

***Wnt-5a* inhibits K⁺ currents in hippocampal synapses through nitric oxide production.**

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Running head: Wnt-5a signaling inhibits potassium current.

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ABSTRACT

Hippocampal synapses play a key role in memory and learning processes by inducing long-term potentiation and depression. The growth of adult synapses is regulated by the *Wnt* signaling pathway, in addition to other pathways. *Wnt* may also regulate the activity of the postsynaptic terminal. We have previously found that *Wnt-5a* induces nitric oxide (NO) production, which modulates NMDA expression in the postsynaptic regions of hippocampal neurons. Because the activity of Kv3.2 channels can be inhibited by NO, we investigated whether *Wnt-5a* can regulate potassium currents in rat hippocampal neurons. *Wnt-5a* activates non-canonical *Wnt*/Ca²⁺ signaling; triggering NO production, which in turn inhibits a sustained voltage-dependent K⁺ current. The inhibition of the potassium current is mediated at least in part by ROR2, one of the three previously identified *Wnt-5a* receptors. Preincubation with the soluble Frizzled receptor protein (sFRP-2), which is a *Wnt* antagonist, and 7-nitroindazole (7-NI), which is a specific inhibitor of neuronal NO synthase, eliminates the inhibitory effect on the K⁺ current. We conclude that *Wnt-5a* produces NO and decreases potassium amplitude currents via NO, demonstrating a novel mechanism by which *Wnt-5a* may regulate the excitability of hippocampal neurons.

Keywords: *Wnt-5a*, ROR2 receptor, K⁺ channel, hippocampal neurons

Introduction

Wnt signaling plays an essential role in synaptic maintenance, function and connectivity in the adult central nervous system (CNS), as well as in the developing mammalian CNS (Budnik & Salinas 2011; Inestrosa & Arenas 2010). *Wnt* ligands are secreted glycoproteins that stimulate the formation of central and peripheral synapses (Budnik & Salinas 2011; Salinas 2012; Oliva et al 2013) by promoting pre-synaptic assembly (Ahmad-Annur et al 2006; Farias et al 2007; Cerpa et al 2008) and the clustering of postsynaptic components (Farias et al 2009; Cuitiño et al 2010).

There are 19 *Wnt* genes identified in the vertebrate genome, all of which signal through Frizzled (FZD) receptors, which have seven transmembrane-spanning domains. Signaling events occur as the direct consequence of FZD activation, via the crucial phosphoprotein Dishevelled (DVL). These receptors, with the co-receptor tyrosine kinase-like orphan receptor 2 (ROR2), are selectively activated by *Wnt-5a* in both mammals and invertebrates (Oishi et al., 2003; Mikels et al., 2009).

Both types of receptors activate at least three alternative signaling pathways: 1) the canonical *Wnt*/β-catenin pathway, involved in the co-activation of *Wnt* target genes with Tcf/Lef transcription factors (Nusse & Varmus 2012); 2) the *Wnt*-Planar Cell Polarity (*Wnt*/PCP) pathway, acting through monomeric GTPases and c-Jun N-terminal kinase (JNK), which regulates cytoskeleton reorganization and 3) the *Wnt*/calcium pathway, which leads to increased intracellular Ca²⁺ levels, activating protein kinase C (PKC), Ca-Calmodulin-dependent protein kinase II (CAMKII) and the phosphatase calcineurin, which are involved in controlling synaptic function and plasticity in the CNS (Kohn & Moon, 2005; Montcouquiol *et al.*, 2006; Simons & Mlodzik, 2008; Rosso & Inestrosa 2013). Among the several *Wnt* ligands described, *Wnt-5a* is of special interest because it has been

reported that *Wnt-5a* induces rapid changes in the clustering of the post-synaptic density protein (PSD-95) through a JNK-dependent signaling pathway. Therefore, the *Wnt-5a*/JNK pathway is responsible for modulating the postsynaptic region of the mammalian synapse (Farias et al 2009). Moreover, *Wnt-5a* increases both the level and retention of cell surface GABA_A receptors and the amplitude of GABA-evoked currents through a mechanism that depends upon the activation of CaMKII, strongly suggesting a role for the *Wnt-5a*/Ca²⁺ signaling pathway in GABA_A receptor trafficking (Cuitino et al 2010).

Nitric oxide (NO) is a ubiquitous, diffusible signaling molecule that plays a wide range of roles in the CNS. Neuronal NO synthase (nNOS) is widely expressed in the mammalian brain and is activated by Ca²⁺ influx, primarily through NMDA receptors; therefore, NO production is tightly related to synaptic activity (Brenman et al 1996). NO signaling also contributes to the fine tuning of ion channel activity and therefore neuron excitability (Steinert et al., 2011). The family of Kv channels, when deregulated, has been linked to CNS diseases including hereditary epilepsy (Otto et al., 2009), episodic ataxia and Alzheimer's disease (Colom et al., 1998; Shieh et al., 2000). Kv3.1 and Kv3.2, members of the Shaw subfamily of voltage-dependent K⁺ channels, have been proposed as neuromodulatory targets of the spiking pattern within interneurons in the CA1 region of the hippocampus and are expressed in these neurons (Lien et al., 2002). These channels are negatively modulated by NO and have fast deactivation rates, which make them quite distinguishable in electrophysiological recordings. Moreover, the expression of Kv3 channels in CHO cells is blocked by NO (Moreno et al., 2001). This channel is found broadly in the brain, including in the hippocampus, in association with NO and glutamatergic signaling (Tansey et al., 2002), suggesting that the nitrergic control of

neuronal excitability may be a ubiquitous mechanism by which a neuron's firing pattern is regulated.

Recently, we found that *Wnt-5a* triggers NO production, which in turn promotes the insertion of the GluN2B subunit of the NMDA receptor into the hippocampal neuronal cell surface (Muñoz et al, 2014). We report here that *Wnt-5a* signaling decreases a voltage-gated K⁺ current through an increase in NO production in hippocampal neurons. The inhibition of the potassium current is mediated at least in part by ROR2. Our results suggest a novel mechanism by which *Wnt-5a* signaling could regulate neuronal excitability in hippocampal neurons.

Materials and Methods

Reagents.

The cell culture reagents and chemicals, including 7-nitroindazole (7-NI), used in our experiments were purchased from Sigma (St. Louis, MO).

Animals and ethical standards.

The experiments were performed on Sprague-Dawley rat fetuses (E18). The experimental procedures were approved by the Bioethical and Biosafety Committee of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile and were conducted in accordance with the guidelines of the National Fund for Scientific and Technological Research (FONDECYT-Chile).

Culture of rat hippocampal neurons.

Hippocampi were dissected from the Sprague-Dawley rats at embryonic day 18, and primary rat hippocampal cultures were prepared. Hippocampal cells were seeded in 24- or 96-well polylysine-coated plates and grown in Neurobasal medium plus B27 supplement; the medium was replaced on days 3 and 7 *in vitro* (DIV). The cells were treated with 2 μ M 1- β -D-arabinofuranosylcytosine (araC) for 24 h once per week, to reduce the numbers of glial cells present in the culture. Fifteen days later (DIV 15), the cultured hippocampal neurons were used for various experiments; the average number of neurons in each cover corresponded to approximately 99% of the total cells present in the cultures (Cerpa et al 2008; Farias et al 2009).

Conditioned Medium containing Wnts Proteins.

The control medium was prepared from L cells (ATCC CRL-2648) and *Wnt-5a*-conditioned media was prepared from L *Wnt-5a* (ATTC CRL-2814) cells. To generate other secreted *Wnt* ligands, Lipofectamine 2000 was used to permanently transfect HEK-293 cells with equal amounts of empty vector pcDNA (control) or the pcDNA vector with sequences encoding *Wnt* ligand or soluble Frizzled receptor protein (HA-sFRP-2) constructs coupled to the sequence encoding a hemagglutinin tag. L *Wnt-5a* cells and transfected HEK-293 cells were grown to 85% confluence. *Wnt*-conditioned media, control media or media containing sFRP-2 were prepared in Neurobasal medium without supplements. *Wnt* secretion was verified by Western blot using an anti-HA antibody (Upstate Biotechnology, Lake Placid, NY). For the electrophysiological studies, the media

containing *Wnt* ligands were diluted or dialyzed in ACSF for 16 h at 4 °C or diluted in external solution.

Lentiviral vector production and in vitro transduction.

Inverted and self-complementary hairpin DNA oligonucleotides encoding short hairpin RNAs targeting the *Rattus norvegicus* receptor tyrosine kinase-like orphan receptor 2 (ROR2) mRNA were chemically synthesized (Life Technologies, Carlsbad, CA), aligned and ligated into a lentiviral vector (pLL3.7) containing a CMV-driven EGFP reporter and a U6 promoter upstream of cloning restriction sites (HpaI and XhoI) to allow the introduction of oligonucleotides. We constructed a control shRNA against luciferase (shLuc) and three different shROR2 constructs targeting the rat ROR2 mRNA. Each DNA oligo consists of a TG nucleotide (nt) pair, a 19-nt sense sequence, a short spacer (TTCAAGAGA), the antisense sequence, 6 Ts (a stop signal for RNA polymerase III), and an XhoI site (Brummelkamp et al., 2002; Rubinson et al., 2003). The oligos were annealed and inserted between the HpaI and XhoI sites of the plasmid. Primer pairs for constructing plasmid shRNAs were as follows: for control shLuc, shLuc forward: TGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCCGGAGAACTTTT TTC, shLuc reverse: TCGAGAAAAAAGTTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTTCG GAGAACA; for shROR2, Forward shRNA-ROR2-#1, 5'TGGACCAGGAAGATGGCTTCTGCCAACCTTATTCAAGAGATAAGGTTGGCAG AAGCCATCTTCCTGGTCCCTTTTTTC3', reverse shRNA-ROR2-#1: 5'TCGAGAAAAAAGGACCAGGAAGATGGCTTCTGCCAACCTTATCTCTTGAATA AGGTTGGCAGAAGCCATCTTCCTGGTCCA3'; for shRNA ROR2-#2, forward shRNA

ROR2-#2: 5'TGCTATCACCAGTG
TTACAACGGCTCATTCAAGAGATGAGCCGTTGTAACACTGGTGATAGCGACCTT
TTTTTC3', reverse shRNA-ROR2-#2:

5'TCGAGAAAAAAGGTCGCTATCACCAGTGTTACAACGGCTCATCTCTTGAATG
AGCCGTTGTAACACTGGTGATAGCGACCA3'; and for shRNA ROR2-#3, forward

shRNA ROR2-#3: 5'TGTCTCCATTGACTCCGA
CATCTGGTCCTATTTCAAGAGAATAGGACCAGATGTCGGAGTCAATGGAGACT
TTTTTC3' and reverse shRNA-ROR2-#3: 5'TCGAGAAAAAAGTCTCCATTGACTCC
GACATCTGGTCCTATTCTCTTGAAATAGGACCAGATGTCGGAGTCAATGGAGA

C3'. The correct insertions of the shRNA cassettes were confirmed by restriction mapping and direct DNA sequencing. The constructed shRNA expression vectors were named pLL3.7-Luc and pLL3.7-ROR2. The production of lentivirus pLL3.7-ROR2 or PLL3.7-Luc was performed in HEK293T cells, using the calcium phosphate method. Briefly, the lentivirus and the packaging vectors were co-transfected and the resulting supernatant was harvested after 60 h. The recovered viral particles were centrifuged to eliminate cell debris, filtered through 0.45-mm cellulose acetate filters, concentrated by ultracentrifugation (1.25 h at 28,000 r.p.m. in a Beckman SW41 rotor) and resuspended in 100 μ l phosphate-buffered saline (PBS). The viral particles were quantified by infecting the HEK293FT cells with serial dilutions of concentrated lentivirus. We determined the GFP expression of the infected cells by flow cytometry 72 h after transduction; for a typical preparation, the titer was approximately $4-10 \times 10^7$ transduction units (TU) per ml. The hippocampal neurons were transfected on DIV10 with a multiplicity of infection (MOI) of 10 for immunofluorescence and morphological analysis and with MOI 50 for analyzing the ROR2

protein knockdown. After 4 days, the EGFP expression was verified with an OLYMPUS IX81 inverted fluorescence microscope. Western blots of protein extracts were performed 4 days after the cells were infected, as indicated in Farías et al., 2009 (Farias et al., 2009). Primary antibodies were prepared in blocking solution at the following dilutions: mROR2 (1:1000), a gift from Dr. Roel Nusse (Department of Developmental Biology and Howard Hughes Medical Institute, Stanford University School of Medicine, Palo Alto, CA); anti-GFP (Abcam Cat# ab6556 RRID: AB_305564, 1:1000); and β -actin (Santa Cruz Biotechnology, Inc. Cat# sc-69879 RRID:AB_1119529, 1:10000) (Santa Cruz Biotechnology, Dallas, TX) and incubated overnight at 4 °C. Secondary antibodies coupled to HRP (Dako) diluted 1:10,000 were incubated for 1 h at room temperature. The immunoreactivity was revealed with a chemiluminescence ECL kit (Thermo Fisher Scientific, Rockford, IL).

Electrical recordings of potassium currents.

The seeded isolated hippocampal neurons (15 DIV) were placed in a recording chamber (total volume approx. 400 μ l) and positioned on the stage of an inverted microscope under constant superfusion (3-5 ml/min) with an external solution containing (in mM): 150 NaCl, 5.4 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 10 glucose, 0.01 Nifedipine (L-type Ca²⁺ channel blocker) and 10 HEPES (pH 7.4) at 32-35 °C and continuously bubbled with air. The bath was grounded with an Ag-AgCl pellet. Voltage-clamp recordings were performed with an Axopatch 200B amplifier (Axon Instruments, CA). The recorded signal was filtered at 2 kHz and acquired at 20 kHz using a Cambridge Electronic Design power

1401 A/D converter and pClamp 9.2 acquisition software (Axon Instruments, CA). Voltage commands were generated using pClamp 9.2 software. Borosilicate glass pipettes (GC150F-10, Warner Instruments Corp) were pulled and polished in a Flaming/Brown P-87 Micropipette Puller (Sutter Instrument Co). Whole-cell current recordings were performed using electrodes filled with an internal solution containing (in mM): 5 NaCl, 135 K-gluconate 1 CaCl₂, 11 EGTA, 10 HEPES, 2 ATP; pH 7.2. Electrode resistance ranged from 4-7 MΩ. Seal formation and membrane breakthrough were monitored by observing the response to a 5 mV voltage pulse lasting 50 msec under voltage-clamp mode. Liquid junction potential between the microelectrodes and bath solution was corrected. Series resistance (usually 5-15 MΩ) and capacitive transients were electronically compensated and periodically monitored throughout the recordings to ensure proper compensation. Based on our previous results, the pipette potential was held at -60 mV. Cellular integrity was tested regularly by monitoring the access resistance (using the seal test pulse described above) and by evaluating the presence of voltage-gated currents (obtained by imposing membrane potentials ranging from -100 to +40 mV, in steps of 10 mV in magnitude and 100 ms in duration). The membrane potential recordings were performed in the current-clamp configuration with no imposed current (I=0, “voltage follower”) and with KCl replacing K-gluconate in the pipette solution. *Wnt-5a* was freshly prepared in HEPES-buffered external solution in which NaCl was replaced by N-methyl-d-glucosamine (NMDG) chloride plus 10 μM Nifedipine (L-type Ca²⁺ channel blocker) and diluted to the desired concentration (see Results). Drugs were applied from different reservoirs maintained at 35 °C by a rapid switch system at 3-5 ml/min. Control experiments showed no effect of the rapid exchange of the control bathing solutions upon the voltage-gated currents. The currents were standardized to the membrane capacity of the cell (expressed as

density current (pA/pF) and measured at the end of the voltage step. The I-V curves were compared using two-way ANOVA followed by the Bonferroni post-hoc test.

Slice Preparation and Electrophysiology.

Hippocampal slices from 2-month-old C57BL6 male mice were prepared according to standard procedures described previously (Cerpa et al 2010; Varela-Nallar et al 2010). Briefly, transverse slices (350 μm) from the dorsal hippocampus were cut under cold artificial cerebrospinal fluid (ACSF, in mM: 124 NaCl, 2.6 NaHCO₃, 10 D-glucose, 2.69 KCl, 1.25 KH₂PO₄, 2.5 CaCl₂, 1.3 MgSO₄, and 2.60 NaHPO₄) using a Vibratome (Leica VT 1000s, Germany) and incubated in ACSF for 1 h at room temperature. In all experiments, 10 μM picrotoxin (PTX) was added to suppress inhibitory GABA_A transmission. The slices were transferred to an experimental chamber (2 ml), superfused (3 ml/min, at 20-22 °C) with gassed ACSF and visualized by trans-illumination with a binocular microscope (MSZ-10, Nikon, Melville, NY). To evoke field excitatory postsynaptic potentials (fEPSPs), we stimulated with bipolar concentric electrodes (Platinum/Iridium, 125 μm OD diameter, FHC Inc., Bowdoin, ME) with a stimulator (Axon 700b, Molecular Devices, Sunnyvale, CA) connected to an isolation unit (Isoflex, AMPI, Jerusalem, Israel). The stimulation site was in the *stratum radiatum* within 100-200 μm from the recording site. The recordings were filtered at 2.0-3.0 kHz, sampled at 4.0 kHz using an A/D converter, and stored with pClamp 10 (Molecular Devices). The evoked postsynaptic responses were analyzed off-line, using analysis software (pClampfit, Molecular Devices) that allowed visual detection of events, computing only those events that exceeded an arbitrary threshold.

Statistical Analysis.

Currents were standardized to the membrane capacity of the cell (expressed as density current (pA/pF)). Data are presented as the mean \pm SEM, and “n” indicates the number of neurons obtained from different animals. The I-V curves were compared using two-way ANOVA followed by the Bonferroni post-hoc test. The significance level was set at $P=0.05$. All curve fitting and statistical calculations were performed with GraphPad Prism 5.0 (GraphPad Software).

Results

Wnt-5a activity inhibits voltage-gated potassium current in hippocampal neurons.

A widespread mechanism for regulating neuronal excitability is the modulation of voltage-gated K^+ currents. Before beginning the electrophysiological experiments, we evaluated NO production in response to the *Wnt-5a* treatment (Fig. 1A). After 3 min of *Wnt-5a* treatment, the NO production increased by 50% (Fig. 1A, black circles). The NO production induced by *Wnt-5a* was prevented by the specific nNOS inhibitor 7-NI (Fig. 1A, white circles) and a general NOS inhibitor L-NAME (Fig. 1A, light gray circles) (Jones et al 1998), as well as by the protein G inhibitor Suramin (Fig. 1A, gray circles) (Freissmuth et al 1999). Simultaneously, we performed conventional whole-cell recordings of isolated voltage-gated K^+ currents (see Methods) during *Wnt-5a* exposure. After 3 min of the bath application of *Wnt-5a*, we observed a significant and reversible reduction in the voltage-gated K^+ current amplitude (Fig. 1B), with no significant difference in the average membrane input resistance (Supplemental Table 1, last column). Moreover, the effect of

Wnt-5a was voltage-dependent because it is significantly different from the control values at test pulses of +10 mV ($p < 0.05$), and the degree of current inhibition was increased at more depolarized values ($p < 0.01$). A maximum inhibition of approximately 50% of the K^+ current (when compared to the baseline conditions) was achieved at +40 mV (Fig. 1B). No further inhibition of the K^+ current was achieved by increasing the *Wnt-5a* concentration. Indeed, the current-voltage relationship of this *Wnt-5a*-sensitive current (given as the difference of baseline - *Wnt-5a*) shows voltage-dependence, with an apparent value of voltage activation between -20 mV and -10 mV (Fig. 1B), suggesting that this *Wnt-5a*-sensitive current has a strong component consisting of a delayed rectifier K^+ current (Fig. 1C arrow). This effect of *Wnt-5a* was reversible, as indicated by the restoration of current responses after 3 min of washing out the ligand (Fig. 1, wash-out). Because previous studies had described a Kv3.2 channel as a delayed rectifier K^+ channel present in the hippocampus (Tansey et al 2002) that can be blocked by NO (Lien et al 2002), we assessed whether the voltage-gated K^+ current expressed in the hippocampal neurons was sensitive to sub-millimolar concentrations of external tetraethyl ammonium (TEA), a pharmacological hallmark of Kv3.2 channels (Lien et al 2002). Accordingly, the voltage-gated K^+ current was inhibited by 0.1 mM TEA (Supplemental Fig. 1, white circle) to a similar extent as that previously achieved by *Wnt-5a*. Consistent with its role as a generic K^+ channel blocker at millimolar levels, 10 mM TEA fully blocks the voltage-gated outward currents in our preparation (Supplemental Fig. 1, grey circle), confirming that we are indeed recording voltage-gated K^+ currents. Together, these data support the idea that the inhibitory effects of *Wnt-5a* may occur via the targeting of a delayed rectifier K^+ channel. Further confirmation of the specific effect of *Wnt-5a* upon the voltage-gated K^+ currents is the fact that *Wnt-5a* superfusion has no effect on either the resting membrane

potential (control: -56.2 ± 2.3 mV; *Wnt-5a*: -57.0 ± 1.8 mV; $p > 0.05$, $n=6$) or the input resistance (control: 201.8 ± 25.5 M Ω ; *Wnt-5a* 220.6 ± 42 M Ω ; $p > 0.05$, $n=6$).

The effect of Wnt-5a on voltage-gated K⁺ currents is specific and depends on nNOS activity.

The effect elicited by *Wnt-5a* signaling on the voltage-gated K⁺ currents (Fig. 2, white circle shows inhibition of the K⁺ current), when compared with the control (black circle), was abolished by co-treatment with soluble 25 μ M Frizzled-related protein 2 (sFRP-2) (Fig. 2, grey circle), a *Wnt* antagonist (Rattner et al 1997), which binds *Wnt* ligands and prevents their interaction with cellular receptors. Next, we evaluated the possible contribution of the increased NO production evoked by *Wnt-5a* on the inhibitory effect upon K⁺ currents by using the nNOS inhibitor 7-NI (Ledo et al 2005; Codocedo et al 2013). The inhibitory effect of *Wnt-5a* on the voltage-gated K⁺ currents (Fig. 2, white circle) was not observed in the presence of 1 μ M 7-NI (Fig. 2, light grey circle), in which case, the K⁺ currents were very similar to the control (Fig. 2, black circle). Further, pre-incubation with 7-NI (0.5-10 μ M) for at least 3 min had no effect on the voltage-gated currents, but it reduced the inhibition of the K⁺ current evoked by *Wnt-5a* in a dose-dependent manner (Table 1). In fact, 1 μ M 7-NI completely prevents the inhibition of the K⁺ current by *Wnt-5a*. These results suggest that *Wnt-5a* specifically enhances NO production (Muñoz et al, 2014) and is responsible for the diminished amplitude of this K⁺ current in cultured hippocampal neurons. However, following the addition of increasing 7-NI concentrations, the NO production was eventually inhibited (Table 1), restoring the K⁺

channel activity to the control levels. This result suggests that a significant component of the activity of this ligand is mediated by delayed rectifier K⁺ currents.

ROR2 is involved in the inhibitory effect of Wnt-5a on the voltage-gated potassium current in hippocampal neurons.

To assess the potential involvement of a specific *Wnt-5a* receptor on the inhibition of voltage-gated potassium currents, we performed whole cell recordings on hippocampal neurons transduced with the lentivirus encoding the shRNA targeting ROR2. We designed and expressed lentivirus-based shRNA constructs specifically directed against rat ROR2 (see Methods), additionally encoding EGFP for the identification of neurons expressing the shRNA. An shRNA construct targeting Renilla luciferase (shLuc) was used as a control. One of the ROR2 shRNA sequences (shROR2 #1) caused the most efficient depletion of the ROR2 protein after the shRNA transduction, as assessed by Western blot (Fig. 3A). The shRNA#1 construct was used in subsequent experiments, and we will refer to it as shROR2. Under conventional whole-cell voltage-clamp recordings on the cultured hippocampal neurons (DIV12; see Methods), neither shROR2 nor the shLuc-infected control neurons showed a difference from the control neurons, whether in terms of membrane potential, input resistance or control I-V curves for the K⁺ currents. The control neurons infected with shLuc showed no changes in the *Wnt-5a* effects in any of the parameters studied when compared to the non-transfected control neurons (shLuc, +40 mV; control = 33.27 ± 1.83 , *Wnt-5a* = 16.25 ± 3.8 , pA/pF, n=3, p< 0. 001; ANOVA, Bonferroni post-test) (Fig. 3B). However, the inhibitory effect of *Wnt-5a* on the K⁺ current was suppressed in the neurons infected with shROR2 (pA/pF, shROR2 +40 mV: control = 41.97

± 3.31 , $Wnt-5a = 26.39 \pm 7.62$, non-significant) (Fig. 3B), indicating that ROR2 is involved in this process and is necessary for the *Wnt-5a*-mediated inhibition of the sustained K⁺ current.

Effect of Wnt-5a upon hippocampal synaptic potentials is suppressed in the presence of the NO synthase inhibitor 7-NI.

Previous evidence shows that *Wnt-5a* augments glutamatergic synaptic transmission (Cerpa et al, 2010; 2011; Varela-Nallar et al, 2010). To examine the NO-mediated effect of *Wnt-5a*, through NO production on synaptic transmission, the Schaffer collaterals (SCs) in the hippocampal slices were stimulated, and we recorded the field excitatory postsynaptic potentials (fEPSPs). *Wnt-5a* treatment induced a fast increment, “early”, in the fEPSP slope (Fig. 4A: black circle); this effect was prevented by the co-incubation of the *Wnt* ligand with the NOS inhibitor (7-NI) (Fig. 4A, upper graph: grey circle). The specificity of the *Wnt-5a* effect was analyzed using sFRP-2. Co-treatment with the ligand and sFRP-2 blocked the effect of *Wnt-5a* on the fEPSP slope to the same extent as the 7-NI blocking effect and to approximately the level of the control; both inhibitors significantly blocked the *Wnt* activity (Fig. 4A: lower graph, * $p < 0.05$). This early effect occurs after the NO production induced by *Wnt-5a* (see Fig. 1A), as well as after the decrease in the K⁺ currents (Fig. 1B, 1C). 7-NI must have blocked the *Wnt-5a* effect during the “early” stage of treatment with the ligand because when *Wnt-5a* induced the first increment in fEPSP, subsequent 7-NI treatment was unable to prevent or revert the effect of *Wnt-5a* (Fig. 4B, upper, grey circle); similar results were also observed during the “late” stage of the treatment (Fig. 4B, lower, light grey bar). The activity of the delayed rectifying K⁺

channels is an important determinant of the activation threshold in dendrites and is thought to have a major impact on neuronal plasticity (Sjostrom et al., 2008). Kv channels have been shown to participate in the regulation of the neuronal excitability of CA3 hippocampal neurons in response to synaptic activity through a NO-dependent mechanism (Steinert et al., 2011). Previously, we have reported that *Wnt-5a* induces NO production in hippocampal neurons to regulate the surface expression of NMDARs (Muñoz et al., 2014); our results suggest a potential common mechanism underlying *Wnt-5a*-dependent changes in post-synaptic excitability and plasticity. In fact, all of the above results show that *Wnt-5a* signaling is at least partly transduced through ROR2, causing the activation of nNOS and NO production, which regulates synaptic function and neuronal activity in rat hippocampal neurons.

Discussion

In the present study, we show for the first time that the ligand *Wnt-5a* causes a marked inhibitory effect on voltage-gated K⁺ currents in cultured rat embryonic hippocampal neurons. This effect appears to depend upon nNOS activation and increased NO levels because it is completely prevented by the nNOS inhibitor 7-NI. In fact, the *Wnt* antagonist sFRP-2 inhibits the effects of *Wnt-5a*, suggesting that this ligand triggers an increase in NO production (Muñoz et al, 2014) that is required for the inhibition of voltage-gated K⁺ currents.

Current evidence for the regulation of plasma membrane ion channels by *Wnt* signaling is sparse; for example, non-canonical *Wnt* ligands have been shown to regulate voltage-dependent calcium channels (Panakova et al., 2010) and TRPC channels, the latter involving the activation of Frizzled receptors and receptor tyrosine kinases (Li et al., 2009).

In the present study, we have shown that *Wnt-5a* has an inhibitory effect on voltage-gated K^+ currents in cultured rat hippocampal neurons and that this effect depends on ROR2 expression. ROR2 has been demonstrated to mediate different signals in response to *Wnt-5a* (Nishita et al., 2010). The signaling mediators reported as downstream of ROR2 include the intracellular adaptor Dishevelled, the monomeric G protein Rac1, the tyrosine kinase Src and the actin-binding scaffold filamin A (Minami et al., 2010; Nishita et al., 2010). ROR2 has been detected in the postsynaptic region, showing co-localization with markers such as postsynaptic density-95 (PSD-95) in hippocampal neurons (Alfaro et al, in preparation). Additional experiments are needed to elucidate the specific pathways downstream of ROR2 that regulate voltage-gated K^+ currents in hippocampal neurons.

The major determinants of the active electrical properties of neurons include voltage-gated channels selective for calcium, sodium and potassium (Hille, 2001). Homologous genes forming a large family encode the diversity of voltage-gated channels. The first voltage-dependent ion channel cloned for sequencing of the primary structure was a sodium channel subunit (Noda et al 1984). Later, various rat Nav subunits were described: Nav1.1, Nav 1.2 and Nav 1.3 (Kayano et al 1988; Noda et al 1984; 1986). Differences in expression, distribution and intrinsic properties define their contributions to membrane excitability. For calcium voltage-dependent channels, the diversity is broader. This classification includes the Cav1 (L-type), Cav2 (P/Q, N and R-type), and Cav3 (T-type) families (Ertel et al 2000); the expression, distribution and roles of the various subtypes of channels define their participation in controlling membrane excitability. For potassium channels, approximately 40 subunit genes grouped into 12 families produce a huge diversity (Coetzee et al 1999), but the pore domain is highly conserved with a high

selectivity for K^+ ion permeation (Doyle et al 1998). The families of Kv receptors differ primarily in their activation conditions. Kv1, Kv4 and Kv7 channels are low-voltage-activated, opening close to the resting potential with small depolarization and regulating the number of action potentials (Coetzee et al 1999; Brew et al 2003). Kv2 channels are high-voltage-activated and are associated with accessory proteins, including the Kv5, Kv6, Kv8 and Kv9 families. Kv3 channels are also high-voltage-activated, requiring voltages achieved only during action potentials, with fast kinetics, and they primarily contribute to the action potential duration and fast firing. Modulation of all these players induces changes in membrane excitability with putative roles controlling connectivity in the brain. Because several reports indicate roles for NO as a modulator of neuronal excitability via the regulation of several ionic conductances (Steinert et al 2008; Bornstein et al 2010; Artinian et al 2012), including potassium conductance, we concentrated our attention on Kv subtype 3.

Bioelectrical activities of the somatosensory cortex and the CA1 hippocampal region are increased under L-NAME and 7-NI treatment, and the putative proconvulsant role of NOS inhibitors is highlighted, with NMDA receptors playing an important role in controlling this effect (Ferraro et al 1999). Electrophysiological recordings in submucosal neurons confirm that the suppression of NO synthesis is associated with increases in EPSPs and decreases in IPSPs (Bornstein et al 2010). Steinert and co-workers showed evidence for the localization of Kv3.2 channels in hippocampal neurons and its importance in the maintenance of the cells' excitability. These authors suggest that NO donors can block/inhibit the Kv3.2 channel and thus generate an overall excitatory effect in the synaptic activity (Steinert et al 2011).

Recently, compelling evidence has shown that *Wnt* signaling plays a key role in several developmental processes during neural differentiation, as well as in synaptic maintenance in the adult nervous system (Inestrosa & Arenas 2010; van Amerongen & Nusse 2009; Budnik & Salinas 2011). *Wnt* signaling also regulates the adult neurogenesis of hippocampal stem cells in rat and human (Lie et al 2005; Varela-Nallar & Inestrosa 2013), and it has been implicated in synaptic plasticity, modulating long-term potentiation (LTP) in mouse hippocampal slices (Wayman et al 2006; Cerpa et al 2011; Chen et al 2006; Oliva et al 2013). The effect of *Wnt* on synaptogenesis and synaptoplasticity involves the modulation of NMDA receptors and calcium permeability (Westenbroek et al 1989; Varela-Nallar et al 2010; Cerpa et al 2011), both of which are associated with membrane excitability.

The extent of the inhibition of potassium currents by a *Wnt-5a* ligand or TEA appears to be similar (Fig. 1 vs. Supplemental Fig. 1). Previous reports (Chen et al 2006; Wayman et al 2006; Cerpa et al 2011; Oliva et al 2013) showed that a *Wnt* ligand induces an increment in synaptic transmission measured via the EPSC amplitude and fEPSP slope in the CA3-CA1 circuit in rodent hippocampus (Cerpa et al 2011; Wayman et al 2006; Chen et al 2006; Oliva et al 2013). Moreover, the effect of TEA on synaptic transmission and LTP was well described several years ago (Huang & Malenka 1993; Hanse & Gustafsson 1994; Song et al 2001). TEA elicits a prolonged synaptic potentiation in the CA1 region of the hippocampus (Huang & Malenka, 1993). NMDA receptor-dependent LTP partially occluded the TEA-induced potentiation; this occlusion was abolished by APV, indicating that one component of the TEA-induced potentiation is NMDA receptor-dependent. However, the partial occlusion is indicative of a second component that is NMDA-receptor-independent and associated with other calcium channels (Hanse &

Gustafsson 1994). Additional experiments showed the anatomical specificity of the TEA effect. The TEA-induced LTP is both NMDA-dependent and NMDA-independent in CA1; however, TEA is unable to induce LTP in the dentate gyrus (DG), where it instead induces NMDA-dependent LTP (Song et al 2001). The reason for these differences between the CA1 and DG appears to be the low expression of type-T VDCCs in the DG (Song et al 2002). The effect of *Wnt* on the DG was studied in the context of neurogenesis, where *Wnt* participates in controlling neuroprogenitor dynamics (Varela-Nallar & Inestrosa 2013). *Wnt-5a* and TEA share at least part of the mechanism of inducing changes in synaptic efficacy and LTP, where the NO component is involved in inducing changes in the hippocampal plasticity, but other mechanisms appear to be involved for each particular effect. It is important to highlight the contribution of nNOS in the induction, or in the early stage, of the effect of *Wnt-5a* on synaptic transmission (Fig. 4A) because the induction is inhibited by an nNOS inhibitor. After the *Wnt-5a* treatment, 7-NI was unable to prevent, or revert, the effect of *Wnt-5a* on synaptic transmission (Fig. 4B); this late effect of *Wnt-5a* does not appear to be modulated by the NO levels and is most likely under the control of another *Wnt* target that participates in synaptic transmission modulation, such as JNK or PKC (Cerpa et al 2011). NO production targets the insertion of the NMDAR channel controlled by *Wnt-5a* (Muñoz et al 2014) within minutes, coincident with the triggering effect of NO controlling synaptic transmission.

Modulation of voltage-gated potassium channels by NO forms an additional control point in the modulation of neuronal excitability by *Wnt* signaling, opening new possibilities in the study of the physiological control of higher brain functions related to neuronal circuit modulation.

Conflict of Interests

The authors declare that they have no conflicts of interest.

Acknowledgments

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Figure Legends

Figure 1. *Wnt-5a* produces NO, and its activity results in the inhibition of sustained K⁺ currents. (A) The ligand *Wnt-5a* induces NO production over time, reaching a plateau after 4 min of stimulation (black circle). When *Wnt-5a* was co-applied with L-NAME (light grey circle), 7-NI (white circle) or Suramin (grey circle), the NO production induced by *Wnt-5a* was blocked. (B) The amplitude of the current density (pA/pF) was diminished for the sustained component after 3 min of *Wnt-5a* superfusion (white circles) and control (black circles) amplitudes were quickly restored after 3 min of washout in the same cell. (C) Representative traces of isolated K⁺ currents (evoked by voltage pulses from -100 to +40 mV; see Methods) recorded from the same cell during baseline (control conditions) (left curves); after 3 min of bath superfusion with *Wnt-5a* (middle); and after 3 min of washing out the ligand (right curves). The difference between the peak outward current and the outward current at the end of the pulse (arrow) was used as a measure of the transient K⁺ current. The results are presented as the mean ± sem. (*p < 0.05, ANOVA, Bonferroni post-test).

Figure 2: *Wnt-5a* treatment decreases the amplitudes of the Potassium current, dependent on nNOS activity. The effects of *Wnt-5a* (white circles) upon K⁺ currents were suppressed when co-applied with 25 μM sFRP-2 (grey circles) or with the nNOS inhibitor 7-NI (light grey circles) to amplitudes similar to the control currents (black circles). The results are presented as the mean ± sem. (n:3; *p < 0.05; **p < 0.01, ***p < 0.001; ANOVA, Bonferroni post-test).

Figure 3: ROR is involved in *Wnt-5a* activity in hippocampal neurons and suppresses the inhibition of the voltage-gated potassium current. (A) Western blot showing the endogenous ROR2 in DIV14 cultured hippocampal neurons and neurons transduced at DIV10 with lentivirus encoding EGFP and ROR2 shRNAs. Three different shRNA sequences targeting ROR2 were tested (1-3), as indicated in the Methods section. Lentiviral shRNA against Renilla Luciferase (shLuc) was used as a control. (B) I-V curves of neurons transduced with shLuc and (C) ROR2 shRNA, control neurons (closed circles) and *Wnt-5a* superfusion for 3 min (open circles). Data represent the mean \pm SE (N=4, *p< 0.05, **p< 0.01, ANOVA, Bonferroni post-test).

Figure 4: *Wnt-5a* requires nNOS activity to induce an increase in fEPSPs in hippocampal slices. (A) **Upper**, Hippocampal slices were exposed to *Wnt-5a* for 10 minutes (black circle) or co-incubated with 7-NI (grey circle), keeping 7-NI in the bath during the rest of the experiment. The plots show the slope of the 1st fEPSP response during the 10 minutes of recording until 60 minutes of recording. **Lower**, Inset shows the representative traces of the different conditions tested. Quantification of the fEPSP slope response during the first 10 min of incubation with *Wnt-5a* (black bar) (labelled as Early response). The cells were co-incubated with *Wnt-5a* plus sFRP-2 (grey bar) or the ligand plus 7-NI (light grey bar). (B) **Upper**, Hippocampal slices were exposed to *Wnt-5a* for 10 minutes (black circle) and then incubated with 7-NI (grey circle) after *Wnt-5a* was removed from the bath. **Lower**, Inset shows representative traces for the different conditions tested.

Quantification of the fEPSP slope response during the last 10 min of incubation with *Wnt-5a* (black bar) (labelled as Late response). The cells were co-incubated with *Wnt-5a* plus sFRP-2 (grey bar) or treated with 7-NI (light grey bar). The bars represent the means \pm SE from 6 different slices. (* $p < 0.05$; ANOVA, Bonferroni post-test).

Supplemental Figure 1. TEA reversibly inhibits voltage-gated K^+ currents in isolated hippocampal neurons. (A) Effect of TEA at 0.1 mM (white circles) or 10 mM (gray circles) on the sustained K^+ current density, compared with the control (black circles). (B) Potassium current at different concentrations was assayed in cultured hippocampal neurons. The results are presented as the mean \pm SEM. (* $p < 0.05$; ANOVA, Bonferroni post-test).

ABBREVIATIONS.

Wnt-5a, Wingless-type family member 5a; NMDA, *N*-methyl D-aspartic acid; NO, Nitric Oxide; 7-NI, 7-nitroindazole; ROR2, receptor tyrosine kinase-like orphan receptor 2; sFRP-2, soluble Frizzled Related Protein 2; DVL, Dishevelled; *Wnt/PCP*, Wnt/planar cell polarity; JNK, c-Jun N-Terminal kinase; CamKII, Ca^{2+} -calmodulin kinase II; CNS, central nervous system; PSD-95, Post-Synaptic Density 95; GABA, γ -Aminobutyric acid; nNOS, neuronal Nitric Oxide Synthase; araC, 1- β -D-arabinofuranosylcytosine; DIV, days in vitro; CMV, Cytomegalovirus; EGFP, enhanced green fluorescent protein; TU, transduction units; MOI, multiplicity of infection; sem, standard error of the mean; PTX, picrotoxin; L-NAME, L-NG-Nitroarginine Methyl Ester; TEA, tetraethylammonium; fEPSP, field excitatory postsynaptic potentials; TRPC, transient receptor potential cation channel; IPSP,

inhibitory postsynaptic potentials, LTP, long term potentiation; VDCC, Voltage-dependent calcium channels; DG, dentate gyrus; PKC, Protein Kinase C.

Fig 1

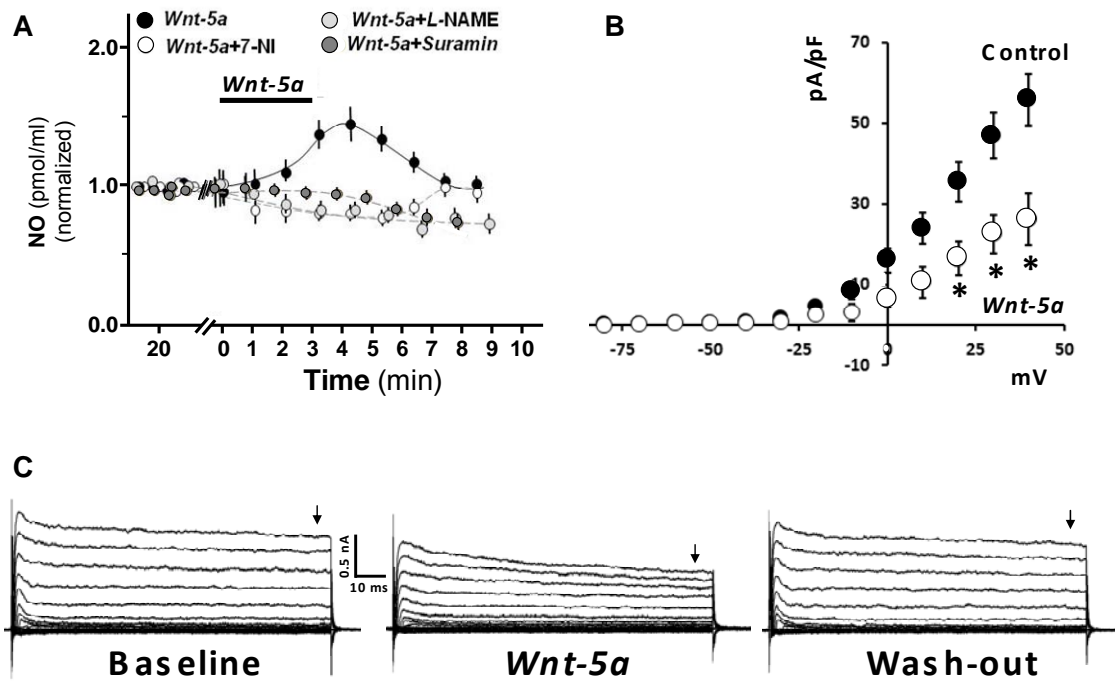


Fig 2

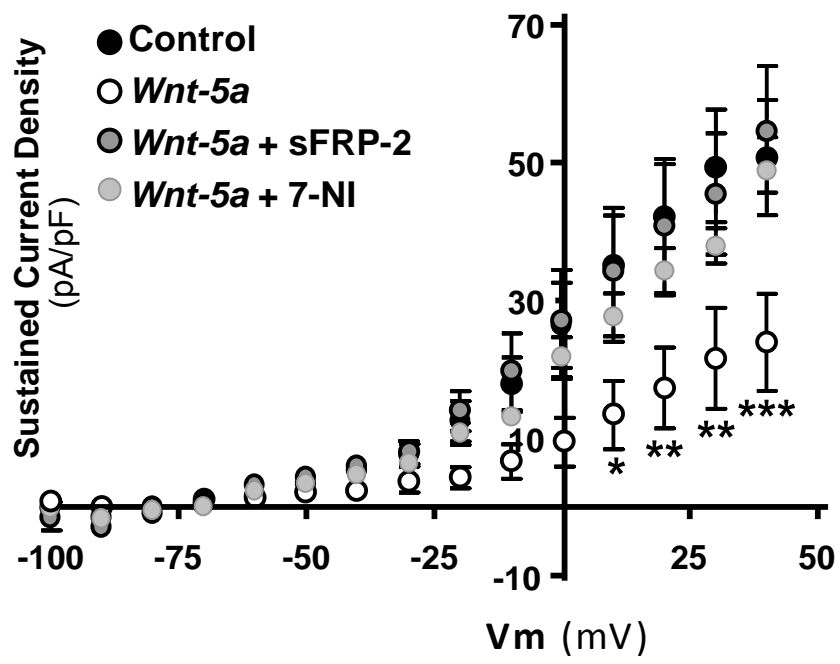


Fig 3

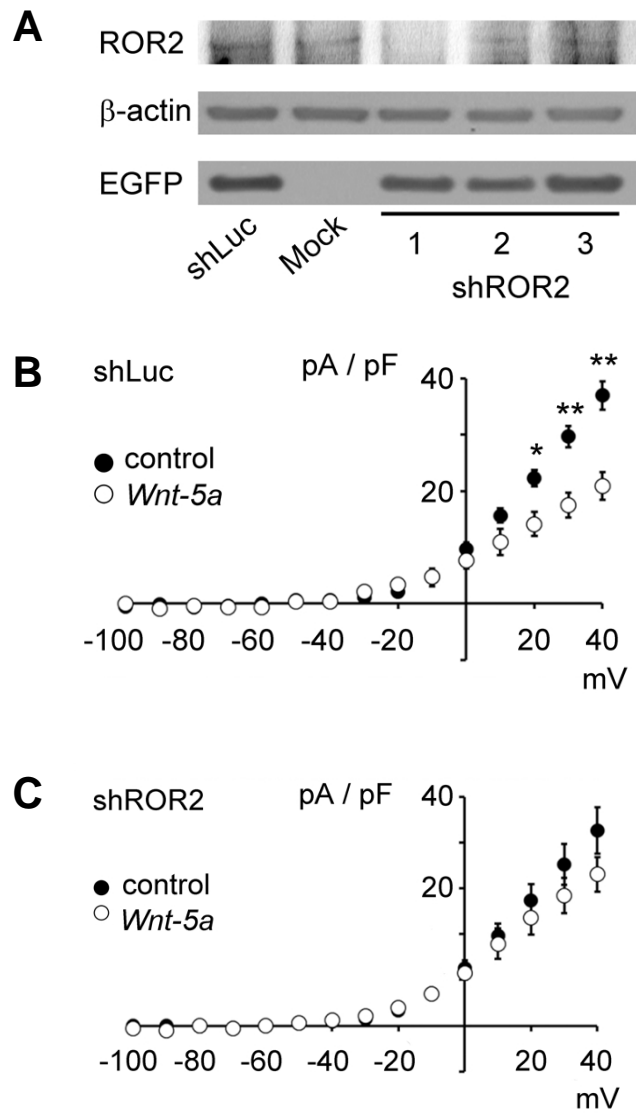


Fig 4

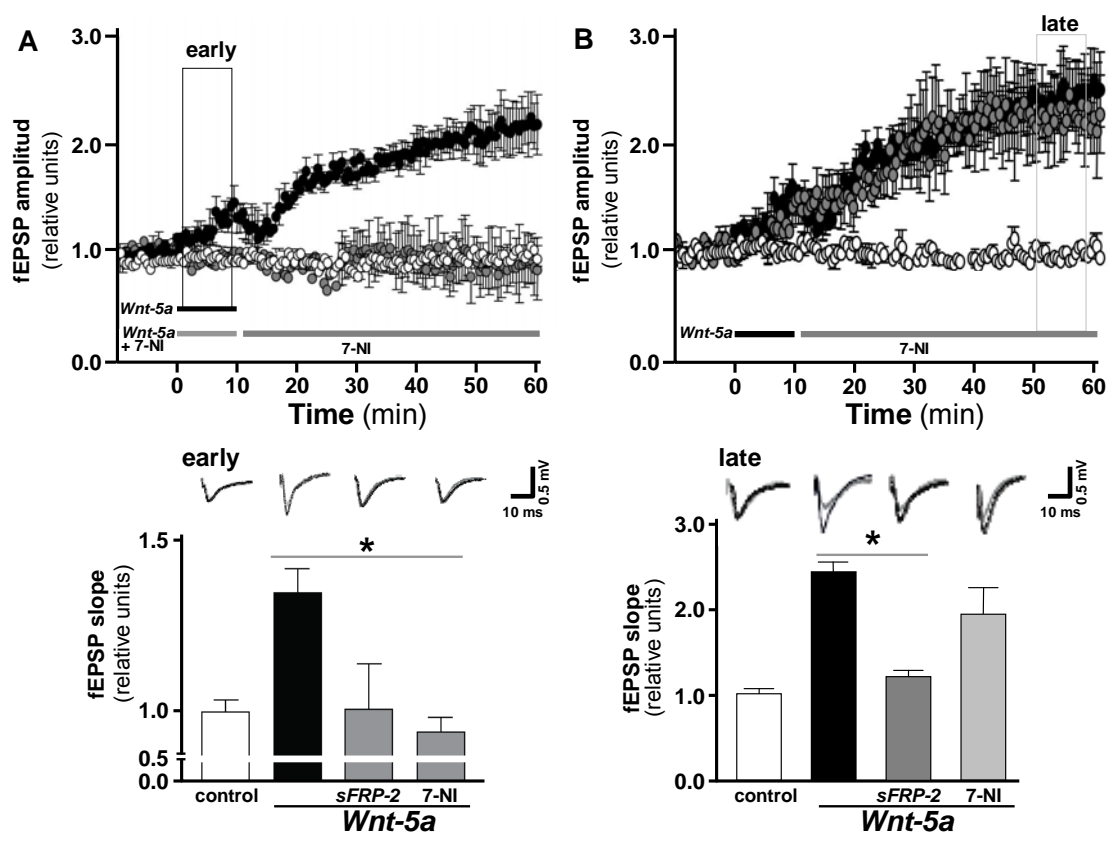


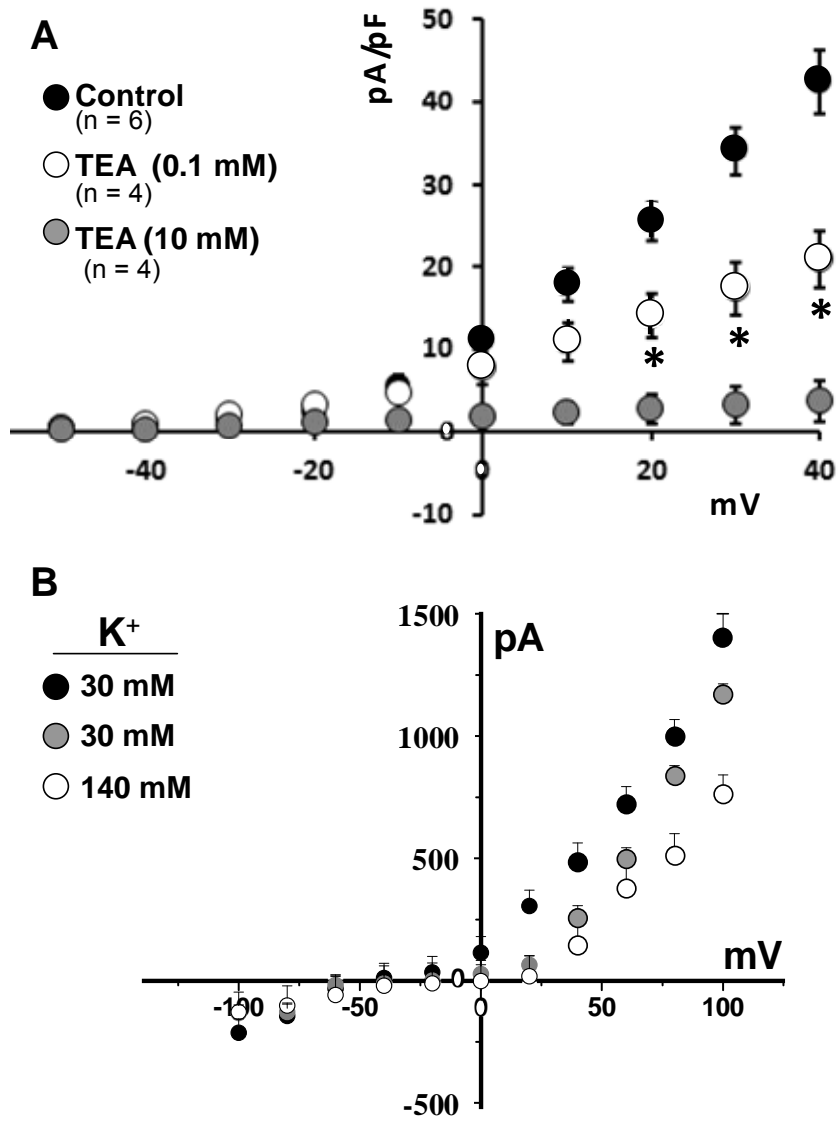
Table 1.

Inhibitions of nNOS with a specific inhibitors (7-NI) reverts the *Wnt-5a* effect over maximum current.

<i>Wnt-5a</i> (2 pM)	7-NI (μ M)	Maximum Current (nA)
-	-	1.31 \pm 0.045 *
+	0.0	0.56 \pm 0.046
+	0.1	0.59 \pm 0.071
+	0.5	0.93 \pm 0.050 *
+	1.0	1.59 \pm 0.036 *
+	10.0	1.81 \pm 0.068 *

Represents * $p < 0.05$ when comparing with control *Wnt-5a* and 0.0, 7-NI concentration.

Fig suppl 1



References

- Ahmad-Annur, A., Ciani, L., Simeonidis, I., Herreros, J., Fredj, N.B., Rosso, S.B., Hall, A., Brickley, S., Salinas, P.C., 2006. Signaling across the synapse: a role for Wnt and Dishevelled in presynaptic assembly and neurotransmitter release. *J Cell Biol* 174,127-39.
- Artinian, L., Zhong, L., Yang, H., Rehder, V., 2012. Nitric oxide as intracellular modulator: internal production of NO increases neuronal excitability via modulation of several ionic conductances. *Eur J Neurosci* 36,3333-43.
- Bornstein, J.C., Marks, K.A., Foong, J.P., Gwynne, R.M., Wang, Z.H., 2010. Nitric oxide enhances inhibitory synaptic transmission and neuronal excitability in Guinea-pig submucous plexus. *Front Neurosci* 4,30.
- Brenman, J.E., Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z, Huang, F., Xia, H., Peters, M.F., Froehner, S.C., Brecht, D.S., 1996. Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell* 84,757-67.
- Brew, H.M., Hallows, J.L., Tempel, B.L., 2003. Hyperexcitability and reduced low threshold potassium currents in auditory neurons of mice lacking the channel subunit Kv1.1. *J Physiol* 548,1-20.
- Brummelkamp, T.R., Bernards, R., Agami, R., 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296, 550-553.
- Budnik, V., Salinas, P.C., 2011. Wnt signaling during synaptic development and plasticity. *Curr Opin Neurobiol* 21,151-9
- Cerpa, W., Farias, G.G., Godoy, J.A., Fuenzalida, M., Bonansco, C., Inestrosa, N.C., 2010. Wnt-5a occludes Abeta oligomer-induced depression of glutamatergic transmission in hippocampal neurons. *Mol Neurodegener* 5,3.
- Cerpa, W., Gambrill, A., Inestrosa, N.C., Barria, A., 2011. Regulation of NMDA-receptor synaptic transmission by Wnt signaling. *J Neurosci* 31,9466-71.
- Cerpa, W., Godoy, J.A, Alfaro, I., Farias, G.G., Metcalfe, M.J., Fuentealba, R., Bonansco, C., Inestrosa, N.C., 2008. Wnt-7a modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons. *J Biol Chem* 283,5918-27.
- Chen, J., Park, C.S., Tang, S.J., 2006. Activity-dependent synaptic Wnt release regulates hippocampal long term potentiation. *J Biol Chem* 281,11910-6.

- Codocedo, J.F., Godoy, J.A., Poblete, M.I., Inestrosa, N.C., Huidobro-Toro, J.P., 2013. ATP induces NO production in hippocampal neurons by P2X(7) receptor activation independent of glutamate signaling. *PLoS One* 8,e57626.
- Coetzee, W.A., Amarillo, Y., Chiu, J., Chow, A., Lau, D., McCormack, T., Moreno, H., Nadal, M.S., Ozaita, A., Pountney, D., Saganich, M., Vega-Saenz de Miera, E., Rudy, B., 1999. Molecular diversity of K⁺ channels. *Ann N Y Acad Sci* 868, 233-85.
- Colom, L.V., Diaz, M.E., Beers, D.R., Neely, A., Xie, W.J., Appel, S.H. 1998. Role of potassium channels in amyloid-induced cell death. *J Neurochem.* 70(5), 1925-34.
- Cuitino, L., Godoy, J.A., Farias, G.G., Couve, A., Bonansco, C., Fuenzalida, M., Inestrosa, N.C., 2010. Wnt-5a modulates recycling of functional GABAA receptors on hippocampal neurons. *J Neurosci* 30,8411-20.
- Doyle, D.A., Morais, Cabral, J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen SL, Chait BT, MacKinnon R., 1998. The structure of the potassium channel: molecular basis of K⁺ conduction and selectivity. *Science* 280,69-77.
- Ertel, E.A., Campbell, K.P., Harpold, M.M., Hofmann, F., Mori, Y., Perez-Reyes, E., Schwartz, A., Snutch, T.P., Tanabe, T., Birnbaumer, L., Tsien, R.W., Catterall, W.A.. 2000. Nomenclature of voltage-gated calcium channels. *Neuron* 25,533-5.
- Farias, G.G., Alfaro, I.E., Cerpa, W., Grabowski, C.P., Godoy, J.A., Bonansco, C., Inestrosa, N.C., 2009. Wnt-5a/JNK signaling promotes the clustering of PSD-95 in hippocampal neurons. *J Biol Chem* 284,15857-66.
- Farias, G.G., Valles, A.S., Colombres, M., Godoy, J.A., Toledo, E.M., Lukas, R.J., Barrantes, F.J., Inestrosa, N.C., 2007. Wnt-7a induces presynaptic colocalization of alpha 7-nicotinic acetylcholine receptors and adenomatous polyposis coli in hippocampal neurons. *J Neurosci* 27,5313-25.
- Ferraro, G., Montalbano, M.E., La Grutta, V., 1999. Nitric oxide and glutamate interaction in the control of cortical and hippocampal excitability. *Epilepsia* 40,830-6.
- Freissmuth, M., Waldhoer, M., Bofill-Cardona, E., Nanoff, C., 1999. G protein antagonists. *Trends Pharmacol Sci* 20.237-45.
- Hanse, E., Gustafsson, B., 1994. TEA elicits two distinct potentiations of synaptic transmission in the CA1 region of the hippocampal slice. *J Neurosci* 14,5028-34.
- Hille, B., 2001. *Ion Channels of Excitable Membranes*: Sinauer Associates, Inc, Edition: Third Chapter 3,4,5.

- Huang, Y.Y., Malenka, R.C., 1993. Examination of TEA-induced synaptic enhancement in area CA1 of the hippocampus: the role of voltage-dependent Ca²⁺ channels in the induction of LTP. *J Neurosci* 13,568-76.
- Inestrosa, N.C., Arenas, E., 2010. Emerging roles of Wnts in the adult nervous system. *Nat Rev Neurosci* 11,77-86.
- Jones, P.A., Smith, R.A., Stone, T.W., 1998., Nitric oxide synthase inhibitors L-NAME and 7-nitroindazole protect rat hippocampus against kainate-induced excitotoxicity. *Neurosci Lett* 249,75-8.
- Kayano, T., Noda, M., Flockerzi, V., Takahashi, H., Numa, S., 1988. Primary structure of rat brain sodium channel III deduced from the cDNA sequence. *FEBS Lett* 228,187-94.
- Kohn, A.D., Moon, R.T., 2005 Wnt and calcium signaling: beta-catenin-independent pathways. *Cell Calcium* 38(3-4), 439-46.
- Ledo, A., Barbosa, R.M., Gerhardt, G.A., Cadenas, E., Laranjinha, J., 2005. Concentration dynamics of nitric oxide in rat hippocampal subregions evoked by stimulation of the NMDA glutamate receptor. *Proc Natl Acad Sci U S A* 102,17483-8.
- Li, L., Hutchins, B.I., Kalil, K., 2009. Wnt5a induces simultaneous cortical axon outgrowth and repulsive axon guidance through distinct signaling mechanisms. *J Neurosci* 29, 5873-5883.
- Lie, D.C., Colamarino, S.A., Song, H.J., Desire, L., Mira, H., Consiglio, A., Lein, E.S., Jessberger, S., Lansford, H., Dearie, A.R., Gage, F.H., 2005. Wnt signalling regulates adult hippocampal neurogenesis. *Nature* 437,1370-5.
- Lien, C.C., Martina, M., Schultz, J.H., Ehmke, H., Jonas, P., 2002., Gating, modulation and subunit composition of voltage-gated K(+) channels in dendritic inhibitory interneurons of rat hippocampus. *J Physiol* 538,405-19.
- Mikels, A., Minami, Y., Nusse, R., 2009. Ror2 receptor requires tyrosine kinase activity to mediate Wnt5A signaling. *J Biol Chem* 284, 30167-30176.
- Minami, Y., Oishi, I., Endo, M., Nishita, M., 2010. Ror-family receptor tyrosine kinases in noncanonical Wnt signaling: their implications in developmental morphogenesis and human diseases. *Dev Dyn* 239, 1-15.
- Montcouquiol, M., Crenshaw, EB 3rd, Kelley MW., 2006. Noncanonical Wnt signaling and neural polarity. *Annu Rev Neurosci.* 29: 363-86.
- Moreno, H., Vega-Saenz de Miera, E., Nadal, M.S., Amarillo, Y., Rudy, B., 2001. Modulation of Kv3 potassium channels expressed in CHO cells by a nitric oxide-activated phosphatase. *Journal of Physiology* 530, 345-358.

- Muñoz, F.J., Godoy, J.A., Cerpa, W., Poblete, I.M., Huidobro-Toro, J.P., Inestrosa, N.C., 2014. Wnt-5a increases NO and modulates NMDA receptor in rat hippocampal neurons. *Biochem Biophys Res Commun* 444,189-94.
- Nishita, M., Itsukushima, S., Nomachi, A., Endo, M., Wang, Z., Inaba, D., Qiao, S., Takada, S., Kikuchi, A., Minami, Y., 2010. Ror2/Frizzled complex mediates Wnt5a-induced AP-1 activation by regulating Dishevelled polymerization. *Molecular and cellular biology* 30, 3610-3619.
- Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H., Numa, S., 1986. Existence of distinct sodium channel messenger RNAs in rat brain. *Nature* 320,188-92.
- Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., 1984. Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. *Nature* 312,121-7.
- Nusse, R., Varmus, H., 2012. Three decades of Wnts: a personal perspective on how a scientific field developed. *EMBO J* 31, 2670-84.
- Oishi, I., Suzuki, H., Onishi, N., Takada, R., Kani, S., Ohkawara, B., Koshida, I., Suzuki, K., Yamada, G., Schwabe, G.C., Mundlos, S., Shibuya, H., Takada, S., Minami, Y., 2003. The receptor tyrosine kinase Ror2 is involved in non-canonical Wnt5a/JNK signalling pathway. *Genes Cells* 8, 645-654.
- Oliva, C.A., Vargas, J.Y., Inestrosa, N.C., 2013. Wnt signaling: Role in LTP, neural networks and memory. *Ageing Res Rev* 12, 786-800.
- Otto, J.F., Singh, N.A., Dahle, E.J., Leppert, M.F., Pappas, C.M., Pruess, T.H., Wilcox, K.S., White, H.S., 2009. Electroconvulsive seizure thresholds and kindling acquisition rates are altered in mouse models of human KCNQ2 and KCNQ3 mutations for benign familial neonatal convulsions. *Epilepsia* 50(7),1752-9.
- Panáková, D., Werdich, A.A., Macrae, C.A., 2010. Wnt11 patterns a myocardial electrical gradient through regulation of the L-type Ca(2+) channel. *Nature* 466(7308), 874-8.
- Rattner, A., Hsieh, J.C., Smallwood, P.M., Gilbert, D.J., Copeland, N.G., Jenkins NA, Nathans J., 1997. A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. *Proc Natl Acad Sci U S A* 94, 2859-63.
- Rosso, S.B., Inestrosa, N.C., 2013. WNT signaling in neuronal maturation and synaptogenesis. *Front Cell Neurosci* 7,103.

- Rubinson, D.A., Dillon, C.P., Kwiatkowski, A.V., Sievers, C., Yang, L., Kopinja, J., Rooney, D.L, Zhang, M., Ihrig, M.M., McManus, M.T., Gertler, F.B., Scott, M.L., Van Parijs, L., 2003. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference, *Nature genetics* 33, 401-406.
- Salinas, P.C., 2012. Wnt signaling in the vertebrate central nervous system: from axon guidance to synaptic function. *Cold Spring Harb Perspect Biol* 4
- Schulte, G., Bryja, V., 2007. The Frizzled family of unconventional G-protein-coupled receptors. *Trends Pharmacol Sci.* 28(10), 518-25.
- Shieh, C.C., Coghlan, M., Sullivan, J.P., Gopalakrishnan, M., 2000. Potassium channels: molecular defects, diseases, and therapeutic opportunities, *Pharmacol Rev.* 52(4), 557-94.
- Simons, M, Mlodzik, M., 2008. Planar cell polarity signaling: from fly development to human disease. *Annu Rev Genet.* 42, 517-40.
- Song, D., Wang, Z., Berger, T.W., 2002. Contribution of T-type VDCC to TEA-induced long-term synaptic modification in hippocampal CA1 and dentate gyrus. *Hippocampus* 12, 689-97.
- Song, D., Xie, X., Wang, Z., Berger, T.W., 2001. Differential effect of TEA on long-term synaptic modification in hippocampal CA1 and dentate gyrus in vitro. *Neurobiol Learn Mem* 76, 375-87.
- Steinert, J.R., Kopp-Scheinflug, C., Baker, C., Challiss, R.A., Mistry, R., Hausteine, M.D., Griffin, S.J., Tong, H., Graham, B.P., Forsythe, I.D., 2008. Nitric oxide is a volume transmitter regulating postsynaptic excitability at a glutamatergic synapse. *Neuron* 60, 642-56.
- Steinert, J.R., Robinson, S.W., Tong H., Hausteine, M.D., Kopp-Scheinflug, C., Forsythe, I.D., 2011. Nitric oxide is an activity-dependent regulator of target neuron intrinsic excitability. *Neuron* 71, 291-305.
- Sjöström, P.J., Rancz, E.A., Roth, A, Häusser, M., 2008. Dendritic excitability and synaptic plasticity. *Physiol Rev* 8 , 769-840.
- Tansey, E.P., Chow, A., Rudy, B., McBain, C.J., 2002. Developmental expression of potassium-channel subunit Kv3.2 within subpopulations of mouse hippocampal inhibitory interneurons. *Hippocampus* 12,137-48.
- van Amerongen, R, Nusse, R., 2009. Towards an integrated view of Wnt signaling in development. *Development* 136, 3205-14.

- Varela-Nallar, L, Alfaro, I.E., Serrano, F.G., Parodi, J., Inestrosa, N.C., 2010. Wingless-type family member 5A (Wnt-5a) stimulates synaptic differentiation and function of glutamatergic synapses. *Proc Natl Acad Sci U S A* 107,21164-9.
- Varela-Nallar, L, Inestrosa, N.C., 2013. Wnt signaling in the regulation of adult hippocampal neurogenesis. *Front Cell Neurosci* 7,100.
- Wayman, G.A., Impey, S., Marks, D., Saneyoshi, T., Grant, W.F., Derkach, V, Soderling, TR., 2006. Activity-dependent dendritic arborization mediated by CaM-kinase I activation and enhanced CREB-dependent transcription of Wnt-2. *Neuron* 50. 897-909.
- Westenbroek, R.E., Merrick, D.K., Catterall, W.A., 1989. Differential subcellular localization of the RI and RII Na⁺ channel subtypes in central neurons. *Neuron* 3, 695-704.

