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Fibroblast Growth Factor 14 Modulates the Neurogenesis of Granule Neurons in the Adult Dentate Gyrus

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Abstract

Adult neurogenesis, the production of mature neurons from progenitor cells in the adult mammalian brain, is linked to the etiology of neurodegenerative and psychiatric disorders. However, a thorough understanding of the molecular elements at the base of adult neurogenesis remains elusive. Here, we provide evidence for a previously undescribed function of fibroblast growth factor 14 (FGF14), a brain disease-associated factor that controls neuronal excitability and synaptic plasticity, in regulating adult neurogenesis in the dentate gyrus (DG). We found that FGF14 is dynamically expressed in restricted subtypes of Sex Determining Region Y-Box 2 (Sox2) positive and doublecortin (DCX) positive neural progenitors in the DG. BrdU incorporation studies and confocal imaging revealed that genetic deletion of *Fgf14* in *Fgf14*^{-/-} mice leads to a significant change in the proportion of proliferating, and immature and mature newly born adult granule cells. This results in an increase in the late immature and early mature

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Author Contributions

M.A.A. and T.K.A.: contributed to the design of the work, the acquisition, analysis, interpretation of the data and wrote the manuscript. M.A.A. and T.K.A.: performed tissue cryosectioning, immunohistochemistry, confocal images and image analysis; M.A.A.: prepared and perfused mouse tissue, performed BrdU treatment, supervised and maintained the animal colony and the animal genotyping in the laboratory. F.S. and M.N.N.: conducted and analyzed electrophysiological experiments. F.L.: all experiments were performed in her laboratory, provided funding, resources, and intellectual support, contributed to editing the manuscript, supervised data analysis, acquisition and interpretation of the manuscript.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest or affiliations, which might be perceived as affecting the objectivity of this review.

population of DCX and calretinin (CR) positive neurons. Electrophysiological extracellular field recordings showed reduced minimal threshold response and impaired paired-pulse facilitation at the perforant path to DG inputs in *Fgf14*^{-/-} compared to *Fgf14*^{+/+} mice, supporting disrupted synaptic connectivity as a correlative read-out to impaired neurogenesis. These new insights into the biology of FGF14 in neurogenesis shed light into the signaling pathways associated with disrupted functions in complex brain diseases.

Keywords

Adult neurogenesis; growth factors; FGF14; axon initial segment; ataxia

Introduction

Adult neurogenesis in the central nervous system is a dynamic multi-stage process that includes proliferation, differentiation, migration, maturation, and functional integration of newborn neurons in the adult brain circuitry (1, 2). This process has recently gained significant attention as a potential cause underlying cognitive pathophysiology in the context of neurological and psychiatric disorders such as Alzheimer's disease, Parkinson's disease, depression, anxiety, schizophrenia, and bipolar disorder (3–9). While observed in several regions of the adult brain, it is adult neurogenesis in the sub-granular zone (SGZ) of the dentate gyrus (DG) within the hippocampal formation to which critical roles for memory formation and cognitive function have been ascribed (3, 4, 10–12). The steady replenishment of DG granule neurons with a pool of newly born cells throughout adult life is required for the maintenance of hippocampal neuronal plasticity, learning and memory and ultimately complex cognitive function (3, 11, 13). Interference with adult neurogenesis processes is considered a hallmark of neurodegenerative and psychiatric disorders (7, 14–16). Yet, despite the emerging interest for adult neurogenesis in the neuroscience field, a complete understanding of the signaling pathways that control each given step of this process remains elusive. Finding new molecular targets underlying adult neurogenesis might improve our understanding of complex brain disorders and provide guidance for new treatment development or biomarker identification.

Abundant evidence indicates that secreted fibroblast growth factors (FGF), such as FGF2 through its FGF tyrosine-kinase receptor pathway, are essential for neuronal development and neurogenesis (17–20). Furthermore, because the *Fgfr1* gene promotes the proliferation of hippocampal progenitors and stem cells during development, conditional deletion of this gene causes a decrease in proliferating radial glial cells in the hippocampal DG in the late embryonic period through adulthood (19, 21). Whether other members of the same gene family have a role in this process has not yet been investigated (22).

The intracellular FGF (iFGF) group includes FGF11–14, factors that share sequence and structure homology with secreted FGFs, but are not secreted and do not act through tyrosine-kinase receptor activation. Instead, iFGFs are retained intracellularly and expressed primarily in neurons (23). Among iFGFs, FGF14 is abundantly expressed in the cerebellum, hippocampus and neocortex of the adult and developing brain (24–26). In central neurons

FGF14 serves as an accessory-regulatory protein of the voltage-gated sodium channels, as a scaffold for kinases (27–32), and possibly a pre-synaptic organizer at excitatory synapses (33). In animal models, genetic deletions and/or dominant negative mutations of *Fgf14* lead to cerebellar deficits and impaired hippocampal synaptic plasticity, neural excitability, and cognitive function (24, 34).

In humans, missense, non-sense, and frameshift mutations along with chromosome translocations and deletion of FGF14 (35–38) are genetic causes of spinocerebellar ataxia type 27 (SCA27) (39), an autosomal dominant neurodegenerative disease characterized by complex motor and cognitive components (26, 40, 41). Clinical profiling of SCA27 patients is partially recapitulated by the *Fgf14*^{-/-} mouse model (42), suggesting loss-of-FGF14 function as a disease component. Recent studies provide additional evidence of FGF14 as a disease relevant gene: several genome-wide association studies (GWASs) reported SNPs in FGF14 as a putative risk factor for schizophrenia, bipolar disorder, and depression, increasing interest in this molecule for a wider range of brain disorders (26, 28, 29, 41–46). Yet, a comprehensive model of FGF14 mechanisms of action in the brain is far from complete.

Based on structural and sequence homology of iFGFs, with their cognate canonical FGF (29, 47–49) abundantly implicated in adult neurogenesis (50), and the phenotypic array of deficits observed in *Fgf14*^{-/-} mice, we hypothesized a role of FGF14 in adult neurogenesis. Through a combination of confocal imaging, BrdU labeling and electrophysiology, we identify a previously unreported role of FGF14 in neurogenesis. Our results show that genetic deletion of *Fgf14* disrupts neurogenesis, reducing the number of proliferating cells and impairing the transition of newly born adult neurons from the late immature to the mature, with implications for DG synaptic connectivity and short-term plasticity. These results shed light into the biology of complex brain disorders associated with FGF14 in which disrupted neurogenesis is a known component of the disease.

Material and Methods

Animals

Fgf14^{-/-} and *Fgf14*^{+/-} male and female mice are maintained on an inbred C57/BL6J background with greater than ten generations of backcrossing to C57/BL6J. Animals were bred in the UTMB animal care facility: either heterozygous *Fgf14*^{+/-} males and females or, in few cases, homozygotes *Fgf14*^{-/-} males with *Fgf14*^{+/-} females; *Fgf14*^{+/-} wild-type mice served as control. Both male and female mice were used in this study at 4–6 months of age, unless otherwise stated. The University of Texas Medical Branch operates in compliance with the United States Department of Agriculture Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and IACUC approved protocols. Mice were housed, n = 5 per cage, kept under a 12-h light/12-h dark cycle with sterile food and water ad libitum. All genotypes described were confirmed by genotyping of the progeny using DNA extraction and PCR amplification following established protocols (26) or conducted at Transnetyx Inc. (Cordova, TN). Both male and female animals were used in this study.

BrdU injections

For the analysis of neural stem cell proliferation and survival, animals received a daily injection of 50 mg/kg thymidine analog bromodeoxyuridine (BrdU) intraperitoneally freshly prepared (Sigma-Aldrich, St. Louis, MO) at a concentration of 10 mg/ml 0.9% NaCl solution for 4 or 5 days; animals were examined 5, 15, and 30 days (see Fig 3I; 6B,C,G for a schematic diagram of injections).

Paraformaldehyde fixation

Age- and gender- matched *Fgf14^{+/+}* and *Fgf14^{-/-}* mice were first deeply anesthetized with 2,2,2-tribromoethanol (250 mg/kg i.p.; Sigma-Aldrich, Saint Louis, MO) and then perfused intracardially with 1X phosphate buffer (PBS), followed by 1% or 4% paraformaldehyde (MasterTech Scientific, Lodi, CA; Sigma-Aldrich) solution freshly prepared. To ensure complete tissue fixation, the brains were removed carefully and transferred into either 1% paraformaldehyde for 30min to 1hr, or 4% paraformaldehyde 24h-48hr at 4°C and then cryopreserved in 20–30% sucrose/PBS in preparation for sectioning.

Volumetric and morphometric analyses

Cresyl violet (Nissl) staining was performed to conduct DG volumetric analysis in age- and gender- matched *Fgf14^{+/+}* and *Fgf14^{-/-}* mice. Two sagittal brain sections (25 µm) were submerged in cresyl violet (Sigma-Aldrich) for 30 min before dehydration. Images were acquired using a Zeiss SteREO Discovery.V20 microscope along with AxioCam MRc5 and AxioVision Imaging System 4.8 software. Data were analyzed with ImageJ US NIH (<http://imagej.nih.gov/ij>).

Immunofluorescence of brain sections

The immunohistochemistry protocol used for this study was slightly modified from previous reports (32). Briefly, brains were sectioned sagittally into 20–25 µm slices using a Leica CM1850 cryostat (Leica Microsystems, Buffalo Grove, IL) and slices stored in a cryoprotectant solution at –20°C. Free floating or glass slide-mounted sections were washed with 1X PBS and 1X TBS, respectively, then pre-incubated with a permeabilizing agent (1% Triton X-100, 0.5% Tween 20 in 1X PBS or acetone 7–10min). For BrdU labeling, sections were treated with 1N HCL for 10 min followed by 2N HCl for 10 min at room temperature then 20 min at 37°C. Then, slices were incubated with borate for pH correction: 0.1 M borate buffer pH 8.5 for 10 min at room temperature. Free floating or glass slide-mounted sections were again washed with 1X PBS and 1X TBS, respectively, incubated for 7min for permeabilization, then washed 5 times with 1X PBS. Sections were then incubated with a blocking buffer (10% normal goat serum NGS (Sigma-Aldrich) or 3–5% donkey serum (Santa Cruz Biotechnology, Dallas, TX) in 1X TBS containing 0.3% Triton X-100 for 1 hr. Finally, sections were incubated overnight at 4°C on an orbital rotator with primary antibodies in 3% bovine serum albumins BSA (Sigma-Aldrich) in 1X PBS containing 0.1% Tween 20. Primary antibodies used in this study were: mouse antibody against FGF14 (1:300, NeuroMabs, catalog number 75–096); mouse antibody against ankyrin-G (1:1000, NeuroMabs, catalog number 75–146); rat antibody against BrdU (1:1000, Abcam, catalog number ab6326); mouse antibody against Nestin (1:300, Millipore, catalog number

MAB353); rabbit antibody against Sox2 (1:1200, Millipore, catalog number AB5603); goat antibody against DCX (1:400, Santa Cruz Biotechnology, catalog number sc-8066); mouse antibody against NeuN (1:750, Millipore, catalog number MAB377); guinea pig antibody against NeuN (1:250, Synaptic System, catalog number 266 004); mouse antibody against calretinin (1:3000, Swant, catalog number 6B3); rabbit antibody against calretinin (1:100, Santa Cruz Biotechnology, catalog number sc-50453) rabbit antibody against calbindin (1:10000, Swant, catalog number CB38); rabbit antibody against active Caspase 3, (cleaved, 1:1000, Millipore, catalog number AB3623). Following the overnight primary antibody incubation, sections were washed five times with 1X PBS or TBS buffer solution, incubated with the appropriate Alexa secondary antibodies at a 1:250 dilution in 3% BSA/PBST, then washed five more times with buffer solution. Prior to mounting on Superfrost® glass microscope slides (Fisher Scientific, Waltham, MA) with ProLong® Gold anti-fade or ProLong® Gold anti-fade mountant with Dapi reagents (Life Technologies, Carlsbad, CA), slices were rinsed with water and counter stained using the nuclear marker Topro-3 (1–3000, Life Technologies).

Confocal microscopy

Confocal Images were acquired using the Zeiss LSM-510 META confocal microscope with a Fluar (5×/0.25) objective, a Plan-Apochromat (20×/0.75na) objective, a C-Apochromat (40×/1.2 W Corr) objective, and Plan-Apochromat (63×/1.46 Oil) objective, with consistent gain and offset settings, as well as a number of confocal image Z-stacks across experimental sets. Multitrack acquisition was performed with excitation lines at 488 nm for Alexa 488, 543 nm for Alexa 568, and 633 nm for A647. Z-series stack confocal images were taken at fixed intervals: 1 µm for 20×, 0.6 µm for 40×, and 0.4 µm for 63× with the same pinhole setting for all three channels; frame size was either 1024 × 1024 or 512 × 512 pixels.

Data acquisition and image analysis

All confocal images were analyzed using ImageJ US NIH (<http://imagej.nih.gov/ij>). For FGF14 sub-cellular localization analysis, a single confocal image slice from each Z-stack was chosen for pixel intensity quantification based on the highest fluorescence intensity for given analytes (i.e., DCX + Sox2; DCX + NeuN). For soma expression level, a region of interest (ROI) corresponding to a line of 3 pixels in width and variable length was highlighted across the cell soma on the overlay image of FGF14, Sox2, and DCX or FGF14, DCX and NeuN staining. For Sox2 soma size and fluorescence intensity analysis, Z-stacks of confocal images were sum-projected and an ROI corresponding to soma was highlighted using an intensity threshold method and quantified. Type I and type II Sox2⁺/nestin⁺ cells were sorted with the integrated Cell Counter plugin for ImageJ ([cell_counter.jar](#)) as described in (51); type I cells correspond to nestin⁺ and Sox2⁺ radial glia-like processes, while type II correspond to non-radial labelled nestin⁺ and Sox2⁺ cells. DCX⁺ cells were identified and counted from Z-stacks of confocal images of DCX immunostaining using the Cell counter plugin and confirmed by an automated fluorescence intensity threshold-based cell counter macros developed in house. Soma size and fluorescence intensity of DCX were obtained from a selection of the three DCX⁺ cells with the largest soma as described in (52). Analysis of migration of DCX⁺ cells was conducted from Z-stacks of confocal images of DCX⁺ cells stained with the nuclear marker Topro-3; layers within the DG (SGZ, GCL and

ML) were defined as described in (52). All other cell counting, soma size and fluorescence intensity analyses conducted in calretinin, calbindin, BrdU, and caspase3 positive cells were conducted as described above for DCX.

Electrophysiological recordings

Age-matched mice (2–3 MO, N=4 per group, n=18–21 independent experiments) were anesthetized with 2,2,2-tribromoethanol (250 mg/kg i.p.; Sigma-Aldrich, Saint Louis, MO) and intracardially perfused with an ice-cold sucrose-based artificial CSF solution containing (in mM 56 NaCl, 100 sucrose, 2.5 KCl, 20 glucose, 5 MgCl₂, 1 CaCl₂, 30 NaHCO₃, 1.25 NaH₂PO₄, and 1 kynurenic acid). Following decapitation, horizontal hippocampal slices (300 µm) were cut with a vibratome VT1200S (Leica, Buffalo Grove, IL) in the sucrose-based artificial CSF solution and transferred to a recovery chamber with 95% O₂ and 5% CO₂ bubbled regular artificial CSF (containing in mM, 125 NaCl, 2.5 KCl, 2 MgCl₂, 2.5 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 20 glucose) at room temperature. After at least 90 min of recovery, recordings were performed in regular artificial CSF in a submerged chamber with addition of GABAergic blocker – bicuculine (10 µM), at 31–32 °C. Recordings of field excitatory post-synaptic potentials (fEPSPs) were performed in the granular cell layer of the hippocampal DG with a tungsten electrode placed in an upper blade of dentate gyrus and connected to an A-M Model 1800 Differential AC Amplifier (A-M Systems, Carlsborg, WA). Perforant path fibers were stimulated by a bipolar tungsten electrode, with 0.1-ms pulses of constant current. The traces were digitized by a Digidata 1200 interface using Clampex 7, and the slopes of the fEPSPs were measured offline with Clampfit 9.0 (PCLamp software; Molecular Devices, Union City, CA).

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) and the statistical significance of observed differences among groups was determined by two-sample Student's t test or the corresponding nonparametric test, Mann-Whitney rank sum test, based on the distribution of the samples underlying the populations. A $P < 0.05$ was regarded as statistically significant. Statistical analysis was performed with SigmaPlot12 and tabulated with Microsoft Excel.

Results

Expression profile of FGF14 during adult hippocampal neural stem cell development

To begin exploring the role of FGF14 in adult hippocampal neurogenesis, we examined FGF14 expression pattern in the DG along with neurogenesis markers. In adult mice, FGF14 immunoreactivity exhibits a specific expression pattern at the axonal initial segment (AIS) of mature neurons (identified by NeuN and calbindin expression) within the granule cell layer where it co-localizes with the AIS resident protein, ankyrin-G (Fig 1A–C, G upper set and H; note the absence of FGF14 staining in *Fgf14*^{-/-} mice Fig 1D–F, G lower set). We then analyzed FGF14 expression in combination with standard markers of early to late stage neurogenesis. As illustrated in Fig 1I–L and S1A–P Fig, FGF14 is differentially expressed in early Sox2⁺ neural stem cells and transiently amplifying progenitors, DCX⁺ migratory immature neurons, NeuN⁺ immature and mature neurons, and calbindin⁺ mature neurons. At

early stages of neural stem cell development, FGF14 immunoreactivity is primarily localized in the soma of sparse Sox2⁺ cells corresponding to type I stem cell and type IIa early progenitor cell lineage (Fig 1I arrow and arrowhead, S1A–E,F Fig blue box). Its expression in these neuronal populations increases as these cells proliferate and migrate. Notably, the expression level and pattern of FGF14 appear to reach a peak and a specialized sub-cellular localization that can be segregated based on DCX⁺ subtypes. At early migratory stages, in DCX⁺/Sox2⁺ cells, FGF14 immunoreactivity was found high in both the nucleus and cytoplasm (Fig 1I yellow line, S1A–E,G Fig red box), a pattern that is lost in Sox2[−]/DCX⁺/NeuN[−] cells (Fig 1K arrowhead, S1A–E,H Fig green box, and S1I–M,N Fig green box). Interestingly, in DCX⁺ progenitors, FGF14 exhibits a distinguished perisomatic cone-like accumulation, visualized by cytoplasmic immunoreactivity (Fig 1I,K yellow line, S1 Fig E Sox2⁺/DCX⁺, M DCX⁺/NeuN⁺). Pixel intensity quantifications indicate that as Sox2 cells become DCX positive, FGF14 pixel intensity increases (Fig 1J, S1G Fig). The same expression pattern of FGF14 has been observed when DCX cells become NeuN positive (Fig 1L, S1O Fig). This correlation was not detected in most Sox2⁺/DCX[−] cells (S1F Fig), Sox2[−]/DCX⁺ (S1H Fig), DCX⁺/NeuN[−] cells (S1N Fig) or DCX[−]/NeuN⁺, where FGF14 expression instead was more pronounced in the AIS (S1P Fig). In calbindin⁺ mature neurons, FGF14 soma expression decreases and becomes almost restricted to the AIS (Fig 1G–H). Thus, FGF14 expression is dynamically regulated, switching from a somatic to axonal pattern in late immature to mature neural progenitor transition (Fig 2).

The effect of FGF14 genetic deletion on the neural stem cell population

Given the FGF14 specialized expression pattern in distinct newly born neurons sub-types, we posited that deletion of the FGF14 gene might have an impact on neurogenesis. Through multichannel confocal imaging in *Fgf14*^{−/−} and *Fgf14*^{+/+} mice, we first analyzed the population of Sox2⁺ cells and found that neither the total number of Sox2⁺ cells ($P=1$, Student's *t* test; Fig 3A,C) or their basic morphological characteristics (i.e., soma size) ($P=0.8$, Mann-Whitney test; Fig 3A,B) were affected upon deletion of *Fgf14*. Though, we did observe a slight reduction in the Sox2 protein content per cell in *Fgf14*^{−/−} compared to *Fgf14*^{+/+} mice ($95.9\% \pm 1.3$, $100\% \pm 1.5$, $P=0.04$, Mann-Whitney test; Fig 3B). Under physiological conditions, reduction of Sox2 expression correlates with the neural stem cell exiting from the cell cycle and entering a differentiation stage. Thus, deletion of *Fgf14* might cause a premature acceleration of early progenitors toward neuronal differentiation (53). We further investigated whether alterations in temporal dynamics of stem cell development might be restricted to a specific sub-type of neuronal stem cells. Neuronal progenitor cells can be morphologically classified into Type I and Type II based on the presence or absence of radial processes, respectively (51, 53) (Fig 3D,E). Quantification based on the combination of Sox2 and nestin immunoreactivity led to the conclusion that neither Type I nor Type II cells were largely affected by deletion of *Fgf14* (Fig 3D,G), albeit a small non statistically significant reduction in Sox2⁺/nestin⁺ Type II cell number was detected in *Fgf14*^{−/−} mice compared to controls ($P=0.06$, Student's *t* test; Fig 3F). Other analysis indicated no reduction in the number of Type IIb cells (54) in *Fgf14*^{−/−} mice compared to controls ($P=0.5$, Student's *t* test; S2A,B Fig). These immunolabeling experiments allowed estimation of the total number of cells within a given cell type population at a steady-state level, but did not provide information on the actual role of FGF14 in the neurogenesis

process. To directly examine whether FGF14 had any significant role in early stages of neurogenesis, we performed BrdU pulse-chase experiments labeling cells 5 days after injection, a commonly used protocol to track putative stem cells and progenitors in the proliferative and early differentiation phases (Type IIa, Type IIb and Type III cells). Our analysis revealed a significant reduction in BrdU⁺ cells in *Fgf14*^{-/-} mice compared to controls ($51.1\% \pm 11.5$, $100\% \pm 13.7$, $P < 0.02$, Student's *t* test; Fig 3H,I).

Ablation of *Fgf14* increases the number of DCX positive immature neurons

Given that no changes were found in any Sox2⁺ cells in *Fgf14*^{-/-} mice, we posited that FGF14 deletion might affect the pool of early differentiated neurons (Type III) that have just lost Sox2 and nestin marker expression and begin to differentiate. To test this hypothesis, we examined the expression level and pattern of DCX, a microtubule binding protein that regulates neuronal migration in pre- and postnatal development, as a marker of neuronal progenitors and early immature neurons (55, 56). Notably, we observed that the DCX⁺ cell number was remarkably increased in *Fgf14*^{-/-} animals compared to *Fgf14*^{+/+} ($124.5\% \pm 7.6$, $100\% \pm 7.4$, $P < 0.03$, Student's *t* test; Fig 4A–B). Furthermore, the DCX protein content per cell soma was significantly higher in *Fgf14*^{-/-} compared to *Fgf14*^{+/+} mice ($119\% \pm 4.9$, $100\% \pm 4.9$, $P < 0.006$, Mann-Whitney test; Fig 4E), despite a comparable soma size in the two genotypes ($P = 0.9$, Mann-Whitney test; Fig 4E). The distance travelled by DCX⁺ cells across GCL specific sub-layers in the two genotypes was comparable, even though in *Fgf14*^{-/-} animals DCX⁺ migratory cells tended to accumulate in the middle of the molecular layer ($P = 0.07$, Student's *t* test; Fig 4C,D).

FGF14 is required for proper maturation and integration

We then examined the effect of *Fgf14* genetic deletion on newborn neurons at a post-mitotic stage. To this end, we sorted late immature and mature granule neurons using calretinin (CR) and calbindin (CB), respectively (57) (Fig 5A,B and Fig S3A–D Fig). In *Fgf14*^{-/-} animals, CR⁺ cells were significantly more abundant than in control *Fgf14*^{+/+} mice ($161.9\% \pm 13.4$, $100\% \pm 9.2$, $P < 0.002$, Student's *t* test; Fig 5C,D) and the CR⁺ cell content per soma, and the soma size, were higher ($112.1\% \pm 3.1$, $100\% \pm 3.7$, $P < 0.02$, Mann-Whitney test; $119.1\% \pm 6.2$, $100\% \pm 5.9$, $P < 0.03$, Student's *t* test, respectively; Fig 5D). Notably, the mature CB⁺ neuron population was unchanged in *Fgf14*^{-/-} animals ($P = 0.2$, Student's *t* test), while the CB protein content was reduced in *Fgf14*^{-/-} compared to *Fgf14*^{+/+} mice ($83.8\% \pm 0.5$, $100\% \pm 0.8$, $P < 0.001$, Mann-Whitney test; Fig 5E,F).

FGF14 genetic deletion affects the early survival phase of newborn neurons

To assess the role of FGF14 in the immature and mature newly born cell population, we injected BrdU once per day for -four or five days and examined cells either fifteen or thirty days later, respectively (see scheme in Fig 6B,C). Fifteen days after injection, the total BrdU⁺ cell population was reduced dramatically in *Fgf14*^{-/-} compared to *Fgf14*^{+/+} mice ($69.7\% \pm 4.3$, $100\% \pm 7.6$, $P < 0.007$, Student's *t* test; Fig 6A,B), but was comparable in the two experimental groups after the thirty day pulse ($P = 0.9$, Student *t* test; Fig 6C–E). Notably, the majority of thirty day treated BrdU⁺ cells were DCX⁻/NeuN⁺ in *Fgf14*^{+/+} mice ($100\% \pm 11.7$, $42.8\% \pm 9.5$, Student's *t* test), but DCX⁺ in *Fgf14*^{-/-} animals ($344\% \pm 70.7$, $100\% \pm 29.1$, Student's *t* test; Fig 6D). Complementary experiments were performed to

determine whether changes in the population of immature and mature newly born neurons could be reconciled with deficits in the apoptotic signaling pathway; BrdU⁺ cells showed no significant difference in somatic active caspase-3 expression between the two genotypes ($P=0.8$, Mann-Whitney test; Fig 6F,G). These results indicate that the pool of survived newly born DG neurons in *Fgf14*^{-/-} mice might be developmentally arrested at the DCX⁺ late immature stage.

We posited that such an aberrant neurogenic process might, over the long-term, impact the DG structure and gross anatomy. Using Nissl staining (Fig 6H), we conducted a volumetric analysis of the entire DG volume. In accordance with previous studies (26), we found no gross anatomical alterations in the two experimental groups ($P=0.5$ in **I**, $P=0.9$ in **K**, Student's *t* test; Fig 6H–K). Nor did we find changes in the total population of DG cells as estimated by nuclear staining (data not shown). DG adult neurogenesis plays a critical role in the maintenance of the trisynaptic hippocampal circuitry, a central station for signal processing in the brain. Thus, even subtle changes in DG circuitry cell composition could have broader consequences for the entire brain structure. Consistent with this hypothesis, we found a slight, but significant reduction in the weight of *Fgf14*^{-/-} compared to *Fgf14*^{+/+} brains ($93.1\% \pm 1.3$, $100\% \pm 1.8$, $P<0.03$, Student's *t* test; Fig 6L,M), a feature that often accompanies brain pathologies associated with neurogenesis deficits (58).

FGF14 genetic deletion results in aberrant DG synaptic activity

We then asked whether the changes in the newly-born neuron population observed in *Fgf14*^{-/-} animals could affect the DG synaptic circuitry (59, 60). To this end, we stimulated perforant path to DG inputs and used electrophysiological field recordings to evoke field excitatory postsynaptic potentials (fEPSPs, Fig 7A). We found that the minimal threshold field response was significantly reduced in *Fgf14*^{-/-} mice compared to control (133.3 ± 9 μ A, $N=4$, $n=18$ in *Fgf14*^{-/-} mice versus 111.9 ± 4.8 μ A in control; $P<0.05$, Fig 7C). Yet, the fEPSP slope was comparable in the two experimental groups for all other given stimuli exceeding minimal stimulation. To study short-term synaptic plasticity, we applied a protocol consisting of paired-pulses with inter-pulse intervals (IPI) of variable duration, and measured the ratio of pulse 2 to pulse 1 for the fEPSP slope (Fig 7D,E). We found that the paired-pulse ratio (PPR) was reduced in the *Fgf14*^{-/-} group at 100 msec IPI compared to control values ($145.5 \pm 3.1\%$ in *Fgf14*^{-/-} mice versus $155.1 \pm 3.5\%$ in control; $P<0.05$ with Student *t*-test; Fig 7E). Notably, PPR values that exceeded 100 msec IPI (i.e. 200, 300 msec) were significantly higher in *Fgf14*^{-/-} mice compared to control (at 200 msec – $127.9 \pm 2.7\%$ in *Fgf14*^{-/-} mice versus $118.7 \pm 3.6\%$ in control; $P<0.05$ with Student *t*-test and at 300 msec – $110 \pm 2.9\%$ in *Fgf14*^{-/-} mice versus $98.7 \pm 3.3\%$ in control; $P<0.05$ with Student *t*-test; Fig 7E).

Together, these results suggest that adult neurogenesis in the DG requires FGF14 expression in newly developing neurons (Fig 7) to guarantee the transition of late immature to early mature neurons, and that disruption of FGF14 function impairs synaptic integration of neurons in the DG circuitry.

Discussion

Adult neurogenesis occurs through distinct stages including proliferation, differentiation, migration, maturation, and integration of newly born neurons. These neurons derive from neural stem cells that steadily integrate into the adult brain circuitry (1, 2). Disruption of this multi-stage process has been implicated in complex cognitive functions and emotional responses that are altered in neurodegenerative and neuropsychiatric disorders (3–5). In the present study we have uncovered a novel role for *Fgf14*, a gene harboring inherited mutations leading to SCA27 and SNPs associated with psychiatric disorders, in adult neurogenesis. Our data demonstrate that FGF14 plays a role in the maturation of adult neural stem cells that may be related to the expression/function of FGF14 during the development of progenitor cells. We found that newly born granule neurons in *Fgf14*^{-/-} mice are dominated by the late immature and early mature population of DCX⁺ and CR⁺ cells, a phenotype that associates with reduced minimal threshold response and impaired paired-pulse facilitation at the perforant path to DG inputs. These results further our understanding of the biology of complex brain disorders associated with deficits in neurogenesis, with implications for advancing their treatment.

The expression pattern level and distribution of FGF14 in the brain has been previously reported in the hippocampal region (32), yet a postnatal time-course study has been lacking. To gain knowledge on a potential role of FGF14 in neurogenesis, we examined the expression pattern of FGF14 along with selected neurogenesis markers in the adult DG. In accordance with previous studies, FGF14 immunoreactivity in mature neurons (NeuN⁺) is high at the AIS. However, at earlier stages its expression level and pattern vary. Our findings indicate that FGF14 expression is regulated dynamically, consistent with other proteins that are implicated in regulating adult neurogenesis such as Alk5, notch, and TrkB (52, 61, 62). Notably, while FGF14 exhibits a more diffused pattern with cell body-localization in Type IIb cells, it exhibits a distinct, perisomatic cone-like pattern in late progenitors. Though the significance of this expression pattern profile remains to be determined, it may indicate developmentally regulated specialized functions of FGF14 at an early post-mitotic phase in which the newly born neuron's axon is formed and dendritic arborization is built (63).

Genetic deletion of *Fgf14* did not affect the early proliferating neural stem cell pool, but caused a significant increase in the number of DCX⁺ differentiating cells. These cells also presented with a higher DCX protein content in *Fgf14*^{-/-} animals compared to controls. Early post-mitotic, newly born granule neurons are transiently positive for CR prior to their full maturation stage characterized by CB expression. *Fgf14*^{-/-} mice exhibit a significant increase in the CR positive population as well as in cell soma size and CR protein content per cell. Yet, later mature stage CB⁺ cells were unaffected by *Fgf14* genetic deletion, despite a significant reduction in the overall level of CB content per cell. In mature neurons, FGF14 is expressed at the AIS. Lack of this molecule at early stages of differentiation might disrupt cell-to-cell contacts mediated by transmembrane proteins of the AIS, locking DCX⁺ and the transiently CR⁺ cell pool in an arrested developmental stage. This aberrant pool of DCX⁺ cells in *Fgf14*^{-/-} mice predominates, even after a thirty day BrdU treatment (Fig 6D); the time by which newly born cells would be fully mature under normal conditions (64). Together, these phenotypes could signify that the FGF14 wild type gene/protein suppresses a

signaling program to exit mitosis and proceed into full maturation. Disruption of this suppressing signal might produce ineffectual neurons that are in a developmentally arrested stage (DCX⁺), and unable to integrate into the hippocampal circuitry at a proper rate. Alternatively, increases in both DCX⁺ and CR⁺ cells might serve as a compensatory response to a pool of reduced Sox2⁺ progenitors (Fig 3H,I) or NeuN⁺ mature neurons (BrdU⁺/DCX⁺) (Fig. 6D). We have detected a slight, but significant reduction in CB content per cell in *Fgf14*^{-/-} animals which may indicate (or be a causal effect) of reduced intracellular Ca²⁺ buffering capability in mature CB⁺ cells. In wild type mice, the population of proliferating BrdU⁺ cells decreases as adult neurogenesis proceeds; only ~50% of proliferating cells survive one month after birth (13). In *Fgf14*^{-/-} mice, the loss of newly born cells incorporating BrdU was evident after a five or fifteen day treatment compared to *Fgf14*^{+/+} animals. However, no differences were found after one month of BrdU treatment. The majority of BrdU⁺ surviving cells in *Fgf14*^{-/-} mice are DCX⁺, and in these animals, no changes in the early proliferating pool of cells (Sox2⁺/nestin⁺) were found (Fig 3A,C). Furthermore, we found no changes in apoptosis levels in *Fgf14*^{-/-} animals. Notably, a small but significant change in Sox2 expression was found in *Fgf14*^{-/-} mice. Decreased Sox2 expression is a biological signal that accompanies the transition from mitosis to differentiation (53). Thus, reduction in FGF14 expression might result in a persistent mitotic exit signal, accelerating the transition of Type II into Type III newly born neurons. In the long-term, loss of FGF14 function might cause structural and functional changes that lead to slow DG circuitry deterioration with influence on other brain areas. Accordingly, *Fgf14*^{-/-} mice exhibit a significant reduction in the whole brain weight that might result from loss of neurons. In agreement with impaired neurogenesis, we detected functional changes in the DG circuit of *Fgf14*^{-/-} animals. Upon stimulation of the perforant pathway, the major extra-hippocampal input to DG granule cells, we observed a reduced minimal threshold response of fEPSPs, and bidirectional changes in paired-pulse facilitation, in *Fgf14*^{-/-} compared to *Fgf14*^{+/+} animals. In addition, PPF was lower in *Fgf14*^{-/-} vs *Fgf14*^{+/+} at 100 ms, but higher for longer inter-pulse intervals. The alterations in time-dependent facilitation observed in *Fgf14*^{-/-} animals are consistent with synaptic input remodeling of the DG circuit, such as granule cell axonal sprouting, homeostatic changes driven by GABAergic inputs or modifications of the release machinery and excitatory pre-synaptic terminals (65, 66). These phenotypes suggest that altered neurogenesis might lead to an over-excitable DG circuit, more prone to an epileptic-like status, or locked in an immature stage (66–68). Future studies are required to confirm this hypothesis.

FGF14 is an integral component of the AIS, a regulator of neuronal excitability, an organizer of the presynaptic release machinery, a scaffold for signaling pathways downstream of tyrosine kinase receptors (24, 28, 30–33, 41, 46, 69–73), and a regulator of synaptic plasticity (26, 74). In *Fgf14*^{-/-} animals, some or all of these functions may be disrupted resulting in aberrant adult neurogenesis. The phenotypes reported in this study might result from cell-autonomous mechanisms that are ascribed to cell-specific and time-dependent expression of FGF14 in the population of developing neurons. For instance, lack of the FGF14 protein in early stages of neurogenesis in immature Sox⁺ cells that are electrically inactive, might be associated with disrupted signaling pathways. In regard to the signaling pathways, FGF14 may trigger downstream mechanisms common to other FGFs. Our and

previous studies implicate FGF14 in neuronal development and neurogenesis, which are well-known phenotypes controlled by canonical FGF signaling (17). Though the endpoint of regulating neurogenesis may be different in canonical versus iFGF, for members of the same family, our data support functional conservation beyond that of transmembrane signaling, which may be part of a global cell program required for organ development. Another signaling pathway classically linked to adult neurogenesis is DISC1, a multifaceted, intracellular scaffolding protein operating through a large macromolecular signaling complex (1, 14, 75–77). One of the DISC1 partners shown to be important for proper neuronal maturation is GSK3 β (14, 78). Previous studies reported FGF 14 as a new target of the GSK3 signaling pathway (32), which may be an indirect pathway by which FGF 14 affects adult neurogenesis.

In electrically active DCX⁺ and CR⁺ neurons (13), lack of the FGF14 protein might have a more prominent effect on neuronal firing, disrupting Nav channel targeting to the AIS, impairing neuronal excitability and impeding or delaying the establishment of neuronal polarity (28, 32). In addition, or as an alternative, to cell-autonomous activity, loss of *Fgf14* may act globally and adversely influence neuronal activity of the entire DG. This would result in a microenvironment that prevents proper maturation and integration of newly born cells in the synaptic circuitry (79–82).

Overall, the phenotypes presented here can be reconciled with immature DG (iDG) (13), with elevation in immature neuronal markers and reduction in mature markers (8, 83, 84). Several animal models of psychiatric disorders including α -CaMKII heterozygous mice, SNAP-25 knock-in, Schnurri-2 knockout and region-specific calcineurin knockout mice (83) exhibit iDG phenotypes associated with cognitive impairment similar to those observed in *Fgf14*^{-/-} mice in the current and previous studies (26, 74). In schizophrenic and bipolar disease patients, cellular and molecular phenotypes of iDG have been reported using immunohistochemistry and gene expression profiling, leading to the premise of iDG traits as an endophenotype of brain disorders (8, 83, 84). Thus, *Fgf14* is likely part of a network of risk-genes for mental diseases that manifest through deficits in adult neurogenesis. Future investigations on the expression levels of FGF14 in human samples will add translational validity to this *Fgf14*^{-/-} model, and feasibility of targeting FGF14 for development of more effective therapeutics against mental disorders.

Future studies will determine whether pathway convergence of these genes contributes to the broad array of cognitive and emotional impairments associated with human neurological and psychiatric disorders.

Conclusions

We have uncovered a novel role for FGF14 in the transition of neuronal progenitors from a late immature to a mature stage in the adult hippocampus. These results contribute to a deeper understanding of the cellular pathways required for neurogenesis and provide new target genes for therapeutic interventions in rare disorders (i.e. SCA27) and complex diseases associated with SNPs in the FGF14 gene, such as schizophrenia, bipolar disease, and depression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Physiological level of FGF14 during postnatal hippocampal neurogenesis

Immunofluorescent staining of a sagittal section of mouse brain shows FGF14 immunoreactivity in the dentate gyrus (DG). **A–F**, Representative confocal Z-stack images of the DG from *Fgf14*^{+/+} and *Fgf14*^{-/-} mice immunostained for FGF14 (gray) in combination with ankyrin-G (blue), and NeuN (green). Quantification of pixel intensity of FGF14 and ankyrin-G fluorescent signals across axon initial segment (AIS) of the DG granular cells, shows an enrichment of FGF14 protein in *Fgf14*^{+/+} **C**, but not *Fgf14*^{-/-} **F**, mice, (n=2 mice per group, 3–4 sections per mouse). **G,H**, Expression of FGF14 at the AIS

of mature neurons (calbindin⁺ cells) in DG from *Fgf14*^{+/+} and *Fgf14*^{-/-} mice. FGF14 expression in different cell types detected in **I,K** from DG of *Fgf14*^{+/+} mice. **I**, shows FGF14 signal in Sox2⁺ (blue) DCX⁺ (green) cells (yellow line) and Sox2⁺ DCX⁻ (arrow), and absent from some population of Sox2⁺ DCX⁻ cells (arrowhead). **J**, pixel intensity of FGF14, Sox2 and DCX across Sox2⁺ DCX⁺ cells. **K**, FGF14 expression in DCX⁺ NeuN⁺ cells (yellow line) and weak expression in DCX⁺ NeuN⁻ cells (arrowhead). **L**, pixel intensity of FGF14, DCX and NeuN across DCX⁺ NeuN⁺ cells (n=2 mice, 3 sections per mouse for **I** and **K**). Abbreviations: DG= dentate gyrus; GCL= granule cell layer; SGZ= subgranular zone. Scale bars represent 40 μ m in D; 20 μ m in E, and G; 10 μ m in H, and K.

Fig 2. Schematic representation of FGF14 expression and function in developing adult neural stem cells in the DG

Neural stem cells, including the slowly dividing radial glial Type I cells and more rapidly amplifying Type II progenitor cells, proliferate in the sub-granular zone (SGZ), migrate, and differentiate over a few weeks into fully mature neurons in the granular cell layer. Expression and localization of FGF14 protein (yellow circles) based on FGF14 immunoreactivity in the DG of tissue sections. FGF14 shows transient and highly dynamic expression in the differentiation and maturation populations. Precise experimental quantification of the protein level and subcellular localization need further investigation. Abbreviations: GCL= granule cell layer; ML= molecular layer.

Fig 3. Effect of *Fgf14* ablation on the early-stage of adult neural stem cell development

A,D Representative immunostaining of early-stage neural stem cells from sagittal sections of DG from *Fgf14*^{+/+} and *Fgf14*^{-/-} mice (4–5 months-old). Cells are identified based on anti-nestin (red) and anti-Sox2 (green). Topro-3 indicates nuclear staining (blue). **B**, Quantification of Sox2⁺ cells soma size and protein intensity in SGZ in *Fgf14*^{+/+} and *Fgf14*^{-/-} mice. **C**, Total Sox2⁺ cells number in the SGZ (n=3 mice, 3–4 sections per mouse). **E**, top, a schematic diagram represents the two types of cells at an early stage of adult neurogenesis in DG; bottom, maximum intensity projection of confocal Z-stack image at

high resolution reveals nestin-positive type-I and type-IIab neural stem cells based on neurite extension. **F,G**, Quantification of type-I (radial glia-like processes) and type IIab (non-radial) nestin⁺/Sox2⁺ cells in *Fgf14*^{+/+} and *Fgf14*^{-/-} mice (n=3 mice, 3–4 sections per mouse, means across genotypes are non-statistically different). **H**, Confocal images of BrdU, Sox2, DCX labeled cells in the dentate gyrus from 6 month-old female *Fgf14*^{+/+} and *Fgf14*^{-/-} mice. **I**, Top, experimental design; bottom part, quantification of BrdU⁺ cells in DG after 4 BrdU (50mg/kg) i.p. injections (n=2 mice per group, 5 sections per mouse). Data are mean ± SEM, *P < 0.05, Student's *t* test. Scale bars represent 50 μm in A, D, and H; 10 μm in E.

Fig 4. Impact of *Fgf14* deletion on immature doublecortin positive neurons in the DG

A, Representative confocal images of immature cells from sagittal sections of adult DG *Fgf14*^{+/+} and *Fgf14*^{-/-} mice. Cells are identified based on anti-doublecortin (DCX, in green) immunoreactivity. **B**, Quantification of DCX labeled newborn neurons in the DG across genotypes (*P < 0.05, Student's t test). **C**, Higher magnification views of DCX⁺ cells in combination with the nuclear label topopro-3 are used to examine soma size, DCX protein intensity and cell migration pattern within the sub-granular zone (SGZ), the inner, middle, and outer zones of the granule cell layer (GCL), and molecular layer (ML). **D**,

Quantification of spatial distribution of DCX+ cells across layers (normalized means across genotypes are non-statistically different). **E**, Quantification of soma size and protein intensity of DCX labeled cells from *Fgf14^{+/-}* and *Fgf14^{-/-}* mice (** $P < 0.01$, Student's t test). Data are from six mice (4–5 months-old) per group and 3 sections per mouse. Data are presented as mean $\pm$ SEM. Scale bars represent 100 μm in A; 40 μm in C.

Fig 5. *Fgf14* is required for late-stage immature to mature transition of adult neuronal stem cells
Immunohistochemical detection and quantification of calretinin (immature neurons) and calbindin (mature neurons) in the DG from *Fgf14*^{+/+} and *Fgf14*^{-/-} mice. **A**, Representative confocal images of triple staining of the entire hippocampus representing calbindin (CB, green), calretinin (CR, red) and topopro-3 nuclear staining (blue) at low magnification of the DG. **B**, Confocal Z-stack images of upper DG blade, view stained with either an anti-CR (**C**) or an anti-CB antibody (**E**). **D,F**, Quantification of cell soma size, fluorescence intensity and cell count for CR⁺ cells (*P 0.05, **P 0.01, Student's *t* test) and CB⁺ cells (**F**, ***P<0.001, Mann-Whitney test) across genotypes. Data are from three mice (4–5 months-

old) per group, 4 sections per mouse and are presented as mean $\pm$ SEM. Scale bars represent 200 μ m in A; 50 μ m in B and E.

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Fig 6. Cell survival and apoptosis level of one month old neurons in the adult DG

A, Representative immunostaining images of the BrdU⁺ cells (red) and the DCX⁺ (green), NeuN⁺ (blue) cells from 1–3 month-old male *Fgf14*^{+/+} and *Fgf14*^{-/-} mice injected i.p. with 4 doses of BrdU (50mg/kg). **B**, Top, schematic representation of 2 week BrdU study design; bottom, quantitative analysis of BrdU⁺ cells in the DG 15 days later (n=3 mice per group, 6–7 sections per mouse, **P < 0.01, Student's *t* test). **C**, Top, schematic representation of one month BrdU study design; bottom, triple staining of DCX (green), BrdU (red) and NeuN (blue) in the DG of 6–7 month-old male *Fgf14*^{+/+} and *Fgf14*^{-/-} mice. **D,E**, The level of

neurogenesis in the DG is assessed by quantification of total BrdU⁺, BrdU⁺/DCX⁺ and BrdU⁺/DCX⁻ cell number in the DG of both genotypes (n=3 mice per group, 4–7 sections per mouse, ** $P < 0.01$, *** $P < 0.001$, Student's t test). **F**, Immunohistochemical detection of caspase 3 activity in BrdU⁺ cells at low (top) and high power (bottom) in the DG from 6–7 month-old *Fgf14*^{+/+} and *Fgf14*^{-/-} mice. **G**, Top, schematic representation of study design; bottom, quantification of caspase 3 fluorescence intensity within BrdU⁺ cells from 6–7 month-old *Fgf14*^{+/+} and *Fgf14*^{-/-} mice (n=3 mice per group, 3–4 sections per mouse, mean is non-statistically different). **H,J**, Cresyl violet (Nissl) staining derived from sagittal brain sections (4–5 months old) revealed normal patterns of gross anatomy in *Fgf14*^{-/-} mice compared to *Fgf14*^{+/+} at low (**H,I**), and a higher magnification of the upper DG blade (**J,K**) (n=1–2 slices/mouse and 7 mice littermates gender-matched). **L**, Representative brain weight from 6–7 month-old *Fgf14*^{+/+} and *Fgf14*^{-/-} mice. **M**, quantification of brain weights of *Fgf14*^{+/+} and *Fgf14*^{-/-} mice (n=4 mice per group, * $P < 0.05$, Student's t test). Data are presented as mean $\pm$ SEM. Scale bars represent 100 μ m in A; 50 μ m in C and J; 40 μ m in F; 200 μ m in H.

Fig 7. Genetic deletion of *Fgf14* impairs field responses and short-term plasticity in DG
A. Representative traces showing field EPSP induced with stimuli of different intensity. **B.** Input-output curve of field EPSP slope in *Fgf14*^{+/+} and *Fgf14*^{-/-} mice. **C.** Minimal stimulus intensity (amount of current needed to induce fEPSP) in *Fgf14*^{+/+} and *Fgf14*^{-/-} mice. **D.** Representative traces showing time-dependence of paired-pulse facilitation. **E.** Paired-pulse facilitation of fEPSP slope presented as a pulse2 fEPSC slope to pulse1 fEPSC slope ratio in

percentage. $P < 0.05$ with Student t -test; “N” represents number of animals and “n” represents number of slices used in experiments.

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