

Wnt-5a/Frizzled9 receptor signaling through the $G\alpha_o$ / $G\beta\gamma$ complex regulates dendritic spine formation.

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*Running Title: Wnt-5a regulates dendritic spines via FZD9 and $G\alpha_o$ - $G\beta\gamma$

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Key words: Wnt signaling; Frizzled Receptor; Dendritic Spine; Heterotrimeric G protein; Hippocampal Neuron.

Wnt ligands play crucial roles in the development and regulation of synapse structure and function. Specifically, Wnt-5a acts as a secreted growth factor that regulates dendritic spine formation in rodent hippocampal neurons, resulting in postsynaptic development that promotes the clustering of the postsynaptic density protein-95 (PSD-95). Here, we focused on the early events occurring after the interaction between Wnt-5a and its Frizzled receptor at the neuronal cell surface. Additionally, we studied the role of heterotrimeric G proteins in Wnt-5a-dependent synaptic development.

We report that Frizzled9 (FZD9), a Wnt receptor related to Williams' syndrome, is localized in the postsynaptic region, where it interacts with Wnt-5a. Functionally, FZD9 is required for the Wnt-5a-mediated increase in dendritic spine density. FZD9 forms a pre-coupled complex with $G\alpha_o$ under basal conditions that dissociates after Wnt-5a stimulation. Accordingly, we found that G-protein inhibition abrogates Wnt-5a-dependent pathway in hippocampal neurons. In particular, the activation of $G\alpha_o$ appears to be a key factor controlling the Wnt-5a-induced dendritic spine density. In addition, we found that $G\beta\gamma$ is required for the Wnt-5a-mediated increase in

cytosolic calcium levels and spinogenesis. Our findings reveal that FZD9 and heterotrimeric G proteins regulate Wnt-5a signaling and dendritic spines in cultured hippocampal neurons.

INTRODUCTION

The Wnt family proteins are secreted growth factors that regulate developmental processes, such as cell fate and polarity and general cell maintenance events, by modulating homeostasis and the cell cycle (1,2). Furthermore, Wnt signaling controls various steps in the differentiation of the central nervous system (3-5), including axon guidance, dendrite development, synapse formation and plasticity (6,7). Certain Wnt ligands, such as Wnt-7a and Wnt-3a, regulate the development and activity of the pre- and postsynaptic regions of glutamatergic synapses (8-10). Similarly, previous results have shown that Wnt-5a regulates the synaptic structure and function by inducing the clustering of PSD-95 through the activation of the c-Jun N-terminal kinase (JNK) (11) and modulates glutamate receptors through nitric oxide production (12). Additionally Wnt-5a promotes the *de novo* formation of dendritic

spines in hippocampal neurons (13) through the non-canonical Wnt/ Ca^{+2} pathway.

The Frizzled (FZD) family has ten members in vertebrates and represents unconventional G protein-coupled receptors (GPCRs) (14). FZD proteins have been identified as Wnt receptors, and they can mediate the signaling triggered by several Wnt ligands. Wnt-5a activity in different model systems has been linked to several FZDs receptors and other co-receptors (15-18). However, the FZD receptor mediating Wnt-5a signaling and spine formation in hippocampal neurons has not yet been identified.

Several signaling cascades can be triggered upon Wnt binding to a FZD receptor, including canonical (i.e., β -catenin-dependent) and non-canonical pathways (19). Consistent with their GPCR identity, recent studies have revealed that FZD receptors can induce Wnt signaling-dependent activation of G proteins (20,21). Heterotrimeric G proteins are composed of a guanine nucleotide-binding α -subunit ($G\alpha$) and a $\beta\gamma$ complex ($G\beta\gamma$). In the resting state, $G\alpha$ is bound to GDP and associated with $G\beta\gamma$ and a GPCR. This complex is dissociated when $G\alpha$ binds to GTP, activating $G\alpha$ and the $G\beta\gamma$ complex, which allows them to regulate their downstream effectors (22). The $G\alpha$ subunits are separated into four families based on sequence homology ($G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12/13}$) (23), each activating different pathways.

It has been shown that FZDs act as guanine nucleotide exchange factors (GEF) for Pertussis toxin (PTX)-sensitive G-proteins *in vivo* ($G\alpha_{i/o}$), mediating both canonical and non-canonical signaling in *Drosophila* (24). In the Wnt/ Ca^{2+} pathway, the G-proteins, particularly the PTX-sensitive $G\alpha$ subunits, have been shown to be functional in several Wnt-dependent processes (25-27). However, the role of the G-proteins in Wnt-5a signaling in hippocampal neurons has not been clarified.

Here, we used hippocampal cultures, confocal fluorescent microscopy and a combination of pharmacological and molecular approaches to characterize the cascade downstream of Wnt-5a that is relevant for dendritic spine development. Based on previous findings showing that FZD9 plays a key role in the formation of neuronal connectivity (28,29) and that FZD9-null mice display hippocampal learning defects (30), we focused on the possible function of this receptor. We report that the activation of the non-canonical cascade by Wnt-

5a required the FZD9 receptor. Also, we found that this receptor is located in the postsynaptic region, suggesting a role in Wnt-5a-induced postsynaptic remodeling. Additionally, the increase in the intracellular Ca^{+2} concentrations produced by Wnt-5a was dependent on FZD9. On the other hand, we found that FZD9 interacts with heterotrimeric G proteins, particularly the $G\alpha_o$, and that this process is crucial for the formation of dendritic spines, postsynaptic remodeling and activation of the signaling cascade. Our results increase the comprehension of the Wnt signaling cascade in hippocampal neurons and confirm the key role of heterotrimeric G proteins in the Wnt-5a pathway.

RESULTS

Postsynaptic distribution of the FZD9 receptor in hippocampal neurons. -To determine whether FZD9 mediates the Wnt-5a synaptogenic effects in hippocampal neurons, we first studied the subcellular localization of this receptor. It has been reported that FZD9 expression increases during embryonic hippocampal development (31), similar to Wnt-5a (13). However, because Wnt-5a regulates synaptic development, we first analyzed the synaptic distribution of FZD9 in cultured hippocampal neurons at DIV14. Our immunolocalization studies showed that FZD9 was distributed in a punctate pattern in the neuronal processes, as previously reported (31), and co-localized with the postsynaptic density scaffolding protein PSD-95 (Fig. 1A). This observation was supported by the Manders overlap coefficient, which represents the number of co-localized pixels expressed as a fraction of the total number of pixels in each channel. The co-localization index between PSD-95 and FZD9 was significantly higher than the co-localization between FZD9 and Piccolo, a presynaptic marker (Fig. 1B). Additionally, PSD-95 displays a higher co-localization index with FZD9 than those with FZD2, FZD3 or FZD7 and PSD-95, suggesting that this co-distribution was specific for FZD9 (Fig. 1C). The postsynaptic distribution of FZD9 was also analyzed biochemically in adult rat brains. Synaptosomal preparations were separated in synaptophysin (SYP)-enriched (Triton X-100 soluble) fraction and the PSD-95-enriched (Triton X-100 insoluble) fraction (Fig.1D). Consistent with the distribution observed in the cultured neurons, western blot showed that FZD9 was highly

enriched in the PSD-95-containing fraction, whereas it was almost undetectable in the SYP-enriched fraction. Together, these data strongly suggest that FZD9 is located in the postsynaptic region of hippocampal neurons.

Wnt-5a interacts with the Wnt-binding domain of FZD9 to increase cytoplasmic Ca^{2+} and dendritic spine density in hippocampal neurons. -In different biological contexts, FZD9 has been shown to be a functional receptor for the ligands Wnt-2, Wnt-7a and Wnt-8 (32-34). To assess whether FZD9 might also mediate the Wnt-5a cascade, we first performed a ligand-receptor binding assay between recombinant Wnt-5a and the cysteine-rich domain of FZD9 (CRD The CRD of mouse -FZD9), which is a Wnt-binding domain (35). FZD9 (Leu24-Asp186) is fused to IgG. We also used the CRDs fused to IgG from FZD1, FZD2 and FZD5 in order to compare the interaction between Wnt-5a and these receptors. Immunoprecipitation of the CRDs-IgG chimera with A/G agarose beads and western blot analysis revealed that Wnt-5a immunoprecipitated with the CRD-FZD9 as other CRD-FZDs (Fig. 2A). The level of immunoprecipitation of Wnt-5a was almost the same with all the CRD-FZD used, suggesting that FZD9 has a similar potential to interact biochemically with Wnt-5a. These ligands-receptors interactions are weak, as they do not interact covalently (Figure 2A, right panel). Our biochemical assays indicate that FZD9 might be a functional receptor for Wnt-5a.

Because Wnt-5a triggers an increase in intracellular Ca^{2+} concentration in hippocampal neurons (13), we attempted to understand the potential role of FZD9 in this effect by measuring the intracellular Ca^{2+} changes using the molecular probe Fura-2AM. To address this possibility experimentally, we measured the intracellular Ca^{2+} changes in hippocampal neurons treated with CRD-FZD9 and Wnt-5a. As expected, Wnt-5a treatment induced a rapid increase in the intensity of the Fura-2AM signal (340/380 ratio), indicating a positive signaling modulation in intracellular Ca^{2+} concentrations. Treatment with CRD-FZD9 blocked the Wnt-5a-mediated increase in the Ca^{2+} concentrations, while CRD-FZD9 have no effect (Fig. 2B). The area under the curve (A.U.C.) decreased from 41.20 ± 2.64 in the Wnt-5a-treated neurons to 6.81 ± 0.72 in Wnt-5a+CRD-FZD9 cells ($p < 0.001$) (Fig. 2C).

Previous observations have shown that Wnt-5a stimulates spinogenesis in cultured

hippocampal neurons (13). Therefore, we next evaluated the potential role of CRD-FZD9 to block this process. To analyze dendritic spines hippocampal neurons were transfected with EGFP at DIV 10 to reveal in detail neuronal morphology. At DIV 14 neurons were fixed and immunostained with anti-PSD-95 to label postsynaptic structures. We observed that almost all the EGFP-protrusions were also labeled with anti-PSD-95, indicating that these protrusions effectively correspond to dendritic spine (Fig 3A). Also, we performed a 3D reconstruction of the dendrite to further analyze the dendritic spine density and the spine width head using Imaris Software (Fig. 3A).

In agreement with previous reports (13) we observed that Wnt-5a increased the dendritic spine density (Fig. 3B). Interestingly, the exogenous addition of CRD-FZD9 inhibited the Wnt-5a-mediated increase in the density of dendritic protrusions after 2 h of Wnt-5a treatment.

To evaluate the potential role of the endogenous FZD9, we selectively decreased FZD9 expression using a short hairpin construct directed against FZD9 (shFZD9-EGFP) or its control vector (Fux-EGFP). Both constructs contained the EGFP gene to label the transfected cells and have been used previously (36). FZD9 knockdown was evaluated by western blotting in the HT22 cell line, in which a marked decrease of FZD9 expression was observed in shFZD9-transfected cells compared with controls, but it failed to decrease the FZD5 and the FZD7 expression (Fig. 4A). Additionally, we observed in hippocampal neurons the efficiency of the FZD9 knockdown, where the number of FZD9 clusters was reduced in shFZD9-EGFP-transfected neurons (Fig. 4B, upper panel).

Thereafter, we analyzed the effect of shFZD9-EGFP or control Fux-EGFP on Wnt-5a-induced intracellular Ca^{2+} increase in 10 DIV hippocampal neurons. In Fux-EGFP-transfected neurons, the Fura-2 AM signal increased after Wnt-5a treatment; notably, this effect was not observed in neurons transfected with shFZD9-EGFP (Fig. 4C, D), as evidenced by the quantitative analysis. A significant reduction in A.U.C from 1.53 ± 1.06 in the Fux-EGFP-transfected neurons to 0.44 ± 0.27 in the shFZD9-EGFP-transfected neurons ($p < 0.05$) was observed. As a control for Ca^{2+} entry, treatment with the Ca^{2+} ionophore ionomycin induced an intracellular Ca^{2+} increase in both neuronal populations without significant differences,

suggesting that both neuron populations can respond and produce a Ca^{2+} increase with a different stimulus than Wnt-5a (Fig. 4C). These experiments reveal that down-regulation of endogenous FZD9 selectively impairs the Wnt-5a-induced rapid increase in intracellular Ca^{2+} concentrations in hippocampal neurons.

Additionally, dendritic spine density was measured in shFZD9-EGFP-transfected neurons after Wnt-5a treatment. As shown in Fig. 4E Wnt-5a treatment induced an increase in the number of dendritic protrusions in Fux-EGFP-transfected neurons, with a maximal effect at 2 h; however, this effect was reduced to basal levels in shFZD9-transfected neurons. To confirm these data, we used a different shRNA against FZD9 (shFZD9c-EGFP, Cod. KH02416G) that is commercially available, that also express the EGFP protein to select the transfected neurons, which reduce the expression of FZD9 (Fig. 4B, lower panel). In the scramble-transfected neurons, there was a significant increase in the density of dendritic spines after the treatment with Wnt-5a (5.69 ± 1.90 spine protrusions per 10 μm dendrite length) compared to control conditions (3.4 ± 1.51). This increase was not observed in shFZD9c-transfected neurons treated with Wnt-5a (4.16 ± 1.07), as compared to the shFZD9c untreated neurons (4.30 ± 1.31) (Figure 4F).

Taken together, our results suggest that FZD9 mediates Wnt-5a signaling inducing a rapid increase in the cytoplasmic Ca^{2+} concentrations and enhancing dendritic spine formation in hippocampal neurons.

Wnt-5a specifically activates $G\alpha_o$ and decouples the FZD9- $G\alpha_o$ - $G\beta\gamma$ complex in hippocampal neurons.- To determine whether Wnt-5a activates G-proteins we performed immunoprecipitation assays using specific antibodies that recognize the GTP-bound $G\alpha_o$ or the $G\alpha_i$ subunits, the active forms of the G α proteins ($G\alpha_o$ -GTP or $G\alpha_i$ -GTP), in cultures of hippocampal neurons that were previously treated with Wnt-5a for different times periods. As a positive control, the untreated lysates were incubated with the non-hydrolysable GTP form GTP γ S, which increase the activation state of G-proteins. Our results show that Wnt-5a induced a sustained activation of the $G\alpha_o$ subunit after 5 min of exposure and remained active for at least 60 min. In contrast, Wnt-5a was unable to alter the level of the active $G\alpha_i$ subunit (Fig. 5A), suggesting that Wnt-5a specifically induces the

activation of $G\alpha_o$. The IgG band shows that an equal amount of the antibody was used for the immunoprecipitations.

Considering that FZD9 mediates the Wnt-5a responses, we next aimed to determine whether FZD9 acts as a GPCR. We treated DIV14 hippocampal neurons with Wnt-5a and then performed co-immunoprecipitation against total $G\alpha_o$. Then the precipitates were analyzed by western blot sequentially against $G\alpha_o$, FZD9 and $G\beta_{1-5}$ (using an antibody that levels the five $G\beta$ isoforms) in the same membrane. We found that FZD9 receptor was markedly co-immunoprecipitated with $G\alpha_o$ in unstimulated neurons (Fig. 5B) suggesting that FZD9 and $G\alpha_o$ interact under basal conditions in neurons, and then acting acts as a GPCR. Notably, when the neurons were treated with Wnt-5a for 30 min, the co-immunoprecipitation of FZD9 was significantly reduced. Additionally, we found that the interaction between $G\alpha_o$ and $G\beta_{1-5}$ decreases after Wnt-5a treatment, suggesting that the $G\beta\gamma$ complex is activated and released after the interaction between Wnt-5a and FZD9 receptor. Taken together, these results suggest that Wnt-5a decouples the interaction between FZD9 and the heterotrimer $G\alpha_o$ and $G\beta\gamma$ proteins.

$G\alpha_{i/o}$ inhibition prevents the activation of the non-canonical Wnt-5a cascade in hippocampal neurons.-Previously, it has been demonstrated that Wnt-5a activates the Wnt/ Ca^{2+} and the Wnt/JNK pathways (37). In addition, Wnt-5a induces the activation of Ca^{2+} /calmodulin-dependent protein kinase II α (CaMKII α) and JNK in a time-dependent manner in hippocampal neurons (11,38). To evaluate whether the effect of Wnt-5a in both cascades is due to the activation of G proteins, we analyzed the effect of PTX, an exotoxin that catalyzes the ADP-ribosylation of the $G\alpha_{i/o}$ subunits precluding its interaction with the GPCR. First, the PTX requires an endosomal uptake and then it is transported to the Golgi and to the endoplasmic reticulum. Finally is released in the cytosol where it ADP-ribosylates the $G\alpha_{i/o}$ subunits (39,40). For this reason, the hippocampal neurons were pre-incubated with PTX for 6 h, and then, Wnt-5a was added for different lengths of time. The hippocampal neurons showed a significant increase in the active form of CaMKII α (p-CaMKII α Tyr286) after 30 min of Wnt-5a treatment compared with the control conditions. This effect was attenuated

by pre-incubation with PTX at all time points (Fig. 6A). In addition, Wnt-5a treatment increased JNK phosphorylation (p-JNK Thr183/Tyr185) after 15 min, with a peak at 30 min, and this effect was also significantly reduced by PTX co-treatment (Fig. 6A). Additionally, we observed that exposing hippocampal neurons to Wnt-5a for 5 min induced an increase in the phosphorylation of protein kinase C β II (PKC β II) at serine 660, a key post-translational modification required for its translocation from the cytoplasm to the membrane and subsequent activation, without affecting total protein levels. Interestingly, this effect was partially blocked by PTX pre-treatment (Fig. 6A). Finally, our findings reveal that neither Wnt-5a nor PTX affected the stabilization of β -catenin in hippocampal neurons, even after 2 h of treatment (Fig. 6A). These experiments confirm that $G\alpha_o$ is required for the activation of the non-canonical Wnt signaling pathways, including CaMKII, JNK and PKC activation.

Because Wnt-5a promotes the clustering of PSD-95 in the postsynaptic region (11), we next analyzed the possible role of $G\alpha_{i/o}$ in this effect. We observed that the density of PSD-95 clustering in DIV14 hippocampal neurons was increased at 1 h and 2 h after Wnt-5a treatment compared with the controls; however, this effect was abrogated by PTX (Fig. 6B). In control experiments, we observed that the total PSD-95 protein levels (Fig. 6A) and PSD-95 clustering were unaffected after PTX treatment alone (Fig. 6B). Thus, our findings suggest that the activation of $G\alpha_{i/o}$ is required for Wnt-5a-induced PSD-95 clustering to promote additional postsynaptic assembly.

Wnt-5a regulates dendritic spine formation by activating $G\alpha_o$. - To identify the heterotrimeric G protein implicated in the Wnt-5a-dependent spine formation, hippocampal neurons were transfected with EGFP at DIV10 and subsequently at DIV14 cultures were treated with Wnt-5a and the effect of $G\alpha_o$, $G\alpha_i$ and $G\alpha_s$ were analyzed. The Wnt-5a-treated hippocampal neurons showed an increased dendritic spine density after 30 min of treatment, with a peak at 2 h (Fig. 7A). Interestingly, 6 h of pre-treatment with PTX prevented the effect of Wnt-5a on dendritic spine formation. Consistent with previous results (13), the width of spine heads was increased after 120 min of Wnt-5a treatment, and this effect was inhibited after PTX treatment

(Fig. 7B). The modulation of the dendritic spine density by Wnt-5a was blocked by co-treatment with soluble Frizzled-related protein 2 (sFRP2; data not shown), a Wnt scavenger that binds to Wnts and prevents their interaction with their receptors. Additionally, the $G\alpha_s$ subunit antagonist NF449 was used to evaluate whether this subunit is involved in the Wnt-5a signaling pathway (41). NF449 was not able to block the Wnt-5a-induced increase in the dendritic spine density, suggesting that the activation of this subunit is not required for the Wnt-5a cascade in hippocampal neurons (Fig. 7C). These data confirm that the spinogenic effect of Wnt-5a is mediated by activation of heterotrimeric $G\alpha_{i/o}$ protein.

To address whether $G\alpha_o$ or $G\alpha_i$ is implicated in the spinogenic effect of Wnt-5a, we co-transfected hippocampal neurons with EGFP and mutants forms of the $G\alpha_o$ ($G\alpha_o$ PTXi) and $G\alpha_i$ ($G\alpha_{i2}$ PTXi or $G\alpha_{i3}$ PTXi) subunits that are insensitive to PTX. As shown in Fig. 7D, transfection with the $G\alpha_o$ PTXi, $G\alpha_{i2}$ PTXi or $G\alpha_{i3}$ PTXi mutants did not affect the spine density. Importantly, the spinogenic effect of Wnt-5a that is abrogated by PTX is completely restored by transfection with the $G\alpha_o$ PTXi, but not with the $G\alpha_i$ PTXi subunits (Fig. 7D), indicating that neither the $G\alpha_{i2}$ nor the $G\alpha_{i3}$ subunits participate in the Wnt-5a-induced cascade. Taken together, these results suggest that $G\alpha_o$ activation is a key factor in the Wnt-5a-mediated regulation of the dendritic spine morphogenesis in hippocampal neurons.

Wnt-5a requires the activation of the $G\beta\gamma$ complex to regulate dendritic spine morphogenesis. - After the activation of a GPCR, the $G\beta\gamma$ complex is released from the heterotrimeric G-proteins to regulate multiple target proteins, including phospholipase C (PLC) and phospho-inositol-3-kinase (PI3K) (42). It has been proposed that the Wnt/ Ca^{2+} signaling pathway activates PLC through the $G\beta\gamma$ complex in astrocytes (43). Therefore, to address whether the activation of the $G\beta\gamma$ complex is involved in Wnt-5a signaling to regulate the dendritic spine density, we used Gallein, a molecule that blocks the $G\beta\gamma$ complex-dependent activation of PLC, but does not block the interaction of $G\beta\gamma$ with the $G\alpha$ subunits (44). Gallein was used at 5 μ M, the lowest effective concentration reported in neurons (45). We measured the intracellular Ca^{+2} concentrations in DIV14 hippocampal neurons treated with Wnt-5a or Wnt-5a plus Gallein. We

determined that Gallein significantly diminished the increase in the Ca^{2+} concentrations after Wnt-5a treatment. Gallein decreased the A.U.C. from 5.04 ± 0.37 in Wnt-5-treated neurons to 2.71 ± 0.21 in Wnt-5a+Gallein-treated neurons ($p < 0.001$; Fig. 8A, B). Additionally, Gallein treatment blocked the Wnt-5a-mediated increase in the dendritic spine density, without disturbing the formation of the dendritic spines themselves (Fig. 8C). Finally, to further confirm the requirement of $G\beta\gamma$ subunit in the spinogenic effect of Wnt-5a, hippocampal neurons were transfected with the β -adrenergic receptor kinase c-terminal peptide (β ARKct) a universal competitor that sequester $G\beta\gamma$ and block its downstream signaling plus EGFP (46). We found that β ARKct expression inhibited the Wnt-5a-mediated increase in the dendritic spine density (Fig. 8D). These data reveal that $G\beta\gamma$ activation is a key step in the Wnt-5a cascade to regulate the non-canonical Wnt signaling pathway and to modulate dendritic spine remodeling.

DISCUSSION

The Wnt-5a ligand has been linked to cognitive and memory functions in the adult brain (7,47). Wnt-5a regulates excitatory synaptic transmission in hippocampal neurons (48-50), including the production of nitric oxide, which triggers the expression of NMDA receptors (12) and promotes the induction of long-term potentiation (LTP) (51). These findings indicate that Wnt-5a is a relevant neural factor for the regulation and maintenance of the structure and function of synapses in the central nervous system. Therefore, understanding the mechanism by which Wnt-5a controls spinogenesis represents a crucial advance in the field.

Because previous studies have established that FZD9 is highly expressed in the hippocampus (31,52), we focused our research on this Wnt receptor. Our findings show that FZD9 co-localizes with postsynaptic proteins in cultured hippocampal neurons, which is consistent with previous data showing the postsynaptic distribution of FZD9 in developing neuromuscular junctions *in vivo* (36). The Wnt-5a pathway mainly regulates postsynaptic structure and function and, consequently, correlates with the distribution of FZD9. Interestingly, the co-receptor Ror2 also plays a role in the formation of the postsynaptic region and in synaptic development (18), which may

suggest a coordinated action between both receptors in Wnt-5a-dependent signaling.

It is known that FZD receptors are able to interact with more than one Wnt ligand, depending on the cellular context. In particular, FZD9 has been shown to bind to Wnt-2 and activates the canonical Wnt pathway, leading to the re-localization of Dvl-1 to the cell membrane in HEK cells (32); however, other studies have indicated that FZD9 interacts with Wnt-7a and activates the Wnt/JNK pathway, mediating the inhibition of lung cancer growth (53). Here, we demonstrated that the CRD of FZD9 was able to bind to Wnt-5a and block Wnt-5a-mediated effects in hippocampal neurons, providing direct evidence that FZD9 acts as a functional partner of Wnt-5a. Similarly, we observed that diminished expression of FZD9 by two different short-hairpin constructs impaired the Wnt-5a-induced increase in dendritic spine density and eliminated the Wnt-5a-mediated increase in intracellular Ca^{+2} concentrations, indicating that the endogenous expression of FZD9 contributes to Wnt-5a-dependent synaptic formation and signaling activation. Even though we do not discard that other FZD receptors could be involved in the Wnt-5a pathway in hippocampal neurons, to our knowledge, this is first time that FZD9 has been associated with Wnt-5a signaling in hippocampal neurons. The physiological relevance of these findings relies on the crucial role that this receptor plays in hippocampal development. Indeed, FZD9 plays an important role in hippocampal development. FZD9 deletion contributes to the neurological symptoms of Williams' syndrome, a developmental neurological disorder causing deficits in hippocampal learning (30). Therefore, understanding the function of FZD9 may provide alternatives for the treatment of Williams' syndrome.

Our present studies reveal a novel role for Wnt signaling in dendritic spine formation, including a strong interaction between $G\alpha_o$ and FZD9, which is decreased upon Wnt-5a treatment. These findings are consistent with the GPCR nature of FZDs (54). Several studies have demonstrated that G proteins are essential transducers of Wnt signaling (55). Moreover, the $G\alpha_o$ subunit has been related to the Wnt pathway in F9 cells (56) and in *D. melanogaster* (24), but its role in cultured hippocampal neurons has not been clarified. Recently, an interaction between FZD7 and $G\alpha_s$ in C2C12 cells was observed (57). Additionally, a pre-coupling between FZD6 and

$G\alpha_i$ has been demonstrated in HEK cells (58). Interestingly, this pre-coupling was significantly reduced when the neurons were treated with Wnt-5a, which is similar to our results in hippocampal neurons.

Dendritic spines are plastic structures that experience striking changes in morphology. New dendritic spines develop as long filopodia, which, during the course of spine maturation, exhibit an increase in the spine head width and retract to the dendritic shaft (59). The increase in the spine head width correlates to increased synaptic strength (60). Several factors have been related to the regulation of dendritic spine density, such as BDNF (61), Wnt-7a (10) and Wnt-5a (13). We found that Wnt-5a significantly increased dendritic spine density and increased dendritic spine head width through a $G\alpha_o$ -activation-dependent mechanism. In addition, we show that the Wnt-5a induced the activation of PKC, CaMKII and JNK through the activation of the $G\alpha_o$ subunit. Together, these data suggest that the $G\alpha_o$ subunit is a key factor in the regulation of the formation of dendritic spines and the related signaling cascades.

G proteins are molecular switches that cycle between active GTP-bound and inactive GDP-bound states. Our study suggests that Wnt-5a activates $G\alpha_o$ without affecting the activation of $G\alpha_i$ and $G\alpha_s$, without excluding the involvement of other subunits. Interestingly, $G\alpha_o$ is highly expressed in the brain (62). The corresponding knockout mice have neurological impairments, such as reduced motor control, hyperactivity, hyperalgesia, and a shortened lifespan (63). In addition, $G\alpha_o$ activation is required for the formation of associative memory in mushroom body neurons in *D. melanogaster* (64) and in hippocampal neurons (65).

Interestingly, previous studies indicated a neuroprotective role of Wnt-5a against the effect of A β oligomers on synaptic depression, as well as in the reduction of PSD-95 clusters in neuronal cultures (49,66). Furthermore, the $G\alpha_o$ subunit has been shown to be involved in Alzheimer's disease, in which the amyloid precursor protein (APP) mediates the neuronal toxicity of A β oligomers through $G\alpha_o$ activation (67). It would be interesting to evaluate how these elements interact and how FZD9 is involved in these processes.

Additionally, we provide strong evidence for the involvement of the $G\beta\gamma$ complex in the spinogenic effect of Wnt-5a. We found that in neurons at rest (non-stimulated), $G\beta\gamma$ interacts

with $G\alpha_o$, as reported previously (68) and that this interaction is reduced after Wnt-5a treatment, suggesting that $G\beta\gamma$ is activated and released. The $G\alpha_o$ protein participates in several transduction cascades, although there is good evidence that PLC is a key downstream enzyme in this cascade, and it has also been suggested that $G\beta\gamma$, which activates PLC, mediates this molecular step (69). Additionally, it has been proposed that $G\beta\gamma$ is involved in canonical Wnt signaling through its ability to recruit Dvl to the plasma membrane. Moreover, $G\alpha_o$ directly interacts with Axin to ensure a robust inhibition of the β -catenin destruction complex to allow the activation of canonical Wnt signaling (70). Further experiments will be required to determine the exact molecular mechanisms by which $G\alpha_o$ - $G\beta\gamma$ mediates Wnt-5a spinogenesis in hippocampal neurons.

In conclusion, we suggest that the cellular mechanism involved in Wnt-5a signaling (Fig. 9) requires the interaction with FZD9 and, consequently, the activation of $G\alpha_o$ and $G\beta\gamma$ to regulate dendritic spine morphogenesis through the non-canonical Wnt pathway.

EXPERIMENTAL PROCEDURES

Reagents-Recombinant Wnt-5a (644-WN-010/CF), CRD-FZD9 (2440-FZ-050), CRD-FZD1 (1120-FZ), CRD-FZD2 (1307-FZ) and CRD-FZD5 (1517-FZ) were purchased from R&D Systems. PTX and Gallein were purchased from Sigma-Aldrich. NF449 was from Merck; Fura-2AM and ionomycin were purchased from Molecular Probes. A/G agarose beads were purchased from Santa Cruz Biotechnology.

Hippocampal neuronal culture-Rat hippocampal cultures were prepared from embryonic day 18 Sprague-Dawley rats of both genders, as previously described (71,72). On day two, the cultured neurons were treated with 2 μ M cytosine arabinoside for 24 h; this method resulted in cultures that were highly enriched for neurons (approximately 5% glia). The primary hippocampal neurons were cultured in Neurobasal media supplemented with 1% B27 (Invitrogen) until the experiments were performed. The Bioethical and Biosafety Committee of the Faculty of Biological Sciences of the Pontificia Universidad Cat3lica de Chile approved the experimental procedures.

Receptor binding assay-Recombinant CRD-FZD1, CRD-FZD2, CRD-FZD5 or CRD-FZD9 (400 ng/ml) were incubated with 20 μ l of A/G agarose beads and recombinant Wnt-5a (400 ng/ml) for 2 h at 4°C in 400 μ l of lysis buffer from New East Biosciences, No. 30303. Protein A/G agarose was collected by centrifugation, and the precipitates were washed 3 times with the lysis buffer. The precipitates from the binding assays were analyzed by SDS-PAGE and probed with goat anti-Wnt-5a AF645 (1:2000) and goat anti-FZD1 (AF1120; 1:1000), goat anti-FZD2 (AF1307; 1:1000), goat anti-FZD5 (AF1617; 1:1000) or goat anti-FZD9 (AF2440; 1:500) antibodies from R&D Systems. For quantifications, the Wnt-5a input band (50 ng) was considered as 100% of signal, and the intensity of the immunoprecipitated bands were compared with the input.

Membrane fractionation from rat brain-Synaptosomes, synaptophysin (SYP)- enriched fraction (Triton X-100 soluble fraction) and PSD-95-enriched fraction (Triton X-100 insoluble fraction) were isolated as previously described, with some modifications (73). Four rat brains were homogenized for each independent experiment in ice-cold homogenization buffer (4 mM HEPES, pH 7.4, containing 320 mM sucrose and 2 mM ethylenediaminetetraacetic acid). The homogenate was centrifuged at 710 $\times g$ for 10 min, and the supernatant was centrifuged at 13,000 $\times g$ for 20 min. Triton X-100 (1%) was added to the pellet for 2h at 4°C, which was then centrifuged at 200,000 $\times g$ for 2 h. The obtained pellet corresponded to the Triton-insoluble fraction enriched in PSD-95, and the supernatant contained the triton-soluble fraction enriched in SYP. The sample was quantified, and 60 μ g of the protein was separated on a 10% SDS-polyacrylamide gel and probed with goat anti-SYP sc-7568 (1:2000) from Santa Cruz Biotechnology, mouse anti-PSD-95 k28/43 (1:5000) from Neuromab and goat anti-FZD9.

Western blotting-Treated and untreated hippocampal neurons were lysed on ice and either immediately processed or frozen at -150°C. Immunoblotting was performed as described previously (74). The following primary antibodies were used: goat anti-FZD9, goat anti-FZD5 (AF1617; 1:500) and goat anti-FZD7 (AF198; 1:500) from R&D; mouse anti-CaMKII α (sc-5306; 1:1000), mouse anti-

phospho Tyr286-CAMKII α (sc-32289; 1:1000), rabbit anti-PKC β II (sc-210; 1:1000), rabbit anti- β -tubulin (sc-9104; 1:1000), mouse anti- β -catenin (sc-7963; 1:1000), and rabbit anti-G β_{1-5} (sc-378; 1:1000) from Santa Cruz Biotechnology; rabbit anti-JNK (#9252; 1:1000) and rabbit anti-phospho-Thr183/Tyr185-JNK (#4668; 1:1000) from Cell Signaling; rabbit phospho-Ser660-PKC β II (ab75837; 1:10000), rabbit anti-G α_o (ab136535; 1:5000), and rabbit anti-G α_i (ab20392; 1:1000) from Abcam; mouse anti-PSD95 (k28/43; 1:10000) from UC Davis/NIH NeuroMab Facility; and mouse anti- β -actin (11978; 1:10000) from Sigma. Equal amounts of protein were loaded (20 μ g).

Immunofluorescence, transfection and image analysis-The hippocampal neurons were cultured on coverslips at a density of 35,000 cells/coverslip, subjected to different treatments, and fixed with a freshly prepared 4% paraformaldehyde plus 4% sucrose in PBS for 20 min at 4°C. The cells were then permeabilized with 0.2% Triton X-100 for 5 min at RT, and then blocked with 1% bovine serum albumin in PBS (blocking solution) for 30 min at 37°C, followed by an overnight incubation with the mouse anti-PSD-95 k28/43 (1:400), rabbit anti-Piccolo 142002 (1:300) from Synaptic Systems and/or goat anti-FZD9 (1:100) primary antibodies at 4°C. The cells were thoroughly washed with PBS and then incubated with phalloidin coupled to Alexa-Fluor 633 (1:500) from Molecular Probes, which was used as a neurite marker, and with secondary antibodies coupled to Alexa-Fluor 488 and Alexa-Fluor 546 (1:1000) from Molecular Probes for 30 min at 37°C. The coverslips were mounted in Fluoromount G mounting media. Digital images were captured with an Olympus FV 1000 confocal microscope. The images were analyzed using NIH ImageJ software. The images of the PSD-95 staining were captured from 10 microscope fields for each condition in 3-4 independent experiments. Each field contained processes for 1–2 neurons, in which 2–3 phalloidin-labeled neurites/neurons were selected and analyzed. We used a previously described protocol to quantify the PSD-95 clusters (11).

To transfect the cultured neurons, we performed magnetofection using NeuroMag (OZ Bioscience) using neurons at DIV10-11 that were plated at 60,000-100,000 cells per 35-mm poly-D-lysine-coated glass-bottom dish. The following constructs were used: enhanced green

fluorescent protein (EGFP; Clontech); Fux (control plasmid); shFZD9-EGFP (36); commercial scramble-FZD9 and shFZD9-EGFP#3 from Qiagen (cat. KH02416G) $G\alpha_o$ PTXi; $G\alpha_{i2}$ PTXi; $G\alpha_{i3}$ PTXi; and β ARKct provided by Dr J. Silvio Gutkind (National Institute of Health, Bethesda). First, the neurons were washed for 30 min with Neurobasal medium. Then, 0.8 μ g of DNA/1.25 μ l of magneto-beads were mixed and incubated at RT for 15 min in 100 μ l of Neurobasal medium. In the co-transfection experiments, the mix was prepared using 0.4 μ g of EGFP plus 0.4 μ g of $G\alpha_o$ PTXi, $G\alpha_{i2/3}$ PTXi or β ARKct plus 1.25 μ l of the beads. Next, the mix was added to the neurons for 15 min with the magnet in the bottom of the plate; it was removed 40 min later, and the transfection medium was replaced with fresh medium. At 14 days *in vitro* (DIV), the hippocampal neurons were starved for 2 h with Neurobasal medium without the B27 supplement before the treatments with recombinant Wnt-5a (300 ng/ml=7,3 nM) with or without PTX (150 ng/ml), Gallein (5 μ M), NF449 (5 μ M) or CRD-FZD9 (300 ng/ml) for different time periods. An Olympus FluoView FV 1000 confocal microscope was used to obtain the digital images at a resolution of 1024 \times 1024. To quantify the dendritic spines and for 3D imaging, the Z-stacks images were reconstructed using the super-pass module of the Imaris software. The dendritic shafts and spines were manually traced using the filament mode. The protrusions below the 3 μ m were considered for the 3D reconstruction. Accurate reconstructions of the diameter of the spine neck and head were created using the diameter function with a contrast threshold of 0.8. Ten microscopic fields were imaged for each condition in 3-5 independent experiments. To determine the spine length and the spine-head width, the mean of all spines from each neurite was measured. The spine density was calculated by measuring the total number of spines per dendrite length (spine density/10 μ m) for each condition.

Measurements of intracellular Ca^{2+} mobilization in hippocampal rat neurons- Ca^{2+} variations were determined in cells loaded with 4.5 μ M Fura-2 AM at 37°C for 30 min (75) using DIV10 hippocampal neurons transfected with Fux-EGFP or shFZD9-EGFP at DIV7 or non-transfected neurons. The experiments were performed with two different isotonic solutions containing (in mM): with calcium, 1.2 $CaCl_2$,

140 NaCl, 5 KCl, 0.5 $MgCl_2$, 5 glucose, and 10 HEPES (305 mOsm/L, pH 7.4 with Tris); and without calcium, 0 $CaCl_2$, 140 NaCl, 2.5 KCl, 1.7 $MgCl_2$, 5 glucose, 0.5 EGTA, and 10 HEPES (305 mOsm/L, pH 7.4 with Tris). Cytosolic Ca^{2+} levels were continuously recorded in the isotonic solution for 1 min before (basal Ca^{2+} level) and 3 min after Wnt-5a application. After 4 min 1 μ M ionomycin was added as a positive control for Ca^{2+} increase. The increases in cytosolic Ca^{2+} were represented as the normalized ratio of the emitted fluorescence at 510 nm after excitation at 340 and 380 nm relative to the ratio measured prior to stimulation (first minute before application of the stimuli) and the area under the curve (A.U.C) after the addition of Wnt-5a was integrated. This integration was performed using the GraphPad Software Prism6. We used the Olympus Spinning Disc IX81 microscope to perform the live Ca^{2+} imaging experiments.

HT22 cell transfections-Immortalized HT22 cells from mouse hippocampal neuronal cells were co-transfected with 0.5 μ g of shFZD9-EGFP or Fux-EGFP plasmids using LipofectamineTM 2000 (Invitrogen) 48 h after plating in six-well culture plates at a density of 1.6×10^6 cells per well.

Immunoprecipitation of $G\alpha_{i/o}$ proteins-We used the $G\alpha_o$ (#80901) and $G\alpha_i$ (#80301) activation assay kits from New East Biosciences. The cultured hippocampal neurons were seeded at a density of 900,000 cells/well at DIV14 and were treated with Wnt-5a (300 ng/ml). After treatment, cells were lysed and the amount of protein was quantified. The same amount of protein was used in each condition for the immunoprecipitation (400ug) with the specific antibodies that recognize the GTP-bound $G\alpha_o$ or the GTP-bound $G\alpha_i$ proteins according to the manufacturer's recommended protocol. GTP γ S was used as a positive control and was added to the neuronal lysate and incubated for 90 min at RT before immunoprecipitation. The total level of protein was detected by immunoblot with the mouse anti- $G\alpha_o$ (1:1000) or mouse anti- $G\alpha_i$ (1:1000) antibodies from the activation kits from New East Bioscience.

For Immunoprecipitation of total $G\alpha_o$ hippocampal cultured neurons were seeded at a density of 600,000 cells/well. The neurons were treated with vehicle or Wnt-5a and then lysed. After protein quantification, 400 μ g of protein was incubated with 5 μ g/ml of mouse anti- $G\alpha_o$

(sc-13532; Santa Cruz biotechnology) and 20 μ l of agarose beads for 1 h at 4°C with orbital rotation. The 10% of the sample was used as input. Next, the lysates were washed 3 times and suspended in 20 μ l of Laemmli 2X loading buffer. The samples were analyzed by immunoblotting with rabbit anti- $G\alpha_o$, goat anti-FZD9 and rabbit anti- $G\beta_{1-5}$ antibodies.

Statistical analyses—Statistical analysis was performed using the statistical software Prism 5 (GraphPad Software). The values are

expressed as the mean \pm standard error of the means. The statistical significance of the differences was assessed using one-way ANOVA with Bonferroni's post-test for multiple comparisons and Student's *t*-test for comparisons between two conditions ($p < 0.05$ was considered significant). The number of independent experiments is indicated in the corresponding figure legend.

Acknowledgments: This work was supported by grants from FONDECYT (No. 1120156) and the Basal Center of Excellence in Science and Technology (CONICYT-PFB12/ 2007) to NCI, a pre-doctoral grant to VTR, and a postdoctoral fellowship from CONICYT (Fondecyt Postdoctorado N°3140355) to ER-F.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: VTR and NCI designed and coordinated the study and wrote the manuscript. VTR performed and analyzed the immunofluorescence and western blot experiments. ER-F performed and analyzed the experiments shown in Fig. 1D, 2B, 2C, 4C, 4D, 8A and 8B. JPH and AF provided technical assistance and contributed to the preparation of the figures and the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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FIGURE LEGENDS

FIGURE 1. FZD9 is located in the postsynaptic region. A. Representative images of DIV14 hippocampal neuron stained with anti-PSD-95 (green) and anti-FZD9 (red) or anti-Piccolo (green). Scale bar=10 μ m. B. Quantification of the Manders co-localization index between FZD9 and PSD-95 or Piccolo. C. Quantification of the Manders co-localization index between PSD-95 and FZD2, -3, -7 and -9 (n=3). D. Detection and quantification of FZD9, PSD-95 and SYP in synaptosomes, Triton X-100 insoluble and Triton X-100 soluble-enriched membrane fractions from rat brain. The graph shows the amount of protein in each fraction normalized by the amount of the same protein in synaptosomes (A.U.) arbitrary units; (n=5). ***p<0.001 **p<0.01, *p<0.05.

FIGURE 2. CRD-FZD9 blocks the calcium increase produced by Wnt-5a. A. Recombinant Wnt-5a was incubated with protein A/G agarose beads and CRD-FZD1, -FZD2, -FZD5 or FZD9 for 2 h. The precipitates were probed with anti-Wnt-5a and anti-FZD1, -FZD2, -FZD5 or -FZD9 antibodies. Wnt-5a (50 ng) or CRD-FZD9 (50 ng) was loaded into the left lanes as input controls (n=3). Quantification of CRD-FZD-Wnt-5a binding assay. The Wnt-5a input was considered as 100%. There was not significant difference between the conditions (n=4). B. Measurement of intracellular Ca^{2+} in Wnt-5a, CRD-FZD9 (300 ng/ml) or Wnt-5a+CRD-FZD9-treated neurons using the Fura-2 AM probe in a 0 Ca^{2+} solution. (n=3 independent experiments, 20 neurons each). C. Quantification of the area under the curve (A.U.C.) (n=3). ***p<0.001, ns, no significant difference.

FIGURE 3. CRD-FZD9 inhibits the spinogenesis of triggered by Wnt-5a treatment. A. *Left*, Immunodetection of PSD-95 (red) in EGFP-transfected neurons (green) maintained for 14 days in culture. The merge image shows the EGFP-protrusion with the PSD-95 puncta stain in the head, indicating that the protrusions are spines. Arrowheads indicate dendrites and arrows indicate an axon. *Right*, 3D reconstruction of the dendrite; Scale bar=25 μ m. B. *Upper panel*, representative images of EGFP-transfected neurons at DIV10 and then treated with Wnt-5a, CRD-FZD9 or Wnt-5a+CRD-FZD9 for 2 h at DIV 14. *Low*, quantification of spine density in hippocampal neurons treated with Wnt-5a and CRD-FZD9; Scale bar= 5 μ m. (n=3; 10 neurons per condition, 2-3 neurites per neuron) **p<0.01, *p<0.05.

FIGURE 4. FZD9 downregulation impairs Wnt-5a signaling. A. Detection of FZD9, FZD5 and FZD7 protein levels in the homogenates of HT22 cells transfected with Fux-EGFP (control) or shFZD9-EGFP (the three FZD receptors were measured in three different membranes). Anti-GFP was used as a transfection control, and anti-actin was used as a loading control (n=3). B. Immunofluorescence of FZD9 (red) in FUX-EGFP/shFZD9-EGFP-transfected (green) neurons and on scramble-EGFP/shFZD9c-EGFP (commercial shFZD9, Cod. KH02416G) transfected neurons; scale bar=8 μ m. C. Measurement of the increase in intracellular Ca^{2+} concentrations in hippocampal neurons transfected with Fux-EGFP (black plots) or shFZD9-EGFP (gray plots) after stimulation with Wnt-5a.

Ionomycin was used as an ionophore to increase intracellular Ca^{2+} levels (n=4). D. Quantification of A.U.C. (n=3). E. *Left panel*, Representative images of the neurites from Fux-EGFP- or shFZD9-EGFP-transfected neurons treated with or without Wnt-5a for 2 h. Scale bar, 3 μ m. *Right panel*, quantification of the spine density in control condition or after 1 or 2 h of Wnt-5a treatment (n=3). F. *Left panel*, Representative images of Scramble-EGFP and shFZD9 transfected neurons (commercial shFZD9, Cod. KH02416G) treated or not with Wnt-5a for 2 h. *right panel*, Quantification of the density of dendritic protrusion per neurite length in hippocampal neurons treated with Wnt-5a; scale bar=3 μ m. ***p<0.001, **p<0.01, *p<0.05. ns, no significant difference compared with the control.

FIGURE 5. Wnt-5a increases the GDP/GTP exchange of the $G\alpha_o$ subunit, and FZD9 is associated with $G\alpha_o$ in DIV14 hippocampal neurons. A. Hippocampal neurons were stimulated with recombinant Wnt-5a for the indicated lengths of time. The lysates were incubated with specific antibodies that recognize $G\alpha_o$ -GTP or $G\alpha_i$ -GTP. As a positive control, untreated hippocampal neuron lysates were incubated with GTP γ S for 90 min at RT and then incubated with the antibody. The lysates were analyzed by SDS-PAGE and immunoblotting with antibodies that recognize the total levels of $G\alpha_o$ or $G\alpha_i$ (n=3). The IgG band shows that an equal amount of the $G\alpha_o$ -GTP antibodies were used for immunoprecipitation. *Left panel*, representative blot images; *Right panel*, blot quantifications. B. DIV13-14 neurons were treated or not with Wnt-5a (30 min) and the lysates were immunoprecipitated with an anti- $G\alpha_o$ antibody and immunoblotted with anti-FZD9 and anti- $G\beta_{1-5}$ (this antibody labels all five $G\beta$ isoforms) (n=4) in the same membrane. No Ab, no antibody was added; *Left panel*, representative blot images; *Right panel*, blot quantifications; **p<0.01, *p<0.05.

FIGURE 6. PTX inhibits the Wnt-5a-induced activation of the non-canonical Wnt pathway in hippocampal neurons. A. Representative western blot and quantification of total and phosphorylated levels of CaMKII (n=4), JNK (n=4) and PKC β II (n=6) and the total levels of β -catenin (n=3) and PSD-95 (n=4) in neurons pre-incubated with PTX for 6 h and then treated with Wnt-5a for the indicated lengths of time. β -actin and β -tubulin were used as loading controls. B. Detection of neurites of hippocampal neurons at DIV14 treated with recombinant Wnt-5a ligand, with PTX or with PTX plus Wnt-5a or untreated for 2 h. PSD-95 is shown in green, and fluorescent phalloidin-labeled actin is shown in blue. Quantification of the number of PSD-95 clusters/10 μ m per neurite after Wnt-5a and/or PTX treatment for the indicated lengths of time (n=3, 10 neurons per condition, 3 neurites per neuron). **p<0.01, *p<0.05.

FIGURE 7. $G\alpha_o$ activation is necessary for the increase in dendritic spine density stimulated by Wnt-5a. A. Quantification of spine density from neurites from EGFP-transfected neurons treated with or without Wnt-5a for 2 h in the presence or absence of PTX. B. Quantification of mean width of the spine heads (n=3; 10 neurons per condition, 3 neurites per neuron). C. Quantification of the spine density from EGFP-transfected neurons treated with or without Wnt-5a for 2 h in the presence or absence of NF449 ($G\alpha_s$ inhibitor) D. DIV10-11 hippocampal neurons were transfected with EGFP or with EGFP plus $G\alpha_o$ PTXi, $G\alpha_{i2}$ PTXi or $G\alpha_{i3}$ PTXi mutants insensitive to PTX. At DIV14, the neurons were pre-treated with PTX for 6 h and then treated with Wnt-5a for 2 h (n=4). Scale bar, 3 μ m ***p<0.001, ** p<0.01, *p<0.05, ns, no significant difference.

FIGURE 8. Gallein blocks the Wnt-5a-induced changes in dendritic spine density. A. Measurement of the increases in intracellular Ca^{2+} concentrations in the hippocampal neurons that were bathed in a Ca^{2+} -free solution and treated with Gallein or Wnt-5a plus Gallein (n=3). B. Quantification of area under the curve of the measurement of Ca^{2+} concentrations. C. Images of the neurites from neurons treated with Wnt-5a and/or Gallein for 2 h. Quantification of the dendritic spine density for all conditions. D. The neurons were transfected with EGFP or EGFP+ β ARKct and treated with Wnt-5a for 2 h. Quantification of the dendritic spine density from all conditions (n=3). Scale bar, 3 μ m, **p<0.001, **p<0.01; ns, no significant difference.

FIGURE 9. Model for the Wnt-5a effects at the post-synaptic region. Wnt-5a induces the activation of the non-canonical Wnt pathway (Wnt/ Ca^{2+}) by interacting with the FZD9 receptor in the

post-synaptic compartment. This interaction facilitates the rapid and transient activation of heterotrimeric G proteins, specifically the $G\alpha_o$ subunit and the $G\beta\gamma$ complex. The activated G proteins participate in the Wnt-5a-induced dendritic spine dynamics and the PSD-95 cluster remodeling through PLC, CaMKII, PKC and JNK activation.

Figure 1.

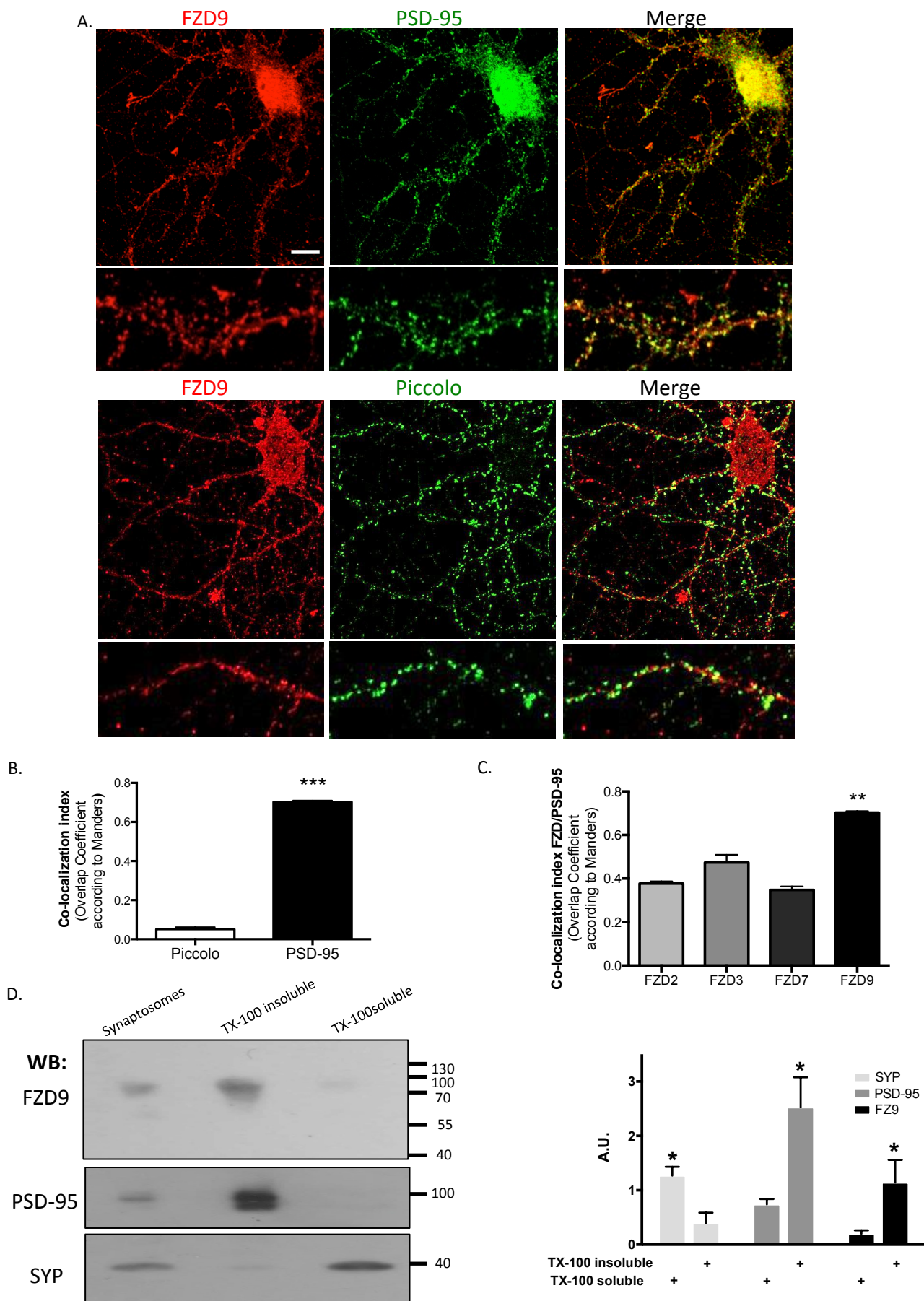


Figure 2.

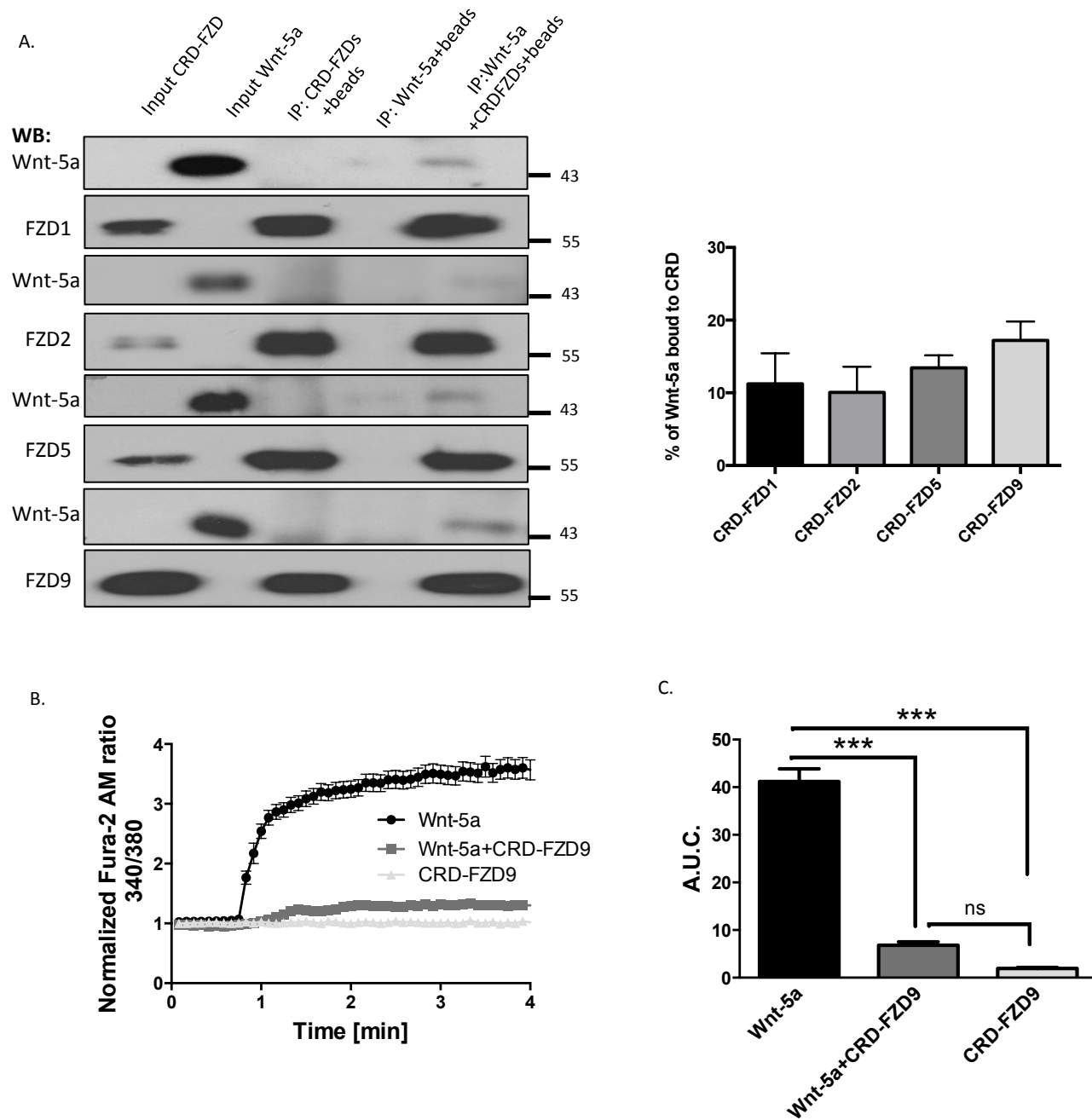
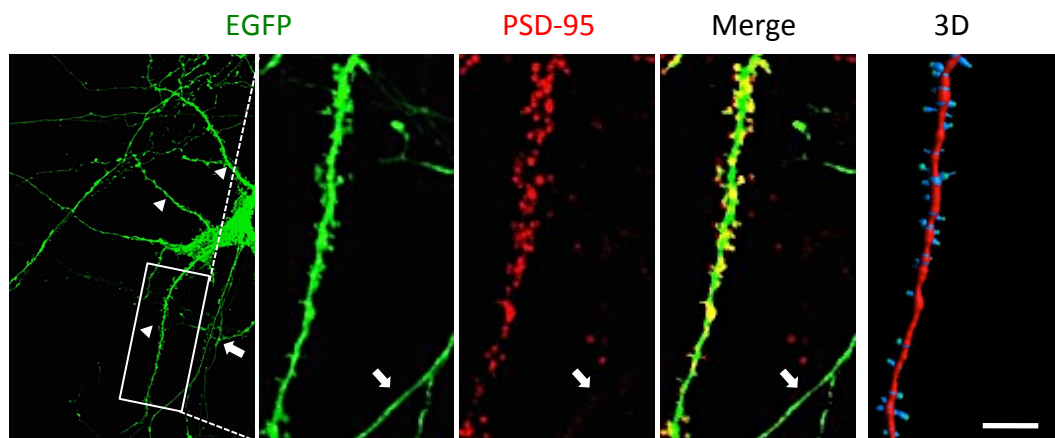


Figure 3.

A.



B.

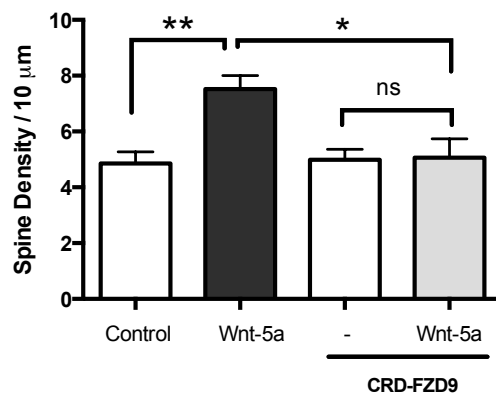
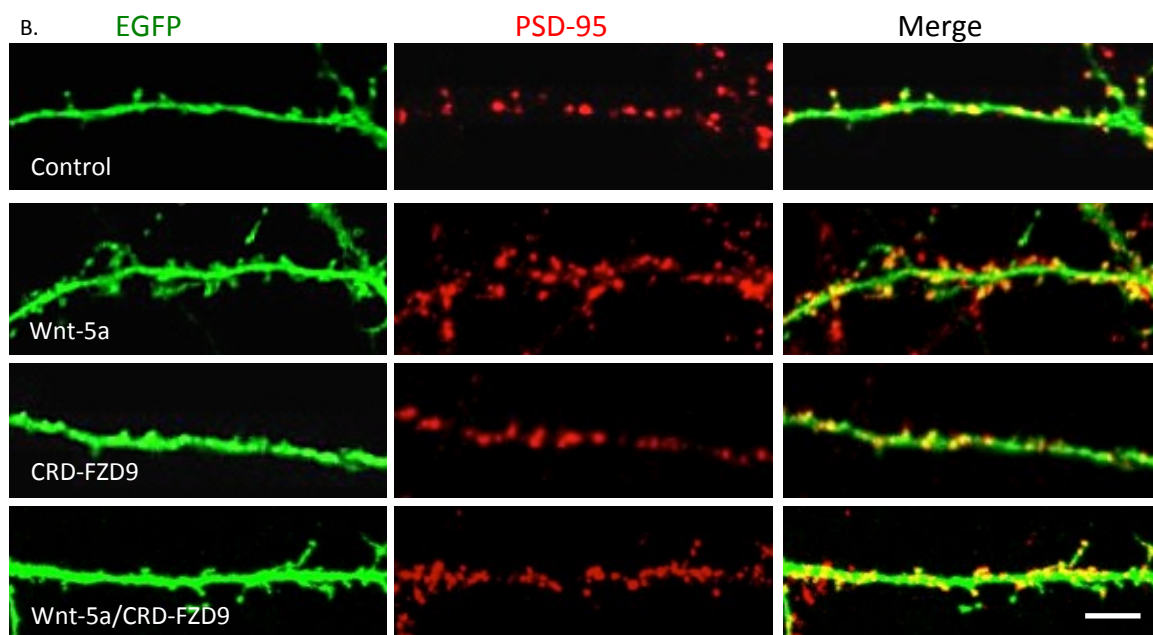


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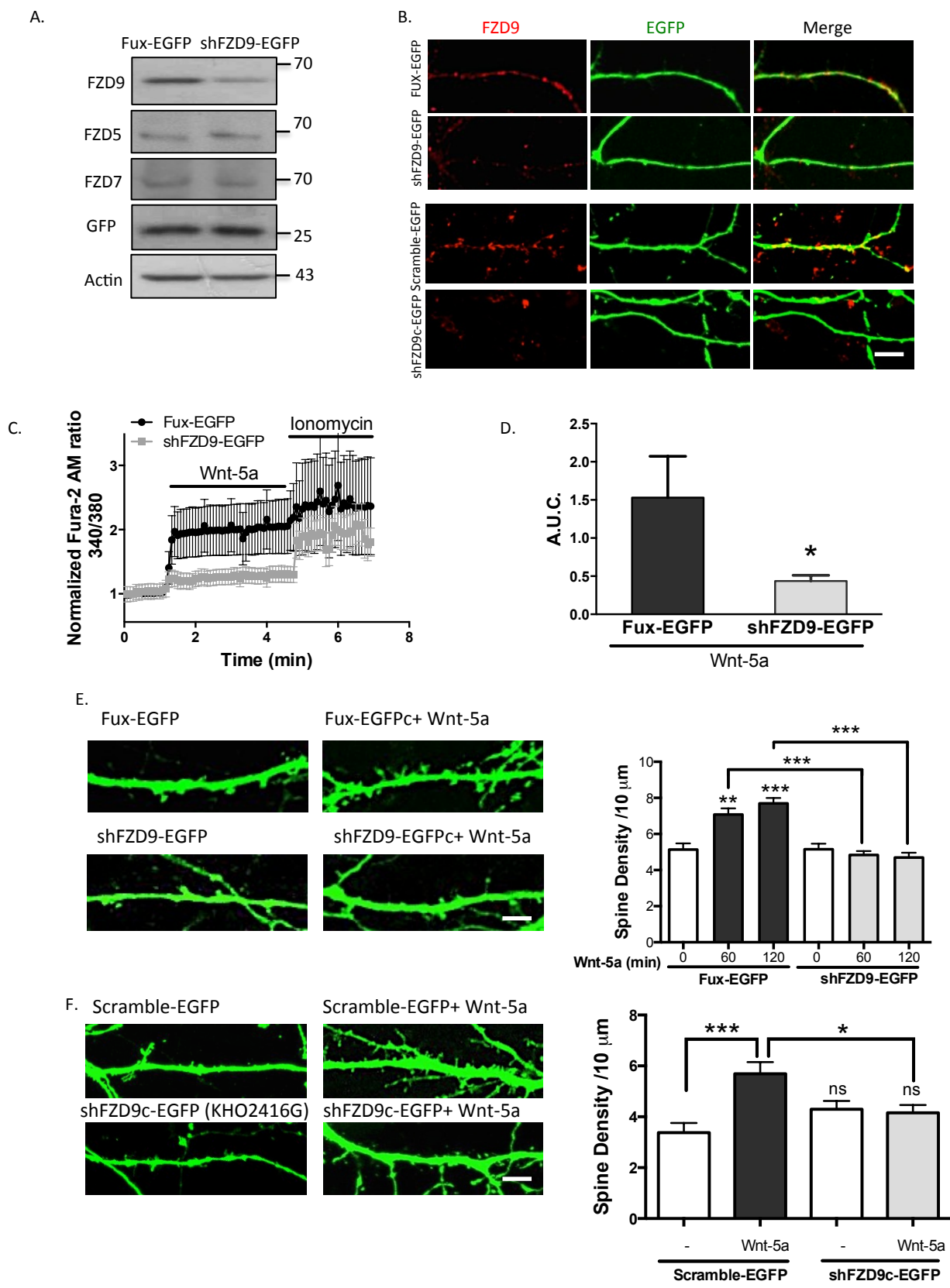


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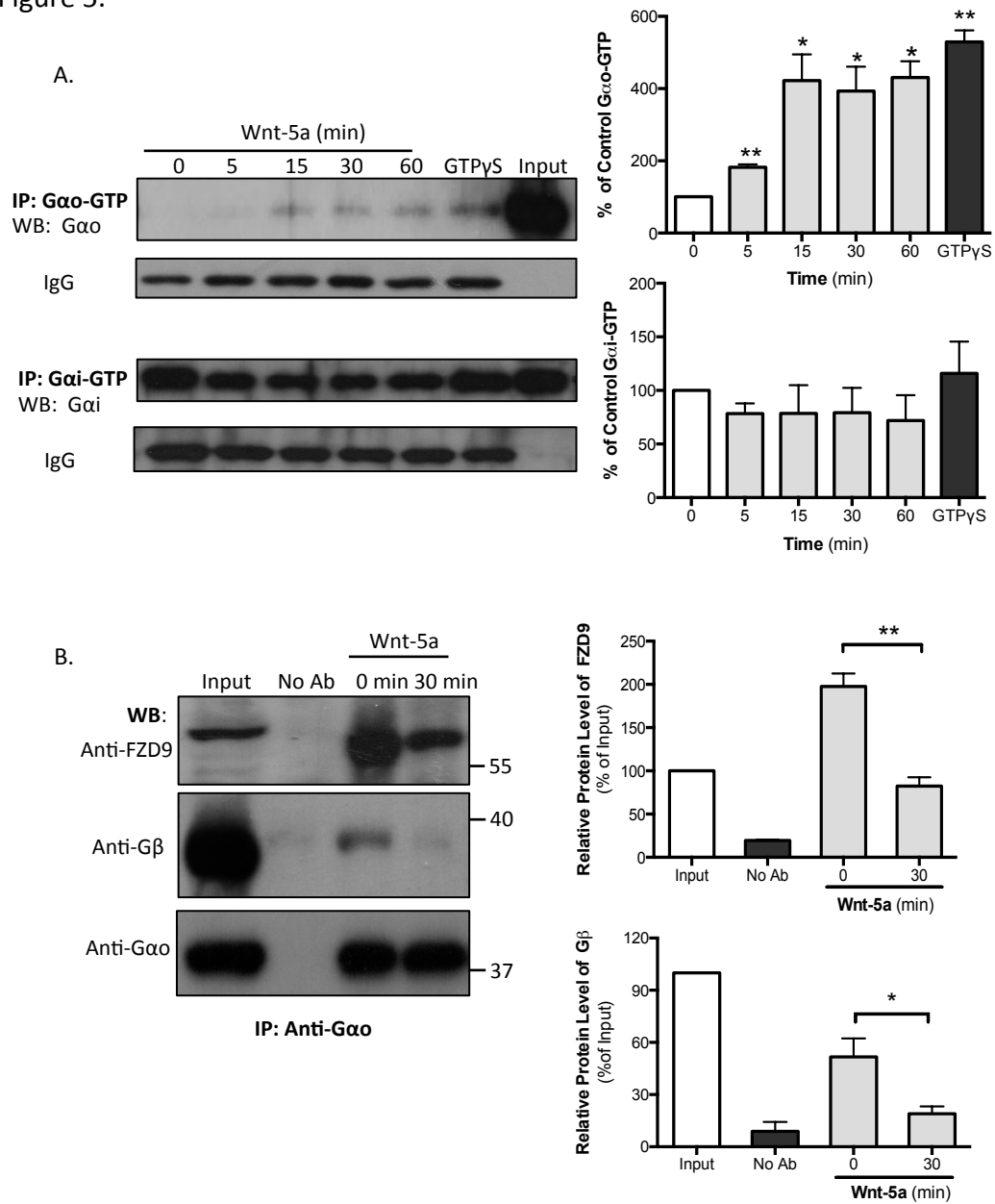
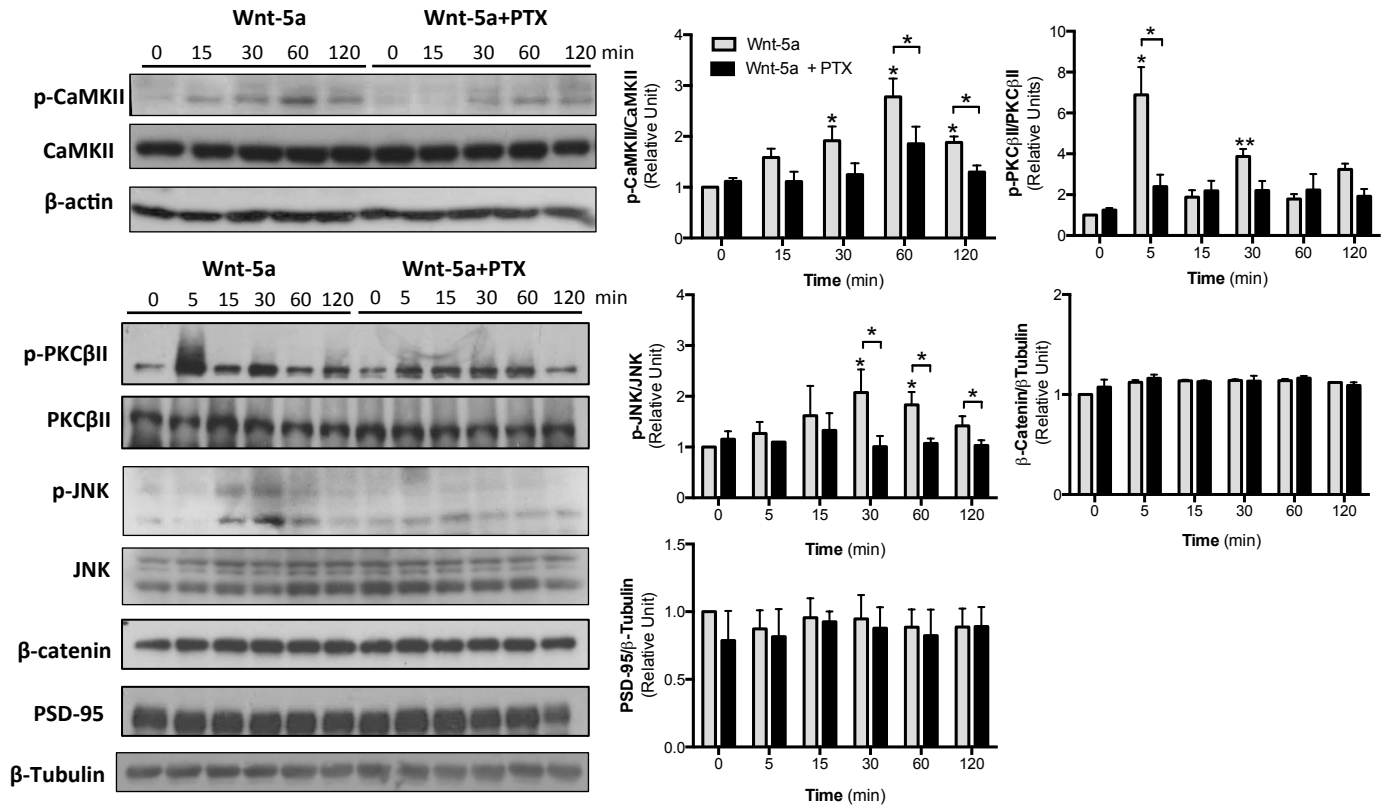


Figure 6.

A.



B.

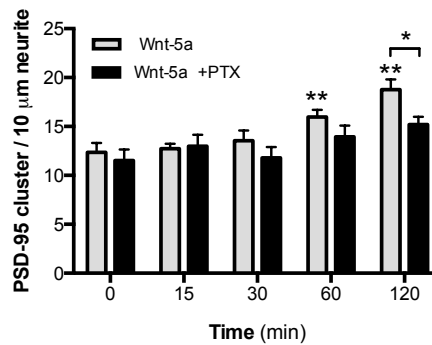
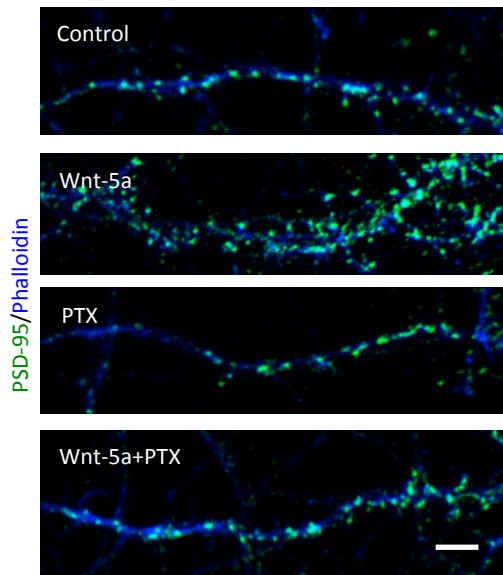


Figure 7.

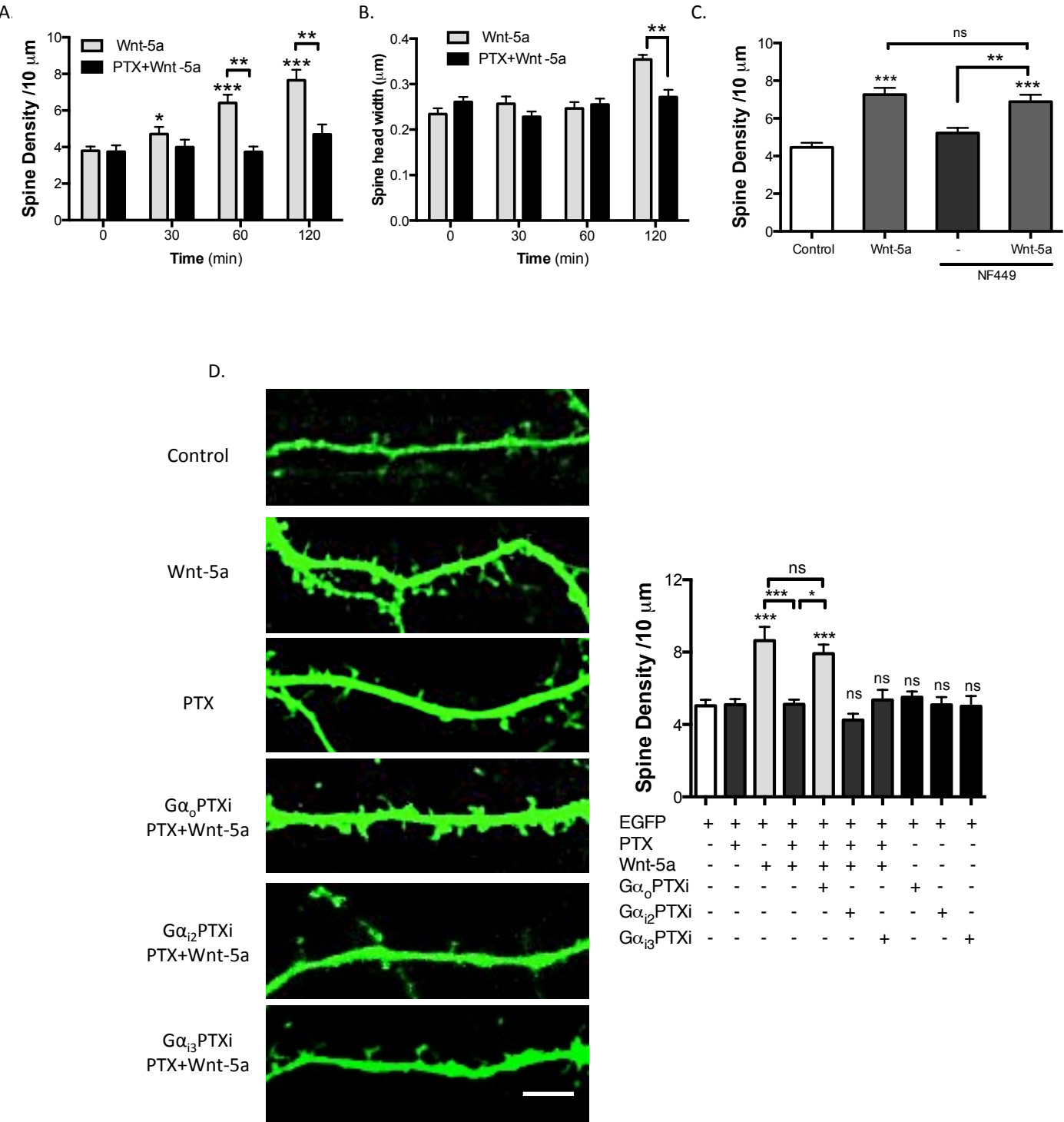


Figure 8.

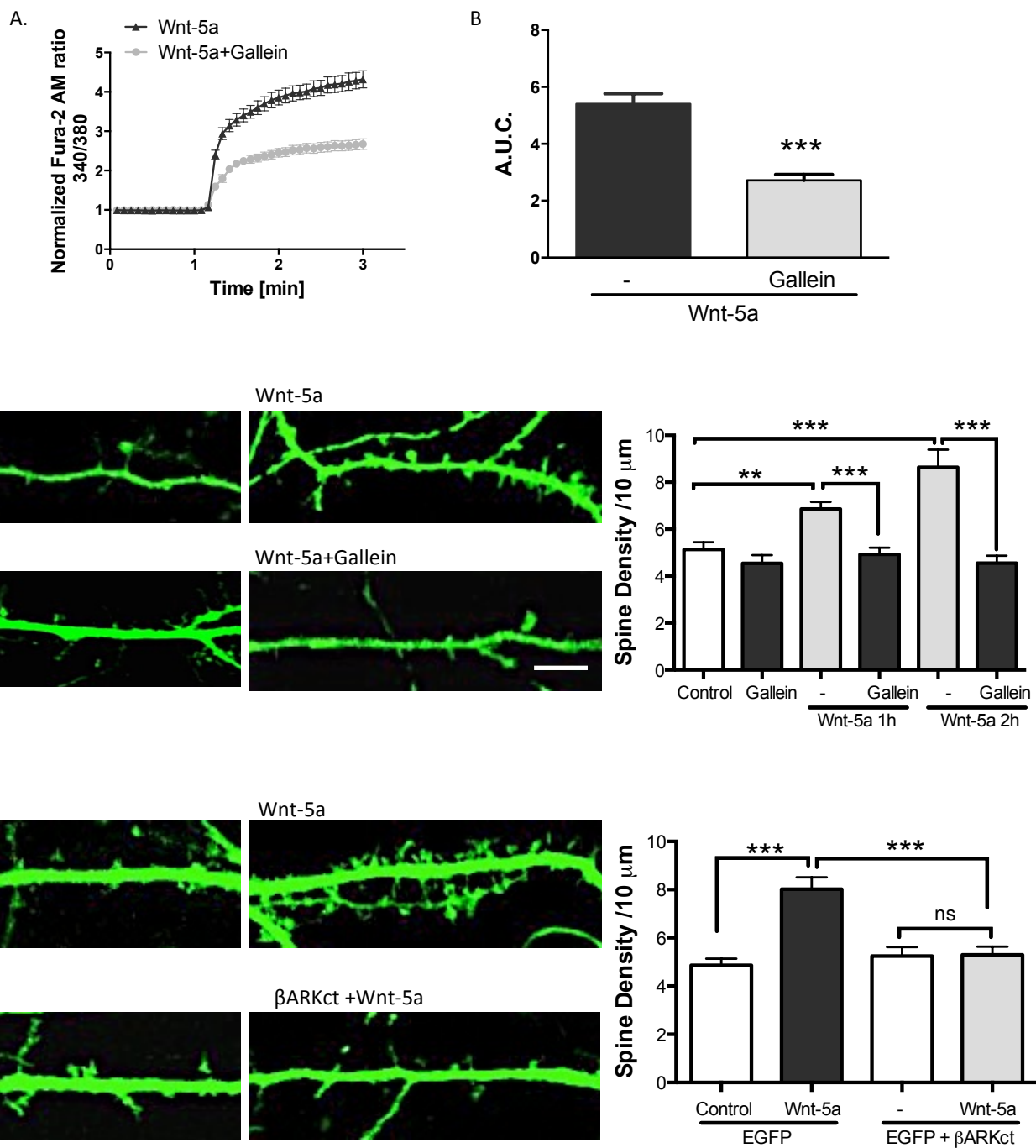
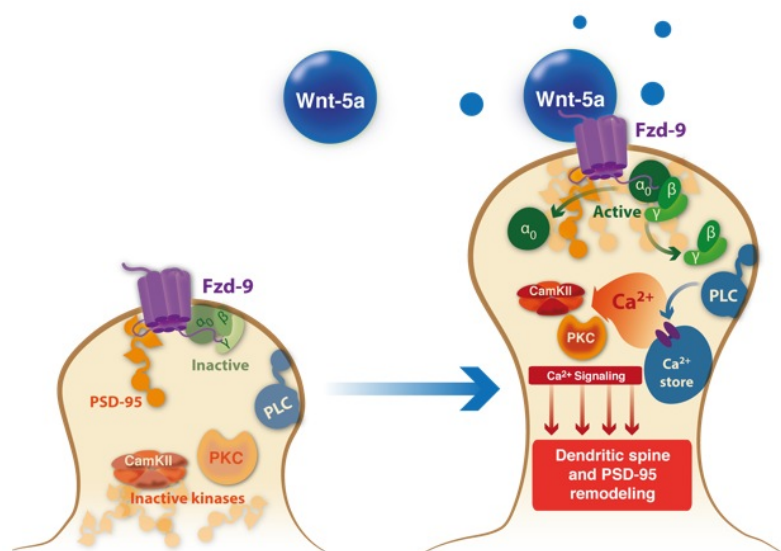


Figure 9.



Wnt-5a/Frizzled9 receptor signaling through the $G\alpha_o/G\beta\gamma$ complex regulates dendritic spine formation.

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J. Biol. Chem. published online July 11, 2016

Access the most updated version of this article at doi: [10.1074/jbc.M116.722132](https://doi.org/10.1074/jbc.M116.722132)

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