CANONICAL WNT3A MODULATES INTRACELLULAR CALCIUM AND ENHANCES EXCITATORY NEUROTRANSMISSION IN HIPPOCAMPAL NEURONS

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A role for Wnt signal transduction in the development and maintenance of brain structures is widely acknowledged. Recent studies have suggested that Wnt signaling may be essential for synaptic plasticity and neurotransmission. However, the direct effect of a Wnt protein on synaptic transmission had not been demonstrated. Here we show that nanomolar (nM) concentrations of purified Wnt3a protein rapidly increase the frequency of miniature excitatory synaptic currents in embryonic rat hippocampal neurons, through a mechanism involving a fast influx of calcium (Ca\(^{2+}\)) from the extracellular space, induction of post-translational modifications on the machinery involved in vesicle exocytosis in the presynaptic terminal leading to spontaneous Ca\(^{2+}\) transients. Our results identify the Wnt3a protein and a member of its complex-receptor at the membrane, the low-density lipoprotein receptor-related protein 6 (LRP6) co-receptor, as key molecules in neurotransmission modulation and suggest a crosstalk between canonical and Wnt/Ca\(^{2+}\) signaling in central neurons.

Throughout mammalian brain development Wnt signaling seems to be spatially confined to specialized regions such as the olfactory bulb, frontal cortex, hippocampal formation and the cerebellum (1-5). In these brain domains Wnt signaling has essential roles in diverse biological processes including neurogenesis (6), axonal remodeling (7), synapse formation and maintenance of pre- and post-synaptic terminals (8-14). Indeed, several studies have begun to show that Wnt signaling may also be involved in excitatory synaptic transmission (15-18). For instance, activation of N-methyl-d-aspartate (NMDA) receptors, after tetanic stimulation in rodent hippocampal slices, induces the release of a pool of vesicles containing Wnt3a in the synaptic terminal modulating long term potentiation events (18). Similarly, double-mutant mice for Wnt7a and Dishevelled-1, which exhibit a decrease in the number of synapses between mossy fibers of the cerebellum and grainy cells, showed marked deterioration in the release of neurotransmitters and in the recycling of vesicles in the existing synapses (15). Furthermore, pharmacological analysis with drugs modulating Wnt signaling in hippocampal neurons showed an acute increase of neurotransmitters released from the presynaptic component resulting in enhanced evoked basal activity and frequency of spontaneous and miniature excitatory currents (16). Finally, it has been reported that conditioned medium containing canonical Wnt7a and to a lesser extent Wnt3a, but not conditioned medium containing the non-canonical Wnt5a, which signals mainly through the Wnt/calcium (Wnt/Ca\(^{2+}\)) pathway (19,20), were found to increase synaptic transmission in CA3-CA1 slices in adult rat hippocampus and to induce the recycling and exocytosis of synaptic vesicles in hippocampal neurons in culture (17).

Although these results indicate that Wnt signaling probably modulates neuronal transmission, however, the direct effect of a Wnt protein on synaptic transmission had not been demonstrated. Here we show that a purified Wnt ligand, the canonical Wnt/\(\beta\)-catenin Wnt3a protein, rapidly increased miniature synaptic currents through a mechanism involving Ca\(^{2+}\) mobilization and post-translational modifications on the
machinery involved in vesicle exocytosis in the presynaptic terminal, suggesting a crosstalk between canonical and Wnt/Ca\(^{2+}\) signaling in central neurons.

**Experimental Procedures**

*Wnt3a purification- The Wnt3a purification was carried out following the protocol previously described (21), which has been implemented in our lab considering the changes suggested by Kishida and co-workers (22). The presence of the Wnt3a protein was detected with an anti-Wnt3a antibody (R&D Systems, Minneapolis, MN, USA) and the positive fractions were pooled and used in the next column. Purity was analyzed by SDS-PAGE (8 %) and stained with Coomassie Blue G250 and analysed through densitometry by using software ImageJ.

*Cultured hippocampal neurons- The animals were treated and handled according to NIH guidelines (NIH, Maryland, USA). Hippocampal neurons were dissociated and maintained as described before (23). Briefly, cells were taken from 18-day pregnant Sprague-Dawley rats and maintained for 12-13 days *in vitro* (DIV) on 35 mm tissue culture dishes with glass coverslips (350,000 cells per dish) coated with poly-L-lysine (Sigma Chemical Co., St, Louis, MO, USA). The neuronal feeding medium consisted of 80% minimal essential medium (MEM, GIBCO, Rockville, MD, USA), 10% heat-inactivated horse serum (GIBCO, Rockville, MD, USA), 10% heat-inactivated bovine fetal serum (GIBCO, Rockville, MD, USA) and a mixture of nutrient supplements. The culture was placed on a shelf in a 37º C humidified CO\(_2\) incubator, and the medium was changed every 3 days.

*Wnt3a functional assays- Purified Wnt3a protein was assessed for its ability to stabilize β-catenin in hippocampal neurons (12-13 DIV), which were incubated with 10 nM of Wnt3a for 0, 15, 30 min and 2 hr. Lithium chloride (LiCl 10 mM; 2 hr; Sigma, St Louis M.O, USA) was used as a control. Then, cells were lysed and 30 µl of lysates were subjected to SDS-PAGE and Western blotting. Similarly, Wnt3a activity was evaluated in pBARL-HT22 cells, a mouse hippocampal cell line stably transfected with the pBARL(Beta-catenin Activated firefly Luciferase) reporter plasmid, which contains 12 elements in response to TCF/LEF transcription factors (24). pBARL-HT22 neurons were incubated with different treatments and after 24 h luciferase activity was measured with the Dual-Luminiscence kit (Promega Madison, Wi, USA), as previously described (25), in a Victor3 Multi-labelled Counter (Perkin-Elmer). Recombinant secreted Frizzled related protein 1 (sFRP1) and Dickkopf 1 (DKK1) were obtained from R&D Systems (Minneapolis, MN, USA).

*Electrophysiology- Whole-cell patch-clamp recordings were performed in 12-13 DIV hippocampal neurons as previously described (26,27). Culture medium was changed to an external solution (ES) containing (in mM): 150 NaCl, 5.0 KCl, 3.0 CaCl\(_2\), 1.0 MgCl\(_2\), 10 HEPES and 10 glucose (pH 7.4, 330 mOsm). The patch pipette solution contained (in mM): 120 CsCl, 4.0 MgCl\(_2\), 10 BAPTA, 10 HEPES and 2 ATP (pH 7.35, 310 mOsm) and a holding potential of -60 mV was used. Stock recordings were obtained using an Axopatch-1D amplifier (Axon Instruments, Inc., Burlingame, CA, USA). Electrodes were pulled from borosilicate capillary glass (WPI, Sarasota, FL, USA) using a horizontal puller (Sutter Instruments, Novato, CA, USA). The current signal was filtered at 2-5 kHz and stored for off line analysis using PC interfaced with a Digidata 1200 acquisition board. In the records of action potentials, the current was set at 0 A. 100 nM tetrodotoxin (TTX) were added when miniature synaptic currents were recorded (2 min segments). Glutamatergic transmission was pharmacologically isolated during the recording of miniature synaptic currents through perfusion of Wnt3a in the presence of either 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 4µM) or d-2-amino-5-phosphonovaleric acid (APV; 50 µM), which were diluted in the ES without Mg\(^{2+}\) for its pre-application at RT (20–24 °C; see Fig. 3). In order to obtain mean average of either cumulative or peak amplitude and frequency, the data was analyzed with Mini Analysis 6.0 program (Synaptosoft, Inc., Leonia, NY, USA) as described previously (26). Decay time histograms were plotted with a bin of 1 pA and the frequency (% total events) was calculated and expressed over control neurons. All reagents were obtained from Sigma (Sigma, St Louis M.O, USA).
**Calcium experiments**- Hippocampal neurons of 12-13 DIV were loaded with 5 µM Fluo-4AM (Molecular Probes, Invitrogen, USA) in external solution for 30 min at 37°C, washed three times with external solution for 5 min and mounted in a perfusion chamber placed on the stage of an inverted fluorescent microscope (Eclipse TE, Nikon). The microscope had a 12 bit CCD camera attached (SensiCam, PCO, Germany). After being incubated for 15 min with different treatments (see Results), neurons were located, selected their somatic region and illuminated (< 0.266 s) by using a Lambda 10-2 filter wheel (Sutter Instruments, Novato, CA, USA) computer-controlled by Axon Instruments Work-Bench 2.2 software (Axon Instrument, Inc., Burlingame, CA, USA). The images at 480 nm were obtained at 2 s intervals during a continuous 5 min period and the frequency of Ca²⁺ transients was determined. Since calcium transients are dependent on neuronal excitability and synaptic transmission, and thus blocked by application of TTX (27,28), Ca²⁺ transient records were performed in the absence of this inhibitor. Finally, a similar protocol was used when examining intracellular Ca²⁺ Release of synaptic vesicles- Treated and control hippocampal neurons (10-13 DIV) were washed with ES and incubated for 5 min in high-K+ solution (30 mM) at 37°C and then loaded with 15 µM probe FM1-43 (Molecular Probes, USA) for 5 min at 37°C, washed with ES for 5 min at RT and mounted on a perfusion chamber. Different treatments were applied by perfusion (see Results) and regions of interest (ROIs) were selected and the decay of fluorescence associated with FM1-43 was continuously measured during 20 min. The recordings were collected with an inverted epifluorescence microscope (Eclipse TE, Nikon, Melville, NY, USA) equipped with a Xenon lamp, 40X and 100X objectives, and a Lambda 10-2 filter wheel (Sutter Instruments, Novato, CA, USA). The microscope had a SensiCam CCD camera (PCO, Kelheim, Germany) and the FM1-43 fluorescence intensity was measured using a 2 x 2 pixels area. FM1-43 was excited at 560 nm and emission was collected with a 620 nm filter. Finally, a similar approach was used when loading for 1 min neurons with 50µM of the fixable probe AM1-43 (Molecular Probes, USA) at 37°C, after which cells were washed with ES for 5 min at RT and mounted on a perfusion chamber for immunocytochemistry.

**Immunocytochemistry**- Treated and control hippocampal neurons (10-13 DIV) were washed with ES and loaded with 50 µM AM1-43 (Molecular Probes, USA) for 1 min at 37°C, washed with ES for 5 min at RT and mounted on a perfusion chamber. Cells were fixed with 4 % paraformaldehyde–PBS (1X, pH 7.4), blocked for 1 h with horse fetal serum (1:10 Hyclone, USA) and permeabilized with PBS1X-Triton X-100 (0.1 %) and incubated for 12 h at 4°C with the following primary antibodies: p-Synapsin I antibody (Ser 553; 1:200; Santa Cruz Biotechnology, USA), LRP6 (1:100; R&D Systems, USA), Synaptophysin (1:500; Zymed, USA) and PSD95 (1:500; Affinity Bioreagent, USA). Then, neurons were washed 3 times and incubated with appropriate secondary antibodies conjugated to Alexa488 (1:500), Cy3 (1:400) and Alexa633 (1:500) (ImmunoResearch Jackson Laboratories, USA). After mounting the samples in Dako (DAKO Corp, USA), fluorescent microscopy was performed (60 X oil immersion objective 1.45 NA) using a TE2000U confocal microscope (Nikon, Japon). Images were acquired with the Nikon software (EZ-C1 V. 3.5).

**Colocalization analysis**- Fluorescent images for each antibody were acquired sequentially on the confocal microscope. We selected ROIs (neuronal process) adjusted to a window/level of 300 pixels and separated the fluorescence channels associated with LR6, Syp and PSD95. The images were deconvoluted and examined for the degree of colocalization between the different channels through qualitative analysis of antibody colocalization using ImageJ (plug-in JacoP). Overlap of the green-red (LRP6-Syp) and green-blue (LRP6-PSD95) channels were visualized in merged images and then overlapping areas were considered as colocalized. The extent of colocalization was further analyzed using the Manders (M1) and Pearsons coefficients (29). Both coefficients range from 0 to 1, with 0 indicating
low colocalization and 1 indicating high colocalization.

Western blot- Proteins were separated in a SDS-PAGE 12% gel and transferred to a nitrocellulose membrane (BioRad, CA, USA). Membranes were blocked with 5% milk in PBS 1X and 0.1 % Tween 20 for 1 h in agitation, then incubated with primary β-catenin or p-Synapsin I (Ser 553) antibodies (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 12 h at 4°C, washed and incubated with appropriate secondary antibody conjugated to HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 4°C. The immunoreactivity of reactive protein was detected using chemoluminescence reagents (Promega, Madison, Wi, USA).

Data Analysis- Data are shown as mean ± s.e.m. and test ANOVA (*p < 0.05, **p < 0.01, ***p < 0.005) was implemented. All analyses were performed using the Origin 6.0 software (Microcal, Inc. Northampton, MA, USA).

RESULTS

Purified Wnt3a enhances excitatory neurotransmission. We purified Wnt3a protein from stable mouse L-cells following standards protocols (21,22) and consistently recovered a fully functional Wnt3a ligand that induced accumulation of its β-catenin target in primary cultures of embryonic rat hippocampal neurons (12-13 DIV) (Fig. 1A-D). Then, to analyze the functional activity of the Wnt3a protein in mature synapses in hippocampal neurons we carried out electrophysiological analysis using the whole-cell patch-clamp technique to record spontaneous synaptic activity in the presence of different concentrations of the purified protein. We found that Wnt3a produced a concentration dependent increase in the frequency of spontaneous synaptic currents (Fig. 2A), which at the cellular level is likely associated to an increase in the neuronal network’s activity, reflecting the sum of action potentials and synaptic potentials. Analyses of characteristic parameters of action potentials (AP; i.e. threshold, amplitude and duration) showed that no significant differences were found in control versus treated neurons with 10 nM Wnt3a for 15 min (Fig. 2B and Table 1). Interestingly, while individual AP remained similar, there was a greater number of repetitive events in Wnt3a-treated neurons (Fig. 2B, bottom trace), reflecting an increase of the neural network excitability and further indicating that the effect of the Wnt3a protein ought to be mainly on synaptic transmission (increased synaptic potential). Moreover, there were no significant differences in input resistance in control and Wnt3a-treated neurons (Table 1). Thus, we decided to use 10 nM of Wnt3a as the effective concentration for subsequent recordings of synaptic potentials (miniature synaptic activity), examined in the presence of 100 nM of the Na+ channel blocker tetrodotoxin (TTX).

Local application of Wnt3a to neurons showed a time-dependent enhancement of miniature synaptic activity compared to control neurons treated with the Wnt3a elution buffer (Fig. 2C). Analysis of individual traces (i.e. mean average peak) revealed that purified Wnt3a affected significantly the frequency, but not the amplitude of the synaptic currents (Fig. 2C-E), effect which was further confirmed by analyses of the cumulative frequency and amplitude between control and Wnt3a-treated neurons (Fig. S1). The enhancing effect on the frequency was apparent after 5 min and peaked at 15 min of incubation (1.1 ± 0.1 Hz in control condition to 3.4 ± 0.3 Hz in the Wnt3a treatment; N=9), and contrary to Wnt3a treated neurons, the application of either denatured Wnt3a (1.5 ± 0.2 Hz; N=8), or the co-application of 2 µg/ml of anti-Wnt3a antibody (1.1 ± 0.01 Hz; N=8), at a dose that blocks Wnt3a activity in Wnt/β-catenin reporter-based luciferase assays (Fig. 1E), were without effects on synaptic activity. Conversely, the inhibitory effect of the anti-Wnt3a antibody was not observed when this antibody was previously denatured (3.6 ± 0.4 Hz; N=5). Moreover, the effect of Wnt3a was reversible as demonstrated after its removal from the bath solution (washout; 1.2 ± 0.5 Hz; N=9; Fig. 2).

Wnt3a-mediated enhancement in the frequency of miniature synaptic currents was completely blocked in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 4 µM), an inhibitor of AMPA receptors (0.7 ± 0.1 Hz; N=5), and partially silenced in the presence of d-2-amino-5-phosphonovaleric acid (APV; 50 µM), a
competitive antagonist of NMDA receptors (0.9 ± 0.1 Hz; N=5), suggesting that modulation of excitatory glutamatergic neurotransmission play a fundamental role in the synaptic effect induced by Wnt3a (Fig. 3A,B). As previously observed, the amplitude of synaptic currents did not differ among treatments (Fig. 3C). Furthermore, analysis of time decay kinetics of synaptic events (26,27) showed that records in the presence of Wnt3a were easily distinguishable as glutamatergic, specifically AMPAergic (93 %; τ = 0.1-10 ms; N=10), in contrast to NMDA/GABAergic kinetics (7 %; τ = 11-40 ms; N=9) (Fig. 3D-F). Therefore, we conclude that low concentrations of purified Wnt3a rapidly enhance the frequency of miniature excitatory synaptic currents and that such effect is specific to the treatments with the purified Wnt3a protein.

Wnt3a induces intracellular Ca2+ influx and the release of presynaptic vesicles. Changes in the frequency of miniature synaptic currents could be interpreted by alterations in the probability of neurotransmitter release and it has been suggested that such release depends on the concentration of Ca2+ within the presynaptic terminal (30). Interestingly, the effect of Wnt3a on the frequency of miniature synaptic activity was completely abolished (0.5 ± 0.05 Hz; N=5) when an external solution with zero nominal Ca2+ was used in the experiments (Fig. 3A-C). Therefore, we next investigated whether there was a relationship between the Wnt3a-induced enhancement in excitatory synaptic transmission and intracellular Ca2+ levels. In order to do so, neurons were loaded with Fluo4-AM and the fluorescent signal related to the frequency of spontaneous calcium transients was recorded using fluorescence microscopy. Notably, as shown in Fig. 4A, neurons incubated with a concentration of 10 nM Wnt3a for 15 min and in the absence of TTX significantly augmented the frequency of spontaneous Ca2+ transients (13.9 ± 0.5 x 10−2 Hz; N=50) when compared to control cells (3.6 ± 0.1 x 10−2 Hz; N=45, p < 0.005). Conversely, neurons treated either with denatured Wnt3a (boiled) or co-incubated with Wnt3a and anti-Wnt3a (2 µg/ml) did not show the above mentioned effect (4.1 ± 0.2 x 10−2 and 4.7 ± 0.6 x 10−2 Hz, respectively). As a way to distinguish among the Ca2+ source required for the synaptic effect of the Wnt3a protein we next examined whether acute perfusion of Wnt3a altered intracellular Ca2+ levels using the fluorescent probe Fluo4-AM (Fig. 4B). Consistent with our previous results, perfusion of Wnt3a acutely augmented the intracellular Ca2+ concentration of neurons loaded with Fluo4-AM, an effect which was partially reversed when Wnt3a was co-incubated with 2 µg/ml of the anti-Wnt3a antibody (decreased to 46 % of the Wnt3a normalized fluorescence signal, N=15), or silenced when a denatured fraction of purified Wnt3a was used after being heated for 10 min at 96° C (decreased to 8 % of normalized fluorescence, N=6). Remarkably, Wnt3a effect on intracellular Ca2+ was dramatically diminished in the presence of 20 µM Cd2+, (12.2 ± 0.8 % of the Wnt3a normalized signal, N=15), which acts as a non-selective Ca2+ channel blocker, or nearly abolished in the absence of extracellular calcium in the working solution (zero nominal calcium; 3.7 ± 1.1 % of the remaining signal, N=18), thus indicating that the extracellular space is the main source of the Ca2+ mobilized by the Wnt3a ligand.

It has been proposed that intracellular Ca2+ concentration is the focal point controlling the exocytosis and trafficking of synaptic vesicles at the presynaptic active zone of nerve terminals (31). Therefore, to examine whether the effect of Wnt3a on the intracellular Ca2+ concentration was sufficient to allow the release of presynaptic vesicles from mature hippocampal neurons, we applied a depolarizing stimulus with a high K+ concentration (30 mM), loaded neurons with FM1-43 and then treatments (i.e. Wnt3a) were applied by perfusion throughout the entire record (Fig. 4C). Indeed, time-course experiments revealed that Wnt3a treatment induced a fast release of synaptic vesicles seen as the decay in the fluorescence associated with FM1-43 after starting the incubation, which subsequently reached a plateau at 12-15 min of stimulation (Fig. 4C). The value of the depleted fraction (∆F/Fi) found after 20 minutes of recording was 0.1 ± 0.04 in the control condition and 0.8 ± 0.02 in neurons treated with Wnt3a. Similarly, such effect was specific to Wnt3a since no significant differences were observed when boiled Wnt3a or co-incubation with the anti-Wnt3a antibody were used as treatments (0.42 ± 0.03 and 0.6 ± 0.03, respectively). In
agreement with the above presented results, fluorescent immunochemistry analysis in hippocampal neurons with the fluorescent probe AM1-43 (equivalent dye to FM1-43 but with the additional property of being fixable) showed that the signal associated to synaptic vesicles strongly decreased after 15 min of treatment with purified Wnt3a (Fig. 4D, upper panel). Collectively, these experiments, which were made in the absence of TTX, indicate that acute incubations with purified Wnt3a allows the influx of Ca\(^{2+}\) from the extracellular space, augmenting the intracellular concentration of Ca\(^{2+}\) and enhancing the release of synaptic vesicles from the presynaptic terminal.

Previous studies have shown that Wnt-7a induced the clustering at remodeled areas of mossy fibers of the neuron-specific phosphoprotein Synapsin I as a preliminary step in synaptogenesis (11). Therefore, we further investigated whether Wnt3a treatment had any effect on such post-translational modification in hippocampal neurons. Indeed, purified Wnt3a induced a substantial increase in the signal of phosphorylated Synapsin I (shown in green) when compared with control neurons (Fig. 4D, lower panel). Finally, Western blot analysis revealed that induction of Synapsin I phosphorylation was specific for the treatment with purified Wnt3a (Fig. 4E), suggesting that this post-translational modification may be an essential step towards the release of synaptic vesicles from presynaptic terminals induced by Wnt3a in hippocampal neurons.

**Involvement of the Wnt/β-catenin complex receptor at the membrane in Wnt3a neurotransmission.** As shown previously, purified Wnt3a induced β-catenin accumulation in hippocampal neurons (Fig. 1C & D). Then, would the molecular machinery normally transducing canonical Wnt3a be the one responsible for the enhancement of intracellular calcium and the fast release of synaptic vesicles from the pre-synaptic terminals? We began to approach this question by interfering Wnt3a signal presentation at the membrane using recombinant proteins that inhibit the activity of its complex receptor Frizzled-LRP5/6. Therefore, the synaptic activity of hippocampal neurons exposed to Wnt3a was recorded either in the presence of 50 nM secreted Frizzled Related Protein 1 (sFRP1), a Wnt antagonist, and 2 µg/ml Dickkopf 1 (DKK1), as an antagonist of the Low Density Receptor Related Protein 6 (LRP6), which acts as a co-receptor (32). Remarkably, we observed blockade of the Wnt3a effect by both sFRP1 and DKK1 on the frequency of miniature synaptic currents (1.6 ± 0.14 Hz; N=6 and 1.2 ± 0.04 Hz; n = 5, respectively; Fig. 5A) and Ca\(^{2+}\) transients (3.8 ± 0.4 x 10^{-2} Hz, N=40 and 6.8 ± 0.09 x 10^{-2} Hz, N=50, respectively; Fig. 5B), suggesting that the Wnt/β-catenin complex receptor is functionally active at the synaptic terminal. No significant increase was observed when sFRP1 and DKK1 proteins were applied alone onto the hippocampal neurons.

DKK1 interferes directly with Wnt3a presentation to the LRP6 receptor itself, instead of being sequestered in the extracellular space as it is the case for the action of sFRP1. Given that we have previously shown that the LRP6 protein is expressed in the human hippocampus (25), therefore we wanted to study whether LRP6 was present in the synaptic terminal. In order to do so, the subcellular localization of the LRP6, as well as pre- and post-synaptic markers synaptophysin (Syp) and PSD95, respectively, was examined in 14-15 DIV hippocampal neurons by confocal microscopy. The results obtained are summarized in Fig. 6, which shows that LRP6 is widely distributed both in the soma and in the neuronal processes of hippocampal neurons, where is co-localized and/or closely apposed to the signal corresponding to synaptophysin and PSD95. Further quantitative analysis of co-localization for LRP6-Syp and LRP6-PSD95 proteins (Fig. 6B & C) revealed that there were no significant differences in the co-localization coefficients Manders (0.34 ± 0.042 and 0.38 ± 0.044, respectively; N=15) or Person (0.54 ± 0.029 and 0.52 ± 0.027, respectively; N=15) (29), suggesting that the LRP6 could be simultaneously present both in pre- and post-synaptic terminals.

**DISCUSSION**

In recent years it has become clear that the signaling cascade activated by any Wnt isoform is highly dependent on cellular context or the complementation between cell surface Wnt receptors (33). Wnt3a belongs to the so called
Wnt/β-catenin or canonical signaling as opposed to the β-catenin independent or non-canonical pathways, which transduce their signals to control asymmetric cell division and morphogenetic movements during vertebrate gastrulation, including the Wnt/Ca\(^{2+}\) pathway (19,20,32). Here we demonstrate that synaptic transmission activity was modulated by direct application of a canonical Wnt3a protein to mature hippocampal neurons, which is in general agreement with the effect of a recombinant Wnt3a preparation used to study LTP events on hippocampal slices (18) and the use of either conditioned medium containing Wnt ligands (15,17) or Wnt/β-catenin small molecule modulators (16). Moreover, the effect was dependent on the incubation with the purified protein (Fig. 2-4), and not observed in the presence of recombinant DKK1 and sFRP1 proteins, classical inhibitors of the Wnt/β-catenin pathway (Fig. 5), suggesting that the canonical complex receptor at the membrane is functionally active at the synaptic terminal.

Although we initially observed that purified Wnt3a induced an increase both in the frequency and the amplitude of spontaneous activity in Wnt3a-treated neurons (Fig. 2A), which could reflect enhanced neuronal excitability, we subsequently observed that the Wnt3a dependent effect was not due to changes in neuronal electrical parameters such as action potentials or input resistance (Fig. 2B and Table 1). Moreover, analyses of miniature post-synaptic currents in TTX-treated neurons indicate that the Wnt3a effect was directly on synaptic transmission, via augmenting the frequency of neurotransmitters release, and not related to the number of activated receptors in the post-synaptic region (Fig. 2C-E; see also Fig. S1).

To investigate whether excitatory or inhibitory neurotransmission is the target of purified Wnt3a, we first evaluated the effects of excitatory AMPA/kainate and NMDA receptors. Our results show that when neurons were treated with 4 µM CNQX (AMPA/kainate antagonist) the Wnt3a effect on the release of synaptic vesicles was dramatically decreased (i.e. similar to that observed in the control neurons) (Fig. 3A-C). Similarly, in the case of the NMDA receptor antagonist APV, the results show that although there was not complete inhibition, no significant differences were observed between APV-treated and control neurons. Finally, considering that the blockade of GABA\(_A\) receptors with bicuculline or strychnine induced an increase in the frequency of miniature AMPA receptor-mediated EPSCs (34) and a change in the network activity of hippocampal neurons (27,34), which were within the time-frame for the Wnt3a effect described here (i.e. > 15 min), therefore, as an indirect way to assess the effects on inhibitory activity, we carried out analyses of time decay kinetics (τ) events. In agreement with previous observations (15-18), our results clearly distinguished the effect of purified Wnt3a as excitatory and not inhibitory and further implicated AMPA receptors as the main effectors controlling the action of the Wnt3a protein at the synapse (Fig. 3D-F).

It is well known that synaptic transmission is affected by changes in presynaptic Ca\(^{2+}\) level (31). Notably, the Wnt3a effect on neurotransmission seems to be mediated through a fast influx of Ca\(^{2+}\) which subsequently induced the phosphorylation of synapsin I and the release of synaptic vesicles from the presynaptic terminal (Fig. 4). Interestingly, the source of this Ca\(^{2+}\) influx appeared to be mainly extracellular. Indeed, the Wnt3a effect was nearly abolished when using an external solution with zero nominal calcium or silenced in experiments in the presence of Cd\(^{2+}\), which suggest that influx through voltage-dependent Ca\(^{2+}\) channels (VDCCs) or transient receptor potential (TRP) ion channels, which are both Cd\(^{2+}\)-sensitive (35-37), could be involved in the Wnt3a evoked effect. Supporting this idea, it has been recently observed that a Wnt/Ca\(^{2+}\) pathway ligand, the Wnt5a protein (19,20), modulates cortical axonal guidance/repulsion processes via augmenting the concentration of intracellular Ca\(^{2+}\) through activation of TRP ion channels (38). Nevertheless, activation of presynaptic neurotransmitter receptors that are permeable to Ca\(^{2+}\), and which enable a sufficient rise in intracellular Ca\(^{2+}\) to trigger neurotransmitter release, should be also examined in further detail when explaining the remaining 12 % in the fluorescent signal associated with the Wnt3a effect in the presence of Cd\(^{2+}\) (Fig. 4B).

The data, which suggest that a cross-talk between Wnt/β-catenin and Wnt/Ca\(^{2+}\) signaling...
could take place in central neurons, is further supported by the following observations: First, we have shown here that purified Wnt3a protein, which is strongly expressed in the hippocampus (39), along with inducing the influx of Ca\(^{2+}\) maintains its activity in the canonical pathway in stabilizing β-catenin and activating a Wnt/β-catenin luciferase-associated gene reporter (Fig. 1) (21,22). Second, it has been observed that in the proliferation of PC12 cells the canonical Wnt1 protein activates intracellular components of the Wnt/Ca\(^{2+}\) pathway, including the protein kinase C (PKC) enzyme (40), which has been previously involved in the regulation of neurotransmitter release (41,42). Third, the Wnt-responsive Dishevelled (Dvl) protein, which acts as a branching point between canonical and non-canonical Wnt signalling pathways, binds to Synaptotagmin and thus participates in the process of endo- and exocytosis of neurotransmitter-containing vesicles in differentiated PC12 cells (43). Fourth, the canonical LRP6 co-receptor, which we have previously found expressed in the human hippocampus and genetically linked to prevalent neurological conditions (25), is seen here localized within pre-and post-synaptic regions (Fig. 6), being essential for inducing the intracellular increase of Ca\(^{2+}\) in order to trigger the Wnt3a-dependent effect on neurotransmission (Fig. 5).

Finally, the experimental data reported in this study does not necessarily imply that the Wnt3a effect on neurotransmission would be independent of the activity mediated by β-catenin/TCF-LEF complexes, which have been previously involved in LTP events (16,18), but rather suggest that such a rapid effect on the neuronal network (i.e. min) involves the cross-talk between receptors and kinases acting as canonical or Wnt/Ca\(^{2+}\) components, which is the molecular machinery associated with or immediately downstream of its complex receptor at the membrane in the Wnt3a effect on synaptic transmission in mature hippocampal neurons.

**REFERENCES**

FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Purification and functional assays of Wnt3a activity. A. Coomassie Blue staining of an SDS-PAGE gel which was loaded with Wnt3a-L-cells conditioned medium (CM) and purified Wnt3a, resulting from a 3-step chromatographic purification (BS: Blue Sepharose; HiL: High Load 16-60 Superdex 200; Hep: Heparin). B & C. Western blotting analysis of purified Wnt3a and time-dependent effect on β-catenin stabilization in hippocampal neurons following different periods of Wnt3a application (15, 30 min and 2 hr); LiCl (10 mM) for 2 hr was used as a control. D. Summary of data as shown in (C). E. β-catenin reporter activity in pBARL-HT22 cells treated with 10 nM Wnt3a, Wnt3a vehicle (buffer), boiled Wnt3a (denatured 10 min at 96 °C), 10 nM Wnt3a plus 50 nM recombinant secreted Frizzled related protein 1 (sFRP1), 50 nM sFRP1, Wnt3a plus 70 nM recombinant Dickkopf 1 (DKK1) and 70 nM DKK1. Control: basal reporter activity in this cell line. Data from 3 independent experiments is shown as mean ± s.e.m. and test ANOVA (*p < 0.05, ***p < 0.005) was implemented.

Fig. 2. Enhancement of miniature synaptic activity by purified Wnt3a in primary cultures of hippocampal neurons. A. Representative traces of spontaneous synaptic currents recorded in the presence of different Wnt3a concentrations (0-10 nM). B. The upper panel shows representative traces of individual events in voltage records (action potentials) obtained from control neurons and those treated with 10 nM of the Wnt3a protein. The lower panel depicts a repetitive event induced by 10 nM Wnt3a. The dotted line marks 0 mV. Records (N=5) were obtained in the absence of TTX and the treatments were applied by perfusion. C. Miniature synaptic activity following the application of Wnt3a (5 and 15 min) and various treatments by perfusion, in the presence of 100 nM TTX (holding potential of -60 mV; 2 min duration). D & E. Summary of miniature synaptic activity data for current frequency and amplitude, respectively. Control: Wnt3a vehicle; Wnt3a: 10 nM Wnt3a; Boiled: denatured Wnt3a (boiled 96 °C); anti-Wnt3a: antibody anti-Wnt3a; Boiled anti- Wnt3a: boiled antibody anti-Wnt3a (96 °C); washout: Wnt3a removed from external solution. Test ANOVA (***; p < 0.005; n = 5). Data are shown as mean ± s.e.m.

Fig. 3. Inhibition of Wnt3a-induced synaptic activity by glutamatergic blockers CNQX and APV. A) The miniature synaptic transmission induced by 10 nM Wnt3a (15 min) in hippocampal neurons was pharmacologically blocked following application of CNQX (4 µM) or APV (50 µM) by perfusion in the presence of ligand and TTX (100 nM) and in the absence of Mg2+. B & C. Data summary for the effects on the frequency and amplitude of the miniature currents, respectively. Control: Wnt3a vehicle; Without Ca2+: zero nominal calcium. Data are shown as mean ± s.e.m. and test ANOVA (*p < 0.05, ***p < 0.005; N=5) was implemented. D-F. Analysis of an extended trace of miniature synaptic activity recorded in the presence of 10 nM Wnt3a. E & F. Stacked bars plot showing event decay-time (ms) distribution histogram and the frequency of total events in Wnt3a treated neurons. Fast AMPAergic events: 0.1 – 10 ms; Slow GABAergic events: 10 – 40 ms.

Fig. 4. Enhancement of intracellular Ca2+ and synaptic vesicle release by Wnt3a in hippocampal neurons. A. Representative fluorescent traces showing spontaneous enhancement of calcium transients following application of Wnt3a and various treatments for 15 min. B. Strokes representing the effect of acute influx of extracellular Ca2+ following perfusion of Wnt3a and various treatments. C & D. Synaptic vesicle
release from pre-synaptic terminals induced by Wnt3a. 

C. Destaining associated to FM1-43 (depleted fraction: \( \Delta F/F_i \)) in the presence or absence of Wnt3a. Treatments were applied by perfusion during the entire record (20 min) and the burden of hippocampal neurons was recorded \( (N=60–80\) neurons). 

D. Immunocytochemical analysis showing the decay of the signal associated with the fixable AM1-43 probe (upper panel) and the enhancement of phosphorylated Synapsin I (Ser 553) after treatment with Wnt3a for 15 min (lower panel). 

E. Western blot of phosphorylated Synapsin I after treatment for 15 min with purified Wnt3a compared to control neurons treated either with boiled Wnt3a or co-incubated with a Wnt3a-specific antibody. All records of Ca\(^{2+}\) changes were made in the absence of TTX. Calibration bar represents 20 \( \mu \)m. Control: Wnt3a vehicle; ENS: external normal solution containing 10 nM Wnt3a; Cd\(^{2+}\): ENS plus 10 nM Wnt3a and 20 \( \mu \)M Cd\(^{2+}\); w/o Ca\(^{2+}\): 10 nM Wnt3a plus external solution without Ca\(^{2+}\) (zero nominal Ca\(^{2+}\)); Boiled: denatured Wnt3a (boiled 10 min to 96 °C); anti-Wnt3a: co-incubation of 10 nM Wnt3a and 2 \( \mu \)g/ml of antibody anti-Wnt3a.

Fig. 5. Evidence for the participation of the Wnt/\( \beta \)-catenin complex-receptor in Wnt3a-induced neurotransmission. 

A & B. Representative traces showing the inhibition of the Wnt3a enhancement in the frequency of miniature synaptic activity (test ANOVA, ***\( p<0.005\); n=5; 100 nM TTX) and calcium transients (test ANOVA, ***\( p<0.005\); n=40-50), respectively, following co-incubation for 15 min of 10 nM Wnt3a with either 50 nM sFRP1 or 70 nM DKK1, which act as inhibitors of the Wnt/\( \beta \)-catenin membrane-associated receptors (see also Fig. 1E). 

C & D. Summary of miniature synaptic activity frequency and calcium transient data, respectively. Data are shown as mean ± s.e.m.

Fig. 6. LRP6 immunoreactivity in the soma and neuronal processes associated with pre- and post-synaptic markers Synaptophysin (Syp) and PSD95. 

A. Confocal images were obtained from neurons of 14-15 DIV using antibodies against LRP6 (green-Alexa488), Syp (red-Cy3) and PSD95 (blue-Alexa633). Arrows show LRP6-Syp and LRP6-PSD95 colocalization. Calibration bar: 20 \( \mu \)m. 

B & C. Mander’s and Pearson’s correlation coefficients for the colocalization of the LRP6 and pre- and post-synaptic markers Syp and PSD95, respectively. An average of coefficients of at least 3 ROIs of 10 neurons was examined.
Table 1. Electrical parameters (action potentials and input resistance) recorded in hippocampal neurons in the absence or presence of Wnt3a (10nM, 15 min).

<table>
<thead>
<tr>
<th></th>
<th>Action Potential</th>
<th>IR (GΩ)</th>
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<tbody>
<tr>
<td></td>
<td>AMP (mV)</td>
<td>TH (mV)</td>
</tr>
<tr>
<td>Control</td>
<td>84 ± 1.4</td>
<td>-46 ± 0.6</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>85 ± 1.5</td>
<td>-44 ± 1.0</td>
</tr>
<tr>
<td>p-value</td>
<td>0.56</td>
<td>0.13</td>
</tr>
</tbody>
</table>

AMP: amplitude; TH: threshold; and AP/2: duration of action potentials; IR: input resistance.
Figure 3
Figure 4

A

Control
Wnt3a
Boiled Wnt3a
Wnt3a + anti-Wnt3a

B

Wnt3a

C

FM1-43 (ΔF/F)

Time (s)

Control
Boiled Wnt3a
Wnt3a + anti-Wnt3a
Wnt3a

D

Control
Wnt3a

AM1-43

p-Synapsin

E

Control
Wnt3a
Boiled Wnt3a
Wnt3a + anti-Wnt3a

p-Synapsin
β-tubulin
Figure 5

A

Control

Wnt3a

Wnt3a + sFRP1

sFRP1

Wnt3a + DKK1

DKK1

B

Control

Wnt3a

Wnt3a + sFRP1

sFRP1

Wnt3a + DKK1

DKK1

C

Miniature Current Frequency (Hz)

D

Calcium Transients (1x10^-2 Hz)

Control

Wnt3a

Wnt3a + sFRP1

sFRP1

Wnt3a + DKK1

DKK1
Canonical Wnt3a modulates intracellular calcium and enhances excitatory neurotransmission in hippocampal neurons
Miguel E. Avila, Fernando J. Sepulveda, Carlos Felipe Burgos, Gustavo Moraga-Cid, Jorge Parodi, Randall T. Moon, Luis G. Aguayo, Carlos Opazo and Giancarlo V. De Ferrari

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