2016

The Level of NMDA Receptor in the Membrane Modulates Amyloid-β Association and Perforation

Christian Peters
Universidad de Concepción

Fernando J. Sepúlveda
University of Concepción

Eduardo Fernández-Pérez
University of Concepción

Robert W. Peoples
Marquette University, robert.peoples@marquette.edu

Luis G. Aguayo
University of Concepción

The Level of NMDA Receptor in the Membrane Modulates Amyloid-β Association and Perforation

Christian Peters
Laboratory of Neurophysiology, Department of Physiology, Universidad de Concepción, Chile
Fernando J. Sepulveda
Laboratory of Neurophysiology, Department of Physiology, Universidad de Concepción, Chile
Eduardo J. Fernandez-Perez
Laboratory of Neurophysiology, Department of Physiology, Universidad de Concepción, Chile
Robert W. Peoples
Laboratory of Biomedical Sciences, Marquette University, Milwaukee, WI
Luis G. Aguayo
Laboratory of Neurophysiology, Department of Physiology, Universidad de Concepción, Chile

Journal of Alzheimer's Disease, Vol 53, No. 1 (2016): pg. 197-207. DOI.

Abstract: Alzheimer's disease is a neurodegenerative disorder that affects mostly the elderly. The main histopathological markers are the senile plaques formed by amyloid-β peptide (Aβ) aggregates that can perforate the plasma membrane of cells, increasing the intracellular calcium levels and releasing synaptic vesicles that finally lead to a delayed synaptic failure. Several membrane proteins and lipids interact with Aβ affecting its toxicity in neurons. Here, we focus on NMDA receptors (NMDARs) as proteins that could be modulating the association and neurotoxic perforation induced by Aβ on the plasma membrane. In fact, our results showed that decreasing NMDARs, using enzymatic or siRNA approaches, increased the association of Aβ to the neurons. Furthermore, overexpression of NMDARs also resulted in an enhanced association between NMDA and Aβ. Functionally, the reduction in membrane NMDARs augmented the process of membrane perforation. On the other hand, overexpressing NMDARs had a protective effect because Aβ was now unable to cause membrane perforation, suggesting a complex relationship between Aβ and NMDARs. Because previous studies have recognized that Aβ oligomers are able to increase membrane permeability and produce amyloid pores, the present study supports the conclusion that NMDARs play a critical protective role on Aβ actions in hippocampal neurons. These results could explain the lack of correlation between brain Aβ burden and clinically observed dementia.
Keywords: Alzheimer's disease, amyloid-beta, glutamate, glycine receptor, hippocampal neurons, membrane damage, membrane pore, NMDA receptor

Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder in the elderly [1]. AD manifests progressively with cognitive and behavioral impairments [2] characterized by loss of memory and learning [3]. One of the major histopathological markers of AD are the senile plaques formed by amyloid-β peptide (Aβ) aggregates [4]. These Aβ aggregates produce a complex cascade of eventsthat finally lead to synaptic failure and neuronal death [5].

A common hypothesis used to explain the toxicity induced by Aβ is the formation of amyloid pores in the plasma membrane. Regarding this, several studies from our and other laboratories support the notion that membrane disruptions are induced by Aβ [6–13]. Furthermore, Aβ perforation allows the entry of small molecules and ions, such as calcium, into the cells [6, 7, 12]. This sustained calcium influx increases the release in synaptic vesicles, leading to a delayed synaptic failure produced by vesicle depletion [6–8].

It is now believed that Aβ association and toxic actions at the membrane level can be affected by the lipid and protein composition [9, 12, 14–18].N-methyl-D-aspartate receptors (NMDARs) are excitatory ligand gated ion channels that have been described as important for some of the toxic effects induced by Aβ, similar to that observed with other membrane proteins like cellular prion, mGluR5, nicotinic receptor, and AβPP [9, 19–25]. The glutamate NMDA receptor is a tetramer composed of different subunit combinations (NR1, NR2A-D) allowing the influx of Na^+ and Ca^{2+} ions into the neurons resulting in excitation [26, 27]. The relationship between AD and NMDARs is widely postulated [28, 29], but the mechanisms involving this relationship are not fully understood.

One of the strongest evidence for the clinical relevance of such interactions between Aβ and NMDARs in AD is that the NMDA receptor antagonist, memantine, is used clinically in the treatment of AD [28]. In fact, other NMDAR antagonists, like (+)MK-801, or the removal of extracellular Ca^{2+}, reduced $A\beta_{1-40}$-induced Ca^{2+} transients, NO production and neurotoxicity in cultured neuroblastoma cells [30]. Moreover, (+) MK-801 partially prevented the decrease in cell viability and the energy impairment induced by $A\beta_{1-42}$ in HEK293 cells transiently expressing NR1/NR2A or NR1/NR2B subunits [29].

Regarding a potential interaction between Aβ and NMDAR, the data is controversial. While some authors indicate that the co-immunoprecipitation of Aβ dodecameric oligomers with NR1 and NR2A is evidence for their interaction [31], others have failed to detect binding of $A\beta_{1-42}$ to any known regulatory sites on glutamate receptors [28]. Furthermore, recent data indicates that such effects of $A\beta_{1-42}$ on NMDA receptors may be due to its binding to postsynaptic anchoring proteins such as PSD-95 or other membrane proteins like prion [21, 32, 33].

Here, in an attempt to clarify a potential role of NMDARs on $A\beta_{1-42}$-induced neurotoxicity, we examined the ability of the peptide to associate to and disrupt plasma membranes, something that has not been studied until now. The results indicate that NMDARs are an important factor controlling Aβ neurotoxicity.

Journal of Alzheimer's Disease, Vol 53, No. 1 (2016): pg. 197-207. DOI.

Materials and Methods

Primary cultures of rat hippocampal neurons

Hippocampal neurons were obtained from 18-day pregnant Sprague-Dawley rats and maintained for10-14 days *in vitro* (DIV) as previously described [34]. All animals were handled in strict accordance with NIH guidelines and approved by the Ethics Committee of the Universidad de Concepción (Concepción, Chile).

Peptide preparation and storage

Human $A\beta_{1-42}$ labeled with FAM (green fluorescence, Aβ-FAM) at its N-terminus, and unlabeled peptides were purchased from Anaspec (CA, USA). The preparation and storage were performed as previously reported by our lab [6]. Briefly, $A\beta_{1-42}$ was dissolved in DMSO (10mg/ml) and stored in aliquots at −20°C. To prepare Aβ oligomers (80 μM), aliquots of the peptide (250 μg in 25 μl of DMSO) were added to 700μl of PBS (Gibco, USA) and vertically agitated (200 RPM at 37°C) for 90min and stored at 4°C until use. Aβ-FAM was dissolved in DMSO (4mg/ml) and immediately stored in aliquots at −20°C.

Transfection

Neuronal transfection was performed using magnetofection with the reagent Neuromag as described by the protocol provided by the manufacturer (Oz Biosciences, France). To decrease the levels of NMDARs, siRNAs for NR1 and NR2B were co-transfected with GFP (2 μg total) to visualize the transfected neurons.

To increase the levels of NMDARs, HEK cells were transfected with the plasmids NR1, NR2B and GFP (2 μg total) using lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. The plasmids and siRNAs used in this study were previously described [35, 36].

Journal of Alzheimer's Disease, Vol 53, No. 1 (2016): pg. 197-207. DOI.

Electrophysiology

Electrophysiological recordings were carried out using the patch clamp technique as previously described [8, 37]. Briefly, culture media was changed for an external solution containing (in mM): 150 NaCl, 5.4 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose and 10 HEPES (pH 7.4). The internal solution consisted of (in mM): 120 KCl, 2.0 $MgCl_2$, 2 ATP-Na_2, 10 BAPTA, 0.5 GTP, 10 HEPES (pH 7.4). The holding potential was fixed at –60 mV and currents were acquired using a Digidata 1200 board and the pClamp10 software (Axon Instruments, Inc.). Recording pipettes were pulled from borosilicate glass (WPI, Sarasota, FL) on a horizontal puller (Sutter Instruments, Novato, CA) having a resistancebetween 5 and 10 MΩ. Perforated recordings were obtained as previously described [6, 8]. Briefly, Aβ was added to the pipette internal solution and a 5 mV pulse was used to monitor the perforation in cell attached configuration. For evoked current recordings, the experiments were performed at room temperature (20–25°C) using a membrane potential of –60 mV. Data are given as mean±S.E.M. and were obtained from more than 5 experiments. Recordings were performed in the presence of 100 nM TTX (tetrodotoxin) to inhibit action potentials.

Immunocytochemistry

Experiments were performed as previously described [9]. The primary antibodies used for 16 hwere: anti-MAP2, 1 : 400 (Santa Cruz Biotechnology, CA, USA); and NR2B, 1 : 200 (Covance, USA). The secondary antibodies conjugated withFITC (ExMax/EmMax = 500/517 nm), Cy3 (ExMax/EmMax = 545/570 nm), or Cy5 (ExMax/EmMax= 649/670 nm) (1 : 400, Jackson ImmunoResearch Laboratories, USA) were incubated for 2 h for fluorescent staining. Finally, samples were mounted in fluorescent mounting medium (DAKO, CA, USA) and images were obtained using a Nikon Eclipse confocal microscope (Nikon, Japan). The immunoreactivity of the proteins was quantified at primary processes with ImageJ software (NIH). Fluorescent signal was quantified as relative units (RU) using a region of interest (ROI).

Journal of Alzheimer's Disease, Vol 53, No. 1 (2016): pg. 197-207. DOI.

Western blots

Equal amounts of proteins were separated on 10–12% SDS-PAGE gels as previously described [7, 8]. Protein bands were transferred onto nitrocellulose membranes, blocked with 5% milk, and incubated with the primary antibody anti-NR2B (1 : 1000; Covance, USA) or *a*-tubulin (Sigma, USA). Immunoreactive bands were detected with secondary antibodies conjugated with HRP 1 : 5000 (Santa Cruz Biotechnology, CA, USA) and visualized with ECL Plus Western Blotting Detection System (PerkinElmer, MA, USA).

Data analysis

Non-lineal analysis was performed using Prism (Graph Pad). The analysis of the cluster size, number, and fluorescence intensity were performed using the ImageJ software package (NIH, USA) and using appropriate and similar intensity thresholds for cluster resolution. Membrane charge was analyzed by integrating the transient capacitative current after subtracting the pipette capacitance. The values are expressed as mean±SEM (standard error mean). Statistical differences were determined using Student's t test or ANOVA. The experiments were performed in triplicate.

Results

We wanted to evaluate the potential role of NMDARs in the mechanism of $A\beta_{1-42}$ association and perforation of the plasma membrane. Therefore, we used experimental protocols that could either decrease or increase the levels of NMDARs in the cell membrane before performing the assays. First, we used a mild enzymatic proteolytic digestion using trypsin (0.00025%, 30 min), a serine protease widely used to remove membrane proteins [38, 39], thus decreasing the levels of NMDARs in the membrane of hippocampal neurons as detected by immunocytochemistry against the NR2B subunit of the receptor (Fig. 1A). More detailed analyses of the immunofluorescence data showed that treatment of hippocampal neurons with trypsin decreased the NMDAR puncta number in primary neuronal processes (Fig. 1B). For instance, control values were 12±1.2 punctas, and these values decreased to 6±1.10 punctas with the

treatment. This result is in agreement with western blot analyses that also showed a decrease of 60±12% in NMDAR total levels after the treatment as compared to control (Fig. 1C,D). To evaluate if the treatment was actually reducing functional membrane NMDAR in the neurons treated with trypsin for 30 min, we performed electrophysiological recordings using patch clamp techniques. The neurons were stimulated with a large concentration of NMDA (100 μM) resulting in current amplitudes of several hundred pA. The results clearly showed a significant reduction in the amplitude of the NMDA-evoked current in trypsin-treated neurons (approximately 20%) as compared to the control condition (Fig. 1E). An internal control using the NMDAR antagonist, D-AP5 (100 μM), showed that the NMDA-evoked current was completely blocked by D-AP5, indicating that the evoked current was only mediated by NMDARs (Fig. 1E). As expected, the NMDA current density (pA/pF) was also significantly lowered in trypsin-treated neurons (4.4 ± 2 pA/pF)as compared to control cells (20 ± 2 pA/pF) (Fig. 1F). No differences were found in the values of membrane capacitance indicating that the treatment did not affect the size of the neurons (Fig. 1G). These data demonstrate that the use of a very low concentration of trypsin (0.00025%) is sufficient to decrease NMDAR levels in the neuronal membrane, making this experimental protocol a good tool for further assays which need decreased levels of this ion channel receptor.

The next step was to evaluate the degree of association of oligomeric $A\beta_{1-42}$ in control and trypsin treated neurons. Figure 2 shows a confocal micrograph of NR2B (red) and MAP2 (blue) together with the fluorescent signal of Aβ (1 h incubation with $A\beta_{1-42}$ coupled to FAM, a green fluorescent tag)in control and trypsin-treated cells. The overall analysis of this data shows that after treating the hippocampal neurons with trypsin, the NR2B signal was reduced and that of Aβ increased (Fig. 2A). The measurement of Aβ clusters in primary neurites corroborated the increase in Aβ-FAM association together with a decrease in NMDAR on trypsin-treated neurons (Fig. 2B). A more detailed analysis indicated that the Aβ-associated clusters were much larger after the treatment (Fig. 2C). In fact, trypsin-treated neurons resulted in an increase in the size of Aβ-FAM clusters together with the number of big clusters (Fig. 2C,D).

Furthermore, to characterize the role of NMDARs in Aβ association using more specific methodologies, we used siRNA for NMDAR subunits NR1 and NR2B to selectively decrease the NMDAR levels in these hippocampal neurons. Neuronal transfections for 48 h with siRNAs also reduced the amplitude of NMDA-evoked currents (100 μM) in patch clamp recordings (Fig. 3A). For instance, the data show that treatment with the siRNA decreased the amplitude of the NMDA current from 720 ± 96 to 347 ± 63 pA (Fig. 3B), resulting in a reduction of approximately 48%. After confirming that the siRNA reduced the expression of membrane NMDARs, we incubated the neurons for 1 h with Aβ-FAM to assess the association to the neuronal membrane (Fig. 3C). The data obtained with immunocytochemistry showed that the siRNA-transfected neurons had an increase in Aβ-FAM association, incrementing the puncta number and the intensity of Aβ-FAM signal in primary processes (Fig. 3D,E), similar to the results obtained in trypsin-treated neurons.

Previous studies have shown that following Aβ association to the plasma membrane, a process of membrane damage begins that produces an increase in membrane current (perforation) and intracellular calcium levels which lead to a delayed synaptic failure [6–8, 40]. Thus, the increase in membrane association of $A\beta_{1-42}$ to neurons depleted of membrane NMDARs could result in an increase in membrane damage. To determine if this was actually occurring, we performed electrophysiological experiments using perforated patch clamp recordings, as previously described [6, 8, 41] (see methods), and found that $A\beta_{1-42}$ increased the peak and charge of the capacitative current after 15 minutes of application in control neurons (Fig. 4A). The effect of $A\beta_{1-42}$ on the amplitude of the capacitative current, on the other hand, developed much quicker in neurons previously treated with trypsin to reduce NMDAR (Fig. 4A). The treatment, however, did not have any effect on the holding current. The time course of $A\beta_{1-42}$ effects on membrane charge in control and after trypsin shows that the treatment caused an increase in the perforation onset (Fig. 4B). Data show that the approximate $t_{1/2}$ of Aβ effect on the perforation was 24 min in control conditions and reduced to 17 min with the treatment. Furthermore, the analysis of the time to acquire an open configuration (perforation) in control and trypsin-treated neurons showed that the effect of $A\beta_{1-42}$ was concentration-dependent, with faster effects at higher concentrations (Fig. 4C). The

latter suggests that removal of membrane proteins, such as NMDARs, facilitates the membrane perforation induced by $A\beta_{1-42}$.

Next, we examined if increasing NMDARs in the cell membrane could have the opposite effect, thus increasing the association of Aβ and the consequent perforation of the membrane. Thus, we overexpressed and electrophysiologically characterizedthe NMDAR subunits NR1 and NR2B in HEK cells (Fig. 5A), and glycine receptor as a control (Fig. 5B), evaluating the association of Aβ-FAM after 1h incubation (Fig. 5C). Data showed that the Aβ-FAM association increased in the cells that overexpressed NMDARs, resulting in a reduced distance between the Aβ-FAM clusters (Control 0.55 ± 0.03, NMDA-R 0.24 ± 0.01 pixels) and an increase in their size (Control 0.46 ± 0.05, NMDA-R 0.97 ± 0.04 μm) (Fig. 5B,C), which is similar to that observed when the levels of NMDARs were decreased (Figs. 2, 3). On the other hand, overexpression of another membrane protein that is associated to inhibitory transmission, the glycine receptor (GlyR), did not produce any change in Aβ-FAM association to the plasma membrane indicating that the effect was selective for NMDARs (Fig. 5). Thereafter, we evaluated the membrane perforation induced by $A\beta_{1-42}$in cells overexpressing NMDARs or GlyRs (Fig. 6A). The data obtained showed that NMDAR overexpression blocked the membrane perforation induced by $A\beta_{1-42}$, while overexpression of GlyR was unable to affect the membrane charge transferred (Control 12.3 ± 5.3, Aβ 155.3 ± 14.3, NMDAR 25.4 ± 4.8, GlyR*α*1 135.9 ± 13.8 fC) (Fig. 6B). As a positive control for membrane perforation, we used a small peptide constructed with the native sequence of $A\beta_{11-17}$ that includes the two histidine residues 13 and 14 (EVHHQKL) [42] which blocked the perforation of the membrane induced by Aβ (Fig. 6B) [6, 41, 42]. The latter suggests that NMDAR presence in the membrane of the cells increases Aβ association but interferes with the perforation induced by the peptide.

Discussion

The presence of a direct or indirect interaction between Aβ and NMDARs is still under discussion, however, the role of NMDARs in AD is becoming more recognized [28]. Previous studies, for example, are focused on the use of NMDAR antagonists, like memantine or (+) MK-

801 as AD treatments [29, 30]. In fact, memantine is one of the few FDA approved drugs for AD [43, 44].

In the present study, we characterized a largely unexplored area of the NMDAR relationship with Aβ regarding membrane association and subsequent perforation and that might have clinical implications. The original idea was to evaluate if the association of Aβ to the plasma membrane was affected by altering the levels of functional NMDARs, thus, cells were treated with trypsin to reduce receptor levels, or transfected with plasmids containing NMDAR subunits to increase the receptor levels. Interestingly, both treatments resulted in higher association of Aβ to the cells, suggesting a more complex role of NMDARs than expected. A simple way of explaining these results of Aβ association is what we call the "forest effect", where the membrane proteins can be likened to trees covering the ground (lipids) (Fig. 7). Thus, decreasing the level of NMDARs, suggested to be important for the interaction of Aβ with the neuronal membrane [21, 31], might be facilitating its association to lipids previously not accessible, resulting in enhanced clustering (Figs. 2 and 3). On the other hand, the increase in Aβ association after the overexpression of NMDARs might be due to enhanced direct or indirect interactions with these excitatory receptors. In agreement, it is now believed that Aβ has promiscuous membrane interactions associating to cellular prion, nicotinic receptors, AβPP, and lipids like GM1 and cholesterol, thus affecting Aβ clustering [9, 15, 17, 19–25]. Therefore, the increased Aβ association after overexpression of NMDARs could be explained by the formation of Aβ/NMDAR complexes [20, 31]. Interestingly, this effect was specific for NMDARs since overexpression of GlyRs did not affect Aβ association to the membrane. This differential effect might be relevant for the disease because GlyRs, unlike NMDARs, are inhibitory proteins that are mainly expressed in spinal cord neurons [45, 46], which are believed to be largely unaffected by the disease.

Interestingly, although decreasing or increasing NMDARs resulted in similar increases in Aβ association to the membrane (clustering), our data showed that the functional impact of altering the levels of NMDARs in the membrane was quite different. For instance, decreasing NMDARs resulted in an accelerated rate of brain membrane damage revealed as an increase in membrane current in the presence of Aβ. This is remarkable because it demonstrates differences on the

Journal of Alzheimer's Disease, Vol 53, No. 1 (2016): pg. 197-207. DOI.

impact that Aβ association has on the plasma membrane. One plausible explanation is that the fine-tuning of Aβ interaction with membrane lipids and proteins affects its capacity to form amyloid pores. Thus, a membrane devoid of some types of proteins (i.e., NMDARs) might be more sensitive to Aβ-induced damage. It was reported that increasing protein levels in the membrane produced a reduction on its fluidity [47], thus our results might also be explained by a reduced level of membrane fluidity. Nevertheless, lipid rafts can also regulate Aβ association to the membrane [15, 17, 18]. For instance, cholesterol in lipid rafts can affect Aβ association and membrane damage by decreasing membrane fluidity as a consequence of reduced phospholipid movement in the bilayer [17, 48].

On the other hand, we found that the decrease in NMDAR levels by siRNAs or trypsin also increased Aβ association. In parallel, the perforation of the membrane was faster when compared to control cells (Fig. 5). We believe that by decreasing the levels of NMDARs, Aβ associates preferentially to membrane lipids facilitating the process of perforation [15, 16, 49]. For example, it is believed that GM1 mediates Aβ association, seeding, fibrillogenesis and membrane disruption [15, 49]. These results could very well explain why some people with high levels of Aβ in the brain do not show dementia and vice versa. In other words, some proteins might be buffering the levels of toxic Aβ [50].

In conclusion, our data support the role of NMDARs as an important mediator for Aβ association and damage/perforation in the plasma memb-rane, actively participating in the membrane toxicity induced by Aβ. These results could explain the lack of correlation between brain Aβ burden and clinically observed dementia.

Acknowledgments

We thank Lauren Aguayo for revising the manuscript and for technical assistance, and Cecilia González for graphic design. This work was supported by FONDECYT grant N° 1140473. EF is a CONICYT fellow.

Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/16-0170r1).

References

[1] Evans DA , Funkenstein HH , Albert MS , Scherr PA , Cook NR , Chown MJ , Hebert LE , Hennekens CH , Taylor JO ((1989)) Prevalence of Alzheimer's disease in a community population of older persons. Higher than previously reported. *JAMA* 262:, 2551–2556.

[2] Faber-Langendoen K , Morris JC , Knesevich JW , LaBarge E , Miller JP , Berg L ((1988)) Aphasia in senile dementia of the Alzheimer type. *Ann Neurol* 23:, 365–370.

[3] Spires TL , Hyman BT ((2005)) Transgenic models of Alzheimer's disease: Learning from animals. *NeuroRx* 2:, 423–437.

[4] Ittner LM , Gotz J ((2011)) Amyloid-beta and tau–a toxic pas de deux in Alzheimer's disease. *Nat Rev Neurosci* 12:, 65–72.

[5] Selkoe DJ ((2002)) Alzheimer's disease is a synaptic failure. *Science* 298:, 789–791.

[6] Sepulveda FJ , Parodi J , Peoples RW , Opazo C , Aguayo LG ((2010)) Synaptotoxicity of Alzheimer beta amyloid can be explained by its membrane perforating property. *PLoS One* 5:, e11820.

[7] Parodi J , Sepulveda FJ , Roa J , Opazo C , Inestrosa NC , Aguayo LG ((2009)) Beta-amyloid causes depletion of synaptic vesicles leading to neurotransmission failure. *J Biol Chem* 285:, 2506–2514.

[8] Peters C , Fernandez-Perez EJ , Burgos CF , Espinoza MP , Castillo C , Urrutia JC , Streltsov VA , Opazo C , Aguayo LG ((2013)) Inhibition of amyloid beta-induced synaptotoxicity by a pentapeptide derived from the glycine zipper region of the neurotoxic peptide. *Neurobiol Aging* 34:, 2805–2814.

[9] Peters C , Espinoza MP , Gallegos S , Opazo C , Aguayo LG ((2015)) Alzheimer's Abeta interacts with cellular prion protein inducing neuronal membrane damage and synaptotoxicity. *Neurobiol Aging* 36:, 1369–1377.

[10] Arispe N , Rojas E , Pollard HB ((1993)) Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: Blockade by tromethamine and aluminum. *Proc Natl Acad Sci U S A* 90:, 567–571.

[11] Arispe N , Pollard HB , Rojas E ((1996)) Zn2+ interaction with Alzheimer amyloid beta protein calcium channels. *Proc Natl Acad Sci U S A* 93:, 1710–1715.

[12] Kawahara M , Ohtsuka I , Yokoyama S , Kato-Negishi M , Sadakane Y ((2011)) Membrane incorporation, channel formation, and disruption

of calcium homeostasis by Alzheimer's beta-amyloid protein. *Int J Alzheimers Dis* 2011:, 304583.

[13] Quist A , Doudevski I , Lin H , Azimova R , Ng D , Frangione B , Kagan B , Ghiso J , Lal R ((2005)) Amyloid ion channels: A common structural link for protein-misfolding disease. *Proc Natl Acad Sci U S A* 102:, 10427–10432.

[14] Lauren J , Gimbel DA , Nygaard HB , Gilbert JW , Strittmatter SM ((2009)) Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature* 457:, 1128–1132.

[15] Kakio A , Nishimoto SI , Yanagisawa K , Kozutsumi Y , Matsuzaki K ((2001)) Cholesterol-dependent formation of GM1 ganglioside-bound amyloid beta-protein, an endogenous seed for Alzheimer amyloid. *J Biol Chem* 276:, 24985–24990.

[16] Yanagisawa K , Odaka A , Suzuki N , Ihara Y ((1995)) GM1 ganglioside-bound amyloid beta-protein (A beta): A possible form of preamyloid in Alzheimer's disease. *Nat Med* 1:, 1062–1066.

[17] Di Scala C , Troadec J-D , Lelièvre C , Garmy N , Fantini J , Chahinian H ((2014)) Mechanism of cholesterol-assisted oligomeric channel formation by a short Alzheimerβ-amyloid peptide. *J Neurochem* 128:, 186–195.

[18] Arispe N , Doh M ((2002)) Plasma membrane cholesterol controls the cytotoxicity of Alzheimer's disease AbetaP (1-40) and (1-42) peptides. *FASEB J* 16:, 1526–1536.

[19] Lorenzo A , Yuan M , Zhang Z , Paganetti PA , Sturchler-Pierrat C , Staufenbiel M , Mautino J , Vigo FS , Sommer B , Yankner BA ((2000)) Amyloid beta interacts with the amyloid precursor protein: A potential toxic mechanism in Alzheimer's disease. *Nat Neurosci* 3:, 460–464.

[20] Um Ji W , Kaufman Adam C , Kostylev M , Heiss Jacqueline K , Stagi M , Takahashi H , Kerrisk Meghan E , Vortmeyer A , Wisniewski T , Koleske Anthony J , Gunther Erik C , Nygaard Haakon B , Strittmatter Stephen M ((2013)) Metabotropic glutamate receptor 5 is a coreceptor for Alzheimer Aβ oligomer bound to cellular prion protein. *Neuron* 79:, 887–902.

[21] Um JW , Nygaard HB , Heiss JK , Kostylev MA , Stagi M , Vortmeyer A , Wisniewski T , Gunther EC , Strittmatter SM ((2012)) Alzheimer amyloid-beta oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. *Nat Neurosci* 15:, 1227–1235.

[22] Fu W ((2003)) β-amyloid peptide activates non- 7 nicotinic acetylcholine receptors in rat basal forebrain neurons. *J Neurophysiol* 90:, 3130–3136.

[23] Cullen WK , Wu J , Anwyl R , Rowan MJ ((1996)) β-Amyloid produces a delayed NMDA receptor-dependent reduction in synaptic transmission in rat hippocampus. *Neuroreport* 8:, 87–92.

[24] He Y , Cui J , Lee JCM , Ding S , Chalimoniuk M , Simonyi A , Sun AY , Gu Z , Weisman GA , Gibson Wood W , Sun GY ((2011)) Prolonged exposure of cortical neurons to oligomeric amyloid-β impairs NMDA receptor function via NADPH oxidase-mediated ROS production: Protective effect of green tea (−)-epigallocatechin-3-gallate. *ASN Neuro* 3:, 13–24.

[25] Li S , Jin M , Koeglsperger T , Shepardson NE , Shankar GM , Selkoe DJ ((2011)) Soluble Aβ oligomers inhibit long-term potentiation through a mechanism involving excessive activation of extrasynaptic NR2B-containing NMDA receptors. *J Neurosci* 31:, 6627–6638.

[26] Chaffey H , Chazot PL ((2008)) NMDA receptor subtypes: Structure, function and therapeutics. *Curr Anaesth Crit Care* 19:, 183–201.

[27] Regehr WG , Tank DW ((1990)) Postsynaptic NMDA receptor-mediated calcium accumulation in hippocampal CA1 pyramidal cell dendrites. *Nature* 345:, 807–810.

[28] Danysz W , Parsons CG ((2012)) Alzheimer's disease,β-amyloid, glutamate, NMDA receptors and memantine - searching for the connections. *Br J Pharmacol* 167:, 324–352.

[29] Domingues A , Almeida S , da Cruz e Silva EF , Oliveira CR , Rego AC ((2007)) Toxicity of beta-amyloid in HEK293 cells expressing NR1/NR2A or NR1/NR2B N-methyl-D-aspartate receptor subunits. *Neurochem Int* 50:, 872–880.

[30] Le WD , Colom LV , Xie WJ , Smith RG , Alexianu M , Appel SH ((1995)) Cell death induced by beta-amyloid 1-40 in MES 23.5 hybrid clone: The role of nitric oxide and NMDA-gated channel activation leading to apoptosis. *Brain Res* 686:, 49–60.

[31] Venkitaramani DV , Chin J , Netzer WJ , Gouras GK , Lesne S , Malinow R , Lombroso PJ ((2007)) Beta-amyloid modulation of synaptic transmission and plasticity. *J Neurosci* 27:, 11832–11837.

[32] De Felice FG , Velasco PT , Lambert MP , Viola K , Fernandez SJ , Ferreira ST , Klein WL ((2007)) Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. *J Biol Chem* 282:, 11590–11601.

[33] Lacor PN , Buniel MC , Furlow PW , Clemente AS , Velasco PT , Wood M , Viola KL , Klein WL ((2007)) Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. *J Neurosci* 27:, 796–807.

[34] Aguayo LG , Pancetti FC ((1994)) Ethanol modulation of the gamma-aminobutyric acidA- and glycine-activated Cl- current in cultured mouse neurons. *J Pharmacol Exp Ther* 270:, 61–69.

[35] Bustos FJ , Varela-Nallar L , Campos M , Henriquez B , Phillips M , Opazo C , Aguayo LG , Montecino M , Constantine-Paton M , Inestrosa NC , van Zundert B ((2014)) PSD95 suppresses dendritic arbor development in mature hippocampal neurons by occluding the clustering of NR2B-NMDA receptors. *PLoS One* 9:, e94037.

[36] Sepulveda FJ , Bustos FJ , Inostroza E , Zuniga FA , Neve RL , Montecino M , van Zundert B ((2010)) Differential roles of NMDA Receptor Subtypes NR2A and NR2B in dendritic branch development and requirement of RasGRF1. *J Neurophysiol* 103:, 1758–1770.

[37] Peters C , Munoz B , Sepulveda FJ , Urrutia J , Quiroz M , Luza S , De Ferrari GV , Aguayo LG , Opazo C ((2011)) Biphasic effects of copper on neurotransmission in rat hippocampal neurons. *J Neurochem* 119:, 78–88.

[38] Lepke S , Passow H ((1976)) Effects of incorporated trypsin on anion exchange and membrane proteins in human red blood cell ghosts. *Biochim Biophys Acta* 455:, 353–370.

[39] Molloy MP , Herbert BR , Walsh BJ , Tyler MI , Traini M , Sanchez JC , Hochstrasser DF , Williams KL , Gooley AA ((1998)) Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. *Electrophoresis* 19:, 837–844.

[40] Peters C , Espinoza MP , Gallegos S , Opazo C , Aguayo LG ((2015)) Alzheimer's Aβ interacts with cellular prion protein inducing neuronal membrane damage and synaptotoxicity. *Neurobiol Aging* 36:, 1369–1377.

[41] Sepúlveda FJ , Fierro H , Fernandez E , Castillo C , Peoples RW , Opazo C , Aguayo LG ((2014)) Nature of the neurotoxic membrane actions of amyloid-β on hippocampal neurons in Alzheimer's disease. *Neurobiol Aging* 35:, 472–481.

[42] Diaz JC , Linnehan J , Pollard H , Arispe N ((2006)) Histidines 13 and 14 in the Abeta sequence are targets for inhibition of Alzheimer's disease Abeta ion channel and cytotoxicity. *Biol Res* 39:, 447–460.

[43] Reisberg B , Doody R , Stoffler A , Schmitt F , Ferris S , Mobius HJ ((2006)) A 24-week open-label extension study of memantine in moderate to severe Alzheimer disease. *Arch Neurol* 63:, 49–54.

[44] Doody RS , Tariot PN , Pfeiffer E , Olin JT , Graham SM ((2007)) Meta-analysis of six-month memantine trials in Alzheimer's disease. *Alzheimers Dement* 3:, 7–17.

[45] Aguayo LG , Castro P , Mariqueo T , Munoz B , Xiong W , Zhang L , Lovinger DM , Homanics GE ((2014)) Altered sedative effects of ethanol in mice with alpha1 glycine receptor subunits that are insensitive to Gbetagamma modulation. *Neuropsychopharmacology* 39:, 2538–2548.

[46] Mariqueo TA , Agurto A , Munoz B , San Martin L , Coronado C , Fernandez-Perez EJ , Murath P , Sanchez A , Homanics GE , Aguayo LG ((2014)) Effects of ethanol on glycinergic synaptic currents in mouse spinal cord neurons. *J Neurophysiol* 111:, 1940–1948.

[47] De Paillerets C , Gallay J , Alfsen A ((1984)) Effect of cholesterol and protein content on membrane fluidity and 3 beta-hydroxysteroid dehydrogenase activity in mitochondrial inner membranes of bovine adrenal cortex. *Biochim Biophys* Acta 772:, 183–191.

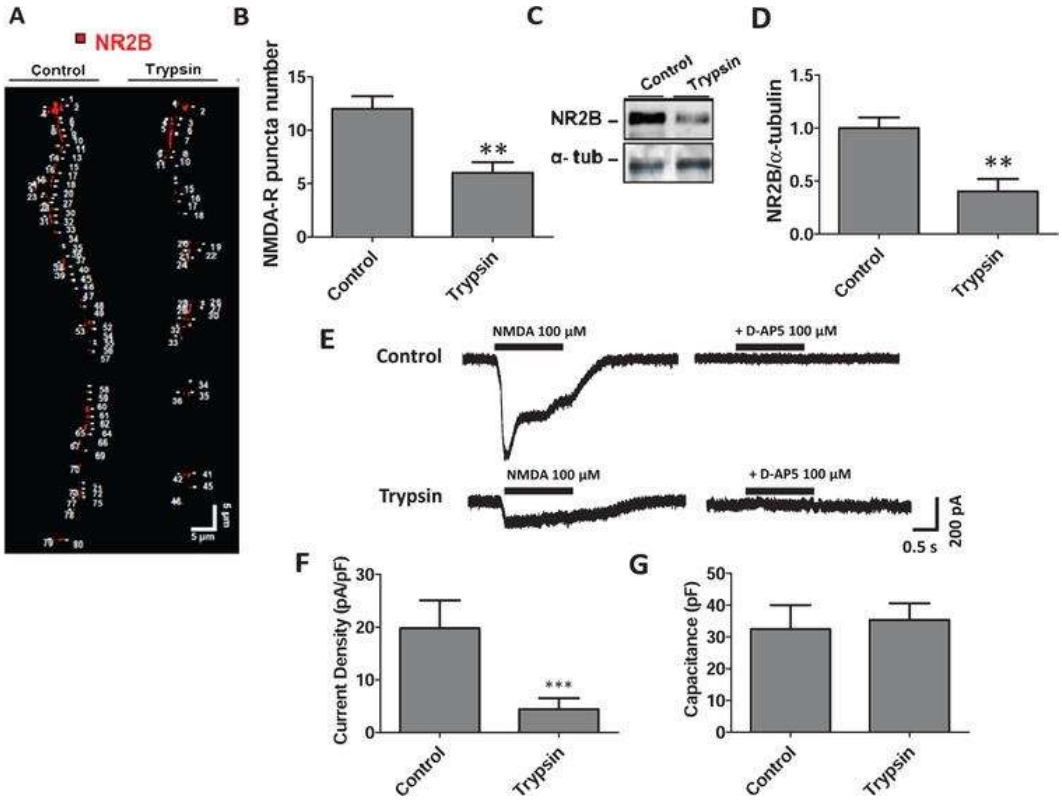
[48] Kawahara M , Kuroda Y ((2001)) Intracellular calcium changes in neuronal cells induced by Alzheimer's beta-amyloid protein are blocked by estradiol and cholesterol. *Cell Mol Neurobiol* 21:, 1–13.

[49] Chi EY , Frey SL , Lee KY ((2007)) Ganglioside G(M1)-mediated amyloid-beta fibrillogenesis and membrane disruption. *Biochemistry* 46:, 1913–1924.

[50] Forman MS , Mufson EJ , Leurgans S , Pratico D , Joyce S , Leight S , Lee VM , Trojanowski JQ ((2007)) Cortical biochemistry in MCI and Alzheimer disease: Lack of correlation with clinical diagnosis. *Neurology* 68:, 757–763.

Figures and Tables

Fig. 1. Treatment with trypsin decreased NMDARs in hippocampal neurons. A) Microphotograph shows NMDAR (NR2B, red) in neuronal primary processes of cultures treated without or with trypsin (0.00025%, 30 min). B) Quantification of NMDAR puncta number shows a decrease in trypsin-treated neurons (0.00025%, 30 min) versus control (not treated). C) Western blot showing the levels of NR2B in control and trypsin-treated neurons (0.00025%, 30 min). *α*-tubulin was used as a loading control. D) Quantification of NR2B levels from the western blot in control and treated neurons. E) Representative evoked currents using NMDA (100 μM) and NMDA plus D-AP5 (100 μM) in control neurons and pre-treatment with trypsin 0.00025% for 30 min. Black bar represents the time of perfusion. F) Plot of current density (pA/pF) in control and trypsin pre-treated neurons showing the current decrease in trypsin-treated neurons. G) The graph shows the capacitance (pF) in control and trypsin-treated cells. $^{**}p<0.01$; $^{***}p<0.001$.



Journal of Alzheimer's Disease, Vol 53, No. 1 (2016): pg. 197-207. DOI.

Fig. 2. Trypsin treatment decreased NMDARs and increased Aβ association in hippocampal neurons. A) Immunofluorescence microphotograph showing the association of Aβ-FAM (1 μM, 1 h) to hippocampal neurons pre-treated with or without trypsin (0.00025%, 30 min). NMDA receptor is shown in red (NR2B subunit), Aβ-FAM in green and MAP2 in blue. Scale bar represents 20 μm. B) Representative images of neuronal primary processes (10 μm) showing the levels of NMDAR (red) and the association of Aβ-FAM (green, 1 h, 1 μM) in control and trypsin pre-treated (0.00025%, 30 min) neurons. The panel at the right displays a zoom of the dendrite. C) Plot shows the quantification of Aβ-FAM cluster size (μm). D) Graph illustrates the relationship between the number and size (nm) of Aβ-FAM clusters in control and treated neurons. $^{**}p < 0.01$.

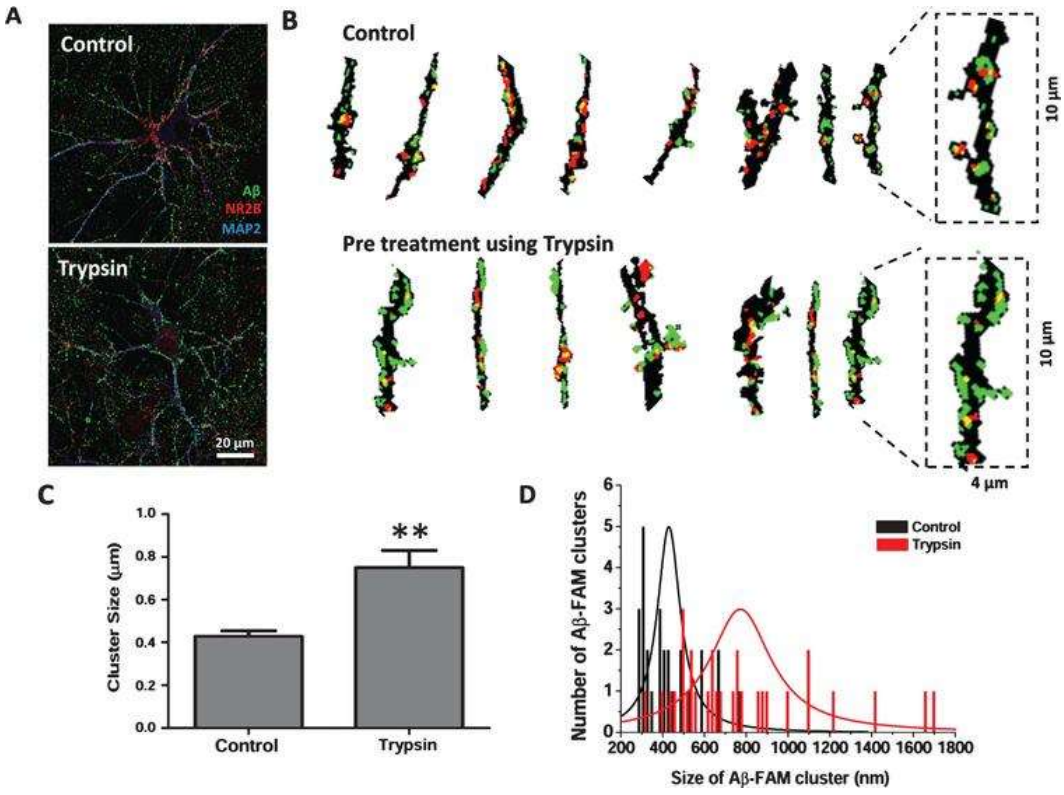


Fig. 3. NMDARs affect Aβ association in hippocampal neurons. A) Representative traces of NMDA-evoked currents in control and after transfection with siRNAs geared towards the NMDAR (NR1 and NR2B subunits). The black bar represents the time of NMDA perfusion (100 μM). B) Plot showing the decrease in the amplitude of NMDA-evoked currents for the siRNA-transfected neurons versus control. C) Immunofluorescence showing Aβ-FAM (green) association (1 μM, 1h) to control and siRNA transfected neurons. MAP2 stained the neurons (blue) and mCherry was used as a control for transfection (red). The white bottom bar represents 20 μm of length. D, E) Plots show the Aβ-FAM puncta number and fluorescence intensity in primary processes (20 μm) for control and transfected neurons. $^{*}p<0.05$, $^{**}p<0.01$.

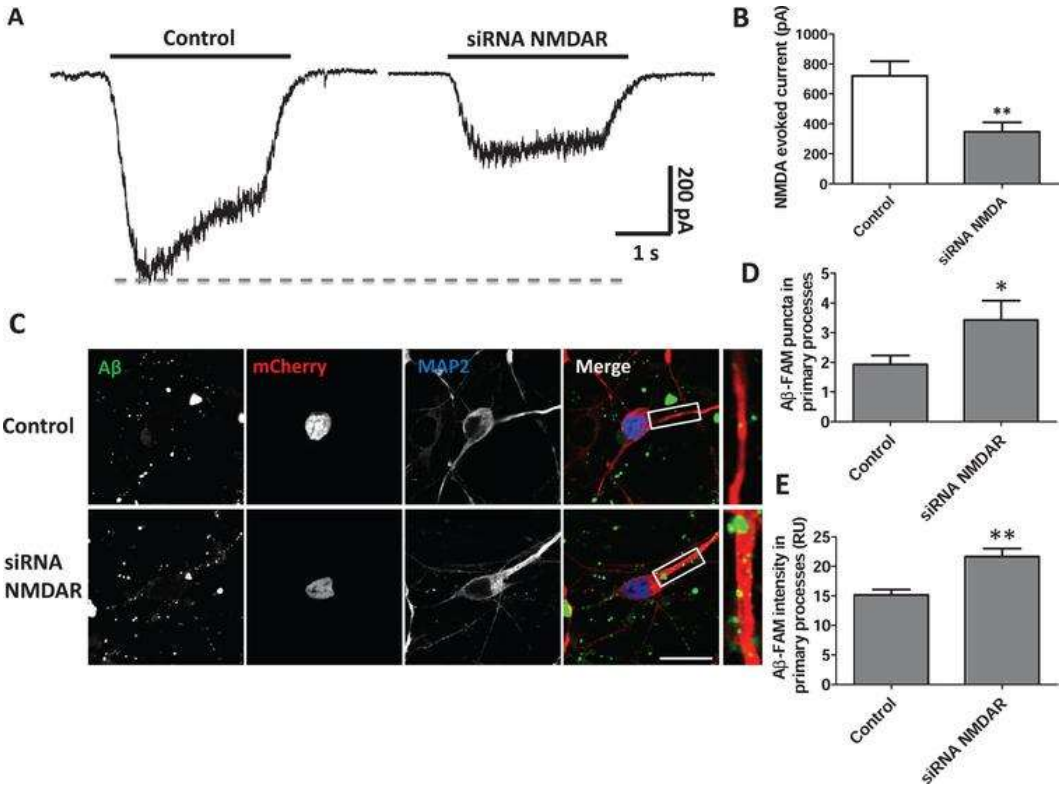
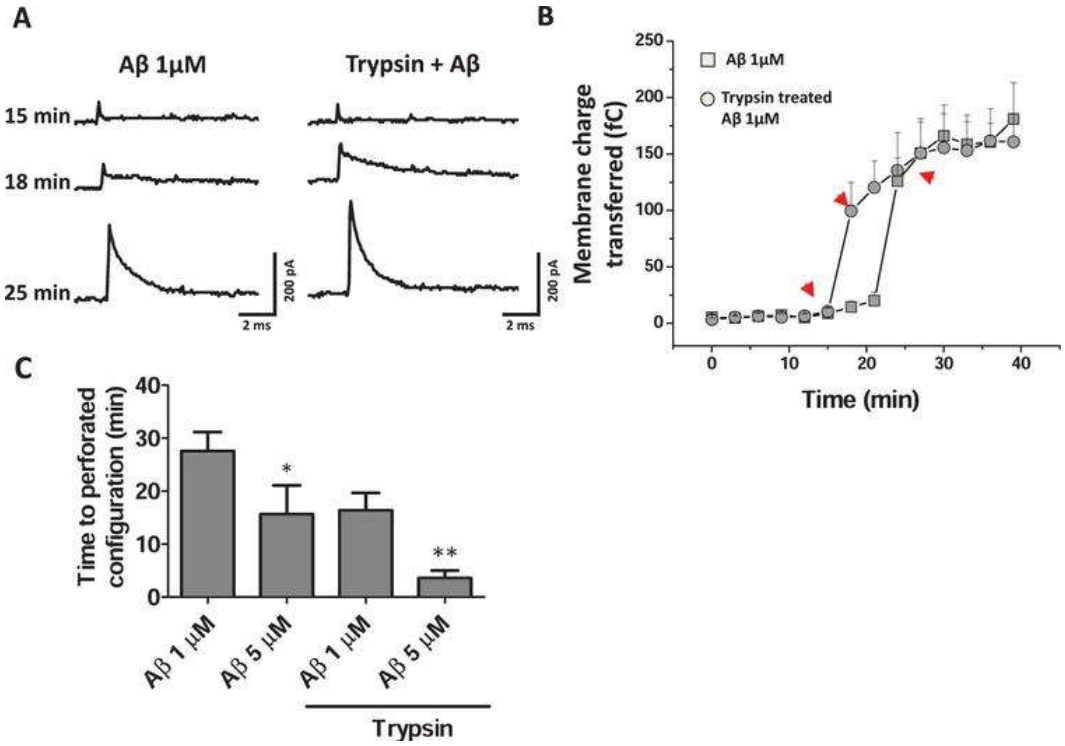
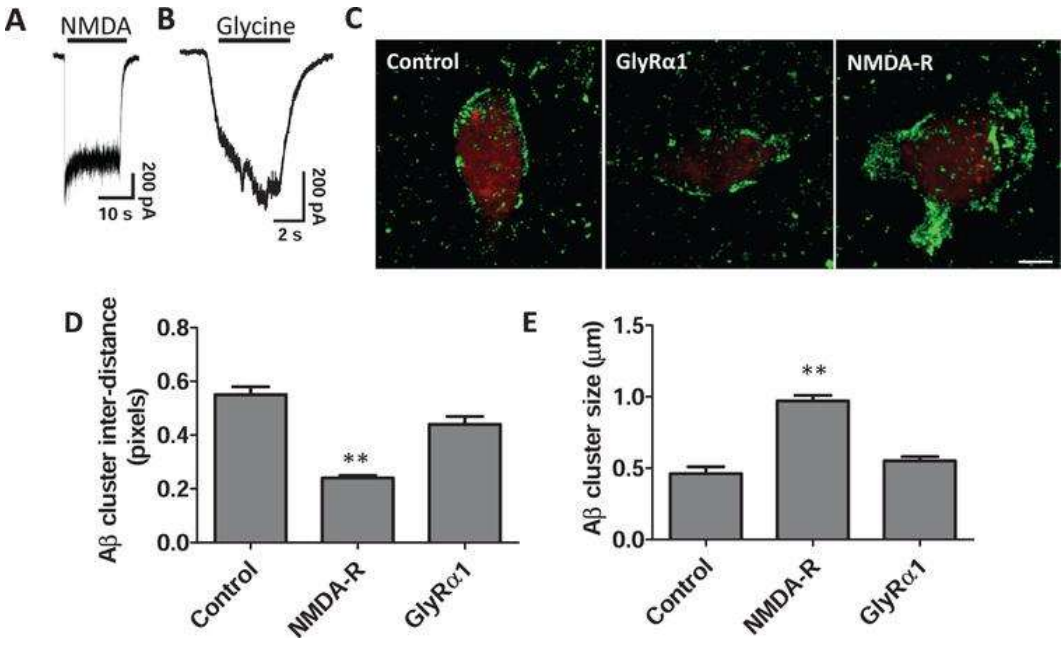


Fig. 4. Trypsin treatment decreased the time to perforated configuration induced by Aβ. A) Representative capacitative currents (5 mV) in the perforated configuration using Aβ (1 μM) in the pipette in HEK cells. B) Plot showing the charge transferred through the membrane of the cell in a time dependent manner, showing that Aβ perforates the membrane faster in trypsin pre-treated cells (0.00025%, 30 min) than in control cells without any treatments. Arrow heads (red) indicate the times of the recordings shown in panel A. C) Graph showing the time needed by Aβ to acquire a perforation configuration. The trypsin pre-treated cells had a faster perforation time. $^{*}p<0.05$, $^{**}p<0.01$.



Journal of Alzheimer's Disease, Vol 53, No. 1 (2016): pg. 197-207. DOI.

Fig. 5. NMDAR overexpression increased Aβ-FAM clustering in HEK cells. A) The trace shows NMDA-evoked current in HEK cells transfected with NR1 and NR2B. B) The trace shows a glycine evoked current in HEK cells transfected with the alpha subunit of the glycine receptor (GlyR*α*1). C) Microphotograph showing Aβ-FAM association (1 μM, 1h) to HEK cells overexpressing NMDAR (NR1/NR2B), GlyR*α*1 and RFP as a control for transfection. D) Plot showing the Aβ-FAM cluster inter-distance. E) Graph showing the increase in Aβ-FAM cluster size in cells that overexpress NMDAR. $^{**}p < 0.01$.



Journal of Alzheimer's Disease, Vol 53, No. 1 (2016): pg. 197-207. DOI.

Fig. 6. NMDAR overexpression inhibited the Aβ-induced membrane perforation. A) Scheme representing the perforated patch clamp configuration used in the experiment. Aβ is added to the patch pipette with the internal solution and the cell-attached configuration is acquired. The recorded cells were previously transfected with GFP, NMDAR or GlyR*α*1. B) Plot showing the membrane charge transferred (fC) in control HEK cells and HEK cells transfected with GFP, NMDAR or GlyR*α*1 after 20 min in the presence of Aβ (1 μM). The cells that overexpressed NMDARs were resistant to Aβ. Na7, a peptide that blocks the amyloid pore, was used as a control for the Aβ perforation. $^{**}p<0.01$, $^{***}p<0.001$.

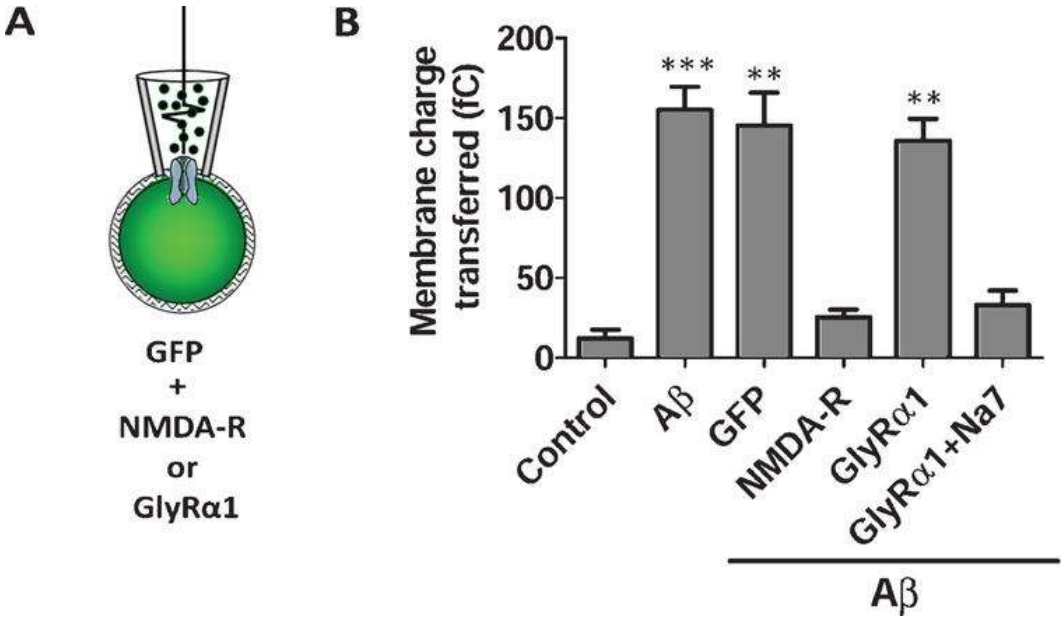
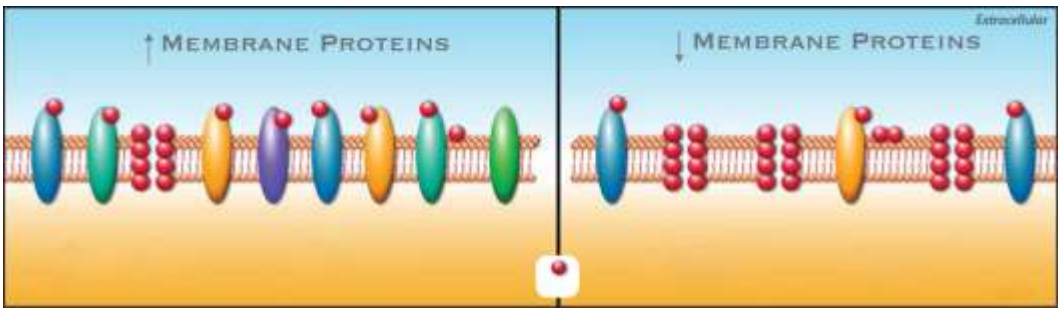


Fig. 7. "Forest effect" of membrane proteins involved in Aβ association and membrane perforation. A) The increase in membrane protein levels, such as NMDAR, augments the association of Aβ (red circles) to these proteins, decreasing the association of Aβ to membrane lipids and therefore the perforation induced by Aβ. B) The decrease in membrane proteins, i.e., NMDAR, results in an increase in Aβ association to the lipids of the plasma membrane, thus increasing the perforation induced by Aβ.



Correspondence: [*] Correspondence to: Dr. Luis G. Aguayo, Department of Physiology, Universidad de Concepción, P.O. Box 160-C, Concepción, Chile. Tel.: +56 41 2203380; Fax: +56 41 2245975; E-mail: laguayo@udec.cl.