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Wnt-5a increases NO and modulates NMDA receptor in rat hippocampal neurons

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ABSTRACT

Wnt signaling has a crucial role in synaptic function at the central nervous system. Here we evaluate whether *Wnts* affect nitric oxide (NO) generation in hippocampal neurons. We found that non-canonical *Wnt-5a* triggers NO production; however, *Wnt-3a* a canonical ligand did not exert the same effect. Co-administration of *Wnt-5a* with the soluble Frizzled related protein-2 (sFRP-2) a *Wnt* antagonist blocked the NO production. *Wnt-5a* activates the non-canonical *Wnt*/Ca^{2+} signaling through a mechanism that depends on Ca^{2+} release from Ryanodine-sensitive internal stores. The increase in NO levels evoked by *Wnt-5a* promotes the insertion of the GluN2B subunit of the NMDA receptor (NMDAR) into the neuronal cell surface. To the best of our knowledge, this is the first time that *Wnt-5a* signaling is related to NO production, which in turn increases NMDARs trafficking to the cell surface.

1. Introduction

The expression of *Wnt* signaling components in the mature central nervous system indicates that this pathway plays a role in synaptic function and connectivity [1,2]. *Wnt* secreted proteins are synaptic organizers that stimulate the formation of central and peripheral synapses [1,3,4] by promoting presynaptic assembly [5,6] and the clustering of postsynaptic components [7]. *Wnt* signaling is also implicated in synaptic plasticity, modulating long-term potentiation (LTP) in mouse hippocampal slices [8,9].

Several *Wnt* genes have been identified in the vertebrate genome, and three alternative signaling pathways have been found. The canonical *Wnt/β-catenin* pathway is characterized by an increase stabilization of the cytoplasmic β-catenin, which enters the nucleus and co-activates transcription of *Wnt* target genes with Tcf/Lef transcription factors [10]. The two non-canonical *Wnt* signaling pathways, depending on the cellular context are, Wnt/PCP (Planar Cell Polarity), acting through monomeric GTPases and c-Jun N-terminal kinase (JNK) [11], and *Wnt*/Ca^{2+}, where *Wnt* ligands activate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC) and calcineurin (phosphatase) [12]. Activation through *Wnt-5a* induces rapid changes in the clustering of the postsynaptic density scaffold protein (PSD-95), through a JNK-dependent signaling pathway [7]. Moreover, *Wnt-5a* increases the level and retention of cell surface GABA**A** receptors as well as the amplitude of GABA-evoked currents; these results are mediated by the activation of CaMKII which confirms that the *Wnt-5a*/Ca^{2+} signaling pathway is involved in GABA**A** receptors trafficking [13]. The *Wnt-5a* signaling acting through the *Wnt*/Ca^{2+} pathway was shown to stimulate the amplitude of

Abbreviations: Wnt-5a, Wingless-type family member 5a; NO, nitric oxide; NMDAR, N-methyl-D-aspartate receptor; sFRP-2, soluble Frizzled related protein-2; *Wnt*/PCP, Wnt/planar cell polarity; JNK, c-Jun N-terminal kinase; CaMKII, Ca^{2+}/calmodulin dependent kinase II; PKC, protein kinase C; Calcineurin, Protein phosphatase; GABA, γ-Aminobutiric acid; nNOS, neuronal NO synthase; SNP, sodium nitroprusside dehydrate; 7-NI, 7-nitroindazole; BAPTA-AM, 2-bis-(o-aminophenoxy)-ethane-N,N,N′,N′tetraaceticacid,tetraacetoxymethyl ester; CRD, cysteine rich domain; AraC, cytosine arabinoside; DIV, days *in vitro*; GFAP, glial fibrillary acidic protein; BSA, bovine serum albumin.

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NMDA spontaneous miniature currents in hippocampal neurons [14]. Similar studies in hippocampal slices confirm that *Wnt-5a* increases NMDAR activity [8].

Nitric oxide (NO) plays a role in numerous functions ranging from neuroendocrine signaling to learning and memory [15,16]. nNOS activation depends on calcium even that released from intracellular stores through the activation of the phosphatase calcineurin [15–19]. Our recent studies indicate that ATP elicited the NO production in hippocampal neurons independently of the NMDAR activity [20].

We report here that *Wnt-5a* increases the NO production that depends of the release of intracellular free Ca^{2+} through a Ryanodine-Ca^{+2}-dependent mechanism. This *Wnt-5a*-evoked NO production promotes GluN2B subunit insertion into the neuronal cell surface. Our results support that *Wnts* act as regulators of synaptic activity.

2. Materials and methods

2.1. Materials

Sodium nitroprusside dihydrate (SNP), 7-nitroindazole (7-NI), Ryanodine, EGTA, BAPTA-AM, cyclosporine, FK-506, cell culture reagents and chemicals (St. Louis, MO, USA); mouse anti-GluN2B-subunit (UC Davis/NIH/NeuroMab Facility, UCLA, Davis, CA, USA); secondary fluorescent antibodies (Molecular Probes, Eugene, OR, USA); Recombinant FzCRD-1, FzCRD-2, FzCRD-5 (R&D Systems, Minneapolis, MN, USA).

2.2. Animals and ethical standards

Experiments were performed on Sprague–Dawley rat fetuses (E18). Rats were housed in the University Facility. The experimental procedures were approved by the Bioethical and Biosafety Committee of the Faculty of Biological Sciences of the Pontificia Universidad Católica de Chile.

2.3. Culture of rat hippocampal neurons

Hippocampi were dissected from Sprague–Dawley rats at 18 embryonic day. Primary rat hippocampal neurons were maintained in Dulbecco's modified Eagle's medium supplemented with 10% horse serum for 2 h. Medium was substituted with Neurobasal medium supplemented with B27, 100 μL/mL streptomycin, and 100 units/mL penicillin. At 3 and 7 days *in vitro* (DIV) cells were treated with 2 μM 1-β-D-arabinofuranosylcytosine (araC) for 24 h to avoid glial cell growth. At DIV 15, neurons were used for experimentation; briefly, cells were maintained and experiments were conducted at 37 °C [6,7]. The percentage of glial contamination was estimated by glial fibrillary acidic protein (GFAP) immunostaining at DIV 14. 30 microscopic fields were analyzed per coverslip from two individual cultures.

2.4. NO measurements in hippocampal neuron cultures

NO sample content was quantified using a Sievers 280 NO analyzer within one hour after concluding the experiment. 50 μL perfused sample were injected into the reaction chamber, and a stream of nitrogen carried the resulting NO to a cell in which the chemoluminiscence generated by the NO-ozone reaction was detected by a photomultiplier. The sensitivity of the setup allows for a detection threshold of 0.5–1 pmol NO (10–20 pmol mL^{-1}). Results are expressed as a pmol/mL and normalized over the control values [20,21].

2.5. Conditioned medium containing Wnt Ligand

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, HEK-293 cells were permanently transfected with Lipofectamine 2000 and equal amounts of empty vector pcDNA (control) or pcDNA containing sequences encoding *Wnt* ligand or soluble Frizzled related protein (HA-sFRP-1) constructs coupled to the sequence encoding a hemaglutinin tag. L cells *Wnt-5a* and transfected HEK-293 cells were grown to 85% confluence and maintained in Neurobasal medium without supplements [6]. *Wnt*-conditioned and, control media or media containing sFRP were prepared. *Wnt* secretion was verified by Western blot using an anti-HA antibody (Upstate Biotechnology, Lake Placid, NY, USA).

2.6. Quantification of neuronal surface GluN2B subunit by a colorimetric ELISA assay

Hippocampal neurons were starved with Neurobasal media without supplement for 2 h. At the end of each experiment, cells were fixed with 4% paraformaldehyde in PBS for 3 min at room temperature and neutralized with 1% glycine at 4 °C for 10 min. Cells were blocked with 3% BSA at 4 °C. To determine the cell surface GluN2B subunit, intact (non-permeabilized) cells were incubated with specific antibody, the total NMDA receptor was detected after 0.2% Triton X-100 treatment. A primary antibody for the GluN2B subunits was then added to the cultures at a dilution of 1:250 and maintained at 4 °C for 12 h. Cells were washed and bound antibody detected using the Ultra-Sensitive ABC Peroxidase Staining Kits (Pierce). The colorimetric reaction using TMB as substrate was measured at 450 nm [13].

2.7. Statistical analysis

Data are presented as a mean ± sem. "*n*" indicates the number of neurons obtained from different animals. The significance level was set at $P = 0.05$. All curve fitting and statistical calculations were performed with GraphPad Prism 5.0 (GraphPad Software).

3. Results

3.1. Wnt-5a increases the NO production in hippocampal neurons

Calcium is a key second messenger in hippocampal neurons that induces the activation of several proteins of the postsynaptic machinery, including nNOS and therefore increasing the NO production [22]. Since *Wnt-5a* induces an increase in intracellular calcium levels [14,22,23], we assessed whether stimulation with *Wnt-5a* was able to increase the NO production in hippocampal neurons from rat embryos. Fig. 1A shows that, *Wnt-5a* stimulated NO production and this effect was maintained by 1 h in the presence of this non-canonical ligand. This effect was specific for *Wnt-5a* since *Wnt-3a*, a canonical ligand, did not trigger detectable NO production (Fig. 1A). The effect elicited by *Wnt-5a* signaling was abolished by co-treatment with soluble Frizzled-related protein 2 (sFRP-2, 25 μM) a *Wnt* antagonist [24] which binds *Wnt* ligands preventing its interaction with cellular receptors (Fig. 1A).

Frizzled receptors have an extracellular N-terminal region that contains a CRD domain consisting of 120–125 residues with 10 conserved cysteine that is necessary for the binding of *Wnt* molecules [25]. When neurons were pre-treated with the soluble cysteine-rich domain of Frizzled-2 receptor [26] the increased NO production evoked by *Wnt-5a* was prevented (Fig. 1B). Interestingly, neither Frizzled-1 (Fz-CRD-1) nor Frizzled-5 (Fz-CRD-5)

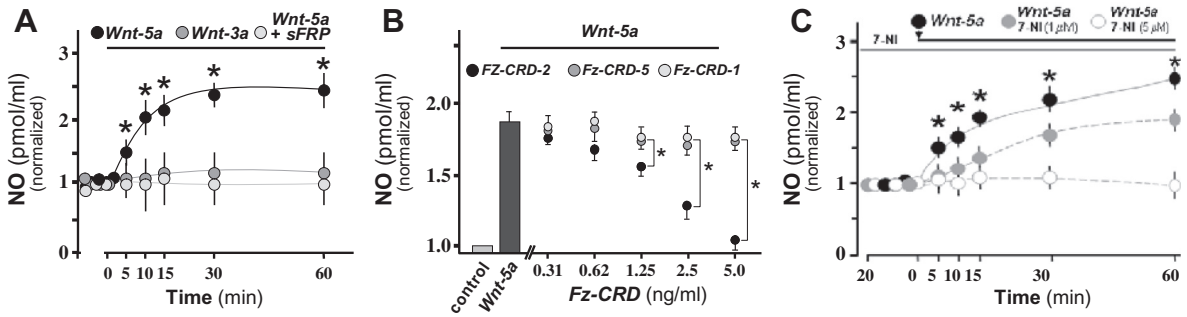

Fig. 1. *Wnt-5a* induces NO production in hippocampal neurons. (A) The *Wnt-5a* ligand induces NO production along the time reaching a plateau after 30 min of stimulation. *Specificity of the Wnt-5a effect:* The production of NO is specific for *Wnt-5a,* a non-canonical Wnt ligand, since canonical ligands such as *Wnt-3* has no effect on the NO production. When the *Wnt-5a* ligand was incubated with *sFRP-2*, a soluble frizzled related protein, the NO production induced by *Wnt-5a* was blocked. (B) *Wnt receptor involved in the Wnt-5a effect.* Several different Fz-CRDs were used to compete with the *Wnt-5a* ligand, to see which Fz-CRD antagonizes the NO production induced by the Wnt ligand. Only Fz-CRD-2 specifically inhibited NO production in a concentration-dependent manner. (C) *Inhibition of NO production*. A selective nNOS inhibitor (7-NI), was used during 60 min after a stabilization period of 20 min at 1 and 5 μM to block the NO production induced by *Wnt-5a*. Results are the mean ± sem of $n = 4–6$ separate experiments performed by triplicate. Student's *t*-test, $^{*}p < 0.05$.

was able to mimic the effect of Fz-CRD-2, suggesting that an Fz-2 receptor might be involved in the observed *Wnt-5a* effects.

When hippocampal neurons were incubated with *Wnt-5a* the NO production increases, however, after the addition of increasing 7-nitroindazole (7-NI) concentrations, an inhibitor of nNOS [27], the NO production was eventually inhibited (Fig. 1C). These results suggest that *Wnt-5a* signaling enhances specifically NO production in cultured hippocampal neurons.

3.2. NO production triggered by Wnt-5a requires calcium release from internal stores

Since the non-canonical *Wnt/Ca^{2+}* pathway acts through intracellular calcium mobilization [12,22,23], we studied the effect of calcium mobilization on NO production. When bathing the neurons with nominal zero calcium buffer plus EGTA (5 mM), the NO production induced by *Wnt-5a* remains unaffected (Fig. 2A). This suggests that extracellular calcium does not contribute to the NO production evoked by Wnt-5a. On the other hand, when we used 5 μM BAPTA-AM, an intracellular calcium chelator, the NO production was completely inhibited, suggesting that intracellular calcium mobilization is required for the increased NO production evoked by *Wnt-5a* (Fig. 2A). We then used the alkaloid Ryanodine (Fig. 2B), a selective modulator of the Ryanodine receptors present in the endoplasmic reticulum (ER) [28,29]. Low concentrations (0.01–0.1 μM) of Ryanodine plus *Wnt-5a* increased NO production up to 180% over control; however, when we raised Ryanodine concentrations to micromolar levels, the increased NO production evoked by *Wnt-5a* was blocked. This latter result fits with the inhibitory effect of Ryanodine at high concentrations on the Ryanodine receptor [29,30]. Considering that nNOS is also activated by calcineurin, a calcium-dependent phosphatase, we used cyclosporine and FK-506, two specific inhibitors of calcineurin [18,19]. As expected, both inhibitors completely prevented the *Wnt-5a*-induced NO production (Fig. 2C). These results suggest that *Wnt-5a* signaling acts at the postsynaptic region mobilizing intracellular calcium, probably activating calcineurin. It enhances nNOS activity and causes the concomitant increase in NO production.

3.3. Wnt-5a increases NMDARs through a NO-dependent mechanism

We evaluated the effect of NO on the NMDAR recycling and activity by incubating hippocampal neurons with *Wnt-5a* in the presence or absence of 7-NI (Fig. 3A). We determined the presence

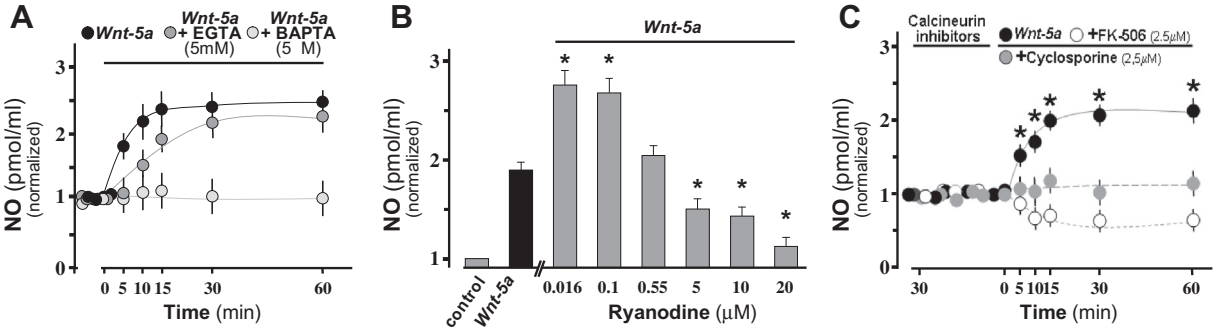

Fig. 2. The increased NO production evoked by *Wnt-5a* requires cytosolic calcium Ryanodine receptors and nNOS activity (A) The graph shows that despite the absence of extracellular calcium (nominal 0 mM Ca^{2+} plus 5 mM EGTA, grey circles), the production of NO induced by *Wnt-5a* was not affected. However, BAPTA-AM (a cell permeable calcium chelator) reduces the production of NO triggered by *Wnt-5a* in hippocampal neurons, suggesting a role for the intracellular calcium in the process. (B) The stimulation of Ryanodine receptors at low doses of Ryanodine (10–100 nM) showed an increase in the production of NO over the increase level trigger by *Wnt-5a*, however at micromolar levels of Ryanodine (10 min incubation), the NO production triggered by *Wnt-5a* was abolished. These results indicated that *mobilization of calcium from internal stores is required for the NO produced by effect of Wnt-5a.* (C) Two calcineurin inhibitors (Cyclosporine and FK-506) decrease NO production induced by *Wnt-5a* in hippocampal neurons. Primary cultures of rat embryo hippocampal neurons were treated by 1 h at 37 °C. *These studies indicated the production of NO requires the activation of a calcium phosphatase (calcineurin).* Results are expressed as percentages of control untreated neurons. Results are the mean ± sem of $n = 4$ separate experiments performed by triplicate. Student's *t*-test, $^{*}p < 0.05$.

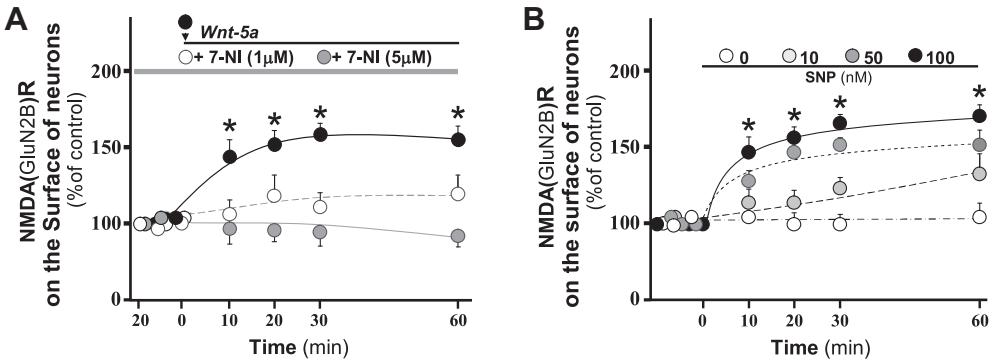

Fig. 3. *Wnt-5a* increases the GluN2B subunit of the NMDAR on the hippocampal neuronal cell surface, mediates by NO production. (A) *Wnt-5a* increases the appearance of the GluN2B subunit of the NMDAR on the cell surface, increasing the 7-NI concentrations reduces the membrane insertion of the NMDAR. (B) The insertion of the NMDAR (GluN2B) was mimicked by SNP (a NO donor), this insertion is dependent on concentration and time of action of the donor. Primary cultures of rat embryo hippocampal neurons were treated by 1 h at 37 °C. Results are expressed as percentages of control neurons (A, B). Results are the mean ± SEM of $n = 4–6$ separate experiments performed by triplicate. Student's *t*-test, $^{*}p < 0.05$.

of the NMDAR on the surface of the hippocampal neuron using an ELISA technique with a specific antibody against the GluN2B subunit, as described previously [13]. We found that *Wnt-5a* increases significantly the presence of the GluN2-containing NMDARs at 5 min in the neuronal cell surface (Fig. 3A), and this effect is maintained for 1 h. Moreover, the increased expression of the GluN2B subunit evoked by *Wnt-5a* was inhibited by 7-NI, a fact consistent with a NO-mediated mechanism of this *Wnt* ligand. Furthermore, when hippocampal neurons were treated with a NO donor (sodium nitropruside; SNP) [20,21], a concentration-dependent increases in the GluN2B subunit expressed in the membrane was observed (Fig. 3B). These findings indicate that *Wnt-5a* induces NO production in hippocampal neurons within few minutes, and that NO mobilized the GluN2B subunit of the NMDAR to the neuronal surface, therefore *Wnt-5a* through NO triggers dramatic changes in the glutamatergic function of the post-synaptic terminal in hippocampal neurons.

4. Discussion

Wnt signaling plays a key role in several developmental processes during neural differentiation as well as in synaptic maintenance in the adult nervous system [1–4,9]. Non-canonical *Wnt-5a* signaling was shown to operate on the postsynaptic regions of rat hippocampal neurons inducing the clustering of PSD-95 [7]. This postsynaptic component can form protein clusters that includes NMDARs [31], voltage-gated K^{+} channels [32,33] and nNOS with guanylate kinase-associated proteins [34,35]. In the present work, we have shown that *Wnt-5a* induces NO production in hippocampal neurons. This effect is specific for *Wnt-5a,* since other *Wnt* ligands such as Wnt-3a were not able to increase the NO formation, and can be inhibited by using sFRP, a Wnt antagonist. Therefore we incubated ligands with CRD, which mimics the cysteine rich domain of the *Wnt* Frizzled receptors and avoids ligand binding with its specific Frizzled receptor. *Wnt-5a* effects over NO production were not affected by Fz-CRD1 or Fz-CRD5, however, they were affected by Fz-CRD2 (partial effect at 1.25 ng/mL) and completely blocked at 5.0 ng/mL.

The role of the *Wnt/*Ca^{2+} pathway in several neuronal processes has been well established [12,22]. In the *Wnt/*Ca^{2+} pathway, the *Wnt-5a* ligand activates PKC, CaMKII and calcineurin, by increasing the intracellular calcium concentration coming from internal stores [12,23]. In fact, we recently established that activation of *Wnt* signaling by the non-canonical *Wnt-5a* ligand-induces mitochondrial dynamics in an ER-calcium related mechanisms [36], moreover, the *Wnt-5a* ligand prevents the Ryanodine receptor-mediated mitochondrial fragmentation induced by the amyloid-β-oligomers [37,38].

Here we demonstrated that the enhanced NO production evoked by *Wnt-5a* is not affected by chelating external Ca^{2+} but it is dose-dependently inhibited by micromolar concentration of Ryanodine. It is a putative novel branch of *Wnt* signaling pathway activated by the non-canonical *Wnt-5a* ligand upon binding to the Fz-2 receptor that involves Ca^{2+} release from Ryanodine-sensitive stores [36] and triggers NO production. It remains to be

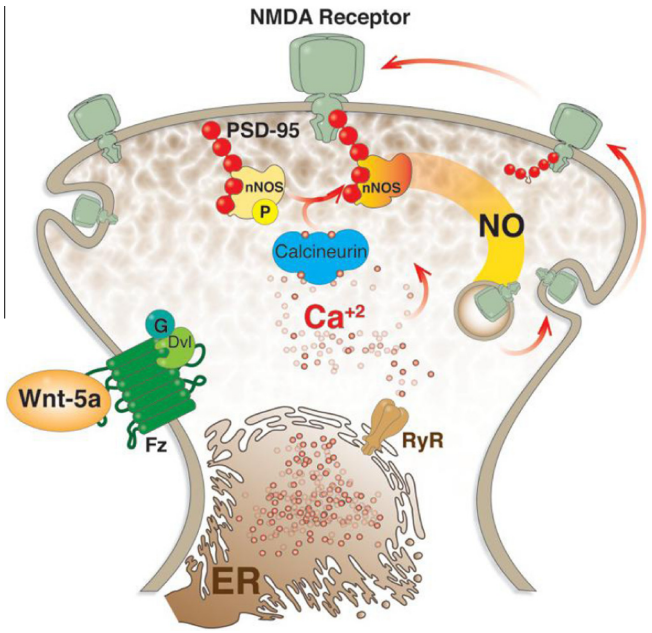

Fig. 4. The target of the NO-mediated *Wnt-5a* effect is an increasing traffic of NMDAR-containing vesicles. In this schematic model, *Wnt-5a* binds to the Fz2 receptor. The Ryanodine receptor is activate and induce Ca^{+2} release (34). The increased cytoplasmic calcium activates the phosphatase-dependent Ca^{++}/calmodulin, Calcineurin which de-phosphorylates nNOS (coupled to plasmatic membrane by PSD-95) with the subsequent NO production, stimulating the trafficking of NMDAR-containing vesicles to the neuronal postsynaptic cell surface. The presence of PSD-95 attached to nNOS allows a close interaction with NMDA receptor forming a signal complex for the effect of NO over NMDA receptor distribution. The de-phosphorylation of nNOS by calcineurin signal, not only induces stabilization of NMDA receptor in the membrane, also participates in the recycling of the receptor perisynaptic and facilitates its incorporation to the final synaptic position.

determined the mechanism by which the activation of the Fz-2 receptor promotes Ca^{2+} release. In addition, our results show that two different specific inhibitors of the phosphatase calcineurin (cyclosporine and FK-506) suppressed the NO-production evoked by *Wnt-5a*, indicating that the required activation of the nNOS is mediated by calcineurin.

NMDAR is critical in memory and learning, the classical hippocampal functions. GluN2B subunit is highly expressed when synapses are growing and interacts directly with PSD-95 and through this scaffold protein with other key proteins in glutamatergic transmission such as nNOS [39]. Our results indicate that this NO-mediated Wnt signaling can trigger a remodeling of synapses by an increased insertion of the GluN2B subunit of the NMDARs in the plasma membrane of hippocampal neurons. Previous studies indicated that *Wnt-5a* increases the NMDARs in rat hippocampus [14] and, that this increases is partially mediates by PKC [8], a result consistent with the release of Ca^{2+} from intracellular pools as observed in the present study. Considering the importance of the regulation of intracellular calcium levels mediated by the activation of the *Wnt/*Ca^{+2} signaling, we propose a model for the possible mechanism of action of *Wnt-5a* on NO production and NMDAR modulation (Fig. 4). In this model, *Wnt-5a* binds to the Fz2 receptor which eventually activate the Ryanodine receptor and induce Ca^{+2} release [36]. The increased cytoplasmic calcium activates the calcium-dependent protein Calcineurin which de-phosphorylates nNOS with the subsequent NO production, stimulating the trafficking of NMDAR [18,40]. Other possibility is that NO induces changes in the number of functional synapses promoting structural changes that incorporate glutamatergic receptor to the synapse. Further studies are necessaries to explore this possibility.

Summarizing we have described for the first time the production of NO in the context of *Wnt-5a* signaling. Further research of this novel NO pathway within the non-canonical *Wnt* signaling could help to understand complex synaptic cellular mechanisms and open the possibility to examine the role of *Wnt/*Ca^{+2}*/NO* pathway in brain pathophysiology.

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