UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of PICK1 and PKC alpha in Alzheimer's Disease

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Neurosciences

by

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The Dissertation of Stephanie Isabel Alfonso is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2016

DEDICATION

I would like to dedicate this thesis to my son and inspiration, Alex Alfonso. Thank you for changing my life and giving me a purpose to live. It is your love and your smile that keep me going everyday and I could never imagine a life without you. This will be the beginning of a major turning point in our lives and I'm glad you'll be there to share it with me.

Tambien le dedico esta tésis a mis padres y mi hermano que siempre han estado alli para ayudarme con todo. Siempre han sido un gran apoyo y nunca les podre pagar lo que han hecho por mi. Gracias por todo el amor que me han dado a mi y a mi hijo.

Los quiero mas a que a nadie en el mundo,

Steph.

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Chapter I is a partial reprint of the material as it appears in European Journal of Neuroscience, Vol. 39, pp. 1225–1233, 2014. The dissertation/thesis author was the primary investigator and author of this paper. Synapto-depressive effects of amyloid beta require PICK1. Alfonso S, Kessels HW, BanosCC, Chan TR, Lin ET, Kumaravel G, Scannevin RH, Rhodes KJ, Huganir R, Guckian KM, Dunah AW, Malinow R.

Chapter II, in part, is currently being prepared for submission for publication of the material. The dissertation/thesis author was the primary investigator and author of this paper. Protein Kinase Cα Required for Synaptic Defects and has Gain of Function Mutations in Alzheimer's Disease. Alfonso S, Callender JA, Hooli B, Antal CE, Mullin K, Leitges M, Newton AC, Tanzi RE, Malinow R. (In Prep)

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PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

The Role of PICK1 and PKC alpha in Alzheimer's Disease

by

Stephanie Isabel Alfonso

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Professor Roberto Malinow, Chair

Beta amyloid (Aβ), a key component in the pathophysiology of Alzheimer's disease (AD) is thought to target excitatory synapses early in the disease. We show that excessive GluA2 receptor endocytosis, mediated by a PDZ ligand-domain interaction with protein interacting with C kinase (PICK1) and protein kinase C alpha (PKCα), is required for Aβ to weaken synapses. In mice lacking PICK1 or PKCα, elevations of Aβ fail to depress synaptic transmission in cultured brain slices. We find that reintroducing PKCα in PKCα null mouse slices rescues the Aβ-induced depression. Moreover, the reintroduction of a PDZ mutant form of PKCα does not rescue the synaptic depression. In addition, analysis of whole genome sequencing (WGS) of late onset AD (LOAD) families' data identified two rare highly penetrant variants in the PKCα gene (*PRKCA*)

that display increased signaling output relative to wild-type PKCα. We conclude that targeting these proteins could accelerate the development of treatments that could prevent, halt or reverse the course of the disease as well as improve its early detection.

INTRODUCTION

AD is a chronic neurodegenerative disease and the most common form of dementia affecting over 46 million people worldwide. It results in the loss of cognitive functioning—thinking, remembering, and reasoning. AD worsens over time and may eventually result in the patient needing full time assistance. AD has no effective treatment. The greatest known risk factor for Alzheimer's is advancing age. The likelihood of developing Alzheimer's doubles about every five years after age 65. The current focus is to accelerate the development of treatments that could prevent, halt or reverse the course the disease as well as improve its early detection.

The amyloid hypothesis (Hardy and Selkoe 2002), proposing that an excessive amount of Aβ peptide is responsible for the cognitive impairment in AD, is the most widely accepted pathophysiological model underlying the disease. AD affects the integrity of neuronal networks by leading to synaptic loss and hindering plasticity mechanisms. Animal models of AD support the idea that synapse shrinkage and loss occur before amyloid plaques and may be early contributors to memory loss. Studies in Tg2576 mice, which express human APP (hAPP), showed decreased dendritic spine density, impaired long-term potentiation (LTP), and behavioral deficits months before plaque deposition (Jacobsen 2006). Other studies have shown deficits in basal synaptic transmission in hAPP mice as early as one month of age (A Y Hsia et al. 1999). Aβ is strongly implicated as a causative agent; however, the functional relationship between continuous exposure to Aβ and depressed synaptic transmission is not well known.

In vitro studies have elucidated a mechanism for the depressed transmission

through which exposure to the Aβ peptide results in a decrease of surface and synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR). Excitatory synapses transmit much information by releasing glutamate onto the AMPARs and thus are the most susceptible to Aβ effects. AMPARs are tetrameric receptors comprised of combinations of four subunits, GluA1-4 (Hollmann and Heinemann 1994; Lomeli et al. 1993; Rosenmund, Stern-Bach, and Stevens 1998). In the hippocampus most of the AMPARs are composed of GluA1/GluA2 and GluA2/GluA3 (Wenthold et al. 1996). Elevated Aβ appears to produce synaptic depression by enhancing the endocytosis of AMPA receptors through a GluA2-mediated process (H Hsieh et al. 2006).

In a similar fashion, the expression of long-term depression of synaptic efficacy (LTD) results in an increase in the regulated endocytosis of GluR subunits that culminates in reduced surface expression of synaptic AMPARs (Luscher et al. 1999). Interestingly, expression of an AMPAR mutant that prevents its LTD-driven endocytosis blocks the morphological and synaptic depression induced by Aβ (H Hsieh et al. 2006). Thus Aβ may hijack the same pathway(s) utilized in LTD.

A candidate GluA2 removal pathway is one through protein interacting with protein kinase c alpha 1 (PICK1), whose PDZ domain can bind to the carboxy termini (CT) of both GluA2 (ESVKI-COOH) and PKCα (LQSAV-COOH) (Staudinger 1997). PICK1-dependent clustering promotes the PKCα phosphorylation of GluA2 by concentrating the kinase and the receptor on a common membrane; this event results in GluA2 surface removal (Seidenman et al. 2003). Interestingly, expression of a GluA2 phosphomimetic mutant [GluA2(R607Q;S880E)] leads to a decrease in AMPAR-mediated synaptic transmission that mimics Aβ overexpression (H Hsieh et al. 2006)

CHAPTER I- SYNAPTO-DEPRESSIVE EFFECTS OF Aβ REQUIRE PICK1

Despite its proposed prominent role, little is known regarding how Aβ produces deleterious effects that lead to AD. There has been considerable interest on the effects of Aβ on synapses (Shankar et al. 2007; Freir et al. 2011), as synapses appear to be an early target in the disease (DeKosky and Scheff 1990; Masliah et al. 2001; Terry et al. 1991). A number of studies indicate that elevated levels of Aβ lead to loss of postsynaptic receptors on excitatory synapses (Kamenetz et al. 2003; Cirrito et al. 2005; H Hsieh et al. 2006; Snyder et al. 2005). We sought to examine this process more carefully. We focused our studies on the GluA2-interacting protein PICK1 (Xu and Xia 2006). Recent studies have indicated that PICK1 is required for the endocytosis of AMPA receptors that occurs in long-term depression (Terashima et al. 2008; Citri et al. 2010) a physiological process that may be hijacked by Aβ to produce synaptic depression (Helen Hsieh et al. 2006; Snyder et al. 2005). Here we test if PICK1 is required for the effects of Aβ on synapses. We find that in tissue from animals lacking PICK1, Aβ fails to depress AMPAR-mediated synaptic transmission and Aβ fails to reduce surface AMPA receptors. Furthermore, a small molecule that blocks the PDZ-domain mediated interaction between GluA2 and PICK1 blocks the effects of Aβ on synaptic transmission and surface receptors. We conclude that a PDZ-domain mediated PICK1 interaction with GluA2 is required for effects of Aβ on synapses.

Methods

Tissue preparation: Experiments were conducted in accordance with and received approval from the Institutional Animal Care and Use Committees at UCSD and Biogen

Idec. The experiments were carried out in accordance with guidelines laid down by the NIH regarding the care and use of animals for experimental procedures.

Hippocampal slice cultures and sindbis virus infection: Organotypic hippocampal slice cultures were made from postnatal day 6 or 7 rat pups as described (Stoppini, Buchs, and Muller 1991). Slice cultures were maintained in culture for 6–8 days then infected using a Sindbis virus (pSinRep5 dp APP-CT100 + tdTomato). Cells were recorded 16-30 hr after Sindbis virus infection.

Dissociated primary neuron cultures : Primary hippocampal cultures were prepared from embryonic day 18 rat brains as described previously (Goslin and G. Banker 1991). Cells were plated on coverslips coated with poly-d-lysine (30 μg/ml) and laminin (2 μg/ml) at a density of 70,000 per well in a 12-well plate. Hippocampal neurons were grown in Neurobasal medium (Invitrogen) and supplemented with B27 (Invitrogen), 0.5 mm glutamine, and 12.5 μm glutamate, and used for the described studies at 21 days *in vitro* (DIV21).

Electrophysiology and pharmacological treatments: Slices were maintained in a solution of ACSF containing in mm: 119 NaCl, 26 NaHCO3, 1 NaH2PO4, 11 d-glucose, 2.5 KCl, 4 CaCl2, 4 MgCl2, and 1.25 NaHPO4 and gassed with 95% O2. In addition, the following drugs were added: 4 μm 2-chloroadenosine (to prevent stimulus induced bursting), and 100 μm picrotoxin (to block inhibitory transmission). Simultaneous whole-cell recordings were obtained from pairs of neighboring (<50 μm) control and infected CA1 pyramidal neurons using 3–5 MΩ glass pipettes with an internal solution containing the following in mM: 115 cesium methanesulfonate, 20 CsCl, 10 HEPES, 2.5 MgCl2, 4 Na2ATP, 0.4 Na3GTP, 10 sodium phosphocreatine, 0.6 EGTA, and 0.1 spermine, at pH

7.25. All recordings were done by stimulating two independent synaptic inputs; EPSCs were recorded while holding the cells at −60 mV, alternating pathways every 8.4 s. Results from each pathway were averaged and counted as n = 1. For pharmacological experiments, slices were incubated 2 hours prior to recordings with 10 μM BIO922; the drug was also added to the recording chamber at the same concentration. To measure rectification, paired recordings were performed (as described above) and cells were held at -60, +40, and 0 mV. In addition, 10 μm gabazine (to block inhibitory transmission) and 100 μm of APV (to block NMDA response) were added to the perfusion chamber. The following equation was used to compute the rectification index: $(EPSC_{-60mV} - EPSC_{0mV})/(EPSC_{+40mV} - EPSC_{0mV})$. The mean rectification of infected cells was normalized by the mean control cell rectification. All data are reported as mean ± SEM. Statistical analysis for paired recordings used the paired t-test, with $p < 0.05$ considered significant.

Biochemical assays: Competition and binding Fluorescence Polarization (FP) assays were used to determine PDZ binding selectivity. For all assays a fixed concentration (5 nM) of FITC-labeled peptides composed of the C-terminal amino acids of GluA2 (875-883) and GluN2B (1474-1482) were used. Binding FP assays were done using increasing concentrations of recombinant full length PICK1, PSD95 PDZ 1-2 and GRIP1 PDZ 4-6 in order to determine sufficient binding for subsequent competition assays. Binding assays were normalized by subtracting tracer only background. Competition FP assays were performed with both fixed FITC-labeled peptides (5 nM) and non-saturating protein concentrations (PICK1, 600 nM; PSD95 PDZ 1-2, 8u M; GRIP1, 10 μM) while changing the concentrations of unlabeled peptides and BIO922

compound from 3-10 μM (data point for 10 μM shown in Figure 2), and normalized by standardizing to both binding and tracer only controls. All FP assay components were diluted in a buffer system containing 50mM Tris pH7.4, 200 mM NaCl, 2 mM DTT, 0.05% PF68 and 5% Glycerol.

Surface staining of hippocampal neurons: Live staining of endogenous AMPA and GABA receptors was performed as previously described (Wyszynski et al. 2002) using antibodies recognizing extracellular regions of GluA1, GluA2 and GABA2/3 subunits. Briefly, hippocampal neurons were incubated with antibody for 15 minutes at 37 °C to decorate surface receptors, fixed under non-permeabilizing conditions in phosphate buffer containing 2% formaldehyde/4% sucrose at room temperature, washed in phosphate buffer, and visualized with Alexa488-conjugated secondary antibody.

Total staining of hippocampal neurons: Neurons were fixed with 4% paraformaldehyde and 4% sucrose in phosphate buffer, permeabilized with 0.25% Triton X-100, and immunolabeled using anti-GluA1, anti-GluA2, anti-GABA2/3 primary antibodies. Staining was visualized with Alexa488-conjugated secondary antibodies.

Image analysis and quantification: Confocal images of immunostained neurons were obtained using a Zeiss LSM 710 confocal microscope objective with sequential acquisition settings at the resolution of 1024 x 1024 pixels. Each image was a z-series of 8-10 spaced at intervals of about 0.5 μm, and the resultant stack was 'flattened' into a single image using a maximum projection. The confocal microscope settings were kept the same for all scans. All analysis and quantifications were performed using MetaMorph image analysis software (Universal Imaging Corporation). Dendrites from experimental groups were randomly selected and carefully traced, and the average intensity of

fluorescence staining was determined for the traced regions. Intensity measurements are expressed in arbitrary units of fluorescence per square area. Blind conditions were used for the acquisition and quantification of images.

Surface biotinylation assay: High-density cortical neurons at DIV21 were used for surface biotinylation as described (Lin et al. 2000). Briefly, neuronal surface proteins were biotinylated with 600 μg/ml Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) in artificial CSF buffer for 20 min at 4°C. Unreacted Sulfo-NHS-SS-Biotin was removed by washing with ice-cold 50 mm glycine in phosphate buffer. Cells were lysed with ice-cold lysis buffer (20 mm sodium phosphate, pH 7.5, 150 mm NaCl, 0.1% SDS, 0.5% NP-40, and 0.5% sodium deoxycholate) containing protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mm PMSF). Biotinylated surface proteins were isolated using immobilized NeurtrAvidine beads (Pierce) and immunoblotted using Anti-GluA2 and Anti-N-cadherin antibodies.

Preparation of Aβ oligomers: Synthetic Aβ42 HFIP peptide was prepared to stock concentration of 200μM by adding 10 μl DMSO to 100μg of Aβ42 HFIP pellet and incubated for 30 minutes at room temperature with occasional mixing. Phosphate buffer was added to final concentration of 1mg/ml and mixed with a pipette. The solution was at room temperature for 2 hours and then used for experiments.

Results

The interaction of phosphorylated GluA2 and PICK1 results in the internalization of surface GluA2 subunits. We hypothesized that the expression of Aβ could enhance this GluA2-PICK1 interaction and thereby increase endocytosis. We virally expressed in

organotypic hippocampal slices for 16-30 hours CT100, the beta-secretase product of APP and precursor to Aβ (Kamenetz et al. 2003). We compared evoked synaptic AMPA-R-mediated transmission between neighboring infected and uninfected CA1 neurons by paired whole-cell recordings. In brain slices prepared from wild-type mice, neurons expressing CT100 displayed significantly depressed excitatory transmission (Figure 1). In contrast, in brain slices prepared from animals lacking PICK1, neurons expressing CT100 showed no significant synaptic depression (Figure 1). In 19 out of 21 paired recordings the cell expressing CT100 displayed depression in control slices while in only 11 out of 20 did the cell expressing CT100 display depression in slices from animals lacking PICK1 ($p< 0.05$, χ^2 test). These results support the view that PICK1 is required for Aβ to produce synaptic depression.

Since PICK1 is known to bind GluA2 (Xu and Xia 2006), we sought to examine if Aβ preferentially acts on GluA2-containing receptors. We measured the rectification index of transmission in neurons expressing CT100. Receptors lacking GluA2 transmit more poorly at positive potentials, and thus display a greater rectification index (see methods). Synaptic transmission onto neurons expressing CT100 showed a larger rectification index (1.6 ± 0.1 in control neurons; 2.6 ± 0.4 in neurons expressing CT100; Figure 1). These results support the view that Aβ preferentially removes synaptic receptors containing GluA2; the remaining transmission thus contains more GluA2-lacking receptors that can explain the increase in rectification index.

To test if an interaction between PICK1 and GluA2 is required for the synaptic effects of Aβ, we used a small molecule (BIO922) that blocks this interaction. BIO922 is an inhibitor (Ki = 98 nM, Figure 2) of the interaction between full length recombinant

PICK1 and GluA2 cytoplasmic domain. BIO922 shows greater than 100-fold selectivity over other related PDZ-domain containing proteins namely, PSD-95 and GRIP (Figure 2). BIO922 was discovered by structure based drug design targeted to the PICK1 PDZ domain (complete discovery of BIO922 will be described elsewhere, manuscript in preparation). Brain slices from wild type animals were infected with a virus producing CT100. After ~16-18 hrs, slices were exposed to media containing 10 μM BIO922 or normal media as a control for 2 hours. We obtained paired whole-cell recordings from infected and non-infected neurons. While slices exposed to normal media displayed the normal synaptic depression in CT100-infected neurons, slices exposed to BIO992 showed no significant synaptic depression in CT100-infected neurons (Figure 3). In 11 out of 12 paired recordings the cell expressing CT100 displayed depression in control slices while in only 10 out of 16 did the cell expressing CT100 display depression in BIO992-exposed slices ($p< 0.05$, χ^2 test). These results support the view that a PDZ-domain interaction between PICK1 and GluA2 is required for Aβ to produce synaptic depression. Since BIO922 was added after synaptic depression occurred, the results indicate that the drug rescued synapses from a depressed state and that Aβ-induced synaptic depression observed at 16-18 hrs is not irreversible.

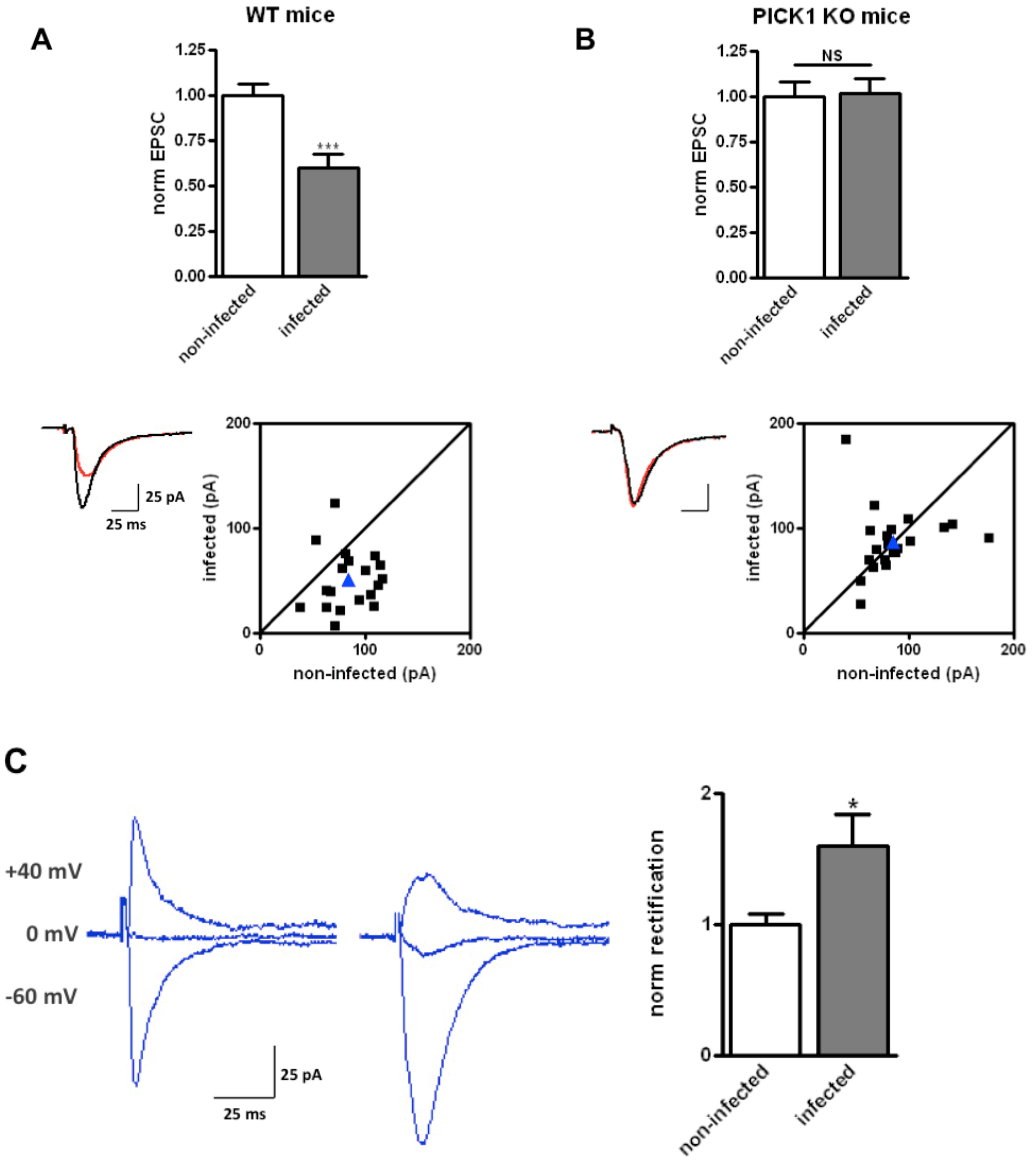


Figure 1: PICK1 KO mice do not show Aβ-induced synaptic depression. A, B, Organotypic hippocampal slices prepared from WT (A) and PICK1 KO (B) mice were infected with CT100 virus to elevate Aβ. EPSCs were recorded from infected and non-infected cell pairs (WT, n = 19 pairs, $p < 0.001$; PICK1 KO, n= 21 pairs, $p = 0.8$). Top, graph of normalized average EPSC amplitudes for infected and non-infected neurons. Lower left, sample traces from infected (red) and non-infected (black) cell pairs. Lower right, dot plot of EPSC amplitude of infected vs non-infected neuron. Each black square represents responses from one cell pair; blue triangle indicates average of all responses. C, Aβ elevation increases rectification of synaptic transmission. Left, sample traces from paired recordings at indicated holding potentials from non-infected (left) and infected (right) cells. Right, graph of normalized rectification index (n = 7 pairs; $p = 0.01$).

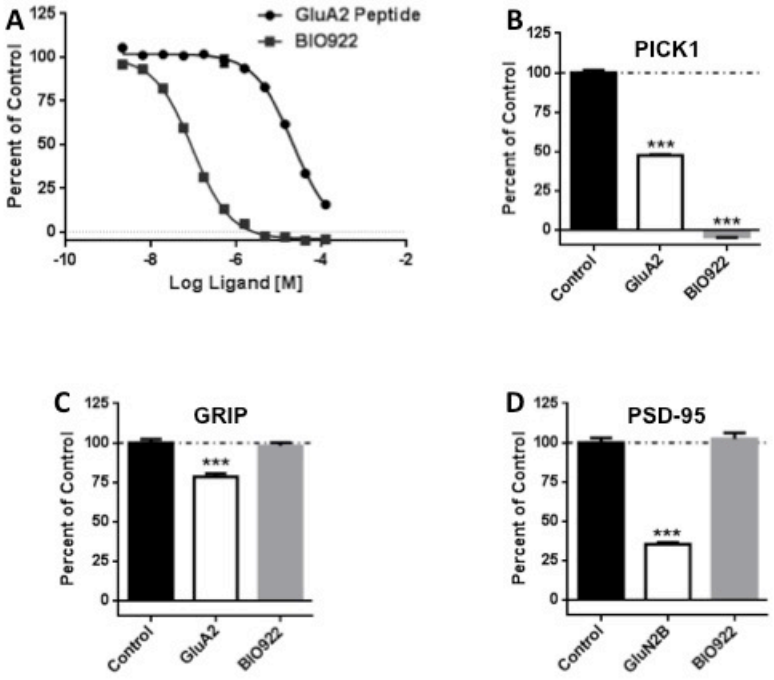


Figure 2: Small molecule inhibitor of PICK1-GluA2 interaction specifically blocks the binding of GluA2 to PICK1 relative to other PDZ domain containing proteins. A, Fluorescence polarization competition binding of PICK1 inhibitor (BIO922) with FITC-labeled BIO424 tracer. B, C, Fluorescence polarization competition assay of FITC-labeled GluA2 peptide with increasing concentrations of unlabeled GluA2 and BIO922 using either recombinant full length PICK1 (B) and purified GRIP PDZ 4-6 proteins (C). D, Fluorescence polarization binding of FITC-labeled GluN2B peptide at increasing concentrations of unlabeled GluN2B and BIO922 using purified PSD-95 PDZ 1-2 protein.

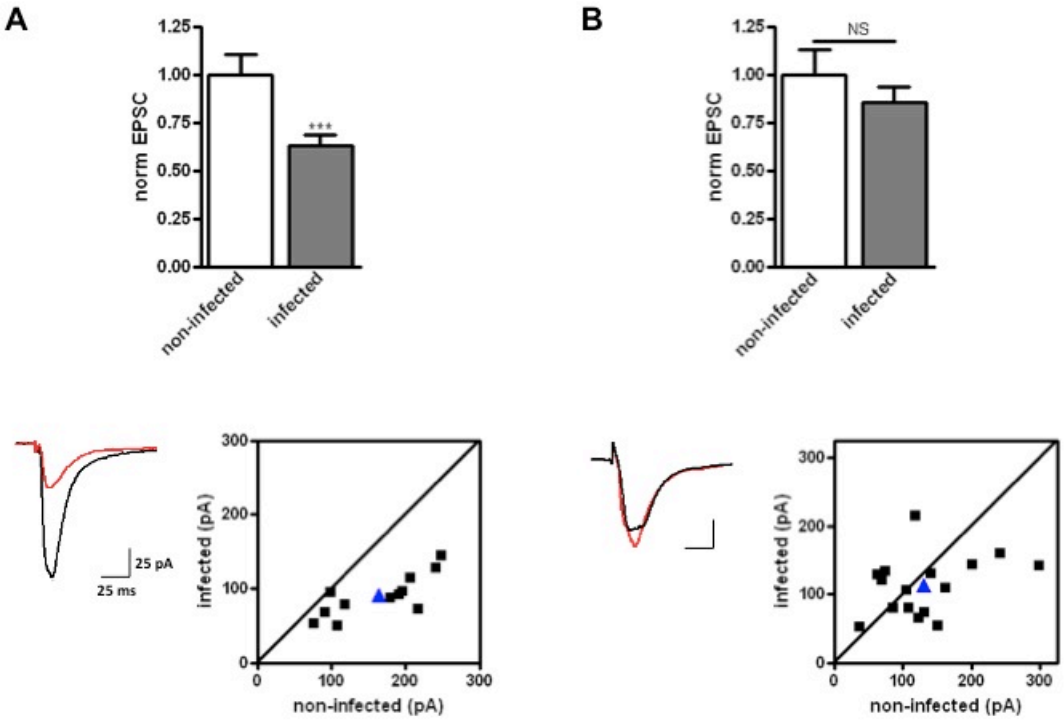


Figure 3: Blocking the interaction between GluA2 and PICK1 PDZ domain rescues Aβ-induced synaptic depression. A, Same as figure 1A for a separate group of cell pairs (n= 12 pairs; $p < 0.001$). B, Same as A, but in the presence of a PICK1 inhibitor (BIO922, 10 μM; n = 16 pairs; $p = 0.3$).

To examine the role of PICK1 on Aβ-induced AMPA-R endocytosis we measured surface AMPA-Rs in dissociated cultured neurons (See methods). Following exposure of dissociated cultured neurons for 24 hours to Aβ, we noted a reduction in surface AMPA-R staining with no effect on total AMPAR staining, consistent with the view that Aβ drives AMPA-R endocytosis (Figure 4). In particular, the effect was more prominent on surface GluA2 compared to surface GluA1 (Figure 4; GluA1 reduction = 70 ± 4%; GluA2 reduction = 41 ± 7%; $p < 0.05$ test). We next examined surface AMPA-Rs in tissue prepared from mice lacking PICK1. We noted that surface staining for GluA2, but not surface GluA1, was elevated in neurons lacking PICK1 compared to wild type neurons (Figure 5). Total GluA2 staining and surface GABA receptor staining were not changed in neurons lacking PICK1. The elevated surface GluA2 was confirmed using surface biotinylation-based assay (See methods; Figure 5). When exposed to Aβ, surface GluA2 staining on wild type neurons was reduced; however Aβ application on neurons lacking PICK1 did not reduce surface GluA2 staining (Figure 6). These findings are consistent with the view that PICK1 normally participates in maintaining a significant fraction of GluA2-containing AMPARs in an intracellular pool. In the absence of PICK1, these GluA2-containing intracellular receptors are released onto the surface. Upon addition of Aβ, PICK1 is required for movement of receptors from the surface to an intracellular pool.

To test if a PDZ-domain interaction between PICK1 and GluA2 is necessary for the actions of Aβ on surface AMPARs, we used BIO922. Dissociated cultured neurons were exposed to Aβ for 24 hrs in the presence or absence of 3 μM BIO922. While neurons exposed to Aβ in the absence of drug showed a significant reduction in surface

GluA2 staining, neurons exposed to Aβ in the presence of BIO922 showed no reduction in surface GluA2 staining (Figure 7). These findings support the view that an interaction between PICK1 and GluA2 is required for Aβ to drive surface AMPAR endocytosis.

In this study we have examined the mechanism by which Aβ affects synapses. We used two different assays to monitor AMPA receptors: synaptic transmission and surface labeling of AMPARs. We confirm that virally-driven elevation of Aβ leads to synaptic depression in organotypic hippocampal slices (H Hsieh et al. 2006). We now find that synaptic transmission remaining after exposure to Aβ displays greater rectification, consistent with the view that Aβ preferentially drives the synaptic removal of AMPARs containing GluA2. We also find that exposure of dissociated cultured neurons to synthetic Aβ drives removal of surface AMPARs, with a greater effect on GluA2 compared to GluA1. Since a significant proportion of AMPARs are thought to contain GluA1 and GluA2 (Wenthold et al. 1996), it is possible that the surface loss of GluA1 is a consequence of GluA2-mediated loss of GluA1/GluA2 heteromers. A significant loss of surface GluA2/GluA3 heteromers could account for the greater effect seen on GluA2 compared to GluA1. Thus, in both assays, GluA2-containing receptors are preferentially targeted for surface and synaptic removal by Aβ.

We have examined the role of PICK1 in the effects of Aβ by using mice lacking PICK1 and BIO922, a drug that targets the interactions between the GluA2 PDZ ligand and the PICK1 PDZ domain. We find that in organotypic slices prepared from mice lacking PICK1, virally-driven elevation of Aβ fails to produce depression of synaptic transmission. We also find that in dissociated cultured neurons prepared from mice lacking PICK1, Aβ fails to drive removal of surface AMPARs. Furthermore, we find that

exposure of slices to BIO922 reverses synaptic depression produced by elevated Aβ, and that exposure of dissociated cultured neurons to BIO922 blocks surface removal of AMPARs produced by Aβ. These findings support a model in which Aβ triggers signaling that increases the interaction between GluA2 cytoplasmic tail and PICK1 PDZ domain, and that such an interaction promotes endocytosis of surface AMPARs. Alternatively, the increased GluA2-PICK1 interaction could stabilize an intracellular pool of AMPARs (Citri et al. 2010; Thorsen et al. 2010) if AMPARs are continually cycling between intracellular and surface locations (Luscher et al. 1999). The finding that BIO922 application to brain slices was able to rescue synaptic depression, is consistent with such AMPAR dynamics. The signaling triggered by Aβ that produces these effects on AMPAR distribution remains to be elucidated, but could include activation of a protein kinase that phosphorylates GluA2 cytoplasmic domain, which has been shown to reduce GluA2 interactions with the synaptic protein GRIP, while maintaining GluA2 interactions with PICK1 (Chung et al. 2000; Thorsen et al. 2010). Our findings suggest that drugs targeting the interaction between GluA2 and PICK1 may be beneficial in offsetting the effects of elevated Aβ, and therefore in the treatment of Alzheimer's disease.

Chapter I is a partial reprint of the material as it appears in European Journal of Neuroscience, Vol. 39, pp. 1225–1233, 2014. The dissertation/thesis author was the primary investigator and author of this paper. Synapto-depressive effects of amyloid beta require PICK1. Alfonso S, Kessels HW, BanosCC, Chan TR, Lin ET, Kumaravel G, Scannevin RH, Rhodes KJ, Huganir R, Guckian KM, Dunah AW, Malinow R.

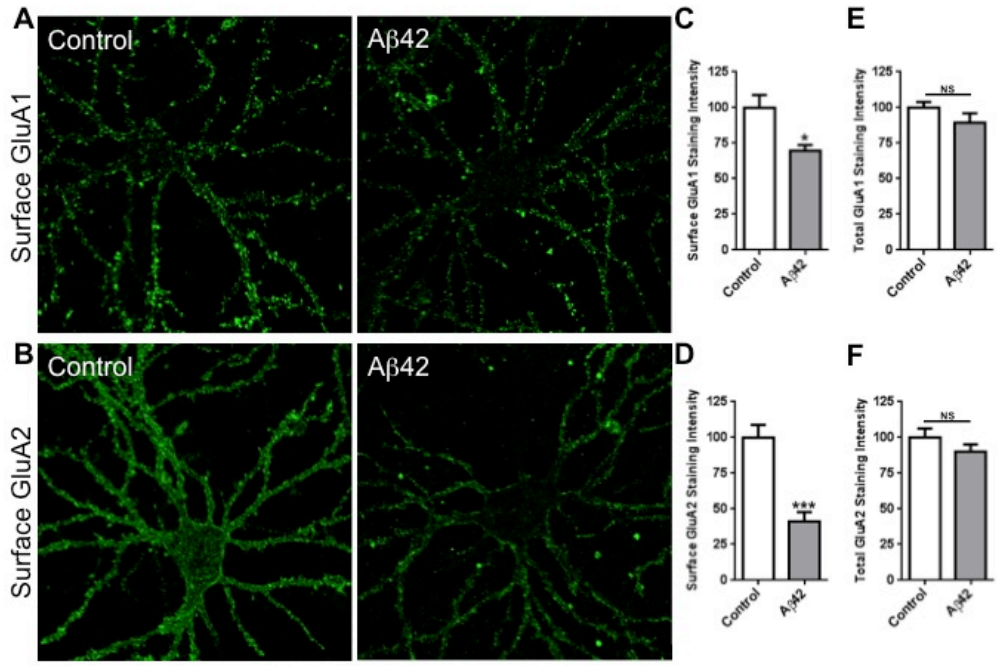


Figure 4: Soluble oligomeric Aβ42 decreases surface AMPA receptors in neurons. A, B; Cultured rat hippocampal neurons were treated with soluble Aβ42 (5 μM) and labeled for surface GluA1 (A) and GluA2 (B). C-F; Quantification of immunofluorescence intensities of surface and total GluA1 and GluA2 subunits, normalized to control group. C, n = 17 control, 17 Aβ42 treated; $p = 0.01$. D, n = 18 control, 18 Aβ42 treated; $p < 0.001$; E, n = 15 control, 15 Aβ42 treated; $p = 0.19$; F, n = 16 control, 16 Aβ42 treated; $p = 0.24$.

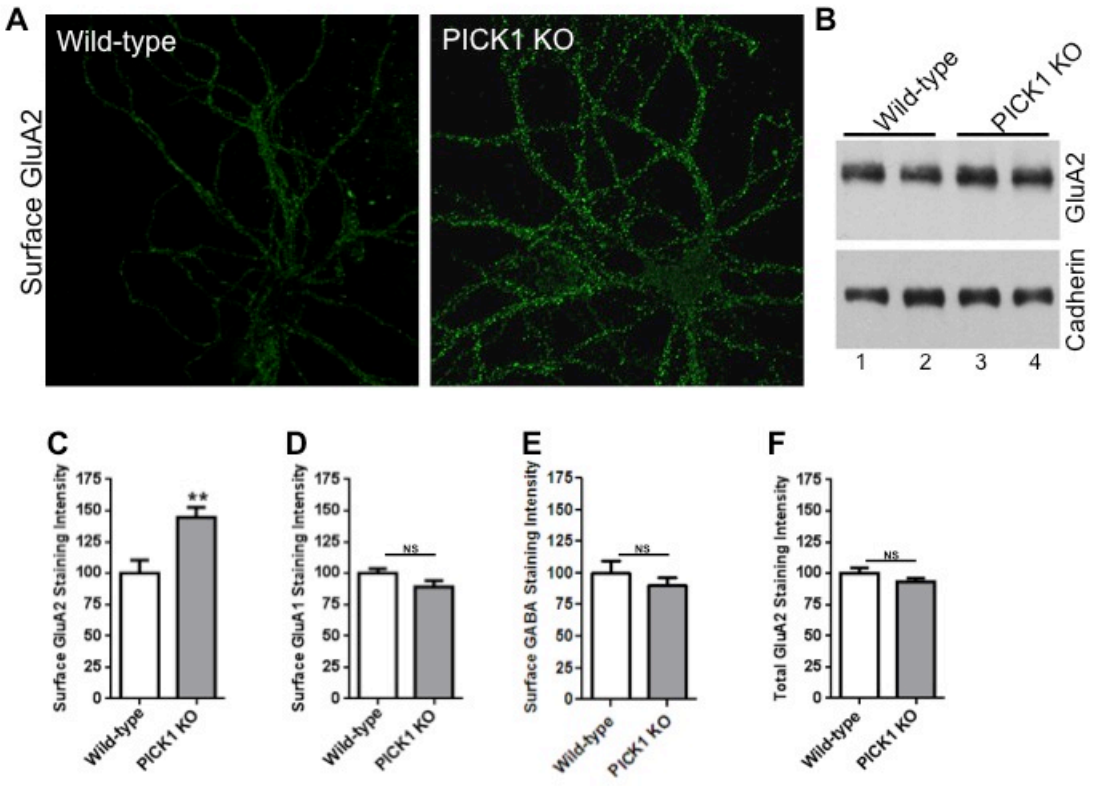


Figure 5: PICK1 deletion increases surface AMPA receptors. A; Cultured hippocampal neurons from wild-type and PICK1 knockout mice were immunostained for surface GluA2. B; Surface biotinylation analysis of GluA2 in cortical neurons cultured from wild-type and PICK1 knockout mice. Western blots were probed as indicated. GluA2 signal intensity normalized to cadherin for wild-type was 1.2 fold in PICK1 knockout mice (from two experiments). C-F; Quantification of staining intensities of surface GluA1 (C), surface GluA2 (D), surface GABA (E) and total GluA2 (F) receptors in wild-type and PICK1 knockout mice neurons normalized to wild-type group. C, n = 18 wild-type, 18 PICK1 KO; p = 0.01. D, n = 16 wild-type, 16 PICK1 KO; p = 0.13; E, n = 15 wild-type, 15 PICK1 KO; p = 0.41; F, n = 16 wild-type, 16 PICK1 KO; p = 0.23.

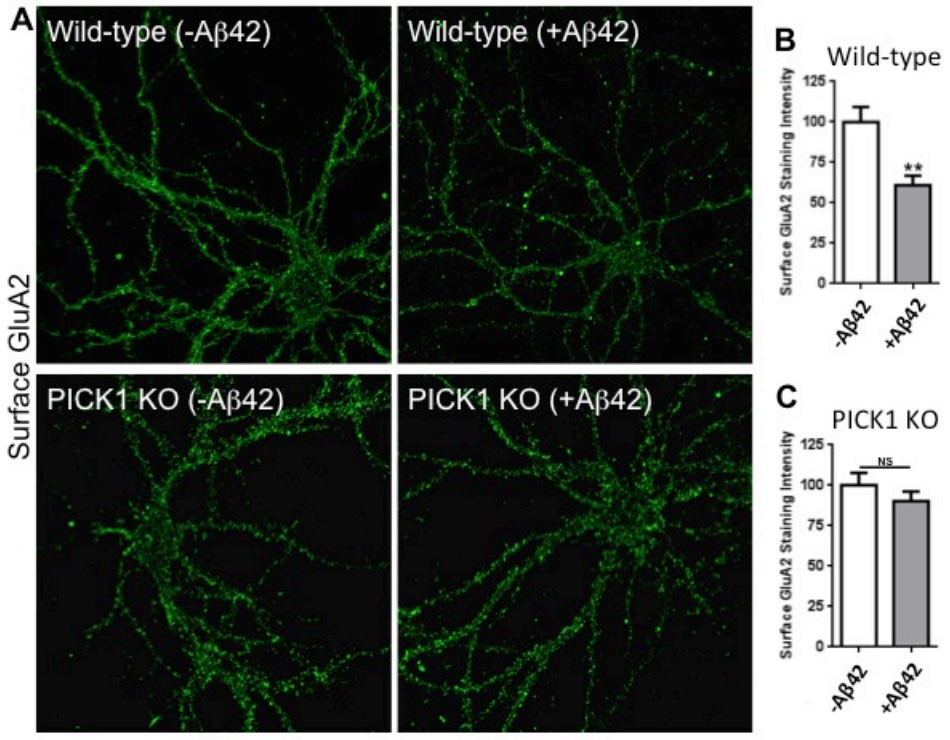


Figure 6: PICK1 deletion blocks Aβ-induced reduction in surface GluA2. A, Hippocampal neurons cultured from wild-type (A) and PICK1 knockout (B) mice were treated with Aβ and labeled for surface GluA2. B, C, Quantification of surface GluA2 immunofluorescence intensities from wild-type and PICK1 knockout mice Aβ42-treated neurons normalized to wild-type values. B, n = 17 wild-type (-Aβ42), 17 WT (+Aβ42); $p < 0.01$. C, n = 18 PICK1 KO (-Aβ42), 18 PICK1 KO (+Aβ42); $p = 0.34$.

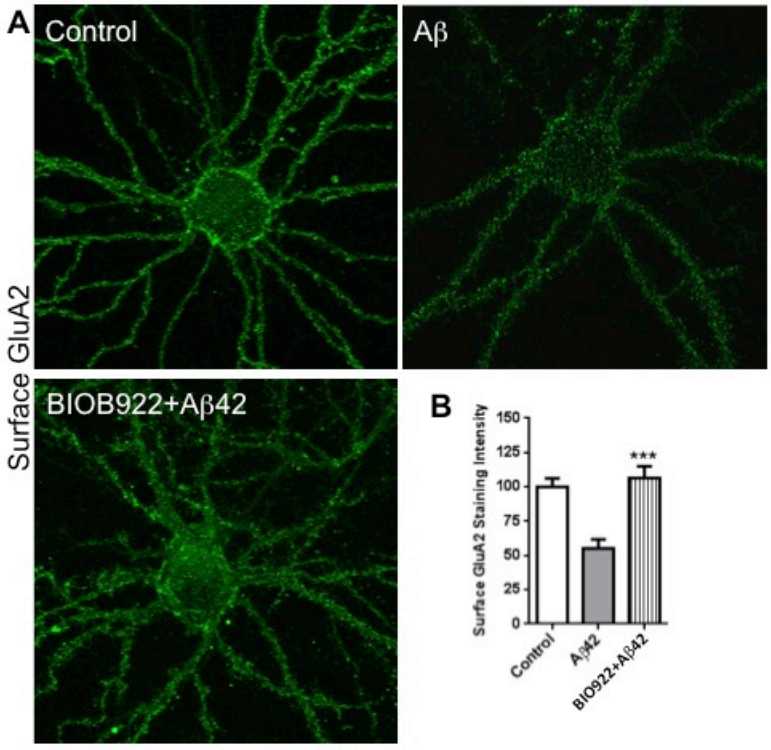


Figure 7: PICK1 inhibitor blocks Aβ-mediated reduction in surface AMPA receptors. A, Hippocampal neurons were treated with no drug (upper left), Aβ42 in presence (lower left) or absence (upper right) of PICK1 inhibitor (BIO922, 3μM) for 24 hours, and immunostained for surface GluA2. B, Quantification of surface GluA2 immunofluorescence intensity normalized to control (non-treated) values. n = 17 control, 17 Aβ42 treated, 17 Aβ42 treated + BIO922, $p < 0.001$ comparing Aβ42 treated with Aβ42 treated + BIO922.

CHAPTER II- PKCα's ROLE IN ALZHEIMER'S DISEASE

Here we examine the role of PKCα, which in many tissues is required to keep cancer in check. We find that in mouse brain tissue, PKCα is required for synaptic degenerative effects of elevated Aβ, a likely early event in AD. Whole genome sequencing of 1345 individuals from 410 LOAD families identified two highly penetrant variants in *PRKCA* in five families. Importantly, both variants displayed increased cellular signaling relative to wild-type PKCα. Thus, whereas loss-of-function mutations in PKCα contribute to cancer, enhanced PKCα activity may participate in AD, reinforcing the importance in maintaining a careful balance in the activity of this enzyme.

Synaptic loss correlates well with the earliest AD symptoms in humans (Terry et al. 1991; Selkoe 2002) and is an early event in rodent models of AD (A. Y. Hsia 1999; Mucke and Selkoe 2012; Oddo et al. 2003). The loss of synapses may result from elevated levels of Aβ (H Hsieh et al. 2006; Wilcox et al. 2011; Kamenetz et al. 2003), a peptide central to AD development. Indeed, familial forms of AD are caused by mutations in genes that increase production of the most toxic form of Aβ (Holtzman, Mandelkow, and Selkoe 2012). Furthermore, a minor allele of APOE (APOE-ε4), which is strongly linked with both the appearance and earlier onset of LOAD (Corder et al. 1993), may act by reducing the clearance of Aβ from the brain (Holtzman, Mandelkow, and Selkoe 2012).

We sought to identify other genes that contribute to LOAD, as their gene products may serve as therapeutic targets. We reasoned that variants of proteins participating in the detrimental effects of Aβ may contribute to the disease. We thus examined the signaling

underlying Aβ-induced synaptic depression. We focused our efforts on PKC (Newton 2010) because an interaction partner, PICK1, is required for the synaptic effects of Aβ (Alfonso et al. 2014) and, additionally, PKC is required for a plasticity form of synaptic depression (Michael Leitges et al. 2004). Furthermore, since reduced PKC activity contributes to increased cell survival (C E Antal et al. 2015), we speculated that increased PKC activity could participate in AD, a disease of neuronal degeneration (Holtzman, Mandelkow, and Selkoe 2012).

Methods

Tissue preparation: Experiments were conducted in accordance with and received approval from the Institutional Animal Care and Use Committees at UCSD. The experiments were carried out in accordance with guidelines laid down by the NIH regarding the care and use of animals for experimental procedures.

Hippocampal slice cultures and Sindbis virus infection: Organotypic hippocampal slice cultures were made from postnatal day 6 or 7 rat pups as described (Stoppini, Buchs, and Muller 1991). Slice cultures were maintained in culture for 6–8 days then infected using a Sindbis virus (pSinRep5 dp APP-CT100 tdTomato). Cells were recorded 16-30 hr after infection. For Figure 2 experiments, slices were made as described above, but from either wild type (wt) or PKC -/- mouse pups and infected with the indicated Sindbis viruses.

Electrophysiology and pharmacological treatments: Slices were maintained in a solution of ACSF containing in mM: 119 NaCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 11 D-glucose, 2.5 KCl, 4 $CaCl_2$, 4 $MgCl_2$, and 1.25 $NaHPO_4$ and gassed with 95% O_2 5% CO_2. In

addition, the following drugs were included: 4 μm 2-chloroadenosine (to prevent stimulus induced bursting), and 100 μm picrotoxin (to block inhibitory transmission). Simultaneous whole-cell recordings were obtained from pairs of neighboring (<50 μm) control and infected CA1 pyramidal neurons using 3–5 MΩ glass pipettes with an internal solution containing the following in mM: 115 cesium methanesulfonate, 20 CsCl, 10 HEPES, 2.5 $MgCl_2$, 4 Na_2ATP, 0.4 Na_3GTP, 10 sodium phosphocreatine, 0.6 EGTA, and 0.1 spermine, at pH 7.25. All recordings were done by stimulating two independent synaptic inputs; EPSCs were recorded while holding the cells at −60 mV, alternating pathways every 8.4 s. Results from each pathway were averaged and counted as n = 1. For pharmacological experiments, slices were incubated overnight prior to recordings with either 0.3 μM Gö 6983 or 3 μM Bisindolylmaleimide IV (Bis IV); the drug was also added to the recording chamber at the same concentration. In addition, 10 μM gabazine (to block inhibitory transmission) was added to the perfusion chamber. All data are reported as mean ± SEM. Statistical analysis employed bootstrap (resampling) methods (Efron 1979). For instance, in Figure 1, we calculated the probability by bootstrap resampling (100,000 times) the data from groups a, b and c, and measuring the frequency with which (A>C or B>C) is true (where caps indicates mean); for Figure 2, we bootstrap resampled (100,000 times) data groups, measuring the frequency with which (A>B or C>B or A>D) is true. For Figure 4, we bootstrap resampled (100,000 times) data groups, measuring the frequency with which [(MV at t=3hrs > wt at t=3hrs) or (MV at t=6hrs > wt at t=6hrs)] is true.

Plasmid Constructs: The C Kinase Activity Reporter (CKAR) and plasma membrane-localized PKC reporter (PM-CKAR) were described previously (Violin et al. 2003). The PSD95 specific PKC reporter (PSD95-CKAR) contains CKAR with PSD95 fused to its N-terminus via a four amino acid linker (EPGQ) in a pcDNA3 vector (Life Technologies). PKC constructs were prepared as described previously (C. E. Antal et al. 2015). For HA-PKCα, human PKCα was N-terminally HA-tagged via Gateway cloning into pDEST-HA generated from ligating the Reading Frame Cassette C into the EcoRV site of pcDNA3-HA. All mutants were generated using QuikChange site-directed mutagenesis (Agilent Technologies).

Antibodies and Reagents: The pan anti-phospho-PKC activation loop antibody (pT497) was previously described (Dutil, Toker, and Newton 1998). The anti-phospho-PKCα/βII (pT638/641; 9375S) and pan anti-phospho-PKC hydrophobic motif (βII pS660; 9371S) antibodies were purchased from Cell Signaling. The α-HA antibody (anti-HA, 11867423001, clone 3F10) purchased from Roche. Phorbol 12,13-dibutyrate (PDBu), Uridine-5'-triphosphate (UTP) trisodium salt, Gö 6983, and Bis IV were obtained from Calbiochem.

Cell Culture, Transfection, and Immunoblotting: All cells were maintained in DMEM (Corning) containing 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin/streptomycin (Corning) at 37˚C, in 5% CO_2. Transient transfection of COS7 was carried out using FuGENE 6 transfection reagent (Roche) for ~24h. Cells were lysed in 50 mM Tris, pH 7.4, 1% Triton X-100, 50 mM NaF, 10 mM $Na_4P_2O_7$, 100 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 50 µg/mL Leupeptin, 1 µM Microcystin, 1 mM DTT, and 2 mM Benzamidine. Whole cell lysates were analyzed by SDS-PAGE and

immunoblotting via chemiluminescence on a FluorChemQ imaging system (ProteinSimple). For cellular dephosphorylation experiments, cells were treated with 200 nM PDBu for the indicated times at 37˚C before lysis.

FRET imaging and analysis: Cells were imaged as described previously . COS7 cells were co-transfected with the indicated mCherry-tagged PKC and either CKAR, plasma membrane-targeted CKAR (PM-CKAR), or CKAR fused to PSD95 (PSD95-CKAR), as specified. Cells were rinsed once with and imaged in Hanks' balanced salt solution containing 1mM $CaCl_2$. Images were acquired on a Zeiss Axiovert microscope (Carl Zeiss Microimaging, Inc.) using a MicroMax digital camera (Roper-Princeton Instruments) controlled by MetaFluor software (Universal Imaging, Corp.). Using a 10% neutral density filter, CFP, YFP, FRET, and mCherry images were obtained every 15 seconds. YFP emission was monitored as a control for photobleaching and mCherry was measured to ensure that overexpressed PKC levels were equal in all experiments. Base-line images were acquired for ≥ 2 min before ligand addition and data were normalized to the baseline FRET ratios. The normalized average FRET ratio is the average of these normalized values ± S.E. Area under curve from 3-6 min was quantified and plotted in the bar graph in Figure 4, and statistical significance was determined as indicated above.

3D PKC Structure Modeling: The PKCα structure was visualized using the PyMOL Molecular Graphics System (Version 1.7.4.1, Schrödinger, LLC).

Genetics. Family cohort: The National Institute of Mental Health (NIMH) Alzheimer's Disease Genetics Initiative Study (Blacker et al. 2003) originally ascertained for the study of genetic risk factors in AD with family-based methods, was used in the WGS analyses in this study. The basis for ascertainment in the NIMH collection was the

existence of at least two affected individuals within a family, typically siblings. The complete NIMH study cohort contains a total of 1,536 subjects from 457 families. For the purpose of this analysis, we assessed 439 multiplex AD families (average onset age of patients: 72, s.d. 8 years) comprising 1,440 subjects (941 definitely affected and 404 definitely unaffected and the remainder could not be determined as definitely unaffected or definitely affected).

To test the likelihood of finding the observed linkage in the two rare PKCα variants, we conducted the following bootstrap analysis. We generated a 'parent' set containing 941 ones (indicating affected) and 404 zeros (indicating unaffected), which is the nature of the definitely assessed population in this cohort. We conducted the following sampling (allowing resampling) procedure: we chose 9, 1, 2 and 1 elements from the 'parent' set to generate 4 subsets, y(1) to y(4). We then tested if sum(y(1))>6 [test 1], sum(y(2))=0 [test2], sum(y(3))=2 [test 3] and sum(y(4))=1 [test 4]. That is, we tested if an affected individual in this population was PKCα variant carrier or APOE-e4 carrier. Note the values in the tests correspond to the observed genotype of the affected and unaffected individuals in our two variant-carrying families. If all tests were true, the result of the procedure was 1; if any of the tests was false, the result of the procedure was 0. This procedure was conducted 100,000 times (run four times). The number of times the result of the procedure was 1, in the four runs was 4859, 4769, 4872 and 4826. Thus the likelihood of finding this linkage distribution is <0.05.

Standard Protocol Approvals, Registrations, and Patient Consents. Diagnosis of AD dementia was established according to NINCDA-ADRDA criteria. Informed consent was provided by all participants, and research approval was established by the relevant

institutional review boards in the study cohort.

WGS Data Generation. 3 μg of total genomic DNA (150 ng/μL) obtained from Rutgers repository was sequenced at Illumina, Inc (San Diego, CA 92122 USA) using their latest HiSeq 2500, paired-end sequencing platform. An average of 48X coverage of 98% of the genme was observed in the resulting 120 GB data from each sample. Genomic variants were called in-house using FreeBayes (v0.9.9.2-18) and GATK best practices method (McKenna et al. 2010)resulting in close to 400 Tera-Bytes of high-quality sequencing data in a fully annotated GEMINI database (Paila et al. 2013).

Results

To test if the activity of PKC is required in Aβ-induced synaptic depression, we initially used PKC antagonists in combination with a previously established method that raises Aβ levels in neurons(Kamenetz et al. 2003; H Hsieh et al. 2006; Wei et al. 2010). Organotypic hippocampal slices were infected sparsely with a virus expressing CT100, the beta-secretase product of amyloid precursor protein (APP) and precursor to Aβ (Haass et al. 2012). 16-24 hours later, synaptic transmission was evoked by stimulating presynaptic axons (orange, Figure 8) and neighboring infected and uninfected postsynaptic CA1 neurons were simultaneously recorded (Figure 8). Neurons expressing CT100 displayed depressed excitatory transmission relative to uninfected neurons (Figure 8A).

To examine the role of PKC in Aβ-dependent synaptic transmission, we incubated slices (for ~12 hours before recordings) with the PKC inhibitors Gö 6983, an ATP-competitive inhibitor, or Bisindolylmaleimide IV (Bis IV), a non-competitive inhibitor.

Gö 6983, the active site inhibitor, was not able to rescue the Aβ-induced depression (Figure 8A, B). Recent work has shown that occupancy of the active site of PKC with an active site-directed inhibitor locks the enzyme in a more phosphatase-resistant conformation and thus prevents the dephosphorylation and subsequent degradation of PKC (Gould et al. 2011). Activation of PKC is achieved by ligand-mediated engagement of its regulatory domains at membranes, which allosterically releases the autoinhibitory pseudosubstrate from its active site, shifting the enzyme into an active and open conformation (Vallentin 2000). Based on these results we employed a regulatory domain inhibitor, BIS IV, which does not protect from dephosphorylation and is effective at inhibiting cellular PKC. We found that this non-competitive inhibitor was able to rescue Aβ-induced synaptic depression (Figure 8A, B).

Because PKC scaffolded near its substrates is refractory to active site inhibitors (Hoshi et al. 2010), these data support a role for scaffolded PKC mediating the synaptic effects of Aβ. Of the PKC isozymes that are modulated by these inhibitors, only PKCα has a PDZ ligand, a 3 amino-acid segment at its carboxyl terminus that binds the PDZ domains of scaffolding proteins (notably PSD95, SAP97 and PICK1). Indeed, expression of CKAR, a genetically-encoded fluorescence-based reporter of PKC activity (Violin et al. 2003), in COS7 cells revealed that endogenous PKC activity at the PSD95 scaffold was effectively inhibited by Bis IV but not Gö 6983, whereas both inhibitors were equally effective at inhibiting bulk PKC activity measured at plasma membrane (Fig 8C).

To test if PKCα is required for the synaptic effects of Aβ, organotypic hippocampal slices were prepared from wild-type (wt) mice as well as mice lacking PKCα (PKCα-/-) (M Leitges et al. 2002). 16-24 hours after infection, neurons expressing

CT100 displayed depressed synaptic transmission compared to nearby neurons in slices from wt mice but showed no difference in PKCα-/- slices (Figure 9A, B). We sought to determine if absence of PKCα, rather than some developmental alteration in PKCα-/- mice, was responsible for the lack of effect by CT100 in neurons from PKCα-/- mice. For this, we used a dual promoter virus to co-express CT100 and PKCα in PKCα-/- organotypic slices. Following expression, transmission onto infected neurons was depressed compared to non-infected neurons, indicating rescue of the effect of CT100 on synapses (Figure 9Ac). To test if the rescue of CT100-induced depression by PKCα co-expression is not due to potentiation of transmission by PKCα expression (which when added to the CT100-induced synaptic depression could normalize transmission), we co-expressed PKCα with CT84, the α-secretase product of APP that does not generate Aβ (Haass et al. 2012), nor cause synaptic depression (Kessels, Nabavi, and Malinow 2013). In this case no significant potentiation was observed, indicating PKCα co-expression rescued the effects of CT100 by enabling CT-100 driven signaling (Figure 9Ae). To test if the effects of Aβ on synapses requires that PKCα act at a scaffold, as suggested by the pharmacological results above, we co-expressed CT100 and PKCα lacking the last 3 amino acids (PKCα (ΔPDZ)), which prevents its binding to PDZ-domain proteins. In contrast to the co-expression of PKCα, co-expression of PKCα (ΔPDZ) did not rescue the effects of CT100 expression (Fig 9Ad). These results indicate that PKCα, acting at a scaffold, is required for the depressive effects of Aβ on rodent synapses.

To test if PKCα plays a role in human AD, we searched for rare variants in whole genome sequencing data for the PKCα gene (*PRKCA*) that were present in any of 410 pedigrees from the NIMH AD Genetics Initiative (Fig 10). In total we identified three

rare variants, of which, two were specifically found in LOAD patients in our families. M489V (rs34406842; Atg/Gtg; minor allele frequency = 0.0005 in 1000 genomes of the general population, http://www.1000genomes.org) was present in seven of nine affected individuals, and was absent in the single unaffected subject in these four families. V636I (rs141376042; Gtc/Atc; minor allele frequency = 0.002 in 1000 genomes) was present in two of three affected subjects in one family [no unaffected subjects available]. The two carriers had an age at onset of 71 and 76, while the non-carrier had onset at 92 and, in contrast to the affected siblings, carried one APOE-ε4 allele. Given that APOE-ε4 is a strong risk factor and reduces the age of onset for LOAD (Corder et al. 1993), it appears that, at least in this family, the V636I *PRKCA* variant was associated with an earlier age of onset. R324W (minor allele frequency = 0.00 in 1000 genomes), was present in one individual in one LOAD pedigree; the individual had a 'questionable unaffected' status at last visit. Neither of the two affected subjects in this pedigree carried the minor allele. Thus, two of these *PRKCA* variants appear to co-segregate with LOAD. A simple bootstrap test (see methods), suggests that the probability of finding such linkage in this population is about 0.03. Given that PKCα activity is necessary for effects of Aβ on synapses (above), and reduced PKC function enhances cell survival (C E Antal et al. 2015), we hypothesized that the rare PKCα variants found in LOAD families would have increased activity.

Both rare variants observed in LOAD patients occur in key regulatory segments of PKC (Newton 2010) : M489V is in the activation loop near the entrance to the active site, and V636I is on the C-terminal segment that clamps over the kinase domain (Figure 11A). Both segments interface with the Ca^{2+}-sensing C2 domain, which maintains PKC

in an autoinhibited conformation (C E Antal et al. 2014). Thus, both mutations have the potential to destabilize autoinhibition. For example, molecular modeling reveals that replacement of the bulky Met at position 489 on the activation loop with the smaller Val loosens the packing of this key regulatory segment (Figure 11A), consistent with reduced autoinhibition.

To examine the functional impact of amino acid changes, we assessed the effect of introducing these LOAD-associated rare variants on the function of PKCα. When expressed in COS7 cells, neither of the two rare variants affected the processing phosphorylation of PKCα, as assessed by Western blot analysis using phospho-specific antibodies to each of the three priming phosphorylations, an indication of proper folding (Keranen, Dutil, and Newton 1995) (Figure 11B). Further analysis of the M489V mutant revealed an enhanced rate of dephosphorylation following stimulation of cells with phorbol esters, potent activators that force PKC into an open, phosphatase-sensitive conformation (Dutil et al. 1994) (Figure 11C). The enhanced rate of dephosphorylation supports the looser packing of the kinase domain resulting from replacement of Met with Val, favoring a more open (i.e. active) conformation of the kinase when challenged with phorbol esters (C E Antal et al. 2014).

To assess the effect of the amino acid changes on the signaling output of PKCα, we co-expressed PKCα variants or wild-type PKCα with cytosolic CKAR (Violin et al. 2003) in COS7 cells. Both rare variants showed enhanced agonist-evoked activity relative to wild-type PKCα (Figure 12). These data are consistent with the mutations destabilizing contacts that maintain PKCα in an inhibited conformation (C E Antal et al. 2014), thus

facilitating activation. Together, these results indicate that the two rare PKCα variants found to co-segregate with LOAD display increased agonist-driven PKCα function.

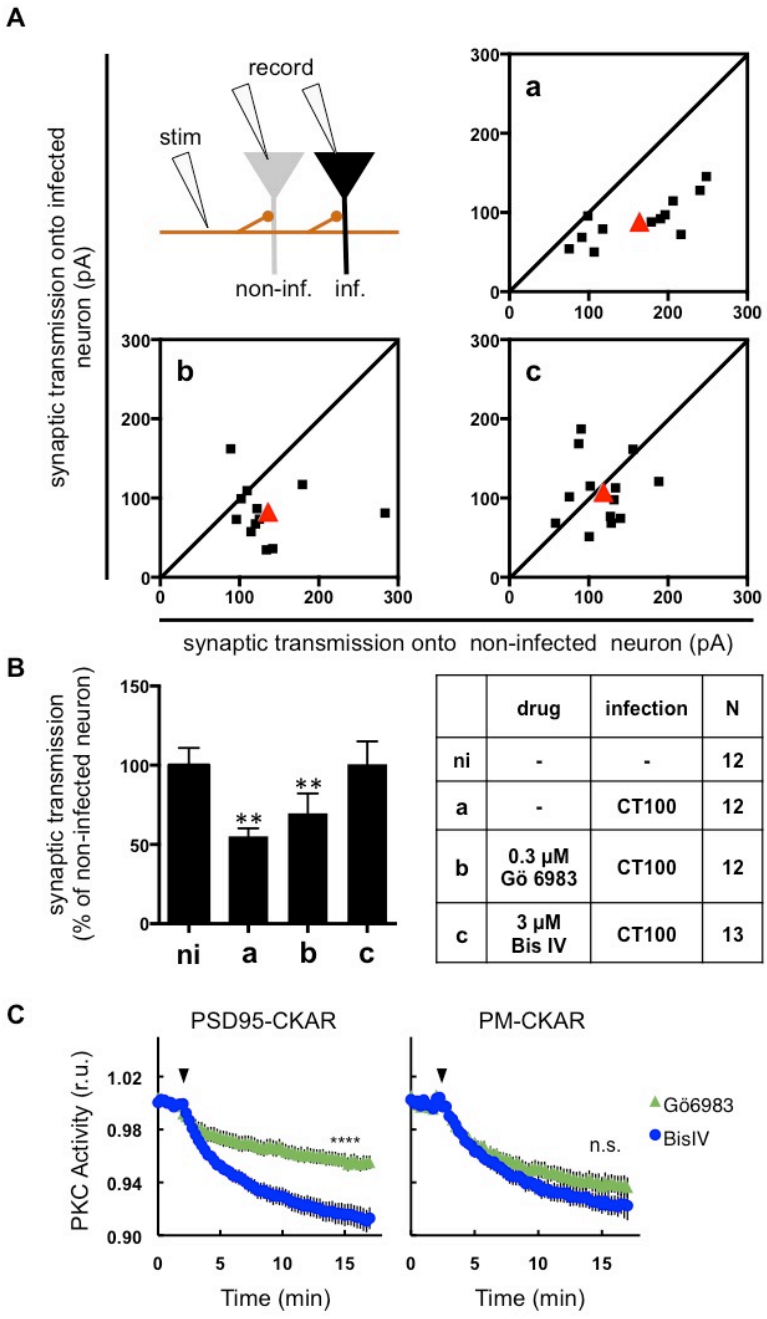
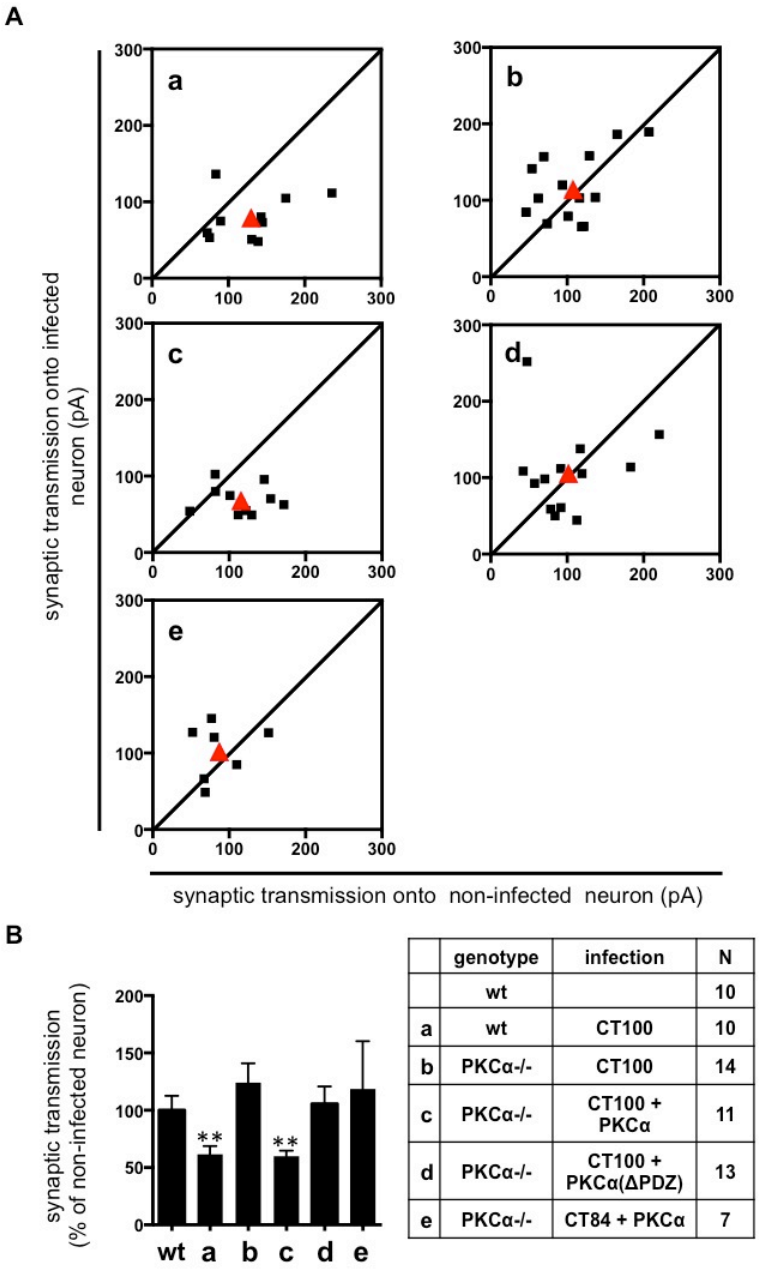


Figure 8: Synaptic depression by Aβ blocked by non-competitive PKC antagonist. A, Top left, experimental design; see text. Aa-c. Plot of evoked synaptic response amplitudes recorded in CT100-infected versus non-infected neuron (conditions and N, indicated in B, table); individual (black) and average (red) of cell pair responses; line, X=Y. B. Bar graph (left; ni, non-infected) for indicated conditions (right). Error bars, SEM throughout; **, $p < 0.03$, bootstrap (see methods). C. Normalized average PKC activity in COS7 cells expressing PKC activity reporter (18) fused to PSD95 (left) or targeted to plasma membrane (right); inhibitor added where indicated (arrow head); N > 16 cells; ****, $p < 0.0001$, bootstrap (see methods).



	genotype	infection	N
	wt		10
a	wt	CT100	10
b	PKCα-/-	CT100	14
c	PKCα-/-	CT100 + PKCα	11
d	PKCα-/-	CT100 + PKCα(ΔPDZ)	13
e	PKCα-/-	CT84 + PKCα	7

Figure 9: Requirement of PKCα for effects of Aβ on synaptic transmission. A, B a-e. Plot of evoked synaptic response amplitudes recorded in infected versus non-infected neurons (a-e); infection and number of cell pairs recorded indicated in table (B, right). Bar graph (B, left) of same data; ** indicates $p<0.03$, bootstrap (see methods).

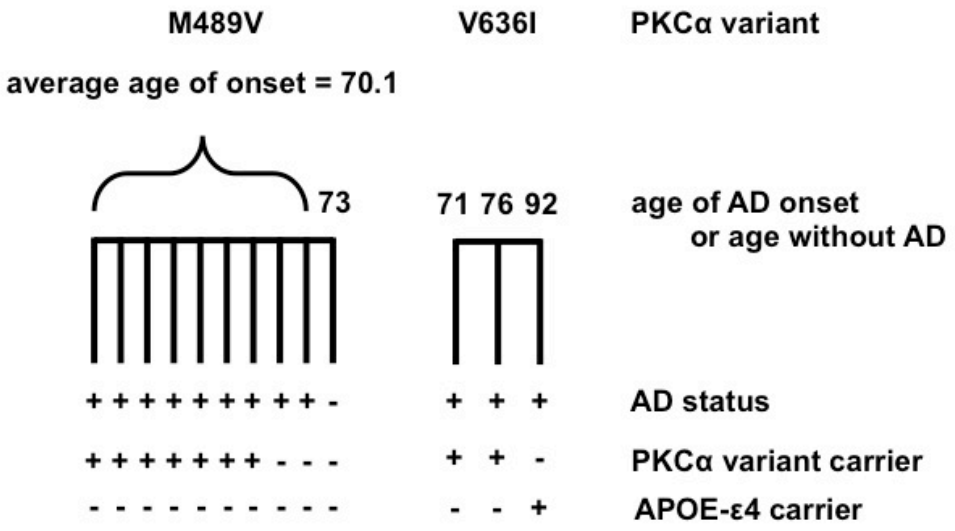


Figure 10: Human genetics of rare PKCα variants. Diagrams indicating genotype, phenotype and ages of individuals in families with M489V or V636I PKCα variants. For p.M489V, data are from all individuals sequenced in four families. For p.V636I, data are from all individuals sequenced in one family. Note the close association between PKCα variant carrier and AD status (bold) and ages/genotypes/phenotypes of V636I family (bold).

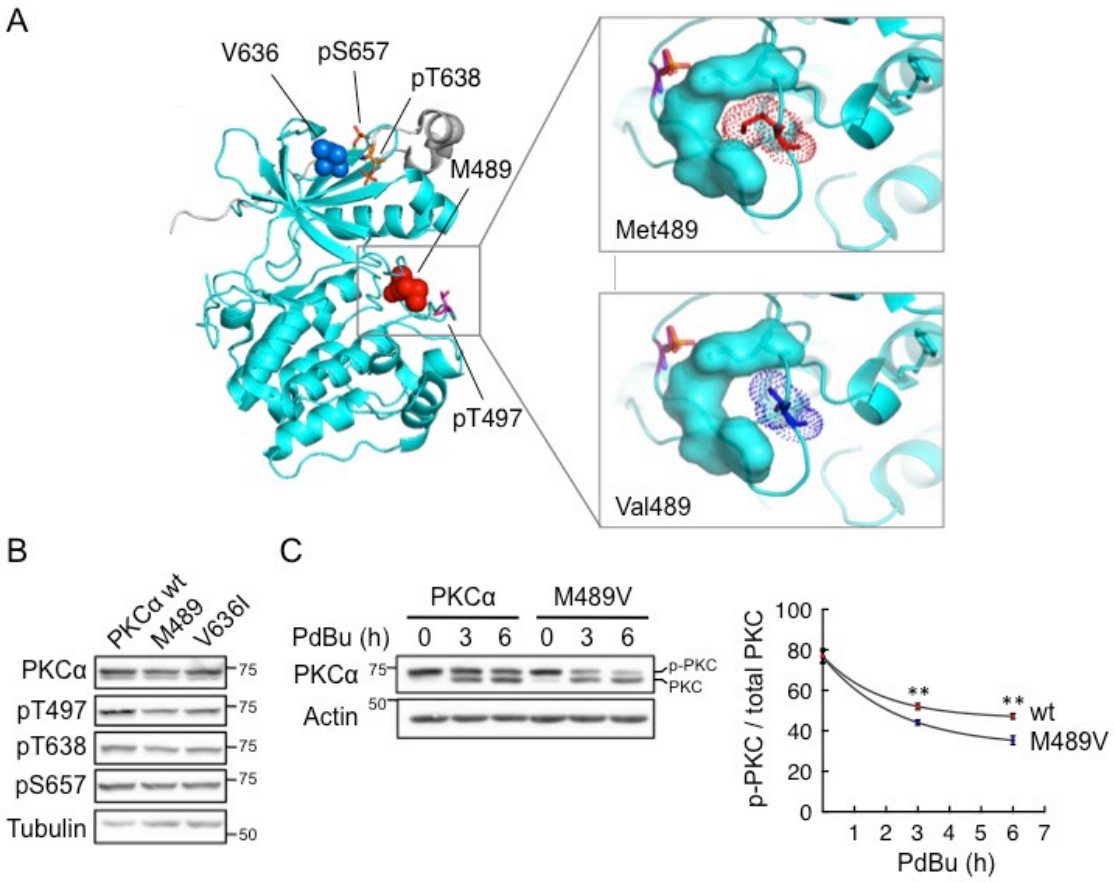


Figure 11: AD associated rare variants in PKCα. A. PKCα kinase domain structure (Wagner et al. 2009) showing the two residues altered in AD: Met489 and Val636. Both are near key regulatory phosphorylation sites (stick representation). Enlargement of activation loop segment (right panels) showing that substitution of Met489 with Val loosens the structural packing of this segment. B. Western blot showing phosphorylation of the indicated HA-tagged PKCα proteins. C. Western blot of COS7 cells expressing wt or M489V PKCα and treated with phorbol dibutyrate (PDBu) for the indicated times, and probed for HA. Quantitative analysis of phosphorylated/total PKC from 5 independent experiments. **, $p < 0.01$, by bootstrap (see methods).

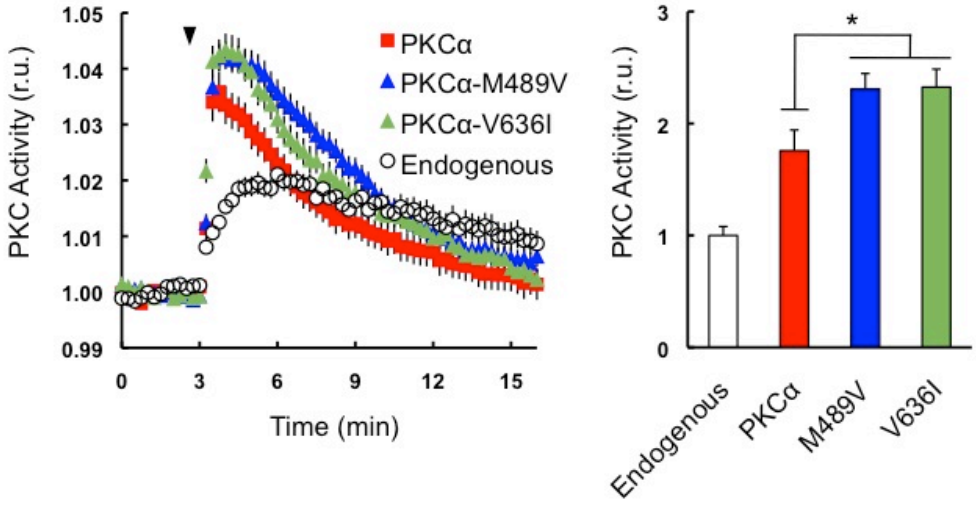


Figure 12: Live cell imaging reveals higher signaling output of both AD-associated rare variants. A. Normalized FRET ratios (mean ± SEM) representing PKC activity of COS7 cells co-expressing a PKC activity reporter {Violin, 2003 #10626} and indicated PKCα. Addition of UTP (100 μM) where indicated (arrow head). N > 25 cells for each construct. Right, area under the curve from 3-6 minutes; *, $p < 0.05$, bootstrap (see methods).

Alzheimer's Disease is a lethal neurodegenerative disorder with increasing prevalence and no effective treatment (Holtzman, Mandelkow, and Selkoe 2012). Elucidating the cellular signaling events that underlie the initiation and progression of LOAD can provide potential targets amenable to therapeutic intervention. Human genetic studies (Guerreiro et al. 2013), particularly family-based studies (Corder et al. 1993) of rare variants (Cruchaga et al. 2014), can identify potentially important molecules in LOAD. However, delineation of LOAD pathophysiology primarily from genetic information from smaller family studies remains challenging (see comments and responses to ref. (Cruchaga et al. 2014)). We therefore sought to combine a large genetic family study with neurophysiological and biochemical analyses to elucidate signaling employed in LOAD pathophysiology.

While the cellular events that initiate LOAD remain unknown (Holtzman, Mandelkow, and Selkoe 2012) elevated levels of Aβ, and the associated synaptic loss, is strongly implicated in its early pathophysiology (Mucke and Selkoe 2012; Querfurth and LaFerla 2010; Terry et al. 1991; Selkoe 2002). Our neurophysiological studies indicate PKCα, and its PDZ-ligand, is required for Aβ to produce synaptic depression (consistent with a prior study demonstrating a requirement of PICK1 (Alfonso et al. 2014), a PDZ-domain protein that binds PKCα). By scanning the genomes of individuals from a large LOAD family study, we identified two rare variants of PKCα that co-segregated with LOAD in five families. When examined in cell-based assays, both variants displayed increased signaling output relative to wild-type PKCα. Furthermore, the more strongly linked M489V mutation, found in seven of nine patients but not an unaffected subject in four LOAD families, also elicited an enhanced rate of dephosphorylation following

stimulation of cells with phorbol esters, consistent with a more open and signaling competent conformation. Thus, PKCα is required for deleterious synaptic effects of Aβ, observed early in LOAD, and increased PKCα activity can contribute to human LOAD.

It is notable that we found activating PKC mutations in LOAD patients whereas human cancer-associated PKC mutations are inactivating (C E Antal et al. 2015). Indeed, the neurodegenerative disease, spinocerebellar ataxia, is caused by mutations in PKCγ that promote the open, active conformation (Verbeek et al. 2008). Thus, our results are consistent with PKC gain-of-function mutations driving neurodegenerative diseases and loss-of-function mutations driving cell survival diseases. Supporting opposing roles of PKC in cancer vs AD, a recent meta analysis of nine independent studies reveals that AD patients exhibit an overall 45% decreased risk of cancer compared with the general population (Do et al. 2015), consistent with earlier reports that AD and cancer display an inverse association (Hirata-Fukae et al. 2008; Driver et al. 2012). It is relevant that inhibitors of PKC have failed in cancer clinical trials (Mochly-Rosen, Das, and Grimes 2012), likely because PKC activity should be restored, rather than inhibited, in cancer therapy (C E Antal et al. 2015). In contrast, repurposing PKC inhibitors for LOAD may prevent the effects of Aβ on synapses and thereby mitigate loss of cognitive function. Together, our findings support inhibition of PKCα as a therapy early in LOAD and suggest that mutations in PKCα can serve as a diagnostic for disease susceptibility.

Chapter II is a partial reprint of a manuscript that is being prepared for submission. The dissertation/thesis author was the primary investigator and author of this paper. Protein Kinase Cα Required for Synaptic Defects and has Gain of Function

Mutations in Alzheimer's Disease. Alfonso S, Callender JA, Hooli B, Antal CE, Mullin K, Leitges M, Newton AC, Tanzi RE, Malinow R.

APPENDIX

Blocking three tyrosine residues in GluA2 c-tail rescues Aβ-induced synaptic depression

LTD expression has been shown to require tyrosine phosphorylation of the C-terminus (CT) of the GluA2 subunit, which results in receptor internalization (Ahmadian et al. 2004). We hypothesized that excessive GluA2 endocytosis might also underlie the depression we see in an AD model. Is the synaptic depression produced by Aβ blocked by inhibiting AMPAR endocytosis?

Organotypic brain slices were infected with a virus in order to constitutively express the 100-amino acid C-terminal fragment of the human amyloid precursor protein (APP CT100) a precursor to A-beta. We then simultaneously recorded from an infected and non-infected cell. The APP-CT100 expressing cell produced a synaptic depression of ~30-40% (Figure 13 top).

To assess the reversibility of the synaptic depression produced by chronic exposure to Aβ, we used a membrane permeable peptide derived from rat GluR2 CT (GluR23Y; aa 869-877), which specifically blocks regulated, but not constitutive, AMPAR endocytosis (Brebner et al. 2005). We found that blocking regulated AMPAR endocytosis ameliorated the depressed synaptic response seen in cells overexpressing APP-CT100 (Figure 13 bottom).

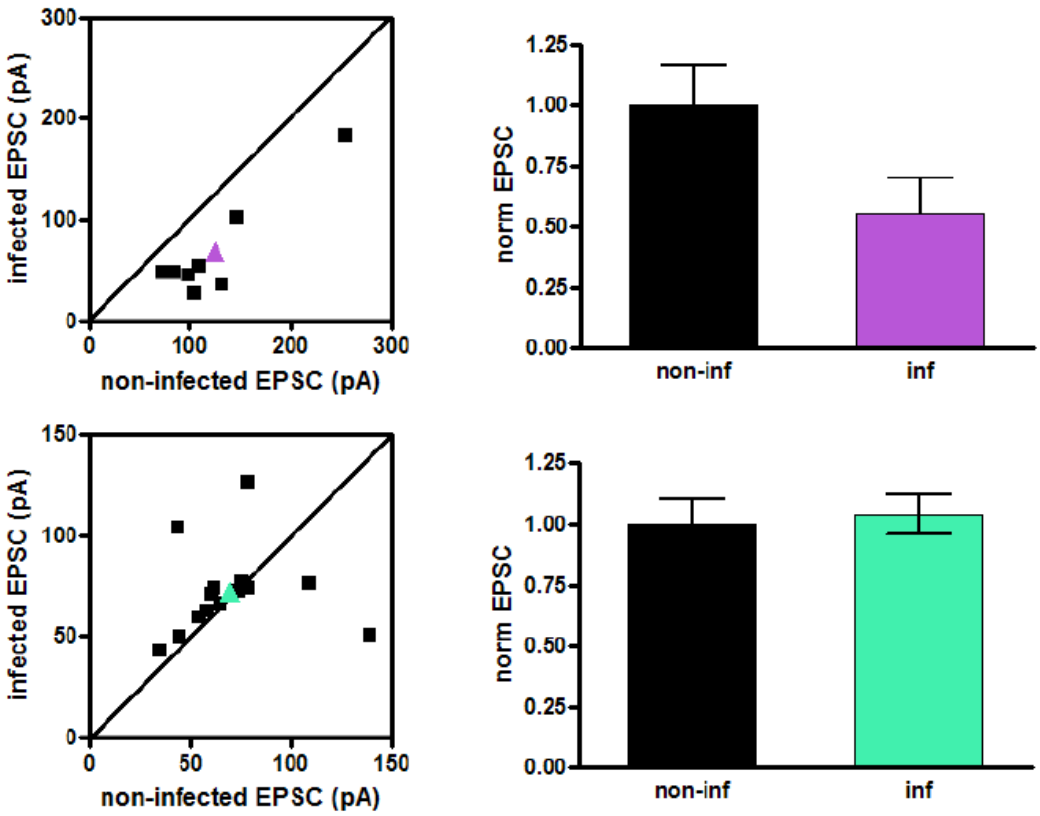


Figure 13: Blocking three tyrosine residues in GluA2 c-tail rescues Aβ-induced synaptic depression. Paired recordings from CA1 pyramidal cells expressing APP-CT100 (infected) and non-infected (control) n=14 pairs showed ~40% reduced response in cells expressing the APP-CT100 (top). In contrast paired recordings from CA1 pyramidal cells expressing APP-CT100 (infected) and non-infected (control) n=8 pairs did not show a significantly different synaptic response when slices were incubated in the GluR23Y peptide (bottom).

PKCα role in LTD

The expression of long-term depression of synaptic efficacy (LTD) results in an increase in the regulated endocytosis of GluA subunits (Luscher et al. 1999). The role of PKCα in LTD is likely to derive from its unique PDZ ligand (QSAV) that allows it to interact and phosphorylate GluA2 subunits, leading to their endocytosis. In addition, studies show that disruptions of the GluA2 CT PDZ domain led to blockade of LTD (Daw et al. 2000). PKCα is known to phosphorylate the GluA2 CT which leads to its association with PICK1 and its dissociation from membrane anchoring proteins like GRIP (Perez et al. 2001). PKC activation leads to an increase in synaptic phospho-GluA2 and thus a decrease of surface GluA2 receptors (Chung et al. 2000).

To test the requirement for PKCα in hippocampal LTD, we recorded from WT and PKCα null mouse organotypic slices. The slices were subjected to an LTD protocol, 1Hz stimulation of Schaffer Collaterals for 15 min and recording in CA1 area (Figure 14).

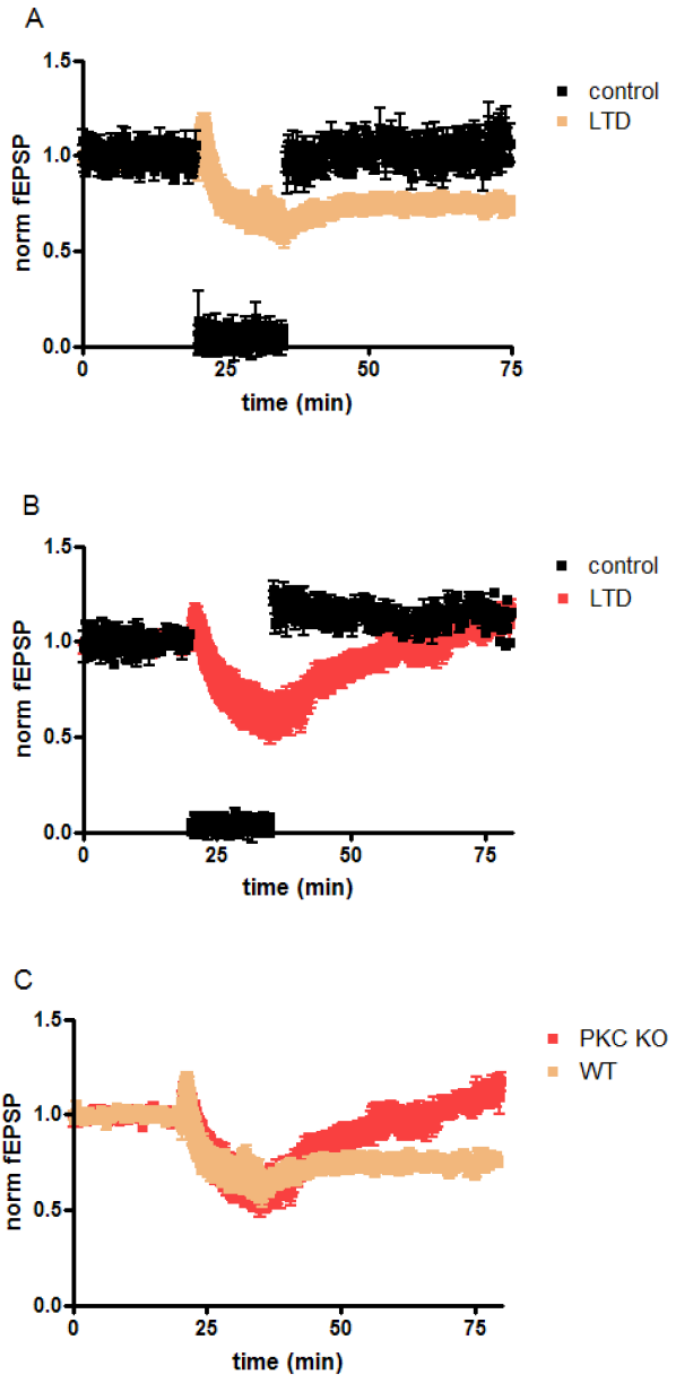


Figure 14: PKCα is required for the expression of LTD. We recorded field excitatory post synaptic potentials (fEPSPs) from WT mouse organotypic slices. The black trace is the control pathway (n=5) that does not receive the LTD stimulation and the peach colored trace is the test pathway (n=13) that receives a 1Hz stimulation for 15 minutes after recording a 20 min stable baseline (A). We also recorded fEPSPs in PKCα -/- mice (B) following the same protocol (n=5 control pathway and n=8 test pathway). (C) Shows an overlay of both test pathways.

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