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Loss of Dickkopf-1 Restores Neurogenesis in Old Age and Counteracts Cognitive Decline

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SUMMARY

Memory impairment has been associated with age-related decline in adult hippocampal neurogenesis. Although Notch, bone morphogenetic protein, and Wnt signaling pathways are known to regulate multiple aspects of adult neural stem cell function, the molecular basis of declining neurogenesis in the aging hippocampus remains unknown. Here, we show that expression of the Wnt antagonist Dickkopf-1 (Dkk1) increases with age and that its loss enhances neurogenesis in the hippocampus. Neural progenitors with inducible loss of Dkk1 increase their Wnt activity, which leads to enhanced self-renewal and increased generation of immature neurons. This Wnt-expanded progeny subsequently matures into glutamatergic granule neurons with increased dendritic complexity. As a result, mice deficient in Dkk1 exhibit enhanced spatial working memory and memory consolidation and also show improvements in affective behavior. Taken together, our findings show that upregulating Wnt signaling by reducing Dkk1 expression can counteract age-related decrease in neurogenesis and its associated cognitive decline.

INTRODUCTION

In the adult brain, neurogenesis takes place in two distinct regions: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Ming and Song, 2011). Neurogenesis encompasses several processes, including cell birth, fate determination, survival, network integration, and the acquisition of functional properties. In the DG, new neurons arise from a population of neural progenitor cells (NPCs) residing in the SGZ (Ming and Song, 2011). The more quiescent neural progenitors (QNPs) are characterized by their potential to self-renew and to differentiate into either neuronal lineage-restricted progenitors or astrocytes, which are characterized by the expression of the astrocytic marker S100β (Encinas et al., 2011). QNPs can be identified by their expression of the intermediate filament protein Nestin or the glial fibrillary acidic protein, as well as the transcription factor Sox2 (Encinas et al., 2011). They give rise to committed amplifying neuronal progenitors (ANPs), which express the transcription factor Tbr2 (a T box transcription factor). Finally, ANPs transform into doublecortin (DCX)-positive neuroblasts (NBs) that lose expression of Tbr2 and migrate into the adjacent granule cell layer (GCL), where they mature into glutamatergic granule neurons that become positive for the neuronal nuclear antigen (NeuN) and project to the CA3 and hilar regions (Ming and Song, 2011).

The unique microenvironment of the SGZ regulates maintenance, activation, and fate choice of adult NPCs through a number of morphogens, including the Notch, Sonic hedgehog homolog, bone morphogenetic protein, and Wnt signaling pathways (Ming and Song, 2011). Astrocytes within the neurogenic niche of the DG produce the Wnt3 ligand, which acts in a paracrine manner on neuronal progenitors to induce expansion of NBs (Lie et al., 2005; Song et al., 2002). Also, Wnt7 has recently been shown to be expressed in adult NPCs in the DG under the control of the orphan nuclear receptor tailless (TLX) to induce their self-renewal (Qu et al., 2010). Binding of Wnt ligands to the frizzled receptors and LRP5 and LRP6 coreceptors induces phosphorylation of LRP5/LRP6, leading to the translocation of Dishevelled and Axin from the β-catenin degradation complex to the plasma membrane (Niehrs, 2006). Consequently, β-catenin is no longer degraded and may shuttle to the nucleus to form transcription activator complexes with the T cell transcription factor (TCF) and lymphoid enhancer binding factor (LEF) family of transcription factors (Niehrs, 2006). The promoter region of the basic helix-loop-helix proneural transcription factor NeuroD1 contains regulatory elements that are repressed by Sox2 and activated by the β-catenin/TCF/LEF activator complex following Wnt activation in adult NPCs (Kuwabara et al., 2009). These Sox2 and LEF binding sites were also found in LINE-1 elements, suggesting the further involvement of Wnt signaling in the generation of neuronal diversity (Kuwabara et al., 2009). However, detailed knowledge of how Wnt activity regulates survival, proliferation, and differentiation in adult neurogenesis is still missing.

Wnt signaling is not only modulated by the presence or absence of Wnt ligands but also by antagonists such as the

Dickkopf family of secreted glycoproteins. Dkk1, Dkk2, and Dkk4 bind to LRP5/LRP6, thereby preventing its interaction with Wnt ligands (Niehrs, 2006). Forced expression of Dkk1 severely reduces neurogenesis in the developing hippocampus (Solberg et al., 2008). Yet, the function of Dkk1 in adult neurogenesis has not been addressed.

In this study, we show that Dkk1 loss in NPCs increases neurogenesis in the SGZ of young and old animals. In addition, we provide evidence that loss of Dkk1 increases Wnt activity in NPCs, leading to enhanced self-renewal and/or survival of the more quiescent neural progenitors and increased numbers of ANPs and NBs. These NBs, as well as the newborn mature neurons, exhibit a more elaborated dendritic morphology and positively impact neuronal activity in the DG. Altogether, these features lead to positive effects on affective behavior, working memory, and memory consolidation.

RESULTS

Loss of Dkk1 Increases Wnt Activity in NPCs and Maintains Neurogenesis in Old Age

To investigate the function of Dkk1 in adult neural stem cells, we generated mutant mice lacking expression of Dkk1 in the brain. To this end, mice with one deleted and one loxP-flanked allele ($Dkk1^{-/f}$) were bred with mice that express the Cre Recombinase under the control of the Nestin promoter, resulting in mice deficient in Dkk1 in neurons, glia, and NPCs (Nestin-Dkk1 mice) (Pietilä et al., 2011). Dkk1 protein and mRNA was expressed in neurospheres derived from the SVZ and SGZ of control—but not of Nestin-Dkk1—mice (Figures 1A and 1B). To assess the effect of Dkk1 loss on Wnt activity, we used a Wnt-reporter plasmid (7TGC) carrying a SV40-mCherry cassette and a 7×Tcf-eGFP Wnt reporter cassette (Fuerer and Nusse, 2010). In the absence of Wnt signals, cells display only red fluorescence, whereas activated Wnt signaling leads to additional expression of eGFP. Loss of Dkk1 resulted in a 4-fold increase in the number of eGFP-expressing NPCs as compared to controls (Figure 1C). Thus, Dkk1 counteracts Wnt transcriptional activity in NPC cultures.

Wnt ligands are reported to be produced within the neurogenic niche and to act on neighboring NPCs (Song et al., 2002). We have shown that Dkk1 is produced by NPCs, but additional secretion by other cells within the niche has not been explored. To selectively address the function of NPC-secreted Dkk1, we bred mice with two loxP-flanked *Dkk1* alleles ($Dkk1^{f/f}$) with mice expressing a tamoxifen (TAM)-inducible Recombinase $CreER^{T2}$ under the control of the Nestin promoter. In the resulting iNestin-Dkk1 mice, TAM administration at adult age induced *Dkk1* deletion in adult NPCs in the DG (Figure 1D). Accordingly, Dkk1 was not expressed in neurospheres derived from these mice (Figure 1E). In addition, transfection of the Wnt-reporter plasmid into NPCs isolated from TAM-treated iNestin-Dkk1 mice revealed that TAM-induced loss of Dkk1 leads to increased Wnt activity in neurosphere cultures (Figure S1 available online). Next, we examined Dkk1 expression and Wnt activity in the DG of control and iNestin-Dkk1 mice 7 months after TAM administration. To this end, we performed in situ hybridization (ISH) of Dkk1 as well as Axin2, a universal reporter gene for canonical Wnt signaling. Expression of Dkk1 as well as Axin2 was detected in the GCL of the DG of control and iNestin-Dkk1 mice (Figure 1F). Notably, whereas iNestin-Dkk1 mice showed many hot spots of Axin2 expression in the SGZ of the DG, these were rare in control mice (Figures 1F and 1F′). Accordingly, expression of Dkk1 mRNA was missing in the SGZ of iNestin-Dkk1 mice and detected as rare hot spots in the SGZ of control mice (Figures 1F and 1F′). Interestingly, some of those Dkk1-empty SGZ regions spread like indentations into the GCL of the DG (Figures 1F and 1F′). Altogether, these data suggest that QNPs and their progeny, once activated, express Dkk1 as a feedback signal onto neighboring clones.

Next, we examined the impact of the observed increase in Wnt activity on the self-renewal of NPCs in vitro with the help of a neurosphere assay. NPCs directly isolated from Nestin-Dkk1 mice generated a higher number of secondary neurospheres than NPCs derived from control mice (Figure 1G). The size of the neurospheres was similar in both groups (Figure 1H). These data suggest that loss of Dkk1 enhances self-renewal of NPCs.

Dkk1 mRNA and protein expression was higher in the DG of old animals than in that of young adult animals (Figures 1I and S1). The observed increase in Dkk1 expression could be one reason underlying the age-related decay of NPCs' self-renewal. Thus, if loss of Dkk1 increases the self-renewal of NPCs in vivo, then loss of Dkk1 would lead to maintenance of neurogenesis in old age. To test this hypothesis, we examined neurogenesis in very old Nestin-Dkk1 mice. Notably, in 2-year-old Nestin-Dkk1 mice, the number of newborn neurons was 80% higher than in age-matched counterparts (Figures 1J, 1K, and S1). Altogether, these data demonstrate that Dkk1 is expressed in NPCs, where it counteracts Wnt activity and the self-renewal of NPCs. Consequently, loss of Dkk1 leads to restoration of neurogenesis in old age.

Deletion of Dkk1 in Adult NPCs Increases NPCs' Self-Renewal and the Number of Neuronal Progenitors

Thereafter, we examined neurogenesis 5 weeks after TAM administration to adult iNestin-Dkk1 mice. These mice exhibited a 2.7-fold increase of proliferating QNPs (here identified as 24hr-$BrdU^+/Tbr2^-/DCX^-$ cells; Figures 2A and 2B) and a 1.7-fold increase in 4 week label-retaining QNPs (identified as 4 week-$BrdU^+/Sox2^+/S100\beta^-$ cells; Figures 2C and 2D), suggesting increased self-renewal of QNPs. In addition, those animals showed a 1.3-fold increase in ANPs (identified as 24hr-$BrdU^+/Tbr2^+/DCX^-$ cells; Figures 2E and 2F) as well as a 1.3-fold higher number of NBs (here identified as 24hr-$BrdU^+/Tbr2^+/DCX^+$ cells; Figures 2G, 2H, and S1) than in control counterparts. The high increase in proliferating and label-retaining QNP numbers in adult iNestin-Dkk1 mice suggests that increased Wnt activity in NPCs increases the number of symmetric divisions and/or the survival of QNPs. Along this line, Wnt signals are reported to increase radial glia self-renewal in the embryonic cortex (Munji et al., 2011).

Notably, quantification of newly generated granule cells (4 week-$BrdU^+/NeuN^+$ cells) or the volume of the granule cell layer in the DG revealed no significant differences between the two groups (Figures 2I and 2J, and S1). These data suggest that a higher proportion of NBs undergo apoptosis in Dkk1-deficient mice than in respective control mice. Indeed, staining of

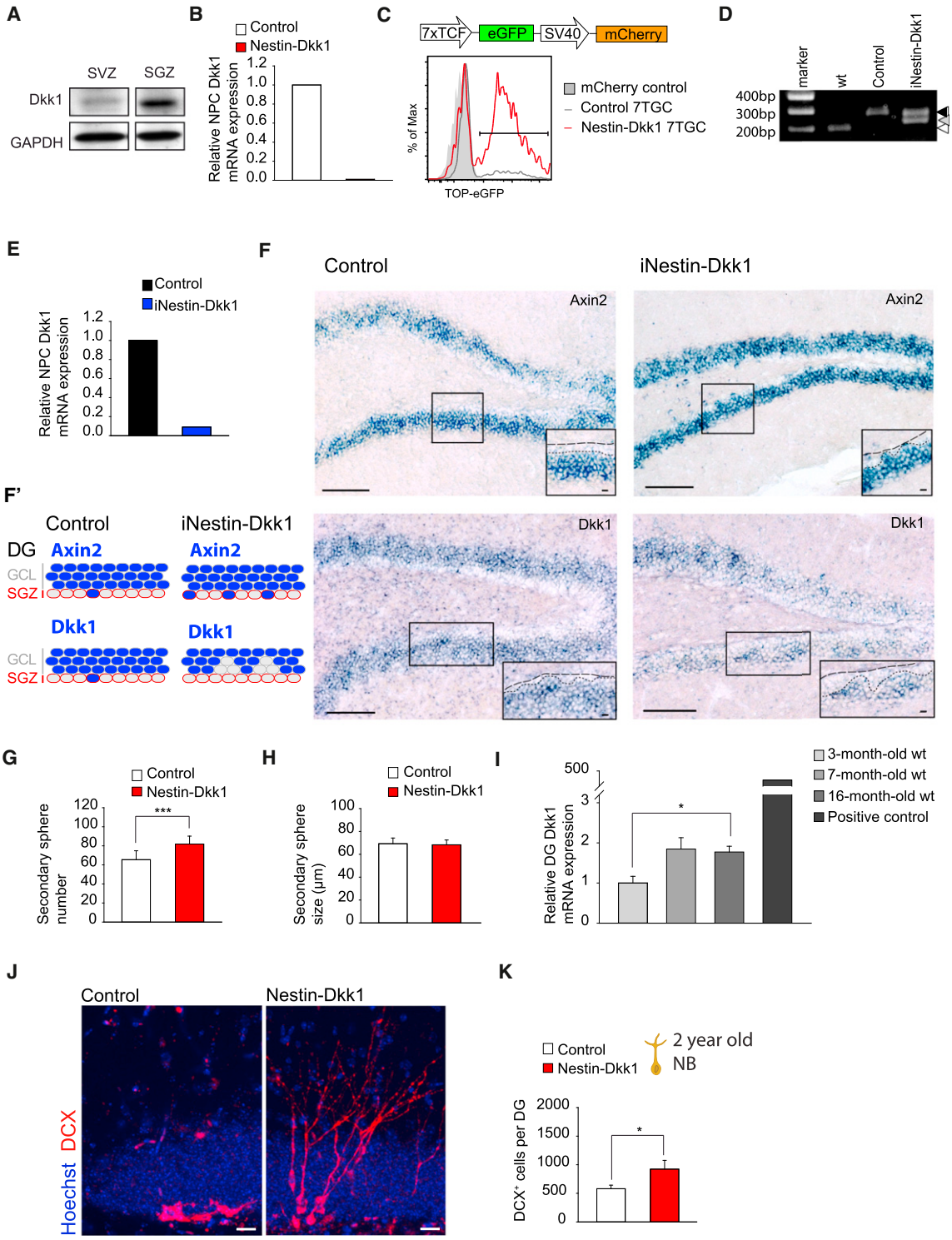

Figure 1. Deletion of Dkk1 Increases Neurogenesis and TCF/LEF Activity

(A) Western blot showing Dkk1 expression in isolated NPCs from the SVZ and HC of WT mice.

(B) Deletion of Dkk1 in neurospheres derived from Nestin-Dkk1 mice used in (C), (G), and (H).

(C) Nestin-Dkk1 NPCs show increased Wnt activity compared to control NPCs, indicated by higher eGFP expression of NPCs transfected with the 7TGC Wnt reporter plasmid (control versus Nestin-Dkk1: 14% versus 59%).

(D) Three-primer Dkk1 PCR on DNA isolated from DG of WT, control, and iNestin-Dkk1 mice to show Dkk1WT sequence (white arrowhead), Dkk1 exon 1- and exon 2-floxed sequence (black arrowhead), and TAM-induced deletion of Dkk1 exon 1 and exon 2 in NPCs (gray arrowhead).

(E) Relative Dkk1 mRNA expression of NPCs isolated from iNestin-Dkk1 and control mice.

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cleaved Caspase-3 revealed a dramatic and selective increase of apoptotic DCX$^+$ cells in iNestin-Dkk1 mice when compared to controls (Figures 2K–2N). Altogether, these data indicate that other signals from the niche are required for long-term integration of newly generated neurons.

Adult NPCs give rise not only to neurons but also to astrocytes. NPCs are reported to give rise to astrocytes either through direct transformation after they lose quiescence or after a serial round of asymmetric neurogenic divisions (Bonaguidi et al., 2011; Encinas et al., 2011). Thus, the maintenance of neurogenesis could be a result of inhibition of gliogenesis by increased Wnt activity. To test this hypothesis, we examined the generation of newborn astrocytes in the DG of control and Dkk1 mutant animals. iNestin-Dkk1 mice and their control counterparts exhibited comparable numbers of astrocytes (4 week label-retaining BrdU$^+$/Sox2$^+$/S100β^+; Figures 2O and 2P). Thus, increased Wnt signaling in the NPC pool increased their self-renewal and the number of neuronal progenitors without a parallel increase of astrocytes.

Loss of Dkk1 Increases Neuronal Dendritic Complexity

The process of aging selectively decreases dendritic integrity in the DG (Yassa et al., 2011). Accordingly, in 2-year-old control mice, we observed that NBs exhibit atrophic dendrites (Figure 1J). By contrast, age-matched Nestin-Dkk1 mice exhibited highly branched NBs. Wnt activity is a major player in dendrite morphogenesis (Ciani and Salinas, 2005). To determine whether loss of Dkk1 additionally influences the dendritic complexity of NBs, we assessed the dendritic length and branching points of DCX$^+$ cells from Dkk1-deficient and control mice. A gain of Wnt activity significantly increased dendritic complexity of DCX$^+$ cells in 2-year-old Nestin-Dkk1 and 4-month-old iNestin-Dkk1 mice as compared to age-matched control counterparts (Figures 3A–3C). We further investigated whether this increased complexity is maintained in 6-week-old neurons, which are reported to modulate DG function (Denny et al., 2012; Ge et al., 2007). To this end, we used iNestin-YFP-Dkk1 mice and respective controls (iNestin-YFP) and traced the dendrites of 6-week-old eYFP$^+$, NeuN$^+$, and DCX$^-$ neurons 6 weeks after TAM administration. These neurons showed more elaborated dendrites than the mature neurons of control animals (Figures 3D–3F). To assess whether the observed higher complexity had an effect on DG neuronal activity, we examined expression of the neuronal-activity marker Arc (Korb and Finkbeiner, 2011). iNestin-Dkk1 mice had significantly more Arc$^+$-, NeuN$^+$-, and DCX$^-$-active neurons at baseline levels per DG than control mice (Figures 3G and 3H). To further assess whether Dkk1's effect on dendrite morphology is specific to newborn neurons, we examined dendritic morphology of granule cells in the neighboring CA3 region of the hippocampus. The number of branching points and the dendrite length of adult CA3 neurons, as revealed by Golgi staining, did not differ between iNestin-Dkk1 mice and their respective controls (Figures 3I–3K). Together, these data indicate that loss of Dkk1 in the NPC compartment increases the dendritic complexity of newborn NBs and mature neurons, leading to higher neuronal activity in the DG.

Deletion of Dkk1 in NPCs Has Beneficial Effects on Mood

Adult-born neurons are required for the efficacy of antidepressants (Santarelli et al., 2003), and mice lacking neurogenesis show increased susceptibility to stress-induced-depression-like behavior (Snyder et al., 2011). We therefore assessed affective behavior in Dkk1 mutant mice. As previously reported (Sahay et al., 2011a), increased neurogenesis did not influence anxiety-like behavior in the open field (OF; Figures 4A, 4B, and S2). The tail suspension test (TST) is a means of assessing affective behavior that can be improved by antidepressants (Cryan et al., 2005). Importantly, old Dkk1-deficient mutant mice exhibited a lower percentage of immobility in the TST (Figures 4C–4D). Thus, increased neuronal activity in the DG produces behavioral responses similar to those elicited by current antidepressant drugs (Nestler and Hyman, 2010). Affective behavior can also be tested by an animal's decreased interest in pleasurable activities, as a measure of anhedonia (Nestler and Hyman, 2010). This approach is based on symptoms of depression rather than on properties of available antidepressants. To assess whether increased neurogenesis counteracts anhedonic behavior, we tested mice's preference for a sucrose-containing solution over water (Nestler and Hyman, 2010; Snyder et al., 2011). Mice were given free access to a 3% sucrose solution and water for 3 days. The animals' preference for sucrose was tested for 70 min after a 6 hr period of water and sucrose deprivation. Notably, this stress-induced sucrose preference was higher in Nestin-Dkk1 mice than in control counterparts (Figure 4E). The animals' motivation across the different groups was similar, as assessed by the amount of water consumption (Figure 4F). Interestingly, 18-month-old animals were found to have decreased anxiety-like behavior in the OF, a lower percentage of immobility in the TST, as well as a higher sucrose preference than their 3-month-old counterparts (Figure S2),

(F) Tissue from 11-month-old animals was processed 7 months after TAM treatment for ISH of Axin2 and Dkk1 mRNA. Representative pictures of Axin2 (upper panels) and Dkk1 (lower panels) in the DG of control and iNestin-Dkk1 mice are shown. Insets show a higher magnification of the SGZ and GCL of the DG; dashed and dotted lines outline the nonimmunoreactive areas.

(F′) The scheme of ISH results shown in (F), blue circles denote mRNA-expressing cells.

(G and H) NPCs from Nestin-Dkk1 mice generate a higher number of secondary spheres (control versus Nestin-Dkk1: 65.44 ± 1.66 versus 81.88 ± 1.47, $p < 0.001$, [G]) that are of comparable sphere size (control versus Nestin-Dkk1: 69.3 ± 0.9 μm versus 68.3 ± 0.8 μm, [H]).

(I) Dkk1 mRNA expression in the DG of WT mice increases with age (3-month-old [1.00 ± 0.16] versus 16-month-old [1.77 ± 0.14] WT mice; $p = 0.024$; 7-month-old WT mice [1.85 ± 0.28]; $n = 3$). Placenta mRNA served as positive control.

(J) Representative images of neuroblasts in 2-year-old control and Nestin-Dkk1 mice.

(K) Two-year-old Nestin-Dkk1 mice show increased numbers of DCX$^+$ NBs compared to controls (Nestin-Dkk1 versus control; 923 ± 153 versus 580 ± 63; $p = 0.042$; $n = 5–7$).

(C) and (G–H) are representative for at least two independent experiments. Scale bars represent 100 μm (F), 10 μm (insets), or 20 μm (J). An unpaired two-tailed Student's t test was used for statistical analysis (*, $p < 0.05$; ***, $p < 0.001$). Results are presented as mean ± SEM.

See also Figure S1 and Tables S1, S2, and S3.

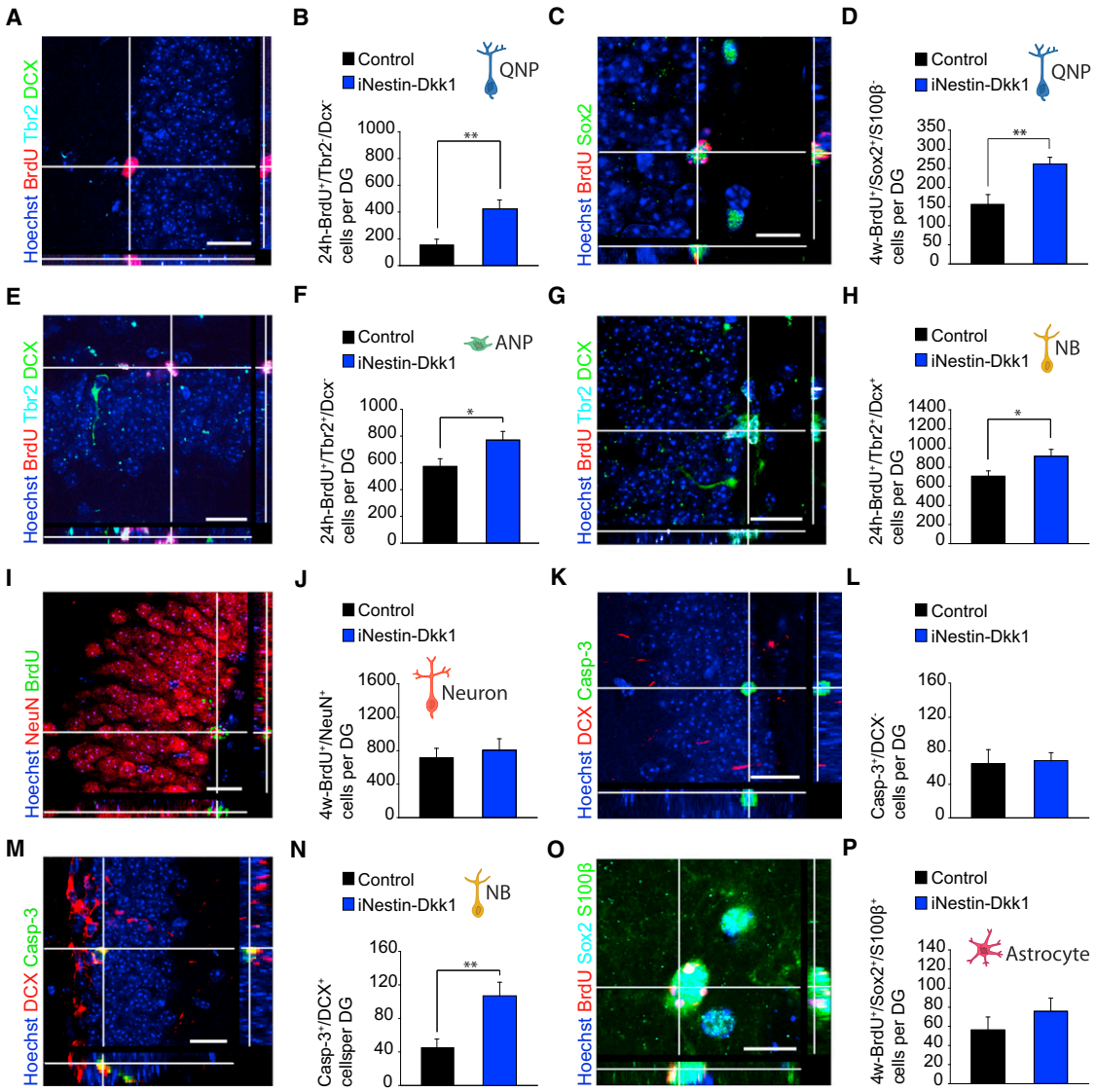

Figure 2. Loss of Dkk1 Increases NPC Self-Renewal and Neuronal Differentiation

(A and B) iNestin-Dkk1 mice show increased numbers of BrdU$^+$/Tbr2$^-$/DCX$^-$ QNPs 24 hr after BrdU labeling compared to controls (424 ± 67 versus 153 ± 45; p = 0.007; n = 6).

(C and D) Increased number of 4 week label-retaining BrdU$^+$/Sox2$^+$/S100β$^-$ QNPs per DG in iNestin-Dkk1 mice compared to controls (261 ± 18 versus 155 ± 26; p = 0.004; n = 7–8).

(E and F) Quantification of 24hr-BrdU$^+$/Tbr2$^+$/DCX$^-$ cells indicates an increase in ANPs per DG in iNestin-Dkk1 mice compared to controls (770 ± 65 versus 573 ± 58; p = 0.047; n = 6).

(G and H) iNestin-Dkk1 mice show increased numbers of newborn NBs (24hr-BrdU$^+$/Tbr2$^+$/DCX$^+$) compared to controls (915 ± 71 versus 705 ± 57; p = 0.043; n = 6).

(I and J) Quantification of 4 week labeled BrdU$^+$/NeuN$^+$ neurons per DG of control and iNestin-Dkk1 mice (714 ± 115 versus 806 ± 137; n = 9–10).

(K and L) Quantification of Casp-3$^+$/DCX$^-$ cells in control and iNestin-Dkk1 mice (65 ± 17 versus 68 ± 10; n = 7–9).

(M and N) iNestin-Dkk1 mice show increased death of immature neurons (Casp-3$^+$/DCX$^+$) compared to control (107 ± 16 versus 45 ± 10; p = 0.009; n = 7–9).

(O and P) Quantification of BrdU$^+$/Sox2$^+$/S100β$^+$ astrocytes four weeks after BrdU administration (control versus iNestin-Dkk1; 56 ± 14 versus 76 ± 14; n = 7–8).

Scale bars represent 20 μm.

Statistical analysis was performed with an unpaired Student's t test (B-J and N-P) or two-tailed Mann-Whitney rank sum test (L). *, p < 0.05; **, p < 0.01. Results are presented as mean ± SEM.

See also Figure S1 and Tables S1, S2, and S3.

indicating that age-related features other than neurogenesis have additional positive effects on affective behavior. Nevertheless, the basal preference for sucrose, as assessed by the sucrose consumption within the first 24 hr, was higher in 11-month-old iNestin-Dkk1 mice than age-matched control mice (Figure S2). Together these data indicate that increased Wnt activity by loss of Dkk1 has an effect on affective behavior in tests that have predictive validity for antidepressive drugs.

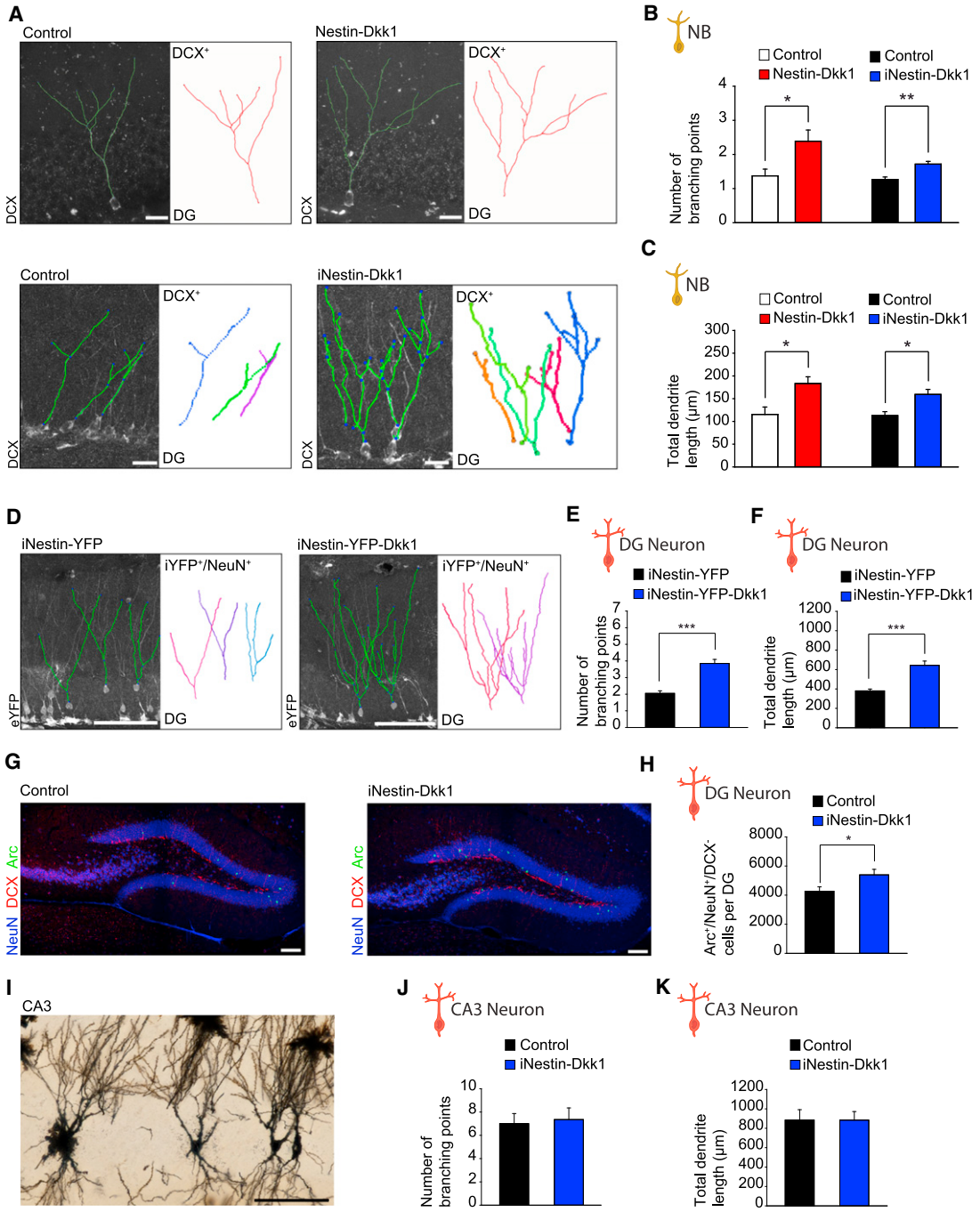

Figure 3. Dkk1 Deletion Increases Dendritic Complexity and Neuronal Activity in the DG

(A) Representative images of maximum projection (left) and 2D projection of 3D reconstruction (right) of DCX-positive immature neurons used for dendritic quantification in 2-year-old Nestin-Dkk1 (upper panels, n = 4–7 animals) or 5-month-old iNestin-Dkk1 mice (lower panels, n = 5 animals) and age-matched controls.

(B) DCX^+ cells of Dkk1 mutant mice have significantly more branching points compared to control mice (control versus Nestin-Dkk1; 1.4 ± 0.2 versus 2.4 ± 0.3; p = 0.049; control versus iNestin-Dkk1; 1.3 ± 0.1 versus 1.7 ± 0.1; p = 0.006).

(C) Dkk1 mutant mice show significantly increased dendritic length of DCX^+ cells (control versus Nestin-Dkk1; 115 ± 16 μm versus 183 ± 15 μm; p = 0.017; control versus iNestin-Dkk1; 113 ± 9 μm versus 159 ± 11 μm; p = 0.016).

(D) Representative images of tracing of 6-week-old neurons of iNestin-YFP and iNestin-YFP-Dkk1 mice. Images of maximum projection (left) and 2D projection of 3D reconstruction (right) are also shown.

(E and F) Newborn neurons, identified as $eYFP^+/NeuN^+/DCX^-$ cells of iNestin-YFP-Dkk1 mice, have significantly more branching points (3.85 ± 0.24 versus 2.05 ± 0.15; p < 0.001; [E]) as well as a significantly increased total dendritic length (643 ± 44 μm versus 380 ± 19 μm; p < 0.001; [F]) compared to neurons from iNestin-YFP mice (n = 2 animals, 10 cells from each animal).

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Loss of Dkk1 Restores Working Memory and Memory Consolidation in Old Age

We have previously shown that animals with reduced neurogenesis exhibit deficits in hippocampus-dependent spatial working memory on the T-maze spontaneous alternation paradigm (Corsini et al., 2009). We therefore hypothesized that the observed increase in the number of neurons with highly complex dendritic arbors in Dkk1 mutant mice would positively impact spatial working memory. Accordingly, increased neurogenesis in iNestin-Dkk1 and Nestin-Dkk1 mice improved working memory in the very same task (Figure 5A). Given that aging decreases neurogenesis as well as some aspects of cognition, we wondered whether spatial working memory is impaired in old mice and, if so, whether it could be restored to levels observed in young animals by the loss of Dkk1. Spontaneous alternation in the T-maze was significantly reduced in 18-month-old animals as compared to 3-month-old animals (Figure 5A). It is noteworthy that the success rate in old Dkk1 mutant animals was similar to the success rate in 3-month-old animals (Figure 5A). Next, we examined learning in the rewarded T-maze and eight-arm radial maze paradigms. Learning of these hippocampus-dependent tasks was comparable in Nestin-Dkk1, iNestin-Dkk1, and control mice (Figure S3). Furthermore, we tested spatial learning in the hippocampus-dependent active place avoidance paradigm, in which the animal is placed on a slowly rotating platform that is open to the room environment and contains spatial cues with a nonrotating 60° shock zone (Figure 5B) (Cimadevilla et al., 2001; Pastalkova et al., 2006). Similarly, spatial learning in this paradigm did not significantly differ between both animal groups (Figure 5C). Importantly, the 24 hr recall of active place avoidance, as measured by the time passed before initial entry into the shock zone, was significantly higher in the iNestin-Dkk1 mice (Figures 5D and S3). The 24 hr recall of place avoidance was significantly lower in 18-month-old animals as compared to 3-month-old animals, whereas learning in the active place avoidance paradigm was similar in both groups (Figures 5E and 5F). Once again, the 24 hr recall was similar in 10-month-old iNestin-Dkk1 mice and 3-month-old wild-type (WT) animals (Figures 5D and 5F). Importantly, to ensure that the observed deficits in the aged group were not related to visual deficits, we performed a novel object recognition task (NOR). Performance in the NOR task was comparable between young and old WT animals, as well as between control and Dkk1 mutant animals (Figure S3). Thus, the observed increase in neuronal activity in the DG positively modulated working memory and long-term retention of stored spatial memory; i.e. memory consolidation. Most importantly, loss of Dkk1 restored working memory and memory consolidation back to levels observed in young animals.

DISCUSSION

In the embryonic brain, Wnt signaling induces self-renewal of radial glia progenitors and differentiation, but not proliferation, of intermediate progenitors (Munji et al., 2011). In adult neurogenesis, the function and site of action of Wnt signals remain controversial. Our study shows that the deletion of Dkk1 in Nestin NPCs results in missing expression of Dkk1 mRNA in the SGZ and also in regions spreading into the GCL. This feature suggests that Dkk1 expression is activated in newly generated clones as a negative feedback signal onto neighboring NPCs. Accordingly, a higher Wnt activity was detected in NPCs but not in cells in the GCL of iNestin-Dkk1 mice. Nevertheless, whether or not the Dkk1-producing NPC is the Axin2-expressing-NPC needs to be addressed by future studies.

Wnt ligands are reported to increase proliferation of adult neural progenitors in vitro and in vivo (Gao et al., 2007; Michaelidis and Lie, 2008; Lie et al., 2005; Qu et al., 2010). However, conditional deletion of β-catenin in Sox2-positive NPCs by retroviral vectors increased the number of neuronal-committed progenitors without influencing the pool of these NPCs (Kuwabara et al., 2009). In contrast, the decreased numbers of NPCs in Wnt7-deficient mice could be rescued by infection with active-β-catenin lentiviral vectors (Qu et al., 2010). Unfortunately, the number of Sox2-positive NPCs in the DG of Wnt7-deficient mice was not examined. The apparent contradiction of in vivo studies on Wnt function in the adult DG is probably due to the use of different viral constructs, which may be biased to infection of specific subsets of NPCs. Conditional deletion of Dkk1 in Nestin-positive NPCs leads to increased self-renewal and/or survival of QNPs and a corresponding increased number of the neurogenic ANPs and NBs. However, there is no corresponding increase of new astrocytes. Interestingly, conditional PTEN deletion in NPCs equally induces increased self-renewal but, as opposed to enhanced Wnt-activity, it also activates astrocytic terminal differentiation (Bonaguidi et al., 2011). This rate of astrocytic differentiation is greater than that of self-renewal, which leads to a net reduction of NPCs (Bonaguidi et al., 2011). Similarly, deletion of the γ2 $GABA_A$ receptor in NPCs increases self-renewal of NPCs but also the number of newborn astrocytes, resulting in long-term NPC depletion (Song et al., 2012). In contrast, loss of Dkk1 does not lead to exhaustion of NPCs, as shown by the enhanced neurogenesis in old Dkk1 mutant mice.

The decrease in adult neurogenesis influences cognitive performance and mood regulation. Increasing adult neurogenesis in young as well as in old animals, as accomplished by voluntary running, has been shown to improve cognitive abilities such as pattern separation (Aimone et al., 2011; Sahay et al., 2011a; Sahay et al., 2011b). Yet, the specific contribution to

(G) Representative images of Arc^+ cells in the DG GCL of Control and iNestin-Dkk1 animals.
(H) iNestin-Dkk1 mice show an increased number of Arc^+-, $NeuN^+$-, and DCX^--active neurons per DG compared to control mice at baseline levels (5,396 ± 376 versus 4,266 ± 302; p = 0.041; n = 6).
(I) Representative image of Golgi staining of CA3 neurons used for dendritic quantification in 10-month-old iNestin-Dkk1 mice.
(J and K) Quantification of branching points (control versus iNestin-Dkk1; 7.0 ± 0.9 versus 7.4 ± 1.0; n = 3; [J]) and dendritic length (control versus iNestin-Dkk1; 884 ± 107 versus 884 ± 88; n = 3; [K]) of CA3 neurons.
Scale bars represent 20 μm (A) or 100 μm (D–I). Statistical analysis was performed with an unpaired two-tailed Student's t test (B, C, and H–K) or a two-tailed Mann-Whitney rank sum test (E and F). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results are presented as mean ± SEM.
See also Tables S1, S2 and S3.

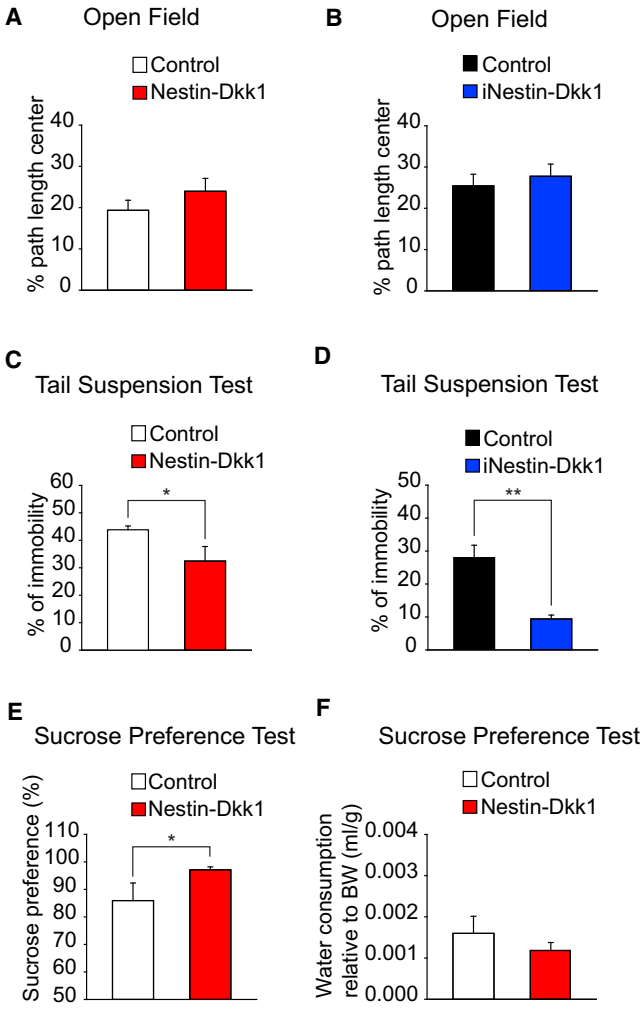

Figure 4. Loss of Dkk1 Has a Beneficial Effect on Affective Behavior

(A and B) Deletion of Dkk1 has no effect on locomotor activity and anxiety-like behavior in the OF in 8-month-old Nestin-Dkk1 and 10-month-old iNestin-Dkk1 mice ("% path length center" displays the proportion of distance traveled in the center to the total distance traveled) (control versus Nestin-Dkk1; 19.4% ± 2.4% versus 24.0% ± 3.1%; n = 5-6 [A]; control versus iNestin-Dkk1; 25.4% ± 2.8% versus 27.8% ± 2.9%; two-tailed Student's t test; n = 9 [B]).

(C and D) Nestin-Dkk1 (C) and iNestin-Dkk1 (D) mice show significantly decreased immobility in the TST compared to control mice (control versus Nestin-Dkk1; 43.9% ± 1.4% versus 32.4% ± 5.3%; p = 0.037; n = 5–7; control versus iNestin-Dkk1; 28.0% ± 3.8% versus 9.4% ± 1.16%; p = 0.002; one-tailed Student's t test; n = 5–7).

(E and F) Nestin-Dkk1 mice show selective increased preference for drinking sucrose-containing water in an acute test (control versus iNestin-Dkk1; 85.9% ± 6.4% versus 97.1% ± 1.1%; p = 0.030; n = 5–8; [E]; control versus iNestin-Dkk1; 0.0016 ± 0.0004 ml/g versus 0.0012 ± 0.0002 ml/g; two-tailed Student's t test; n = 5–8; [F]).

Statistical analysis was performed with an unpaired Student's t test (A, B, and D, and F) or Mann-Whitney rank sum test (C and E). BW, body weight. *, $p < 0.05$; **, $p < 0.01$. Results are presented as mean ± SEM.

See also Figure S2.

these functions of the young versus the mature newly generated neurons remains a matter of debate. At 14 days of age, adult-born neurons can already be considered as functionally relevant to the network and, at up to 6 weeks of age, they exhibit distinct electrophysiological properties such as hyperexcitability and heightened synaptic plasticity of their glutamatergic inputs. Together, these features predict a unique contribution to information processing of these young neurons (Aimone et al., 2010; Ge et al., 2007). Expansion of the population of newborn neurons by genetic deletion of the apoptosis inducer Bax in adult NPCs increases pattern separation in a contextual fear discrimination paradigm (Sahay et al., 2011a). Furthermore, we previously showed that increased neurogenesis by voluntary running enhances working memory (Corsini et al., 2009). However, in those studies, increased neurogenesis was always accompanied by a corresponding increase of mature newborn neurons. The present study shows, on one hand, that loss of Dkk1 enlarges the pool of immature neurons, which could already impact the activity of the neuronal network in the DG and affective behavioral responses, such as pattern separation, at the age of 3 weeks (Nakashiba et al., 2012). On the other hand, we show that increased complexity of the dendritic arbors of newborn neurons, but not of their number, might be another way to positively impact neuronal activity in the DG and performance in spatial memory tasks. In this issue of *Cell Stem Cell*, Jang et al. (2013) show that neuronal activity regulates neurogenesis via another Wnt antagonist, secreted frizzled-related protein 3 (sFRP3). DG neuronal activity induces expression of sFRP3 on mature DG granule neurons, which in turn reduces the self-renewal activity of NPCs and the speed of dendritic maturation in newborn neurons without affecting NPCs' fate choice. These two papers illustrate two different negative feedback signals to neurogenesis that are linked to DG neuronal activity with apparently different sources but with some shared and distinct actions in NPCs and their progeny.

Whereas the dorsal hippocampus is more involved in memory and cognitive processing, the ventral part is rather involved in complex behavior, such as stress or emotions, and as such is strongly implicated in schizophrenia and depression (Fanselow and Dong, 2010). Ablation of adult hippocampal neurogenesis decreases the beneficial effects of some antidepressant drugs (David et al., 2009; Santarelli et al., 2003). In addition, chronic antidepressant treatment enhances neurogenesis in the DG (Hanson et al., 2011). However, disruption of neurogenesis does not produce depression-like behavior (Jayatissa et al., 2009; Surget et al., 2008). Notably, disruption of neurogenesis does increase stress-induced-depression-like behavior (Snyder et al., 2011). Interestingly, chronic stress has been shown to induce Dkk1 expression in the DG (Matrisciano et al., 2011). Hence, Dkk1 might be a major player in stress-related depression. Here, we show that enhanced neurogenesis and neuronal activity decrease immobility in the TST. Similarly, Kim et al. (2012) recently showed that an increase of neuronal activity in the hippocampal CA1 region by knockout of HCN1 produced anxiolytic- and antidepressant-like effects. In contrast to our data, increased neurogenesis by genetic deletion of Bax did not influence immobility in the forced swimming test (FST). We believe that discrepancies between the two studies are due to the higher sensitivity of the TST versus the FST in mice (Duman, 2010). Nevertheless, the observed reduced behavioral despair in the TST is further supported by the increased hedonic behavior exhibited by Dkk1-deficient mice.

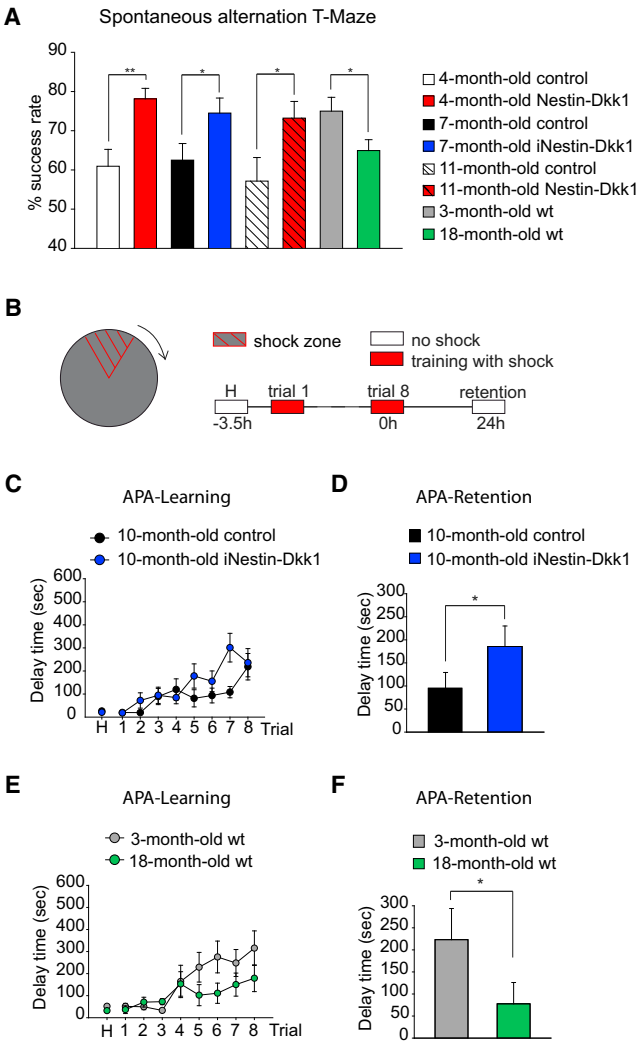

Figure 5. Loss of Dkk1 Restores Age-Related Memory Decline

(A) Here, 4- and 11-month-old Nestin-Dkk1 and 7-month-old iNestin-Dkk1 mice show significantly increased success rates in the spontaneous alternation in the T-maze compared to control mice (4-month-old control versus Nestin-Dkk1: 60% ± 4% versus 78% ± 3%, p = 0.007, n = 10; 7-month-old control versus iNestin-Dkk1:63% ± 4% versus 75% ± 4%, p = 0.04996, n = 10; 11-month-old Nestin-Dkk1 versus control: 57% ± 6% versus 73% ± 4%, p = 0.0496, n = 7). The 18-month-old WT animals perform significantly worse in this test compared to 3-month-old WT mice (65% ± 3% versus 75% ± 4%; p = 0.035; n = 12). Loss of Dkk1 rescues age-related decrease in working memory and restores it to levels observed in young animals (4-month-old Nestin-Dkk1, 7-month-old iNestin-Dkk1, and 11-month-old Nestin-Dkk1 versus 3-month-old WT; p = 0.569, p = 0.924, and p = 0.755, respectively).

(B) Schematic outline of the active place avoidance paradigm, presenting the shock zone on a rotating platform with surrounding spatial cues. One shock-free habituation trial is followed by eight shock-containing learning trials. One shock-free retention trial was performed after 24 hr.

(C) iNestin-Dkk1 mice show no difference in delay times in entering the shock zone during learning trials compared to control mice (two-way repeated-measures ANOVA of group and trial: $F_{(1,33)} = 2.17$, p = 0.150 [group]; $F_{(5,148)} = 2.13$, p = 0.072; n = 17–18 [group × trial]).

(D) iNestin-Dkk1 mice show significantly increased delay times compared to controls in entering the shock zone in the retention trial (controls versus iNestin-Dkk1; 95 ± 34 s versus 186 ± 45 s; p = 0.036; n = 17–18).

(E) Here, 3- and 18-month-old WT mice show comparable delay times in entering the shock zone during learning trials (two-way repeated-measures ANOVA of group and trial; $F_{(1,22)} = 2.50$, p = 0.128 [group]; $F_{(5,99)} = 1.55$, p = 0.188; n = 12 [group × trial]).

(F) Here, 3-month-old WT mice show significantly increased delay times in entering the shock zone in the retention trial compared to 18-month-old WT mice (3- versus 18-month-old mice: 223 ± 71 s versus 78 ± 48 s, p = 0.030, n = 12). Deletion of Dkk1 in NPCs rescues age-related decreased memory consolidation and restores young levels (10-month-old iNestin-Dkk1 versus 3-month-old WT: 186 ± 45 s versus 223 ± 71 s, p = 0.832). All four groups—10-month-old Dkk1 mutants and respective controls as well as 3- and 18-month-old WT animals— show similar capacity to learn the active place avoidance paradigm (two-way repeated-measures ANOVA of group and trial; $F_{(3,55)} = 1.77$, p = 0.163 [group]; $F_{(14,261)} = 1.48$, p = 0.117 [group × trial]). H, habituation; APA, active place avoidance.

Statistical analysis was performed with an unpaired two-tailed Student's t test (A), with two-way repeated-measures ANOVA (C and E), or with two-tailed Mann-Whitney rank sum test (D and F). *, p < 0.05; **, p < 0.01. Results are presented as mean ± SEM.

See also Figure S3.

The age-related decline of adult neurogenesis has been strongly associated with memory impairments in the elderly population. Functional MRI studies of aging human subjects, aging rhesus monkeys, and aging mice show that aging itself preferentially affects the DG, as opposed to Alzheimer's disease, which instead affects the entorhinal cortex (Small et al., 2011). In WT mice, the number of proliferating cells and NBs in the SGZ of the DG declines exponentially in the first 9 months of life, with a 40% decline in the number of proliferating cells already observed in 2-month-old animals as compared to 1-month-old animals, until it is barely present in 18-month-old mice (Ben Abdallah et al., 2010). The number of NPCs in old animals is slightly lower than in younger counterparts. In particular, an 80% decrease of the more proliferative population of NPC has been observed in aged mice (Lugert et al., 2010). Along this line, activated progenitors disappear with time through terminal differentiation into astrocytes (Lugert et al., 2010; Encinas et al., 2011). Accordingly, neurogenesis decreases with age while gliogenesis does not (Jinno, 2011). In addition, remaining NPCs are more quiescent and endorsed with shorter telomeres due to reduced telomerase activity (Jaskelioff et al., 2011). One recognized cause of the DG differential vulnerability to the process of aging is its high levels of mineralocorticoid receptors, which make the DG especially sensitive to the high levels of circulating corticosteroids in old individuals (Cameron and McKay, 1999; Sloviter et al., 1989). Other blood-borne factors that similarly increase with age, such as CCL11, reduce neurogenesis (Villeda et al., 2011). Besides an aging systemic milieu, the aged neurogenic niche produces fewer neurotrophic factors, becoming less conducive for neurogenesis (Lee et al., 2012). Adrenalectomy and exercise are therefore interventions that restore neurogenesis in old age. Here, we report that Dkk1 is another factor that increases with age and that loss of Dkk1 re-establishes the ability of aged NPCs to self-renew and generate new neurons. Neutralizing antibodies to Dkk1 are in clinical trials for enhancement of Wnt signaling in osteoporosis (Rey and Ellies, 2010). Our study raises the possibility that neutralization of Dkk1 might be beneficial in counteracting depression-like behavior and improving cognitive decline in the aging population. Altogether, this study defines Dkk1 as a major cause of age-related decline in neurogenesis. It also helps to clarify the role of Wnt activity in adult

neurogenesis. The contribution of newly generated young neurons to memory and affective behavior opens tantalizing opportunities for the prevention of affective impairments and age-related cognitive decline.

EXPERIMENTAL PROCEDURES

Animals

Dkk1-floxed (Dkk1$^{f/f}$), Dkk1$^{+/-}$ (Pietilä et al., 2011), and eYFP$^{f/f}$ mice [B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J] were bred with Nestin-Cre or Nestin-CreER^{T2} mice, both on a C57BL/6 background, to generate Dkk1$^{-/f}$ Nestin-Cre (Nestin-Dkk1), Dkk1$^{f/f}$ Nestin-CreER^{T2} (iNestin-Dkk1), and eYFP$^{+/f}$ Nestin-CreER^{T2} (iNestin-YFP) mice. Controls for Nestin-Dkk1 mice were Dkk1$^{+/+}$, Dkk1$^{+/f}$, Dkk1$^{+/-}$, or Dkk1$^{+/f}$ Nestin-Cre mice. Next, 8- to 12-week-old Nestin-CreER^{T2} mice and respective controls (Dkk1$^{f/f}$ or Dkk1$^{+/+}$ Nestin-CreER^{T2}) were intraperitoneally (i.p.) injected twice a day for 5 days with Tamoxifen (TAM; 2 mg/day). In vivo neurogenesis was examined for 5 weeks and neuronal morphology analysis for 6 weeks after TAM administration. For behavioral experiments, 3- and 18-month-old C57BL/6 mice (WT mice) were used.

Animals were housed in the animal facilities of the German Cancer Research Center (DKFZ) at a 12 hr dark/light cycle and had free access to food and water. For experiments, animals were age-matched. All animal experiments were performed in accordance with the institutional guidelines of the DKFZ and were approved by the Regierungspräsidium Karlsruhe (DKFZ206 and G-179/10), Germany.

In Vivo Neurogenesis

In vivo examination of neurogenesis was performed as previously described (Corsini et al., 2009). In brief, for examination of ongoing adult hippocampal neurogenesis, age-matched iNestin-Dkk1 and control mice were injected with 300 mg/kg BrdU and perfused after 24 hr for analysis of BrdU-positive cells. Alternatively, for the study of newborn cell survival, mice were injected on three consecutive days with 300 mg/kg/day BrdU and perfused 4 weeks later for the analysis of BrdU-positive cells. Nestin-Dkk1 and control mice were perfused with 4% PFA. For each animal, confocal stacks of six vibratome coronal brain slices (50 μm thick, 250 μm apart) were acquired on a Leica TCS-SP5 or a Nikon C1S1 confocal microscope and cells were counted. For Figures 1J, 1K, 3A–3C (Nestin-Dkk1), and S1D, sagittal brain sections were used. Cell numbers were normalized to the volume of the DG granule cell layer measured by ImageJ.

Neuronal Morphology Analysis

For analysis of neuronal morphology of DCX-positive or eYFP-positive cells in the DG, the Amira Filament Editor (Visage Imaging) was used as described previously (Corsini et al., 2009). In brief, for each mouse, three brain slices (50 μm thick, 250 μm apart) were stained for DCX or eYFP, DCX, and NeuN and high-resolution confocal stacks were acquired on a Leica TCS-SP5 or Nikon C1S1. For analysis of neuronal morphology of mature CA3 neurons, PFA-fixed brains were cut into two hemispheres and stained with the FD Rapid GolgiStain Kit (FD NeuroTechnologies). Brains were cut with a vibratome into 100-μm-thick slices, mounted onto gelatine-coated glass slides, and embedded with Eukitt. Stacks were recorded on a Leica TCS-SP5 microscope. Branching points and total dendrite length were measured with the use of the Amira Filament Editor.

Behavioral Tests

Detailed descriptions of behavioral experimental procedures are described in the Supplemental Information.

Statistics

Statistical analyses were performed with the use of Sigma Plot, the program package ADAM from the Biostatistics Unit of the DKFZ, and the *car* package in the R Project for Statistical Computing (http://www.r-project.org/). An unpaired two-tailed Student's t test was used for normal distributed data. For non-normal distributed data or data sets with different variances, the Mann-Whitney rank sum test was used. For learning data sets, a two-way repeated-measures ANOVA (analysis of variance) was used. Sphericity was tested with Mauchly's test. The Greenhouse-Geisser correction was used to correct for departures from sphericity. The alpha level for all tests was 5%. Results are presented as mean ± SEM. A summary of cell count with respective statistical analysis can be found in Table S3.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, three figures, and three tables and can be found with the article online at http://dx.doi.org/10.1016/j.stem.2012.11.010.

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