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Frizzled Receptors in Neurons: From Growth Cones to the Synapse

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The Wnt signaling pathway has been implicated in several different aspects of neural development and function, including dendrite morphogenesis, axonal growth and guidance, synaptogenesis and synaptic plasticity. Here, we studied several Frizzled Wnt receptors and determined their differential expression during hippocampal development. In cultured hippocampal neurons, the cellular distributions of Frizzleds vary greatly, some of them being localized at neurites, growth cones or synaptic sites. These findings suggest that the Wnt signaling pathway might be temporally and spatially fine tuned during the development of neuronal circuits through specific Frizzled receptors. © 2012 Wiley Periodicals, Inc

Key Words: frizzled receptors, Wnt signaling, hippocampal neurons, growth cone, synapse

Introduction

In recent years, the Wnt signaling pathway has been shown to be essential for the development and function of the central nervous system [Salinas and Zou, 2008; Inestrosa and Arenas, 2010]. Different Wnt signaling pathways have been described. Activation of the Wnt/β-catenin pathway prevents the degradation of β-catenin through activation of Dishevelled (Dvl), allowing the transcription of Wnt target genes [Gordon and Nusse, 2006]. On the other hand, activation of β-catenin-independent pathways, or non-canonical Wnt pathways, may induce either an increase in intracellular calcium concentration (Wnt/Ca^{2+} pathway) or activation of the c-Jun-N-terminal kinase (JNK) cascade (Wnt/JNK pathway) [van Amerongen et al., 2008; Angers and Moon, 2009].

Activation of Wnt cascades regulates several events in developing neurons and in the adult brain. Different Wnt ligands modulate neural morphogenesis by regulating dendrite arborization, axonal growth and guidance [Rosso et al., 2005; Li et al., 2009; Blakely et al., 2011; Hutchins et al., 2011]. Also, Wnts regulate synaptogenesis, synaptic function and synaptic plasticity [Ahmad-Annuar et al., 2006; Chen et al., 2006; Cerpa et al., 2008; Varela-Nallar et al., 2010; Cerpa et al., 2011]. Considering the different roles of Wnt ligands in the central nervous system, it may be necessary to specifically regulate Wnt molecular machinery to enhance a specific functional effect. In such a context, the first line of regulation of Wnt signaling may be linked to Wnt receptor function.

Wnt signaling is activated by the interaction of a Wnt ligand with members of the Frizzled (Fz) family of seven-pass transmembrane cell-surface receptors [Gordon and Nusse, 2006; Schulte, 2010]. In addition to the 10 Fz receptors that have been identified in mammals, Ror2 and Ryk have been identified more recently as alternative Wnt receptors [Oishi et al, 2003; Keeble and Cooper, 2006]. Moreover, Ror2 may act in cooperation with Fzs to activate non-canonical Wnt signaling [Grumolato et al., 2010; Nishita et al., 2010]. Fz receptors have been implicated in several developmental processes that involve the nervous system: Fz1 regulates synaptic differentiation in hippocampal neurons [Varela-Nallar et al., 2009]; Fz3 is required for axonal outgrowth and guidance in the central nervous system [Wang et al., 2002; Lyuksyutova et al., 2003] and controls neural tube closure [Wang et al., 2006]; Fz4 plays a role in the maintenance of the structure and function of the cerebellum [Wang et al., 2001]; Fz5 is required for the survival of mature neurons in the parafascicular nucleus of the thalamus [Liu et al., 2008] and modulates synaptogenesis in the hippocampus [Sahores et al., 2010]; and Fz9 is important for hippocampal development [Zhao et al., 2005].

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We previously determined that Wnt-3a/Fz1 signaling modulates the structure and function of the presynaptic compartment in hippocampal neurons, where Fz1 is specifically located at the presynaptic region [Varela-Nallar et al., 2009]. That study suggested a correlation between the localization and function of Fz1. In the present work, we studied some of the other Fz receptors in hippocampal neurons, with an emphasis on pinpointing changes in their expression and subcellular localization that may be implicated in the fine-tuning of the Wnt signaling pathway in the developing and mature hippocampus.

Results

Different Expression of Fz Receptors During Hippocampal Development

First, and in order to determine if there is a regulated expression of Fzs during development, the overall protein levels of several Fzs were analyzed in whole protein extracts from rat hippocampus at different stages of development, from embryonic day 18 (E18) until postnatal day 60 (P60) (Fig. 1A). As shown by immunoblot analysis using specific antibodies, there is a differential expression pattern for the Fzs analyzed during hippocampal development (Fig. 1B). As previously shown, Fz1 expression increases during development, with the highest expression level occurring in the adult hippocampus [Varela-Nallar et al., 2009]. A similar change in the expression pattern was observed for Fz9, which began to be enriched after P15. In contrast, Fz7 expression decreases during hippocampal development. In the case of Fz2, there is an increase during early postnatal stages until postnatal day 15, after which, there is a gradual decrease in Fz2 levels until it becomes completely undetectable at the adult stages (Fig. 1A).

Distribution of Fz7 and Fz9 in Developing Cultured Hippocampal Neurons

We studied the localization of Fz7 and Fz9 during the development of cultured neurons since these receptors show different patterns of expression during hippocampal development (Fig. 1). At 3 days *in vitro* (DIV) Fz7 shows a diffuse staining in the soma and all neurites (Fig. 2A), and Fz9 shows a somatodendritic distribution with a higher immunoreactivity at the tip of some neural processes (Fig. 2B, arrows). At DIV7, Fz7 shows a somatodendritic staining (Fig. 2C), however it also seems to be present in neurites that are negative for the somatodendritic marker MAP2, suggesting that this receptor is also present in axons (Fig. 2C, arrows). At DIV7, Fz9 assumes a punctate pattern of staining in the somatodendritic compartment (Fig. 2D). At 14 DIV, we observed a lower staining of Fz7 (not shown), suggesting that in cultured neurons Fz7 levels decreased

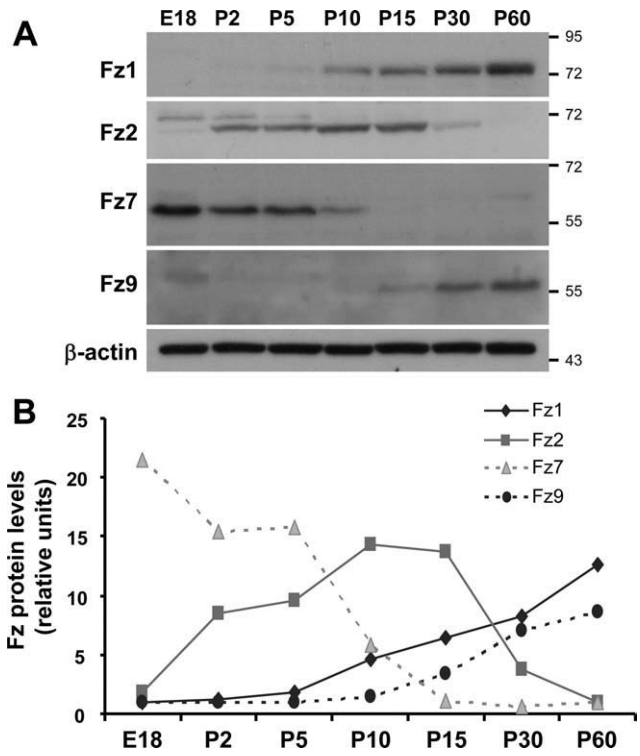

Fig. 1. Differential expression of Fz receptors during hippocampal development. (**A**) Immunodetection of different Fz receptors in total protein extracts from hippocampi of rats at embryonic day 18 (E18) and from postnatal day 2 (P2) to P60. The same amount of protein was loaded in each lane. Molecular weight standards are indicated on the right. (**B**) Densitometric analysis of bands shown in (A). The graph indicates the levels of Fz proteins relative to the lane where the lowest level of the receptor is observed.

during development, as observed in the hippocampus (Fig. 1). At this stage, Fz7 maintains a distribution very similar to that observed earlier in development, showing a clustered distribution in the soma and all neurites (Fig. 2E). At 14 DIV Fz9 exhibits a more punctate staining than at early stages (Fig. 2F), and as compared to Fz7. The staining of both receptors is very different; Fz7 is present in the shaft of neurites as a diffuse and vesicular pattern (Fig. 2E), while Fz9 is present in puncta along the processes (Fig. 2F).

Fz9 is Enriched at the Growth Cone During Early Development

At 3 DIV Fz9 showed higher immunoreactivity at the tip of some neural processes (Fig. 2B, arrows), suggesting that in hippocampal neurons Fz9 may be present at the growth cones, as previously observed in regenerating adult spiral ganglion neurons [Shah et al., 2009]. By triple labeling with phalloidin and the axonal protein phosphorylated MAP1B (PMAP1B) we determined that Fz9, but not Fz7, is concentrated at the peripheral domain of axonal growth cones at 3 DIV (Figs. 3A and 3B). We studied whether other Fzs might share this distribution in growth cones,

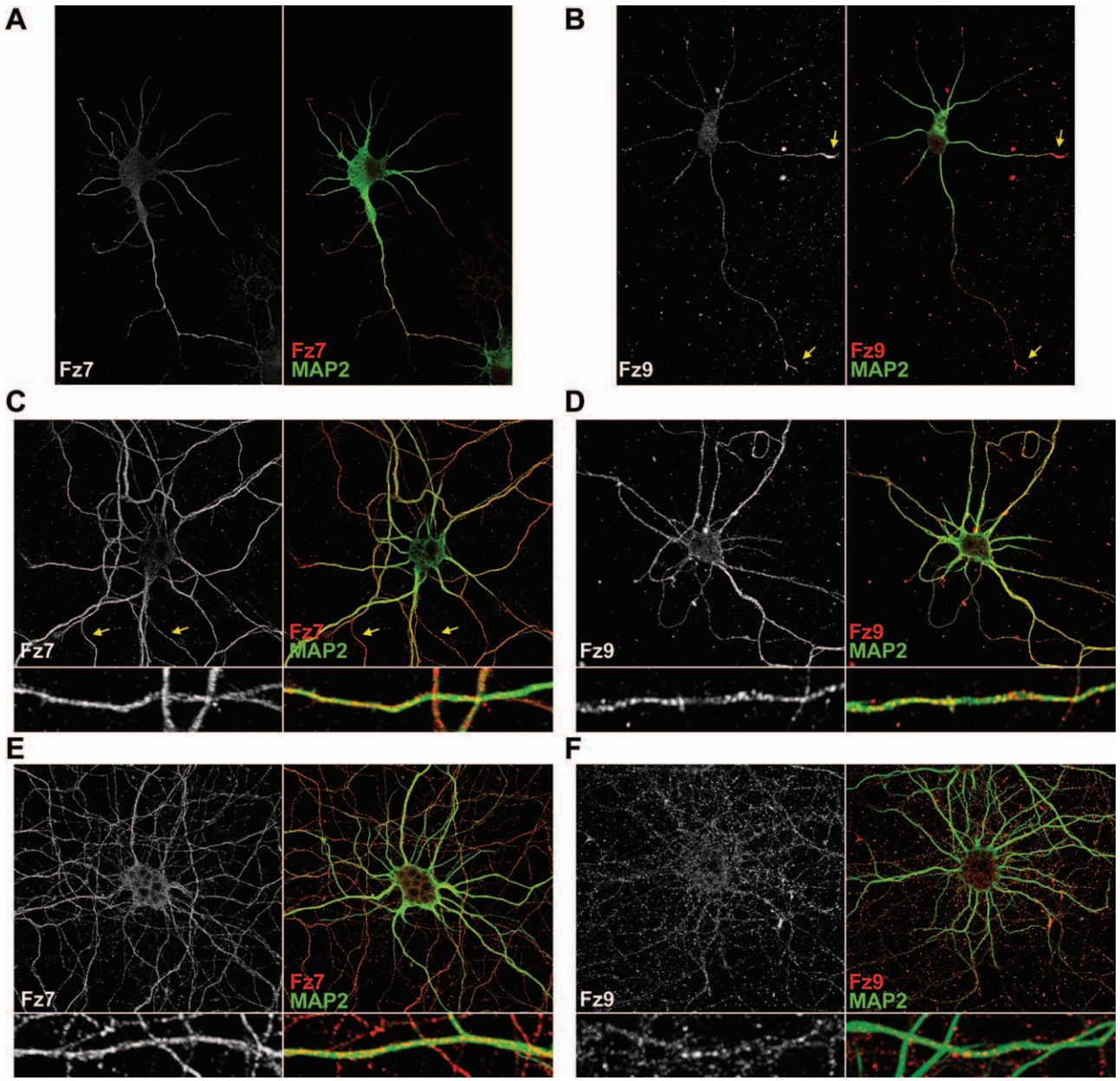

Fig. 2. Distribution of Fz7 and Fz9 in cultured hippocampal neurons during development. (**A-F**) Immunodetection of Fz7 (A,C,E), Fz9 (B,D,F) and the somatodendritic protein MAP2 in hippocampal neurons at day in vitro 3 (A,B), 7 (C,D) or 14 (E,F). Arrows indicate higher immunoreactivity of Fz9 at the tip of some neurites (B) or Fz7 immunoreactivity in neurites negative for MAP2 staining (C). Bottom panels in C-F show higher magnifications of the images shown.

and determined that Fz5 shows a very particular distribution being specifically concentrated at the peripheral domain of growth cones, where it codistributes with actin microfilaments (Fig. 3C). Positive labeling for Fz9 was observed within the axon (Fig. 3A), while Fz5 is exclusively observed in the growth cone and seems not to be codistributed with microtubules within the axon (Fig. 3C). Fz1 was previously observed in the axon in young cultured hippocampal neurons [Varela-Nallar et al., 2009], however neither Fz1 or Fz2 are concentrated at growth cones (not shown). These findings indicate that early in neuronal development there is a specific localization of Fzs, with some of them being specifically located in growth cones.

Synaptic Distribution of Fz Receptors in Cultured Hippocampal Neurons

It was previously shown that Fz1 is highly expressed in hippocampal neurons at 14 DIV [Varela-Nallar et al., 2009], and it is located in a clustered distribution mainly at synaptic sites co-localizing with the presynaptic marker synapsin 1 (Syn-1) (Figs. 4A and 4B). On the other hand, at 14 DIV Fz2 is mainly located at the neuronal soma where it

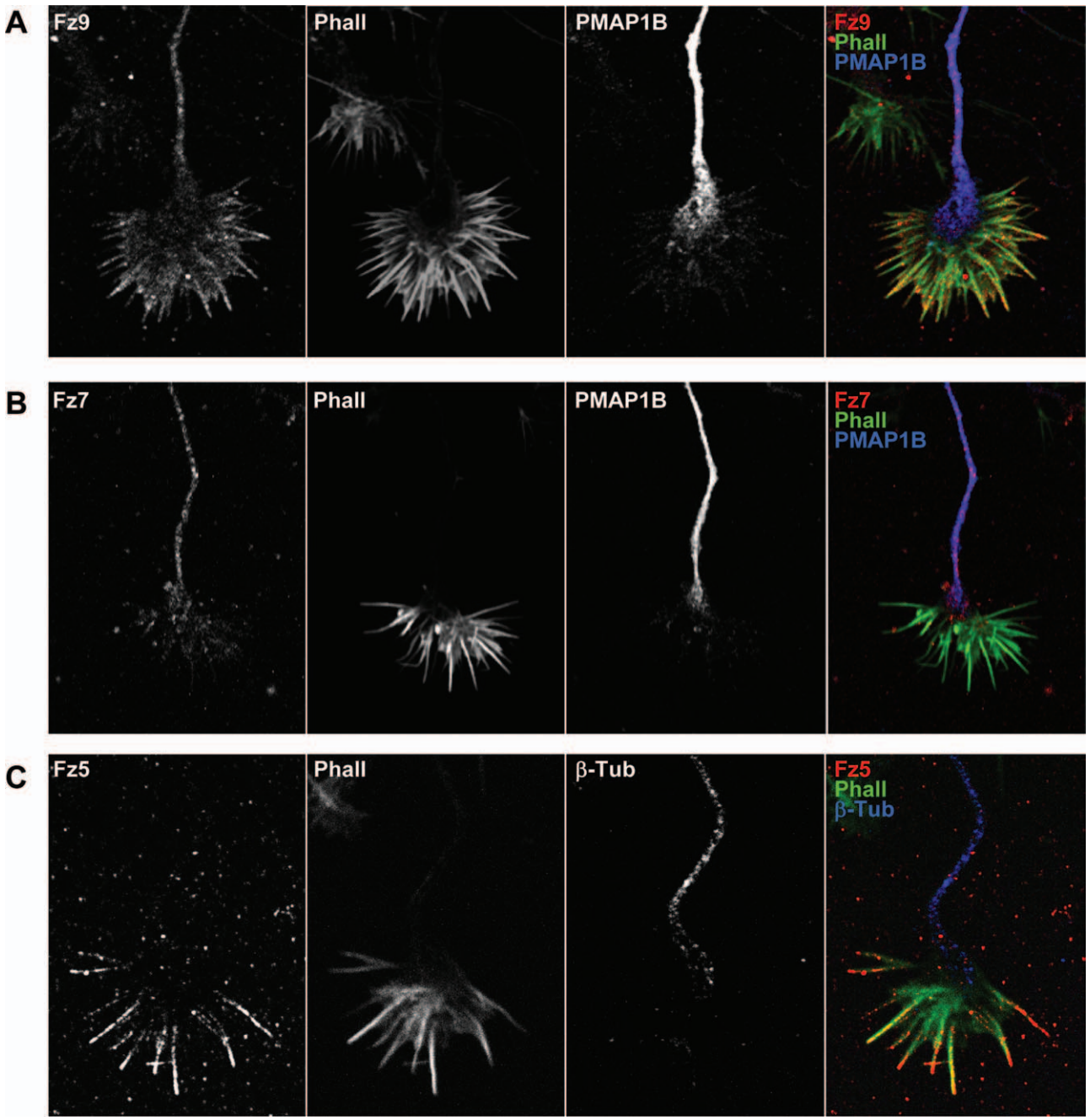

Fig. 3. Some Fz receptors are located in the axonal growth cone during early development. (**A, B**) At 3 DIV, Fz9 (A) but not Fz7 (B) is present in growth cones identified with the axonal marker PMAP1B and phalloidin (Phall). (**C**) At 3 DIV, Fz5 was detected in growth cones at the tips of actin filaments stained with Phall. β-Tubulin (β-Tub) staining was used to visualize microtubules.

exhibits a clustered staining and also shows a lower and diffuse staining in dendrites (Fig. 4C). This receptor does not show co-localization with synaptic markers such as the presynaptic protein Syn-1, suggesting Fz2 is not present at the synapse (Fig. 4D). Fz3 shows a clustered distribution in dendrites (Fig. 4E) and by co-staining with Syn-1 and the postsynaptic scaffold protein PSD-95, it was shown that this receptor is also present at the synapse (Fig. 4F, arrows). These observations indicate that some, but not all Fz receptors have a synaptic distribution.

Discussion

Wnt signaling has important roles in the development and maintenance of the central nervous system, participating in the formation and maintenance of neuronal circuits [Salinas and Zou, 2008; Inestrosa and Arenas, 2010]. Here, we have shown that Fz receptors, which are key elements for triggering the activation of Wnt cascades, have different patterns of expression during hippocampal development, suggesting that the activation of Wnt cascades

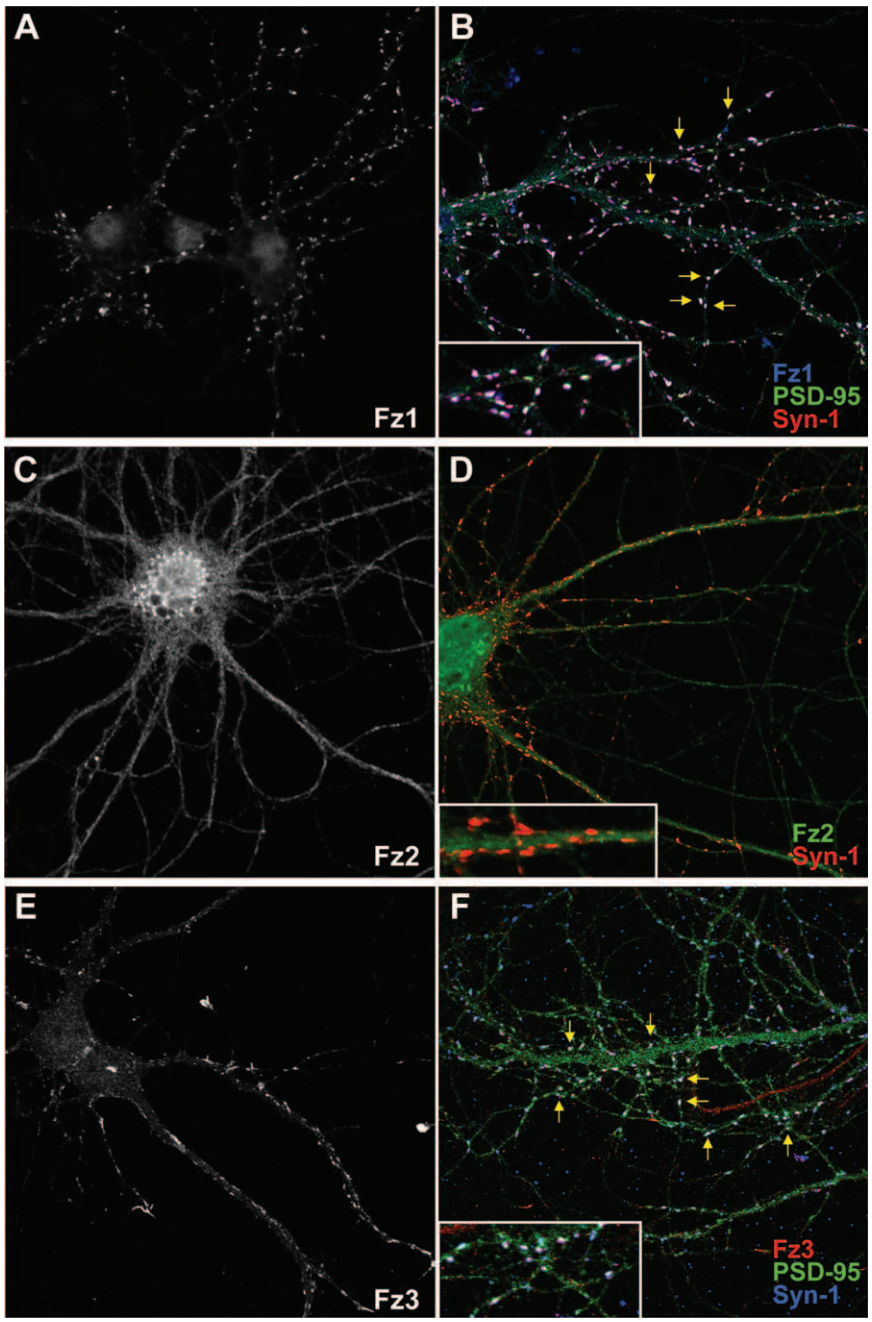

Fig. 4. Synaptic distribution of Fz receptors in cultured hippocampal neurons. Immunofluorescence analysis of Fz localization using specific antibodies in neurons at 14 days in vitro. (**A, B**) Fz1 shows a clustered distribution in neurites (A) that co-localize with synaptic proteins PSD-95 and Syn-1 (B, arrows). (**C, D**) Fz2 staining is mainly detected at the cell soma and diffusely distributed in dendrites (C), and this receptor does not co-localize with the synaptic protein Syn-1 (D). (**E, F**) Fz3 shows a clustered distribution (E) that is co-localized with synaptic protein markers (F). Insets show higher magnifications.

may be regulated during development by the presence of specific receptors. It is well established that Fzs can interact with more than one ligand and one ligand can interact with more than one Fz [Schulte, 2010], which increases the complexity of the mechanisms controlling the specificity of the pathway. In fact, most of the functional interactions of Wnt and Fzs are still unknown. The segregated temporal expression of receptors may be relevant for the specificity of the interaction and activation of Wnt signaling by particular ligands.

As mentioned, Wnt ligands modulate different neuronal processes. Wnt-7b regulates dendritic development in young neurons through the JNK cascade [Rosso et al., 2005]. Wnt-2 can also stimulate the dendritic arborization in hippocampal neurons [Wayman et al., 2006]. It has been suggested that Fz9 binds to Wnt-2 and activates the

canonical Wnt pathway and causes the re-localization of Dvl-1 to the cell membrane [Karasawa et al., 2002]. The early expression of Fz in the somatodendritic compartment could regulate the dendritic effect of the Wnt ligands.

On the other hand, Wnt-5a regulates axon growth and guidance in different types of neurons, and these effects are dependent on Ryk and Fz receptors [Li et al., 2009; Blakely et al., 2011]. Wnt-7a and Wnt-3a have also been shown to regulate axon behaviour [Hall et al., 2000; Purro et al., 2008]. The specific distribution of Fz5 and Fz9 in growth cones, as determined in this work, could allow local transduction of Wnt signals into the developing axon. Actually, it has been reported that Wnt-7a interacts with Fz9 to activate the Wnt/JNK pathway [Winn et al., 2005].

Over the past few years, increasing evidence indicates that the Wnt pathway regulates synaptic structure and function. Different Wnt ligands modulate presynaptic differentiation, postsynaptic protein clustering and synaptic plasticity in mammals [Ahmad-Annuar et al., 2006; Chen et al., 2006; Cerpa et al., 2008; Farias et al., 2009; Varela-Nallar et al., 2010], and also the Wnt/Wingless pathway regulates the assembly of pre- and post-synaptic regions in peripheral synapse differentiation in Drosophila [Packard et al., 2002]. Synaptic effects of Wnts could be locally regulated by synaptic Fz receptors, as was shown for Wnt-3a/Fz1 [Varela-Nallar et al., 2009], and more recently for Wnt-7a/Fz5 [Sahores et al., 2010].

In summary, we have determined that in hippocampal neurons Fz receptors show different temporality and localization, suggesting that these receptors could be important regulators for the specific activation of the Wnt signaling cascades during the development of hippocampal circuits. The specific Wnt/Fz interactions that could be relevant in controlling neuronal functions, both in development and in adult neurons may be identified in the future. Additionally, it will also be interesting to determine whether Wnt co-receptors can be differentially expressed and/or localized, thus providing an additional level of regulation for the control of Wnt signaling activation in neurons.

Materials and Methods

Immunoblot Analysis

Hippocampi obtained from rat brains at different ages were lysed in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate) supplemented with protease inhibitors. The homogenates were maintained in ice for 30 min and centrifuged at 15,000 x *g* for 10 min at 4°C. The supernatant was recovered and protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL). Proteins were resolved in SDS-PAGE (10% polyacrylamide), transferred to PVDF membrane and reacted with primary antibodies. The reactions were followed by incubation with peroxidase labeled secondary antibodies (Pierce) and developed using the ECL technique (PerkinElmer, Waltham, MA). Primary antibodies used were: goat anti-Fz1 (R&D Systems, Minneapolis, MN), goat anti-Fz2 (R&D Systems), goat anti-Fz7 (R&D Systems), goat anti-Fz9 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and mouse anti-β-actin (Sigma-Aldrich, St Louis, MO).

Primary Culture of Rat Hippocampal Neurons

Rat hippocampal cultures from Sprague-Dawley rats at embryonic day 18 were prepared as previously described [Caceres et al., 1984; Varela-Nallar et al., 2009]. Dissociated hippocampal cells were seeded onto poly-L-lysine-coated 24-well culture plates at a density of 2.5×10^4 cells per well. Cultures were maintained at 37°C in 5% CO_2 with neurobasal growth medium (GIBCO, Rockville, MD) supplemented with B27 (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. At day 2, cultured neurons were treated with 2 μM cytosine arabinoside (AraC) for 24 h, to obtain cultures highly enriched for neurons (~ 5% glia).

Immunofluorescence

Cells were rinsed twice in ice-cold PBS and fixed with a freshly prepared solution of 4% paraformaldehyde + 4% sucrose in PBS for 20 min and permeabilized for 5 min with 0.2% Triton X-100 in PBS. After several rinses in ice-cold PBS, cells were incubated in 1% BSA in PBS for 30 min at room temperature, followed by an overnight incubation at 4°C with primary antibodies. Cells were extensively washed with PBS and then incubated with Alexa-conjugated secondary antibodies (Invitrogen/Molecular Probes, Carlsbad, CA) for 30 min at 37°C. Coverslips were mounted in Fluoromount G (Electron Microscopy Sciences, Hatfield, PA) and analyzed on a Zeiss LSM 5 Pascal confocal microscope. Primary antibodies used were the same used for immunoblot analyses plus: rabbit anti-Fz3 (LifeSpan BioSciences Inc., Seattle, WA), goat anti-Fz5 (R&D Systems), rabbit anti-Synapsin I (Santa Cruz Biotechnology Inc.), monoclonal anti-PMAP1B antibody (Sternberger Monoclonals, Baltimore, MD), rabbit anti-MAP2 (Millipore, Billerica, MA), rabbit anti-β-tubulin antibody (Santa Cruz Biotechnology Inc.), and monoclonal anti-PSD95 antibody (UC Davis/NIH NeuroMab Facility, Davis, CA). Alexa-conjugated Phalloidin (Invitrogen/Molecular Probes) was incubated with secondary antibodies.

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