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Impact of Swiprosin-1/Efhd2 on Adult Hippocampal Neurogenesis

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SUMMARY

Swiprosin-1/Efhd2 (Efhd2) is highly expressed in the CNS during development and in the adult. EFHD2 is regulated by Ca^{2+} binding, stabilizes F-actin, and promotes neurite extension. Previous studies indicated a dysregulation of EFHD2 in human Alzheimer's disease brains. We hypothesized a detrimental effect of genetic ablation of *Efhd2* on hippocampal integrity and specifically investigated adult hippocampal neurogenesis. *Efhd2* was expressed throughout adult neuronal development and in mature neurons. We observed a severe reduction of the survival of adult newborn neurons in *Efhd2* knockouts, starting at the early neuroblast stage. Spine formation and dendrite growth of newborn neurons were compromised in full *Efhd2* knockouts, but not upon cell-autonomous *Efhd2* deletion. Together with our finding of severe hippocampal tauopathy in *Efhd2* knockout mice, these data connect *Efhd2* to impaired synaptic plasticity as present in Alzheimer's disease and identify a role of *Efhd2* in neuronal survival and synaptic integration in the adult hippocampus.

INTRODUCTION

SWIPROSIN-1/EFHD2 (EFHD2) is an F-actin-stabilizing protein, whose coiled-coil domain mediates dimerization in a Ca^{2+}-dependent manner. EFHD2 also acts as putative cytoskeletal adaptor interacting with the microtubule-associated protein TAU (Ferrer-Acosta et al., 2013; Hagen et al., 2012; Kwon et al., 2013). We have previously shown that EFHD2 is highly expressed in the CNS throughout embryonic development and in the adult (Purohit et al., 2014).

The serine-threonine protein kinase cyclin-dependent kinase 5 (CDK5) regulates the Ca^{2+}-binding affinity of EFHD2 by phosphorylation of EFHD2 at Ser74 (Vázquez-Rosa et al., 2014). CDK5 activity is dysregulated in Alzheimer's disease (AD) and thereby promotes the formation of amyloid beta and the hyperphosphorylation of TAU (Liu et al., 2016). A potential involvement/dysregulation of EFHD2 in AD was described previously in AD mouse models and AD postmortem studies (Tseveleki et al., 2010; Twine et al., 2011; Vega et al., 2008; Ferrer-Acosta et al., 2013; Borger et al., 2014; Vega, 2016). These studies suggested that EFHD2 interacts with TAU and TAU-associated proteins in AD.

Adult neurogenesis, the generation of new neurons in the adult brain, persists throughout life within the dentate gyrus (DG) of the hippocampus (Eriksson et al., 1998; Spalding et al., 2013). *Cdk5* is a modulator of adult neurogenesis (Jessberger et al., 2008), and knock down of overactivated *Cdk5* in mice transgenic for amyloid precursor protein rescued impaired neurogenesis (Crews et al., 2011).

Given (1) the link of both *Efhd2* and *Cdk5* to AD, (2) the interaction between *Cdk5* and *Efhd2*, and (3) the dysregulation of adult neurogenesis in AD models and upon *Cdk5* deletion, we hypothesized that deletion of *Efhd2* negatively affects adult neurogenesis. *Efhd2* is broadly expressed in the adult neurogenic niche. We demonstrate a severe decrease of adult neurogenesis in the DG of $Efhd2^{-/-}$. There is defective integration of newborn neurons in $Efhd2^{-/-}$. We propose a cell-extrinsic mechanism of *Efhd2* deficiency mediated by increased levels of phosphorylated TAU in the hippocampus of $Efhd2^{-/-}$ mice. Our findings describe a role of *Efhd2* in the adult hippocampal stem cell niche and have potential implications for tauopathies such as AD.

RESULTS

Efhd2 Is Expressed during Adult Neuronal Development

To investigate the role of *Efhd2* in the adult hippocampus, we first studied hippocampal expression in adult $Efhd2^{-/-}$ knockout/*LacZ* knockin mice. In this model, both *Efhd2* alleles were replaced by a *LacZ* reporter gene downstream of the *Efhd2* promoter (Brachs et al., 2014). In the hippocampus, we found high *Efhd2* gene expression in the cornu

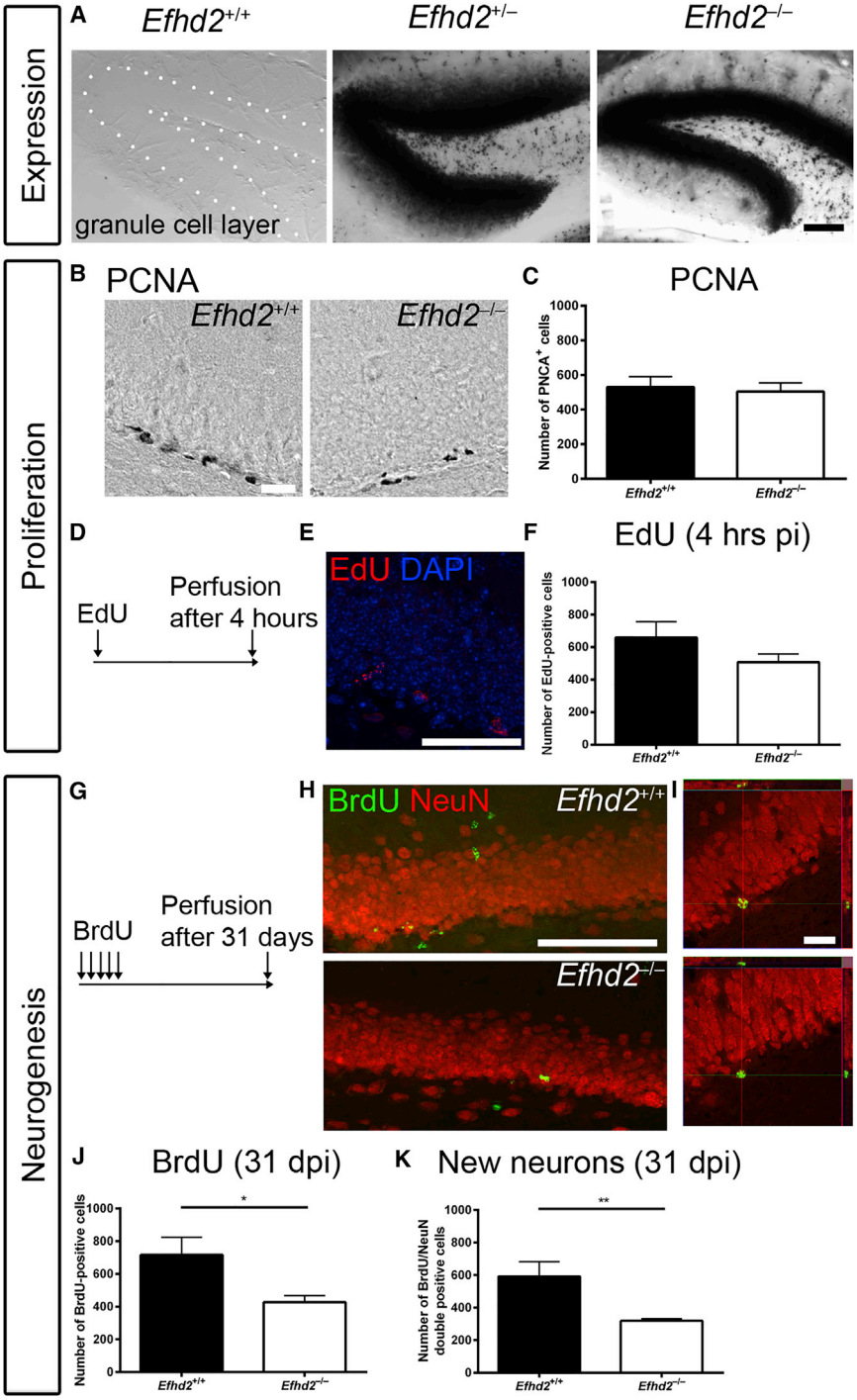

Figure 1. *Efhd2* Is Expressed in the Mouse Hippocampus and *Efhd2* Knockout Impairs Survival of Newborn Neurons

(A) Beta-Gal staining of the DG of $Efhd2^{+/+}$ (outline indicated by white dots), $Efhd2^{+/-}$, and $Efhd2^{-/-}$ mice showing expression of the *Efhd2* locus in the adult DG (immunohistochemistry for LacZ is shown in detail in Figure S1).

(B–F) Analysis of proliferation at the age of 4 months. (B) Immunohistochemistry for PCNA. (C) There was no significant difference of PCNA numbers in $Efhd2^{-/-}$ compared with $Efhd2^{+/+}$. (D) In a separate experiment, animals received a single injection of EdU. (E) EdU-positive cells in the dentate gyrus were quantified after 4 hr. (F) Quantification of EdU-positive cells showed no change in $Efhd2^{-/-}$.

(G–K) Survival and neuronal differentiation were analyzed 31 days after BrdU labeling. (H) Low- and (I) high-magnification images of BrdU/NeuN colabeling. (J) In $Efhd2^{-/-}$, the number of BrdU-positive cells was significantly reduced after 31 days. (K) The number of BrdU/NeuN double-positive cells (newborn neurons) was significantly reduced in $Efhd2^{-/-}$. dpi, days after injection; hrs pi, hours after injection. $^{*}p < 0.05$; $^{**}p < 0.01$.

Data are represented as mean ± SEM, n = 5 animals per genotype for all quantifications. For all analyses, animals were 4 months of age. Scale bars, (A) and (H) 100 μm, (B), (E), and (I) 25 μm.

ammonis regions CA1 and CA2 and in the DG (Figures 1A, S1A, and S1B).

In the DG, *Efhd2* was not only expressed in NeuN-positive mature neurons, but also in Sox2-positive radial glia-like cells and DCX-positive neuroblasts (Figures S1C–S1G). The observed expression in adult neural stem cells correlates with expression of *Efhd2* in murine embryonic stem cells (data not shown). As shown previously (Fischer et al., 2005; Jessberger et al., 2008), p35, the regulatory subunit of CDK5, was expressed in the DG, including its expression in DCX-positive neuroblasts (Figure S1H).

Observing widespread expression of *Efhd2* in the DG, we analyzed total DG volumes. DG volume was comparable between *Efhd2*$^{-/-}$ and *Efhd2*$^{+/+}$, suggesting that the overall structure of the DG was unchanged in *Efhd2*$^{-/-}$ mice (Figures S2A and S2B; Table S1). The total number of NeuN-positive neurons in the granule cell layer was unchanged (Figure S2C) and zinc-transporter-3-based analysis of the mossy fiber pathway area showed comparable sizes of the axonal compartment of DG neurons in *Efhd2*$^{-/-}$ and controls (Figures S2D–S2G). These results suggest that the overall anatomy of the DG is unchanged in *Efhd2*$^{-/-}$.

Compromised Survival of Adult Newborn Neurons in Efhd2$^{-/-}$

Observing expression of *Efhd2* throughout adult neuronal development, we hypothesized a role of *Efhd2* for adult neurogenesis. We first analyzed proliferation within the DG using the marker PCNA, which was not altered in 4-month-old *Efhd2*$^{-/-}$ mice (Figures 1B and 1C). This result was confirmed by 5-ethynyl-2′-deoxyuridine (EdU) labeling of proliferating cells, which was unchanged 4 hr after labeling in *Efhd2*$^{-/-}$ (Figures 1D–1F). Next, bromodeoxyuridine (BrdU) was applied to 3-month-old *Efhd2*$^{-/-}$ mice and controls. We observed a severely decreased number of BrdU-positive cells in *Efhd2*$^{-/-}$ 1 month after BrdU-injection (427 ± 92 cells per hemisphere ± SD in EFhd2$^{-/-}$ versus 716 ± 241 in controls; $p < 0.05$; Figures 1G–1J). The fraction of NeuN-positive cells among BrdU-positive cells in *Efhd2*$^{-/-}$ was similar to controls. This indicates that the rate of neuronal differentiation is not altered (Table S1). When calculating the number of newborn neurons, we found a 46% reduction of the total number of BrdU/NeuN double-positive newborn neurons after 1 month (318 ± 28 in *Efhd2*$^{-/-}$ versus 592 ± 201 in controls; $p < 0.01$; Figure 1K). Given this profound reduction of surviving newborn neurons despite unchanged DG volume and neuron numbers, our findings suggest that neurogenesis is compromised in *Efhd2*$^{-/-}$ specifically in the adult.

Loss of Neuroblasts and Increased Cell Death in Efhd2$^{-/-}$ Mice

We next aimed to determine the stage at which adult-born neurons are lost in the *Efhd2*$^{-/-}$ DG. We analyzed the number of BrdU-positive cells 14 days after labeling and observed a significant decrease by 60% in *Efhd2*$^{-/-}$ (Figures 2A and 2B). Immature adult-generated hippocampal neurons undergo well-defined morphological changes during their maturation (Brown et al., 2003; Couillard-Despres et al., 2005) which allows the identification of developmental subpopulations (Figure 2E). Comparing the numbers of early, intermediate, and late-stage DCX-positive cells, we found a significant reduction in *Efhd2*$^{-/-}$ persisting from the early through to the late stage (Figures 2C and 2D). To determine whether there is an additional selective loss of intermediate or late-stage neuroblasts, we calculated the relative loss of DCX-positive cells and found no significant differences (Table S1).

As we observed a marked decrease of newly born neurons despite unchanged proliferation, we next analyzed whether cell death was altered in the *Efhd2*$^{-/-}$ hippocampus. Indeed, the number of cells positive for activated caspase-3 was significantly increased in the DG of *Efhd2*$^{-/-}$ mice (Figures 2F–2H).

Efhd2-Deletion Affects Dendrite Outgrowth and Spine Formation of Adult Newborn Neurons

To determine whether *Efhd2* knockout also impacts the morphogenesis of surviving newborn neurons, we stereotactically delivered a GFP retrovirus into the DG at the age of 3 months to label newborn neurons, and analyzed their morphology after 1 month of survival (Figures 3A and 3B; Zhao et al., 2006). Total dendrite length of GFP-labeled neurons was decreased by 38% (328 ± 149 μm [mean ± SD] in *Efhd2*$^{-/-}$ animals versus 526 ± 170 in controls; $p < 0.05$). This finding was substantiated by Sholl analysis which showed a significant reduction of dendrite complexity at intermediate distances from the soma, i.e., at 100–150 μm (Figure 3E). The number of branching points was unchanged between groups (Figure 3C) indicating intact branching of newborn neurons' dendrites.

The density of dendritic spines was significantly reduced by 59% in *Efhd2*$^{-/-}$ (0.501 ± 0.220 spines per μm ± SD in *Efhd2*$^{-/-}$ DG versus 1.21 ± 0.271 in controls; $p < 0.05$; Figure 3F). The subpopulation of mushroom spines was identified based on morphological criteria of spine head size (Zhao et al., 2006). We found that the density of mushroom spines was also significantly decreased by 54% in the absence of *Efhd2* (14.5 ± 8.5 mushroom spines per nm ± SD in *Efhd2*$^{-/-}$ DG versus 31.3 ± 1.6 in controls; $p < 0.01$; Figure 3G). This reduction of spines, and in particular of mushroom spines, provides further evidence for an impairment of dendritic integration of newborn neurons into the molecular layer of *Efhd2*$^{-/-}$ mice.

To separate cell-autonomous from cell-non-autonomous effects in *Efhd2*$^{-/-}$ mice, we made use of a newly generated *Efhd2*$^{fl/fl}$ mouse model, where exon 2 of the *Efhd2* gene had been flanked by *loxP* sites and is deleted upon Cre-mediated recombination (Figure S3). Conditional deletion of *Efhd2* in adult newborn cells in the DG was induced by a retrovirus expressing both GFP and Cre (GFP-Cre). As only few cells are infected using this approach, while the majority of surrounding neurons maintains normal *Efhd2* expression, it allows the specific investigation of cell-autonomous

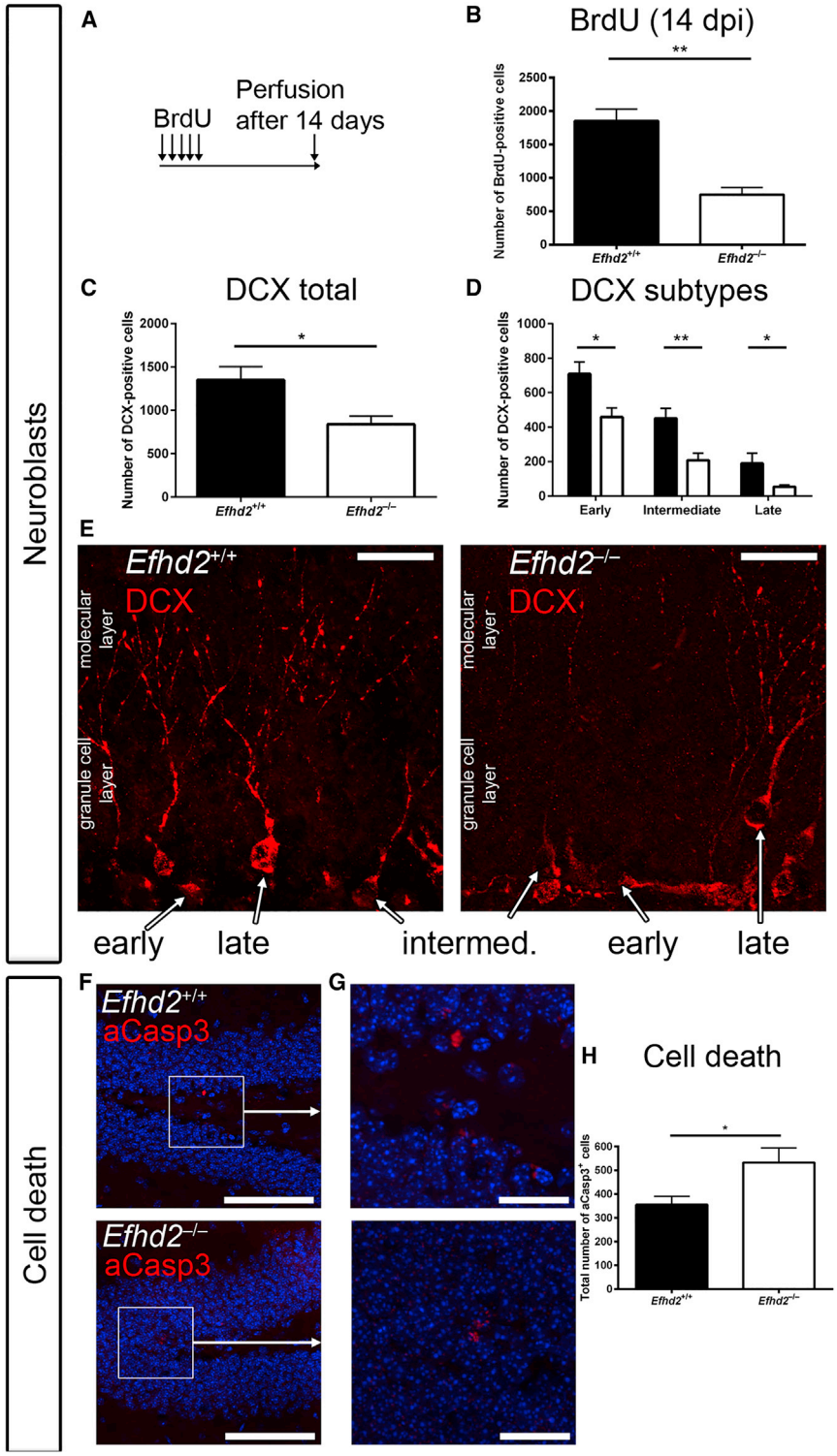

Figure 2. Loss of Neuroblasts and Increased Cell Death in the Adult *Efhd2*$^{-/-}$ Dentate Gyrus

(A) To analyze early survival of newly generated cells, animals were killed on day 14 after BrdU labeling.

(B) There was a significantly reduced survival of BrdU-positive cells at 14 days in *Efhd2*$^{-/-}$.

(C) The total number of DCX-positive cells was significantly reduced in *Efhd2*$^{-/-}$ mice.

(D and E) Different developmental stages during neuroblast maturation were distinguished based upon morphological criteria. Early-stage neuroblasts are located in the subgranular zone, form clusters, and exhibit no processes or processes with parallel orientation. Intermediate neuroblasts are located in the granule cell layer and exhibit one or more processes confined to the granule cell layer. Late neuroblasts are located in the granule cell layer and display processes extending into the molecular layer. (D) There was a significant reduction of early, intermediate, and late neuroblasts in *Efhd2*$^{-/-}$ mice. (E) Overview images of doublecortin-positive neuroblasts in controls and *Efhd2*$^{-/-}$ mice at different stages, as described above.

(F and G) Low-magnification images of activated caspase-3 immunostaining in the dentate gyrus of *Efhd2*$^{+/+}$ and *Efhd2*$^{-/-}$ (F) and magnifications from boxed areas (G).

(H) The number of activated caspase-3-positive cells was significantly increased in the dentate gyrus of *Efhd2*$^{-/-}$ mice.

Data are represented as mean ± SEM, n = 5 animals per genotype, aged 4 months for all analyses. Scale bars, 100 μm (F), 25 μm (E and G). *p < 0.05, **p < 0.01.

Efhd2 deletion in newborn neurons. Comparing the morphologies of adult newborn neurons infected with GFP-Cre versus GFP only, we observed no differences in dendritic branching points, dendrite length, spine density, and mushroom spine density (Figures 3H–3N). This finding strongly indicates a cell-non-autonomous mechanism of impaired dendritic morphology of newborn neurons in the adult hippocampus of *Efhd2*$^{-/-}$ mice.

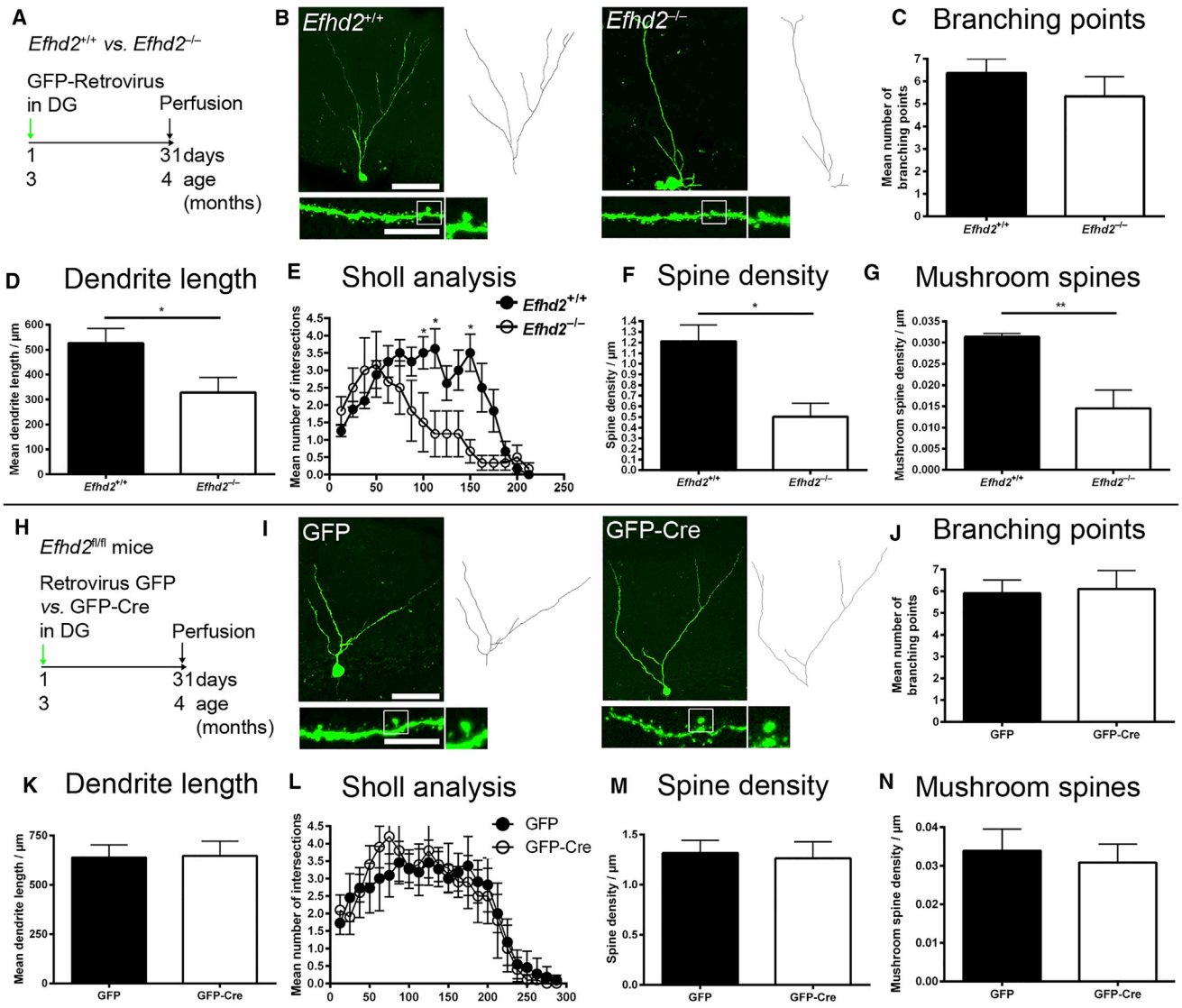

Figure 3. Compromised Dendrite Length and Spine Density of Mature Newborn Neurons in *Efhd2*$^{-/-}$, but Not Upon Conditional *Efhd2* Deletion

(A) CAG-GFP retrovirus was stereotactically injected into the hippocampus of 3-month-old wild-type and *Efhd2*$^{-/-}$ mice, perfusion was performed 31 days later.

(B) Representative images of retrovirally labeled GFP-expressing newborn neurons in wild-type and *Efhd2*$^{-/-}$ mice, as well as spine micrographs and mushroom spine magnifications (boxed).

(C and D) The number of dendritic branching points is unchanged in *Efhd2*$^{-/-}$ mice (C), but the total length of the dendritic tree is significantly reduced (D).

(E) Sholl analysis shows that there are significantly less dendritic branches in a distance of 100 and 150 µm from the soma.

(F) The density of dendritic spines is significantly reduced in *Efhd2*$^{-/-}$ mice.

(G) The density of mushroom spines is significantly reduced in *Efhd2*$^{-/-}$ mice.

(H) Conditional knock out of *Efhd2* in newborn neurons by application of a retrovirus encoding GFP and Cre recombinase (GFP-Cre) in 3-month-old mice, where the *Efhd2* locus had been floxed (*Efhd2*$^{fl/fl}$; see also Figure S3).

(I) Representative images of GFP- and GFP-Cre-expressing newborn neurons in *Efhd2*$^{fl/fl}$ mice, as well as spine micrographs and mushroom spine magnifications (boxed).

(J and K) There is no difference in the number of branching points and the length of the dendritic tree.

(L) Sholl analysis of GFP versus GFP-Cre overexpressing neurons.

(legend continued on next page)

Increased Levels of Phosphorylated TAU in the Hippocampus of Efhd2$^{-/-}$

Multiple lines of evidence suggest a role of *Efhd2* in AD pathogenesis (Mielenz and Gunn-Moore, 2016; Vega, 2016). We thus analyzed expression of pSer202/pThr205 TAU. Using immunohistochemistry, we found that phospho-TAU levels were significantly increased in the hippocampus of *Efhd2*$^{-/-}$ compared with controls, including all hippocampal areas analyzed (molecular layer, DG, and hilus; Figures 4A–4D). Moreover, western blot of whole-brain lysates showed abundant presence of phospho-TAU in *Efhd2*$^{-/-}$, compared with very low expression levels in controls (Figures 4E and 4F). We conclude that lack of *Efhd2* leads to increased levels of phospho-TAU and may be directly connected with decreased survival and dendritic integration of adult newborn neurons.

DISCUSSION

In summary, we show that *Efhd2* is strongly expressed in the adult hippocampal neurogenic niche. In *Efhd2*$^{-/-}$ mice, the numbers of neuroblasts and of newborn neurons are markedly reduced. Surviving neurons exhibit impaired dendrite morphology and a reduced number of spines and mushroom spines. This defective dendritic integration is due to a cell-extrinsic mechanism of *Efhd2* deficiency and is accompanied by increased levels of phosphorylated TAU in the hippocampus of *Efhd2*$^{-/-}$ mice. Our findings thus broaden the knowledge about the emerging role of *Efhd2* in TAU-related neurodegeneration and describe a function of *Efhd2* in adult neurogenesis.

A multiplicity of regulatory factors impacting the different stages of adult neurogenesis have been identified, highlighting the exact balance that is needed for the proper function of adult newborn neurons (Gonçalves et al., 2016). Our results demonstrate a role of *Efhd2* in the adult hippocampus, specifically for survival and postsynaptic integration of newborn neurons. Mushroom spines are sites of stable synaptic input and are thus major mediators of memory engram. Adult newborn neurons are critical for memory encoding involving pattern-separation tasks (Aimone et al., 2014). Particularly in light of the animals' increased levels of phospho-TAU, characterization of memory function in *Efhd2*$^{-/-}$ mice will be an important future experiment.

Volumetry of the DG and of the mossy fiber pathway, as well as the total number of neurons in the granule cell layer, showed no alterations in *Efhd2*$^{-/-}$ mice. In line with this intact overall morphology, the embryonic development of the CNS in terms of brain volumes and synaptic marker expression were unchanged at the macroscopic level in *Efhd2*$^{-/-}$ mice (Purohit et al., 2014).

We observe that *Efhd2*$^{-/-}$ neurons display normal dendrite/spine morphology when allowed to develop in a wild-type hippocampal neurogenic niche. These observations imply that cell-extrinsic factors play a major role in the integration deficit observed in *Efhd2*$^{-/-}$ mice.

We describe major survival deficits of newborn neurons at an early stage. We presently cannot distinguish whether the loss of newborn neurons is caused by cell-intrinsic or -extrinsic mechanisms or by both. In adult-born neurons, intracellular Ca^{2+} levels are influenced by excitatory stimuli and are critical for survival (Deisseroth et al., 2004). The Ca^{2+}-binding protein EFHD2 operates downstream of CDK5. Intriguingly, CDK5 was reported to cell autonomously regulate survival of adult-born neurons (Lagace et al., 2008). These observations raise the interesting possibility that a CDK5 EFHD2 pathway may be involved in regulating the survival of adult-born neurons.

Strikingly, we observe an increased phospho-TAU expression in the *Efhd2*$^{-/-}$ hippocampus. Multiple lines of evidence have shown alterations of adult neurogenesis in neurodegenerative diseases including AD (Winner et al., 2011). Loss of dendritic spines is a hallmark of AD and is also found in adult newborn neurons in transgenic AD models (Sun et al., 2009). A deficit of the total rate of neurogenesis precedes neuropathology in both the triple-transgenic (*APP/PSEN1/TAU*) and in the genomic *TAU* mouse model (Demars et al., 2010; Komuro et al., 2015). This indicates that adult neurogenesis may already be affected during early-stage AD pathological events.

Our results of defective integration of newborn neurons in the *Efhd2*$^{-/-}$ DG and increased phospho-TAU expression thus raise the intriguing possibility that this adult hippocampal *Efhd2*-related phenotype reflects an early neuritic phenotype of neurodegeneration, and that EFHD2, with its actin-binding ability, has a neuroprotective function. It will be interesting to investigate TAU-associated neurodegeneration in aging *Efhd2*$^{-/-}$ mice in more detail in the future. Increased levels of phospho-TAU in the hippocampus of *Efhd2*$^{-/-}$ animals suggest a neuroprotective role of *Efhd2*.

(M and N) There were no significant differences in spine density and mushroom spine density between GFP- and GFP-Cre-overexpressing neurons.

Data are represented as mean ± SEM, n = 5 per group (paradigm A), n = 4 per group (paradigm H). *p < 0.05, **p < 0.01. Scale bars, 50 μm (upper line) and 10 μm (lower line) in (B and I).

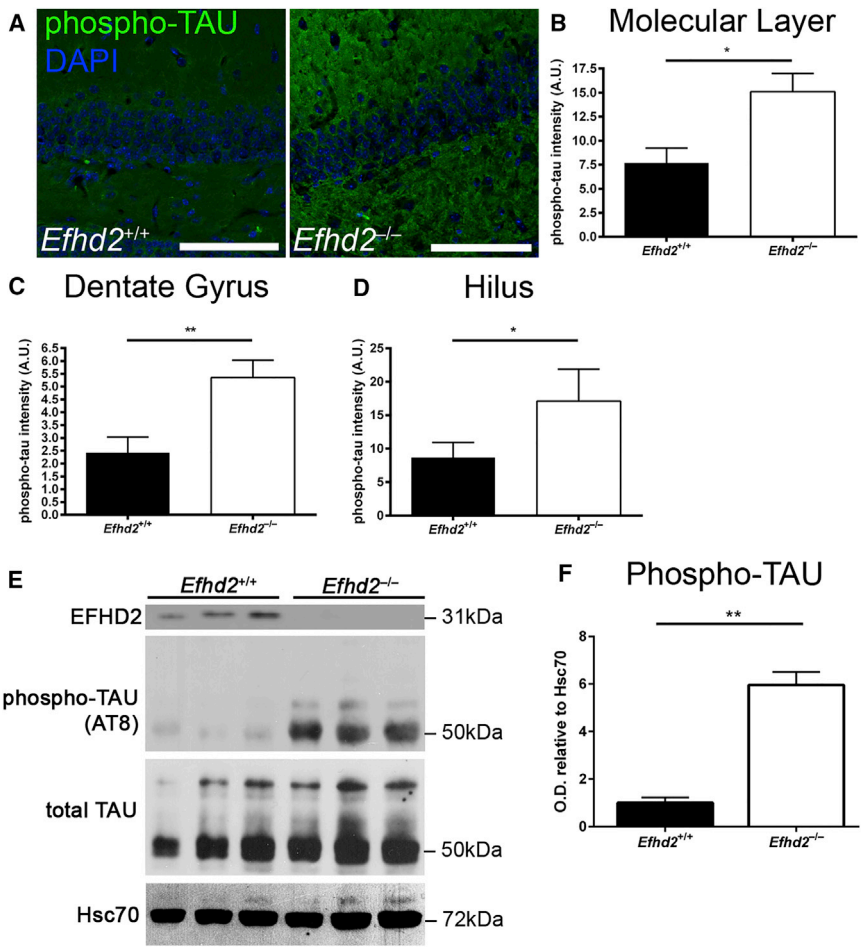

Figure 4. Increased Phospho-TAU Levels in *Efhd2*$^{-/-}$

(A–D) Representative images of immunohistochemistry for pSer202/pThr205-TAU (AT8 antibody, green) in controls and *Efhd2*$^{-/-}$ mice, respectively (A). Analysis of the integrated density of phospho-TAU staining shows significantly increased levels in the molecular layer (B), the dentate gyrus (C), and the hilus (D) of *Efhd2*$^{-/-}$ mice.

(E) Western blot for pSer202/pThr205-TAU shows prominent expression of TAU and predominantly phospho-TAU in *Efhd2*$^{-/-}$, but not in controls. Hsc70 served as loading control.

(F) Quantification of the optical density of phospho-TAU in *Efhd2*$^{-/-}$, normalized to Hsc70.

Data are represented as mean ± SEM. (B–D) n = 4 per genotype (age 4 months); (F) n = 3 per genotype (age 3 months).

Scale bars, 100 μm. *p < 0.05, **p < 0.01.

EXPERIMENTAL PROCEDURES

Animals

Animal experiments were conducted in accordance with the European Communities Council Directive of November 24th, 1996, and were approved by the local governmental administration for animal health (permit numbers: 54-2532.1-21/09 and 54.2532.1–18/14). In *Efhd2*$^{-/-}$ mice, the entire *Efhd2* gene was replaced by a *β-Gal* reporter gene as described elsewhere (Brachs et al., 2014).

Retrovirus-Mediated Labeling and Analysis of Newborn Neurons

A Moloney murine leukemia retrovirus-based CAG-GFP plasmid was used as described earlier (Zhao et al., 2006). CAG-GFP drives expression of EGFP by the compound promoter CAG. A CAG-GFP-IRES-CRE plasmid had been generated from the original CAG-GFP vector by insertion of an IRES-Cre coding sequence (Jagasia et al., 2009). A concentrated viral solution was titered to 4 × 10^8 pfu/mL. A stereotaxic frame (Kopf Instruments) was used for sequential bilateral infusion into the DG (AP –2.00 mm, ML ± 1.6 mm from bregma, DV –2.3 mm from skull; n = 5 per genotype for *Efhd2*$^{+/+}$ versus *Efhd2*$^{-/-}$; n = 4 per construct for GFP versus GFP-Cre). A total volume of 1.0 μL was slowly infused (0.2 μL/min).

Statistical Analysis

For statistical analysis with Prism (GraphPad Software), significance level was assumed at 0.05. All parameters (cell counts, volumes, dendrite length, number of branching points, spine density, and mushroom spine density) were compared using unpaired, two-sided Student's t tests.

Details on the generation of *Efhd2*$^{fl/fl}$ mice, BrdU/EdU treatment, tissue processing, immunohistochemistry, microscopy, and western blot can be found in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at https://doi.org/10.1016/j.stemcr.2017.12.010.

AUTHOR CONTRIBUTIONS

Conceptualization, B.W. and D.M.; Methodology, M.R. and B.W.; Investigation, M.R., S.L., D.C.L., I.P., S.B., and D.R.; Resources, D.C.L., D.M., and B.W.; Writing – Original Draft, M.R.; Writing – Review & Editing, D.C.L., D.M., and B.W.; Funding Acquisition, M.R., D.M., and B.W.

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