## Plenary Lecture 2

# Ginsenoside (20S)Rg3 Ameliorates Synaptic and Memory Deficits in an Animal Model of Alzheimer's Disease

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## ABSRACT

The amyloid β-peptide (Aβ), which originates from the proteolytic cleavage of amyloid precursor protein (APP), plays a central role in the pathogenesis of Alzheimer's disease (AD). Mounting evidence indicates that different species of Aβ, such as Aβ oligomers and fibrils, may contribute to AD pathogenesis via distinct mechanisms at different stages of the disease. Importantly, elevation and accumulation of soluble Aβ oligomers closely correlate with cognitive decline and/or disease progression in animal models of AD. In agreement with these studies, oligomers of Aβ have been shown to directly affect synaptic plasticity, a neuronal process that is known to be essential for memory formation. Our previous studies showed that Aβ induces the breakdown of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), a phospholipid that regulates key aspects of neuronal function. PI(4,5)P2 breakdown was found to be a key step toward synaptic and memory dysfunction in a mouse model of AD. To this end, we seek to identify small molecules that could elevate the levels of PI(4,5)P2 and subsequently block Aβ oligomer-induced breakdown of PI(4,5)P2 and synaptic dysfunction.. We found that (20S)Rg3, an active triterpene glycoside from heat-processed ginseng, serves as an agonist for

phosphatidylinositol 4-kinase IIalpha (PI4KIIalpha), which is a lipid kinase that mediates a rate-limiting step in PI(4,5)P2 synthesis. Consequently, (20S)Rg3 stimulates PI(4,5)P2 synthesis by directly stimulating the activity of PI4KIIalpha. Interestingly, treatment of a mouse model of AD with (20S)Rg3 leads to reversal of memory deficits. Our data suggest that the PI(4,5)P2-promoting effects of (20S)Rg3 may help mitigate the cognitive symptoms associated with AD.

## INTRODUCTION

Alzheimer's disease (AD) is the most common form of late-onset dementia and is a major health issue facing the aging population. Almost 50% of people who reach age 85 will develop the disease. The pathogenic hallmark of AD is the accumulate ion of amyloid β-peptide (Aβ) and subsequent deposition of Aβ in plaques (1, 2). A β is produced by two sequential proteolytic cleavages of β-amyloid precursor protein (APP) by β- and γ-secretases (3, 4). Aβ1-40 (Aβ40) is the predominant cleavage product, although the longer and less abundant cleavage product, Aβ42, is more amyloidogenic and thus believed to be a key pathogenic agent in AD (1, 2). While AD affected brains generally contain amyloid plaques that consist of insoluble aggregates of Aβ, mounting evidence indicates that different conformations of Aβ, such as Aβ oligomers and fibrils, may contribute to AD pathogenesis via distinct mechanisms at different stages of the disease (5,6). Importantly, accumulation of soluble oligomeric forms of Aβ closely correlates with cognitive decline and/or disease progression in animal models and AD patients (5-9). Thus, it is crucial to understand specific and early synaptic/neuronal changes associated with the exposure of neurons to soluble oligomers. Current studies in the field have determined that the oligomer of Aβ42 is the primary toxic and synapse-impairing form of Aβ (10, 11). Aβ42 (e.g. oligomeric form of Aβ42) has been shown to directly affect synaptic plasticity and trigger the loss of synaptic dendritic spines. For instance, Aβ 42 treatment of hippocampal slice preparations impairs long-term potentiation (LTP)

(10, 12, 13) and facilitates the induction of long-term synaptic depression (LTD) (14).

Phosphatidylinositol-(4,5)-bisphosphate, PI(4,5)P2, is an important signaling lipid at the synapse involved in ion channel regulation, exocytosis, endocytosis, actin cytoskeleton rearrangement and signaling (15). PI(4,5)P2 has also been shown to be AD pathogenesis (16-18). Brain specific metabolic enzymes for PI(4,5)P2 synthesis and degradation have been identified (Fig. 1). Synthesis of PI(4,5)P2 is primarily mediated by phosphatidylinositol phosphate kinase type I γ (PIPK1γ), which phosphorylates PI(4)P on the 5' position of the inositol ring (23, 26-29) (Fig. 1). PI(4)P is the immediate precursor for PI(4,5)P2 synthesis and its formation, mediated by phosphatidylinositol 4-kinase, is also required for maintenance of PI(4,5)P2 levels.

Type II PI4-kinase (phosphatidylinositol 4-kinase type IIα, or PI4KIIα) is a brain-enriched PI 4-kinase that serves as a rate-limiting kinase for the synthesis of PI(4,5)P2 (19). Overexpression of this enzyme was shown to promote the synthesis of PI(4)P as well as PI(4,5)P2 (20). Several studies indicate that both PI(4,5)P2 and the presenilins, the catalytic components of γ-secretase complex, may be enriched in detergent-resistant membrane domains (DRMs), also referred to as lipid rafts (21, 22, 19). Thus, it is conceivable that PI(4,5)P2 influences the properties of the γ-secretase by either modification of the lipid environment or sequestration of γ-secretase component(s) in DRMs. A recent study suggests that phosphoinositides may inhibit γ -secretase activity by regulating the interaction between lipid substrate and enzyme complex (23). Thus, these observations indicate the therapeutic potential of lipid modulation in AD.

## METHODS

### Cell culture

PC12 cells are maintained in Dulbecco's modified Eagle's medium with sodium

pyruvate (Invitrogen) supplemented with 5% fetal bovine serum, 10% horse serum, glutamine (4 mM), penicillin (200 units/mL), streptomycin (200 μg/mL); N2a cells will be maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and glutamine, penicillin, and streptomycin as described above. Cells will be maintained at 37°C in 5% CO2. Twenty-four hours before transfection, PC12 cells will be plated (at 50% confluence) on coverslips pre-coated with polylysine (20 ?g/mL) for 1 hr at 37°C. Transfections of the GFP fusion of the PH domain of human phospholipase Cδ1 (amino acids Met1-Ile175) will be accomplished using Lipofectamine 2000 (Invitrogen).

**CREB assay**

Neurons will be stimulated with 10 ?M NMDA (24) for 10 minutes and immediately scraped into Laemmli sample buffer and heated for 10 minutes at 99oC. Proteins will be separated on Tris/Glycine gels 4-20% and transferred to PVDF for western analysis. The antibody to detect p-CREB is from Invitrogen and the antibody to detect TUJ-1 is from Covance. Infrared detection of secondary antibodies will be accomplished using the OdesseyTM Imaging System and integrated intensity for pCREB for each sample was normalized to the signal for TUJ-1 immunoreactivity.

**Confocal microscopy**

After 24 hr of transfection with GFP- PHPLC δ 1 domain, PC12 cells are incubated with 200 nM Aβ42 oligomers, 2 μM ionomycin (Sigma-Aldrich), 200 nM of Aβ42Rev (inverted peptide) or 200 nM of Aβ38. Cells will be analyzed after being treated for different time lengths: 0, 10 min, 30 min and 120 min. Cells will be washed in phosphate buffer and fixed with 4% paraformaldehyde. Confocal z-stack images (0.5 μm) of PC12 will be obtained using Nikon EZ-C1.2.30 confocal microscope, (X100) oil immersion objective. Quantification of GFP intensity will be calculated using the ImageJ software: for each cell in a given image, a line intensity profile across the cell was obtained. The relative decrease in plasma membrane

localization will be calculated as the ratio between the plasma membrane fluorescence intensity and the average cytosolic fluorescence intensity. The same kind of analysis will be performed for PC12 cells transfected for 24 hr with GFP-PHPLCδ1 domain after a 2 hr incubation with oAβ42 in the presence of the compounds.

## Electrophysiology

Transverse hippocampal slices (400 μm) will be cut with a tissue chopper (EMS, PA) and maintained in an interface chamber at 29°C for 90 min prior to recording, as previously reported (25). CA1 field-excitatory post-synaptic potentials (fEPSPs) will be recorded by placing both the stimulating and the recording electrodes in CA1 stratum radiatum (32, 43). Basal synaptic transmission (BST) will be evaluated either by plotting the stimulus voltages (V) against slopes of fEPSP, or by plotting the peak amplitude of the fiber volley against the slope of the fEPSP, to generate input-output relations. During baseline recordings, responses will be evoked at an intensity of approximately 35% of the maximum evoked response. PPF will be tested using different interstimulus intervals, and defined as the second fEPSP slope expressed as a percentage of the first. LTP will be induced using a θ-burst stimulation (4 pulses at 100 Hz, with bursts repeated at 5 Hz and each tetanus including 3 ten-burst trains separated by 15 sec). oAβ42 will be applied for 20 min prior to the θ-burst.

## RESULTS & DISCUSSION

### PI4KIIα overexpression inhibits the Aβ-induced depletion of PI(4,5)P2 in the plasma membrane

A large pool of PI(4,5)P2 is shown to be concentrated at the plasma membrane (26). Our recent paper showed that treatment of PC12 cells expressing PI(4,5)P2 probe (GFP-conjugated pleckstrin homology domain of phospholipase C δ1; PH-PLC δ1) with oligomeric Aβ leads to the substantial loss of the fluorescent signal of the

PI(4,5)P2 probe from the plasma membrane due to the breakdown of PI(4,5)P2 (Fig. 2A). We found that overexpression of PI4KIIα leads to the inhibition of PI(4,5)P2 breakdown induced by the Aβ treatment (Fig. 2B, C). In contrast, PI4KIIα-KD (kinase inactive mutant) did not confer any protective effects against the Aβ-induced PI(4,5)P2 breakdown. Thus, these results suggest that increased PI4KIIα levels protect the neural cells from Aβ-induced depletion of PI(4,5)P2 and the catalytic activity of PI4KIIα is required for the observed protective effect.

**(20S)Rg3, Aβ42-reducing natural compound, is an activator of PI4KIIα**

We next tested if (20S)Rg3 (Fig 3A) could influence the levels of PI(4,5)P2 in the cells. We found that only (20S)Rg3 greatly elevates the levels of the steady state levels of PI(4)P and PI(4,5)P2 measured by HPLC analysis (17, 18; data not shown). We next performed lipid kinase/phosphatase assays to determine if (20S)Rg3 directly influences the activity of the enzyme(s) involved in PI(4)P and PI(4,5)P2 metabolism, such as PI4KIIα, PIP5K1γ and synaptojanin 1 (Synj1). We found that the activities of PIP5K1γ and Synj1 were not affected by the incubation with (20S)Rg3 up to 100 uM (data not shown). In contrast, (20S)Rg3 potentiated the activity of PI4KIIα when the recombinant PI4KIIα was incubated with PI and [γ 32P-ATP] (Fig. 3B, C). The (20S)Rg3-enhanced formation of PIP was not detected when (20S)Rg3 was incubated with the inactive (kinase-dead) mutant (PI4KIIα-KD). Thus, we conclude that (20S)Rg3 potentiates the activity of PI4KIIα.

**(20S)Rg3, an activator of PI4KIIα, confers dual inhibitory effects on Aβ42 production as well as on Aβ-induced synaptic dysfunction**

Since the overexpression of PI4KIIα leads to the suppression of Aβ-induced PI(4,5)P2 depletion in the plasma membrane (Fig. 2B, C), we next tested if (20S)Rg3, an activator of PI4KIIα could exert a similar effect. We found that (20S)Rg3 treatment inhibits the Aβ-induced PI(4,5)P2 loss (Fig. 3D). Thus, these results indicate that (20S)Rg3 may interfere with the signaling cascade that is triggered by Aβ oligomers. We hypothesize that (20S)Rg3 promotes the activity of

PI4KIIα and therefore increases the relevant 'pool' of PI(4,5)P2. To this end, we determined the effects of (20S)Rg3 on Aβ-induced synaptic dysfunction. We found that (20S)Rg3 treatment prevents the loss of of CREB phosphorylation (a transcription factor that is linked to synaptic plasticity and memory), which is suppressed by treatment with Aβ oligomers (Fig. 3E, F). Furthermore, incubation of acute hippocampal slices with (20S)Rg3 was able to block the Aβ-induced suppression of LTP (Fig. 3G), suggesting that (20S)Rg3 can interfere with Aβ -triggered dysfunction at the synapse.

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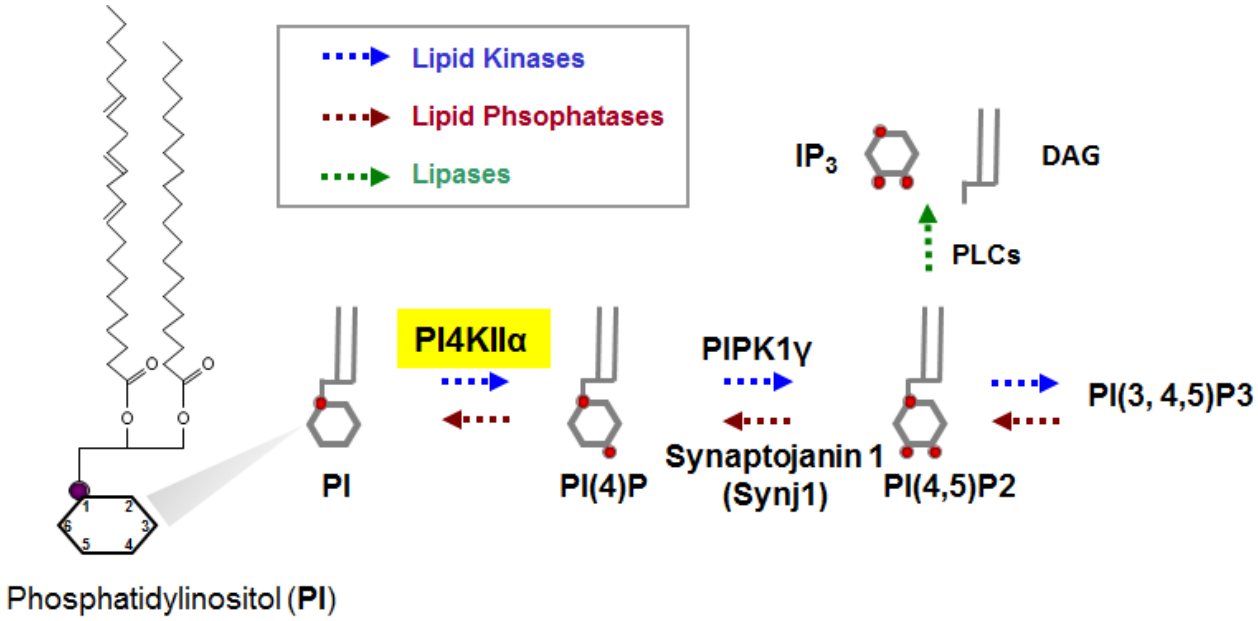


**Figure 1. The metabolism of the major phosphoinositides in the brain.**

Phosphoinositides are phosphorylated derivatives of the minor membrane lipid phosphatidylinositol (PI). A series of PI kinases and phosphatases mediate inter-conversion between different PI species, including PI(4)P, PI(4,5)P2 and PI(3,4,5)P3, by reversible phosphorylation at the 3,4,5-position of inositol ring. Phosphatidylinositol 4-kinase type IIα (PI4KIIα) mediates the generation of PI(4)P from PI. Phospholipase C (PLC) mediates hydrolysis of PI(4,5)P2 to generate second messengers IP3 and DAG. Minor PIs are not shown.

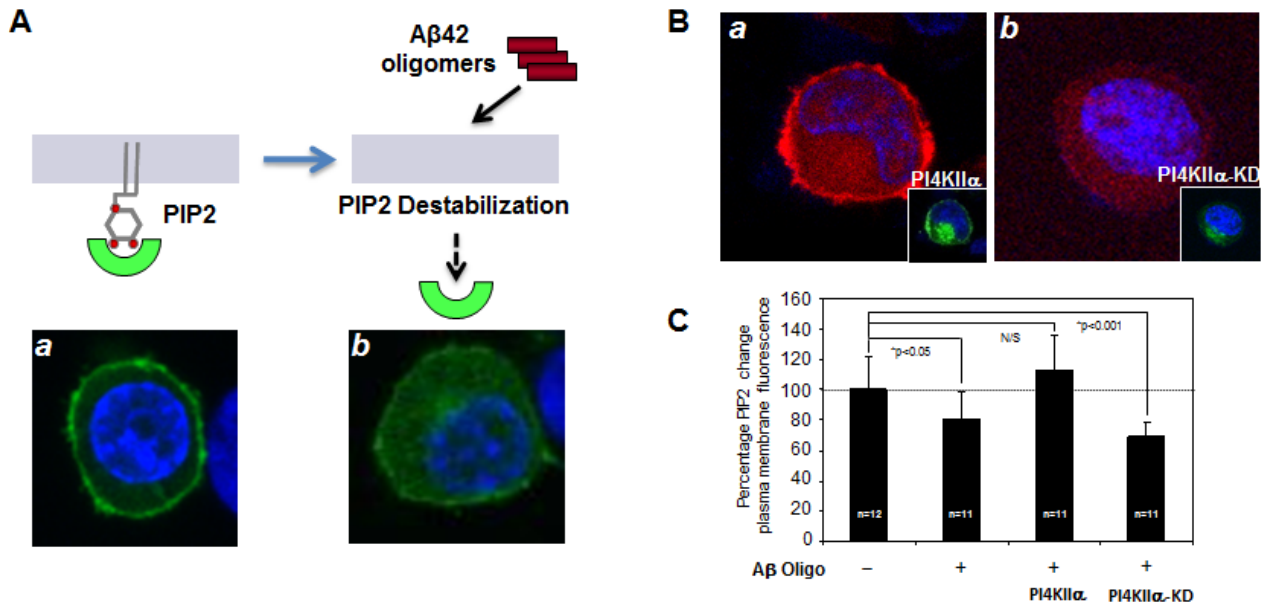


**Figure 2. Effect of PI4KIIα on the Aβ-induced PIP2 deficiency at the plasma membrane.**

(A) Schematic illustration of GFP-PHPLCδ1 as a probe to detect PI(4,5)P2 at the plasma membrane. The PH domain of PLC-δ1 was fused with GFP and expressed in PC12 cells. Treatment of PC12 cells with 200 nM Aβ42 oligomers led to the depletion of PI(4,5)P2 (PIP2) and redistribution of the PI(4,5)P2 probe into cytosol (bottom panels: a, b). (B) PC12 cells co-transfected with RFP-PHPLCδ1 and either GFP-PI4KIIα (a) or GFP-PI4KIIα-KD (b) in the presence of 200nM Aβ42 oligomer (n=11). Expression of either GFP-PI4KIIα or GFP-PI4KIIα-KD are shown in the inset. (C) The relative PI(4,5)P2 change in plasma membrane fluorescence was calculated as described previously. Graph showing the decrease in plasma membrane fluorescence of RFP-PHPLCδ1 after Aβ42 oligomer (n=11) treatment. Overexpression of GFP-PI4KIIα was able to restore the Aβ42-induced PIP2 deficiency, while the kinase-dead mutant (GFP-PI4KIIα-KD) did not reverse the PIP2 deficiency. Average percent change in plasma membrane/cytosol fluorescence ratio normalized to DMSO control (error bars represent standard deviation, significance was determined with an unpaired Student's t-test)

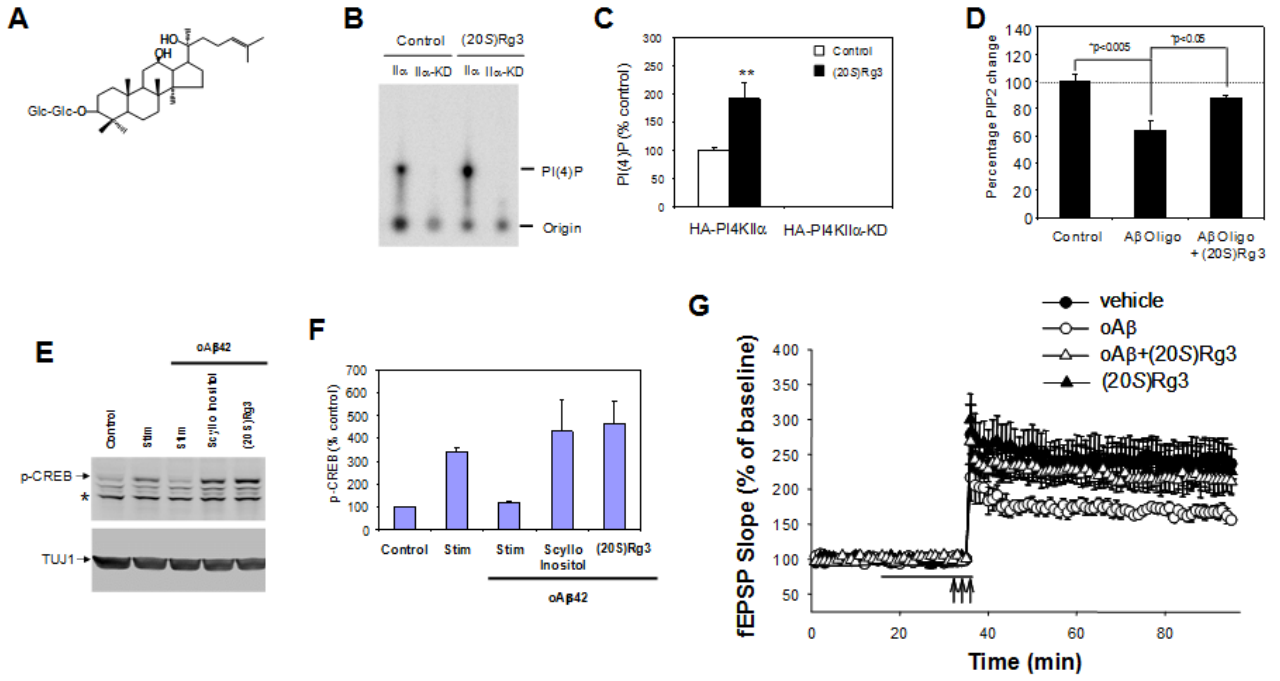


**Figure 3. (20S)Rg3, a natural compound that increases the activity of PI4KIIα, inhibits the Aβ-induced PI(4,5)P2 depletion and synaptic abnormalities.**

(A) Chemical structure of (20S)Rg3. (B) Lipid kinase assay and TLC analysis using either wild-type (HA- PI4KIIα) or kinase-dead mutant (HA- PI4KIIα-KD) forms of PI4KIIα. The assay was performed in the absence or presence of 50 μM (20S)Rg3. (C) Quantification of TLC data on PI(4)P in (B). Data are expressed as mean ± s.d. (n=3, **p<0.01). (D) (20S)Rg3 inhibits Aβ42-induced PIP2 deficiency. PC12 cells transfected with GFP-PHPLCδ1 probe were incubated with Aβ oligomers as described in Figure 2. Relative distribution of the probe in the plasma membrane vs. cytosol is shown as % control (untreated with Aβ). Representative intensity profiles of the GFP-PHPLCδ1 probe with treatment of DMSO (n=25, control), 200nM Aβ42 oligomer (n=28) and Aβ42 oligomer plus 30?M (20S)Rg3 (n=25) for 30 min are shown as mean ± s.d., n=3. (E) (20S)Rg3 prevents the loss of CREB phosphorylation in stimulated neurons treated with oligomeric Aβ (200 nM). Primary cortical neurons were stimulated (stim) with a condition that caused increased neuronal activity (10 ?M NMDA; ref. 38). Note that scyllo-inositol (a known Aβ blocker, positive control) and (20S)Rg3 blocked the Aβ-induced decrease in the

levels of phophorylated CREB at Ser-133 (p-CREB). The asterisk (*) indicates a location of band that was previously described as crossreactivity with ATF. (F) Bar graphs are shown as % control normalized to the levels of Tuj-1 (neuron-specific tubulin- βIII). (G) (20S)Rg3, an activator of PI4KIIα, blocks the suppression of hippocampal long-term potentiation (LTP) induced by Aβ oligomers . LTP profile after treatment with vehicle (n = 7), Aβ oligomers (n = 8), (20S)Rg3 alone (n = 7), or Aβ oligomers together with (20S)Rg3 (n = 10). The slices treated with (20S)Rg3 (30 ?M) exhibit normal LTP despite of the presence of Aβ oligomers.