

1-21-2022

## A PDK-1 Allosteric Agonist Neutralizes Insulin Signaling Derangements and Beta-amyloid Toxicity in Neuronal Cells and In Vitro

Henry Querfurth  
*Tufts Medical Center*


John Marshall  
*Brown University*

Keykavous Parang  
*Chapman University, parang@chapman.edu*

Mengia S. Rioult-Pedotti  
*Brown University*

Rakesh Tiwari  
*Chapman University, tiwari@chapman.edu*

Follow this and additional works at: [https://digitalcommons.chapman.edu/pharmacy\\_articles](https://digitalcommons.chapman.edu/pharmacy_articles)

 [a next page for additional authors](#)

Part of the [Endocrine System Diseases Commons](#), [Medicinal and Pharmaceutical Chemistry](#)

[Commons](#), [Nervous System Diseases Commons](#), and the [Other Pharmacy and Pharmaceutical Sciences Commons](#)

### Recommended Citation

Querfurth H, Marshall J, Parang K, Rioult-Pedotti MS, Tiwari R, Kwon B, et al. (2022) A PDK-1 allosteric agonist neutralizes insulin signaling derangements and beta-amyloid toxicity in neuronal cells and in vitro. *PLoS ONE* 17(1): e0261696. <https://doi.org/10.1371/journal.pone.0261696>

This Article is brought to you for free and open access by the School of Pharmacy at Chapman University Digital Commons. It has been accepted for inclusion in Pharmacy Faculty Articles and Research by an authorized administrator of Chapman University Digital Commons. For more information, please contact [laughtin@chapman.edu](mailto:laughtin@chapman.edu).

---

# A PDK-1 Allosteric Agonist Neutralizes Insulin Signaling Derangements and Beta-amyloid Toxicity in Neuronal Cells and In Vitro

## Comments

This article was originally published in *PLoS ONE*, volume 17, issue 1, in 2022. <https://doi.org/10.1371/journal.pone.0261696>

## Creative Commons License



This work is licensed under a [Creative Commons Attribution 4.0 License](https://creativecommons.org/licenses/by/4.0/).

## Copyright

The authors

## Authors

Henry Querfurth, John Marshall, Keykavous Parang, Mengia S. Rioult-Pedotti, Rakesh Tiwari, Bumsup Kwon, Steve Reisinger, and Han-Kyu Lee

## RESEARCH ARTICLE

# A PDK-1 allosteric agonist neutralizes insulin signaling derangements and beta-amyloid toxicity in neuronal cells and in vitro

Henry Querfurth<sup>1</sup>\*, John Marshall<sup>2</sup>, Keykavous Parang<sup>3</sup>, Mengia S. Rioult-Pedotti<sup>2,4</sup>, Rakesh Tiwari<sup>3</sup>, Bumsup Kwon<sup>5</sup>, Steve Reisinger<sup>6</sup>, Han-Kyu Lee<sup>1</sup>

**1** Department of Neurology, Tufts Medical Center, Boston, MA, United States of America, **2** Department of Molecular Pharmacology, Physiology, and Biotechnology, Brown University, Providence, RI, United States of America, **3** Center for Targeted Drug Delivery, Chapman University, School of Pharmacology, Irvine, CA United States of America, **4** Department of Neurology, Clinical Neurorehabilitation, University of Zurich, Zurich, Switzerland, **5** Department of Neurology, Rhode Island Hospital, Providence, RI, United States of America, **6** Med Chem Partners, Lexington, MA, United States of America

\* These authors contributed equally to this work.

\* [henry.querfurth@tufts.edu](mailto:henry.querfurth@tufts.edu)



## OPEN ACCESS

**Citation:** Querfurth H, Marshall J, Parang K, Rioult-Pedotti MS, Tiwari R, Kwon B, et al. (2022) A PDK-1 allosteric agonist neutralizes insulin signaling derangements and beta-amyloid toxicity in neuronal cells and in vitro. *PLoS ONE* 17(1): e0261696. <https://doi.org/10.1371/journal.pone.0261696>

**Editor:** Jaya Padmanabhan, H Lee Moffitt Cancer Center and Research Institute, UNITED STATES

**Received:** May 31, 2021

**Accepted:** December 8, 2021

**Published:** January 21, 2022

**Copyright:** © 2022 Querfurth et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the paper and its [Supporting Information files](#).

**Funding:** This study was funded by the National Institute of Health, National Institute on Aging (NIA): NIH AG044871-01 (HWQ), The Brown Institute for Brain Science and Norman Prince Neuroscience Institute (HWQ, JM), and through an award from the Bennett Foundation (HWQ). The funders had no role in study design, data collection

## Abstract

The Alzheimer's brain is affected by multiple pathophysiological processes, which include a unique, organ-specific form of insulin resistance that begins early in its course. An additional complexity arises from the four-fold risk of Alzheimer's Disease (AD) in type 2 diabetics, however there is no definitive proof of causation. Several strategies to improve brain insulin signaling have been proposed and some have been clinically tested. We report findings on a small allosteric molecule that reverses several indices of insulin insensitivity in both cell culture and *in vitro* models of AD that emphasize the intracellular accumulation of  $\beta$ -amyloid ( $A\beta$ ). PS48, a chlorophenyl pentenoic acid, is an allosteric activator of PDK-1, which is an Akt-kinase in the insulin/PI3K pathway. PS48 was active at 10 nM to 1  $\mu$ M in restoring normal insulin-dependent Akt activation and in mitigating  $A\beta$  peptide toxicity. Synaptic plasticity (LTP) in prefrontal cortical slices from normal rat exposed to  $A\beta$  oligomers also benefited from PS48. During these experiments, neither overstimulation of PI3K/Akt signaling nor toxic effects on cells was observed. Another neurotoxicity model producing insulin insensitivity, utilizing palmitic acid, also responded to PS48 treatment, thus validating the target and indicating that its therapeutic potential may extend outside of  $\beta$ -amyloid reliance. The described *in vitro* and *cell based-in vitro* coupled enzymatic assay systems proved suitable platforms to screen a preliminary library of new analogs.

## Introduction

Clinically-based Alzheimer's Disease (AD) currently affects 5.8 million or 1 in 10 adults (10%) in the U.S.A. over age 65 and 32% in the >85 age group. Several phase III clinical trials of promising agents to prevent AD progression, based primarily on the amyloid hypothesis, have yielded disappointing overall results. These included anti-amyloid agents such as  $\gamma$ -secretase

and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have no competing interests or financial conflicts to declare. HQ and HKL contributed equally to this work. All authors directly contributed to the data set.

(Semagacestat) [1] and BACE (Verubecestat) [2] inhibitors and passive immunotherapies (Bapineuzumab, Gantenerumab, Solanezumab, IVIG) [3–6]. However, some recent positive outcome measures resulting in an FDA approval, with allowance for study design issues (Aducanumab), support continued efforts (e.g. Donanemab) [7, 8]. These drug trial outcomes call for targets that are not only based on the supply side or removal of secreted  $\beta$ -amyloid ( $A\beta$ ), but directly address its toxic effects on critical neuronal metabolic, plasticity and survival signal pathways. One less often considered offender, intra-neuronal  $\beta$ -amyloid peptide ( $A\beta_i$ ) accumulation, may be relevant to the early pathogenesis of AD [9–11] and considered a target. A related and potentially remediable inciting risk factor for both amyloid formation and AD progression is systemic insulin resistance (IR) and type 2 diabetes mellitus (T2DM) [12]. Moreover, there is wide recognition that the AD brain is itself an insulin resistant end-organ, a so-called ‘type III diabetes’ condition [13, 14].

While the relationship of brain insulin resistance to the accumulation of cellular and plaque  $A\beta$  and the cross-talk between T2DM, peripheral and central IR continue to be explored, there is ample experimental data that peripheral IR can drive Alzheimer pathology. Animal models of T2DM or bearing an AD transgene and made pre-diabetic on an oil rich or high fat diet, show ensuing brain IR and amyloidogenesis [15–17]. Loss of peripheral insulin signaling can further result in the central hyperphosphorylation of Tau [18, 19]. Targeted disruption of insulin signaling within the CNS by genetic means or intracerebral streptozotocin injection also leads to AD-like degeneration and tau hyperphosphorylation [20–22]. In turn, these pathologic changes are rescued by insulin treatments.

The essential roles of brain insulin and the mechanism behind IR in AD have been extensively studied (for reviews [23, 24]). Levels of insulin, insulin-like growth factor I (IGF-I) and cognate receptors, become deregulated in AD brain [25–29]. Normally, these promote energy metabolism, neuronal survival, synaptic plasticity [30] and memory formation [31, 32]. Insulin/IGF-1 receptors populate synapses [33] where they signal through IRS-1 (insulin receptor substrate-1) and the phosphatidylinositol 3-kinase / Protein kinase B (PI3K/Akt) and MAPK pathways [34]. The insulin-PI3K/Akt activation sequence brings together phosphoinositide-dependent protein kinase-1 (PDK-1) and Akt in a sub-membrane complex [35]. Activated Akt maintains post-mitotic cell viability by phosphorylating several pro-apoptotic mediators, including glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) [36, 37]. Conversely, dephosphorylation (inhibition) of Akt sensitizes the cell to environmental stressors [38, 39]. IGF-1/Akt regulate transcription factors to support hippocampal progenitor neurogenesis [40, 41] and learning/memory, e.g. via CREB [42, 43]. Insufficient insulin signaling impacts the activity of mTOR, a suppressor of autophagy, lowers levels of IDE, known to degrade  $A\beta$  [17, 44] and negatively affects translocation of GLUT-3/4, glucose transporter proteins [45, 46].

$A\beta_{42}$  accumulation in excess produces neuronal IR and PI3K/Akt axis disruption by several mechanisms. The inhibitory effect of  $A\beta_{42}$  oligomers on hippocampal LTP and PI3K/Akt is reversed by insulin [47, 48]. One mechanism is the caspase-mediated cleavage of Akt1 [49]. Next, extracellular  $A\beta$  inhibits the binding of insulin to its receptor [50] and results in their downregulation (removal) from the neuronal membrane [47, 51].  $A\beta$  oligomers are further shown to inhibit insulin-induced phosphorylations of both insulin receptor [51] and Akt [48]. Lastly, IR in AD brain is linked to inhibitory feedback phosphorylations of IRS-1 (S616 and S636) by pS6K [27, 52].  $A\beta$  provokes IR in this way by first activating (de-repressing) mTOR via phosphorylation of the inhibitory subunit, PRAS40 [53]. mTOR target pS6K becomes indirectly stimulated [53–55]. The end result is a decrease in IRS-1 levels [27, 56]. Interestingly, the inflammatory cytokine TNF $\alpha$  mediates the same outcome [57, 58].

PI3K/PDK/Akt signaling in AD brain is reported to be abnormally stimulated [52, 59–63]. Accordingly, over-activation of downstream mTORC1 is found [28, 64–67] alongside loss of

autophagy markers [68, 69]. Intra-hippocampal injections of anti-A $\beta$  antibody or immunization normalized the hyper-activation of Akt and mTOR in transgenic AD mice [53, 70]. The mechanisms underlying the paradoxical hyperactivity of Akt and mTOR under basal conditions, are not completely understood. In addition to direct activation of mTOR noted above, A $\beta$  can directly inactivate PTEN (phosphatase and tensin homolog), thereby disinhibiting PI3K [69, 71]. One seminal study found all IRS-1 -S and -Y sites were hyperphosphorylated in live AD hippocampal and cerebellar tissue, essentially isolating IRS-1 from binding to insulin receptors and p85-PI3K. Intrinsic over-activation of mTOR/S6K and other kinases was held responsible. Importantly, their work proved resistance to insulin/IGF-1 action in AD; a 90% decrease in Akt, IRS-1, IR, and mTOR phospho-activations in response to insulin stimulation [52].

There is opposing evidence gathered from several AD models that basal Akt is *deactivated*, which is also consistent with IR in AD. Inhibited Akt is further noted in post-mortem tissue from AD [64, 72], Huntington's and Parkinson's diseases [67, 73–75]. In two AD models, the inhibition of PTEN instead rescued synaptic and cognitive impairments, mediated through the stimulation of PI3K/Akt [76]. Conversely, PTEN over-expression led to synaptic depression. A $\beta$  peptides applied to hippocampal neurons induced the same synaptic defects and dephosphorylation of Akt by recruiting PTEN to dendritic spines [76]. In 2576 AD mice, where cellular A $\beta$  is co-localized to mTOR, it was actually found to have an inhibitory role [72]. Moreover, reduction in mTOR signaling markers and basal phospho-Akt levels/enzymatic activities, were found in 2xAPP/PS1 mice and in AD brain. These were correlated with oxidatively damaged synaptic Akt. Akt enhancement rescued BDNF-induced protein translation [77]. Deactivation of Akt is reported in rat PCNs and N2a cells exposed to oligomeric A $\beta$ , resulting in inhibition of normal BDNF-induced Akt/mTOR activation [78, 79].

Due to conflicting reports and the paucity of preclinical and clinical data on direct Akt/PDK-1 intervention in AD models, we tested the hypothesis that targeting insulin resistance at this step may be beneficial. In a previous study, intraneuronal A $\beta$ 42 (A $\beta$ i) expression led to a decrease in the levels of p-Akt and activity, causing p-Tau accumulation and apoptosis [80]. A $\beta$ i inhibited the association of PDK-1 with Akt, resulting in the loss of normal insulin-stimulated pathway activation [64, 81]. This added mechanism for IR presents a novel target for the treatment of AD. We reasoned that an allosteric ligand acting on the Akt/PDK-1/mTORC2 interaction complex could normalize insulin sensitivity and restore the imbalance in Akt activity. Promising results from early clinical trials in MCI and mild AD of insulin sensitizers (metformin, [82]), GLP-1 receptor agonist/ incretin analogs (liraglutide, [83]), intranasal (IN) insulin [84, 85] and insulin-sensitizing PPAR- $\gamma$  agonists that target genes such as IRS-1, GLUT-4 and PI3K [86, 87] (Rosiglitazone, [88]; Pioglitazone, [89]), support finding druggable targets in this pathway and several relevant ongoing trials: Metformin (phase 3, NCT04098666), liraglutide (phase2b, NCT 01843075, [90]) and semaglutide (phase 3, NCT 04777396). However, some phase 3 trials have not met their primary endpoints or been terminated for lack of efficacy, e.g. IN insulin [91], Rosiglitazone [92, 93] and Pioglitazone [94, 95]. Acknowledging this uncertainty, an approach to reestablish insulin sensitivity in AD need not be dependent on the amyloid hypothesis or any of these interventions in particular to be useful. We report preclinical findings using PS48, a chlorophenyl pentenoic acid and allosteric activator of PDK-1 [96, 97].

## Materials and methods

### Ethics

Animal research ethics for this work were approved by the IACUC at Rhode Island Hospital under Clinical, Biochemical and Electrophysiologic Investigations into Neurodegeneration,

no. 462439-3/2013, PI: HWQ. All personnel and collaborators involved in these experiments, performed on mice and rats, were included. All procedures, tissue collection, biohazard uses, recombinant DNA, husbandry, special diets, breeding and euthanasia were covered. Euthanasia was by either sodium pentothal (120–200 mg/Kg IP) or ketamine (80–100 mg/Kg IP) followed by decapitation.

## Cell culture and reagents

Undifferentiated neuroblastoma cell lines SH-SY5Y (human) and N2a (mouse) (ATCC, Manassas, VA; Sigma, St. Louis, MO) were grown in DMEM, 10% FBS, 25mM glucose, at or below 80% confluence. SH-SY5Y were left undifferentiated. Mouse C<sub>2</sub>C<sub>12</sub> cells (ATCC) were grown in Dulbecco's modified Eagle medium (DMEM), 20% fetal bovine serum (FBS) (Invitrogen), and maintained for passage below 60% confluence. Cultures at or above 90% confluence were then differentiated in DMEM, 2% adult horse serum (DM) for 3 days before use. Primary rat cortical neurons (PCNs) were cultured from E18 Sprague-Dawley rat fetal cortex (Charles River, Wilmington, MA) as described [98]. Briefly, isolated fetal cerebral cortex was dissociated into single cells and then seeded into 6-well plates coated with poly-D-lysine at  $1 \times 10^6$  cells per well. PCNs were cultured in neurobasal medium (Invitrogen, Carlsbad, CA) containing 2% B27 without insulin, 25 mM D-glucose, 0.5 mM L-glutamine and 1% penicillin/streptomycin for 7 days before experiments.

Antibodies used were: goat anti-Akt-1 and Actin (Santa Cruz Biotechnology); anti-p-Akt (Ser473 and Thr308), p-GSK-3 $\alpha/\beta$  (Ser21/9), and GSK-3 $\alpha/\beta$  (Cell Signaling); mouse anti-PDK-1 (BD Biosciences); 6E10 (Covance, Co); R1282 (gift from Dr. D. Selkoe); anti phospho-CREB (Ser 133) 87G3 rabbit mAb Cell Signaling #9198); anti-mTOR (Cell Signaling, recognizing C1 and C2), p-mTOR (Ser2448, Ser2481), neuron-specific enolase (NSE, SantaCruz), 6E10 (anti A $\beta$ , Covance). GSK-3 fusion peptide (crosstide-paramyosin), a 27 kDa substrate for Akt phosphorylation (Cell Signaling #9237). See [S1 Table](#) for dilutions. Fluorophore-labeled A $\beta$ 42 is carboxy-fluorescein conjugated to the N<sup>7</sup> terminus (FAM-A $\beta$ 42) purchased from Anaspec (Fremont CA). Recombinant human Akt1 (inactive) and PDK-1 (active) proteins were obtained from Amsbio. PS48 and PS47, from SIGMA and Axon MedChem; dipalmitoyl-PIP3 (Matreya, State College, PA); ATP (Adenosine-5'-triphosphate, disodium salt) is supplied as a 10 mM solution in doubly distilled water (Cell Signaling). Protein A/G PLUS-Agarose (Roche). Human Insulin, recombinant, dry or 10 mg/ml solution, was purchased from Sigma-Aldrich.

## Infection of SY5Y and C<sub>2</sub>C<sub>12</sub> myotubes with adenoviruses

Adv TetOn and TRE-A $\beta$ 42 viruses were described previously [80]. SY5Y and C<sub>2</sub>C<sub>12</sub> myotubes were infected with Adv A $\beta$ 42/TetOn (4:1 ratio) 24–36 hr before doxycycline induction (1  $\mu$ g/ml) for an additional 24–36 hr. Insulin (10 or 40 nM) was added in the last 20–30 min before harvest. PS48 (Sigma), PS47 (Axon Medchem, Reston VA), and 501-1-x compounds were added 5 min before adding insulin. Cell extracts were prepared in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% NP-40, 10% Glycerol, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1  $\mu$ g/ml Leupeptin, 1  $\mu$ g/ml Pepstatin A, 1  $\mu$ g/ml Aprotinin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Roche)] and were stored at -80°C until use.

## Cell viability

SH-SY5Y Cells were washed twice in warm DPBS and incubated in 1 ml DMEM containing 0.5 mg (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT or WST; Molecular Probes, Eugene, OR) for 2–3 h at 37°C and 5% CO<sub>2</sub>. The medium was aspirated

and the cells were washed twice with pre-warmed DPBS. The formazan salts were dissolved in 1 ml pure ethanol before use. Cells were homogenized by repetitive pipetting and centrifuged for 5 min at 4500 rpm, and the supernatant collected. Absorbance was read against an ethanol blank at 590 nm.

### A $\beta$ and ADDL preparation

A $\beta$  peptides were obtained from BioSource as dried trifluoroacetic acid salts. Monomeric A $\beta$  peptides were prepared by solubilization in 5% dimethyl sulfoxide (DMSO); 25 mM Tris-HCl, pH 7.4, and used fresh or flash frozen. A $\beta$ -derived diffusible ligands (ADDLs) were prepared as detailed previously [64] and as according to Lambert et al., [99] and Klein et al., [100]. Briefly, A $\beta$  peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma) and evaporated on a Speedvac. The A $\beta$  film was resuspended in 100% anhydrous DMSO, diluted to 5mM in F12 medium lacking phenol red (BioSource), and incubated at 4°C for 24 to 48hr. Following incubation and centrifugation at 14,000 g for 10 min at 4°C, the supernatant containing ADDL-enriched A $\beta$  was transferred to a new tube.

### Western blot analysis

Whole-cell extracts were used directly for western blot analysis (20–30  $\mu$ g). Extracts from cultured cells prepared in lysis buffer, were diluted into Laemmli sample buffer, heated (95°C, 10 min), cleared by centrifugation, separated on SDS-PAGE and transferred to PVDF membrane (Immobilon-P; Millipore). Membranes were blocked in TBS containing 0.3% Tween-20 and 5% (wt/vol) non-fat dry milk. After incubation with primary antibodies (18 hr at 4°C in buffer containing 5% BSA and 0.05% NaN<sub>3</sub>), blots were washed and incubated in HRP-conjugated secondary antibodies (1:2000 dilution; Cell Signaling). Signals were detected using ECL reagents and quantified using a Kodak Image Station 4000R.

### In vitro p-Akt and activity levels

Immunoprecipitations (IPs) of PDK and Akt1 were prepared from 100  $\mu$ g of either SH-SY5Y, C<sub>2</sub>C<sub>12</sub> myotubes or from insulin-treated cultures. Alternatively, commercial recombinant Akt (100 ng) and PDK (10ng) proteins were used. PIP<sub>3</sub> (50 nM) in the role of activating phosphoinositide lipid, GSK-3 $\beta$ -paramyosin fusion protein (1  $\mu$ g/50  $\mu$ l, 1.0  $\mu$ g), kinase buffer and synthetic A $\beta$ 42 peptide oligomers were added. ATP (200  $\mu$ M) started the reaction (50  $\mu$ l) that continued for 30 minutes at 30°C. The reaction was stopped by adding 40  $\mu$ l of Laemmli buffer. 20  $\mu$ l of sample was loaded onto a 10% polyacrylamide gel.

An *in vitro* radio assay (EMD Millipore, KinaseProfiler) was also adapted as follows. PKB $\alpha$  (human, recombinant, inactive, 209 nM) is incubated in 8 mM MOPS pH 7.0, 0.2 mM EDTA with 30  $\mu$ M GSK3 $\alpha/\beta$  consensus sequence GRPRTSSFAEGKK and PDK1 (human, recombinant, 285 nM).  $\beta$ -amyloid peptide (oligomerized, 5  $\mu$ M final) and PS48 are added. Final DMSO is 2%. 10 mM Mg Acetate and [ $\gamma$ -<sup>33</sup>P- ATP] (specific activity approx. 500 cpm/pmol) are prepared. The reaction is initiated by the addition of the Mg ATP mix (200  $\mu$ M ATP final). After incubation for 40 minutes at 37°C, the reaction is stopped by adding 3% phosphoric acid solution. 10  $\mu$ l of the reaction is spotted onto a P30 filtermat and washed three times for 5 minutes in 75 mM phosphoric acid and once in methanol, prior to drying and scintillation counting.

### Electrophysiology

Minor modifications were made to a previously published procedure [101, 102]. Deeply anesthetized rats (pentobarbital, 50 mg/kg) were decapitated, their brains quickly removed and

immersed in cold (5–7°C), oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 1 MgSO<sub>4</sub>, 2 CaCl<sub>2</sub>, 26 NaHCO<sub>3</sub>, 10 dextrose. Coronal slices of prefrontal cortex from day 14 rat pups (400  $\mu$ m) were perfused with A $\beta$  oligomers (40 nM) or A $\beta$  plus PS48 (10  $\mu$ M) x 60 min. before applying the high frequency stimulation (HFS) protocol. Control treatment is DMSO in ACSF (artificial cerebrospinal fluid, 20  $\mu$ M bicuculline). Extracellular postsynaptic field potentials were recorded using an AxoClamp2B amplifier (Axon instruments) and EX1 differential amplifier (Dagan), and digitized at 10 kHz. Data was acquired using Igor Pro (Wave Metrics) and Neuromatic ([www.neuromatic.thinkrandom.com](http://www.neuromatic.thinkrandom.com)). The stimulus intensity eliciting 50% of the maximum amplitude (~32  $\mu$ A) was used for all measurements before and after LTP induction. Baseline amplitudes were recorded for 20–30 minutes using single field stimuli applied every 30 sec (2Hz) to layer IV-V using concentric bipolar electrodes. Following a stable baseline period, LTP was induced by two sets of high-frequency stimulation (HFS) at 100 Hz, 60  $\mu$ A (twice stimulus intensity), for 1 sec, 20 sec apart. Extracellular postsynaptic field potentials were measured from layer II-III using glass micropipettes filled with 0.9% NaCl. The amplitude rather than the slope of evoked FPs was used as a measure of the population excitatory synaptic response because in the neocortex the initial slope is contaminated by antidromic stimulation. The last 10 minutes (20 points) of the stimulated EPSP recordings were normalized to baseline and then averaged. LTP values were expressed as a percentage of mean baseline EPSP  $\pm$  SEM or % change normalized to baseline. ANOVA with Tukey's multiple comparisons and paired two-tailed t-tests were used for statistical analysis.

### Binding in solution: Fluorescence polarization

A procedure based on Lynch et al., 1997 [103] was modified and adopted from Tiwari et al. [104]. FAM-labeled A $\beta$ 42 peptide (probe) is mixed with wild type A $\beta$ 42 (1 mM total in HFIP) in a 1:2 molar ratio, evaporated to film, then solubilized to 5 mM in 100% DMSO and bath sonicated. It is then diluted to a 100  $\mu$ M stock in HAMS F12, pH7.4, 2% DMSO and incubated 4°C for 24 hrs to oligomerize A $\beta$  (checked by western blot against 6E10 and directly by UV light-western). The recombinant protein binding targets, PDK-1 (59 kDa) or Akt-1 (60 kDa) are stocked as 500  $\mu$ M. Final probe concentration is fixed at 200 nM. Final target concentrations (0–10  $\mu$ M) are increased in successive samples until saturation is reached. The reaction is carried out manually in 0.6 cc quartz cuvettes (Suprasil Micro cells, 5 mm path length) for 30 min, 25°C, in 1X buffer: 100 mM NaCl, 20 mM phosphate pH7.4, 2 mM DTT, 0.1% BSA, 2% DMSO. Final volume 600  $\mu$ l. Where PS48 was added, final concentrations tested were 10, 20 and 100  $\mu$ M. Absolute fluorescence polarization (FP) values were read off a LS55 PerkinElmer luminescence spectrophotometer, excitation 485/ emission 530 nm. FAM-A $\beta$ 42 was prepared as ADDLs and incubated with recombinant Akt-1 or PDK-1 (final [A $\beta$ 42] 200 nM, Akt from 0 to 12.5  $\mu$ M. Immunoprecipitation of A $\beta$ 42 with 6E10 and western developed with anti Akt and re-probed with R1282.

### Drug screening

Two focused libraries of PS48-family compounds were designed based on known and hypothesized structure-activity relationships and tested using an *in vitro* screen carried out in 48 well plates as follows (in order of rapid additions, final concentrations): 10x Kinase buffer, recombinant PDK-1 (5  $\mu$ l, pre-immunoprecipitated onto agarose beads using monoclonal IgG), PIP3 50 nM, A $\beta$ 42 (as ADDL oligomers, 10 $\mu$ M), recombinant Akt-1 (5  $\mu$ l, pre-immunoprecipitated onto beads using polyclonal goat IgG, treated with PP2A to dephosphorylate Akt and washed), compound or PS48/ PS47 (solubilized in DMSO then diluted with H<sub>2</sub>O, 10  $\mu$ M),



ATP (to initiate Akt activation, 200  $\mu$ M). Incubation proceeded for 15 min. GSK-tide (Cell Signaling) was then added and the reaction allowed to carry for 20 min more before termination in sample buffer and fractionation on SDS gel. Transfers were probed with anti-p-GSK, anti phospho-473 and -308Akt and total Akt. The *in vitro* results were validated using a cell culture-based assay, as above. Briefly, adenovirus-infected PCNs (2 days) were induced with doxycycline (48 hrs) to express A $\beta$ 42. The compound was added for 12 hrs. The cells were stimulated with insulin before harvest.

## Statistical

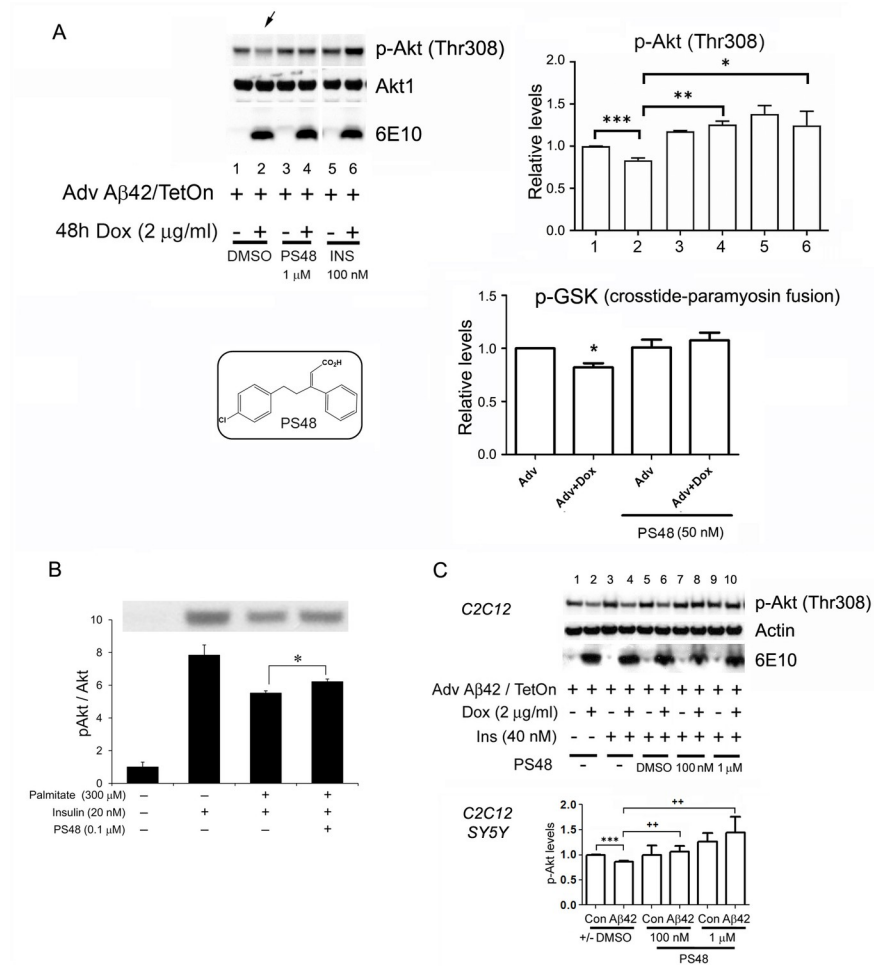
Where quantified, experiments were carried out in triplicate unless otherwise stated. Mean, standard errors and significance levels using students t- test were computed in Excel or Prism. *In vitro* Akt activation assay data (see above), in which the % inhibitory effects of A $\beta$ 42 monomers and ADDLs were tested, was fitted using a 2 site (hyperbolic), non-linear algorithm (Prism) to obtain  $I_{max}$  and  $K_{0.5}$  equilibrium constants. Western signal intensities were all quantified by densitometry. Akt activation (phosphorylation) and activity (GSK phosphorylation) western results (stimulation or inhibition) were for the most part, concordant and equivalent in the fraction of change versus control. Therefore, where both endpoints were evaluated, their normalized values were combined in the quantification as indicated. ANOVA (1 way; between treatment groups or columns and 2 way; between treatments groups and between repeated measures or rows) was carried out on Western, cell viability and LTP experiments. Where indicated, Dunnett's or Tukey's multiple comparisons were applied. Effect sizes for A $\beta$  and drug additions are given as mean differences ( $\pm$  SE difference) and 95% confidence intervals.

## Results

We had previously shown that cellular  $\beta$ -amyloid expression inhibits PI3K-PDK1-Akt signaling [80, 81, 105]. To summarize, *in vivo* assays of phospho-Akt/total Akt and downstream substrate, phospho-GSK3 $\beta$  levels were carried out on extracts from cultured neurons exposed to an inducible adenoviral vector encoding A $\beta$ 42 [64, 80]. Cells were pretreated with insulin for 20 min prior to harvest in order to activate Akt, finding that insulin-stimulated p-Akt levels were reduced to baseline in the presence of A $\beta$ 42 expression. To confirm this, Akt enzymatic activity was measured in cell lysates using a coupled assay; immuno-precipitated (IP) Akt from insulin-stimulated cells, phosphorylated a synthetic substrate peptide bearing the phosphorylation consensus sequence GSK3 $\beta$  fused to paramyosin (crosstide). An *in vitro* kinase assay was also developed (see methods), finding that 5  $\mu$ M A $\beta$  peptide inhibited the Akt-dependent phosphorylation of the target substrate, accompanied by an expected reduction in pSer473Akt. Further data [64] indicated that A $\beta$  inhibits the PDK-1- dependent activation of Akt by disrupting their interaction.

The pathological target identified in these *in vivo* and *in vitro* platforms suggested that a small molecule could be found that modulates the insulin-PDK-Akt activation cycle in such a way to relieve the inhibitory amyloid effect. A chemical data base search identified an allosteric activator of PDK-1 (CAS 1180676-32-7, PS48), a chlorophenyl pentenoic acid having a MW of 286.7 [106] (Fig 1 top). Furthermore, it has an inactive 'E' isomer, PS47, for control use [96, 97]. It is unique in its action to bind the hydrophobic motif/PIF binding pocket of PDK and not the ATP binding site. The compound has other possible beneficial actions that may translate to improve hippocampal neurogenesis [107].

Employing the above assays, the SHSY5Y neuroblastoma line was used for most cell-based experiments. First, to model insulin resistance in these cells, Akt (PKB $\alpha$ ) activation with increasing insulin concentrations applied shortly before harvest was established (S1A Fig in S1 File). Then at the same doses, cellular expression of A $\beta$ 42 over the preceding 48 hours was



**Fig 1. PS48 or Insulin restores Akt activation in  $\beta$ -amyloid expressing cells.** **1A, left.** SH-SY5Y cultures were infected with Adenovirus (Adv) encoding A $\beta$ 42 (24–36 hrs) and induced with Doxycycline (46 additional hrs). Amyloid bearing cells show inhibited Akt phosphorylation (lane 2 arrow). PS48 (1  $\mu$ M) or high dose Insulin (100 nM) added 2 hrs before doxycycline induction (pre-treatment) restores Akt activation levels in cells expressing A $\beta$ 42 (lane 4 and 6). Low dose Insulin 40 nM (stimulation) is added to all cultures 20 min prior to harvest. PS48 alone does not over-stimulate Akt (lane 3 vs. 1). **1A, right.** Quantification of restored inhibited Akt activation by PS48 and Insulin. pT308 Akt levels are normalized RFU, (relative fluorescence units). n = 3 experiments. Error bars are 1 SE relative to lane 1 (control). Brackets indicate individual t-tests comparing A $\beta$  expression result (lane/bar2) to: control (lane/bar1), PS48 (lane/bar 4) and Insulin (lane/bar 6) additions. \*\*\* p < .001, \*\*p = .01, \* p < .05, t-test. Insulin increases pAkt T308 over control (p = .02, lane/bar 5 vs.1). PS48 also has a stimulatory effect (p < .01, lane/bar 3 vs.1). ANOVA 1-way: P = .03, F3,7; 2-way: P = .06, F = 3.1 (between groups), P = .49 (within treatments, ie. replicates). **1A, below, in vivo-in vitro** coupled assay of Akt activity. Akt was immunoprecipitated from SH-SY5Y cells infected with Adv. Phosphorylation of GSK3 $\alpha$ / $\beta$  consensus peptide proceeded *in vitro* after adding 200  $\mu$ M ATP to the immunoprecipitate. PS48 pre-treatment (50 nM), added to cells 2 hrs. before Doxycycline addition, prevented the inhibition of peptide phosphorylation by A $\beta$ 42. (Western not shown, n = 3, \*p < .05 vs. control (Adv. alone). PS48 structure, left. **1B.** Non-amyloid-based insulin resistance model. Primary rat cortical neurons (PCN) exposed to fatty acid neurotoxin, palmitate (300  $\mu$ M). PS48 (100 nM, 24 hrs) partly reversed inhibition of Akt Ser473 phosphorylation by palmitate, in insulin-stimulated cells (20nM, 15 mins before lysis). Identical result in ceramide (50  $\mu$ M)-treated PCNs, not shown). (\*p < .05, n = 3). **1C.** SH-SY5Y and C<sub>2</sub>C<sub>12</sub> cells (representative Western) were used to test dose dependency of PS48 (1  $\mu$ M, 100 nM, 10 nM). A $\beta$ 42 expression inhibits the insulin-stimulated phosphorylation (activation) of Akt (lanes 4 vs.3 and 6 vs.5 were combined to correspond to bar 2 vs.1). PS48-, doxycycline-, Insulin-additions and harvest as above. Data from both cell types were combined for the bar graph. PS48 100 nM and 1  $\mu$ M (10  $\mu$ M, not shown) each significantly overcome the A $\beta$ 42 effect (lanes 8 and 10 vs. 4 or 6). Bar graph: \*\*\* p < .005, A $\beta$  vs Con; ++ p < .01, PS48/A $\beta$  (bars 4 and 6) vs. vehicle/A $\beta$ 42 (bar 2). DMSO is vehicle control. (n = 3).

<https://doi.org/10.1371/journal.pone.0261696.g001>

introduced to produce insulin resistance. At moderate insulin doses (20 nM), Akt activation is inhibited by A $\beta$ 42, whereas high doses of insulin (100 nM) overcame the effect. Thus A $\beta$ 42 was shown to desensitize insulin action, raising the insulin concentration threshold to achieve an equivalent response (S1B Fig in S1 File). In this system, we find that PS48 exposure did not intrinsically activate basal Akt. However, in the presence of low dose insulin (3 nM) where Akt activation is subthreshold, PS48 augmented the response. This is in line with its purported allosteric action to positively modulate PDK activity (S1C Fig in S1 File). At moderate insulin doses, that produce robust Akt phosphorylation, PS48 did not further enhance it. Neither did PS48 intrinsically affect at least one critical downstream factor in this pathway, mTOR (S1D Fig in S1 File). These properties make it ideal to test if it will protect insulin signaling against amyloid peptide toxicity, while not over-regulating the pathway.

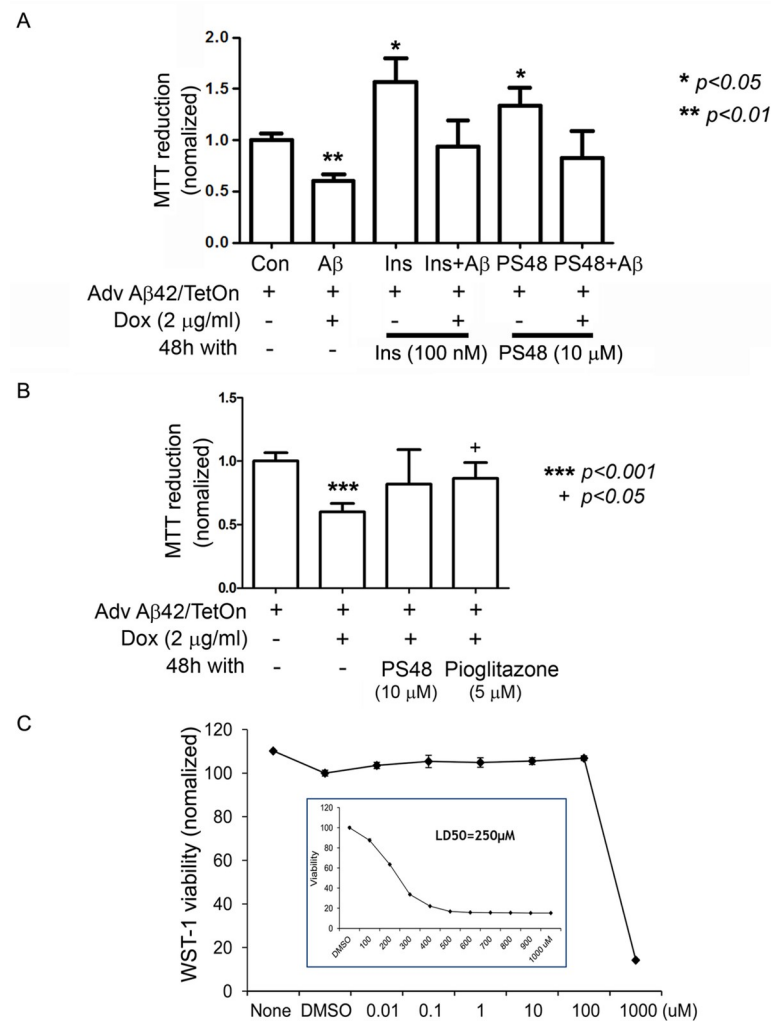
First we tested PS48 (1  $\mu$ M) in cultured SH-SY5Y cells, where it is shown to normalize  $\beta$ -amyloid expression-induced inhibition of Akt activation (pT308 phosphorylation) by subacute moderate dose insulin (40 nM) applied 2 hours before harvest (Fig 1A). Notably, it did not overactivate basal levels of phosphoAkt to a statistically significant extent (lane/bar 3 vs 1). Live cells were pre-treated with either PS48 (1  $\mu$ M) or high dose Insulin (100 nM) for 2 hrs. before doxycycline was added. A $\beta$ 42 expression proceeded over the next 46 hrs. PS48 and high dose insulin had similar protective actions for the duration of expression. pAkt levels were quantified densitometrically, shown right. A $\beta$  expression at moderate toxic levels to the cell, reduced Akt phosphorylation with an effect size of  $-0.17 \pm 0.02$  relative units ( $p < .001$  t-test; mean difference of  $1.00 - 0.83 \pm 0.03$  RU). PS48 addition recovered pAkt to control levels with an effect size of  $0.42 \pm 0.04$  RU ( $p < .01$  vs. A $\beta$  expression; mean difference of  $1.25 \pm 0.04 - 0.83$ ), as did insulin (effect size  $0.41 \pm 0.19$ ;  $p < .05$ ; mean difference of  $1.24 \pm 0.17 - 0.83$ ; lane/bars 4 or 6 vs. 2). ANOVA yielded  $P = .03$ ,  $F = 3.7$  (1-way);  $P = .06$ ,  $F = 3.1$  (2-way). To confirm, we next tested Akt enzymatic activity to phosphorylate a consensus substrate peptide corresponding to phospho-Ser21/9 sites on GSK3- $\alpha/\beta$ . We obtained the same outcome, mainly that lower dose PS48 (50 nM) also protected Akt activity from inhibitory cellular A $\beta$ 42 expression. We also tested the effect of PS48 to reverse a non-amyloid dependent model of insulin pathway toxicity, exposure to the long-chain saturated fatty acid, palmitate, in rat primary cortical neurons. In Fig 1B, insulin (20–40 nM) given just 20 min prior to harvest, stimulates Akt phosphorylation, which palmitate (300  $\mu$ M) partly blocked (~30%). PS48 significantly corrects some of this inhibition. In Fig 1C, we confirm and expand results of 1A, testing various doses of PS48; 100 nM, 1  $\mu$ M, (10  $\mu$ M not shown) in both SH-SY5Y and C<sub>2</sub>C<sub>12</sub> myotube cell lines. A dose dependent effect to reverse A $\beta$ 42- provoked inhibition of insulin-stimulated Akt phosphorylation became apparent at 100 nM. Insulin-stimulated pAkt levels were reduced from baseline in the presence of A $\beta$  expression with an effect size of  $-0.14 \pm 0.09$  relative units ( $p < .001$ ; mean difference of  $1.00 - 0.85 \pm 0.03$  RU). 100 nM PS48 recovered pAkt to control levels with an effect size of  $0.20 \pm 0.15$  RU ( $p < .01$  vs. A $\beta$  expression), as did 1  $\mu$ M PS48 (effect size  $0.58 \pm 0.13$ ;  $p < .01$  t-test and  $p = .0003$  Dunnett's. ANOVA yielded  $P = .0013$ ,  $F = 5.1$  (1-way);  $P = .013$ ,  $F = 3.8$  (2-way).

Several downstream effectors and substrates of Akt were examined for sensitivity to A $\beta$  toxicity and PS48. CREB, the cAMP response element binding transcription factor, has pleiotropic actions to promote neuronal survival, progenitor proliferation, neurite outgrowth and differentiation. It is also well documented to control the activity-driven and neurotrophin-dependent expression of proteins essential to long term memory formation (LTM) and synaptic plasticity (LTP) (see reviews by [42, 108, 109]). It is situated in the PI3K/Akt/CREB pathway to transduce effects of Insulin, IGF-1 and BDNF on protein expression critical to neurogenesis and plasticity [40, 43, 110]. CREB supports LTM by stabilizing synaptic strength, regulating intrinsic neuronal excitability and recruiting subsets of neurons in the hippocampus and amygdala that encode the memory trace [111–114]. We focused on CREB because it can be directly activated by Akt [115,

[116], is protective against neuronal apoptosis [117, 118] and supports LTP [119]. We found consistent inhibition of insulin-stimulated CREB phosphorylation (pS133) by intracellular A $\beta$ 42 and this was also stabilized by PS48 (50 nM) (S2A Fig in S1 File).

Previous work had shown sensitivity of endogenous GSK3 $\alpha/\beta$  (inhibitory S9A phosphorylation) to viral expressed A $\beta$ 42 [80], however the current experiments under combined A $\beta$ 42 pressure and PS48 proved inconclusive. Nevertheless, PS48 had no effect on resting cellular pGSK levels (S2B Fig in S1 File). Finally, in testing for changes in activating phospho-levels of indirect downstream substrate and metabolic sensor mTOR, we found that neither A $\beta$  expression (also shown in [105]) nor PS48 applications had any effect (S2C Fig in S1 File).

In parallel experiments, PS48 pre-treatment partially restored the A $\beta$ -induced decrement in cell viability. (Fig 2A, MTT reduction to formazan assay). SH-SY5Y cells were exposed to Adv



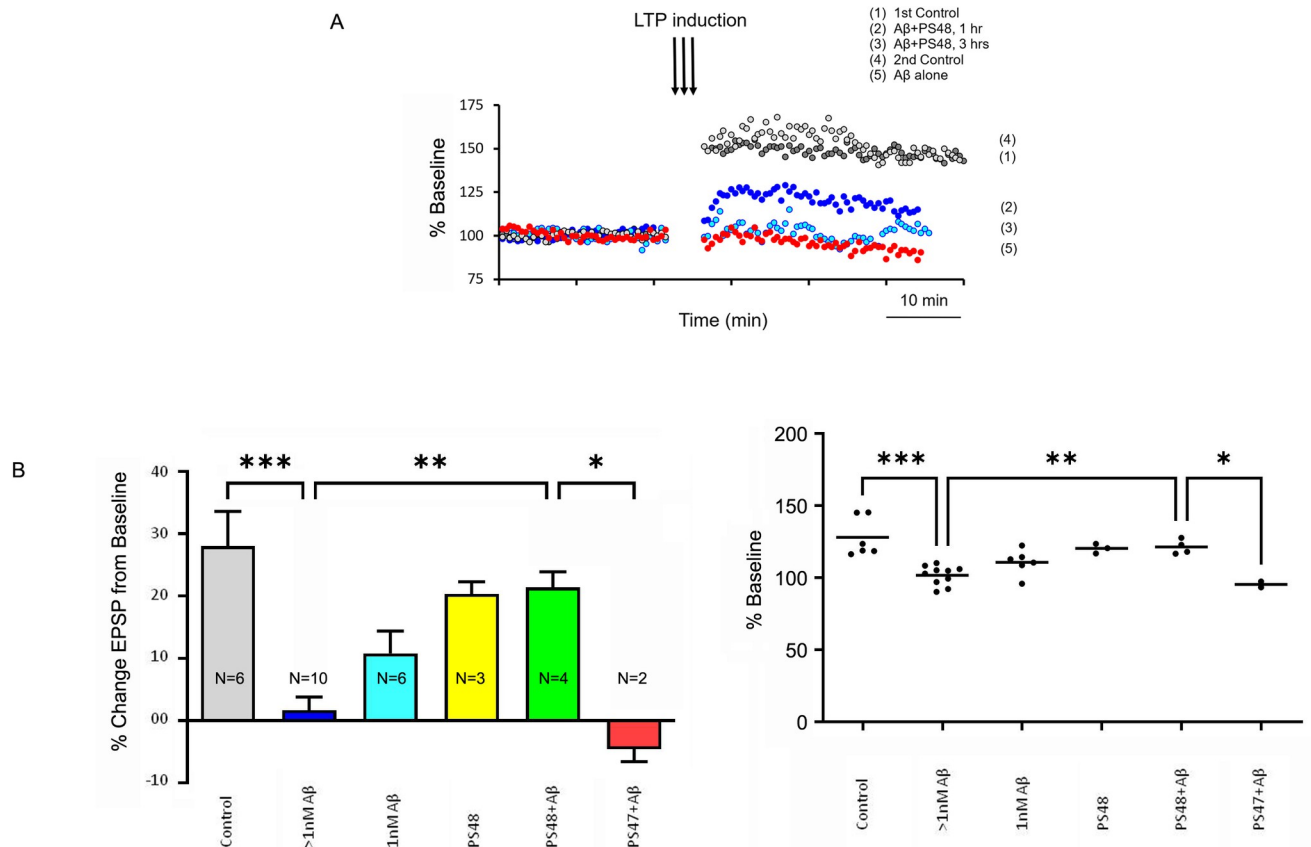
**Fig 2. PS48 or Insulin partly restores partial viability in  $\beta$ -amyloid expressing cells.** 2A. SH-SY5Y cultures infected with Adenovirus encoding A $\beta$ 42 and induced with Doxycycline. Amyloid bearing cells are less viable in an MTT reduction assay (bar 2 vs. 1; t-test,  $p < .01$ ). Pretreatment with insulin or PS48 partially reverses cell death to control viability levels (bars 4 and 6 compared to Con). Insulin or PS48 alone are also neurotrophic (bars 3 and 5 compared to Con, \*  $p < .05$ ,  $n = 3$ ). ANOVA 1-way:  $P = .003$ ; 2-way  $P < .0001$  (column effect). See text for treatment effect sizes (given as mean differences). 2B. In C<sub>2</sub>C<sub>12</sub> myotubes, 48 hrs pretreatment with PS48 (10  $\mu$ M) is comparable to pioglitazone (5  $\mu$ M). +  $p < .05$  vs. bar 2. 2C. Using a WST viability assay, cultured N2a cells are unaffected by PS48 at doses up to 100  $\mu$ M, estimated LD50 is 250  $\mu$ M. 24 hrs. exposure,  $n = 4$ .

<https://doi.org/10.1371/journal.pone.0261696.g002>

TRE-A $\beta$  and  $\pm$  doxycycline to induce A $\beta$ 42, then harvested at 48 hrs. Amyloid bearing cells are less viable compared to control (bar 2 vs. control bar 1; t-test \*\*  $p < .01$ ; effect size (mean difference) = -0.41, confidence intervals [-0.20 to -0.62]). Pretreatment of live cell cultures (before adding doxycycline) with high dose insulin (100 nM) or PS48 (10  $\mu$ M) reverts cells in the direction of normal (basal) conditions (bars 4 and 6 vs. con bar 1 were not significantly different). However, they were also not significantly different from A $\beta$ -laden cells (bar 2). Effect sizes (and 95% CI) for Ins+A $\beta$  vs. A $\beta$  and for PS48+A $\beta$  vs. A $\beta$  are 0.33 [-0.12 to 0.79] and 0.22 [-0.27 to 0.71], respectively (ANOVA ns). We therefore conclude a trend effect. Nevertheless, in the absence of A $\beta$  expression, both Insulin and PS48 appear to have significant trophic effects (bars 3 and 5 vs. con bar 1; \*  $p < .05$ ). Overall, ANOVA yielded a  $P = .003$  (1-way) and  $P < .0001$  (2-way). The same cytoprotection was obtained in C<sub>2</sub>C<sub>12</sub> myotubes. In Fig 2B, PS48 also compared favorably with a PPAR agonist, pioglitazone. The toxicity profile of PS48 itself was determined in a similar reduction assay (WST-1) and is well tolerated by cells (Fig 2C, LD50 250  $\mu$ M).

PS48 was further tested *in vivo* and found to partially reverse the inhibition of long term potentiation (LTP) caused by added oligomers of synthetic A $\beta$ 42 peptide (Fig 3). Acute pre-frontal rat cortical slices were super-perfused with A $\beta$ 42 peptide (0.5  $\mu$ M) prepared as amyloid diffusible ligands (ADDLs), shown to be largely comprised of oligomeric species. LTP was measured as % baseline EPSP amplitudes. Representative single experiments showing EPSP amplitudes (expressed as % baseline) under the following conditions: control LTP (1), inhibition after A $\beta$  5 nM (5), A $\beta$  plus PS48 10  $\mu$ M after 1 (2) and 3 hrs. (3) exposure (showing a run-down effect) and reversibility to control LTP after washout (4), are superimposed in Fig 3A. Quantification of data from  $n = 3$  to 10 independent experiments is given in chart (mean of means as EPSP % change above baseline) and scatter (individual means comprising the group mean, as EPSP % baseline) graphic forms, respectively (Fig 3B). LTP was completely abrogated by all concentrations of A $\beta$ 42 > 1nM (2.5–500 nM) (\*\* $p < .0001$ , 1-way Tukey's multiple comparisons), whereas A $\beta$  at 1 nM partially did so ( $p < .05$ , Tukey's). 10  $\mu$ M PS48 alone produced a small, nonsignificant decrement in mean LTP compared to control. However, in the presence of higher dose A $\beta$  (>1 nM), this level of LTP was sustained by PS48, representing a significant improvement (bar 5 vs. 2, \*\* $p < .01$ , Tukey's). For instance, the mean effect size of PS48 (10  $\mu$ M) to improve LTP induction at a fixed A $\beta$  concentration of 5 nM, is a net 18% ([95% CI; 27.1 to 8.9%], t-test  $p = .0065$ ,  $n = 3$  and 4 respectively). The control inactive stereoisomer PS47 provided no benefit compared to PS48 in the presence of A $\beta$  (\* $p < .05$  bar 4 vs. 5, Tukey's), in fact slightly accentuated the A $\beta$  effect. ANOVA results: 1-way:  $P < .0001$ ,  $F = 10.2$ ; 2-way  $P = .0018$  for group treatments,  $P = 0.8$  for row (within group) effect.

We next tested PS48 in an *in vitro* assay of both Akt activation (phosphorylation of T308) and enzyme activity. In Fig 4A, recombinant Akt and PDK-1 were added to a reaction mixture containing synthetic A $\beta$ 42 peptide oligomers (10  $\mu$ M) and ATP to start the reaction. Some experiments employed added PI3P and/or pre-dephosphorylation of Akt by treatment using PP2A, with variable improvements in the efficiency of activation. A GSK fusion peptide was added as substrate for the enzymatic readout (Westerns of phospho-S9 GSK3 $\alpha/\beta$ ). Initial experiments used high dose PS48 (100  $\mu$ M) and tested if added before (pre) or after (post) the A $\beta$  peptide made a difference. pAkt-T308 levels were reduced in the presence of A $\beta$ , similar to the cell-based experiments. PS48 restored Akt phosphorylation. Moreover, it had greater efficacy when added after A $\beta$  peptide equilibration. So, this procedure was followed in Fig 4B, that alternatively used recombinant Akt and immunoprecipitated PDK-1. PS48 was found to be active at 0.1 and 10  $\mu$ M in reversing the inhibition of GSK phosphorylation. The pooled results from same experiments over a range of PS48 concentrations are quantified in Fig 4C. In the presence of 10  $\mu$ M A $\beta$ 42, ~30% of Akt activation is inhibited (expressed as a remaining

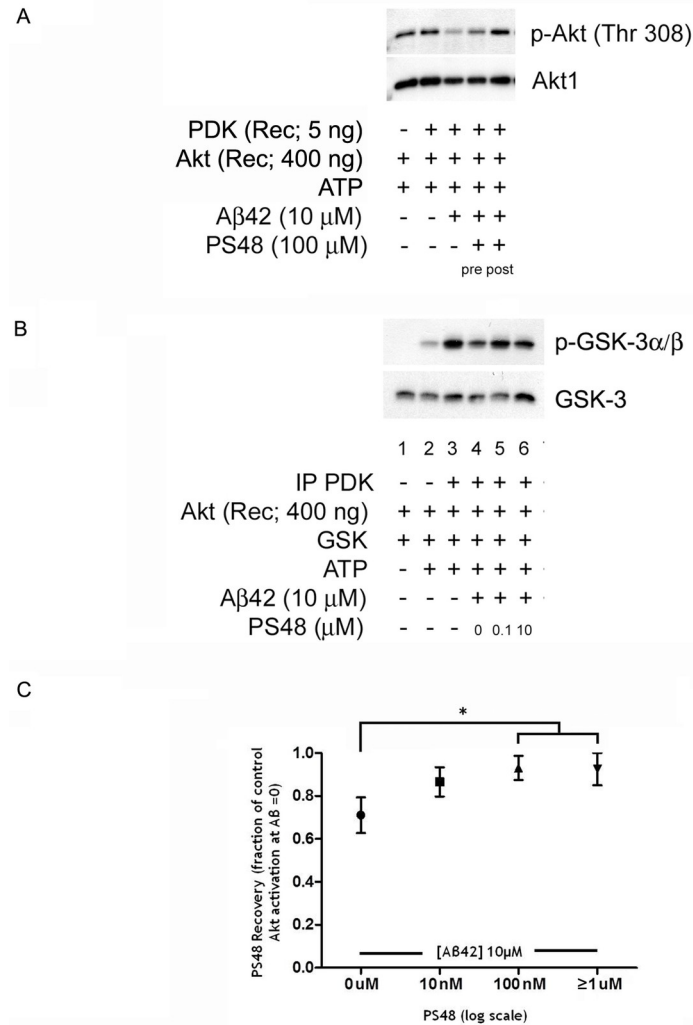


**Fig 3. PS48 partly restores the suppression of LTP by A $\beta$ 42.** **A.** Day 14 rat prefrontal cortex (PFC) exposed to A $\beta$  oligomers (0.5  $\mu$ M) or A $\beta$  plus PS48 (10  $\mu$ M). Superimposed representative single recordings made under conditions 1 through 5, as noted. Control is DMSO-vehicle. EPSPs recorded from layer 2; stimuli (32  $\mu$ A =  $\frac{1}{2}$  max ampl., 2 Hz) applied to layer 5. A $\beta$  completely suppressed LTP (5 vs. 1). PS48 in the presence of A $\beta$  restored 68  $\pm$  18% of control potentiated EPSP (1 hour perfusion, condition 2). A 3 hour application however showed near complete run-down (3). A $\beta$  and A $\beta$ /PS48 experiments were restored to control LTP levels after washout (4). **B.** Quantification of LTP data. Bar graph (left). LTP induction shown as mean % change (increase or decrease) from baseline ( $\pm$  SEM). n independent replicates are noted. Scatter graph (right) gives means of individual experiments and group averages as unadjusted % baseline values (normalized at 100%). All concentrations A $\beta$  > 1 nM (high dose, 2.5–500 nM) completely abrogated LTP. PS47 is an inactive isomer control (n = 2). n replicates as shown. \*\*\* p < .0001 A $\beta$  alone (high dose) vs control, \*\* p < .01 PS48 treatment vs A $\beta$  alone (high doses), \* p < .05 PS48 treatment vs. P47 control (A $\beta$  high doses); ANOVA and Tukey's tests (see text for additional statistical details).

<https://doi.org/10.1371/journal.pone.0261696.g003>

fraction of the control (absent A $\beta$ , set to 1.0), thus  $0.70 \pm 0.09$ . Beginning at 10nM PS48, activation/activity is increasingly normalized until a maximum of  $0.95 \pm 0.08$  of control is reached at  $\geq 1 \mu$ M PS48 (includes 10 and 100  $\mu$ M data points). When all values  $\geq 0.1 \mu$ M are combined (n = 8), the trend toward normalization reached significance (p < .02, compared to 0 nM PS48, t-test, n = 6).

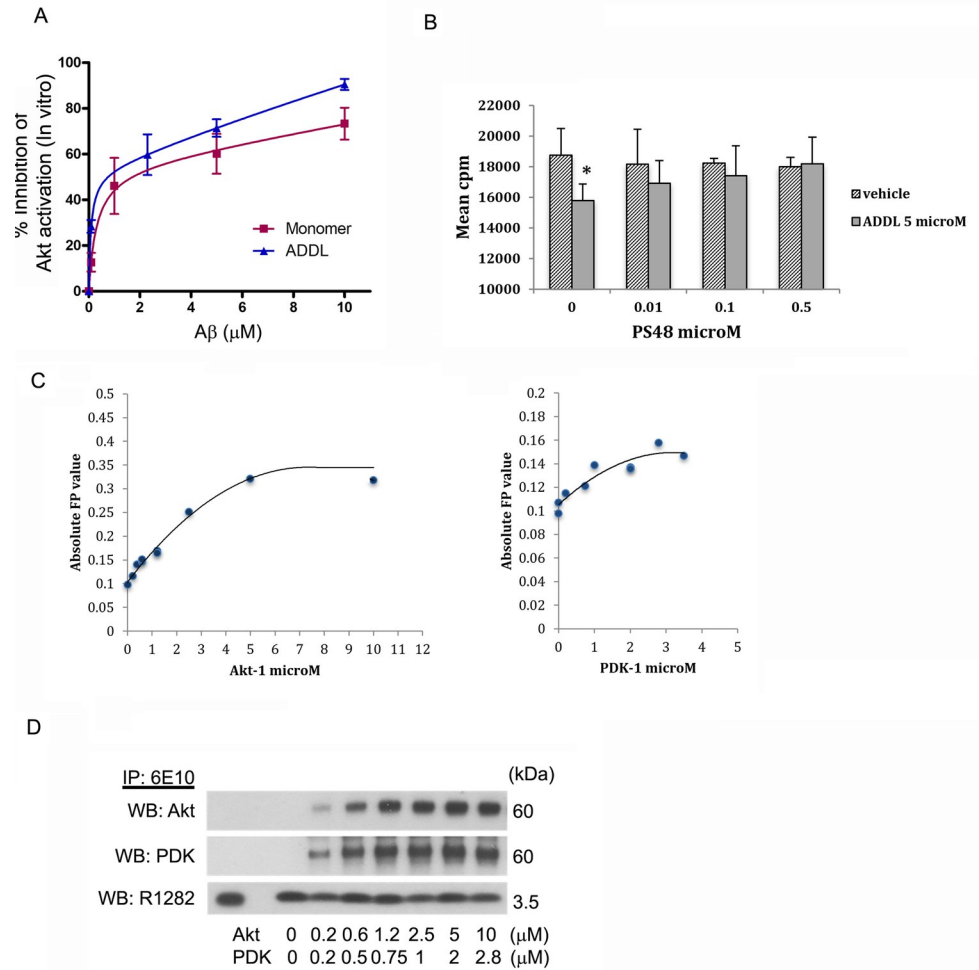
To further explore target engagement, we probed the characteristics of A $\beta$ 42 interaction with PDK-1 and/or Akt-1. We conducted equilibrium A $\beta$ 42 dosage and binding experiments in solution to the respective kinase targets. First, the *in vitro* paradigm above was used to test whether A $\beta$ 42 peptide exhibits a dose-dependant specificity to inhibit the activation (phosphorylation of T308) and enzymatic activity of Akt (to phosphorylate its consensus substrate). The combined Western densitometry data in Fig 5A suggests the possibility that either A $\beta$  monomers or oligomers act to inhibit either one or both of the two kinases, separately or in complex, and in a saturable manner. Using a 2 site, non-linear fit algorithm a  $I_{max}$  value of  $\sim 52\%$  inhibition of control activation is obtained for both species, however the oligomers



**Fig 4. PS48 partly restores inhibited Akt phosphorylation (Akt activation) and GSK3 $\beta$  phosphorylation (Akt activity).** In vitro additions of recombinant PDK (5 ng; **4A**.) or pre-immunoprecipitated PDK-1 (**4B**.). **4A.** A $\beta$  peptide addition near completely results in Akt dephosphorylation (lane 3). PS48 added after A $\beta$  peptide (post) in the reaction mixture completely reverses the loss of Akt phosphorylation (lane 5 vs. 3). When pre-added (pre), PS48 partially restores pAkt levels (lane 4). Note commercial recombinant Akt lots come variably phosphorylated (lane 1), but lack enzymatic activity in control reactions until PDK is added (see Figs **4B** and **6B** and text) and also undergo further phosphorylation. **4B.** Restoration of Akt-catalyzed phosphorylation of GSK3 $\beta$  peptide (lanes 5 and 6 vs. 4). **4C.** Quantified trend to reverse inhibited Akt activation and activity by PS48 in the presence of 10  $\mu$ M A $\beta$ 42 oligomers (ADDLs). PS48 is active in the 10 nM range. Data from pAktT308, pAktS473 and pGSK3 $\alpha/\beta$  S9 experiments were normalized and pooled (n = 3–6 ea.). 'Fraction of control Akt activation' is the [A $\beta$  ( $\pm$  PS48 presence) /Control] densitometry signals ratio, where 1.0 represents full restoration. \* p < .02, t-test, A $\beta$  10 $\mu$ M/0 $\mu$ M PS48 (n = 6) vs. combined 100 nM and  $\geq$  1  $\mu$ M (1, 10 and 100  $\mu$ M) data (n = 8). The mean difference is 0.23  $\pm$  0.08, 95% CI = [0.41 to 0.05].

<https://doi.org/10.1371/journal.pone.0261696.g004>

showed greater affinity (ADDLs: K<sub>0.5</sub> = 0.08  $\mu$ M, monomers: K<sub>0.5</sub> = 0.31). To independently confirm the reversal of the PDK/Akt activation sequence by A $\beta$ 42 peptide, we employed a modified radiolabelled-based assay in **Fig 5B**. Again, PS48 dose-dependently reduced the inhibition of P<sup>32</sup>-labelled phosphate addition to a consensus peptide. The effect was first noticed at 10 nM (**Fig 5B**), consistent with **Fig 4C**. Next, a novel assay was adapted to determine if A $\beta$ 42 bound to either PDK-1 or Akt-1 in solution and to discern any effect of PS48 on this,



**Fig 5. Equilibrium enzymatic and binding studies.** PDK-1 and Akt-1 are  $A\beta$ 42 targets. **5A.**  $A\beta$ 42 monomers and ADDL-oligomer preparations, at concentrations shown, exhibit saturation effects to inhibit the activation of Akt by PDK-1. In vitro assay data are presented as % inhibition of either activation of Akt (phospho-T308) by PDK-1 or of its subsequent activity to phosphorylate a GSK3 $\beta$  consensus peptide fused to paramyosin ('crosstide'). Densitometry results from both western blots were combined.  $I_{max}$  ~ 52% for both  $A\beta$  preparations: ADDL,  $K_{0.5}$  = 0.08  $\mu$ M; monomers,  $K_{0.5}$  = 0.31  $\mu$ M.  $n$  = 2–7 experiments each point,  $\pm$  1 SEM. **5B.** In vitro radioassay (see methods). PS48 from 10 nM to 0.5  $\mu$ M, gradually diminishes the inhibitory effect of  $A\beta$  (5  $\mu$ M ADDL alone, bar 2, \* $p$  < .05 vs. vehicle) on the phosphorylation of GSK peptide (measured in cpm). The trend is toward control activity, not significant from bar 1 or other vehicle controls. **5C.** Quantification of  $A\beta$ 42 binding to kinase targets by fluorescence polarization (FP). The probe was FAM-tagged- $A\beta$ 42. Recombinant kinase titrations shown along bottom. The spectrophotometric polarization signal increases as the probe becomes more restricted by receptor binding.  $B_{max}$ : Akt 0.32; PDK 0.16.  $IC_{50}$ : Akt ~2  $\mu$ M, PDK ~1  $\mu$ M. **5D.** Direct binding of recombinant PDK-1 and Akt-1 to tagged  $A\beta$ 42 in solution is confirmed and exhibits saturation characteristics.  $A\beta$ 42 was immunoprecipitated (6E10), fractionated by Western and co-precipitates are detected using anti- Akt and -PDK.  $A\beta$ 42 concentration was fixed at 200 nM and detected using polyclonal R1282. Lane 1 is  $A\beta$  peptide control, lane 2 is beads without 6E10 control.

<https://doi.org/10.1371/journal.pone.0261696.g005>

using the fluorescence polarization (FP) technique. A spectrophotometric filter detects the fluorescent signal from a probe that becomes polarized once restricted by receptor binding. We found that the FP signal increases as the concentration of either recombinant PDK or Akt are increased. The probe concentration (FAM tagged- $A\beta$  = 200 nM) is fixed in this procedure. Results shown are from 2 experiments each. Saturable binding to  $A\beta$  is shown by both target molecules, where the  $K_d$  (1/2 max constant) for PDK shows slightly increased affinity over

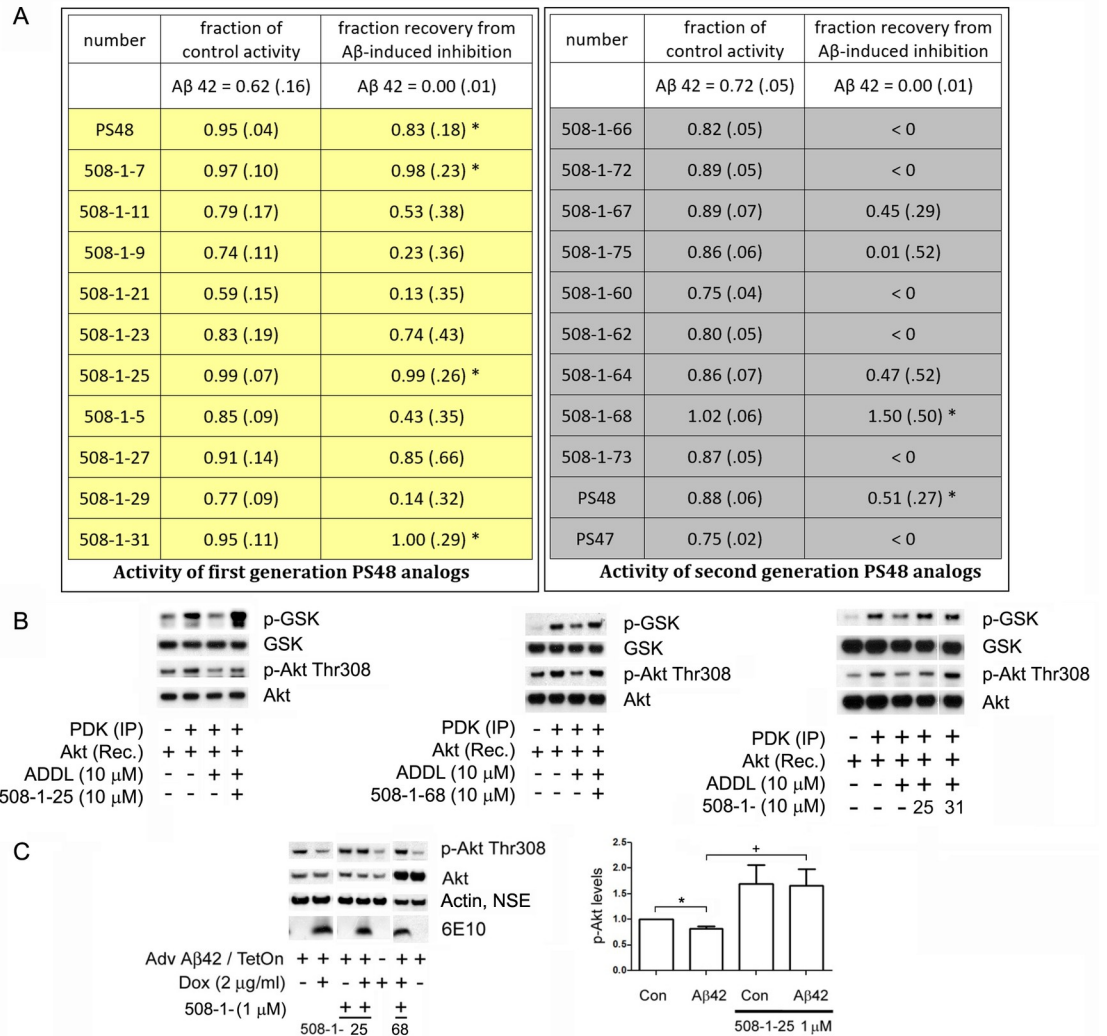


Akt, but fewer binding sites (Fig 5C). PS48 additions did not affect either polarization signals, concluding that A $\beta$ 42 is probably not competitively occupying the pocket site (results not shown). The in-solution FP assay was validated by co-immunoprecipitating the bound reaction products. IP of FAM-labeled A $\beta$ 42 with anti-6E10 pulls down increasing amounts of Akt and PDK according to the titration until saturation (Fig 5D). A $\beta$  concentrations were constant as shown in the anti-R1282-developed blot shown below. Previous work has shown that A $\beta$  peptide in AD brain co-immunoprecipitated with PDK and Akt and that cellular expression of A $\beta$ 42 disrupts Akt-PDK interaction [64].

Lastly, we launched drug screening experiments using a focused library of novel compounds that were synthesized using PS48 as the starting scaffold. Two generations were created based on structure-activity relations (SAR) in an attempt to optimize performance. The quantified results of western-based *in vitro* assays and signal intensities, compared to PS48, are shown in Fig 6A. The activity of each compound against the A $\beta$ 42 effect to inhibit the PDK-Akt activation sequence is shown as two indices: 1) 'fraction of control activity', as remaining in the presence of A $\beta$  with drug [A $\beta$  with drug]/[control], where the control is absent A $\beta$  and normalized to 1.00. The fraction of control activity observed in reactions having just A $\beta$  alone (maximal inhibition, no drug) was  $0.62 \pm 0.16$  (top left), and 2) 'fraction recovery to control level from A $\beta$ 42-induced inhibition', where the index is normalized instead to the A $\beta$  inhibitory effect size [A $\beta$  with drug—A $\beta$ ] / [control—A $\beta$ ] signals. The fraction recovery with just A $\beta$  alone (or no drug effect) is therefore 0.0 on this scale (top right). Using either index, a value of  $\geq 1.00$  is a complete reversal. Based on this data, a rough SAR is beginning to emerge where it appears that the greatest effect is realized by modifying the acidic portion of PS48, e.g. extension from the linker and aryl groups. An increase in potency over PS48 was achieved with compounds 7, 25, 31 and 68. Examples of *in vitro* assay performance are shown below (Fig 6B) for compounds no. 25 and 31 (generation 1) and 68 (generation 2). These were then validated as correcting the inhibition of Akt phosphorylation in the cell-based model of A $\beta$  toxicity (as in Fig 1), shown at the bottom for compounds 25 (with quantification) and 68 (Fig 6C). The control stereoisomer PS47 had no effect in these *in vitro* reactions (table bottom right). The new 'hit' compounds also had no direct or indirect effects on potential downstream off-targets such as mTOR (protein levels and phosphorylation status, results not shown). Moreover, the LD50 of several promising compounds in N2A neural cultures proved even higher than PS48 (e.g. no. 25; 350  $\mu$ M, WST assay, result not shown).

## Discussion

Akt (PKB) is an essential kinase in the insulin/IGF signal cascade having pleotropic influence over many cell survival and metabolic pathways. Its co-crystal structure in complex with a substrate peptide (GSK3 $\beta$ ) and an ATP analog, reveals the structural relationship between the C-terminal hydrophobic motif (HM) and the activating phosphorylation of Akt on the Thr 308 residue by PDK-1 [120]. The co-crystal structure of PDK-1 in complex with ATP reveals the HM-binding pocket (PIF domain located on the N terminus) and phosphoSer-binding pocket through which it docks with its many substrates including: Akt, SGK, S6K, PKC and RSK [121]. Interestingly, Akt is the only substrate not requiring docking at the PIF-pocket site to undergo catalytic activation by PDK-1 [122, 123]. PS48 and family of its analogs are small molecule allosteric activators of PDK-1, binding within the PIF pocket [97], thereby facilitating Akt activation by IGF-1 [96, 106, 124]; for review see Xu et al. [125]. These features are presented in schematic in S3 Fig in S1 File. Additional actions of this allosteric binding include supporting the induction of pluripotent stem cells from somatic cells [107].



**Fig 6. In vitro screen of focused compound library based on PS48 scaffold. 6A.** Compounds numbered according to SAR. Left, first generation; Right, second generation compounds. Two indices of effectiveness are labeled: 'Fraction of control activity' represents the observed amount (or fraction) of activation of Akt by PDK in the presence of compound with amyloid, relative to the no drug, no amyloid control (full activity = 1.00,  $A\beta$ 42 alone is 0.62). 'Fraction of recovery' is an index measure of the ability of a compound to restore the activation of Akt by PDK in the presence of  $A\beta$ , relative to the level of inhibition from  $A\beta$  alone (drug- $A\beta$ )/(control- $A\beta$ ) signals ratio. (full activity = 1.00,  $A\beta$ 42 alone is 0.0). \*  $p < 0.01$  for both measures,  $n = 5$  independent experiments each compound. PS47 is the inactive stereoisomer of PS48, bottom right. Final concentrations of both  $A\beta$  (ADDLs) and drug were  $10 \mu\text{M}$ . Results are combined phosphorylations of Akt (T308, Akt activation) and pGSK3 $\beta$  consensus peptide substrate (S21/9, Akt enzymatic activity). **6B.** Selected Westerns of in vitro reaction mixture by-products, highlighting significant GSK peptide (S21/9) and Akt (T308) phosphorylations in the presence of  $A\beta$  by compounds 508-1-25 and -31 (1st generation) and 508-1-68 (2nd generation). These 'hits' restored activations at or better than PS48. **6C.** In vivo verification studies in SH-SY5Y cells. Addition of Doxycycline results in intracellular amyloid accumulation (6E10 blots). Compounds 25 and 68 ( $1 \mu\text{M}$ ) intrinsically boost Akt phosphorylation to similar degrees, that remain sustained under  $A\beta$  pressure (as the case with PS48). Phospho-Akt levels, both T308 (as exemplated) and S473 (not shown) were quantified and pooled for the bar graph, featuring 508-1-25. All cultures were stimulated with insulin ( $40 \text{ nM}$ ,  $240 \text{ ng/ml}$ ) in the last 20 minutes of the  $A\beta$ - expression period.  $n = 3$  experiments. \*  $p < .05$  vs. Con, +  $p < .01$  vs.  $A\beta$ 42.

<https://doi.org/10.1371/journal.pone.0261696.g006>

Herein, we characterized PS48 action in the context of insulin signaling using neuroblastoma cell lines and then demonstrated its ability to partially or wholly normalize  $A\beta$ 42 oligomer induced insulin resistance and toxicity using an adenoviral expression system. First, a dose dependency of Akt activation by insulin in PCN and N2a cultures was established.

Intracellular expression of A $\beta$ 42 oligomers resulted in a reduction of sensitivity to insulin, such that higher insulin doses were required to overcome the resistance. At low, subthreshold doses of insulin (3 nM), PS48 pretreatment appeared to sensitize Akt activation. Next, PS48 (0.1 to 1  $\mu$ M) is shown to significantly counter the inhibitory effect of A $\beta$ i expression on submaximal insulin-induced Akt phosphorylation in live cells, similar to the effect of a higher insulin dose (100 nM) alone. The same findings were obtained in another insulin responsive cell line, C2 myotubes. Moreover, PS48 partially overcame insulin resistance in a non-amyloid model of cellular toxicity, to the saturated fatty acid palmitate. A downstream effector and substrate of Akt, CREB, is also hypophosphorylated after A $\beta$  expression, and accordingly corrected by treatment with PS48. On the other hand, another effector, phospho-mTOR, remained unaffected by either treatment. The lead compound furthermore partially prevented A $\beta$ -induced cell death in a neuroblastoma cell line, as did high dose insulin and pioglitazone treatments. Next, it significantly normalized the effect of synthetic A $\beta$  peptide (ADDL oligomers) to inhibit LTP in rat prefrontal cortical slices. To test the purported cellular step involved in this mechanism of insulin resistance, we performed *in vitro* reactions using recombinant Akt and constitutive active PDK kinases to phosphorylate a GSK3 $\alpha/\beta$ -based consensus peptide substrate. A $\beta$  oligomers (ADDLs) inhibit Akt activation and crosstide phosphorylation and PS48 (10 nM to 1  $\mu$ M) restored this to ~90% of control levels. Mechanistically, tagged A $\beta$ 42 is shown to bind to both recombinant Akt and PDK using an in-solution fluorescence polarization assay, the two kinases having different affinities and saturation levels. Finally, *in vitro* and cell-based assay platforms were employed in a focused medicinal chemistry effort to probe the structure-activity characteristics of the parent molecule. Other analogs were found that reversed the inhibited Akt activity by better than 90%.

The two main physiological readouts of A $\beta$ i toxicity in this study that were partially protected by PS48 were cell death and inhibited synaptic plasticity (LTP). We show that PS48 and analogs in development significantly improved Akt activation by insulin from inhibition by A $\beta$ i accumulation. Akt is critical to both neuronal survival [126] and LTP as demonstrated in prefrontal cortex, amygdala and hippocampus, [127–129]. Hippocampal LTP is particularly sensitive to A $\beta$  oligomers [48, 49]. Among the possible effectors of these two outcomes, we find the CREB link plausible because this result mirrored the Akt responses to A $\beta$ i and PS48 (Fig 1). CREB is activated by several canonical receptor-activated kinase pathways (i.e. PKA, CaMK, MAPK). In particular, BDNF/TrkB receptor activation has been well studied [130–132]. However, insulin and IGF-1 also phosphorylate CREB via PI3K/Akt [43], and this can occur directly (see also [133, 134]). Another effector intervening between PI3K/PDK/Akt and CREB is GSK3 $\alpha/\beta$ . Akt stimulation results in GSK3 inactivation, resulting in CREB de-repression (via inhibitory pS129: [135, 136]). Moreover, GSK3 activation promotes apoptosis [137, 138] and depresses spatial learning and LTP in mice [139, 140]. Although our results were inconclusive on cellular GSK, the Akt activity assay data using GSKtide could still be consistent with a partial role for this mechanism in our endpoints.

Several limitations of this work will require clarification in future studies. Practical ones include finding the optimum concentrations of drug to test, which depends on the assay employed. Taking for instance *in vitro* drug screening, the optimum may actually lie between 100 nM and the 10  $\mu$ M concentrations reported here (Figs 4C and 6). The same applies to cell viability assays (Fig 2). Another issue that arose during the *in vitro* reactions was the basal phosphorylation status of commercial, recombinant Akt. It was generally high and only partially decreased by PP2A treatment. However, we believe this does not affect our results because drug efficacy was based on: 1. maximal stimulation with PDK (recomb Akt was always further phosphorylated after PDK (constitutively active) addition, regardless of its basal phospho state), 2. the level to which A $\beta$  inhibited this, and 3. the recovery from that by drug.

Moreover, regardless the phospho level of unstimulated recombinant Akt, it had no enzymatic activity (Figs 1A and 6B), which further supports its use for the same reasons. Nevertheless, experiments using more completely dephosphorylated Akt are a consideration to see if an even greater therapeutic effect size can be realized. Next, we found adding PS48 *after* A $\beta$  peptide slightly more effective in the same *in vitro* experiments. This interesting result raises the speculation that an A $\beta$ -induced conformational change in the Akt-PDK complex, makes the allosteric PS48 modification more effective thereafter. Preventative and treatment roles for PS48 therefore deserve further study. Finally, the AD model used here is based on cellular A $\beta$ 42 and is only one of many others. While our data are limited to this view of pathogenesis, the electrophysiology data on *ex vivo* slices exposed to soluble oligomeric A $\beta$  species recommend that PS48 could also be tested in models emphasizing extracellular A $\beta$ , or for that matter Tau, accumulations.

The results of published studies have been mixed with respect to the state of Akt activation in AD brain and models, reporting either over or under phosphorylation or activity. Far fewer reports specifically address the 3-phosphoinositide-dependent kinase, PDK-1 in AD or neurodegeneration. Pietri and colleagues [62, 141] found PDK-1 activity increased in neurons infected with prion protein PrP<sup>Sc</sup> or in transgenic mice affected by  $\beta$ -amyloid pathology, as well as in AD brain. In a novel but complicated mechanism, PDK-1 overactivation is held responsible for loss of TACE-mediated APP and PrPc  $\alpha$ -secretase cleavages, from accelerated TACE internalization. The result is an over-production of A $\beta$  and TNF-mediated neurotoxicity and memory deficit. Accordingly, PDK-1 silencing or inhibition restored survival and memory and reversed pathology parameters. Both PrP<sup>Sc</sup> and A $\beta$  were hypothesized to stimulate PrPc to recruit Src and PI3K kinases to overactivate PDK-1. The relevance of these changes to the insulin/Akt axis was however not explored. In contradistinction, a PrP<sup>Sc</sup>-like peptide (106–126) inactivated Akt and caused death in SHSY5Y and primary granule cells, outcomes confirmed in a PrP<sup>Sc</sup> infected mice model. These were reversed by constitutive activation of Akt or insulin treatment [142].

It remains plausible but clinically untested if a strategy to restore Akt responsiveness to insulin, has value in prevention or treatment of AD. Based on our data targeting the PDK-Akt activation sequence, PS48 or a structurally similar allosteric analog may be a viable candidate. Importantly, PS48 does not itself over-stimulate normal insulin signaling in PCNs, nor over-activate basal Akt, lessening potential oncogenesis concerns [143, 144]. Notably, PS48 was not toxic to cells (LD<sub>50</sub> = 250  $\mu$ M). This is possibly due to the purported allosteric modulatory action of this compound, which has also been observed in various other drugs of this class [145, 146]. Other bi-aryl, halogenated carboxylic acids have a safe record in humans, for instance, Tolfenamic acid, used for the treatment of migraines [147, 148].

In support of efforts to facilitate Akt/PDK signaling, other interventions have had similar action on the insulin /Akt transduction pathway to mitigate A $\beta$  toxicity. For instance,  $\alpha$ 7nAChR stimulation (nicotine on PCNs) activates PI3K and pAkt to block A $\beta$ -enhancement of mitochondrial AIF release/nuclear translocation [149], as well as block A $\beta$ -mediated glutamate toxicity and prevent mitochondrial apoptosis [150–152]. We note with interest several recent reports that direct pharmacological activation of Akt in A $\beta$ -injected and in 5X FAD AD mice, resolved memory impairments and synaptic LTP deficits and restored inhibited Akt to control levels [153]. Activation of Akt/PI3K in primary mouse neurons also proved protective against transfected mutant APP and improved locomotor activity in an A $\beta$ 42-drosophila model [154]. The aforementioned insulin pathway clinical trials were all supported by robust preclinical cell and animal data, e.g. GLP-1 mimetics [155–158], IN insulin [159–162], PPAR agonists [14], as well as by epidemiological data, e.g. metformin [163, 164]. Recent reviews of these drug

classes in AD prevention endorse continued clinical trials, where supported by basic studies [165–167].

Our future studies will focus on an expanded class of modified biphenyl pentanoic acids and optimization of the PS48 pharmacophore with the goal to push potency into nM range and improve cell permeability. PS48 also has a low toxicity profile in preliminary animal testing, and further clinical data will be reported separately.

## Supporting information

### S1 File.

(PDF)

### S1 Table.

(PDF)

### S1 Raw images.

(PDF)

## Acknowledgments

We thank Drs. Shaomin Li and Dennis Selkoe for early establishment of feasibility to measure field EPSP in rodent prefrontal cortex and test for amyloid peptide effects on LTP.

## Author Contributions

**Conceptualization:** Henry Querfurth.

**Data curation:** Henry Querfurth, John Marshall, Keykavous Parang, Mengia S. Rioult-Pedotti, Bumsup Kwon, Steve Reisinger, Han-Kyu Lee.

**Formal analysis:** Rakesh Tiwari, Bumsup Kwon, Han-Kyu Lee.

**Funding acquisition:** Henry Querfurth.

**Investigation:** Han-Kyu Lee.

**Methodology:** Rakesh Tiwari, Han-Kyu Lee.

**Resources:** John Marshall, Keykavous Parang, Steve Reisinger.

**Supervision:** Henry Querfurth.

**Validation:** Han-Kyu Lee.

**Visualization:** Han-Kyu Lee.

**Writing – original draft:** Henry Querfurth.

**Writing – review & editing:** Henry Querfurth, Keykavous Parang, Steve Reisinger, Han-Kyu Lee.

## References

1. Doody RS, Raman R, Farlow M, Iwatsubo T, Vellas B, Joffe S, et al. A phase 3 trial of semagacestat for treatment of Alzheimer's disease. *N Engl J Med*. 2013; 369(4):341–50. Epub 2013/07/26. <https://doi.org/10.1056/NEJMoa1210951> PMID: 23883379.
2. Kennedy ME, Stamford AW, Chen X, Cox K, Cumming JN, Dockendorf MF, et al. The BACE1 inhibitor verubecestat (MK-8931) reduces CNS beta-amyloid in animal models and in Alzheimer's disease patients. *Sci Transl Med*. 2016; 8(363):363ra150. Epub 2016/11/04. <https://doi.org/10.1126/scitranslmed.aad9704> PMID: 27807285.

3. Honig LS, Vellas B, Woodward M, Boada M, Bullock R, Borrie M, et al. Trial of Solanezumab for Mild Dementia Due to Alzheimer's Disease. *N Engl J Med*. 2018; 378(4):321–30. Epub 2018/01/25. <https://doi.org/10.1056/NEJMoa1705971> PMID: 29365294.
4. Ostrowitzki S, Lasser RA, Dorflinger E, Scheltens P, Barkhof F, Nikolcheva T, et al. A phase III randomized trial of gantenerumab in prodromal Alzheimer's disease. *Alzheimers Res Ther*. 2017; 9(1):95. Epub 2017/12/10. <https://doi.org/10.1186/s13195-017-0318-y> PMID: 29221491; PubMed Central PMCID: PMC5723032.
5. Relkin NR, Thomas RG, Rissman RA, Brewer JB, Rafii MS, van Dyck CH, et al. A phase 3 trial of IV immunoglobulin for Alzheimer disease. *Neurology*. 2017; 88(18):1768–75. Epub 2017/04/07. <https://doi.org/10.1212/WNL.0000000000003904> PMID: 28381506; PubMed Central PMCID: PMC5409846.
6. Salloway S, Sperling R, Fox NC, Blennow K, Klunk W, Raskind M, et al. Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease. *N Engl J Med*. 2014; 370(4):322–33. Epub 2014/01/24. <https://doi.org/10.1056/NEJMoa1304839> PMID: 24450891; PubMed Central PMCID: PMC4159618.
7. Mintun MA, Lo AC, Duggan Evans C, Wessels AM, Ardayfio PA, Andersen SW, et al. Donanemab in Early Alzheimer's Disease. *N Engl J Med*. 2021; 384(18):1691–704. Epub 2021/03/16. <https://doi.org/10.1056/NEJMoa2100708> PMID: 33720637.
8. Tolar M, Abushakra S, Hey JA, Porsteinsson A, Sabbagh M. Aducanumab, gantenerumab, BAN2401, and ALZ-801—the first wave of amyloid-targeting drugs for Alzheimer's disease with potential for near term approval. *Alzheimers Res Ther*. 2020; 12(1):95. Epub 2020/08/14. <https://doi.org/10.1186/s13195-020-00663-w> PMID: 32787971; PubMed Central PMCID: PMC7424995.
9. Wisniewski HM, Wegiel J. The neuropathology of Alzheimer's disease. *Neuroimaging clinics of North America*. 1995; 5(1):45–57. Epub 1995/02/01. PMID: 7743084.
10. Yankner BA. Mechanisms of neuronal degeneration in Alzheimer's disease. *Neuron*. 1996; 16(5):921–32. Epub 1996/05/01. [https://doi.org/10.1016/s0896-6273\(00\)80115-4](https://doi.org/10.1016/s0896-6273(00)80115-4) PMID: 8630250.
11. Gouras GK, Tampellini D, Takahashi RH, Capetillo-Zarate E. Intraneuronal beta-amyloid accumulation and synapse pathology in Alzheimer's disease. *Acta neuropathologica*. 2010; 119(5):523–41. Epub 2010/04/01. <https://doi.org/10.1007/s00401-010-0679-9> PMID: 20354705; PubMed Central PMCID: PMC3183823.
12. Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM. Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology*. 1999; 53(9):1937–42. Epub 1999/12/22. <https://doi.org/10.1212/wnl.53.9.1937> PMID: 10599761.
13. de la Monte SM, Tong M, Cohen AC, Sheedy D, Harper C, Wands JR. Insulin and insulin-like growth factor resistance in alcoholic neurodegeneration. *Alcoholism, clinical and experimental research*. 2008; 32(9):1630–44. Epub 2008/07/12. <https://doi.org/10.1111/j.1530-0277.2008.00731.x> PMID: 18616667.
14. de la Monte SM, Tong M, Lester-Coll N, Plater M Jr., Wands JR. Therapeutic rescue of neurodegeneration in experimental type 3 diabetes: relevance to Alzheimer's disease. *J Alzheimers Dis*. 2006; 10(1):89–109. Epub 2006/09/22. <https://doi.org/10.3233/jad-2006-10113> PMID: 16988486.
15. Arnold SE, Lucki I, Brookshire BR, Carlson GC, Browne CA, Kazi H, et al. High fat diet produces brain insulin resistance, synaptodendritic abnormalities and altered behavior in mice. *Neurobiol Dis*. 2014; 67:79–87. Epub 2014/04/02. <https://doi.org/10.1016/j.nbd.2014.03.011> PMID: 24686304; PubMed Central PMCID: PMC4083060.
16. Ho L, Qin W, Pompl PN, Xiang Z, Wang J, Zhao Z, et al. Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. *FASEB J*. 2004; 18(7):902–4. Epub 2004/03/23. <https://doi.org/10.1096/fj.03-0978fje> PMID: 15033922.
17. Zhao L, Teter B, Morihara T, Lim GP, Ambegaokar SS, Ubeda OJ, et al. Insulin-degrading enzyme as a downstream target of insulin receptor signaling cascade: implications for Alzheimer's disease intervention. *J Neurosci*. 2004; 24(49):11120–6. Epub 2004/12/14. <https://doi.org/10.1523/JNEUROSCI.2860-04.2004> PMID: 15590928; PubMed Central PMCID: PMC6730264.
18. Kimura N. Diabetes Mellitus Induces Alzheimer's Disease Pathology: Histopathological Evidence from Animal Models. *Int J Mol Sci*. 2016; 17(4):503. Epub 2016/04/09. <https://doi.org/10.3390/ijms17040503> PMID: 27058526; PubMed Central PMCID: PMC4848959.
19. Yarchoan M, Toledo JB, Lee EB, Arvanitakis Z, Kazi H, Han LY, et al. Abnormal serine phosphorylation of insulin receptor substrate 1 is associated with tau pathology in Alzheimer's disease and tauopathies. *Acta Neuropathol*. 2014; 128(5):679–89. Epub 2014/08/12. <https://doi.org/10.1007/s00401-014-1328-5> PMID: 25107476; PubMed Central PMCID: PMC4304658.
20. Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S. Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. *J*

- Neurochem. 2007; 101(3):757–70. Epub 2007/04/24. <https://doi.org/10.1111/j.1471-4159.2006.04368.x> PMID: 17448147.
21. Lester-Coll N, Rivera EJ, Soscia SJ, Doiron K, Wands JR, de la Monte SM. Intracerebral streptozotocin model of type 3 diabetes: relevance to sporadic Alzheimer's disease. *J Alzheimers Dis.* 2006; 9(1):13–33. Epub 2006/04/22. <https://doi.org/10.3233/jad-2006-9102> PMID: 16627931.
  22. Schubert M, Brazil DP, Burks DJ, Kushner JA, Ye J, Flint CL, et al. Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation. *J Neurosci.* 2003; 23(18):7084–92. Epub 2003/08/09. <https://doi.org/10.1523/JNEUROSCI.23-18-07084.2003> PMID: 12904469; PubMed Central PMCID: PMC6740672.
  23. Arnold SE, Arvanitakis Z, Macauley-Rambach SL, Koenig AM, Wang HY, Ahima RS, et al. Brain insulin resistance in type 2 diabetes and Alzheimer disease: concepts and conundrums. *Nat Rev Neurol.* 2018; 14(3):168–81. Epub 2018/01/30. <https://doi.org/10.1038/nrneuro.2017.185> PMID: 29377010; PubMed Central PMCID: PMC6098968.
  24. Ferreira LSS, Fernandes CS, Vieira MNN, De Felice FG. Insulin Resistance in Alzheimer's Disease. *Front Neurosci.* 2018; 12:830. Epub 2018/12/14. <https://doi.org/10.3389/fnins.2018.00830> PMID: 30542257; PubMed Central PMCID: PMC6277874.
  25. Craft S, Peskind E, Schwartz MW, Schellenberg GD, Raskind M, Porte D Jr. Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: relationship to severity of dementia and apolipoprotein E genotype. *Neurology.* 1998; 50(1):164–8. Epub 1998/01/27. <https://doi.org/10.1212/wnl.50.1.164> PMID: 9443474
  26. Messier C, Teutenberg K. The role of insulin, insulin growth factor, and insulin-degrading enzyme in brain aging and Alzheimer's disease. *Neural Plast.* 2005; 12(4):311–28. <https://doi.org/10.1155/NP.2005.311> PMID: 16444902.
  27. O'Neill C, Kiely AP, Coakley MF, Manning S, Long-Smith CM. Insulin and IGF-1 signalling: longevity, protein homeostasis and Alzheimer's disease. *Biochemical Society transactions.* 2012; 40(4):721–7. Epub 2012/07/24. <https://doi.org/10.1042/BST20120080> PMID: 22817723.
  28. Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, et al. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease—is this type 3 diabetes? *J Alzheimers Dis.* 2005; 7(1):63–80. Epub 2005/03/08. <https://doi.org/10.3233/jad-2005-7107> PMID: 15750215.
  29. Zhao WQ, Townsend M. Insulin resistance and amyloidogenesis as common molecular foundation for type 2 diabetes and Alzheimer's disease. *Biochimica et biophysica acta.* 2009; 1792(5):482–96. Epub 2008/11/26. <https://doi.org/10.1016/j.bbadis.2008.10.014> PMID: 19026743.
  30. van der Heide LP, Kamal A, Artola A, Gispen WH, Ramakers GM. Insulin modulates hippocampal activity-dependent synaptic plasticity in a N-methyl-d-aspartate receptor and phosphatidylinositol-3-kinase-dependent manner. *J Neurochem.* 2005; 94(4):1158–66. Epub 2005/08/12. <https://doi.org/10.1111/j.1471-4159.2005.03269.x> PMID: 16092951.
  31. Benedict C, Hallschmid M, Schmitz K, Schultes B, Ratter F, Fehm HL, et al. Intranasal insulin improves memory in humans: superiority of insulin aspart. *Neuropsychopharmacology.* 2007; 32(1):239–43. Epub 2006/08/29. <https://doi.org/10.1038/sj.npp.1301193> PMID: 16936707.
  32. Causes Hoyer S. and consequences of disturbances of cerebral glucose metabolism in sporadic Alzheimer disease: therapeutic implications. *Adv Exp Med Biol.* 2004; 541:135–52. Epub 2004/02/24. [https://doi.org/10.1007/978-1-4419-8969-7\\_8](https://doi.org/10.1007/978-1-4419-8969-7_8) PMID: 14977212.
  33. Lee CC, Huang CC, Wu MY, Hsu KS. Insulin stimulates postsynaptic density-95 protein translation via the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway. *J Biol Chem.* 2005; 280(18):18543–50. <https://doi.org/10.1074/jbc.M414112200> PMID: 15755733.
  34. Bondy CA, Cheng CM. Signaling by insulin-like growth factor 1 in brain. *European journal of pharmacology.* 2004; 490(1–3):25–31. <https://doi.org/10.1016/j.ejphar.2004.02.042> PMID: 15094071.
  35. Brunet A, Datta SR, Greenberg ME. Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol.* 2001; 11(3):297–305. [https://doi.org/10.1016/s0959-4388\(00\)00211-7](https://doi.org/10.1016/s0959-4388(00)00211-7) PMID: 11399427.
  36. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell.* 1999; 96(6):857–68. Epub 1999/04/02. [https://doi.org/10.1016/s0092-8674\(00\)80595-4](https://doi.org/10.1016/s0092-8674(00)80595-4) PMID: 10102273.
  37. Zheng WH, Kar S, Quirion R. Insulin-like growth factor-1-induced phosphorylation of the forkhead family transcription factor FKHL1 is mediated by Akt kinase in PC12 cells. *J Biol Chem.* 2000; 275(50):39152–8. Epub 2000/09/21. <https://doi.org/10.1074/jbc.M002417200> PMID: 10995739.
  38. Ugi S, Imamura T, Maegawa H, Egawa K, Yoshizaki T, Shi K, et al. Protein phosphatase 2A negatively regulates insulin's metabolic signaling pathway by inhibiting Akt (protein kinase B) activity in 3T3-L1

- adipocytes. *Molecular and cellular biology*. 2004; 24(19):8778–89. Epub 2004/09/16. <https://doi.org/10.1128/MCB.24.19.8778-8789.2004> PMID: 15367694; PubMed Central PMCID: PMC516764.
39. Gao T, Furnari F, Newton AC. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol Cell*. 2005; 18(1):13–24. Epub 2005/04/06. <https://doi.org/10.1016/j.molcel.2005.03.008> PMID: 15808505.
  40. Peltier J O'Neill A, Schaffer DV. PI3K/Akt and CREB regulate adult neural hippocampal progenitor proliferation and differentiation. *Dev Neurobiol*. 2007; 67(10):1348–61. Epub 2007/07/20. <https://doi.org/10.1002/dneu.20506> PMID: 17638387.
  41. Yuan H, Chen R, Wu L, Chen Q, Hu A, Zhang T, et al. The regulatory mechanism of neurogenesis by IGF-1 in adult mice. *Mol Neurobiol*. 2015; 51(2):512–22. Epub 2014/04/30. <https://doi.org/10.1007/s12035-014-8717-6> PMID: 24777577.
  42. Benito E, Barco A. CREB's control of intrinsic and synaptic plasticity: implications for CREB-dependent memory models. *Trends Neurosci*. 2010; 33(5):230–40. Epub 2010/03/13. <https://doi.org/10.1016/j.tins.2010.02.001> PMID: 20223527.
  43. Leininger GM, Backus C, Uhler MD, Lentz SI, Feldman EL. Phosphatidylinositol 3-kinase and Akt effectors mediate insulin-like growth factor-I neuroprotection in dorsal root ganglia neurons. *FASEB J*. 2004; 18(13):1544–6. Epub 2004/08/21. <https://doi.org/10.1096/fj.04-1581fje> PMID: 15319368.
  44. Farris W, Mansourian S, Chang Y, Lindsley L, Eckman EA, Frosch MP, et al. Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. *Proc Natl Acad Sci U S A*. 2003; 100(7):4162–7. <https://doi.org/10.1073/pnas.0230450100> PMID: 12634421.
  45. Grillo CA, Piroli GG, Hendry RM, Reagan LP. Insulin-stimulated translocation of GLUT4 to the plasma membrane in rat hippocampus is PI3-kinase dependent. *Brain Res*. 2009; 1296:35–45. Epub 2009/08/15. <https://doi.org/10.1016/j.brainres.2009.08.005> PMID: 19679110; PubMed Central PMCID: PMC2997526.
  46. Uemura E, Greenlee HW. Insulin regulates neuronal glucose uptake by promoting translocation of glucose transporter GLUT3. *Exp Neurol*. 2006; 198(1):48–53. Epub 2005/12/13. <https://doi.org/10.1016/j.expneurol.2005.10.035> PMID: 16337941.
  47. De Felice FG, Vieira MN, Bomfim TR, Decker H, Velasco PT, Lambert MP, et al. Protection of synapses against Alzheimer's-linked toxins: insulin signaling prevents the pathogenic binding of Abeta oligomers. *Proc Natl Acad Sci U S A*. 2009; 106(6):1971–6. Epub 2009/02/04. <https://doi.org/10.1073/pnas.0809158106> PMID: 19188609; PubMed Central PMCID: PMC2634809.
  48. Townsend M, Mehta T, Selkoe DJ. Soluble Abeta inhibits specific signal transduction cascades common to the insulin receptor pathway. *J Biol Chem*. 2007; 282(46):33305–12. Epub 2007/09/15. <https://doi.org/10.1074/jbc.M610390200> PMID: 17855343.
  49. Jo J, Whitcomb DJ, Olsen KM, Kerrigan TL, Lo SC, Bru-Mercier G, et al. Abeta(1–42) inhibition of LTP is mediated by a signaling pathway involving caspase-3, Akt1 and GSK-3beta. *Nat Neurosci*. 2011; 14(5):545–7. Epub 2011/03/29. <https://doi.org/10.1038/nn.2785> PMID: 21441921.
  50. Xie L, Helmerhorst E, Taddei K, Plewright B, Van Bronswijk W, Martins R. Alzheimer's beta-amyloid peptides compete for insulin binding to the insulin receptor. *J Neurosci*. 2002; 22(10):RC221. Epub 2002/05/15. <https://doi.org/10.1523/JNEUROSCI.2002-02.2002> PMID: 12006603; PubMed Central PMCID: PMC6757630.
  51. Zhao WQ, De Felice FG, Fernandez S, Chen H, Lambert MP, Quon MJ, et al. Amyloid beta oligomers induce impairment of neuronal insulin receptors. *FASEB J*. 2008; 22(1):246–60. Epub 2007/08/28. <https://doi.org/10.1096/fj.06-7703com> PMID: 17720802.
  52. Talbot K, Wang HY, Kazi H, Han LY, Bakshi KP, Stucky A, et al. Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *The Journal of clinical investigation*. 2012; 122(4):1316–38. Epub 2012/04/06. <https://doi.org/10.1172/JCI59903> PMID: 22476197; PubMed Central PMCID: PMC3314463.
  53. Caccamo A, Maldonado MA, Majumder S, Medina DX, Holbein W, Magri A, et al. Naturally secreted amyloid-beta increases mammalian target of rapamycin (mTOR) activity via a PRAS40-mediated mechanism. *The Journal of biological chemistry*. 2011; 286(11):8924–32. Epub 2011/01/27. <https://doi.org/10.1074/jbc.M110.180638> PMID: 21266573; PubMed Central PMCID: PMC3058958.
  54. Caccamo A, Majumder S, Richardson A, Strong R, Oddo S. Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-beta, and Tau: effects on cognitive impairments. *The Journal of biological chemistry*. 2010; 285(17):13107–20. Epub 2010/02/25. <https://doi.org/10.1074/jbc.M110.100420> PMID: 20178983; PubMed Central PMCID: PMC2857107.
  55. Majumder S, Caccamo A, Medina DX, Benavides AD, Javors MA, Kraig E, et al. Lifelong rapamycin administration ameliorates age-dependent cognitive deficits by reducing IL-1beta and enhancing NMDA signaling. *Aging cell*. 2012; 11(2):326–35. Epub 2012/01/04. <https://doi.org/10.1111/j.1474-9726.2011.00791.x> PMID: 22212527; PubMed Central PMCID: PMC3306461.



56. Kapogiannis D, Boxer A, Schwartz JB, Abner EL, Biragyn A, Masharani U, et al. Dysfunctionally phosphorylated type 1 insulin receptor substrate in neural-derived blood exosomes of preclinical Alzheimer's disease. *FASEB J*. 2015; 29(2):589–96. Epub 2014/10/25. <https://doi.org/10.1096/fj.14-262048> PMID: 25342129; PubMed Central PMCID: PMC4314222.
57. Lourenco MV, Clarke JR, Frozza RL, Bomfim TR, Forny-Germano L, Batista AF, et al. TNF-alpha mediates PKR-dependent memory impairment and brain IRS-1 inhibition induced by Alzheimer's beta-amyloid oligomers in mice and monkeys. *Cell Metab*. 2013; 18(6):831–43. Epub 2013/12/10. <https://doi.org/10.1016/j.cmet.2013.11.002> PMID: 24315369.
58. Rui L, Aguirre V, Kim JK, Shulman GI, Lee A, Corbould A, et al. Insulin/IGF-1 and TNF-alpha stimulate phosphorylation of IRS-1 at inhibitory Ser307 via distinct pathways. *J Clin Invest*. 2001; 107(2):181–9. Epub 2001/02/13. <https://doi.org/10.1172/JCI10934> PMID: 11160134; PubMed Central PMCID: PMC199174.
59. Griffin RJ, Moloney A, Kelliher M, Johnston JA, Ravid R, Dockery P, et al. Activation of Akt/PKB, increased phosphorylation of Akt substrates and loss and altered distribution of Akt and PTEN are features of Alzheimer's disease pathology. *J Neurochem*. 2005; 93(1):105–17. <https://doi.org/10.1111/j.1471-4159.2004.02949.x> PMID: 15773910.
60. Moloney AM, Griffin RJ, Timmons S, O'Connor R, Ravid R, O'Neill C. Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling. *Neurobiol Aging*. 2010; 31(2):224–43. Epub 2008/05/16. <https://doi.org/10.1016/j.neurobiolaging.2008.04.002> PMID: 18479783.
61. Pei JJ, Khatoun S, An WL, Nordlinder M, Tanaka T, Braak H, et al. Role of protein kinase B in Alzheimer's neurofibrillary pathology. *Acta Neuropathol (Berl)*. 2003; 105(4):381–92. <https://doi.org/10.1007/s00401-002-0657-y> PMID: 12624792.
62. Pietri M, Dakowski C, Hannaoui S, Alleaume-Butaux A, Hernandez-Rapp J, Ragagnin A, et al. PDK1 decreases TACE-mediated alpha-secretase activity and promotes disease progression in prion and Alzheimer's diseases. *Nat Med*. 2013; 19(9):1124–31. Epub 2013/08/21. <https://doi.org/10.1038/nm.3302> PMID: 23955714.
63. Rickle A, Bogdanovic N, Volkman I, Winblad B, Ravid R, Cowburn RF. Akt activity in Alzheimer's disease and other neurodegenerative disorders. *Neuroreport*. 2004; 15(6):955–9. Epub 2004/04/13. <https://doi.org/10.1097/00001756-200404290-00005> PMID: 15076714.
64. Lee HK, Kumar P, Fu Q, Rosen KM, Querfurth HW. The insulin/Akt signaling pathway is targeted by intracellular beta-amyloid. *Mol Biol Cell*. 2009; 20(5):1533–44. Epub 2009/01/16. <https://doi.org/10.1091/mbc.e08-07-0777> PMID: 19144826; PubMed Central PMCID: PMC2649265.
65. Liu Y, Liu F, Grundke-Iqbal I, Iqbal K, Gong CX. Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes. *The Journal of pathology*. 2011; 225(1):54–62. <https://doi.org/10.1002/path.2912> PMID: 21598254.
66. Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA. Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nature genetics*. 2004; 36(2):131–7. Epub 2004/01/28. <https://doi.org/10.1038/ng1296> PMID: 14745448.
67. Colin E, Regulier E, Perrin V, Durr A, Brice A, Aebischer P, et al. Akt is altered in an animal model of Huntington's disease and in patients. *Eur J Neurosci*. 2005; 21(6):1478–88. Epub 2005/04/23. <https://doi.org/10.1111/j.1460-9568.2005.03985.x> PMID: 15845076.
68. Perluigi M, Pupo G, Tramutola A, Cini C, Coccia R, Barone E, et al. Neuropathological role of PI3K/Akt/mTOR axis in Down syndrome brain. *Biochimica et biophysica acta*. 2014; 1842(7):1144–53. Epub 2014/04/17. <https://doi.org/10.1016/j.bbadis.2014.04.007> PMID: 24735980; PubMed Central PMCID: PMC4062876.
69. Tramutola A, Triplett JC, Di Domenico F, Niedowicz DM, Murphy MP, Coccia R, et al. Alteration of mTOR signaling occurs early in the progression of Alzheimer disease (AD): analysis of brain from subjects with pre-clinical AD, amnesic mild cognitive impairment and late-stage AD. *Journal of neurochemistry*. 2015; 133(5):739–49. Epub 2015/02/04. <https://doi.org/10.1111/jnc.13037> PMID: 25645581.
70. Chiang ACA, Fowler SW, Savjani RR, Hilsenbeck SG, Wallace CE, Cirrito JR, et al. Combination anti-Abeta treatment maximizes cognitive recovery and rebalances mTOR signaling in APP mice. *J Exp Med*. 2018; 215(5):1349–64. Epub 2018/04/08. <https://doi.org/10.1084/jem.20171484> PMID: 29626114; PubMed Central PMCID: PMC5940263.
71. Bhaskar K, Miller M, Chludzinski A, Herrup K, Zagorski M, Lamb BT. The PI3K-Akt-mTOR pathway regulates Abeta oligomer induced neuronal cell cycle events. *Molecular neurodegeneration*. 2009; 4:14. Epub 2009/03/18. <https://doi.org/10.1186/1750-1326-4-14> PMID: 19291319; PubMed Central PMCID: PMC2663563.

72. Ma T, Hoeffler CA, Capetillo-Zarate E, Yu F, Wong H, Lin MT, et al. Dysregulation of the mTOR pathway mediates impairment of synaptic plasticity in a mouse model of Alzheimer's disease. *PloS One*. 2010; 5(9):e12845. Epub 2010/09/24. <https://doi.org/10.1371/journal.pone.0012845> PMID: 20862226; PubMed Central PMCID: PMC2942840.
73. Guttuso T Jr., Andrzejewski KL, Lichter DG, Andersen JK. Targeting kinases in Parkinson's disease: A mechanism shared by LRRK2, neurotrophins, exenatide, urate, nilotinib and lithium. *J Neurol Sci*. 2019; 402:121–30. Epub 2019/05/28. <https://doi.org/10.1016/j.jns.2019.05.016> PMID: 31129265.
74. Humbert S, Bryson EA, Cordelieres FP, Connors NC, Datta SR, Finkbeiner S, et al. The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves Huntingtin phosphorylation by Akt. *Dev Cell*. 2002; 2(6):831–7. Epub 2002/06/14. [https://doi.org/10.1016/s1534-5807\(02\)00188-0](https://doi.org/10.1016/s1534-5807(02)00188-0) PMID: 12062094.
75. Sekar S, Taghibiglou C. Elevated nuclear phosphatase and tensin homolog (PTEN) and altered insulin signaling in substantia nigral region of patients with Parkinson's disease. *Neurosci Lett*. 2018; 666:139–43. Epub 2017/12/31. <https://doi.org/10.1016/j.neulet.2017.12.049> PMID: 29288045.
76. Knafo S, Sanchez-Puelles C, Palomer E, Delgado I, Draffin JE, Mingo J, et al. PTEN recruitment controls synaptic and cognitive function in Alzheimer's models. *Nat Neurosci*. 2016; 19(3):443–53. Epub 2016/01/19. nn.4225 [pii] <https://doi.org/10.1038/nn.4225> PMID: 26780512.
77. Ahmad F, Singh K, Das D, Gowaikar R, Shaw E, Ramachandran A, et al. Reactive Oxygen Species-Mediated Loss of Synaptic Akt1 Signaling Leads to Deficient Activity-Dependent Protein Translation Early in Alzheimer's Disease. *Antioxid Redox Signal*. 2017; 27(16):1269–80. Epub 2017/03/08. <https://doi.org/10.1089/ars.2016.6860> PMID: 28264587; PubMed Central PMCID: PMC5655421.
78. Chen TJ, Wang DC, Chen SS. Amyloid-beta interrupts the PI3K-Akt-mTOR signaling pathway that could be involved in brain-derived neurotrophic factor-induced Arc expression in rat cortical neurons. *Journal of neuroscience research*. 2009; 87(10):2297–307. Epub 2009/03/21. <https://doi.org/10.1002/jnr.22057> PMID: 19301428.
79. Jimenez S, Torres M, Vizueté M, Sanchez-Varo R, Sanchez-Mejias E, Trujillo-Estrada L, et al. Age-dependent accumulation of soluble amyloid beta (A $\beta$ ) oligomers reverses the neuroprotective effect of soluble amyloid precursor protein-alpha (sAPP(alpha)) by modulating phosphatidylinositol 3-kinase (PI3K)/Akt-GSK-3beta pathway in Alzheimer mouse model. *The Journal of biological chemistry*. 2011; 286(21):18414–25. Epub 2011/04/05. <https://doi.org/10.1074/jbc.M110.209718> PMID: 21460223; PubMed Central PMCID: PMC3099658.
80. Magrane J, Rosen KM, Smith RC, Walsh K, Gouras GK, Querfurth HW. Intraneuronal beta-amyloid expression downregulates the Akt survival pathway and blunts the stress response. *J Neurosci*. 2005; 25(47):10960–9. <https://doi.org/10.1523/JNEUROSCI.1723-05.2005> PMID: 16306409.
81. Suhara T, Magrane J, Rosen K, Christensen R, Kim HS, Zheng B, et al. A $\beta$ 42 generation is toxic to endothelial cells and inhibits eNOS function through an Akt/GSK-3beta signaling-dependent mechanism. *Neurobiol Aging*. 2003; 24(3):437–51. Epub 2003/02/26. [https://doi.org/10.1016/s0197-4580\(02\)00135-5](https://doi.org/10.1016/s0197-4580(02)00135-5) PMID: 12600720.
82. Luchsinger JA, Perez T, Chang H, Mehta P, Steffener J, Pradabhan G, et al. Metformin in Amnesic Mild Cognitive Impairment: Results of a Pilot Randomized Placebo Controlled Clinical Trial. *J Alzheimers Dis*. 2016; 51(2):501–14. Epub 2016/02/19. <https://doi.org/10.3233/JAD-150493> PMID: 26890736; PubMed Central PMCID: PMC5079271.
83. Vargas-Soria M, Carranza-Naval MJ, Del Marco A, Garcia-Alloza M. Role of liraglutide in Alzheimer's disease pathology. *Alzheimers Res Ther*. 2021; 13(1):112. Epub 2021/06/14. <https://doi.org/10.1186/s13195-021-00853-0> PMID: 34118986; PubMed Central PMCID: PMC8199799.
84. Craft S, Baker LD, Montine TJ, Minoshima S, Watson GS, Claxton A, et al. Intranasal insulin therapy for Alzheimer disease and amnesic mild cognitive impairment: a pilot clinical trial. *Arch Neurol*. 2012; 69(1):29–38. Epub 2011/09/14. <https://doi.org/10.1001/archneurol.2011.233> PMID: 21911655; PubMed Central PMCID: PMC3260944.
85. Reger MA, Watson GS, Frey WH, 2nd, Baker LD, Cholerton B, Keeling ML, et al. Effects of intranasal insulin on cognition in memory-impaired older adults: modulation by APOE genotype. *Neurobiol Aging*. 2006; 27(3):451–8. Epub 2005/06/21. <https://doi.org/10.1016/j.neurobiolaging.2005.03.016> PMID: 15964100.
86. Kintscher U. Pharmacological differences of glitazones: does peroxisome proliferator-activated receptor-alpha activation make the difference? *J Am Coll Cardiol*. 2008; 52(10):882–4. Epub 2008/08/30. <https://doi.org/10.1016/j.jacc.2008.06.012> PMID: 18755354.
87. Miller BW, Willett KC, Desilets AR. Rosiglitazone and pioglitazone for the treatment of Alzheimer's disease. *Ann Pharmacother*. 2011; 45(11):1416–24. Epub 2011/10/27. <https://doi.org/10.1345/aph.1Q238> PMID: 22028424.

88. Risner ME, Saunders AM, Altman JF, Ormandy GC, Craft S, Foley IM, et al. Efficacy of rosiglitazone in a genetically defined population with mild-to-moderate Alzheimer's disease. *Pharmacogenomics J*. 2006; 6(4):246–54. Epub 2006/02/01. <https://doi.org/10.1038/sj.tpj.6500369> PMID: 16446752.
89. Sato T, Hanyu H, Hirao K, Kanetaka H, Sakurai H, Iwamoto T. Efficacy of PPAR-gamma agonist pioglitazone in mild Alzheimer disease. *Neurobiol Aging*. 2011; 32(9):1626–33. Epub 2009/11/20. <https://doi.org/10.1016/j.neurobiolaging.2009.10.009> PMID: 19923038.
90. Femminella GD, Frangou E, Love SB, Busza G, Holmes C, Ritchie C, et al. Evaluating the effects of the novel GLP-1 analogue liraglutide in Alzheimer's disease: study protocol for a randomised controlled trial (ELAD study). *Trials*. 2019; 20(1):191. Epub 2019/04/05. <https://doi.org/10.1186/s13063-019-3259-x> PMID: 30944040; PubMed Central PMCID: PMC6448216.
91. Craft S, Raman R, Chow TW, Rafii MS, Sun CK, Rissman RA, et al. Safety, Efficacy, and Feasibility of Intranasal Insulin for the Treatment of Mild Cognitive Impairment and Alzheimer Disease Dementia: A Randomized Clinical Trial. *JAMA Neurol*. 2020; 77(9):1099–109. Epub 2020/06/23. <https://doi.org/10.1001/jamaneurol.2020.1840> PMID: 32568367; PubMed Central PMCID: PMC7309571.
92. Gold M, Alderton C, Zvartau-Hind M, Egginton S, Saunders AM, Irizarry M, et al. Rosiglitazone monotherapy in mild-to-moderate Alzheimer's disease: results from a randomized, double-blind, placebo-controlled phase III study. *Dement Geriatr Cogn Disord*. 2010; 30(2):131–46. Epub 2010/08/25. <https://doi.org/10.1159/000318845> PMID: 20733306; PubMed Central PMCID: PMC3214882.
93. Harrington C, Sawchak S, Chiang C, Davies J, Donovan C, Saunders AM, et al. Rosiglitazone does not improve cognition or global function when used as adjunctive therapy to AChE inhibitors in mild-to-moderate Alzheimer's disease: two phase 3 studies. *Curr Alzheimer Res*. 2011; 8(5):592–606. Epub 2011/05/20. <https://doi.org/10.2174/156720511796391935> PMID: 21592048.
94. Burns DK, Alexander RC, Welsh-Bohmer KA, Culp M, Chiang C, O'Neil J, et al. Safety and efficacy of pioglitazone for the delay of cognitive impairment in people at risk of Alzheimer's disease (TOMMORROW): a prognostic biomarker study and a phase 3, randomised, double-blind, placebo-controlled trial. *Lancet Neurol*. 2021; 20(7):537–47. Epub 2021/06/20. [https://doi.org/10.1016/S1474-4422\(21\)00043-0](https://doi.org/10.1016/S1474-4422(21)00043-0) PMID: 34146512.
95. Geldmacher DS, Fritsch T, McClendon MJ, Landreth G. A randomized pilot clinical trial of the safety of pioglitazone in treatment of patients with Alzheimer disease. *Arch Neurol*. 2011; 68(1):45–50. Epub 2010/09/15. <https://doi.org/10.1001/archneurol.2010.229> PMID: 20837824.
96. Hindie V, Stroba A, Zhang H, Lopez-Garcia LA, Idrissova L, Zeuzem S, et al. Structure and allosteric effects of low-molecular-weight activators on the protein kinase PDK1. *Nat Chem Biol*. 2009; 5(10):758–64. Epub 2009/09/01. <https://doi.org/10.1038/nchembio.208> PMID: 19718043.
97. Stroba A, Schaeffer F, Hindie V, Lopez-Garcia L, Adrian I, Frohner W, et al. 3,5-Diphenylpent-2-enoic acids as allosteric activators of the protein kinase PDK1: structure-activity relationships and thermodynamic characterization of binding as paradigms for PIF-binding pocket-targeting compounds. *J Med Chem*. 2009; 52(15):4683–93. Epub 2009/07/18. <https://doi.org/10.1021/jm9001499> PMID: 19606904.
98. Magrane J, Smith RC, Walsh K, Querfurth HW. Heat shock protein 70 participates in the neuroprotective response to intracellularly expressed beta-amyloid in neurons. *J Neurosci*. 2004; 24(7):1700–6. <https://doi.org/10.1523/JNEUROSCI.4330-03.2004> PMID: 14973234.
99. Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, et al. Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proc Natl Acad Sci U S A*. 1998; 95(11):6448–53. Epub 1998/05/30. <https://doi.org/10.1073/pnas.95.11.6448> PMID: 9600986; PubMed Central PMCID: PMC27787.
100. Klein WL. Abeta toxicity in Alzheimer's disease: globular oligomers (ADDLs) as new vaccine and drug targets. *Neurochem Int*. 2002; 41(5):345–52. [https://doi.org/10.1016/s0197-0186\(02\)00050-5](https://doi.org/10.1016/s0197-0186(02)00050-5) PMID: 12176077.
101. Marshall J, Szmydynger-Chodobska J, Rioult-Pedotti MS, Lau K, Chin AT, Kotla SKR, et al. TrkB-enhancer facilitates functional recovery after traumatic brain injury. *Sci Rep*. 2017; 7(1):10995. Epub 2017/09/10. <https://doi.org/10.1038/s41598-017-11316-8> PMID: 28887487; PubMed Central PMCID: PMC5591207.
102. Molina-Luna K, Pekanovic A, Rohrich S, Hertler B, Schubring-Giese M, Rioult-Pedotti MS, et al. Dopamine in motor cortex is necessary for skill learning and synaptic plasticity. *PLoS One*. 2009; 4(9):e7082. Epub 2009/09/18. <https://doi.org/10.1371/journal.pone.0007082> PMID: 19759902; PubMed Central PMCID: PMC2738964.
103. Lynch BA, Loiacono KA, Tiong CL, Adams SE, MacNeil IA. A fluorescence polarization based Src-SH2 binding assay. *Anal Biochem*. 1997; 247(1):77–82. Epub 1997/04/05. <https://doi.org/10.1006/abio.1997.2042> PMID: 9126374.

104. Tiwari R, Brown A, Narramaneni S, Sun G, Parang K. Synthesis and evaluation of conformationally constrained peptide analogues as the Src SH3 domain binding ligands. *Biochimie*. 2010; 92(9):1153–63. Epub 2010/01/30. <https://doi.org/10.1016/j.biochi.2010.01.017> PMID: 20109515.
105. Lee HK, Kwon B, Lemere CA, de la Monte S, Itamura K, Ha AY, et al. mTORC2 (Rictor) in Alzheimer's Disease and Reversal of Amyloid-beta Expression-Induced Insulin Resistance and Toxicity in Rat Primary Cortical Neurons. *J Alzheimers Dis*. 2017; 56(3):1015–36. Epub 2016/12/31. <https://doi.org/10.3233/JAD-161029> PMID: 28035937; PubMed Central PMCID: PMC5577393.
106. Engel M, Hindie V, Lopez-Garcia LA, Stroba A, Schaeffer F, Adrian I, et al. Allosteric activation of the protein kinase PDK1 with low molecular weight compounds. *EMBO J*. 2006; 25(23):5469–80. Epub 2006/11/18. <https://doi.org/10.1038/sj.emboj.7601416> PMID: 17110931; PubMed Central PMCID: PMC1679772.
107. Zhu S, Li W, Zhou H, Wei W, Ambasadhan R, Lin T, et al. Reprogramming of human primary somatic cells by OCT4 and chemical compounds. *Cell Stem Cell*. 2010; 7(6):651–5. Epub 2010/11/30. <https://doi.org/10.1016/j.stem.2010.11.015> PMID: 21112560; PubMed Central PMCID: PMC3812930.
108. Alberini CM. Transcription factors in long-term memory and synaptic plasticity. *Physiol Rev*. 2009; 89(1):121–45. Epub 2009/01/08. <https://doi.org/10.1152/physrev.00017.2008> PMID: 19126756; PubMed Central PMCID: PMC3883056.
109. Carlezon WA Jr., Duman RS, Nestler EJ. The many faces of CREB. *Trends Neurosci*. 2005; 28(8):436–45. Epub 2005/06/29. <https://doi.org/10.1016/j.tins.2005.06.005> PMID: 15982754.
110. Barco A, Marie H. Genetic approaches to investigate the role of CREB in neuronal plasticity and memory. *Mol Neurobiol*. 2011; 44(3):330–49. Epub 2011/09/29. <https://doi.org/10.1007/s12035-011-8209-x> PMID: 21948060.
111. Josselyn SA, Shi C, Carlezon WA Jr., Neve RL, Nestler EJ, Davis M. Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. *J Neurosci*. 2001; 21(7):2404–12. Epub 2001/03/27. <https://doi.org/10.1523/JNEUROSCI.21-07-02404.2001> PMID: 11264314; PubMed Central PMCID: PMC6762400.
112. Lisman J, Cooper K, Sehgal M, Silva AJ. Memory formation depends on both synapse-specific modifications of synaptic strength and cell-specific increases in excitability. *Nat Neurosci*. 2018; 21(3):309–14. Epub 2018/02/13. <https://doi.org/10.1038/s41593-018-0076-6> PMID: 29434376; PubMed Central PMCID: PMC5915620.
113. Restivo L, Tafi E, Ammassari-Teule M, Marie H. Viral-mediated expression of a constitutively active form of CREB in hippocampal neurons increases memory. *Hippocampus*. 2009; 19(3):228–34. Epub 2008/11/13. <https://doi.org/10.1002/hipo.20527> PMID: 19004015.
114. Sekeres MJ, Neve RL, Frankland PW, Josselyn SA. Dorsal hippocampal CREB is both necessary and sufficient for spatial memory. *Learn Mem*. 2010; 17(6):280–3. Epub 2010/05/25. <https://doi.org/10.1101/lm.1785510> PMID: 20495061.
115. Brami-Cherrier K, Valjent E, Garcia M, Pages C, Hipskind RA, Caboche J. Dopamine induces a PI3-kinase-independent activation of Akt in striatal neurons: a new route to cAMP response element-binding protein phosphorylation. *J Neurosci*. 2002; 22(20):8911–21. Epub 2002/10/22. <https://doi.org/10.1523/JNEUROSCI.22-20-08911.2002> PMID: 12388598; PubMed Central PMCID: PMC6757682.
116. Li XY, Zhan XR, Liu XM, Wang XC. CREB is a regulatory target for the protein kinase Akt/PKB in the differentiation of pancreatic ductal cells into islet beta-cells mediated by hepatocyte growth factor. *Biochem Biophys Res Commun*. 2011; 404(2):711–6. Epub 2010/12/16. <https://doi.org/10.1016/j.bbrc.2010.12.048> PMID: 21156158.
117. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science*. 1999; 286(5443):1358–62. Epub 1999/11/13. <https://doi.org/10.1126/science.286.5443.1358> PMID: 10558990.
118. Walton M, Woodgate AM, Muravlev A, Xu R, Doring MJ, Dragunow M. CREB phosphorylation promotes nerve cell survival. *J Neurochem*. 1999; 73(5):1836–42. Epub 1999/10/28. PMID: 10537041.
119. Barco A, Alarcon JM, Kandel ER. Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. *Cell*. 2002; 108(5):689–703. Epub 2002/03/15. [https://doi.org/10.1016/s0092-8674\(02\)00657-8](https://doi.org/10.1016/s0092-8674(02)00657-8) PMID: 11893339.
120. Yang J, Cron P, Good VM, Thompson V, Hemmings BA, Barford D. Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. *Nat Struct Biol*. 2002; 9(12):940–4. Epub 2002/11/16. <https://doi.org/10.1038/nsb870> PMID: 12434148.
121. Biondi RM, Komander D, Thomas CC, Lizcano JM, Deak M, Alessi DR, et al. High resolution crystal structure of the human PDK1 catalytic domain defines the regulatory phosphopeptide docking site. *EMBO J*. 2002; 21(16):4219–28. Epub 2002/08/10. <https://doi.org/10.1093/emboj/cdf437> PMID: 12169624; PubMed Central PMCID: PMC126174.

122. Biondi RM, Kieloch A, Currie RA, Deak M, Alessi DR. The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO J.* 2001; 20(16):4380–90. Epub 2001/08/14. <https://doi.org/10.1093/emboj/20.16.4380> PMID: 11500365; PubMed Central PMCID: PMC125563.
123. Collins BJ, Deak M, Arthur JS, Armit LJ, Alessi DR. In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *EMBO J.* 2003; 22(16):4202–11. Epub 2003/08/13. <https://doi.org/10.1093/emboj/cdg407> PMID: 12912918; PubMed Central PMCID: PMC175797.
124. Biondi RM, Cheung PC, Casamayor A, Deak M, Currie RA, Alessi DR. Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA. *EMBO J.* 2000; 19(5):979–88. Epub 2000/03/04. <https://doi.org/10.1093/emboj/19.5.979> PMID: 10698939; PubMed Central PMCID: PMC305637.
125. Xu X, Chen Y, Fu Q, Ni D, Zhang J, Li X, et al. The chemical diversity and structure-based discovery of allosteric modulators for the PIF-pocket of protein kinase PDK1. *J Enzyme Inhib Med Chem.* 2019; 34(1):361–74. Epub 2019/02/09. <https://doi.org/10.1080/14756366.2018.1553167> PMID: 30734603; PubMed Central PMCID: PMC6327997.
126. Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, et al. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science.* 1997; 275(5300):661–5. Epub 1997/01/31. <https://doi.org/10.1126/science.275.5300.661> PMID: 9005851
127. Lai WS, Xu B, Westphal KG, Paterlini M, Olivier B, Pavlidis P, et al. Akt1 deficiency affects neuronal morphology and predisposes to abnormalities in prefrontal cortex functioning. *Proc Natl Acad Sci U S A.* 2006; 103(45):16906–11. Epub 2006/11/02. <https://doi.org/10.1073/pnas.0604994103> PMID: 17077150; PubMed Central PMCID: PMC1636552.
128. Lin CH, Yeh SH, Lin CH, Lu KT, Leu TH, Chang WC, et al. A role for the PI-3 kinase signaling pathway in fear conditioning and synaptic plasticity in the amygdala. *Neuron.* 2001; 31(5):841–51. Epub 2001/09/25. [https://doi.org/10.1016/s0896-6273\(01\)00433-0](https://doi.org/10.1016/s0896-6273(01)00433-0) PMID: 11567621.
129. Pen Y, Borovok N, Reichenstein M, Sheinin A, Michaelevski I. Membrane-tethered AKT kinase regulates basal synaptic transmission and early phase LTP expression by modulation of post-synaptic AMPA receptor level. *Hippocampus.* 2016; 26(9):1149–67. Epub 2016/04/14. <https://doi.org/10.1002/hipo.22597> PMID: 27068236.
130. Alonso M, Vianna MR, Izquierdo I, Medina JH. Signaling mechanisms mediating BDNF modulation of memory formation in vivo in the hippocampus. *Cell Mol Neurobiol.* 2002; 22(5–6):663–74. Epub 2003/02/15. <https://doi.org/10.1023/a:1021848706159> PMID: 12585686.
131. Pizzorusso T, Ratto GM, Putignano E, Maffei L. Brain-derived neurotrophic factor causes cAMP response element-binding protein phosphorylation in absence of calcium increases in slices and cultured neurons from rat visual cortex. *J Neurosci.* 2000; 20(8):2809–16. Epub 2001/02/07. <https://doi.org/10.1523/JNEUROSCI.20-08-02809.2000> PMID: 10751432; PubMed Central PMCID: PMC6772214.
132. Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME. Ca<sup>2+</sup> influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron.* 1998; 20(4):709–26. Epub 1998/05/15. [https://doi.org/10.1016/s0896-6273\(00\)81010-7](https://doi.org/10.1016/s0896-6273(00)81010-7) PMID: 9581763.
133. Du K, Montminy M. CREB is a regulatory target for the protein kinase Akt/PKB. *J Biol Chem.* 1998; 273(49):32377–9. Epub 1998/11/26. <https://doi.org/10.1074/jbc.273.49.32377> PMID: 9829964.
134. Pugazhenti S, Boras T, O'Connor D, Meintzer MK, Heidenreich KA, Reusch JE. Insulin-like growth factor I-mediated activation of the transcription factor cAMP response element-binding protein in PC12 cells. Involvement of p38 mitogen-activated protein kinase-mediated pathway. *J Biol Chem.* 1999; 274(5):2829–37. Epub 1999/01/23. <https://doi.org/10.1074/jbc.274.5.2829> PMID: 9915817.
135. Grimes CA, Jope RS. CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. *J Neurochem.* 2001; 78(6):1219–32. Epub 2001/10/02. <https://doi.org/10.1046/j.1471-4159.2001.00495.x> PMID: 11579131; PubMed Central PMCID: PMC1947002.
136. Horike N, Sakoda H, Kushiyaama A, Ono H, Fujishiro M, Kamata H, et al. AMP-activated protein kinase activation increases phosphorylation of glycogen synthase kinase 3beta and thereby reduces cAMP-responsive element transcriptional activity and phosphoenolpyruvate carboxykinase C gene expression in the liver. *J Biol Chem.* 2008; 283(49):33902–10. Epub 2008/09/20. <https://doi.org/10.1074/jbc.M802537200> PMID: 18801732; PubMed Central PMCID: PMC2662216.
137. Beurel E, Jope RS. The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. *Prog Neurobiol.* 2006; 79(4):173–89. Epub 2006/08/29. <https://doi.org/10.1016/j.pneurobio.2006.07.006> PMID: 16935409; PubMed Central PMCID: PMC1618798.
138. Hetman M, Cavanaugh JE, Kimelman D, Xia Z. Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. *J Neurosci.* 2000; 20(7):2567–74. Epub 2000/03/24. <https://doi.org/10.1523/JNEUROSCI.20-07-02567.2000> PMID: 10729337; PubMed Central PMCID: PMC6772233.

139. Dewachter I, Ris L, Jaworski T, Seymour CM, Kremer A, Borghgraef P, et al. GSK3beta, a centre-staged kinase in neuropsychiatric disorders, modulates long term memory by inhibitory phosphorylation at serine-9. *Neurobiol Dis.* 2009; 35(2):193–200. Epub 2009/04/22. <https://doi.org/10.1016/j.nbd.2009.04.003> PMID: 19379814.
140. Hernandez F, Borrell J, Guaza C, Avila J, Lucas JJ. Spatial learning deficit in transgenic mice that conditionally over-express GSK-3beta in the brain but do not form tau filaments. *J Neurochem.* 2002; 83(6):1529–33. Epub 2002/12/11. <https://doi.org/10.1046/j.1471-4159.2002.01269.x> PMID: 12472906.
141. Ezpeleta J, Baudouin V, Arellano-Anaya ZE, Boudet-Devaud F, Pietri M, Baudry A, et al. Production of seedable Amyloid-beta peptides in model of prion diseases upon PrP(Sc)-induced PDK1 overactivation. *Nat Commun.* 2019; 10(1):3442. Epub 2019/08/03. <https://doi.org/10.1038/s41467-019-11333-3> PMID: 31371707; PubMed Central PMCID: PMC6672003.
142. Simon D, Herva ME, Benitez MJ, Garrido JJ, Rojo AI, Cuadrado A, et al. Dysfunction of the PI3K-Akt-GSK-3 pathway is a common feature in cell culture and in vivo models of prion disease. *Neuropathol Appl Neurobiol.* 2014; 40(3):31–26. Epub 2013/06/08. <https://doi.org/10.1111/nan.12066> PMID: 23741998.
143. Adamo B, Deal AM, Burrows E, Geradts J, Hamilton E, Blackwell KL, et al. Phosphatidylinositol 3-kinase pathway activation in breast cancer brain metastases. *Breast cancer research: BCR.* 2011; 13(6):R125. Epub 2011/12/03. <https://doi.org/10.1186/bcr3071> PMID: 22132754.
144. Lo HW. EGFR-targeted therapy in malignant glioma: novel aspects and mechanisms of drug resistance. *Current molecular pharmacology.* 2010; 3(1):37–52. Epub 2009/12/25. <https://doi.org/10.2174/1874467211003010037> PMID: 20030624.
145. Zorn JA, Wells JA. Turning enzymes ON with small molecules. *Nature chemical biology.* 2010; 6(3):179–88. Epub 2010/02/16. <https://doi.org/10.1038/nchembio.318> PMID: 20154666.
146. Brotz-Oesterhelt H, Beyer D, Kroll HP, Endermann R, Ladel C, Schroeder W, et al. Dysregulation of bacterial proteolytic machinery by a new class of antibiotics. *Nat Med.* 2005; 11(10):1082–7. Epub 2005/10/04. <https://doi.org/10.1038/nm1306> PMID: 16200071.
147. Liggett JL, Zhang X, Eling TE, Baek SJ. Anti-tumor activity of non-steroidal anti-inflammatory drugs: cyclooxygenase-independent targets. *Cancer letters.* 2014; 346(2):217–24. <https://doi.org/10.1016/j.canlet.2014.01.021> PMID: 24486220; PubMed Central PMCID: PMC3965613.
148. Hansen PE. Tolfenamic acid in acute and prophylactic treatment of migraine: a review. *Pharmacology & toxicology.* 1994;75 Suppl 2:81–2. <https://doi.org/10.1111/j.1600-0773.1994.tb02005.x> PMID: 7816790
149. Yu W, Mechawar N, Krantic S, Quirion R. alpha7 Nicotinic receptor activation reduces beta-amyloid-induced apoptosis by inhibiting caspase-independent death through phosphatidylinositol 3-kinase signaling. *J Neurochem.* 2011; 119(4):848–58. Epub 2011/09/03. <https://doi.org/10.1111/j.1471-4159.2011.07466.x> PMID: 21884524.
150. Kawamata J, Shimohama S. Stimulating nicotinic receptors trigger multiple pathways attenuating cytotoxicity in models of Alzheimer's and Parkinson's diseases. *J Alzheimers Dis.* 2011; 24 Suppl 2:95–109. Epub 2011/03/16. <https://doi.org/10.3233/JAD-2011-110173> PMID: 21403387.
151. Kihara T, Shimohama S, Sawada H, Honda K, Nakamizo T, Shibasaki H, et al. alpha 7 nicotinic receptor transduces signals to phosphatidylinositol 3-kinase to block A beta-amyloid-induced neurotoxicity. *J Biol Chem.* 2001; 276(17):13541–6. Epub 2001/03/30. <https://doi.org/10.1074/jbc.M008035200> PMID: 11278378.
152. Shimohama S, Kihara T. Nicotinic receptor-mediated protection against beta-amyloid neurotoxicity. *Biol Psychiatry.* 2001; 49(3):233–9. Epub 2001/03/07. [https://doi.org/10.1016/s0006-3223\(00\)01100-8](https://doi.org/10.1016/s0006-3223(00)01100-8) PMID: 11230874.
153. Yi JH, Baek SJ, Heo S, Park HJ, Kwon H, Lee S, et al. Direct pharmacological Akt activation rescues Alzheimer's disease like memory impairments and aberrant synaptic plasticity. *Neuropharmacology.* 2018; 128:282–92. Epub 2017/10/29. <https://doi.org/10.1016/j.neuropharm.2017.10.028> PMID: 29079294.
154. Zhang B, Wang Y, Li H, Xiong R, Zhao Z, Chu X, et al. Neuroprotective effects of salidroside through PI3K/Akt pathway activation in Alzheimer's disease models. *Drug Des Devel Ther.* 2016; 10:1335–43. Epub 2016/04/23. <https://doi.org/10.2147/DDDT.S99958> PMID: 27103787; PubMed Central PMCID: PMC4827895.
155. Batista AF, Forny-Germano L, Clarke JR, Lyra ESNM, Brito-Moreira J, Boehnke SE, et al. The diabetes drug liraglutide reverses cognitive impairment in mice and attenuates insulin receptor and synaptic pathology in a non-human primate model of Alzheimer's disease. *J Pathol.* 2018; 245(1):85–100. Epub 2018/02/13. <https://doi.org/10.1002/path.5056> PMID: 29435980; PubMed Central PMCID: PMC5947670.

156. Bomfim TR, Forny-Germano L, Sathler LB, Brito-Moreira J, Houzel JC, Decker H, et al. An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease-associated A $\beta$  oligomers. *J Clin Invest*. 2012; 122(4):1339–53. Epub 2012/04/06. <https://doi.org/10.1172/JCI57256> PMID: 22476196; PubMed Central PMCID: PMC3314445.
157. McClean PL, Holscher C. Liraglutide can reverse memory impairment, synaptic loss and reduce plaque load in aged APP/PS1 mice, a model of Alzheimer's disease. *Neuropharmacology*. 2014; 76 Pt A:57–67. Epub 2013/08/27. <https://doi.org/10.1016/j.neuropharm.2013.08.005> PMID: 23973293.
158. McClean PL, Parthasarathy V, Favier E, Holscher C. The diabetes drug liraglutide prevents degenerative processes in a mouse model of Alzheimer's disease. *J Neurosci*. 2011; 31(17):6587–94. Epub 2011/04/29. <https://doi.org/10.1523/JNEUROSCI.0529-11.2011> PMID: 21525299; PubMed Central PMCID: PMC6622662.
159. Chen Y, Zhao Y, Dai CL, Liang Z, Run X, Iqbal K, et al. Intranasal insulin restores insulin signaling, increases synaptic proteins, and reduces A $\beta$  level and microglia activation in the brains of 3xTg-AD mice. *Exp Neurol*. 2014; 261:610–9. Epub 2014/06/12. <https://doi.org/10.1016/j.expneurol.2014.06.004> PMID: 24918340.
160. Guo Z, Chen Y, Mao YF, Zheng T, Jiang Y, Yan Y, et al. Long-term treatment with intranasal insulin ameliorates cognitive impairment, tau hyperphosphorylation, and microglial activation in a streptozotocin-induced Alzheimer's rat model. *Sci Rep*. 2017; 7:45971. Epub 2017/04/07. <https://doi.org/10.1038/srep45971> PMID: 28382978; PubMed Central PMCID: PMC5382700.
161. Mao YF, Guo Z, Zheng T, Jiang Y, Yan Y, Yin X, et al. Intranasal insulin alleviates cognitive deficits and amyloid pathology in young adult APP<sup>swe</sup>/PS1<sup>dE9</sup> mice. *Aging Cell*. 2016; 15(5):893–902. Epub 2016/07/28. <https://doi.org/10.1111/acer.12498> PMID: 27457264; PubMed Central PMCID: PMC5013027.
162. Rajasekar N, Nath C, Hanif K, Shukla R. Intranasal Insulin Administration Ameliorates Streptozotocin (ICV)-Induced Insulin Receptor Dysfunction, Neuroinflammation, Amyloidogenesis, and Memory Impairment in Rats. *Mol Neurobiol*. 2017; 54(8):6507–22. Epub 2016/10/13. <https://doi.org/10.1007/s12035-016-0169-8> PMID: 27730514.
163. Shi Q, Liu S, Fonseca VA, Thethi TK, Shi L. Effect of metformin on neurodegenerative disease among elderly adult US veterans with type 2 diabetes mellitus. *BMJ Open*. 2019; 9(7):e024954. Epub 2019/08/02. <https://doi.org/10.1136/bmjopen-2018-024954> PMID: 31366635; PubMed Central PMCID: PMC6677947.
164. Sluggett JK, Koponen M, Bell JS, Taipale H, Tanskanen A, Tiihonen J, et al. Metformin and Risk of Alzheimer's Disease Among Community-Dwelling People With Diabetes: A National Case-Control Study. *J Clin Endocrinol Metab*. 2020;105(4). Epub 2019/11/30. <https://doi.org/10.1210/clinem/dgz234> PMID: 31778170.
165. Cao B, Rosenblat JD, Brietzke E, Park C, Lee Y, Musial N, et al. Comparative efficacy and acceptability of antidiabetic agents for Alzheimer's disease and mild cognitive impairment: A systematic review and network meta-analysis. *Diabetes Obes Metab*. 2018; 20(10):2467–71. Epub 2018/05/24. <https://doi.org/10.1111/dom.13373> PMID: 29790638.
166. Munoz-Jimenez M, Zaarki A, Garcia-Arnes JA, Garcia-Casares N. Antidiabetic Drugs in Alzheimer's Disease and Mild Cognitive Impairment: A Systematic Review. *Dement Geriatr Cogn Disord*. 2020; 49(5):423–34. Epub 2020/10/21. <https://doi.org/10.1159/000510677> PMID: 33080602.
167. Saunders AM, Burns DK, Gottschalk WK. Reassessment of Pioglitazone for Alzheimer's Disease. *Front Neurosci*. 2021; 15:666958. Epub 2021/07/06. <https://doi.org/10.3389/fnins.2021.666958> PMID: 34220427; PubMed Central PMCID: PMC8243371.