Wnt-5a/JNK Signaling Promotes the Clustering of PSD-95 in Hippocampal Neurons

During the formation of synapses, specific regions of pre- and postsynaptic cells associate to form a single functional transmission unit. In this process, synaptogenic factors are necessary to modulate pre- and postsynaptic differentiation. In mammals, different Wnt ligands operate through canonical and non-canonical Wnt pathways, and their precise functions to coordinate synapse structure and function in the mature central nervous system are still largely unknown. Here, we studied the effect of different Wnt ligands on postsynaptic organization. We found that Wnt-5a induces short term changes in the clustering of PSD-95, without affecting its total levels. Wnt-5a promotes the recruitment of PSD-95 from a diffuse dendritic cytoplasmic pool to form new PSD-95 clusters in dendritic spines. Moreover, Wnt-5a acting as a non-canonical ligand regulates PSD-95 distribution through a JNK-dependent signaling pathway, as demonstrated by using the TAT-TI-JIP peptide in mature hippocampal neurons. Finally, using adult rat hippocampal slices, we found that Wnt-5a modulates glutamatergic synaptic transmission through a postsynaptic mechanism. Our studies indicate that the Wnt-5a/JNK pathway modulates the postsynaptic region of mammalian synapse directing the clustering and distribution of the physiologically relevant scaffold protein, PSD-95.

3 The abbreviations used are: PSD, postsynaptic density; DIV, day(s) in vitro; HEK-293, human embryonic kidney 293; JNK, c-Jun NH2-terminal kinase; ACSEF, artificial cerebrospinal fluid; EPEPS, field excitatory postsynaptic potential; PPF, paired pulse facilitation; BSD-X, bisindolylmaleimide-X; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HA, hemagglutinin.

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nucleus, where it co-activates transcription of Wnt target genes (24, 25). Non-canonical Wnt signaling pathways have been described that do not affect gene transcription through β-catenin but instead mediate several cellular processes through different molecular intermediates, including monomeric GTPases of the Rho family, mitogen-activated protein kinases like Jun N-terminal kinase (JNK), and changes in intracellular calcium levels (26).

In the present study, we tested whether different Wnt ligands are able to modulate the assembly and differentiation of the postsynaptic regions in mature hippocampal neurons. We report here that Wnt-5a, but not Wnt-7a, regulates postsynaptic structure and protein localization, directing the clustering of PSD-95 (postsynaptic density protein-95) through the Wnt/JNK pathway. Moreover, we show that Wnt-5a modulates the synaptic transmission. These results suggest that the noncanonical Wnt/JNK pathway controls synaptic structure and function of the excitatory postsynaptic compartment of mammalian synapse.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Formylated hexapeptide was obtained from Genemed Synthesis, Inc. (South San Francisco, CA); fumagillin, anisomycin, palmitate, and 2-bromopalmitate were from Sigma; bisindolylmaleimide-X (BSD-X), SP600125, KN-93, and TAT-TI-JIP-(153–163) were from Calbiochem; rabbit anti-synaptophysin, mouse anti-β-tubulin, rabbit anti-anisomycin, mouse anti-N-cadherin, mouse anti-CAMKII, goat anti-PSD-95, and goat anti-Wnt-5a were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mouse anti-PSD-95 was from the University of California/NINDS/NIMH, National Institutes of Health (Davis, CA); rabbit anti-JNK and rabbit anti-phospho-JNK were from Cell Signaling Technology (Beverly, MA); antibodies and secondary antibodies labeled with 488Alexa, 543Alexa, or 633Alexa were from Affinity Bio Reagents Inc. (Golden, CO); and phalloidin labeled with 633Alexa was from Molecular Probes, Inc. (Eugene, OR).

**Constructions**—Green fluorescent protein (GFP) was from Clontech (Mountain View, CA); the different hemagglutinin-Wnt constructs were a kind gift of several individuals that made this work possible. HA-Wnt-7a construct was a gift from Dr. Patricia Salinas (University College London, London, UK); HA-Wnt-5a was a gift of Dr. Randall Moon (University of Washington, Seattle, WA); and sFRP-1 (soluble Frizzled receptor protein-1) was a gift of Dr. Jeremy Nathans (The Johns Hopkins University School of Medicine, Baltimore, MD).

**Conditioned Medium Containing Wnt Ligands**—To generate secreting Wnt ligands, Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), 100 µg/ml streptomycin, and 100 units/ml penicillin. HEK-293 cells were transiently transfected by calcium phosphate precipitation with constant and equal amounts of empty vector pcDNA (control) or pcDNA containing sequences encoding Wnt-7a, and Wnt-5a and sFRP-1-HA constructs (27) or stably transfected HEK-293 cells containing pcDNA or Wnt-5a ligands were used. Then transfected HEK-293 cells were maintained in neurobasal medium supplemented with 100 µg/ml streptomycin and 100 units/ml penicillin for 3 days. The presence of Wnt ligands in the conditioned medium was verified by Western blot analysis using an antibody against the HA epitope. To block Wnt ligand function, we co-incubated the different Wnt ligands with sFRP-1 for 30 min before treatment of the neurons.

**Hippocampal Neuronal Cultures and Transfection**—Rat primary hippocampal neurons were obtained from 18-day-old Sprague-Dawley rat embryos (as described previously) (27) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum for 2 h. The culture medium was then substituted with Neurobasal medium supplemented with B27, 100 µg/ml streptomycin, and 100 units/ml penicillin. Cells were treated with 2 µM 1,4-bis(d-arabinofuranosyl)cytosine for 24 h on day 3 to reduce the number of proliferating non-neuronal cells. Experiments were performed at 14–18 DIV in the presence or absence of pcDNA, Wnt-5a, Wnt-7a, or sFRP conditioned medium, preincubation with or without fumagillin (10–100 nM) for 16 h, KN-93 (10 µM), BSD-X (1 µM), SP600125 (0.5 µM), anisomycin (1 µg/ml), and TAT-TI-JIP (0.1–1 µM) co-incubated or not with Wnt-5a conditioned medium for 1 h. Hippocampal neurons expressing GFP were transfected with enhanced GFP at 3 DIV with Lipofectamine and treated with the conditioned media at 14 DIV or fumagillin.

**Immunofluorescence and Image Analysis**—Hippocampal neurons were subjected to different treatments while on coverslips within 24-well plates at a plating density of 30,000 cells/coverslip, fixed with 4% paraformaldehyde, 4% sucrose in PBS for 20 min, permeabilized with 0.2% Triton X-100 for 5 min, blocked with 0.2% gelatin, and stained with PSD-95 antibody. Phalloidin coupled to Alexa 633 was used as a neurite marker. Digital images of neurons on coverslips were captured with a Zeiss confocal microscope using a ×63/1.4 numerical aperture oil immersion objective. Images used for quantification were taken with identical microscope settings and analyzed using Image J software (National Institutes of Health, Baltimore, MD). PSD-95 images from 10 microscope fields for each condition of three independent experiments were registered. Each field containing processes for 1–2 neurons, in which 2–3 neurites/neurons staining with phalloidin to label neuronal processes, were selected. To quantify PSD-95 cluster parameters, images of individual neurites were isolated, background for neurite-free fields was subtracted, and the mean intensity of diffuse dendritic PSD-95 staining was obtained using multiple regions of interest from dendritic shafts (28). In each image, punctate PSD-95 staining was segmented using a value of 3 times the local mean intensity of diffuse shaft staining as a lower limit threshold. PSD-95 cluster number, mean intensity, integrated intensity, and size were obtained with the particle analysis tool using size particle limits of 0.04–1 µm². Cluster number was normalized against neurite length to obtain cluster density. Integrated intensities of PSD-95 clusters were added and normalized against total dendritic PSD-95 integrated intensity to obtain relative cluster integrated intensity. For quantification of dendritic spine density, images of neurites of transfected neurons were captured with a 2× digital zoom. Dendritic protrusions below 3 µm in length showing visible
spine heads and positives for PSD-95 immunoreactivity were quantified from using the Image J software (National Institutes of Health) from at least 30 neurites for each condition in three independent experiments.

Subcellular Cell Fractionation—Hippocampal neurons were treated with Wnt-5a ligand or pcDNA for 2 h or with palmatine (10 μM) for 8 h or 2-bromopalmitate (10 μM) for 4 h. Then total protein was lysed in a buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 1 mM dithiothreitol, supplemented with protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin). Lysates were incubated in ice for 30 min and centrifuged at 700 × g for 5 min at 4 °C. Supernatants were centrifuged at 100,000 × g for 1 h at 4 °C, and the pellet (equivalent to membrane or particulate fraction) was resuspended in a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 0.15 mM NaCl, and the above protease inhibitor mixture (27). The supernatant (equivalent to the cytoplasmic fraction) and the enriched membrane fraction were resolved using SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membranes, and immunoblotting was done using monoclonal anti-PSD-95. Controls of fractionation were performed using anti-GAPDH and -tubulin antibodies (used as markers of soluble proteins) and anti-transferrin receptor and anti-N-cadherin antibodies (used as markers of membrane-associated proteins).

Slice Preparation and Electrophysiology—Hippocampal slices were prepared according to standard procedures from 22–30-day-old Sprague-Dawley rats. Transverse slices (250–300 μm) from the dorsal hippocampus were cut under cold artificial cerebrospinal fluid (ACSF) using a Vibroslice microtome (VSL, WIPI) and incubated in ACSF for more than 1 h at room temperature. In all experiments, picrotoxin (10 μM) was added to ACSF perfusion media in order to suppress inhibitory GABA_A transmission. Then slices were transferred to an experimental chamber (2 ml), superfused (3 ml/min at 22–26 °C) with gassed ACSF, and visualized by transillumination with a binocular stereo microscope (MSZ-10; Nikon). The experiments were carried out at room temperature (21–22 °C), measured at the recording chamber. Extracellular field potential recordings (23) were made with glass pipettes, connected to an AC amplifier (P-5 Series; Grass), with gain X10,000, LP filter (3.0 kHz), and HP filter (0.30 Hz) that was placed in the middle of the stratum radiatum of CA1. The electric pulses (50 μs, 0.3 Hz, 20–100 μA) were applied on Schaeffer collaterals, eliciting compound action potentials from the presynaptic axons (fiber volley) followed by field excitatory postsynaptic potentials (fEPSPs). To evoke fEPSPs, Schaeffer collateral fibers were activated by bipolar cathodic stimulation, generated by a stimulator (Master 8; AMPI) connected to an isolation unit (Isoloflex; AMPI). The bipolar concentric electrodes (platinum/iridium, 125 μm OD diameter; FHC Inc.) were placed in the stratum radiatum within 100–200 μm of the recording site.

The paired pulse facilitation (PPF) index was calculated by \((R2 − R1)/R1\), where \(R1\) and \(R2\) are the peak amplitudes of the first and second fEPSPs, respectively. Evoked postsynaptic responses were analyzed off-line, using analysis software (Mini-analysis; Synaptosoft), which allowed visual detection of events, computing only those events that exceeded an arbitrary threshold. Data are expressed as means ± S.E. (number of slices), unless otherwise noted. Student’s t test analysis at \(p < 0.05\) determined significant differences between control and experimental treated groups.

Statistical Analysis—Data were expressed as the mean ± S.E. of the values from the number of experiments, as indicated in the corresponding figures. Data were evaluated statistically by using Student’s t test, with \(p < 0.01\) or \(p < 0.05\) considered significant.

RESULTS

Wnt-5a Affects Clustering of PSD-95—Several Wnt ligands, including Wnt-4, Wnt-5a, Wnt-7a, and Wnt-11, are expressed in the hippocampus of adult rats and in hippocampal neuron cultures using a prototype mammalian Wnt gene for screening (23). Previously, we have shown that short term exposure to Wnt-7a but not Wnt-5a ligand modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons (23). Here, we examined whether Wnt ligands can regulate the postsynaptic region in mature hippocampal neurons. Neurons exposed to Wnt-7a ligand for 1 h did not show any changes in the clustering of the postsynaptic protein PSD-95, with respect to the control (Table 1) under conditions in which the clustering of the presynaptic protein synaptophysin was increased (Table 1), as described previously (23). However, when hippocampal neurons were incubated with the Wnt-5a ligand, an increase in the PSD-95 density was observed (Fig. 1A, a, b, and d, and Table 1) without affecting the clustering of the presynaptic protein synaptophysin (Table 1).

To establish the specificity of the Wnt-5a ligand conditioned medium effect, Wnt-5a ligands were incubated with a soluble Fz receptor protein (sFRP) antagonist. sFRP binds to the Wnt ligands, thereby preventing their interaction with cellular membrane-bound receptors (29). The co-treatment of Wnt-5a/sFRP prevented the increase of the number of PSD-95 clusters triggered by Wnt-5a (Fig. 1A, b, c, and d). Moreover, to ensure that the effect in PSD-95 clustering is due to Wnt-5a and not to the presence of other proteins contained in the conditioned medium, we used a formylated hexapeptide ligand derived from the sequence of the Wnt-5a molecule in other systems (30). We found a similar increase in the number of PSD-95 clusters when hippocampal neurons were exposed to 50 μM formylated hexapeptide ligand for 1 h of treatment under conditions in which the hexapeptide ligand did not affect the clustering of the presynaptic protein synapsin-1 (Fig. S1).

The short term effect of the Wnt-5a ligand in the clustering of PSD-95 was evaluated; a time-dependent increase in PSD-95

| Treatment | PSD-95 clustering | Synaptophysin clustering |
|-----------|-------------------|--------------------------|
|           | number/100 μm neurite | number/100 μm neurite |
| pcDNA     | 59.67 ± 7.72       | 45.32 ± 8.93             |
| Wnt-7a    | 64.17 ± 10.03      | 86.07 ± 16.35*           |
| Wnt-5a    | 110.72 ± 19.01*    | 48.79 ± 12.16            |

*p < 0.01.
clusters was evidenced. A 25% increase after 30 min was observed, reaching plateau values of 50% over control after 120 min of Wnt-5a exposure (Fig. 1B). To evaluate whether the increase in the PSD-95 cluster density is due to an increase in the expression of total PSD-95 protein, we evaluated the levels of PSD-95 protein in a total extract of hippocampal neurons. Wnt-5a did not change the total levels of PSD-95 in the same temporal course in which we observed the increase in the number of PSD-95 clusters (Fig. 1C).

The presence of PSD-95 clusters in excitatory neurons is well correlated with the number of mature dendritic spines (6, 31). To confirm that Wnt-5a induces clustering of PSD-95 in dendritic spines, neurons were transfected with the enhanced GFP to analyze the PSD-95 contained in spines. Dendritic protrusions below 3 μm in length showing visible spine heads were evaluated for the presence of PSD-95 immunoreactivity and quantified. Hippocampal neurons exposed to Wnt-5a for 2 h presented an 80% increase in the number of PSD-95-containing spines (Fig. 1D, a–g). These results indicate that Wnt-5a but not Wnt-7a affects the postsynaptic region of mammalian synapse by promoting the clustering of PSD-95.

**New PSD-95 Clusters Induced by Wnt-5a Come from a Diffuse Pool**—Since an increase in the clustering of PSD-95 was observed but the total amount of this protein did not change, we asked whether Wnt-5a increases the number of PSD-95 clusters due to a redistribution of existing PSD-95. To evaluate changes in the PSD-95 distribution, two hypotheses were considered. First, the new clusters of PSD-95 induced after Wnt-5a ligand are due to the splitting of existing PSD-95 clusters (Fig. 2A). Second, Wnt-5a induces the clustering of PSD-95 through recruitment of free PSD-95 from a diffuse pool (Fig. 2B). Hippocampal neurons were incubated with Wnt-5a, and different parameters of PSD-95 clusters were analyzed. The mean cluster intensity was evaluated to determine whether Wnt-5a decreases the intensity of PSD-95 clusters. Results show that Wnt-5a did not affect the mean intensity of PSD-95 clusters (Fig. 2C), indicating that the increase in the number of PSD-95 clusters is not due to a decrease in the amount of PSD-95 contained in preexistent clusters. Then in order to analyze whether Wnt-5a induces the splitting of existing PSD-95 clusters, the mean PSD-95 cluster area was measured. Moreover, we also quantified the relative PSD-95 cluster integrated intensity. If Wnt-5a decreases the mean PSD-95 cluster area and the relative PSD-95 cluster integrated intensity, it is possible that splitting of PSD-95 clusters occurs. However, if the integrated intensity of PSD-95 increases, it will suggest that new PSD-95 clusters come from a diffuse pool. Results show that Wnt-5a did not affect the mean area of PSD-95 (Fig. 2D) but increased the inte-
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3, B and D) and palmitate redistributes PSD-95 from cytoplasm (Fig. 3, B and D) to the membrane-enriched compartment (Fig. 3, A and C), as evidenced previously (33, 34). Therefore, we evaluate whether Wnt-5a induces a change in the localization of PSD-95. We found that hippocampal neurons treated with Wnt-5a for 2 h showed increased levels of PSD-95 in the membrane-enriched fraction with respect to the control (Fig. 3, A and C) (as assessed by the presence of N-cadherin and transferrin receptor and the absence of GAPDH proteins; data not shown) and a concomitant decrease in the PSD-95 levels in the cytoplasmic fraction (Fig. 3, B and D) (as assessed by the presence of β-tubulin and GAPDH and the absence of N-cadherin and transferrin receptor proteins; data not shown). These results are consistent with the idea that new clusters of PSD-95 come from a diffuse dendritic cytoplasmic pool.

Wnt-5a Is a Non-canonical Wnt Ligand in Rat Hippocampal Neurons—Different Wnt ligands act through canonical or non-canonical Wnt pathways, depending on the cellular context (35). To evaluate whether the effect of Wnt-5a versus Wnt-7a in the clustering of PSD-95 is due to the activation of different Wnt pathways, we incubated mature hippocampal neuron with the Wnt-5a and Wnt-7a ligands and analyzed the main effectors of the Wnt pathways. Previously, we showed that Wnt-7a induces the stabilization of β-catenin in a time-dependent manner, where the accumulation of β-catenin became evident after 30 min (23, 27).

Hippocampal neurons exposed to Wnt-5a did not show any change in the stabilization of β-catenin even after 2 h of treatment (Fig. 4A). Moreover, when Wnt-5a and Wnt-7a ligands were compared in their capacity to activate the canonical Wnt pathway, Wnt-7a but not Wnt-5a stabilized β-catenin (Fig. 4, A and B). The co-incubation of the Wnt-7a ligand with sFRP prevented the increase in the β-catenin levels at 2 h of treatment (Fig. 4B). Since Wnt-5a but not Wnt-7a induces the clustering of PSD-95, these results suggest that Wnt-5a ligand does not require the activation of the canonical Wnt pathway to promote the clustering of PSD-95.

Wnt ligands can act through the non-canonical Wnt pathways, such as Wnt/Ca2+ and Wnt/JNK (12, 26). To assess whether Wnt-5a activates non-canonical Wnt pathways in hippocampal neurons, we evaluated CAMKII activation, a downstream component on the Wnt/Ca2+ pathway and JNK activation, a downstream component on the Wnt/JNK pathway (26).

Hippocampal neurons incubated with Wnt-5a showed phosphorylation of CAMKII after 5 min, with a peak at 15 min, and then phosphorylation decreased at 30 min and remained at similar levels at 1 h with respect to control conditions (Fig. 4C). When the phosphorylation state of JNK was evaluated, we observed that Wnt-5a increased the phosphorylation of JNK (46-kDa isoform) after 30 min with a peak at 60 h (Fig. 4D), similar to the observed increase in the PSD-95 clustering. The total levels of JNK were not affected by Wnt-5a treatment (Fig. S2). The effect on JNK phosphorylation was antagonized by co-incubation with the Wnt antagonist sFRP at 2 h of treatment (Fig. S3).

Since we have previously not found changes in the clustering of PSD-95 in the presence of Wnt-7a ligand, we examined whether Wnt-7a ligand can activate the Wnt/JNK pathway.
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Interestingly, we have not observed phosphorylation/activation of JNK by Wnt-7a at 1 h of treatment (data not shown) (23), suggesting that Wnt-5a signaling through a noncanonical Wnt pathway promotes the clustering of PSD-95 in hippocampal neurons.

The Non-canonical Wnt/JNK Pathway Is Involved in the PSD-95 Clustering Induced by Wnt-5a—To determine the contribution of the non-canonical Wnt pathways in the clustering of PSD-95 induced by Wnt-5a, we used fumagillin, a synthetic inhibitor of the non-canonical Wnt pathways that acts downstream of the Wnt receptors but upstream of CAMKII and JNK without affecting the canonical Wnt/β-catenin pathway (36) (Fig. 5A). In hippocampal neurons, fumagillin blocked the activation of JNK induced by Wnt-5a and did not affect the β-catenin stabilization (Fig. 5B). Interestingly, fumagillin inhibited the clustering of PSD-95 induced by Wnt-5a at 1 h, without affecting the clustering of PSD-95 (Fig. 5C) or the dendritic spine formation (Fig. S4) with fumagillin alone. To dissect whether Wnt-5a induces the clustering of postsynaptic proteins via Wnt/Ca²⁺ or Wnt/JNK pathways, we have used inhibitors for the three well known effectors of these pathways: KN-93 and BSD-X to inhibit the activity of CAMKII and protein kinase C, respectively, of the Wnt/Ca²⁺ pathway and SP600125 to inhibit the activity of JNK of the Wnt/JNK pathway (Fig. 5A).

Hippocampal neurons were incubated with Wnt-5a ligand for 1 h in the presence of KN-93 or BSD-X. However, the Wnt/Ca²⁺ pathway inhibitors did not affect significantly the PSD-95 clustering induced by Wnt-5a (Fig. 6, A and B). However, when neurons were co-incubated with Wnt-5a in the presence of SP600125, the clustering of PSD-95 induced by Wnt-5a was completely blocked (Fig. 7, A and B). To confirm that the Wnt/JNK pathway is implicated in the clustering of PSD-95, we used anisomycin, a JNK activator (37, 38). Anisomycin induced the clustering of PSD-95 at 1 h of treatment in a way similar to that of Wnt-5a ligand (Fig. 7, A and B).

Since pharmacological reagents as SP600125 and anisomycin may cause nonspecific effects, we used a small peptide that inhibits JNK activity, and it is structurally unrelated to SP600125. TAT-TI-JIP is a smaller 11-mer peptide of JIP conjugated to Tat peptide that perturbs the interaction of JIP-JNK and, as a consequence, affects the JNK activity (39). We observed that TAT-TI-JIP prevented the increase in the number of PSD-95 clusters induced by Wnt-5a in a concentration-dependent manner (Fig. 7, C and D) under conditions in which TAT-TI-JIP alone at this concentrations did not affect the clustering of PSD-95 (Fig. 7, C and D). These results indicate that the non-canonical Wnt/JNK pathway is required for the clustering of the PSD-95 induced by the Wnt-5a ligand.

Wnt-5a Increases Synaptic Amplitude of the Glutamatergic Transmission without Affecting Paired Pulse Facilitation—To examine the effects of Wnt-5a on excitatory glutamatergic transmission evoked by stimulation of Schaeffer collaterals in adult hippocampal slices, we recorded the fEPSP in presence of picrotoxin (10 μM) to block GABA_A-mediated inhibitory synaptic transmission. The fEPSP amplitude increased within 20 min after the addition of Wnt-5a conditioned medium to ACSF perfusion medium, without changing either the fiber volley (fV) amplitude (Fig. 8, B and E) or PPF (Fig. 8D). This effect was antagonized completely in the presence of an anti-Wnt-5a, a generic antibody against the Wnt-5a domain (Fig. 8, A and C). On average, Wnt-5a induced an increase 205.2% ± 42.4 of the fEPSP amplitude, which was blocked in the presence of anti-Wnt-5a (Fig. 8C), whereas the mean values of the PPF and the fiber volley amplitude did not change significantly with respect to their controls in any of these conditions (Fig. 8, B, D, and E).
These results indicate that Wnt-5a does not affect the presynaptic region to induce an increase in the fEPSP, suggesting that Wnt-5a plays a functional role in the postsynaptic region.

**DISCUSSION**

Maturation of functional postsynaptic compartments involves gradual recruitment of scaffold molecules, receptors, and a specialized signaling machinery for clusters in dendrites, which then will form part of functional synapses or be eliminated (40–42). This process is highly regulated for transynaptic adhesion molecules and secreted factors that ensure the coordination with the presynaptic differentiation status or activity. So far, agrin and narp are the only soluble synaptogenic factors that modulate postsynaptic differentiation in developing central nervous system synapses acting only on the postsynaptic side (4). Previous studies of Budnik and co-workers (43) on the Drosophila neuromuscular junction indicated that the loss of Wingless results in aberrant development of postsynaptic specializations.

Here, for the first time it is shown that a Wnt ligand is able to modulate the postsynaptic region in the mammalian central nervous system. Since the presynaptic and postsynaptic regions strongly interact, alterations in structuring the presynaptic terminal or the postsynaptic region are accompanied by a parallel change in the opposite synaptic side. Our studies were made after short term exposure to Wnt ligands; however, we do not know whether a long term exposure to Wnt ligands affects the presynaptic counterpart as a consequence of postsynaptic differentiation.

Ataman et al. (44) found that Wg (Wingless) acts through different pathways to regulate pre- and postsynaptic boutons in glutamatergic Drosophila neuromuscular junction. However, in mammals, different Wnt ligands are expressed. Interestingly, our group have found that different Wnt ligands operate in the central nervous system synapse. Wnt-7a and Wnt-3a, but not Wnt-5a, activate the canonical Wnt pathway and regulate the presynaptic region modulating the clustering of presynaptic proteins, including α7-nAChR (27) and the synaptic vesicle cycle in hippocampal neurons (23). Here, we showed that Wnt-5a but not Wnt-7a regulates the postsynaptic region promoting the clustering of PSD-95 protein through activation of the non-canonical Wnt pathway. However, other Wnt ligand could be involved in the modulation of the postsynaptic region through activation of non-canonical Wnt pathways.

The increase in the number of PSD-95 clusters induced by Wnt-5a observed in mature hippocampal neurons suggests an increase in the number of dendritic spines. We found that almost 80% of dendritic spines contain PSD-95 clusters under control conditions (data not shown) and that Wnt-5a induces the number of total protrusions (data not shown) and protrusions containing PSD-95 by ~60%. It is possible that the increase in the number of clusters of PSD-95 will be contained in newly formed protrusions;
however, it is necessary to evaluate the kinetics of the event to know for sure whether the PSD-95 is recruited as a cluster concomitantly or after the protrusion formation.

Interestingly, we found that Wnt-5a can increase the number of PSD-95 clusters without changing the total expression of PSD-95 and that Wnt-5a increases the density of PSD-95 clusters due to a redistribution of existing PSD-95. Two ideas for de novo cluster formation of PSD-95 have been suggested. It has been observed that splitting of PSD-95 clusters can occur in neuron cultures (45). On the other hand, de novo PSD-95 clusters can be formed by recruitment from a diffuse pool of PSD-95 into the synapse with no obvious involvement of discernible discrete transport particles (46).

Our quantitative analysis of PSD-95 clusters suggests that splitting of PSD-95 cannot explain the increase in PSD-95 clustering induced by Wnt-5a, because the relative cluster integrated intensity of PSD-95 was increased and the mean size of the PSD-95 cluster did not change. These results are consistent with the idea that new PSD-95 clusters induced by Wnt-5a could be formed from a diffuse pool of PSD-95. Subcellular fractionation corroborates our results. In fact, we observed an increase in the membrane-attached PSD-95 pool (particulate fraction) and a decrease in the diffuse PSD-95 pool (cytoplasmic fraction). The monoclonal antibody to PSD-95 cross-reacted with bands of slower migration, which have been described as part of the MAGUK family (47). These proteins also are affected by the Wnt-5a ligand, suggesting that PSD-95-related members are modulated by Wnt-5a; however, more studies are required to clarify this issue.

In this study, we showed that SP600125, a specific JNK inhibitor (48), and TAT-TI-JIP, a small peptide that perturbs the interaction of JIP-JNK affecting the JNK activity (39), prevent the increase in the clustering of PSD-95 induced by Wnt-5a, suggesting that a Wnt/JNK signaling pathway is required by Wnt-5a to induce the clustering of PSD-95 in hippocampal neurons. Moreover, we have demonstrated that the treatment of hippocampal neurons with a JNK activator (anisomycin) and a mimetic formylated hexapeptide can imitate both the effect of Wnt-5a on PSD-95 clustering. A higher concentration of SP (5–20 μM) as used by Rosso et al. (37) and Kim et al. (38) or...
TAT-TI-JIP (5 μM), affected the clustering of PSD-95 (data not shown), according to an endogenous JNK activity that maintains the postsynaptic structure in hippocampal neurons.

The increase in the clustering of PSD-95 induced by the Wnt-5a ligand is correlated with the increase in mature dendritic spines with PSD-95 positive in their heads, suggesting that the non-canonical Wnt/JNK signaling participates in the maturation of the overall postsynaptic structure. Previous evidence suggests that PSD-95 palmitoylation is required to move PSD-95 from a diffuse pool to the synaptic membrane (33). A recent study also suggests that phosphorylation of Ser-295 of PSD-95 from a diffuse pool to the synaptic membrane (33). Several publications showed that the activation of the Wnt pathway has relevance in the synaptic process. Wnt-3a has a regulatory role in long-term potentiation (17), an electrophysiological correlate of memory encoding, suggesting a role for Wnt signaling in the regulation of synaptic plasticity (5). Small synthetic molecules mimic Wnt, leading to both increased spontaneous and evoked neurotransmission that occurs in a transcription-independent fashion (51). Functionally, our results indicate that Wnt-5a is able to increase the amplitude of fEPSP after 20 min of treatment of hippocampal slices with no effect on presynaptic activity, suggesting that Wnt-5a plays a functional role in the postsynaptic region, probably as a consequence of the increase on PSD-95 clustering.

In the canonical Wnt pathway, it has been reported that members of the LRP family of single pass transmembrane proteins are also required for the reception of the Wnt signal transduction pathway and that both Fz and LRP function as co-receptors for Wnt proteins (52). A recent study indicates that Wnt-5a either activates or inhibits β-catenin-TCF signaling, depending on the receptor context (35), suggesting that a Wnt ligand may act in a canonical or non-canonical form, depending on the corresponding characteristics of the receptor present in the cell. In hippocampal neurons, the Wnt-5a ligand and its formylated mimetic were able to modulate non-canonical Wnt pathway components but did not affect canonical Wnt pathway molecules, such as β-catenin. Therefore, it is possible that the LRP receptor may not be present in a context that allows Wnt-5a to act as a canonical Wnt ligand in hippocampal neurons.

Interestingly, it has been reported that the canonical Wnt pathway is required specifically in the presynaptic region to promote synaptic differentiation at the Drosophila neuromuscular junction and that a loss of function of this pathway in the postsynaptic region does not affect this process (53). In our laboratory, we showed that Wnt-7a ligand increases the frequency of fEPSP and field excitatory postsynaptic currents through presynaptic mechanisms (23), and our experiments showed that Wnt-5a increases the amplitude of the fEPSP, suggesting that the mechanism of Wnt-5a is postsynaptic because the PPF is not affected. This evidence together with our results suggest that the canonical and non-canonical Wnt pathways are compartmentalized to act locally and coordinate the differentiation of the pre- and postsynaptic regions, respectively.

The fact that Wnt-5a and their formylated hexapeptide-derived ligands promote the clustering of PSD-95 suggests that the non-canonical Wnt signaling pathway may be a target for the treatment of neurodegenerative diseases that affect synaptic integrity, such as Alzheimer disease (32, 50, 54).

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