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Par Carla MONTECINOS OLIVA

AMPA receptor stabilization mediated by non-canonical Wnt signaling protects against $A\beta_{42}$ oligomers synaptotoxicity

Sous la direction de Nibaldo INESTROSA
et de Daniel CHOQUET

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Membres du Jury :

M. Alain BUISSON	Université Grenoble-Alpes	Président
M. Waldo CERPA	Université Catholique du Chili	Examinateur
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Mme. Mireille MONTCOUQUIOL	Université de Bordeaux	Invité

La stabilisation des récepteurs AMPA médiée par une signalisation Wnt non canonique protège de la synaptotoxicité des oligomères $A\beta_{42}$

Les récepteurs AMPA (AMPARs) sont les principaux responsables de la transmission excitatrice rapide dans le système nerveux central, aussi les neurones d'hippocampe étudiés ici. AMPARs sont très dynamiques dans la membrane. Au sein des épines dendritiques, ils peuvent se déplacer par traffic membranaire entre les compartiments intracellulaires et la membrane plasmique. Une fois à la surface, ils se déplacent par diffusion latérale et peuvent s'ancrer réversiblement avec des protéines de la densité postsynaptique ou retourner dans des compartiments endocytaires. Les oligomères Aβ (oAβ) augmentent l'endocytose des AMPARs, diminuent la densité des épines dendritique et provoquent des défaillances globales dans la transmission synaptique. Ces effets, sont englobés dans le terme "synaptotoxicité des oAβ " et sont un domaine principal d'étude de l'étiologie de la maladie d'Alzheimer. Wnt5a un ligand Wnt endogène connu pour activer la voie non-canonique dans les neurones d'hippocampe, génère une augmentation des courants excitateurs et des aggrégats de PSD95 et protége les neurones de la synaptotoxicité des oAβ. Compte tenu du fait que Wnt5a semble contrecarrer les effets nocifs causés par les oligomères Aβ, nous avons procédé à l'étude du mécanisme par lequel Wnt5a protège de la synaptotoxicité des oAβ. Cela nous a conduit à évaluer l'effet de Wnt5a sur l'un des facteurs dans la transmission glutamatergique, la dynamique des AMPARs. Par microscopie à super-résolution dans les neurones d'hippocampe vivants, nous avons trouvé que Wnt5a module la dynamique et la localisation des récepteurs AMPA. Plus précisément, Wnt5a stabilise les AMPAs dans les sites spine et dendrite. Ceci est corrélé avec une augmentation de la co-localisation et de l'interaction entre GluA2 et PSD95. Ces effets ne sont exercés que par l'activation non-canonique de la signalisation Wnt, à travers le ligand Wnt5a et non par les effets canoniques de Wnt7a. Remarquablement, la pré-incubation de Wnt5a prévient la toxicité des oAβ et maintient la dynamique basale des AMPARs. Nos données suggèrent que Wnt5a empêche la synaptotoxicité des oAβ en favorisant leur stabilisation dans les sites synaptiques.

Mots-clés : Wnt, dinamique du récepteur AMPA, Aβ oligomers, synaptotoxicité, neuroprotection.

La estabilización de receptores AMPA, mediada por Wnt5a, protege contra los efectos sinaptotóxicos de los oligómeros $A\beta_{42}$

Los receptores AMPA (AMPARs) son los principales responsables de la respuesta excitatoria rápida en el sistema nervioso central, incluyendo neuronas hipocampales, estudiadas en esta tesis. A diferencia de otros receptores glutamatérgicos, los AMPARs son altamente dinámicos. Dentro de las espinas dendríticas, se pueden mover hacia y desde compartimentos endocíticos y hacia la membrana plasmática. Una vez en la superficie, a través de difusión lateral, se pueden anclar a proteínas de la densidad postsináptica o regresar a compartimentos endocíticos. Por otro lado, los oligómeros Aβ (oAβ) aumentan la endocitosis de AMPARs, disminuyen la densidad de espinas dendríticas y causan una falla generalizada de la transmisión sináptica excitatoria. Estos efectos, entre otros, se engloban en el término "sinaptotoxicidad por oAβ" y es uno de los principales puntos de estudio en la etiología de la enfermedad de Alzheimer. Al contrario, Wnt5a un ligando endógeno conocido por activar la vía no canónica en neuronas hipocampales, genera un aumento en corrientes excitatorias y en los clusters de PSD95 y protege a las neuronas contra la sinaptotoxicidad causada por oAβ. Debido a esto, procedimos a estudiar el mecanismo por el cual Wnt5a protege de la sinaptotoxicidad causada por Aβ. Esto nos llevó a evaluar los efectos de Wnt5a en uno de los principales factores en la transmisión glutamatérgica, la dinámica de los AMPARs. Con el uso de microscopía de super-resolución en neuronas hipocampales vivas, encontramos que Wnt5a modula la dinámica y localización de los AMPARs. Específicamente, Wnt5a estabiliza los AMPARs en espinas y dendritas. Lo cual se correlaciona con un aumento en la co-localización e interacción entre GluA2 y PSD95. Estos efectos son causados únicamente por la activación no-canónica de la vía Wnt, a través del ligando Wnt5a y no por los efectos canónicos de Wnt7a. De manera interesante, la pre-incubación de Wnt5a previene la toxicicidad de los oligómeros Aβ y mantiene la dinámica basal de los AMPARs. Esta data sugiere que Wnt5a promueve la estabilización de AMPARs, previniendo los efectos synaptotóxicos de los oAβ .

Palabras clave : Wnt, dinámica de receptores AMPA, oligómeros Aβ, sinaptotoxicidad, neuroprotección.

AMPA receptor stabilization mediated by non-canonical Wnt signaling protects against $A\beta_{42}$ oligomers synaptotoxicity

AMPA receptors (AMPARs) are responsible for most fast excitatory synaptic transmission in the central nervous system, including hippocampal neurons, studied here. AMPARs are highly dynamic in the plasma membrane. Within dendritic spines, they transition by membrane trafficking between intracellular compartments and the plasma membrane. Once at the surface, they move through lateral brownian diffusion and can reversibly anchor to postsynaptic density proteins or return to endocytic compartments. Aβ oligomers (oAβ) increase endocytosis of AMPARs, diminish dendritic spine density and cause overall failures in excitatory transmission. These effects, among others, are englobed in the term “oAβ synaptotoxicity” and are a main focus on the study of Alzheimers disease ethiology. On the contrary, Wnt5a - an endogenous Wnt ligand known to activate the non-canonical pathway in hippocampal neurons - generates an increase in excitatory currents, clusters of PSD95 and protects neurons against oAβ synaptotoxicity. Given the fact that Wnt5a seems to counteract the distresses caused by oAβ, we proceeded to study the mechanism through which Wnt5a protects from oAβ synaptotoxicity. This led us to evaluate the effect of Wnt5a on one of the most important factors in glutamatergic transmission, i.e. AMPARs dynamics. By using super-resolution microscopy in live hippocampal neurons, we found that Wnt5a modulates the dynamic and localization of AMPARs. Specifically, Wnt5a stabilizes AMPARs in spine and dendritic compartments. This correlates with an increase in co-localization and interaction between GluA2 and PSD95. These effects are exerted only by non-canonical activation of Wnt signaling, through Wnt5a ligand and not by the canonical effects of Wnt7a. Interestingly, pre-incubation of Wnt5a prevents toxicity of oAβ and maintains basal AMPARs dynamics. Our data suggest that Wnt5a prevents oAβ effects by promoting their stabilization in synaptic sites.

Keywords: Wnt, AMPA receptor dynamics, Aβ oligomers, synaptotoxicity, neuroprotection.

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Université de Bordeaux
Centre Broca Nouvelle Aquitaine
146 Rue Léo Saignat
33076, Bordeaux
France

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1. CV SUMMARY

1. PUBLICATIONS

1. AMPARs stabilization mediated by non-canonical Wnt signaling.
   Carla Montecinos-Oliva, Daniel Choquet, Nibaldo C. Inestrosa. (in preparation).

2. Wnt in the Central Nervous System: New Insights in Health and Disease. **(2018)**. Carolina A. Oliva Gutiérrez, **Carla Montecinos-Oliva**, Nibaldo C. Inestrosa. Progress in Molecular Biology and Translational Science. Elsevier.

3. Cheril Tapia-Rojas, Carolina B. Lindsay, **Carla Montecinos-Oliva**, Macarena S. Arrazola, Rocio M. Retamales, Daniel Bunout, Sandra Hirsch and Nibaldo C. Inestrosa **(2015)**. Is L-methionine a trigger factor of Alzheimer's like-neurodegeneration? Changes in Abeta oligomers, tau phosphorylation, synaptic proteins, Wnt signaling and behavioral impairment in wild-type mice. Mol Neurodegener. 10(1):62

4. Parodi J, **Montecinos-Oliva C**, Varas R, Alfaro IE, Serrano FG, Varas-Godoy M, Muñoz FJ, Cerpa W, Godoy JA, Inestrosa NC **(2015)**. Wnt5a inhibits K+ currents in hippocampal synapses through nitric oxide production. Mol Cell Neurosci. 24; 68:314-322.

5. Cisternas P, Salazar P, Serrano FG, **Montecinos-Oliva C**, Arredondo SB, Varela-Nallar L, Barja S, Vio CP, Gomez-Pinilla F, Inestrosa NC **(2015)**. Fructose consumption reduces hippocampal synaptic plasticity underlying cognitive performance. Biochim Biophys Acta. 1852(11):2379-90

6. Codocedo JF, **Montecinos-Oliva C** and Inestrosa NC **(2015)**. Wnt-related SynGAP1 is a neuroprotective factor of glutamatergic synapses against oAβ. Front Cell Neurosci. 9 (227).

7. **Carla Montecinos-Oliva,** Andreas Schüller, Nibaldo C. Inestrosa **(2015)**. Tetrahydrohyperforin - a neuroprotective modified natural compound against Alzheimer's disease. *Neural Regeneration Research,* 10 (4):552-554.

8. **Carla Montecinos-Oliva**, Andreas Schüller, Jorge Parodi, Francisco Melo, Nibaldo C. Inestrosa **(2014)**. Effect of Tetrahydrohyperforin on Hippocampal Mice Slices: Neuroprotection, LTP and TRPC channels, *Current Medicinal Chemistry*, 21 (30):3494-3506.

9. Nibaldo C. Inestrosa, **Carla Montecinos-Oliva**, Marco Fuenzalida **(2012)**. Wnt signaling: role in Alzheimer disease and Schizophrenia, *Journal of* Neuroimmune *Pharmacology*, 7 (4):788-807.

2. BOOK CHAPTERS

New Discoveries on the Hyperforin Derivative, Tetrahydrohyperforin: Closer to and Alzheimer's Disease Treatment? **(2015) Carla Montecinos-Oliva**, Cheril Tapia-Rojas, Patricia V. Burgos and Nibaldo C. Inestrosa. Nova Publishers.

3. SCIENTIFIC MEETINGS ATTENDANCE

1. Society for Neuroscience Annual Meeting San Diego, 3-7 November 2017
Poster: **"oAβ42 deregulate AMPA receptor membrane trafficking"**
Carla Montecinos-Oliva, Daniel Choquet, Nibaldo C. Inestrosa.

2. Society for Neuroscience Annual Meeting Washington DC, 11-15 November 2017
Poster: "**AMPARs stabilization mediated by non-canonical Wnt signaling protects synapses against $A\beta_{1-42}$ oligomers synaptotoxicity". Carla Montecinos-Oliva**, Daniel Choquet, Nibaldo C. Inestrosa.

3. XIII Annual Meeting of Neuroscience Society of Chile Castro, 1-3 August 2017
Young Neuroscientist Symposium: **"Wnt5a stabilizes AMPARs, causing neuroprotection against oAβ". Carla Montecinos-Oliva**, Daniel Choquet, Nibaldo C. Inestrosa.

4. 10th FENS Forum of Neuroscience Copenhagen, 2-6 July 2016
Poster: **"The Wnt5a ligand favors immobilization of AMPARs in hippocampal neurons". Carla Montecinos-Oliva**, Daniel Choquet, Nibaldo C. Inestrosa.

5. 7th EMCCS-FENS Satellite and 1st EBBS-EMCCS Copenhagen, 30-1 June-July 2016
Poster: **"The Wnt5a ligand favors immobilization of AMPARs in hippocampal neurons". Carla Montecinos-Oliva**, Daniel Choquet, Nibaldo C. Inestrosa.

6. 9th FENS Forum of Neuroscience Milan, 5-9 July 2014
Poster: **"Impact of the Wnt non-canonical signaling pathway on AMPARs synaptic enrichment". Carla Montecinos-Oliva**, Anne-Sophie Hafner, Daniel Choquet, Nibaldo C. Inestrosa.

7. XXVII Cellular Biology Society of Chile Annual Meeting Pto. Varas 23-27 October 2013
Poster: "**Non-canonical Wnt signaling increases AMPARs clusters in hippocampal neurons". Carla Montecinos-Oliva** & Nibaldo C. Inestrosa.

8. XXVII Cellular Biology Society of Chile Annual Meeting Pto.Varas, 23-27 October 2013
Poster: **"Chronic treatment of wild-type mice with high doses of methionine induces neurodegeneration".** Cheril Tapia-Rojas, **Carla Montecinos-Oliva**, Carolina B. Lindsay, Sandra Hirsch, Nibaldo C. Inestrosa.

4. GRANTS, AWARDS AND FELLOWSHIPS

US/CRC Fellowship December 2017
Society for Neuroscience and US/CRC of International Brain Organization

Young Neuroscientist Award September 2017
Chilean Society for Neuroscience

Latin-American Training Program Fellowship March 2017- November 2018
Society for Neuroscience and International Brain Organization

Claude Gay Program for Doctoral Studies November 2016 - December 2017
External Affairs Ministry, French Government

Advanced Human Capital, National PhD Program Fellowship January 2015 - August 2017
National Commission for Science and Technology (CONICYT), Chilean Government

5. COURSES ATTENDANCE

1. **Neurobiology** May 31st - July 28th 2018
   Marine Biological Laboratory – The University of Chicago
   Woods Hole, MA, USA.

2. **Signal Processing: from single molecules to brain circuits** March 2017 - April 2017
   Society for Neuroscience, International Brain Organization and Universidad del Valle. Cali, Colombia.

3. **Cell Biology Summer Course** January 2015
   Institute Curie and Fundación Ciencia y Vida.
   Santiago, Chile

2. COMMONLY USED ABBREVIATIONS

Aβ: amyloid-β

AD: Alzheimer's disease

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

AMPARs: AMPAR-type receptor

CAM: Cell-adhesion molecule

CaMKII: calcium-calmodulin dependen kinase II

CNS: Central Nervous System

CP-AMPARs: calcium permeable AMPARs

DIV: days *in-vitro*

eLTP: early Long-Term Potentiation

EPSP: Excitatory Post-Synaptic Potential

HFS: High Frequency Stimulation

JNK: Jun-N-terminal kinase

KARs: kainate-type glutamate receptors

KO: knock-out

lLTP: late Long-Term Potentiation

LTD: Long-Term Depression

LTP: Long-Term Potentiation

MAGUK: Membrane-associates guanylate kinase

NMDA: N-Methyl-D-aspartic acid

NMDAR: NMDA-type glutamate receptor

oAβ: oligomers Aβ

PALM: Photo-Activated Localization Microscopy

PCP: Planar Cell Polarity

PDZ: PSD-95/DLG/ZO-1

PIP_2: phosphatidil inositol di-phosphate

PPF: Paired-Pulse Facilitation

PSD: Postsynaptic density

PSD95: Postsynaptic density 95

PTM: Post-translational modification

sFRP: soluble Frizzled Related Protein

STORM: Stochastic Optical Reconstruction Microscopy

STP: Short-Term Potentiation

TARP: Transmembrane AMPARs Regulatory Protein

TBS: Theta-Burst Stimulation

uPaint: universal Point Accumulation in the Nanoscale Topography

Wnt: Wingless-type

3. INTRODUCTION

In the intricate machinery that gives shape to the synapse, the identity and amount of molecules (neurotransmitters, receptors, scaffolding proteins, kinases, etc.) present is fundamental and have been widely studied. However, to have an efficient synaptic communication, those elements are not the only variables to keep in mind. It is also crucial for those elements to be located in the right place, at the right moment.

For efficient synaptic transmission to occur, it is necessary that the system adapts rapidly to new and ever changing inputs. This is called synaptic plasticity, the ability to change the firing properties of a synapse, and it relies on many factors, one of them being the fast, initial response to neurotransmitter release. Specifically, in glutamatergic synapses, AMPA receptors (AMPARs) are the main mediators of fast excitatory response and variations in the amount of AMPARs account for short term plasticity (STP) and long term potentiation (LTP) (Benke et al., 1998; Makino and Malinow, 2009; Penn et al., 2017). The study of how AMPARs respond to endogenous inputs could help us to unveil mechanisms to recover from synaptic failure like the one occurring in Alzheimer's disease (AD).

3.1. Glutamatergic synapses

Glutamatergic synapses are characterized by the presence of glutamate as the main neurotransmitter. Glutamate, is a non.essential aminoacid, is synthesized from glutamine and is the main excitatory neurotransmitter in the vertebrate central nervous system (CNS) (Purves et al., 2004). In excitatory synapses, the presynaptic site releases excitatory neurotransmitters and their binding to receptors causes membrane depolarization and an excitatory post-synaptic potential (EPSP) that increases the probability to generate an action potential. Glutamate receptors can be divided into metabotropic and ionotropic receptors. Metabotropic glutamate receptors are associated to G-proteins and transduct signals through second messengers like PIP_2 and β-arrestin. There are three types of metabotropic glutamate receptors: group 1, group 2 and group 3. On the other hand, ionotropic receptors contain a channel allowing ions to pass in/out from the cell. There are three types of ionotropic glutamate receptors: AMPAR, NMDA and kainate receptors (KARs). This thesis focuses on the study of AMPARs, for reasons that will be addressed in the following paragraphs.

A characteristic feature of glutamatergic neurons, is the presence of dendritic spines. These protrusions are found in the dendrites and constitute the contact point with presynaptic sites. Like this, in dendritic spines there is an entire machinery to respond accordingly to presynaptic inputs. Dendritic spines are dynamic structures, that vary in shape and quantity throughout short and long periods of time. They can be modulated by different inputs and, as will be further addressed, Wnt signaling is one of them.

3.2. AMPA receptors

AMPARs are highly conserved and appear early on in evolution (Chiu et al., 1999). Probably, this is a reflection of the relevance of AMPARs in synaptic transmission. AMPARs are mainly expressed in the hippocampus, followed by cerebellum and cortex (Schwenk et al., 2014). They are highly abundant in hippocampal neurons, compared to other hippocampal cell types. In basal conditions, it has been reported that the ratio of PSD95:AMPARs:NMDAR molecules is 15:3:1 (Sheng and Hoogenraad, 2007). In hippocampal neurons, AMPARs located in dendritic spines, in conjuction with other glutamate receptors (NMDARs and KARs), generate EPSPs which are transmitted into the soma of the neuron. If the summation of EPSPs reaches the membrane potential treshold (around -30 mV), an action potential will be generated. As a consequence, the lack of surface-expressed AMPARs into synapses translates into a lower probability of action potential generation and decreased synaptic activity. A synapse that does not contain surface-expressed AMPARs in the postsynaptic site, is refered to as a silent synapse (Isaac, 2003; Liao et al., 1995). After trains of high frequency stimulation (HFS) (i.e., tetanic stimulation), the number of AMPARs increases largely at synapses, underlying in part, activity dependent synaptic potentiation (Makino and Malinow, 2009; Penn et al., 2017; Wu et al., 2017). Alternatively, low frequency stimulation (LFS) induces a loss in AMPAR from synapses leading to synaptic depression (Fujii et al., 2018; Wu et al., 2017; Zhou et al., 2018) . For these various reasons, the study of the localization and dynamic of AMPARs is crucial to understand the physiology and patophysiology of synaptic transmission.

AMPARs are ionotropic glutamate receptors. They are composed of 4 subunits (GluA1-4) and are usually hetero-tetramers, meaning they contain 2 doublets of homodimers. The most

common composition in hippocampal neurons is by ~80% GluA1/GluA2 hetero-tetramers followed by ~16% GluA2/GuA3 (Lu et al., 2009). AMPARs are mainly permeable to Na^+ and K^+, althought depending on their subunit composition, they can also permeate Ca^{2+}. Any receptor that contains GluA2 subunit will be impermeable to Ca^{2+}, because this subunit contains a reentrant loop in the channel forming trasmembrane segment that blocks Ca^{2+} entry. Therefore, homotetramers formed by GluA1/GluA1 subunits are calcium permeable AMPARs (CP-AMPARs), and are of great importance in the generation of long-term potentiation (LTP). The subunit composition of AMPARs present in postsynaptic sites can vary depending on activity. Particularly, it is proposed that GluA1 homotetramers (CP-AMPARs) are inserted into the plasma membrane after high frequency stimulation or theta-burst stimulation (TBS), allowing an extra Ca^{2+} influx on top of the one caused by NMDAR currents, and an increase in the firing pattern frequency (Liu and Savtchouk, 2012; Park et al., 2016b). In fact, it is proposed that Ca^{2+} influx via CP-AMPARs causes fast activity-dependent postsynaptic plasticity (Kim and Von Gersdorff). It has even been proposed that Ca^{2+} influx from activated CP-AMPARs causes inhibition of NMDA currents (Rozov and Burnashev, 2016).

Recently, it has been shown that AMPARs organize themselves into nanodomains within the dendritic spines (Fukata et al., 2013; MacGillavry et al., 2013; Nair et al., 2013). This contrasts with the previous idea that they were homogenously distributed along the membrane of dendritic spines (Baude et al., 1995; Nusser et al., 1994). In fact, now we know that AMPARs at the plasma membrane are in a dynamic equilibrium between retention sites within nanodomains and freely moving outside of nanodomains (Barrera-Ocampo and Chater, 2013; MacGillavry et al., 2013; Nair et al., 2013). Each dendritic spine contains between 1.4-2.5 AMPARs nanodomains, depending on the super-resolution technique used to study it. Nanodomains are about ~70 nm and contain ~20 AMPARs, but their shape and position can change during time, although most of them are stable for up to 1 h (Nair et al., 2013).

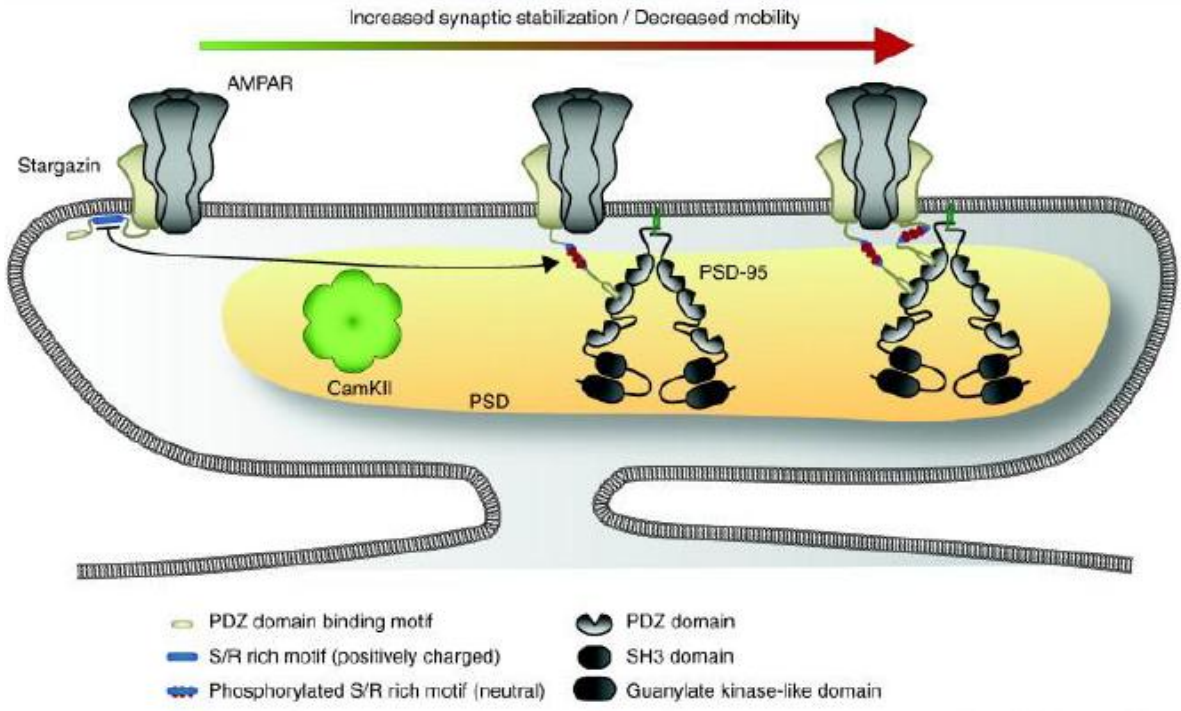
Another important characteristic of AMPARs is the fact that once inserted into the plasma membrane, they can be found in at least, three different conformational states: closed, open or desensitized. On the close state, there is no or very low glutamate bound to AMPARs, and no

current is generated. The binding of at least two glutamate molecules activates AMPARs, opening the channel and allowing an inward Na^+ current. Since the affinity of AMPARs to glutamate is low (~EC_{50} 1.1 ± 0.3 mM), the ligand will rapidly disociate from the receptor (Kessler et al., 2008). However, AMPAR can also enter a desensitized state that has a high affinity for glutamate (Dürr et al., 2014). In this state, AMPARs can still bind glutamate, but no current will be generated from it. In addition, each GluA subunit on the tetramer can bind one molecule of glutamate, this adds complexity to the system and generates a differential responses (low/high conductance) depending on the amount and type of subunits binding to glutamate (Dürr et al., 2014; Kessler et al., 2008).

3.2.1. The AMPARs-TARP complex.

Interestingly, AMPARs are part of a bigger molecular complex in which they are partnered with transmembrane-AMPARs associated protein (TARPs). The C-terminal tail of AMPARs does not bind directly to the main scaffolding protein of synaptic densities, PSD95. For this reason, the presence of a TARP able to bind AMPARs, allows stabilization of the complex on PSD95 through the TARP C-terminal domain, creating an indirect interaction between AMPARs and PSD95. The process in which AMPARs-TARP complex binds to PSD95, is called “trapping” and can lead to persistent or transitory immobilization of AMPARs (Ehlers et al., 2007; Opazo et al., 2010). TARPs can be phosphorylated and this constitutes a new regulatory mechanism of AMPARs activity, trafficking and pharmacology (Jackson and Nicoll, 2011; Kato et al., 2010; Ziff, 2007).

There is a variety of TARPs, TARP-γ2 (Stargazin) is the most studied. Stargazin is highly expressed in the cerebellum, while still present in hippocampus but with low expression levels. On the other hand, TARP-γ8 is highly enriched in the hippocampal formation (Fukaya et al., 2006). It has important roles in regulating surface expression of AMPARs (Zheng et al., 2015) as well as their distribution (Rouach et al., 2005). It is also involved in synaptic plasticity (Sumioka et al., 2011) and LTP generation (Park et al., 2016a). In the present work, we do not investigate the identity of the TARP involved in the effects of Wnt5a, but this information will be necessary to understand the results and perspectives surrounding them.



from (Opazo et al., 2012)

TARP-dependent regulation of AMPAR immobilization to scaffolding proteins. The figure shows Stargazin (TARP-γ2), but the principle applies to every TARP. Phosphorylation on the C-terminal tail of Stargazin, by Ca^{2+}-activated CaMKII, causes a change on the net charge of it. By becoming more negative (due to the addition of phosphate groups), the C-tail is repelled by the instrinsically negative internal membrane of the plasma membrane and suffers a conformational change that allows the interaction with PDZ-domains of scaffolding proteins, in this case PSD95, with the PDZ-binding domains of the TARP C-terminal tail. This will cause the trapping of the AMPAR-TARP complex into synaptic sites, and the formation of the trimeric AMPAR-TARP-PSD95 complex. Importantly, each GluA subunit has the ability to bind a TARP, and each TARP can bind a PDZ domain. Thus adding different degrees of regulation and trapping.

3.2.2. Role of AMPARs dynamic in synaptic plasticity

Synaptic plasticity is defined as the ability of synapses to accommodate their synaptic efficacy by a change in the firing properties; that is, to potentiate or depress the synaptic response depending on the input (Nicoll, 2017). The main paradigms in synaptic plasticity are short-term potentiation (STP), long-term potentiation (LTP) and long-term depression (LTD). Both forms of

long-term plasticity (LTP and LTD) have been suggested to be the molecular correlates for memory and learning (Morris, 1989; Morris et al., 1986). In all of these processes, the role of synaptic receptors and their location on the synapse, is crucial. Next, the role of AMPARs in STP, LTP and LTD will be addressed.

In the classic view of synapses, over 20 years ago, synaptic receptors were considered to be statically located at a certain position in the postsynapse, waiting to bind a neurotransmitter and exert their action. Under this view, receptors could be exocytosed or endocytosed to and from the plasma membrane, directly at synaptic sites (Lüscher et al., 1999). In 2002, a third trafficking route was suggested by Daniel Choquet, according to which AMPARs could also be in an intermediate location; the plasma membrane but at extrasynaptic sites (Borgdorff and Choquet, 2002). From there, they could move through lateral diffusion, always in the plane of the membrane, to synaptic sites. Presently, and after many studies backing up this hypothesis (Ashby et al., 2006; Esteves da Silva et al., 2015; Heine et al., 2008; Makino and Malinow, 2009; Penn et al., 2017), it is widely accepted that surface mobility of AMPARs is fundamental both at rest and at different steps of synaptic plasticity (Hastings and Man, 2018).

Within dendritic spines, AMPARs can be found in endocytic compartments or inserted in the plasma membrane. The fast dynamic of AMPARs helps to tune synaptic response (Heine et al., 2008). This occurs mainly by regulating the ratio of naïve functional/desensitized AMPARs present in the PSD. Depending on the type of stimuli, AMPARs can be either endocytosed or inserted into the plasma membrane at extrasynaptic sites, from where they can move to the PSD through lateral diffusion (Opazo and Choquet, 2011). This process is fast, AMPARs can move from extrasynaptic to synaptic sites, within seconds (Heine et al., 2008). Once present in the membrane, AMPARs only reside at the neuronal surface, on average, for tens of minutes (Heine et al., 2008). In addition, within the synapse, mobile receptors represent between 50-80% of all AMPARs and, if not anchored to PSD, reside only for a few seconds (~2 s) in the synapse (Heine et al., 2008; Opazo et al., 2012). On the other hand, AMPARs anchored in the PSD, remain immobile in the synapse and can remain confined for minutes within PSD95 nanodomains (Nair et al., 2013).

Donald Hebb, in 1949 published “The Organization of Behaviour”, setting the basis to our current understanding of synaptic plasticity. He introduced what became known as the Hebbian rule, he postulated that “…the persistence or repetition of a reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability.[…] When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.” (Hebb, 1949), more easily remembered as the corollary “cells that wire together, fire together”. Interestingly, Hebb was introducing many concepts like synaptic plasticity, spike-timing-dependent plasticity and engrams. Theorical concepts that were later proof to be true by Eric Kandel, using the *Aplysia* model (Kandel and Tauc, 1965).

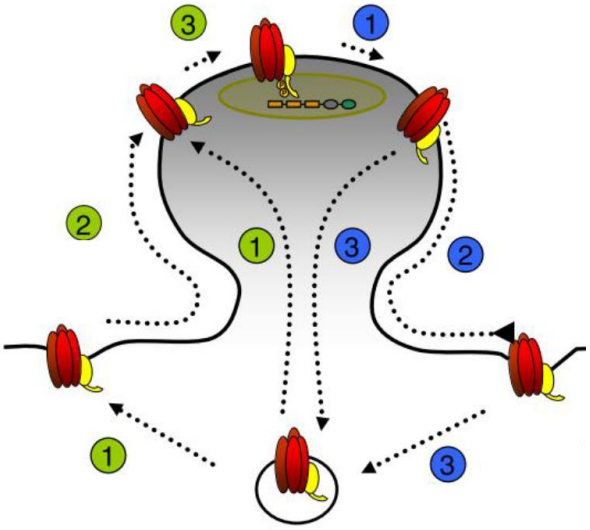
Taking the Hebbian principle with what we have learned in the last 70 years, we know that upon strong presynaptic stimulation (i.e., increased release of neurotransmitter), like the one occurring during LTP induction, there is a rapid lateral diffussion of CP-AMPARs from extrasynaptic to synaptic compartments. This rapid change is a proccess of STP (Rozov and Burnashev, 2016). Therefore, STP depends greatly on the recruitment of CP-AMPARs from extrasynaptic pools, by lateral diffusion (Penn et al., 2017). CP-AMPARs have a faster Ca^{2+}-entry onset than NMDARs and the close proximity of both receptors, usually colocalizing, allows local activation of NMDARs (Rozov and Burnashev, 2016).

Following, NMDA-dependent activation of CaMKII and PKC, there is insertion of new AMPARs from endocytic compartments to the plasma membrane, on extrasynaptic sites. Then, lateral diffusion of AMPARs from extrasynaptic to synaptic sites, causes an enrichment in AMPARs in the PSD. There, AMPARs can get stabilized through interactions between TARPs and the PDZ domains of PSD95 (Opazo and Choquet, 2011) or other scaffolding proteins (see section 6.2.1.). If they are persistently stabilized into the PSD, the postsynaptic excitatory transmission becomes more efficient in response to glutamate. Overall, generating a more persistent synaptic connection in time, giving rise to LTP, similar to what was proposed by the Hebbian theory.

LTP can be subdivided into 2 different stages, which involve different molecular players and proccessess: early LTP (eLTP) which accounts the changes that occur 1 hour after stimulation

and late LTP (lLTP) which happens after 1 hour of stimulation and can last for days. eLTP is protein synthesis-independent, while lLTP is protein synthesis-dependent (Frey et al., 1988) .

The role of extrasynaptically located AMPARs, has proof to be fundamental for both STP and eLTP (Penn et al., 2017). Therefore supporting the important role of lateral diffusion of AMPARs in synaptic plasticity. Interestingly, while lateral diffusion is necessary and sufficient for STP, lateral diffusion is not sufficient for eLTP (Penn et al., 2017). In eLTP, following lateral diffusion of already existent membrane-expressed AMPARs at extrasynaptic sites, exocytosis of new AMPARs into the extrasynaptic membrane is necessary to increase and maintain the pool of AMPARs. Therefore eLTP requires at least two steps; exocytosis of AMPARs to extrasynaptic sites and their lateral diffusion to synaptic sites (Penn et al., 2017). This is explained next, in the three-step model for AMPARs mobility (Opazo and Choquet, 2011).



modified from (Opazo and Choquet, 2011)

Representation of the three step model for AMPARs synaptic recruitment in synaptic plasticity. During LTP (on green), the AMPARs-TARP complex is inserted into the plasma membrane (step 1), either at extrasynaptic (dendritic shaft) or synaptic sites (close to the PSD) in the spine head. From there, it moves through the plane of the membrane (step 2) towards the PSD and finally AMPARs get immobilized on PSD slots, through indirect interaction between TARP and PSD95. During LTD (on blue), the AMPARs-TARP complex gets destabilized from the PSD (step 1), moves in the plane of the membrane to extrasynaptic sites (step 2) and finally it gets endocytosed either directly from the spine head or from the dendritic shaft (step 3).

Recently, the fundamental role of AMPARs diffusion on LTP has been proof in *in-vitro* and *in-vivo* experiments. The recruitment of new AMPARs to synapses by lateral surface diffusion is critical for the LTP to occur (Penn et al., 2017). By blocking lateral AMPAR diffusion and LTP, fear-conditioning learning also gets impaired, proving a correlate between AMPAR lateral mobility and learning proccessess (Penn et al., 2017).

More generally, our view not only about the dynamics, but also of the biophysics itself behind AMPARs conductance has changed in the last few years. It has been proof that after a single subthreshold synaptic activation, most of the sodium entering the neuron does it through AMPARs, and not NMDA or voltage-gated sodium channels (Miyazaki and Ross, 2017). Sodium, along with potassium, are the main contributors to generate EPSP and action potentials, from a purelly electrical point of view. Like this, the relevance of AMPARs becomes even clearer. LTP requires stabilization, lateral diffusion towards synaptic sites and exocytosis of new AMPARs. But not only are AMPARs crucial players in STP and LTP processess, they are fundamental in diverse forms of synaptic plasticity. LTD, for example, depends on the unstabilization, release from synaptic sites and endocytosis of AMPARs (Opazo and Choquet, 2011). Therefore, by studying the elements that stabilize or destabilize AMPARs to and from synaptic sites, we are studying the basis of synaptic plasticity.

3.3. Alzheimer's disease

Alzheimer's disease (AD) was first described in 1907 by Alois Alzheimer. It is a progressive neurodegenerative disease and the most common cause of dementia in the elderly population, accounting for an estimated 50-70% of all late-life dementia cases (Feldman et al., 2014). Clinically, is characterized by cognitive failure and loss of encephalic volume, both features correspond to late phase states of the disease. AD becomes more and more prevalent since worldwide, life expectancy increases and populations age. For example, it is estimated that in western Europe by the year 2040, the population over 60 years of age, with dementia, will increase from a current 5.4 to 9.9 million, while in Latinamerica the increase would be from 4.6 to 9.1 million (Ferri et al., 2005)

3.3.1. The tau and the amyloid hypothesis on AD

AD has two well defined pathological hallmarks, the intracellular neurofibrillary tangles formed by hyperphosphorylated tau protein and the extracellular amyloid plaques, mainly formed of Aβ protein (Crews and Masliah, 2010; Selkoe, 2013). Therefore, giving rise to the two main hypothesis regarding the aetiology of AD; the tau hypothesis and the amyloid cascade hypothesis. There is compeling evidence for both hypothesis, and the determination of which one triggers the disease, remains a subject of debate. Next, a short description of both hypothesis will be given, to later focus our study on the amyloid hypothesis, as subject of this thesis work.

Tau is a microtubule-associated protein (MAP) and it has over 80 posible phosphorylatoin sites (Ksiezak-Reding et al., 1992). In AD brains, the amount of phosphorylated tau has been found to be 3-times higher than in healthy, same age brains (Köpke et al., 1993). It is unclear how the increase in phosphorylation of tau promotes the formation of neurofibrillary tangles, but it has been proposed that hyperphosphorylated tau has a lower affinity to microtubules. Considering the fundamental role of microtubules in neuronal organization and function, differential treafficking of proteins, compartimentalization of neurons, etc. any disruption of microtubules would be of great damage. Therefore, tau hyperphosphorylation decreases the affinity of tau to stabilize microtubules and forms intracellular neurofibrillary tangles, that disrupts the normal shape and function of neurons (Guerrero-Muñoz et al., 2015).

On the other hand, we find the amyloid cascade hypothesis, on which base we conducted our experiments. The amyloid cascade considers Aβ as the trigger element of the pathology. The rational behing the Aβ hypothesis is that the abnormal extracellular accumulation of Aβ in the brain parenquima leads to synaptic failure and synaptotoxicity followed by neuronal death, inflammation, cognitive failure and dementia. However, there is low correlation between the presence of senile plaques and the severity of cognitive deficit. What has been revealing, is the fact that the presence of a certain species of soluble Aβ, named Aβ oligomers (oAβ) does show a higher correlation with neurotoxicity (Benilova et al., 2012; Lue et al., 1999; Tomic et al., 2009).

Today, there is no consensus on whether Aβ has a direct causative role on AD, and if targeting Aβ has a significant impact on the progression of the disease. However, there is evidence that oAβ are able to cause tau hyperphosphorylation (Bilousova et al., 2016; Miller et al., 2014; Sarko et al., 2017) and mislocalization of tau from axon to dendrites (Miller et al., 2014). Therefore, supporting the idea that oAβ could be the first insult into AD, causing a cascade of effects in a Aβ dependent – tau mediated fashion. Unfortunately, a number of clinical trials that were promising in the laboratory and pre-clinical studies have resulted inconclusive (Umar and Hoda, 2018).

In order to understand more deeply what this oligomeric species are and how they relate to early synaptic failure, we will discuss the processing of APP that gives rise to Aβ.

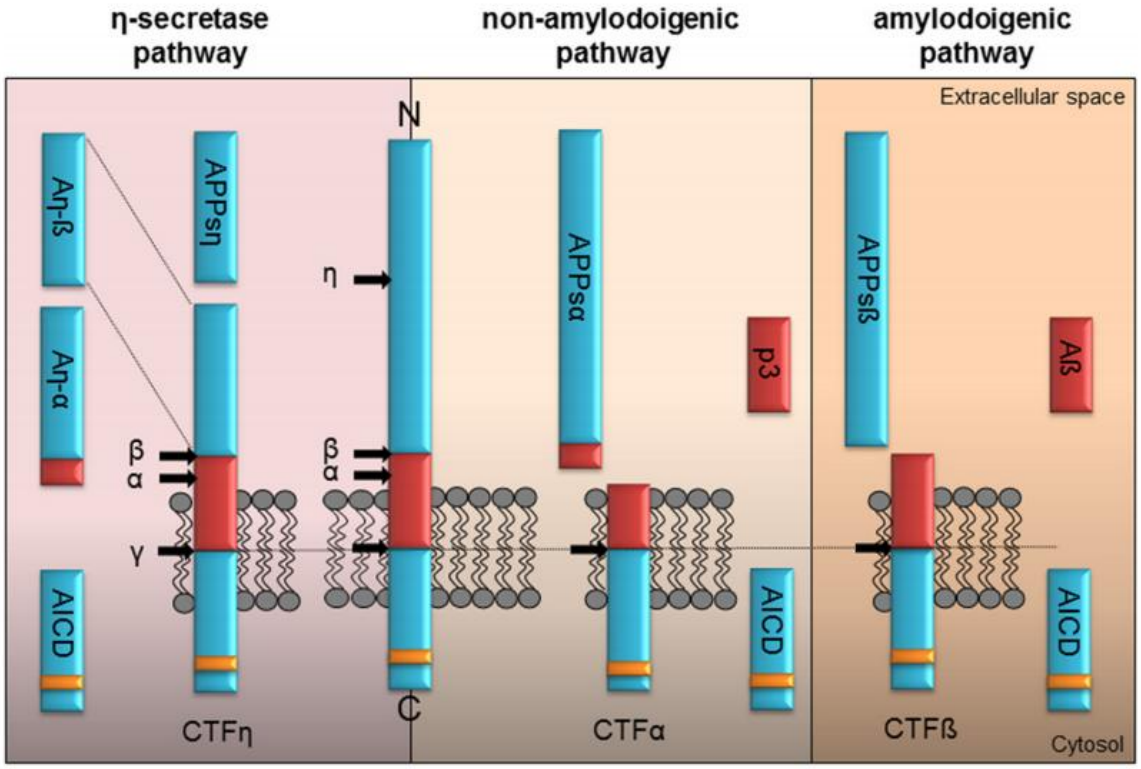
3.3.2. APP Processing

The Amyloid Precursor Protein (APP) is a type I transmembrane glycoprotein. In humans, the gene for APP is located in chromosome 21 and has three splice variants, being APP695 the predominant isoform in excitatory neurons and inhibitory interneurons (Hick et al., 2015), while their presence in astrocytes remains unclear. Full length APP is a cell-adhesion molecule (CAM) and therefore regulates cell-cell interactions in *trans.* APP, like most CAM proteins, regulate cell-cell contacts by interacting extracellularly with other CAMs or extracellular matrix components (i.e., laminin, reelin, etc.) and intracellularly with cytoskeleton machinery. APP is able to regulate the guidance of the axonal growth cone, the forming and maintenance of synaptic contacts (Ludewig and Korte, 2017). An important difference between full-length APP and other CAMs is that APP is found in highly dynamic cellular subdomains like the growth cone and dendritic spines, whereas other CAMs (ie., cadherins, integrin β1, etc) are forming more stable, tight adhesions. The role of APP in spine dynamics is directly linked to synaptic plasticity (Hick et al., 2015; Korte et al., 2012; Ludewig and Korte, 2017). In fact, KO mice for APP have a deficit in synaptic plasticity, mainly in LTP, that becomes evident with age, while no changes are seen at young ages (Ring et al., 2007; Seabrook et al., 1999). It is important to notice that there are two mammalian homologous of APP, APP-like 1 (APLP-1) and APP-like 2 (APLP-2). The three homolog forms show high structural conservancy and high functional

redundancy. One explanatio for the lack of phenotype at young ages is function compensation given by the redundant APLP2 (the isoform with the highest degree of sequence homology with APP).

APP has physiological roles as a full-length protein and also the different proteolytics fragments product of secretase cleaving have different functions. APP can be cleaved by 4 different secretases: α-, β-, γ- and η-secretase, giving rise to different proteolitic pathways and products. In the figure below, we see three possible pathways for APP cleaving: η-secretase pathway, non-amyloidogenic pathway dependent on α-secretase and the amyloidogenic pathway, dependent on β- and γ-secretases to produce Aβ species.

It has been proposed that presynaptically located APP can have roles in synaptic vesicle release and short-term plasticity (Korte et al., 2012). APP also interacts with vesicle proteins like Rab, AP2, synaptotagmin and clathrin, among others (Ludewig and Korte, 2017).



from (Ludewig and Korte, 2017)

Proteolytic processing of APP. Full-length APP can be processed by different sequential combinations of α-, β-, η-, and γ-secretases (arrows indicate cleaving sites), giving raise to three different pathways. On the left, it is shown the **η-secretase processing of APP**. First, η-secretase cleavage releases the soluble APPsη, while CTFη remains in the plasma membrane. CTFη is further processed by α- or β-secretase generating An-α or An-β. Resulting of the cleavage of CTFη γ-secretase yields the APP intracellular domain (AICD) containing the highly conserved interaction motif (YENPTY, yellow box) or the short extracellular peptides Aβ (red) seen in the amyloidogenic or p3 (red) within the non-amyloidogenic pathway. **The non-amyloidogenic pathway** shown in the middle, is driven by the action of α-secretase liberating APPsα in the extracellular space. Subsequently processing of membrane tethered CTFα by γ-secretase generates the p3 peptide and cytoplasmic AICD. The right panel illustrates the **amyloidogenic processing** of APP, led by β-secretase resulting initially in the release of the APPsβ ectodomain. Next, γ-secretase makes a second cut of the membrane tethered CTFβ and the Aβ peptide is secreted along with AICD to the cytoplasm.

3.3.3. oAβ: isoform, agreggation status and concentration.

When referring to Aβ species, it is important to notice at least three different characteristics: isoform, agreggation status and concentration used. Experimentally, has been shown that biological effects can vary notably depending on these factors (Gulisano et al., 2018; Puzzo et al., 2012). Next, an explanation on the differences his characteristics make on toxicity and correlation to cognitive impairment, as well as a rationale for the use of $A\beta_{1-42}$ oligomers in the experiments of this thesis.

oAβ, is a term used to describe soluble subproducts of the amyloidogenic cleavage of APP. Particularly, soluble products ranging between 2-12 peptides (Ballard et al., 2011). During many years the main focus of research was on the amyloid plaques. The study of oligomeric species of Aβ became relevant after the realization of the poor correlation existent between fibrillar plaque deposits and cognitive impairment in both animal models and AD human patients (Esparza et al., 2013) . On the other hand, soluble oAβ show a high correlation with cognitive decline (Benilova et al., 2012; Lue et al., 1999; Tomic et al., 2009). A study comparing post-mortem cortex lysates from non-demented patients with Aβ plaque pathology and AD patients with Aβ plaque pathology (Esparza et al., 2013). Interestingly, this study showed that even though the two groups of patients had equivalent Aβ plaque pathology, only in the AD group there was a high correlation ($r = 0.88$) between Aβ oligomer concentrations and Aβ plaque coverage, whereas in the non-AD Aβ plaque group, there was a very weak correlation ($r = 0.30$) (Esparza et al., 2013). Meaning that despite both groups having Aβ plaques, only in the AD group the concentration of oAβ was higher. For this reason, we will focus on the study of soluble oligomeric forms of Aβ and not, for instance, fibrillary forms.

Now, the isoform of oligomeric Aβ species can vary greatly. A study, using high-resolution mass spectrometry in post-mortem frontal cortex of six confirmed cases of AD found a diversity of 26 different Aβ proteoforms (Wildburger et al., 2017). Interestingly, the canonical forms of $A\beta_{1-40}$ and $A\beta_{1-42}$ were present in samples from all patients with $A\beta_{1-42}$ presenting the highest relative abundance (Wildburger et al., 2017). It is precisely this proteoform, $A\beta_{1-42}$ that will be

used on this thesis. Although, it is important to notice that other forms of soluble Aβ have also shown toxicity, specially $A\beta_{1-40}$ (Lue et al., 1999) has been also widely used in experimentation.

Finally, the concentration of $oA\beta_{42}$ used is probably hardest to define. It is of course, desirable to use a concentration closer to the patophysiological concentrations found in AD patients. In this regard, the most reported measurements correspond to oAβ in cerebrospinal fluid. Even though it can be very helpful marker of Aβ levels in the brain, the truth is that it does not shade light on the actual concentration of Aβ in the brain parenquima, concentration to which neurons are being subjected to.

This subject will be further analyzed in the results and discussion sections.

3.3.4. oAβ affect synaptic plasticity, the AMPAR view

Synaptic plasticity is greatly affected in hippocampal neurons treated with oAβ. Although oAβ cannot fully account for the etiology of AD, evidence in the last decades agrees on the relevance of oAβ accumulation as a hallmark for AD (Dinamarca et al., 2008; Lacor et al., 2004; Walsh and Selkoe, 2007). Recent work proposes that oAβ generate early synaptic failure and synaptotoxicity leading to neuronal death and progressively, cognitive loss. Also, it has been reported that oAβ could be accountable for generating other hallmarks of AD, like neurofibrillary tangles caused by tau hyperphosphorylation (Jin and Selkoe, 2015) and can lead to tau mislocalization (Miller et al., 2014).

How do oAβ cause synaptotoxicity?, there is no definitive and single answer. Among the many effects observed after oAβ exposure into hippocampal neurons are: mitochondria damage (Paula-Lima et al., 2011), pore opening in the plasma membrane (Arrázola et al., 2017), AMPARs endocytosis (Hsieh et al., 2006; Jin and Selkoe, 2015), over-activation of NMDA receptors and calcium homeostasis deregulation (Shankar et al., 2007), decrease in density and maturity of dendritic spines (Wu et al., 2012), decrease in excitatory currents (Cerpa et al., 2008a; Dinamarca et al., 2008), LTP impairment (Shankar et al., 2007; Townsend et al., 2006; Walsh et al., 2002) and many others.

In fact, at least a couple of publications have described how human derived oAβ, from AD patients, can directly inhibited LTP, when injected into rat brains and tested *in-vivo* (Shankar et al., 2008; Walsh et al., 2002). Probably giving the most crucial proof in favor of the amyloid hypothesis as the trigger of AD pathology. Moreover Aβ producing cells were treated with a γ-secretase inhibitor, to decrease the production of oAβ and favor monomeric Aβ in the cell medium. When the conditioned media was injected into the rat brains, LTP was no longer disrupted (Walsh et al., 2002) .

It has been widely reported, that oAβ cause endocytosis of AMPARs (Hsieh et al., 2006; Miller et al., 2014; Miñano-Molina et al., 2011). Particularly, $oA\beta_{42}$ induces a calcineurin-dependent dephosphorylation of S845 residue in GluA1, causing endocytosis of AMPARs (Miller et al., 2014).

Not only calcinuerin is affected by oAβ, also, the activity of CaMKII can be jeopardized by it. It has been found that in animal models for AD, the amount of CaMKII clusters is lower and the same happened in *in-vitro* treatments with oAβ, leading to endocytosis of AMPARs and a decrease in AMPA current density (Gu et al., 2009). Under different experimental conditions, it has been proposed that Aβ-dependent synaptotoxicity is in fact an NMDA-dependent metaplasticity phenomena involving AMPAR desestabilization (Opazo et al., 2018) . The proposed mechanism is as follows: within 30 min of treatment with oAβ a NMDAR-dependent activation of CaMKII occurs, specifically through GluN2B subunits. This initial and fast CaMKII activation occludes new the further activation of new rounds of CaMKII (Opazo et al., 2018). A metaplasticity response causes AMPAR desestabilization and a decrease in dendritic spine volume, the precise mechanism causing this final effect, remains to be solved (Opazo et al., 2018).

3.4. Wnt signaling

Wnt ligands are glycoproteins, members of a wide family of morphogenes involved in cellular organization. They were first discovered in the *Drosophila melanogaster* and initially, their role was thought to be exclusively on the pattern formation of the antero-posterior axis in development. Wnt signaling is highly conserved in the animal kingdom. In rats and humans, for

example, there are 19 different Wnt ligands and 10 Frizzled receptors, with orthologue pairs and share ~94% amino acids (Bjarnadóttir et al., 2006). In recent years, there has been a novel interest in studying the roles of Wnt signaling at postnatal stages. Particularly, the study of Wnt signaling on adults has focused in cancer research and in the postnatal maturation and maintenance of neurons in the CNS. Nowadays, we know that Wnt signaling plays a role in processess, as diverse as: adult neurogenesis in dopaminergic (Andersson et al., 2013), cerebellum (Subashini et al., 2017) and hippocampal neurons (Lie et al., 2005; Okamoto et al., 2011), dendritic spine growth (Ciani et al., 2011) and dendritic maintenance (Chen et al., 2017), clustering of PSD95 (Ciani et al., 2011; Farías et al., 2009) and of other synaptic proteins like Bassoon (Gogolla et al., 2009; Varela-Nallar et al., 2009) , vGlut1 (Varela-Nallar et al., 2009) and SynGAP (Codocedo et al., 2015) to mention a few. Wnts can also modulate synaptic activity, it has been reported they play a role in presynaptic neurotransmitter release (Cerpa et al., 2008b), increase in glutamatergic currents (Cerpa et al., 2010a) and LTP activity (Cerpa et al., 2011, 2015; Ciani et al., 2011) and it has also been involved in neuroprotection (Cerpa et al., 2010a; Zhang et al., 2015b).

Next, information regarding the biogenesis of mature Wnt ligands, their release and the effects of different Wnt signaling pathways on the mature CNS, will be addressed.

3.4.1. Biogenesis and post-translational modification of Wnt ligands

There are 19 different Wnt ligands in humans. In general, Wnt ligands are composed of around 350-400 aminoacids with a molecular weight of ~40-45 kDa, with 23 conserved cysteine residues in their N-terminal domain, allowig for intramolecular disulphide bonds to form (Rijsewijk et al., 1987).

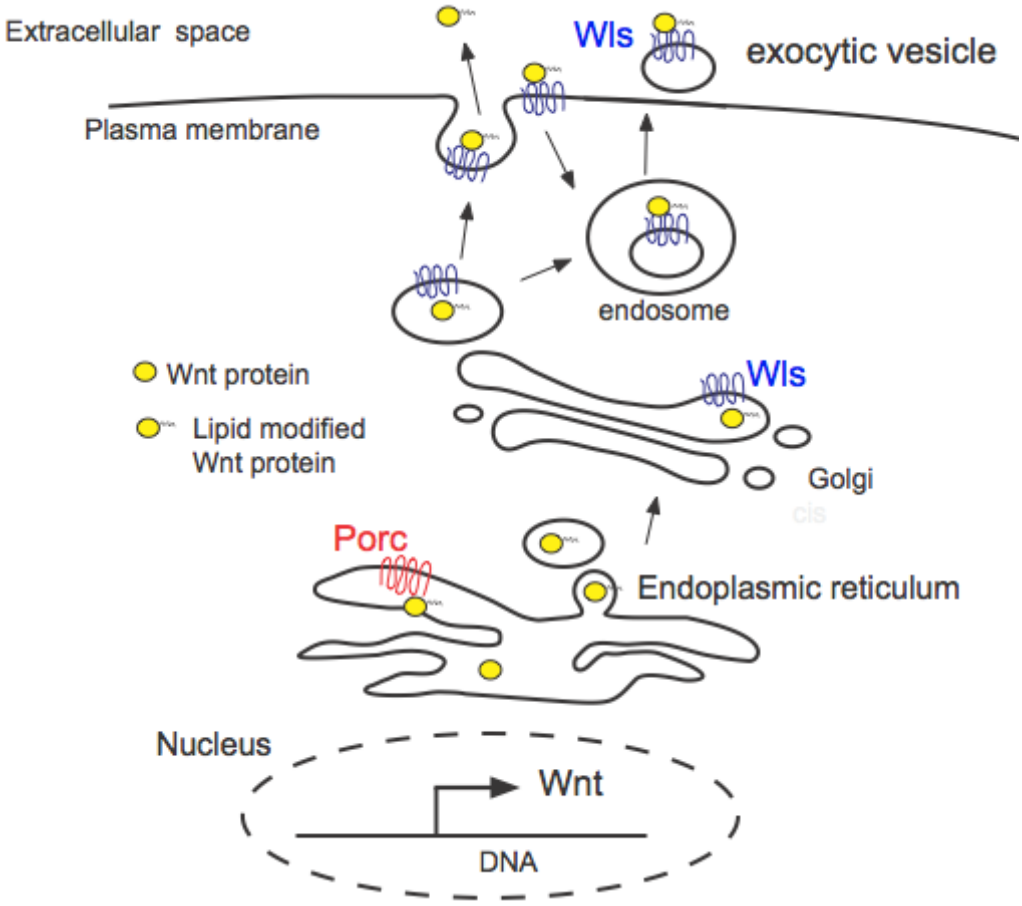
Wnt proteins suffer from different two post-translational modifications (PTM), that are key for their secretion and posterior binding to Frizzled receptors. N-glycosilation and palmitoylation, the addition of palmitate or palmitoleic acid (Komekado et al., 2007; Kurayoshi et al., 2007). Once these two PTM occur, Wnt proteins are considered to be mature and can be refered as Wnt ligands. Wnt proteins are translated in the ER and immediately after translation, they suffer

PTM (glycosylation and palmitoylation) directly at the ER. In charge of catalising the glycosylation and palmitoylation of newly synthesized Wnt proteins, is Porcupine, an eight transmembrane domain protein, resident of the ER and member of the membrane-bound-O-acyl-transferases family (Herr et al., 2012). Both modifications are independent but it has been described that glycosylation follows palmitoylation (Komekado et al., 2007). The role of glycosylations is unclear, but is generally accepted that unglycosylated Wnt proteins are retained in the ER, thus are not secreted (Komekado et al., 2007). Also, it has been described that glycosylation plays a role in Wnt ligand binding to its Frizzled receptor. More conclusive evidence shows the fundamental role of palmitoylation in ligand secretion and enhancing their ability to bind to Frizzled receptors and co-receptors (Komekado et al., 2007). Porcupine palmitoylates Wnt3a proteins at least at two different residues, Cys77 and Ser209, this action has found to be crucial for the secretion of Wnt ligands (Komekado et al., 2007; Takada et al., 2006). In the case of Wnt5a, Cys104 residues are palmitoylated and several asparagine residues get glycosylated (Kurayoshi et al., 2007). Different Wnt proteins may have different palmitoylation sites, but the overall mechanism and function, its the same. Either, Porcupine mutations or site specific mutations on the palmitoylation residues are associated with intracellular accumulation of Wnt protein (Takada et al., 2006).

Once PTM are completed, Wnt ligands continue the secretory pathway and migrate to the Golgi Apparatus. There, mature Wnt ligands bind to Wntless, a transmembrane protein essential for the secretion of Wnt ligands found associated with plasma membrane at the Golgi Apparatus. It is considered to be a Wnt transpoter protein. The Porcupine-dependent palmitoylation step previously described seems to be a prerequisite for Wnt binding to Wntless (Komekado et al., 2007).

Wnt ligands can be secreted into small vesicles, probably exosomes (Gross et al., 2012), which would allow Wnts to travel longer distance and not interacting with extracellular matrix components like heparan sulfate proteoglycans, which due to their highly lipidic mature form, is very likely to happen. Nonethelesss, Wnts can also act on a shorter range distance, suggesting that could be also be released from their binding to Wntless, therefore the plasma membrane

and be secreted freely. When this happens, usually Wnt ligands act in a short distance paracrine or autocrine manner.



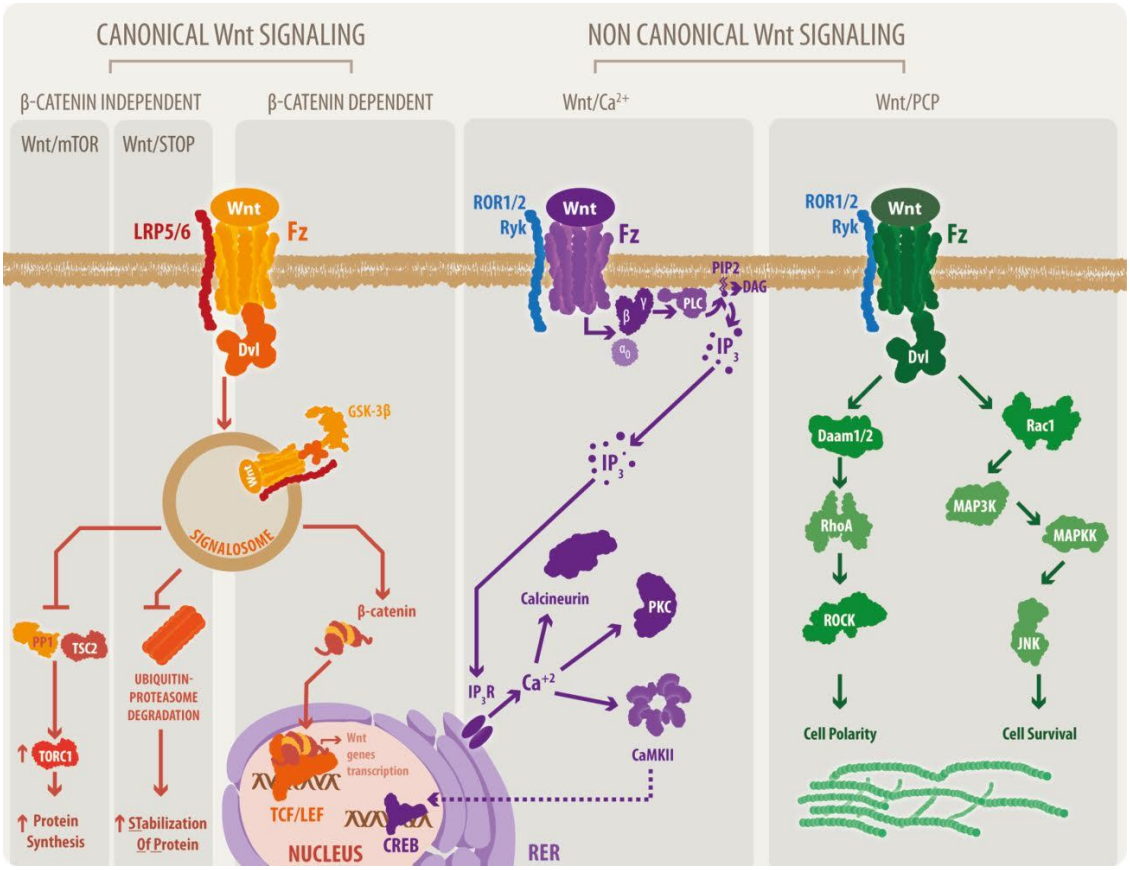
from The Wnt homepage (https://web.stanford.edu/group/nusselab/cgi-bin/wnt/node/263) by Roel Nusse.

The secretory pathway of Wnt ligands. Upon translation at the ER, Wnt proteins (in yellow) suffer a series of post-translational modifications. The first one being the addition of lipids, which occurs also at the ER and it is catalyzed by the enzime Porcupine. The palmitoyltransferase activity of Porcupine, adds a palmitate (lipid) group to the protein. Following on the secretory pathway, the lipid modified Wnt (palmitated) will continue into the Golgi Apparatus where only lipid-modified Wnts will bind to the membrane-bound Wntless protein. Wnt and Wntless will remain attached together into secretory vesicles, or Wnt ligands will be freely-secreted into the extracellular space.

3.4.2. Wnt Pathways

Wnt signaling can be roughly divided in two different pathways, noting that this classification is not straight forward due to the variable interactions of Wnt ligands with different receptors and co-receptors. The first one, called canonical Wnt pathway, can be subdivided depending on the requirement of β-catenin and consequent activation of gene transcription (β-catenin dependent), or the activation of the Wnt/mTOR or Wnt/STOP pathways, the first one leading to increased protein synthesis and the second one to protein stabilization. On the other hand, the non-canonical Wnt pathway can be subvided into Wnt/Ca^{2+}, which increases intracellular Ca^{2+} concentrations from internal stores and activates CaMKII, PKC and the phosphatase calcineurin. The other branch of the Wnt non-canonical pathway is the Wnt/Planar Cell Polarity (PCP), also refered as Wnt/JNK, and causes activation of a plethora ot small GTPases like Rac1, Daam1/2 and ROCK, and leads to cell polarity and survival. There is evidence arguing for a loss on the regulation and balance between canonical and non-canonical Wnt signaling in the early stages of AD. And has even been proposed that a deregulation in the Wnt pathway triggers the onset of the disease.

In all cases, a Wnt ligand binds to a Frizzled receptor and this binding favor the coupling of a co-receptor, forming a signaling complex. At least 5 families of co-receptors have been described. LRP5/6 is related to canonical signaling whereas Ror1/2 or Ryk1, are related to non-canonical signaling, as depicted on the figure below. It has been proposed then, that co-receptor binding to the Wnt-Frizzled complex, will confer specificity to the downstream response and add regulatory mechanisms for the fine tunning of responses (Verkaar and Zaman, 2010). More recently, new co-receptors has been discovered, like: PTK7, RYK and heparan sulphate proteoglycan (HSPG)(Niehrs, 2012; Verkaar and Zaman, 2010).



from (Oliva et al., 2018)

The Wnt signaling pathways. The Wnt signaling pathways can be divided into two main branches, canonical (GSK-3β dependent) and non-canonical (GSK-3β independent). In the canonical, binding of a Wnt ligand causes internalization of the Frizzled (Fz) receptor and co-receptor (LRP5/6), forming an intracellular signalosome that binds GSK-3β and disassembles the constitutively active β-catenin destruction complex, allowing cytoplasmatic β-catenin accumulation. Next, β-catenin migrates to the cell nucleus and binds to TCF/LEF transcription factors, favoring the transcription of Wnt-target genes. The second branch of the canonical (GSK-3β dependent) Wnt pathway is β-catenin independent. It diverges from the signalosome and leads to proteosome inhibition and Stabilization Of Proteins (Wnt/STOP). Also, can cause the inhibition of TCS2 which will lead to activation of TORC1 and an increase in protein synthesis (Wnt/mTOR). Non-canonical pathways are independent on GSK-3β and β-catenin action. The co-receptor can be either Ror1/2 or Ryk. In the Wnt/Ca^{2+} signaling, Fz receptor activation leads to G-protein and PLC activation, which will cleave PIP_2 into DAG and IP_3, This will activate IP_3 receptors in the rugose endoplasmic reticulum (RER) allowing Ca^{2+} release. The increase in cytoplasmic Ca^{2+} activates kinases like CaMKII and PKC or the phosphatase calcineurin. CaMKII activation, into successive steps (dashed line), leads to the activation of the transcription factor CREB and consequent gene transcription. Finally, activation of the non-canonical Wnt/PCP pathway acting on small GTPases like RhoA and Rac1, activates kinases like ROCK, MAPK and JNK to cause cytoskeleton rearrangements and cell survival.

3.4.3. Wnt signaling in the mature central nervous system

Since the discovery of Wnt signaling, as a crucial developmental signaling pathway, much of the focus was put in its role in embryonic patterning formation. However, in the last decade a new interest on the roles of Wnt signaling in the central nervous system, and Particularly in the already formed, mature CNS, has come to light (Oliva et al., 2018).

That is how it is clear now that Wnt ligands continue on playing a fundamental role in adult stages and throughout the entire lifespan of an animal. Particularly, in the mature CNS, Wnt signaling is key on regulating neurogenesis (Schneider et al., 2016; Yao et al., 2016), maintenance of dendritic arborization (Chen et al., 2017), neurotransmitter release (Cerpa et al., 2008b; Ciani et al., 2015), among many other functions that are listed in Table 1.

Most of the evidence that will be presented comes from *in-vitro* experimentation, which in many cases has found *in-vivo* confirmation. In any case, increasing evidence, specially in the last few years, converge on the relevance of Wnt signaling in the physiological functioning of the CNS.

Table 1. Reported effects of Wnt ligands in the adult central nervous system

Ligand	Effects	Cell Type/Tissue/Model	Pathway
Wnt1			
	Favors neurogenesis	Adult midbrain SC (in vivo)/progenitor neurons (in vitro)	Canonical (β-catenin)
	Protects against oxidative stress	Hippocampal neurons (in vitro)/rats (in vivo)	Canonical (β-catenin-Akt)
	Protects against neurodegeneration	Dopaminergic neurons/substantia nigra	Canonical (β-catenin)
	Necessary for neurogenesis	Midbrain dopaminergic neurons (in vitro)	Canonical (β-catenin)
Wnt2			
	Mediate antidepressant effect	Ventral hippocampus	Canonical (β-catenin)
	Dendritogenesis and spinogenesis	Cortical neurons (in vitro)	nd.
Wnt3			
	Mediates antidepressant effect	Ventral hippocampus	Canonical (β-catenin)
	Promotes neurogenesis	Dentate gyrus	Canonical (β-catenin)
Wnt3a			
	Increases basoon clusters	Hippocampus (in vivo)/hippocampal neurons (in vitro)	Canonical (β-catenin)
	Increases vGlut1 clusters	Hippocampal neurons (in vitro)	Canonical (β-catenin)
	Increases synaptic vesicle release	Hippocampal neurons (in vitro)	Canonical (β-catenin)
	Regulates oscillatory activity	mECx-CA1-CA3 (ex vivo)	nd.
	Favors neurogenesis	Midbrain dopaminergic neurons (in vitro)	nd.
	Induces exosome secretion	Microglia (in vitro)	Noncanonical (nd.)
	Counteracts proinflammatory lipopolysaccharides	Microglia (in vitro)	nd.
	Regulates inhibition of mPTP opening	Isolated mitochondria from hippocampal slices (in vitro)	Canonical (β-catenin) mitochondrial GSK-3β
Wnt5a			
	Increases clustering of PSD95	Hippocampal neurons (in vitro)	Noncanonical (JNK)
	Increases clustering of SynGAP	Hippocampal neurons (in vitro)	Noncanonical (nd.)
	Stimulates synaptic differentiation	Hippocampal neurons (in vitro)	Noncanonical (CaMKII)
	Increases AMPA and NMDA currents	Hippocampal neurons (in vitro)	Noncanonical (CaMKII)
	Increases fEPSP transmission	Hippocampal neurons (in vitro)	Noncanonical (CaMKII)
	Protects against Aβ oligomers synaptoxicity	Hippocampal neurons (in vitro)	Noncanonical (nd.)
	Modulates NO production	Hippocampal neurons (in vitro)	Noncanonical (Ror2)
	Increases dendritic spine density	Hippocampal neurons (in vitro)	Noncanonical (Ca^{2+}, nd.)
	Favors dendritic maintenance/ plasticity	Hippocampal neurons (in vitro)	Noncanonical (CaMKII)
	Recycling of functional $GABA_A$ receptors	Hippocampal neurons (in vitro)	Noncanonical (CaMKII)
	Long-term information storage	Hippocampus (in vivo)	nd.
	Prevents memory impairment	Rats (in vivo)	nd.
	Regulates oscillatory activity	mECx-CA1-CA3 (ex vivo)	nd.
	Favors neurogenesis	Midbrain dopaminergic neurons (in vitro)	nd.
	Differentiation, axonal growth, and repulsion	Midbrain dopaminergic neurons (in vitro)	nd.
Wnt7a			
	Promotes dendritic spine growth	Hippocampal neurons (in vitro)/ hippocampus (in vivo)	Non-canonical (CaMKII)

(continues on next page)

	Induces synapse formation	Hippocampal neurons (in vitro)	Canonical (β-catenin-cyclin D1)
	Increases clustering of PSD95	Hippocampal neurons (in vitro)	Canonical (β-catenin-NGN2)
	Vesicle cycle	Hippocampal neurons (in vitro)	Noncanonical (CaMKII)
	Modulates α7-nAChR expression/localization	Hippocampal neurons (in vitro)	Noncanonical (CaMKII)
	Increases clustering of vGlut	Hippocampal neurons (in vitro)	Noncanonical (CaMKII)
	Increases clustering of Synaptophysin	Hippocampal neurons (in vitro)	nd.
	Long-term information storage	Hippocampus (in vivo)	nd.
	Induces clustering of bassoon (Wnt7a/b)	Hippocampus (in vivo)	nd.
	Modulates excitatory neurotransmitter release	Hippocampus (ex vivo)	Canonical (β-catenin)
	Increases clustering of synapsin 1	Cerebellar neurons	nd.
	Induces stem cell proliferation	Ventral midbrain and dentate gyrus	Canonical (β-catenin)
	Induces neural stem cell maturation	Dentate gyrus	Canonical (β-catenin)
Wnt7b			
	Promotes dendritic growth	Hippocampal neurons	Noncanonical (Rac-JNK)
	Induces clustering of VAMP2	Mossy fibers	nd.
	Induces clustering of Bassoon	Mossy fibers	nd.

from (Oliva et al., 2018)

In this thesis, we will focus on the role of Wnt5a in hippocampal neurons, where it acts as a non-canonical Wnt ligand (Varela-Nallar et al., 2010) . Cumulative evidence suggests that Wnt5a has an important role in the normal development, maturity and maintenance of hippocampal neurons. But also has the ability to cause synaptic- and neuroprotection against oAβ.

3.5. Wnt5a and its neuroprotective role against oAβ

Wnt5a is a member of the Wnt ligands family and has been unequivocally described as an activator of the non-canonical Wnt pathway in hippocampal neurons. Therefore, several studies by us and others, have studied the roles of Wnt5a in the organization and maturation of postnatal and adult hippocampal activity. Within the rat and human brain, Wnt5a is highly expressed in hippocampus and it is the only non-canonical ligand expressed at detectable levels (Allen Mouse Brain Atlas webpage). In contrast, Wnt3a and Wnt7a which are also expressed in the hippocampus, but are considered to act mainly as canonical Wnt ligands. Interestingly, in mouse and rat hippocampus, Wnt5a is expresed only from postnatal stages, between P7-P10, with parallel expression of important synaptic proteins, and not earlier in

development (Chen et al., 2017). Once expressed, Wnt5a is present throughout the entire lifespan of animals (Chen et al., 2017; Varela-nallar et al., 2010).

Studies show that in hippocampal neurons, Wnt5a increases miniature excitatory postsynaptic currents, both for AMPAR and NMDA receptors (Varela-Nallar et al., 2010), promotes the clustering of PSD95 (Farías et al., 2009) and SynGAP (Codocedo et al., 2015), increases de density of dendritic spines (Ramírez et al., 2016), among other roles described in Table 1. It has been shown that Wnt5a alone, has a crucial role in synaptic plasticity and structural maintenance of dendritic arborization (Chen et al., 2017). A more recent study showed that conditional knock-down expression of Wnt5a in adult mice hippocampus causes severe memory impairment, which correlates with the decrease in dendritic arborization (Chen et al., 2017). Demonstrating that Wnt5a, in adult hippocampus, has a functional relevance in processess of learning and memory. On Table 1 are described the effects of Wnt5a in mature CNS (Oliva et al., 2018).

Several studies have described a neuroprotective role for Wnt5a against oAβ. *In-vitro* experiments show that Wnt5a can revert the effects of oAβ42 by recovering: PSD95 clusters, SynGAP clusters (Codocedo et al., 2015), dendritic spines density, excitatory synaptic currents (Cerpa et al., 2010a). In hippocampal neurons, Wnt5a activates CaMKII and JNK, which as mentioned above are fundamental in the regulation of AMPARs-TARPs interaction with PSD95 (Opazo and Choquet, 2011; Opazo et al., 2010).Therefore, we reasoned that Wnt5a promotes the stabilization of AMPARs in rat hippocampal neurons. And by doing so, Wnt5a protects synaptic organization and functionality, against $A\beta_{42}$ oligomers.

In-vivo administration of $A\beta_{25-35}$ oligomers reduce LTP response in hippocampal CA1 region, withouth affecting paired pulse facilitation (PPF). This effect, is blocked when Wnt5a is administrated before $A\beta_{25-35}$ oligomers. This effects correlate also with a decrease in spatial learning and memory when animals are treated with $A\beta_{25-35}$ oligomers, which again is reversed by Wnt5a (Zhang et al., 2015a). Also *in-vivo*, it has been proof that administrating a mimic of Wnt5a (Foxy5) through an osmotic pump implanted in the hippocampus of APP/PS1 mice, a commonly used model for AD, rescue their memory impairment (Vargas et al., 2014).

3.6. What we know so far

Next, a list of facts, all of them were above mentioned in detail, regarding the two main topics that this thesis pretends to study and unite. The study of Wnt ligands as modulators of AMPA receptors is still in its youth, so there is not a great body of evidence that directly links them. There is, nonethelesss, considerable evidence relating Wnt signaling and excitatory synaptic transmission.

In the case of Wnt signling and oAβ synapto- and neuroprotection, a greater body of evidence shows that Wnt signaling (mainly Wnt5a) is able to protect from the toxic effects of oAβ.

3.6.1. Wnt signaling and AMPA receptors

Not much information exists directly relating Wnt signaling to AMPARs. That's one of the reasons this research is of novelty and importance to the field. Next, a few bullet points describing known facts over Wnt signaling and AMPA receptor modulation:

- Wnt5a increases the EPSCs of both AMPA and NMDA receptors (Cerpa et al., 2010b).

- Wnt5a increases mEPSCs of both AMPA and NMDA receptors (Varela-Nallar et al., 2010).

- Wnt7a rapidly promoted synaptic AMPAR recruitment and trapping (Mcleod et al., 2018).

- Wnt7a increases the presence of extrasynaptic AMPARs, in a CaMKII and PKA mediated manner (Mcleod et al., 2018).

3.6.2. Wnt signaling and oAβ-dependent synapto- and neurotoxicity

- oAβ_{42} significantly reduces AMPA and NMDA currents, with a greater effect on NMDA currents (Cerpa et al., 2010b).

- Wnt5a treatments (40 min) occlude the synaptic transmission depression induced by oAβ (Cerpa et al., 2010b). In this case, it was not determined if the protective effect from Wnt5a was by action (directly or indirectly) over AMPA or NMDA receptors.

- Wnt5a is able to recover the loss of several synaptic proteins, induced by oAβ treatments. Some of this proteins are: PSD95 (Cerpa et al., 2010b), SynGAP (Codocedo et al., 2015).

- *In-vivo* activation of Wnt signaling, through a mimic of Wnt5a, causes memory improofment in animal mouse model of AD (Vargas et al., 2014).

- Wnt3a prevents oAβ-induced mitochondrial permeability transition pore opening in living neurons. Like this, it prevents mitochondria swelling, mitochondrial membrane potential loss and cytochrome c release. By preventing this sequence of events, it is proposed that Wnt3a causes neuroprotection against oAβ (Arrázola et al., 2017).

- *In-vitro* experiments have shown that inhibition of Wnt signaling leads to an increase in the amyloidogenic processing of APP. Thus, linking loss of Wnt signaling to an increase in oAβ content and amyloid depositions (Tapia-Rojas et al., 2016).

3.7. The problem and the line of attack

Considering the information provided throughout the introduction and what we know about Wnt signaling in regard to AMPA receptors and oAβ_{42} (sections 6.6.1 and 6.6.2.), our working hypothesis is as follows: “Wnt5a causes protection against oAβ_{42} by modulating AMPARs dynamics”.

This thesis presents a study on the mechanism by which Wnt5a is able to cause synaptic protection against oAβ. Throughout this project, we used mainly super-resolution microscopy techniques, but also biochemical techniques and confocal microscopy. We performed a sedulous attempt to first study the dynamic of AMPARs in response to Wnt5a and oAβ, independently. Once these responses were characterized, we analyzed the effect of applying Wnt5a followed by oAβ, to test if there is a preventive effect of Wnt5a. Like this, we discovered that Wnt5a immobilizes AMPARs and by this means, helps to prevent the effects of Aβ_{42} oligomers.

4. MATERIALS AND METHODS

Molecular Biology

eGFP-Homer 1c plasmid is a gift from S. Okabe (Tokyo University, Tokyo). Coding DNA for GluA1 was first subcloned in the eukaryotic vector prK5 before SEP insertion at its amino-terminal end by subcloning.

Primary Neuronal Cultures and Transfection

Hippocampal cells of Sprague Dawley embryos E18 were dissected and plated at a density of 75 cells/mm^2 on poly lysine-coated glass coverslips. Coverslips were placed upside-down over a layer of astrocytes, to modulate physiological conditions and increase cell growth and viability, following a Banker protocol. Neurons were transfected at DIV 9-11 with calcium phosphate method or Effectene (Qiagen, 301427) according to the protocol suggested by the supplier. Shortly, cDNA and 300 µL buffer solution were mixed with 16 µL of enhancer and 50 µL Effectene. After every addition, the mix was gently vortexed and left at room temperature for 10 min. On a separate dish, the final mix was added to coverslips and left on incubator (37°C, 95% O_2) for 50-80 min. Afterwards, coverslips were returned to the original dishes and back to the incubator. For PALM-STORM experiments, cells were transfected using the Ca^{2+}-phosphate method (Jiang and Chen, 2006). cDNA constructs used were: 1 µg of Homer1C::GFP, 0.5 µg GluA1-HA and 1 µg of XpH15::mEos. All experiments were performed at 14-16 DIV.

Recombinant Proteins and reagents

Recombinant Wnt5a (645-WN/CF), recombinant Wnt7a (3008-WN/CF) and recombinant sFRP2 (1169-FR/CF) were acquired from R&D Systems, MN. All recombinant proteins were reconstituted in PBS 1X to a stock solution of 100 µg/mL. rWnt5a was used at a final concentration of 300 ng/mL, rWnt7a was used at 300 ng/mL or 600 ng/mL, and sFRP-2 was used at 1 µg/mL or 2 µg/mL. All stocks were stored at -20°C and used within 3 months of reconstitution. KN93 (Tocris) was pre-incubated for 2h in incubator (37°C, 5% CO_2) at 10µM. Wnt5a + sFRP2 complex was formed by mixing 300 ng/mL Wnt5a with 1 µg/mL sFRP2 and left in agitation for 30 min, at 37°C.

oAβ preparation

Amyloid-β (Aβ) protein 1-42 (H-1368, Bachem AG, Switzerland) was dissolved in hexafluor-2-propanol (HFIP) to 1 mM and let rest at room temperature for 30 min and aliquoted. Tubes were left under hood, overnight so traces of HFIP evaporate. To remove traces of HFIP, tubes were taken to SpeedVac and dry down for 1 h. Dried peptides were stored at -20°C until use. One day before experimentation, the pellet is dissolved in DMSO, mixed carefully with pipette and sonicated for 1 min. Finally, Neurobasal medium is added to a final concentration of 100 µM, vortexed and incubated for 24 h at 4°C to allow oligomerization. oAβ were used at 1 µM or 5 µM with a calculated final concentration of DMSO <0.002%.

Co-immunoprecipitation and Western Blot

Hippocampal cells of Sprague Dawley embryos E18 were dissected and plated at a density of 7500 cells/mm^2. For co-immunoprecipitation, cells were washed with PBS and lysed in ice cold HEPES lysis buffer, supplemented with EDTA, phosphatase and protease inhibitors. Afterwards, the lysate was centrifuged for 15 min at 14.000 rpm, 4°C and the supernatant was collected. Twenty microliters of beads was added to the lysate along with 0.5 µL of anti-PSD95, the mix was left incubating in slow rotation for 2 h at 4°C. Washings were performed on the same lysis buffer used previously, all supernatant is carefully removed. Loading buffer 2x is added to the final sample and heated at 95°C for 5 min and loaded into a 10% acrylamide SDS-PAGE gel. Non-denaturing gel was run without adding SDS, β-mercaptoethanol or heating samples. Tris-tricine gel was run to separate Aβ species. Primary antibodies used are: Wnt5a (Abcam, ab72583), PSD95 (Neuromab, clone K28/43), GluA2 (Neuromab, clone L21/31), GluN2B (Neuromab, clone N59/36), CaMKII (Santa Cruz, SC5306), pCaMKII (Santa Cruz, SC32289), JNK (Santa Cruz, SC474), pJNK (Cell Signaling, #9251S), 6E10 (BioLegend, S803004) and tubulin (Santa Cruz, H235). Bands were quantified by pixel density using ImageJ (NIH).

Live-stainning, immunofluorescence and epifluorescence imaging

After super-resolution imaging, coverslips were removed from the Ludin chamber and placed on a droplet of Tyrode-1% BSA for 2 min in incubator (37°C, 95% O_2). Following, neurons were

incubated on GluA2 antibody (1:500) in Tyrode-1% BSA, for 6 min in incubator conditions. Excess antibody was removed by washing in a droplet of Tyrode-1% BSA, for a few seconds. Once live-stainning protocol for GluA2 was ready, cells were fixated in freshly prepared PFA 4%-Sucrose 4% and rinsed in PBS 1X. Cells were washed with 50 mM PBS-NH_4Cl for 10 min in order to quench the reactive aldehyde groups, and rinsed with PBS 1X. Blockade was made with PBS-1% BSA for 30 min at room temperature. Permeabilization was done using PBS-0.3% Tx-100 for 5 min. Primary antibodies PSD95 (1:500) and VAMP2 (1:2000) were incubated for 60 min at room temperature. Then, rinsed with PBS 1X three times and blocked with PBS-2% BSA for 30 min.

Secondary isotope-specific antibodies were incubated (1:500) for 30 min at room temperature. For STORM samples, a second blocking step is done with PBS-1% BSA for 30 min. After secondary antibody incubation, a second fixation is done with PFA 2%-Sucrose 2%. Finally, neurons were rinsed with PBS 1X, distilled water and kept on PBS 1X at 4°C for STORM. For DM5000, neurons were mounted in slides with Fluoromount G-DAPI and kept overnight on a dark chamber at room temperature. Within a week, images were acquired in microscope or a DM5000 epifluorescence microscope. For the latter, a 63x oil immersion objective was used, 8-10 steps of 0.2 µm in z-axis were obtained. Images were passed by StackReg plugin for ImageJ (NIH), to correct any possible drift on the z-stack images. For co-localization analysis the plugin Just another Co-localization Plugin (JaCOP) (Bolte and Cordelieres, 2006) was used and for synaptic contacts the plugin SynapcountJ, running in ImageJ. JaCOP allows the gathering of different co-localization parameters like: Mander's coefficients 1 and 2, ICA, R, Pearson's coefficient, among others. All of these coefficients measure the co-localization of two labelings, and they have their advantages and drawbacks. We used Mander's coefficient, which integrates in its algorithm Pearson´s coefficient as a correlation measurement, but allows easier interpretation of the results (Dunn et al., 2011). Mander's overlap coefficient (MOC), as is formally known, is calculated as follows:

$$\mathrm{MOC} = \frac{\sum_i (R_i \times G_i)}{\sqrt{\sum_i R_i^2 \times \sum_i G_i^2}}$$

Where, Ri and Gi refer to the intensity values on the red and green channel, respectively.
MOC measures the fraction of pixels with positive values for both channels. MOC values range from 0 to 1, 0 being no co-localization and 1 reflecting 100% co-localization.

uPaint microscopy

Throughout this thesis work, the use of super-resolution microscopy has been a fundamental part of the experimentation. Particularly, a technique called universal Point Accumulation in on the Nanoscale Topography (uPaint) developed by. This technique combines two type of microscopy; Photoactivated Localization Microscopy (PALM) and Total Internal Reflection Fluoroscence Microscopy (TIRFM). Next, a short explanation of both microscopy techniques (PALM and TIRF) will be presented individually. Following, a specific description of the uPaint technique, data analysis and results interpretation, will be explained.

Super-resolution microscopy techniques, do not break the resolution limit; the size of a incident laser wavelength cannot be changed, and that is the ultimate limit for light microscopy, according to:

$$\boldsymbol{r}_{(x,y)} = \frac{\mathbf{0.61}\ \boldsymbol{\lambda}}{\boldsymbol{NA}} \qquad \text{(Equation 1)}$$

Where: r is the resolution limit on the x,y axis; λ is the incident light wavelenght; NA is the numeric aperture of the lens.

What super-resolution microscopy does, is to bend the resolution limit through clever techniques. In PALM microscopy, a subset of fluorophores is stochastically switch from an on to an off state. This allows that at a certain time point, the distance between two on-state fluorophores is higher than the resolution limit. Like this, each point can be individually located and mathematically corrected by the Point Spread Function (PSF). A large amount of individual particles are obtained over time and summated into one reconstructed super-resolved image. This reconstruction takes into account the particular Point Spread Function (PSF) to mathematically correct the size and location of the fluorescent particle and therefore, increase the resolution of the image.

TIRF microscopy was first developed by in . Its principle is the use a critical angle, called TIRF angle, in which the incident illumination over the sample, mediums with different refraction indexes, allows the formation of an evanescent wave. The evanescent wave allows for a small size of the sample, in proximity with the interface between glass and sample, to receive part or all the light in a small section. Usually, this allows to visualize into 100-200 nm deep of a particular sample. This feature increases the signal-to-noise ratio, allowing better detection and resolution, overall cleaner images. For this reason, TIRF microscopy is widely used to observe particles in the plasma membrane.

The uPaint technique uses PALM and TIRF in conjugation to obtain live labeling at super-resolution in plasma membrane proteins. A main difference between uPaint and other super-resolution techniques is that labeling itself is performed at low density and therefore, the detection will be at low density as well. Instead, in techniques like PALM and STORM, the labeling is performed at high density, but due to the use of photoswitchable dyes, detection can be performed at low density. The combination of these techniques results in the following advantages:

1. The longest observation time of individual probes, allowing recordings of minutes tracking the same population of receptor.
2. Versatility of endogenous molecules labeling, since the labeling system basically depends on a primary antibody coupled to a fluorescent dye.
3. No need of photoswitchable dyes, which can be toxic and harder to produce.

Once the acquisitions are obtained, the offline analysis is as follows:
Trajectories are reconstructed by connecting single particle detections from consecutive frames. In order to avoid false positives, individual particles are detected for a minimum of ten frames are included in into the analysis, the ones not complying to this parameter are not considered for analysis. All trajectories are summed into one super resolution reconstructed image. To identify the position of each single particle at any given time, the following equation is used:

$$\boldsymbol{r}_{(x,y)} = \mathbf{I_0}\,\mathbf{exp} - \frac{(\mathbf{x}-\mathbf{x_0})^2+(\mathbf{y}-\mathbf{y_0})^2}{\mathbf{2\sigma^2}} \qquad \text{(Equation 2)}$$

Where : I_0 is the amplitude of the Gaussian; σ is related to the Full Width at Half Maximum (FWHM) if the diffraction pattern by FWHM = 2.35 σ; the parameters x_0 and y_0 set up the central position of the located spot.

Mean Square Displacement (MSD), measures the area covered by a single particle in a fixed time frame (500 milliseconds). Normally, a freely diffusing particle with no constrictions but temperature (like in brownian movement) shows a linear MSD behaviour, while particles with regulated diffusion display a curved MSD, reaching a plateau over time. Therefore, the more confined the particles are, the lower the MSD curve.

$$\boldsymbol{MSD}_{(n,\Delta t)} = \sum_{i=1}^{N-n} \frac{\left[x((i+n)*\Delta t) - x(i*\Delta t)\right]^2 + \left[y((i+n)*\Delta t) - y(i*\Delta t)\right]^2}{N-n} \qquad \textbf{(Equation 3)}$$

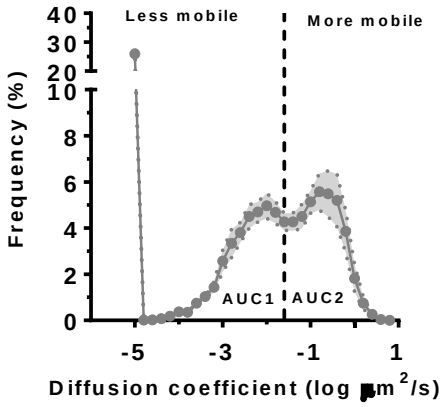
uPaint microscopy (Giannone et al., 2010) was used to detect and track exogenous GluA1 or endogenous GluA2 subunits, in single molecule tracking. Neurons were imaged at 37°C in a Ludin chamber (Life Imaging Services, Basel, Switzerland) in a caged microscope, and bathed on Tyrode extracellular solution (15 mM D-glucose, 108 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES; pH adjusted to 7.4, 280 mOsm) and mounted in an inverted motorized PALM microscope (Nikon, TI-eclipse) equipped with a NA plan-achromat 100x 1.49 NA oil immersion objective and a perfect focus system, mounted on an anti-vibrational table (TMC, USA). Laser diodes of 488 nm and 642 nm, were used. Images were acquired in total internal reflection (TIRF; Ilas, Roper Scientific, Evry, France) configuration, to manipulate tha illumination angle. Signals were detected with an EMCCD camera (Evolve, Roper Scientific, Evry, France). With the 488 nm laser, detection of Homer1c::eGFP allowed to identify synapses and segment according to the labeling of the tagged postsynaptic marker. Within the dendrite, those regions lacking Homer1c:eGFP were considered to be dendrites.Anti-SEP antibodies were used to detect GluA1-SEP molecules. Anti-GluA2 was in-house made and a gift from Dr. Eric Gouaux (Portland, USA). All antibodies were coupled to Atto-647N-NHS-ester (Atto-Tec, Siegen, Germany). Single-molecule fluorescent spots were localized in each frame and tracked over time. Metamorph software (Molecular Devices, CA, USA) was used for acquisition and analysis of images. A total of 8000 frames with an exposure time of 20 ms, were obtained per

cell. The pointing accuracy, a measurement of the precision on locating the detected points, was calculated as ~50 nm PALM tracer, a plugin running with Metamorph, was used to derive quantitative data on protein localization and dynamics.

Reliable data analysis depends on a good signal/noise ratio. For this reason, with the help of PALM Tracer, a threshold is manually set up to determine signal from background. Gaussian fitting is used to detect particles on each frame of the acquisition video. Also, a watershed algorithm is used to discriminate close particles and identify them as singles. Once single particle detections are done, trajectories are reconstructed by connecting detections from one image to the next.

As a result of the analysis on Metamorph, not only we obtain a super-resolved image of the trajectories of AMPARs. Also, we obtain a file with the diffusion coefficient (D, $\mu m^2/s$) which is the area that a given particle travels, per second. We can also obtain the MSD of each particule.

Next, a brief explanation on how each of the paramenters and graphs are obtained. Also, how to interpret the results.



LogD histogram: off-line analysis by calculating LogD and their relative frequency compared to the LogD of the entire population of particles gives an histogram (frequency %) of LogD of AMPARs dynamics for a given condition, depending on the experimental approach. From this histogram, different analysis can be made:

Completely immobile : corresponding to the % of AMPARs that have a LogD equal to -5, in the far left end of the LogD histogram. Meaning that those AMPARs move 0.00001 $\mu m^2/s$. And therefore are considered to be immobile. Biologically, this means that during the entirety of the recording (around 2 min) those AMPARs were completely immobile, probably anchored to a protein. It is graphed separately for better understanding.

Mobility fraction: by calculating the area under the curve (AUC) of the less mobile population of AMPARs (AUC1), from the more mobile population of AMPARs (AUC2) and obtaining the relation between AUC2/AUC1, the mobility is obtained. This parameter is a good representation on the behavior of the entire population of AMPARs and allows a simple comparison between two conditions.

Trajectories: Quantifying and graphing the amount of trajectories detected is important because it gives a good idea on the efficacy on the antibody binding. Also, by comparing the change on the amount of trajectories, we can have an idea if we have more or less labeling between two time points on the same experiment. In general, it is not desirable to have a significant increase or decrease on the detected trajectories. This could an artifactual effect or a biological response that would mask the effect on membrane dynamics of AMPARs and lead to incorrect interpretation. Due to the high amount of detections, trajectories are graphed in thousands (x1000).

Data Analysis and Statistics

All experimental data is presented as mean ± SEM. For statistical analysis, Prism GraphPad 7.0 was used. Grubb's test was used to detect outliers in data sets. t-test was used to determine differences between two conditions. One-way ANOVA was used to determine differences in the mobility between three or more groups. In all cases a minimum of 3 independent experiments were performed and n represents the number of total replicates. The significance value used was $*P<0.05$.

5. RESULTS

5.1. Wnt5a immobilizes GluA1-containing AMPARs in a CaMKII-independent manner.

First we tested if, as reported before, Wnt5a (300 ng/mL) activates the non-canonical Wnt signaling on mature hippocampal neurons. Effectively, Wnt5a treatments activate two important kinases involved in the non-canonical pathway. CaMKII and JNK are rapidly activated by treatment with Wnt5a (Figure 1A-B). The time course reveals that already at 15 min there is a significant increase of pCaMKII/CaMKII (Figure 1B, black circles). Similarly, pJNK/JNK at 54 and 48 kDa show an increase already at 5 min of treatment with Wnt5a (Figure 1B, grey circles). In order to test our hypothesis, that Wnt5a affects AMPARs dynamics, we evaluated if GluA1-containing AMPARs mobility is altered by treatments with Wnt5a. Neurons were co-transfected with Homer1C:eGFP to visualize the postsynapse and with GluA1-HA in order to track them with an anti-HA-Atto-647 antibody (Figure 1C). The epitope for anti-HA is located on the N-terminal (extracellular) domain of GluA1, therefore allowing live cell tracking without affecting membrane integrity (Figure 1C). In every case, basal acquisition corresponds to the dynamic of AMPARs before Wnt5a exposure. After ligand addition, acquisitions were made at 15 and 30 min of continuous Wnt5a treatment. We observed that the effect of Wnt5a is time-dependent. At 15 min we see a decrease in mobilization but only at 30 min after treatment, the effect is statistically significant (Supplementary Figure 1). For this reason, although in all cases experiments were done at basal, 15 and 30 min, we will contrast data at basal vs 30 min. This set of experiments are unpaired, meaning that acquisitions were not made in the same dendrite before/after treatment, but in different dendrites of the same coverslip.

Figure 1D shows the selected GFP neuron (positively transfected with Homer1C::eGFP) and the ROI, trajectories and confinement of the detected AMPARs. Figure 1E shows an histogram of the different diffusion coefficients of detected AMPARs. This analysis reveals that compared to basal condition (grey), Wnt5a treatment (red) shifts the curve to the left, into less mobile coefficients. This proofs that there is a decrease in the mobile population and an increase in the immobile population of AMPARs after Wnt5a treatment. The percentage of receptors that have a diffusion coefficient (LogD) equal to -5; meaning that the diffusion coefficient of that population of receptors is too slow to be detected therefore, is considered to be completely immobile. In

this case, Wnt5a seem to increase the completely immobile population of GluA1-containing AMPARs, but shows no statistically significant differences (Figure 1F). Nonethelesss, compared to 15 min after Wnt5a, there is a time-dependence on the effect (Supplementary Figure 1B). The ratio between mobile/immobile, gives away the mobility of the entire population of AMPARs. Figure 1G evidences a significant decrease in the mobility after Wnt5a treatment. Again, this effect is stronger at 30 min than at 15 min (Supplementary Figure 1C). MSD expresses a measurement for confinement of single particles. In this case, we see a decrease in the MSD values after Wnt5a treatment, compared to basal MSD (Figure 1H). This means, that AMPARs are more confined, covering less distance per unit of time, due to Wnt5a treatment. Interestingly, when Wnt5a is boiled (10 min at 95°C) and then applied to neurons, the previously observed shift to the left after Wnt5a is lost (Figure 1I), no difference in completely immobility (Figure 1J) or mobility (Figure 1K). This proofs that the effect of the native functional Wnt5a protein is responsible for the observed changes in AMPARs dynamic.

Finally, it has been reported that the activation of CaMKII, one of the main downstream effectors of Wnt5a, causes phosphorylation of TARP-γ2 (Stargazin), promoting the interaction between PSD95 and AMPARs leading to receptor stabilization in synaptic sites (Opazo and Choquet, 2011; Opazo et al., 2010). Therefore, we performed the same experiment using a CaMKII inhibitor, KN93. Interestingly, it seems that the stabilization of AMPARs through Wnt5a is independent on the activity of CaMKII, because we continue to see the shift of the curve (Figure 1L). Also, the use of KN93 does not affect the completely immobile % of AMPARs due to Wnt5a treatment (Figure 1M), similarly to what is shown in Figure 1F. The independence on CaMKII action is clearly seen in the ratio mobility where there is a clear tendency towards immobilization between basal KN93 and KN93 + Wnt5a ($P=0.08$) and no difference between Wnt5a (same from 1G) and KN93 + Wnt5a ($P=0.66$) (Figure 1N). In order to test the possibility of KN93 having an effects on its own, control experiments comparing basal mobility with basal mobility after KN93 pre-treatment were performed, and no effect was detected (Figure 1N). Overall, we argue that although CaMKII may be a part of the effects of Wnt5a, its activity is not necessary for the immobilization effect over AMPARs, caused by Wnt5a. In conclusion, Wnt5a stabilizes GluA1-containing AMPARs in a CaMKII- independent manner.

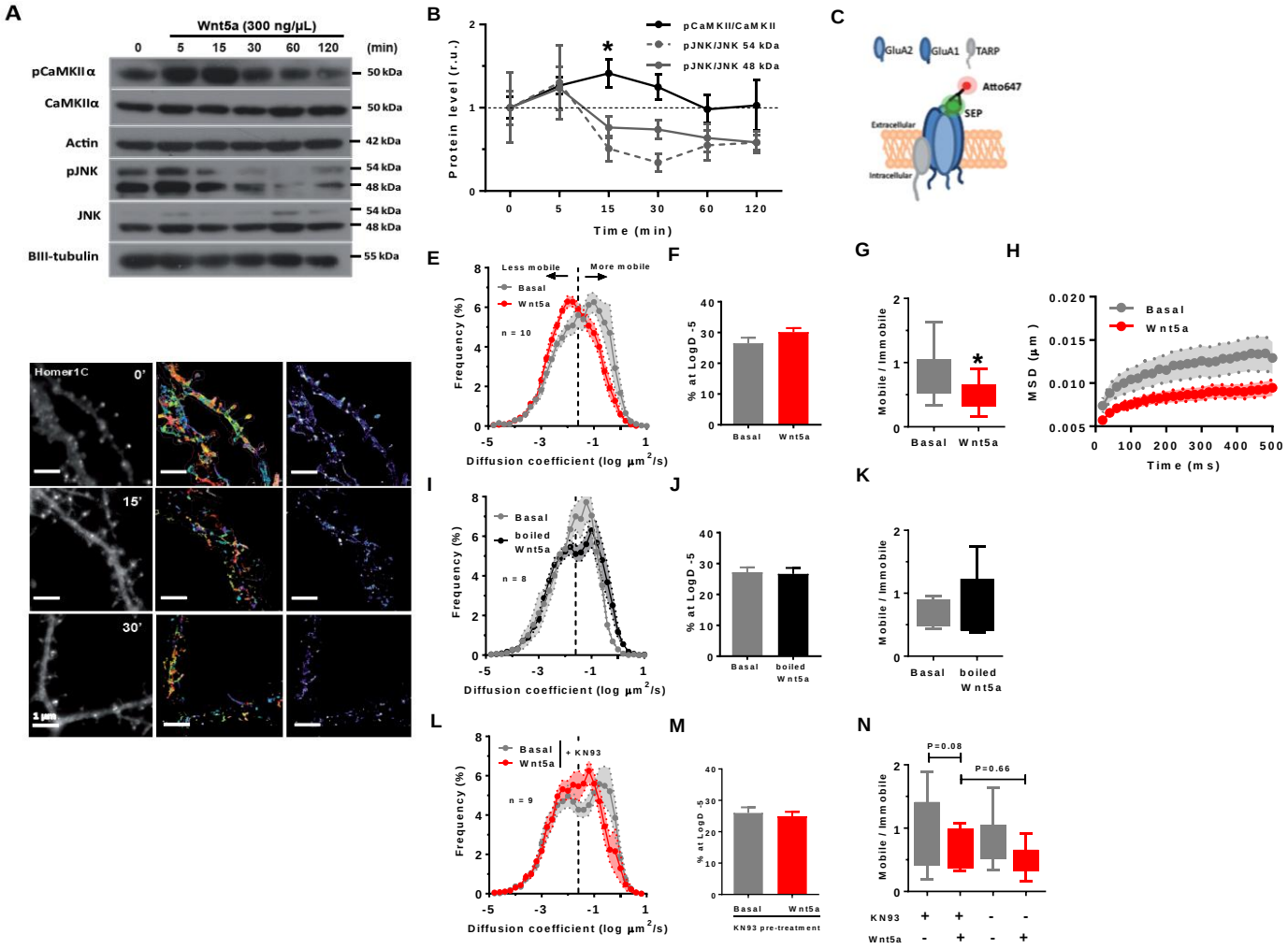


Figure 1. Activation of non-canonical Wnt signaling immobilizes GluA1-containing AMPARs in hippocampal neurons. Detection of overexpressed GluA1 on hippocampal neurons before/after Wnt5a treatment. **A)** Wnt5a activates non-canonical Wnt signaling, as seen by increased phosphorylation of JNK and CaMKII on hippocampal neurons. **B)** Quantification of pCaMKII and pJNK (54 and 48 kDa), in each case proteins are standardized against its corresponding total protein. **C)** Simplified model of the labeling method for detection of GluA1-SEP overexpressed protein on AMPARs, anti-SEP-Atto647 was used to track GluA1 subunits. **D)** GFP image to detect Homer1C:GFP, second column shows trajectories and third column, the confinement for GluA1 under basal conditions and after 15 and 30 min of exposure to Wnt5a. **E)** Histogram shows diffusion coefficients of GluA1-containing AMPARs before (basal, grey) and after treatment with Wnt5a (red). Vertical dotted line separates immobile and mobility s. **F)** Completely immobility , corresponding to the frequency of particles with a diffusion

coefficient equal to -5. % of completely immobile AMPARs for basal and 30 min of Wnt5a treatment. **G)** Mobility for basal and after 30 min of Wnt5a, according to E). **H)** Mean Square Displacement (MSD) determines the confinement of the particles in basal and Wnt5a conditions. **I)** Histogram of diffusion coefficients for basal versus 30 min boiled (denatured) Wnt5a. **J)** % of completely immobile receptors under basal and 30 min boiled Wnt5a. **K)** mobility according to I). **L**) Histogram of diffusion coefficient of KN93 alone (grey) and KN93+Wnt5a (red). **M)** % completely immobile AMPARs under basal (KN93 alone) and KN93+Wnt5a treatments. **N)** Mobility according to I). In each case, a minimum of 5 independent experiments were performed, n represents the total amount of cells registered. Unpaired t-test. P*<0.05.

5.2. Non-canonical activation of Wnt signaling stabilizes endogenous GluA2-containing AMPARs.

Considering the immobilizing effect of Wnt5a on overexpressed GluA1-containing AMPARs, we wanted to test if similar effects are seen on endogenous GluA2-containing AMPARs. To do so, we used an antibody able to detect endogenous GluA2 subunits on living hippocampal neurons. As before, the epitope is located on the N-terminal domain of GluA2, allowing live tracking (Figure 2A). Since GluA2 is the most abundant AMPARs subunit in the hippocampus (Schwenk et al., 2014), this experiment not only complements our previous finding but is also a better representation on the effects of Wnt5a over the bulk population of receptors. To test if this effect is ubiquitous for other Wnt ligands, we also used Wnt7a. This ligand has been described to activate the Wnt canonical pathway in hippocampal neurons (Cerpa et al., 2008b; Davis et al., 2008). We therefore observe a parallel between non-canonical (300 ng/mL Wnt5a) and canonical (300 ng/mL Wnt7a) effects.

In this set of experiments, as for the rest of the thesis, paired acquisitions were obtained, meaning that in each case, a direct comparison can be made because a single dendrite was imaged before/after treatment.

Similar to the results seen before for GluA1 tracking (Figure 1), we observe shorter and more confined trajectories of AMPARs after 30 min of exposure to Wnt5a (Figure 2B). On the contrary, there is no effect between basal mobility of AMPARs and 30 min exposure to Wnt7a

(Figure 2C). Quantification of the diffusion coefficients show a clear change in the curve from basal (grey) to Wnt5a (red) condition (Figure 2D). Notably, the completely immobile receptors (LogD equals -5) frequency is significantly increased after Wnt5a treatment, while no difference is seen after Wnt7a treatment (Figure 2E). In fact, due to Wnt5a there is a ~7% increase in the completely immobile AMPARs, while there is virtually no difference caused by Wnt7a (Figure 2E). Analyzes of mobility shows a significant decrease after Wnt5a treatment and again, no difference is seen after Wnt7a treatment (Figure 2F). Here, the effect is tracked on the same ROI (neurite) before and after exposure to Wnt5a, represented as points connected by a line (Figure 2E). Cell by cell analysis shows that ~65% (11 out of 17) of all neurons treated with Wnt5a decreased their mobility while only ~31% (4 out of 13) of all neurons treated with Wnt7a showed a decrease in the ratio. The amount of trajectories detected did not change between different time points (Figure 2G). This is an important point to discard any artifactual effects, like antibody feeding, this will be further addressed in the discussion section. Finally, we see a small non-significant decrease in MSD due to Wnt5a, while again, there is no difference what so ever in MSD after Wnt7a treatment (Figure 2H). Altogether, Wnt5a causes a significant decrease in the mobility of AMPARs, while under the same conditions, Wnt7a has no effect.

To make sure the lack of effect given by Wnt7a is consistent, we doubled the concentration of Wnt7a used (to 600 ng/mL). We observed the same effect of Wnt7a, for both low (300 ng/mL) and high (600 ng/mL) concentration. Therefore, we can safely conclude that there is no effect of Wnt7a over AMPARs mobilization. Results shown are pooled together.

It is clear that Wnt5a generates immobilization of AMPARs, while Wnt7a, a canonical ligand on hippocampal neurons, has no effect on the mobility of AMPARs. Overall, this data proofs that the activation of the non-canonical Wnt signaling through Wnt5a ligand causes immobilization of AMPARs in hippocampal neurons.

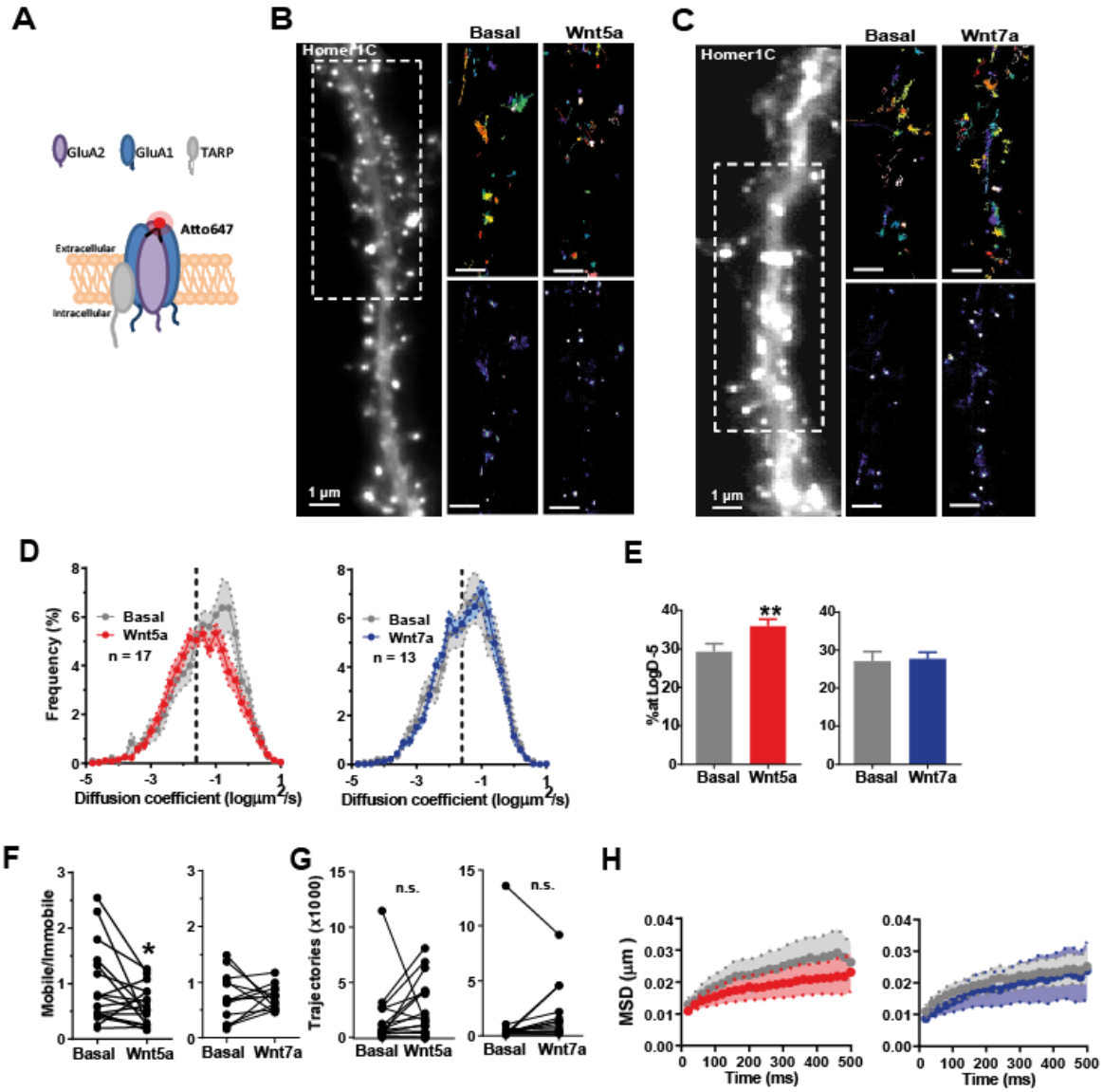


Figure 2. Activation of non-canonical Wnt signaling immobilizes endogenous GluA2-containing AMPARs. Detection of endogenous GluA2 on hippocampal neurons before/after Wnt5a or Wnt7a treatment. **A)** Simplified model of the labeling method for detection of endogenous GluA2-containing AMPARs. **B)** GFP image to detect Homer1C::eGFP, second column shows trajectories and third column, confinement for endogenous GluA2 under basal conditions and after 30 min of exposure to Wnt5a. **C)** GFP image to detect Homer1C:GFP, second column shows trajectories and third column, confinement for endogenous GluA2 under basal conditions and after 30 min of exposure to Wnt7a. **D)** Histograms shows diffusion coefficient of endogenous GluA2-containing AMPARs under basal (grey) or after 30 min of treatment with Wnt5a (red) or Wnt7a (blue). **E)** Completely immobility (corresponding to the frequency of particles with a diffusion coefficient equal to -5) for Wnt5a (red) or Wnt7a (blue). **F)** mobility of AMPARs after 30 min od treatment with of Wnt5a and Wnt7a, each compared with its

basal mobility according to to D). **G)** Amount of trajectories detected (in thousands), in each experiment, no significant differences. **H)** MSD for Wnt5a (red) or Wnt7a (blue) treated neurons. In every case, in grey is shown the basal dynamique of receptors. In each case, a minimum of 5 independent experiments were performed, n represents the total amount of cells registered. Paired t-test. n.s;. non significative, P*<0.05.

5.3. The immobilization effect is dependent on the binding of Wnt5a to its receptor. In order to test how specific is the effect of AMPARs immobilization to Wnt5a action, we used a tool to interrupt the binding of Wnt5a to Frizzled receptors. Soluble Frizzled Related Protein 2 (sFRP2) is an endogenous protein with homology to the N-terminal ligand-binding domain of Frizzled receptors, we used recombinant sFRP2. By co-incubating Wnt5a (300 ng/mL) with an excess of sFRP2 (1 µg/mL) for 30 min at 37°C in agitation, a complex (Wnt5a + sFRP2) is formed, blocking the Frizzled binding site on the Wnt5a ligand, preventing Wnt5a to bind to Frizzled. Like this, we prevent the formation of the Wnt5a-Frizzled complex and block signal initiation. Because sFRP2 is used in excess (>3 times more sFRP2 than Wnt5a), there is no or little free-Wnt5a to interact with Frizzled receptors. As a proof of this we ran a non-denaturing gel for the exact amount of Wnt5a used for experiments (lane 1) and for Wnt5a + sFRP2 complex (lane 2) and immunoblot against Wnt5a (Figure 3A). Recombinant Wnt5a is labeled in at ~41 kDa, while on lane 2 there is very weak labeling for Wnt5a at ~41 kDa, suggesting there is little amount of free-Wnt5a available, when is complexed with sFRP2. Also, there is labeling around ~80 kDa (arrow) which is consistent with the predicted molecular weight for the complex Wnt5a + sFRP2 (~41 kDa + ~31.6 kDa) on lane 2. We estimate there is less than a third of free-Wnt5a available on lane 2 compared to lane 1, and therefore if any free Wnt5a is available in our experiments, it is probably at non-functional low concentrations. This is a rough estimation and further experiments should be performed to properly establish the efficacy of complex formation. Wnt5a + sFRP2 complex was formed as described in Materials and Methods.

Representative figures of Homer1C::eGFP, trajectories and confinement are shown (Figure 3B). Following the same experimental design as before, it is evident that when the Wnt5a + sFRP2 complex is added to live hippocampal neurons, there is no change on the dynamics of

AMPARs. As seen in the histogram (Figure 3C) and in the completely immobility (Figure 3D), there is no difference on the diffusion coefficients between basal and Wnt5a + sFRP2. Also, the mobility remains unchanged when compared to basal (Figure 3E). In this case, there is a significant difference on the amount of trajectories detected (Figure 3F). This was not expected and further experiments should be performed to corroborate the effect.

Depending on the cellular context, sFRP2 can have different effects on Wnt signaling or even other signaling pathways (i.e., BMP) (Kongkham et al., 2010; Mii and Taira, 2011; Tokuda et al., 2014). Therefore, it is necessary to test if by adding sFRP2 alone, the mobility of AMPARs is affected, or not. Since we are adding an excess of sFRP2 we needed to test if the extra sFRP2, not bound to Wnt5a, could be exerting any unwanted effects (i.e., complexing with endogenous Wnt5a, interacting with other Wnt ligands, binding to other molecules, etc). To test this, the same concentration of sFRP2 (1 µg/mL) used to form the complex, was directly added. Results show there is no effect on the mobility of AMPARs when comparing basal with 30 min of incubation with sFRP2 (Figure 3G-H), on the completely immobility (Figure 3I) or in the mobility (Figure 3J). To further proof the point, a higher concentration (2 µg/mL) of sFRP2 was also tested and still failed to cause an effect on AMPARs dynamics. Results show both concentrations pooled together. No differences on the amount of trajectories detected (3K).

We can extract three main conclusions: 1) the immobilization effect reported before (Figure 2) is dependent on the binding of Wnt5a to its Frizzled receptor, 2) the use of sFRP2 itself does not alter the dynamics of AMPARs and 3) the effect on immobilization is not caused by an artifactual effect like antibody feeding, which was also corroborated by the fact that Wnt7a does not change AMPARs dynamics (Figure 2). This will be further analyzed on the discussion section. Overall, this data corroborates the fact that Wnt5a induces stabilization of GluA1 and GluA2-containig AMPARs.

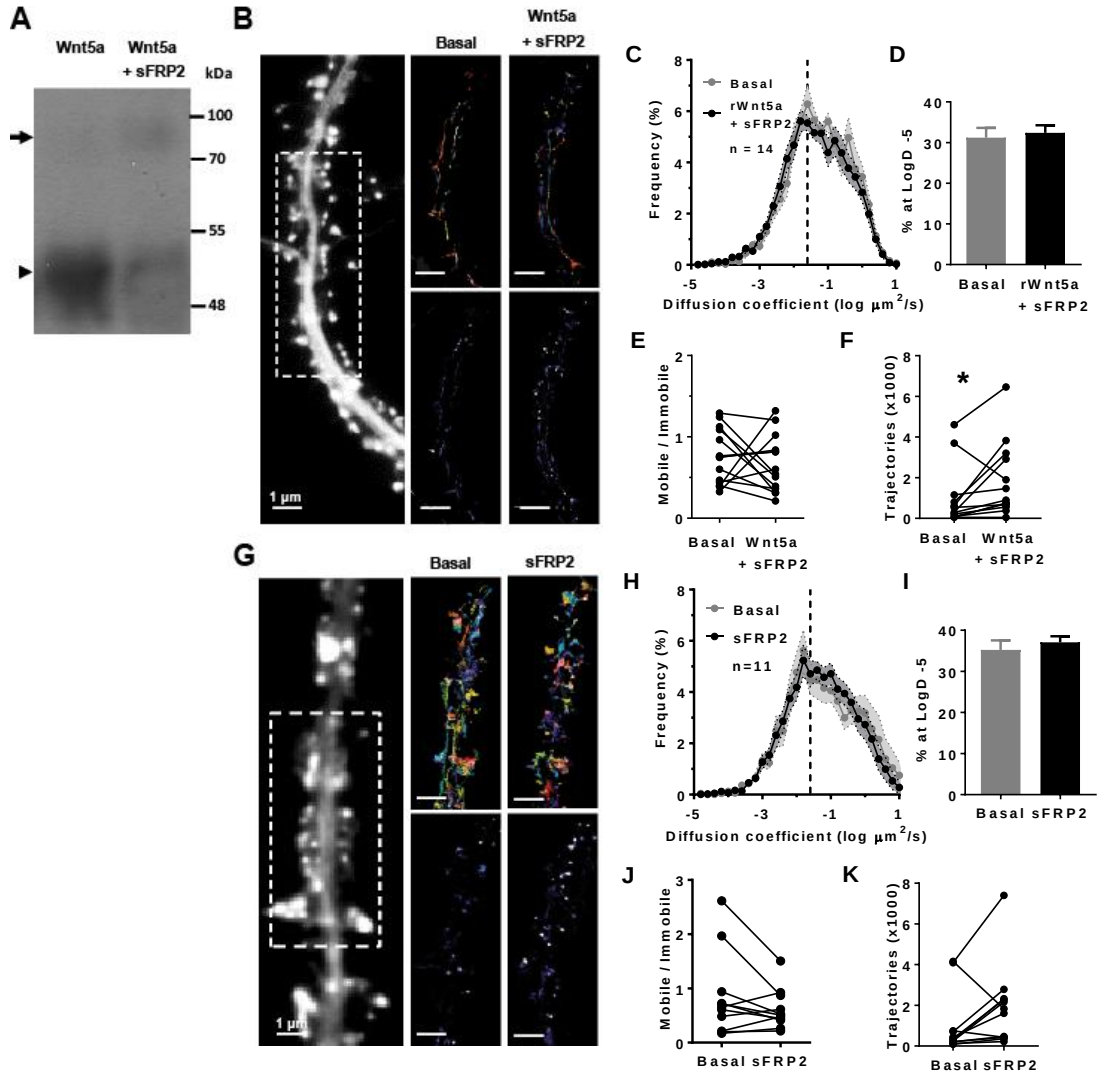


Figure 3. The immobilization of AMPARs due to Wnt5a is dependent on the binding of Wnt5a to a Frizzled receptor. Detection of endogenous GluA2-containing AMPARs in hippocampal neurons treated with Wnt5a + sFRP2 complex or sFRP2 alone. **A)** Immunoblot against Wnt5a for Wnt5a alone and the Wnt5a + sFRP2 complex, arrow indicates labeling at approximately 80 kDa (estimated weight for the complex) and arrowhead shows labeling for Wnt5a at approximately 40 kDa. **B)** Representative images for Homer::eGFP, trajectories and confinement for GluA2 endogenous receptors of neurons under basal and Wnt5a + sFRP2. **C)** Frequency distribution of neurons under basal condition (grey) and treated with the Wnt5a+ sFRP2 complex (black). **D)** Completely immobility (corresponding to the frequency of particles with a diffusion coefficient equal to -5) for basal (grey) or after 30 min of treatment with Wnt5a+sFRP2 complex (black). **E)** mobility of the frequency distribution according to C). **F)** Amount of detections (in thousands), in each experiment. **G)** Representative images for

Homer1C::eGFP, trajectories and confinement for GluA2 under basal conditions and after 30 min of exposure to sFRP2 alone. **H)** Frequency distribution of neurons under basal (grey) and sFRP2 (black). **I)** Completely immobility for basal (grey) or after 30 min of treatment with sFRP2 alone (black). **J)** mobility according to G). **K)** Amount of particle detections (in thousands), in each experiment. In each case, a minimum of three independent experiments were performed, n represents the total amount of cells registered. Paired t-test. P*<0.05.

5.4. Wnt5a-induced stabilization of AMPARs occurs in synaptic and extrasynaptic sites.

Since Wnt5a causes a significant decrease in AMPARs mobility (Figure 2). We want to establish where in the neuron is this immobilization occurring. In that line, we dissected the previous analysis by separating synaptic and extrasynaptic trajectories, corresponding to the mobility of endogenous AMPARs found in synaptic or extrasynaptic sites. To differentiate synaptic trajectories, ROIs surrounding Homer1C::eGFP labeling were done. Everything not compressed into synaptic sites (Homer1C::eGFP) was considered to be extrasynaptic. Since uPaint technique gives us a spatial resolution of 0.1 µm and on average a dendritic spine is 0.5-2 µm long (Morgan Sheng, 2001), we can easily differentiate between dendrite and spines.

Three different dendritic spines, of the same dendrite are shown under basal conditions and after 30 min of treatment with Wnt5a, trajectories shown on top and confinement on bottom (Figure 4A). Spine analysis of the diffusion coefficient shows that Wnt5 causes an important shift to the left compared to basal conditions, indicating immobilization of AMPARs (Figure 4B). Analysis of the completely immobility , reveals no significant difference in the immobilization of AMPARs, compared to basal conditions (P = 0.48) (Figure 4C). However, there is a significant difference in mobility analysis between both groups, cell to cell analysis shows that 80% (8/10) of neurons treated with Wnt5a had a decrease in the mobility of AMPARs (Figure 4D). The number of trajectories showed no significant difference (Figure 4E). Although there is a tendency towards lower valuers of MSD after Wnt5a treatment, no clear difference is found on the MSD curves (Figure 4F).

The extrasynaptic analysis also shows immobilization of AMPARs after 30 min of treatment with Wnt5a (Figure 4G). Wnt5a causes a decrease in the diffusion coefficient of AMPARs (Figure 4H). We observe a significant increase in the amount of AMPARs considered to be completely immobile in Wnt5a-treated neurons (Figure 4I). Also, there is a strong and statistically significant decrease on the mobility after treatment with Wnt5a, cell to cell analysis shows that ~83% (10/12) of neurons treated with Wnt5a decreased the mobility of their AMPARs (Figure 4J). The number of trajectories showed no significant difference (Figure 4K). Interestingly, Wnt5a generates a significant decrease on the MSD curve, which indicates the increased confinement of AMPARs (Figure 4L). The difference on MSD results for synaptic and extrasynaptic analysis might be an indicative of a preponderant role of Wnt5a on extrasynaptic AMPARs immobilization. This will be further analyzed in the discussion section.

We can conclude that Wnt5a promotes the immobilization of AMPARs in synaptic and extrasynaptic sites. Since the localization of AMPARs in synaptic and extrasynaptic sites is differentially regulated, it implies that the effect of Wnt5a is not limited to one action mechanism and several intermediaries might be involved.

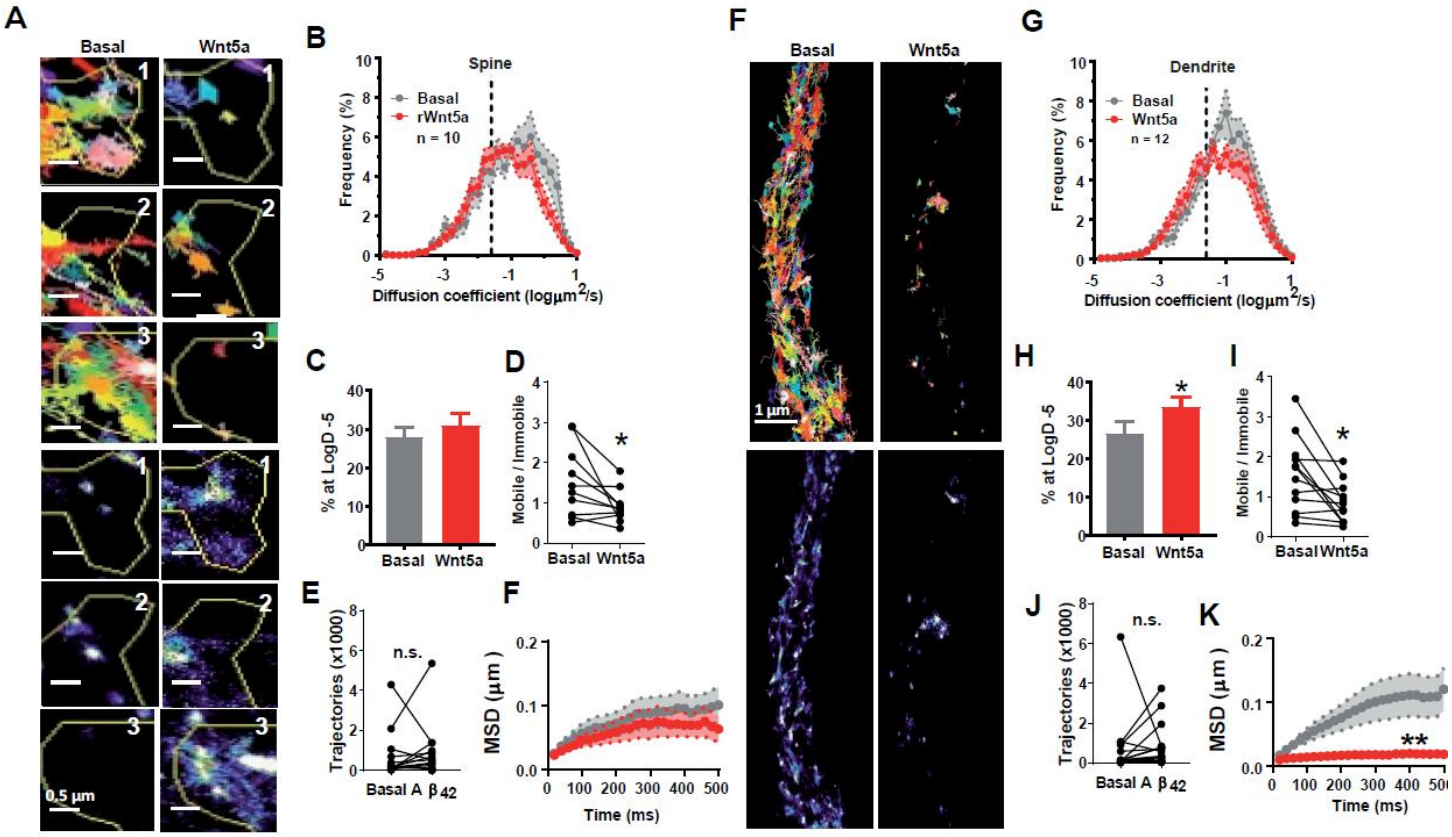


Figure 4. Non-canonical activation of Wnt signaling causes de immobilization of AMPARs at synaptic and extrasynaptic sites. Synaptic/extrasynaptic analysis of endogenous GluA2 on hippocampal neurons before/after rWnt5a treatment. **A)** Trajectories and confinement of endogenous GluA2-containing AMPARs of three different spines (one per line) under basal conditions and after 30 min of treatment with Wnt5a, numbers indicate same spines. **B)** Histogram shows diffusion coefficient of endogenous GluA2-containing AMPARs in dendritic spines. **C)** Completely immobility for synaptic endogenous GluA2-containing AMPARs. **D)** Quantification of mobility according to B). **E)** Amount of particle detection (in thousands), in each experiment. **F)** Mean Square Displacement (MSD) for basal (grey) for synaptic analysis. **G)** Trajectories and confinement of endogenous GluA2-containing AMPARs in dendrite, under basal conditions and after 30 min of treatment with Wnt5a. **H)** Histogram shows diffusion coefficient of GluA2 for extrasynaptic analysis. **I)** Completely immobility for extrasynaptic analysis. **J)** Quantification of mobility according to G). **K)** Amount of particle detection (in thousands), in each experiment. **L)** Mean Square Displacement (MSD) for extrasynaptic

analysis. In each case, a minimum of five independent experiments were performed, n represents the total amount of cells registered. Paired t-test. n.s.; non significative P*<0.05, P*<0.01.

5.5. Wnt5a increases AMPARs-PSD95 co-localization in dendritic spines.

Following uPAINT experiments, coverslips were removed from the microscope chamber and neurons were live stained for GluA2. After fixation and permeabilization, cells were also labeled with PSD95 and VAMP2 antibodies, dyed for DAPI and observed under a DM5000 epifluorescence microscope. This way, performing parallel experiments on the same coverslips for uPaint and fluorescence microscopy. Co-localization analysis on GluA2-PSD95 was performed on the complete dendrite (including spines) and spines alone. As shown, control, Wnt5a and Wnt5a + sFRP2 treated neurons were evaluated, in the merge (GluA2/PSD95) image circles show the spines evaluated for co-localization and representative images are shown aside (Figure 5A).

In the dendritic analysis, we observe no statistically significant difference in the co-localization of GluA2-containing AMPARs and PSD95 (data not shown). But, when spines are analyzed independently of the dendritic shaft, we observe that Wnt5a causes a small but statistically significant increase (13 ± 0.01%) of GluA2-PSD95 co-localization on dendritic spines, as seen by Mander's 1 coefficient (M1) (Figure 5B, left). Also, there is a similar change on Mander's 2 coefficient (M2) shows a similar change on co-localization of PSD95-GluA2 (~15% ± 0.03%) (Figure 5B, middle). This suggests that after 30 min of exposure to Wnt5a, there is an increase of AMPARs within dendritic spines, colocalizing with PSD95. For both coefficients, there is no increase in co-localization when neurons are treated with the Wnt5a + sFRP2 complex (as used before). This, not only supports the effects of Wnt5a, but also correlates with our observations on uPaint experiments (Figure 4A-E). Interestingly, we also observed a difference between the amount of co-localization clusters (i.e. times in which both proteins were co-localized, independent on the level of co-localization) occurring between conditions. Therefore, we quantified the percentage of clusters that are both PSD95 immunoreactive and GluA2 immunoreactive ($PSD95^{+}/GluA2^{+}$), independent of the level of co-localization (which is shown

with Mander's coefficients). Meaning, those PSD95 clusters that have GluA2-containing AMPARs. To our surprise, Wnt5a significantly increases the percentage of clusters that contain both proteins, when compared to basal conditions (Figure 5B, right). Again, we observe a significant decrease to basal levels when neurons are treated with the Wnt5a + sFRP2 complex (Figure 5B, right). Through this analysis, it is impossible to examine if those "new" AMPARs into PSD95 are functional AMPARs, or not, so we cannot refer to this difference as an increase of active synapses, but we believe that is a strong indication of that, this point will be addressed in more detail on the discussion section. To further study the interaction between GluA2-PSD95, we performed co-immunoprecipitation assays from lysates of hippocampal neurons treated with Wnt5a and/or sFRP2 and Wnt7a ligand. Pull-down of PSD95 and detection of GluA2 shows that after 30 min of incubation with Wnt5a there is an increase association of GluA2 with PSD95 of around 20% (Figure 5C). Although this increase fails to be statistically significant it coincides with our previous observation of AMPARs increased co-localization with PSD95, in dendritic spines. Importantly, the total amount of PSD95 and GluA2 in total hippocampal neuron lysates, are not significantly changed by any treatment (Figure 5 D). This corroborates the idea that the changes observed are a product of redistribution of existing AMPARs, and not a change in their expression level. It is worth mentioning, that this type of analysis is not adequate to measure interaction of GluA2 with extrasynaptic proteins. Therefore, we cannot conclude effects on extrasynaptic site. We can conclude that activation of the non-canonical Wnt signaling pathway through Wnt5a causes an increase of AMPARs at synaptic sites.

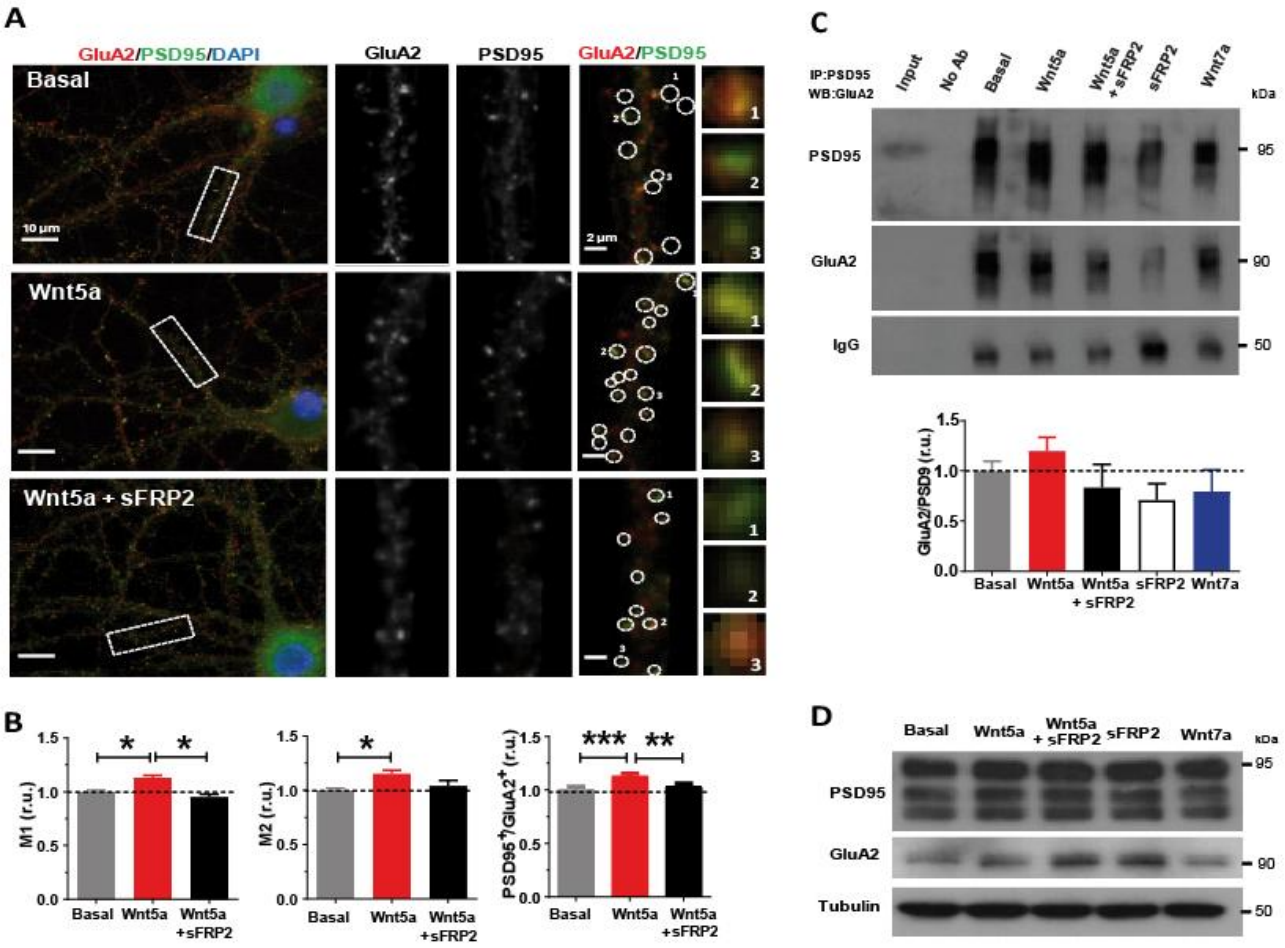


Figure 5. Wnt5a increases co-localization of GluA2 and PSD95 on dendritic spines, without affecting total levels of protein. Following uPAINT experiments, hippocampal neurons were live-stained for GluA2, fixed, permeabilized and labeled for PSD95 and images in an DM-5000 epifluorescence microscope. **A)** Live staining of GluA2, following fixation and permeabilization, neurons were labeled with PSD95 and DAPI. Dendrites (dashed rectangle) and spines were selected (dashed circles) according to the labeling for PSD95. Co-localization of GluA2 and PSD95 on spines was assessed. Magnification of the representative spines are shown, three per condition. **B)** Left graph shows the quantification of Mander's 1 (M1, GluA2 over PSD95), middle graph shows quantification of Mander's 2 (M2, PSD95 over GluA2) coefficient and right graph shows the % of clusters positive for PSD95 and for GluA2, meaning postsynaptic densities containing AMPARs. **C)** After treatment and lysis of neurons, PSD95 was pulled-down and GluA2 detected by immunoblot. Below it is shown the pixel density quantification for each treatment, normalized to PSD95 and compared to basal levels. **D)**

Immunoblot analysis of total lysates for PSD95, GluA2 and tubulin. A minimum of three independent experiments were done, with between 7-10 neurons per condition.Two-way ANOVA and Bonferronoi post-test. n.s. non significative, P*<0.05, P**<0.01, P***<0.001.

5.6. Wnt5a does not affect the clusters of PSD95 or GluA2.

In order to corroborate previous findings, we wanted to test if our treatment causes the reported increase of PSD95 clustering, after Wnt5a treatment (Farías et al., 2009). To do so, we followed the same approach as described above. Following uPAINT experiments, under basal conditions or with treatments for 30 min of Wnt5a or Wnt5a + sFRP2, cells were live-stained for GluA2, fixed, permeabilized and labeled for PSD95 (Figure 6A). Cluster analysis shows that there is no significant differences for any of the parameters analyzed. That is, after 30 min of treatment with Wnt5a, the amount of clusters per 100 µm of dendrite was not affected either for PSD95 (Figure 6B) or GluA2 (Figure 6E), the fluorescence intensity of the clusters was not significantly affected either (Figure 6C-F), which it is usually considered to be an indicator that there are no changes in the total amount of proteins present in the clusters. Therefore, a 30 min treatment of Wnt5a would not be affecting total protein levels of PSD95 or GluA2. Finally, we see no significant changes on the size of the clusters for PSD95 (Figure 6D). For GluA2, the amount of clusters remains the same but show a tendency towards a decrease in their size (Figure 6G). This data does not corroborate previous findings and the possible explanations will be addressed on the discussion section.

Importantly, treatment with Wnt5a seem to affect mainly post-synaptic architecture, which is consistent with the fact that does not affect paired-pulse ratio, therefore suggesting that the pre-synaptical terminal is not affected (Cerpa et al., 2010a). This is consistent with our findings that the amount of synaptic contacts is not altered by treatments with Wnt5a (Supplementary Figure 2).

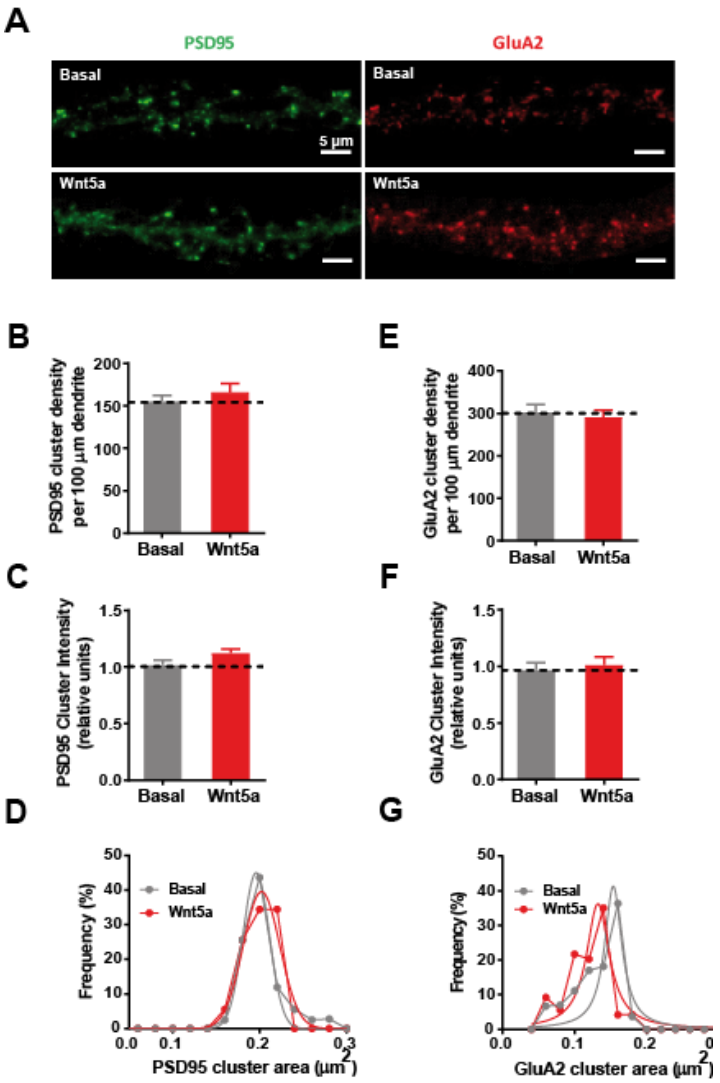


Figure 6. Wnt5a does not affect the amount of clusters of PSD95 or GluA2. Following uPAINT experiments, hippocampal neurons were live-stained for GluA2, fixed, permeabilized and labeled for PSD95 and images in an DM-5000 epifluorescence microscope. **A)** Immunofluorescence of PSD95 (green) and GluA2 (red) for neurons under basal condition and after 30 min Wnt5a treatment. **B)** Average PSD95 cluster density, normalized to 100 µm of dendrite. **C)** Average fluorescence intensity of PSD95 clusters. **D)** Histogram of PSD95 cluster size shows a small, shift to the right. **E)** Average GluA2 cluster density, normalized to 100 µm of dendrite. **F)** Average fluorescence intensity of GluA2 clusters. **G)** Histogram shows a small decrease in GluA2 cluster size. A minimum of three independent experiments were done, with between 7-10 neurons per condition.

5.7. oAβ42 cause a decrease in the mobility of synaptic AMPARs.

In the last years, accumulated evidence supports the idea that oAβ are the main and primary effectors on the synaptotoxicity seen in early stages of AD (Jin and Selkoe, 2015; Shankar et al., 2007, 2008; Walsh et al., 2002). On recent years, it seems that the evidence has gone further to propose that this could be occurring even before the appearance of neurofibrillary tangles (Miller et al., 2014). One of the mechanisms proposed on the action of oAβ42 over synaptic function involves the endocytosis of AMPARs (Hsieh et al., 2006; Miñano-Molina et al., 2011) and the consequent decrease of AMPAR currents and overall failure on glutamatergic activity.

We used an $A\beta_{42}$ aggregation protocol that enriches soluble oligomeric species, as shown by electron microscopy (Figure 7A, top), tris-tricine separation and detection with 6E10 antibody (Figure 7A, bottom). Neurotoxicity was tested by incubating 5 μM $A\beta_{42}$, for 24h at 37°C, in incubator (Supplementary Figure 3). Confirming, we are using a preparation mainly composed of $A\beta_{42}$ oligomers. First, we used 1 μM oAβ42 and evaluated the effects on AMPARs dynamic (Figure 7B, C, D). This concentration has been reported to cause endocytosis of AMPARs and a decrease in AMPAR currents, when applied for an hours-to-days period (Gu et al., 2009). Our experimental design studies the effects on the short-term exposure (≤30 min) to $A\beta_{42}$ oligomers. Nonethelesss, since AMPARs dynamic has not been studied we wanted to established if there might be an earlier effect at this given concentration. Analysis of the global dynamic reveals no change between basal and after 30 min of treatment with 1 μM $A\beta_{42}$ oligomers. No changes were detected on the dynamic of AMPARs, relative frequency of completely immobile receptors, mobility or number of trajectories (Figure 7B). But, when we examined the synaptic dynamic of AMPARs, we see a small, but consistent, tendency towards immobilization of AMPARs, with no changes in the completely immobile receptors and an interesting tendency towards immobilization in the mobility (Figure 7C). The extrasynaptic analysis reveals no changes on AMPARs dynamic, completely immobile receptors or mobility (Figure 7D).

To verify the tendency seen before, we studied the effects using a higher concentration (5 μM) of oAβ42 (Figure 7E, F, G). This concentration has been reported to cause calcineurin-

dependent failures in the insertion of AMPARs to the cell membrane (Miñano-Molina et al., 2011). On the global analysis, we observe a strong immobilization of AMPARs (Figure 7E). Interestingly, this immobilization is not increasing the frequency of completely immobile AMPARs. Instead, it affects the mobility, therefore immobilizing receptors but not to a complete stop. Noteworthy, at the synaptic level we also observe a strong immobilization of AMPARs (Figure 7F), which confirms the tendency seen with 1 µM oAβ42 (Figure 7C) and shows the existence of a concentration-dependent effect. To our surprise, there is no effect whatsoever on the dynamic of AMPARs at extrasynaptic levels, either at the completely immobile receptors or mobility (Figure 7G). At this point, it is very important to emphasize that oAβ42 seem to be acting only at a synaptic level, this because neither at 1 or 5 µM there is an effect at extrasynaptic levels. This data agrees with recent publications showing that oAβ bind and cluster at excitatory synaptic sites (Sinnen et al., 2016). Another interesting observation is that already at 15 min the immobilization reaches a peak, since there is virtually no difference between 15 and 30 min exposure to oAβ42 (Supplementary Figure 4). This leads to the idea that oAβ42 are causing synaptic immobilization of AMPARs, which occurs rapidly and steadily at ≤15 min. In every experimental condition and analysis, the average number of detected trajectories remained unaltered.

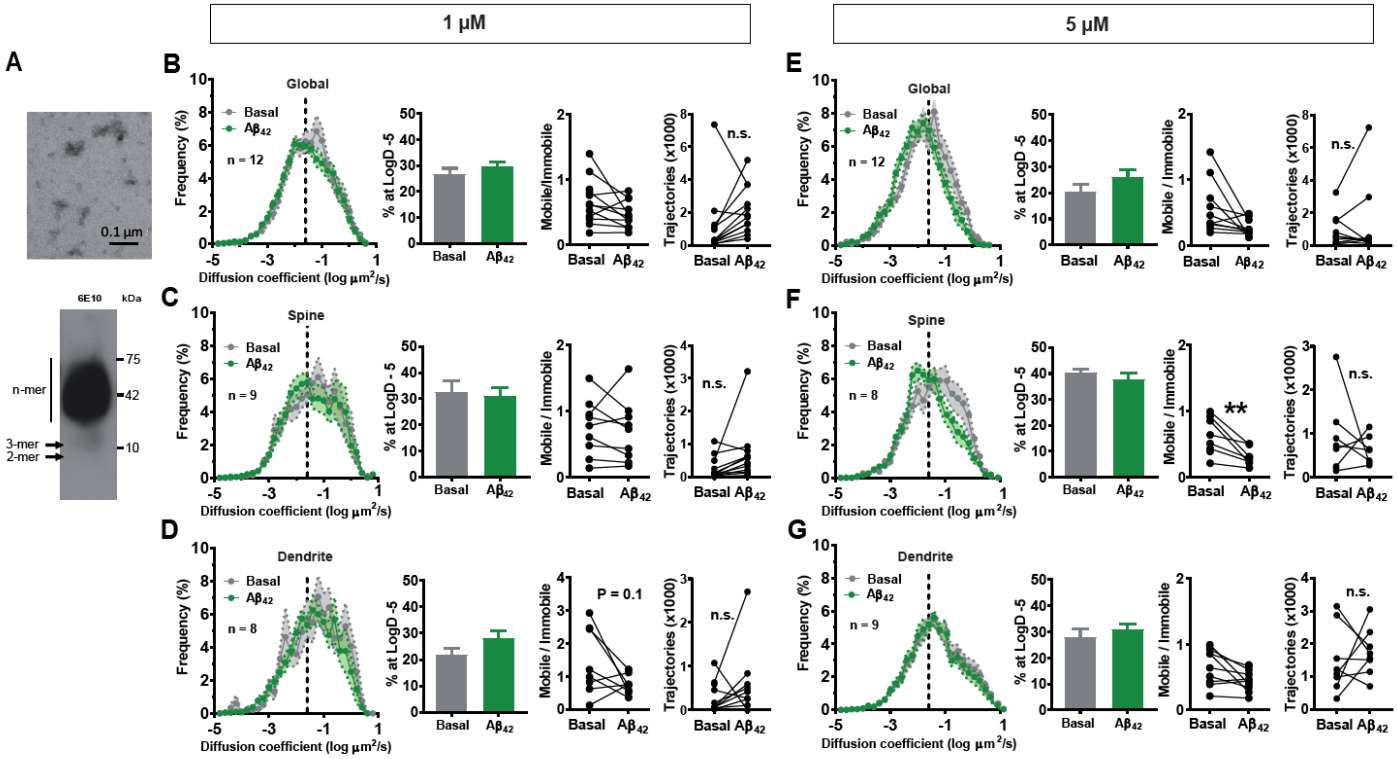


Figure 7. Short exposure to oAβ42 (5 µM) affects the dynamic of synaptic AMPARs. Hippocampal neurons were treated with 1 or 5 µM of oAβ42 and dynamic of AMPARs was measured at basal, 15 and 30 min of treatment. In each case, diffusion coefficient distribution, completely immobile %, mobility and amount of trajectories detected, are graphed. A) Top image shows electronic microscopy of $A\beta_{42}$ used, corroborating the presence of oligomeric forms and not fibrills. Bottom image shows immunoblot against 6E10, an epitope for Aβ, labeling shows the preparation is enriched in oligomeric species between 30-72 kDa (n-mer) and not higher or dimers (2-mer) or trimers (3-mer). In every case, from left to right is: Histogram for coefficient distribution, completely immobility , mobility and number of trajectories detected in each condition, for: B) 1 µM of $A\beta_{42}$ global analysis C) 1 µM of $A\beta_{42}$ spine analysis, D) 1 µM of $A\beta_{42}$ dendritic analysis, E) 5 µM of $A\beta_{42}$ global analysis, F) 5 µM of $A\beta_{42}$ spine analysis and G) 5 µM of $A\beta_{42}$ dendritic analysis. n represents number of cells, a minimum of 3 independent experiments were performed, for each condition. Paired t-test. n.s. non significative, P*<0.05, P**<0.01.

5.8. Wnt5a prevents the effects caused by oAβ in AMPARs dynamics.

We have already established that Wnt5a causes stabilization of AMPARs at synaptic and extrasynaptic sites. This effect increases with time exposure to Wnt5a and reaches significance at 30 min of treatment. We also know, that 5 µM of oAβ42 immobilizes AMPARs, but only at a synaptic level and reaching a maximum at 15 min of treatment (no difference between 15 and 30 min of treatment). Since our main hypothesis is that the effects of Wnt5a against the sinaptotoxicity of oAβ42 is related to a change in the mobility of AMPARs. Therefore, and according to the time frames established above, we pre-treated neurons with Wnt5a for 15 min, after wish we added $A\beta_{42}$ oligomers, as presented in the scheme (Figure 8A). Like this, giving enough time for both molecules to act and allowing us to determine if Wnt5a prevents the effects of 5 µM $A\beta_{42}$ oligomers. Representative images of the same dendrite throughout the experiment progression, showing complete dendrite and three individual dendritic spines, for trajectories and confinement (Figure 8B). Global analysis (Figure 8C, left) shows that after 15 min of treatment with Wnt5a there is a shift from to the left, compared with basal conditions, indicating some degree of stabilization of AMPARs, as expected. Interestingly, after 15 min of co-treatment with oAβ42 there is no further stabilization of AMPARs. At the same time, analysis of the completely immobile (% at logD -5) shows no significant changes (Figure 8C, middle). Mobility denotes a clear tendency towards immobilization of AMPARs, in synchrony with what it is seen in the histogram (Figure 8C, right). Next, as done before, synaptic and extrasynaptic dynamic of AMPARs was examined. Histogram of synaptic analysis shows no important differences on the dynamic of AMPARs (Figure 8D, left). Similarly, no significant differences are found for the % at logD -5, meaning that there are is no change on the percentage of completely immobile receptors for Wnt5a (15 min) alone or Wnt5a co-treated with oAβ42 (Figure 8D, middle). Except for one neuron, there is no significant differences in the mobility of AMPARs dynamics (Figure 8D, right). Finally, extrasynaptic analysis was performed (Figure 8E). From the histogram (Figure 8E, left), it is clear there is a small shift to the left, probably caused by Wnt5a compared to basal conditions. Interestingly, after co-treatment with oAβ42 there is a shift to the left, indicating that AMPARs get stabilized in extrasynaptic sites (Figure 7E). In fact, % at logD -5 shows a tendency to increase due to Wnt5a + $A\beta_{42}$ co-treatment (Figure 7E). This correlates with a decrease in mobility for most of the neurons examined (Figure 7E).

Overall, this data indicates that after co-treatment of Wnt5a + oAβ42 there is stabilization of AMPARs only at extrasynaptic sites, not affecting synaptic dynamic of AMPARs. As seen before, Wnt5a stabilizes AMPARs at synaptic and extrasynaptic sites (Figure 4), while oAβ42 do it only at synaptic sites (Figure 7B). Therefore, we believe that Wnt5a can compensate for the synaptic effects of $A\beta_{42}$ oligomers. Individual application of Wnt5a and oAβ42 seem to have the same response in synaptic sites; stabilization of AMPARs. Since synaptic and extrasynaptic effect vary, we believe the mechanisms involved are different, this will be further address on the discussion section. In conclusion, Wnt5a prevents the synaptotoxic effects of oAβ42 on AMPARs dynamic and this would explain the synapto- and neuro-protective effects reported for Wnt5a against oAβ toxicity.

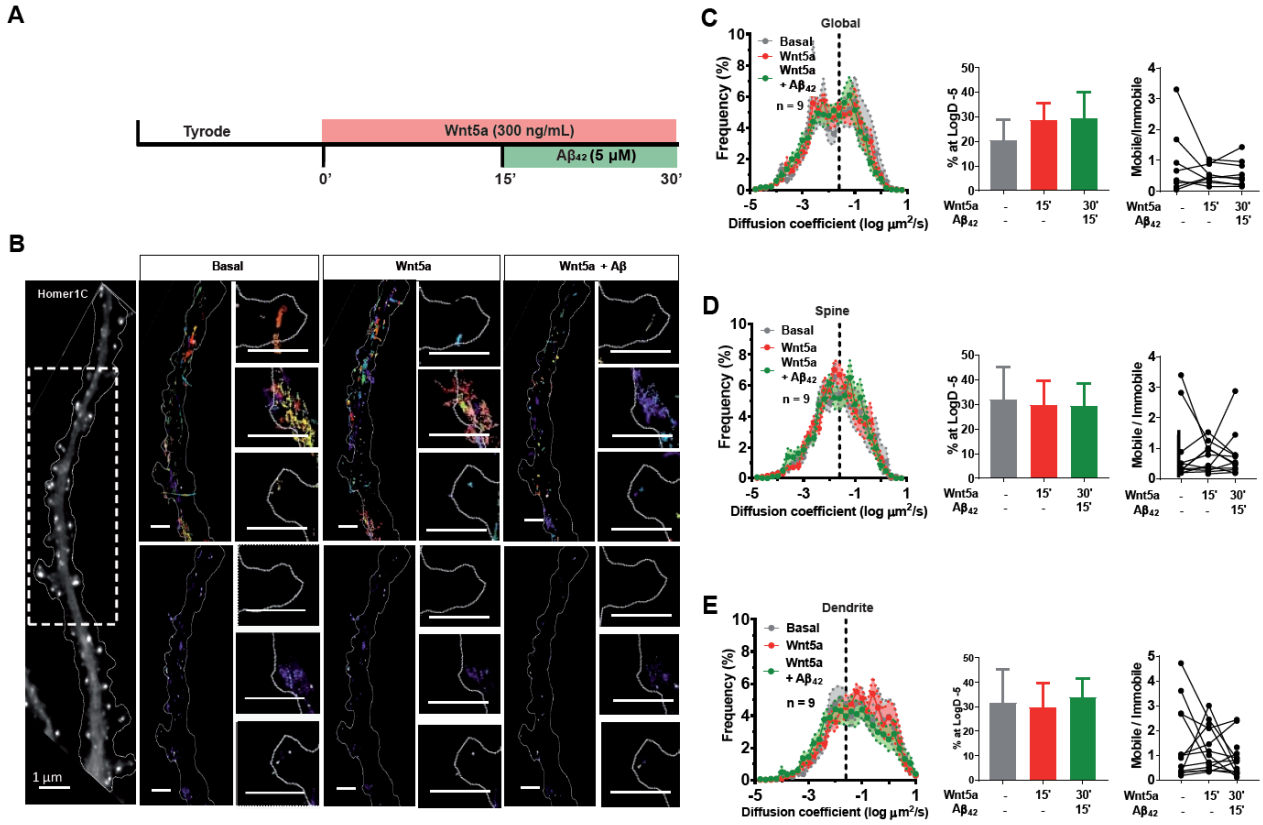


Figure 8. Wnt5a prevents the effects of oAβ42 on synaptic immobilization of AMPARs. Co-treatment of hippocampal neuron. **A)** Scheme showing the treatment of neurons, basal acquisition of AMPARs dynamic at time zero, addition of Wnt5a (300 ng/mL) with a first acquisition at 15 min, followed by addition of oAβ42 with a final acquisition at 30 min of Wnt5a exposure and 15 min of $A\beta_{42}$ oligomers. **B)** Representative images of the selected dendrite (Homer1c::eGFP), trajectories (top) and confinement (bottom) of AMPARs under basal, Wnt5a

or Wnt5a + $A\beta_{42}$ conditions. In every case, basal condition is shown in grey, Wnt5a (15 min treatment) in red and Wnt5a + oAβ42 (30 min and 15 min of exposure, respectively). **C)** Global analysis showing frequency distribution, completely immobile receptors (% LogD -5) and mobility. **D)** Synaptic analysis showing frequency distribution, completely immobile receptors (% LogD -5) and mobility. **E)** Extrasynaptic analysis showing frequency distribution, completely immobile receptors (% LogD -5) and mobility.

6. DISCUSSION

In this research, we have proof for the first time that Wnt5a immobilizes AMPARs in synaptic and extrasynaptic sites of hippocampal neurons. While oAβ42 immobilizes AMPARs only at synaptic sites, consistent with previous reports arguing an increase in endocytosis caused of oAβ42 exposure (Almeida et al., 2005; Hsieh et al., 2006; Miñano-Molina et al., 2011) .

Our main finding is that Wnt5a immobilizes GluA1-containing AMPARs (Figure 1E-H) and endogenous GluA2-containing AMPARs (Figures 2 and 3). This was proof by different controls like denatured recombinant Wnt5a (Figure 1I-K), Wnt7a (Figure 2) and use of sFRP2 complexed with Wnt5a (Figure 3A-F). The capacity of recombinant Wnt5a to activate CaMKII and JNK in hippocampal neurons has been proof by our lab and many others, but it is essential to test every batch of recombinant protein and in our particular experimental conditions. Activation of these molecules represent the quintessential proof of activation of Wnt non-canonical signaling in hippocampal neurons. We proof that under our working conditions recombinant Wnt5a is able to activate non-canonical downstream effectors like CaMKII and JNK in a time dependent manner (Figure 1A). Therefore, there is no doubt that recombinant Wnt5a is activating Wnt non-canonical signaling in hippocampal neurons.

We used recombinant Wnt5a to treat neurons. This has the advantage that is a purified form of the protein and that at all times, we know the exact concentration used in the experiments, making them more reliable and reproducible than, for instance, conditioned medium. On the down side, recombinant Wnt5a lacks palmitoylations and glycosylations found in the endogenously produced Wnt5a ligand. Lack of glycosylation should not be of importance since this post-transcriptional modification is involved in secretion of the ligand and we are exogenously adding it to the extracellular medium already. But, by not being palmitoylated there is a percentage of Wnt5a that will not bind to Frizzled receptors. Therefore, it raises the question on whether the effects seen are actually product of a smaller proportion of the ligand, interacting with Frizzled receptors. This point is indirectly answered by the fact that when the Wnt5a + sFRP2 complex is added, the effect of Wnt5a on AMPAR mobility, is completely

ablated (Figure 3B-F) and sFRP2 on its own is not causing any effects on AMPARs mobility (Figure 3G-K).

Molecular interactors and action mechanism of Wnt5a-dependent AMPARs immobilization

Here, we do not identify the type of receptor, either Frizzled or co-receptors (i.e. ROR2, LRP5/6 and Ryk, to mention a few) involved in the signaling of Wnt5a. Nonethelesss, the literature suggests that in hippocampal neurons, Wnt5a acts through binding to Frizzled9 (Ramírez et al., 2016), Frizzled2 (Sato et al., 2010) or Frizzled4 (Bian et al., 2015). sFRP2 has a higher affinity to Wnt5a and it has been extensively used to block the effect of Wnt5a, although it is not specific to Wnt5a. Since we are working with recombinant Wnt5a, this molecule is the only predominant form of Wnt5a in our working conditions and the complex formation solution includes only Wnt5a and sFRP2. So the only possibility for unwanted effects of sFRP2 would be if freely available sFRP2 binds to other endogenous Wnt ligands. For this reason, we tested if sFRP2 alone has an effect on AMPARs mobility. Our data shows that there is no effect over AMPARs mobility after 30 min treatment with sFRP2 (used at the same concentration as found in the complex) (Figure 3 G-K). Also, since we do not observe any difference in mobility when adding only sFRP2, we can presume that there is no, or low quantity, of endogenous Wnt ligands. This can be explained by several facts: 1. By changing the neurobasal growing medium for Tyrode extracellular solution, we deplete all secreted Wnt and factors from the experimental conditions and 2. The little amount of Wnt that could be released by neurons during experimentation is even less significant since it is considered that astrocytes are the main secretors of Wnt ligands.

The aim of this study was to determine the effect of Wnt5a on AMPARs dynamics. Therefore, we did not dissect the specific molecules involved in the immobilization effect. One alternative would be that Wnt5a causes modulation of TARPs. It has been described that TARP-γ2 (Stargazin) (Bats et al., 2007; Bedoukian et al., 2006; Chen et al., 2000) and TARP-γ8 control AMPARs number, distribution and function (Rouach et al., 2005). Earlier work reported CaMKII

to be fundamental on the increased interaction of the C-terminal Stargazin with PSD95 (Opazo et al., 2010). Since the pre-incubation of cells with KN93 did not prevent the Wnt5a immobilization of AMPARs (Figure 1 L-N), we do not consider CaMKII activation a necessary step for Wnt5a-mediated immobilization of AMPARs. Consequently, Stargazin is not the TARP mediating the immobilization of AMPARs caused by Wnt5a in hippocampal neurons. Interestingly, the 2h KN93 pre-incubation does not cause changes in the mobility of GluA1-containing AMPARs compared to basal mobility (Figure 1 L-N), which questions the role of CaMKII in the Wnt5a-dependent immobilization of AMPARs. Since it was not the aim of this work to dissect the particular signaling pathway underlying the effects of Wnt5a, further experiments should be performed to corroborate the CaMKII-independence of Wnt5a-dependent AMPAR immobilization. Specially, since there are publications that show a role of CaMKII on synaptic recruitment of AMPARs (Opazo and Choquet, 2011; Opazo et al., 2010). A different auxiliary protein, TARP-γ8 is expressed in higher numbers than TARP-γ2 in hippocampus (Schwenk et al., 2012). Therefore, although we did not test the involvement of TARP-γ8, Wnt5a could be acting over TARP-γ8 to cause immobilization of AMPARs. Considering that TARP-γ8 is mainly found in extrasynaptic sites (Rouach et al., 2005), this could explain the extrasynaptic immobilization we report (Figure 4 G-L). The possibility that TARP-γ8, and not TARP-γ2 mediates these effecst, could be tested in the future experiments.

Another alternative on the mechanism behind Wnt5a actions, relies on the activation of small GTPases, which would be in agreement with cytoskeleton rearrangements caused by activation of Wnt non-canonical pathway. It is described that Wnt5a activates Rac1, leading to actin reorganization on dendritic spines (Chen et al., 2017). Therefore, it is plausible for Wnt5a to cause AMPARs immobilization through actin reorganization on dendritic spines. This idea would need to be further analyzed.

These data provides evidence suggesting that the mechanisms involved in the immobilization of AMPARs in synaptic/extrasynaptic sites are different. First, after Wnt5a treatment the shape of the curves is different between extrasynaptic and synaptic analysis (compare Figure 4B and 4H, red curves). Secondly, the fact that there is immobilization of AMPARs in synaptic and extrasynaptic sites, but we only observe an increase in the GluA2-PSD95 co-localization in

synaptic sites (Figure 5 A-B) with no changes on extrasynaptic sites (not shown). This suggests that extrasynaptic immobilization is not mediated by binding to PSD95. Which is supported by literature, that claims other interactor to be involved in the anchoring of AMPARs outside the PSD, like 4.1N (for GluA1) and NSF (for GluA2). Finally, there is no reason to think there is only one pathway involved in the immobilization of AMPARs by Wnt5a. It is feasible, for instance, that TARP - γ8 is involved in the extrasynaptic immobilization and that actin reorganization through Rac1 or other small GTPases (i.e. Cdc42 and RhoA) are acting at a synaptic level. An hypothesis that is currently under investigation by our laboratory. Like this, Wnt5a could be mobilizing AMPARs from endocytic to extrasynaptic compartments and from there, increasing their presence on synaptic compartments.

Overexpressed GluA1-SEP versus endogenous GluA2 labeling

We noticed a difference on the experiments done using GluA1 overexpression versus those detecting endogenous GluA2. The overall effect of immobilization of the receptors is maintained, but it seems that the difference shown in the histograms are bigger when detecting GluA1 (Figure 1E) than GluA2 (Figure 2D). However, when comparing the completely immobile fraction after Wnt5a treatment, there is a significant increase for GluA2 (Figure 2E) whereas in the GluA1 detection is only increased by ~2%. These difference can be for several reasons. First, GluA1 overexpression gives away a higher detection of particles, because of the higher presence of GluA1-containing receptors. Also, we cannot rule out the possibility that the mechanisms leading to immobilization of GluA1-containing AMPARs and GluA2-containing AMPARs are different. Since we did not evaluate the effects of Wnt5a on synaptic plasticity but only basal activity, we cannot distinguish differences on GluA1 versus GluA2 activity (i.e. calcium permeability, conductance, inactivation time, etc).

GluA2-containing AMPARs and their interaction with PSD95

When examining the co-localization of AMPARs to PSD95 before and after treatment, it was obvious that Wnt5a increases the co-localization on both Mander's coefficients (Mander's 1 and

2). Importantly, when neurons are treated with Wnt5a + sFRP2 complex, co-localization returns to basal levels (Figure 5A-B). Although there is no important change in the amount of clusters for PSD95 or GluA2 (Figure 6). It is even more interesting the fact that the percentage of PSD95 clusters containing AMPARs is significantly increased compared to basal conditions, while again the Wnt5a + sFRP2 treatment causes no change from basal conditions (Figure 5B). This suggests a possible effect of Wnt5a in turning a silent synapse into an active synapse. Electrophysiological experiments are mandatory in order to confirm this idea. But if true, it would be a major finding in the regulation of synaptic plasticity.

The fact that we were not able to replicate the effect of Wnt5a over PSD95 clusters is conflicting. Previous reports used the same concentration of Wnt5a, similar immunofluorescence protocol and imaging. The main difference is the type of cell culture used, they used hippocampal neurons without astrocytes, while in our experiments Banker cultures were used. This could cause a difference, mainly on the basal state of the neurons. Like this, Banker-grown neurons could basally have a higher amount of PSD95 clusters, due to the enriched medium caused by astrocytic factors. Effectively, that is the case, in the paper from Farias et al., under basal conditions they detect an average of 80 PSD95 clusters per 100 µm of dendrite (Farías et al., 2009; see Figure 1B), while under our experimental conditions we detect an average of 150 PSD95 clusters per 100 µm of dendrite (Figure 6B), almost twice the amount detected before. Indeed, after 30 min of treatment they detected a significant increase on the amount of clusters for PSD95, corresponding to ~95 clusters per 100 µm of dendrite. A count that is much lower than ours either for basal or Wnt5a treated conditions. For this reason, we think that the difference between culture types (only neurons vs Banker cultured neurons), could account for the different results for PSD95 cluster density.

It has been described that Wnt5a causes a decrease in presynaptic terminals in hippocampal neurons (Davis et al., 2008). Therefore, we examined if in our working conditions we observe a change in synaptic contacts. To do so, hippocampal neurons were treated for 30 min with Wnt5a at 37°C and 5% CO2, fixed and labeled for immunofluorescence. PSD95 and VAMP2 were used as post- and pre-synaptic markers, respectively and DAPI was used to check nucleus integrity. Results show that there is no difference on the the amount of synaptic

contacts, when comparing control and Wnt5a treated neurons (Supplementary Figure 2). This confirms the idea that the changes seen due to Wnt5a action are occurring at a post-synaptic level. Still, it is possible that with longer Wnt5a incubations periods there are changes in synaptic contacts. But it is clear that under our working conditions, the immobilization of AMPARs is not dependent on changes on the amount of synaptic contacts.

How specific is the effect of Wnt5a versus Wnt7a on AMPAR immobilization?

We have shown that Wnt5a immobilizes AMPARs in synaptic and extrasynatic sites (Figure 4). The fact that Wnt7a exerts no effects on the dynamics of AMPARs (Figure 2), proofs that only the non-canonical activation of Wnt signaling, through Wnt5a is able to affect AMPARs mobility. In hippocampal neurons, Wnt5a acts as a non-canonical ligand and Wnt7a as a canonical one. Although there is a report arguing non-canonical postsynaptic effects of Wnt7a, like increase spine size, CaMKII activation and increase excitatory transmission (Ciani et al., 2011). There are big differences in their study and ours. First, they treated neurons for 3-16h, we treated them for a maximum of 30 min. Second, they used a range of 50-100 ng/mL of recombinant Wnt7a while we used throughout all the experiments 300 ng/mL. Wnt7a was acquired from the same supplier (R&D Systems), but they do not explicit if they used the carrier (bovine serum albumin) or carrier free presentation. Which in our hands, exerts different results. For this reason and in order to avoid any unwanted interference with our results, we always used the carrier free recombinant proteins.

Recently, it has been reported that Wnt7a modulates spine plasticity and regulates AMPAR localization (Mcleod et al., 2018), the data on this points is robust and in tone with previously reported Wnt7a effects, by the same group (Ciani et al., 2011; Sahores et al., 2010). More broadly, by means of blocking endogenous Wnt ligands with a mixture of broadly acting sFRPs and by shFzd7 expression, authors show that Wnt signaling activation is necessary for LTP activity (Mcleod et al., 2018). In this two observations, there is no direct relation with any Wnt ligand Particularly and could easily be related to Wnt7a as well as Wnt5a, as the two main Wnt ligands in the hippocampus. Also, the relationship between Wnt7a-Fz7 receptor is not exclusive, as Fz7 can interact with Wnt1, Wnt3/3a and Wnt7a/b, as reported by CRISPR targeting

(Voloshanenko et al., 2017). Even more, it has also been reported thart Fz7 as a strong interaction with Wnt5a, also (Dijksterhuis et al., 2015). For this reason, we believe that limiting the effect of any particular Wnt ligand to unequivocally to one Frizzled receptor, is a mistake. The Wnt-Frizzled interaction, is intrinsically ambiguous and promiscuous. One piece of information that directly relates to the results from our work is the data on AMPARs mobility. We performed Wnt7a studies on AMPA receptor mobility and found no difference compared to basal conditions (Figure 2), while McLeod et al., also detected no difference on the general dynamic of AMPA receptors, but only a difference in the completely immobility . To our opinion, the observations on AMPA receptor dynamics made by McLeod at al., are not conclusive and there is no parallel study performed with Wnt5a (Mcleod et al., 2018). That being said, we believe our data and their data complement each other on the important role of Wnt ligands on the physiology of mature hippocampal neurons.

oAβ42 immobilize AMPARs only at synaptic sites: possible action mechanisms

Extracellularly applied oAβ cause a decrease in the AMPAR currents, mainly due to endocytosis of the receptor (Gu et al., 2009; Hsieh et al., 2006; Liu et al., 2010; Miller et al., 2014; Miñano-Molina et al., 2011; Roselli et al., 2005; Zhao et al., 2010). In our experiments, we use two different concentrations of $A\beta_{42}$ oligomers; 1 and 5 μM (Figure 7). Previous research shows that treatment with 1 μM oAβ42 for 3 days, causes endocytosis of AMPARs and a decrease in AMPARs ionic currents, an effect dependent on CaMKII activation (Gu et al., 2009). However, a 5 μM oAβ42 concentration has been used for shorter term effects, within 30-60 min (Miñano-Molina et al., 2011). The rapid effects of 5 μM oAβ42 are dependent on the activity of calcineurin, which dephosphorylates Ser845 of GluA1 subunits (Miñano-Molina et al., 2011). Phosphorylation of Ser845 by PKA causes synaptic insertion of GluA1-containing AMPARs to extrasynaptic sites. Therefore, oAβ42 (through dephosphorylation of GluA1-Ser845) would actually be decreasing the extrasynaptic population of AMPARs. Like this, oAβ42 could be preventing LTP from occurring and at the same time, promoting LTD, which has been shown on several researches (Li et al., 2009; Shankar et al., 2008; Townsend et al., 2006). In our investigation, we observe an effect of oAβ only at synaptic and not at extrasynaptic sites (Figure 7F vs 7G), which is consistent with recent work that establishes that oAβ bind and clusters to

excitatory synaptic sites (Sinnen et al., 2016). It is important to notice that we track endogenous GluA2-containing AMPARs, nonetheless since the most common conformation of AMPARs in hippocampal neurons is the GluA1/GluA2 heterotetramer, we are indirectly tracking endogenous GluA1 as well. Therefore, the hypothesis of Aβ acting over the phosphorylation state of GluA1-Ser845, is still reasonable. Our findings are supported by published evidence that oAβ42 cause a decrease in the activity of AMPARs (Hsieh et al., 2006).

uPAINT: Technical considerations

This data also allows to examine the existence of any effects on the antibody over the mobility of AMPARs. The type of antibody we use to live-track AMPARs is a bivalent IgG-antibody of ~15 nm. The small size of the antibody allows for it to go in and out of the synaptic cleft. An issue that has to be addressed regarding live cell imaging techniques and antibody use is the fact that exposure to antibodies alone could be generating an unwanted effect on AMPARs mobility. Particularly it has been shown that binding of antibodies to certain motifs in the extracellular domain of membrane receptors causes endocytosis of the receptor. This is called “antibody feeding” and has been used to study endocytic and recycling pathways. We can discard the possibility that antibodies causes endocytosis of AMPARs, creating artificial immobilization, we have relevant controls that confirm our observations. First, when testing GluA1-AMPARs mobility, we boiled the recombinant Wnt5a protein and under the same experimental design, we saw no effect of the denatured Wnt5a. Second, when testing GluA2-AMPAR receptor mobility, the use of recombinant Wnt7a caused no effect whatsoever on the mobility of AMPARs. Third, when using the Wnt5a+sFRP2 complex, we deplete the effect seen with Wnt5a alone. Finally, we do not observe changes in the number of trajectories detected. This proofs that the effects here reported are not artifactual and that effectively, Wnt5a promotes immobilization of AMPARs. It is expected that live staining by means of antibody use (antibody feeding) causes by itself a certain degree of receptor immobilization. But the fact that sFRP2 depletes the effect of Wnt5a alone, supports the idea of AMPARs stabilization being caused by Wnt5a.

NMDA receptors

Here, we did not examine the effect of Wnt5a on the dynamics of NMDA receptors. However, that could be interesting to address because, as mentioned above, Wnt5a also affects the mEPSC of NMDA receptors. We cannot discard that Wnt5a also affects NMDARs, but since oAβ seem to cause endocytosis only on AMPARs, we focused on study them. Also, we believe that in case Wnt5a affects both receptors (AMPAR and NMDA receptors) dynamics, probable that the effects over NMDA receptors are not as rapid as the effects over AMPARs.

The contribution of this research into the field

This investigation and the recent work done by others (Chen et al., 2017; Subashini et al., 2017) are paving the way into exploring the multiple roles of Wnt5a in the functioning of excitatory synapses in hippocampus. A field of study that will for sure continue on expanding.

7. HIGHLIGHTS

1. **Wnt5a stabilizes GluA1- and GluA2-containing AMPARs.**

   This effect is partially seen after 15 min of treatment and it is statistically significantly after 30 min of treatment with Wnt5a. It is dependent on the native structure of Wnt5a, proof by the lack of effect in denatured Wnt5a. Another proof of the specificity of Wnt5a is the fact that when complexed with sFRP2, no effect is seen. Also, Wnt7a which is commonly considered as a canonical ligand in hippocampal neurons, has no effect on AMPARs dynamic. Although it was not a goal of this work to elucidate the molecular cascades involved in the Wnt5a-dependent stabilization of AMPARs, it seems that is not CaMKII-dependent.

2. **Wnt5a stabilizes AMPARs at synaptic and extrasynaptic sites.**

   Further analysis showed that Wnt5a stabilizes AMPARs in synaptic and extrasynaptic sites. Although the overall effect seems to be the same, the dramatic difference in MSD at extrasynaptic sites argue for a more critical effect of Wnt5a in extrasynaptic sites compared to synaptic sites. Nevertheless, we still observe an increase in stabilization at synaptic sites, this correlates with an increase of AMPARs co-localization with PSD95 in dendritic spines. Also, co-immunoprecipitation assays suggest an increased interaction between GluA2 and PSD95, after 30 min of treatment with Wnt5a.

3. **oAβ immobilize AMPARs only at synaptic sites.**

   Two concentrations of oAβ42 were used, 1 or 5 μM and effects were evaluated at 15 and 30 min. Although 1 μM oAβ42 showed a tendency towards immobilization of AMPARs on global and synaptic analysis, no significant effects were observed and no changes were seen at extrasynaptic sites. At 5 μM the before mentioned tendency was confirmed; oAβ42 immobilize AMPARs at synaptic sites and again, no changes on extrasynaptic sites were observed. Another important fact is that oAβ exerted their effect rapidly, more rapidly than Wnt5a. Already at 15 min of treatment with oAβ the

immobilization effect seems to reach a plateau. For this reason, 5 µM of oAβ for 15 min, were the conditions used for next experiments.

4. Wnt5a compensates the synaptic effects of $A\beta_{42}$ oligomers.

Co-treatment analysis of Wnt5a + oAβ42 shows that AMPARs are stabilized at extrasynaptic sites, with no changes on synaptic mobility. These results suggest that there is a compensation of the synaptic effects of oAβ by Wnt5a. This would explain the functional changes caused by Wnt5a, meaning increase in excitatory currents and LTP induction, among others. Furthermore, it explains the synapto- and neuro- protective effects of Wnt5a against oAβ toxicity.

8. FUTURE DIRECTIONS

We believe this work helps on the understanding of the fine-regulation of synaptic transmission through endogenous molecules and made a contribution to the knowledge of the mechanistics behind AD. Knowing in more detail the action mechanism of Wnt5a on synapto- and neuro-protective function against toxic elements, like oAβ, supports its use in the treatment of neurodegenerative diseases, specially AD. A recent publication uses intranasal administration of Wnt5a into mice to treat them after ischemia stroke, results are promising and shows there is a simple, non-invasive administration method for Wnt5a.

Currently, there are non-invasive intranasal treatments to introduce small molecules into the brain. Already, Wnt3a has been administrated into rats by this mean, reaching mainly the olfactory bulb, an area which is early and greatly affected by Aβ accumulation. Therefore, the possibility of using Wnt5a has treatment for Aβ synaptotoxicity, on AD murine models. Of course, more studies are needed to find out a way to locally administrate areas particularly affected by amyloidosis in humans, not affecting other areas.

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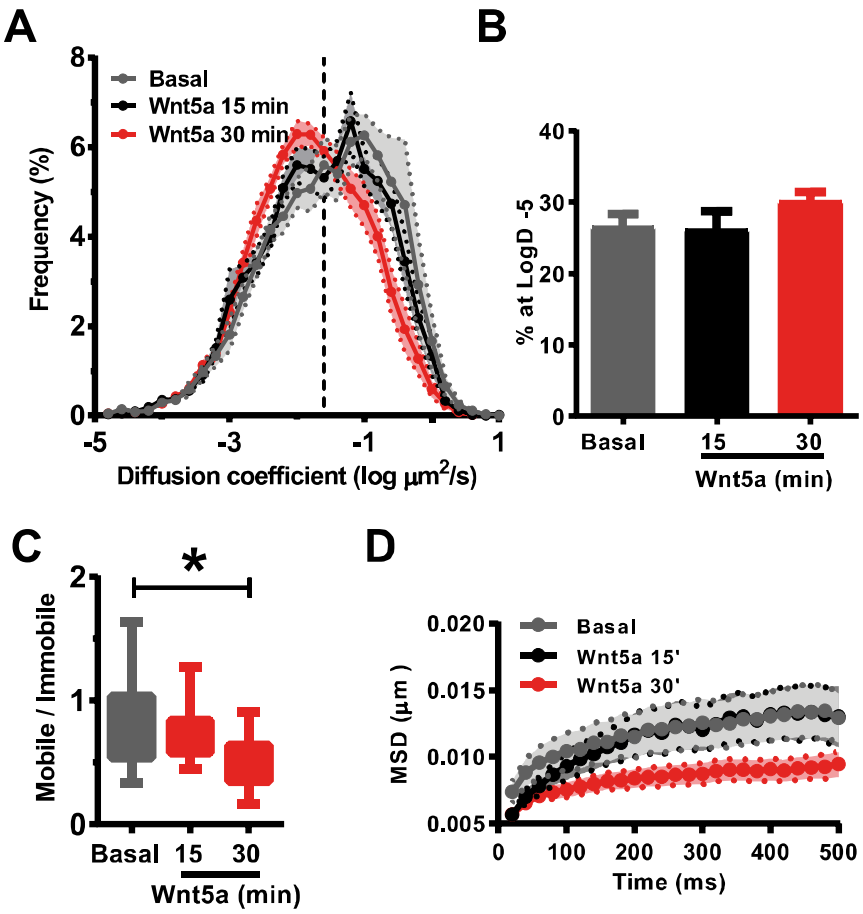
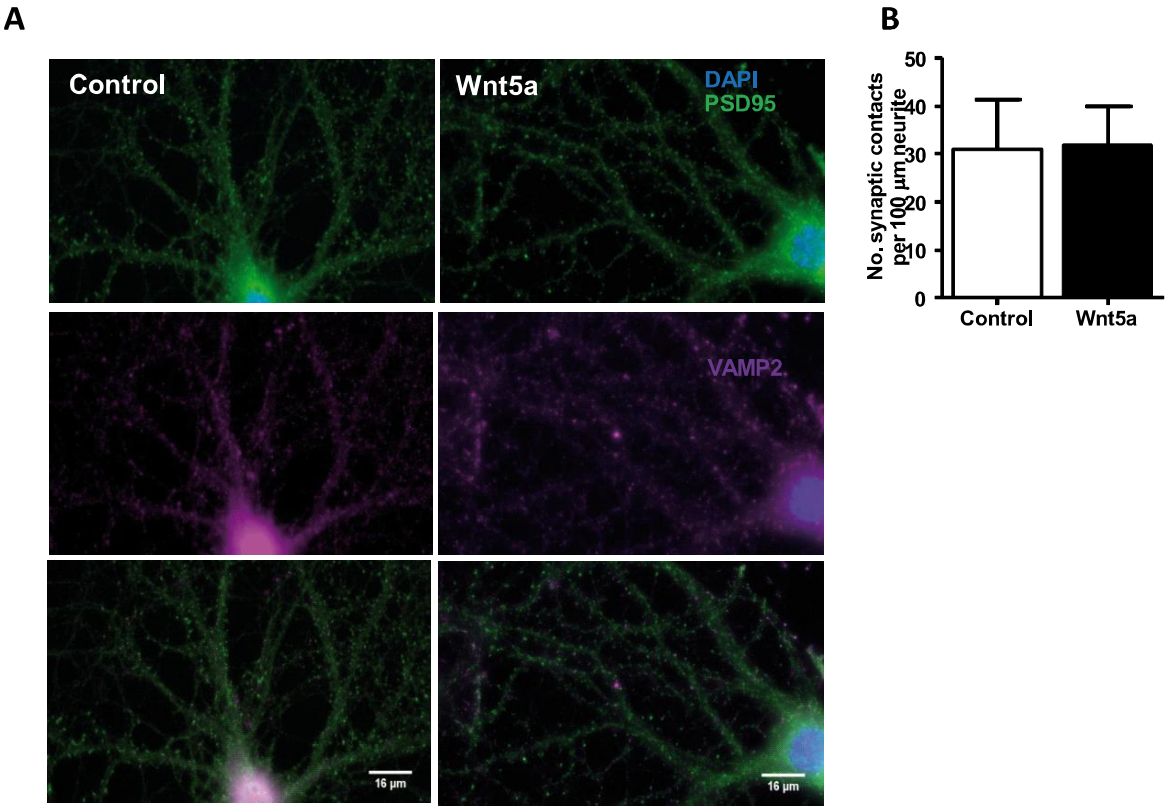
10. APPENDIXES

10.1. APPENDIX 1: Supplementary Figures

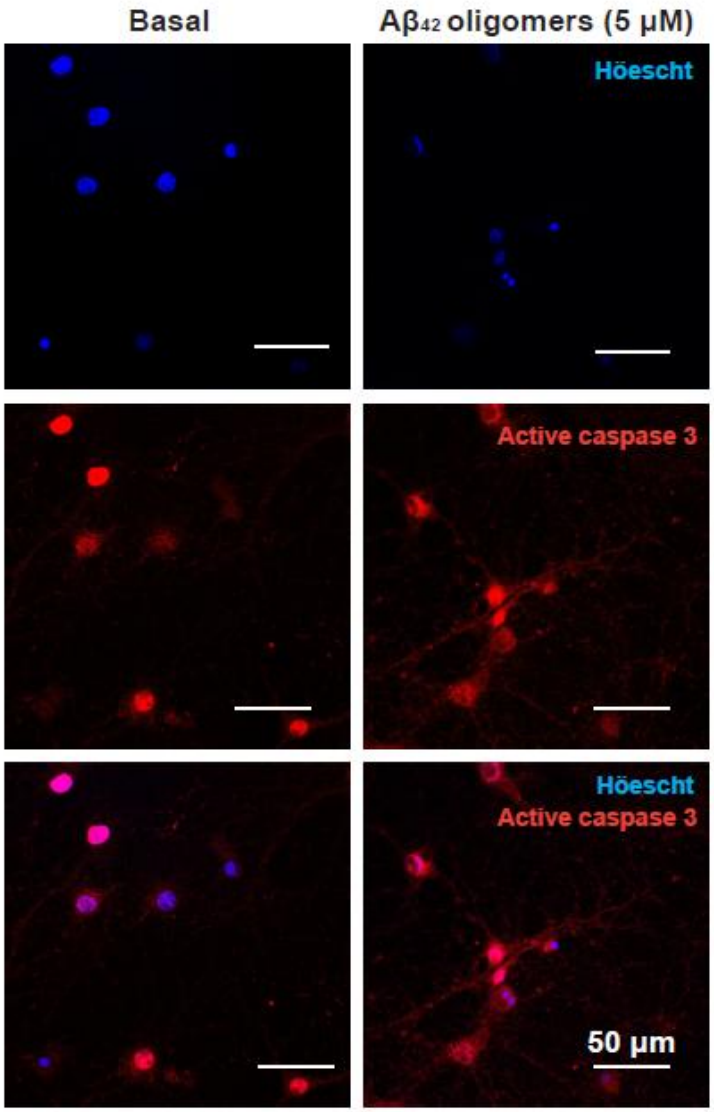


Supplementary Figure 1. Wnt5a causes immobilization of GluA1-containing AMPARs in a time-dependent manner. In every case, the effect of Wnt5a was evaluated at basal condition, 15 and 30 min after Wnt5a addition, but effects are significant only after 30 min. **A)** Histogram of GluA1 tracking. **B)** Completely immobile receptors at different time points. **C)** mobility of AMPARs diminishes with exposure to Wnt5a, it is only statistically significant at 30 min. **D)** Mean Square Displacement (MSD) is also significantly diminished after 30 min exposure to Wnt5a. Endogenous GluA2 tracking. One-way ANOVA and Bonferroni post-test, P*<0.05, P***<0.001.



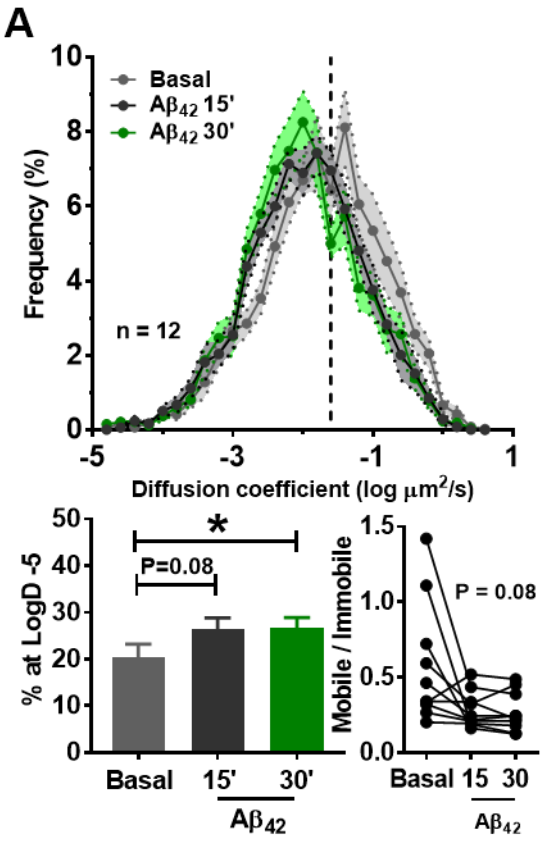
Supplementary Figure 2. Wnt5a does not affect the amount of synaptic contacts.

Following uPAINT experiments, hippocampal neurons were fixed, permeabilized and labeled for PSD95 and VAMP2, as post-synaptic and pre-synaptic markers, respectively. DAPI was used to confirm viability. **A)** Control and 30 min Wnt5a-treated neurons labeled for PSD95-DAPI (top), VAMP2-DAPI (middle) and PSD95-VAMP2-DAPI, to test apposition of PSD95-VAMP2 labeling. **B)** Quantification shows no difference in the amount of synaptic contacts under control or Wnt5a conditions.



Supplementary Figure 3. oAβ42 identity and neurotoxicity effects.

To confirm the identity of the Aβ species we produce with the aggregation protocol described above, we approach three methods. **A)** Electron microscopy shows the shape and length and shape expected for oligomeric species. **B)** Tris-tricine gel to identify with 6E10 antibody (β-amyloid detection), confirms that the species are mainly found in the range of 75-30 kDa (n-mers), with a smaller population around 10 kDa, consistent of dimers (2-mers) and trimers (3-mers). **C)** Hippocampal neuron were exposed to 24h of oAβ42 (5 μM), Höescht stainning and active-caspase3 were observed, before and after treatment.



Supplementary Figure 4. 5 μM oAβ42 immobilizes AMPARs rapidly in peaking fast.

Global analysis of 5 μM oAβ42. In every case, the effect was evaluated at basal condition, 15 min and 30 min after treatment. **A)** Histogram of diffusion coefficients at basal, already at 15 min a shift to the left indicates an increase immobilization of AMPARs. **B)** Completely immobile fraction, shows a significant difference against basal and after 30 min of treatment. **C)** Mobility indicates a P-value of 0.08 between basal and 30 min of treatment, there is no significant difference between 15 and 30 min of treatment.

10.2. APPENDIX 2: Publications

Wnt signaling in the Central Nervous System: New Insights in Health and Disease

Carolina A. Oliva*, Carla Montecinos-Oliva*†, Nibaldo C. Inestrosa*,†,‡,§,1

* Center for Aging and Regeneration (CARE-UC), Pontifical Catholic University of Chile, Santiago, Chile
† Interdisciplinary Institute for Neuroscience (IINS), University of Bordeaux, Bordeaux, France
‡ Center for Healthy Brain Ageing, University of New South Wales, Sydney, NSW, Australia
§ Center of Excellence in Biomedicine of Magallanes (CEBIMA), University of Magallanes, Punta Arenas, Chile
1 Corresponding author. E-mail address: ninestrosa@bio.puc.cl

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MOLECULAR NEURODEGENERATION

RESEARCH ARTICLE Open Access

Is L-methionine a trigger factor for Alzheimer's-like neurodegeneration?: Changes in Aβ oligomers, *tau* phosphorylation, synaptic proteins, *Wnt* signaling and behavioral impairment in wild-type mice

Cheril Tapia-Rojas[1†], Carolina B. Lindsay[1†], Carla Montecinos-Oliva[1], Macarena S. Arrazola[1], Rocio M. Retamales[1], Daniel Bunout[2], Sandra Hirsch[2] and Nibaldo C. Inestrosa[1,3,4,5,6*]

Abstract

Background: L-methionine, the principal sulfur-containing amino acid in proteins, plays critical roles in cell physiology as an antioxidant and in the breakdown of fats and heavy metals. Previous studies suggesting the use of L-methionine as a treatment for depression and other diseases indicate that it might also improve memory and propose a role in brain function. However, some evidence indicates that an excess of methionine can be harmful and can increase the risk of developing Type-2 diabetes, heart diseases, certain types of cancer, brain alterations such as schizophrenia, and memory impairment.

Results: Here, we report the effects of an L-methionine-enriched diet in wild-type mice and emphasize changes in brain structure and function. The animals in our studypresented 1) higher levels of phosphorylated *tau* protein, 2) increased levels of amyloid-β (Aβ)-peptides, including the formation of Aβ oligomers, 3) increased levels of inflammatory response,4) increased oxidative stress, 5) decreased level of synaptic proteins, and 6) memory impairment and loss. We also observed dysfunction of the *Wnt* signaling pathway.

Conclusion: Taken together, the results of our study indicate that an L-methionine-enriched diet causes neurotoxic effects *in vivo* and might contribute to the appearance of Alzheimer's-like neurodegeneration.

Keywords: L-Methionine, Amyloid, Tau, Memory impairment, Alzheimer's disease

Background

Methionine and cysteine are considered to be the principal sulfur-containing amino acids in proteins, and they play critical roles in cell metabolism. Methionine aids in the breakdown of fats by both preventing their accumulation in the arteries [1], aiding the digestive system and facilitating the elimination of heavy metals from the body, which can be converted into cysteine to prevent toxic damage in the liver. Methionine is also an important antioxidant because its sulfur group can inactivate free radicals [2–5], and it is one of the three amino acids that the body needs to produce creatine, which is an essential compound for energy production and muscle building [6]. In addition, it may be useful in the treatment of depression [7, 8]; some studies have indicated that methionine might improve memory recall and suggested a key role for this aminoacid in brain processes. Methionine deficiencies can trigger

* Correspondence: ninestrosa@bio.puc.cl
†Equal contributors
[1]Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, P. Centro de Envejecimiento y Regeneración (CARE), Pontificia Universidad Católica de Chile, Santiago, Chile
[2]Centre for Healthy Brain Ageing, School of Psychiatry, Faculty of Medicine, University of New South Wales, Sydney, Australia
Full list of author information is available at the end of the article

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Wnt5a inhibits K^+ currents in hippocampal synapses through nitric oxide production

Jorge Parodi [a,1], Carla Montecinos-Oliva [a], Rodrigo Varas [b], Iván E. Alfaro [c,d], Felipe G. Serrano [a], Manuel Varas-Godoy [c], Francisco J. Muñoz [e], Waldo Cerpa [f], Juan A. Godoy [a,e], Nibaldo C. Inestrosa [a,g,h,i,*]

[a] Centro de Envejecimiento y Regeneración (CARE), Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile
[b] Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Talca, Chile
[c] Fundación Ciencia y Vida, Santiago, Chile
[d] Facultad de Ciencias Naturales y Exactas, Universidad de Playa Ancha, Valparaíso, Chile
[e] Laboratory of Molecular Physiology and Channelopathies, Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona, Spain
[f] Laboratorio de Función y Patología Neuronal, Departamento de Biología Celular y Molecular, Facultad de Ciencias Biológicas, P. Universidad Católica de Chile, Santiago, Chile
[g] Centre for Healthy Brain Ageing, School of Psychiatry, Faculty of Medicine, University of New South Wales, Sydney, Australia
[h] Centro UC Síndrome de Down, Pontificia Universidad Católica de Chile, Santiago, Chile
[i] Centro de Excelencia en Biomedicina de Magallanes (CEBIMA), Universidad de Magallanes, Punta Arenas, Chile

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ABSTRACT

Hippocampal synapses play a key role in memory and learning processes by inducing long-term potentiation and depression. Wnt signaling is essential in the development and maintenance of synapses via several mechanisms. We have previously found that Wnt5a induces the production of nitric oxide (NO), which modulates NMDA receptor expression in the postsynaptic regions of hippocampal neurons. Here, we report that Wnt5a selectively inhibits a voltage-gated K^+ current (Kv current) and increases synaptic activity in hippocampal slices. Further supporting a specific role for Wnt5a, the soluble Frizzled receptor protein (sFRP-2; a functional Wnt antagonist) fully inhibits the effects of Wnt5a. We additionally show that these responses to Wnt5a are mediated by activation of a ROR2 receptor and increased NO production because they are suppressed by the shRNA-mediated knockdown of ROR2 and by 7-nitroindazole, a specific inhibitor of neuronal NOS. Together, our results show that Wnt5a increases NO production by acting on ROR2 receptors, which in turn inhibit Kv currents. These results reveal a novel mechanism by which Wnt5a may regulate the excitability of hippocampal neurons.

1. Introduction

Wnt signaling plays an essential role in synaptic maintenance, function and connectivity in the developing and adult mammalian central nervous system (CNS) (Budnik and Salinas, 2011; Inestrosa and Arenas, 2010; Inestrosa and Varela-Nallar, 2014). Wnt ligands are secreted glycoproteins that stimulate the formation of central and peripheral synapses (Budnik and Salinas, 2011; Salinas, 2012) by promoting pre-synaptic assembly (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Farias et al., 2007) and the clustering of postsynaptic components (Cerpa et al., 2010; Cuitino et al., 2010; Farias et al., 2009).

Nineteen Wnt genes have been identified in the vertebrate genome, all of which signal through the seven-transmembrane-domain Frizzled (FZD) receptors (Schulte and Bryja, 2007). Signaling events occur as the direct consequence of FZD activation, via the phosphoprotein dishevelled (DVL). These receptors, with the co-receptor tyrosine kinase-like orphan receptor 2 (ROR2), are selectively activated by Wnt5a in both mammals and invertebrates (Mikels et al., 2009).

Both types of receptors activate at least three alternative signaling pathways: 1) the canonical Wnt/β-catenin pathway, involved in the co-activation of Wnt target genes with Tcf/Lef transcription factors (Nusse and Varmus, 2012); 2) the Wnt-Planar Cell Polarity (Wnt/PCP) pathway, acting through monomeric GTPases and c-Jun. N-terminal kinase (JNK), which regulates cytoskeleton reorganization (Simons and

Abbreviations: Wnt5a, wingless-type family member 5a; NMDA, N-methyl D-aspartic acid; NO, nitric oxide; 7-NI, 7-nitroindazole; ROR2, receptor tyrosine kinase-like orphan receptor 2; sFRP-2, soluble frizzled related protein 2; DVL, Dishevled; Wnt/PCP, Wnt/planar cell polarity; JNK, c-Jun. N-terminal kinase; CamKII, Ca^{2+}-calmodulin kinase II; CNS, central nervous system; PSD-95, post-synaptic density 95; GABA, γ-aminobutyric acid; nNOS, neuronal nitric oxide synthase; araC, 1-β-D-arabinofuranosylcytosine; DIV, days in vitro; CMV, cytomegalovirus; EGFP, enhanced green fluorescent protein; TU, transduction units; MOI, multiplicity of infection; SEM, standard error of the mean; PTX, picrotoxin; L-NAME, L-NG-nitroarginine methyl ester; TEA, tetraethylammonium; fEPSP, field excitatory postsynaptic potentials; TRPC, transient receptor potential cation channel; IPSP, inhibitory postsynaptic potentials; LTP, long term potentiation; VDCC, voltage-dependent calcium channels; DG, dentate gyrus; PKC, protein kinase C.

* Corresponding author at: CARE Biomedical Research Center, Pontificia Universidad Católica de Chile, Av. Alameda 340, Santiago, Chile.
E-mail address: ninestrosa@bio.puc.cl (N.C. Inestrosa).
[1] Laboratorio de Fisiología de la Reproducción, Escuela de Medicina Veterinaria, Núcleo de Investigación en Producción Alimentaria, Facultad de Recursos Naturales, Universidad Católica de Temuco, Temuco, Chile.

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Fructose consumption reduces hippocampal synaptic plasticity underlying cognitive performance

Pedro Cisternas[a], **Paulina Salazar**[a], **Felipe G. Serrano**[a], **Carla Montecinos-Oliva**[a], **Sebastián B. Arredondo**[b], **Lorena Varela-Nallar**[b], **Salesa Barja**[c], **Carlos P. Vio**[a], **Fernando Gomez-Pinilla**[d], and **Nibaldo C. Inestrosa**[a,e,f,g,*]

[a]Centro de Envejecimiento y Regeneración (CARE), Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

[b]Center for Biomedical Research, Faculty of Biological Sciences and Faculty of Medicine, Universidad Andres Bello, Santiago, Chile

[c]Departamento de Pediatria, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile

[d]Department of Integrative Biology and Physiology, University of California, Los Angeles, CA, USA

[e]Centre for Healthy Brain Ageing, School of Psychiatry, Faculty of Medicine, University of New South Wales, Sydney, Australia

[f]Centro de Excelencia en Biomedicina de Magallanes (CEBIMA), Universidad de Magallanes, Punta Arenas, Chile

[g]Centro UC Síndrome de Down, Pontificia Universidad Católica de Chile, Santiago, Chile

Abstract

Metabolic syndrome (MetS) is a global epidemic, which involves a spectrum of metabolic disorders comprising diabetes and obesity. The impact of MetS on the brain is becoming to be a concern, however, the poor understanding of mechanisms involved has limited the development of therapeutic strategies. We induced a MetS-like condition by exposing mice to fructose feeding for 7 weeks. There was a dramatic deterioration in the capacity of the hippocampus to sustain synaptic plasticity in the forms of long-term potentiation (LTP) and long-term depression (LTD). Mice exposed to fructose showed a reduction in the number of contact zones and the size of postsynaptic densities (PSDs) in the hippocampus, as well as a decrease in hippocampal neurogenesis. There was an increase in lipid peroxidation likely associated with a deficiency in plasma membrane excitability. Consistent with an overall hippocampal dysfunction, there was a subsequent decrease in hippocampal dependent learning and memory performance, i.e., spatial learning and episodic memory. Most of the pathological sequel of MetS in the brain was reversed three month after discontinue fructose feeding. These results are novel to show that MetS triggers a cascade of molecular events, which disrupt hippocampal functional plasticity, and specific

*Corresponding author at: CARE Biomedical Center, Faculty of Biological Sciences, Pontificia Universidad Católica de Chile, Alameda 340, P.O. Box 114-D, Santiago, Chile. ninestrosa@bio.puc.cl (N.C. Inestrosa).

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Wnt-related SynGAP1 is a neuroprotective factor of glutamatergic synapses against Aβ oligomers

*Juan F. Codocedo[1], Carla Montecinos-Oliva[1] and Nibaldo C. Inestrosa[1,2,3,4]**

[1] Molecular Neurobiology Lab and Center for Aging and Regeneration Center, Department of Cell and Molecular Biology, Faculty of Biological Sciences, Pontifical Catholic University of Chile, Santiago, Chile, [2] Center for Healthy Brain Ageing, School of Psychiatry, Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia, [3] Centro de Excelencia en Biomedicina de Magallanes, Universidad de Magallanes, Punta Arenas, Chile, [4] Centro UC Síndrome de Down, Pontificia Universidad Católica de Chile, Santiago, Chile

Wnt-5a is a synaptogenic factor that modulates glutamatergic synapses and generates neuroprotection against Aβ oligomers. It is known that Wnt-5a plays a key role in the adult nervous system and synaptic plasticity. Emerging evidence indicates that miRNAs are actively involved in the regulation of synaptic plasticity. Recently, we showed that Wnt-5a is able to control the expression of several miRNAs including miR-101b, which has been extensively studied in carcinogenesis. However, its role in brain is just beginning to be explored. That is why we aim to study the relationship between Wnt-5a and miRNAs in glutamatergic synapses. We performed *in silico* analysis which predicted that miR-101b may inhibit the expression of synaptic GTPase-Activating Protein (SynGAP1), a Ras GTPase-activating protein critical for the development of cognition and proper synaptic function. Through overexpression of miR-101b, we showed that miR-101b is able to regulate the expression of SynGAP1 in an hippocampal cell line. Moreover and consistent with a decrease of miR-101b, Wnt-5a enhances SynGAP expression in cultured hippocampal neurons. Additionally, Wnt-5a increases the activity of SynGAP in a time-dependent manner, with a similar kinetic to CaMKII phosphorylation. This also, correlates with a modulation in the SynGAP clusters density. On the other hand, Aβ oligomers permanently decrease the number of SynGAP clusters. Interestingly, when neurons are co-incubated with Wnt-5a and Aβ oligomers, we do not observe the detrimental effect of Aβ oligomers, indicating that, Wnt-5a protects neurons from the synaptic failure triggered by Aβ oligomers. Overall, our findings suggest that SynGAP1 is part of the signaling pathways induced by Wnt-5a. Therefore, possibility exists that SynGAP is involved in the synaptic protection against Aβ oligomers.

Keywords: Wnt-5a, microRNAs, SynGAP, CamKII, Alzheimer disease

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****Correspondence:***
Nibaldo C. Inestrosa, Molecular Neurobiology Lab and Center for Aging and Regeneration Center, Department of Cell and Molecular Biology, Faculty of Biological Sciences, Pontifical Catholic University of Chile, Avenue Alameda 340, Santiago, Region Metropolitana-Santiago, Chile
ninestrosa@bio.puc.cl

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Introduction

Dendritic spines compartmentalize biochemical cascades at the postsynaptic level and are enriched in neurotransmitter receptors, ion channels, and components of various signaling pathways (Chen and Sabatini, 2012; Colgan and Yasuda, 2014), including the Wnt

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PERSPECTIVE

Tetrahydrohyperforin: a neuroprotective modified natural compound against Alzheimer's disease

According to the World Health Organization (WHO), a total of 35.6 million cases of dementia were estimated in 2010, with close to 7.7 million new cases each year. In 2008, WHO declared dementia a priority condition. 90% of all dementia cases are considered to be Alzheimer's disease (AD). Therefore, great effort was made to understand the etiology of the disease and stop or slow down AD progression.

Several different therapies have been developed, including active and passive immunotherapy against the amyloid-β (Aβ) protein, drugs targeting cholinergic synapses (*i.e.*, donezepil and rivastigimine) and glutamatergic synapses (*i.e.*, memantine), and microtubule stabilization by targeting tau protein, among many others. Unfortunately, these drugs do not directly attack the disease, but rather act by compensating the synaptic transmission loss, and therefore, show only short-term beneficial effects.

For over ten years we have studied the effects of tetrahydrohyperforin, a semisynthetic derivative of the natural compound hyperforin found in St. John's wort (*Hypericum perforatum*). Hyperforin shows low stability and bioavailability. The chemically reduced derivative tetrahydrohyperforin is more stable and therefore more efficient in treatments. Injecting tetrahydrohyperforin into wild-type and double transgenic mice that model AD ($APP_{swe}/PS1^{A\beta 5}$), we have found positive effects at a molecular, cellular and cognitive level. Among the effects of tetrahydrohyperforin, we found that it prevents deposition of Aβ peptides, prevents abnormal tau phosphorylation, and mitigates synaptotoxicity. It also increases adult neurogenesis in wild-type and $APP_{swe}/PS1^{A\beta 5}$ mice. These molecular and cellular changes are most likely related to tetrahydrohyperforin's ability to prevent cognitive deficits when administered to young or old $APP_{swe}/PS1^{A\beta 5}$ mice.

In the present perspective, we are going to present and compare the effects of tetrahydrohyperforin and hyperforin, propose a mechanism of action based on recently published data, and finally, will discuss future possibilities of tetrahydrohyperforin in AD therapy.

Tetrahydrohyperforin is a derivative of the natural compound hyperforin: St. John's wort has been used since ancient times for its antidepressive, antiinflammatory and antiseptic properties as part of popular medicine traditions. The main active compound of this plant is hyperforin, a bicyclic polyprenylated acylphloroglucinol. Once extracted, the compound is highly unstable when exposed to light, heat, or air. More stable analogues of hyperforin were sought by systematic chemical modification and resulted in the semisynthetic compound tetrahydrohyperforin, where two carbonyl groups were reduced to hydroxyl groups. Tetrahydrohyperforin shows greater chemical stability and oral bioavailability.

Most of the pharmacological effects described for hyperforin have been described for tetrahydrohyperforin, as well. In particular, among the shared effects described for the central nervous system (CNS), there are: enhanced performance in memory paradigm tests, reduction in Aβ plaque size, and decrease of astrogliosis and various inflammation markers in brain.

Even more interesting are the discoveries of the neuroprotective properties of tetrahydrohyperforin. Next, we are going to describe the molecular effects of tetrahydrohyperforin in a mouse model of AD and how these effects prevent memory loss.

Tetrahydrohyperforin generates neuroprotective effects and prevents memory loss in double transgenic mice modeling AD: In our laboratory, we established that hyperforin is able to disaggregate Aβ peptides *in vitro* and have shown that it prevents Aβ neurotoxicity in rat hippocampus *in vivo* (Dinamarca et al., 2006). We have also reported that intraperitoneal administration of this drug in the most commonly used mouse model of AD ($APP_{swe}/PS1^{A\beta 5}$) occludes cognitive deficit, reducing the Aβ plaque size and the oxidative damage in cortex and hippocampus (Cerpa et al., 2010). Once the effects of hyperforin were clear, we aimed at studying tetrahydrohyperforin to elucidate whether it had similar effects.

Experiments showed that in $APP_{swe}/PS1^{A\beta 5}$ mice, tetrahydrohyperforin promotes non-amyloidogenic amyloid precursor protein (APP) processing by inhibiting γ-secretase-mediated cleavage of APP's C-terminal fragment, C99 (Inestrosa et al., 2011). As mentioned before, it also increases neurogenesis in the dentate gyrus of wild-type and $APP_{swe}/PS1^{A\beta 5}$ mice (Abbott et al., 2013). It was further established that tetrahydrohyperforin prevents mitochondrial Ca^{2+} overload, and therefore protects against mitochondrial dysfunction (Zolezzi et al., 2013). Overall, tetrahydrohyperforin both *in vitro* and *in vivo* has synapto- and neuroprotective effects against Aβ oligomers.

Although the data obtained was relevant, one important issue was not solved: the mechanism of action through which tetrahydrohyperforin could be generating these effects. Several hypotheses have been proposed during the years. One of them takes into consideration the fact that hyperforin can act as an N-methyl D-aspartate (NMDA) receptor antagonist, and therefore, could block reactive oxygen species (ROS) formation (Kumar et al., 2006). Unfortunately, there is no further advance in this work. Another possibility is that tetrahydrohyperforin, similar to hyperforin, could activate the transient receptor potential cation channel subfamily 6 (TRPC6) (Leuner et al., 2007). TRPC channels are non-selective cationic channels thought to be composed of six membrane spanning segments with intracellular amino- and carboxy-termini. Based on sequence similarity these were divided into two subfamilies: TRPC1/4/5 and TRPC3/6/7. TRPC6 channels are principally Ca^{2+} permeable channels ubiquitously found in the body, but importantly located in the postsynaptic density of hippocampal neurons.

Within the CNS, TRPC3/6 channels have key roles in neuronal survival by transmitting brain-derived neurotrophic factor (BDNF) signaling and promoting gene expression *via* the cAMP response element-binding protein (CREB). TRPC3 channels are involved in hippocampal excitability. TRPC7 channels are found only in embryonic stages, so they

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Effects of Tetrahydrohyperforin in Mouse Hippocampal Slices: Neuroprotection, Long-term Potentiation and TRPC Channels

C. Montecinos-Oliva[1], A. Schüller[2,3], J. Parodi[1], F. Melo[2,3] and N.C. Inestrosa*[,1,4,5]

[1]Centro de Envejecimiento y Regeneración (CARE), Departamento de Biología Celular y Molecular; Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile; [2]Molecular Bioinformatics Laboratory, Millennium Institute on Immunology and Immunotherapy; [3]Departamento de Genética Molecular y Microbiología; Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile; [4]Center for Healthy Brain Ageing, School of Psychiatry, Faculty of Medicine, University of New South Wales, Sydney, Australia; [5]Centro de Excelencia en Biomedicina de Magallanes (CEBIMA), Universidad de Magallanes, Punta Arenas, Chile

Abstract: Tetrahydrohyperforin (IDN5706) is a semi-synthetic compound derived from hyperforin (IDN5522) and is the main active principle of St. John's Wort. IDN5706 has shown numerous beneficial effects when administered to wild-type and double transgenic (APPswe/PSEN1ΔE9) mice that model Alzheimer's disease. However, its mechanism of action is currently unknown. Toward this end, we analysed field excitatory postsynaptic potentials (fEPSPs) in mouse hippocampal slices incubated with IDN5706 and in the presence of the TRPC3/6/7 activator 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), the TRPC channel blocker SKF96365, and neurotoxic amyloid β-protein (Aβ) oligomers. To study spatial memory, Morris water maze (MWM) behavioural tests were conducted on wild-type mice treated with IDN5706 and SKF96365. *In silico* studies were conducted to predict a potential pharmacophore. IDN5706 and OAG had a similar stimulating effect on fEPSPs, which was inhibited by SKF96365. IDN5706 protected from reduced fEPSPs induced by Aβ oligomers. IDN5706 improved spatial memory in wild-type mice, an effect that was counteracted by co-administration of SKF96365. Our *in silico* studies suggest strong pharmacophore similarity of IDN5706 and other reported TRPC6 activators (IDN5522, OAG and Hyp9). We propose that the effect of IDN5706 is mediated through activation of the TRPC3/6/7 channel subfamily. The unveiling of the drug's mechanism of action is a necessary step toward the clinical use of IDN5706 in Alzheimer's disease.

Keywords: Aβ oligomers, Alzheimer's Disease, neuroprotection, hippocampus, tetrahydrohyperforin, TRPC channels.

INTRODUCTION

Alzheimer's disease (AD) is characterised by a progressive loss of cognitive abilities, eventually leading to the death of the individual [1]. Accumulation of the amyloid β-protein (Aβ), a product of the processing of the amyloid precursor protein (APP), is believed to play a key role in the cognitive deficits observed in AD [2]. The mechanisms involved in the pathogenic changes triggered by Aβ oligomers are not clearly understood. Aβ oligomers trigger neuronal dysfunction and cytoskeletal alterations, early manifestations that lead to aberrant remodelling of dendrites and axons, synaptic loss [3], and eventually, a progressive loss of neuronal populations [4]. Synaptic failure is correlated with a reduction in synaptic proteins and alterations in synaptic function [2, 5].

Hyperforin is a prenylated phloroglucinol derivative and the primary active molecule responsible for the antidepressant activity of St. John's Wort (*Hypericum perforatum*)[6]. It has been used for centuries in Chinese traditional medicine as a sedative, antimalarial and diuretic substance [7]. Hyperforin has been suggested to enhance memory in rodents [8] and may have additional anti-inflammatory, antibacterial, antiangiogenic and antitumoral effects. Accordingly, we have previously shown that hyperforin reduces the behavioural alterations induced by intra-hippocampal injection of Aβ fibrils in an acute rat model of AD [9]. Hyperforin, a natural compound, is chemically instable, easily oxidised, sensitive to heat and light, and degrades quickly as well as its bioactivity may be rapidly lost during storage [10, 11]. Tetrahydrohyperforin (IDN5706) is a semi-synthetic derivative of hyperforin that has higher stability and increased oral bioavailability [12], while maintaining its neuroprotective properties [13].

So far, the mechanism of action of IDN5706 that explains its memory and behaviour altering effects is not known. Studies indicate that the canonical transient receptor potential 6 (TRPC6) channel, a tetrameric, non-selective cation channel, is specifically activated by hyperforin [14]. TRPC6 channels have also been found to promote dendritic growth [15] and play a role in the formation of excitatory synapses [16]. Here, we report that IDN5706-mediated activation of TRPC channels improves the synaptic response measured by a reversible increase in field excitatory postsynaptic potential (fEPSP), has neuroprotective effects on Aβ oligomers, and improves spatial memory in wild-type

*Address correspondence to this author at the CARE Biomedical Center, Pontificia Universidad Católica de Chile, Av. Alameda 340, Santiago, Chile; Tel: + (56)-26862724; Fax: + (56)-2-6862959;
E-mail: ninestrosa@bio.puc.cl

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