

Wnt5a is essential for hippocampal dendritic maintenance and spatial learning and memory in adult mice

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Stability of neuronal connectivity is critical for brain functions, and morphological perturbations are associated with neurodegenerative disorders. However, how neuronal morphology is maintained in the adult brain remains poorly understood. Here, we identify Wnt5a, a member of the Wnt family of secreted morphogens, as an essential factor in maintaining dendritic architecture in the adult hippocampus and for related cognitive functions in mice. Wnt5a expression in hippocampal neurons begins postnatally, and its deletion attenuated CaMKII and Rac1 activity, reduced GluN1 glutamate receptor expression, and impaired synaptic plasticity and spatial learning and memory in 3-mo-old mice. With increased age, Wnt5a loss caused progressive attrition of dendrite arbors and spines in Cornu Ammonis (CA)1 pyramidal neurons and exacerbated behavioral defects. Wnt5a functions cell-autonomously to maintain CA1 dendrites, and exogenous Wnt5a expression corrected structural anomalies even at late-adult stages. These findings reveal a maintenance factor in the adult brain, and highlight a trophic pathway that can be targeted to ameliorate dendrite loss in pathological conditions.

autocrine Wnt signaling | dendrite arbors | adult hippocampus

Long-term structural maintenance of neuronal networks is essential for sustaining brain functions. The size and pattern of dendrite arbors dictate the ability of neurons to receive and integrate synaptic inputs and are thus critical determinants of information processing in the brain. Established by periods of dynamic growth during development, dendrite arbors and spines are thought to be largely stable in adulthood and are maintained for the lifetime of an organism (1). The significance of this maintenance phase for normal brain functions is underscored by evidence that late-onset retraction of dendritic arbors and spine loss are the most consistent morphological correlates of several neurological and psychiatric disorders, including schizophrenia, major depressive disorder, anxiety, and Alzheimer's disease (2–5). Thus, specific molecular signals must exist to ensure the maintenance of neuronal morphology and synaptic connectivity in the adult nervous system.

To date, understanding of the molecular cues that maintain adult dendritic patterns has been limited, and largely originates from studies of developmental signals that are deployed later during postnatal life to regulate maturation or stability (1, 6). Brain-derived neurotrophic factor (BDNF) provides an example of an extrinsic signal that is required throughout life for both the establishment and maintenance of neuronal connectivity. Conditional deletion of either *BDNF* or its receptor, *TrkB*, leads to a reduction in dendritic complexity of adult cortical neurons (7, 8). Intriguingly, deletion of *BDNF* or *TrkB* does not affect dendrite architecture in the adult hippocampus, a brain structure critical for spatial learning and memory and anxiety (7, 9–13). Currently, little is known about dendrite support mechanisms in adult hippocampal neurons. Cornu Ammonis (CA)1 pyramidal neurons in the hippocampus are particularly vulnerable in Alzheimer's disease

and exhibit extensive dendrite arbor loss that correlates with the degree of cognitive decline (14). Identification of adult maintenance mechanisms would be highly relevant to the understanding of the structural basis of hippocampus-dependent behaviors, as well as the etiology of neurodegenerative diseases where extensive dendritic anomalies are manifested late in life.

Here, using neuron-specific deletion in mice, we identify *Wnt5a*, a member of the Wnt family of developmental morphogens, as an essential factor for the long-term stability of dendritic architecture in the adult hippocampus. Previous studies have implicated *Wnt5a* in regulating developmental axon and dendrite outgrowth and synapse formation in cultured hippocampal neurons (15–20). Here, we report that *Wnt5a* deletion does not compromise hippocampal development or maturation in vivo, but results in striking adult-onset defects in dendrite arborization, lengths, and spine densities in CA1 hippocampal pyramidal neurons that manifest after 4 mo of age in mice. *Wnt5a* is required cell-autonomously in adult neurons to maintain dendritic architecture. Loss of *Wnt5a* impairs hippocampal synaptic plasticity and spatial learning and memory in adult mice before the onset of dendritic regression,

Significance

The maintenance of neuronal morphology in the adult brain is an understudied area. Here, using tissue-specific deletion in mice, we reveal *Wnt5a*, a member of the Wnt family of developmental morphogens, as an essential factor for the long-term stability of dendritic architecture in the adult hippocampus. *Wnt5a* influences synaptic plasticity and related cognitive functions in the mature hippocampus through CaMKII-mediated signaling, Rac1-dependent actin dynamics, and cyclic AMP-responsive element binding-mediated NMDA receptor biosynthesis. In the long-term, *Wnt5a*-mediated regulation of cytoskeletal signaling and excitatory synaptic transmission is responsible for the maintenance of dendritic arbors and spines in adult CA1 pyramidal neurons. These findings provide insight into the poorly understood structural maintenance mechanisms that exist in the adult brain.

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although the behavioral deficits are exacerbated with the appearance of structural abnormalities. *Wnt5a* acts via calcium- and cytoskeletal-mediated signaling in the adult hippocampus, and unexpectedly, via cyclic AMP-responsive element binding (CREB)-mediated transcription of the obligatory NMDA receptor subunit, GluN1. Finally, we demonstrate that late expression of *Wnt5a*, even after substantial structural loss, fully restores neuronal morphology, highlighting the growth-promoting capacity of this pathway in the adult brain. Together, these findings reveal that noncanonical autocrine Wnt signaling maintains adult hippocampal connectivity and synaptic plasticity, and provide a trophic pathway that can be targeted to counter structural deficits in pathological situations.

Results

Cell-Autonomous Requirement for *Wnt5a* in Maintaining Adult CA1 Dendrite Arbors. We observed *Wnt5a* expression in the mouse hippocampus at 1 wk after birth, which increased prominently by 2 wk and was sustained at adult stages (Fig. 1*A* and *B*). *Wnt5a* mRNA was localized throughout the hippocampal formation, and was enriched in the dentate gyrus and the CA1 region. The expression pattern in the hippocampus is consistent with previous findings (21) and that in the Allen Brain Atlas (www.brain-map.org/), where *Wnt5a* expression has also been noted in the cerebellum and to a lesser degree in the cerebral cortex and olfactory bulb in the adult mouse brain. The onset of *Wnt5a* expression in hippocampal neurons correlated with the appearance of pre- and postsynaptic proteins (Fig. 1*B*).

That prominent hippocampal *Wnt5a* expression is detected only at postnatal stages was intriguing, given the reported roles of *Wnt5a* in embryonic processes in the brain (15, 22–25). *Wnt5a* expression in the postnatal hippocampus, together with evidence supporting the role of Wnts in regulating morphological changes in cultured hippocampal neurons (26, 27), prompted us to address the functions of *Wnt5a* in hippocampal neurons in vivo. To accomplish this end, we crossed floxed *Wnt5a* (*Wnt5a*^{fl/fl}) mice with the pan-neuronal *Nestin-Cre* line (28) to delete *Wnt5a* in all neurons starting at embryonic stages (Fig. S1*A*). *Wnt5a* was undetectable in the *Nestin-Wnt5a*^{fl/fl} hippocampus throughout postnatal ages, including postnatal day 21 (P21) by in situ hybridization (Fig. S1*B*). Importantly, quantitative PCR (qPCR) analysis showed that levels of other *Wnts* were unaltered in the absence of *Wnt5a* (Fig. S1*C* and *D*), indicating that *Wnt5a* loss did not elicit compensatory changes in the expression of other *Wnt* genes in the hippocampus.

Despite the early deletion of *Wnt5a* (Fig. S1*A–C*), there were no obvious differences in gross morphology and projections of hippocampal layers between *Nestin-Wnt5a*^{fl/fl} mice and control *Wnt5a*^{fl/fl} littermates at 1 mo (Fig. S1*E*), when hippocampal neural circuit establishment should be complete (29–31). Detailed examination of dendritic morphology of CA1 hippocampal pyramidal neurons revealed normal dendrite length, complexity, and spine densities in 1-mo-old mutant mice (Fig. S1*F–J*). These results show that *Wnt5a* is dispensable for establishing dendritic arbors and for spine formation in CA1 pyramidal neurons in vivo, contrary to published reports that *Wnt5a* promotes neuronal morphogenesis in cultured hippocampal neurons (15, 18, 19, 32).

To next address if *Wnt5a* might function in the mature hippocampus, we crossed *Wnt5a*^{fl/fl} mice with calcium-calmodulin kinase II (*CaMKII*) α -*Cre* mice, where *Cre* expression starts at 2.5 wk after birth and is restricted to forebrain excitatory neurons in the hippocampus and cortex (33). *Wnt5a* deletion was near complete in the *CaMKII-Wnt5a*^{fl/fl} hippocampus by 3 mo of age (Fig. S2*A* and *B*), whereas other *Wnts* showed normal expression (Fig. S2*C*). Surprisingly, we observed a decrease in the thickness of the CA1 dendritic layers in 6-mo-old *CaMKII-Wnt5a*^{fl/fl} mice using MAP2 immunostaining, although hippocampal cyto-architecture and axonal projections were unaffected (Fig. S2*D*). To better visualize the morphologies of indi-

vidual CA1 neurons and their processes and to pinpoint the onset of dendritic defects triggered by the postnatal loss of *Wnt5a*, we crossed *CaMKII-Wnt5a*^{fl/fl} mice with *Thy1-GFP-M* transgenic mice that have mosaic GFP expression in the hippocampus (34), and analyzed neuronal structure at different ages from 3 to 12 mo (Fig. 1*C*). Sparsely labeled CA1 pyramidal neurons in *Thy1-GFP;CaMKII-Wnt5a*^{fl/fl} mice had normal dendrite arbor complexities and lengths at 3 mo of age (Fig. 1*D, H, L, and M*). However, by 4.5 mo, *Thy1-GFP;CaMKII-Wnt5a*^{fl/fl} neurons showed striking dendritic deficits that progressively declined in older animals (Fig. 1*E–G, and I–M*). Based on Sholl analyses, distal dendrites at distances 50 μ m and farther from the soma were more severely affected than proximal dendrites (Fig. 1*I–K*). In control mice, the total dendritic length remained remarkably stable between 3 and 12 mo of age (4,866 \pm 175 μ m at 3 mo vs. 4,978 \pm 162.7 μ m at 12 mo) (Fig. 1*L*). In contrast, *CaMKII-Wnt5a*^{fl/fl} mice exhibited a pronounced decrease in dendritic length between 3 and 12 mo of age (4,780 \pm 132.4 μ m at 3 mo vs. 2,810 \pm 62.36 μ m at 12 mo) (Fig. 1*L*). Quantification of dendritic spine densities also revealed a significant reduction (31.5% decrease) in 6-mo-old *CaMKII-Wnt5a*^{fl/fl} mice compared with controls (Fig. S2*E* and *F*). Despite the profound dendritic shrinkage in adult *CaMKII-Wnt5a*^{fl/fl} mice, there was no overt loss of neurons in these animals even at 12 mo (Fig. S2*G* and *H*). Together, these results indicate a requirement for *Wnt5a* in the maintenance of dendrite arbors and spine densities in adult CA1 neurons.

Wnts are known to act either as autocrine (cell-autonomous) or paracrine (noncell-autonomous) secreted factors. Because *Wnt5a* is deleted from all excitatory hippocampal neurons in *CaMKII-Wnt5a*^{fl/fl} mice, we are unable to distinguish between these two modes of signaling using the conditional mutants. To determine whether *Wnt5a* has a cell-autonomous or noncell-autonomous role in the hippocampus, we performed mosaic analyses by lentiviral delivery of GFP-T2A-*Cre* or GFP alone into subsets of hippocampal neurons in *Wnt5a*^{fl/fl} animals. Viral infections were done at 2 mo and mice harvested 3 mo later for morphological analyses (Fig. 1*N*). We found stunted dendritic arbors in sparse GFP-labeled CA1 neurons with significant reductions in branch complexity and length in *Wnt5a*^{fl/fl} animals infected with GFP-T2A-*Cre* but not GFP alone (Fig. 1*O–R*). Thus, mosaic *Wnt5a* elimination causes poorly branched and diminished dendritic arbors in isolated *Cre*-expressing neurons despite the presence of non-*Cre*-expressing neighboring neurons that are capable of releasing *Wnt5a*. These results suggest that *Wnt5a* secreted from adult CA1 neurons maintains neuronal morphology in an autocrine manner, likely because of limited diffusibility attributed to the lipid-modified and hydrophobic nature of Wnts (35).

***Wnt5a* Is Essential for Hippocampal Synaptic Plasticity.** The dramatic changes in adult neuronal morphology with *Wnt5a* loss prompted us to ask whether *Wnt5a* is essential for hippocampal synaptic transmission in vivo. We performed electrophysiological recordings in *CaMKII-Wnt5a*^{fl/fl} mice at 3 mo of age, a time when CA1 neuronal morphology is still intact, and at 6 mo when the morphology is impaired. We observed that basal synaptic transmission is normal in *CaMKII-Wnt5a*^{fl/fl} mice at 3 mo but not at 6 mo, consistent with impaired dendritic structures at this age (Fig. 2*A–D*). We also measured the probability of synaptic release at presynaptic sites by paired pulse facilitation (PPF) analyses and found that PPF was comparable between *CaMKII-Wnt5a*^{fl/fl} mice and control littermates at both 3 and 6 mo of age (Fig. S3). The normal presynaptic properties in *Wnt5a*-deficient mice suggests that *Wnt5a* acts primarily at postsynaptic sites.

Hippocampal neurons exhibit prominent synaptic plasticity in which activity-dependent modulation of the strength of synaptic connections underlies learning and memory (36). Previously, broad-spectrum Wnt antagonists have been shown to affect synaptic structure, plasticity, and cognitive functions in adult

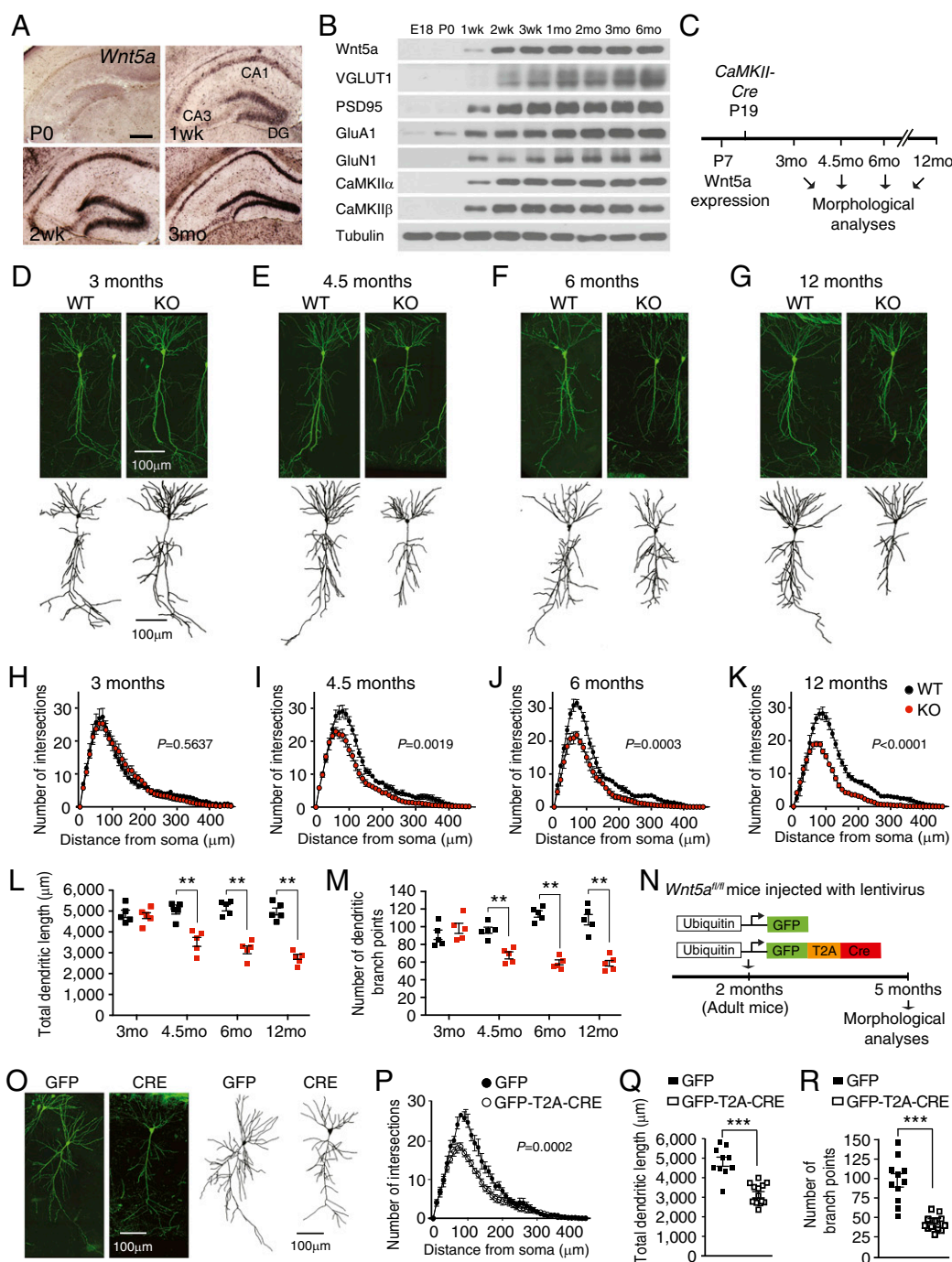


Fig. 1. *Wnt5a* is required cell-autonomously for maintenance of adult CA1 dendrite architecture. (A) In situ hybridization shows *Wnt5a* transcript in the mouse hippocampus at postnatal stages. *Wnt5a* is absent at the day of birth (P0) but is detected by 1 wk and is abundant in the 3-mo-old hippocampus. (Scale bar, 300 μ m.) (B) Onset of *Wnt5a* protein expression coincides with the appearance of pre- and postsynaptic proteins in hippocampal neurons. Hippocampal homogenates from mice of different ages (ranging from E18 to 6 mo) were immunoblotted with antibodies against *Wnt5a*, VGLUT1, PSD95, GluA1, GluN1, CaMKII α , CaMKII β , and tubulin. (C) Schematic of the strategy to assess effects of postnatal *Wnt5a* deletion on neuronal morphologies in adult mice at different ages using *CaMKII α -Cre* transgenic mice. (D–G) Representative images of GFP⁺ CA1 pyramidal neurons show that dendritic arbors are normal at 3 mo, but become progressively stunted with age in *Thy1-GFP;CaMKII-Wnt5a^{fl/fl}* (KO) mice compared with control *Thy1-GFP;Wnt5a^{fl/fl}* mice (WT). (Scale bar, 100 μ m.) Shown below are 3D reconstructions of neuronal soma and dendrites from WT and KO mice at 3, 4.5, 6, and 12 mo using Imaris. (H–K) Sholl analyses show that dendritic complexity is unaffected in 3-mo-old KO mice but significantly decreased by 4.5 mo and declines in older KO mice. Results are mean \pm SEM from five neurons traced per animal and a total of five mice per genotype. Two-way ANOVA with Bonferroni post hoc test. (L and M) Total dendrite length and number of dendritic branch points are unaltered in 3-mo-old KO mice, but progressively decreases from 4.5 mo to 12 mo in older KO mice. Results are mean \pm SEM from five neurons traced per animal and a total of five mice per genotype. *** P < 0.01 two-tailed t test. (N) *Wnt5a^{fl/fl}* mice were stereotactically injected with lentiviral vectors expressing GFP-T2A-CRE or GFP at 2 mo and dendritic morphologies analyzed after 3 mo. (O) Dendritic arbors were stunted in Cre-expressing neurons compared with GFP-expressing neurons. (Scale bar, 100 μ m.) (P) Sholl analysis shows decreased dendrite arbor complexity in sparsely labeled Cre-expressing neurons compared with controls. Two-way ANOVA with Bonferroni post hoc test. (Q and R) Dendritic lengths and branch points are reduced in Cre-expressing neurons compared with controls. Results are mean \pm SEM from 14 GFP-T2A-CRE- and 10 GFP-infected neurons from a total of 10 *Wnt5a^{fl/fl}* mice. *** P < 0.001, two-tailed t test.

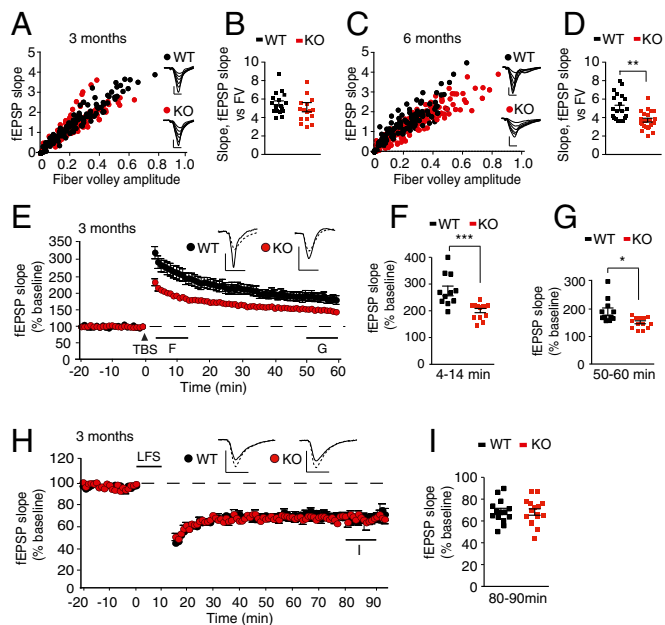


Fig. 2. Wnt5a loss impairs LTP in the absence of changes in basal synaptic transmission or neuronal morphology. (A–D) Basal synaptic transmission is unaffected in *CaMKII-Wnt5a^{fl/fl}* (KO) mice at 3 mo but impaired at 6 mo compared with *Wnt5a^{fl/fl}* litter-mates (WT). The input–output relationships and slopes of input–output curves are normal in 3-mo-old KO mice, but significantly different from WT at 6 mo. Results are mean \pm SEM from five animals per genotype ($n = 18$ slices for WT and 17 slices for KO mice) at 3 mo, and from five WT mice ($n = 21$ slices) and four KO mice ($n = 23$ slices) at 6 mo. $**P < 0.01$, two-tailed t test. [Scale bars, 1 mV (vertical), 2.5 ms (horizontal) for all sample traces.] (E) Impaired LTP at the Schaffer collateral–CA1 synapses in 3-mo-old KO mice. $n = 11$ slices from 5 WT mice and $n = 13$ slices from 6 KO mice. Sample traces represent fEPSPs (field excitatory postsynaptic potentials) right before (dashed line) and 1 h after (solid line) θ -burst stimulation. [Scale bars, 1 mV (vertical), 10 ms (horizontal) for both sample traces.] (F and G) Induction and maintenance of LTP are attenuated in KO mice. Results are mean \pm SEM from five WT mice ($n = 11$ slices) and six KO mice ($n = 13$ slices). $*P < 0.05$, $***P < 0.001$, two-tailed t test. (H) Low-frequency stimulation-induced LTD at Schaffer collateral–CA1 synapses is unaltered in 3-mo *CaMKII-Wnt5a^{fl/fl}* mice. Sample traces represent fEPSPs right after (dashed line) and 1 hr after (solid line) stimulation. [Scale bars, 1 mV (vertical), 10 ms (horizontal) for both sample traces.] (I) Mean fEPSP slopes are comparable between the genotypes. Results are mean \pm SEM from five *Wnt5a^{fl/fl}* mice ($n = 14$ slices) and five *CaMKII-Wnt5a^{fl/fl}* mice ($n = 16$ slices).

organisms (37–41). However, there are 19 vertebrate Wnts, and which Wnt is essential for these functions in the adult brain in vivo remains unknown. To assess the role of Wnt5a in NMDA receptor-dependent long-term potentiation (LTP), an electrophysiological correlate of strengthening of synaptic transmission, we used θ -burst stimulation to induce LTP at Schaffer collateral–CA1 synapses in 3-mo-old mice. Recordings from *Wnt5a^{fl/fl}* control slices revealed a robust induction of LTP and a sustained maintenance phase (Fig. 2 E–G). In contrast, *CaMKII-Wnt5a^{fl/fl}* slices showed a significant reduction in both induction ($274.3 \pm 18.2\%$ in control slices vs. $201 \pm 7.9\%$ in *CaMKII-Wnt5a^{fl/fl}* slices, $P = 0.0002$) and maintenance ($190.8 \pm 13.3\%$ in control slices vs. $151.9 \pm 5.4\%$ mutant slices, $P = 0.01$) phases of LTP (Fig. 2 E–G). The impairment in LTP detected at 3 mo in *CaMKII-Wnt5a^{fl/fl}* mice when neuronal structure and basal synaptic transmission are still intact, suggests that synaptic plasticity is more susceptible to the loss of Wnt5a.

To address if Wnt5a contributes to NMDA receptor-dependent long-term depression (LTD), an electrophysiological correlate of weakening of synaptic transmission, we used a standard low-frequency stimulation paradigm to induce LTD in the CA1 hippocampus. In contrast to the LTP defect, we found no differences in LTD between mutant and control mice at 3 mo of age (Fig. 2 H and I).

Taken together, these results indicate a specific role for Wnt5a in the potentiation of synaptic efficacy.

Wnt5a Is Essential for Spatial Learning and Memory. Synaptic plasticity is widely considered to be a cellular mechanism that underlies learning and memory (42, 43). In addition, structural maintenance of synaptic connectivity has been postulated to be critical for life-long memories (44). Given the decreased CA1 LTP in 3-mo-old *CaMKII-Wnt5a^{fl/fl}* mice, we subjected *Wnt5a* mutant mice to behavioral paradigms to evaluate cognitive functions. In the novel-object recognition test, which evaluates the preference of mice to explore a new over a familiar object (45), adult *Wnt5a^{fl/fl}* mice showed a significant preference for the novel object, with 3- and 6-mo-old mice spending $70.8 \pm 5.5\%$ and $68.1 \pm 8.1\%$ of time exploring the novel object, respectively. However, *CaMKII-Wnt5a^{fl/fl}* mice showed no preference for the novel object at both 3 and 6 mo of age (Fig. 3 A and B), indicating a deficit in recognition memory.

To test hippocampus-dependent spatial learning in these mice, we used the Morris water maze to test an animal's ability to use spatial cues to locate a hidden platform in a tank of water (46) (Fig. S4A). Importantly, *CaMKII-Wnt5a^{fl/fl}* mice had visual acuity and swimming speeds comparable to littermate controls (Fig. S4 B and C). Three-month-old *CaMKII-Wnt5a^{fl/fl}* mice took significantly longer time to locate the hidden platform during the initial training period of 12 consecutive days using four trials per day (see schematic in Fig. 3C), compared with age-matched controls, eventually achieving similar latencies as control mice on the sixth day (Fig. 3D). Six-month-old *CaMKII-Wnt5a^{fl/fl}* mice also required significantly more time to find the platform. However, 6-mo-old mutants did not achieve similar latencies as control mice, even when tested on the 12th day (Fig. 3E). These results show that acquisition of spatial learning is impaired in the absence of structural deficits in 3-mo-old *Wnt5a* mutant mice, but that learning deficits are exacerbated with the appearance of morphological abnormalities in older animals.

To evaluate reference memory, we conducted a probe trial on day 13 in which the platform was removed and measured the amount of time that mice spent in the original target quadrant (Fig. 3C). Both 3- and 6-mo-old mutant mice spent significantly less time in the target quadrant and made fewer platform crossings (Fig. 3 F and G and Fig. S4 D and E). We then performed a reversal training by relocating the hidden platform to the opposite quadrant from days 14–25 (Fig. 3C). As in the initial training phase, 3-mo-old *Wnt5a* mutant mice required more days of training to find the new platform, but eventually reached similar latencies as control mice (Fig. S4F). However, 6-mo-old mutant mice took significantly longer to find the platform compared with control littermates even on day 25 (Fig. S4G). We then conducted probe trials for 7 d (days 26–32) to assess memory retention. Three-month-old mutants maintained a preference for the target quadrant for the 7 d of the probe trial (Fig. 3H), whereas this preference was lost in 6-mo-old mutants by the fifth day (Fig. 3I), suggesting a marked decay in memory retrieval in older mutant animals.

Taken together, the findings from the Morris water maze test support an essential role for Wnt5a in the acquisition of spatial learning and memory storage in adult animals. Notably, the cognitive dysfunction in 3-mo-old *CaMKII-Wnt5a^{fl/fl}* mice were consistent with the LTP defects observed at this age but appeared before the onset of anatomical impairments. The more pronounced behavioral defects in 6-mo-old *CaMKII-Wnt5a^{fl/fl}* mice suggest a progressive decline in cognitive functions with the manifestation of dendritic abnormalities.

Wnt5a Loss Disrupts Calcium and Cytoskeletal Signaling Pathways and CREB-Mediated Transcription of Glutamate Receptors. Wnts are known to exert their effects by signaling through three

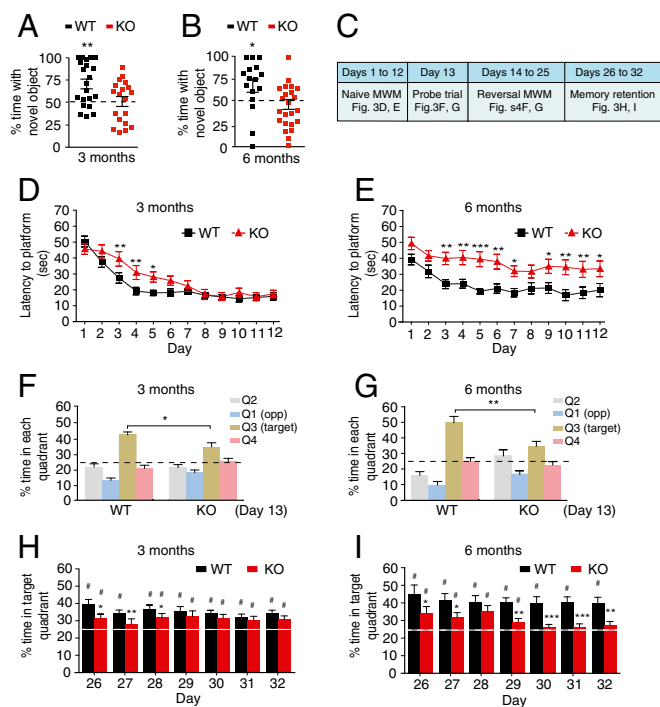


Fig. 3. Adult *Wnt5a* mutant mice show a progressive decline in hippocampus-mediated behaviors. (A and B) WT mice show a significant preference for exploring a new object in the novel-object recognition task, whereas *Wnt5a* KO mice spent similar amounts of time with familiar and new objects. Deficits in recognition memory were evident in both 3- and 6-mo-old mutants. Dashed line indicates equal amount of time spent exploring new and familiar objects. Results are mean \pm SEM from $n = 21$ WT mice and $n = 19$ KO mice at 3 mo, and $n = 16$ WT mice and $n = 23$ KO mice at 6 mo. $*P < 0.05$, $**P < 0.01$ significantly different from 50% time spent with the novel-object, two-tailed t test. (C) Timeline for the Morris water maze (MWM) tasks. (D) Three-month-old KO mice show impaired spatial learning in the Morris water maze test. Results are mean \pm SEM from $n = 20$ WT and $n = 19$ KO mice. $*P < 0.05$, $**P < 0.01$, two-way ANOVA with Fisher's least-significant difference (LSD) post hoc test. (E) Six-month-old KO mice exhibit more severe deficits in learning and fail to acquire the latency of control animals even on day 12. Results are mean \pm SEM from $n = 18$ WT and $n = 20$ KO mice. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-way ANOVA with Fisher's LSD post hoc test. (F and G) During probe trials, 3- and 6-mo-old KO mice showed less preference for the target quadrant compared with WT mice. Results are mean \pm SEM, $*P < 0.05$, $**P < 0.01$, two-tailed t test. (H and I) During the memory retention test, both 3- and 6-mo-old KO mice spent less time in the target quadrant compared with control littermates. However, older mutant mice exhibited a marked decline in memory retrieval by the fifth day of the probe trial. White dashed line indicates 25% of time that mice spent in the target quadrant. Results are mean \pm SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ significantly different from control littermates; $^{\#}P < 0.05$ significantly different from 25% line, two-tailed t test.

effector pathways: the canonical β -catenin-dependent pathway, a Ca^{2+} -dependent pathway, and the planar cell polarity pathway (47). We found comparable levels of nuclear β -catenin and *Axin2*, *c-myc*, and *NeuroD1*, all transcriptional targets of canonical β -catenin signaling (48), between *CaMKII-Wnt5a*^{fl/fl} and control *Wnt5a*^{fl/fl} hippocampus at 3 mo (Fig. S5 A–C), indicating that canonical Wnt signaling is unaffected by *Wnt5a* depletion in the mature hippocampus.

We next assessed the Wnt-calcium pathway, where Wnt ligands promote an increase of cytoplasmic Ca^{2+} (49, 50). Strikingly, *Wnt5a* treatment acutely elicited a calcium response in 92% of cultured rat hippocampal neurons transfected with GCaMP3, whereas only 43% of neurons responded to control treatment. Furthermore, the number of calcium transients was fivefold higher in *Wnt5a*-treated neurons (Fig. 4 A–C and Movies S1 and S2). We then performed biochemical analyses to assess activa-

tion of CaMKII, a critical regulator of hippocampal connectivity and functions (51, 52), in vivo, in young adult *CaMKII-Wnt5a*^{fl/fl} mice at 3 mo before the appearance of structural anomalies. Using a phospho-specific antibody that detects activated CaMKII (threonine 286 phosphorylation on CaMKII α and T287 on CaMKII β) (53), we found a pronounced attenuation of phosphorylated CaMKII α (59% decrease) and CaMKII β (57% decrease) in *CaMKII-Wnt5a*^{fl/fl} mice (Fig. 4 D and E). CaMKII-mediated phosphorylation of the GluA1 subunit of AMPA-type glutamate receptors at a critical serine 831 site (54, 55) has been functionally linked to synaptic plasticity and retention of spatial memory in mice (56, 57). We found a marked decrease in phospho-S831-GluA1 in postsynaptic density fractions from the mutant hippocampus (Fig. 4 D and E). These results suggest decreases in phosphorylation of CaMKII and GluA1 as the molecular underpinnings for the impairments in synaptic plasticity and spatial memory in *CaMKII-Wnt5a*^{fl/fl} mice.

Calcium signaling within synapses could couple to transcriptional responses via shuttling of a Ca^{2+} /CaM/CaMKII γ complex to the nucleus to promote phosphorylation of CaMKIV, which then phosphorylates and activates the transcription factor CREB (58). Phosphorylation of CaMKIV and CREB were significantly reduced in nuclear fractions from 3-mo-old *CaMKII-Wnt5a*^{fl/fl} hippocampal tissues (Fig. 4 F and G). Because *Wnt5a* deletion altered nuclear CREB phosphorylation, we assessed levels of several synaptic proteins (Fig. S5 D and E) and found that only GluN1, the obligatory NMDA receptor subunit, was decreased (Fig. 4 H–J), raising the possibility that *GluN1* transcription is CREB-dependent. We did not observe any changes in levels of other NMDA receptor subunits, GluN2a/2b, that are coexpressed and coassembled in the endoplasmic reticulum (ER) with GluN1, in the mature hippocampus (Fig. 4 H–J). We identified three putative CRE sites (*CRE1* at –212 bp, *CRE2* at –238 bp, and *CRE3* at –770 bp) in a 1-kb region upstream of the transcription start site in the mouse *GluN1* promoter (Fig. 4K). In a dual luciferase assay, *Wnt5a* stimulation of hippocampal neurons for 6 h significantly increased luciferase activity compared with control treatment (Fig. 4 K and L). Mutation of just the two proximal CRE elements (–212 to –216 bp and –238 to –242 bp) abolished *Wnt5a*-induced luciferase activity (Fig. 4L). These results reveal an unexpected role for *Wnt5a* in enhancing *GluN1* transcription through a noncanonical pathway that involves calcium-CaMKII-CREB activation.

We finally examined the planar cell polarity pathway where noncanonical Wnts induce cytoskeletal dynamics by activating small GTPases, such as Rac1 and JNK signaling (59). Rac1 is a critical regulator of the actin cytoskeleton in dendrites and spines (60, 61). Active Rac1-GTP and phospho-JNK levels were significantly reduced in hippocampal homogenates prepared from 3-mo-old *CaMKII-Wnt5a*^{fl/fl} mice (Fig. 4 M–P). Rac1 activity can also be influenced by CaMKII activity via CaMKII-mediated phosphorylation of the Rac1-specific GEFs, Tiam1 and Kalirin-7 (62, 63). Taken together, these results suggest that *Wnt5a* signals via CaMKII and Rac1-mediated signaling, as well as CREB-mediated GluN1 synthesis to maintain synaptic plasticity and structure in the adult hippocampus.

Late Induction of *Wnt5a* Reverses Dendrite Attrition. Our results show that adult *Wnt5a*-deficient mice have profound defects in hippocampal synaptic plasticity, dendrite morphology, and related molecular changes. Could restoring *Wnt5a* expression prevent or even correct synaptic signaling and dendritic defects in adult *CaMKII-Wnt5a*^{fl/fl} mice? To address this question, we expressed *Wnt5a* using an adeno-associated virus (AAV) virus carrying a Cre-dependent *Wnt5a* transgene, DIO-*Wnt5a*, in *CaMKII-Wnt5a*^{fl/fl} mice. First, to address if *Wnt5a* expression rescues signaling defects observed at 3 mo, we delivered *Wnt5a* at this time point and performed biochemical analyses at 2 wk

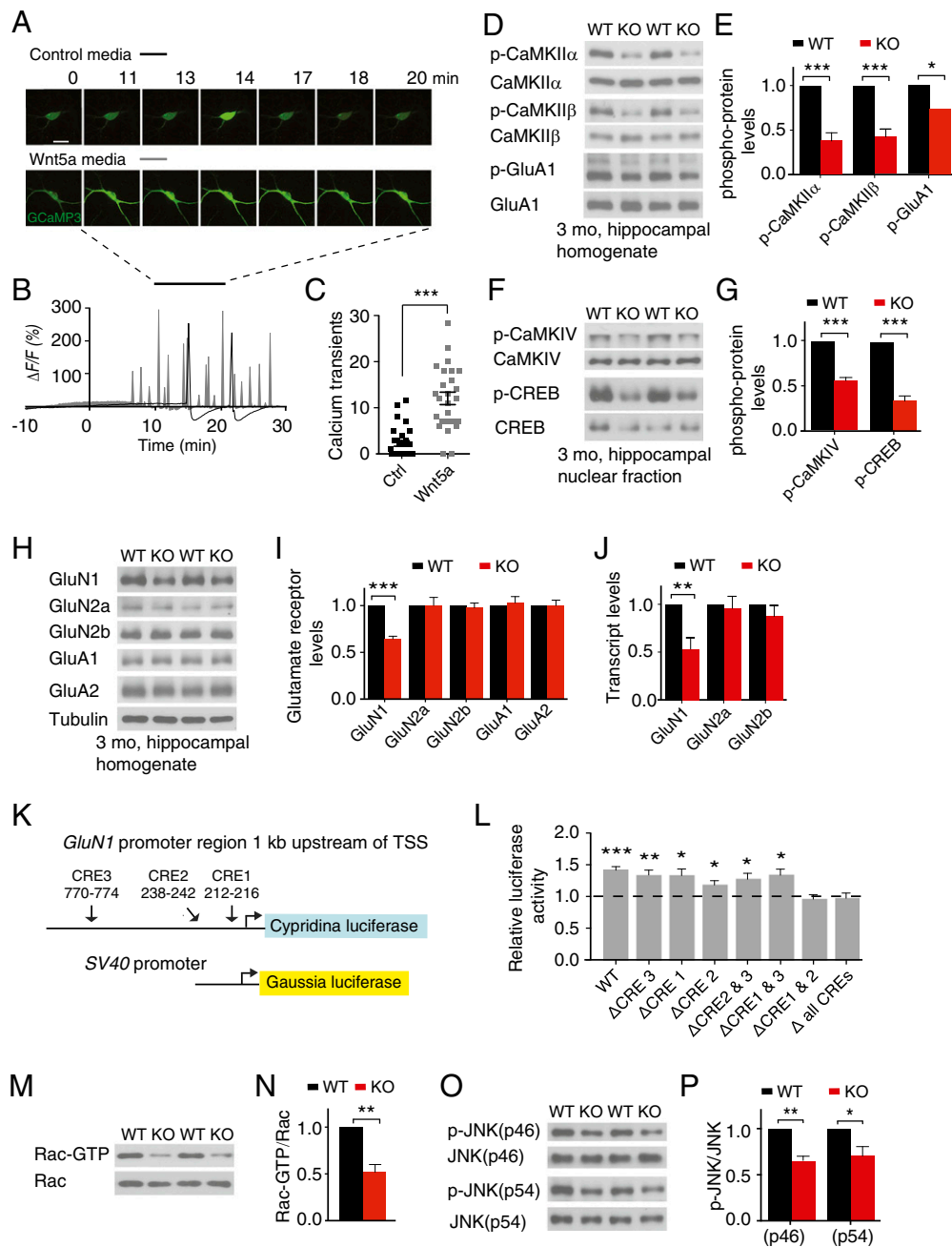


Fig. 4. Wnt5a loss disrupts calcium and cytoskeletal signaling and decreases CREB-mediated GluN1 synthesis. (A) Wnt5a elicits a robust increase in calcium transients in hippocampal neurons. Rat hippocampal neuron cultures were transfected with GCaMP3 and treated with control L- or Wnt5a-conditioned media for 30 min. (Scale bar, 20 μ m.) (B) Representative traces of calcium transients. (C) Wnt5a elicits a fivefold increase in calcium transients compared with control media. Results are mean \pm SEM from $n = 3$ independent experiments (total of $n = 31$ L media-treated cells and $n = 26$ Wnt5a-treated neurons); $***P < 0.001$, two-tailed t test. (D and E) Phosphorylated CaMKII α/β and phosphorylation of GluA1^{Ser831} are significantly reduced in *CaMKII-Wnt5a^{fl/fl}* (KO) hippocampus compared with *Wnt5a^{fl/fl}* litter-mates (WT) at 3 mo. Immunoblots were stripped and reprobed for total CaMKII α , β , and GluA1 for normalization. Results are mean \pm SEM from $n = 6$ mice per genotype; $*P < 0.05$, $***P < 0.001$, two-tailed t test. (F and G) CaMKIV and CREB phosphorylation are attenuated in hippocampal nuclear fractions from 3-mo-old KO mice. Blots were reprobed for total CaMKIV and CREB. Results are mean \pm SEM from $n = 6$ and $n = 5$ mice per genotype; $***P < 0.001$, two-tailed t test. (H and I) GluN1, the obligatory NMDA receptor subunit, is significantly decreased in 3-mo KO hippocampus, but levels of other NMDA receptor subunits, GluN2a/2b, and AMPA-type glutamate receptor subunits, GluA1 and GluA2, are unaltered. Immunoblots were stripped and reprobed for tubulin. Results are mean \pm SEM from $n = 6$ mice per genotype; $***P < 0.001$, two-tailed t test. (J) qPCR analysis shows decrease in *GluN1* but not *GluN2a/b* transcripts in the KO hippocampus. Results are mean \pm SEM from $n = 6$ mice per genotype; $**P < 0.01$, two-tailed t test. (K) A 1-kb region upstream of the mouse *GluN1* promoter harbors three putative CRE sites. Hippocampal neurons were cotransfected with luciferase reporter constructs, *Cypridina* luciferase driven by the *GluN1* promoter region, and *Gaussia* luciferase driven by an SV40 promoter as a control. Neurons were treated with control or Wnt5a media for 6 h and then harvested to measure luciferase activity. (L) Wnt5a treatment significantly increases *GluN1* promoter-driven luciferase activity. Deletion of all three CRE sites or just the two proximal binding sites (CRE1 and CRE2) alone abolishes the Wnt5a-mediated response. Results are mean \pm SEM for $n = 6$ independent experiments; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-tailed t test. (M and N) Rac1-GTP levels are reduced in 3-mo KO mice. Hippocampal homogenates were subjected to GST-PAK-PBD-agarose pull-downs for active Rac-GTP and immunoblotted for Rac1. Rac1-GTP signal intensities were normalized to total Rac1. Results are mean \pm SEM from $n = 6$ mice per genotype; $**P < 0.01$, two-tailed t test. (O and P) Phospho-JNK levels are significantly decreased in hippocampal homogenates from 3-mo KO mice. Results are mean \pm SEM from $n = 6$ mice per genotype; $*P < 0.05$, $**P < 0.01$, two-tailed t test.

after viral infection (Fig. 5A). Immunoblotting of hippocampal homogenates revealed that AAV-mediated Wnt5a expression was sufficient to correct the impairments in CaMKII and CREB phosphorylation, GluN1 expression, and Rac1 activity in *CaMKII-Wnt5a^{fl/fl}* mice (Fig. 5B–E). These results suggest a direct and acute role for Wnt5a in regulating calcium and Rac1 activity and enhancing CREB-mediated synthesis of NMDA-type glutamate receptor subunits in the mature hippocampus.

We next addressed whether the dendrite atrophy in adult *CaMKII-Wnt5a^{fl/fl}* neurons was permanent or could be reversed

by Wnt5a administration well after the onset of structural abnormalities. Thus, we delivered AAV-DIO-Wnt5a into *Thy1-GFP;CaMKII-Wnt5a^{fl/fl}* mice at 6 mo when *CaMKII-Wnt5a^{fl/fl}* mice exhibit pronounced regression of dendritic arbors, and mice were harvested 3 mo after AAV infections for morphological analyses (Fig. 5F). We simultaneously delivered AAV-DIO-mCherry as a means to label infected neurons that are also GFP⁺ to facilitate tracing of neuronal morphologies in isolated neurons. Strikingly, AAV-mediated expression of Wnt5a in *Thy1-GFP;CaMKII-Wnt5a^{fl/fl}* resulted in dendritic arbors that were

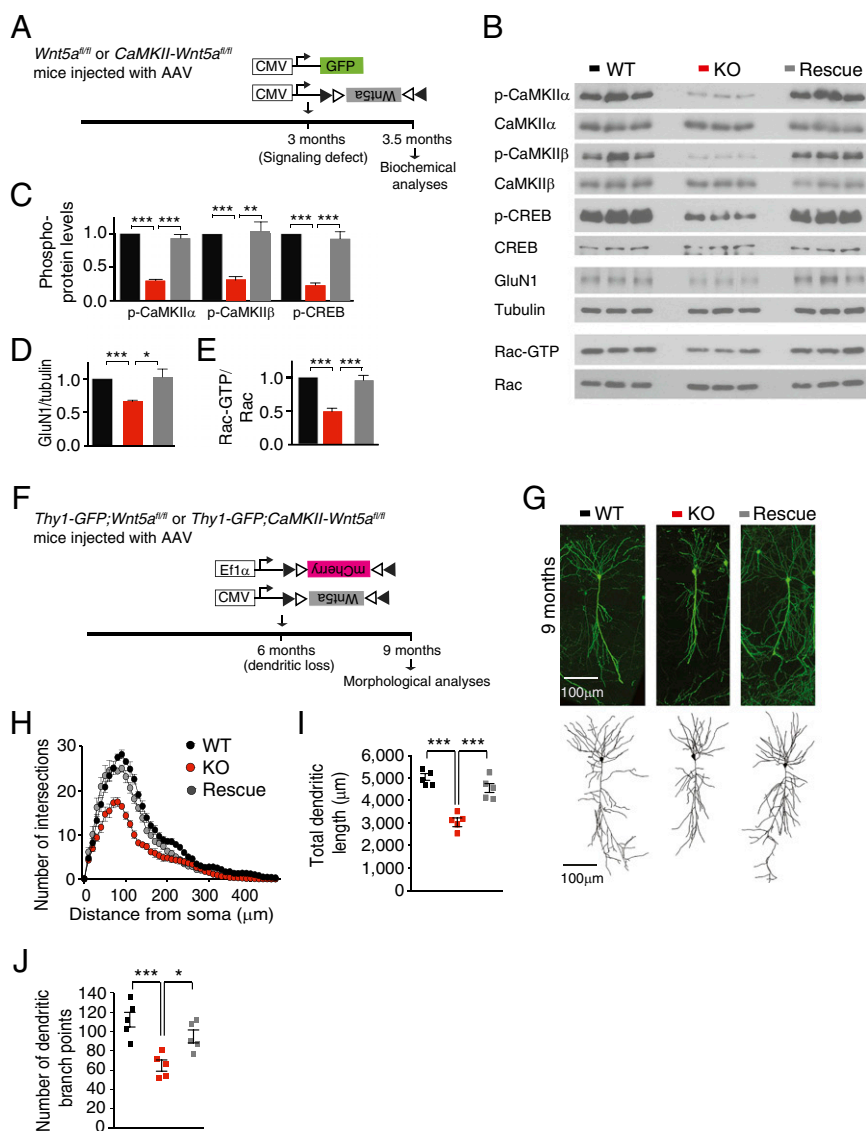


Fig. 5. Reversal of dendritic attrition by late induction of Wnt5a expression. (A) Schematic of the strategy to assess effects of Wnt5a expression on synaptic signaling in *CaMKII-Wnt5a^{fl/fl}* mice. Cre-dependent AAV expressing Wnt5a (AAV-DIO-Wnt5a) was injected into *CaMKII-Wnt5a^{fl/fl}* mice at 3 mo and biochemical analyses performed 2 wk later. AAV-DIO-Wnt5a infected mutant mice (Rescue) were compared with *CaMKII-Wnt5a^{fl/fl}* mice (KO) or control *Wnt5a^{fl/fl}* mice (WT) infected with AAV-GFP. (B–E) Wnt5a expression fully restored CaMKII and CREB phosphorylation, GluN1 expression, and Rac1 activity in *CaMKII-Wnt5a^{fl/fl}* hippocampi. Results are mean \pm SEM from $n = 6$ mice per group; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, one-way ANOVA followed by Tukey's post hoc test. (F) Schematic of the strategy to assess effects of Wnt5a expression on neuronal morphology in adult *Thy1-GFP;CaMKII-Wnt5a^{fl/fl}* mice. AAV-DIO-Wnt5a was delivered into *Thy1-GFP;CaMKII-Wnt5a^{fl/fl}* hippocampus at 6 mo when there is marked dendritic regression, and morphological analyses were performed at 9 mo. Infected neurons were identified by coinfection of AAV-DIO-mCherry. GFP fluorescence was used for imaging. (G) CA1 dendritic arbors in Wnt5a-infected *Thy1-GFP;CaMKII-Wnt5a^{fl/fl}* mice (Rescue) were comparable to control *Thy1-GFP;Wnt5a^{fl/fl}* mice (WT). However, *Thy1-GFP;CaMKII-Wnt5a^{fl/fl}* neurons (KO) infected with AAV-DIO-mCherry had stunted dendritic arbors, as expected. (Scale bar, 100 μ m.) (H) Sholl analysis shows that AAV-mediated Wnt5a expression for 3 mo corrects dendrite complexity defects in 9-mo-old *Thy1-GFP;CaMKII-Wnt5a^{fl/fl}* mice. (I and J) Dendritic lengths and dendritic branch points are comparable between WT and Rescue neurons, but significantly decreased in KO neurons in 9-mo-old mice. Results are mean \pm SEM from $n = 5$ neurons traced per animal and a total of five mice per group; $*P < 0.05$, $***P < 0.001$, one-way ANOVA followed by Tukey's post hoc test.

comparable in complexity and lengths to control *Thy1-GFP*; *Wnt5a^{fl/fl}* neurons (Fig. 5 G–J). As expected, with infection with AAV-DIO-mCherry alone, we observed substantial decreases in dendrite complexity and lengths in *Thy1-GFP*; *CaMKII-Wnt5a^{fl/fl}* CA1 neurons (Fig. 5 G–J). These findings indicate that dendritic attrition in adult mutant neurons can be reversed, and reveals that Wnt5a, remarkably, promotes substantial dendritic growth and branching in the adult brain when neuronal connectivity is thought to be largely immutable.

Discussion

Wnts are evolutionarily conserved signaling molecules that have been classically associated with embryonic patterning and establishment of neural circuits (47, 64, 65). That these classic developmental cues may have critical functions in the adult brain has been implied by recent findings that broad-spectrum blockade or activation of Wnt pathway components affects synaptic structure, plasticity, and cognitive functions in adult animals (37–41). However, surprisingly little is known about which of the 19 vertebrate Wnts is essential for adult nervous system functions *in vivo*. Furthermore, manipulation of the Wnt pathway through overexpression of antagonists, such as Dickkopf-1 (38, 41), deletion of the Lrp6 coreceptor (40), or the cytoplasmic effector, β -catenin (37), may have consequences on neuronal connectivity and function that are independent of Wnt ligands, via effects on cell–cell adhesion, JNK signaling, and GPCR-mediated cAMP signaling (66–68). Here, we show that deletion of a single Wnt family member, Wnt5a, is sufficient to elicit profound disruptions in synaptic plasticity, structural maintenance, and learning and memory in adult mice, identifying the importance of this particular noncanonical Wnt in later-life functions. Thus, the loss of Wnt5a cannot be compensated for by other Wnts in the adult hippocampus. Together, our results, summarized in the model in Fig. 6, define a causal sequence of events where Wnt5a first influences synaptic plasticity and related cognitive functions in the adult hippocampus through CaMKII-mediated signaling, Rac1-dependent actin dynamics, and CREB-mediated NMDA receptor biosynthesis. In the long-term, Wnt5a-mediated regulation of cytoskeletal signaling and excitatory synaptic transmission is responsible for the maintenance of dendritic arbors and spines. These findings provide insight into the poorly understood structural maintenance mechanisms that exist in the adult brain, and suggest Wnt5a signaling as a molecular target in ameliorating dendrite shrinkage and cognitive decline associated with pathological situations.

The finding that embryonic deletion of Wnt5a in neurons did not elicit any structural abnormalities in CA1 pyramidal neurons during development suggests that neuronal Wnt5a is dispensable for the establishment or maturation of hippocampal connectivity *in vivo*. These results were surprising in the context of reported developmental functions for Wnt5a in cultured hippocampal neurons, and in embryonic processes in other brain regions (15, 18, 19). In hippocampal neurons, several signaling pathways have been shown to influence dendrite morphogenesis, maturation, and stability *in vitro* and *in vivo* (1, 6, 69). Thus, in the absence of Wnt5a, other signaling mechanisms, including other Wnt molecules (26, 27), could provide trophic support to hippocampal CA1 dendrite arbors and spines at least for the first several months of life in mice. Alternatively, Wnt5a derived from non-neuronal sources may support hippocampal formation in the absence of neuron-derived Wnt5a. However, the profound defects in adult mice lacking Wnt5a suggest that these mechanisms are unable to compensate for Wnt5a loss at later stages of life. Notably, we demonstrate that Wnt5a, derived from CA1 pyramidal neurons themselves, is critical for sustaining dendritic architecture in the adult hippocampus, implying that specificity for neuronal wiring is intrinsic to active neurons themselves in hippocampal circuits. To date, our limited understanding of the

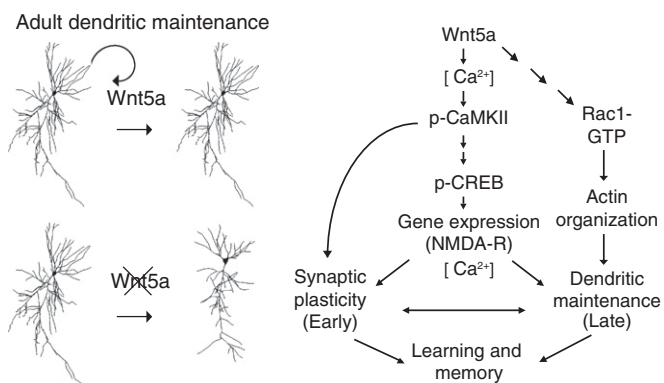


Fig. 6. Model for adult-specific roles for autocrine Wnt5a signaling in maintenance of CA1 dendrite architecture and function. Wnt5a influences synaptic plasticity and related cognitive functions in the adult hippocampus through CaMKII-mediated signaling and NMDA receptor biosynthesis, and also Rac1-dependent actin dynamics in dendritic spines. In the long-term, Wnt5a-mediated regulation of cytoskeletal signaling and excitatory synaptic transmission is responsible for maintenance of dendritic structure.

molecular cues that influence neuronal morphology in adult animals has largely come from analyses of cortical neurons in genetically modified mice. Among the examples are adult mice with deletion of BDNF and its receptor TrkB (7, 8), the adhesion molecule δ -catenin (70), and the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (71). Our findings identifying Wnt5a as being essential for the maintenance of adult CA1 hippocampal neurons is relevant to understanding the structural bases of hippocampus-dependent behaviors.

We found that synaptic plasticity is most susceptible to the postnatal depletion of Wnt5a. *CaMKII-Wnt5a^{fl/fl}* mice had impaired CA1 LTP and related behavioral defects at 3 mo of age, a time when basal synaptic transmission and dendritic morphology are intact. The normal presynaptic properties indicate that Wnt5a acts primarily at postsynaptic sites. Recombinant Wnt5a has previously been shown to acutely modulate NMDAR-mediated synaptic transmission in rat hippocampal slices (72). Our results suggest that Wnt5a likely modifies synaptic strength through CaMKII-mediated signaling events, including the phosphorylation and subsequent trafficking/conductance of AMPA-type glutamate receptors, Rac1-dependent regulation of actin dynamics in dendritic spines, and regulation of NMDA receptor biosynthesis. Attenuation of small GTPase-mediated signaling and excitatory synaptic transmission, both postulated to be critical determinants in stabilizing neuronal connectivity (61, 73, 74), may underlie the gradual attrition of dendritic arbors and spines in later life. Because mice with forebrain-specific deletion of *GluN1* have impairments in plasticity at CA1 synapses and spatial memory acquisition (75), these findings suggest that down-regulation of NMDA receptor synthesis contributes, in part, to the functional and behavioral defects that we observed in *CaMKII-Wnt5a^{fl/fl}* mice. Although NMDA receptor-mediated LTD is impaired in mice with *CaMKII-Cre*-mediated deletion of *GluN1* (75), that we observed normal LTD responses in *CaMKII-Wnt5a^{fl/fl}* mice can be attributed to the fact that residual *GluN1* expression in Wnt5a mutant mice may still allow sufficient Ca^{2+} influx to promote LTD, consistent with the view of differential Ca^{2+} requirements for LTD versus LTP (76, 77). Previously, targeted *GluN1* deletion has also been reported to result in modest (~35%) decreases in *GluN2a/2b* protein expression, but unaltered levels of *GluN2a/2b* mRNA (78, 79). The decrease in *GluN2a/2b* subunits was attributed to their aberrant retention in the ER and protein degradation when *GluN1* is

unavailable (78). That we did not observe any changes in GluN2a/2b protein levels in *CaMKII-Wnt5a^{fl/fl}* mice is likely because the GluN1 depletion in our study (34%) is less robust compared with the near-complete depletion previously reported in *GluN1* conditional null mice (78, 79). Thus, residual GluN1 expression in *CaMKII-Wnt5a^{fl/fl}* mice might be sufficient to avoid ER-associated degradation of the GluN2a/2b subunits. Although the total GluN2a/2b protein content was unaltered in the *CaMKII-Wnt5a^{fl/fl}* hippocampus, it is possible that their synaptic localization might be affected by Wnt5a loss, given previous findings that Wnt5a modulates the surface expression of GluN2b in cultured hippocampal neurons (80).

Currently, the Wnt5a receptors that mediate effects on dendritic maintenance and synaptic functions in the adult hippocampus remain to be determined, although likely candidates include the Ror1/2 receptor tyrosine kinases and Frizzled-9. Ror1/2 have been demonstrated to be bona fide Wnt5a receptors *in vivo* (81). Ror2 is abundantly expressed in mature CA1 dendrites, promotes dendritic activation of noncanonical Wnt signaling and, notably, is necessary for Wnt5a-mediated potentiation of NMDAR currents in acute hippocampal slices (82). Ror2 may function in coordination with Frizzled receptors; in particular, Frizzled-9 is localized to postsynaptic sites in hippocampal neurons, binds Wnt5a via its cysteine-rich domain in biochemical analyses, and the Frizzled-9 cysteine-rich domain is required for Wnt5a-mediated changes in spine densities in cultured hippocampal neurons (32).

The early LTP defects and the cognitive decline followed by retraction of dendrites and spine loss observed in adult *Wnt5a* mutant mice bear similarities to the progression of events in animal models of Alzheimer's disease (83). Recent genetic evidence implicates deficiencies in Wnt signaling, largely the canonical arm, in the synaptic dysfunction and cognitive impairments in Alzheimer's disease (40, 84, 85). Our study emphasizes that noncanonical Wnt signaling is essential for maintaining synaptic function and connectivity in the adult brain. That late induction of Wnt5a expression even after the onset of substantial neuronal atrophy, remarkably restores dendrite morphology in

adult neurons, highlights the capacity of the adult nervous system to undergo large-scale structural changes, and suggests a Wnt5a-dependent trophic pathway that could be harnessed for therapeutic purposes in pathological situations.

Materials and Methods

Animals. All procedures relating to animal care and treatment conformed to The Johns Hopkins University and NIH guidelines. Animals were housed in a standard 12:12 light:dark cycle. The generation of *Wnt5a^{fl/fl}* mice has been previously described (86). Hippocampal neuron cultures were established from embryonic day 18 (E18) rat pups, as previously described (87).

Neuronal cell counts were performed as described in Ramanan et al. (88). Lentiviral or AAV vectors were stereotaxically delivered to the hippocampus using coordinates that were previously described (89). Golgi-based analyses of dendrite arbors and spines were performed as described previously (90). Further details of dendrite reconstructions and analyses are included in *SI Materials and Methods*. Details of *in situ* hybridization, real-time PCR primers and assays, Rac1 GTPase activity, electrophysiology, calcium imaging, luciferase assays, and the novel-object recognition test can be found in *SI Materials and Methods*. The Morris water maze test was performed as previously described (91), and the visual acuity was measured as previously described (92).

Statistical Analyses. All Student's *t* tests were performed using two-tailed, unpaired, and a confidence interval of 95%. One-way or two-way ANOVA analyses were performed when more than two groups were compared. Statistical analyses were based on at least three independent experiments and are described in the figure legends. All error bars represent the SEM.

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