Pontificia Universidad Católica de Chile
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INVOLVEMENT OF *Wnt* SIGNALING PATHWAY IN THE ADULT HIPPOCAMPAL SYNAPSES: ROLE IN NEUROPROTECTION

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To my beloved parents, Juan and Bidita
and my little brother, Christian.

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INDEX

FIGURES AND TABLES INDEX

ABBREVIATIONS

α7-nAChR: α7-nicotinic acetylcholine receptor

Aβ: amyloid-β peptide

ACSF: artificial cerebrospinal fluid

AD: Alzheimer's disease

AMPA-R: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

APC: adenomatosis polyposis coli

APP: amyloid precursor protein

APV: (2R)-amino-5-phosphonovaleric acid

CA1: *Cornu Ammonis* area 1

CA3: *Cornu Ammonis* area 3

CaMKII: Ca^{2+}-calmodulin-dependent protein kinase type II

CK-1α: casein kinase-1α

CNS: central nervous system

Dkk-1: dickkopf-1

Dvl: disheveled

fEPSP: field excitatory postsynaptic potential

FOXY-5: formylated *Wnt-5a*-derived hexapeptide

Fz: frizzled receptor

GABA: γ-aminobutyric acid

GSK-3β: glycogen synthase kinase-3β

HFS: high frequency stimulation

JNK: c-Jun N-terminal kinase

LTD: long-term depression

LTP: long-term potentiation

l-LTP: late-LTP

mEPSC: miniature excitatory postsynaptic current

NMDA-R: N-Methyl-D-aspartate receptor

GluN2B: NMDA receptor subunit 2B

PHF-1: tau epitope paired-helical filament-1

PPF: paired-pulse facilitation

PSD-95: postsynaptic density protein-95

PS-1: presenilin-1

sFRP: soluble Fz-related protein

Syn-1: synapsin-1

Syp: synaptophysin

Syt: synaptotagmin

TCF/LEF: T cell factor/lymphoid enhancer factor

WASP-1: *Wnt*-Activating Small molecule Potentiatior-1

WT: wild-type mice.

ABSTRACT

Wnt ligands are signaling proteins expressed in several brain regions, where they function as activators of the *Wnt* signaling pathway. During the nervous system development, *Wnt* ligands play a key role in modulating axonal remodeling, dendritogenesis and synaptogenesis. In the adult brain, components of *Wnt* signaling are still expressed, but little is known about its role in mature synapses yet. Emerging *in vitro* studies have implicated *Wnt* signaling in synaptic plasticity of the adult brain. Additionally, it has been shown that activation of *Wnt* signaling protects against amyloid-β-induced synaptic impairment. In our laboratory, we have previously shown that some *Wnt* ligands are able to regulate synaptic transmission and specifically to induce modification at either the presynaptic or the postsynaptic structure. Besides, *in vitro* studies performed in our laboratory have revealed that some *Wnt* ligands are also able to protect against amyloid-β-induced cytotoxic and synaptotoxic insults. Now, in this work we have studied the involvement of *Wnt* signaling in the regulation of adult normal cognitive function, *in vivo*. Moreover, the beneficial effect caused by the activation of *Wnt* signaling in the cognitive function of double transgenic mouse, APPswe/PS1dE9, model of Alzheimer's disease, has also been evaluated herein.

The effects of *Wnt* signaling activation on the cognitive function of wild type and transgenic mice, was accomplished through the chronic administration of the *Wnt* signaling modulators WASP-1 and FOXY-5. As shown here, both treatments improve behavioral performance of mice evaluated for episodic memory tests. Electrophysiological recordings of hippocampal slices from animals treated with WASP-1 or FOXY-5 show an increase in both the excitatory synaptic transmission and the synaptic plasticity, compares to control mice. Furthermore, WASP-1 prevents synaptic protein loss, reduces the *tau* phosphorylation and blocks amyloid-β aggregation in transgenic mice. Altogether, these results suggest that *Wnt* signaling could play a key role in regulating synaptic plasticity of neural circuits and therefore controlling the outgoing cognitive function of adult mice. Moreover, the activation of *Wnt* signaling could rescue memory lost and improve amyloid-β-induced synaptic dysfunction in transgenic mice, thus *Wnt* signaling could be a promising new therapeutic target for Alzheimer's disease treatment.

RESUMEN

Los ligandos *Wnt* son proteínas de señalización que se expresan en distintas regiones del cerebro, donde funcionan activando la vía de señalización *Wnt*, que modula los procesos de remodelamiento axonal, dendritogénesis y sinaptogénesis, durante el desarrollo del sistema nervioso. En el cerebro adulto, continúan siendo expresados los ligandos *Wnt*, así como también, los distintos componentes de la vía señalización, pero aún se desconoce qué función desempeñan en el sistema nervioso maduro. Evidencias recientes sugieren que la vía *Wnt* funciona en el cerebro adulto, modulando la plasticidad sináptica. Previamente, en nuestro laboratorio se demostró que distintos ligandos *Wnt* son capaces de regular la transmisión sináptica glutamatérgica y modificar de forma específica la estructura pre y post-sináptica. Además, otros estudios de nuestro laboratorio han mostrado que la activación de la vía *Wnt* protege frente al daño sináptico ocasionado por el péptido β-amiloide, *in vitro*. En esta tesis, se estudió *in vivo* la participación de la vía *Wnt* en la regulación de la función cognitiva de ratones adultos. Igualmente, se evaluó el efecto benéfico producido por la activación de la vía *Wnt* en ratones doble transgénico APPswe/PS1dE9, modelo de la enfermedad de Alzheimer. El efecto de la activación de la vía *Wnt* sobre la función cognitiva de ratones silvestres y transgénicos, se estudió mediante la administración crónica de los moduladores de la vía *Wnt*: WASP-1 y FOXY-5. Ambos tratamientos produjeron una mejora en la memoria episódica de los animales tanto silvestres como transgénicos, sometidos a distintas pruebas conductuales. El análisis de los registros electrofisiológicos en rebanadas de hipocampo obtenidas de los animales tratados con WASP-1 o FOXY-5, reveló que ambos tratamientos inducen un aumento en la trasmisión sináptica y en la potenciación sináptica a largo plazo, en comparación con el grupo control. Además, WASP-1 previene la pérdida de proteínas sinápticas, reduce la fosforilación de la proteína *tau* y bloquea la agregación del péptido β-amiloide, en los ratones transgénicos. Todos estos resultados sugieren que la vía *Wnt* podría regular la plasticidad de los circuitos cerebrales y por lo tanto, mejorar la función cognitiva en ratones adultos. Asimismo, la activación de la vía *Wnt* podría rescatar la pérdida de memoria observada en los ratones transgénicos y disminuir el daño sináptico ocasionado por el péptido β-amiloide, lo que coloca a esta vía como un prometedor blanco terapéutico para el tratamiento de la enfermedad de Alzheimer.

1. INTRODUCTION

1.1. *Wnt* signaling pathway

Wnts are extracellular ligands that play a key role as developmental regulators involved in several cellular processes, including cell proliferation, differentiation, migration, adhesion, survival and apoptosis (Moon et al., 2002; Nusse, 2012; Willert and Nusse, 2012).

Wnts are palmitoylated glycoproteins, mostly conformed by about 350 amino acids long and 40 kDa molecular weight (Coudreuse and Korswagen, 2007). *Wnts* act as signaling molecules in all metazoan organisms, controlling a wide spectrum of cellular processes during the development and in the adulthood (van Amerongen and Nusse, 2009).

The mechanisms involved in the synthesis and secretion of *Wnts* have been first and foremost studied for *Drosophila Wnt* homolog, Wingless (Wg). Thus, through this model, *Wnt* secretion has been proposed to take place mainly by a constitutive pathway, which could be involved in a short-range signaling from the *Wnt*-producing cell (Ching et al., 2008; Hausmann et al., 2007). Alternatively, a small fraction of *Wnt* binds to lipoprotein particles possibly within specialized endosomal compartments, during the secretion route (Harterink and Korswagen, 2011; Panakova et al., 2005), and the *Wnt*-lipoprotein complex formed may participate in a long-range signaling after been released outside of its producing cell

(Hausmann et al., 2007; Lorenowicz and Korswagen, 2009). Although, there is not enough evidence that support signaling activity of the *Wnt*-lipoprotein complex, it is possible that this complex allows *Wnts* to signal along the morphogenetic field (Bartscherer and Boutros, 2008).

Released *Wnt* ligands bind to specific receptors at the cell membrane surface. Frizzled receptor (Fz), a protein that have seven transmembrane-spanning domains, was the first receptor described as responsible for *Wnt* signal transduction (Schulte and Bryja, 2007). The binding of *Wnt* to Fz, activates the cytoplasmic protein Disheveled (Dvl), which in turn modulates the activity of other components of the pathway (Nusse, 2012). Downstream of Dvl, *Wnt* signaling cascade diverges into three different branches: *Wnt*/β-catenin pathway, also called canonical *Wnt* pathway, *Wnt*/JNK pathway and *Wnt*/Ca^{2+} pathway, known together as non-canonical *Wnt* pathway (Ciani and Salinas, 2005) (see Appendix I for a schematic model of *Wnt* pathways).

In the *Wnt*/β-catenin pathway, Dvl activation induces the disassembly of "β-catenin destruction complex" formed by glycogen synthase kinase-3β (GSK-3β), casein kinase-1α (CK-1α), adenomatosis polyposis coli (APC) and axin (Moon, 2005). In the absence of *Wnt*, β-catenin is sequentially phosphorylated by CK-1α and GSK-3β, targeting β-catenin for ubiquitination and proteasome degradation (Moon, 2005; Nusse and Varmus, 2012). The activation of this pathway starts when *Wnt* binds to Fz receptor and to its co-receptor, the low-density lipoprotein receptor-related protein 5/6 (LRP-5/6), inducing recruitment of GSK-3β binding protein (GBP) by Dvl, which causes sequestration of the enzyme and prevents β-catenin phosphorylation (Clevers and Nusse, 2012). Thus, β-catenin is stabilized and accumulated in the cytoplasm, but then β-catenin enters the nucleus and interacts with members of the DNA-binding T cell factor/lymphoid enhancer factor (TCF/LEF) family

proteins to activate transcription of *Wnt* target genes (Archbold et al., 2011). Several *Wnt* target genes are activated in this process, including: c-myc (He et al., 1998), cyclin-D1 (Shtutman et al., 1999), cyclo-oxygenase-2 (Howe et al., 1999), neurogenin-1 (Hirabayashi et al., 2004) and Ca^{2+}- calmodulin-dependent protein kinase type IV (CaMKIV) (Arrazola et al., 2009). Therefore, activation of *Wnt*/β-catenin pathway regulates gene transcription.

The *Wnt*/JNK pathway was first described in *Drosophila*, where it controls planar cell polarity (PCP), hence it is also known as *Wnt*/PCP pathway (De Ferrari and Moon, 2006). In this pathway, the binding of *Wnt* to Fz is followed by the activation of both Rho and Rac small GTPases, which in turn stimulate c-Jun N-terminal kinase (JNK) (Widelitz, 2005). The downstream effects of this pathway include regulation of the actin cytoskeleton and microtubules organization, cell migration and gene expression, through activation of JNK-dependent transcription factors, such as the activating transcription factor-2 (ATF-2) (Kikuchi et al., 2011; Simons and Mlodzik, 2008).

In the *Wnt*/Ca^{2+} pathway, the binding of *Wnt* to Fz, results in the stimulation of heterotrimeric G-proteins, which in turn activates phospholipase-C (PLC) inducing the generation of diacylglycerol (DG) and inositol trisphosphate (IP_3). Then, IP_3 increases the intracellular Ca^{2+} release and decreases cyclic guanosine monophosphate (cGMP), resulting in the activation of Ca^{2+}-calmodulin-dependent protein kinase type II (CaMKII), calcineurin and protein kinase-C (PKC) (De, 2011). Downstream of this pathway, the transcription factor cAMP response element-binding protein-1 (CREB), is activated (Kohn and Moon, 2005).

The activation of a *Wnt* pathway in particular can depend, among other factors, on the binding of a specific *Wnt* ligand to its Fz receptor. Thus, *Wnts* are usually classified as canonical ligands if they activate *Wnt*/β-catenin signaling or non-canonical ligands, if they activate either *Wnt*/JNK pathway or *Wnt*/Ca^{2+} pathway, instead (Widelitz, 2005).

Until now, 19 different *Wnt* ligands and 10 members of Fz receptors family have been described in mammals (Kikuchi et al., 2011). Therefore, signaling specificity can depend not only on the type of ligand bound, but also on the receptors and co-receptors involved.

Additionally, several secreted antagonists that act extracellularly to block or to reduce *Wnt* signal response can regulate this pathway (Nusse, 2012). For instance, *Wnt* signaling can be inhibited, among other molecules, by soluble Fz-related proteins (sFRP), which compete with Fz receptors for binding to *Wnts*; or by Dickkopf (Dkk), a protein that specifically inhibits *Wnt*/β-catenin signaling through interaction with the co-receptor LRP-5/6 and inducing its endocytosis (Ahn et al., 2011; Filipovich et al., 2011; Mii and Taira, 2011).

In general, certain *Wnts* preferentially activate a specific signaling pathway in a particular cell type (Widelitz, 2005). The specificity of triggered response to *Wnt* might depend on: a) the presence of a particular *Wnt* ligand, b) the receptors and co-receptors located on the cell surface, c) the secretion of extracellular antagonists, and d) the expression of intracellular *Wnt* signaling components. Moreover, the timing, specificity and availability of all these factors also contribute to modulate *Wnt* signaling.

1.2. Role of *Wnt* signaling in the developing central nervous system (CNS)

Wnt proteins have a key role during embryonic development, acting as important mediators of intercellular communication involved in the normal development of various systems, including the nervous system (Ciani and Salinas, 2005).

At the cellular level, *Wnt* signaling plays an important role in controlling neuronal connectivity through the regulation of neuronal polarity, axonal remodeling, dendritic morphogenesis and synapse formation (Budnik and Salinas, 2011; Li et al., 2005). Accordingly, *Wnt-3a* ligand increases growth cone size and promotes axonal branching, by controlling terminal arborization in Neurotrophin-3-responsive neurons (Krylova et al., 2002). Moreover, it has been reported that both *Wnt-7a* ligand and the inhibition of GSK-3β, induce axonal branching through microtubule remodeling during postnatal cerebellar development (Lucas et al., 1998; Lucas and Salinas, 1997). Also, *Wnt-3a* ligand has been involved in the regulation of axon guidance and growth cone remodeling through changing directionality of microtubules (Purro et al., 2008). These observations suggest that *Wnts* are able to control axonal behavior by modulating the cytoskeleton organization and dynamics.

Besides participating in axonal remodeling, *Wnts* also might stimulate dendritic morphogenesis by acting in a paracrine manner, if they are released by the incoming axons, or in an autocrine manner, if they are released by the same responsive neurons (Ciani and Salinas, 2005). Interestingly, *Wnt-7b* ligand increases the length and branching of dendrites in hippocampal neurons, and this effect can be mimicked by the expression of Dvl or blocked by the *Wnt* antagonist, sFRP-1 (Rosso et al., 2005). In order to accomplish this effect *Wnt-7b* and Dvl signal through a noncanonical pathway in which Rac and JNK activation are involved (Rosso et al., 2005). Also, it has been shown that neuronal activity induces the expression of *Wnt-2* ligand, which in turn stimulates dendritic arborization in developing hippocampal neurons (Wayman et al., 2006). This activity-dependent dendritic outgrowth and branching is mediated by the activation of Ca^{2+}-calmodulin-dependent protein kinase type I (CaMKI) and the enhanced CREB-dependent transcription of *Wnt-2* ligand (Wayman et al., 2006). Furthermore, increased intracellular levels of β-catenin, N-cadherin and α-N-

catenin, members of the cadherin/catenin complex, enhances dendritic arborization in rat hippocampal neurons through a mechanism that does not require *Wnt*/β-catenin-dependent transcription (Yu and Malenka, 2003).

The effects of *Wnts* on axonal remodeling and dendritic morphogenesis are early events that precede synapse formation and maturation. However, during CNS development, *Wnts* are also involved in synaptogenesis, a process that requires crosstalk between presynaptic and postsynaptic cells. In the mouse cerebellum, the *Wnt-7a* ligand is expressed by granule cells during synaptogenesis with mossy fibers (Lucas and Salinas, 1997). Exogenous administration of *Wnt-7a* ligand to mossy fiber axon growths from explants increases the clustering of Synapsin-1 (Syn-1), a synaptic vesicle protein involved in synapses formation and function (Lucas and Salinas, 1997). Furthermore, *Wnt-7a* mutant mice show less remodeling of mossy fiber axons and delayed accumulation of Syn-1, compared to control animals (Hall et al., 2000). Together, these observations indicate that *Wnt-7a* ligand acts as a retrograde signal that regulates axon behavior and synapse formation in the cerebellum. However, the role as a synaptogenic factor is not restricted to *Wnt-7a* ligand or the cerebellum. As shown in an *in vitro* study, canonical ligands *Wnt-3a*, *Wnt-7a* and *Wnt-7b*, as well as, the stabilization of β-catenin, increases the clustering of the vesicular glutamate transporter-1 (vGLUT-1), during synaptogenesis in hippocampal neurons (Davis et al., 2008). Instead, the inhibition of the canonical pathway by Dkk-1, or the activation of the non-canonical pathway by *Wnt-5a* ligand, affects synapse formation through reducing the number of clusters of vGLUT-1 and consequently decreasing the number of presynaptic terminals (Davis et al., 2008). These observations suggest that canonical *Wnt* pathway could positively influence synapse formation, while the non-canonical *Wnt* pathway could induce anti-synaptogenic effects.

Not only *Wnts* have been identified as synaptogenic factors, but also some Fz receptors have been involved in synapse formation. For instance, Fz-4 receptor expressed by Purkinje cells in the cerebellum, participates in synapse formation between Purkinje cells and granule cells (Wang et al., 2001). More recently, Fz-5 receptor has been identified as a possible intermediary of *Wnt-7a* synaptogenic effects in hippocampal neurons (Sahores et al., 2010). Likewise, it has been reported that Ror-1 and Ror-2 receptors modulate synapse formation in hippocampal neurons, because of the interaction with *Wnt-5a* ligand (Paganoni et al., 2010).

When synaptic contact between presynaptic and postsynaptic sites has been done, rapid assembly of synaptic components is required for the establishment of functional synapses. *Wnt* signaling also regulates synapse maturation through retrograde and anterograde signals, and in an autocrine manner (Ataman et al., 2008). Evoked activity induces *Wnt* release from synaptic boutons, which regulates both cytoskeletal dynamics at the presynaptic terminal and the assembly of the postsynaptic apparatus (Ataman et al., 2008). This bidirectional effect of *Wnt* signaling, allows rapid changes in the synapse structure that are necessary to undergo synaptic plasticity (Budnik and Salinas, 2011).

1.3. Expression of *Wnt* signaling components in the adult brain

Although the expression of *Wnt* genes is mainly observed during embryonic brain development, it has been reported that *Wnts*, Fz receptors and several components of *Wnt* pathway remain expressed in the postnatal and adult brain (Parr et al., 1993; Shimogori et al., 2004). The expression of *Wnts* in the adult brain have been identified in the major subdivisions of the cerebral cortex: the olfactory bulb, hippocampus, and neocortex

(Shimogori et al., 2004). Interestingly, the expression of some *Wnts* is particularly high in those brain areas where neurons are continuously renewed, such as the olfactory bulb and the dentate gyrus in the hippocampus (Shimogori et al., 2004). However, the expression of *Wnts* in the adult brain is not restricted to neurogenic regions. An *in situ* hybridization analysis showed that *Wnt-2b* ligand is expressed in both somatosensory and entorhinal cortex, while *Wnt-5a* ligand is expressed in the prefrontal, anterior cingulate, insular and entorhinal cortex of postnatal and young adult mice (Shimogori et al., 2004). In the cortex, *Wnts* are expressed following a specific layers and regions pattering, as *Wnt-2b* ligand that is expressed in layers 4 and 6 of the somatosensory cortex and in layer 6 of the visual and auditory cortex, while *Wnt-5a* ligand is expressed in the layers 2, 3 and 5 of parietal and temporal neocortex (Shimogori et al., 2004). In the adult mouse hippocampus the expression of *Wnt-1*, *Wnt-2*, *Wnt-4*, *Wnt-5a*, *Wnt-7a*, *Wnt-7b*, *Wnt-8b* and *Wnt*-11 has been reported (Cerpa et al., 2008; Gogolla et al., 2009; Shimogori et al., 2004; Wayman et al., 2006). Specifically, the expression of *Wnt-2* ligand is most prominent in the CA1, CA2 and CA3 hippocampal regions (Wayman et al., 2006), while the expression of *Wnt-5a*, *Wnt-7a* and *Wnt-8b* is confined to specific layers of the dentate gyrus, related to adult neurogenesis (Shimogori et al., 2004). Immunofluorescence analysis of hippocampal expression of *Wnt-5a* and *Wnt-7a* in adult rats has shown that both ligands are mainly expressed in the pyramidal layer of CA1 hippocampal region (Oliva et al., 2013a) (Appendix II).

In addition, different Fz receptors are also expressed in several regions of the mature brain. Accordingly, Fz-1, Fz-2, Fz-8, Fz-9 and Fz-10 are ubiquitously expressed in the cortex and hippocampus from early postnatal ages to young adulthood (Shimogori et al., 2004). Interestingly, the expression of Fz-1, Fz-2 and Fz-9 resembles the expression of *Wnt-5a* ligand in the olfactory bulb and the hippocampal dentate gyrus (Shimogori et al., 2004).

Moreover, other components of *Wnt* pathway, such as sFRP antagonists and TCF/LEF transcription factors, are also found in the postnatal brain (Shimogori et al., 2004). Remarkably, hippocampal expression of several components of *Wnt* pathway is associated with synaptic regions. Thereby, the expression of *Wnt-3a* ligand colocalizes with the expression of Fz-4 and the postsynaptic density protein-95 (PSD-95) (Chen et al., 2006).

The widespread expression of *Wnt* pathway components in the adult brain, suggests that *Wnt* signaling plays a key role in the functioning of mature CNS (Budnik and Salinas, 2011; Inestrosa and Arenas, 2010; Shimogori et al., 2004).

1.4. Role of *Wnt* signaling in mature synapses

At the presynaptic site, *Wnts* modulate the clustering and trafficking of several presynaptic proteins (Budnik and Salinas, 2011; Inestrosa and Arenas, 2010; Oliva et al., 2013b). In mature hippocampal neurons, canonical *Wnt-7a* ligand regulates presynaptic localization of α7-nicotinic acetylcholine receptor (α7-nAChR) at the surface of the plasma membrane, through a mechanism that is independent of transcription of *Wnt* target genes, but requires the recruitment of APC (Farias et al., 2007). Furthermore, hippocampal neurons exposed to *Wnt-7a* ligand exhibit an increase in the number of clusters of several synaptic vesicle proteins, such as Syn-1, SV-2, synaptophysin (Syp) and synaptotagmin (Syt) (Cerpa et al., 2008; Farias et al., 2007). Conversely, loss of *Wnt-7a* function induces defects in the localization and clustering of Syn-1, Syp and SV-2 in the cerebellar synapse, and these effects may be exacerbated by loss of function of Dvl-1 (Ahmad-Annuar et al., 2006). Thus, double mutant *Wnt-7a*$^{-/-}$/Dvl-1$^{-/-}$ mice exhibit an enhanced defect in the localization of presynaptic

proteins when compared with single mutant Dvl-1$^{-/-}$ mice. (Ahmad-Annuar et al., 2006). Although the effect of *Wnt-7a* ligand on the distribution of presynaptic proteins, correlates with β-catenin stabilization, it does not involve changes in the expression levels of *Wnt* target genes, indicating that activation of *Wnt*/β-catenin signaling could promote rapid changes in the presynaptic structure through a "divergent *Wnt* canonical pathway" mechanism (Budnik and Salinas, 2011). Unexpectedly, GSK-3β is also not necessary for *Wnt-7a*-induced clustering of presynaptic proteins, which indicates that the mechanism responsible for this effect occur upstream in the signaling pathway (Cerpa et al., 2008).

Furthermore, *Wnt-7a* ligand increases recycling rate and synaptic vesicles exocytosis in both hippocampal and cerebellar cultured neurons labeled with FM 1-43 fluorescent dye (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). These observations are consistent with the finding that *Wnt-7a* ligand increases the frequency of the miniature excitatory postsynaptic currents (mEPSC) (Ahmad-Annuar et al., 2006; Cerpa et al., 2008), and enhances the amplitude of the field excitatory postsynaptic potentials (fEPSP), while decreases the paired-pulse facilitation (PPF) rate (Cerpa et al., 2008), which indicate that *Wnt-7a* ligand could modulate neurotransmitters release dynamic. Moreover, *Wnt-3a* ligand has shown to induce similar effects on synaptic vesicles recycling and exocytosis, as producing by *Wnt-7a* ligand (Avila et al., 2010; Cerpa et al., 2008). Purified *Wnt-3a* ligand increases mEPSC frequency through a mechanism that involve a fast influx of calcium from the extracellular space (Avila et al., 2010). Although most of *Wnts* that modulate the presynaptic structure and function, act through the *Wnt*/β-catenin signaling, this later evidence shows that *Wnt*/Ca^{2+} signaling could be also involved (Avila et al., 2010), suggesting that some components associated to non-canonical *Wnt* pathway might also participate in the modulation of the presynaptic terminal.

At the postsynaptic site, *Wnts* promote the assembly of the postsynaptic apparatus by increasing the clustering of postsynaptic proteins (Cuitino et al., 2010; Farias et al., 2009). In fact, *Wnt-5a* ligand acting through *Wnt*/JNK signaling induces the clustering of the scaffold protein, PSD-95, and its localization in dendritic spines (Farias et al., 2009). Additionally, *Wnt-5a* ligand acting through *Wnt*/Ca^{2+} signaling, promotes dendritic spines morphogenesis by inducing the formation of new ones and/or by increasing the size of pre-existing ones (Varela-Nallar et al., 2010). Interestingly, these changes in the structure of the postsynaptic apparatus result in an increased efficacy of glutamatergic synapses in mature hippocampal neurons (Farias et al., 2009; Varela-Nallar et al., 2010). Indeed, *Wnt-5a* ligand acting through *Wnt*/JNK signaling increases excitatory postsynaptic currents (EPSCs), specifically through the glutamate receptor, N-methyl-$_D$-aspartate receptor (NMDA-R) (Cerpa et al., 2011). However, the effects of *Wnt-5a* ligand are not restricted to glutamatergic synapses in the hippocampus. Recently, it was reported that *Wnt-5a* ligand also regulates the assembly of inhibitory synapses through inducing the clustering of γ-aminobutyric acid receptor type A ($GABA_A$-R) and its insertion in the membrane surface, by a mechanism that involves the activation of *Wnt*/Ca^{2+} signaling (Cuitino et al., 2010). In addition, *Wnt-5a* ligand increases the efficacy of inhibitory synapses in the hippocampus by increasing the amplitude of GABA-currents (Cuitino et al., 2010).

Electrophysiological recordings in slices and cultured neurons indicate that the effects of *Wnts* on both presynaptic and postsynaptic structures could lead to changes in the synaptic function. In fact, the activation of *Wnt* signaling not only regulates excitatory (Ahmad-Annuar et al., 2006; Avila et al., 2010; Cerpa et al., 2008; Farias et al., 2009), and inhibitory (Cuitino et al., 2010) synaptic transmission but also affects synaptic plasticity through modulating long-term potentiation (LTP) (Beaumont et al., 2007; Cerpa et al., 2011; Chen et

al., 2006). Thus, *Wnt-3a* ligand acting through *Wnt*/β-catenin signaling, facilitates the induction and maintenance of LTP in hippocampal slices after tetanic stimulation (Chen et al., 2006). Likewise, *Wnt-5a* ligand also increases the LTP magnitude but through the activation of *Wnt*/Ca^{2+} signaling (Cerpa et al., 2011). Interestingly, the inhibition of *Wnt* signaling blocks the LTP induction, suggesting that endogenous activation of this pathway, is required for LTP establishment (Cerpa et al., 2011; Chen et al., 2006).

1.5. *Wnt* signaling in Alzheimer's disease

Deregulation of *Wnt* signaling has been implicated in the appearance and progression of several neurological disorders, such as autism (Moon et al., 2004), schizophrenia (De Ferrari and Moon, 2006; Inestrosa et al., 2012), mood disorders (Hu et al., 2011; Okerlund and Cheyette, 2011), frontotemporal dementia (Korade and Mirnics, 2011), and Alzheimer's disease (Boonen et al., 2009; da Cruz e Silva et al., 2010; De Ferrari and Inestrosa, 2000).

1.5.1. Alzheimer's disease

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by progressive deterioration of the individual cognitive functions, mainly caused by synaptic damage and neuronal death in specific brain regions (Mattson, 2004; Selkoe, 2001a; Sheng et al., 2012). Two distinctive pathological features are observed in AD brains: a) senile plaques, composed by extracellular deposits of amyloid-β (Aβ) peptide, and b) neurofibrillary tangles, composed by intracellular aggregates of hyper-phosphorylated *tau* protein (Mayeux and Stern, 2012).

Aβ deposits are commonly located in the hippocampus, cerebral cortex and other brain areas linked to cognitive functions (Selkoe, 2001a). The Aβ peptide is produced through proteolytic cleavage of the amyloid precursor protein (APP) by a group of proteases, including α-, β- and γ-secretase (Selkoe, 2008). Mutations in APP or in the proteins presenilin-1 (PS-1) and presenilin-2 (PS-2), both involved in γ-secretase proteolytic processing of APP, result in an increased production of Aβ peptide, predominantly in its oligomeric form $A\beta_{1-42}$ (Pradier et al., 1999; Selkoe, 2001b; Xia, 2000). The oligomeric specie $A\beta_{1-42}$, has been identified as the primarily amyloidogenic form of Aβ peptide and it can aggregate to form diffusible oligomeric assemblies of Aβ peptide or neuritic amyloid plaques (McLean et al., 1999; Selkoe, 2002). Also, soluble aggregates of Aβ oligomers have been recognized as responsible for synaptic dysfunction associated with early stages of AD (Sakono and Zako, 2010). In fact, it has been reported that Aβ oligomers isolated from AD brains, impair spatial memory and disrupt synaptic plasticity when injected into the hippocampus of healthy rats (Shankar et al., 2008). Moreover, it has been shown that Aβ oligomers can block the induction of LTP (Walsh et al., 2002; Wang et al., 2004), but this effect can be reversed by inhibitors of Aβ oligomerization (Walsh et al., 2005). These evidences suggest that AD cognitive impairment might be produced by a direct effect of Aβ oligomers on synaptic transmission. Indeed, Aβ oligomers have shown to disrupt excitatory synaptic transmission by reducing the amplitude of fEPSPs and EPSCs in hippocampal CA3-CA1 synapses (Cerpa et al., 2010). Although, in normal conditions Aβ peptide is naturally released in an activity-dependent manner, triggering synapse facilitation, uncontrolled Aβ release causes aberrant effects, initially by producing over-excitation of synaptic transmission but at long-term causing depression of neuronal activity, which correlates to an important loss of cognitive function (Palop and Mucke, 2010).

1.5.2. Deregulation of *Wnt* signaling in Alzheimer's disease

Several evidences suggest that *Wnt* signaling disruption contributes to AD pathogenesis (Inestrosa and Varela-Nallar, 2014; Purro et al., 2014). In the AD brains a marked decrease in β-catenin levels has been observed (Zhang et al., 1998), as well as, an increased expression of Dkk-1 (Caricasole et al., 2004), in comparison to healthy individuals. In addition, pathogenic mutations in PS-1 gene, linked to early-onset of familial AD, cause β-catenin destabilization and a consequent increase in its degradation (Zhang et al., 1998). Also, genetic variations of LRP-6 co-receptor, involved in *Wnt*/β-catenin signaling, have been associated to late-onset AD (De Ferrari et al., 2007).

Interestingly, it has been reported that Aβ peptide could bind directly to Fz receptors, therefore inhibiting *Wnt* signaling (Magdesian et al., 2008). Moreover, loss of function of *Wnt*/β-catenin signaling enhances neuronal vulnerability to Aβ-induced apoptosis (Zhang et al., 1998). In fact, Aβ-mediated toxicity requires an increased GSK-3 activity, and could be reversed by the inhibition of either the expression or activity of GSK-3β (Alvarez et al., 1999; Takashima et al., 1996). Furthermore, exposure to Aβ induces an increased expression of Dkk-1 (Caricasole et al., 2004; Purro et al., 2014), as well as, decreased expression of β-catenin in hippocampal neurons (Alvarez et al., 2004). In the AD brains, the expression of Dkk-1 has been associated to the presence of neurofibrillary tangles and dystrophic neurites (Caricasole et al., 2004). Indeed, chronic overexpression of Dkk-1 induces an increase in the phosphorylation of *tau* and activates apoptosis in cortical neurons exposed to Aβ (Caricasole et al., 2004). Conversely, knock-down of Dkk-1, attenuated neuronal death and almost completely prevented the increase in *tau* phosphorylation in Aβ-treated neurons (Caricasole et al., 2004).

However, the activation of *Wnt* pathway has a protective effect against cytotoxic damage caused by exposure to Aβ (Cerpa et al., 2009; De Ferrari et al., 2003; Shruster et al., 2011). In fact, *Wnt-3a* ligand prevents Aβ-induced neuronal death of hippocampal neurons, through specific binding to Fz-1 receptor (Alvarez et al., 2004; Chacon et al., 2008), while *Wnt-5a* ligand acting through the *Wnt*/JNK signaling, prevents both synaptic transmission depression and loss of PSD-95 induced by Aβ oligomers (Cerpa et al., 2010; Varela-Nallar et al., 2012). On the other hand, the *in vivo* activation of *Wnt* signaling by lithium, a pharmacological activator of *Wnt*/β-catenin, reduces memory loss in double transgenic APP/PS1 mice, model of AD (Toledo and Inestrosa, 2010). In addition, lithium treatment shows a significant decrease in Aβ plaque deposition and in the total levels of Aβ in the cerebral cortex and the hippocampus of APP/PS1 mice (Toledo and Inestrosa, 2010). Moreover, lithium treatment reduces Aβ plaques formation in the brains that overproduce APP and blocks the increment in Aβ levels in the brains that overexpress GSK-3β (Phiel et al., 2003; Su et al., 2004). The overexpression of GSK-3β blocks the LTP induction and impairs spatial memory in conditional transgenic mice (Hernandez et al., 2002; Hooper et al., 2007). Since these animals have also shown a reduction in the intranuclear levels of β-catenin (Hernandez et al., 2002), it is possible that the activation of canonical *Wnt* pathway could protect them from synaptic and cognitive damage causing by GSK-3β overexpression. In effect, chronic treatment with lithium rescues LTP deficits in transgenic mice that conditionally overexpress GSK-3β (Hooper et al., 2007).

Since the activation of *Wnt* signaling has a neuroprotective effect against Aβ-induced damage, the search for molecules that modulate the activation of this pathway could lead to new therapies for the prevention and/or recovery of neuronal dysfunction in AD.

1.6. What is the physiological role of *Wnt* signaling in the adult brain?

Several *in vitro* studies have shown that *Wnt* signaling is involved in the regulation of synaptic plasticity in mature synapses (Ahmad-Annuar et al., 2006; Beaumont et al., 2007; Cerpa et al., 2011; Cerpa et al., 2008; Cuitino et al., 2010; Chen et al., 2006; Varela-Nallar et al., 2010). However, there is not enough *in vivo* evidences to determine how *Wnt* signaling modulates synaptic function in the adult brain, specifically during active behavior.

Recent studies suggest a role for *Wnt*/β-catenin signaling in memory acquisition and consolidation (Maguschak and Ressler, 2008, 2011). Interestingly, a selective increase in the hippocampal levels of *Wnt-7* ligand during consolidation and spatial memory recall in wild-type mice has been reported (Tabatadze et al., 2012), suggesting that *Wnt*/β-catenin signaling might control long-term information storage. Moreover, Dkk-1 injection into the basolateral amygdala has shown to impair long-term memory consolidation without affecting short-term memory (Maguschak and Ressler, 2011). Remarkably, the injection of *Wnt-1* ligand also impairs long-term memory consolidation by blocking the transient decrease of *Wnt-1* mRNA that occurs immediately after fear conditioning (Maguschak and Ressler, 2011). Moreover, a transient increase in the levels of β-catenin mRNA in the basolateral amygdala also occurs during fear memory consolidation (Maguschak and Ressler, 2008). These findings suggest that a dynamic regulation of *Wnt*-related genes should be critical for fear memory expression.

All these evidences suggest that the activation of *Wnt*/β-catenin signaling is involved in memory formation and memory consolidation in both the amygdala and the hippocampus (Oliva et al., 2013a). Yet, it is still unknown whether other *Wnt* pathways, besides *Wnt*/β-catenin signaling, are also involved in learning and memory.

In this thesis, we investigated for the first time the effects of *in vivo Wnt* signaling activation on adult hippocampal cognitive function, as well as, the neuroprotective effects against Aβ-induced synaptic damage. The data presented here suggest that *Wnt* signaling plays a key role in the regulation of synaptic function that could be used as a therapeutic target for AD treatment.

1.7. Hypothesis and Objectives

1.7.1. Hypothesis:

The activation of *Wnt* signaling pathway enhances synaptic plasticity and improves hippocampal-dependent memory in adult mice, therefore causing a reduction of cognitive deficits observed in double transgenic APP/PS1 mice, model of AD.

1.7.2. Objectives:

General Objective:

To study the effects of i*n vivo Wnt* signaling activation on the cognitive function of adult mice.

Specific Objectives:

1. To evaluate the effects of *in vivo Wnt* signaling activation on the episodic memory of wild-type and APP/PS1 mice.
2. To evaluate the effects of *in vivo Wnt* signaling activation on hippocampal synaptic function of wild-type and APP/PS1 mice.

2. CHAPTER I

In the next section we present the results obtained in order to accomplish partially the Specific Objectives N° 1 and 2, previously described.

The manuscript presented below, was published last February in the 34th Volume of Journal of Neuroscience (J. Neurosci. 2014; 34(6):2191-202). doi: 10.1523/JNEUROSCI.0862-13.2014.

As is shown below, this manuscript describes the effects on memory and synaptic plasticity of two *Wnt* signaling activators: WASP-1, a small molecule that potentiates *Wnt*/β-catenin signaling, and FOXY-5, a *Wnt-5a*-based peptide that activates *Wnt*/JNK signaling. Both *Wnt* signaling activators were administered *in vivo* into the hippocampus of either adult wild-type mice, to study the effect of *Wnt* signaling activation on normal cognitive function, or adult APP/PS1 transgenic mice, to study the protective effect of *Wnt* signaling activation against Aβ-induced cognitive deficit. The data presented below, strongly suggest that *Wnt* signaling activation up-regulates hippocampal cognitive function of adult mice, therefore contributing to reduce synaptic and memory impairments, linked to AD.

In vivo activation of *Wnt* signaling pathway enhances cognitive function of adult mice and reverses cognitive deficits in an Alzheimer's diseases model.

Abbreviated title: *Wnt* signaling improves cognitive function.

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Abstract

The role of *Wnt* signaling pathway during synaptic development has been well established. In the adult brain, different components of *Wnt* signaling are expressed, but little is known about its role in mature synapses. Emerging *in vitro* studies have implicated *Wnt* signaling in synaptic plasticity. Furthermore, activation of *Wnt* signaling has shown to protect against amyloid-β-induced synaptic impairment. The present study provides the first evidence that *in vivo* activation of *Wnt* signaling improves episodic memory, increases excitatory synaptic transmission and enhances long-term potentiation in adult wild-type mice. Moreover, the activation of *Wnt* signaling also rescues memory loss and improves synaptic dysfunction in APP/PS1 transgenic mice that model the amyloid pathology of Alzheimer's diseases. These findings indicate that *Wnt* signaling modulates cognitive function in the adult brain and could be a novel promising target for the Alzheimer's diseases therapy.

Introduction

Wnt ligands are secreted glycoproteins that participate as signaling molecules in diverse cellular processes (Nusse and Varmus, 2012). *Wnt* ligands signal through at least three different pathways: the canonical or *Wnt*/β-catenin pathway and the two non-canonical *Wnt*/JNK and *Wnt*/Ca^{2+} pathways (van Amerongen and Nusse, 2009). In addition to the important roles that *Wnt* signaling play during the nervous system development (Ciani and Salinas, 2005), *Wnt* ligands and other components of *Wnt* signaling are also expressed in most regions of the adult brain (Cerpa et al., 2008; Shimogori et al., 2004), but the role of this pathway in mature nervous system is still unclear.

Recent evidences suggest that *Wnt* ligands might modulate the efficacy of the excitatory and inhibitory synaptic transmission (Budnik and Salinas, 2011; Park and Shen, 2012). Indeed, *Wnt-7a* ligand enhances synaptic transmission by increasing the probability of neurotransmitter release in cerebellar and hippocampal synapse (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). While, *Wnt-5a* ligand potentiates synaptic transmission by promoting the clustering of postsynaptic proteins and stimulating dendrite spine morphogenesis (Cuitino et al., 2010; Farias et al., 2009; Varela-Nallar et al., 2010). Moreover, activation of *Wnt* signaling facilitates long-term potentiation (LTP), whereas blockage of *Wnt* signaling impairs it (Beaumont et al., 2007; Cerpa et al., 2011; Chen et al., 2006). These evidences leaded to postulate the hypothesis that *Wnt* signaling might play a key role in the modulation of synaptic plasticity at mature synapse.

On the other hand, deregulation of *Wnt* signaling has been implicated in Alzheimer's diseases (AD) pathology (Anderton et al., 2000; Boonen et al., 2009; De Ferrari and Inestrosa, 2000). AD is a neurodegenerative disorder characterized by a progressive deterioration of the cognitive functions (Selkoe, 2001; Sheng et al., 2012). A remarkable feature in the brains of AD patients is the accumulation of amyloid-β (Aβ) peptide, that is associated with synaptic failure at early stage and neuronal loss at later stages of the disease (Selkoe, 2002). Several studies have revealed a relationship between loss of *Wnt* signaling and Aβ-induced neurotoxicity (De Ferrari et al., 2003; Garrido et al., 2002; Zhang et al., 1998). In fact, blockage of *Wnt/*β-catenin signaling by the *Wnt* antagonist Dickkopf-1 (Dkk1), increases neuronal death and synaptic loss induced by Aβ (Caricasole et al., 2004; Purro et al., 2012). Conversely, activation of *Wnt/*β-catenin signaling by *Wnt-3a* ligand protects against Aβ-induced cytotoxic insults (Alvarez et al., 2004; Shruster et al., 2011). Moreover, lithium treatment, a pharmacological activator of *Wnt/*β-

catenin signaling, has shown to reduce memory loss in APP/PS1 mice, model of AD (Toledo and Inestrosa, 2010).

Here, we evaluated for the first time the *in vivo* activation of *Wnt* signaling in the hippocampus of adult wild-type and APP/PS1 mice. We found that chronic activation of *Wnt* signaling improves episodic memory, enhances basal excitatory synaptic transmission, and facilitates LTP in both mice. In summary, our results suggest that *Wnt* signaling not only plays a key role in synaptic plasticity of mature nervous system but it is also a promising therapeutic target for AD treatment.

Materials and Methods

Animals. The subjects were male of seven-months-old double transgenic APPswe/PSEN1DE9 mice (known as APP/PS1 in this study) and male age-matched wild-type littermates, purchased from Jackson Laboratory (Bar Harbor, ME). All the animals were housed in the Animal House Facility of P. Universidad Católica de Chile in temperature, humidity and light-controlled rooms, with food and water *ad libitum* until the end of the treatments. Procedures for animal care, surgery and slice preparation were in accordance with the guidelines for the care and use of laboratory animals adopted by the Society for Neuroscience.

Reagents. FOXY-5 (Formyl-MDGCEL) was obtained from Genemed Synthesis Inc. (South San Francisco, CA). JNK Inhibitor VII (TAT-TI-JIP$_{153-163}$) was obtained from EMD Millipore (Billerica, MA). TCS-183 was obtained from Tocris Bioscience (Bristol, UK). WASP-1 (2-(2,7-diethoxy-9H-fluoren-9-ylidene) hydrazinecarboximidamide), was obtained from Chemdiv Inc. (San Diego, CA).

Infusion system preparation. Animals received bilateral chronic infusion into the CA1 hippocampal region with: (a) WASP-1 (*Wnt*-activating small molecule), a potentiator of the canonical *Wnt* signaling, which require the activation of the signaling by endogenous *Wnt-3a* ligand (Beaumont et al., 2007), (b) TCS-183 a competitive inhibitor of GSK-3β (Ser9) phosphorylation, (c) FOXY-5 (formylated *Wnt-5a*-derived hexapeptide) an activator of the non-canonical *Wnt* signaling that mimics the effect of *Wnt-5a* ligand (Safholm et al., 2006; Safholm et al., 2008), (d) TAT-TI-JIP an cell-permeable JNK inhibitor, or (e) vehicle solution (artificial cerebrospinal fluid, ACSF). Sterile reagents were diluted in ACSF and 0.01% DMSO to reach a final concentration of 5 μM WASP-1, 300 μM TCS-183, 50 μM FOXY-5 and 1 μM TAT-TI-JIP. These concentrations were chosen since have been previously reported as effective to activate or inhibit *Wnt* signaling and to produce *in vitro* changes in the synaptic function (Beaumont et al., 2007; Cuitino et al., 2010; Farias et al., 2009; Peineau et al., 2007; Varela-Nallar et al., 2012). Chronic delivery was achieved via Alzet osmotic mini-pump (Alzet 1004, Durect Co., Cupertino, CA). The assembly and pre-incubation of the mini-pump were performed according to the manufacturer's instructions. Briefly, mini-pumps were filled and connected to a double brain infusion cannula (Plastics One Inc., Roanoke, VA) by catheter tubing (Durect Co., Cupertino, CA) and a bifurcation cannula (Plastics One Inc., Roanoke, VA). The assembled infusion system was incubated in sterile saline at 37 ºC for 48 h before surgery.

Surgical procedures. For implantation of the infusion system, APP/PS1 and wild-type mice were anesthetized using 1.5-2.5 % isoflurane. The head was shaved and the animal was placed in a small animal stereotaxic frame (Stoelting Co., Wood Dale, IL) with non-traumatic ear-bars to hold the skull in place. The skull was exposed from several millimeters anterior and posterior

to bregma and lambda. Bore holes were made above the left and right hippocampus (coordinates: -2.46 mm anterior to the bregma, ±1.0 mm lateral, -1.5 mm relative to dura mater). Brain infusion cannula was stereotactically inserted into the hippocampus and fixed to the skull surface using binary dental cement. The mini-pump was inserted beneath the skin at the dorsum of the animal and the wound was closed using cyanoacrylate. Body temperature during anesthesia was maintained at 37°C by means of an isothermal heating pad. Mice were allowed to recover for 1 week before testing. Infusion was applied up to 21 days.

Behavioral tests. For the *memory flexibility* test (Chen et al., 2000; Toledo and Inestrosa, 2010), infused animals were trained in a circular water maze (1.2 m diameter, opaque water, 50 cm deep, 19–21°C). To evaluate the performance of the mice in the water maze, we used the visible platform test (four trials of 60 s top, plus 10 s on platform at the end of trials). For test episodic memory, each animal was trained for one pseudo-random location of the platform per day, with the platform hidden 1 cm below water. Mice were trained for 4 days, with a new platform location each day. Training was conducted up to 15 trials per day, until the criterion of 3 successive trials with an escape latency of < 20 s was reached. Data were collected using a video tracking system for water maze (HVS Imagen, Hampton, UK). For the *novel object recognition* test (Hillen et al., 2010), mice were habituated to the experimental room in the experimental cages for 3 consecutive days (30 min each time and 1 h on testing day). Testing was performed in 25 cm × 25 cm opaque walled cages. During memory acquisition phase, mice were allowed to explore for 10 min, two identical objects (blue balls of 4 cm diameter). After this, animals were returned to their home cages for 2.5 h. During memory retention phase, mice were exposed for 10 min, to the presence of one familiar object and a new object (yellow cube of 4 cm^3).

Cages were routinely cleaned with ethanol before each testing/habituating of mice. Object preferences were analyzed using the Anymaze (Stoelting Co., Wood Dale, IL) video tracking software. Object preference index was determined by calculating the time spent near the novel object divided by the cumulative time spent with both familiar and novel objects.

Western Blot. Following behavioral test, hippocampus of treated and control mice were dissected and homogenized in RIPA buffer using a Potter homogenizator. Samples were centrifuged at 14000 r.p.m. at 4 ºC for 5 min. Supernatants were used. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL). Equal amounts of protein of each sample were resolved by 10% SDS-PAGE. Proteins were transferred to PVDF membranes, and immunoblotting was done using mouse anti-PSD-95, mouse anti-NR2B (Neuromab, Davis, CA), rabbit anti-Syn-1, mouse anti-β-catenin, mouse anti-c-myc, mouse anti-CaMKII, mouse anti-phospho-CaMKII (Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-Syp, rabbit anti-GSK-3β, rabbit anti-phospho-Ser9-GSK-3β, rabbit anti-JNK, rabbit anti-phospho-JNK, mouse anti-β-actin (Cell Signaling Technology Inc., Danvers, MA) antibodies. Immunoreactivity was visualized using a chemiluminescent substrate (Thermo Fisher Scientific Inc., Rockford, IL), and optical densities were quantified using NIH ImageJ software.

Electrophysiological recording. Slices preparation and field electrophysiological recordings were performed as previously described (Bonansco et al., 2011; Cerpa et al., 2008). Hippocampal slices were prepared from 15-20 days infused mice. Animals were decapitated and the brain was removed and immediately submerged in cold ACSF (~ 4 ºC) bubbled with

carbogen (95% O_2, 5% CO_2). Coronal slices from the hippocampus (250 μm) were cut using a vibroslice microtome (World Precision Instruments Inc., Sarasota, FL) and incubated in carbogen-bubbled ACSF for 1 h at room temperature (22-24 °C). Slices were transferred into a chamber and superfused (3 ml/min, at 22–26 °C) with gassed ACSF. In all experiments picrotoxin (10 μM) was added to ACSF perfusion solution to suppress inhibitory ϒ-aminobutyric acid (GABA), type-A transmission. Recording of field excitatory postsynaptic potentials (fEPSP) was carried out with a glass pipette (2–4 megaohms, filled with ACSF), placed in the middle of stratum radiatum of CA1, and connected to an AC amplifier (P-5 Series, Grass), with gain 10,000 ×, low pass filter 3.0 kHz, and high pass filter 0.30 Hz. A bipolar concentric electrode (platinum/iridium, 125 μm outer diameter, FHC Inc.) was placed in the stratum radiatum at 100–200 μm away from the recording site. Schaeffer collaterals fibers were activated by bipolar cathodic stimulation, generated by a stimulator (Master 8, AMPI) connected to an isolation unit (Isoflex, AMPI). Electric pulses (50 μs, 0.3 Hz, 20–100 μA) were applied on Schaeffer collaterals. Basal excitatory synaptic transmission was measured by using an *input/output curve* protocol (Hsia et al., 1998), consisting of eight stimuli ranging from 200 to 900 μA (interval between stimuli 10 s). To elicit LTP in the adult mice we used a *high frequency stimulation* (HFS) protocol (Hu et al., 2006; Maglio et al., 2006) consisting of two trains of 100 Hz for 500 ms separated by 1s, applied after at least 15 min of stable baseline recordings. Analyses of fEPSPs were performed with Clampfit 10 (pCLAMP, Molecular Devices) software.

Statistical analysis. Data was expressed as mean ± S.E. of the values from the number of experiments as indicated in the corresponding figures. Data was evaluated statistically by using

a Student's *t*-test or ANOVA with Tukey's post hoc test to determine differences among more than two groups. Differences were considered significant at $*p<0.01$ or $**p<0.001$.

Results

WASP-1 and FOXY-5 activate *Wnt* signaling *in vivo*. In order to study the role of *Wnt* signaling in the cognitive function of adult mice, we used WASP-1, a potentiator of *Wnt*/β-catenin signaling (Beaumont et al., 2007) and FOXY-5, an activator of both *Wnt*/JNK and *Wnt*/Ca^{+2} signaling (Cuitino et al., 2010; Farias et al., 2009; Varela-Nallar et al., 2012). Administration of WASP-1, FOXY-5 or ACSF (control) was carried out into the hippocampus of infused animals. Chronic treatment with WASP-1 increases hippocampal levels of phospho-Ser9-GSK-3β, β-catenin and c-myc (Fig. 1*A*). The WASP-1-induced increase of phospho-Ser9-GSK-3β, an inhibitory form of glycogen synthase kinase-3β (GSK-3β, a component of the β-catenin destruction complex), might lead to an increment in the levels of β-catenin that could account for the increment of c-myc, a target gene of *Wnt*/β-catenin signaling. The occurrence of these events suggests that infusion of WASP-1 *in vivo* activates *Wnt*/β-catenin signaling. On the other hand, the intra-hippocampal infusion of FOXY-5 increases JNK phosphorylation without changing the phosphorylation state of the Ca^{2+}-sensitive protein calmodulin-dependent protein kinase II (CaMKII) or the β-catenin levels (Fig. 1*B*), indicating that FOXY-5 treatment activates *Wnt*/JNK signaling *in vivo*.

WASP-1 and FOXY-5 improve episodic memory. Recent reports have found that activation of *Wnt*/β-catenin signaling can modulate amygdala-dependent fear memory formation (Maguschak and Ressler, 2008, 2011). We hypothesized that activation of *Wnt* signaling could

also participate in hippocampal-dependent learning and memory processes. To determine whether WASP-1 and FOXY-5 have an effect on memory, we compared memory acquisition and retention abilities of treated and control wild-type (WT) mice in tests for episodic memory. We first assessed the performance of mice in a visible platform test, variant of the Morris water maze (MWM) to test for baseline differences of visual and motivational performance (Okun et al., 2010). WASP-1, FOXY-5 and control mice exhibit similar escape latencies for the visible platform test (Fig. 1*G*) suggesting no baseline differences in vision or motivation among groups. In order to measure hippocampus-dependent episodic memory we tested the mice in a memory flexibility test (Chen et al., 2000). Animals treated with WASP-1 and FOXY-5 show a reduction in the number of trials to reach criterion in comparison to control mice (Fig. 1*C*). The average of the four days of training indicates that WASP-1 and FOXY-5 treatments cause a significant reduction in the number of trials to reach criterion (Fig. 1*D*). Indeed, WASP-1 and FOXY-5 treated animals showed improved strategies to reach the platform, compared to control mice (Fig. 1*E*). The fact that there are no differences in swim speed among WASP-1, FOXY-5 and control groups (Fig. 1*F*), indicates that the reduction in the number of trials to reach criterion observed in WASP-1 and FOXY-5 treated mice is due to memory improvement.

We also test the mice for novel object recognition, to evaluate non-spatial short-term memory of a novel object in a familiar setting (Bevins and Besheer, 2006). No significant difference in total exploration time during object familiarization among control, WASP-1 and FOXY-5 treated mice (Fig. 1*H*) was observed. However, WASP-1 and FOXY-5 treated mice exhibit significantly higher preference for a novel object than control mice during the memory retention phase of the test (Fig. 1*I*).

In summary, these results suggest that chronic *Wnt* signaling activation enhances short-term memory retention improving episodic memory in adult mice. This is consistent with previous evidence indicating that an increase on hippocampal levels of *Wnt* ligands would serve to enhance consolidation of new memories (Tabatadze et al., 2012).

WASP-1 and FOXY-5 enhance synaptic function and plasticity. Recent studies have implicated *Wnt* signaling in synaptic plasticity of the mature nervous system (Budnik and Salinas, 2011; Inestrosa and Arenas, 2010). Electrophysiological recordings of hippocampal slices or cultured neurons suggest that *Wnt* ligands can enhance synaptic transmission and increase the magnitude of LTP (Ahmad-Annuar et al., 2006; Beaumont et al., 2007; Cerpa et al., 2011; Cerpa et al., 2008; Cuitino et al., 2010; Chen et al., 2006; Varela-Nallar et al., 2010). Now, we evaluate the effect of *in vivo Wnt* signaling activation on excitatory synaptic transmission and LTP in adult WT mice. First we tested whether WASP-1 and FOXY-5 treatments could change basal excitatory synaptic transmission by using an input/output analysis. Since the activation of *Wnt* signaling has shown to increase the number of excitatory synapses (Ciani et al., 2011; Farias et al., 2009; Gogolla et al., 2009; Varela-Nallar et al., 2010), we hypothesized that WASP-1 and FOXY-5 treatments will enhance synaptic responses if the increase in synapse density corresponds to the formation of functional synapses. Therefore, we compared fEPSP input/output relations in WASP-1 and FOXY-5 treated mice versus control mice (Fig. 2). In Fig. 2*A* superimposed field responses of WASP-1, FOXY-5 and control animals are shown. For equal fiber volley amplitudes (arrow) the elicited synaptic response are larger in WASP-1 and FOXY-5 treated mice (Fig. 2*A*). As Fig. 2*B* shows, for given fiber volley amplitudes comparatively more robust synaptic responses are exhibit by WASP-1 and FOXY-

5 treated mice than control mice. Since the fiber volley is a measure of the number of axons activated (Hsia et al., 1998), the observed increase in the magnitude of fEPSP amplitude suggest that WASP-1 and FOXY-5 treatments enhance synaptic strength between CA3-CA1 hippocampal connection. Indeed, WASP-1 and FOXY-5 treatments display enhanced input/output relationship in response to increasing stimulus intensity (Fig. 2*D*). Measurements of fEPSP amplitude showed that WASP-1 and FOXY-5 treated mice exhibit a significant increase of basal synaptic transmission in comparison to control mice (Fig. 2*C*, 2*D*). For example, at the highest stimulus intensity tested (900 μA) the mean value of fEPSP were 5.04 ± 0.22 mV, 5.86 ± 0.23 mV and 3.84 ± 0.28 mV in WASP-1 (n = 10 slices/ 6 mice), FOXY-5 (n = 10 slices/ 5 mice) and control (n = 10 slices/ 7 mice) animals, respectively. As shown in Fig. 2C, for several stimulus intensities, control mice exhibit synaptic responses of similar amplitude to that of the fiber volley, while WASP-1 and FOXY-5 treatments evoke synaptic responses of larger amplitude than the accompanying fiber volley. In fact, the fiber volley amplitude elicited by a given stimulus intensity are decreased in WASP-1 and FOXY-5 treated mice (Fig. 2E), suggesting that less axon fibers than in control mice should be activated to elicit synaptic responses of similar amplitude. Altogether, these findings strongly suggest that *in vivo* activation of *Wnt* signaling induces an enhancement of the basal excitatory synaptic strength.

Considering the effects found on behavioral performance (Figs. 1*C* - 1*I*) and the fact that chronic activation of *Wnt* signaling increases the efficacy of basal synaptic transmission (Fig. 2), we next investigated the effect of WASP-1 and FOXY-5 treatments on synaptic plasticity. Using a high frequency stimulation (HFS) protocol we studied the effect of WASP-1 and FOXY-5 on hippocampal LTP. In Fig. 3A, superimposed field responses of WASP-1 and FOXY-5 treated mice show that HFS evokes fEPSP of larger amplitude than in control mice.

As the fEPSP monitored for 10 min before HFS were generally stable, their mean value was determined as the baseline (dotted line in Fig. 3*B*, 3*C*). A robust potentiation of the synaptic responses were produced and maintained for over 90 min after HFS in control (n = 10 slices/ 7 mice) animals (Fig. 3*B*). Interestingly, fEPSP amplitudes evoked by the same paradigm, were comparatively larger in WASP-1 (n = 9 slices/ 6 mice) and FOXY-5 (n = 9 slices/ 5 mice) treated mice than in control animals (Fig. 3*B*). Moreover, this effect was sustained for more than 1 h after LTP induction (Fig. 3*B*). Thus, the LTP magnitude at 30 or 90 minutes after HFS was significantly enhanced in WASP-1 (148.12 ± 2.68 % and 147.94 ± 2.04 % respectively) and FOXY-5 (162.44 ± 1.94 % and 158.44 ± 5.02 % respectively) than in control (121.54 ± 2.24 % and 129.18 ± 1.90 % respectively) mice (Fig. 3*C*). Increased potentiation of fEPSP in both WASP-1 and FOXY-5 treatments compared to control, suggest that chronic activation of *Wnt* signaling facilitates long-term synaptic plasticity at the Schaffer collateral-CA1 synapses.

In order to determine possible downstream effectors of the *Wnt* pathway involved in the effects elicited by WASP-1 and FOXY-5 treatments on hippocampal LTP, we performed chronic infusions of *Wnt* inhibitors in the presence and absent of WASP-1 or FOXY-5. We used TCS-183, a competitive inhibitor of the inactivating GSK-3β phosphorylation, to block *Wnt*/β-catenin pathway. In Fig. 4*A* representative field responses of WASP-1 (n = 9 slices/ 6 mice), TCS-183 (n = 6 slices/ 3 mice) and TCS-183 plus WASP-1 (n = 6 slices/ 3 mice) treated mice, before and after HFS, are shown. The co-infusion of TCS-183 plus WASP-1 significantly decrease LTP magnitude in comparison to the infusion of WASP-1 alone (Figs. 4*A* - 4*C*). In fact, LTP magnitude at 30 or 60 min after HFS was significantly reduced in TCS-183 plus WASP-1 (111.23 ± 8.96 % and 102.21 ± 5.97 % respectively) than in WASP-1 (148.12 ± 2.68 % and 147.89 ± 1.76 % respectively) mice (Fig. 4*C*). Interestingly, infusion of TCS-183 alone

was able to block LTP, indicating that blocking the inhibition of endogenous GSK-3β is sufficient to impair hippocampal LTP (Figs. 4*A* - 4*C*), which is in accordance to previous evidence showing that inhibition of GSK-3β facilitates the induction of LTP (Hooper et al., 2007; Peineau et al., 2007). On the other hand, to establish a possible effector implicated in the effect of FOXY-5 on LTP, we used the JNK inhibitor, TAT-TI-JIP, since we found that chronic treatment with FOXY-5 activates *Wn*t/JNK pathway but no *Wnt*/Ca^{2+} pathway (Fig. 1*B*). In Fig. 4*D* representative field responses of FOXY-5 (n = 9 slices/ 5 mice), TAT-TI-JIP (n = 6 slices/ 3 mice) and TAT-TI-JIP plus FOXY-5 (n = 5 slices/ 3 mice) treated mice, before and after HFS, are shown. Both treatments, TAT-TI-JIP alone and TAT-TI-JIP plus FOXY-5 significantly decrease LTP magnitude in comparison to FOXY-5 treatment (Figs. 4*D* - 4*F*). Indeed, LTP magnitude at 30 or 60 min after HFS was significantly reduced in TAT-TI-JIP (120.94 ± 3.52 % and 116.29 ± 5.43 % respectively) and TAT-TI-JIP plus FOXY-5 (109.37 ± 4.27 % and 108.42 ± 1.62 % respectively) compared to FOXY-5 (162.44 ± 1.94 % and 166.67 ± 5.17 % respectively) mice (Fig. 4*E*). The fact that infusion of TAT-TI-JIP alone causes blockage of LTP, indicates that JNK activity is necessary for LTP induction, as has been previously proposed (Chen et al., 2005; Seo et al., 2012). Interestingly, co-infusion of TAT-TI-JIP did not block the effect of WASP-1 on LTP, as well as the co-infusion of TCS-183 did not cause significant changes over the effect of FOXY-5 (data not shown). These results suggest that GSK-3β could be a downstream effector of WASP-1 but not FOXY-5, while JNK could be mediating the *in vivo* effects of FOXY-5 but it is not involved in the effects of WASP-1. Together with data shown in Figs. 1*A* and 1*B*, these findings indicate that WASP-1 selectively activates *Wnt*/β-catenin pathway, while FOXY-5 activates *Wnt*/Ca^{2+} pathway.

Our findings are consistent with previous *in vitro* studies indicating that *Wnt* ligands can modulate synaptic transmission and plasticity (Budnik and Salinas, 2011; Inestrosa and Arenas, 2010). Here, we demonstrated that *in vivo* activation of *Wnt* signaling increase synaptic efficacy of excitatory synapses and reduce the threshold for LTP induction in the adult hippocampus. Moreover, we show here that endogenous activation of *Wnt* signaling is required for induction and maintenance of hippocampal LTP, since infusion of *Wnt* inhibitors alone, were able to impair LTP without affecting post-tetanic potentiation (Fig. 4*B*, 4*C*).

Since the activation of *Wnt* signaling can regulate the expression of genes that are involved in memory and synaptic plasticity (Arrazola et al., 2009), it is possible that the effects of WASP-1 and FOXY-5 on hippocampal LTP could be mediated by the modulation of gene expression. Actually, previous studies have shown that late phase LTP (l-LTP) requires protein translation and/or gene transcription (Kelleher et al., 2004; Raymond, 2007; Richter and Klann, 2009). Using acute hippocampal slice preparation, we tested the effects of WASP-1 and FOXY-5 on the LTP magnitude, in the presence or absence of a gene transcription inhibitor, Actinomycin-D (AMD, 25 μM), and the protein synthesis inhibitor, Cycloheximide (CH, 40 μg/ml). In accordance to previous reports, we found that perfusion of AMD or CH alone affects l-LTP maintenance (Table 1). Interestingly, transcription inhibition only impairs FOXY-5-induced LTP at 120 min after HFS application, but it does not affect WASP-1-induced LTP at any time assayed; however, translation inhibition impairs LTP induced by either WASP-1 or FOXY-5 treatments only at 120 min after HFS application (Table 1). These results indicate that the effect of WASP-1 on the maintenance of l-LTP is dependent on protein synthesis, but it does not involve gene transcription, while, the effect of FOXY-5 requires both.

WASP-1 and FOXY-5 increase synaptic protein levels. Previous studies have shown that activation of *Wnt* signaling promotes the assembly of pre- and postsynaptic sites in developing and mature synapses (Ciani and Salinas, 2005; Farias et al., 2010). Indeed, canonical *Wnt* ligands have been implicated in the assembling of presynaptic proteins (Inestrosa and Arenas, 2010). Thus, *Wnt-7a* ligand induces the clustering of the α_7-nicotinic acetylcholine receptor (α_7-nAChR), SV2, Synaptotagmin (Syt) (Farias et al., 2007), Synapsin-1 (Syn-1) (Hall et al., 2000) and Synaptophysin (Syp) (Cerpa et al., 2008), while *Wnt-7b* increases the number of clusters of Bassoon, VAMP2 and Syt (Ahmad-Annuar et al., 2006). Conversely, non-canonical *Wnt* ligand, *Wnt-5a*, promotes the clustering of postsynaptic proteins as the GABA-$_A$ receptor (Cuitino et al., 2010) or postsynaptic density protein-95 (PSD-95) (Farias et al., 2009). Here, we studied whether chronic treatment with WASP-1 and FOXY-5, also has an effect on hippocampal synaptic proteins levels of adult mice. As shown in Fig. 5*A*, WASP-1 treatment significantly enhanced the levels of the presynaptic proteins Syp and Syn-1, as well as the postsynaptic protein *N-Methyl-D-aspartate* (NMDA) receptor subunit 2B (NR2B) without affecting the levels of PSD-95. Instead, FOXY-5 treatment increased the levels of NR2B protein but did not induce changes on the levels of PSD-95 or any assayed presynaptic protein (Fig. 5*B*). These results indicate that chronic activation of *Wnt* signaling might modulate synaptic structure of pre- and postsynaptic sites *in vivo*. Even though, FOXY-5 treatment only affected the levels of the postsynaptic protein NR2B, the activation of canonical *Wnt* signaling by WASP-1 (Fig. 1*A*) raised the levels not only of presynaptic but also postsynaptic proteins. This dual effect on the synaptic structure could be responsible for the functional changes observed in the hippocampal CA3-CA1 synapses (Fig. 2, 3).

WASP-1 and FOXY-5 rescue cognitive impairment in adult APP/PS1 mice. Several studies suggest that *Wnt* signaling is involved in AD pathology (Caricasole et al., 2004; De Ferrari et al., 2007; Toledo and Inestrosa, 2010; Zhang et al., 1998). Moreover, numerous *in vitro* studies have shown that activation of *Wnt* signaling has a neuroprotective effect against Aβ-induced synaptic damage (Alvarez et al., 2004; Cerpa et al., 2010; De Ferrari et al., 2003; Shruster et al., 2011). To evaluate the effect of *in vivo Wnt* signaling activation on cognitive impairment associated to AD, we used here APP/PS1 double transgenic mice that coexpress mutant APPswe (K595N/M596L) and PSEN1 (exon 9 deletion of the presenilin-1 gene). The episodic memory performance in APP/PS1 mice begins to be affected as early as three-months-old, while spatial memory is impaired only after six-months-old (Trinchese et al., 2004). Therefore, to address whether WASP-1 and FOXY-5 treatments can rescue non-spatial episodic memory decline, we examined behavioral performance of seven-months-old APP/PS1 mice in the novel object recognition task. Exploration time during familiarization of the mice to the objects did not differ significantly among control, WASP-1 and FOXY-5 treated APP/PS1 mice (Fig. 6*A*). Upon introduction of a novel object, WASP-1 and FOXY-5 treated APP/PS1 mice exhibited stronger preference for the novel object than control APP/PS1 mice (Fig. 6*B*). Remarkably, performance of WASP-1 and FOXY-5 treated mice is similar to that of WT animals (Fig. 6*B*), indicating that chronic activation of *Wnt* signaling successfully overcome memory impairment in adult APP/PS1 mice. Although, APP/PS1 mice treated with WASP-1 and FOXY-5 almost reached control WT performance, still exhibited lower preference index than treated WT mice (Fig. 6*C*).

Defective learning and memory in APP/PS1 mice has been associated with synaptic plasticity deficits (Trinchese et al., 2004). Indeed, APP/PS1 mice show decreasing on basal synaptic transmission at late ages of five to six-months-old, whereas hippocampal LTP

beginning to impair as early as three-months-old (Trinchese et al., 2004). Thus, we next investigated whether WASP-1 and FOXY-5 treatment could also rescue synaptic function deficit in seven-months-old APP/PS1 mice. In order to do that, we initially looked for changes of basal synaptic transmission between CA3-CA1 neurons in hippocampal slices of APP/PS1 mice treated with WASP-1 and FOXY-5. We found that synaptic strength in WASP-1 and FOXY-5 treated mice is increased compared to control mice (Fig. 7*B*), since larger synaptic responses are evocated by an equal fiber volley amplitude (Fig. 7*A*). In Fig. 7*C*, representative traces of fEPSP show changes in the amplitude induced by WASP-1 and FOXY-5 treatments in comparison to APP/PS1 and WT control mice. With these treatments, an enhanced input/output correlation is observed upon increasing stimulus intensity (Fig. 7*D*). For the highest intensity assayed (900 μA), the mean fEPSP amplitude registered for APP/PS1 control (n = 8 slices/ 6 mice) mice was 3.50 ± 0.3 mV, while for WT control (n = 10 slices/ 7 mice) mice was 3.84 ± 0.28 mV, showing not significant differences between APP/PS1 and WT control mice. Notably, fEPSP amplitude in WASP-1 (n = 8 slices/ 5 mice) and FOXY-5 (n = 8 slices/ 4 mice) treated animals significantly enhanced up to 4.54 ± 0.30 mV and 4.72 ± 0.26 mV, respectively. Even though, we found not differences in the input/output relations of APP/PS1 and WT control mice, both WASP-1 and FOXY-5 treatments significantly improved basal excitatory synaptic strength in APP/PS1 mice. On the other hand, the mean fiber volley amplitude was significantly decreased by WASP-1 and FOXY-5 treatments (Fig. 7*E*), indicating that less synaptic fibers were activated by the same stimuli. This decreasing in the fiber volley amplitude induced by WASP-1 and FOXY-5 treatments almost reaches values near to those of the WT mice. These data suggest that WASP-1 and FOXY-5 treatments effectively improve basal excitatory synaptic transmission in APP/PS1 mice.

Since LTP is reduced in the adult APP/PS1 mouse (Trinchese et al., 2004), we studied next the ability of WASP-1 and FOXY-5 treatments to undergo plastic changes on impaired hippocampal synapses of seven-months-old APP/PS1 mice. In Fig. 8*A*, representative wave form changes of fEPSP, before and after HFS, are shown for control WT (n = 10 slices/ 7 mice) and WASP-1 (n = 8 slices/ 5 mice), FOXY-5 (n = 8 slices/ 4 mice) and control (n = 10 slices/ 6 mice) APP/PS1 groups. As shown in Fig. 8*B*, WASP-1 and FOXY-5 consistently increased the LTP magnitude of APP/PS1 treated mice in comparison to APP/PS1 control mice. Interestingly, this induced enhancement of synaptic potentiation was maintained for more than 1 h after the application of HFS protocol (Fig. 8*B*, 8*C*). Indeed, the LTP magnitude at 30 or 70 minutes after HFS was higher in WASP-1 (122.00 ± 3.22 % and 120.82 ± 3.08 % respectively) and FOXY-5 (130.95 ± 4.05 % and 127.04 ± 4.84 % respectively) than in control (101.28 ± 5.82 % and 100.28 ± 4.62 % respectively) APP/PS1 mice (Fig. 8*C*). The analysis of fEPSP amplitudes at 30 and 70 min after induction of LTP reveals that WASP-1 and FOXY-5 treatments significantly rescue LTP deficit observed in APP/PS1 control mice, reaching similar values to those shown by WT (121.54 ± 2.24 % at 30 min and 127.75 ± 1.60 % at 70 min after HFS) mice (Fig. 8*C*). These results indicate that loss of synaptic function in AD pathology could be recovered by WASP-1 and FOXY-5 treatments. However, a comparison of the mean LTP magnitude between APP/PS1 and WT mice treated either with WASP-1 (Fig. 8*D*) or FOXY-5 (Fig. 8*E*), shows that rescue of LTP impairment in APP/PS1 mice is not sufficient to reach those levels exhibited by WT treated animals.

Together, our findings suggest that chronic activation of *Wnt* signaling in adult APP/PS1 mice could reverse the hippocampus-dependent memory deficits and rescue synaptic impairments. Therefore, we propose *Wnt* activators as a promising therapy for AD treatment.

Discussion

Even though, several *in vitro* data suggest that *Wnt* signaling participate in synaptic plasticity of the adult brain (Budnik and Salinas, 2011; Inestrosa and Arenas, 2010; Rosso and Inestrosa, 2013)**,** until now there were not *in vivo* studies that investigated whether *Wnt* signaling has a role in the adult hippocampal cognitive function. Here we provided the first evidence that *in vivo* activation of *Wnt* signaling enhances synaptic plasticity and improves memory in adult mice. Moreover, we found that activation of *Wnt* signaling can rescue synaptic and memory impairments in adult APP/PS1 mice. These results suggest that *Wnt* signaling could have a role in synaptic function that underlies memory in the adult brain.

***Wnt* signaling could be an important pathway regulating memory of adults.** The role of *Wnt* signaling in adult memory has been previously suggested (Oliva et al., 2013). Recent studies have implicated *Wnt* signaling in the amygdala-dependent fear memory consolidation and the regulation of hippocampal long-term information storage (Maguschak and Ressler, 2011; Tabatadze et al., 2012). In fact, the infusion of Dkk1 into the amygdala has shown to impair fear memory consolidation (Maguschak and Ressler, 2011), while infusion of Dkk1 into the hippocampus impairs object recognition memory consolidation (Fortress et al., 2013). In addition, other proteins of *Wnt* signaling have been involved in memory processing. For instance, adenomatous polyposis coli (APC) heterozygous knockout mice display age-dependent working memory deficits (Koshimizu et al., 2011).

Here, we provide evidences that *Wnt* signaling also enhances short-term information storage of both spatial and non-spatial memories in adult mice. Remarkably, we found that not only the activation of *Wnt*/β-catenin signaling has an effect on memory consolidation, as it has

been previously reported (Fortress et al., 2013; Maguschak and Ressler, 2008), since the activation of non-canonical *Wnt* signaling by FOXY-5 has a similar effect.

***Wnt* signaling is involved in synaptic plasticity of the adult brain.** LTP, an activity-dependent enhancement of synaptic strength, is considered one of the physiological mechanisms that underlie learning and memory in the hippocampus (Citri and Malenka, 2008). Recent studies have shown that activation of *Wnt* signaling facilitates hippocampal LTP, whereas blockade of this signaling impairs it (Cerpa et al., 2011; Chen et al., 2006). Furthermore, *Wnt* signaling activation also increases excitatory synaptic transmission in hippocampal neurons (Beaumont et al., 2007; Cerpa et al., 2008; Varela-Nallar et al., 2010). These findings suggest a role for *Wnt* signaling in regulation of synaptic plasticity on mature synapses, but *in vivo* studies are still required. Here we present evidences that *in vivo* activation of *Wnt* signaling into the adult hippocampus results in an enhancement of basal excitatory synaptic efficacy as well as the LTP magnitude.

Previously, our lab showed that both canonical and non-canonical *Wnt* ligands are able to increase hippocampal excitatory synaptic transmission (Cerpa et al., 2011; Cerpa et al., 2008; Varela-Nallar et al., 2010). Consistent with this observation, we show here that *in vivo* activation of canonical and non-canonical *Wnt* signaling equally enhance basal excitatory transmission of adult mice. Even though canonical and non-canonical *Wnt* ligands have the same effect on excitatory synaptic transmission, it seems that different mechanisms are involved in each effect. For instance, canonical *Wnt-7a* ligand modulates presynaptic vesicles recycling and neurotransmitters release through a mechanism that diverges from the transcription-dependent pathway (Cerpa et al., 2008), whereas non-canonical *Wnt-5a* ligand regulates the postsynaptic

structure by promoting the formation of new spines or increasing the size of preexisting ones, in a Ca^{2+}-dependent manner (Varela-Nallar et al., 2010).

In addition, it has been reported that either canonical *Wnt-3a* or non-canonical *Wnt-5a* ligands facilitates the induction of LTP (Cerpa et al., 2011; Chen et al., 2006). Here we compared the effect of chronic activation of both canonical and non-canonical *Wnt* pathways on hippocampal LTP. Our data show that although both WASP-1 and FOXY-5 treatments increase the LTP magnitude, FOXY-5 has a stronger effect than WASP-1. It is possible that these differences arise from dissimilar mechanism involved in the activation of the pathway and/or in the effects downstream. Indeed, while FOXY-5 directly activates non-canonical *Wnt* signaling (Safholm et al., 2006), WASP-1 requires the presence of *Wnt-3a* ligand to synergistically activate canonical *Wnt* signaling (Beaumont et al., 2007), suggesting that endogenous *Wnt-3a* ligand is locally released at the hippocampus and allows to WASP-1 exerts its effect. Besides, the effects of canonical and non-canonical Wnt signaling on synaptic plasticity occur through different mechanisms. Thus, canonical *Wnt* ligands could regulate synaptic plasticity by influencing neurotransmitters release through modulation of the interaction between proteins of *Wnt* pathway and molecules implicated in synaptic plasticity and memory formation. In fact, it has been reported the interaction between β-catenin and cadherins (Maguschak and Ressler, 2008), Dvl and Syt (Kishida et al., 2007), or APC and presynaptic α_7-nAChRs (Farias et al., 2007). Conversely, non-canonical *Wnt* ligands could modulate synaptic plasticity by inducing a rapid increase in the intracellular Ca^{2+} concentrations and the activation of CaMKII (Cerpa et al., 2011; Varela-Nallar et al., 2010).

Moreover, the effects of *Wnt* signaling on synaptic plasticity are also mediated by the modulation of gene expression. Indeed, we found here that the effect of WASP-1 on l-LTP

depends on synthesis of new proteins, but it does not depend on new RNA synthesis, while the effect of FOXY-5 on l-LTP requires both transcription and translation (Table 1). Following the categorization of LTP in LTP1, LTP2 and LTP3 in accordance to persistence and the mechanisms involved in each form (Racine et al., 1983; Raymond and Redman, 2006), WASP-1 seems to elicit LTP2, which is only dependent on protein synthesis from pre-existing mRNA located in the dendrites; while FOXY-5-induced LTP is consistent with LTP3, since both protein translation and gene transcription are required to l-LTP maintenance. These results suggest that the mechanisms elicited by canonical and non-canonical *Wnt* signaling to influence hippocampal l-LTP, have different requirements.

***Wnt* signaling is implicated in the remodeling of synaptic structures.** Since structural modifications of synaptic sites are associated and might be required for synaptic plasticity and memory (De Roo et al., 2008), *Wnt* signaling could therefore play a role in synaptic remodeling of mature synapses. Supporting this hypothesis, our results show that *in vivo* activation of *Wnt* signaling induces an improvement on hippocampal cognitive function in adult mice that correlates with an increase in synaptic protein expression. Previously, it was proposed that canonical *Wnt* ligands selectively modulate presynaptic sites, while non-canonical *Wnt* ligands would modulate the postsynaptic structure (Cerpa et al., 2009). However, we found here that activation of canonical *Wnt* signaling could change the levels of synaptic proteins at both pre- and postsynaptic sites. This dual effect has been previously reported for canonical *Wnt-7a* ligand: on one hand, a role for *Wnt-7a* ligand in promoting the clustering of several presynaptic proteins has been demonstrated (Cerpa et al., 2008; Farias et al., 2007); on the other hand, recent evidences suggest that *Wnt-7a* ligand also increases the density and maturity of dendritic spines

(Ciani et al., 2011; Gogolla et al., 2009). This dual effect could be explained assuming that a single *Wnt* ligand can activate different signaling branches. Indeed, *Wnt-3a* ligand activates both canonical and non-canonical pathways, but the type of *Wnt* signaling activated depends on ligand concentration (Nalesso et al., 2011).

***Wnt* signaling dysfunction in AD.** There are several components of *Wnt* pathway that are altered in AD (Inestrosa et al., 2012; Moon et al., 2004). Indeed, β-catenin levels in AD brains are considerably downregulated (Zhang et al., 1998), while the expression of Dkk1 is upregulated (Caricasole et al., 2004) compared to healthy brains. Moreover, a recent study has shown that Aβ-induced Dkk1 expression depends on clusterin, a susceptibility factor for late-onset AD (Killick et al., 2012). In addition, several *in vitro* studies have demonstrated a relationship between loss of *Wnt* signaling and Aβ neurotoxicity (Alvarez et al., 2004; Purro et al., 2012). In fact, Aβ has shown to inhibit *Wnt*/β-catenin signaling by directly binding to several Frizzled receptors (Magdesian et al., 2008).

The activation of canonical *Wnt* signaling protects against Aβ-dependent cytotoxic effects in hippocampal cultured neurons (Alvarez et al., 2004) and reduces spatial memory deficit and Aβ deposition in APP/PS1 mice (Toledo and Inestrosa, 2010). Also, the activation of non-canonical *Wnt* signaling by *Wnt-5a* ligand prevents Aβ-induced depression of excitatory synaptic transmission in hippocampal slices (Cerpa et al., 2010).

Here we add evidences that activation of both canonical and non-canonical *Wnt* signaling enhance basal synaptic transmission and rescue hippocampal LTP impairment in adult APP/PS1 mice. Moreover, *in vivo* activation of *Wnt* signaling also improved short-term memory of APP/PS1 mice, as has been proposed in other models of memory deficits (Guo et al., 2012; King

et al., 2013) . However, from our results it is not possible to conclude whether rescue of memory and synaptic deficits by activation of *Wnt* signaling occurs by a direct action on mechanisms impaired by Aβ, or whether it is an additive effect independent of Aβ-induced damage, therefore, more studies are required to clarify this issue.

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Figure 1.

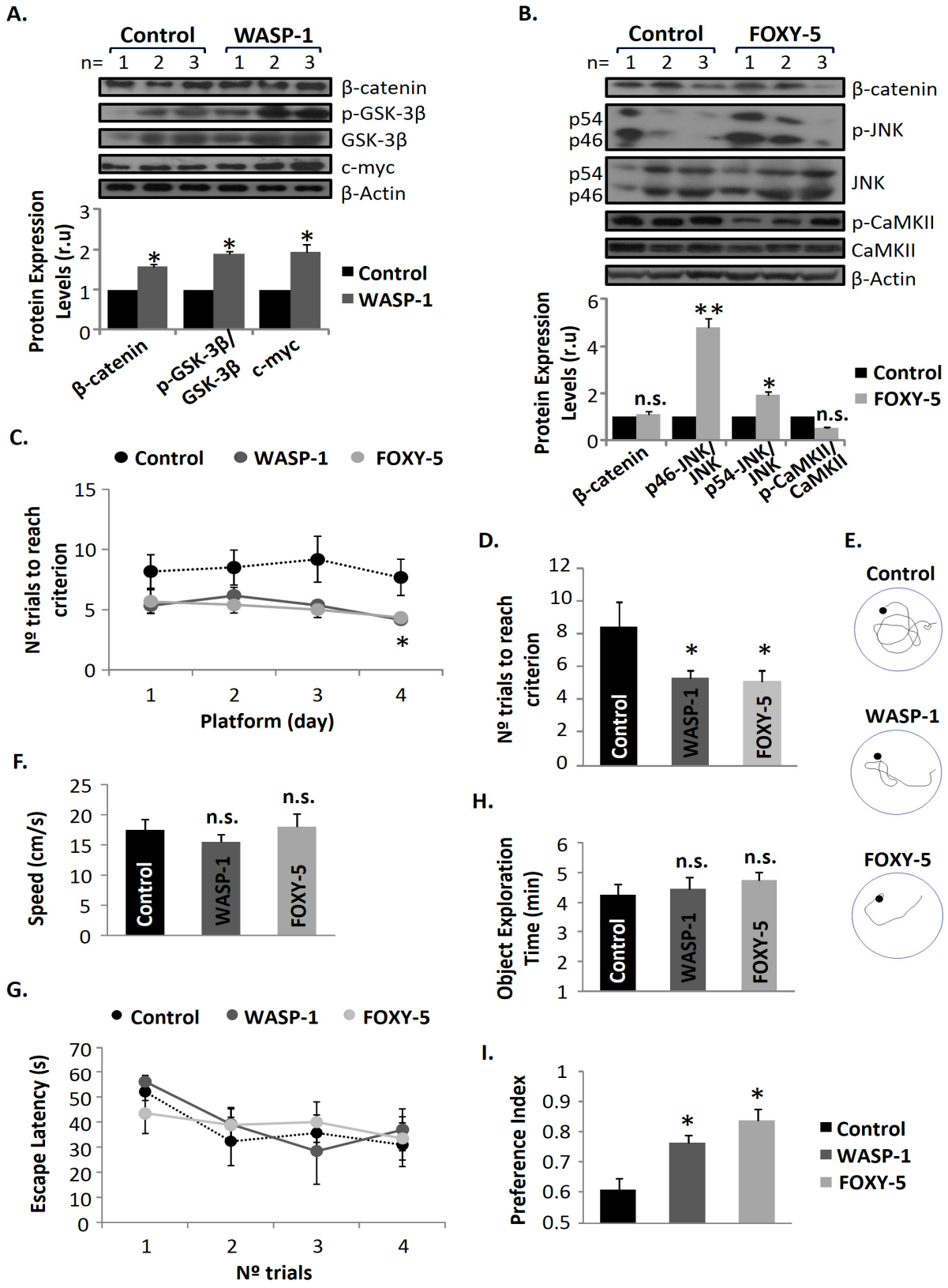
A.
Control
WASP-1
n= 1 2 3 1 2 3
β-catenin
p-GSK-3β
GSK-3β
c-myc
β-Actin
Protein Expression Levels (r.u)
Control
WASP-1
β-catenin
p-GSK-3β/ GSK-3β
c-myc
B.
Control
FOXY-5
n= 1 2 3 1 2 3
β-catenin
p54
p46
p-JNK
JNK
p-CaMKII
CaMKII
β-Actin
Protein Expression Levels (r.u)
n.s.
Control
FOXY-5
β-catenin
p46-JNK/ JNK
p54-JNK/ JNK
p-CaMKII/ CaMKII
C.
Control
WASP-1
FOXY-5
Nº trials to reach criterion
Platform (day)
D.
Nº trials to reach criterion
Control
WASP-1
FOXY-5
E.
Control
WASP-1
FOXY-5
F.
Speed (cm/s)
n.s.
Control
WASP-1
FOXY-5
H.
Object Exploration Time (min)
n.s.
Control
WASP-1
FOXY-5
G.
Control
WASP-1
FOXY-5
Escape Latency (s)
Nº trials
I.
Preference Index
Control
WASP-1
FOXY-5

Figure 1. *In vivo* administration of WASP-1 and FOXY-5 activate *Wnt* signaling and improve memory in adult wild type mice. (A-B) Immunoblotting for *Wnt* pathway proteins of hippocampal homogenates from WASP-1 (n = 3), FOXY-5 (n = 3) and control (n = 3) mice. (A) Above: western blot of control and WASP-1 treated mice for the canonical *Wnt* signaling proteins β-catenin, GSK-3β and its inhibitory isoform phospho-Ser-9, following by the target gene c-myc. Below: quantification of protein band intensities, after β-actin normalization. Graphic shows fold increase versus control. (B) Above: western blot of control and FOXY-5 treated mice for β-catenin and the non-canonical *Wnt* signaling proteins JNK and CaMKII and their corresponding phosphorylated isoforms. Below: quantitative analysis as described in A. (C-G) Behavioral performance of WASP-1 (n =7), FOXY-5 (n = 6) and control (n = 7) mice in the memory flexibility test. (C) Daily progression on each platform location. (D) Comparison of the mean number of trials necessary to reach criterion. Graphic shows the mean value of the four days of testing for each group. (E) Representative tracks of the last day of testing show different swimming strategies among groups. (F) Comparison of the mean swim speed showing no significant differences among groups. (G) Comparison of the escape latency in the visible platform test showing no significant differences among groups for any trial. (H-I) Behavioral performance of WASP-1 (n = 7), FOXY-5 (n = 6) and control (n = 7) mice in the novel object recognition test. (H) Total object exploration time during acquisition memory phase of the test, showing no significant differences among groups. (I) Comparison of the preference index for a novel object during retention memory phase of the test. Preference index was calculated as the time spent exploring the new object compared with both objects.
No significant differences are indicated by n.s.

Figure 2.

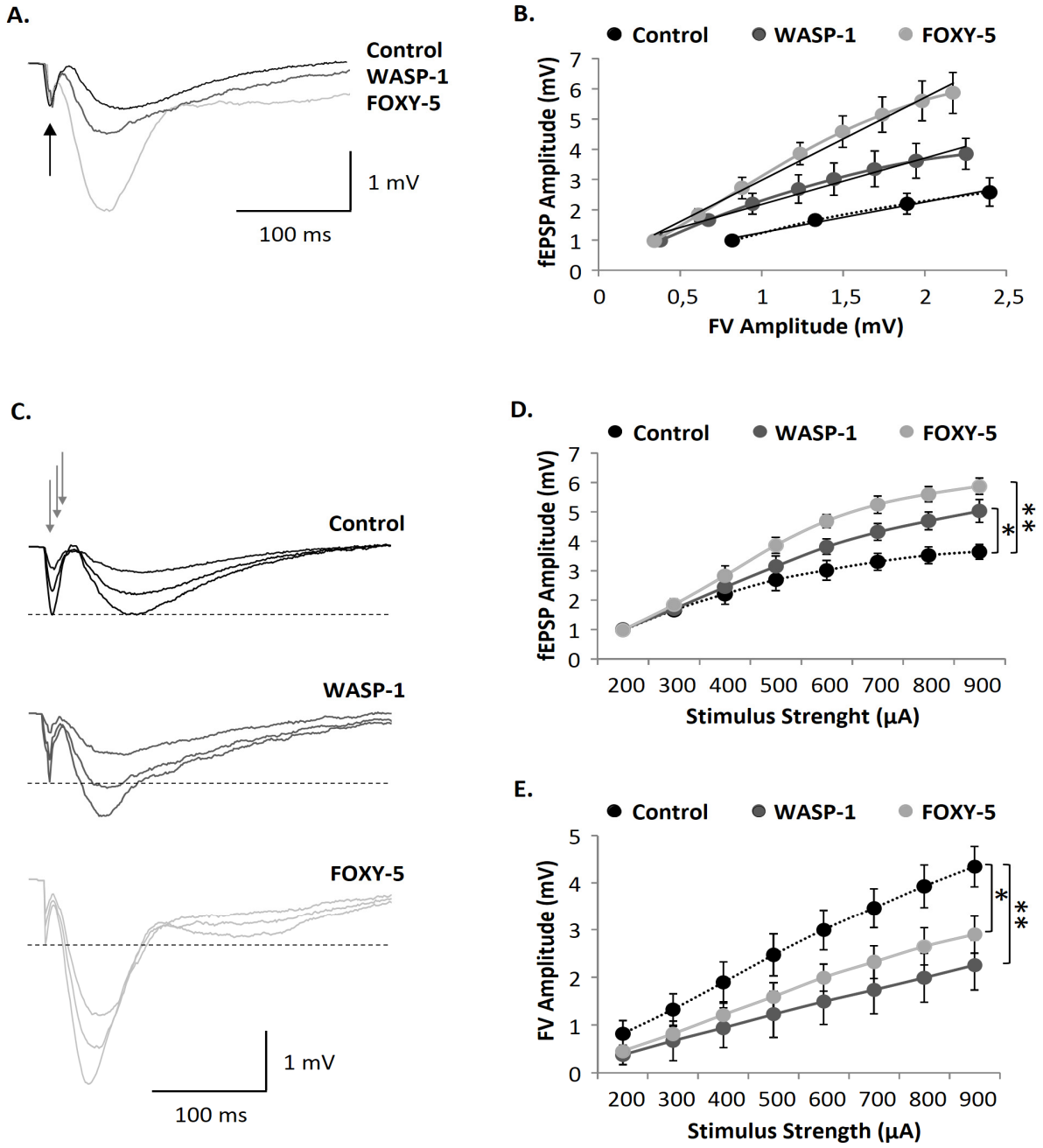

Figure 2. *Wnt* activators enhance basal excitatory synaptic transmission in adult wild type mice. (A-E) Field recordings of hippocampal slices from WASP-1 (n = 10), FOXY-5 (n = 10) and control (n = 10) mice. (A) Representative synaptic responses for equal fiber volley (FV) amplitude (black arrow). (B) Correlation of fEPSP and FV amplitudes for each group. Black lines are the regression line for fEPSP (output) and FV (input). The correlation coefficients (R^2)

in the control, WASP-1 and FOXY-5 mice were 0.9855, 0.9714 and 0.9869, respectively. (C) Average traces of fEPSP at three different intensities of stimulation (grey arrows). Dotted lines indicate FV amplitude of 1 mV. (D) Plot of fEPSP amplitude versus stimulus intensity (from 200 to 900 μA). (E) Plot of FV amplitude versus stimulus intensity (from 200 to 900 μA).

Figure 3.

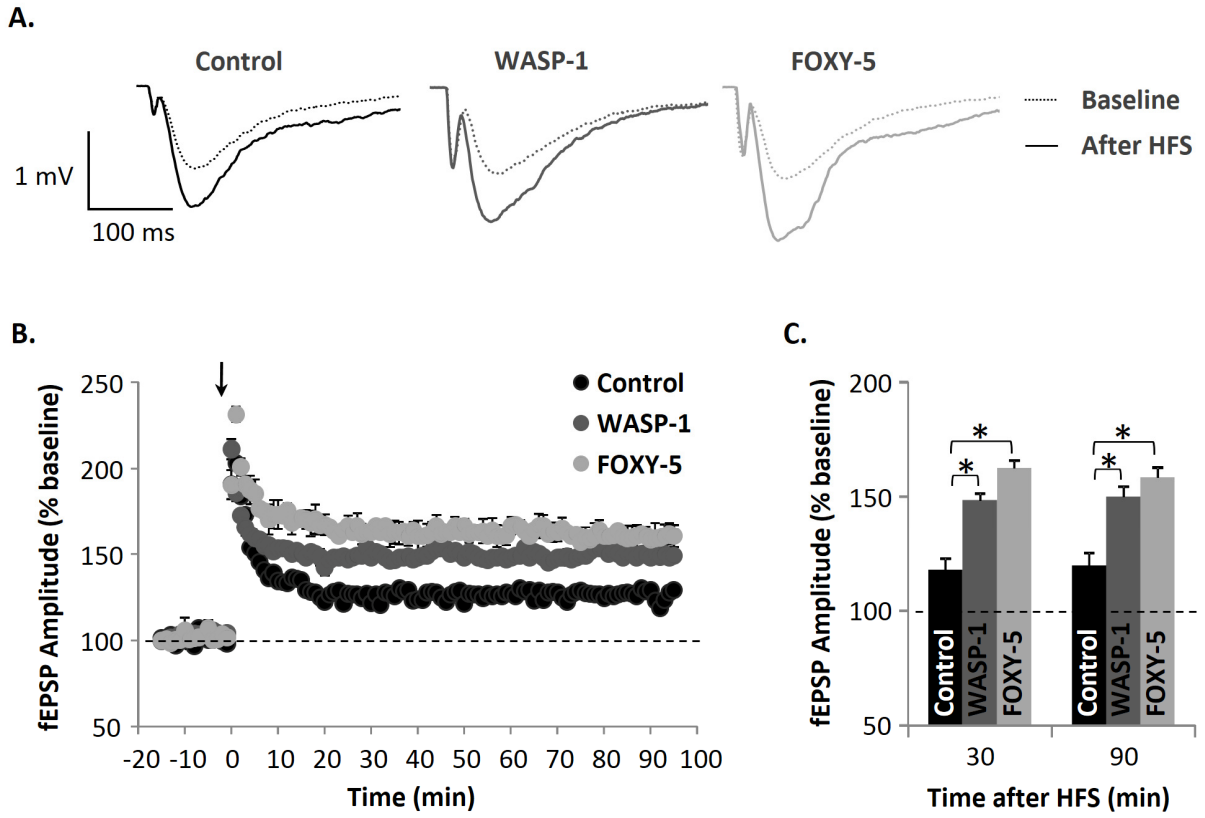

Figure 3. *Wnt* activators enhance hippocampal LTP in adult wild type mice. (A-C) Field recordings of hippocampal slices from WASP-1 (n = 9), FOXY-5 (n = 9) and control (n = 10) mice. (A) Average traces of fEPSP before (dotted lines) and after (filled lines) HFS. (B) Plot of fEPSP amplitude versus time (from 10 min before HFS (arrow) up to 90 min after). Graphic shows percent increase of fEPSP amplitude compared to baseline (dotted line). (C) Comparison of mean fEPSP amplitude among groups at 30 or 90 min after HFS showing percent increase versus baseline.

Figure 4.

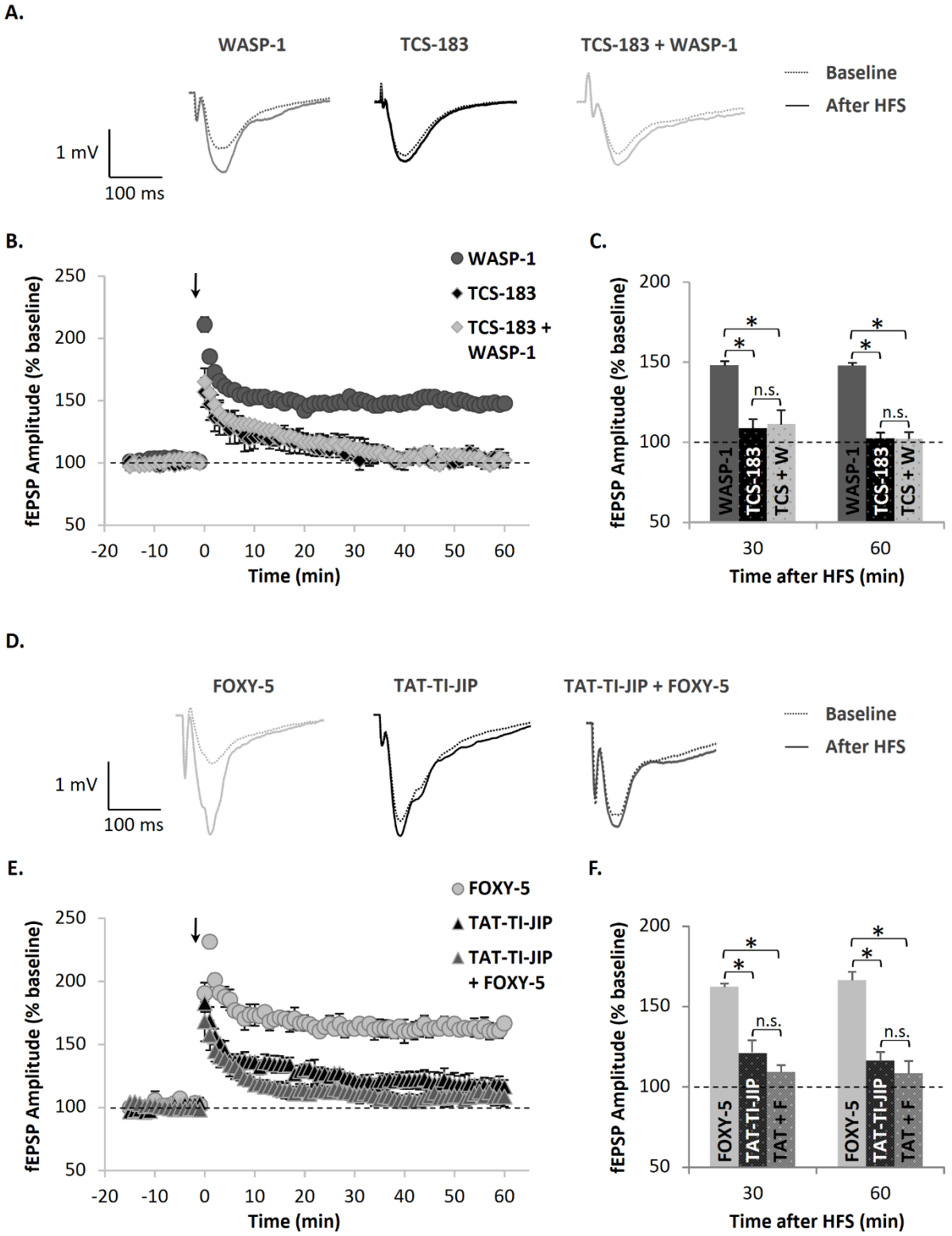
A.
WASP-1
TCS-183
TCS-183 + WASP-1
Baseline
After HFS
1 mV
100 ms
B.
WASP-1
TCS-183
TCS-183 + WASP-1
fEPSP Amplitude (% baseline)
Time (min)
C.
fEPSP Amplitude (% baseline)
WASP-1
TCS-183
TCS + W
n.s.
Time after HFS (min)
D.
FOXY-5
TAT-TI-JIP
TAT-TI-JIP + FOXY-5
Baseline
After HFS
1 mV
100 ms
E.
FOXY-5
TAT-TI-JIP
TAT-TI-JIP + FOXY-5
fEPSP Amplitude (% baseline)
Time (min)
F.
fEPSP Amplitude (% baseline)
FOXY-5
TAT-TI-JIP
TAT + F
n.s.
Time after HFS (min)

Figure 4. Inhibition of *Wnt* pathway impairs hippocampal LTP in adult wild type mice. (A-C) Field recordings of hippocampal slices from WASP-1 (n = 9), TCS-183 (n = 6) and TCS-183 plus WASP-1 (n = 6) treated mice. (A) Representative traces of fEPSP before (dotted lines) and after (filled lines) HFS. (B) Time course of normalized fEPSP amplitude from 15 min before HFS (arrow) up to 60 min after. Graphic shows percent increase of fEPSP amplitude compared to baseline (dotted line). (C) Comparison of mean fEPSP amplitude among treated mice groups at 30 or 60 min after HFS showing percent increase versus baseline. (D-F) Field recordings of hippocampal slices from FOXY-5 (n = 9), TAT-TI-JIP (n = 6) and TAT-TI-JIP plus FOXY-5 (n= 5) treated mice. (D) Representative traces of fEPSP before (dotted lines) and after (filled lines) HFS. (E) Time course of normalized fEPSP amplitude as described in B. (F) Comparison of mean fEPSP amplitude as described in C.

Figure 5.

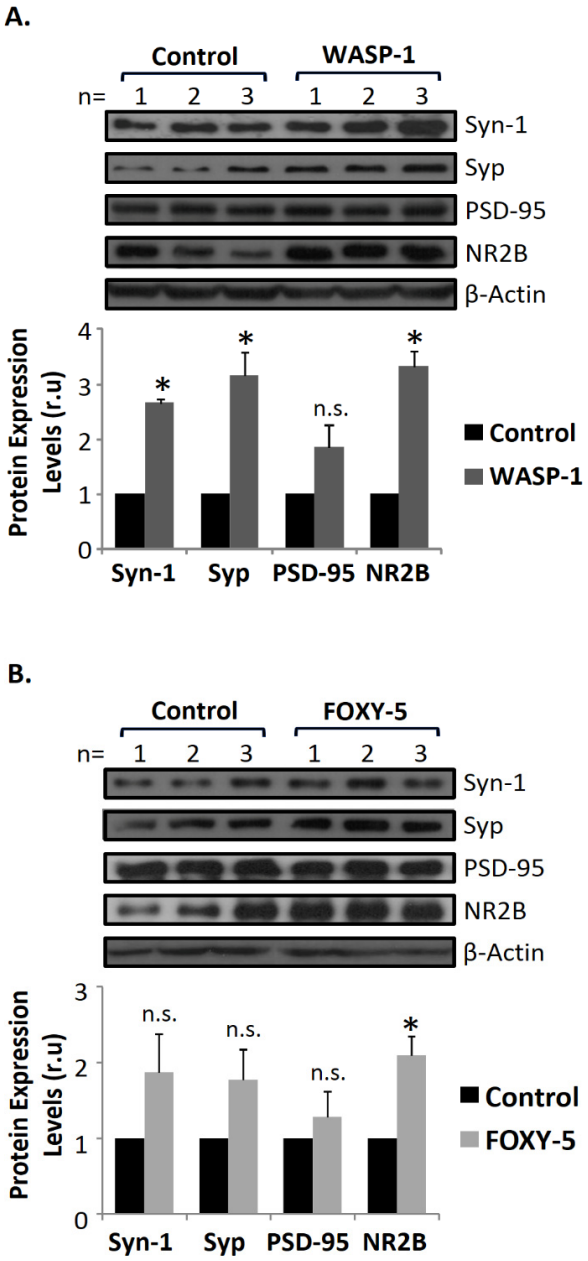

Figure 5. *Wnt* activators increase hippocampal synaptic protein levels in adult wild type mice. (A-B) Immunoblotting of presynaptic (Syn-1 and Syp) and postsynaptic (PSD-95 and NR2B) proteins. (A) Above: western blot of hippocampal homogenates from WASP-1 (n = 3) and control (n = 3) mice. Below: quantification of protein band intensities, after β-actin normalization. Graphic shows fold increase versus control. (B) Above: western blot of hippocampal homogenates from FOXY-5 (n = 3) and control (n = 3) mice. Below: quantitative analysis as described in A. No significant differences are indicated by n.s.

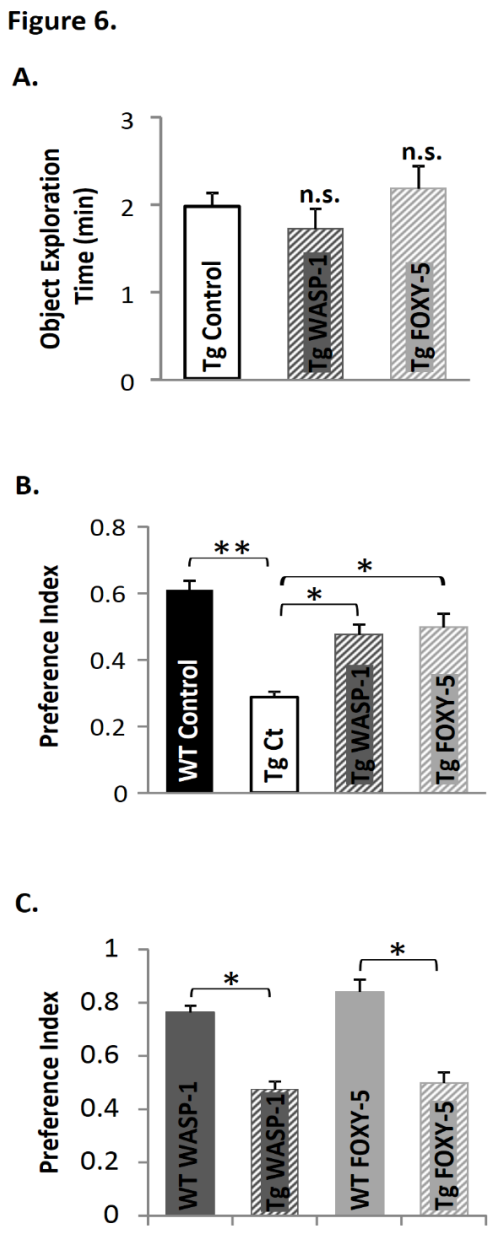

Figure 6. *Wnt* activators rescue memory impairment in adult APP/PS1 mice. (A-C) Behavioral performance of APP/PS1 mice treated with WASP-1 (n = 7), FOXY-5 (n = 6) and control (n = 7), evaluated by the novel object recognition test. (A) Total object exploration time during acquisition memory phase of the test, showing no significant differences (n.s.) among groups. (B) Preference index for the novel object during retention memory phase of the test. Comparison among control WT mice and the APP/PS1 groups. (C) Comparison of preference index for the novel object between WT and APP/PS1 mice treated with WASP-1 or FOXY-5 during retention memory phase of the test.
Tg: transgenic APP/PS1 mice, WT: wild type mice, Ct: control.

Figure 7.

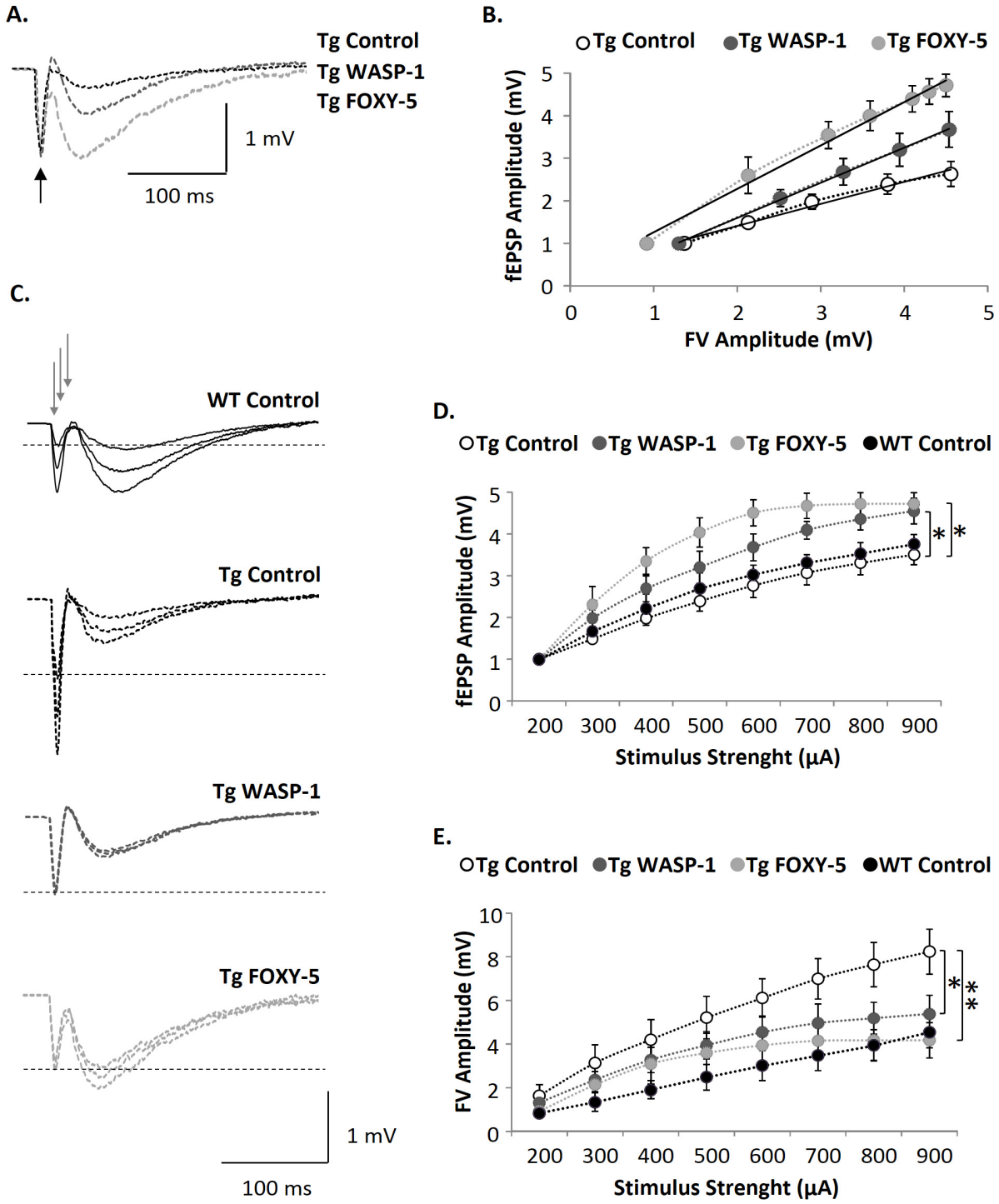
A.
Tg Control
Tg WASP-1
Tg FOXY-5
1 mV
100 ms
B.
Tg Control
Tg WASP-1
Tg FOXY-5
fEPSP Amplitude (mV)
FV Amplitude (mV)
C.
WT Control
Tg Control
Tg WASP-1
Tg FOXY-5
1 mV
100 ms
D.
Tg Control
Tg WASP-1
Tg FOXY-5
WT Control
fEPSP Amplitude (mV)
Stimulus Strenght (µA)
E.
Tg Control
Tg WASP-1
Tg FOXY-5
WT Control
FV Amplitude (mV)
Stimulus Strenght (µA)

Figure 7. *Wnt* activators enhance basal excitatory synaptic transmission in adult APP/PS1 mice. (A-E) Field recordings of hippocampal slices from APP/PS1 animals treated with WASP-1 (n = 8), FOXY-5 (n = 8) or control (n = 8). (A) Representative waveforms of fEPSP amplitude for given fiber volley (FV) amplitude (black arrow). (B) Correlation of fEPSP and FV amplitudes for each group. Black lines are the regression line for fEPSP (output) and FV (input). The correlation coefficients (R^2) in the control, WASP-1 and FOXY-5 mice were 0.9829, 0.9993 and 0.9893, respectively. (C) Average traces of fEPSP at three different intensities of stimulation (grey arrows). Dotted lines indicate FV amplitude of 1 mV. (D) Plot of fEPSP amplitude versus stimulus intensity (from 200 to 900 μA). (E) Plot of FV amplitude versus stimulus intensity (from 200 to 900 μA).
Tg: transgenic APP/PS1 mice, WT: wild type mice.

Figure 8.

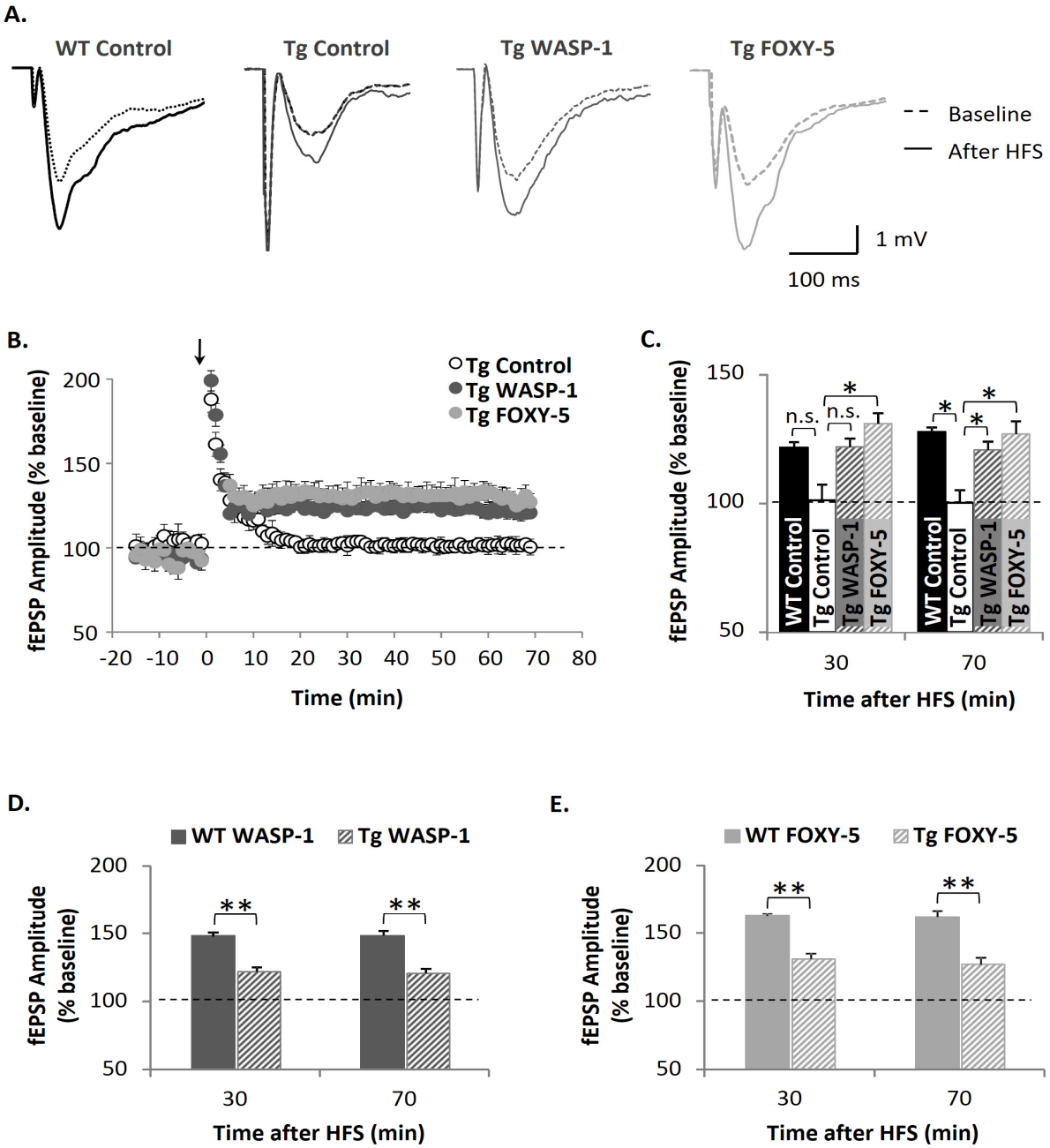

Figure 8. *Wnt* activators rescue LTP deficit in adult APP/PS1 mice. (A-B) Field recordings of hippocampal slices from APP/PS1 animals treated with WASP-1 (n = 8), FOXY-5 (n = 8) and control WT (n = 10) or APP/PS1 (n =9) mice. (A) Representative traces of fEPSP from APP/PS1 and control WT mice, before (dotted lines) and after (filled lines) HFS. (B) Plot of fEPSP amplitude versus time (from 10 min before HFS (arrow) up to 70 min after). Graphic shows

percent increase of fEPSP amplitude compared to baseline (dotted line). (C-E) Mean fEPSP amplitude mice at 30 or 70 min after HFS. Graphs show percent increase of fEPSP amplitude versus baseline (dotted line). (C) Comparison among control and treated APP/PS1 groups versus control WT mice. No significant differences are indicated by n.s. (D) Comparison between WT and APP/PS1 mice treated with WASP-1. (E) Comparison between WT and APP/PS1 mice treated with FOXY-5.
Tg: transgenic APP/PS1 mice, WT: wild type mice.

Table 1. Effect of transcription and translation inhibitors on hippocampal LTP enhancement induced by WASP-1 and FOXY-5 treatments.

Time after HFS (min)	*Treatments*								
	CONTROL			**WASP-1**			**FOXY-5**		
	Vehicle	AMD	CH	Vehicle	AMD	CH	Vehicle	AMD	CH
30	134.6 ± 4.8 %	131.1 ± 7.1 %	139.1 ± 3.1 %	145.6 ± 2.3%	142.4 ± 3.4 %	144.7 ± 2.1 %	153.9 ± 3.1 %	159.6 ± 4.2 %	151.8 ± 2.6%
60	128.8 ± 5.2 %	117.3 ± 7.6 %	113.3 ± 5.6 %	140.8 ± 6.4 %	147.2 ± 5.8 %	136.9 ± 4.3 %	148.0 ± 2.8 %	143.0 ± 5.5 %	149.9 ± 5.6 %
120	121.2 ± 6.8 %	98.2 ± 4.2 % *	100.5 ± 6.1 % *	129.2 ± 3.2%	132.4 ± 7.2 %	101.7 ± 4.1 % *	139.0 ± 3.5 %	102.2 ± 4.6% *	103.4 ± 6.2 % *

Comparison of mean fEPSP amplitude from acute hippocampal slices perfused with vehicle (n = 4), AMD (n = 5), CH (n = 4), WASP-1 alone (n = 6), WASP-1 plus AMD (n = 4), WASP-1 plus CH (n = 5), FOXY-5 alone (n = 6), FOXY-5 plus AMD (n = 4), and FOXY-5 plus CH (n = 4). Data show percent increase of fEPSP amplitude versus baseline at 30, 60 and 120 after HFS. Significant differences in comparison to vehicle treatment in each group are indicated.

3. CHAPTER II

In the next section we present the results obtained in order to accomplish partially the Specific Objectives N° 1 and 2, previously described.

The manuscript presented below, was submitted last February to Molecular Neurodegeneration (Manuscript ID: 1095115749121568).

As is shown below, this manuscript describes the neuroprotective effect of WASP-1, a *Wnt*/β-catenin signaling potentiator, on Aβ-impaired synapses. The effects of WASP-1 were evaluated both *in vitro* and *in vivo*, using several experimental assays. First, the effects of WASP-1 on normal hippocampal synaptic function of adult mice, was evaluated. Then, the ability of WASP-1 to protect against Aβ-induced synaptic damage, was explored. Finally, a potential model of WASP-1 mechanism at normal and Aβ-impaired synapses, was proposed. The data presented below, indicate that WASP-1 treatment rescues synaptic impairments induced by Aβ oligomers exposure, therefore suggesting that WASP-1 could be a potential agent for AD therapy.

WASP-1, a canonical *Wnt* signaling potentiator, rescues hippocampal synaptic impairments induced by Aβ oligomers.

Running title: WASP-1 rescues Aβ-induced synaptic dysfunction.

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Abstract.

Background: Amyloid-β (Aβ) oligomers are a key factor in Alzheimer's disease (AD)-associated synaptic dysfunction. Aβ oligomers block the induction of hippocampal long-term potentiation (LTP) in rodents. Activation of *Wnt* signaling prevents Aβ oligomers-induced neurotoxic effects. The compound WASP-1 (*Wnt*-Activating Small molecule Potentiatior-1), has been described as a synergist of the *Wnt-3a* ligand, which enhances activation of *Wnt*/β-catenin signaling. We report here that WASP-1 is neuroprotective against Aβ-induced damage.

Results: Herein, we show that activation of canonical *Wnt*/β-catenin signaling by WASP-1 increases synaptic transmission and rescues hippocampal LTP impairments induced by Aβ oligomers. Also, intra-hippocampal administration of WASP-1 in double transgenic APPswe/PS1dE9 mouse model of AD prevents synaptic protein loss and reduces the *tau* phosphorylation. Moreover, we found that WASP-1 blocks Aβ aggregation *in vitro* and reduces hippocampal levels of Aβ oligomers *in vivo*.

Conclusions: The activation of *Wnt* signaling by WASP-1 treatment, successfully rescued synaptic impairments both *in vitro* and *in vivo*. These results indicate that targeting canonical *Wnt* signaling with WASP-1 could be a potential therapeutic agent for treating AD.

Keywords.

Alzheimer's disease, Aβ oligomers, synaptic plasticity, synaptic proteins, WASP-1, *Wnt* signaling.

Introduction.

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive deterioration of cognitive abilities, mainly caused by synaptic impairments and neuronal death in specific regions of the brain (Mattson, 2004; Selkoe, 2001). Accumulation of amyloid-β peptide (Aβ) in senile plaques mostly located in hippocampus, cortex and other brain areas linked to cognitive processes, is considered one of the major pathological hallmarks of AD brains (Selkoe, 2001). Most recent evidence indicates that soluble Aβ oligomers rather than plaques determine cognitive decline (Lacor et al., 2007; Shankar et al., 2007). Actually, the severity of dementia in AD patients is strongly correlated with the levels of soluble Aβ oligomers (Lue et al., 1999; McLean et al., 1999). Thus, the current view of AD considers Aβ oligomers as a key factor in synaptic dysfunction linked to early stages of AD (Sakono and Zako, 2010). Indeed, Aβ oligomers isolated of AD brains can affect memory and disrupt hippocampal synaptic plasticity through inhibition of long-term potentiation (LTP) and increasing long-term depression (LTD) (Cleary et al., 2005; Shankar et al., 2008). However, Aβ-induced blockade of LTP can be overcome by inhibitors of Aβ oligomerization (Walsh et al., 2005), suggesting that AD cognitive impairment might be due to a direct effect of Aβ oligomers on the synaptic region. In fact, Aβ oligomers can affect excitatory synaptic transmission by reducing the amplitude of the field excitatory postsynaptic potentials (fEPSP) at hippocampal synapses (Cerpa et al., 2010; Hermann et al., 2009).

Several lines of evidence indicates that disruption of *Wnt* signaling pathway contributes to AD pathogenesis (da Cruz e Silva et al., 2010; Inestrosa et al., 2000). In the central nervous system, *Wnt* signaling plays an important role during development and also in the maintenance and function of mature synapses (Ciani and Salinas, 2005; Inestrosa and Arenas, 2010). Until

now, a number of *Wnt* pathways has been identified (Nusse, 2012). In particular, canonical *Wnt* pathway, also called *Wnt*/β-catenin pathway, has been involved in the modulation of the hippocampal synaptic plasticity (Cerpa et al., 2008; Chen et al., 2006).

In AD brains a marked decrease of β-catenin levels (Zhang et al., 1998) and an increased expression of the *Wnt*/β-catenin signaling antagonist, Dickkopf-1 (Dkk-1), (Caricasole et al., 2004) has been observed. Also, common genetic variations within the low-density lipoprotein receptor-related protein 6 (LRP6), a co-receptor for *Wnt*/β-catenin signaling, have been associated with late-onset AD (De Ferrari et al., 2007). Moreover, several studies have shown a relationship between the loss of *Wnt*/β-catenin signaling and an increase in Aβ-related neurotoxicity (Alvarez et al., 2004; De Ferrari et al., 2003). Specifically, loss of *Wnt*/β-catenin signaling increases neuronal vulnerability to Aβ-induced apoptosis (Zhang et al., 1998). Furthermore, Aβ exposure increases Dkk-1 expression (Caricasole et al., 2004; Purro et al., 2012) causing a reduction in the number of synapses and the size of pre- and postsynaptic compartments (Purro et al., 2012). However, activation of *Wnt*/β-catenin signaling has shown to protect against Aβ-induced cytotoxic effects (De Ferrari et al., 2003; Inestrosa et al., 2012). Indeed, incubation with the canonical *Wnt-3a* ligand prevents neuronal cell death induced by Aβ exposure (Alvarez et al., 2004). Also, treatment with lithium, a pharmacological activator of *Wnt*/β-catenin signaling, rescues memory loss and reduces Aβ deposition in the brain of a transgenic mouse model of AD (Toledo and Inestrosa, 2010). Moreover, treatment with lithium also rescues LTP deficits in a transgenic mouse that conditionally overexpress glycogen synthase kinase-3β (GSK-3β), a key enzyme that blocks *Wnt*/β-catenin signaling (Hooper et al., 2007).

These data suggest that direct stimulation of *Wnt*/β-catenin signaling or the inhibition of their endogenous antagonists could become a potential therapeutic approach to treat AD. Here, we study the beneficial effects of WASP-1, a synthetic molecule that potentiates *Wnt*/β-catenin signaling (Beaumont et al., 2007) on AD-related synapses damage. Through *in vitro* and *in vivo* approaches, we found that WASP-1 treatment effectively protects against Aβ-induced synaptic dysfunction.

Results.

WASP-1 potentiates *Wnt*/β-catenin signaling and enhances synaptic plasticity. WASP-1 was identified as a transcriptional activator of β-catenin-dependent TopFlash reporter (Korinek et al., 1997), which in the presence of the *Wnt-3a* ligand causes a synergistic increase of the reporter activation (Beaumont et al., 2007). Interestingly, it seems that WASP-1 exerts its effect by acting downstream of Frizzled (the receptor for *Wnt* ligand) (Beaumont et al., 2007). Here, we tested the effect of WASP-1 on the stability of β-catenin, a key protein in the canonical *Wnt* pathway. Using primary culture of hippocampal neurons of 18 days *in vitro*, we tested the ability of WASP-1 treatment to increase the β-catenin levels, in the presence or absence of *Wnt-3a* ligand (Fig. 1A). We found that only in the presence of *Wnt-3a*, WASP-1 induces a significant increase of β-catenin in comparison to the control (Fig. 1A). This raise in the β-catenin levels could be a dose-dependent effect, since the expression of β-catenin is higher when cells were incubated with 5 μM WASP-1, than with 1 μM WASP-1 (Fig. 1A). Since WASP-1 alone was not able to induce significant changes in the β-catenin levels (Fig. 1A), we conclude that *Wnt-3a* is required for the effect of WASP-1. Even though, we found that the levels of β-catenin are higher in the presence of *Wnt-3a* plus 5 μM WASP-1, than in the presence of *Wnt-3a* alone (Fig.

1A), this enhancement of the *Wnt*/β-catenin signaling is not synergistic as was previously described using a TopFlash reporter (Beaumont et al., 2007). Our findings suggest that WASP-1 potentiates *Wnt*/β-catenin signaling in hippocampal neurons exposed to the *Wnt-3a* ligand. Considering these results, we used 5 μM WASP-1 to perform both electrophysiological recordings and *in vivo* experiments, presented below. For those experiments, we used WASP-1 alone since endogenous expression and release of *Wnt-3a* ligand in the rodent hippocampus has been already demonstrated (Cerpa et al., 2008; Chen et al., 2006).

As has been shown for several *Wnt* ligands, WASP-1 can also modulate excitatory synaptic transmission by increasing synaptic strength of CA3-CA1 hippocampal synapses (Beaumont et al., 2007). Here we show that perfusion of 5 μM WASP-1 alone is enough to significantly increase the amplitude of the field excitatory postsynaptic potentials (fEPSP) in hippocampal slices from adult wild-type (WT) mice (Fig. 1B-b). Using a paired pulse test, we addressed whether WASP-1 exerts its effect through a presynaptic mechanism as has been described for canonical *Wnt* ligands (Cerpa et al., 2008). In Figure 1B-a, superimposed field responses shows that WASP-1 elicits larger synaptic responses than those observed in control slices. This increase in fEPSP amplitude occurs concomitant with a decrease of the paired-pulse facilitation (Fig. 1B-c). Since changes in the paired-pulse facilitation are considered to be of presynaptic origin (Clark et al., 1994; Kuhnt and Voronin, 1994), this result suggests that WASP-1 acts at the presynaptic terminal. Moreover, we found that WASP-1 perfusion does not change the fiber volley amplitude in comparison to control conditions (Figs. 1B-d, -e), indicating that WASP-1 does not modify the number of presynaptic fibers activated and suggests that the effect of WASP-1 on the paired-pulse facilitation is due to a change in the neurotransmitter release.

A previous study has presented evidence that WASP-1 also enhances hippocampal LTP magnitude by facilitating the LTP-maintenance for at least 2 h after LTP-induction (Beaumont et al., 2007), a fact that is consistent with our results (Fig. 2A). As Figure 2A-a shows, a high frequency stimulation (HFS) protocol evoked larger fEPSP responses in WASP-1 perfused slices than in control slices. Indeed, the average LTP magnitude was significantly enhanced in WASP-1 perfused slices (148.5 ± 4.9 %) in comparison to control slices (130.1 ± 5.6 %). Moreover, WASP-1-dependent potentiation of the synaptic responses was maintained for over 120 min after HFS (Fig. 2A-b).

Considering: a) maintaining of LTP for more than 1-2 h after induction, a phase known as late LTP (l-LTP), requires local dendritic protein synthesis as well as gene transcription (Citri and Malenka, 2008), and, b) activation of *Wnt*/β-catenin signaling can regulate the expression of genes that are involved in synaptic plasticity (Arrazola et al., 2009); we hypothesized that WASP-1 effects on hippocampal LTP could be mediated by translation and/or transcription. Therefore, we tested the effects of WASP-1 on the LTP magnitude, in the presence of the protein synthesis inhibitor, Cycloheximide (CH, 40 μg/ml), or the gene transcription inhibitor, Actinomycin-D (AMD, 25 μM). Interestingly, we found that perfusion of either CH alone or CH plus WASP-1 did not affect LTP-induction but, both blocked LTP-maintenance since fEPSP amplitude returns to basal levels (indicated by the dotted line), at 120 min after HFS application (Fig. 2B-a). However, significant differences in the LTP magnitude between slices perfused with CH or CH plus WASP-1 were found at 60 min after HFS application (Fig. 2B-b). The fact that co-perfusion of CH and WASP-1 causes blockage of l-LTP, indicates that protein translation is necessary for the effect of WASP-1 on the maintaining of LTP. Additionally, we found that perfusion of AMD alone also blocked LTP-maintaining without affecting LTP-

induction (Fig. 2C-a) as has been previously reported (Calixto et al., 2003; Gelinas et al., 2008). However, co-perfusion of AMD and WASP-1 did not affect neither LTP-induction nor LTP-maintenance since HFS-evoked fEPSP amplitude was significantly different from baseline (dotted line) at any time assayed (Fig. 2C-a, -b). Together, these results suggest that WASP-1 induces an increase of hippocampal LTP magnitude in a way that depends on local protein synthesis, but it does not involve gene transcription. In view of these results and considering the categorization of LTP in LTP1, LTP2 and LTP3, according to persistence and the mechanisms involved in each form (Racine et al., 1983; Raymond and Redman, 2006), WASP-1 seems to elicit LTP2, which only requires local protein synthesis from pre-existing mRNA.

On the other hand, it has been reported that tetanic stimulation could induces release of endogenous *Wnt-3a* ligand from hippocampal synapses by following the activation of the glutamate receptor, N-Methyl-D-aspartate receptor (NMDA-R) (Chen et al., 2006). Since, WASP-1 requires endogenous release *Wnt-3a* ligand in order to exerts its effect (Beaumont et al., 2007), then, we asked whether NMDA-Rs are involved in the effect of WASP-1 on hippocampal LTP. To test this possibility, we measured the magnitude of LTP in hippocampal slices perfused with the NMDA-R antagonist DL-2-amino-5-phosphonovaleric acid (APV) alone or in the presence of WASP-1 (Fig. 2D-a). We found that the fEPSP amplitude, at 30 or 60 min after HFS application, is not significantly different from baseline (dotted line) when slices were perfused either with 50 μM APV alone (n = 5 slices) or with 50 μM APV plus 5 μM WASP-1 (n = 5 slices) (Fig. 2D-b). Moreover, synaptic responses evoked at 30 or 60 min after HFS, in slices treated with APV plus WASP-1 (110.2 ± 7.4 % and 107.7 ± 10.1 % respectively) were markedly reduced in comparison to those exhibited by the slices treated with WASP-1 alone (145.6 ± 2.3 % and 140.8 ± 6.4 % respectively), indicating that blockage of NMDA-Rs

inhibits the LTP enhancement induced by WASP-1. This result suggests that activation of NMDA-Rs is required for the effect of WASP-1 on LTP.

WASP-1 rescues Aβ-induced hippocampal synaptic dysfunction. Several studies have shown that activation of *Wnt* signaling has a neuroprotective effect against Aβ-induced synaptic damage (Alvarez et al., 2004; Cerpa et al., 2010; De Ferrari et al., 2003; Shruster et al., 2011). Since the administration of WASP-1 has shown to enhances excitatory synaptic transmission and facilitates hippocampal LTP (Beaumont et al., 2007), as we showed in Figures 1B and 2A, we then hypothesized that WASP-1 perfusion could also rescues synaptic impairment of excitatory transmission induced by Aβ oligomers. To address this issue, we tested the effects on synaptic transmission (Fig. 3A) and LTP (Fig. 3B) in hippocampal slices perfused with Aβ oligomers alone or Aβ oligomers plus WASP-1. We found that perfusion of Aβ oligomers alone causes a strong reduction of the fEPSP amplitude in comparison to control condition (Fig. 3A-b), that starts after 10 min of Aβ perfusion (Fig. 3A-a). Indeed, average fEPSP amplitude of Aβ perfused slices (62.3 ± 6.4 %) was significantly diminished in comparison to control slices (101.3 ± 1.9 %). Moreover, Aβ oligomers also cause a strong inhibition of hippocampal LTP (Fig. 3B). Actually, after a brief post-tetanic potentiation evoked by a HFS protocol, fEPSP amplitude of Aβ-perfused slices returns to basal levels (dotted line) in less than 20 min (Fig. 3B-a). These results are consistent with previous reports indicating that Aβ oligomers are able to induce a decrease of hippocampal excitatory synaptic transmission and LTP (Li et al., 2011; Shankar et al., 2008; Walsh et al., 2002; Wang et al., 2004). Interestingly, we found that slices perfused with Aβ oligomers plus WASP-1 exhibit an increase of fEPSP amplitude in comparison to baseline (dotted line) that reach a plateau at 15 min after co-perfusion begins

(Fig. 3A-a). Comparison of the average fEPSP amplitude between Aβ alone and Aβ plus WASP-1 treatments reveals that WASP-1 rescues Aβ-induced impairment of excitatory synaptic transmission (Fig. 3A-b). In fact, average fEPSP amplitude of Aβ plus WASP-1-perfused slices (128.7 ± 2.8 %) was significantly larger than Aβ-perfused slices (62.3 ± 6.4 %). Furthermore, in contrast to what is observed in Aβ-perfused slices, we found that HFS application is able to induce LTP in hippocampal slices co-perfused with Aβ oligomers plus WASP-1 (Fig. 3B-a), indicating that WASP-1 could overcome Aβ-induced LTP inhibition. Remarkably, WASP-1-induced rescuing effect of Aβ-impaired LTP was maintained for at least 1 h after the application of HFS protocol (Fig. 3B-b). Indeed, the LTP magnitude at 30 or 60 min after HFS was higher in Aβ plus WASP-1-perfused slices (129.5 ± 7.8 % and 128.8 ± 7.2 %, respectively) than Aβ-perfused slices (110.8 ± 6.6 % and 104.6 ± 7.4 %, respectively). These results indicates that WASP-1 treatment significantly rescues LTP deficit caused by Aβ oligomers exposure.

Together, our findings suggest that potentiation of the canonical *Wnt*/β-catenin signaling by WASP-1 could successfully overcome Aβ-induced impairments on both, the excitatory synaptic transmission and the hippocampal synaptic plasticity.

WASP-1 rescues loss of synaptic proteins and β-catenin in APP/PS1 mice. Several *in vitro* studies have shown that activation of *Wnt*/β-catenin signaling has a neuroprotective effect against Aβ-induced synaptic damage (Alvarez et al., 2004; De Ferrari et al., 2003; Shruster et al., 2011). Here we found that the activation of *Wnt*/β-catenin signaling by WASP-1 rescues synaptic transmission and LTP deficits in Aβ-perfused hippocampal slices (Fig. 3). Now, we asked whether WASP-1 could also rescue Aβ-induced synaptic impairments *in vivo*. To evaluate the effect of WASP-1 *in vivo*, we used double transgenic APP/PS1 mice (APPswe/PS1dE9:

carrying the Swedish (K595N/M596L) mutation of the amyloid precursor protein (APP) and the exon 9 deletion of presenilin-1 (PS1) gene), that show the Aβ pathology of AD (Garcia-Alloza et al., 2006). Administration of WASP-1 was achieved by using an osmotic pump system (see Methods section for details) that allowed the chronic intra-hippocampal infusion of WASP-1 in APP/PS1 and WT mice.

As it has been previously shown, APP/PS1 animals display reduced levels of several synaptic proteins in both cortex and hippocampus (Inestrosa et al., 2011). Therefore, we tested whether WASP-1 treatment could reverses synaptic proteins loss in the hippocampus of APP/PS1. We found that chronic administration of WASP-1 in adult APP/PS1 mice significantly increases hippocampal levels of the presynaptic protein, SYP (synaptophysin) and the postsynaptic proteins, GluN2B (N-Methyl-D-aspartate (NMDA) receptor subunit 2B) and PSD-95 (postsynaptic density protein-95), in comparison to control APP/PS1 mice (Fig. 4A). Even though SYP levels in the hippocampus of APP/PS1 mice are not significantly reduced in comparison to WT mice (Inestrosa et al., 2011), as Figure 4A-b shows, WASP-1 treatment increases SYP levels in APP/PS1 mice above WT levels. This change in the levels of a presynaptic protein is consistent with the results shown in Figure 1B, indicating that WASP-1 could act through a presynaptic mechanism. However, we found that WASP-1 treatment also rescues decreased levels of GluN2B and PSD-95 in APP/PS1 mice (Fig. 4A). We thought that WASP-1-induced increase of postsynaptic proteins levels could be caused by the activation of the non-canonical *Wnt* signaling pathways, that have been shown to modulate the structure of the postsynaptic compartment (Farias et al., 2009; Varela-Nallar et al., 2010), and could be activated by low concentrations of the canonical *Wnt-3a* ligand (Nalesso et al., 2011). Since WASP-1 treatment raised the levels of both presynaptic and postsynaptic proteins, it seems that

chronic administration of WASP-1 could cause a dual effect on the hippocampal synaptic structure of APP/PS1 mice.

In comparison to healthy brains, a marked reduction of β-catenin levels has been reported in the AD patients (Zhang et al., 1998). Moreover, APP/PS1 mice also shown lower hippocampal β-catenin levels than WT mice (Toledo and Inestrosa, 2010). Here we tested whether WASP-1 treatment could rescue loss of β-catenin levels in the hippocampus of APP/PS1 mice. Interestingly, we found that APP/PS1 mice chronically treated with WASP-1 shows a significant increase on hippocampal β-catenin levels in comparison to control APP/PS1 mice (Fig. 4B). As Figure 4B-b shows, WASP-1 was able to rescue the decreased β-catenin levels observed in APP/PS1, almost reaching the WT levels of the protein.

Our results show that *in vivo* administration of WASP-1 increases synaptic protein levels and reverses β-catenin loss into the hippocampus of adult APP/PS1 mice.

WASP-1 reduces *tau* phosphorylation and blocks Aβ aggregation. The main pathological hallmarks found in the AD brain are senile plaques and neurofibrillary tangles (Sorrentino and Bonavita, 2007). Senile plaques are primarily formed by extracellular deposits of Aβ peptide, while neurofibrillary tangles are constituted by intracellular aggregates of hyper-phosphorylated *tau* protein (Mayeux and Stern, 2012). According to the amyloid hypothesis, Aβ aggregation is the key influence driving AD pathogenesis, whereas neurofibrillary tangles formation is proposed to be a result from an imbalance between Aβ production and Aβ clearance (Hardy and Selkoe, 2002). Here, we asked whether WASP-1 could down regulates both hyper-phosphorylated *tau* levels and Aβ aggregation. First, we tested whether *in vivo* administration of WASP-1 in the brain of APP/PS1 mice could reduce hippocampal levels of phosphorylated

tau. The phosphorylation of *tau*, particularly the presence of PHF-1 epitope (phosphorylated Ser-396 and Ser-404) was assessed by immunoblotting (Fig. 5A-a). WASP-1 treatment significantly decreases phosphorylation of *tau* (Fig. 5 A-b), since a reduction on PHF-1 epitope levels was observed in the hippocampus of adult APP/PS1 mice. In view of this result, we evaluated whether WASP-1 treatment was able to affect the Aβ oligomeric species in the hippocampus of APP/PS1 animals. An immunoblotting assay was carried out using the 6E10 antibody to detect the presence of Aβ oligomeric species in hippocampal extract from APP/PS1 mice treated with WASP-1 or vehicle (Fig. 5B-a). We found that chronic treatment with WASP-1 reduces the levels of Aβ oligomers, specifically of Aβ*56 (a 56kDa oligomer) specie (Fig. 5B-b), which is a soluble Aβ assembly that impairs memory in AD transgenic mice independently of plaques formation or neuronal loss (Lesne et al., 2006). Since, it is possible that the WASP-1-induced decrease of Aβ*56 oligomeric form could occur by blocking Aβ aggregation, we evaluated *in vitro* whether WASP-1 could interferes with the Aβ aggregation process. Interestingly, we found that both concentrations assayed of WASP-1 (0.5 μM and 5 μM) decreases Aβ fibril formation in a time-dependent manner (Fig. 5C). After 8 h incubation, WASP-1 induces a 45-50 % reduction of Aβ fibril formation compared to Aβ alone (Fig. 5C). Altogether, these results indicate that WASP-1 is able to reduce Aβ aggregation both *in vitro* and *in vivo*.

Discussion.

WASP-1 has been previously described as a small molecule that potentiates canonical *Wnt*/β-catenin signaling and enhances excitatory transmission in mature hippocampal synapses (Beaumont et al., 2007). Since, the activation of *Wnt*/β-catenin signaling has a neuroprotective

effect against Aβ-induced cytotoxic and synaptotoxic insults (Boonen et al., 2009; Cerpa et al., 2009; Moon et al., 2004), we tested here the ability of WASP-1 to rescue functional and structural damage of hippocampal synapses exposed to Aβ oligomers. Interestingly, we found that WASP-1 was able to reduce Aβ-induced synaptic impairments both *in vitro* and *in vivo*. Specific effects of WASP-1 observed in this study include: the rescuing of Aβ-induced disruption of excitatory synaptic transmission and the overcome of the Aβ-induced blockage of hippocampal LTP, reversing synaptic proteins loss, reduction of Aβ oligomeric aggregates and reduction in the levels of *tau* phosphorylation. Taken together, our data suggest that WASP-1 could be of therapeutic relevance in AD.

Several studies have reported that Aβ oligomers negatively modulate synaptic plasticity (Cleary et al., 2005; Li et al., 2011; Walsh et al., 2002). Previously, we showed that Aβ oligomers also reduce synaptic efficacy and impair hippocampal synaptic transmission mainly by decreasing NMDA and AMPA receptors currents, probably caused by a reduction in PSD-95 levels and synaptic contacts (Cerpa et al., 2010). Our present findings indicate that WASP-1 rescues impaired synaptic transmission and plasticity in hippocampal slices exposed to Aβ oligomers. Although, we do not know the precise mechanism of action of WASP-1, present data strongly suggests that WASP-1 effects on Aβ-decreased hippocampal synaptic responses could be the result of the increment in synaptic proteins, including SYP, GluN2B (NMDA-R) and PSD-95 levels. The fact that WASP-1 increases the levels, not only of the presynaptic protein SYP, but also of the postsynaptic proteins GluN2B (NMDA-R) and PSD-95 was unexpected, since the paired pulse facilitation analysis revealed that WASP-1 has only a presynaptic effect. WASP-1-induced increases in the levels of SYP (a synaptic vesicle protein), is consistent with the finding that WASP-1 modulates the release of neurotransmitters at the presynaptic terminal.

However, WASP-1-induced increases in the levels of GluN2B (NMDA-R) and PSD-95 could be explained by assuming that long-lasting administration of WASP-1 could cause a dual effect on the synaptic structure that affect both presynaptic and postsynaptic sites. This dual effect has been previously reported for the canonical *Wnt-7a* ligand, which can promotes the clustering of several presynaptic proteins (Cerpa et al., 2008; Farias et al., 2007) and, at the same time, increases the density and maturity of dendritic spines (Ciani et al., 2011; Gogolla et al., 2009). A possible cause for this dual effect is that different signaling pathways acting on different synaptic structures can be activated by a single *Wnt* ligand. Indeed, it has been shown that *Wnt-3a* ligand can activates both the canonical (acting at the presynaptic region), as well as, the non-canonical *Wnt* signaling (acting at the postsynaptic region) (Cerpa et al., 2009), and the activation of each pathway might depends on the ligand concentration (Nalesso et al., 2011).

Interestingly, we have shown here that the effects of WASP-1 on synaptic plasticity could be modulated by protein synthesis. Despite the fact that co-perfusion of WASP-1 plus a transcription inhibitor did not affect LTP-maintenance, we found that co-perfusion of WASP-1 plus a translator inhibitor completely blocked LTP-maintenance without change LTP-induction. These findings indicate that WASP-1-induced facilitation of l-LTP depends on the synthesis of new proteins, but it does not require the synthesis of new mRNA. Following the categorization of LTP in LTP1, LTP2 and LTP3 according to the differences in the durability and the molecular mechanisms involved in each one (Racine et al., 1983; Raymond and Redman, 2006), WASP-1 seems to elicit LTP2, which depends on protein synthesis from pre-existing mRNA located in the dendrites and is independent of gene transcription (Raymond, 2007).

On the other hand, positive effects of WASP-1 on Aβ-impaired LTP-induction and LTP-maintenance could be related with WASP-1-induced reduction of hippocampal levels of Aβ

oligomers. We found here that WASP-1 also reduces hippocampal levels of the Aβ*56 oligomeric specie in APP/PS1 mice, probably by blocking Aβ aggregation. These findings are consistent with the notion that blockade of Aβ oligomerization could rescues LTP impairment (Walsh et al., 2005). In the present study, WASP-1 was also shown to reduce *tau* pathology, another key hallmark of AD, besides Aβ aggregates (Sheng et al., 2012). The reduction of phosphorylated *tau* levels could be a consequence of WASP-1-induced decreases of Aβ accumulation and/or a side effect of the inhibition of GSK-3β, a kinase which activity is down regulated by the activation of *Wnt*/β-catenin signaling (Clevers and Nusse, 2012), and that is involved in *tau* phosphorylation (Hooper et al., 2008). Indeed, we found previously that WASP-1 could modulates the activity of GSK-3β in the hippocampus of WT mice (Vargas et al., 2014).

In AD pathology, several components of *Wnt*/β-catenin signaling have shown to be altered (Inestrosa et al., 2012; Moon et al., 2004; Oliva et al., 2013). Particularly, β-catenin levels in AD brains are considerably down-regulated compared to healthy brains (Zhang et al., 1998). Moreover, there is relationship between familial onset AD and the de-regulation of β-catenin. In fact, pathogenic mutations in the PS1 gene, one of the major causes for early-onset AD, have shown to affect the stability of β-catenin increasing its degradation (Zhang et al., 1998). Here we have shown that hippocampal β-catenin levels are significantly reduced in APP/PS1 mice compared to the levels in WT animals, however, WASP-1 treatment almost completely rescued this loss, suggesting that WASP-1 could influence β-catenin stability. Indeed, we reported here that co-incubation of hippocampal neurons with WASP-1 and the canonical *Wnt-3a* ligand strongly increases β-catenin stability, but this effect cannot be achieved by the incubation of WASP-1 alone. These findings suggest that WASP-1 requires the presence of *Wnt-3a* ligand to exert its effect (Beaumont et al., 2007). Therefore, the endogenous *Wnt-3a* ligand should be

locally released in the hippocampus of both WT and APP/PS1 mice in order to the beneficial effects of WASP-1 can be produced. A previous work showed that releasing of endogenous *Wnt-3a* ligand from hippocampal synapses is dependent on NMDA-R activation (Chen et al., 2006). Moreover, we found here that blockage of NMDA-R activation occludes positive effects of WASP-1 on LTP, which indicates that WASP-1 could facilitate a NMDA-R-dependent form of LTP, known as the Hebbian plasticity (Citri and Malenka, 2008). However, the obstruction of WASP-1-induced enhancement of LTP also could be due to a reduction in *Wnt-3a* ligand availability, since blockade of NMDA-R activation could interfere with the release of *Wnt-3a* ligand as well. These findings suggest that WASP-1 in cooperation with the endogenous *Wnt-3a* ligand, could act on hippocampal neurons to potentiate *Wnt*/β-catenin signaling and consequently to produce functional improvement of the synapses in both normal and Aβ-induced pathologic conditions.

Altogether, these data suggest that WASP-1, through *Wnt* signaling, can rescues Aβ-induced synaptic impairments and could be potentially used in the treatment of patients with neurodegenerative diseases such as AD.

Conclusions.

In this study, we show that WASP-1 potentiates the activation of *Wnt*/β-catenin signaling in hippocampal neurons, therefore increasing normal synaptic function or rescuing Aβ-induced synaptic impairment (Fig. 6). Under normal conditions, WASP-1 could potentiate the effect of endogenous *Wnt-3a* ligand and increase β-catenin stability. At the presynaptic terminal, WASP-1 enhances excitatory synaptic transmission by increasing neurotransmitters release. At the postsynaptic compartment, WASP-1 elicits a form of LTP that requires the activation of

NMDA-R for LTP-induction and depends on translation of pre-existing mRNA at the dendritic spines for LTP-maintaining (Fig. 6A). In Aβ-impaired synapses, WASP-1 could reduce both aggregation of Aβ oligomers and *tau* phosphorylation. At the presynaptic terminal, WASP-1 enhances synaptic transmission, probably by increasing the levels of the synaptic vesicle protein, SYP. At the postsynaptic compartment, WASP-1 overcomes LTP impairments probably by rescuing diminished levels of PSD-95 and the GluN2B subunit of NMDA-R (Fig. 6B). In summary, our findings suggest that WASP-1 could be of therapeutic interest for AD treatment.

Material and Methods.

Reagents. WASP-1 (2-(2,7-diethoxy-9H-fluoren-9-ylidene) hydrazine-carboximidamide) from Chemdiv, Inc. Synthetic $A\beta_{1-42}$ peptide corresponding to the human Aβ wild-type from Genemed Synthesis, Inc. Actinomycin-D and Cycloheximide purchased from Sigma-Aldrich, Inc. Primary antibodies used are as follows: mouse anti-β-actin and rabbit anti-synaptophysin (Cell Signaling Technology, Inc.), mouse anti-Aβ (6E10; Chemicon International, Inc.), mouse anti-β-catenin (Santa Cruz Biotechnology, Inc.), mouse anti-*tau* epitope paired-helical filament-1 (PHF-1) was a gift of Dr. Peter Davis, from the Albert Einstein College of Medicine, NY. Monoclonal antibodies: mouse anti-PSD-95 and mouse anti-GluN2B were purchased from Neuromab, Davis/NIH NeuroMab Facility.

Primary neuronal cell culture. Hippocampal neurons were obtained from Sprague-Dawley rats on embryonic day 18. Hippocampi were dissected and primary cultures were prepared as described previously (Farias et al., 2007) and maintained in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10% horse serum for 2 h. The culture medium was replaced by Neurobasal medium supplemented with B27, 100 μg/ml streptomycin, and 100 U/ml penicillin. On day 3 cells were treated with 2 μM AraC for 24 h to reduce the number of proliferating non-neuronal cells. Experiments were performed on day 18 in the presence or absence of WASP-1 and *Wnt-3a*-conditioned media.

Conditioned medium preparation. HEK-293 cells were transiently transfected by calcium phosphate precipitation with equal amounts of empty vector pcDNA (control) or pcDNA containing sequences encoding *Wnt-3a* ligand. Cells were grown to 85% confluence and maintained in Neurobasal medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin for 60 h. Conditioned media were prepared as described previously (Farias et al., 2007).

Chronic treatments. Double transgenic APPswe/PS1dE9 male mice (known as APP/PS1 in this study) were purchased from Jackson Laboratory and wild-type (C57/BL6) mice were obtained from a colony of the Animal House Facility of P. Universidad Católica de Chile. Seven months-old mice received bilateral intra-hippocampal infusion of 5 μM WASP-1 or artificial cerebrospinal fluid (ACSF) during 21 days. Chronic delivery was achieved via Alzet osmotic mini-pump (Alzet 1004, Durect Co.). The assembly and pre-incubation of the pumps were performed as described previously (62), following the manufacturer's instructions. Pumps were filled with WASP-1 or ACSF and then connected to a double brain infusion cannula (Plastics One Inc.). The infusion system was incubated in sterile saline at 37 ºC for 48 h before surgery.

Surgical procedures. For implantation of the infusion system, mice were anesthetized by isoflurane inhalation (1.5-2.5 %) and placed in a small animal stereotaxic frame (Stoelting Co.). The brain infusion cannula was inserted in the hippocampus according to coordinates: -2.46 mm anterior to the bregma, 1.0 mm lateral, and 2.0 mm ventral to the skull surface. The cannula was fixed to the skull surface using binary dental cement. The mini-pump was inserted beneath the skin at the dorsum of the animal and the wound was closed using cyanoacrylate (Vargas et al., 2014). Mice were allowed to recover for 1 week before testing.

Electrophysiological recordings. Slices preparation and field electrophysiological recording was performed as described previously (Bonansco et al., 2011; Cerpa et al., 2008). Briefly, hippocampal slices were prepared from 7 months-old WT mice. Animals were decapitated and the brain was submerged in cold ACSF bubbled with carbogen (95% O_2, 5% CO_2). Coronal slices from the hippocampus (250 μm) were cut using a Vibroslice microtome (World Precision Instruments Inc.) and incubated in gassed ACSF for 1 h at room temperature (24 °C). Slices were transferred into a chamber and superfused (3 ml/min) with gassed ACSF, unless otherwise noted. In all experiments picrotoxin (10 μM) was added to suppress inhibitory γ-aminobutyric acid (GABA), type-A transmission. Schaeffer collaterals fibers were activated through bipolar cathodic stimulation, generated by a stimulator (Master 8, AMPI) connected to an isolation unit (Isoflex, AMPI). Electric pulses (50 μs, 0.3 Hz, 20–100 μA) were applied on Schaeffer collaterals. Recording of fEPSP was carried out with a glass pipette (2–4 megaOhms, filled with ACSF) with a bipolar concentric electrode (platinum/iridium, 125 μm outer diameter, FHC Inc.), placed in the middle of CA1 stratum radiatum and connected to an AC amplifier (Grass Inc.) with 10,000 × gain, 3.0 kHz low pass filter, and 0.30 Hz high pass filter. To evaluate WASP-1

regulation on fEPSP amplitude, we estimated changes in the paired pulse facilitation which are considered to be of presynaptic origin (Clark et al., 1994). The paired pulse facilitation index was calculated by (R2-R1)/R1, where R1 and R2 are the peak amplitudes of the first and second postsynaptic responses, respectively. To elicit LTP we used a high frequency stimulation (HFS) protocol (Bozdagi et al., 2010), consisting of two trains of 500 msec, 100 Hz, separated by 1 sec. Analysis of fEPSPs were performed with Clampfit 10 (pCLAMP software, Molecular Devices).

Amyloid species preparation. Aβ peptide stock solution was prepared by dissolving freeze-dried aliquots of Aβ in dimethyl sulfoxide (DMSO) at 15 mg/ml. Aβ oligomers were prepared by dissolving Aβ peptide in 0.5% PBS for a final concentration of 50 μM and then subjected to a basic shock adding 2N NaOH to reach pH 12 and neutralized with 1N HCl. The mixture was incubated at room temperature under constant agitation during 1 h to obtain the Aβ oligomers. Aβ oligomer formation has been previously visualized by electron microscopy (Dinamarca et al., 2010).

Amyloid aggregation assay. An aliquot of Aβ peptide stock solution (15 μg/μl) was added to the aqueous PBS buffer pH 7.4. A 100 μM solution was prepared from the Aβ stock solution added to a buffer containing 500 nM, 5 μM or 50 μM WASP-1. Samples were stirred continuously at room temperature at 1300 r.p.m. and sterile aliquots were taken at different time points for analysis. Aliquots were taken to measure Aβ fibril formation by Thioflavine-T (Th-T) fluorescence.

Th-T based fluorimetric assay. Aβ aggregates obtained were characterized by Th-T fluorimetric assay (LeVine, 1993). Th-T binds specifically to amyloid fibrils, thereby producing a shift in its emission spectrum and an increase in the fluorescent signal, proportional to the amount of amyloid fibrils formed. Following incubation of Aβ alone or co-incubated with WASP-1, 50 mM sodium phosphate buffer pH 6.0 and 0.1 mM Th-T in a final volume of 2 ml were added. Fluorescence was monitored at excitation 450 nm and emission 485 nm using a JASCO FP-6200 Spectrofluorometer, as described previously (Dinamarca et al., 2010).

Western Blot. Hippocampi of chronically treated mice or cultured hippocampal neurons were homogenized in RIPA buffer. Samples were centrifuged at 14000 r.p.m. at 4 ºC for 5 min. Supernatants were tested for protein concentrations using the BCA Protein Assay Kit (Pierce Chemical Co.). Equal amounts of protein of each sample were resolved using either Tris-Tricine-SDS gel (16% acrylamide) to identify peptides or 10% SDS-PAGE to identify proteins. Samples were transferred to PVDF membranes, and immunoblotting was conducted. Immunoreactivity was visualized using a chemiluminescent substrate (Thermo Fisher Scientific Inc.), and optical densities were quantified using NIH Image J software.

Statistical Analysis. Data was expressed as mean ± S.E. of the values from the number of experiments as indicated in the corresponding figures. Statistical analysis was performed using Student's two-tailed *t*-test after normal distribution of the data was verified using Shapiro-Wilk normality test (SigmaPlot 12.0). Differences were considered significant at $*p<0.05$ or $**p<0.01$.

Competing interests.

The authors declare that they have no competing interests.

Authors' contributions.

J.Y.V. carried out the experiments, participated in the study design, performed statistical analysis and wrote the manuscript. J.A. and M.S.A. carried out experiments and performed statistical analysis. M.F. and N.C.I. conceived the study, participated in its design and coordination, revised and helped to draft the manuscript.

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Figure 1.

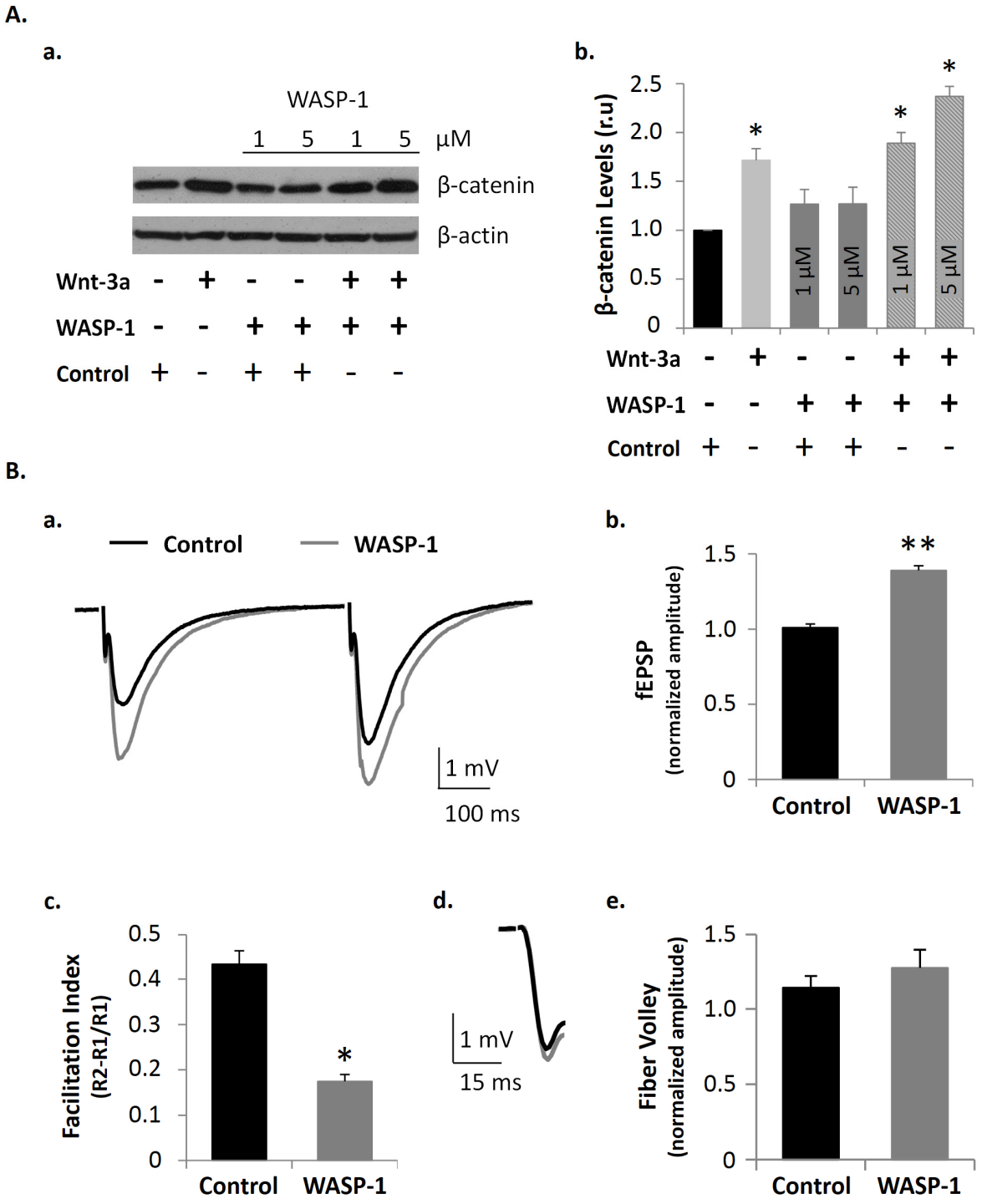
A.
a.
WASP-1
1 5 1 5 μM
β-catenin
β-actin
Wnt-3a - + - - + +
WASP-1 - - + + + +
Control + - + + - -
b.
β-catenin Levels (r.u)
2.5
2.0
1.5
1.0
0.5
0
1 μM
5 μM
1 μM
5 μM
Wnt-3a - + - - + +
WASP-1 - - + + + +
Control + - + + - -
B.
a.
Control
WASP-1
1 mV
100 ms
b.
fEPSP
(normalized amplitude)
1.5
1.0
0.5
0
Control
WASP-1
c.
Facilitation Index
(R2-R1/R1)
0.5
0.4
0.3
0.2
0.1
0
Control
WASP-1
d.
1 mV
15 ms
e.
Fiber Volley
(normalized amplitude)
1.5
1.0
0.5
0
Control
WASP-1

Figure 1. WASP-1 potentiates canonical *Wnt* signaling activation and enhances glutamatergic synaptic transmission by a presynaptic mechanism. (A) Western blot analysis of β-catenin levels in hippocampal cultured neurons treated with *Wnt-3a* or control vector conditioned media alone or in the presence of 1 μM or 5 μM WASP-1. (a) Immunoblotting of β-catenin in a representative experiment. (b) Densitometric analysis for the relative levels of β-catenin after β-actin normalization. The graph shows fold increase versus control (black bar). Data corresponds to mean ± SE for four experiments. (B) Excitatory synaptic transmission in mouse hippocampal slices (n= 6) treated with 5 μM WASP-1. (a) Average traces (60 recordings) of fEPSP evoked by paired pulse protocol before (control) and after 30 min of continued perfusion with WASP-1. (b) Average values of normalized fEPSP amplitudes for the first response in control and WASP-1 treated slices. (c) Facilitation index measured before and after WASP-1 application. (d) Average traces of fiber volley in control and after WASP-1 treatment. (e) Normalized fiber volley amplitude in control and after WASP-1 treatment.

Figure 2.

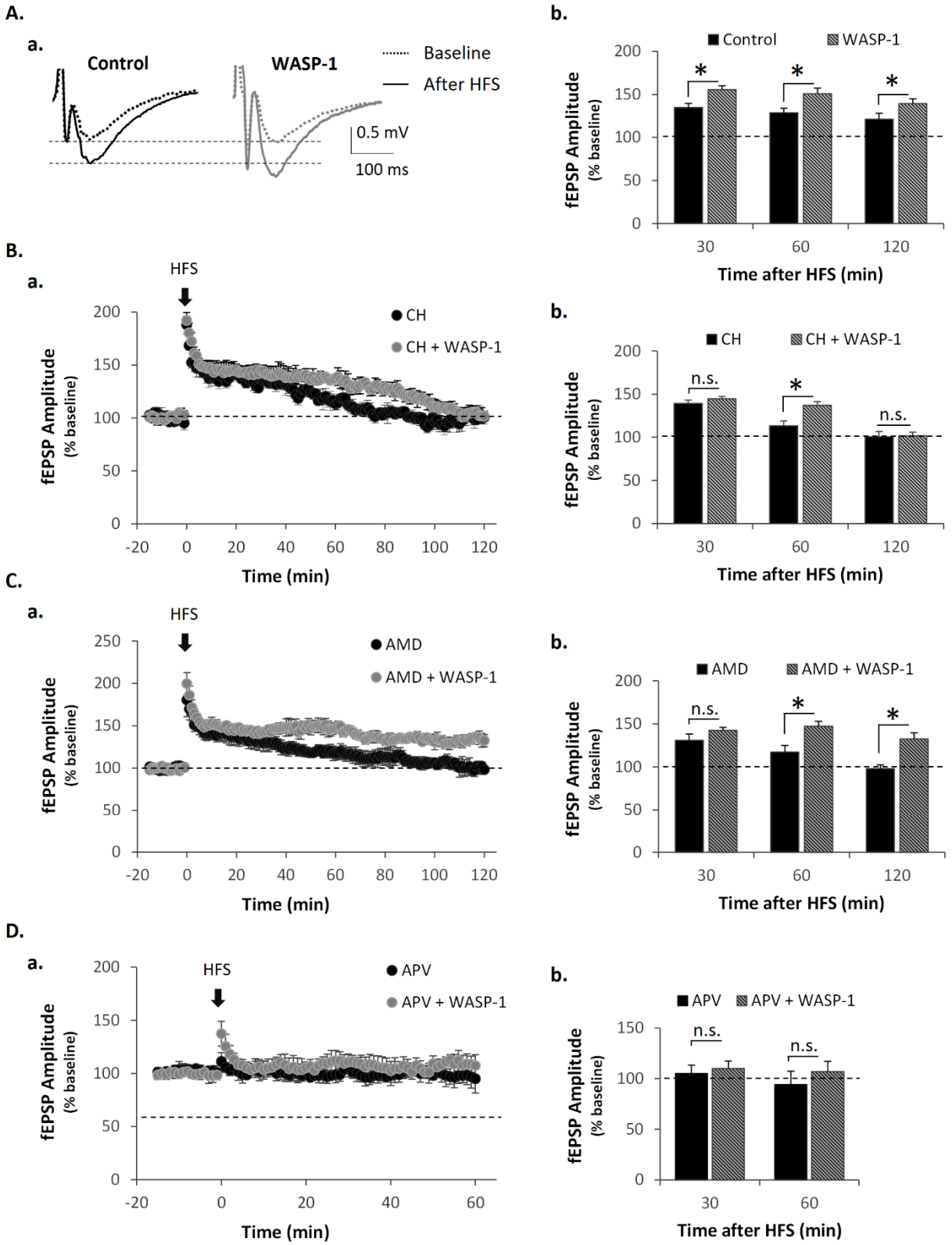
A.
a.
Control
WASP-1
Baseline
After HFS
0.5 mV
100 ms
b.
Control
WASP-1
fEPSP Amplitude (% baseline)
Time after HFS (min)
B.
a.
HFS
CH
CH + WASP-1
fEPSP Amplitude (% baseline)
Time (min)
b.
CH
CH + WASP-1
n.s.
fEPSP Amplitude (% baseline)
Time after HFS (min)
C.
a.
HFS
AMD
AMD + WASP-1
fEPSP Amplitude (% baseline)
Time (min)
b.
AMD
AMD + WASP-1
n.s.
fEPSP Amplitude (% baseline)
Time after HFS (min)
D.
a.
HFS
APV
APV + WASP-1
fEPSP Amplitude (% baseline)
Time (min)
b.
APV
APV + WASP-1
n.s.
fEPSP Amplitude (% baseline)
Time after HFS (min)

Figure 2. WASP-1 enhances hippocampal LTP through a mechanism that requires protein translation and the activation of NMDA receptor. (A) fEPSP recordings of hippocampal slices perfused with WASP-1 (n = 7) or vehicle (n = 8). (a) Representative traces of fEPSP before (dotted lines) and after (filled lines) HFS. (b) Comparison of mean fEPSP amplitude between WASP-1 and control slices at 30, 60 and 120 min after HFS. Graphic shows percent increase of fEPSP amplitude compared to baseline (dotted line). (B) Effect of perfusion of a protein translator inhibitor (CH) on WASP-1-enhanced LTP. Time course of normalized fEPSP amplitude (a) and comparison of mean fEPSP amplitude at 30, 60 and 120 min after HFS (b), between CH (n = 4) or CH plus WASP-1 (n = 5) treated slices, showing percent increase versus baseline (dotted line). (C) Effect of perfusion of a gene transcription inhibitor (AMD) on WASP-1-enhanced LTP. Time course of normalized fEPSP amplitude (a) and comparison of mean fEPSP amplitude at 30, 60 and 120 min after HFS (b), between AMD (n = 5) or AMD plus WASP-1 (n = 4) treated slices, showing percent increase versus baseline (dotted line). (D) Effect of perfusion of a NMDA-R inhibitor (APV) on WASP-1-enhanced LTP. Time course of normalized fEPSP amplitude (a) and comparison of mean fEPSP amplitude at 30 and 60 min after HFS (b), between APV (n= 5) or APV plus WASP-1 (n= 6) treated slices, showing percent increase versus baseline (dotted line).

Figure 3.

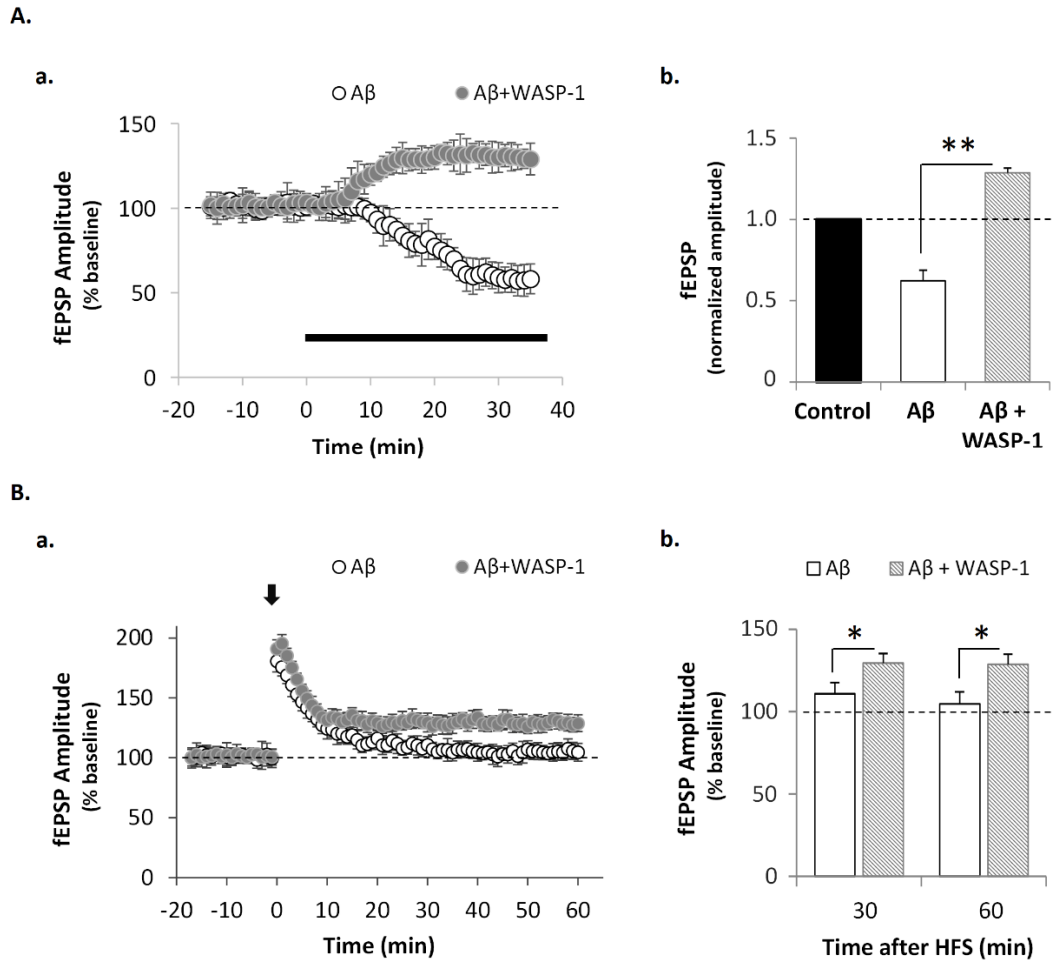

Figure 3. WASP-1 rescues Aβ-induced synaptic impairment of glutamatergic synapses in mouse hippocampal slices. (A) fEPSP recordings before (control) and after 30 min of continued perfusion either with 50 µM Aβ alone or 50 µM Aβ in the presence of 5 µM WASP-1. (a) Time course of effect of Aβ (white circles) or Aβ plus WASP-1 (gray circles) on fEPSP peaks amplitude. The black bar indicates the perfusion duration of both treatments and the dotted line indicates baseline level. (b) Average values of normalized fEPSP amplitude measured before (control) and after 15 min perfusion of Aβ alone (n= 4) or Aβ plus WASP-1 (n= 5) treatment application. (B) fEPSP recordings of hippocampal slices before (baseline) and after application of HFS protocol. (a) Time course of normalized fEPSP amplitude (from 15 min before HFS (arrow) up to 65 min after), in slices perfused with 50 µM Aβ alone or 50 µM Aβ in the presence of 5 µM WASP-1. Graphic shows percent increase of fEPSP amplitude in Aβ (white circles) or Aβ plus WASP-1 (gray circles) treated slices compared to baseline (dotted line). (b) Comparison of mean fEPSP amplitude between Aβ (n= 5) or Aβ plus WASP-1 (n= 5) treated slices, at 30 or 90 min after HFS, showing percent increases from baseline (dotted line).

Figure 4.

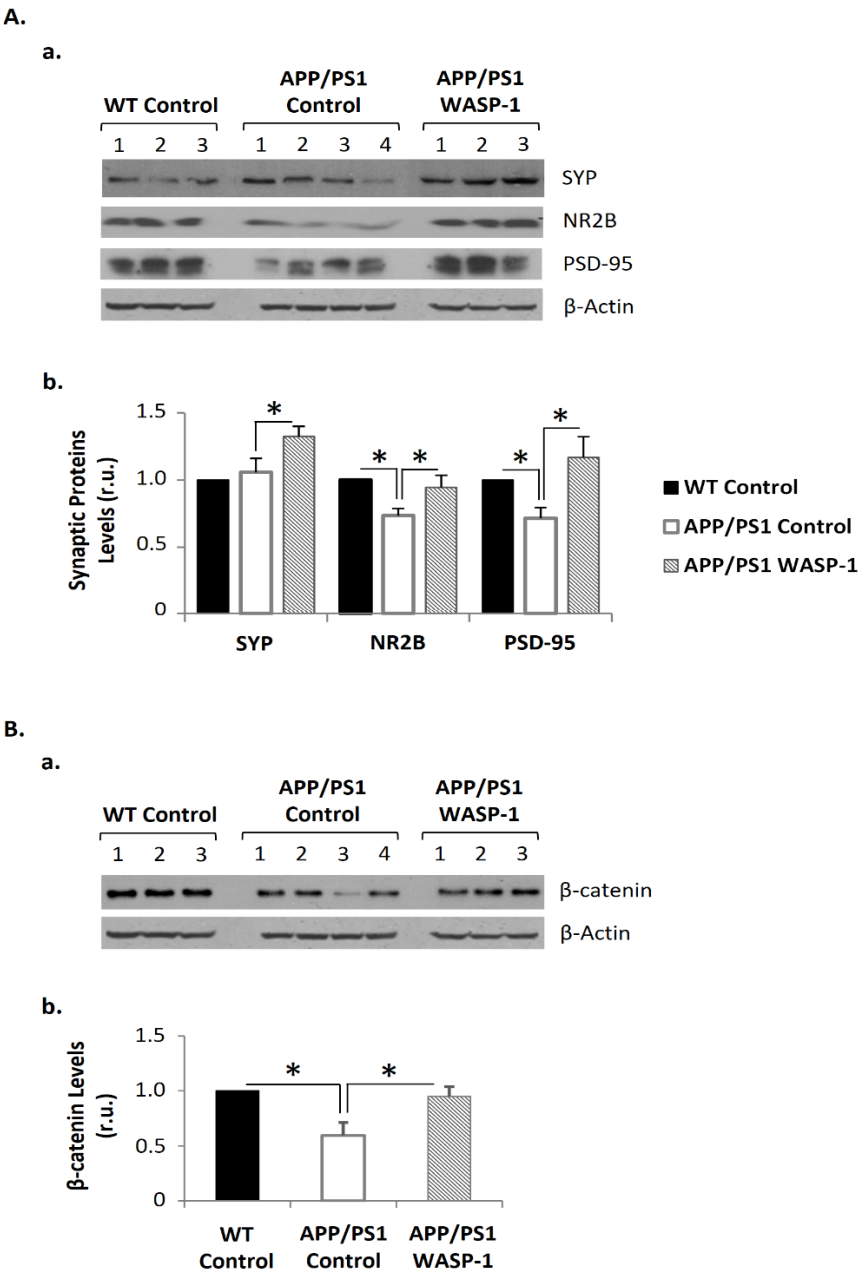

Figure 4. WASP-1 rescues loss of synaptic proteins and β-catenin in the hippocampus of APP/PS1 double transgenic mice. Western blot analysis of hippocampal homogenates from WT control mice (n = 3), APP/PS1 control mice (n = 4) and APP/PS1 mice treated with WASP-1 (n = 3) to detect presynaptic (SYP) and postsynaptic (PSD-95 and NR2B) proteins (A) or β-catenin levels (B). In (a) immunoblotting of indicated proteins. In (b) densitometric analysis for the relative levels of indicated protein after β-actin normalization. The graphs show fold of increase versus WT control (black bar) and significant differences among groups are also indicated.

Figure 5.

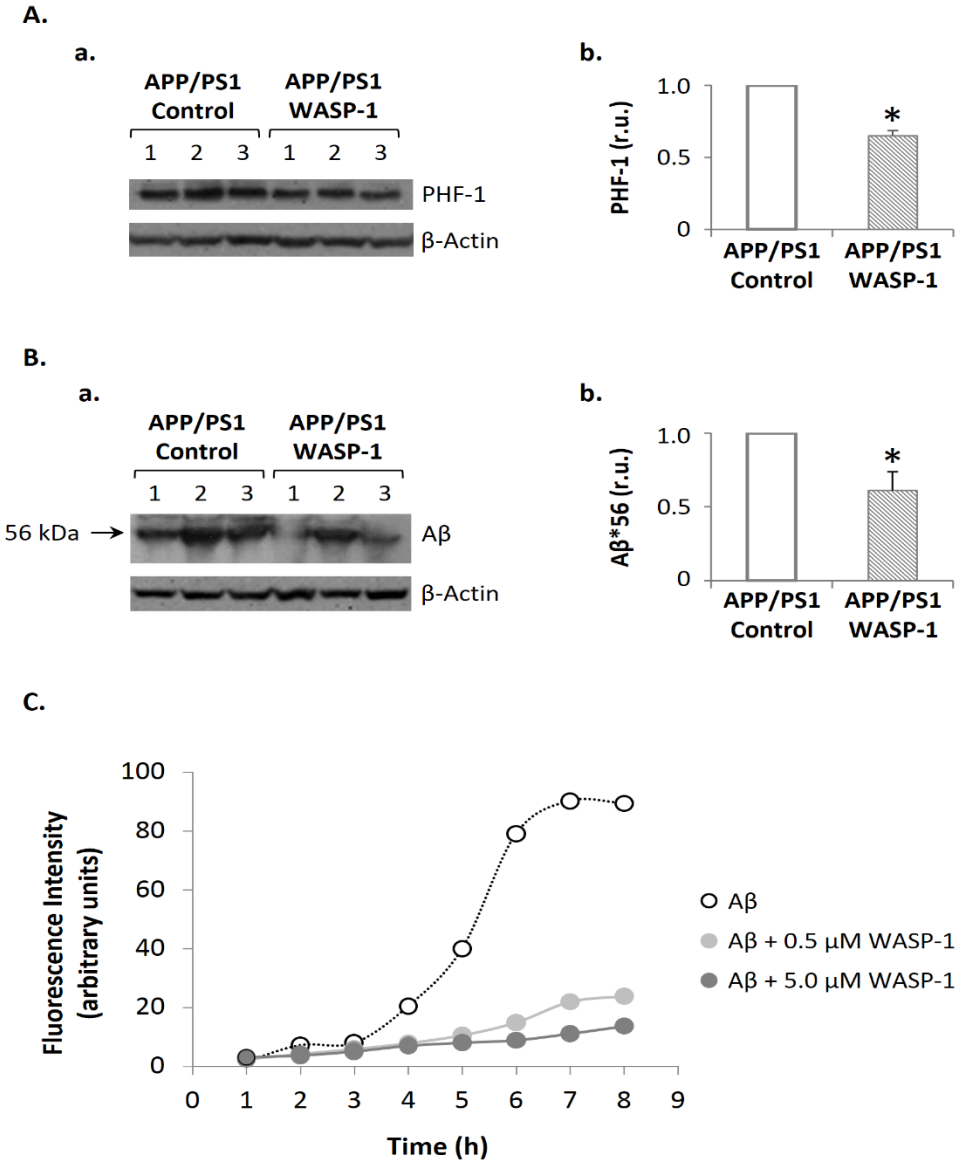

Figure 5. WASP-1 reduces *tau*-phosphorylation in the hippocampus of APP/PS1 mice and blocks Aβ aggregation. Western blot analysis of PHF-1 (A) or Aβ*56 oligomeric form (B) in hippocampal homogenates from APP/PS1 mice treated with or without WASP-1. In (a) immunoblotting of indicated protein. In (b) densitometric analysis for the relative levels of indicated protein after β-actin normalization of n = 3 per experimental group. The graph shows fold of decrease of protein levels in APP/PS1 mice treated with WASP-1 (gray bar) versus APP/PS1 control mice (white bar). (C) *In vitro* amyloid aggregation assay carry out with 100 μM Aβ solution alone or in the presence of 0.5 μM or 5 μM WASP-1. Graphic shows time course of fluorescence intensity among different treatments.

Figure 6.

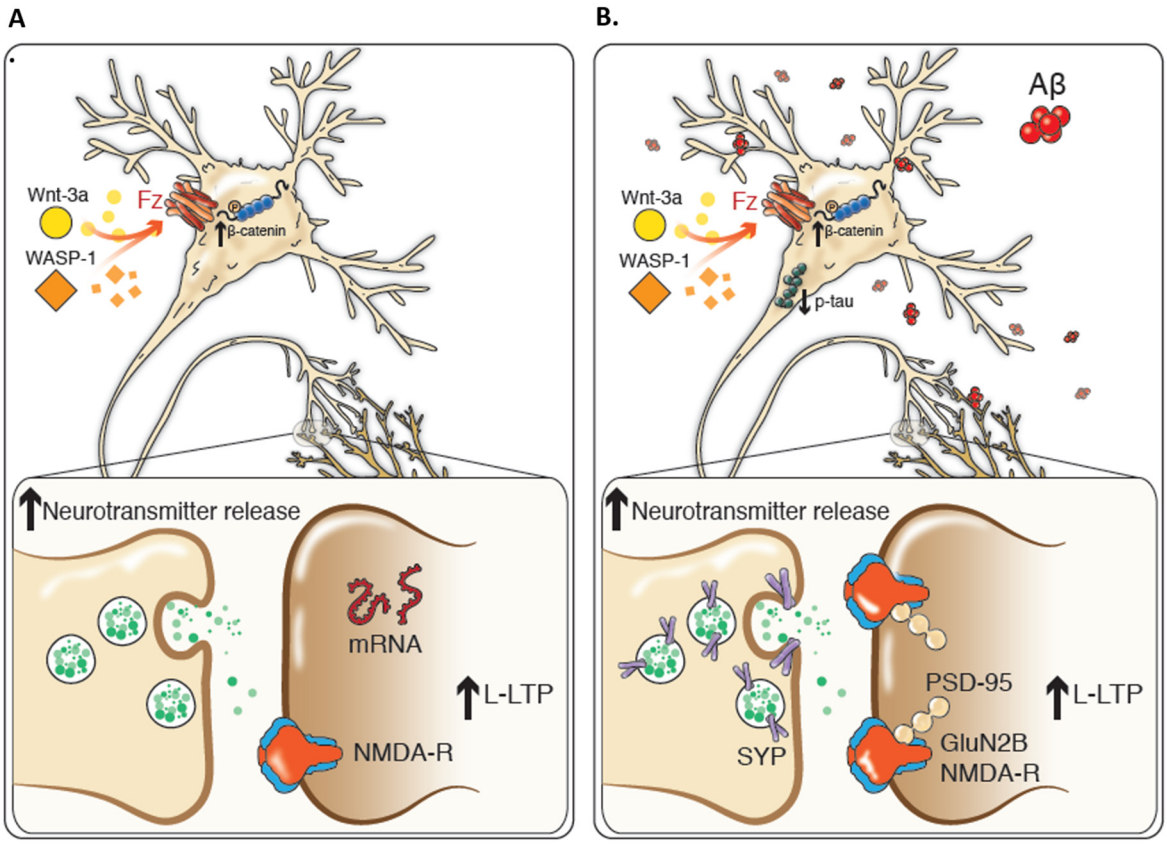

Figure 6. Scheme of the effects of WASP-1 over *Wnt-3a* signaling in hippocampal synapses. WASP-1 enhances synaptic transmission by triggering an increase in the neurotransmitter release and it potentiates synaptic plasticity by inducing an increase of LTP magnitude. The scheme, summarizes the main ideas of the present study. (A) Under normal conditions, WASP-1 potentiates *Wnt* signaling, expressed by an increase of β-catenin, which in turn, activates exocytosis of synaptic vesicles with an increase of glutamate release at the pre synaptic region, while, at the postsynaptic region, the activation of NMDA-R allows the potentiation of the synapse and the expression of the LTP-2. The pre-existent mRNA for some of the NMDA-R subunits is showed in the postsynaptic region. (B) Under pathological conditions, such as those present in AD, where the loss of *Wnt* signaling function is apparent and the synaptic plasticity is partially or totally blocked by Aβ oligomers, the protective effect of WASP-1 is fully expressed, by potentiating *Wnt*/β-catenin signaling. The activation of this signaling leads to the inhibition of GSK-3β, therefore increasing β-catenin and reducing *tau*-phosphorylation. At the same time, the Aβ aggregation is prevented by WASP-1, and the formation of the Aβ oligomers decreases, facilitating both an increase in the release of glutamate, as well as, the recovery of the LTP. Thanks to WASP-1-induced increase of SYP at the presynaptic region, a further commitment of synaptic vesicles for the release of glutamate is achieved. At the postsynaptic

region, WASP-1-induced increase of the scaffold PSD-95 protein facilitates the incorporation of vesicles containing newly synthetized GluN2B subunit of the NMDA-R (possibly translated at the dendritic spines from pre-existent mRNA) into the postsynaptic membranes. Together, all these WASP-1-induced features allow the recovery of the Aβ-impaired synaptic plasticity and contribute to improve neurodegenerative conditions observed in AD.

4. DISCUSSION

Previously, several *in vitro* studies have suggested that *Wnt* signaling participates in modulating synaptic plasticity of the adult brain (Budnik and Salinas, 2011; Inestrosa and Arenas, 2010; Rosso and Inestrosa, 2013) but, so far there were not *in vivo* evidences that support a role for *Wnt* signaling in the adult hippocampal cognitive function. Now we provided the first evidence that *in vivo* activation of *Wnt* signaling enhances synaptic plasticity and improves memory in adult mice. Moreover, we found that activation of *Wnt* signaling can rescue synaptic and memory impairments in adult APP/PS1 mice, also causing a reduction of several markers of AD pathology. These results suggest that *Wnt* signaling could have a role in synaptic function that underlies memory in the adult brain.

4.1. *Wnt* signaling as a key factor in synaptic remodeling.

Wnt ligands have been linked to the assembly of structural components at both presynaptic and postsynaptic sites (Farias et al., 2010; Oliva et al., 2013a). Several *in vitro* studies have shown that canonical *Wnt* ligands could modulate the structure of the presynaptic compartment by inducing axonal spreading and increasing growth cone size and branching during neuronal development, but also by leading the accumulation of synaptic

proteins at the presynaptic terminal of mature neurons (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008; Farias et al., 2007; Hall et al., 2000; Lucas and Salinas, 1997). On the other hand, non-canonical *Wnt* ligands have shown to modulate the assembly of the postsynaptic compartment by increasing the clustering of postsynaptic proteins, while changing the postsynaptic structure through inducing *de novo* formation of dendritic spines or increasing the size of pre-existent ones (Cuitino et al., 2010; Farias et al., 2009; Varela-Nallar et al., 2010). In summary, these evidences seem to suggest that canonical *Wnt* ligands selectively modulate the presynaptic sites, while non-canonical *Wnt* ligands would modulate the postsynaptic structure (Cerpa et al., 2009). However, by using an *in vivo* approach, now we found that the long-term activation of canonical has an impact in both presynaptic and postsynaptic sites. Indeed, we found that WASP-1 chronic treatment rises the levels of hippocampal presynaptic and postsynaptic proteins in both wild-type and APP/PS1 mice, indicating that canonical *Wnt* signaling could have a bidirectional synaptic effect. This dual effect has been previously reported for the canonical *Wnt-7a* ligand, which, on one hand can promote the clustering of several presynaptic proteins (Cerpa et al., 2008; Farias et al., 2007), and on the other hand, can increase the density and maturity of dendritic spines (Ciani et al., 2011; Gogolla et al., 2009). This effect could be explained by assuming that a single *Wnt* ligand can activate different signaling branches. Supporting this idea, a recent study has demonstrated that canonical *Wnt-3a* ligand could activate both canonical and non-canonical pathways, but the type of *Wnt* signaling activated depends on ligand concentration (Nalesso et al., 2011). Thus, low concentrations of *Wnt-3a* ligand could trigger *Wnt*/Ca^{2+} signaling, whereas, high concentrations could activate *Wnt*/β-catenin signaling instead (Kestler and Kuhl, 2011; Nalesso et al., 2011).

Even though, we found that chronic activation of non-canonical *Wnt* signaling by FOXY-5 treatment, was not able to produce significant changes in the levels of assayed presynaptic proteins, a tendency to an up-regulation was observed, suggesting that more studies are required to understand and analyses the involving of non-canonical *Wnt* signaling in the modulation of the postsynaptic structure.

Interestingly, *Wnt*-induced structural modifications of synaptic sites could be associated to synaptic plasticity events. Indeed, it was demonstrated that the remodeling of the hippocampal synaptic structures induced by an enriched environment exposure, could be mimicked by the activation of *Wnt* pathway or inhibited by the blockage of this signaling (Gogolla et al., 2009). Moreover, we found here that the activation of *Wnt* signaling induces an improvement on hippocampal cognitive function of adult mice that correlates with an increase in synaptic protein expression. However, more studies are required to fully understand how *Wnt*-induced synaptic remodeling could later translate into cognitive improvements, and the mechanism that underlies this process.

4.2. *Wnt* signaling as a synaptic plasticity regulator.

Synaptic plasticity is considered as the foundation of complex brain functions, such as learning and memory. In this phenomenon, the ups and downs in evoked neural activity lead to long-term modifications of synaptic efficacy, frequently accompanied by changes in the synaptic structure (Citri and Malenka, 2008). The fact that *Wnt* ligands are released at the synapses in an activity-dependent manner to induce modification in the synaptic structure and function (Ataman et al., 2008; Chen et al., 2006a), suggest a role for *Wnt* signaling in the regulation of synaptic plasticity on mature synapses. Support for this hypothesis came from

evidences showing that activation of *Wnt* signaling facilitates hippocampal LTP, while the blockage of this signaling impairs it (Cerpa et al., 2011; Chen et al., 2006a). Furthermore, *Wnt* signaling activation has also shown to increase excitatory synaptic transmission in hippocampal neurons (Beaumont et al., 2007; Cerpa et al., 2008; Varela-Nallar et al., 2010). Here we presented evidences that *in vivo* activation of *Wnt* signaling results in an enhancement of basal excitatory synaptic efficacy as well as the hippocampal LTP facilitation.

Previously, it has been shown that both canonical and non-canonical *Wnt* ligands can enhance hippocampal glutamatergic transmission (Cerpa et al., 2011; Cerpa et al., 2008; Varela-Nallar et al., 2010). Consistent with this observation, our results show that *in vivo* activation of canonical and non-canonical *Wnt* signaling equally enhance basal excitatory synaptic transmission of adult mice. Even though the activation of both pathways have the same effect on the excitatory transmission, it seems that different mechanisms are involved in each effect. Thus, canonical *Wnt* ligands could modulate synaptic transmission by controlling presynaptic vesicles recycling and neurotransmitters release through a mechanism that diverges from the transcription-dependent pathway (Cerpa et al., 2008). On the other hand, non-canonical *Wnt* ligands could regulate synaptic transmission through inducing modifications on the postsynaptic structure by promoting morphogenesis and remodeling of dendritic spines, in a Ca^{2+}-dependent manner (Varela-Nallar et al., 2010).

Interestingly, *Wnt* ligands not only have an influence on synaptic transmission, but also, they can change long-term synaptic plasticity by facilitating the induction and maintenance of LTP (Cerpa et al., 2011; Chen et al., 2006a). As we showed here, *in vivo* activation of both canonical and non-canonical *Wnt* signaling induces an enhancement of the LTP magnitude, which agrees and validates those previous findings. In order to determine whether *Wnt*

signaling activation is required for LTP expression in the mouse hippocampus, we performed some experiments that allowed us to test *in vivo* the effects of *Wnt* loss of function. To block *Wnt*/β-catenin signaling, we used a competitive inhibitor of the inactivating GSK-3β phosphorylation, and to block *Wnt*/JNK signaling, we used a JNK inhibitor. We found that both *Wnt* inhibitors were able to interfere the hippocampal LTP facilitation induced by WASP-1 and FOXY-5, when the activator and the matching inhibitor were administered together. Interestingly, we found that the administration of both *Wnt* inhibitors alone were enough to almost completely block the LTP induction, suggesting that endogenous *Wnt* activity is necessary for normal synaptic plasticity events take place.

We found that the enhancement of the LTP magnitude induced by the *in vivo* activation of *Wnt* signaling is accompanied by a significant improvement of the animal's performance in two different episodic memory task. However, in order to establish a better correlation between *Wnt*-induced LTP facilitation and cognitive enhancement, *in vivo* electrophysiological recordings in freely behaving animals, are required. Even though, our results show that the activation of either canonical or non-canonical pathways has a positive effect on hippocampal LTP, we found that the activation of non-canonical *Wnt* signaling by FOXY-5 treatment has a stronger effect on the LTP magnitude in comparison to the activation of canonical *Wnt* signaling by WASP-1 treatment. These differences could arise from dissimilar mechanism involved in the activation of the pathway and/or in the effects downstream. Indeed, while FOXY-5 directly activates non-canonical *Wnt* signaling (Safholm et al., 2006), WASP-1 requires the presence of *Wnt-3a* ligand to synergistically activate canonical *Wnt* signaling (Beaumont et al., 2007), suggesting that endogenous *Wnt-3a* ligand is locally released at the hippocampus, allowing that WASP-1 can exerts its effect. Besides, it is possible that the effects of canonical and non-canonical *Wnt* signaling on synaptic

plasticity took place through different mechanisms. Thus, canonical *Wnt* ligands could regulate synaptic plasticity by influencing neurotransmitters release through modulation of the interaction between proteins of *Wnt* pathway and molecules implicated in synaptic plasticity and memory formation. In fact, it has been reported the interaction between β-catenin and cadherins (Maguschak and Ressler, 2008), Dvl and Syt (Kishida et al., 2007), or APC and presynaptic α_7-nAChRs (Farias et al., 2007). Conversely, non-canonical *Wnt* ligands could modulate synaptic plasticity by inducing a rapid increase in the intracellular Ca^{2+} concentrations and the activation of CaMKII (Cerpa et al., 2011; Varela-Nallar et al., 2010).

Moreover, the effects of *Wnt* signaling on synaptic plasticity are also mediated by the modulation of gene expression. Indeed, we found here that the effect of WASP-1 on l-LTP depends on synthesis of new proteins, but it does not depend on synthesis of new RNA, while the effect of FOXY-5 on l-LTP requires both transcription and translation. Following the categorization of LTP in LTP1, LTP2 and LTP3 in accordance to persistence and the mechanisms involved in each form (Racine et al., 1983; Raymond and Redman, 2006), WASP-1 seems to elicit LTP2, which is only dependent on protein synthesis from pre-existing mRNA located in the dendrites; while FOXY-5-induced LTP is consistent with LTP3, since both protein translation and gene transcription are required to l-LTP maintenance. These results suggest that the mechanisms elicited by canonical and non-canonical *Wnt* signaling to influence hippocampal l-LTP, have different requirements.

Interestingly, a regulating feedback between *Wnt* signaling and synaptic plasticity events has been reported. Thus, LTP induces changes in the mRNA levels and the expression patterns of several *Wnt* proteins, such as *Wnt-3a* ligand, Fz-4, β-catenin and Dvl-3 (Chen et al., 2006). LTP also induces the accumulation of β-catenin in the nucleus and a subsequent

increase in the levels of several *Wnt* target genes, suggesting that tetanic stimulation could induce the activation of canonical *Wnt* signaling (Chen et al., 2006). Moreover, the size and number of *Wnt-3a* label at hippocampal synapses, diminishes after tetanic stimulation, probably because *Wnt-3a* ligand is being locally released at the synapse and in a way that depends on NMDA-Rs activation (Chen et al., 2006). Here we found that blockage of NMDA-Rs inhibits the LTP enhancement induced by WASP-1, suggesting that endogenous release of *Wnt-3a* ligand is required to WASP-1 can exert its effect. This finding indicate that LTP expression could be endogenously modulated by *Wnt* signaling.

4.3. Role of *Wnt* signaling in learning and memory.

Recent studies have implicated the canonical *Wnt* signaling in the amygdala-dependent fear memory consolidation and the regulation of hippocampal long-term information storage (Maguschak and Ressler, 2011; Tabatadze et al., 2012). In fact, the infusion of Dkk-1 into the amygdala has shown to impair fear memory consolidation (Maguschak and Ressler, 2011), while infusion of Dkk-1 into the hippocampus impairs object recognition memory consolidation (Fortress et al., 2013). The fact that Dkk-1 impairs two distinct types of memories when infused into multiple brain regions suggests a more general role for canonical *Wnt* signaling in mediating memory consolidation. In addition to Dkk-1, other proteins of the canonical *Wnt* pathway have been involved in memory processing. For instance, APC heterozygous knockout mice display an age-dependent working memory deficit, which suggest a role for APC in this type of memory (Koshimizu et al., 2011).

We showed here that *Wnt* signaling also enhances short-term information storage of both spatial and non-spatial memories in adult mice. Remarkably, we found that not only the

activation of canonical *Wnt* signaling has an effect on memory consolidation, as it has been previously reported (Fortress et al., 2013; Maguschak and Ressler, 2008), since the activation of non-canonical *Wnt* signaling by FOXY-5 has a similar effect. Our results agree with a previous evidence showing that there is a selective increase in the hippocampal levels of *Wnt-7* and *Wnt-5a* ligands, but not *Wnt-3* isoform, during consolidation and recall of spatial memories (Tabatadze et al., 2012). The levels of *Wnt-7* in the hippocampus of animals trained in a water maze hidden platform, were enhanced in comparison to the animals trained with a visible platform, suggesting that activation of *Wnt* signaling could be necessary for memory consolidation (Tabatadze et al., 2012). Moreover, animals trained in the hidden platform also exhibited better retention memory after 30 days of training than those trained in the visible platform, and this performance also correlates to elevated levels of *Wnt-7* ligand (Tabatadze et al., 2012). Similar results were found using a novel object recognition paradigm. After object training, animals show a rapid increase in the hippocampal levels of GSK-3β phosphorylation (Ser-9), β-catenin and cyclin-D1, suggesting that object learning could regulate canonical *Wnt* signaling (Fortress et al., 2013). Conversely, the administration of Dkk-1 impairs hippocampal-dependent novel object recognition memory by rapidly decreasing protein levels of β-catenin, cyclin-D1, c-myc, *Wnt-7a* ligand and PSD-95 (Fortress et al., 2013). Interestingly, we found that several of these proteins were upregulated by the intrahippocampal administration of the canonical *Wnt* signaling potentiator, WASP-1, and this enhancement correlates with a better performance in the novel object recognition test and the memory flexibility test, when compared to vehicle delivered animal's behavior.

In a previous study has been shown that the administration of *Wnt-1* ligand during fear memory formation interferes with memory consolidation in the amygdala, through avoiding a transient decrease in the levels of *Wnt-1* ligand that occurs immediately after fear

conditioning (Maguschak and Ressler, 2011). Even though, *Wnt-1* ligand is reduced in the amygdala after fear learning, the levels in the hippocampus did not change after novel object familiarization (Fortress et al., 2013; Maguschak and Ressler, 2011). A possible explanation for these differences could be that *Wnt* signaling modulates learning and memory in the amygdala and hippocampus through different mechanisms. In any case, all these evidences strongly suggest that *Wnt* signaling has a critical role in memory formation and consolidation. In particular, our results suggest an important new role for canonical *Wnt* signaling in the consolidation of short-term hippocampal memory.

4.4. *Wnt* signaling as a therapeutic target in Alzheimer's disease.

In AD pathology, several components of the *Wnt* signaling have shown to be altered (Inestrosa et al., 2012; Moon et al., 2004; Oliva et al., 2013b). Also, a relationship between loss of *Wnt* signaling and Aβ neurotoxicity has been demonstrated (Alvarez et al., 2004; Purro et al., 2012). Moreover, Aβ peptide can inhibit *Wnt* signaling by directly binding to several Frizzled receptors (Magdesian et al., 2008).

Here we showed that the activation of *Wnt* signaling rescues Aβ-induced disruption of excitatory synaptic transmission and overcomes hippocampal LTP impairments using both *in vitro* and *in vivo* models. Moreover, we found that the activation of *Wnt* signaling also rescues impaired-episodic memory in APP/PS1 mice. In addition, *Wnt* signaling reverses synaptic proteins loss, reduces Aβ oligomeric aggregates and decreases the levels of *tau* phosphorylation. Even though, our data strongly suggest that *Wnt* signaling could be of therapeutic relevance in AD treatment, from our results is not possible to conclude whether beneficial effects of *Wnt* signaling occur by a direct action on mechanisms impaired by Aβ,

or whether they are independent of Aβ-induced damage. Therefore, more studies are required to clarify this issue. However, we found that WASP-1, but not FOXY-5, blocks Aβ aggregation *in vitro* and also reduces the levels of Aβ-oligomers *in vivo*, indicating that the beneficial effects of WASP-1 could be the consequence of the direct prevention of Aβ plaque formation, but also the enhancement of mice cognitive function could be independent of Aβ-induced synaptic impairments.

Several studies have reported that Aβ oligomers negatively modulate synaptic plasticity (Cleary et al., 2005; Li et al., 2011; Walsh et al., 2002). Previously, our lab showed that Aβ oligomers also reduce synaptic efficacy and impair hippocampal synaptic transmission mainly by decreasing NMDA and AMPA receptors currents, probably caused by a reduction in PSD-95 levels and synaptic contacts (Cerpa et al., 2010). Now, our findings indicate that *Wnt* signaling rescues impaired synaptic transmission and plasticity in hippocampal slices exposed to Aβ oligomers, but more importantly, in adult APP/PS1 mice. Although, we do not know the precise mechanism of action involved in these effects, our data suggests that *Wnt* signaling could enhance synaptic responses by inducing an increase in the levels of synaptic proteins, such as SYP, GluN2B (NMDA-R) and PSD-95. Moreover, the rescue of synaptic transmission and LTP impairments in APP/PS1 mice treated with WASP-1 and FOXY-5, correlates with an improvement on episodic memory. Interestingly, our results agree with the finding that the activation of canonical *Wnt* signaling by inhibiting GSK-3β can reverses the hippocampus-dependent memory deficits in diabetic mice, as well as, in a mouse model of the X fragile syndrome (Guo et al., 2012; King et al., 2013).

Interestingly, we found here that WASP-1 enhances hippocampal LTP magnitude and at the same time reduces hippocampal levels of the Aβ*56 oligomeric specie in APP/PS1 mice, probably by blocking Aβ aggregation. These findings are consistent with the notion that

blockade of Aβ oligomerization could rescue LTP impairments (Walsh et al., 2005). Furthermore, WASP-1 treatment also showed to reduce *tau* pathology, another key hallmark of AD, besides Aβ aggregates (Sheng et al., 2012). The reduction of phosphorylated *tau* levels could be a consequence of WASP-1-induced decreases of Aβ accumulation and/or a side effect of the inhibition of GSK-3β, a kinase which activity is down regulated by the activation of canonical *Wnt* signaling (Clevers and Nusse, 2012), and that is involved in *tau* phosphorylation (Hooper et al., 2008). Indeed, we found here WASP-1 could modulate the activity of GSK-3β in the hippocampus of WT mice. Our results are consistent with previous findings showing that the activation of *Wnt*/β-catenin signaling through inhibition of GSK-3β, leads to neuroprotection in both hippocampal cultured neurons and APP/PS1 mice (Alvarez et al., 2004; De Ferrari et al., 2003; Garrido et al., 2002; Inestrosa et al., 2002; Quintanilla et al., 2005).

Altogether, these data suggest that the activation of *Wnt* signaling, can rescues Aβ-induced synaptic impairments and could be potentially used in the treatment of patients with neurodegenerative diseases such as AD.

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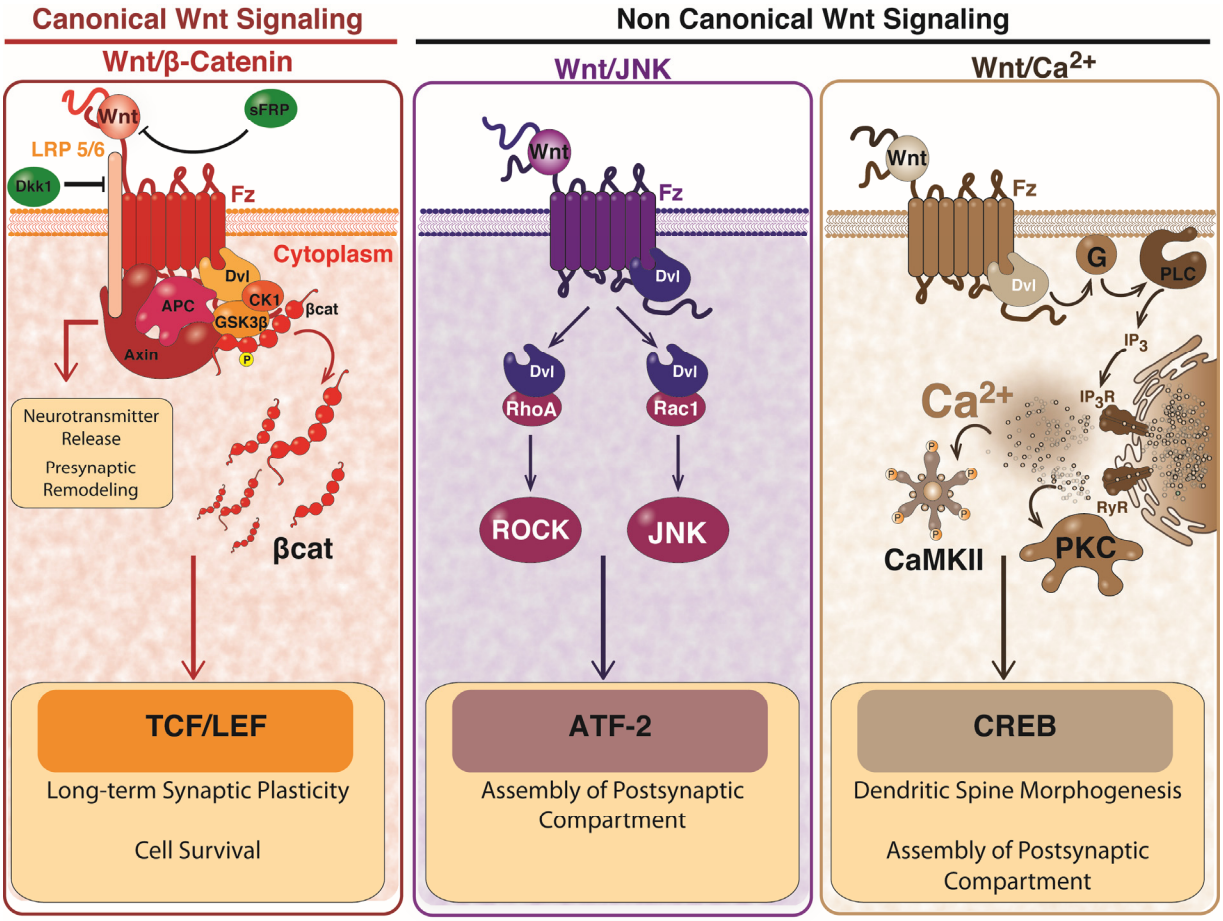
6. APPENDICES

Appendix I.

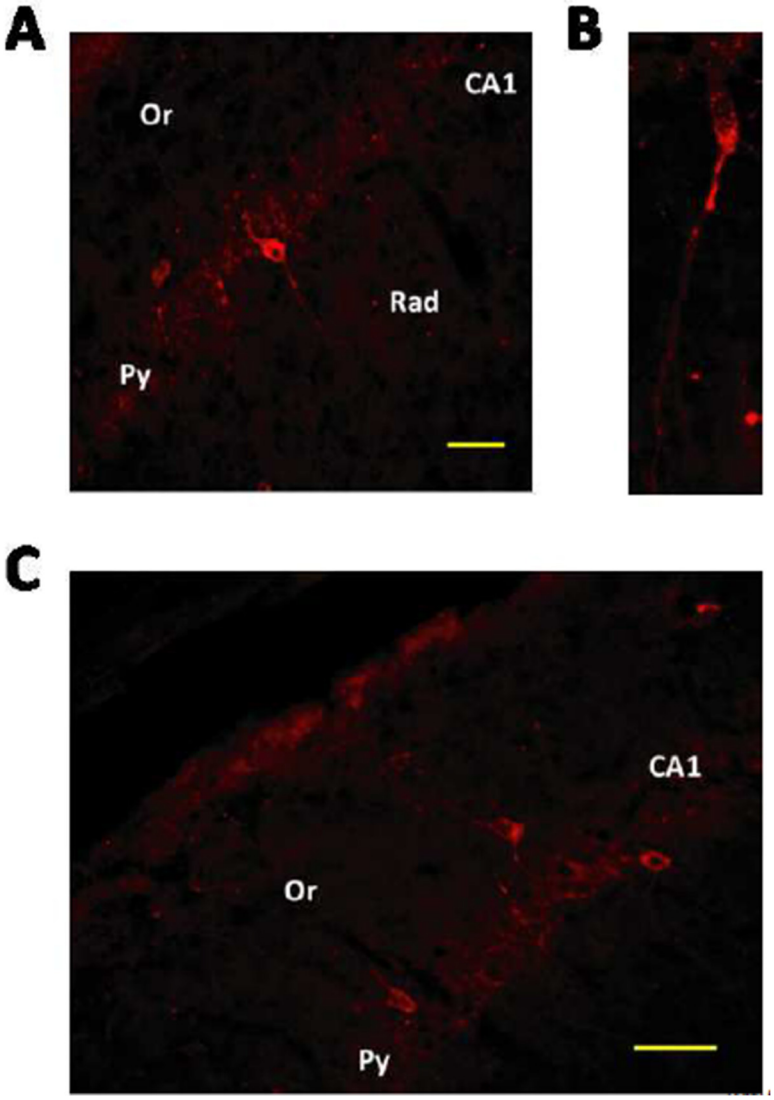


<u>*Wnt* signaling pathways and the effects in mature CNS synapses</u>. Left: The activation of *Wnt*/β-catenin signaling starts with the binding of *Wnt* ligand to Frizzled (Fz) receptor and the low-density lipoprotein receptor-related protein 5/6 (LRP 5/6), causing the recruitment of Dishevelled (Dvl) and the subsequent inhibition of the "β-catenin destruction complex" formed by Axin, adenomatous polyposis coli (APC), glycogen synthase kinase-3β (GSK3β) and casein kinase-1α (CK1). The inhibition of the "β-catenin destruction complex" causes the accumulation of β-catenin, which is no longer phosphorylated and then translocate to the nucleus to activate the transcription of *Wnt* target genes mediated by T-cell specific factor (TCF)/lymphoid-enhancing factor (LEF). Canonical *Wnt* pathway participates in synaptic plasticity and cell survival. A divergent pathway involving proteins of the "β-catenin destruction complex" participates in the clustering of presynaptic proteins and the neurotransmitter release. The *Wnt*/β-catenin pathway can be blocked by Dickkopf-1 (Dkk-1) that binds to LRP co-receptor, or by the secreted Frizzled-related protein (sFRP) that binds directly to *Wnt* ligands. Center: In

the *Wnt*/JNK pathway, activation of Dvl by the binding of *Wnt* to Fz, induces the activation of Rho and Rac small GTPases. Activation of Rho signals through the Rho-associated kinase (ROCK) and the activation of Rac signals to the c-Jun N-terminal kinase (JNK). This pathway is involved in the clustering of postsynaptic proteins and it can conclude in gene transcription mediated by the activating transcription factor 2 (ATF2). This pathway is involved in the assembly of the postsynaptic compartment. Right: In the *Wnt*/Ca^{2+} pathway, binding of *Wnt* to Fz, activates heterotrimeric G-proteins which in turns signal through phospholipase-C (PLC) and inositol-1,4,5-thriphosphate (IP_3) to induce the release of intracellular Ca^{2+} and the activation of both protein kinase C (PKC) and Ca^{2+}-calmodulin-dependent protein kinase type-II (CaMKII). In this pathway the cAMP Response Element-Binding Protein-1 (CREB) is activated. *Wnt*/Ca^{2+} pathway is involved in the clustering of postsynaptic proteins and dendritic spine morphogenesis.

This figure was taken from: Oliva, C.A., Vargas, J.Y. and Inestrosa, N.C. (2013). *Wnts* in adult brain: from synaptic plasticity to cognitive deficiencies. Front Cell Neurosci **7**: 224.

Appendix II.



Fluorescent immunostaining against *Wnt-5a* and *Wnt-7a* in brain slices of 4-month-old rats. (A) *Wnt5a* immunoreactive cells at the CA1 hippocampal region. Scale bar 50 µm. (B) Pyramidal cell expressing endogenous *Wnt-5a* ligand. (C) *Wnt-7a* immunoreactive cells at CA1 hippocampal region. Scale bar 50 µm.
Or = stratum oriens, Py = pyramidal layer, Rad = stratum radiatum.

This figure was taken from: Oliva, C.A., Vargas, J.Y. and Inestrosa, N.C. (2013). *Wnt* signaling: Role in LTP, neural networks and memory. Ageing Res Rev. **12**(3): 786-800.