santoro NF, Etgen AM** 2005 Attenuation of preoptic
421 area glutamate release correlates with reduced luteinizing hormone secretion in
422 middle-aged female rats. Endocrinology146:4331–4339
423
424 10. **Huang HH, Marshall S, Meites J** 1976 Capacity of old versus young female rats to
425 secrete LH, FSH and Prl. Biol. Reprod. 14:538-543.
426
427 11. **Neal-Perry G, Nejat E, Dicken C** 2010 The neuroendocrine physiology of female
428 reproductive aging: An update. Maturitas. 67: 34–38
429
430 12. **Rubin BS, Lee CE, King JC** 1994 A reduced proportion of luteinizing hormone
431 (LH)-releasing hormone neurons express Fos protein during the preovulatory or
432 steroid-induced LH surge in middle-aged rats. Biol Reprod 51:1264–1272.
433
434 13. **Hoffman GE, Finch CE** 1986 LHRH neurons in the female C57BL/6J mouse brain
435 during reproductive aging: no loss up to middle age. Neurobiol Aging 7:45–48.
436

437 14. **Krajnak K, Rosewell KL, Wise PM** 2001 Fos-induction in gonadotropin-releasing
438 hormone neurons receiving vasoactive intestinal polypeptide innervation is reduced in
439 middle-aged female rats. Biol Reprod 64:1160–1164.
440
441 15. **Rubin BS, Lee CE, Ohtomo M, King JC** 1997 Luteinizing hormone-releasing
442 hormone gene expression differs in young and middle-aged females on the day of a
443 steroid-induced LH surge. Brain Res 770:267–276.
444
445 16. **Rubin BS, Bridges RS** 1989 Alterations in luteinizing hormone-releasing hormone
446 release from the mediobasal hypothalamus of ovariectomized, steroid-primed middle-
447 aged rats as measured by push-pull perfusion. Neuroendocrinology 49: 225–232.
448
449 17. **Miller BH, Gore AC** 2001 Alterations in hypothalamic insulin-like growth factor-I
450 and its associations with gonadotropin releasing hormone neurones during
451 reproductive development and ageing. J Neuroendocrinol 13:728–736.
452
453 18. **Le W-W, Wise PM, Murphy AZ, Coolen LM, Hoffman GE** 2001 Parallel Declines
454 in Fos Activation of the Medial Anteroventral Periventricular Nucleus and LHRH
455 Neurons in Middle-Aged Rats. Endocrinology 142:4976–4982.
456
457 19. **Wise PM, Smith MJ, Dubal DB, Wilson ME, Rau SW, Cashion AB, Böttner M,**
458 **Rosewell KL** 2002 Neuroendocrine modulation and repercussions of female
459 reproductive aging. Recent Prog Horm Res 57:235–256
460

461 20. **Neal-Perry GS, Zeevalk GD, Shu J, Etgen AM** 2008 Restoration of the luteinizing
462 hormone surge in middle-aged female rats by altering the balance of GABA and
463 glutamate transmission in the medial preoptic area. Biol Reprod 79:878–888.
464
465 21. **Jarry H, Wise PM, Leonhardt S, Wuttke W** 1999 Effects of age on GABA turnover
466 rates in specific hypothalamic areas in female rats. Exp Clin Endocrinol Diabetes
467 107:59–62.
468
469 22. **Brann DW, Mahesh VB** 2005 The aging reproductive neuroendocrine axis. Steroids
470 70:273–283.
471
472 23. **Krajnak K, Kashon ML, Rosewell KL, Wise PM** 1998 Aging alters the rhythmic
473 expression of vasoactive intestinal polypeptide mRNA but not arginine vasopressin
474 mRNA in the suprachiasmatic nuclei of female rats. J Neurosci 18:4767–4774.
475
476 24. **Le Greves M, Le Greves P, Nyberg F** 2005 Age-related effects of IGF-1 on the
477 NMDA-, GH- and IGF-1-receptor mRNA transcripts in the rat hippocampus. Brain
478 Res Bull 65:369–374.
479
480 25. **Sonntag WE, Bennett SA, Khan AS, A Thornton PL, Xu X, Ingram RL, Brunso-**
481 **Bechtold JK**.2000 Age and insulin-like growth factor-1 modulate Nmethyl-D-
482 aspartate receptor subtype expression in rats. Brain Res Bull 51:331–338.
483

484 26. **Hiney JK, Srivastava VK, Pine MD, Les Dees W** 2009 Insulin-like growth factor-I
485 activates KiSS-1 gene expression in the brain of the prepubertal female rat.
486 Endocrinology 150:376–384.
487
488 27. **Lara JI, Lorenzo MJ, Cacicedo L, Tolón RM, Balsa JA, López-Fernández J,**
489 **Sánchez-Franco F** 1994 Induction of vasoactive intestinal peptide gene expression
490 and prolactin secretion by insulin-like growth factor I in rat pituitary cells: evidence
491 for an autoparacrine regulatory system. Endocrinology 135:2526–2532.
492
493 28. **Servoss SJ, Lee SJ, Gibney G, Gozes I, Brenneman DE, Hill JM**. 2001 IGF-I as a
494 mediator of VIP/activity-dependent neurotrophic factor-stimulated embryonic growth.
495 Endocrinology 142:3348–3353.
496
497 29. **Carro E, Torres-Aleman I** 2006 Serum insulin growth factor I in brain function;
498 IKeio J Med 55: 59-63
499
500 30. **Hereñu CB, Cristina C, Rimoldi OJ, Becú-Villalobos D, Cambiaggi V, Portiansky**
501 **EL, Goya RG** 2007 Restorative effect of Insulin-like Growth Factor-I gene therapy in
502 the hypothalamus of senile rats with dopaminergic dysfunction; Gene Therapy; 14:
503 237-245
504
505 31. **Hereñu CB, Sonntag WE, Morel GR, Portiansky EL, Goya RG** 2009 The
506 ependymal route for insulin-like growth factor-1 gene therapy in the brain.
507 Neuroscience 163: 442-447
508

509 32. **Nishida F, Morel GR, Hereñú CB, Schwerdt JI, Goya RG, Portiansky EL** 2011
510 Restorative effect of intracerebro-ventricular Insulin-like Growth Factor-I gene
511 therapy on motor performance in aging rats. Neuroscience 177**:** 195–206
512
513 33. **Zolotukhin S, Potter M, Zolotukhin I, Sakai Y, Loiler S, Fraites TJ, Jr., Chiodo**
514 **VA, Phillipsberg T, Muzyczka N, Hauswirth WW, Flotte TR, Byrne BJ, Snyder**
515 **RO** 2002 Production and purification of serotype 1, 2, and 5 recombinant adeno-
516 associated viral vectors. Methods 28:158-167.
517
518 34. **Grimm D, Kern A, Rittner K, Kleinschmidt JA** 1998 Novel tools for production
519 and purification of recombinant adenoassociated virus vectors. Hum Gene Ther
520 9:2745-2760.
521
522 35. **Paxinos G, Watson C** 1998 The rat brain in stereotaxic coordinates. San Diego:
523 Academic Press.
524
525 36. **Marcondes F, Bianchi F, Tanno A** 2002 Determination of the estrous cycle phases of
526 rats: some helpful considerations. Braz J Biol 62: 609–614.
527
528 37. **Astwood EB 1939** Changes in the weight and water content of the uterus of the
529 normal adult rat. Am J Physiol 126: 162-170.
530
531 38. **Yoshida M, Sanbuissyo A, Hisada S, Takahashi M, Ohno Y, Nishikawa A** 2009
532 Morphological characterization of the ovary under normal cycling in rats and its
533 viewpoints of ovarian toxicity detection. J Toxicol Sci. 34 Suppl 1:189-197 .

534

535 39. **Røste LS, Taubøll E, Berner A, Isojärvi JI, Gjerstad L** 2001 Valproate, but not
536 lamotrigine, induces ovarian morphological changes in Wistar rats. Exp Toxicol
537 Pathol 52:545-552
538
539 40. **Huang HH, Meites J** 1975 Reproductive capacity of aging female rats.
540 Neuroendocrinology 17:289-295
541
542 41. **Lu JKH, Hopper BR, Vargo TM, Yen SSC** 1979 Chronological changes in sex
543 steroid, gonadotropin and prolactin secretion in aging female rats displaying different
544 reproductive states. Biol Reprod 21: 193-203
545
546 42. **Aschheim P** 1961 Repetitive pseudopregnancy in senile rats (in French) CR Acad Sci
547 253: 1988-1990
548
549 43. **Huang HH, Steger RW, Bruni J, Meites J** 1978 Patterns of sex steroid and
550 gonadotropin secretion in aging female rats. Endocrinology. 103: 1855-1859
551
552 44. **Lu JKH, Kledzik GS** 1981 Chronological changes in ovarian function and
553 morphology in aging rats and their relation to neuroendocrine responses. In: Schwartz
554 NB, Hunzicker-Dunn M, eds. Dynamics of Ovarian Function New York: Raven
555 Press ; 291-296

556

557 45. **Lu, JKH** 1983 Changes in ovarian function and gonadotropin and prolactin secretion
558 in aging female rats. In: Meites J, ed., Neuroendocrinology of Aging. New York:
559 Plenum Press;103-122
560
561 46. **Goya RG, Lu JKH, Meites J** 1990 Gonadal function in aging rats and its relation to
562 pituitary and mammary pathology. Mech Age Dev 56:77-88
563
564 47. **Quesada A, Romeo HE, Micevych P** 2007 Distribution and localization patterns of
565 estrogen receptor-beta and insulin-like growth factor-1 receptors in neurons and glial
566 cells of the female rat substantia nigra: localization of ERbeta and IGF-1R in substantia
567 nigra. J Comp Neurol 503: 198-208
568
569 48. **Cardona-Gomez GP, DonCarlos L, Garcia-Segura LM** 2000 Insulin-like growth
570 factor I receptors and estrogen receptors colocalize in female rat brain. Neuroscience
571 99: 751–760
572
573 49. **Méndez P, Wandosell F, Garcia-Segura LM** 2006 Cross-talk between estrogen
574 receptors and insulin-like growth factor-I receptor in the brain: Cellular and molecular
575 mechanisms. Front. Neuroendocr **27:**391–403 (2006)
576
577 50. **Nass TE, La Polt PS, Judd HL, Lu JKH** 1984 Alterations in ovarian steroid and
578 gonadotrophin secretion preceding the cessation of regular oestrous cycles in ageing
579 female rats; J Endocrinol 100: 43-50
580

581 51. **Lu JKH, Huang HH, Chen H, Kurcz M, Mioduszewski R, Meites J** 1977 Positive
582 feedback by estrogen and progesterone on LH release in old and young rats. Proc Soc
583 Exp Biol Med 154:82-85
584
585 52. **Lu JKH, Gilman DP, Meldrum DR, Judd HL, Sawyer CH** 1981 Relationship
586 between circulating estrogens and the central mechanisms by which ovarian steroids
587 stimulate LH secretion in aged and young female rats. Endocrinology. 108:836- 841
588
589 53. **Peng MT, Huang HH** 1972 Aging of hypothalamic-pituitary-ovarian function in the
590 rat. Fertil Steril 23: 535–542.
591
592 54. **Yin W, Gore AC** 2006 Neuroendocrine control of reproductive aging: roles of GnRH
593 neurons. Reproduction. 131:403-414.
594
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606 **FIGURE LEGENDS**

607 **Figure 1. Effect of long-term MBH IGF-I gene therapy on the frequency of regular,**
608 **irregular and CE cycles in M-A female rats.-** Rats had their vaginal cytology assessed daily
609 from 34.5 to 49.5 weeks of age. Vector injection in the MBH was performed on week 36.2
610 (except in the intact animals) and the number of regular, irregular and CE cycles per week
611 was calculated for the pre-treatment period (weeks 34.5 to 36.2) and the post-treatment period
612 (weeks 36.2 to 49.5). N was 6 for the intact group and 13 for both the DsRed and the IGF-I
613 groups. Asterisks indicate significant (* $P<0.05$) or highly significant (** $P<0.01$) differences
614 versus respective pre-injection control for a two-tailed t-test for equal SD. Dagger (†)
615 indicates significant difference for a one-tailed t-test for significantly different SD's.
616

617 **Figure 2. Right panels-** Histology of representative ovaries from control and experimental
618 49.5 weeks months old M-A rats submitted to long-term IGF-I gene therapy in the MBH.
619 Ovarian section from an intact animal (upper panel). It was assigned **grade 1** and shows large
620 ovarian cysts (OC) but no corpora lutea (CL) or mature follicles (MF).
621 Ovarian section from a DsRed rat (middle panel). It was assigned **grade 2** and shows
622 numerous atretic follicles (AF) but no CL or MF. Apoptotic cells can be observed in the
623 follicular space (arrow). Interstitial connective tissue is abundant and highly cellular. Ovarian
624 section from an animal submitted to IGF-I gene therapy (bottom panel). It was assigned
625 **grade 3** and shows developing follicles (DF) and CL of normal aspect as well as some AF.
626 Scale bar, 300 µm; OM, ovarian medulla
627 **Left panels-** Histomorphometric assessment of the ovaries of control and RAd-IGF-I-treated
628 MA rats. The number of follicular cysts **(A)**, and corpora lutea **(B)** per section as well as the
629 histologic grade **(C)** was assessed in the Intact (N=6), DsRed (N=13) and IGF-I (N=13)

630 groups. Asterisks refer to differences versus the IGF-I group (ANOVA followed by the
631 Tukey´s test), ** ($p<0.01$), *($p<0.05$).

632

633 **Figure 3. Serum hormone levels in M-A rats before and after IGF-I gene therapy.** Blood
634 samples were collected from the tail veins at the beginning (wk 35-36, pre-treatment) and at
635 the end (wks 48-49, post-treatment) of the study. Numbers in parentheses above columns
636 indicate the number of samples assessed. Column height and bar above represents × and
637 SEM, respectively of each data point. * indicates $P<0.05$ and **, $P<0.01$ versus respective
638 pre-treatment value.

639

Figure 1-cycles per week
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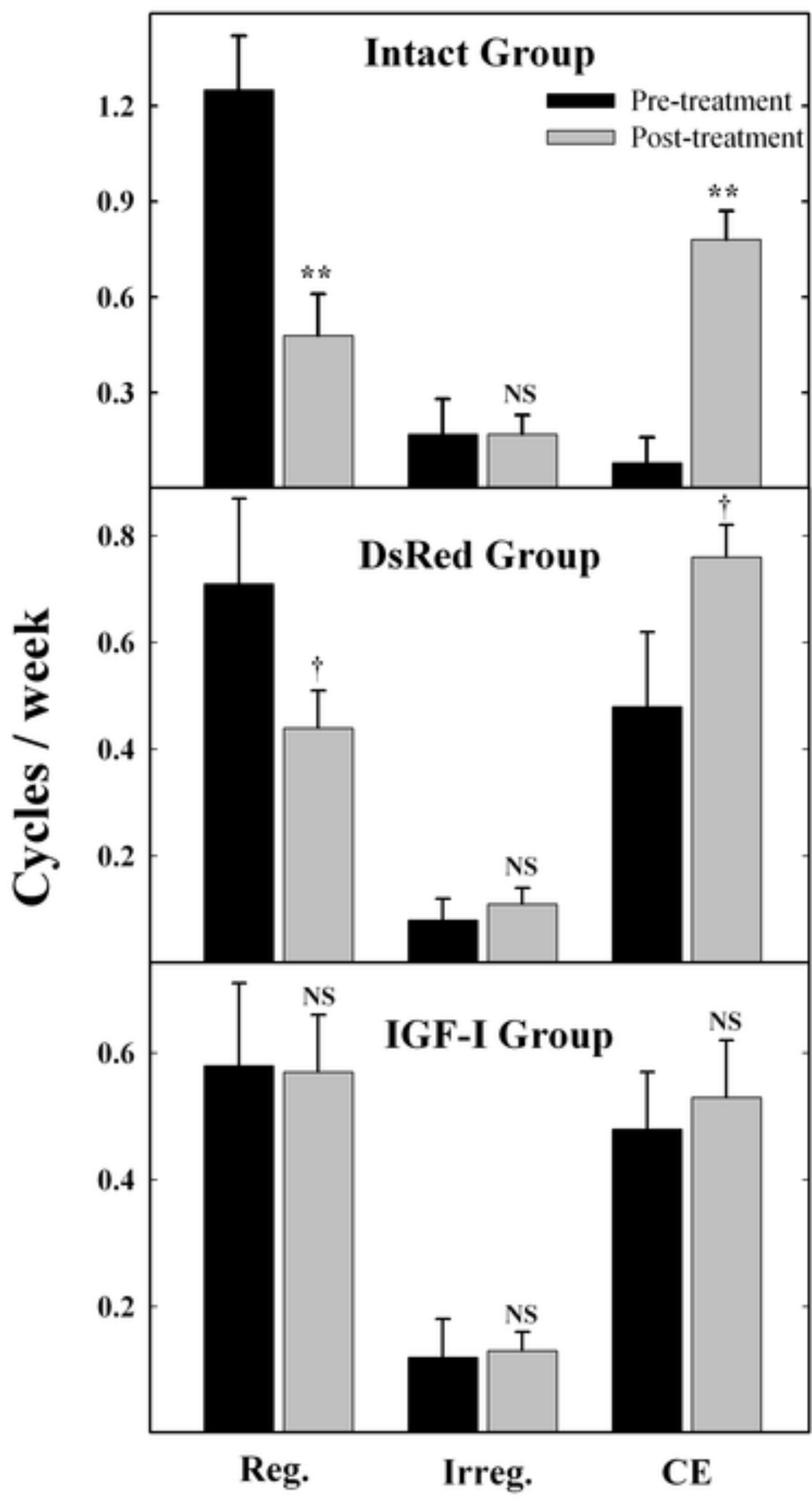

Figure 2-ovarian morphology
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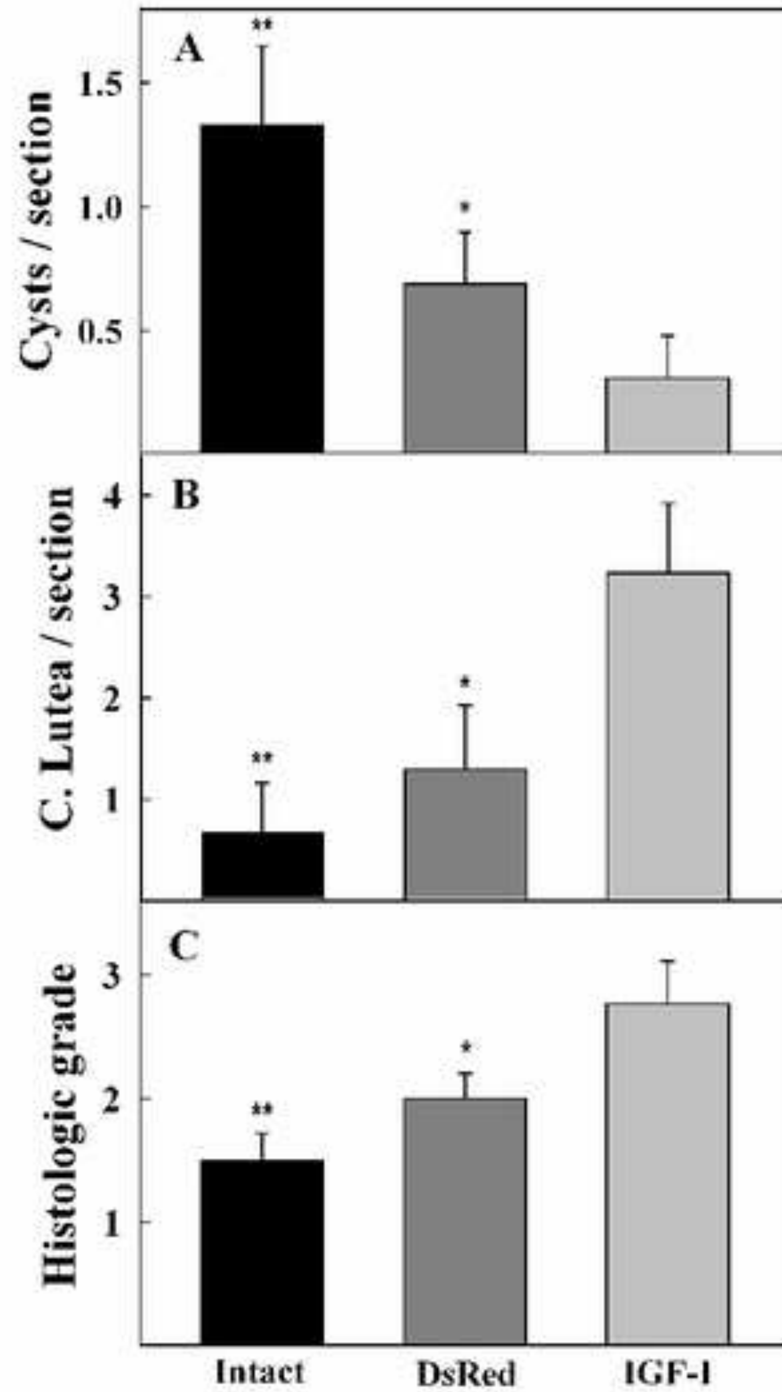


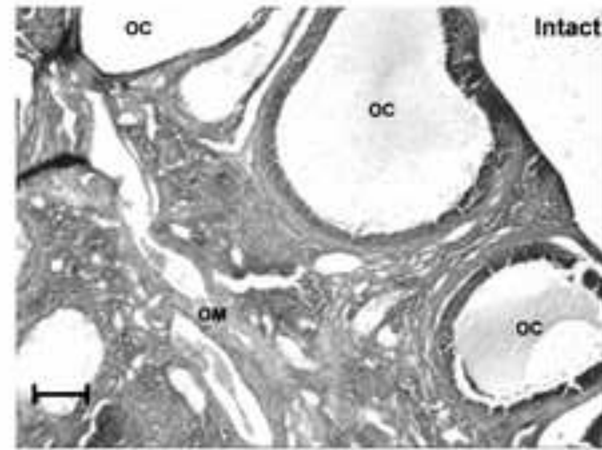


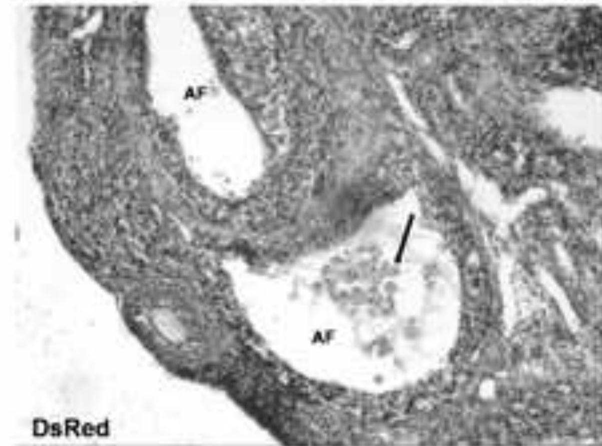


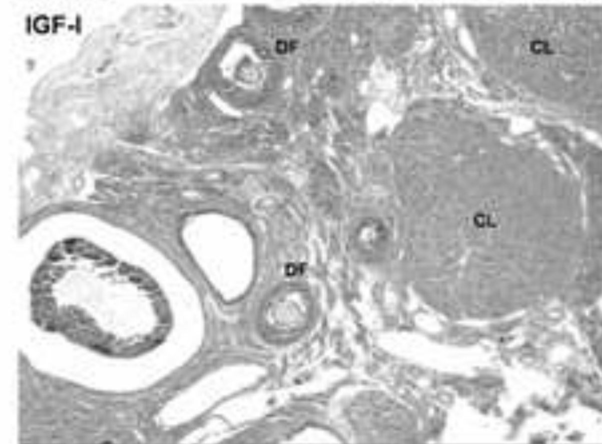

Figure3- LH & PRL fata

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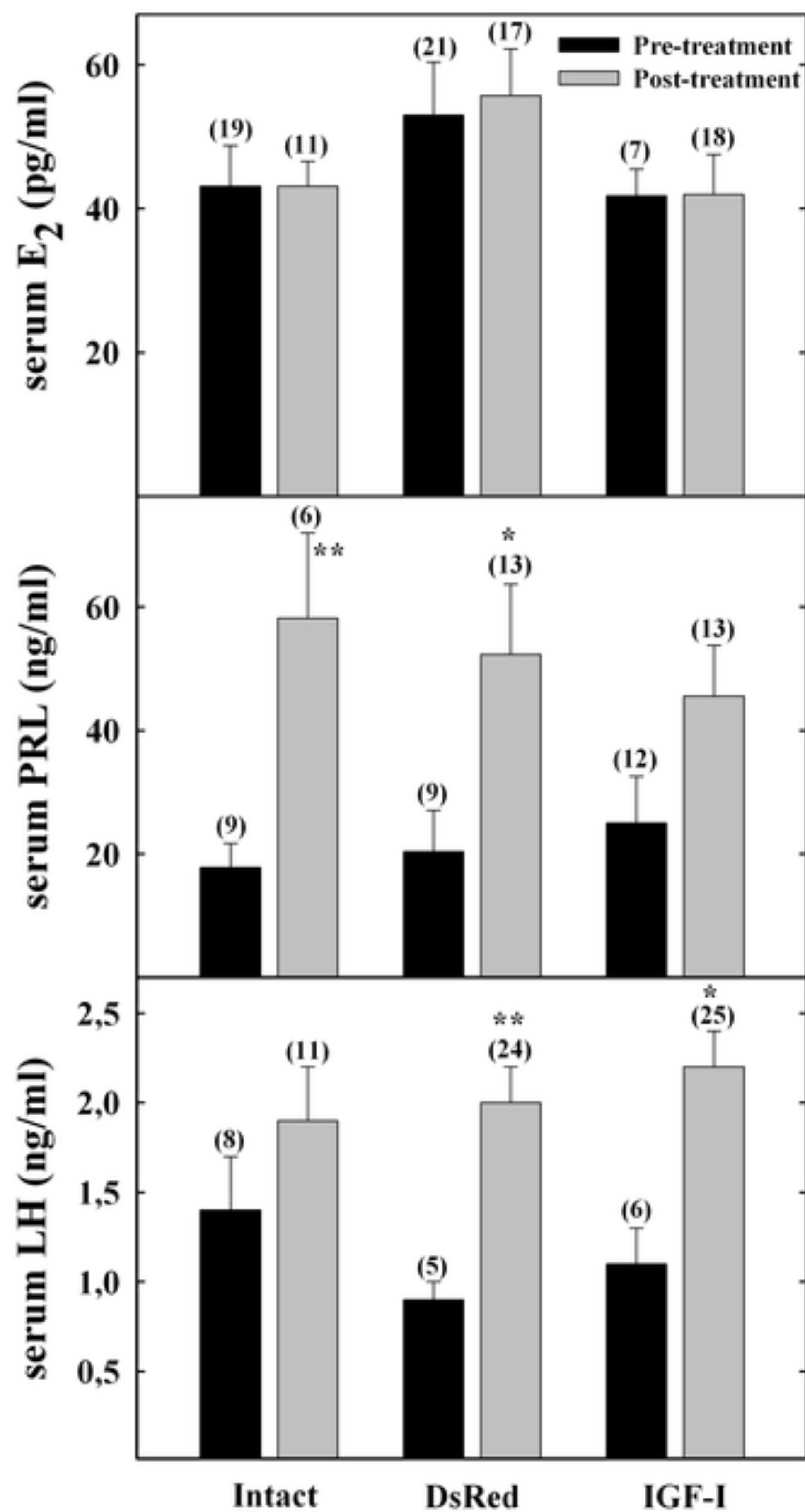

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