Development 137, 2215-2125 (2010) doi:10.1242/dev.046722

Frizzled-5, a receptor for the synaptic organizer Wnt7a, regulates activity-mediated synaptogenesis

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SUMMARY

Wnt proteins play a crucial role in several aspects of neuronal circuit formation. Wnts can signal through different receptors including Frizzled, Ryk and Ror2. In the hippocampus, Wnt7a stimulates the formation of synapses; however, its receptor remains poorly characterized. Here, we demonstrate that Frizzled-5 (Fz5) is expressed during the peak of synaptogenesis in the mouse hippocampus. Fz5 is present in synaptosomes and colocalizes with the pre- and postsynaptic markers vGlut1 and PSD-95. Expression of Fz5 during early stages of synaptogenesis increases the number of presynaptic sites in hippocampal neurons. Conversely, Fz5 knockdown or the soluble Fz5-CRD domain (Fz5CRD), which binds to Wnt7a, block the ability of Wnt7a to stimulate synaptogenesis. Increased neuronal activity induced by K^+ depolarization or by high-frequency stimulation (HFS), known to induce synapse formation, raises the levels of Fz5 at the cell surface. Importantly, both stimuli increase the localization of Fz5 at synapses, an effect that is blocked by Wnt antagonists or Fz5CRD. Conversely, low-frequency stimulation, which reduces the number of synapses, decreases the levels of surface Fz5 and the percentage of synapses containing the receptor. Interestingly, Fz5CRD abolishes HFS-induced synapse formation. Our results indicate that Fz5 mediates the synaptogenic effect of Wnt7a and that its localization to synapses is regulated by neuronal activity, a process that depends on endogenous Wnts. These findings support a model where neuronal activity and Wnts increase the responsiveness of neurons to Wnt signalling by recruiting Fz5 receptor at synaptic sites.

KEY WORDS: Wnt signalling, Synaptic assembly, Hippocampus, Dendrite, Neuronal activity, Mouse

INTRODUCTION

After axons reach their targets, secreted and membrane proteins activate signalling cascades to initiate the assembly of synaptic sites. At central synapses, secreted signals such as Wnts, FGFs and neurotrophins activate specific receptors on target cells, resulting in structural and functional changes in the presynaptic terminal and on the postsynaptic receptive dendrite (Ciani and Salinas, 2005; Fox and Umemori, 2006; Hardin and King, 2008). Little is known, however, about the localization and identity of the receptors involved.

Wnt proteins function as synaptic organizers in both vertebrates and invertebrates (Salinas and Zou, 2008; Speese and Budnik, 2007). In a retrograde or anterograde manner, Wnts regulate axon remodelling, required for the conversion of growth cones into synaptic boutons (Hall et al., 2000; Packard et al., 2002), and induce the recruitment of pre- and postsynaptic components to future synaptic sites (Hall et al., 2000; Packard et al., 2002). Wnts induce presynaptic differentiation through the activation of the canonical or β-catenin pathway (Davis et al., 2008; Hall et al., 2000). Studies in *Drosophila* and *C. elegans* have shown that Wnts activate the seven transmembrane Frizzled (Fz) receptors to regulate synapse formation (Ataman et al., 2008; Klassen and Shen, 2007). At vertebrate synapses, by contrast, the receptors activated by Wnts during synaptic assembly remain elusive.

Three distinct receptor families mediate Wnt signalling: Fzs, the single-pass LRP5/6 co-receptors and the atypical tyrosine kinase receptors Ror2 and Ryk (Angers and Moon, 2009; van Amerongen et al., 2008). In the nervous system, Fzs mediate a range of functions from neuronal differentiation (Van Raay et al., 2005) to cell polarity (Prasad and Clark, 2006), cell migration (Pan et al., 2006; Vivancos et al., 2009), axon guidance (Lyuksyutova et al., 2003; Wang et al., 2002) and cell survival (Wang et al., 2001). In *Drosophila*, the Wnt protein Wingless (Wg) activates the Fz2 receptor, present at both sides of the neuromuscular synapse, to regulate pre- and postsynaptic signalling (Ataman et al., 2008; Packard et al., 2002). In *C. elegans*, by contrast, activation of the extrasynaptic Fz receptor Lin-17 inhibits synapse formation (Klassen and Shen, 2007). At vertebrate central synapses, Wnts such as Wnt7a, Wnt3a and Wnt5a stimulate synapse formation in the cerebellum, spinal cord and hippocampal neurons (Ahmad-Annuar et al., 2006; Davis et al., 2008; Hall et al., 2000; Krylova et al., 2002). In cultured hippocampal neurons, Fz1 is present at presynaptic sites and mediates Wnt3a-induced synapse formation (Varela-Nallar et al., 2009). However, the receptors for key synaptogenic factors such as Wnt7a remain poorly characterized.

Neuronal activity plays a crucial role in synapse formation and function. Activity regulates synapse formation through changes in axonal and dendritic filopodia dynamics (Hua and Smith, 2004) and by recruiting pre- and postsynaptic proteins to synaptic sites (Craig et al., 2006; McAllister, 2007). In addition, activity regulates the localization of membrane proteins that control the formation and function of neuronal circuits. Depolarization or induction of long-term potentiation (LTP) recruits N-cadherin (Bozdagi et al., 2000; Tanaka et al., 2000), as well as AMPA and NMDA receptors (Friedman et al., 2000; Hayashi et al., 2000; Heynen et al., 2000) to synaptic sites, whereas field stimulation or depolarization

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Accepted 30 April 2010

increases TrkB surface levels (Du et al., 2000; Meyer-Franke et al., 1998). By contrast, low-frequency stimulation (LFS)-evoked long-term depression (LTD) is associated with internalization of synaptic AMPA and NMDA receptors (Carroll et al., 2001; Shi et al., 1999) as well as TrkB receptors (Du et al., 2003). However, little is known about the role of neuronal activity in the localization of Wnt receptors.

Here, we report that the Wnt receptor Fz5 is expressed in the mouse hippocampus and in cultured hippocampal neurons during the period of synaptogenesis, where it localizes to both pre- and postsynaptic sites. Gain-of-function studies demonstrate that Fz5 increases the number of synapsin I and bassoon puncta, a hallmark of presynaptic assembly. In addition, Wnt7a binds to the extracellular cysteine-rich domain (CRD) of Fz5, which is crucial for binding to Wnts and for signalling (Povelones and Nusse, 2005; Rulifson et al., 2000). Importantly, Fz5 knockdown or the soluble Fz5-CRD domain (Fz5CRD) block the synaptogenic activity of Wnt7a. In addition, neuronal activity induced by depolarization or high-frequency stimulation (HFS) increases the mobilization of Fz5 to the cell surface and its localization to synapses. By contrast, LFS decreases the trafficking of Fz5 to the surface and its insertion to synaptic sites. Fz5CRD or the Wnt antagonists Sfrp1 and Sfrp3, block the activity-dependent localization of Fz5 at synapses. Moreover, HFS-induced synapse formation is completely blocked by Fz5CRD, suggesting a role for Wnt-Fz5 signalling in this process. We therefore propose that activation of Fz5 by Wnt7a, together with neuronal activity, regulate synaptic assembly in hippocampal neurons and that both neuronal activity and Wnts regulate Fz5 localization to synapses.

MATERIALS AND METHODS

Neuronal cultures and cell transfection

Primary hippocampal cultures were prepared from embryonic day 18 (E18) Sprague-Dawley rat embryos and cultured as described previously (Rosso et al., 2005). Cells were cultured at a density of 30-50 cells/mm^2 for immunostaining or at 250 cells/mm^2 for biotinylation assays. Hippocampal neurons were transfected at 3-5 days in vitro (DIV) with EGFP or hFz5-HA constructs using Lipofectamine 2000 (Invitrogen), treated as indicated and fixed at 9-11 DIV. Transfected neurons were exposed to recombinant Wnt7a (50 ng/ml, R&D Systems) for 16 hours. Untransfected neurons (9-12 DIV) were exposed for 16 hours to control or Fz5CRD-containing conditioned media obtained from transfected QT6 cells.

Surface receptor staining, immunofluorescence and immunohistochemistry

Cultured neurons were fixed with 4% paraformaldehyde (PFA) in PBS with 4% sucrose, and stained with primary antibodies against β-III tubulin (Tuj-1; Chemicon), HA (Roche), GFP (Molecular Probes and Upstate), Myc (Sigma), bassoon (Bioquote Limited), synapsin I (Transduction Labs), vGlut-1 (Chemicon), NR1 (Synaptic Systems), MAP-2 (Sigma) or frizzled 5 (Abcam). Secondary antibodies were from Molecular Probes. For surface expression, anti-Fz5 antibody was added to live cells and incubated for 30 minutes on ice or 15 minutes at 37°C. Mouse brains were fixed with 4% PFA in PBS at 4°C and then incubated in 30% sucrose in PBS at 4°C. Cryostat sections (16 mm) were incubated in blocking solution (0.2% gelatine, 0.25% Triton X-100 in PBS) followed by primary antibodies against vGlut1, PSD-95 (Affinity Bioreagents) and Fz5 and finally by appropriate secondary antibodies. Fluorescence images were captured with an Olympus BX60 microscope or with a Leica TCS SP1 confocal microscope. Images were acquired with either Metamorph or Leica software and analyzed using Metamorph or Volocity software. Single-plane confocal images or maximal projections obtained with the 3D feature on Volocity were used to determine colocalization.

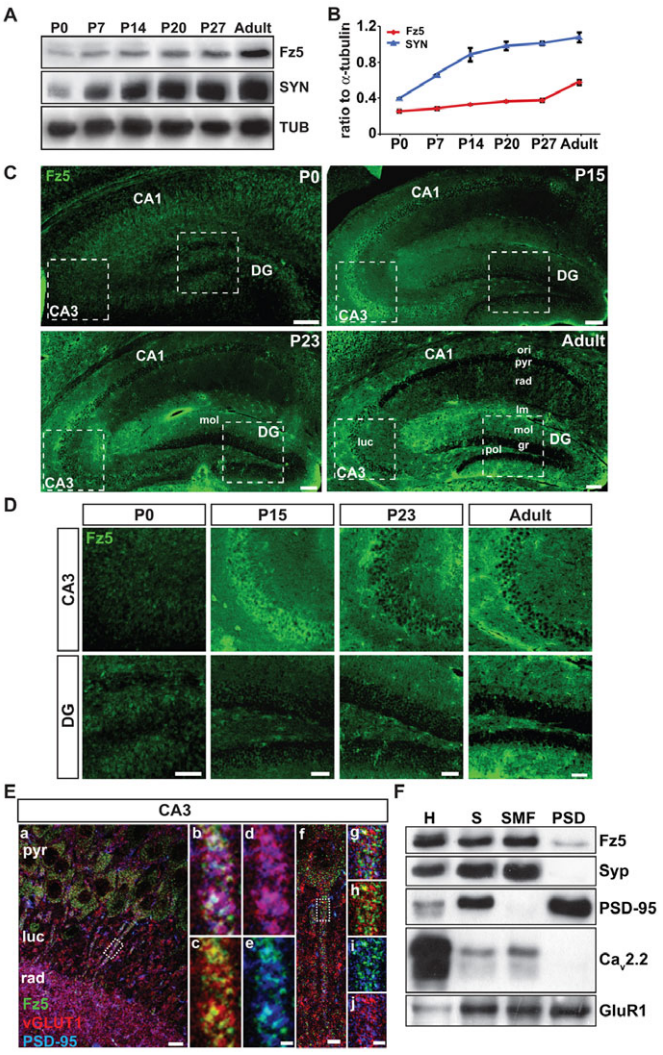

Fig. 1. Localization of Fz5 in the mouse hippocampus at different developmental stages. (**A**) Representative blots from brain homogenates of P0, P7, P14, P20 and adult mice showing the expression levels of Fz5, synapsin I (SYN) and α-tubulin (TUB). (**B**) Fz5/α-tubulin and synapsin I/α-tubulin ratios as determined by densitometric analysis of mouse brain homogenates (*n*=3 brains for each age). (**C**) Expression pattern of Fz5 in the hippocampus of P0, P15, P23 and adult mice (*n*=3 brains for each stage). gr, granular cell layer of the dentate gyrus (DG); lm, stratum lacunosum moleculare; luc, stratum lucidum of CA3; mol, molecular layer of the DG; ori, stratum oriens; pol, polymorphic layer of the DG; pyr, pyramidal cell layer; rad, stratum radiatum. (**D**) Enlarged boxed areas from C. (**E**) (**a**) Maximal projection of confocal images obtained with the 3D feature of Volocity showing Fz5, vGlut1 and PSD-95 staining in the CA3 region of P15 mice (*n*=3 experiments). (**b-e**) Enlarged boxed area from a with different stainings as indicated in a. (**f**) Single confocal plane of a cell also present in a, showing the colocalization of Fz5 with vGlut1 and PSD-95. (**g-j**) Enlarged boxed area from f with different stainings as indicated in a. (**F**) Fz5 distribution in adult mouse brain homogenates (H), synaptosomes (S), the synaptosomal membrane fraction (SMF) and the PSD fraction (PSD. *n*=5 experiments. Synaptophysin (Syp) and PSD-95 are pre- and postsynaptic markers, respectively. N-type Ca^{2+} channel ($Ca_v2.2$) and GluR1 were used to show receptor distribution. Scale bars: 100 μm in C; 50 μm in D; 10 μm in Ea; 5 μm in Ef; 1 μm in Eb-d,g-j.

Western blot analysis

Brain homogenates were prepared with RIPA buffer using a tissue homogenizer at 0°C. Equal amounts of protein (Lowry assay) were analyzed by SDS-PAGE and western blot using antibodies for synapsin I (Chemicon), Fz5 and α-tubulin (Sigma). Membranes were probed with HRP-coupled secondary antibodies and developed with ECL reagent (Amersham). Quantification of band intensity was performed using ImageJ software.

Synaptosomal preparation

Synaptosomes were prepared as previously described (Cohen et al., 1977), with minor modifications (Ahmad-Annuar et al., 2006). In brief, synaptosomes were treated with TX-100, followed by centrifugation at 82,500 ***g*** for 45 minutes. Then the supernatant, corresponding to the synaptosomal membrane fraction (SMF), was removed and the pellet, corresponding to the postsynaptic density (PSD) fraction, was resuspended in buffer B. Equal amounts of proteins were loaded onto an SDS-PAGE. Primary antibodies against Fz5, synaptophysin (Chemicon), PSD-95, N-type Ca^{2+} channel (α_{1B} subunit, Sigma) and GluR1 (Upstate) were used.

Binding assay

QT6 cells were used to prepare Wnt7a-HA-containing conditioned media. Cos-7 cells were transfected with the CRD domain of human Fz5 or *Drosophila* Dfz2, both containing a glycosyl-phosphatidylinositol (GPI) sequence (Fz5CRD-myc-GPI and Dfz2CRD-myc-GPI). Live transfected Cos-7 cells were incubated for 1 hour at RT with control or Wnt7a-HA-containing conditioned media. Cells were fixed with 4% PFA and 4% sucrose in PBS, and incubated with primary antibodies to HA and Myc followed by incubation with biotinylated secondary antibodies (Amersham), Alexa 488 conjugated secondary antibodies and Hoechst (Molecular Probes). HRP staining was developed according to the manufacturer's protocol (Vectastain Elite ABC Kit, Vector Laboratories).

RNA interference to Fz5

Fz5 knockdown was achieved by using SureSilencing shRNA plasmids to rat Fz5 (SABiosciences). The kit contains 4 shRNA vectors specifically directed to the rat Fz5 and an shRNA vector with a scrambled artificial sequence as a negative control (nc-shRNA). These plasmids also contain the GFP gene to identify transfected cells. NRK cells were transfected with the shRNAs using Lipofectamine 2000. NRK cells were fixed and processed for immunostaining or lysed and prepared for western blot analysis 48 hours after transfection. Primary hippocampal neurons were transfected at 4 DIV with either the mixture of Fz5 shRNAs or nc-shRNA using Lipofectamine 2000. At 9 DIV, these neurons were incubated with recombinant Wnt7a (50 ng/ml) for 16 hours, fixed and processed for immunocytochemistry.

Cell surface biotinylation

Surface Fz5 was detected by cell surface biotinylation using Sulfo-NHS-LC-LC-biotin (Pierce) and Streptavidin Sepharose High Performance (GE Healthcare) (Du et al., 2000). Cell lysates were prepared from 14 DIV hippocampal neurons after K^+ depolarization, HFS or LFS.

Stimulation of cultured hippocampal neurons

For K^+ depolarization studies, 14 DIV neurons were treated with 50 mM NaCl or KCl. For HFS and LFS studies, neurons were electrically stimulated using a Grass48 stimulator (Grass Instruments). Trains of HFS were used, with train duration of 400 milliseconds containing 20 consecutive pulses at 50 Hz. The interval between trains lasted 5 seconds. LTD-like LFS used a constant stimulation frequency of 4 Hz. Electrical stimulation was delivered for 1 hour at 37°C in a 6 cm-diameter dish via two Ag/AgCl electrodes. Neurons were exposed to control or Fz5CRD-containing conditioned media obtained from transfected QT6 cells or to recombinant Sfrp1 and Sfrp3 (2.5 mg/ml and 250 ng/ml respectively, R&D).

To assess depolarization and HFS, the levels of CaMKII phosphorylation, which increase under these experimental conditions, were analyzed by western blot (Blitzer et al., 1998; Pettit et al., 1994). The level of CaMKII activation was determined by its phosphorylation at threonine 286 and normalized to total CaMKII levels (Cell Signaling).

To assess the effect of LFS, the levels of NR1 were analyzed. Following LFS, cells displayed a 54% reduction in the number NR1 puncta (data not shown) and a 49% reduction in the number of synapses (Fig. 7J), indicative that LTD-like induction was achieved (Heynen et al., 2000).

Statistical analyses

Values given are mean ± s.e.m. Statistical significance was determined using two-tailed Student's *t*-test and ANOVA. Levels of significance were labelled as follows: ****, $P<0.0001$; ***, $P<0.001$; **, $P<0.01$ and *, $P<0.05$.

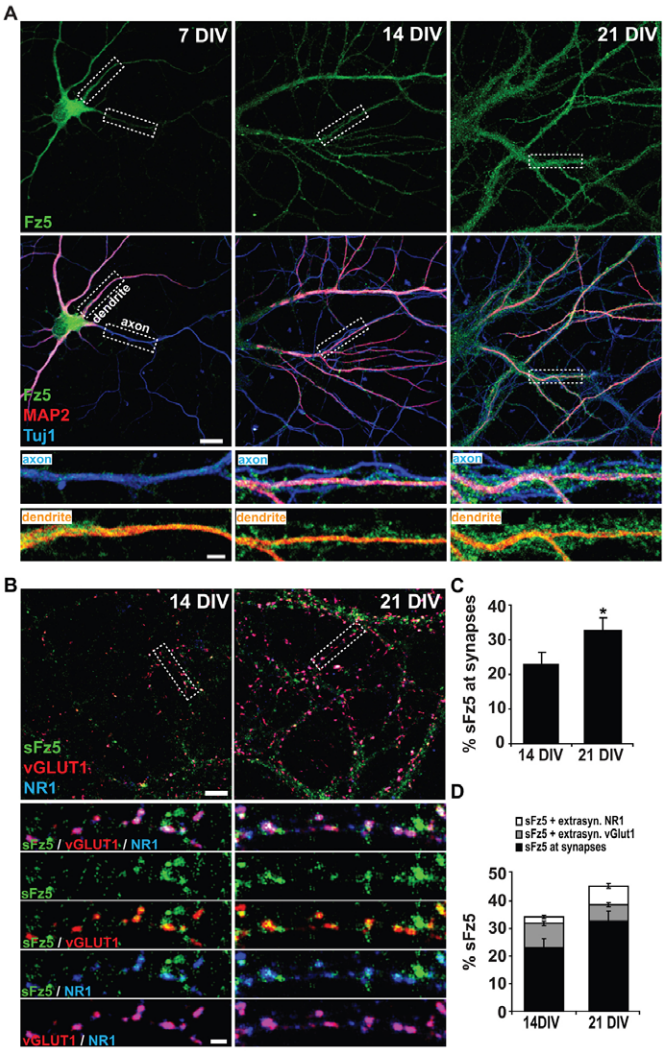

Fig. 2. Fz5 distribution in cultured hippocampal neurons. (**A**) Expression of Fz5 in 7, 14 and 21 DIV cultured hippocampal neurons (*n*=3 experiments). Middle panels: Fz5 colocalization with MAP-2 and tubulin (Tuj1). Lower panels: enlarged boxed areas. (**B**) Representative images showing the colocalization of sFz5 with vGlut1 and NR1. Lower panels: enlarged boxed areas. (**C**) Percentage of sFz5 that colocalizes with both vGlut1 and NR1 in 14 and 21 DIV cultured neurons (*n*=3 experiments). (**D**) Percentage of sFz5 that colocalizes with both synaptic and extrasynaptic vGlut1 (grey) and NR1 (white) in 14 and 21 DIV neurons (*n*=3 experiments). Scale bars: 10 μm (top and middle panels) and 2 μm (lower panels) in A; 5 μm (upper panels) and 1 μm (lower panels) in B. *, $P<0.05$.

RESULTS

Fz5 is expressed during synapse formation in the hippocampus

To determine whether Fz receptors play a role in central synapse formation, we first examined the protein levels of Fz5 in brain homogenates from different stages. Fz5 is expressed at low levels in newborn mice but it increases during the postnatal period, reaching a plateau at postnatal day 27 (P27), with a further increase in the adult brain (Fig. 1A). When compared with synapsin I, a presynaptic protein and an indicator of synaptogenesis (Chin et al., 1995; Rosahl et al., 1995), Fz5 expression increases with a slight delay (Fig. 1B). These results indicate that Fz5 expression follows synaptic development and maturation.

We next examined the distribution of Fz5 in the mouse hippocampus by immunofluorescence microscopy. Fz5 is particularly enriched in neurons and neuropil (Fig. 1C,D). At P0, Fz5 is present in the cell body and processes of pyramidal neurons (CA1 to CA3 regions) and granule cells of the dentate gyrus (DG; Fig. 1C,D). From P15, a progressive increase in Fz5 levels is observed. In the CA3 region, there is a clear elevation in Fz5 expression at P15 that is maintained throughout development. In the DG, however, the increase in Fz5 expression is more gradual and reaches a maximum in the adult. Interestingly, Fz5 exhibits a laminar-selective distribution, which becomes more evident from P23. Fz5 is present at higher levels in the molecular than in the granular cell layer of the DG (Fig. 1C,D). In CA3 and CA1 regions, Fz5 is highly localized to the stratum lacunosum moleculare (Fig. 1C). At P23, this pattern is maintained. In the adult hippocampus, Fz5 is highly expressed in the polymorphic layer of the DG and it is more evident in the stratum oriens of the CA1 region (Fig. 1C,D). Together, these results demonstrate that Fz5 expression increases during postnatal hippocampal development.

A pool of Fz5 localizes to synapses

Given that Fz5 is expressed by neurons, we investigated whether it localizes to synapses. In P15 mice, Fz5 presents a punctate pattern and colocalizes with the pre- and postsynaptic markers vGlut1 and PSD-95, respectively, in CA3 pyramidal neurons (Fig. 1E). Fz5 also colocalizes with vGlut1 and PSD-95 in the polymorphic layer of the DG, presumably along the axons of the granule cells (see Fig. S1 in the supplementary material). Consistent with its synaptic localization, Fz5 is present in synaptosomes isolated from adult mouse brain. Synaptophysin, N-type Ca^{2+} channel, PSD-95 and GluR1 were used as markers for different synaptic fractions. Fz5 is present in synaptosomes (S), and a pool of Fz5 is present in the synaptosomal membrane fraction (SMF) and also in the postsynaptic density (PSD) fraction (Fig. 1F). These results indicate that Fz5 is present at both pre- and postsynaptic sites.

We next examined the distribution of Fz5 in cultured hippocampal neurons. At 7 DIV, Fz5 is present in the soma of pyramidal neurons (Fig. 2A) and exhibits a punctate appearance along dendrites (MAP-2 positive) and axons (MAP-2 negative), which becomes more evident as neurons mature. At 14 and 21 DIV, Fz5 is clearly present along neurites and particularly at high levels in areas where axons wrap around dendrites (Fig. 2A). Thus, Fz5 localization is developmentally regulated in cultured hippocampal neurons, as occurs in vivo.

We next examined the distribution of surface Fz5 (sFz5) at different stages of synaptogenesis by following its colocalization with the pre- and postsynaptic markers vGlut1 and NR1. Synaptic localization was defined by the receptor colocalization with both vGlut1 and NR1. At 14 DIV, at the peak of synaptogenesis, 23% of sFz5 colocalizes with both synaptic markers but increases to 33% by 21 DIV (Fig. 2B,C). The percentage of the receptor associated with NR1 puncta, which is not in contact with vGlut1

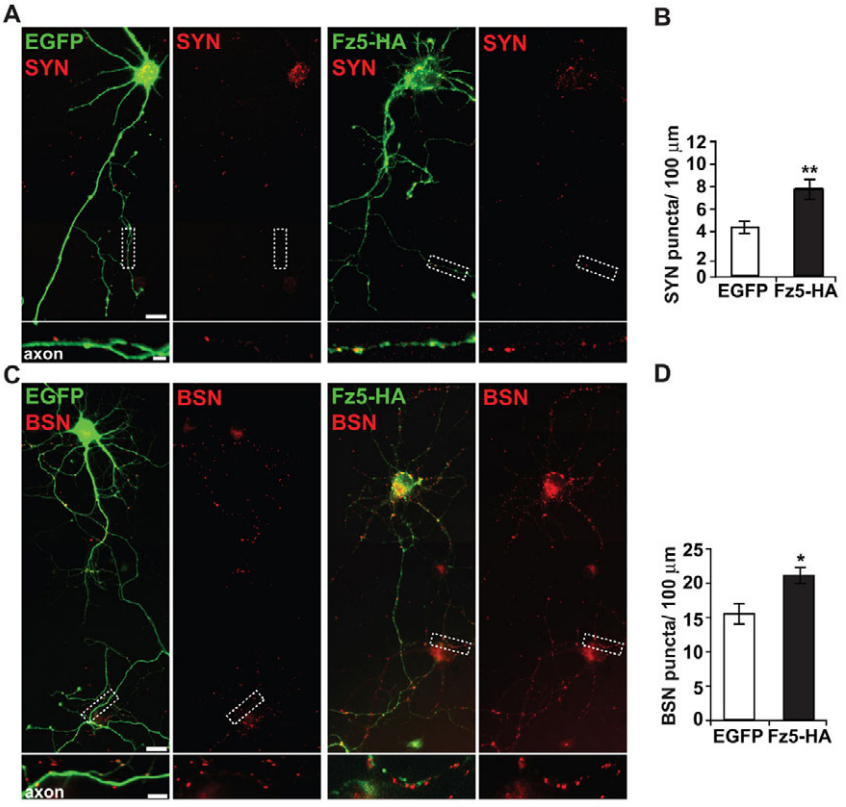

Fig. 3. Fz5 stimulates the formation of presynaptic sites. (**A**) Representative images showing synapsin I puncta (SYN) in hippocampal neurons expressing EGFP or Fz5-HA. Lower panels: enlarged boxed areas. (**B**) Number of synapsin 1 clusters in hippocampal neurons expressing EGFP or Fz5-HA (*n*=4 experiments; 8-12 cells were used per condition for each experiment). (**C**) Microscopy images showing bassoon puncta (BSN) in hippocampal neurons expressing EGFP or Fz5-HA. Lower panels: enlarged boxed areas. (**D**) Number of bassoon puncta in cultured neurons expressing EGFP or Fz5-HA (*n*=4 experiments; 8-12 cells were used per condition for each experiment). Scale bars: 20 μm (upper panels) and 5 μm (lower panels) in A; 20 μm (upper panels) and 5 μm (lower panels) in C. *, $P<0.05$; **, $P<0.01$.

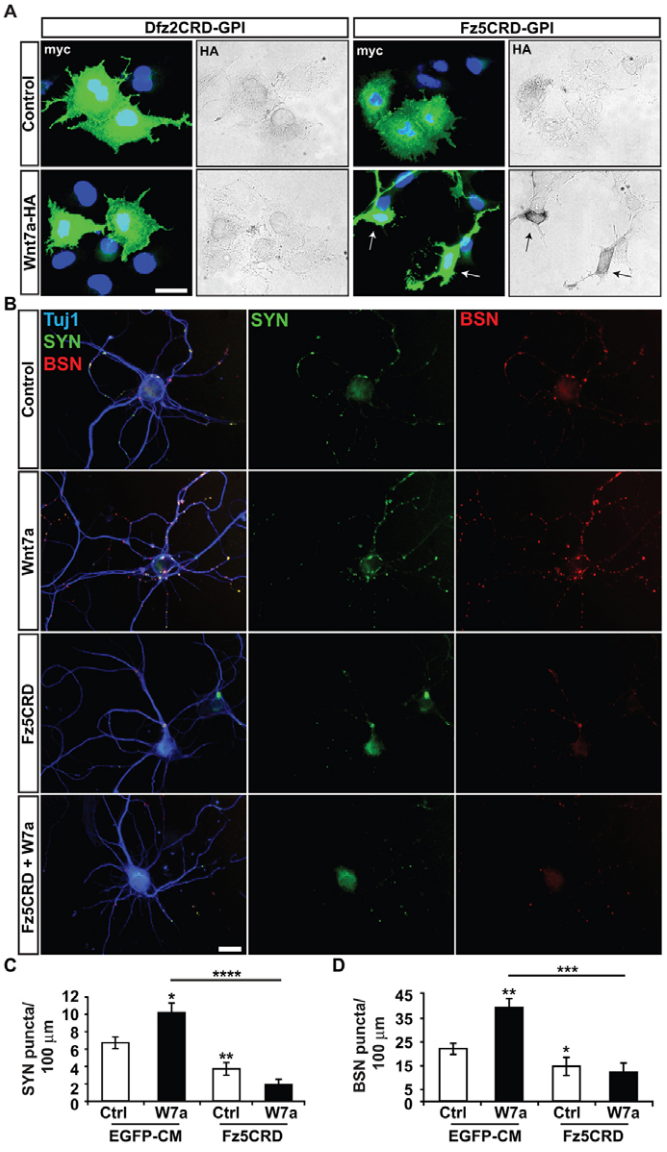

Fig. 4. Fz5 promotes synapse formation by binding to Wnt7a. (**A**) Wnt7a-HA binding (dark precipitate, black arrows) to Fz5CRD-myc-GPI (white arrows) but not to Dfz2CRD-myc-GPI-expressing Cos-7 cells (*n*=3 experiments). Blue: Hoechst nuclei staining. (**B**) Synapsin I (SYN) and bassoon (BSN) clustering in cultured hippocampal neurons treated with Fz5CRD and/or Wnt7a. Tubulin: Tuj1. (**C**) Number of synapsin 1 clusters in hippocampal neurons treated with Fz5CRD and/or Wnt7a (*n*=3 experiments; 8-12 cells were used per condition for each experiment). EGFP-CM: conditioned medium from EFGP-transfected QT6 cells. (**D**) Bassoon cluster number in cultured neurons treated with Wnt7a and/or Fz5CRD (*n*=3 experiments; 8-12 cells were used per condition for each experiment). Scale bars: 20 µm in A,B. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001; ****, *P*<0.0001.

puncta, also increases during neuronal maturation (Fig. 2D). Although the number of sFz5 and extrasynaptic vGlut1 puncta increases between 14 and 21 DIV, the percentage of sFz5 that colocalizes with extrasynaptic vGlut1 puncta does not significantly change during this period (Fig. 2D). Together, our data suggest that Fz5 is present at synaptic sites and that its synaptic localization increases during synapse formation.

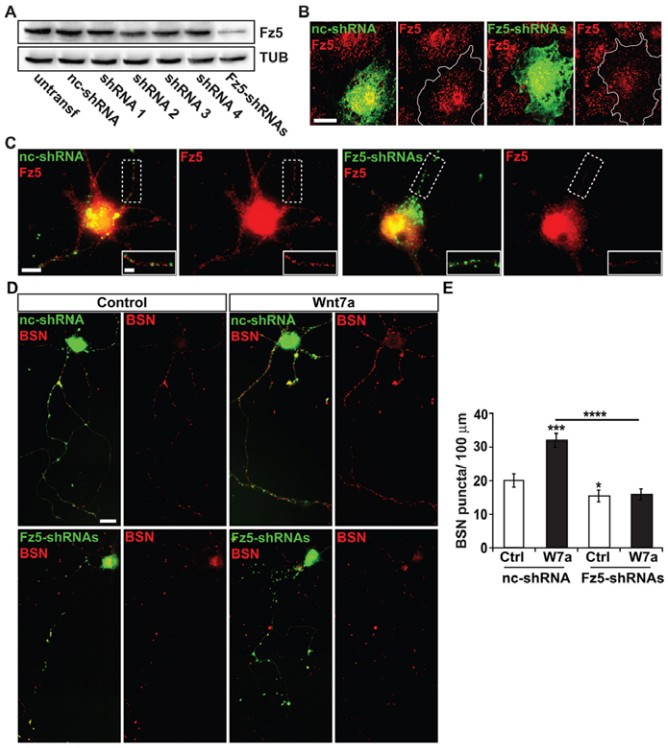

Fig. 5. Fz5 knockdown abolishes Wnt7a-induced synapse formation. (**A**) Representative blots showing Fz5 and α-tubulin (TUB) protein levels in NRK cells transfected with shRNAs to Fz5 (*n*=3 experiments). nc-shRNA, negative control shRNA; shRNA 1 to 4, Fz5shRNAs; untransf, untransfected cells; Fz5-shRNAs, mixture of Fz5shRNAs. (**B**) Confocal images show Fz5 expression in NRK cells transfected with Fz5-shRNAs or nc-shRNAs (*n*=3 experiments). (**C**) Fz5 expression in cultured hippocampal neurons transfected with Fz5-shRNAs or nc-shRNA (*n*=3 experiments). Insets show an enlarged view of boxed areas. (**D**) Bassoon (BSN) puncta in cultured hippocampal neurons transfected with Fz5-shRNAs or nc-shRNAs and treated with Wnt7a. (**E**) Number of bassoon clusters in hippocampal neurons treated with Wnt7a and transfected with Fz5-shRNAs or nc-shRNAs (*n*=3 experiments; 8-12 cells were used per condition for each experiment). Scale bars: 10 µm; 5 µm in inset in C. *, *P*<0.05; ***, *P*<0.001; ****, *P*<0.0001.

Fz5 stimulates the formation of synaptic sites

The synaptic localization of sFz5 led us to test its possible role in synaptogenesis by performing gain-of-function studies. Hippocampal neurons (9-11 DIV) expressing control EGFP or Fz5 (Fz5-HA) were examined for the formation of presynaptic puncta (labelled with the synaptic vesicle protein synapsin I and the active zone protein bassoon) along axons (Fig. 3A,B). Axonal Fz5 expression induces a 78% and a 36% increase in the number of synapsin I and bassoon puncta, respectively (Fig. 3C,D). Thus, expression of Fz5 in axons induces presynaptic differentiation in a similar manner to the synaptogenic factor Wnt7a.

Fz5 functions as a receptor for Wnt7a during synapse formation

Wnt7a induces the formation of synapses in hippocampal neurons (Cerpa et al., 2008) but the receptor mediating this effect has not been identified. Therefore, we tested whether Fz5 functions as a

receptor for Wnt7a. Previous studies have shown that Wnt ligands bind to the cysteine-rich domain (CRD) of Fz and that this domain is required for signalling (Liu et al., 2008; Povelones and Nusse, 2005). Importantly, the CRD domain of Fz5 can block Wnt signalling during early embryonic patterning (Kemp et al., 2007; Liu et al., 2008). We expressed the CRD domains of human Fz5 (Fz5CRD-GPI) and the *Drosophila* Dfz2 (Dfz2CRD-GPI) in Cos-7 cells. Transfected cells were then incubated with control or Wnt7a-HA-containing conditioned medium. Immunocytochemical assays reveal that Wnt7a binds to cells expressing Fz5CRD-GPI but not Dfz2CRD-GPI or EGFP (Fig. 4A; data not shown). These results demonstrate that Wnt7a specifically binds to the CRD domain of Fz5.

We then investigated whether Fz5 functions as a receptor for Wnt7a in hippocampal neurons. To test this, we examined whether a soluble version of the CRD domain of Fz5 (Fz5CRD) has an effect on synaptogenesis. Hippocampal neurons were exposed to control or Fz5CRD-containing conditional media either with or without recombinant Wnt7a for 16 hours. Fz5CRD alone decreased the number of synapsin I and bassoon puncta by 45% and 34%, respectively, when compared with controls (Fig. 4B-D). These findings suggest that Fz5CRD blocks endogenous synaptogenic Wnts. We next tested if Fz5CRD could block the ability of exogenous Wnt7a to regulate synaptogenesis. Wnt7a alone induced a 52% and a 79% increase in the number of synapsin I and bassoon puncta, respectively (Fig. 4B-D). By contrast, neurons exposed to both Wnt7a and Fz5CRD exhibited a decrease of 82% in synapsin I puncta and 70% in bassoon puncta, when compared with Wnt7a-treated cells (Fig. 4B-D). Therefore, Fz5CRD completely blocks the synaptogenic activity of Wnt7a, suggesting that Fz5 mediates Wnt7a function at synapses.

To confirm that Fz5 is a receptor for Wnt7a in hippocampal neurons, we knocked down its expression using shRNAs. The level of knockdown was assessed by transfecting each of the four different shRNAs (Fz5-shRNA 1 to 4) or a mixture of the four into NRK cells. A scrambled artificial sequence shRNA (nc-shRNA) was used as control. Expression of the EGFP gene present in the shRNA plasmids allowed identification of transfected cells. Western blot analyses and immunocytochemistry indicated that expression of each single shRNA does not affect the levels of endogenous Fz5 (Fig. 5A). By contrast, combined expression of the four Fz5-shRNAs decreased the expression of endogenous Fz5 when compared with untransfected cells or cells expressing nc-shRNA (Fig. 5A,B). In hippocampal neurons, the Fz5 shRNA mixture significantly decreased the level of endogenous Fz5, whereas nc-shRNA had no effect (Fig. 5C).

To test the effect of Fz5 knockdown in synapse formation, hippocampal neurons were transfected with Fz5-shRNAs or nc-shRNA. We found that Fz5-shRNAs decreased the number of bassoon puncta by 23% when compared with nc-shRNA-expressing cells (Fig. 5D,E). These findings demonstrate that endogenous Fz5 regulates synaptogenesis. A significant increase in the number of Bassoon clusters (59%) was observed in the presence of Wnt7a in nc-shRNA-transfected neurons (Fig. 5D,E). However, Fz5 knockdown abolished the ability of exogenous Wnt7a to induce the bassoon clustering (Fig. 5D,E). These results demonstrate that Fz5 functions as a receptor for Wnt7a during synapse formation in hippocampal neurons.

K^+ depolarization increases the levels of Fz5 at the cell surface and at synaptic sites

The localization of receptors can be regulated by neuronal activity (Bozdagi et al., 2000; Du et al., 2000; Hayashi et al., 2000). We therefore examined the possible effect of high K^+ depolarization on Fz5 localization. Neuronal depolarization was assessed by the level of CaMKII phosphorylation (Blitzer et al., 1998; Pettit et al., 1994; Wang et al., 2003). K^+ depolarization induced a 56% increase in the levels of phosphorylated CaMKII on Thr286 when compared with controls (Fig. 6A,B). Importantly, depolarization increased the level of sFz5 by 58%, as determined by biotinylation, without affecting the total levels of the protein in cultured hippocampal neurons (Fig. 6C,D).

Confocal microscopy revealed that K^+ depolarization induced a 40% increase in the number, a 113% increase in the volume and a 75% increase in intensity of sFz5 puncta (Fig. 6E-H). Synaptic localization of sFz5, as determined by its colocalization with the

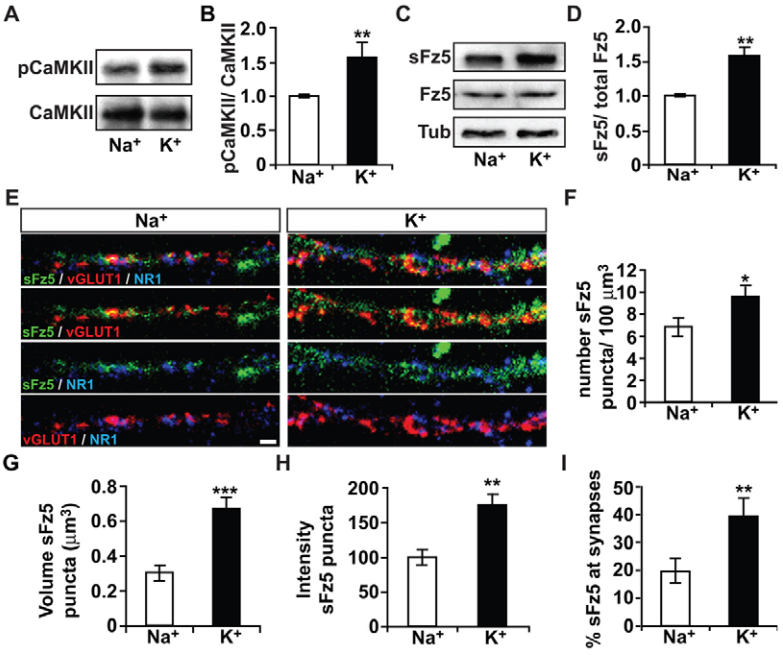

Fig. 6. K^+ depolarization increases Fz5 levels at the surface and at synapses. (**A**) Representative blots showing the effects of K^+ depolarization on CaMKII phosphorylation in cultured hippocampal neurons. Upper panel: CaMKII phosphorylated on Thr286. Lower panel: total CaMKII. (**B**) pCaMKII/total CaMKII ratio in Na^+ and K^+-treated neurons as determined by densitometric analysis (n=3 experiments). (**C**) Levels of sFz5 following K^+ depolarization. Upper panel: biotinylated sFz5. Middle panel: total Fz5. Lower panel: α-tubulin. (**D**) Surface Fz5/total Fz5 ratio used to determine the effect of K^+ depolarization in cultured neurons (n=3 experiments). (**E**) Representative images showing the localization of sFz5, vGlut1 and NR1 following K^+ depolarization. (**F-I**) Effects of K^+ depolarization on the number (F), volume (G), intensity (H) and synaptic localization (I) of sFz5 puncta (n=3 experiments; 8-12 cells were used per condition for each experiment). Scale bar: 1 µm in E. *, P<0.05; **, P<0.01; ***, P<0.001.

synaptic markers vGlut1 and NR1, demonstrated that depolarization increases the level of sFz5 at synapses from 20% to 39% (Fig. 6E,I). Together, these results demonstrate that depolarization not only increases the trafficking of Fz5 to the cell surface but also stimulates its mobilization to synapses.

Different patterns of electrical stimulation differentially modulate Fz5 levels at the cell surface and synaptic sites

To further investigate the role of neuronal activity on Fz5 localization, we examined the effect of patterned electrical stimulation, shown to regulate the distribution of a number of receptors (Du et al., 2000; Hayashi et al., 2000; Rodriguez et al., 2008; Shi et al., 1999). We used two different electrical stimuli to induce neuronal activity. Intermittent high-frequency stimulation (HFS), where a regular stimulation pattern comprising trains of 20 stimuli at 50 Hz (10 millisecond interval) delivered every 5 seconds was used to mimic average neuronal activity in vivo (an average of 4 action potentials per second) (Schoenbaum et al., 1999; Attwell and Laughlin, 2001). We also used low-frequency (LFS) stimulation at 4 Hz (250 millisecond interval), known to induce LTD (Zamani et al., 2000; Dudek and Bear, 1992). Neuronal activation induced by HFS was confirmed by a 74% increase in the levels of CaMKII Thr286 phosphorylation when compared with unstimulated cells (Fig. 7A,B). Then, sFz5 levels were measured by biotinylation after HFS or LFS. Surface Fz5 increased by 52% in HFS cells, whereas LFS induced a 35% decrease in sFz5, without changing the total level of Fz5 (Fig. 7C,D). These results show that patterned electrical stimulation can differentially regulate the amount of Fz5 present at the cell surface.

We next examined the effect of HFS and LFS on sFz5 by confocal microscopy. These two stimuli exert opposing effects on sFz5 localization. HFS increased the number of sFz5 puncta by 47%, the volume by 90% and their intensity by 57% (Fig. 7E-H), whereas LFS decreased the number of sFz5 puncta by 61% without changing their volume or intensity (Fig. 7E-H). In addition, HFS increased the amount of sFz5 at synapses from 21% to 43%, whereas LFS decreased synaptic sFz5 to 15% (Fig. 7E,I). When the number of synapses was analyzed (as determined by the colocalization of vGlut1 and NR1), we found that HFS increased the number of synapses by 152%, whereas LFS decreased the synapse number by 49% (Fig. 7E,J), consistent with previous reports (Bastrikova et al., 2008; Antonova et al., 2001; Bozdagi et al., 2000). Under basal conditions, 45% of synapses contained sFz5, whereas after HFS, 65% of synapses contained sFz5 (Fig. 7E,K). Following LFS, however, only 20% of synapses contained sFz5 (Fig. 7E,K). These results demonstrate that neuronal activity modulates the localization of sFz5 to synapses and that the outcome depends on the stimulation frequency used. Neuronal activity triggered by HFS enhances the trafficking of Fz5 to the cell surface and its localization to synaptic sites. By contrast, LFS significantly reduces the amount of sFz5 and impairs its localization to synapses.

Wnts contribute to HFS-induced synapse formation and synaptic localization of Fz5

Neuronal activity regulates the expression of Wnts in hippocampal neurons (Wayman et al., 2006; Yu and Malenka, 2003; Gogolla et al., 2009). Therefore, HFS could modulate the mobilization of Fz5 through changes in endogenous Wnt proteins. To test this hypothesis, we blocked endogenous Wnts that bind to Fz5 by using Fz5CRD. Cultured neurons were exposed to control or Fz5CRD-containing conditional media during HFS. Surface biotinylation indicated that, in unstimulated cells, Fz5CRD decreased the amount of receptor present at the surface by 40%, whereas it completely abolished the increase in sFz5 induced by HFS without affecting the total level of the protein (Fig. 8A,B). Using confocal microscopy, we found that, under basal conditions, Fz5CRD decreases the number, volume and intensity of sFz5 puncta (by 41%, 47% and 41%, respectively), while it completely blocks the effect of HFS on sFz5 levels (Fig. 8C-F).

As Fz5CRD blocked Wnt-mediated synaptogenesis after 16 hours of exposure (Fig. 4), we tested whether it affects the increase in synapse number induced after 1 hour of HFS. In unstimulated cells, Fz5CRD did not significantly change the number of synapses (Fig. 8C,G). By contrast, Fz5CRD prevented the increase in synapse number that follows HFS (Fig. 8C,G), suggesting that Wnts participate in the formation of synapses induced by activity. In addition, Fz5CRD completely abolished

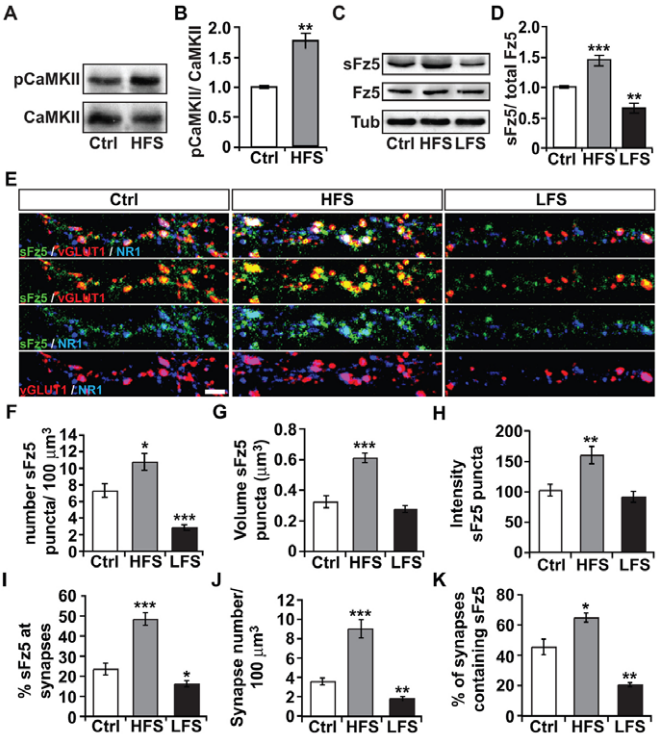

Fig. 7. High-frequency stimulation and low-frequency stimulation have opposite effects on Fz5 levels at the surface and at synapses. (**A**) Effect of high-frequency stimulation (HFS) on CaMKII phosphorylation in cultured hippocampal neurons. Upper panel: CaMKII phosphorylated on Thr286. Lower panel: total CaMKII. (**B**) Quantification showing the level of phosphorylation of Thr286CaMKII after normalization to total CaMKII. (**C**) Surface Fz5 levels following HFS and low-frequency stimulation (LFS). sFz5, biotinylated sFz5; Fz5, total Fz5; Tub, α-tubulin. (**D**) Surface Fz5/total Fz5 ratio calculated to show the effects of HFS and LFS on Fz5 levels. (**E**) Representative images of sFz5, vGlut1 and NR1 staining following HFS and LFS. (**F-I**) Effects of HFS and LFS on the number (F), volume (G), intensity (H) and synaptic localization (I) of sFz5 puncta. (*n*=3 experiments; 8-12 cells were used per condition for each experiment). (**J**) Quantification of synapse number following HFS and LFS (*n*=3 experiments; 8-12 cells were used per condition for each experiment). (**K**) Percentage of synapses containing sFz5 after HFS and LFS (*n*=3 experiments; 8-12 cells were used per condition for each experiment). Scale bar: 2 µm in E. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001.

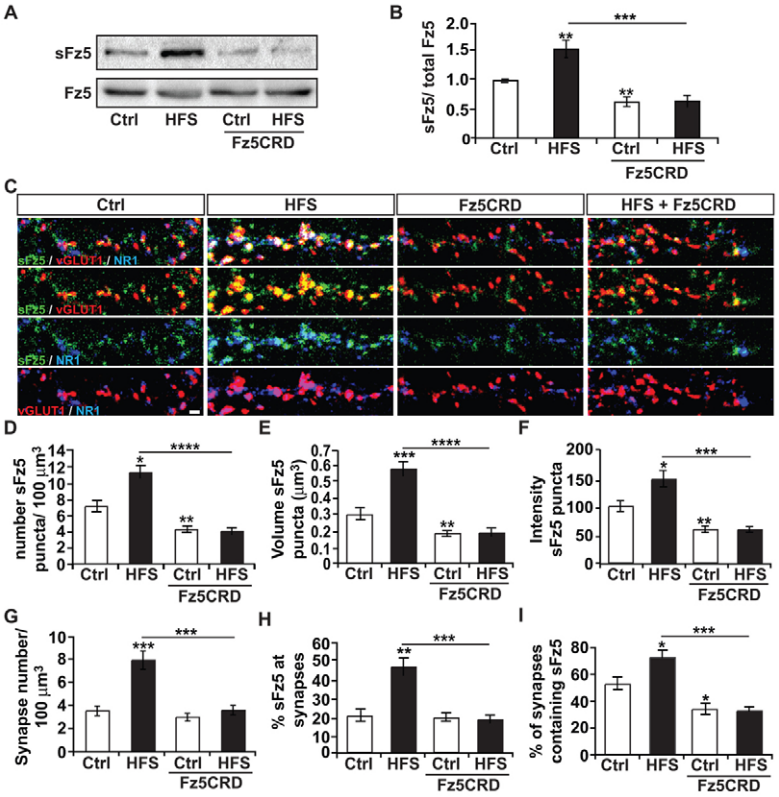

Fig. 8. Fz5CRD abolishes the effect of HFS on the localization of Fz5 at the surface and the formation of synapses. (**A**) Representative blots showing the effect of Fz5CRD on sFz5 in stimulated and non-stimulated neurons. sFz5, biotinylated sFz5; Fz5; total Fz5. (**B**) Surface Fz5/total Fz5 ratio that shows sFz5 levels in stimulated or unstimulated cells incubated with Fz5CRD (*n*=3 experiments). (**C**) Unstimulated or HFS neurons incubated with Fz5CRD followed by staining for sFz5, vGlut1 and NR1. (**D-F**) Quantification showing the effect of Fz5CRD on the number (D), volume (E) and intensity (F) of sFz5 puncta in unstimulated or stimulated cells (*n*=3 experiments; 8-12 cells were used per condition for each experiment). (**G-I**) Effect of Fz5CRD on the total number of synapses (G), synaptic sFz5 (H) and percentage of synapses containing sFz5 (I) in unstimulated or stimulated cells (*n*=3 experiments; 8-12 cells were used per condition for each experiment). Scale bar: 1 µm in C. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001; ****, *P*<0.0001.

the increase in synaptic sFz5 induced by HFS (Fig. 8C,H). The percentage of synapses that contained sFz5 decreased in the presence of Fz5CRD, from 52% to 33% in unstimulated cells and from 72% to 32% in HFS cells (Fig. 8C,I). Therefore, under basal conditions, Fz5CRD decreases the amount of synapses that contain sFz5 by merely decreasing the amount of receptor present at the cell surface. In stimulated cells, Fz5CRD decreases the amount sFz5 located at synapses and also blocks the increase in synapse number induced by HFS. These data suggest that endogenous Wnts play a role not only in Fz5 localization but also in HFS-induced synaptogenesis.

To further confirm that Wnt proteins play a role in Fz5 localization following HFS, endogenous Wnts were blocked using two secreted Wnt antagonists, Sfrp1 and Sfrp3 (Sfrps). Neurons were treated with Sfrps during the HFS protocol. We found that Sfrps did not affect the levels of sFz5 under basal conditions or in stimulated cells, as determined by surface biotinylation (see Fig. S2A,B in the supplementary material). Confocal microscopy revealed a similar effect: Sfrps did not change the number, volume or intensity of Fz5 puncta in either non-stimulated or stimulated cells (see Fig. S2C-F in the supplementary material). These results show that Sfrps do not significantly affect the changes in surface levels of Fz5 induced by neuronal activity.

As Sfrps block the synaptogenic activity of Wnts in cultured neurons (Hall et al., 2000; Krylova et al., 2002; Rosso et al., 2005), we investigated whether Sfrps affect the number of synapses in our experimental model. Under basal conditions, Sfrps decreased the number of synapses by 34% and partially blocked the ability of HFS to induce synapse formation (see Fig. S2C,G in the supplementary material). In addition, in unstimulated cells, Sfrps significantly decreased the amount of sFz5 located at synapses from 21% to 15% but did not change the percentage of synapses containing sFz5 (see Fig. S2C,I in the supplementary material). In HFS cells, the Wnt antagonists partially blocked the increase in synaptic sFz5 (see Fig. S2C,H in the supplementary material). In addition, Sfrps decreased the percentage of synapses that contained sFz5 in stimulated cells (from 75% to 66%; see Fig. S2C,I in the supplementary material). Thus, blockade of endogenous Wnts with Sfrp1 and 3 decreases synaptic sFz5 without changing the surface levels of the receptor in stimulated neurons.

DISCUSSION

Signalling pathways activated by synaptogenic factors such as Wnts are beginning to be understood. However, the identity and localization of their receptors remain poorly characterized. Here, we report that the Wnt receptor Fz5 is present at synapses and is upregulated during synaptogenesis in the hippocampus. Fz5, by binding Wnt7a, induces presynaptic differentiation. Neuronal activity differentially regulates the surface levels of Fz5 and its localization at synapses. Importantly, blockade of Fz5-mediated signalling abolishes both the insertion of Fz5 at synapses and the formation of new synapses induced by HFS. Thus, under these conditions, Wnt signalling is necessary for HFS-induced synaptogenesis in hippocampal cultures. In summary, neuronal activity through endogenous Wnts modulates the distribution of surface Fz5 at synapses and the formation of synaptic sites.

In the nervous system, Fz receptors mediate a range of functions from neuronal differentiation (Van Raay et al., 2005) to cell survival (Liu et al., 2008; Wang et al., 2001), cell polarity (Prasad and Clark, 2006), cell migration (Pan et al., 2006; Vivancos et al., 2009), axon guidance (Lyuksyutova et al., 2003; Wang et al., 2002) and synapse formation (Varela-Nallar et al., 2009; Klassen and Shen, 2007). Here, we focus our attention on Fz5, previously shown to regulate neuronal development (Liu and Nathans, 2008; Liu et al., 2008). In the hippocampus, Fz5 is developmentally upregulated during synaptogenesis. Fz5

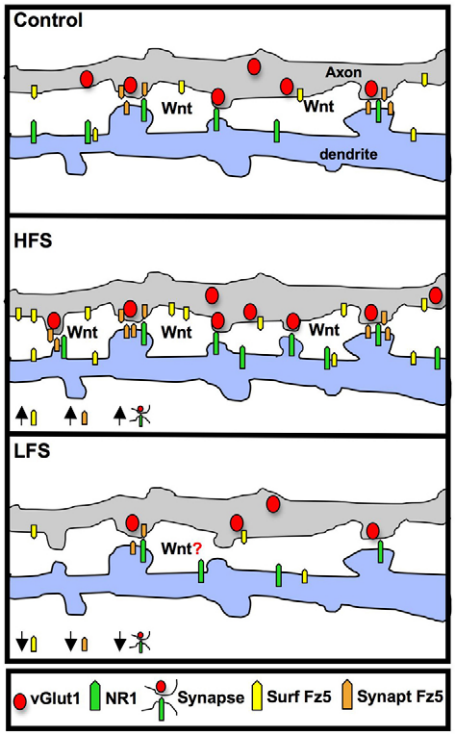

Fig. 9. Regulation of sFz5 localization and synapse formation by neuronal activity and secreted Wnts. The insertion of Fz5 to synapses is differentially regulated by distinct temporal patterns of synaptic activation. LFS drives Fz5 out of synapses, whereas HFS, through a mechanism that involves secreted Wnts, increases the levels of Fz5 at the surface and at synaptic sites. In addition, Wnt-Fz5 signalling participates in the formation of new synapses induced by neuronal activity.

exhibits a punctate distribution in axons, where it colocalizes with vGlut1, and in dendrites, where it colocalizes with PSD-95 and NMDA receptors. Fz5 is also present at sites where both pre- and postsynaptic markers colocalize. Consistently, Fz5 is found in synaptosomes, the SMF and the PSD. Therefore, Fz5 is present at both pre- and postsynaptic sites at the peak of synaptogenesis, where it could mediate bidirectional Wnt signalling to regulate synaptic assembly.

Fz5 is required for Wnt7a-mediated synapse formation in hippocampal neurons. Several pieces of evidence suggest that Fz5 functions as a receptor for Wnt7a. First, Wnt7a (Davis et al., 2008) and Fz5 are expressed in the hippocampus during the peak of synaptogenesis. Second, Wnt7a binds to the CRD domain of Fz5, crucial for signalling (Povelones and Nusse, 2005). Third, axonal Fz5 expression induces the clustering of presynaptic markers as observed with Wnt7a (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). Fourth, Fz5 knockdown or Fz5CRD blocks the ability of Wnt7a to induce presynaptic differentiation. Fz5 might not be the only receptor that mediates the synaptogenic effect of Wnt7a, as Fz1 also stimulates synapse formation in hippocampal neurons (Varela-Nallar et al., 2009). However, our findings strongly support the idea that endogenous Fz5 functions as a receptor for Wnt7a to regulate synapse formation in hippocampal neurons.

Neuronal activity regulates the localization of transmembrane proteins to sub-cellular compartments. For example, activity increases the level of surface TrkB receptors (Du et al., 2000; Meyer-Franke et al., 1998) and the mobilization of N-cadherin to synapses (Bozdagi et al., 2000; Tanaka et al., 2000). Neuronal activity also drives the synaptic incorporation of AMPAR and NMDAR and their lateral diffusion between synaptic and extrasynaptic sites (Lau and Zukin, 2007; Newpher and Ehlers, 2008; Heynen et al., 2000). Here, we show that neuronal activity has a profound effect on the distribution and synaptic localization of Fz5 (Fig. 9). Depolarization and HFS increase the levels of Fz5 at the cell surface and the percentage of synapses that contain Fz5. By contrast, LFS decreases the amount of Fz5 at the surface and the percentage of synaptic Fz5. The total levels of Fz5 remain unaffected, suggesting that neuronal activity regulates the trafficking of the receptor from intracellular stores to the plasma membrane and to synaptic sites. Importantly, in these experiments, the patterns of HFS and LFS were chosen such that they both deliver, on average, 4 stimulation pulses per second. As the cultures received the same number of stimuli, it is clear that distinct temporal patterns of synaptic activation, such as HFS and LFS, modulate the insertion of Fz5 receptors in opposing ways.

How does neuronal activity induce Fz5 recruitment to synapses? Activity has previously been shown to regulate the expression and/or secretion of Wnt proteins. Depolarization stimulates Wnt2 transcription in cultured hippocampal neurons, resulting in increased dendritic arborization (Wayman et al., 2006). The Wnt inhibitor Dkk1 blocks depolarization-induced dendrite growth in cultured hippocampal neurons (Yu and Malenka, 2003). Moreover, experience-induced plasticity enhances Wnt7a/b expression, which regulates hippocampal axon remodelling (Gogolla et al., 2009). At the *Drosophila* neuromuscular junction, neuronal activity increases the secretion of Wg to stimulate pre- and postsynaptic assembly as well as dendritic refinement (Singh et al., 2010; Ataman et al., 2008). Thus, neuronal activity modulates the levels of Wnts, which in turn could regulate Fz receptor localization. Indeed, Wnt proteins control the localization of Fz receptors in neurons (Ataman et al., 2008; Klassen and Shen, 2007). However, a role for neuronal activity in Fz localization to synapses has not been documented. By blocking Wnts using two different approaches, we demonstrate that secreted Wnts contribute to the regulation of surface Fz5 localization to synapses elicited by neuronal activity.

Neuronal activity regulates the formation and maintenance of synapses (Craig et al., 2006; Blankenship and Feller, 2010). Here, we demonstrate that Wnts and Fz5 are necessary for stimulation-evoked synaptogenesis in 14 DIV hippocampal cultures, as blockade of Fz5 signalling with Fz5CRD completely abolishes the synaptogenic effect of HFS. We propose a model where neuronal activity modulates the secretion of Wnts, which then stimulate the formation of synapses through binding to Fz5. Our results provide a link between neuronal activity, Wnt-Fz5 signalling and synapse formation.

Acknowledgements
We thank Drs Jeremy Nathans and Xi He for constructs. We also thank members of our laboratory for useful discussion and comments on the manuscript. The Wellcome Trust and MRC supported this work. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.046722/-/DC1

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