Brain IGF-I regulates LTP, spatial memory, and sexual dimorphic behavior

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Insulin-like growth factor-I (IGF-I) exerts multiple actions, yet the role of IGF-I from different sources is poorly understood. Here, we explored the functional and behavioral consequences of the conditional deletion of Igf-1 in the nervous system (Igf-1Δ/Δ), and demonstrated that long-term potentiation was impaired in hippocampal slices. Moreover, Igf-1Δ/Δ mice showed spatial memory deficits in the Morris water maze, and the significant sex-dependent differences displayed by Igf-1Δ/Δ mice disappeared in Igf-1Δ/Δ mice in the open field and rota-rod tests. Brain Igf-1 deletion disorganized the granule cell layer of the dentate gyrus (DG), and it modified the relative expressions of GAD and VGLUT1, which are preferentially localized to inhibitory and excitatory presynaptic terminals. Furthermore, Igf-1 deletion altered protein modules involved in receptor trafficking, synaptic proteins, and proteins that functionally interact with estrogen and androgen metabolism. Our findings indicate that brain Igf-1 is crucial for long-term potentiation, and that it is involved in the regulation of spatial memory and sexual dimorphic behaviors, possibly by maintaining the granule cell layer structure and the stability of synaptic-related protein modules.

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Introduction

Insulin-like growth factor-I (IGF-I) is a critical factor that regulates cell and tissue homeostasis at various levels, such as energy allocation, body and organ growth, life span, and tissue responses to stress (Liu et al., 1998; Pichelin et al., 2003; Kappeler et al., 2008; Wu et al., 2009; Fernandez & Torres-Alemán, 2012; Bartke et al., 2013; Carlson et al., 2014; Nieto-Estevez et al., 2016a; Santi et al., 2018; Fernandez de Sevilla et al., 2022). In the central nervous system, the activities of IGF-I influence neural stem cell and progenitor cell proliferation (Arsenijevic et al., 2001; Aberg et al., 2003; Nieto-Estevez et al., 2016b), the survival, differentiation, and maturation of neurons, astrocytes and oligodendrocytes (Carson et al., 1993; Arsenijevic & Weiss, 1998; Cheng et al., 1998; Camarero et al., 2001; Vicario-Abejon et al., 2003, 2004; Otaegi et al., 2006; Cao et al., 2011; Corvin et al., 2012); the correct positioning of migrating neurons (Hurtado-Chong et al., 2009; Onuma et al., 2011; Li et al., 2012; Maucksch et al., 2013; Littlejohn et al., 2020); adult neurogenesis, synaptogenesis, neuron firing, and long-term potentiation (LTP) (Aberg et al., 2000; O’Kusky et al., 2000; Sun et al., 2005; Trejo et al., 2007; Llorens-Martin et al., 2010; Dyer et al., 2016; Hu et al., 2016; Nieto-Estevez et al., 2016a; Ogundele et al., 2018; Pristera et al., 2019; Maglio et al., 2021); and cognition, olfactory memory, spatial and skill learning, fear extinction memory, and anxiety (Trejo et al., 2007; Muller et al., 2012; Gazit et al., 2017; Liu et al., 2017; Frater et al., 2018; Farias Quiplidor et al., 2019; Pristera et al., 2019; Zegarra-Valdivia et al., 2019; Maglio et al., 2021). The resulting net biological effect of IGF-I binding to the IGF-I receptor depends on the cell and tissue type, the age and sex of the animal, and the context or status in terms of stress (e.g., inflammation, neurodegeneration, neurovascular hypoxia or traumatic brain injury) (Gontier et al., 2015; Lopez et al., 2015; Nieto-Estevez et al., 2016a; Munive et al., 2016; Ashpole et al., 2017; De Magalhaes Filho et al., 2017; Gubbi et al., 2018; Santi et al., 2018; Cardoso et al., 2021).

Experiments on mice carrying a conditional deletion of Igf-1 in the liver challenged the prevalent concept that blood IGF-I (mostly from the liver) was essential for normal postnatal growth. Rather, these studies appeared to emphasize the importance of locally synthetized IGF-I acting through autocrine and/or paracrine mechanisms (Sjogren et al., 1999; Yakar et al., 1999; Ohlsson et al., 2009; Wu et al., 2009). This latter concept was confirmed in more
recent studies using mice carrying a conditional Igf-I deletion in brain cells (Nieto-Estevez et al., 2016b; Pristera et al., 2019).

We previously found that brain IGF-I plays a major role in promoting the correct generation, migration, and maturation of neurons from neural stem cells during adult hippocampal neurogenesis (Nieto-Estevez et al., 2016b), although electrophysiological or behavioral phenotypes were not investigated in that study. Here, we show that the lack of brain IGF-I almost completely abrogates hippocampal LTP, and altering spatial memory, sex-dependent behavior, and granule cell layer (GCL) organization, and inducing major changes in the hippocampal proteome.

Results

Brain IGF-I is essential for hippocampal synaptic plasticity

The IGF-I signaling pathway fulfills a crucial role in the activity and plasticity of hippocampal synapses, and in hippocampus-dependent learning and memory (Trejo et al., 2007; Stern et al., 2014; Gazit et al., 2016), although the contribution of locally synthesized IGF-I to these processes remains to be fully elucidated. Here, we first evaluated the impact of local brain IGF-I deficiency on hippocampal synaptic plasticity, monitoring its potential consequences on LTP, a well-known cellular mechanism underlying learning and memory processes (Pauslen & Sejnowski, 2000). We recorded neuronal activity by measuring field excitatory postsynaptic potentials in the dentate gyrus (DG) before and after stimulating the perforant pathway using a high frequency stimulation (HFS) protocol (Fig 1A). HFS evoked robust synaptic potentiation in IGF-I+/- slices relative to the baseline (***P < 0.001, one way-ANOVA, with post hoc Tukey’s test: Fig 1B–D), yet synaptic potentiation was not evident in hippocampal slices from IGF-I−/− mice but rather, they displayed a significant depression of synaptic activity after the HFS protocol relative to the baseline (***P < 0.001, one way-ANOVA, with a post hoc Tukey's test: Fig 1B–D). The difference in the responses to HFS in the hippocampal slices from either mice was significant (###P < 0.001: Fig 1D) and supports a critical role for brain-synthesized IGF-I in long-lasting synaptic plasticity at the hippocampal DG.

Brain IGF-I regulates spatial memory and sexually dimorphic behavior

Because IGF-I depletion impairs hippocampal LTP, we tested whether two hippocampal-dependent tasks were affected in IGF-I−/− mice: spatial learning and memory (Figs 2 and 3). In the Morris water maze (MWM), IGF-I−/− mice displayed relatively higher escape latencies in the acquisition trials than the IGF-I+/+ mice, yet these increases were only significant on day 4 (\(*P < 0.05, t\) test) when the mouse genotype alone was considered in the analysis (Fig 2). The escape latency also increased in IGF-I−/− females on day 3 (\(*P < 0.05, t\) test), whereas possible differences in IGF-I−/− males did not reach statistical significance (\(P = 0.0509\) on day 2: Fig S1A and B).

We observed significant changes in the performance of IGF-I−/− mice relative to the IGF-I+/+ mice during the transfer test when analyzed independently of the animal’s sex (Fig 3A–C). These differences were evident through reductions in both the relative time spent in the platform (P) quadrant (\(*P < 0.01, t\) test: Fig 3B) and the relative distance swum in that quadrant (\(*P < 0.001, t\) test: Fig 3C). Moreover, the time spent by IGF-I+/+ mice in each quadrant

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**Figure 1.** Conditional deletion of brain insulin-like growth factor-I (IGF-I) abolishes hippocampal long-term potentiation. (A) Schematic representation of the experimental approach in hippocampal slices. The extracellular-recording pipette (Erec) was placed at the granular layer of dentate gyrus and to activate synaptic inputs, the stimulation electrode (Estim) was located in the perforant pathway. (B) Representative average traces of field excitatory postsynaptic potential (fEPSP) recordings before (pre) and 60 min after (post) high-frequency stimulation (HFS) in IGF-I+/+ and IGF-I−/− mice. (C) Relative fEPSP slope recordings (from basal values) from dentate gyrus hippocampal slices of 14–15 mo-old IGF-I+/+ (black circles) and IGF-I−/− mice (white circles). The red arrow denotes the initiation of the HFS protocol. Note the absence of synaptic potentiation but also of significant depression of excitatory synaptic transmission in IGF-I−/− slices after HFS. (D) The average of the relative changes in the fEPSP slope 60 min after initiating HFS in slices from control (n = 3 mice, seven slices) and IGF-I−/− mice (n = 4 mice and seven slices), and the different responses to HFS of hippocampal slices from both mice. The results are represented as the mean ± SEM: ***P < 0.001 and ###P < 0.001, where “#” refers to the analysis in relation to the base line and “*” refers to the analysis between the two experimental groups.
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A series of analyses were then performed to determine whether the animal’s sex might influence the spatial memory of Igf-I−/− mice in the hope that this may help explain the electrophysiological and behavioral phenotypes observed. As such, hippocampal proteostasis was measured using tandem mass tags (TMTs) coupled to tandem mass spectrometry (MS), detecting 65 differentially expressed proteins (DEPs) in female Igf-I−/− mice and 58 DEPs in male Igf-I−/−, relative to their respective Igf-I+/+ mice (Fig 5A and Table S1). No proteins were deregulated uniformly across both cohorts. However, most of the DEPs mapped to vesicle-related compartments, such as the TGN, presynapses, vesicle tethering complexes, and the early endosome (Igf-I−/−/+), with membrane trafficking the only biological activity disrupted at the protein level in both the female and male Igf-I−/− hippocampi. This conclusion was also reached by performing a bioinformatics analysis that highlighted different functional profiles between the sexes of these mice (Fig S4).

Interlocking the differentially expressed proteomes with the SYNGO and Ingenuity Pathway Analysis repositories revealed alterations to synaptic proteins and to proteins involved in LTP. Specifically, Igf-I deficiency in males modulated a synaptic protein module comprised of FLOT1, RABBA, RAB2A, BAIAP2, PLCB3, RPL7A, LG1, PENK, CTNNA2, ADGRL1, VPS45, RHEB, FBXO2, PICK1, NF1, and ATP6AP1, whereas Igf-I−/− female mice displayed changes in expression of a quite different set of synaptic proteins.

Hippocampal proteome and the structure of the GCL rely on brain Igf-I

To examine the consequences of Igf-I deficiency on hippocampal homeostasis, we applied shotgun proteomics to obtain novel information about the site-specific molecular signature of male and female Igf-I−/− mice in the hope that this may help explain the electrophysiological and behavioral phenotypes observed. As such, hippocampal proteostasis was measured using tandem mass tags (TMTs) coupled to tandem mass spectrometry (MS), detecting 65 differentially expressed proteins (DEPs) in female Igf-I−/− mice and 58 DEPs in male Igf-I−/−, relative to their respective Igf-I+/+ mice (Fig 5A and Table S1). No proteins were deregulated uniformly across both cohorts. However, most of the DEPs mapped to vesicle-related compartments, such as the TGN, presynapses, vesicle tethering complexes, and the early endosome (Igf-I−/−/+), with membrane trafficking the only biological activity disrupted at the protein level in both the female and male Igf-I−/− hippocampi. This conclusion was also reached by performing a bioinformatics analysis that highlighted different functional profiles between the sexes of these mice (Fig S4).

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Figure 3. Conditional deletion of brain insulin-like growth factor-I (IGF-I) produces spatial memory deficits.

Adult male and female IGF-I Ctrl and IGF-I Δ/Δ mice (6–16 mo old) were assayed in the Morris water maze test (A). The images show representative traces of the routes swum by the different mice in the four quadrants of the pool (P, LP, OP, and RP). (B, C) Significant differences between IGF-I Δ/Δ and IGF-I Ctrl mice were observed in the relative time spent (**P < 0.01 (B)) and relative distance swum (**P < 0.01 (C)) in the platform quadrant (P). (B) As seen from the graphs, IGF-I Δ/Δ mice spent a similar relative time in the platform quadrant compared to IGF-I Ctrl mice. (C) IGF-I Δ/Δ mice swum a significantly greater distance in the platform quadrant compared to IGF-I Ctrl mice. (D, E) Graphs showing the distribution of times and distances spent by male and female mice in the platform quadrant (P).
proteins that included PRKCE, RAB6A, CASK, VAMP7, ERC1, GPHN, RPL6, MYO6, CTNN2, KCNQ1, LYN7C, and NBEA (Table S2). These alterations were accompanied by changes in protein intermediates of LTP and long-term depression, such as PAAH1B2 and PRKCE in Igf-I−/− males, and BAIAP2/RSp53 together with PPP2R5A in Igf-I−/− males. We analyzed the relationships between the altered hippocampal proteomes and the estrogen/androgen pathways or neurogenesis using the STRING and BioGRID tools (Szlárczyk et al., 2019; Oughtred et al., 2021). Proteins involved in estrogen and androgen metabolisms were functionally connected to some of the deregulated hippocampal proteins (Fig 6). Furthermore, according to the BioGrid repository, 25% of the altered estrogen and androgen metabolisms were functionally connected to some of the deregulated hippocampal proteins (Fig 6). These alterations were accompanied by changes in protein intermediates of LTP and long-term depression, such as PAFAH1B2—MYO6, AGFG1, KHSRP, CEP170B, COX4I1, LIN7C, LUZP1, MINK1, PDK3, TPP1, RPL6, CASK, CTNN2, PSMC1, and NDUFB3 or ESRI (16 proteins—MYO6, AGFG1, KHSRP, CEP170B, COX4I1, LIN7C, LUZP1, MINK1, PDK3, TPP1, RPL6, CASK, CTNND2, PSMC1, and NDUFB3) or ESRI (17 proteins—MYO6, AGFG1, KHSRP, CEP170B, COX4I1, LIN7C, LUZP1, MINK1, PDK3, TPP1, CTBZ, ESR1, MCCC2, UGTT, SFN1, and PSMAS). Significantly, none of the hippocampal proteins altered in male Igf-I−/− mice is currently considered to interact with androgen receptors.

To complement our study, we wanted to determine whether we could obtain functional evidence of a relationship between genes involved in neurogenesis (Nieto-Estevez et al., 2016b) and the discohesive proteome detected at the level of the hippocampus in Igf-I−/− mice. As such, both the differential proteomes were interlocked with HES5, NEUROG2, CALB1, and DSCALML1, and considered to interact with androgen receptors.

To search for specific targets affected by Igf-I deletion, we decided to investigate changes in individual hippocampal cells and proteins in Igf-I−/− mice. Because we previously found that Igf-I deletion affected the positioning of Prox1+ neurons in the GCL of postnatal day 49 Igf-I−/− mice (Nieto-Estevez et al., 2016b), we sought to investigate whether this phenotype was maintained in old mice (6–16 mo old). Immunohistochemistry (IHC) with an antibody against Prox1 identified more ectopic Prox1+ neurons (arrowheads) in the molecular layer (ML, 2.5-fold, *P < 0.01, t test: Fig 8A and B) and hilus (Hi, 2.5-fold, *P < 0.05, t test: Fig 8A and C) of Igf-I−/− than in Igf-I+/+ mice. Therefore, local synthesis of Igf-I is necessary for the correct positioning of granule cells in the GCL throughout the animal’s life.

Having seen that Igf-I deletion altered the hippocampal proteome, we assessed whether this deletion produces changes in the relative expression of specific proteins in Western blots and using IHC. There was less RAB6A in the hippocampus of Igf-I−/− mice, a small GTPase involved in LTP regulation (Gerges et al., 2005; Haussser & Schlett, 2019), gephyrin (Cho & Ko, 2015; Ravasenga et al., 2022) and VAMP7 (Kandachar et al., 2018), although the data dispersion in Igf-I−/− mice was not significant (Figs 9B–D and SSA–C). Moreover, no differences were detected between males and females (Figs 9A–D and SSA–C). To investigate whether RAB6A might be specifically modulated in neurons rather than in the hippocampus as a whole, we immunostained sections with antibodies against RAB6A and MAP2ab, the latter a general marker of neurons (Vicario-Abejon et al., 1998). We evaluated the co-localization of both these proteins in neurons located in the Hi, as they were easily identified (Fig S6). The fluorescence intensity was expressed in relative fluorescence units (RFU), and it was similar in both Igf-I−/− and Igf-I+/+ mice. Moreover, no differences were found in the proportion of neurons immunoreactive for both MAP2 and RAB6A relative to the total number of MAP2+ neurons in the Hi. Similarly, no change in the RFU was evident when sections from Igf-I−/− and Igf-I+/+ mice were immunostained with an antibody against the postsynaptic protein gephyrin (Fig S7), and against Synapsin-1 and PSD95, a pre and a postsynaptic protein, respectively (Fig S8) (Vicario-Abejon et al., 2002), or an antibody against aromatase (an enzyme that catalyses oestrogen synthesis from androgens, Fig S9) (Sarcia-Segura et al., 2010). By contrast, we detected significant changes when hippocampal sections from Igf-I−/− and Igf-I+/+ mice were immunostained with antibodies against GAD65 and VGUT1, two markers of inhibitory and excitatory neurons, respectively (Vicario-Abejon et al., 2002). In fact, GAD65 RFU was 36% higher in Igf-I−/− than in Igf-I+/+ mice (*P < 0.05, t test: Fig 10A and B), whereas VGUT1 RFU was a 12% higher in Igf-I−/− than in Igf-I+/+ mice (*P < 0.05, t test: Fig 10A and C), suggesting that Igf-I deletion could provoke an imbalance in the inhibitory/excitatory ratio in the DG. Because parvalbumin (PVA) expressing neurons in the Hi drive synaptic inhibition in DG granule cells (Afrasiabi et al., 2022), we assessed the distribution of PVA in sections using an antibody raised against this protein (Fig 11). However, although the average number of PVA* neurons in the Hi, ML, and GCL were all higher in Igf-I−/− than Igf-I+/+ mice, these increases were not significant.

Together, these findings suggest that Igf-I deletion alters the structure of the GCL, the inhibitory/excitatory ratio, and hippocampal protein modules, which could ultimately lead to impairing LTP, producing deficits in spatial memory formation and affecting sexual dimorphic behaviors.

**Discussion**

Eliciting the role of circulating (largely derived from the liver) and locally synthesized Igf-I both during brain maturation and in its functioning has been the focus of many studies, although this issue remains unresolved. Early studies suggested
that most of IGF-I’s activities were carried out by circulating IGF-I, acting as an endocrine hormone on brain cells (Russo et al., 2005; Ohlsson et al., 2009; Wu et al., 2009; Fernandez & Torres-Aleman, 2012). However, the finding that mice deficient in liver Igf-I develop a relatively mild phenotype (Sjogren et al., 1999; Yakar et al., 1999) prompted studies into the autocrine and/or paracrine role of locally synthesized IGF-I. Nonetheless, it has not been easy to address this possibility because of the lack of suitable animal models.

Using a conditional Igf-I knockout in which brain IGF-I synthesis is severely diminished (Nieto-Estevez et al., 2016b), we show that this growth factor is essential for hippocampal LTP and the acquisition of new spatial memories, and that it regulates sexual dimorphic behaviors. Furthermore, brain IGF-I is critical to maintain

Figure 4. Conditional deletion of brain insulin-like growth factor-I (IGF-I) alters sexual dimorphic behaviors. (A, B, C, D) Conditional deletion of brain IGF-I produces a loss of sexual dimorphism in the open field test. Adult male and female IGF-I knockout mice (6–10 mo old) were assayed in the open field test, and the significant differences between male and female IGF-I Ctrl mice were found in deambulatory horizontal activity (A), vertical activity (B), stereotypic activity (C), and the time spent in the center of the arena (D) were lost in IGF-I knockout mice. The data are represented as the means ± SEM (n = 5–14 mice per condition). *P < 0.05.

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Figure 5. Hippocampal protein homeostasis is disrupted by insulin-like growth factor-I (IGF-I) deficiency.
(A) Heatmap representation showing both the clustering and the degree of change for the differentially expressed proteins (DEPs) in the hippocampus of 10–15 mo-old female (65 DEPs) and male (58 DEPs) Igf-I<sup>Δ/Δ</sup> mice relative to the Igf-I<sup>Ctrl</sup> mice (n = 3 mice per condition). Note that the same proteins were not deregulated in mice of both sexes. (B) Functional clustering of DEPs in the hippocampus of female and male Igf-I<sup>Δ/Δ</sup> mice relative to the Igf-I<sup>Ctrl</sup> mice according to the subcellular distribution (upper) and pathway mapping (lower).
Figure 6. Functional interactomes of the deregulated hippocampal proteomes in Igf-
Δ/Δ mice. (A) Functional relationships between differentially expressed proteins in female Igf-
Δ/Δ mice with protein intermediates involved in estrogen metabolism (ESR1, ESR2, and GPER1: highlighted in red), including ADCY9 and NQO1. (B) Functional relationships between differentially expressed proteins in male Igf-
Δ/Δ mice with protein intermediates involved in androgen metabolism (SRD5A1, SRD5A2, and SRD5A3: highlighted in red), including CCAR1, PRMT5, and UBL4A. Protein interactomes were constructed using the STRING tool: ESR1 and ESR2, estrogen receptors 1 and 2; GPER1, G protein-coupled estrogen receptor 1; SRD5A1, SRD5A2, and SRD5A3, steroid-5 alpha reductases 1, 2, and 3.
the hippocampal proteome, the cellular organization of the DG (particularly that of the GCL), and possibly, the balance between inhibitory and excitatory synaptic activities.

A crucial finding here is that the loss of brain *Igf-I* impairs the formation of new spatial memories, which links the failure to express LTP with a hippocampal-related behavior (Paulsen & Sejnowski, 2000; Lynch, 2004). It is important to emphasize that there is a strong reduction in the *Igf-I* expression in the brains of the *Igf-I*Δ/Δ mice studied here although these animals have normal serum IGF-I levels, supporting the conclusion that the loss of LTP and the impaired spatial memory are because of the lack of locally synthesized IGF-I. Because a liver IGF-I deficiency disrupted both hippocampal LTP and spatial memory (Trejo et al, 2007), our study adds further insights to the regulation of these processes by IGF-I via autocrine and/or paracrine mechanisms. This could be critical to link activity-dependent IGF-I synthesis in the hippocampus with synaptic plasticity and with the formation of new spatial memories.

Notably, female and male *Igf-I*Δ/Δ mice have a completely distinct set of DEPs, with membrane trafficking being the only biological activity commonly disrupted in the hippocampus of both sexes. Moreover, alterations to synaptic proteins occur in both female and male mice, and proteins involved in LTP are also altered. These include the small GTPase Rab6A in females and Rab2A in males, both of which play important roles in regulating ionotropic AMPA glutamate receptor trafficking from the Golgi apparatus to the postsynaptic membrane during LTP (Gerges et al, 2005; Hausser & Schlett, 2019). In this regard, less RAB6A was detected in Western blots of the *Igf-I*Δ/Δ hippocampus, although apparently not significantly *P* = 0.0554. In addition, our bioinformatics analysis reveals a number of pre and postsynaptic proteins whose function could be altered, affecting synaptic activity in both males and females. However, no changes were observed in gephyrin, VAMP7, synapsin-I or PSD95. By contrast, we observed changes in the relative expressions of GAD and VGLUT1, which are preferentially localized to inhibitory and excitatory presynaptic terminals, respectively (Vicario-Abejon et al, 2002). Because *Igf-I*Δ/Δ mice appear to have relatively stronger GAD staining than that of VGLUT1, it is tempting to hypothesize that there is an imbalance in the inhibitory to excitatory ratio in the *Igf-I*Δ/Δ hippocampus that could affect LTP and synaptic plasticity. In

![Figure 7. Functional relationships between proteins involved in neurogenesis and the dysregulated hippocampal proteome of female *Igf-I*Δ/Δ mice. The orange circle indicates specific connections with calbindin (CALB1), and they include CASK1, NEUROG2, and gephyrin (GPHN). No functional relationships were observed in the male *Igf-I*Δ/Δ mice.](image-url)
addition, our data suggest that the disruption of synaptic protein modules may also underlie the failure to induce hippocampal LTP in the Igf-IΔ/Δ hippocampus. Furthermore, the GCL of Igf-IΔ/Δ mice is disorganized because of the presence of ectopic Prox1+ neurons in the ML and Hi. Because the recording electrode in such studies is placed in the GCL, this disorganization, together with the impaired dendritic complexity and differentiation of newly born granule neurons and the reduced size of the DG (Nieto-Estevez et al, 2016b), could potentially affect the efficacy of the synaptic network in response to HFS. By contrast, elevating IGF-I levels in the brain enhances neurogenesis and neuron survival, and it promotes synaptic plasticity by regulating AMPA glutamate receptor phosphorylation and trafficking (Nishijima et al, 2010; Fernandez & Torres-Aleman, 2012; Littlejohn et al, 2020; Williams et al, 2022).

Notably, these processes appear to be partially influenced by the animal's sex, as suggested by the significant differences observed in Igf-IΔ/Δ mice submitted to the MWM test which are not evident in Igf-IΔ/Δ mice. Furthermore, the conditional Igf-I deletion in our mouse model eliminates the sexual dimorphic performance observed between control males and females in the OFT and rota-rod test, which could be in part because of the more
Figure 9. The effect of brain insulin-like growth factor-I (IGF-I) deletion on the expressions of RAB6A, RAB2A, gephyrin, and VAMP7.

Proteins extracted from the hippocampus of adult male and female Igf-I Ctrl or Igf-I Δ/Δ mice (6–16 mo-old) were separated by gel electrophoresis and transferred to membranes that were probed with specific antibodies against: (A) RAB6A, (B) RAB2A, (C) gephyrin, (D) VAMP7, and β-actin (A, B, C, D). Antibody binding was revealed with appropriate secondary antibodies and visualized using Odyssey CLx imaging system (see images on the left). The small decrease in the relative protein levels detected in Igf-I Δ/Δ versus Igf-I Ctrl mice were not significantly different (t-test), although a clear trend was observed for RAB6A (P = 0.0554). The results are the mean ± SEM (n = 8 mice when data from males and females were combined) and (n = 4, when data from males and females were analyzed independently).

### Table 1: Protein expressions

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<tr>
<th>Protein</th>
<th>Males</th>
<th>Females</th>
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<tr>
<td>RAB6A</td>
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<td>42 kDa</td>
</tr>
<tr>
<td>RAB2A</td>
<td>23 kDa</td>
<td>42 kDa</td>
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<tr>
<td>Gephyrin</td>
<td>93 kDa</td>
<td>42 kDa</td>
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<tr>
<td>VAMP7</td>
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limited performance of \( \text{Igf-I}^{\Delta/\Delta} \) female mice in terms of horizontal and vertical activities, stereotypy time, the time they spent in the central area, and the time they remained on the rota-rod. Therefore, brain-synthesized IGF-I is necessary to drive the sexual dimorphism in terms of the formation of new spatial memories, exploratory behavior, and motor learning and coordination.

Although our IHC assays revealed similar aromatase expression in the hippocampus of \( \text{Igf-I}^{\Delta/\Delta} \) and \( \text{Igf-I}^{\text{Ctrl/Ctrl}} \) mice, 25% of the hippocampal DEPs in female \( \text{Igf-I}^{\Delta/\Delta} \) mice have been characterized as proteins that physically interact with estrogen receptors according to the BioGrid repository, whereas none of the hippocampal proteins altered in male \( \text{Igf-I}^{\Delta/\Delta} \) mice are currently considered to interact with androgen receptors. Nonetheless, we found functional relationships between the DEPs in female and male \( \text{Igf-I}^{\Delta/\Delta} \) mice with protein intermediates involved in estrogen and androgen metabolism, respectively. In addition, our analysis indicates the existence of functional relationships between proteins involved in neurogenesis and the altered hippocampal proteome of female but not of male \( \text{Igf-I}^{\Delta/\Delta} \) mice.

The IGF-I signaling system has previously been implicated in the regulation of synaptic plasticity (Trejo et al., 2007; Fernandez & Torres-Aleman, 2012; Gazit et al., 2016; Pristera et al., 2019; Maglio et al., 2021; Williams et al., 2022) and in sexual dimorphic responses (Munive et al., 2016; Ashpole et al., 2017; Pinto-Benito et al., 2022), yet whether these effects were because of the autocrine/paracrine actions of this growth factor has been difficult to investigate. Using a genetic strategy, we present novel evidence that brain-synthesized IGF-I is crucial for hippocampal synaptic plasticity, and that it is involved in the regulation of spatial memory and sexual dimorphic behaviors that require exploratory activity and motor coordination. We identified alterations to the GCL organization, the inhibitory/excitatory ratio, and modules of synaptic proteins, which potentially underlie these processes. Because patients diagnosed with Alzheimer’s disease show deficits in synaptic plasticity and in the acquisition of new memories (Wilson et al., 2023), our findings suggest that locally synthesized IGF-I is critical to maintain a healthy functional brain.
Materials and Methods

**Ethical statement**

All animal care and handling was carried out in accordance with European Union guidelines (directive 2010/63/EU) and Spanish legislation (Law 32/2007 and RD 53/2013). The protocols were approved by the Ethics Committees of the Consejo Superior de Investigaciones Científicas (CSIC, Madrid) and the Comunidad de Madrid (reference, PROEX 025/17). The animals were administered food and water ad libitum, and they were maintained under strictly controlled environmental conditions: 12-h light/dark cycle, temperature 22°C, and humidity 44%. All efforts were made to ameliorate any animal suffering.

**Conditional Igf-I knock-out mice, Nestin-Cre::Igf-I (Igf-I^+/Δ)**

The PCR genotyping and the general characteristics of the Nestin-Cre::Igf-I mice were described previously in detail (Nieto-Estevez et al., 2016b). In brief, the Igf-I^+/Δ mice grow normally although their hippocampal DG and the GCL are significantly smaller and hippocampal neurogenesis is altered. In the present work, we analyzed 31 conditional Igf-I KO mice (Igf-I^+/Δ) and 32 control mice. The latter included Igf-I^+/+, Igf-I^+/Δ, Igf-I^+/Δ, and Igf-I^+/Δ Cre^+ mice. Statistically,
significant differences were not found between the different control mice (hereafter referred to as Igf-^-I^CR^L and Igf-^-I^PL^A mice (14–15 mo old) after the animals had been anaesthetized and decapitated. To reduce the swelling and damage to the superficial layers of the slices from the adult/ageing mice, their brains were removed rapidly and placed in an ice-cold modified NMDG artificial cerebrospinal fluid (ACSF) gassed with 95% O2/5% CO2. This solution is used as a substitute for sodium ions over a wide range of adult ages and for different applications (Ting et al., 2014), and it contains (in mM): NMDG 93, KCl 2.5, NaH2PO4 1.2, NaHCO3 30, HEPES 20, MgSO4 10, CaCl2 0.5, glucose 25, Sodium Ascorbate 5, thiourea 2, sodium pyruvate 3, (pH 7.3–7.4). Hippocampal slices (400 μm) were obtained in NMDG-ACSF and incubated for 10 min at 32°C before being transferred to regular ACSF at RT for 1 h before recording. This regular ACSF was also gassed with 95% O2/5% CO2 and it was made up of (in mM): NaCl 124, KCl 2.69, NaH2PO4 1.2, NaHCO3 26, HEPES 20, MgSO4 2, CaCl2 2, glucose 10 (pH 7.3–7.4). The slices were then transferred to a recording chamber and superfused with regular gassed ACSF. The hippocampus was visualized under an Olympus immersion recording chamber and superfused with regular ACSF. The velocity and freezing time were also assessed. The tracking file obtained was examined with the Tracking Analyzer module and the data collected were analyzed statistically with GraphPad Prism 8 software (GraphPad).

**Electrophysiological slice recordings**

Hippocampal slices were obtained from Igf-^-I^CR^L and Igf-^-I^PL^A mice (14–15 mo old) after the animals had been anaesthetized and decapitated. To reduce the swelling and damage to the superficial layers of the slices from the adult/ageing mice, their brains were removed rapidly and placed in an ice-cold modified NMDG artificial cerebrospinal fluid (ACSF) gassed with 95% O2/5% CO2. This solution is used as a substitute for sodium ions over a wide range of adult ages and for different applications (Ting et al., 2014), and it contains (in mM): NMDG 93, KCl 2.5, NaH2PO4 1.2, NaHCO3 30, HEPES 20, MgSO4 10, CaCl2 0.5, glucose 25, Sodium Ascorbate 5, thiourea 2, sodium pyruvate 3, (pH 7.3–7.4). Hippocampal slices (400 μm) were obtained in NMDG-ACSF and incubated for 10 min at 32°C before being transferred to regular ACSF at RT for 1 h before recording. This regular ACSF was also gassed with 95% O2/5% CO2 and it was made up of (in mM): NaCl 124, KCl 2.69, NaH2PO4 1.2, NaHCO3 26, HEPES 20, MgSO4 10, glucose 25, Sodium Ascorbate 5, thiourea 2, sodium pyruvate 3, (pH 7.3–7.4). The slices were then transferred to an immersion recording chamber and superfused with regular gassed ACSF. The hippocampus was visualized under an Olympus BX50WI microscope and extracellular recordings were obtained at 32°C. The stimulation and recording electrodes (2–4 MO, filled with NaCl 3 mM) were placed on the ML and GCL of the DG, respectively. Excitatory synaptic activity was isolated with the GABAa receptor antagonist picrotoxin (50 μM), and field excitatory postsynaptic potentials were bandpass filtered between 0.3 Hz and 1.0 kHz, and digitized at 10.0 kHz. Ten-min baseline responses at 0.1 Hz were recorded before LTP protocol, which was achieved with 100 Hz trains delivered four times at 0.05 Hz. The pCLAMP 10 software recorded before LTP protocol, which was achieved with 100 Hz trains delivered four times at 0.05 Hz. The pCLAMP 10 software (Molecular Devices) was used to generate the stimuli, and for the data display, acquisition, storage, and analysis.

Normality tests were performed before performing statistical comparisons. Then, one-way ANOVA followed by Tukey’s post hoc test were applied for multiple comparisons.

**Behavioural tests**

**MWM**

To assess spatial learning and memory, animals (6–16 mo old) were placed in a water pool on successive days (Morris, 1984). On the pretraining day, the mice were submitted to a habituation session in the absence of a platform to determine if they have a preference for a particular quadrant of the pool and to rule out any motor problems. The acquisition phase was then carried out over four consecutive days during which the mice had to swim to reach a platform in the pool within a maximum time of 1 min, measuring the average escape latency (seconds) from the water to the platform in four trials each day. If the animal did not reach the platform, it was guided to the platform and it remained there for 30 s. Finally, in the transfer test on day 6, the animals were placed in the pool without the platform, and the time and distance they swam in the platform (P) quadrant or in the other three quadrants were measured using the AnimalTracker application (Gulyas et al., 2016) and ImageJ software (NIH, Bethesda, MD). The velocity and freezing time were also assessed. The tracking file obtained was examined with the Tracking Analyzer module and the data collected were analyzed statistically with GraphPad Prism 8 software (GraphPad).

**OFT**

Mice (6–10 mo old) were introduced into a 42 × 42 × 30 cm arena (Versamax: AccuScan Instruments, Inc.) for 5 min, and the time spent exploring the center of the open field, and the total distance travelled, were quantified automatically with the manufacturer’s software.

**Rota-rod test**

Motor learning and coordination were assessed using a rota-rod apparatus (Ugo Basile) in six consecutive 5-min trials with constant acceleration. Animals were familiarized with the procedure under a constant rod speed the day before the tests started.

**Elevated plus maze**

To assess anxiety-like/coping behavior, mice were introduced in a maze of 40 cm from the floor with two opposing, closed and open, arms. The test measures anxiety as a function of time spent in the open arms versus the closed arms. Time and entries in open and closed arms were scored, with more time spent in the open arms indicating less anxiety. Each animal was introduced into the middle of the apparatus for 5 min. Stress was scored as time spent in the closed arms, whereas coping behavior was estimated by time spent in the open arms. All measures were recorded (Video Tracking Plus Maze Mouse; Med Associates), and analyzed as described (Munive et al., 2019). For statistical analysis, the data were analyzed with GraphPad Prism 6.0 or 8.0 software, first assessing a normal distribution of the data using a Kolmogorov–Smirnov test or a Shapiro–Wilks test (for a small number of samples). The data were then analyzed with a t test with Welch correction for different variances, or one-way ANOVA followed by a Tukey post hoc test for equal variances, a Welch’s one-way ANOVA test followed by Games–Howell post hoc test for different variances, or a two-way ANOVA followed by a Bonferroni or Sidak post hoc tests. P < 0.05 was considered significant and the ROUT method was applied to score potential data outliers.

**Sample preparation for hippocampal proteomics**

Adult mice (10–15 mo old) were placed in a CO2 atmosphere and then decapitated. After carefully removing the skull, the hippocampus was dissected out, frozen rapidly in liquid nitrogen and stored at −80°C until processing. ~0.5 mg of hippocampal tissue from the Igf-^-I^CR^L (or Igf-^-I^ control) or Igf-^-I^PL^A (or Igf-^-I^ conditional knockout -cko- mice) was homogenized in lysis buffer containing 7 M urea, 2 M thiourea, and 50 mM DTT. The homogenates were centrifuged at 100,000g for 1 h at 15°C. Protein precipitation was performed using the ReadyPrep 2-D Cleanup Kit (Bio-Rad) following...
the manufacturer’s instructions. Then, protein concentration was measured using the Bradford assay kit (Bio-Rad).

**Protein digestion, peptide TMT labelling, and off-line fractionation**

Two independent TMT-based quantitative proteomic experiments were performed on three biological replicates from male and female igf-I<sup>cKO</sup>-1 and igf-I<sup>R/A</sup> mice. TMT labelling of each sample was performed following the manufacturer’s protocol (Thermo Fisher Scientific) and briefly, equal amounts of protein (600 μg) from each sample were reduced for 1 h at 55°C with 200 mM tris (2-carboxymethyl) phosphate. Cysteine residues were alkylated with 375 mM iodoacetamide at RT for 30 min and protein trypsin cleavage (1:40, w/w; Promega) was carried out at 37°C for 16 h. Peptide desalting was performed using Pierce Peptide Desalting Spin Columns according to the manufacturer’s instructions. For TMT experiments, each tryptic digest was labelled with one isobaric amine-reactive tag as follows: (i) TMT-Plex-1 (male mice)—Tag126, igf-I control-1; Tag127, igf-I control-2; Tag128, igf-I control-3; Tag129, igf-I cKO-1; Tag130, igf-I cKO-2; Tag131, igf-I cKO-3; (ii) TMT-Plex-2 (female mice)—Tag126, igf-I control-1; Tag127, igf-I control-2; Tag128, igf-I control-3; Tag129, igf-I cKO-1; Tag130, igf-I cKO-2; Tag131, igf-I cKO-3. After 1 h of incubation at RT, the reactions were stopped by adding 5% hydroxylamine, and the labelled samples corresponding to the same plex were independently pooled, desalted, and evaporated in a vacuum centrifuge. The peptide pools were reconstituted with 40 μl of ammonium bicarbonate (ABC, 5 mM) at pH 9.8, and injected into an AKTA pure protein purification 25 system (GE Healthcare Life Sciences) with a high pH stable X-Terra RP18 column (C18, 2.1 × 150 mm, 3.5 μm; Waters). The mobile phases were 5 mM ammonium formate in 90% acetonitrile (ACN) at pH 9.8 (buffer B) and 5 mM ammonium formate in water at pH 9.8 (buffer A). The column gradient was established as an 80-min three-step gradient: from 5% B–30% B over 5 min, 30% B–60% B over 40 min, 15 min in 60% B, and 60% B–90% B over 20 min. The column was equilibrated in 95% B for 30 min and 2% B for 10 min. 12 fractions were collected and evaporated under vacuum.

**Mass spectrometry data acquisition**

Before MS analysis, the peptide fractions were reconstituted to a final concentration of 0.5 μg/μl in 2% ACN, 0.5% formic acid (FA), and 97.5% ddH2O. The peptide mixtures were then separated by reverse phase chromatography using an Eksigent nanoLC ultra 2D pump fitted with a 75-μm iD column (0.075 × 250; Eksigent). Samples were first loaded for desalting and concentrating onto a 2 cm, 100 μm iD precolumn packed like the separating column. The mobile phases were 100% water/0.1% FA (buffer A) and 100% ACN/0.1% FA (buffer B). Non-modified peptide fractions were analyzed according under the following conditions. The column gradient was established as a 135-min three-step gradient from 2% B–30% B over 90 min, from 30% B–40% B over 10 min, and from 40–80% over 10 min. The column was equilibrated with 97% B for 3 min and 2% B for 23 min. Throughout the process, the precolumn was in line with the column and the flow was maintained along the gradient at 300 nL/min. The peptides eluted from the column were analyzed using a 5,600 Triple-TOF system (SCIEX) and the data were acquired upon a survey scan performed in a mass range from 350 up to 1,250 m/z, and with a scan time of 250 ms. LC-MS/MS control and data acquisition were performed using the Analyst TF1.7 software (SCIEX). The top 35 peaks were selected for fragmentation and a minimum accumulation time for MS/MS was set at 11 ms, giving a total cycle time of 3.8 s. The product ions were scanned in a mass range from 100 m/z up to 1,500 m/z and excluded for further fragmentation for 15 s.

**Proteomic data analysis**

The raw MS/MS spectra were processed using the MaxQuant v1.6.17 software (Tyanova et al, 2016a) and used to search the Uniprot proteome reference for mouse. The parameters employed were: initial maximum precursor (25 ppm); fragment mass deviations (40 ppm); variable modification (methionine oxidation and N-terminal acetylation), and fixed modification (MMTS); enzyme (trypsin) with a maximum of 1 missed cleavage; minimum peptide length (seven amino acids); false discovery rate for peptide-spectrum match and protein identification (1%). Frequently observed laboratory contaminants were removed. Protein identification was considered valid with at least one unique or “razor” peptide. Protein quantification was calculated using at least two razor plus unique peptides, and statistical significance was calculated with a two-way t test (P < 0.05), with a 1.3-fold change cut-off. Proteins with TMT ratios below the low range threshold (0.77) were considered to be down-regulated, whereas those above the high range threshold (1.3) were considered to be up-regulated. The Perseus v1.6.14.0 software (Tyanova et al, 2016b) was used for statistical analysis and data visualization. The pathway and interactome analyses were performed using the BioGrid 4.4 (Oughtred et al, 2021), STRING 11.5 (Szklarczyk et al, 2019), Ingenuity (QIAGEN), and Metascape tools (Zhou et al, 2019).

**Tissue collection and Western blotting**

Animals (6–16 mo old) previously used in the MWM test were euthanized and their brains were removed from the skulls. The hippocampus was isolated using a magnifying glass and homogenized mechanically in RIPA buffer (R0278; Sigma-Aldrich, Merck) supplemented with protease and phosphatase inhibitor cocktails (Roche, Merck). The extracts were then centrifuged at 16,000g at 4°C for 10 min, collecting the supernatants and storing them at −80°C until further use. The concentration of the proteins obtained was determined by the BCA method (BCA Protein assay kit, 23227; Thermo Fisher Scientific). Hippocampal samples were then diluted in Laemmli buffer (60 mM Tris–HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue), boiled for 5 min at 100°C, and resolved on precast 4–20% SDS-polyacrylamide gels (Mini-Protein TGX gels, 4561094; Bio-Rad). The proteins were transferred to a 0.2-μm nitrocellulose membrane (Amersham Protran nitrocellulose blotting membrane, 10600094; Merck) and the membrane was blocked for 1 h at RT with 5% nonfat dry milk in TBS plus Tween 20 (20 mM Tris–HCl [pH 7.6], 140 mM NaCl and 1% Tween 20). The membrane was probed with the primary
antibodies overnight at 4°C. anti-RAB6A (1:2,000: NBPT-33110; Novus biologicals), anti-RAB2A (1:2,000: A95802; Antibodies.com), anti-Gephyrin (1:2,000: P5AS-29036; Invitrogen, Thermo Fisher Scientific), anti-VAM7 (1:1,000; 232003; Synthetic Systems), and anti-β-actin (1:10,000: A2228; Sigma-Aldrich, Merck). Antibody binding was detected with IRDye 800CW and 680RD secondary antibodies (1:20,000; LI-COR Biosciences) for 1 h at RT and the images were acquired with the Odyssey CLx imaging system (LI-COR Biosciences). Denstometric quantification was performed using Image Studio Lite acquisition software (LI-COR Biosciences), subtracting the background and normalizing the signal was to that of β-actin as an internal control. Each sample was assayed in triplicate and the statistical analysis was performed using a t test to compare delta/delta with Ctrl/Ctrl, and with a two-way ANOVA and Sidak post hoc test to include the sex variable, all performed using Graph Pad 8.0. The ROUT method was applied previously to determine potential outliers.

Tissue collection and immunohistochemistry

Adult mice (12 mo old) were anaesthetized with an intraperitoneal (ip) pentobarbital and then perfused transcardially with 0.9% NaCl followed by 4% PFA in PB. The animal (ip) pentobarbital and then perfused transcardially with 0.9% NaCl followed by 4% PFA in PB. The animal's brain was post-fixed for 48 h, embedded in 3% agarose, and 40 μm vibratome serial sections were processed for immunohistochemistry. Sections were permeabilized in a solution containing 0.4% Triton X-100 and 10% normal donkey serum, and then incubated overnight at RT with a primary antibody against: aromatase (kindly provided by Dr Pablo Méndez, Instituto Cajal, Madrid, Spain); GAD65 (1:50: Cat# MAB351, RRID:AB_2263126; Millipore); gephyrin (1:500: Cat# P5AS-29036, RRID:AB_2546512; Invitrogen/Thermo Fisher Scientific); MAP2 (1:200–1:500; Cat# M1406, RRID:AB_477171; Sigma-Aldrich); Prox1 (1:750: Cat# AB5475, RRID:AB_177485; Millipore); PSD95 (1:500: Cat# 18258, RRID:AA_2200400; Millipore); VGLUT1 (1:500; Cat# M1406, RRID:AB_477171; Sigma-Aldrich); Prox1 (1:750: Cat# AB5475, RRID:AB_177485; Millipore); PSD95 (1:500: Cat# AB5905, RRID:AB_2301751; Millipore). Subsequently, the samples were washed with PBS and incubated for 4 h at RT in the dark with the appropriate Alexa Fluor secondary antibodies. The sections were then washed, exposed to Hoechst (1 μg/ml: Cat# H3570; Thermo Fisher Scientific), and mounted on glass microscope slides in Mowiol. No specific signal was detected in the absence of the primary antibodies but in the presence of the secondary antibodies.

Cell counting and analysis of the relative fluorescence intensity (RFU)

Neurons located in the DG and that express aromatase, MAP2, Prox1, PVA, and RAB6A were counted in confocal images taken from serial sections. Individual z-planes were collected at a resolution of 1,024 × 1,024 every 1–2 μm with a 40x objective. The entire Z-stack was counted to calculate the number of immunoreactive cells, and to assess the co-localization between MAP2 and RAB6A and between MAP2 and aromatase. Co-localization was confirmed by analyzing each individual plane of each section using ImageJ software. The results are shown as the mean (±SEM) of MAP2+ RAB6A+ neurons and of MAP2+/aromatase+ neurons relative to the total number of MAP2+ neurons or as the mean (±SEM) of the labelled cells per volume as determined using the ImageJ software on confocal Z-stacks. For all the IHC experiments, two sections per animal from three Igf-ICtrl and five to six Igf-IΔ/Δ animals were analyzed per condition (*n* value = 3–6).

The fluorescence intensity (given as the RFU) of the orthogonal projection images of the DG was measured using Fiji-imageJ2 after subtracting the intensity from the negative controls (i.e., sections incubated with secondary antibodies alone). Briefly, individual z-planes at a resolution of 1,024 × 1,024 were taken every 0.5–1.0 μm with a 40x objective and the maximal orthogonal projection per z-stack was obtained. After establishing the threshold from the images from negative controls, the average fluorescence intensity was measured and annotated. This value was subtracted from each projection image of the sections incubated with both primary and secondary antibodies. The threshold was established, selecting pixels with specific labelling and measuring the average fluorescence intensity. Blood vessels and autofluorescent aggregates were excluded from the analysis.

An unpaired two-tailed *t* test was used to compare the mean ± SEM values from Igf-IΔ/Δ and Igf-IΔ/+ mice, with a Welch’s correction when the F-test indicated significant differences between the variances of both groups.

Data Availability

The files of the MS data and search results were deposited in the Proteome Xchange Consortium via the JPOST partner repository ([https://repository.jpostdb.org/](https://repository.jpostdb.org/))—[Okuda et al, 2017](https://doi.org/10.26508/lsa.202201691 vol 6 | no 10 | e202201691)—with the identifier PXD028458 for ProteomeXchange and JPST001316 for JPOST.

Supplementary Information

Supplementary information is available at [https://doi.org/10.26508/lsa.202201691](https://doi.org/10.26508/lsa.202201691).

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Author Contributions

R Herrero-Labrador: formal analysis, investigation, and methodology. J Fernández-Irigoyen: data curation, formal analysis, supervision, investigation, and methodology.
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R Vecino: software, formal analysis, validation, investigation, visualization, methodology, and writing—review and editing.  
C González-Arias: formal analysis, investigation, and methodology.  
K Ausin: investigation and methodology.  
I Crespo: investigation and methodology.  
FJ Fernández Acosta: formal analysis, validation, investigation, visualization, and writing—review and editing.  
V Nieto-Estévez: methodology.  
M Román: investigation and methodology.  
G Perea: conceptualization, resources, formal analysis, supervision, funding acquisition, methodology, and writing—original draft and project administration.  
I Torres-Aleman: conceptualization, resources, formal analysis, supervision, funding acquisition, methodology, and writing—original draft and project administration.  
E Santamaria: conceptualization, resources, data curation, formal analysis, funding acquisition, methodology, and writing—original draft and project administration.  
C Vicario: conceptualization, resources, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, and writing—original draft and project administration.  

Conflict of Interest Statement  
The authors declare that they have no conflict of interest.  

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