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ORIGINAL ARTICLE

Liver X Receptor Agonist GW3965 Regulates Synaptic Function upon Amyloid Beta Exposure in Hippocampal Neurons

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Abstract

Alzheimer's disease (AD) is a devastating neurodegenerative disease characterized by beta-amyloid (Aβ) accumulation and neurofibrillary tangles formation in the brain which are associated to synaptic deficits and dementia. Liver X receptor (LXR) agonists have been demonstrated to revert of pathologic and cognitive defects in murine models of AD through the regulation of Apolipoprotein E, *ATP-Binding Cassette A1* (ABCA1), by dampening neuroinflammation and also by reducing the levels of amyloid-β (Aβ) accumulation in the brain. However, the role of LXR with regard to the regulation of synaptic function remains relatively understudied. In the present paper, we analyzed the in-vitro effect of the LXR agonist GW3965 on synaptic function upon exposure of primary hippocampal cultures to oligomeric amyloid-β (oAβ(1–42)). We showed that oAβ(1–42) exposure significantly decreased the density of mature (mushroom shaped) dendritic spines density and synaptic contacts number. oAβ(1–42) also modulates the expression of pre- (VGlut1, SYT1, SV2A) and post-synaptic (SHANK2, NMDA) proteins, it decreases the expression of PINK1, and increases ROCKII, and activates of caspase-3; these changes were prevented by the pre-treating neuronal cultures with GW3965. These results show further support the role of the LXR agonist GW3965 in synaptic physiology and highlight its potential as an alternative pharmacological strategy for AD.

Keywords Alzheimer's disease · Hippocampal neurons · Synapsis · Amyloid-β · Liver X receptors · GW3965

Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the world (Kukull et al. 2002). The amyloid cascade hypothesis postulates that the accumulation of amyloid beta peptides (Aβ) either as monomers, oligomers or fibrils, promotes a series of events which are finally detrimental to neurons. These include tangle formation, neurite dystrophy, synaptic deficits, neuronal death and dementia (Hardy and Allsop 1991). So far, no clear therapeutic alternatives targeting one of these events and able to delay AD progression have been designed (Armstrong 2011).

Dendritic spines represent the main synaptic inputs in neurons (Yuste 2015; Hering and Sheng 2001) and are considered highly specialized dynamic structures able to acquire different shapes, sizes and densities as a result of changes in the functional properties of neurons (Arellano et al. 2007; Sorra and Harris 2000). The process of dendritic spines maturation follows a continuum depending on their synaptic activity from an immature spine (less synaptically active or filopodium shaped), intermediate spine (thin and stubby shaped), to mature spines (more synaptically active or mushroom shaped) (Hering and Sheng 2001). Dendritic spines have several distinctive characteristics: (1) they are mainly composed by an actin cytoskeleton, with varying ratios of G-actin/F-actin depending on their morphology and functional activity (Cingolani and Goda 2008; Korobova and Svitkina 2010); (2) they are characterized by the presence of a presynaptic compartment enriched in synaptic vesicles and diverse associated proteins such as the vesicular glutamate transporter 1 (VGlut1) which is important for the transport of glutamate inside vesicles; and by the Synaptic Vesicle Protein 2A

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(SV2A) and Synaptotagmin-1 (SYT1), critical for the release of neurotransmitters; and (3) a post-synaptic compartment characterized by the presence of a post-synaptic density (PSD) structure composed by many proteins including post-synaptic density-95 protein (PSD-95), ionotropic (NMDA and AMPA) and metabotrophic (mGluR5) glutamate receptors, and the PSD scaffold protein SHANK2 (SH3 and Kyrin repeat domain protein 2) (Tashiro and Yuste 2003).

Recently, the impact of Aβ on synapsis biology and function has raised increasing interest. Literature widely report that Aβ exposure caused alterations in neuronal synaptic plasticity (Li et al. 2010), in synaptic proteins, and in number and morphology of dendritic spines and synaptic contacts (Lacor et al. 2007; Knobloch and Mansuy 2008; Bittner et al. 2010; Penzes et al. 2011). In addition, Aβ exposure has also been associated with changes in neurotransmitter transporters (Chen et al. 2011) and glutamate receptors (Gong et al. 2009; Dewar et al. 1991) expression, which were in part mediated by the interaction of Aβ with the prion protein (Laurén et al. 2009). As mitochondrial impairment is an early pathological feature of AD, the impact of Aβ exposure on neuronal survival pathways that regulate the proper mitochondrial function and dynamics such as the PI3K/AKT and PINK1/PARKIN pathways is still unknown. It has been demonstrated that absence of PINK1 cause alterations in the NMDA excitatory postsynaptic currents (Pearlstein et al. 2016), and recently it was shown that PINK1 signaling rescues amyloid pathology in models of AD (Du et al. 2017), suggesting a possible link between glutamate receptors signaling, Aβ exposure and PINK1 function. All these changes in the synaptic biology and neuronal survival pathways associated with neuronal exposure to Aβ may be important during the early stages of AD, and could be a potential target for new therapeutic strategies for the disease (Citron 2010).

It has been recently demonstrated that the member of the nuclear receptor superfamily of transcription factor liver X receptor (LXR) agonists, revert pathologic and cognitive changes in murine models of AD through the regulation of the expression of Apolipoprotein E and *ATP-Binding Cassette A1* (ABCA1), and also by reducing neuroinflammation and Aβ levels in the brain (Donkin et al. 2010; Jiang et al. 2008; Sandoval-Hernández et al. 2016). In addition, LXR agonists promote synaptic plasticity and neurorestauration in stroke models (Chen et al. 2010; Cui et al. 2013), while in Parkinson's disease models they protect dopaminergic neurons against diverse toxic insults (Dai et al. 2012). However, the role of LXR agonist on synaptic function regulation in AD models still remains understudied. By using the triple transgenic mouse model of AD (3xTg-AD), we have recently demonstrated that the LXR agonist GW3965 restores cognitive deficits by regulating gene expression and, in particular, apolipoprotein E (APOE) and ATP-binding cassette transporter (ABCA1). The cognitive improvement conferred by the LXR agonist was associated with the modulation of the methylation status of different genes and notably, the most important changes were observed in synaptic genes such as Synapsin-1 (Syn-1) (Sandoval-Hernández et al. 2016).

In the present paper, we analyzed the in vitro effect of a LXR agonist (GW3965) on the synaptic function of primary hippocampal neurons upon exposure to amyloid-β oligomers (oAβ(1–42)), the main pathogenic form of Aβ (Haass and Selkoe 2007). We demonstrate that oAβ(1–42) exposure significantly decreases the density of mature dendritic spines (mushroom shape) and the number of synaptic contacts, and alters the expression of pre- (VGlut, SYT1, SV2A) and post-synaptic (SHANK2, NMDA) proteins. In addition, oAβ(1–42) also caused a decrease in the expression of PTEN-induced kinase-1 (PINK1), which is critical for mitochondrial dynamics and neuroprotection, and an increased in ROCKII and active caspase-3. Notably, pre-treatment of neurons with GW3965 modulate all these changes associated to oAβ(1–42) exposure, further supporting the role of LXR agonist as a potential pharmacological alternative for the treatment of AD.

Materials and Methods

Ethical Approval All animal procedures were performed in concordance with the Animal Research Reporting in Vivo Experiments (ARRIVE) guidelines (Kilkenny et al. 2010). In addition to following the Guide for the Care and Use of Laboratory Animals, 8th edition, published by the National Institutes of Health (NIH) and procedures were conducted according to Colombian standards (law 84/1989 and resolution 8430/ 1993). All procedures were approved by Ethics Committee for Animal Experimentation of the Universidad Nacional de Colombia, Bogotá-Colombia.

Primary Culture of Hippocampal Neurons Primary cultures of hippocampal neurons were directly cultured from C57BL/6 E18 pups, as described previously (Frassoni et al. 2005; Beaudoin et al. 2012). Briefly, hippocampus were dissociated and collected by trypsin treatment (0.5% durante 15 min at 37 °C), followed by trituration using a Pasteur pipette. Dissociated cells were then seeded in glass coverslips previously treated with poly-L-lysine, at a density of 90,000 cells on 24 mm diameter coverslip and 50,000 cells in 18 mm diameter coverslips. Cells were grown in Neurobasal medium supplemented with B27 (Gibco, USA), 1% penicilin/streptomicin (Sigma, USA), ultra-glutamine (Lonza, USA). Cells were grown for 15 days in vitro (DIV), ensuring the medium was changed by half every 3 days.

Preparation of Aβ(1–42) Oligomers and GW3965 Aβ(1–42) oligomers (oAβ) were prepared as previously described. Briefly, Aβ(1–42) *HCl* salt peptides (*American Peptide Company*) were diluted in 50 μl of cold dimetilsulfoxide (DMSO, Biotium # 90082) to a final concentration of 2 mM; aliquots were frozen until use. On the day of the experiment, 1 μL of the initial Aβ(1–42) solution was further diluted in 99 μL of aggregation buffer (100 mM $NaHPO_4$ + 50 mM NaCl). The preparation was left at room temperature for 20 min and then added to the neurons at a final concentration of 200 nM for 6 h.

GW3965 was prepared as previously described (Cui et al. 2013). GW3965 hydrochloride (Sigma # G6295 5 mg) ($C_{33}H_{31}F_3ClNO_3 \cdot HCl$) was diluted in 161.6 μL of DMSO at a final concentration of 50 mM, aliquoted and frozen until use. On the day of the experiment, the aliquot was vortexed and added to neurons at a final concentration of 1 μM for 18 h previous to oAβ(1–42) addition.

Transfection of Primary Hippocampal Neurons A transient transfection of primary hippocampal cultures was done using 2 μg plasmid DNA at 10 DIV by using Lipofectamine 3000® (Invitrogen cat: L3000–015). Briefly, 250 μL of transfection mix was added to independent wells of a 12-well plate, and was incubated at 37 °C for 45 min. Transfection mix was then removed and Neurobasal medium was added and incubated at 37 °C until used. Transfection was confirmed after 2 days under a fluorescence microscope (Nikon C1 Plus) using a FITC filter. Neurons were then treated at 14 DIV with GW3965 for 18 h followed by oAβ(1–42) exposure for 6 h.

Immunofluorescence Analysis After 15 DIV, neurons were transfected as described above with pEGFP-N1 and fixed by using paraformaldehyde (4% + sucrose 4%). Cells were then permeabilized by using goat serum dilution buffer (GSDB), followed by incubation with primary antibodies at 4 °C overnight: Guinea pig anti VGLUT1 (1:800; Synaptic System, Goettingen, Germany) and rabbit anti-SHANK2 (1:400, Synaptic System, Goettingen, Germany). Subsequently, cells were incubated with secondary antibodies for 2 h at room temperature: Anti-Guinea Pig Alexa Fluor 637 (Millipore Bioscience Research Reagents) and Anti-Rabbit Alexa Fluor 543 (Millipore Bioscience Research Reagents). Setup was then done using mounting medium containing DAPI staining at a concentration of 1 μM (Cat. No. 4083 Cell Signaling).

Western Blotting Analysis Following treatments, the cells were scraped, pelleted, and lysed at 4 °C for 10 min using lysis buffer (RIPA-buffer- Sigma–Aldrich, St. Louis, MO, USA) containing 1% protease inhibitor cocktail (Complete Mini-Roche Molecular Biochemicals; Mannheim, Germany) and 1% phosphatase inhibitor cocktail (Phospho-STOP-Roche Molecular Biochemicals; Mannheim, Germany). Lysates were sonicated and centrifuged at 13,000 rpm at 4 °C for 20 min. The protein concentration was determined using the BCA protein assay kit (Thermo Scientific, Rockford IL, USA) with bovine serum albumin (BSA) as the standard. Fifty μg of protein samples were run in SDS-PAGE at 100 V. After electrophoresis, proteins were transferred to a hydrophobic Polyvinylidene difluoride (PVDF) membrane (Hybond-LFP from GE Healthcare, formerly Amersham Biosciences, and Piscataway NJ, USA) and incubated in a blocking buffer containing 5% *w/v* powdered skim milk in Tween 20 Tris-buffered saline (TTBS) for 150 min at room temperature. The PVDF membrane was then incubated overnight at 4 °C with 5 ml monoclonal primary antibodies all from Synaptic System, Goettingen, Germany: anti PSD95, anti SHANK2, anti- NMDAR1 and NMDAR2B, anti-GLUTA2, anti-mGLUR5, anti-Syt1, anti SV2A, anti-VGLUT1, anti-Clived Caspase3, anti-PINK1, anti-ROCKII, anti-APOE, anti-Actin, all in a 1:1000 dilution in the blocking buffer. The next day the membranes were washed 3 times in TTBS (5 min/wash). This was followed by incubation with peroxidase-conjugated secondary anti-mouse (Santa Cruz Biotechnology, Santa Cruz CA, USA) or anti-rabbit (Cell Signaling Technology, Danvers MA, USA) in a 1:2000 dilution in blocking buffer for 1 h at room temperature, followed by washing 2 times with TBS 1X (3 min/wash). Bound antibodies were detected using the BioRad system (USA) with ChemiDocTM MP Imaging System (Bio-Rad). Quantitation was performed by densitometry using the NIH Image program (ImageJ).

Analysis of Dendritic Spines Density and Morphology by Confocal Microscopy An analysis of dendritic spines was performed in pEGFP-N1-transfected DIV15 hippocampal cultures. The analysis was done in 20 neurons per experimental group of two replications, for a total of 40 neurons analyzed per group. Images were captured using a 60X oil immersion objective (PLAN APO VC 60X OIL DIC N2) (1024 X 1024 pixels), using a Nikon C1 Plus Confocal Microscopy with the following lasers potencies (laser 408: 6.2%; laser 488: 6.2%; laser 568: 17.3%; laser 633: 18.5%), laser exposure of 4 μs, photomultiplicating gain (515/30: 6; 590/50: 8.5; 650 LP: 6) and 30 μm pinhole. Each image is the final results of a group of Z plane projection images of the neuron, approximately 14 to 18 sections or focal planes, separated each other by 0.2 μm in the Z axis. The total length of the dendrite segment analyzed was 80–100 mm per neuron, as shown in Fig. 2.

Quantitative analysis and classification of spines (filopoda + thin, stubby, mushroom, cup shape and atypical spines) was completed according to Frassoni et al. (2005), using the ImageJ software (v. 1.48, National Institutes of Health, USA) and Nis Elements (Nikon corporation, USA). All dendritic protuberances exhibiting a clearly recognized neck connected to the dendritic axis were classified as a spine.

Statistically differences were calculated using a one-way ANOVA followed by Bonferroni posttest whereas $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, by using GraphPad Prism 6 software (La Jolla, CA 92037 USA).

Results

GW3965 Regulates Neuronal Survival Through PINK1, ROCKII and Decrease of Caspase 3

In order to assess oAβ(1–42) toxicity on primary hippocampal neurons, we measured the release of lactate dehydrogenase (LDH), a biomarker for cellular cytotoxicity and cytolysis, from neuronal cultures after oAβ(1–42) exposure. oAβ(1–42) treatment induced a dose dependent LDH release from hippocampal neurons (Fig. 1a) which was significantly increased after 6 h at a concentration of 500 nM oAβ(1–42). Instead, the treatment with GW3965 did not increase LDH release at the different doses and times analyzed (Fig. 1b). Thus, for subsequent analyses, we used oAβ(1–42) at a concentration of 200 nM for 6 h which is not toxic to neurons, pre-treating or not neurons with GW3965 (at a concentration of 1μM for 18 h). Bright field images in Fig. 1c–f show that hippocampal neurons treated with oAβ(1–42), GW3965 and GW3965 + oAβ(1–42) (at the doses specified above) display a healthy morphology.

Although not significant, oAβ(1–42) (200 nM for 6 h) has a tendency to increase in LDH release (Fig. 1g) associated to a subtle activation of caspase-3 (Fig. 1h), increases that were completely prevented by pre-treatment with GW3965, decreasing to levels below the control. (Fig. 1g, h).

In addition, western blot analysis showed that neuronal cultures exposed to oAβ(1–42) for 6 h had significantly decreased PINK1 (Fig. 1i) and APOE (Fig. 1k), and increased ROCKII (Fig. 1j) expression levels. GW3965 itself was able to increase the expression levels of PINK1 and APOE proteins (Fig. 1i and k), while it did not have an effect on ROCKII (Fig. 1j). However, when neurons were pre-treated with GW3965 and subsequently exposed to oAβ(1–42), GW3965 was capable to reverse the changes in the expression of PINK1, ROCKII and APOE induced by oAβ(1–42) (Fig. 1i-k).

GW3965 Impacts on Dendritic Spine Density and Morphology and Modulates oAβ(1–42) Effects

In order to analyze the effect of GW3965 at the synaptic level, hippocampal neuronal cultures were exposed to 200 nM oAβ(1–42) for 6 h pretreating or not neurons with the LXR agonist GW3965 and the density and the morphology of the dendritic spines were analyzed (Fig. 2a–g). No significant changes in dendritic spines density was detected upon oAβ(1–42) exposure as compared to vehicle treated neurons (Fig. 2a–f); while a significant increase in the total spines number was observed upon pre-treatment with GW3965 both in the presence or in the absence of oAβ(1–42) (Fig. 2c–f). Morphological analysis of dendritic spines showed a significant decrease in mature spines (mushroom shaped) upon oAβ(1–42) exposure as compared to vehicle treated neurons (Fig. 2e and g); while pre-treatment with GW3965 upon oAβ(1–42) exposure partially restored mushroom spines density (Fig. 1g).

GW3965 Increases the Number of Synaptic Contacts and Regulates Synaptic Proteins Expression

Synaptic contacts were assessed by colocalization analysis of VGlut1 and Shank2 positive puncta by immunoreactivity (Fig. 3a and b). oAβ(1–42) treatment induced a significant decrease inVGlut1 and Shank2 colocalizing puncta as compared to the control treated neurons (Fig. 3a and c), and was associated to a decrease in VGlut1 (Fig. 3d) and increased Shank2 (Fig. 3e) expression as evaluated by western blot. In line with previous experiments, pre-treatment with GW3965 restores the number of colocalizing synaptic contacts by preventing the decrease induced by oAβ(1–42) exposure (Fig. 3a and c). Also, GW3965 pre-treatment reverted the changes in VGlut1 and Shank2 protein expression. These findings highlight the role played by GW3965 in regulating the glutamatergic system of synaptic contacts and its involvement in the generation of new spines and synapsis.

In order to analyze the effect mediated by oAβ(1–42) and GW3965 on other important pre-synaptic proteins, we analyzed by western blot the expression levels of SYT-1 (Fig. 4a) and SV2A (Fig. 4b). oAβ(1–42) treatment induced a significant increase in the expression level of these two presynaptic proteins, while GW3965 prevented the observed increase restoring protein levels to the control (Fig. 4a and b). oAβ(1–42) exposure for 6 h also induced a significant increase in the postsynaptic density protein 95 (PSD95) (Fig. 4c), a post-synaptic protein, which is functionally related to glutamate ionotropic (NMDA) and metabotrophic (mGluR5) receptors. The LXR agonist pre-treatment was able to revert the PSD95 increase mediated by oAβ(1–42) (Fig. 4c). Also, oAβ(1–42) exposure caused an increase in the expression level of the NR2B subunit of NMDA receptor (Fig. 4e), and on the GluA2 subunit of the AMPA receptor (Fig. 4g). However, oAβ(1–42) did not change the expression of the NR1 subunit of the NMDA receptor (Fig. 4d), but did decrease the expression of mGlutR5 (Fig. 4f). On the other hand, pre-treatment with GW3965 reverted the changes in levels of expression of proteins associated to oAβ(1–42) exposure: decrease in the expression of NR2B and GlutA2 and an increase in the expression of NR1 and mGluR5 (Fig. 4d–g). In addition, GW3965 by itself was able to significantly increase the expression level of NR1 and decrease those of NR2B (Fig. 4d,

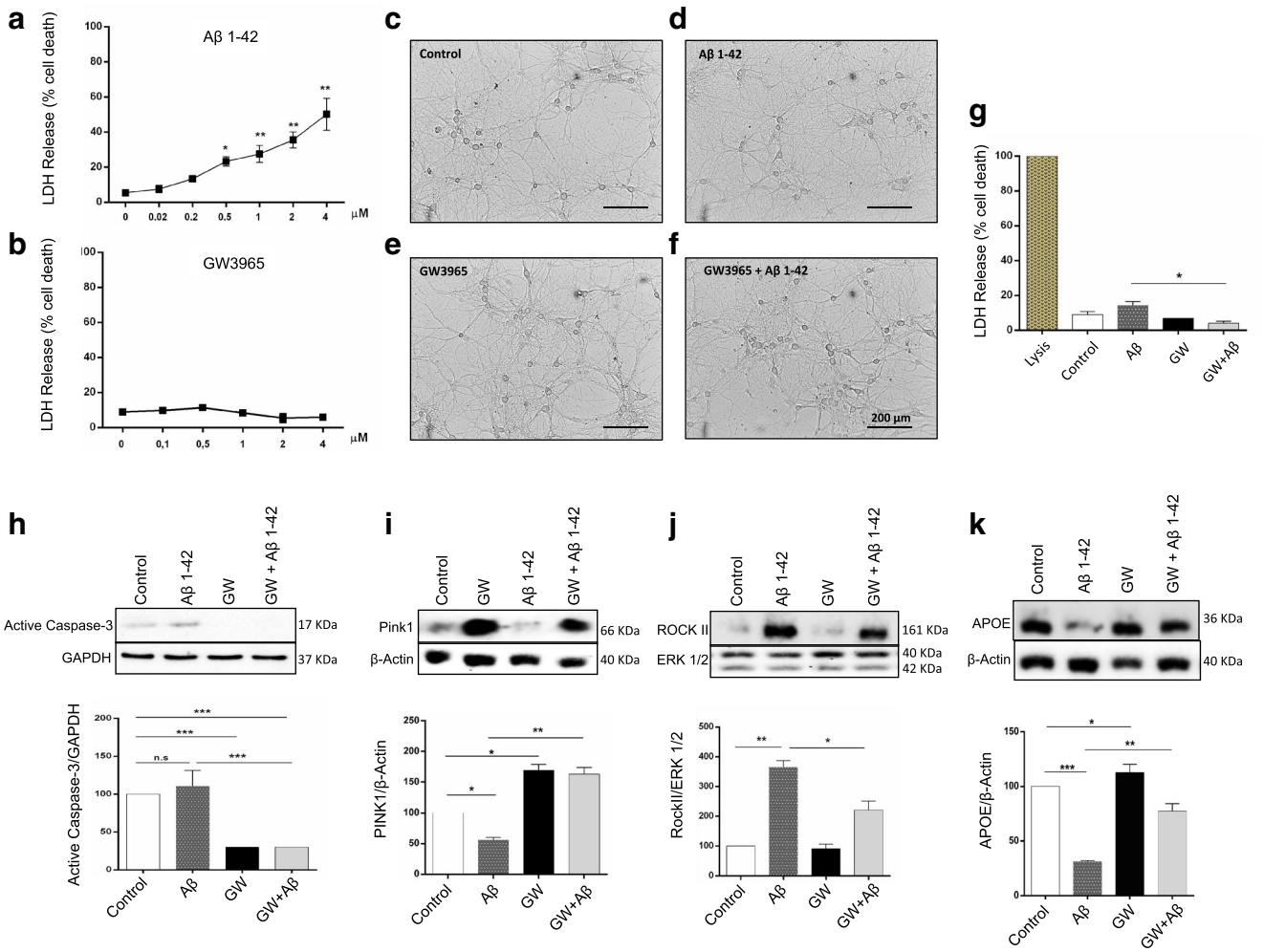

Fig. 1 GW3965 regulates cell death and caspase-3 activation upon oAβ(1–42) exposure. Effect of increasing concentrations of (**a**) oAβ(1–42) (0 nM, 20 nM, 200 nM, 500 nM, 1μM, 2μM, 4μM) and (**b**) GW3965 (0 nM, 0.1 nM, 0.5 nM, 1μM, 2μM, 4μM), on LDH release after 6 and 18 h of treatment respectively. oAβ(1–42) caused a significant increase in LDH release on hippocampal neurons starting at 500 nM; GW3965 did not cause a significative release of LDH at the different concentrations analyzed. (**c**–**f**) Phase contrast microscopy images taken under a 10X magnification of hippocampal neurons in: (**c**) control, (**d**) oAβ(1–42) (200 nM for 6 h), (**e**) GW3965 (1μM for 18 h) and (**f**) pre-treament with GW3965 + oAβ(1–42). Scale bar is 200μm. Under the different treatments, hippocampal neurons show a healthy morphology under the concentrations used. (**g**) LDH release assay upon oAβ(1–42) (200 nM for 6 h) and GW3965 (1μM for 18 h). Although not significant, oAβ(1–42) has a tendency to increase in LDH release that is completely prevented by pre-treatment with GW3965. Data is presented as mean + s.e.m. ($n = 3$ independent experiments), by using one-way ANOVA, followed by Bonferroni test ($^*p < 0.05$; $^{**}p < 0.01$). (**h**) Analysis of Caspase-3 activation by western blotting. oAβ(1–42) (200 nM for 6 h) increases the 17KDa fragment of active caspase-3, while pre-treatment with GW3965 (1 μM for 18 h) completely regulates this process, even to lowers levels as compared to control. Shown is the densitometric analyses of the blot. Data is presented as mean + s.e.m. ($n = 3$ independent experiments), by using one-way ANOVA, followed by Bonferroni test ($^{***}p < 0.001$; ns: not significant). (**i**, **j**) Exposure of oAβ(1–42) to hippocampal neurons significantly decrease the expression level of (**i**) PINK1, and increase the expression of (**j**) ROCKII; while pretreatment with GW3965 was capable of reversing these expression changes in PINK1 and ROCKII upon oAβ(1–42) exposure (**i** and **j**). Shown are densitometric analyses of each blot. Data is presented as mean + s.e.m. ($n = 3$ independent experiments), by using one-way ANOVA, followed by Bonferroni test ($^*p < 0.05$; $^{**}p < 0.01$)

e), while it did not change the expression levels of mGluR5 and GluA2 (Fig. 4f, g).

Discussion

Early synaptic deficit has been associated to the memory loss symptoms observed in AD (Canas et al. 2014; DeKosky and Scheff 1990), and therefore the studies of the dynamics and morphology of neuronal dendritic spines, synaptic contacts and synaptic proteins in cellular models of AD are important for the development of new therapeutic targets and interventions that could impact the progression of this disease. Results from recent studies, including our own, have demonstrated that in murine models of AD, the LXR agonist treatment improves cognitive function

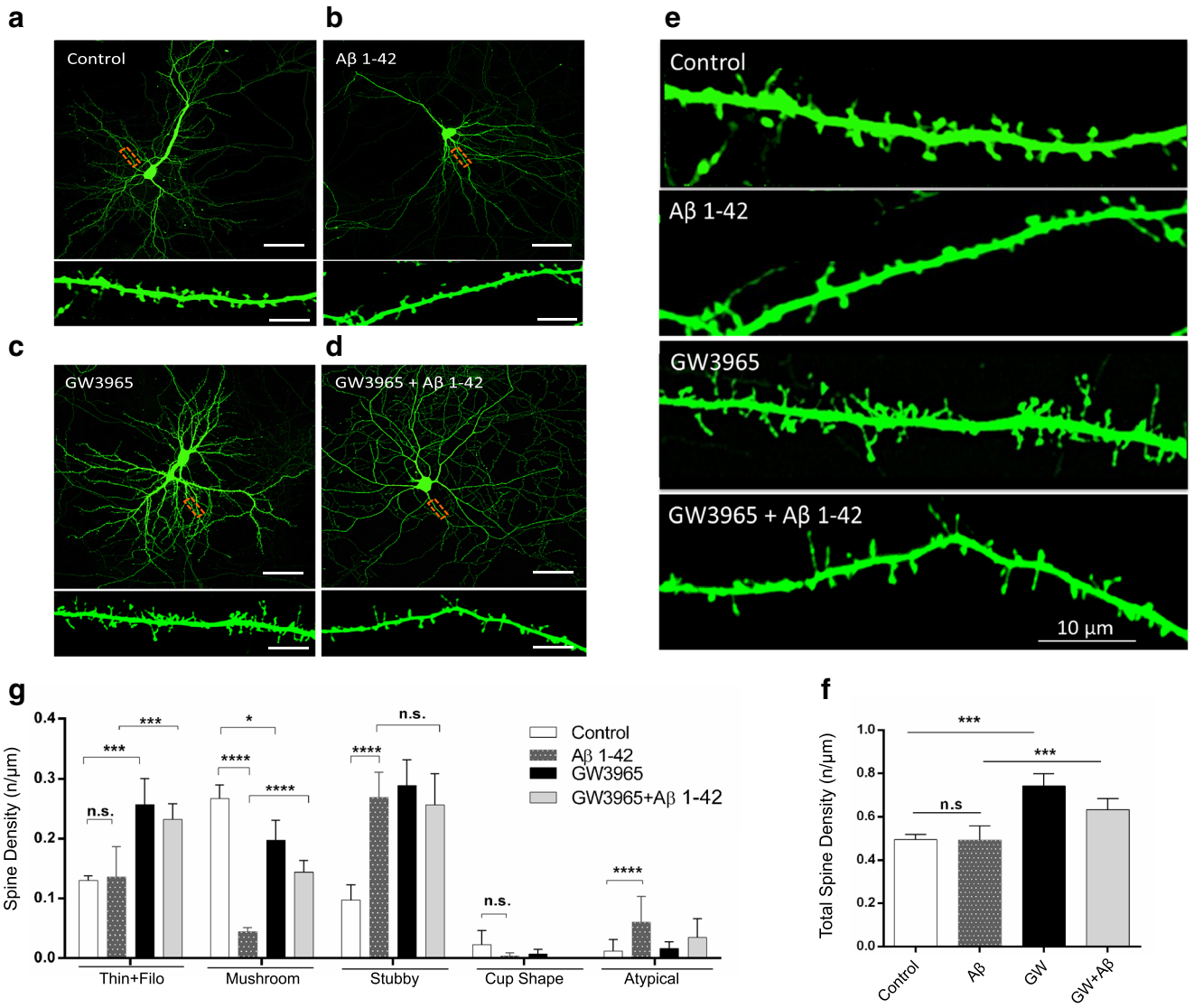

Fig. 2 GW3965 increases dendritic spine density and modulates their morphology upon oAβ(1–42) exposure. **a**–**d** Representative images of GFP label hippocampal neurons (top panel) and amplification of the dendrites analyzed (bottom panel). (**a**) Control; (**b**) oAβ(1–42) treated; (**c**) GW3965 treated; (**d**) GW3965 pre-treatment + oAβ(1–42) exposure. Scale bar, 50 µm; amplification, 10 µm. (**e**) Dendritic segment amplify (5 times) from Fig. 2a–d. (**f**) Quantitative analysis of dendritic spine density of primary hippocampal neuronal cultures from Fig. 1e. Data is presented as mean + s.e.m. ($n = 2$ independent experiments; $n = 40$ neurons per treatment; $n = 160$ neurons analyzed in total) by using one-way ANOVA, followed by Bonferroni test (***$p < 0.001$; ns: not significant). (**g**) Analysis of spine density according to their morphology. Dendritic spines were classified according to their shape in: thin and philopodia, mushroom, stubby, cup shape and atypical. Neurons exposure to oAβ(1–42) showed a significant decrease in mushroom shape dendritic spines as compared to control. Pre-treatment with GW3965 prevented the oAβ(1–42)-dependant decreased in mushroom shape dendritic spines. Histogram depicts the number of dendritic spines per µm. Data is presented as mean + s.e.m. ($n = 2$ independent experiments; $n = 40$; neurons per treatment; $n = 160$ neurons analyzed in total), by using two-way ANOVA, followed by Bonferroni test (**$p < 0.01$; ****$p < 0.0001$; ns: not significant)

(Sandoval-Hernández et al. 2015; Koldamova et al. 2005; Vanmierlo et al. 2011; Jiang et al. 2008; Zelcer et al. 2007) associated to increase expression of APOE and ABCA1, and changes in the methylation status of the genes important in synaptic processes (Sandoval-Hernández et al. 2016). However, the impact of LXR agonists in synaptic function has yet to be elucidated.

Functional alterations in synapsis following exposure to oAβ(1–42) in AD models have been previously described, including the impairment of long-term potentiation (LTP) (Sandoval-Hernández et al. 2015; Cullen et al. 1997) and facilitation of long-term depression (LTD) (Li et al. 2009, 2010). These alterations have been associated with the disruption of normal release and recycling of neuronal glutamate (Alberdi

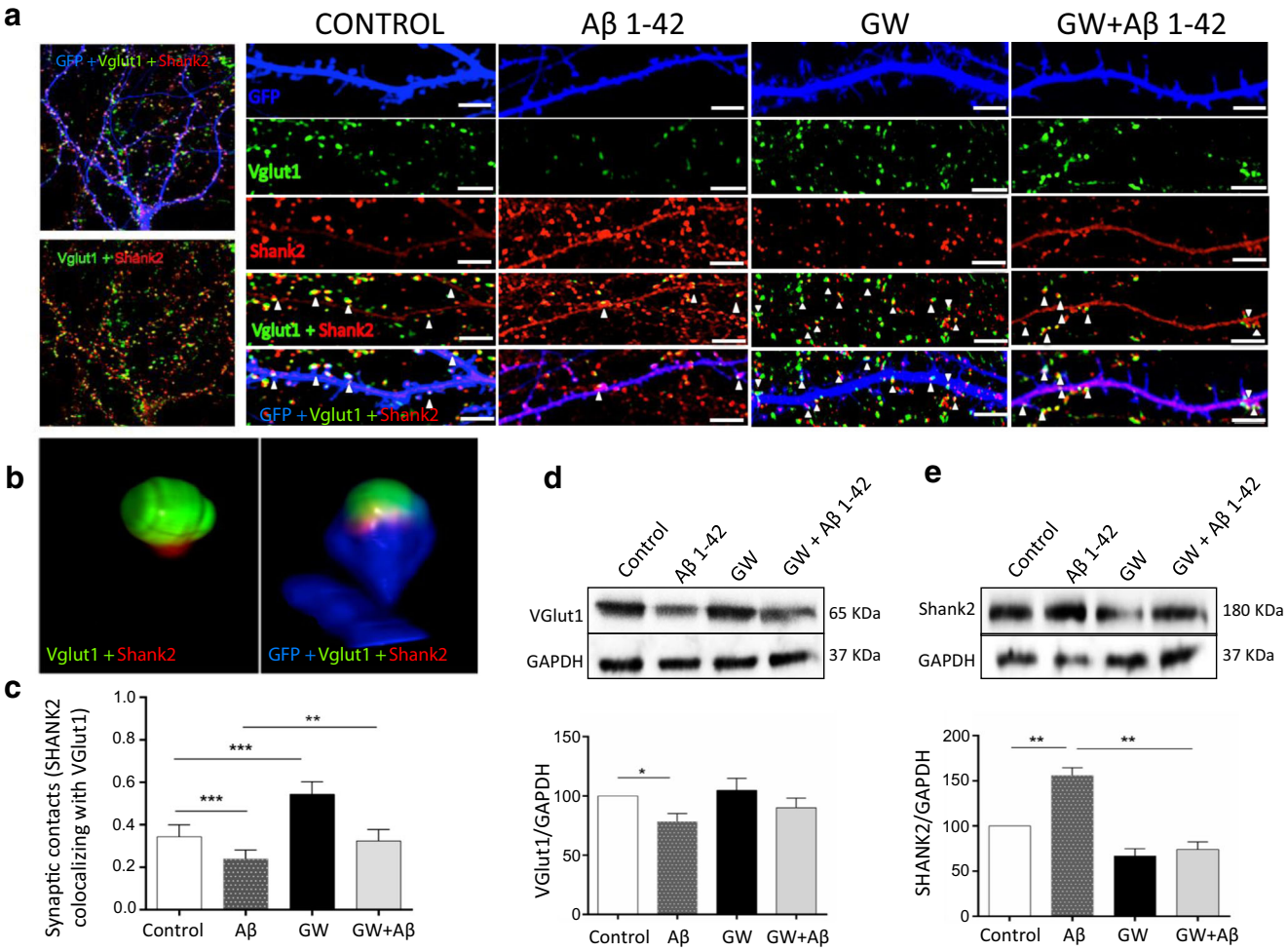

Fig. 3 GW3965 increases synaptic contacts upon oAβ(1–42) exposure. **a** Left panels: representative images of dendritic segments analyzed under confocal microscopy stained for Vglut1 (green), Shank2 (red), and cytoskeleton (GFP: blue). Right panels: amplification of the dendrites analyzed in control, oAβ(1–42) treated; pre-treated with GW3965 + oAβ(1–42); treated with GW3965. White arrowheads indicate co-localization between Vglut1 and Shank2, corresponding to synaptic contacts. Scale bar is 5 μm. **b** Tridimensional representation of a synaptic contact on a mushroom dendritic spine. Scale bar is 0.5 μm. **c** Quantification of colocalization of Vglut1 and Shank2 using the Pearson correlation coefficient. oAβ(1–42) exposure induced a significant decrease in synaptic contacts that was prevented by pre-treatment with GW3965. Data is presented as mean + s.e.m. (n = 2 independent experiments; $n = 40$; neurons per treatment; $n = 160$ neurons analyzed in total), by using one-way ANOVA, followed by Bonferroni test (**$p < 0.01$; ***$p < 0.001$). **d**, **e** Western blotting and densitometric quantification of expression of Vglut1 and Shank2. oAβ(1–42) exposure induced a significant decrease in Vglut1 (**d**) and increase in Shank2 (**e**) expression; these changes that were prevented by pre-treatment with GW3965. Data is presented as mean + s.e.m. ($n = 3$ independent experiments), by using one-way ANOVA, followed by Bonferroni test (*$p < 0.05$ **$p < 0.01$)

et al. 2010; Kashani et al. 2008; Jacob et al. 2007; Kabogo et al. 2010) to changes in morphology and density of dendritic spines (Li et al. 2010; Penzes et al. 2011). In the present paper, we demonstrated that oAβ(1–42) exposure in primary hippocampal cultures caused important alterations in synaptic dynamics, including (1) decrease in mature mushroom type spines; (2) decrease in the number of total synaptic contacts; (3) changes in pre-synaptic proteins, such as a decrease in VGlut1 and increase in the expression levels of SYT1 and SV2A, most probably leading to an increase in the docking of synaptic vesicles and an increase in release of glutamate (Lai et al. 2014), as has been described previously upon oAβ(1–42) treatment (Kabogo et al. 2010); (4) changes in the post-synaptic proteins, such as increased Shank2, PSD95, NR2B and GluA2; and (5) decrease in the expression of PINK1, activation of caspase-3 and increased ROCKII. Alterations of expression of diverse pre- and post-synaptic proteins have been previously demonstrated in AD patients, including down regulation of SYT1 in the hippocampus and Vglut1 in the cortex (Sze et al. 2000; Kirvell et al. 2006); while others have shown an increase in Shank 2 at the PSD (Gong et al. 2009). All changes in dendritic spines and expression of synapsis proteins in the present model were prevented or restored to control levels using pre-treatment with the LXR

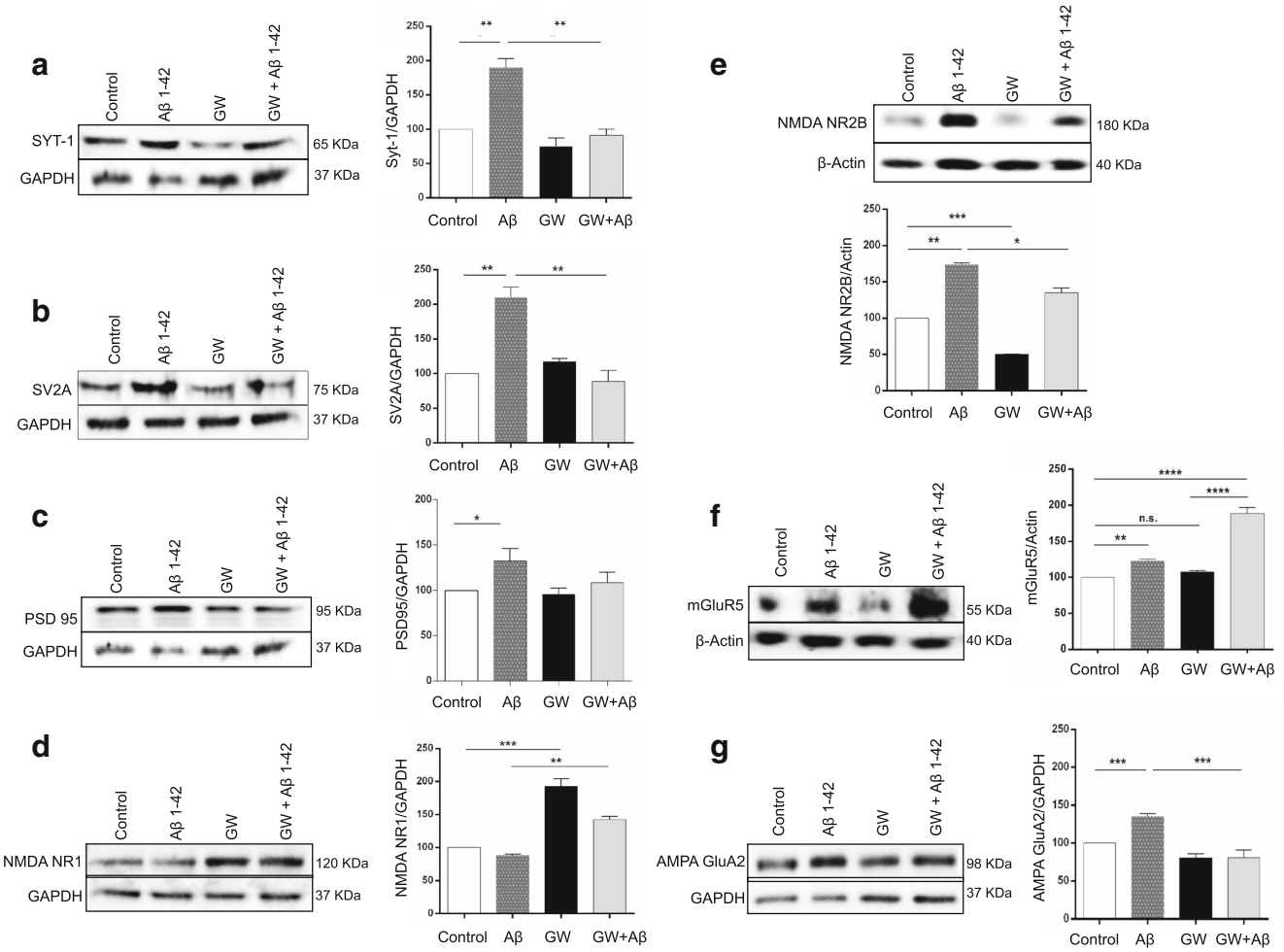

Fig. 4 GW3965 regulates the expression level of pre- and post-synaptic proteins upon oAβ(1–42) exposure. **a**–**g** Western blot analysis of expression of (**a**) SYT1, (**b**) SV2A, (**c**) PSD95, (**d**)NMDA NR1, (**e**) NMDA NR2B, (**f**) mGluR5, (**g**) AMPA GluA2. Shown are densitometric analyses of each blot. Data is presented as mean + s.e.m. ($n = 3$ independent experiments), by using one-way ANOVA, followed by Bonferroni test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$)

agonist GW3965, which suggests a critical role of LXR in the proper functioning and maintenance of dendritic spines and synapsis.

The beneficial effects of LXR agonist upon synaptic function could be explained by different alternatives: (1) As a key regulator of transcription and translation of some pre- and post-synaptic genes. As previously mentioned, LXR agonist clearly increases the expression of APOE in diverse models, which plays a critical role in the favorable effects of these molecules. Recent studies have demonstrated the importantance of APOE in the maintainance of synaptic integrity by its ability to transport and deliver cholesterol to neurons (Tachibana et al. 2016). In addition, it has been demonstrated that bexarotene, a retinoid X receptor (RXR) agonist related to LXR, was able to restore the decrease in GluR1, NR1 and PSD95 observed during normal mouse aging, which was found to be dependent on increased expression of APOE and its receptor LRP1 (Tachibana et al. 2016). In a cerebral ischemia-reperfusion model, it has been shown that GW3965 was able to increase the expression of synaptophysin, promote synaptic plasticity and axonal regeneration (Cui et al. 2013); 2) By regulation of epigenetic changes in DNA. We have recently demonstrated that GW3965 causes changes in the methylation pattern of DNA in the triple transgenic mouse model of AD, in particular changes in synaptic genes such as Syn-1 and Syp (Sandoval-Hernández et al. 2016); and 3) By non-genomic regulation of synapsis, including the regulation of proper transport, sorting and function of synaptic proteins (Fig. 5). All these observations require further analysis.

The regulation of neuronal survival by PINK1 has been associated to the maintenance of proper mitophagy

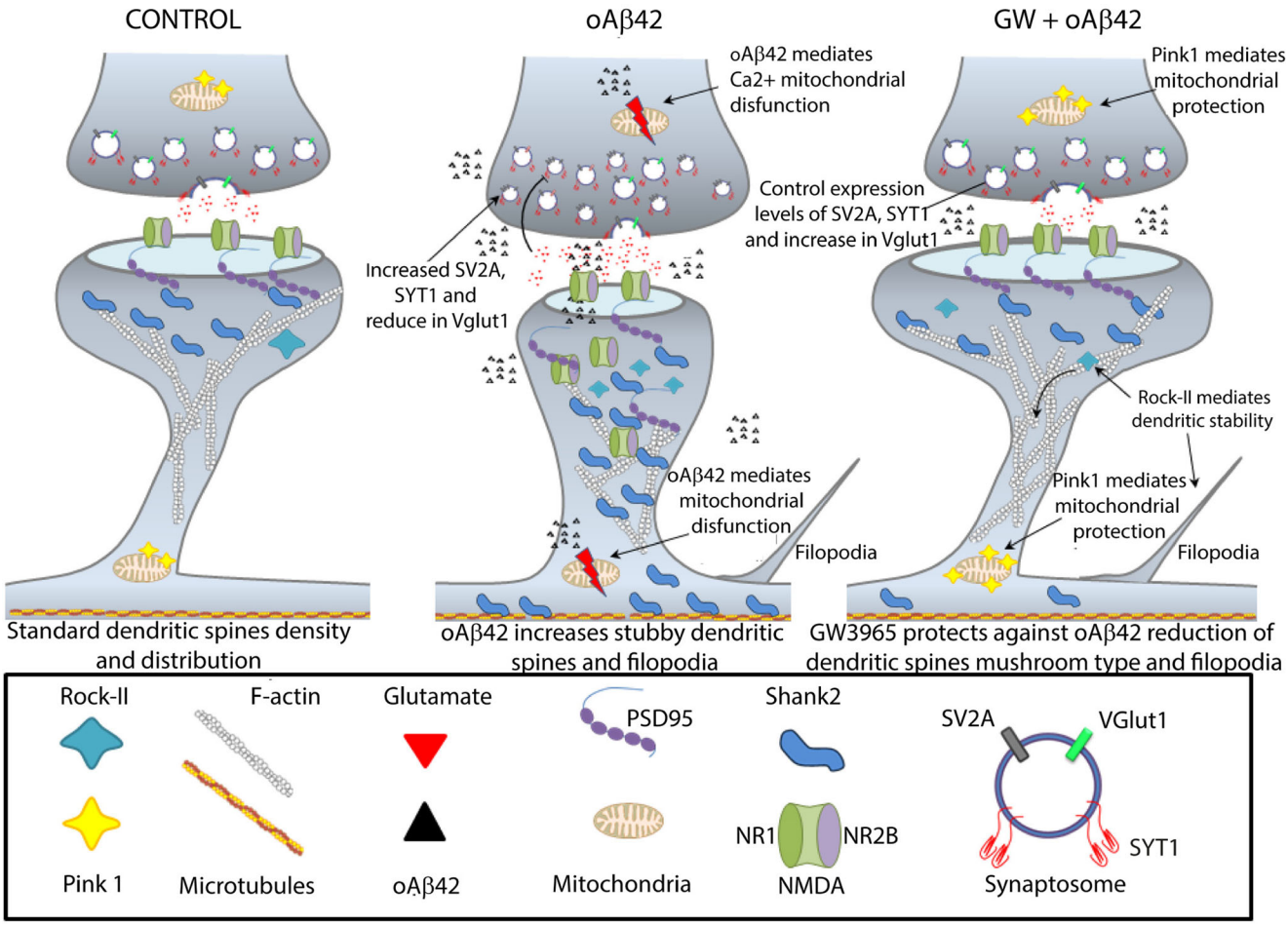

Fig. 5 Model proposed for the mechanism of action of oAβ(1–42) and GW3965 on synaptic function of hippocampal neurons. (Left panel) Control neurons exhibit a proper synaptic function. (Middle panel) Exposure of oAβ(1–42) causes diverse changes in synapsis: (1) Increase in the expression of Rock II is associated to damage of microtubules and transport of mRNA towards the spine; (2) increase SYT1 and SV2A most probably associated to increase in the pre-synaptic activity and release of glutamate; (3) increase in NR2B and GluA2. Maybe associated to post-synaptic damage; and (4) decreased mature spines. (Right panel) Pre-treatment with GW3965 prevents most changes associated to oAβ(1–42) exposure on synapsis: (1) GW3965 may modulate the genomic expression of synaptic proteins, including modulation of RockII, SYT1 and SV2A, and other proteins such as PINK1, which in conjunction favors dendritic spine stability, maduration and proper synaptic connection and function

(Beilina and Cookson 2015) and also to the regulation of growth factor receptor activation, such as the insulin-like growth factor-1 (IGF-1) effect upon ligand binding (Contreras-Zárate et al. 2015). The relationship between oAβ(1–42) exposure and PINK1 remains unknown. However, recent studies in the oxygen-glucose deprivation (OGD) model showed that overactivation of the NR2B-containing NMDA receptors induced PINK1 reduction (Shan et al. 2009). In addition, absence of PINK1 has been shown to cause alterations in the NMDA excitatory postsynaptic currents in the substantia nigra in a mouse model (Pearlstein et al. 2016), suggesting a possible bidirectional regulation of glutamate signaling/toxicity and PINK1 function. Moreover, a recent report has demonstrated that PINK1-dependent signalling promotes the rescue of amyloid pathology and amyloid-β-mediated mitochondrial and synaptic dysfunctions (Du et al. 2017). Therefore, the role of LXR agonist as a regulator of the expression of PINK1, opens the possibility of PINK1-mediated regulation of NMDA receptors function in the context of oAβ(1–42) exposure, as a plausible therapeutic target. These observations require further investigation.

In conclusion, the present results demonstrate that the LXR agonist GW3965 restores density and mature morphology of dendritic spines and regulates the expression of diverse pre- and post-synaptic and survival proteins upon oAβ(1–42) exposure to hippocampal neuronal cultures. These observations are important to increase the understanding of the beneficial effects LXR agonists may have, which can be investigated as a plausible treatment for AD.

Contribution of Each Author Báez-Becerra C: performed most experiments and contributed to manuscript writing.

Filipello F: performed dendritic spine analysis.

Sandoval-Hernández A: performed western blott experiments.

Arboleda H: contributed to desing of the experiments and writing of the manuscript.

Arboleda G: desing the experiments and wrote the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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