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**IGF-I GENE THERAPY IN AGING RATS MODULATES
HIPPOCAMPAL GENES RELEVANT TO MEMORY FUNCTION**

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3 **IGF-I GENE THERAPY IN AGING RATS MODULATES HIPPOCAMPAL**
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5 **GENES RELEVANT TO MEMORY FUNCTION**
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52 **Running title:** IGF-I and hippocampal transcriptome in old rats

53 **Keywords:** aging – IGFI - spatial memory – hippocampal transcriptome - Barnes maze
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59 * These authors contributed equally to this work.
60

ABSTRACT

In rats, learning and memory performance decline during normal aging, which makes this rodent species a suitable model to evaluate therapeutic strategies. In aging rats, insulin-like growth factor-I (IGF-I), is known to significantly improve spatial memory accuracy as compared to control counterparts. A constellation of gene expression changes underlie the hippocampal phenotype of aging but no studies on the effects of IGF-I on the hippocampal transcriptome of old rodents have been documented. Here, we assessed the effects of IGF-I gene therapy on spatial memory performance in old female rats and compared them with changes in the hippocampal transcriptome. In the Barnes maze test, experimental rats showed a significantly higher exploratory frequency of the goal hole than controls. Hippocampal RNA-sequencing showed that 219 genes are differentially expressed in 28 months old rats intracerebroventricularly injected with an adenovector expressing rat IGF-I as compared with placebo adenovector-injected counterparts. From the differentially expressed genes, 81 were down and 138 upregulated. From those genes, a list of functionally relevant genes, concerning hippocampal IGF-I expression, synaptic plasticity as well as neuronal function was identified. Our results provide an initial glimpse at the molecular mechanisms underlying the neuroprotective actions of IGF-I in the aging brain.

INTRODUCTION

In humans and rats, brain aging is associated with a progressive deterioration of spatial learning and memory, which makes this rodent species a suitable model to evaluate therapeutic strategies of potential value for correcting age-related cognitive deficits. Some of these strategies involve the administration of neurotrophic factors, one of which, insulin-like growth factor-I (IGF-I), is emerging as a promising molecule that plays a physiologic role in neuroprotection. Intracerebroventricular (ICV) infusion of IGF-I in the lateral ventricles improves reference and working memory in aging rats (1). Also, it has been documented that IGF-I protects hippocampal neurons from the toxic effects of amyloid peptides (2). Furthermore, IGF-I treatment markedly reduced the brain burden of A β amyloid in transgenic mice expressing a mutant A β amyloid peptide (3).

Gene therapy for IGF-I in the central nervous system (CNS) of senile rats has shown promising results. Thus, a recombinant adenoviral vector (RAd) harboring the gene for rat IGF-I was used to implement IGF-I gene therapy in the hypothalamus of aging female rats displaying tuberoinfundibular dopaminergic neurodegeneration and chronic hyperprolactinemia. The treatment reversed hyperprolactinemia and increased the number of dopaminergic neurons in the hypothalamus of the aging rats (4). The ependymal route is particularly suitable for RAd-mediated gene delivery as it can effectively increase IGF-I levels in the cerebrospinal fluid (CSF) of rats (5). Taking advantage of this fact, we performed ICV IGF-I gene therapy in very old female rats and achieved a significant amelioration of their motor performance (6). In aging rats, it has been recently shown that IGF-I gene therapy significantly improves spatial memory accuracy as compared to control counterparts. Furthermore, in the dentate gyrus (DG) of

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3 the old rats submitted to IGF-I gene therapy there was a higher number of immature
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5 neurons than in the old controls (7).

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7 There is clear evidence that a constellation of gene expression changes underlie the
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9 hippocampal phenotype of aging. Thus, gene expression studies in aging rodents have
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11 documented significant changes in hippocampal genes related to cholesterol synthesis,
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13 inflammation, transcription factors, neurogenesis and synaptic plasticity (8-12). While
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15 those studies revealed that aging itself is associated with the majority of gene expression
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17 changes, a smaller portion of the transcriptional differences in the hippocampus are
18
19 related to changes in learning and spatial memory performance. The above evidence
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21 prompted us to assess the effect of ICV IGF-I gene delivery on the hippocampal
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23 transcriptome in old rats compared with placebo vector-treated counterparts.
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29 MATERIALS AND METHODS

30 Animals

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32 Twenty three 25-months old female Sprague-Dawley rats weighing 267 ± 5 g were used.
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34 Rats were housed in a temperature-controlled room ($22 \pm 2^\circ\text{C}$) on a 12:12 h light/dark
35
36 cycle. Food and water were available *ad libitum*. We used a commercial diet whose
37
38 percentual composition was: humidity, 12%; protein, 23%; lipids, 7%; raw fiber, 6%;
39
40 Total minerals, 10%, Ca, between 1.0 and 1.4%; P, between, 0.5 and 0.8%; Cl, 0.3%;
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42 Na, 0.2%; K, 0.7%; Mg, 0.2%; S, 0.16%. All experiments with animals were performed
43
44 in accordance to the Animal Welfare Guidelines of NIH (INIBIOLP's Animal Welfare
45
46 Assurance No A5647-01). The ethical acceptability of the animal protocols used in this
47
48 study was approved by our institutional IACUC (Protocol # T09-01-2013).
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Spatial memory assessment

The modified Barnes maze protocol used here has been previously documented (7,13).

It consists of an elevated (108 cm to the floor) black acrylic circular platform, 122 cm in diameter, containing twenty holes around the periphery. The holes are of uniform diameter (10 cm) and appearance, but only one hole is connected to a black escape box (tunnel). The escape box is 38.7 cm long x 12.1 cm wide x 14.2 cm in depth and it is removable. A white cylindrical starting chamber (an opaque, 25 cm in diameter and 20 cm high, open-ended chamber) is used to place rats on the platform with a random orientation of their bodies.

Four proximal visual cues are placed in the room, 50 cm away from the circular platform. The escape hole is numbered as hole 0 for graphical normalized representation purposes, the remaining holes being numbered 1 to 10 clockwise, and -1 to -9 counterclockwise. During the whole experiment, hole 0 remained in a fixed position, relative to the cues in order to avoid randomization of the relative position of the escape box. A 90-dB white-noise generator and a white-light 500 W bulb provided the escape stimulus from the platform. At the beginning of the experiment, rats were habituated to the task. The habituation routine consists of placing the animals in the starting chamber and escape box during 180 s. The purpose of habituation consists of accustoming animals to new environments and lowers the level of anxiety. An acquisition trial (AT) consists of placing a rat in the starting chamber, located at the center of the platform, for 30 s; the chamber is then raised, the aversive stimuli (bright light and high pitch noise) are switched on and the rat is allowed to freely explore the maze for 120 s. The purpose of ATs is to train the rats on finding the escape hole. A probe trial (PT) is similar to an AT except that the escape box has been removed, the purpose being to assess recent spatial memory retention. During PTs, rats explore the maze for 90 s. The behavioral

performances were recorded using a computer-linked video camera mounted 110 cm above the platform. The performance of the subjects was determined using the Kinovea v0.7.6 (<http://www.kinovea.org>) software. The behavioral parameters assessed were as follows.

(a) Escape box latency: time (in s) spent by an animal since its release from the starting chamber until it enters the escape box (during an AT) or until the first exploration of the escape hole (during a PT). A shorter escape box latency time indicates a better learning.

(b) Nongoal hole exploration (errors): number of explorations of holes different from the escape one. Each exploration of an incorrect hole is counted as an error, provided that the rat lowers its nose below the plane of the table surface.

(c) Exploration frequency: the times the rat explores every hole of the maze during the allotted time (90 s). A higher exploration frequency of the goal hole and the holes close to it indicates higher spatial memory accuracy.

Helper-dependent adenovectors (HD-RA_d)

HD-RA_ds were constructed using a kit sold by Microbix Biosystems (Ontario, Canada). The kit provides the shuttle plasmid pC4HSU, the helper virus H14 and the 293 Cre4 cell line. The construction procedure followed the guidelines of the Microbix manual and those described by Oka and Chan (14). Briefly, an expression cassette containing the gene for either rat IGF-I or the red fluorescent protein DsRed, both under the control of the murine cytomegalovirus (mCMV) promoter, was cloned in pC4HSU, a plasmid that consists of the ITRs for Ad 5 virus, the packaging signal and part of the E4 adenoviral region plus a stuffer noncoding DNA of human origin which keeps a suitable size (28-31 Kbp) of the viral DNA so that it is efficiently packaged into the capsids

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3 during vector generation but bands at sufficient distance from helper virus H14 in CsCl
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5 gradients, thus minimizing the risk of contamination of the newly generated vector. The
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7 shuttle vector harboring the expression cassette of interest was transfected in 293 Cre4
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9 cells which were then infected with the helper virus Ad H14 whose packaging signal is
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11 flanked by lox P sites recognized by the Cre recombinase expressed by the 293 Cre4
12
13 cells. Therefore, the helper virus provides in trans all of the viral products necessary for
14
15 generation of the desired HD-RAd. Following iterated coinfections with the HD-RAd
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17 and H14 virus, a sufficiently high concentration of HD-RAd is generated whereas very
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19 low levels of H14 are produced due to the cleavage of the packaging signal of H14
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21 effected by the Cre recombinase. The new adenovectors, termed **HD-RAd-IGF-I** and
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23 **HD-RAd-DsRed** as appropriate, were purified by ultracentrifugation in a CsCl gradient
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25 and titrated for adenoviral particles.
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32 **Experimental design**

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34 We used a modified version of the protocol for the Barnes maze paradigm already
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36 described in our laboratory (7, 13). On experimental day -10 the animals were
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38 habituated as described above. The task was organized into 3 separate sessions at one
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40 month interval. Every session lasted 9 days and consisted of four ATs per day, done
41
42 every fifteen minutes. On the last day of each session, fifteen minutes after the last AT a
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44 PT was conducted to assess spatial memory as preference for the goal hole (hole 0). On
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46 experimental day -10, ten rats were allotted to group "DsRed" (injected with placebo
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48 Adenovirus) and 12 rats were allotted to group "IGF-I" (injected with an IGF-I
49
50 Adenovirus) (see below). On experimental day 0 rats were ICV injected with the IGF-I
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52 or DsRed vector as appropriate and on day 80, CSF was obtained from the great
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54 cerebral cistern by puncture as previously documented (6). On experimental day 80, at
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3 age 28 months, animals were euthanized by rapid decapitation and their hippocampi
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5 microdissected and stored at -80°C until RNA extraction (see below) (**Fig 1A**).
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9 **Stereotaxic injections.** DsRed and IGF-I rats were anesthetized with ketamine
10 hydrochloride (40 mg/kg; ip) plus xylazine (8 mg/kg; im) and placed in a stereotaxic
11 apparatus. In order to access the lateral ventricles (LV), the tip of a 26G needle fitted to
12 a 10µl syringe was placed at the following coordinates relative to the bregma: -0.8 mm
13 anteroposterior, 4.1 mm dorsoventral and ±1.5 mm mediolateral (**15**). The animals were
14 injected bilaterally with 8 µl per side of a suspension containing 1.7×10^{12} viral particles
15 of the appropriate vector.
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19 **IGF-I assay.** IGF-I was extracted from CSF samples (20 µl) by acid-ethanol
20 cryoprecipitation and was radioimmunoassayed using antibody UB2-495 from L.
21 Underwood and J.J. Van Wyk, which is distributed by the Hormone Distribution
22 Program of the National institute of Diabetes and Digestive and Kidney diseases
23 (NIDDK, Bethesda), National Hormone and Pituitary Program. Recombinant human
24 IGF-I (rh IGF-I, Chiron Corp., Emeryville, CA) was used as tracer and unlabeled
25 ligand. Intra and inter-assay coefficients of variation were 7.2 and 12.8%, respectively.
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43 **RNA extraction, library preparation, and sequencing**

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45 The brains from four rats per group (i.e., DsRed and IGF-I groups) were rapidly
46 removed after euthanasia and their right hippocampus dissected for transcriptome
47 analysis. The hippocampus was stored at -80°C until RNA extraction.
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51 Tissues were homogenized in TRIzol Reagent (Life Technologies). The quality of the
52 isolated RNA was assessed by measuring the RIN (RNA Integrity Number) using the
53 Fragment Analyzer. Library preparation for RNA-Seq was performed using the truSeq
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3 RNA Sample Preparation Kit (Illumina, Cat. N° RS-122-2002) starting from 500 ng of
4 total RNA. Accurate quantitation of cDNA libraries was performed using the
5 QuantiFluor™ dsDNA System (Promega). The size range of final cDNA libraries
6 was 280-320 bp and was determined applying the DNA Chip for NGS Libraries using
7 the Fragment Analyzer (Advanced Analytical). cDNA libraries were amplified and
8 sequenced by using the cBot and HiSeq2000 from Illumina (SR; 50 bp; ca. 30–35
9 million reads per sample). Raw datasets have been submitted to NCBI GEO database.
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20 **RNA-Seq data analysis**

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22 Illumina HiSeq 2000 fluorescence images were transformed to BCL files with the
23 Illumina BaseCaller software and samples were demultiplexed to FASTQ files with
24 CASAVA (version 1.8.2). Sequencing quality was checked and approved via the
25 FastQC software. Sequences were aligned to the genome reference sequence of *Rattus*
26 *norvegicus* (RGSC assembly v5.0) using the STAR alignment software (16) allowing
27 for 2 mismatches within 50 bases. Subsequently, resulting SAM files were converted to
28 sorted BAM files, filtering of unique hits and counting was conducted with SAMtools
29 (17) and HTSeq (18).
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40 We used the Bioconductor package edgeR for differential expression analysis of reads
41 counts arising from RNAseq between hippocampal samples from IGF-I and DsRed rats.
42 (19). The list of differentially expressed genes (DEG) was established from a Log Fold
43 Change > 0.5 and a p adj. value <0.05.
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49 Functional enrichment analyses of DEG were performed using the databases for
50 annotation, visualization and integrated discovery (DAVID,
51 <http://david.abcc.ncifcrf.gov/>), Enrichr (<http://amp.pharm.mssm.edu/Enrichr/>), and
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GeneMania resources (<http://genemania.org/>). Data integration and plots visualization were done with R and the MultiExperiment Viewer software (MeV v4.9) (20).

Q RT-PCR

Total RNA was treated with gDNA wipeout and cDNA was synthesized with the Qiagen QuantiTect Reverse transcription kit (#205310). qPCR was performed with the MESA BLUE qPCR MasterMix Plus for SYBR Assay Low ROX on a Stratagene Mx3000P qPCR system. The primers used are listed in **Suppl Table 1**. The $2^{(-\Delta\Delta CT)}$ method was employed for measuring the gene variation between DsRed and IGF-I rats.

Statistics

Behavioral data were analyzed with the GraphPad Prism 6 Software. Latency to escape box and errors made were analyzed by Two way ANOVA, considering AT and treatment factors. When ANOVA was significant, comparisons between means and AT1 were performed with the Sidak post hoc test. Unpaired t-test was used for IGF-I levels, goal hole exploration in PTs and qRT-PCR. For RNAseq data analysis, as described above, we used the statistical language R and the analysis packages from Bioconductor.

RESULTS

Effects of IGF-I gene therapy on spatial memory

Latency and errors to escape box. The treatment did not induce significant changes in either latency or errors to escape box, two parameters that are a measure of learning ability. During the first series of AT sessions, (before treatment), both parameters fell significantly at comparable rates in the two groups (Two way ANOVA, Treatment factor $p=0.19$, AT factor $p<0.0001$, interaction $p=0.34$) (Sidak post-hoc test AT2

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3 onwards vs AT1 $p < 0.0001$). During sessions 2 and 3 both latency and errors to escape
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5 box remained low in both groups, indicating that the animals remembered the location
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7 of the escape hole as well as at the end of session 1 (**Fig. 1B&C**).

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10 **Hole exploration frequency.** As expected, hole exploration frequency showed
11
12 overlapping bell-shaped distributions around hole 0 in the pre-treatment PT (**Fig. 1D**).
13
14 Thirty-eight days after vector injection the distribution of exploration frequency
15
16 remained comparable in both groups (**Fig. 1E**), but at 77 days post-treatment,
17
18 exploration frequency of the goal hole (hole 0) in the IGF-I group was significantly
19
20 higher than in the DsRed counterparts (unpaired t-test $t = 2.644$, $df = 20$, $p = 0.016$). (**Fig.**
21
22 **1F**). The higher exploration frequency displayed in PT3 by the IGF-I rats at the goal
23
24 hole suggests that at this time point, the treatment increased the accuracy of spatial
25
26 memory in the aged animals.
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29 **Ventricular transgene expression and IGF-I levels**

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31 Two days after ICV DsRed adenovector injection there was a strong expression of
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33 DsRed in the ependymal cells lining the cerebral ventricles (**Fig 2A, B, C and D**).
34
35 Eighty days after vector injection (on the day of sacrifice) transgene expression in the
36
37 ependymal cell layer was still observable (**Fig 2E, F and G**). On experimental day 80
38
39 CSF levels of IGF-I were significantly higher in the IGF-I group than in the DsRed
40
41 counterparts (unpaired t-test $t = 4.967$, $df = 5$, $p = 0.0042$) (**Fig 2H**). The results of this
42
43 section indicate that transgene expression of the adenovector genome in the ependymal
44
45 cells remains active for at least 80 days after injection.
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49 **Hippocampal genes whose expression is modified by IGF-I gene therapy in old rats**

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51 Analysis of the hippocampal transcriptome of old rats revealed that after long-term IGF-
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53 I gene therapy, 219 genes were significantly ($P < 0.05$) differentially expressed, 81 down
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55 and 138 up (**Suppl table 2**). We performed quantitative RT-PCR analysis of 4
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3 representative transcripts namely, Itga 8, Sypl2, Dusp1 and Nnat. The results were in
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5 line with the RNAseq data (**Fig 3D**).

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7 From those genes, a list of functionally relevant genes, concerning hippocampal IGF-I
8
9 expression, synaptic plasticity as well as neuronal function was identified (**Table 1**).
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11 They were grouped as follows.

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13 **Hippocampal genes related to IGF-I expression and transport-** Expression of IGF-I
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15 and its binding protein IGFBP-6, in the hippocampus was significantly upregulated by
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17 ICV IGF-I gene therapy. In contrast, the treatment downregulated the expression of the
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19 gene encoding IGF binding protein IGFBP-4. Data analysis using the Kyoto
20
21 Encyclopedia of Genes and Genomes (KEGG) database collections revealed that the
22
23 IGF-I gene is functionally linked to a number of metabolic pathways and to several of
24
25 the hippocampal genes differentially expressed by the treatment (**Fig. 3**).

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27 **Genes involved in synaptic plasticity and neurogenesis-** Three genes related with
28
29 synaptic processes were up-regulated after IGF-I gene therapy. The up-regulated genes
30
31 were Synaptophysin-like protein 2 (Sypl2), Neuronatin (Nnat) and integrin $\alpha 8$ subunit
32
33 (Itga-8). Sypl2 is an integral component of synaptic vesicle membrane, cellular calcium
34
35 ion homeostasis, transporter activity and substantia nigra development (see the
36
37 Discussion section for further details).

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39 Nnat mRNA is abundant in dendrites particularly in rat brain embryos.
40
41 ITGA8 expression in brain mediates cell-cell interactions and regulates neurite
42
43 outgrowth of sensory and motor neurons. The neurogenesis related gene DCX, that
44
45 encodes the cytoskeletal protein doublecortin, was significantly upregulated by the
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47 treatment.
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51 **Micro RNA 186 (miRNA 186) and β -site amyloid precursor cleavage-** IGF-I gene
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53 therapy up-regulated miRNA-186 in the hippocampus of 28 months old female
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3 Sprague-Dawley rats. miR-186 is a potent negative regulator of β -site amyloid
4 precursor protein-cleaving enzyme 1 (BACE1) in neuronal cells (see Discussion for
5 further details and references).
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10 **Miscellaneous genes downregulated by IGF-I gene therapy in old rats- DUSP1 and**
11 **Nr4a1** encode for DUSP1 (phosphatase) and Nr4a1 (transcription factor), two proteins
12 induced when the glucocorticoid receptor is phosphorylated and activated.
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16 Taken together, the results of this section show that among the hippocampal genes
17 whose expression was modified by the treatment there is a set of genes functionally
18 relevant to a number of hippocampal activities affected by aging.
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24 25 **DISCUSSION**

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27 Cognitive aging leads to a progressive decline in memory function. There is a reduced
28 persistence of experimentally induced, long-term potentiation (LTP) of hippocampal
29 synapses, which is correlated with faster behavioral forgetting of spatial information
30 (21). Spatial memory of aged rats is also impaired as revealed by various tests of spatial
31 learning and memory (13,22-24). Hippocampal neurogenesis is important for certain
32 types of memory and falls significantly in aged rats (13,25). The present results confirm
33 our previous findings on the restorative ability of IGF-I gene therapy on spatial memory
34 accuracy (7,26).
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45 **Gene-expression changes in the hippocampus during aging**

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47 There is clear evidence that a constellation of gene expression changes underlie
48 hippocampal phenotype aging. Thus, gene expression studies in aging rodents have
49 documented significant changes in hippocampal genes related to cholesterol synthesis,
50 inflammation, transcription factors, neurogenesis and synaptic plasticity. In rodents,
51 aging and, to a lesser extent, deficits in memory performance have been associated with
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3 changes in hippocampal gene expression (8-12). These differences consist mostly of
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5 gene upregulation in middle-aged mice (15-mo old) as compared with 2-mo old
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7 counterparts (12). In the CA1 hippocampal region of old male rats 233 genes were
8
9 found to be differentially expressed with aging, 60% upregulated and 40%
10
11 downregulated (8). We have recently found that in the entire hippocampus of female
12
13 rats, 210 transcripts are differentially expressed in old animals when compared with
14
15 young counterparts, with 61% being downregulated and 39% upregulated (27).
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20 21 **Hippocampal transcriptome changes induced by IGF-I gene therapy in old rats**

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23 To our knowledge, there are no documented studies on the effects of neuroprotective
24
25 factor treatment on the hippocampal transcriptome of aging rats. Since we have
26
27 characterized the restorative effect of IGF-I gene therapy on cognitive performance in
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29 aging female rats (7), we were interested in correlating the transcriptome changes
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31 induced by long-term IGF-I gene therapy on the hippocampus of aging female rats with
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33 hippocampal function improvement. To this end, we performed RNA-seq analysis of
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35 the whole female rat hippocampus of old rats ICV injected with a helper dependent-
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37 adenovector expressing rat IGF-I comparing the results with placebo vector-treated
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39 counterparts.
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43 From the 219 genes significantly differentially expressed by IGF-I gene therapy in the
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45 hippocampus of old rats, we could identify a short list of genes relevant to IGF-I
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47 expression and transport, synaptic plasticity and neurogenesis as well as neuronal
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49 function.
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54 **Genes related to IGF-I-** Since our IGF-I adenovector was delivered via ICV, the IGF-I
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56 gene upregulation recorded in the hippocampus must reflect the expression of the
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3 endogenous rat gene, implying that transgenic IGF-I directly or indirectly stimulated
4 hippocampal IGF-I production. Increased expression of IGF-I in the hippocampus is
5 likely to have played a significant role in the deregulation of a number of hippocampal
6 genes in the experimental old rats (**Fig. 3**). The IGFBP4 and IGFBP6 binding proteins
7 are members of the insulin-like growth factor binding protein family. They bind and
8 prolong life in blood of both insulin-like growth factors (IGFs) I and II and alter their
9 interaction with cell surface receptors (**28,29**).

10
11 The treatment downregulated the expression of IGFBP4 and upregulated the expression
12 of IGFBP6. Consequently, hippocampal tissue levels of free IGF-I and IGF-II are likely
13 to have changed as a result of the altered proportion of these two binding proteins.
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27 **Genes involved in synaptic plasticity and neurogenesis-** Four genes related with
28 synaptic processes and neurogenesis were up-regulated after IGF-I gene therapy,
29 namely Sypl2, Nnat, Itga-8 and DCX.
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33 Sypl2, mouse alias Mitsugumin 29, is an integral component of synaptic vesicle
34 membrane, it regulates cellular calcium ion homeostasis, transporter activity and
35 substantia nigra development (**30**). Sypl2 is a distinctly inducible gene also in human
36 astrocytes surrounding A β -containing senile plaques in vivo. In lesions of Alzheimer's
37 Disease (AD) brain, increased expression of Sypl2 is detected only in activated
38 astrocytes. But in quiescent astrocytes in non-AD brain and in lesion-free areas of AD
39 brains, the expression of this gene is controlled at a low level (**31**). We hypothesize that
40 overexpression of Sypl2 in astrocytes could play a neuroprotective role, preventing the
41 development of senile plaques.
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54 Nnat is a maternal imprinted gene, which encodes a membrane protein in the
55 endoplasmic reticulum (**32,33**). mRNA levels are highest early in brain development
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3 and decrease postnatally (34). However, traces of neuronatin mRNA continue to be
4 present even in the adult brain including the hypothalamus, hippocampus and pituitary
5 gland (35-37). NNAT, shown to be enriched in isolated dendrites, provides a means for
6 rapidly eliciting site-specific changes in protein levels during neuronal development and
7 synaptic plasticity (35,36,38). Importantly, NNAT levels increase during neurogenesis
8 (between E16–19) (35). Oyang and col. reported that NNAT is indeed dendritically
9 translated in mature hippocampal neurons during homeostatic plasticity and that it likely
10 regulates dendritic calcium by modulation of intracellular Ca²⁺ stores by antagonizing
11 SERCA pump activity (37). Although high embryonic and early postnatal expression
12 has suggested significant roles for NNAT during neuronal development and
13 neurogenesis, its function in mature neurons has not been examined. Here, we show that
14 IGF-I up-regulated NNAT in the senile hippocampus, which suggests that this peptide
15 may be playing a significant modulatory role in neuronal plasticity during aging.

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A8-integrin (ITGA8) expression in the brain mediates cell-cell interactions and
regulates neurite outgrowth of sensory and motor neurons (39,40). Accumulating
evidence has implicated integrin function in the CNS physiology underlying synaptic
and behavioral plasticity. Mice deficient in $\alpha 8$ -integrin in the forebrain are impaired
specifically in the expression of hippocampal LTP (40). In this context, the up-
regulation of $\alpha 8$ -integrin in the hippocampus of senile rats reported here could
contribute to restoring LTP and consequently, improve spatial memory.

Doublecortin is a cytoskeleton-associated protein (41) present in immature neurons. As
such, DCX expression in the hippocampal DG indicates quantity of immature neuron
count (neurogenesis). In a previous study, we observed that IGF-I gene therapy prompts
an increase in DCX (+) neuron number in the DG of senile rats (7). Thus, the
upregulation reported herein is in line with our previous findings.

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3 **miRNAs role in aging brain and Alzheimer disease pathology-** Studies on the roles
4 that microRNAs (miRNAs) play in brain aging and AD pathogenesis have only recently
5 been initiated (42-45). MiRNAs are endogenous small RNA molecules that control gene
6 expression post-transcriptionally, primarily through binding to complementary target
7 sequences in the 3' untranslated regions (UTRs) of mRNAs. Age is significantly
8 associated with a decline in miRNA expression levels in the brains of fish (46), mice
9 (45,47,48), rats (49), chimpanzees (44), rhesus macaques (42,44), and even humans
10 (42,44). Moreover, low miRNA levels are likely to contribute to loss of brain
11 functioning and neurodegeneration (50-52).

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23 In this context, our finding that IGF-I gene therapy up-regulates miR-186 in the
24 hippocampus of 28 months old female Sprague-Dawley rats is likely to be relevant for
25 improvement of memory function. miR-186 expression is significantly decreased in
26 mouse cortices at 13 months of age, compared to 2 months of age, and it shows a trend
27 to further decrease at 24 months of age. In the brain, miR-186 is broadly expressed
28 across multiple brain subregions in mice (53). miR-186 is a potent negative regulator of
29 β -site amyloid precursor protein-cleaving enzyme 1 (BACE1) in neuronal cells and it
30 may be one of the molecular links between brain aging and the increased risk for AD
31 during aging (53). Importantly, miR-186 over-expression significantly decreases A β
32 level by suppressing BACE1 expression in cells expressing human pathogenic mutant
33 amyloid precursor protein (53). In this context, our finding that IGF-I up-regulates miR-
34 186 levels in the hippocampus of senile rats suggests a neuroprotective role.

51 52 **Concluding Remarks**

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54 The present study shows for the first time, to the best of our knowledge, that IGF-I gene
55 therapy in the brain of aging rats induces significant changes in the expression of a large
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3 number of hippocampal genes (219). Although we cannot determine the functional
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5 relevance of all of them in hippocampal function, we could identify a limited number of
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7 upregulated genes that play a significant role in synaptic plasticity and neurogenesis.
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9 Interestingly, IGF-I upregulates the expression of miRNA 186, a micro RNA whose
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11 production declines with age and which exerts a neuroprotective action by inhibiting the
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13 activity of BACE1 protease. Furthermore, the IGF-I gene and one of its binding proteins
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15 were also upregulated. Our results provide an initial glimpse at the molecular
16
17 mechanisms underlying the neuroprotective actions of IGF-I in the aging brain.
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41 2015).
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AUTHORS' CONTRIBUTIONS

JP, GRM and OMO did the cognitive studies and the analysis of the behavioral data.

MA, EL, TFO and RGG performed the analysis and interpretation of the RNA-seq data.

The qRT-PCR experiments were done by IP. JP, MA and EL designed the different plots and graphs. JP, GRM, MA, EL, TFO and RGG wrote different sections of the manuscript. JP, GRM, TFO and RGG assembled the final version of the paper.

We hereby declare that none of the authors has potential competing interests.

LIST OF ABBREVIATIONS

AD: Alzheimer's disease

AT: acquisition trial

BM: Barnes Maze

CSF: cerebrospinal fluid

DG: dentate gyrus

ICV: intracerebroventricular

IGF-I: Insulin like growth factor I

GO: gene ontology analysis

PT: probe trial

RAAd: Recombinant adenoviral vector

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



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Table 1

Effect of IGF-I gene therapy on functionally relevant hippocampal genes of old rats

Gene	Biological function	Expression modulation	Reference
Igf1 	Neuroprotection	↑	<i>Pardo et al., 2016</i>
Dcx 	Neurogenesis	↑	<i>Gleeson et al., 1999</i>
Itga-8	Cell-cell interaction	↑↑↑	<i>Bossy et al., 1991; Chan et al., 2010</i>
Sypl2	Synaptic function	↑↑	<i>Shimuta et al., 1998</i>
Nnat		↑↑	<i>Dou et al., 1996; Kagitani et al., 1997</i>
Igfbp4 	IGF transport in blood	↓	<i>Shimazaki et al., 1990</i>
Igfbp6 		↑	<i>Ehrenborg et al., 1999</i>
Ang2 	Blood vessel growth	↓↓↓	<i>Yuan et al, 2009</i>
Dusp1	Glucocorticoid response	↓↓	<i>Arango-Lievano et al, 2016</i>
Nr4a1		↓	
MiRNA 186 	β-amyloid processing	↑	<i>Kim et al, 2016</i>

The indicated references are included in the general Reference list at the end of the paper. The number of arrows for each gene indicates the magnitude of its deregulation in the old rats of the IGF-I group versus the DsRed counterparts.

FIGURE LEGENDS

Figure 1. Effect of IGF-I gene therapy on the performance of old rats in the Barnes Maze.- **Panel A**, shows a diagram illustrating the experimental design used. Learning ability was assessed by performing 3 sessions of 9 days each (4 AT per day) with a 30-day interval between each session. On the last day of each session a probe trial (PT) was conducted. DsRed or IGF-I adenovectors were ICV injected on Experimental Day 0 (syringe icon). Eut= Euthanize. **Panels B and C** show latency to escape hole and error number during the three sessions performed during the experiment. Arrows indicate vector injection day. **Panels D, E and F** show hole exploration frequency in probe trials 1, 2 and 3. Notice the sharp increase in exploratory frequency of hole #0 (escape hole) in the IGF-I group at PT3. Data are represented as mean \pm SEM. Comparisons between IGF-I versus DsRed are made for each pair of IGFI – DsRed time points. *: $P < 0.05$. N was 10 for DsRed group and 12 for IGF-I group.

Figure 2. Transgene expression and IGF-I levels in the CSF of DsRed and IGF-I rats. **Panel A** shows a brightfield image of the LV of an old rat 2 days after DsRed adenovector injection. **Panel B** shows expression (red fluorescence) of DsRed in the ependymal layer of the LV. **Panel C** represents a magnification of the yellow framed region shown on Panel B. **Panel D** shows a diagrammatic representation of the ependymal cell layer shown on Panel C. **Panels E, F and G** show DsRed fluorescence, DAPI staining and merge of the two colors, respectively, in the 3V at the end of the experiment (Exp. Day 80). **Panel H** shows CSF IGF-I levels on Exptl. day 80 in DsRed and IGF-I animals. N was 3 and 4 for the DsRed and IGF-I groups, respectively. LV, lateral ventricle; 3V, third ventricle; CPu, caudate Putamen. Scale bar for panel

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3 B=250 μ m which also applies to panel A; scale bar for panel G=100 μ m which also
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5 applies to panels C, E, and F.
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10 **Figure 3. A.** Heatmap representation of the 219 DEG (138 up & 81 down) genes
11 between DsRed and IGF-I rats ordered according to the LogFC values. **B.** Functional
12 enrichment of the upmodulated genes in IGF-I rats, based on Kegg-2016 gene set
13 library. **C.** Network representation of IGF-I related genes obtained from the list of
14 upmodulated genes. Red nodes indicate genes from the query gene list, whereas blue
15 nodes indicates genes related to the query. **D.** Bar plots representing qRT-PCR of four
16 representative genes induced and repressed by IGF-I gene therapy: N=4 for both groups.
17 Bars represent mean \pm SD. * p<0.05, **p<0.01, ***p<0.001.
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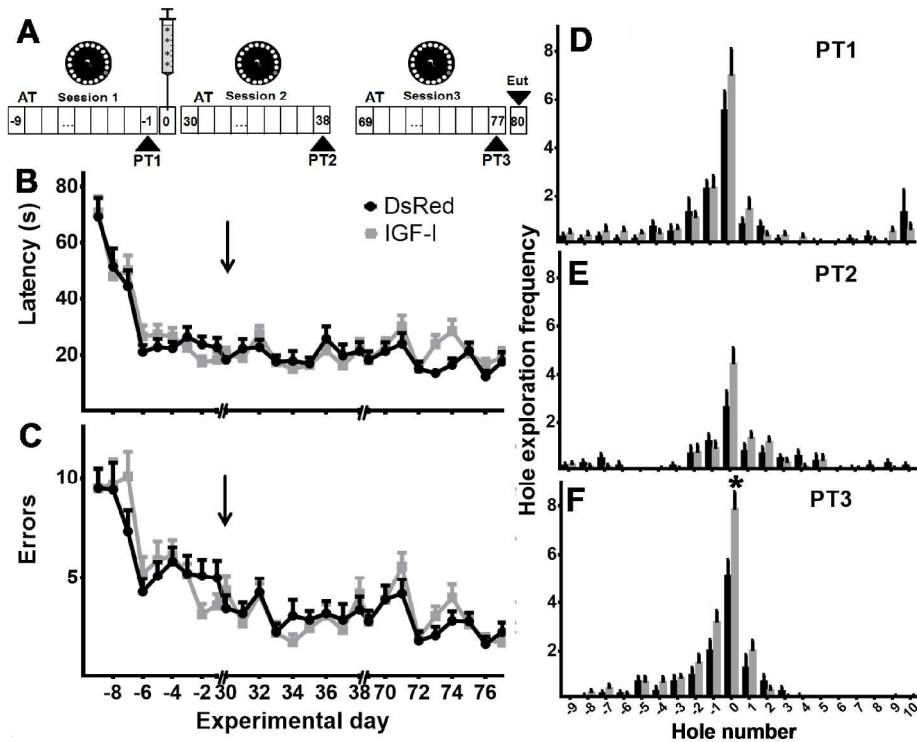


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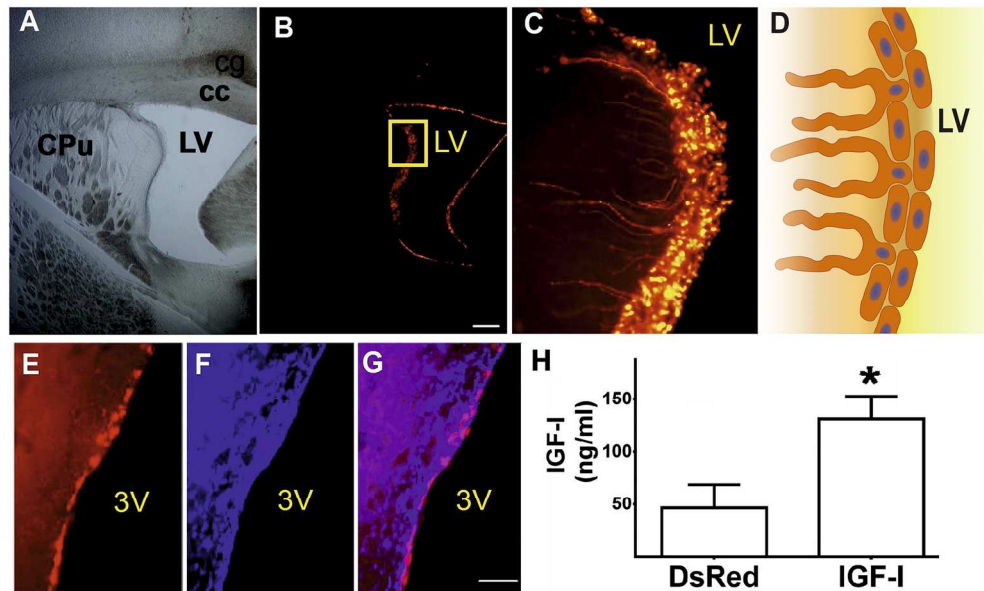


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173x102mm (300 x 300 DPI)

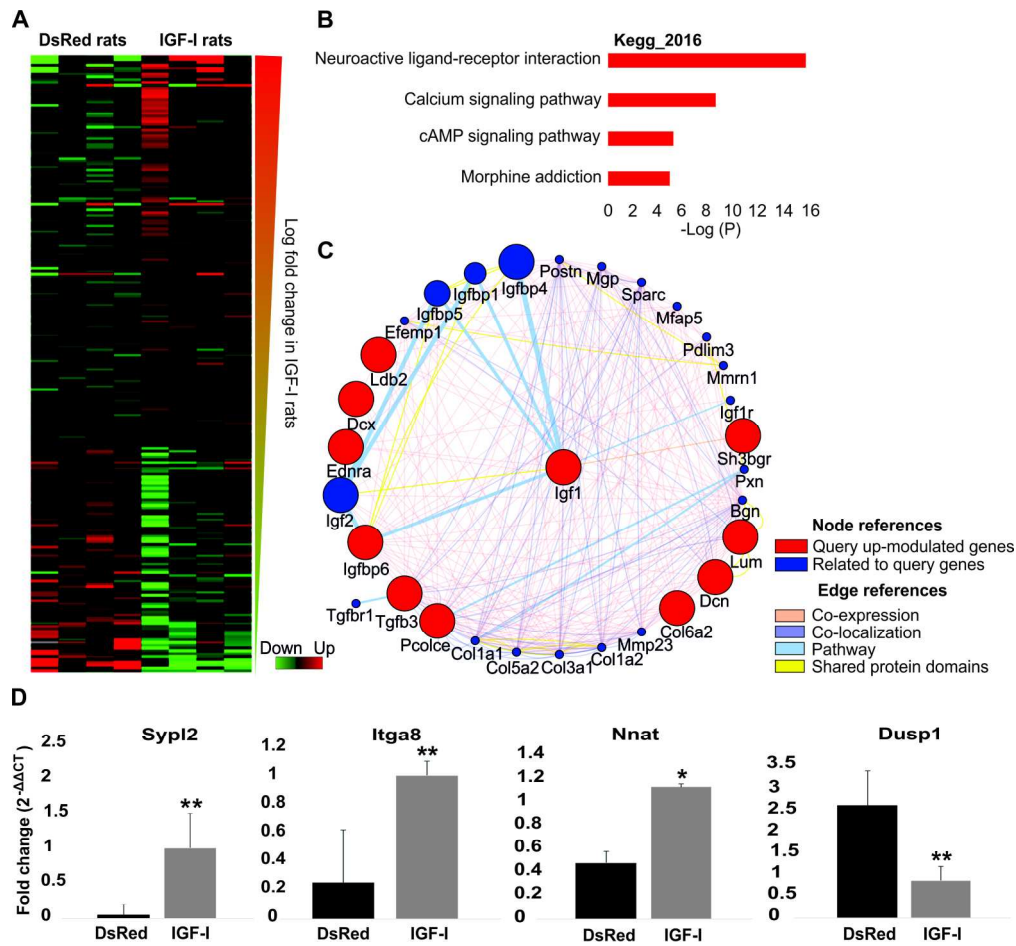


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Gene	Forward primer
Itga8	TGATTACCCAGATTTACTTGTCGG
Sypl2	GACTGATGTCAAAGGGGCCA
Dusp1	TGATCAACGTCTCGGCAAT
Nnat	CTCTTGCTGTCCCTTGCCTAT
Bactin	GGGAAATCGTGCGTGACATT

For Peer Review

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Reverse primer	Amplicon size (bp)
AGCTGGGCATCCACTGTTAC	90
AGCCAAAGAGCACAGAGATGT	134
TCACGAACTCAAAGGCCTCG	258
CTGCGTGAGACCAGGGATAAG	204
ATGGTGGTGCCGCCAGACAG	149

For Peer Review

edgeR Analysis Result

List of DEG between IGF1 and DsRED rats (LogFC>1.5; pvalue<0.05)

Gene_Name	logFC	logCPM	PValue	FDR	Significance
Alox15	-3.544625037	2.060801424	1.78404E-05	0.017324325	Down in IGF1
Tr	-2.851922036	5.445466731	9.63022E-05	0.068906789	Down in IGF1
S100a8	-2.703027443	1.641117278	0.000263423	0.143249649	Down in IGF1
S100a9	-2.675150871	2.604995881	1.54801E-05	0.017324325	Down in IGF1
AABR07051735.1	-1.860949763	1.356074144	1.72897E-05	0.017324325	Down in IGF1
Apold1	-1.771191413	5.223560107	1.63337E-12	2.22057E-08	Down in IGF1
Alas2	-1.639376162	1.350656007	7.37866E-06	0.011145881	Down in IGF1
Spink5	-1.594578794	-0.025886869	5.72801E-05	0.045807223	Down in IGF1
Mfrp	-1.5932081	1.635883717	0.003348393	0.65211732	Down in IGF1
Clic6	-1.568614911	1.423830396	0.003919178	0.701068698	Down in IGF1
Cxcl9	-1.556173259	1.255514069	0.069885506	1	Down in IGF1
Slc4a5	-1.539433839	0.946849141	0.007960898	0.852687314	Down in IGF1
Hba1	-1.374973885	6.752948896	2.95623E-06	0.005741412	Down in IGF1
AABR07054319.1	-1.331411003	6.487702149	5.30864E-05	0.045106878	Down in IGF1
Hba2	-1.309422158	5.264746182	7.29506E-06	0.011145881	Down in IGF1
Sik1	-1.233674974	2.863215367	1.07016E-09	3.63722E-06	Down in IGF1
Hbb-b1	-1.151898075	1.481864752	0.002073635	0.525052326	Down in IGF1
Ccl5	-1.146444014	0.098535456	0.03007257	1	Down in IGF1
Ifit3	-1.048794622	1.947829407	0.005839419	0.770746642	Down in IGF1
Loxl4	-0.907352347	0.733147865	0.00814876	0.854422119	Down in IGF1
Aqp1	-0.90090843	0.320749275	0.013164854	0.944967997	Down in IGF1
Klf2	-0.892603602	3.687472932	6.39918E-10	2.8999E-06	Down in IGF1
AC128800.1	-0.892365642	2.243955219	1.40286E-05	0.017324325	Down in IGF1
Ibsp	-0.891670142	0.375587901	0.004908365	0.741060084	Down in IGF1
Atf3	-0.878008739	0.728349008	0.006636121	0.810426825	Down in IGF1
Hif3a	-0.875898282	1.919379991	0.018077797	1	Down in IGF1
Tmem252	-0.867531069	1.587577663	0.00871796	0.878567081	Down in IGF1
C2cd4a	-0.857634989	0.195161352	0.030807778	1	Down in IGF1
Lrg1	-0.84840963	2.482627098	0.025645719	1	Down in IGF1
Dusp1	-0.846454656	5.549043965	1.38277E-08	3.75976E-05	Down in IGF1
Cd3e	-0.825037335	3.269563149	0.256578733	1	Down in IGF1
Nts	-0.813083912	4.788617728	0.021579663	1	Down in IGF1
Angpt2	-0.802477629	1.107380965	0.041501758	1	Down in IGF1
Fmo3	-0.785456999	2.949919704	0.000663266	0.28294506	Down in IGF1
	-0.763355542	1.540103178	0.038280967	1	Down in IGF1
RGD1566307	-0.761078087	1.492068638	0.151779932	1	Down in IGF1
Btg2	-0.75895809	4.60712286	2.34596E-11	1.59467E-07	Down in IGF1
Cyr61	-0.743046809	1.461317051	0.011056952	0.920218261	Down in IGF1
	-0.739099361	1.525405148	0.00185802	0.502763925	Down in IGF1
Tmem207	-0.730003781	1.400930675	0.005369777	0.741060084	Down in IGF1
Serpib1a	-0.723279954	1.757542585	0.00682534	0.810426825	Down in IGF1
Ccdc37	-0.714731088	1.241198871	0.090102049	1	Down in IGF1
Tp73	-0.713877408	2.632606413	0.059418913	1	Down in IGF1
Grp2	-0.712420791	1.720281004	0.01267595	0.944967997	Down in IGF1
Pla2g3	-0.71007318	2.935655759	0.049246815	1	Down in IGF1
Napsa	-0.708808721	0.066079829	0.063651604	1	Down in IGF1
	-0.700103802	2.29824211	0.167569602	1	Down in IGF1
Trpm1	-0.693073958	0.923783155	0.142461402	1	Down in IGF1
	-0.680552274	3.709354869	0.011526556	0.943997182	Down in IGF1

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edgeR Analysis Result

Klf4	-0.678354385	2.974922755	0.001769512	0.501088366	Down in IGF1
Igfbp4	-0.66356559	4.592135446	0.000121215	0.082396194	Down in IGF1
Tspo	-0.661856563	1.470184417	0.006128005	0.793430781	Down in IGF1
Spata18	-0.658465803	1.965104688	0.173114289	1	Down in IGF1
Lingo3	-0.653712797	4.432203214	0.001562115	0.473768859	Down in IGF1
Agbl2	-0.648497905	0.974741027	0.079647384	1	Down in IGF1
Fam84b	-0.646932698	1.051909505	0.064554999	1	Down in IGF1
Fam110d	-0.642343354	1.609041313	0.019754661	1	Down in IGF1
C4b	-0.638914615	1.85333263	0.020140057	1	Down in IGF1
Nr4a1	-0.635575264	5.87118157	2.6537E-07	0.000601283	Down in IGF1
AABR07004112.1	-0.627239117	1.651615012	0.110022524	1	Down in IGF1
RT1-CE16	-0.619805513	4.045552928	0.063176251	1	Down in IGF1
Fos	-0.614310109	3.761988541	0.000792568	0.326514057	Down in IGF1
Tmem114	-0.608994049	0.869890465	0.049787689	1	Down in IGF1
Ackr2	-0.608417181	1.741117729	0.037060207	1	Down in IGF1
Cdkn1a	-0.606971958	3.55431549	0.009282299	0.894984764	Down in IGF1
	-0.603176065	0.922732575	0.05272995	1	Down in IGF1
Synpo2	-0.602503735	1.877775341	0.021906462	1	Down in IGF1
Wfs1	-0.599160178	8.730176448	0.053205217	1	Down in IGF1
Yjefn3	-0.599033063	3.348197876	0.002603278	0.570831675	Down in IGF1
Epha8	-0.596428785	6.568671071	0.04968537	1	Down in IGF1
Enpp3	-0.595218689	0.279249923	0.050471895	1	Down in IGF1
P2ry2	-0.590213175	0.843695183	0.02709475	1	Down in IGF1
Lrrc74b	-0.589457765	0.725313715	0.195512468	1	Down in IGF1
Acer2	-0.588152042	4.983052133	0.001130644	0.404502735	Down in IGF1
Ppp1r32	-0.583553856	1.810783199	0.282273698	1	Down in IGF1
Il21r	-0.583147762	0.353602312	0.063308701	1	Down in IGF1
LOC686662	-0.583037599	0.1281437	0.060685311	1	Down in IGF1
	-0.58044193	3.970696447	0.123985346	1	Down in IGF1
Lcn2	-0.578935833	1.624565178	0.082828525	1	Down in IGF1
Klk11	-0.576549352	1.230646859	0.01999798	1	Down in IGF1
Dll4	-0.569498795	1.814425846	0.004785515	0.741060084	Down in IGF1
Clca4	1.154917846	1.486819572	0.241098165	1	Up in IGF0
Cbx2	0.503300414	1.248829283	0.040513049	1	Up in IGF1
Jag1	0.506842971	2.655892949	0.030967667	1	Up in IGF1
Pzp	0.511828278	0.248301887	0.133290254	1	Up in IGF1
Fcgbp	0.512177956	2.164738293	0.123883942	1	Up in IGF1
Rgs4	0.513448798	5.497601811	0.04385449	1	Up in IGF1
AABR07016841.1	0.515817426	3.90623261	0.000227563	0.128905241	Up in IGF1
Ldb2	0.519328715	4.984802214	0.018490745	1	Up in IGF1
AC142180.1	0.5196826	0.38808575	0.083861002	1	Up in IGF1
Tgfb3	0.522408949	3.943710973	0.049262109	1	Up in IGF1
Sema3f	0.522813229	1.391828332	0.095660004	1	Up in IGF1
Pde4c	0.524345729	1.271721427	0.037740869	1	Up in IGF1
Hpcal1	0.525480804	4.709904177	0.029672137	1	Up in IGF1
Fras1	0.527580304	3.834111057	0.108833038	1	Up in IGF1
Dcx	0.53293664	5.069111987	0.010834067	0.909192252	Up in IGF1
Atp8b3	0.533018128	1.969583485	0.08243029	1	Up in IGF1
Dysf	0.536576029	1.198032323	0.032273646	1	Up in IGF1
Ceacam3	0.540113924	0.905068927	0.030406217	1	Up in IGF1
Gabrg3	0.540406371	1.014689649	0.040776547	1	Up in IGF1

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4	Pspn	0.544208945	0.263200117	0.072343317	1	Up in IGF1
5	Tnnc2	0.552307259	2.341824551	0.04113891	1	Up in IGF1
6	Ccdc33	0.555361178	1.64600212	0.077173629	1	Up in IGF1
7	Ednra	0.556699595	2.041736967	0.069900052	1	Up in IGF1
8		0.559020746	0.933754891	0.045044335	1	Up in IGF1
9	Sh3bgr	0.559333188	0.821871644	0.038068715	1	Up in IGF1
10	Exph5	0.561651296	2.029391302	0.025145468	1	Up in IGF1
11	Fhod3	0.562819632	3.770273662	0.020771737	1	Up in IGF1
12	Col6a1	0.564442006	5.895618182	0.064991845	1	Up in IGF1
13	Meis1	0.564834855	1.468228087	0.06143654	1	Up in IGF1
14	Gpr88	0.565005743	2.97736489	0.057905145	1	Up in IGF1
15	Rasgef1c	0.567542456	1.53032321	0.057816687	1	Up in IGF1
16	Odf3l1	0.568555969	0.471378681	0.11614033	1	Up in IGF1
17	Tbx18	0.569413158	1.47700926	0.107537773	1	Up in IGF1
18	Trpc7	0.571883188	1.463657892	0.084676075	1	Up in IGF1
19	Prss22	0.572231769	0.869213389	0.194743967	1	Up in IGF1
20	Bend7	0.572712821	0.060011544	0.076372374	1	Up in IGF1
21	Srms	0.576482119	0.240203815	0.102710812	1	Up in IGF1
22	Arhgap18	0.576989017	2.845648919	0.037468835	1	Up in IGF1
23	Lpin3	0.579490772	3.757202176	0.054639072	1	Up in IGF1
24	Olr951	0.581542868	2.341219579	0.041694467	1	Up in IGF1
25	Dnase1l2	0.58164211	1.31424808	0.034001048	1	Up in IGF1
26	Bves	0.584296241	1.49981091	0.060617247	1	Up in IGF1
27	Ccdc150	0.584540103	0.017636734	0.072155718	1	Up in IGF1
28	LOC100360835	0.591093657	0.158646414	0.047084625	1	Up in IGF1
29	Baiap3	0.593999849	2.924132241	0.146074614	1	Up in IGF1
30	Rxfp3	0.596222165	1.015352475	0.037631514	1	Up in IGF1
31	Igf1	0.596687354	1.671675382	0.028460293	1	Up in IGF1
32	Kcnj16	0.596991071	3.710742114	0.038342359	1	Up in IGF1
33	Sertad3	0.601110635	0.812453453	0.018407795	1	Up in IGF1
34	Dpp10	0.601764512	4.010507056	0.003980537	0.702797459	Up in IGF1
35	Cpne7	0.602345844	7.605625771	0.054497569	1	Up in IGF1
36	Igfbp6	0.603565868	2.355389946	0.058803951	1	Up in IGF1
37	Pcolce	0.606622421	0.664139863	0.094483477	1	Up in IGF1
38	RGD1560034	0.612510599	0.248306549	0.03804199	1	Up in IGF1
39	Avil	0.61939762	1.92297136	0.043909598	1	Up in IGF1
40	Klhdc8a	0.619994476	1.947888609	0.072554979	1	Up in IGF1
41	Hcrt	0.623099529	1.131257734	0.010340342	0.909192252	Up in IGF1
42	Igsf1	0.624620696	5.768781819	0.006855394	0.810426825	Up in IGF1
43	Gpat2	0.628326699	-0.0659915	0.094200445	1	Up in IGF1
44	RGD1561149	0.633479636	2.570684806	0.049133499	1	Up in IGF1
45		0.634771968	1.695962499	0.025178686	1	Up in IGF1
46	Igkc	0.637492451	7.203284062	0.360383631	1	Up in IGF1
47	Tpd52l1	0.640373189	3.906079272	0.021200065	1	Up in IGF1
48	Aplnr	0.644937702	1.161506246	0.039982498	1	Up in IGF1
49		0.651946613	2.614323453	0.021993828	1	Up in IGF1
50	Glt8d2	0.653939403	0.823845647	0.013808048	0.944967997	Up in IGF1
51	Adra2a	0.656304237	2.878980305	0.079003568	1	Up in IGF1
52	Fxyd6	0.660775368	2.491020658	0.022356098	1	Up in IGF1
53	Gng4	0.664563099	0.345040298	0.031374516	1	Up in IGF1
54	Efcab6	0.664791109	3.501350927	0.028510026	1	Up in IGF1
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4	Chad	0.665207444	0.721992495	0.020040354	1	Up in IGF1
5	RT1-M1-2	0.673667751	2.308004624	0.021168791	1	Up in IGF1
6	Mir186	0.675605551	1.236760674	0.003099804	0.619732876	Up in IGF1
7	Tmie	0.675780689	2.948934567	0.007258352	0.850666296	Up in IGF1
8	Slc7a3	0.679894245	1.422460321	0.050188449	1	Up in IGF1
9	Gabra6	0.679965213	2.564311167	0.082985055	1	Up in IGF1
10	Abi3bp	0.682501834	0.778079286	0.079511305	1	Up in IGF1
11	Atp6ap1l	0.684591711	1.039506462	0.108701253	1	Up in IGF1
12	RGD1562726	0.688151479	2.006304017	0.054381386	1	Up in IGF1
13	Kcnk9	0.689514914	3.485841633	0.039832796	1	Up in IGF1
14	Cplx3	0.693455286	0.600443572	0.116912211	1	Up in IGF1
15	Camk2d	0.695530345	5.683447008	0.002266416	0.540559985	Up in IGF1
16	Magel2	0.695849037	1.966803212	0.084016623	1	Up in IGF1
17	Ecel1	0.704186932	0.71180937	0.173047863	1	Up in IGF1
18	Npy2r	0.704643176	2.944290823	0.040536583	1	Up in IGF1
19	Susd2	0.722301303	4.691010298	0.012447433	0.944967997	Up in IGF1
20		0.722997189	5.531492902	0.345915413	1	Up in IGF1
21	Gpr101	0.728637868	0.820334106	0.139862325	1	Up in IGF1
22	Fam179a	0.729563513	1.421099479	0.150723668	1	Up in IGF1
23	Dcn	0.733961982	3.658363714	0.010537023	0.909192252	Up in IGF1
24		0.737790855	1.610530337	0.007931522	0.852687314	Up in IGF1
25	Plekhg4	0.741694764	0.43405304	0.02323636	1	Up in IGF1
26	Osr1	0.743756167	0.415502984	0.05667885	1	Up in IGF1
27	Pnlcd1	0.758001818	0.253204221	0.018316807	1	Up in IGF1
28	Sstr1	0.758701597	3.613394785	0.033385692	1	Up in IGF1
29	Nppa	0.758855321	3.222644521	0.02277798	1	Up in IGF1
30	Cntnap3	0.788045557	1.636444973	0.045234852	1	Up in IGF1
31	Eppin	0.791524462	0.700663619	0.007700348	0.852687314	Up in IGF1
32	Slc38a4	0.795113378	0.790364492	0.106778843	1	Up in IGF1
33	AABR07009357.1	0.804199795	0.563807887	0.059482014	1	Up in IGF1
34	Klhl14	0.809559257	2.300729753	0.022737129	1	Up in IGF1
35	Syp12	0.82338483	1.688608836	0.002393796	0.551587466	Up in IGF1
36	Rpl10l	0.830381891	1.467258079	0.006780029	0.810426825	Up in IGF1
37	Rps4y2	0.847002651	1.363984194	0.008170274	0.854422119	Up in IGF1
38	Bace2	0.862946555	1.576478739	0.003842872	0.696584551	Up in IGF1
39	Stra6	0.864899934	2.200608676	0.001392929	0.452763642	Up in IGF1
40	Atp2b4	0.901205513	5.03292495	0.011624875	0.944967997	Up in IGF1
41	Gli1	0.904381531	2.178516407	0.025875365	1	Up in IGF1
42	Arhgap6	0.912599166	3.291369476	0.002314028	0.54240022	Up in IGF1
43	Nr2f2	0.919140676	3.874062551	0.00637719	0.810426825	Up in IGF1
44	Cpne2	0.920595536	3.829272586	0.017022	1	Up in IGF1
45	Pcdh11x	0.92932649	0.6456199	0.014046043	0.944967997	Up in IGF1
46	Col6a2	0.945652123	4.553955368	0.019137021	1	Up in IGF1
47	Ush1g	0.961313946	-0.012397496	0.013181043	0.944967997	Up in IGF1
48	Nnat	0.979102067	8.186459949	0.005258732	0.741060084	Up in IGF1
49	Tacr3	0.985738265	1.674987592	0.063571538	1	Up in IGF1
50	Tpbp	1.014052169	2.745578495	0.02409323	1	Up in IGF1
51		1.015668417	2.611587192	0.004930062	0.741060084	Up in IGF1
52	Rxrg	1.031133107	2.697068312	0.026453724	1	Up in IGF1
53	Tac1	1.035848363	2.016402508	0.006651954	0.810426825	Up in IGF1
54	Itga8	1.037108992	3.383053124	0.013941849	0.944967997	Up in IGF1
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4	Mx1	1.044799726	2.240562138	0.02124564	1	Up in IGF1
5	Tcerg1l	1.05362326	2.455553058	0.019142952	1	Up in IGF1
6		1.096129457	1.532675268	0.00612712	0.793430781	Up in IGF1
7	Kcnf1	1.098109486	2.613163035	0.03788608	1	Up in IGF1
8	RGD1311744	1.106999345	2.207978455	0.027107842	1	Up in IGF1
9	Htr2c	1.125706548	5.438136984	0.014052105	0.944967997	Up in IGF1
10	Trhr	1.131738805	1.541432031	0.009235951	0.894984764	Up in IGF1
11	Cdhr1	1.184990172	1.790412841	0.026143422	1	Up in IGF1
12	Olr59	1.289216772	0.672503751	0.000861696	0.344551554	Up in IGF1
13	Slc6a5	1.290180474	1.066525458	0.00435839	0.71388326	Up in IGF1
14	Lum	1.41001991	2.143715398	0.00160026	0.473768859	Up in IGF1
15	Igj	1.427644154	3.527686377	0.005681698	0.757281205	Up in IGF1
16	Igh-1a	1.638593475	6.635969662	0.011989283	0.944967997	Up in IGF1
17	Plagl1	1.656532652	1.954949774	0.00776037	0.852687314	Up in IGF1
18	Ighm	2.139870407	4.933950804	0.004275374	0.71388326	Up in IGF1
19	AABR07072761.1	2.636817615	2.985622637	0.000560966	0.262977194	Up in IGF1
20	LOC365791	3.140251872	2.102807849	8.56317E-06	0.011641627	Up in IGF1
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RESPONSES TO REVIEWERS**Reviewer: 1**

1) Grammar and sentence structure need to be reviewed, some conjunction in sentences are missing.

a. Line 14 of the abstract and 12 of the introduction "...underlie the hippocampal phenotype of aging..."

We have corrected the sentence in the Abstract and Introduction as the reviewer recommends. Page 2 line 6 from top and page 4 lines 3-4 from top.

2) Use of the term deregulated on lines 32 and 5 is a bit unclear, use different terminology i.e. differentially expressed.

We have replaced the term "deregulation" by "differentially expressed" in the revised version of the manuscript.

3) Add a brief sentence at the end of Escape box latency and exploration frequency indicating what a positive outcome is i.e. "short escape box latency time indicates better learning..."

As recommended, we added a short sentence at the end of Escape box latency and Exploration frequency, explaining how to interpret the values they take. Page 6 lines 7_8 and 13_14 from top.

4) Method section mentions that RNA was extracted from 4 rats per group on line 28, please indicate what the groups are at this point in the manuscript for clarity. Also indicate the age of the animals at time of sacrifice and RNA isolation.

As recommended, we have now indicated what the groups are. As indicated in the revised manuscript, rats were sacrificed 80 days post adenovector injection (28 months of age). Page 8 line 1 from top and page 8 line 6 from bottom.

5) Analysis of variance between the 4 samples in each group should be provided as a control.

Following the reviewer's suggestion, homoscedasticity between samples along all genes was estimated. Results demonstrate the equality of variances for the 4 samples in each group.

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
IGF.I_16	13595	1001123.491	73.63909457	316598.6918
IGF.I_24	13595	1037597.863	76.3220201	310851.7393
IGF.I_27	13595	989975.5446	72.81909118	217186.4508
IGF.I_29	13595	1015073.515	74.66520889	246754.6292

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	92945.92414	3	30981.97471	0.11355036	0.952209853	2.605072539
Within Groups	14836376201	54376	272847.8778			
Total	14836469147	54379				

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
DsRed_7	13595	1008745.059	74.19971013	227038.8892
DsRed_12	13595	988382.3669	72.70190268	178083.9198
DsRed_22	13595	990273.8746	72.84103528	190786.6145
DsRed_25	13595	970285.5989	71.37076859	162182.0955

ANOVA

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	54533.94544	3	18177.98181	0.095914445	0.962316553	2.605072539
Within Groups	10305496109	54376	189522.8798			
Total	10305550643	54379				

6) Have you preformed a stepwise linear regression to see if the addition of IGF-1 virus can predict the outcome of the rat memory tests? Use the presence or absence of IGF as a dependent variable.

In the first part of the question, the reviewer suggests performing a stepwise linear regression using the outcome of the memory test as dependent variable, whereas in the second part of the question the reviewer suggests using IGF-I addition as dependent variable.

Thus, with the Statistica 8 Software (Tulsa, USA) we have performed both approaches for the PT3, the time point at which the IGF-I rats displayed an increase in memory accuracy. We have set the variable “goal hole exploration” as memory outcome, whereas for “IGF-I addition” we have set the value equal to 1 for IGF-I subjects and equal to 0 for DsRed subjects.

When setting IGF-I addition as independent variable and goal hole exploration as dependent variable, we obtained a curve with a $R^2=0.25$, as shown below in the regression summary document.

Regression Summary for Dependent Variable: Goal Hole exploration (Spreadsheet1)						
R= ,50895856 R ² = ,25903881 Adjusted R ² = ,22199075						
F(1,20)=6,9920 p<,01556 Std.Error of estimate: 2,4142						
N=22	Beta	Std.Err. of Beta	B	Std.Err. of B	t(20)	p-level
Intercept			5,100000	0,763435	6,680331	0,000002
IGF-I addition	0,508959	0,192479	2,733333	1,033696	2,644233	0,015560

When setting IGF-I addition as independent variable and goal hole exploration as dependent variable, we obtained a curve with a $R^2=0.25$, as shown below in the regression summary document.

Regression Summary for Dependent Variable: IGF-I addition (Spreadsheet1)						
R= ,50895856 R ² = ,25903881 Adjusted R ² = ,22199075						
F(1,20)=6,9920 p<,01556 Std.Error of estimate: ,44953						
N=22	Beta	Std.Err. of Beta	B	Std.Err. of B	t(20)	p-level
Intercept			-0,079168	0,254923	-0,310556	0,759351
Goal Hole exploration	0,508959	0,192479	0,094770	0,035840	2,644233	0,015560

Thus, we think that the stepwise linear regression does not fit to our data.

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3 Overall, we believe the stepwise linear regression is not an appropriate statistical
4 method to show our results. We would rather use an unpaired t test to compare mean
5 goal hole exploration between DsRed and IGF-I groups. We have already used unpaired
6 t test to compare goal hole exploration in a previous study [Pardo et al., 2017].
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10 11 **Reference**

12 Pardo J, Abba MC, Lacunza E, Francelle L, Morel GR, Outeiro TF, Goya RG.
13 Identification of a conserved gene signature associated with an exacerbated
14 inflammatory environment in the hippocampus of aging rats. Hippocampus. 2017 Jan
15 13. doi: 10.1002/hipo.22703. [Epub ahead of print] PubMed PMID: 28085212.
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21 **7) The fold change cut off is 0.5? Do you mean to report the log2 fold change from**
22 **DESeq2? Does not seem stringent enough for your cut off. Also, fold change is not**
23 **the DESeq output format. Please indicate how the cut off values were calculated.**
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28 We apologize for the mistake introduced in Materials & Methods section regarding the
29 method employed in RNAseq data analysis. The Bioconductor package used for the
30 differential expression analysis was edge R instead of DESeq2.
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33 The FC output format corresponds to an analysis of edgeR. In addition, the significance
34 level to get the DEG was established at $\text{Log}_2 \text{FC}$ (instead of FC) > 0.5 and p value
35 < 0.05 .
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38 The corresponding corrections were introduced in the manuscript. Page 9 lines 4_7 from
39 bottom and page 22 lines 3-5 from top.
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43 **8) A subset of genes should be validated for changes in gene expression by rt-PCR**
44 **or other direct measure of mRNA abundance.**
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46 Following reviewer suggestion, we have validated a subset of the more relevant genes
47 by qRT-PCR. Materials and methods and results have been included in the revised
48 manuscript and in figure 3D. Page 10 lines 3-8 from top, page 11 line 1 from bottom,
49 page 12 lines 1-2 from top, page 28 lines 1-3 from bottom. Primers are listed in Suppl.
50 Table 1.
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56 **9) Add suggesting statement at the end of Hole exploration frequency results**
57 **section, what do these results indicate?**
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3 As recommended, at the end of Hole exploration frequency in the Results section, we
4 have added a brief interpretation of the data obtained. Page 11 lines 10-12 from top.
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8 **10) More detail in results section is required for general audience understanding**
9 **and clarity.**

10 We have added short interpretative paragraphs at the end of each subsection in Results.
11 We believe that the new text will serve better to improve the understandability and
12 clarity of our results that would including further technical details. Page 11 lines 5-7
13 from bottom and page 13 lines 7-9 from top.
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20 **11) More explanation and references needed to explain how you got lists of genes**
21 **used to pull out functional groups of genes is needed.**

22 We performed a search of the literature in order to identify the differentially expressed
23 genes that were functionally significant concerning, IGF-I itself, neurogenesis, memory
24 and synaptic plasticity. In the Discussion we provided a not too extensive review of the
25 known role of each of the differentially expressed genes that we selected. Each
26 statement was accompanied by relevant references.
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33 **12) Reduce the amount of data presented in all lists and tables at the end of the**
34 **manuscript to only those that meet the statistical thresholds or are meaningful to**
35 **the results section of the manuscript. Full lists of data can be supplementary or**
36 **posted on a public domain. It is not useful or helpful to provide a list of 20K genes**
37 **with no annotation or legend.**

38 As suggested by the reviewer, the now supplementary table 2 was modified and now
39 contains the requested information.
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46 **13) Label each list/table of RNA seq and DsRed Data and provide legend with**
47 **description.**

48 Table 1 in the manuscript carries a title and a footnote (legend). The label of the Suppl.
49 Table 2 is “**List of DEG between IGF1 and DsRED rats (LogFC>1.5;**
50 **pvalue<0.05)**”. It is embedded within the Table on the headings grid.
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56 **14) The data sets must be deposited in a public database for distribution.**

57 We are now in the process of depositing RNA seq data in GEO database.
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3 **Reviewer: 2**

4 **1. Abstract, Results and Discussion: The authors should consider using a term**
5 **other than deregulated to describe gene expression changes induced by IGF-1.**
6 **Deregulation is sometimes used to describe dysregulation or lack of regulation. In**
7 **this study, it doesn't appear that IGF-1 is causing dysfunctional gene expression**
8 **but rather appears to simply alter the expression of some genes. This change in**
9 **gene expression may actually be beneficial to the animal.**

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14 As indicated to reviewer 1, we have now replaced the term deregulation by differential
15 expression.
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20 **2. Materials and Methods (p. 4): Information about the diet supplied to the mice**
21 **should be given in the Materials and Methods section. Diet may influence gene**
22 **transcription, and it would be helpful to have diet information available when**
23 **comparing the results of different studies.**

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26 The diet composition (%) of the animals is now provided in M&M. Page 4 lines 4-7
27 from bottom.
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31 **3. Materials and Methods (p. 7) and Figure 1: Were animals euthanized on day 80**
32 **or 78? The Methods section indicates that the rats were euthanized on day 80**
33 **while Figure 1 indicates the animals were euthanized on day 78.**

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36 We apologize for this error. Animals were euthanized on day 80. This has now been
37 corrected (Fig 1A).
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41 **4. Discussion (p. 14): It would be helpful if the authors provided more**
42 **information about the expected outcome of downregulation of Igfbp4 and**
43 **upregulation of Igfbp6. Is there sufficient information in the literature to allow**
44 **speculation about the net impact of these binding proteins changing in opposite**
45 **directions?**

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49 We believe that there is not enough information in the literature to allow speculation
50 about the net impact of these binding proteins changing in opposite directions. Please,
51 consider that this is, to our knowledge, the first report on the effects of IGF-I gene
52 therapy on the hippocampal transcriptome. As already indicated in the Discussion, our
53 data are insufficient to allow a prediction of the net effect of these opposite changes on
54 the net hippocampal IGF-I tissue levels in our old rats.
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5 **5. Discussion: Do the authors have a theory as to why a significant difference in**
6 **hole exploration frequency was only observed 77 days (PT3) after IGF-1 gene**
7 **therapy?**
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10 In a previous short-term IGF-I therapy study [Pardo et al., 2016] performed on 28
11 months old female rats, we observed an increase in goal hole exploration frequency
12 comparable to that induced by the treatment in the present study when the rats reached
13 28 months of age. In the present study, the old rats did not show any improvement in
14 goal hole exploration frequency at earlier ages (26 and 27 months). Therefore, we
15 speculate that 28 months of age might constitute a time window where the sensitivity of
16 the animals to IGF-I increases concerning goal hole exploration frequency. This is just a
17 possibility that would need experimental confirmation. Since there is no experimental
18 support for this hypothesis we prefer not to include it in the MS.
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26 **Reference**

27
28 Pardo J, Uriarte M, Console GM et al. Insulin-like growth factor-I gene therapy
29 increases hippocampal neurogenesis, astrocyte branching and improves spatial memory
30 in aging rats; European Journal of Neuroscience. 2016. 44(4):2120-2128.
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