IGF2 acts as a mediator of FGF22-induced differentiation of excitatory synapses in the hippocampus

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Abstract

Two prominent types of synapses in the brain are excitatory and inhibitory, also known as glutamatergic and GABAergic synapses, respectively. A precise balance between these two types of synapses is required for the proper functioning of the brain. Disturbances to this balance have been shown to lead to the development of a number of neurological disorders such as schizophrenia, Tourette's syndrome, autism, and epilepsy. Due to this fact, the study of the differential formation of excitatory and inhibitory synapses is important in order to to gain a better understanding of the pathology of these diseases. By deciphering the molecular mechanisms of correct synapse formation, we can gain insight that will allow for the development of effective treatments. In the hippocampus, a specific type of excitatory synapse is formed between the axons of the dentate granule cells (DGCs) and dendrites of the CA3 pyramidal neurons. Fibroblast growth factor-22 (FGF22) has previously been identified as a target-derived presynaptic organizer that promotes the differential formation of these glutamatergic synapses. However, FGF22 does not work alone in this process, but rather it initiates a cascade of molecules that act as mediators of its synaptogenic effect. At present, little is known about the mechanism by which FGF22 promotes presynaptic differentiation, including the factors involved. Here, I have shown insulin-like growth factor-2 (IGF2) to be a strong potential candidate. Through the use of various biochemical techniques, I have gathered data that suggests FGF22 regulates IGF2 expression at a specific stage of synaptic development. Along with this, my results indicate that IGF2 is required for FGF22-induced synaptic vesicle accumulation at the presynaptic terminals on DGC axons. The results of this study further the knowledge on this specific synaptic differentiation process, and have taken one step closer to finding effective treatments for diseases in which it is involved.

Introduction

The brain is the most complex organ in the body. As the center of the nervous system, the brain is responsible for storing our memories, gifting us with the ability to have complex thoughts, processing sensory information from the world around us, coordinating our movements, and giving us emotion and personality. This organ is truly exceptional in that it is able to do all of these things at the same time, with astonishing speed and accuracy. So, what exactly allows the brain to precisely govern all of these processes? Simply put, the brain is endowed with such complexity because it is composed of a precise architecture of over 100 billion neurons, which all interact at particular points of contact called synapses. Accurate brain function is controlled by specific patterns of synaptic connectivity formed by various types of neurons[3]. Synapses begin to form after growing axons recognize their appropriate targets, and the resulting communication between the two is required for synaptic differentiation. Molecules released from each side of the synapse, often termed axon and target-derived factors or pre-and postsynaptic organizers, are the means of communication between developing synaptic partners[1]. Moreover, the recruitment of synaptic components occurs explicitly at axon-target contact sites, implying that molecules exchanged between contacting neurons function as synaptic organizers[3].

Different types of synapses have different components and structures, and serve different functions. These differences suggest that the differential formation of each type of synapse involves a distinct signaling pathway and a characteristic set of numerous molecular factors[3]. One major category of synapses is glutamatergic, which stimulate excitatory outputs in their target neurons. An exact balance between the excitatory outputs and opposing inhibitory signals in the hippocampus is vital for proper brain functioning[3]. Recent studies have revealed the existence of glutamatergic and GABAergic synapse-specific organizers[3].

The differentiation of glutamatergic synapses is accomplished by the successful passing of neurons through critical developmental stages, which involve processes facilitated by synaptic organizers[3]. Fibroblast growth factors (FGFs) have previously been identified as a target-derived presynaptic organizers[2]. These proteins are secreted by certain subpopulations of post-synaptic neurons and promote the local differentiation of presynaptic axons into functional nerve terminals at sites of synaptic contact[2]. This involves multiples processes, including the clustering of synaptic vesicles, the formation of active zones, cytoskeletal restructuring and the assembly of vesicle recycling machinery[5].

In vivo and *in vitro* studies have indicated that one such FGF, FGF22, is involved in the differentiation of glutamatergic synapses formed between axons of dentate gyrus (DG) and dendrites of CA3 pyramidal neurons in the hippocampus[5] (Fig. 1). Previous *In vivo* studies have indicated that FGF22, expressed and postsynaptically secreted by CA3 pyramidal neurons, drives presynaptic differentiation early on in development[1].

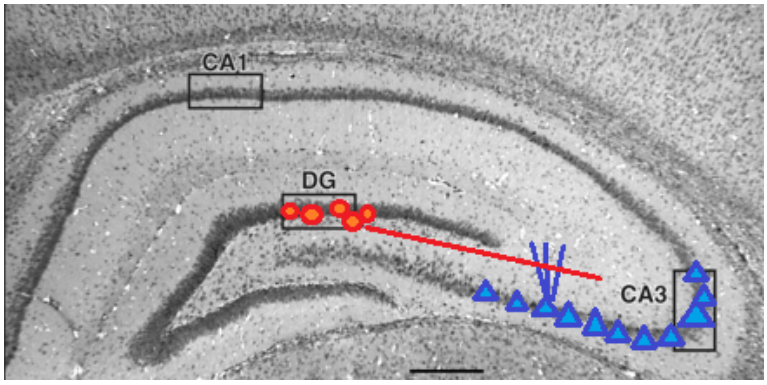


Figure 1| Hippocampal section showing the circuit between the DG and CA3. The dentate granule cell axons (red) form synapses with the dendrites of the CA3 pyramidal neurons (blue).

In situ hybridization has revealed that fgf22 messenger RNAs (mRNAs) are strongly expressed in the CA3 of the mouse hippocampus at 8 days after birth (P8)[5], approximately the time of the onset of synaptogenesis[1]. Additionally, FGF22 protein localizes to synapse-rich areas of the CA3, the stratum radiatum and stratum lucidum. Both of these findings support the idea that FGF22 is involved in synapse formation in the CA3[5]. In order to determine whether FGF22

is required for presynaptic differentiation, the accumulation of synaptic vesicles in FGF22 knock out (KO) mice, in which FGF22 expression is defective, was measured relative to wild-type (WT) mice. Clustering of synaptic vesicle protein SV2 in the CA3 was shown to be significantly decreased in FGF22KO mice at P14, when synaptogenesis is at its peak[5]. In other areas of the hippocampus, such as the CA1, synaptic vesicle clustering was not effected[5]. Furthermore, measurement of the clustering of the active-zone marker bassoon was not significantly different between FGF22KO and WT mice. This finding indicated that FGF22 is not involved in active zone formation in the development of these synapses[5]. These results suggest that FGF22 is specifically involved in the synaptic vesicles clustering step of presynaptic differentiation exclusively in the CA3.

Synaptic vesicle clustering in FGF22KO mice was not impaired in all synapses of the CA3, indicating that FGF22 acts only on a certain subgroup[5]. The effect of FGF22, particularly on glutamatergic presynaptic differentiation in this region, was investigated. This was done by measuring the clustering of vesicular glutamate transporter 1 (VGLUT1), which is a protein associated with synaptic vesicle membranes and functions in glutamate transport. Clustering of vesicular GABA transporter (VGAT), which is an integral membrane protein of synaptic vesicles responsible for GABA uptake and storage at inhibitory nerve terminals, was also measured to rule out the possibility that FGF22 acts on inhibitory synapse differentiation. At P14 VGLUT1, but not VGAT, staining was significantly decreased in the CA3 of FGF22KO mice[5]. Western blots demonstrated that the total amount of VGLUT1 in the CA3 of FGF22KO mice was not decreased from its level in WT mice. This suggested that the deficits in presynaptic differentiation in FGF22KO mice are due to defects in synaptic vesicle recruitment and not their formation[5]. Additionally, overexpression of FGF22 in cultured hippocampal neurons was shown

to induce a significant increase in the clustering of VGLUT1 puncta at the synaptic terminals, along the dendrites of FGF22-expressing neurons. These results demonstrate that FGF22's effect on synaptic vesicle clustering is limited to glutamatergic synapses in the CA3[5].

So, if FGF22 is a target-derived presynaptic organizer, the differentiation of glutamatergic presynaptic terminals on the dendrites of CA3 pyramidal neurons should be specifically impaired in FGF22KO mice. This hypothesis was shown to be correct in an *in vitro* study that utilized the measurement of synaptic vesicle clustering at the presynaptic nerve terminals as an indicator of synaptic differentiation. CA3 pyramidal neurons were identified using the Py antibody, which probes for their dendrites and cell bodies. An anti-VGLUT1 antibody was used to measure synaptic vesicle accumulation in these cells. At 14 days *in vitro* (DIV14), the clustering of VGLUT1 on the dendrites of the CA3 was significantly diminished in FGF22KO cultures relative to WT. Furthermore, it was shown that this presynaptic defect in FGF22KO cultures could be rescued by exogenous FGF22 expression through bath application. These results supported the hypothesis that FGF22 is critical for the proper differentiation of excitatory presynaptic terminals formed on the CA3[5].

To better understand the mechanism that mediates FGF22's synaptogenic effects, its synaptic localization was determined by transfecting hippocampal cell cultures with a plasmid encoding enhanced green fluorescent protein (EGFP)-tagged FGF22. It was found that FGF22 localizes to CA3 dendrites, as was shown by staining with anti-microtubule-associated protein 2 (MAP2) antibodies. In addition, more than 85% of FGF22-EGFP puncta colocalized with postsynaptic density protein 95 (PSD95) but not with Gephyrin[5], which are postsynaptic scaffolding proteins at glutamatergic and GABAergic synapses, respectively. Thus, FGF22 specifically localizes to the postsynaptic side of glutamatergic synapses[5].

The functional consequences of impaired presynaptic differentiation in FGF22KO neurons were investigated in two ways. First, miniature excitatory and inhibitory postsynaptic currents were measured in FGF22KO hippocampal cultures. It was found that the frequency, but not the amplitude, of only excitatory postsynaptic currents were decreased in FGF22KO cultures relative to WT[5]. Second, since a synaptic imbalance in the hippocampus is hypothesized to contribute to the development of various neurological disorders, such as epilepsy, FGF22KO and control animals were subjected to the kindling protocol. Animals were injected with a GABA receptor antagonist to induce epileptic seizures. None of the FGF22KO mice were kindled within the period examined whereas half of the control mice were. It seems likely that FGF22 plays a role in the formation or maintaining the excitatory-inhibitory balance in the hippocampus. Moreover, FGF22KO mice are resistant to induced seizures, probably due to their impaired formation of excitatory synapses and therefore greater relative level of inhibitory synapses whose correct formation do not depend on FGF22[5].

Taken together, the outcomes of these experiments suggest that FGF22 plays a vital role in glutamatergic presynaptic differentiation in the CA3 of the hippocampus. Yet, the details of molecular mechanism by which FGF22 exerts its effects are not entirely understood. As a target-derived factor, FGF22 is secreted from the CA3 pyramidal neurons and diffuses across the synaptic cleft to the presynaptic nerve terminals of DG axons where it binds to its receptor and subsequently induces some intracellular cascade. This cascade ultimately results in the clustering of synaptic vesicles at the presynaptic nerve terminals. The study presented here began with the intent of identifying other factors involved in this mechanism.

In order to identify factors that could be involved in the mechanism by which FGF22 induces synaptic vesicle clustering during glutamatergic presynaptic differentiation, qRT-PCR

was used to screen for genes that are downregulated in the dentate gyrus of FGF22KO mice. One factor that was found to have decreased mRNA levels in the DG was Insulin-like growth factor-2 (IGF2). IGF2 was chosen as a strong candidate for a number of reasons. Most importantly it has been found that IGF2KO mice are resistant to induced seizures, similar to FGF22KO mice. Furthermore, recent studies have provided proof that IGF2 is involved in various processes of neurogenesis and synaptogenesis in the hippocampus. IGF2 is involved in adult hippocampal neurogenesis by selectively controlling the proliferation of dentate granule neural stem cells[7], it facilitates a fear extinction pathway in the hippocampus[6], and it enhances memory retention and prevents forgetting[8].

Likewise, hippocampal formation has been shown to have some of the most intense and abundant IGF2/MP6 receptor (IGF2R) immunoreactivity in the brain[10]. IGF2R staining demonstrates that it is strongly expressed in nearly all CA3 pyramidal neurons[10]. The fact that the receptor for IGF2 has been shown to be located in this area of the hippocampus strongly suggests that IGF2 has some influence on the cells in this area.

Although the following experiment were performed mostly by me, I am also including experiments performed by Akiko Terauchi, my supervisor, directly before I began work on this project. With her help, I have developed the following model (Fig. 2) for the role of IGF2 as a molecular mediator of excitatory presynaptic differentiation in the hippocampus, including its relation to FGF22: Upon binding of FGF22 at its receptor (FGF22R) on DGCs a signal cascade is induced that leads to an upregulation of IGF2. IGF2 expression ultimately results in the recruitment of synaptic vesicles to the axon terminals of glutamatergic synapses.

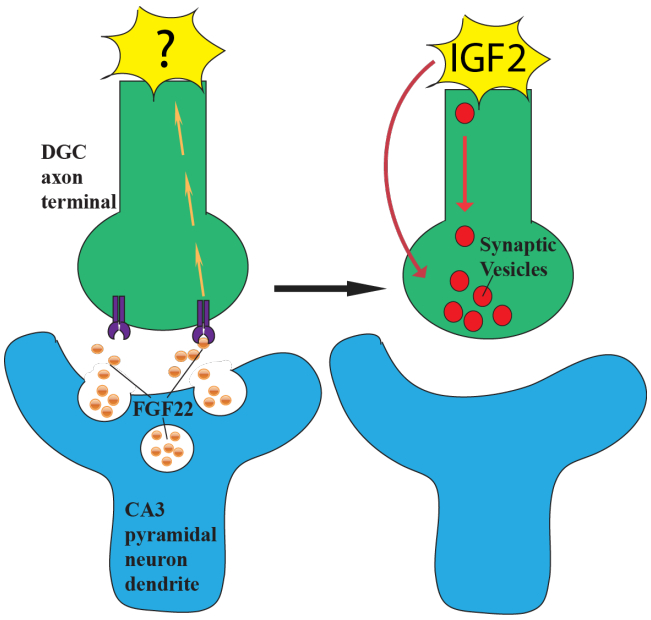


Figure 2| Model for IGF2-dependent synaptic vesicle accumulation. First, FGF22 (orange) is released from the postsynaptic cell (CA3 pyramidal neuron, blue) and binds to its receptor (purple) on the presynaptic cell (DGC. green). Upon binding to the receptor, a message is relayed within the cell that signals the upregulation of some molecular factor(s) required for synaptic vesicle clustering at the axon terminal. I propose that IGF2 is a key molecular factor involved in synaptic vesicle clustering at DGC axon terminals, and that its expression regulated by FGF22.

Materials and Methods

Knockout mice. FGF22KO mice were generated by replacing exon 1 and a part of exon 2 of the fgf22 gene with the neomycin-resistant gene by means of homologous recombination.

Primary Neuronal Cultures. Dissociated hippocampal cultures were prepared from the brains of P0 mice. Using a dissecting microscope, hippocampi were dissected out in a dish containing a solution of neurobasal (NB) media and a penicillin/streptomycin mixture then immediately placed into a 15-mL falcon tube containing a neurobasal media. The hippocampi-containing tubes were placed into a centrifuge (0.5 rcf, 2 minutes, 4˚C). Next, the NB was removed and replaced with a trypsin solution (2.5% Trypsin, 0.1% DNase, 0.5% Glucose in HBSS) for cell dissociation. The tubes were then placed into a 37˚C water bath and allowed to incubate for 15 minutes, after which time they were centrifuged (1.0 rcf, 3 minutes, 4˚C) again. The trypsin solution was then removed completely and the hippocampi were washed two times with HBSS

(10 mL). HBSS was then removed from the tubes and NB/B27 solution was added (500 µL; 0.01% L-Glutamine, 0.02% B27 supplement (Invitrogen), 0.01% Penicillin/Streptomycin in NB media). Single-cell suspension was performed by pipetting the hippocampi up and down 5-10 times using a 1 mL pipette tip until no chunks of tissue remained. Cells were counted and then re-suspended in the appropriate amount of NB/B27 solution to reach the desired cell concentration ($3x10^4$ cells/coverglass or $5x10^4$ cells/coverglass for cultures to be transfected). Neurons were plated onto poly-D-lysine coated glass coverslips (diameter 12-14mm) and maintained in neurobasal medium with B27 supplement in a 5% CO_2 incubator at 37°C.

Transfection. Transfections were performed on cultured hippocampal neurons using the calcium phosphate method. Neurons were isolated from the dentate gyrus and CA3 regions of the hippocampus ($5 X 10^4$ cells/coverslip) and cultured for 1 to 3 days before each coverglass was transferred into a single well of a 24 well plate. Each well was filled with 50 µL of transfection media (B27 and L-Glutamine in NB). The culture media from each dish was stored in a tube and saved for use in a later step. Cells were then incubated in a 5% CO_2 incubator for 30 minutes at 37°C. During incubation, cDNA solutions were prepared in 1.5 µL centrifuge tubes at a concentration of 1-2 µg per coverglass. Additionally, one 1.5 µL centrifuge tube was filled with 100 µL of HEPES buffer solution for each cDNA tube. The cDNA solutions were then added into the HEPES buffer-containing tubes. 1/8 of the cDNA solution was pipetted gently into the HEPES buffer at a time and the tubes were vortexed for about 2 seconds following each addition. cDNA solutions were allowed to incubate in the HEPES buffer for 20 minutes prior to transfection. 50 µL of transfection solution was added to each coverglass and then the 24-well plate was placed back into the 5% CO_2 incubator for 1 hours and 5 minutes. After incubation, the

transfection solution was removed from each well and 10% CO_2 saturated NB/B27, prepared at least 3 hours prior to use, was added. This was followed by incubation in a 10% CO_2 incubator for 15 minutes, and then for 5 minutes in a 5% CO_2 incubator. Wells were washed two times with NB and finally, 350 μL of the saved NB/B27 culture media was added onto each coverglass. Transfected cultures were then allowed to grow in a 5% CO_2 incubator for the 6-12 days.

Plasmids.

For experiments in which IGF2 was overexpressed, two plasmids were used, the first was a plasmid encoding IGF2 protein and the second was a synaptophysin-yfp plasmid that allowed synaptophysin protein to be viewed by fluorescence microscopy. A plasmid construct encoding short hairpin RNA (shRNA) for IGF2 was used as method of RNA interference (RNAi) in experiments in which IGF2 expression was knocked down. By this method, IGF2 mRNA was targeted for cleavage by the RISC complex of transfected cells, inhibiting translation of the mRNA into IGF2 protein. A control shRNA plasmid was also used for these experiments.

Immunocytochemistry. Cultured neurons were fixed with either 4% paraformaldehyde (PFA) for 10 min at 37°C, methanol for 5 min at -20°C, or acetone for 2 min at -20°C depending on the primary antibodies being used. Neurons were blocked for 30 min in 2% BSA, 2% normal goat serum, 0.1% Triton X-100, followed by incubation overnight with primary antibodies at 4°C. For goat polyclonal antibodies, neurons were blocked for 30 min in 2% BSA, 0.1% Triton X-100, and then incubated with primary antibodies. Secondary antibodies were applied for 1 h at 25°C and slides were mounted onto glass slides with *p*-phenylenediamine (PPD). Slides were kept in a

-20 ˚C freezer. For experiments in which anti-CR or anti-CB antibodies were used, prox1 was stained using the Zenon labeling method (Zenon labeling kit: molecular probes).

Dilutions of antibodies used are as follows: anti-VGLUT1 (1:4,000), anti-VGAT (1:1,000), anti-PSD95 (1:700), anti-Gephyrin (1:500), anti-Calbindin (1:250 or 1:300 or 1:1,000), anti-Calretinin (1:250 or 1:200), anti-Prox1 (1:2,000 or 1:500), anti-GFP (1:100 or 1:500 or 1:3,000), anti-Neurofiliment (1:1,000 or 1:2,500), anti-MAP2 (1:4,000), anti-IGF2 (1:50), anti-IGF2R (1:70 or 1:150), and the Py antibody (1:20).

Microfluidic Chamber Culture. Coverslips were initially washed and stored at 4˚C in a bottle containing 70% ethanol. Before use, coverslips were additionally washed in 10 cm culture dishes 3 times using sigma water, thoroughly dried, and coated with PDL diluted in boric buffer (4x concentration, 8ml/coverslip) overnight in a culture hood. The next day the PDL solution was aspirated and coverslips were washed 3 times with sigma water after which they were soaked in sigma water for 1-2 hours in a sterile culture hood. After, the water was aspirated and each coverslip was placed into a 6 cm culture dish and allowed to dry overnight in a culture hood. The preparation of the microfluidic devices began on days after coverslips began to be coated with PDL. Devices were washed by placing them into a 10 cm culture dish with their 'bridge' side facing up and soaking them in 200-proof ethanol for 5-10 minutes. Excess ethanol was then removed and the devices were dried overnight in a culture hood. The next day, devices were placed onto PDL coated coverslips in 6 cm dishes with the 'bridge' side facing down. A reversible bond was made by gently pressing down on each device. Hippocampal neurons isolated from the DG/CA3, cultured as previously described, were immediately loaded (7,000 cells/μL, 5μL) into the somal side of the devices following their attachment to the coverslips.

Culture medium was added to the axonal chambers. Cells were then incubated for 10-15 minutes in a 37°C, 5% CO_2 incubator to facilitate adhesion. Culture medium was then added to both sides of the device (100 µL to the axonal side, 150 µL to the somal side). At DIV3, FGF22 was added to the axonal side of the device (2nM**,** 10 µL). Culture medium (30 µL) was also added to the somal side to maintain the volume difference between the two chambers. At DIV9**,** the devices were removed from the coverslips by pouring warm neurobasal (4 ml/dish) into the 6 cm dishes and gently detaching them using sterile forceps. Cells were fixed in acetone for 2 minutes at -20°C and then washed 3 times with PBS for 5 minutes each. Cells were then staining using the methods previously described.

Imaging. Twelve-bit images were acquired on an epifluorescence microscope (BX61; Olympus) using 10x, 20x, or 40x objective lenses and an F-View II CCD Camera (Soft Imaging System) at 1,376 pixels X 1,032 pixels resolution, or on a confocal microscope (FV1000; Olympus) using a 40X objective lens at 1,024 pixels X 1,024 pixels resolution. For each experiment, all images were acquired with identical settings for laser power, detector gain and amplifier offset. Confocal images were acquired as a z-stack (10-25 optical sections, 0.5µm step size). For quantification, neurites or random fields were selected, thresholded (the intensity of the dendritic shaft in control cultures was calculated as the background fluorescence), and the average size, density (per length), and average intensity of puncta were calculated with Metamorph Software. Metamorph software was also used for determining the colocalization indices. Images of single fluorescence channels were thresholded and binarized. Puncta in one channel were considered to be colocalized if their area was covered by the signal a second channel.

Results

IGF2 mRNA expression is impaired in the dentate gyrus of FGF22KO mice

In accordance with the proposed model, IGF2 expression in the DG should be decreased by impaired expression of FGF22 in developing CA3 pyramidal neurons. This was verified by an experiment done by Akiko Terauchi using *in situ* hybridization for the *Igf2* gene in the hippocampus. *Igf2* mRNA levels were measured in FGF22KO mice relative to WT mice at P7 and P14. At P7, there was no significant difference in the amount of IGF2 mRNA between FGF22KO and WT mice. However, expression at P14 it was diminished in the inner molecular layer of dentate granule cells (DGCs) in the DG of FGF22KO mice. These results support the belief that FGF22 regulates presynaptic IGF2 expression from the DG during a specific period of synaptogenesis.

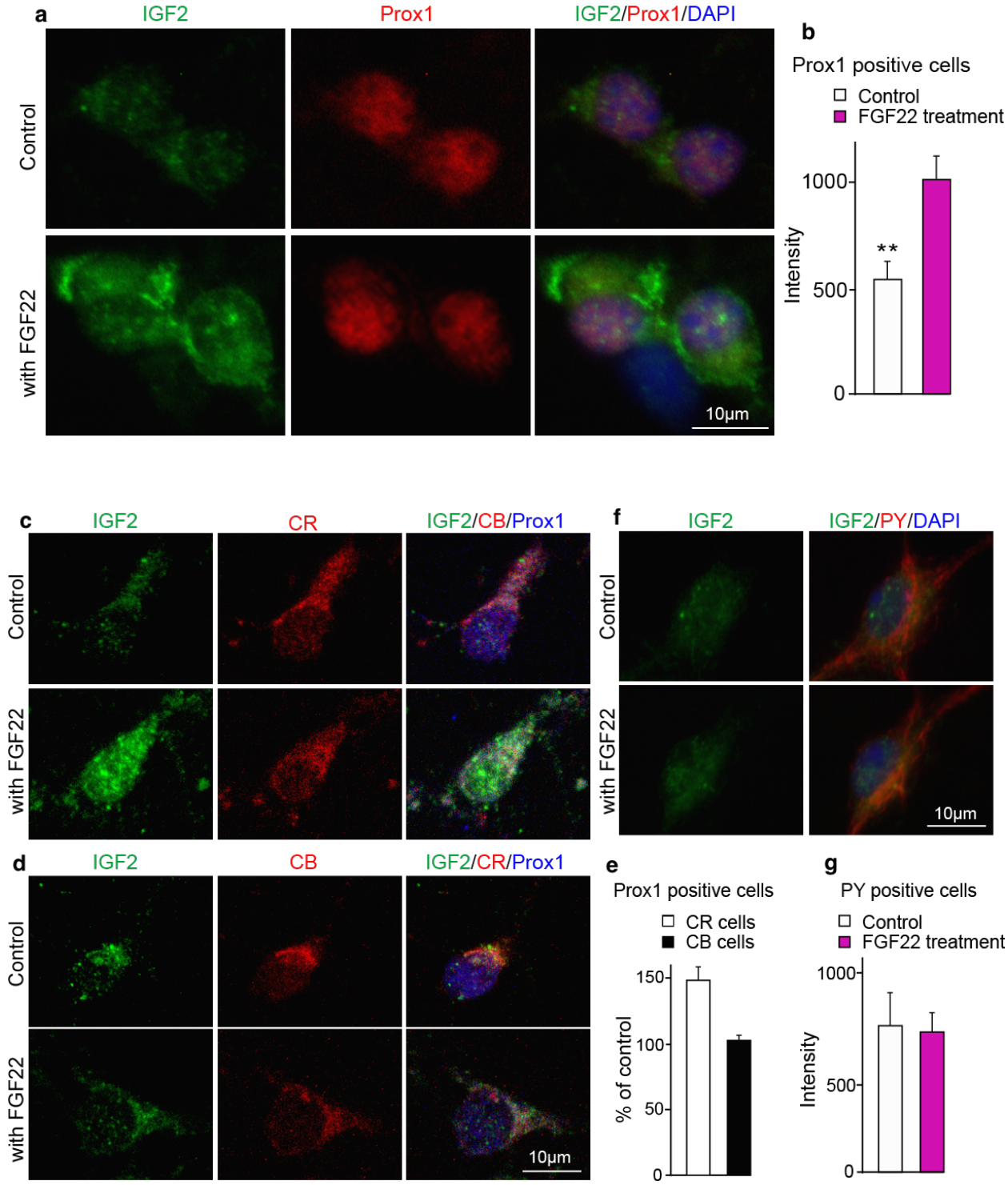
IGF2 protein levels are decreased in the inner molecular layer of the DG in FGF22KO mice during a specific stage of synaptogenesis.

Since IGF2 expression was decreased exclusively in the inner cell layer of the DG of FGF22KO mice brain sections, this layer-specific expression was investigated by Akiko Terauchi. She immunostained WT and FGF22KO brain slices at P7 and P14 with an anti-IGF2 antibody in addition to ether an anti-Calretinin (CR), to mark the immature inner cell layer of the DG, or anti-Calbindin (CB) antibody, to mark the mature outer cell layer. At P7, IGF2 levels did not show any appreciable change between FGF22KO and WT brain sections in both the CR and CB-positive cell layers. By contrast, at P14 IGF2 expression was significantly reduced in the CR-positive cell layer of the DG in FGF22KO mice. No decrease was seen in the CB-positive

cell layer. These observations suggested that IGF2 is only regulated by FGF22 at a later stage of synaptogenesis and that its regulation does not continue in mature DGCs.

Overexpression of FGF22 results in increased IGF2 expression in Calretinin positive dentate granule cells

To investigate the relationship between FGF22 and IGF2 expression in the hippocampus from another perspective, I overexpressed FGF22 protein in cultured hippocampal neurons. In this case, the model predicts that FGF22 overexpression should cause increased IGF2 expression. I bath applied FGF22 to cultured DG/CA3 neurons at DIV1 and compared IGF2 expression to the WT control cultures with no added FGF22. Cells were fixed and stained at DIV7. An anti-Prox1 antibody was used to immunolabel DGCs so IGF2 expression could be specifically visualized in these cells. The intensity of IGF2 antibody immunoreactivity in Prox1-positive cell bodies was used as a measure of IGF2 protein expressed in these cells (Fig. 3a). It was found that IGF2 expression in FGF22-treated cultures was significantly increased compared to the control (Fig. 3b). These results verify the prediction that FGF22 treatment will upregulate the expression of IGF2.


a
IGF2
Prox1
IGF2/Prox1/DAPI
Control
with FGF22
10μm
b
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Control
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PY positive cells
Control
FGF22 treatment
Intensity
1000
500
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Figure 3| Bath application of FGF22 protein increases IGF2 expression in immature dentate granule cells. Cultured hippocampal neurons treated and not treated with FGF22. **a,** Prox1 (red) labeled neurons displayed increased IGF2 (green) expression following bath application of FGF22. Data for the experiments is shown in **b**. **c,** FGF22 treatment enhanced IGF2 expression in Calretinin-positive DGCs (red), but did not cause increased expression in Calbindin-positive DGCs, **d** (also red). Quantification of these two results is shown in **e**. **f,** IGF2 expression showed no change with FGF22 treatment in CA3 pyramidal neurons immunolabeled with the PY antibody (red). Corresponding data is displayed graphically in **g**. Data shown in **b** was obtained from 93-116 cells from 3 independent experiments. **e,** Average IGF2 intensities, displayed as % of control, were measured in 3 independent experiments from 5-9 cells per experiment. Data in **g** is from 38-45 cells from 4 independent experiments. Significant difference from control: **P< 0.01 by t-test.

Subsequent experiments were carried out in which anti-CR and anti-CB antibodies were used to differentiate between immature and mature Prox1-positive cells, respectively. For these experiments, Prox1 was stained using the Zenon labeling method. These experiments gave insight into the developmental timing of FGF22's control over IGF2 expression. As expected, IGF2 expression was increased by FGF22 treatment only in CR-positive cells while no change was seen in CB-positive cells (Fig. 3c, e). To assure that FGF22 treatment was not influencing the expression of IGF2 in the CA3 pyramidal neurons, some cell cultures were probed with anti-Py antibody and IGF2 expression was analyzed. Bath applied FGF22 had no effect on IGF2 expression in these cells (Fig. 3d, f), demonstrating that IGF2 specifically acts on the DGCs. So, FGF22 seems to be exerting its effects on IGF2 expression only in DGCs during early synapse formation, but not later in mature cells.

IGF2 localizes to presynaptic terminals in the axons of dentate granules cells

The model includes that FGF22 localizes to the CA3 pyramidal neuron dendrites, which has already been proven, while IGF2 should localize to DGC axons. To examine the localization

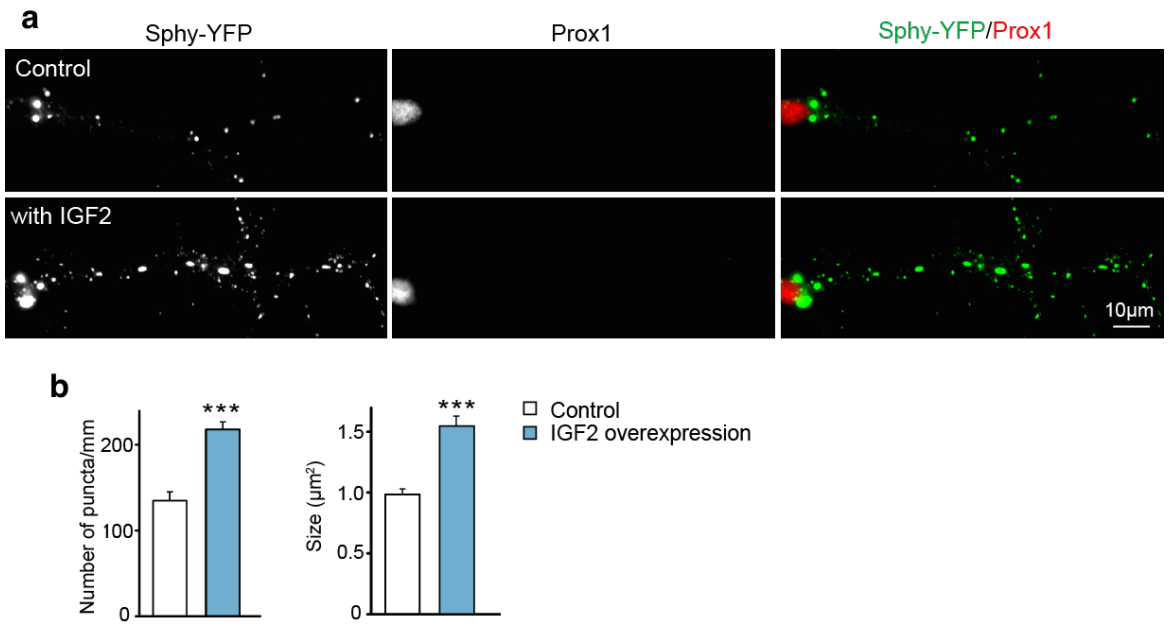
of IGF2, Akiko Terauchi generated an EGFP-tagged IGF2 expression plasmid, which was used to tag IGF2 with enhanced green fluorescence protein. Cultures were probed with an anti-Prox1 primary antibody to mark DGCs. In addition, half of the cultures were stained with an anti-Neurofiliment (NF) antibody and the other half with an anti-MAP2 antibody to mark axons and dendrites, respectively. The results demonstrated that IGF2 localizes to the axons of Prox1-positive cells where it is expressed with a punctated pattern. This punctated pattern implied that IGF2 was being sequestered at discrete locations along the axon, possibly at nerve terminals. By contrast, IGF2 was not localized at the dendrites as expected.

Following the results of the previously mentioned experiment, Akiko Terauchi investigated whether IGF2 is located at the axon terminals of the DGCs. To do this, she co-transfected cultured hippocampal neurons with two plasmids, one encoding synaptophysin-mCherry (syt-mCherry) and the other encoding IGF2-EGFP. She then looked to see if their expression was co-localized. Cultures were also stained with Prox1 to ensure that the cells we were observing were DGCs. The results are in compliance with the model, as the majority of IGF2-EGFP puncta were co-localized with syt-mCherry puncta, suggesting that IGF2 localizes to the synaptic terminals of the DGCs.

IGF2 overexpression in dentate granule cells increases synaptic vesicle clustering at presynaptic terminals

I proposed that IGF2 has a direct effect on synaptic vesicle accumulation and will therefore, if overexpressed, increase the number of synaptic vesicles recruited to the axon

terminals of DGCs. Akiko Terauchi and I carried out experiments in order to test this. A non-tagged IGF2 expression plasmid was created and at DIV3, it was co-transfected into hippocampal cultures with a plasmid encoding syt-YFP, as described previously, using the calcium-phosphate method to assure that the two plasmids were taken up into cells simultaneously. The purpose of the IGF2 expression plasmid was not to label IGF2 protein but only to enhance the expression of IGF2 so that we could focus solely on the expression of syt-YFP. Cultures were fixed and stained with Prox1 at DIV10. As shown in Figure 4b, syt-YFP puncta were significantly increased in cells transfected with the IGF2 expression plasmid compared to the control group, which did not receive the IGF2 expression plasmid. These results confirm the proposal that IGF2 directly regulates synaptic vesicle accumulation at glutamatergic presynaptic terminals. We also wanted to see if overexpressing IGF2 had any effect on synaptic vesicle clustering in cells outside of the DG. So, we performed the same experiment, this time focusing on syt-YFP puncta in Prox1 negative cells. Fig 4d graphically depicts the results seen in 4c. The shIGF2 plasmid did not have an effect on syt-YFP puncta in non-DGCs.



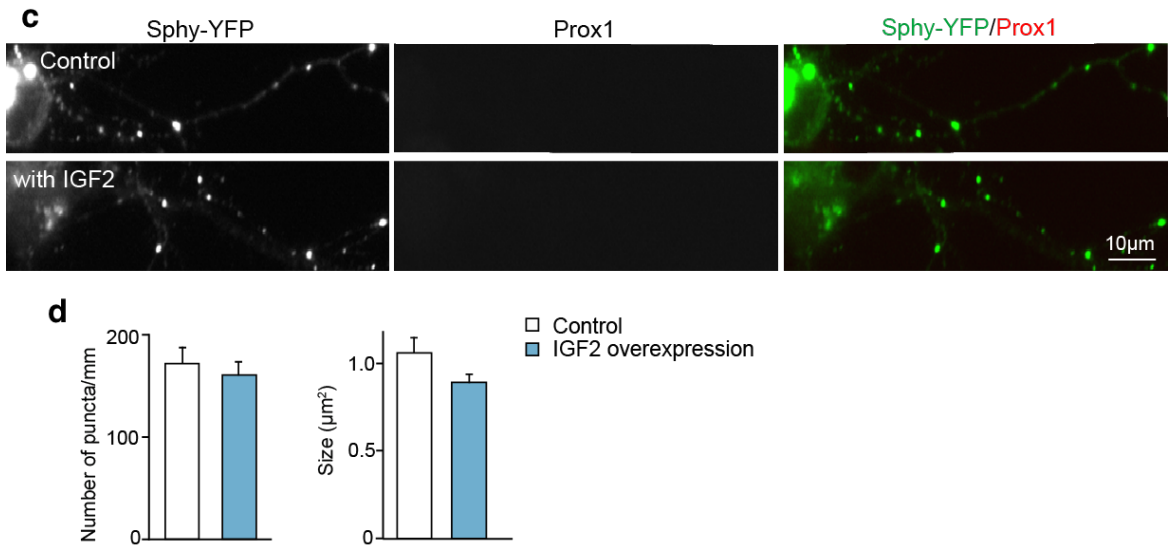


Figure 4| Dentate granule cells transfected with an IGF2 overexpression plasmid demonstrate enhanced presynaptic differentiation. Cultured hippocampal neurons transfected with the IGF2 expression plasmid along with the synaptophysin-YFP plasmid. **a,** Synaptophysin-mCherry (in green) puncta clustering was increased in prox1 positive cells (in red) transfected with the IGF2 overexpression plasmid compared to the control. **b,** Graphs for the number and size of synaptophysin-YFP puncta. Included 36-44 cells from 5-7 experiments. **c,** In cultured cells that were prox1 negative, overexpression of IGF2 did not change clustering of synaptophysin-mCherry. **d,** Graphs quantifying the number and size of synaptophysin-YFP puncta of 11-13 cells from 2-3 experiments. Error bars are s.e.m. Significant difference from control at ***P < 0.0001 by t-test. Scale bars, 10 μm.

Impaired synaptic vesicle accumulation at glutamatergic synapses in the dentate gyrus of FGF22KO mice is recued by bath applied IGF2

The following experiments not only investigated the relationship between IGF2 and FGF22, but also the order of their roles in the pathway to induce the accumulation of synaptic vesicles at the axon terminals of DGCs. I hypothesized that the application of IGF2 protein to FGF22KO DG/CA3 hippocampal neurons *in vitro* should restore synaptic vesicle accumulation

to a level very close or identical to that found in the DG/CA3 neurons of WT mice. A bath application method was used to introduce IGF2 into hippocampal cell cultures of FGF22KO and WT mice at DIV1. Untreated FGF22KO cultures displayed impaired synaptic vesicle clustering, as determined by staining them with an anti-VGLUT1 antibody. I then looked to see if these defects in synaptic vesicle accumulation found could be rescued in FGF22KO cultured by IGF2 treatment. Following fixation at DIV13, I compared the expression of VGLUT1 in FGF22KO and WT cultured Py-positive neurons with and without IGF2 added. The CA3 pyramidal neurons were isolated by staining with the Py antibody, which is specific to their axons and dendrites. The results display that VGLUT1 accumulation in FGF22KO cultures treated with IGF2 was virtually the same as it was in WT cultures (Fig. 5b, a). From this I was able to conclude that bath applied IGF2 rescues defects in VGLUT1 accumulation at the presynaptic terminals caused by knocking down FGF22. Bath application of IGF2 did not effect VGAT (Fig. 5c,d), PSD95 (Fig. 5e,f), or Gephyrin (Fig. 5g,h) localization and accumulation, as expected. Therefore, IGF2 does not have an effect on excitatory postsynaptic differentiation or inhibitory pre and postsynaptic differentiation, indicating that it is most likely not involved in synaptic differentiation in the hippocampus in a broader sense.

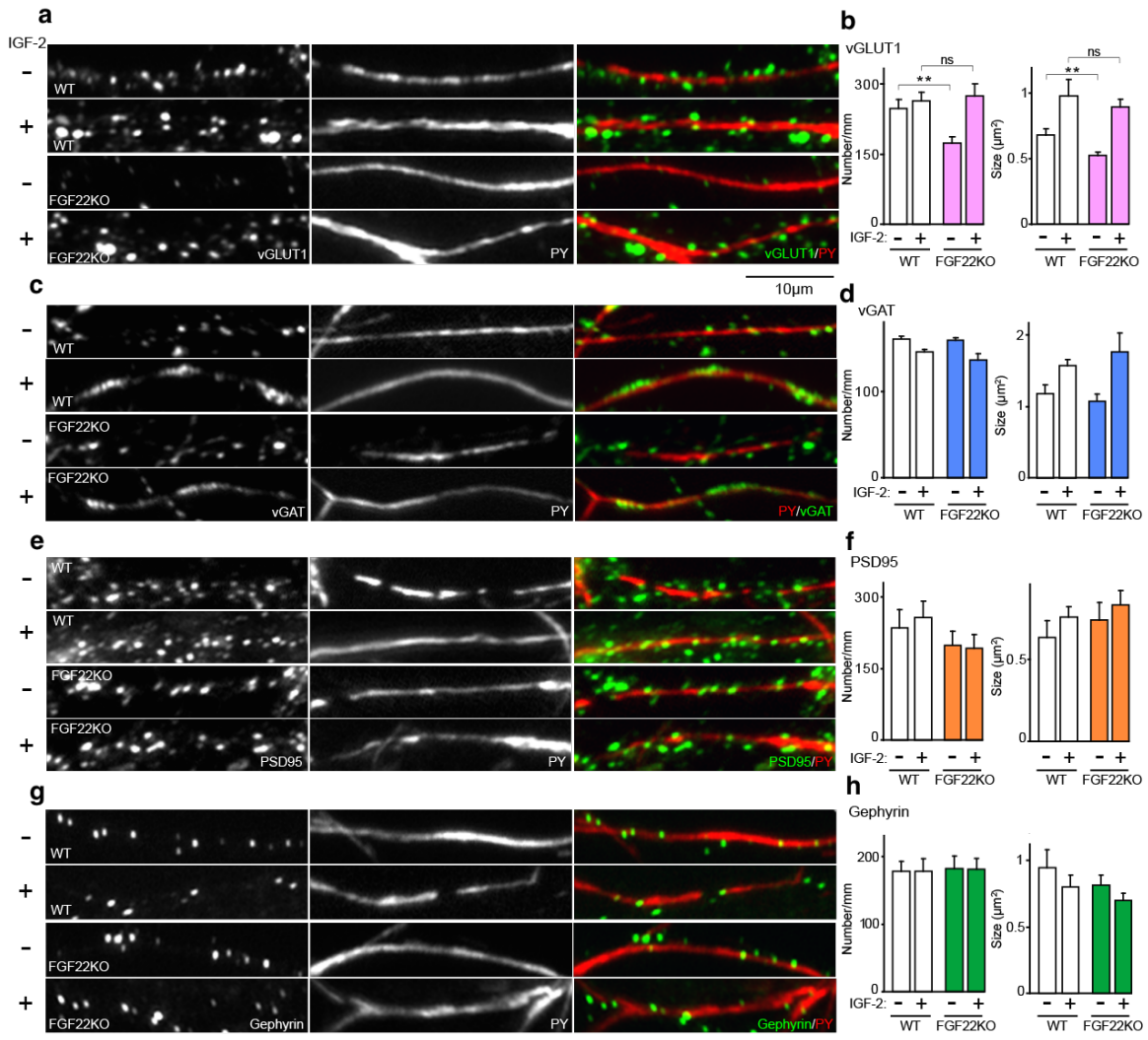


Figure 5| Impairment of synaptic vesicle accumulation at excitatory presynaptic terminals of FGF22KO mice is restored to normal levels by bath applied IGF2. WT and FGF22KO cultured neurons treated and not treated with IGF2. **a,** VGLUT1 clustering on the dendrites of CA3 pyramidal neurons (labeled with Py antibody, red) of WT and FGF22KO mice. Significant defects in clustering of VGLUT1 puncta on the CA3 dendrites resulted from knocking down FGF22. However, bath application of IGF2 was able to rescue these defects, as demonstrated the restoration of VGLUT1 accumulation to essentially the amount found normally in WT mice. Graphs of the density and size of VGLUT1 puncta are shown in **b**. **c-h,** FGF22KO mice did not display significant changes in VGAT, PSD95, or Gephyrin clustering as a result of knocking down FGF22 or by treatment with IGF2. Error bars are s.e.m. Data was obtained from 3-10 cells from 4-6 independent experiments. Significant difference from control: **P<0.01 by ANOVA followed by Tukey test.

IGF2 is required for FGF22-induced synaptogenesis after initial synapse formation

To verify the order of IGF2-mediated effects on synapse formation in relation to FGF22 that is proposed by the model, I performed essentially the opposite of the previous experiment. The dentate gyrus/CA3 pyramidal neuron region of the hippocampus was dissected from WT mice at P0. Endogenous IGF2 expression was knocked down in hippocampal cell cultures using shRNA-mediated gene silencing. Cultures we transfected at DIV1 or DIV2 with either the shIGF2 plasmid or a control plasmid. Afterwards, FGF22 protein was bath applied to half of the experimental and control group cultures to determine if it is able to rescue any defects in synapse formation caused by knocking down IGF2 expression. I expected that FGF22 application should not restore any defects in synaptic vesicle clustering at DG axon terminals that result from the knocking down of IGF2 expression. This is because, in my proposed mechanism, FGF22 is expressed prior to, and is the cause of IGF2 expression in the DG. Cell cultures were then either fixed at DIV6 or DIV12 to see if there were any differences in the results at these two stages of synapse development. Fig. 6 demonstrates that knocking down IGF2 significantly reduced synaptic vesicles clustering at DIV12, but not at DIV6. In addition, FGF22 did not have the same effect on IGF2 expression at DIV6 and DIV12. At DIV12 (Fig. 6b, d) FGF22 was not able to induce synaptic vesicle accumulation in cultures with IGF2 expression knocked down, depicted by a reduced number of synaptophysin-YFP puncta/mm. However, at DIV6 (Fig. 6a, c) FGF22 was still able to increase synaptic vesicle accumulation even in cultures with IGF2 knocked down. These results were interpreted to mean that the pathway by which FGF22 induces synaptic vesicle accumulation only involves IGF2 during a certain time period during synapse formation.

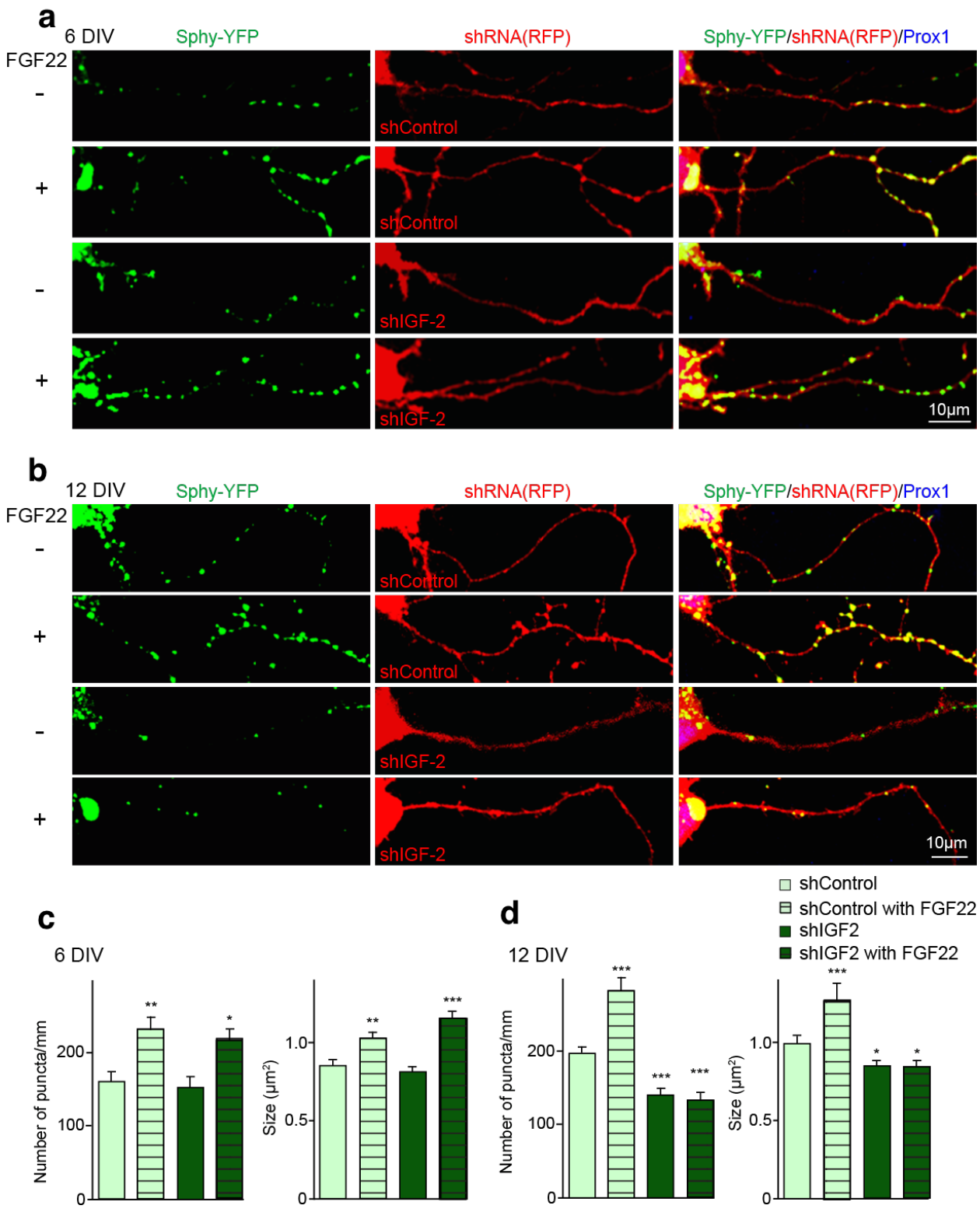

a
6 DIV
Sphy-YFP
shRNA(RFP)
Sphy-YFP/shRNA(RFP)/Prox1
FGF22
-
+
-
+
shControl
shControl
shIGF-2
shIGF-2
10μm
b
12 DIV
Sphy-YFP
shRNA(RFP)
Sphy-YFP/shRNA(RFP)/Prox1
FGF22
-
+
-
+
shControl
shControl
shIGF-2
shIGF-2
10μm
c
6 DIV
Number of puncta/mm
Size (μm²)
d
12 DIV
Number of puncta/mm
Size (μm²)
shControl
shControl with FGF22
shIGF2
shIGF2 with FGF22

Figure 6| FGF22 acts as a mediator of FGF22-induced excitatory presynaptic differentiation only during a later phase of synaptogenesis. Cultured neurons from the DG/CA3 region of the hippocampus transfected with plasmids expressing synaptophysin-YFP and either control shRNA of IGF2-shRNA. **a,** At DIV6, syt-YFP (green) clustering in DGCs (labeled with a prox1 antibody, red) was not significantly effected by knocking down IGF2. FGF22 treatment increased the density and size of syt-YFP puncta to the same extent in control and shIGF2-treated cultures. The data from which this conclusion was drawn is depicted graphically in **c. b,** At DIV12, prox1 positive cells demonstrated a substantial decrease in size and number of syt-YFP puncta when IGF2 expression was knocked down. This impairment was not rescued by FGF22 bath application. Quantification of the density and size of syt-YFP puncta are shown in **d**. Error bars are s.e.m. Data are from 3-9 cells from 4-8 independent experiments. Significant difference from control: *P<0.05, **P<0.01, ***P<0.001 by ANOVA followed by Tukey test.

In addition, to demonstrate that the observed results could not be attributed to any negative effects of the shIGF2 constructs on overall wellbeing of DGCs, we analyzed cell morphology (Fig. 7). After comparing the cells transfected with the control shRNA and the IGF2-shRNA, it was determined that the morphology of transfected cells was the same in both cases. Therefore, the shIGF2 plasmid did not seem to be decreasing the observed number and size of syt-YFP puncta by negatively impacting cell health.

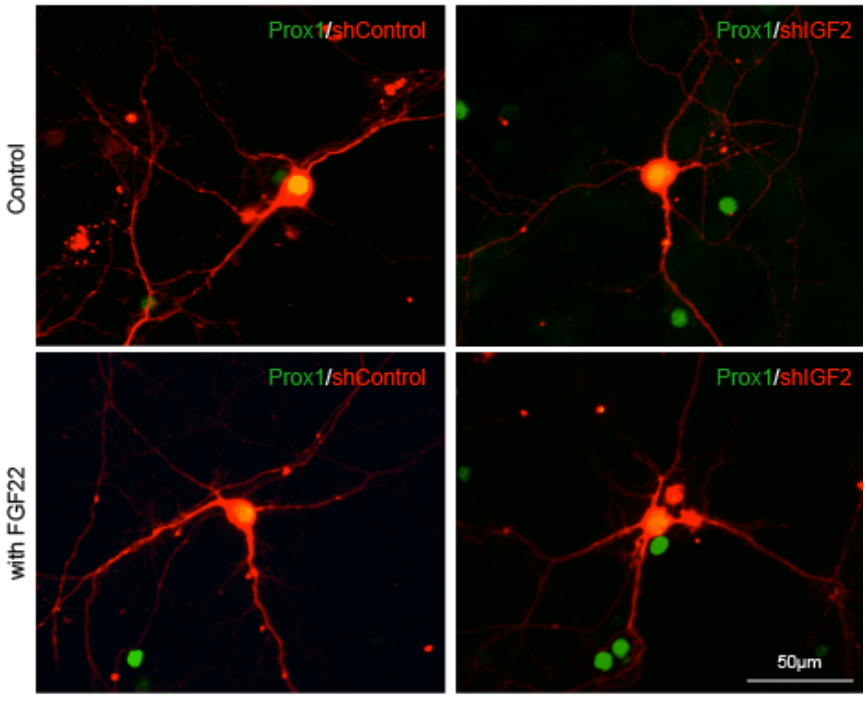


Figure 7| Knocking down shIGF2 by shRNA-mediated gene silencing had no effect on DGC morphology. Neurons obtained from the DG/CA3 region of the hippocampus of P0 mice were cultured and then transfected at DIV3. Cultured were fixed and stained at DIV10 for morphological observation. Cells transfected with control shRNA (left) and cells transfected with IGF2-shRNA (right) displayed identical morphology. DGCs were labeled with a prox1 antibody (green) and transfected cells are shown in red.

FGF22 application to axons of DGCs using microfluidic culture system

To further demonstrate the validity of the proposed model, which says that FGF22 is secreted from the CA3 pyramidal neurons and initiates a signaling cascade in the pre-synaptic DGCs by binding to its receptor on their axons that leads to the downstream activation of IGF2 expression and induction of synaptic vesicle accumulation, I searched for a way to isolate neuronal axons. I decided to use a microfluidic culture system, for which I devised a protocol that allowed me to apply FGF22 directly and exclusively to the cell axons in DG/CA3 cultures. Microfluidic culture devices have two chambers separated by a bridge with very narrow grooves that only allow for the extension of axons from cells plated in the 'somal chamber' into the 'axonal chamber'. Neurons were dissected from P0 mice, treated with FGF22 at DIV2, and fixed at DIV9. If mechanism proposed by the model is correct, then FGF22 treatment to the axons should increase IGF2 expression in prox1 positive cells. This experiment has not yet been completed, but my initial observations (Fig. 8) have shown that it is likely that FGF22 is causing increased IGF2 expression in DGCs. The completion of these experiments will provide the strongest evidence that FGF22 acts specifically at DGC axons to upregulate IGF2, if that is found to be the case.

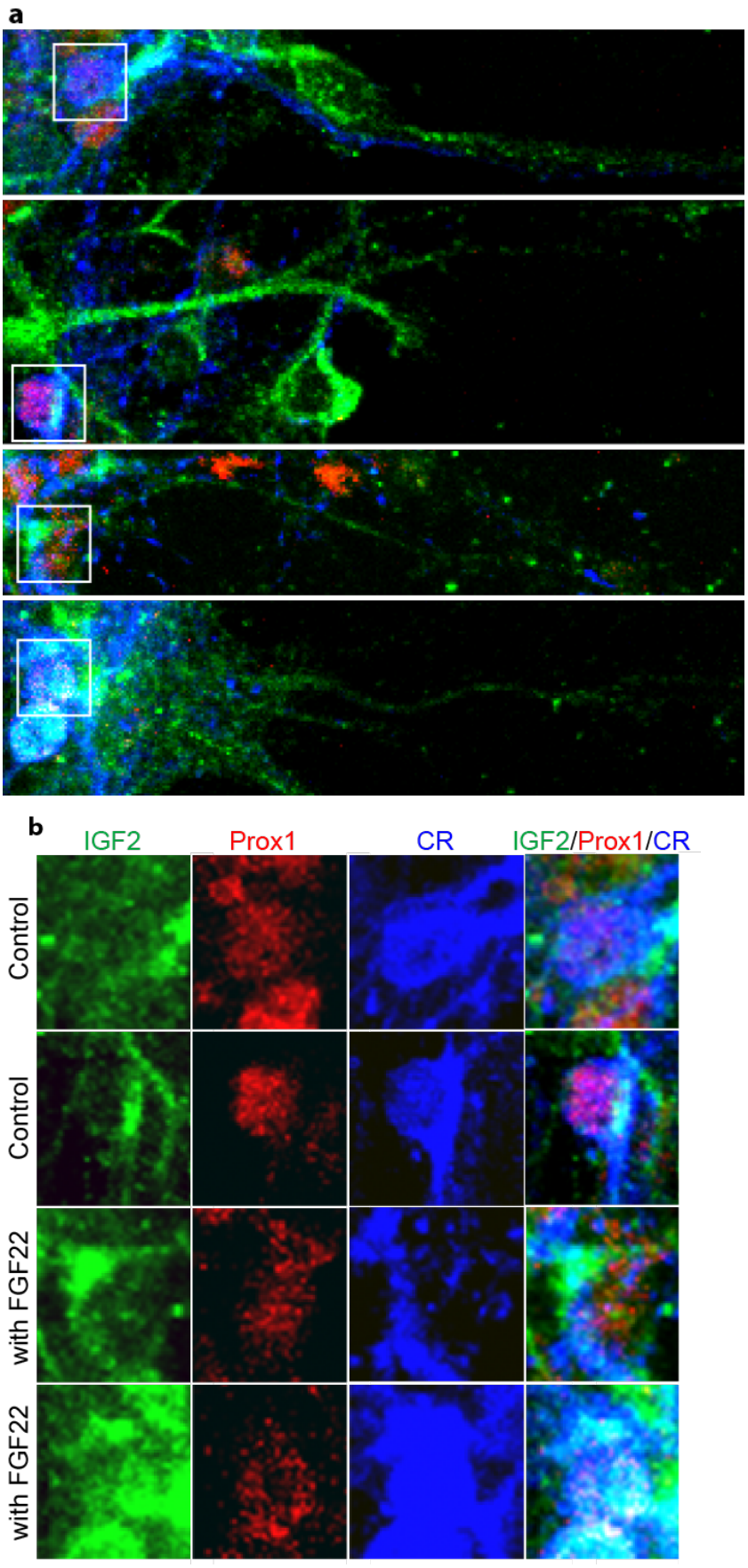


Figure 8| Bath application of FGF22 to the axonal chamber of microfluidic cultured neurons may be increasing IGF2 expression in DGCs. Two cells, were chosen out of the control and FGF22-treated groups to represent the results that have been obtained so far, one having about the highest observed IGF2 intensity and the other about the lowest intensity for each group. Cell cultures were stained with the following primary antibodies: IGF2 (green), Prox1 (red), Calretinin (blue), along with corresponding fluorescent secondary antibodies. **a**, Prox1 and Calretinin positive cells that seemed to be extending their axons through the bridge to the axonal chamber (as determined by Calretinin staining, where FGF22 was applied. **b,** higher magnification images of the white boxed in areas shown in **a**. As can be seen from these images, Calretinin positive DGCs to which FGF22 was applied appear to have a greater IGF2 intensity in the cell bodies. However, more data must be collected and analysis needs to be

The IGF2 receptor displays a punctated expression pattern in the axons of dentate granule cells and localizes to presynaptic terminals

Determining which cells express the IGF2 receptor (IGF2R) is also important because it will indicate where IGF2, once expressed and secreted from the presynaptic cells, has it its effects. To address this question, I dissected neurons from the DG/CA3 of the hippocampus and allowed cultures to grow until DIV12 at which time they were fixed and stained. To start, I stained for both Neurofiliment (NF), an intermediate filament protein found in cell axons, and Map2, a microtubule associated protein that stabilizes dendritic shape during neuron development, along with the IGF2 receptor. As can be seen in Fig. 9a, in prox1 positive cells the receptor expressed a punctated pattern in axons and a diffused pattern in the dendrites. This expression pattern correlates with the expression pattern of IGF2 itself, which is also punctated in axons and diffused in dendrites. This could indicate that IGF2 is acting in an autocrine manner, in that it is secreted from the DG presynaptic axon terminals and then subsequently binds to its receptor, also located along the axons, thereby triggering a pathway that activates synaptic vesicle accumulation.

In addition, I looked for potential colocalization of the IGF2 receptor with VGLUT1 (Fig. 9b, top three panels), VGAT (Fig. 9b, bottom three panels), PSD95 (Fig. 9c, top three panels), and Gephyrin (Fig. 9c, bottom three panels), using the immunocytochemistry as described in the methods section. The results of these experiments demonstrate that it is likely that the IGF2R is colocalized with VGLUT1 and definitely not colocalized with VGAT. It was more difficult to determine the extent to which IGF2R puncta colocalize with PSD95 and Gephyrin puncta due to the quality of the IGF2R antibody and its possible incompatibility with the fixation methods

used. It seemed possible that IGF2R puncta could be colocalized with PSD95 puncta to some extent, but did not appear to colocalize at all with Gephyrin.

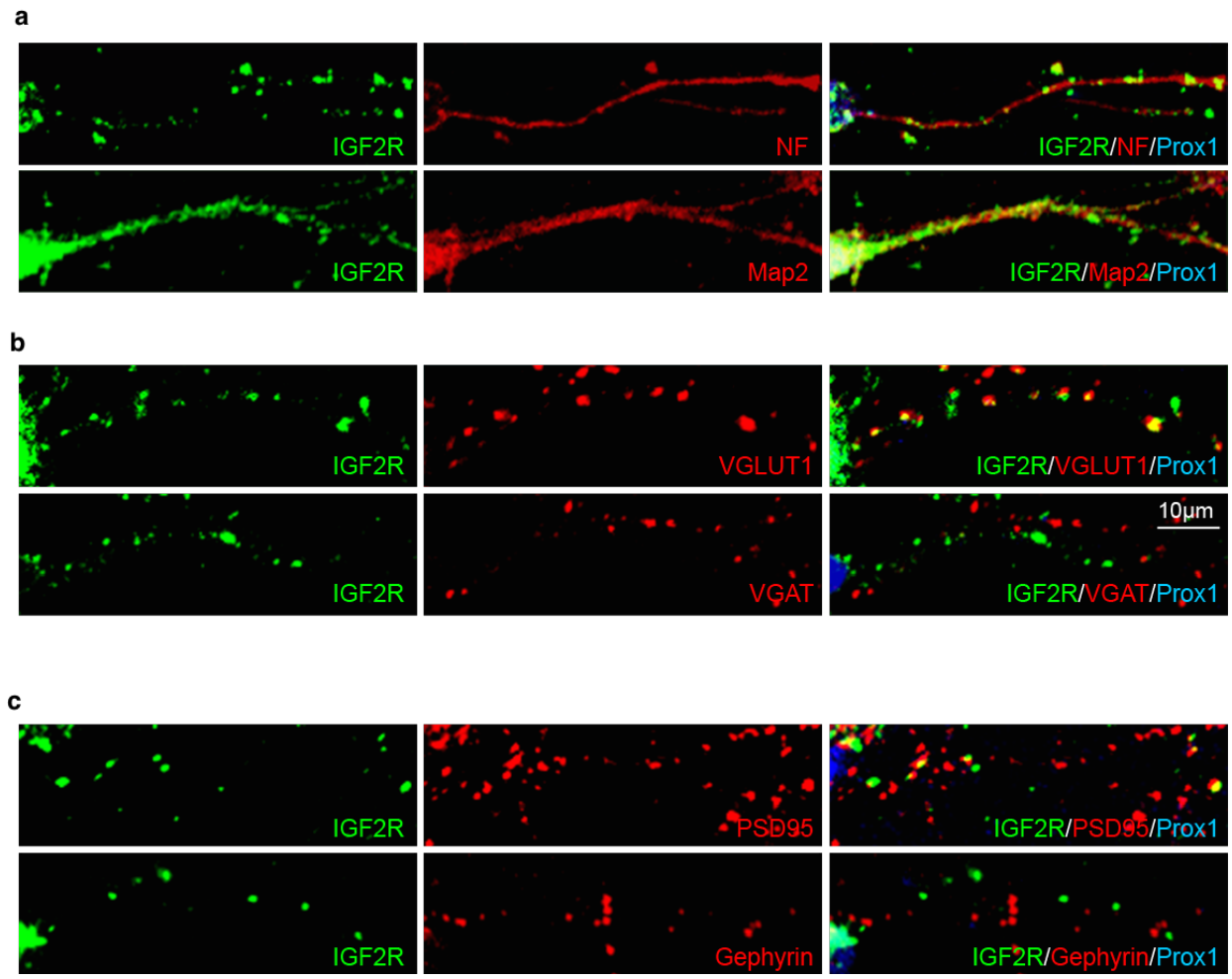


Figure 9| The IGF2 receptor localizes to the presynaptic terminals of DGCs and displays a punctated expression pattern in the axons. Neurons from the hippocampus of P0 mice were cultured and later fixed and stained at DIV12. The localization of the IGF2 receptor (green) was observed in prox1 positive neurons (blue). The extent to which the IGF2R colocalized with VGLUT, VGAT, PSD95, and Gephyrin (all shown in red) was also noted. **a, The** IGF2R expression pattern was punctated in the DGC axons (immunopositive for NF, in red) but diffused in the dendrites (immunopositive for Map2, also in red). **b,** The majority of IGF2R puncta appeared to colocalize with VGLUT1 puncta, but not with VGAT puncta. **c,** Gephyrin (bottom panel) also did not seem to be notably colocalized with IGF2R puncta. However, in the case of PSD95, IGF2R puncta did seem to colocalize, although to a lesser extent than they did with VGLUT1 puncta.

Discussion

By conducting the experiments in this study, I have been able to verify the role of IGF2 in glutamatergic presynaptic differentiation. More directly, I have shown that IGF2 is upregulated by FGF22 during a specific stage of synaptogenesis to act as a mediator in synaptic vesicle accumulation at DGC nerve terminals. By knocking down and overexpressing FGF22 it has been shown that IGF2 expression correspondingly is decreased and increased, but only at a later stage of synaptogenesis. This regulation of IGF2 by FGF22 was not observed early on during synaptic differentiation. Consistent with my proposed hypothesis, IGF2 was shown to localize to DGCs where it appeared to aggregate at presynaptic terminals along the axons. The role of IGF2 specifically in synaptic vesicle accumulation at excitatory presynaptic terminals was elucidated by observing its effect on VGLUT1 clustering and later synaptophysin-YFP clustering. IGF2 appeared to have no effect on synaptic vesicle accumulation in non-prox1 positive cells. The order of the roles of FGF22 and IGF2 in this pathway was clarified by observing that FGF22 was not able to rescue defects in synaptic differentiation after knocking down IGF2, but IGF2 was able to rescue defects in cells lacking FGF22 expression. These results imply that FGF22 regulated IGF2 expression, but IGF2 does not regulated FGF22 expression. Furthermore, the observed colocalization of the IGF2R with VGLUT1 and to a lesser extent PSD95 provided information regarding the cells on which IGF2 acts after it is expressed and secreted from the DGCs. Colocalization with VGLUT1 suggests that IGF2 acts in an autocrine manner, binding to its receptor on the presynaptic cells and thereby exerting its effect on DGCs. On the other hand, colocalization of the IGF2R with PSD95 implies paracrine mode of action for IGF2, in that it binds it receptor on the postsynaptic cells and initiates some molecular cascade in the CA3 pyramidal neurons. Alternatively, IGF2 could be acting on both the pre- and

postsynaptic cells. Further work is needed to discern between these possibilities. Ultimately, the results of this study are significant because they have shed light on the molecular mechanism controlling the differentiation of this type of glutamatergic synapse and how defects in this mechanism are able to cause impaired synapse formation. Now that this information is known we can investigate the role of this specific mechanism in the development of neurological disorders, such as epilepsy, and use it for the development of possible treatments.

The results of this study have illuminated the need for further research on this mechanism and the specific roles of FGF22 and IGF2. It still must be determined exactly where IGF2 acts after being secreted from the DGCs, and whether IGF2's effect is direct or whether other factors are recruited by IGF2 to induce synaptic vesicle accumulation. In addition, the expression of FGF22 and IGF2 in epileptic patients and/or in animal models of epilepsy is should be examined for any abnormalities. Moreover, treatments involving the application of IGF2 and FGF22 should be investigated for the ability to reduce the occurrence of seizures or alleviate them completely. In addition, corresponding factors involved in inhibitory synapse formation in the hippocampus should also be found.

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