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Overexpression of Protein Kinase M ζ in the Hippocampus Mitigates Alzheimer's Disease-Related Cognitive Deficit in Rats

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Abstract

Accumulation of amyloid beta ($A\beta$) soluble forms in the cerebral parenchyma is the mainstream concept underlying memory deficit in the early phase of Alzheimer's disease (AD). PKM ζ plays a critical role in the maintenance of long-term memory. Yet, the role of this brain-specific enzyme has not been addressed in AD. We examined the impact of hippocampal PKM ζ 's overexpression on AD-related memory impairment in rats. Oligomeric form of $A\beta$ (o $A\beta$) or vehicle was bilaterally microinjected into the dorsal hippocampus of male Wistar rats under stereotaxic surgery. One week later, 2 μ l of lentiviral vector (10^8 T.U. / ml.) encoding PKM ζ genome was microinjected into the dorsal hippocampus. Seven days later, behavioral performance was assessed using shuttle box and Morris water maze. The expression levels of GluA1, GluA2 and KCC2 were determined in the hippocampus using western blot technique. Our data showed that o $A\beta$ impairs both passive avoidance and spatial learning and memory. However, overexpression of PKM ζ in the dorsal hippocampus restored the behavioral performance. This improving effect was blocked by microinjection of ZIP, a PKM ζ inhibitor, into the hippocampus. o $A\beta$ or PKM ζ did not significantly change GluA1 level in the hippocampus. Furthermore, PKM ζ failed to not restore elevated KCC2 level induced by o $A\beta$. However, o $A\beta$ decreased GluA2 level, and overexpression of PKM ζ restored its expression toward the control level. In conclusion, hippocampal overexpression of PKM ζ restored memory dysfunction induced by amyloidopathy in part, through preserving hippocampal GluA2 containing AMPA receptors. PKM ζ 's signaling pathway could be considered as a therapeutic target to battle memory deficits in the early phase of AD.

Keywords: Alzheimer's disease, amyloid beta, PKM ζ , memory, hippocampus, lentiviral vector

Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is the most common cause of dementia. Worldwide prevalence of AD is about 30 million, and the number is estimated to fourfold by 2050 [25]. The most common early symptom of Alzheimer's disease is amnesia, which is characterized by difficulty in acquiring and maintaining new memories, and later, gradual loss of established memories [32, 43].

Accumulation of amyloid beta ($A\beta$) in the cerebral parenchyma is the mainstream concept underlying memory deficit in AD [9, 22], suggesting that excessive $A\beta$ accumulation may set off a complex downstream cascade leading to a range of events from synaptic dysfunction to progressive neuronal loss and cognitive deficit [20]. $A\beta$ is a small (4-KDa) hydrophobic polypeptide which is produced by proteolytic cleavage of transmembrane amyloid precursor protein (APP) [9]. Some studies indicate that soluble forms of $A\beta$, including soluble oligomers, are the most toxic form of $A\beta$ to the synaptic function and also show the best correlation to cognitive impairment [49]. Loss of synapses is prominent in AD prior to considerable neuronal death [27], suggesting that synaptic deficits might precede neuronal loss specially in the early stages of AD. Synapses are continuously remodeling, partly, through postsynaptic content of the glutamate receptor α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), known as AMPA trafficking[1]. Many studies on AD have illustrated that expression of AMPA receptors is altered in AD [6]. Furthermore, there is an ample pieces of evidence that $A\beta$ molecules, specifically the soluble $A\beta$ oligomers, tend to bind to AMPA [26], and N-methyl D-aspartate (NMDA) [44] receptors causing them to internalize and prohibit the long term potentiation (LTP). Protein kinase $M\zeta$ (PKM ζ), which is a brain-specific atypical isoform of protein kinase C, has been suggested to have a critical role in regulating AMPA receptor trafficking [10, 33, 39], and maintenance of LTP and long-term memory [33, 41].

KCC2, a neuron-specific K^+ - Cl^- cotransporter, has been suggested playing an important role in the glutamatergic excitatory synaptic transmission [3, 19]. Further, many studies have indicated that KCC2 functions near excitatory synapses and promotes spine development by interacting with the cytoskeleton [15, 17]. Interestingly, it has been reported that KCC2 expression and phosphorylation was significantly enhanced in a signaling pathway dependent to the PKM ζ [50].

On the other hand, a recent study indicates the involvement of APP in GABA neurotransmission through KCC2 [45]. But, the mechanism in which how KCC2 modulates excitatory glutamatergic pathway has not yet been clarified.

The hippocampus encodes and initially stores experience-dependent spatial information [34] and is one of the earliest regions affected in AD. Persistent activity of PKM ζ has been suggested to underlie many forms of long-term memory in a wide variety of neural circuits. In this line, long term memories including spatial memories [21, 41], basolateral amygdala mediated aversive memories [16], trace memories in the hippocampus [31], appetitive memories in the nucleus accumbens [8], neocortex mediated skilled sensorimotor memories [48] and extinction [23] were all disrupted by inhibiting PKM ζ . Furthermore, PKM ζ has been shown to sustain late-LTP and long-term memory by modifying the trafficking of AMPA receptors in favor of increasing the GluA2 containing receptor number at the postsynaptic sites [51]. Although there are many reports indicating the PKM ζ importance in the process of memory retention, the role of this enzyme in the pathophysiology of AD has not been yet described. On the other hand, strong evidence indicates that KCC2 is implicated in the AD and it is also shown that KCC2 through an interaction with actin cytoskeleton, regulates the local confinement of AMPA receptors and synaptic potency [17]. Therefore, the aim of the present study was to examine the PKM ζ overexpression in the dorsal hippocampus on behavioral performance and glutamatergic synaptic related molecules in rat model of AD.

Materials and Methods

Preparation of A β Oligomers

Oligomeric A β was prepared from synthetic A β 1–42 (TOCRIS, UK). Briefly, the peptide was dissolved in 1mM hexafluoroisopropanol, HFIP, (Sigma-Aldrich, Germany), dried at room temperature. The dried A β was dissolved in dimethyl sulfoxide, DMSO, (Sigma-Aldrich, Germany) to make a 5mM concentration, then aliquoted and kept in–80°C. One day before use, an aliquot was dissolved with sterilized PBS, vortexed for 30 seconds and incubated at 4°C for 12 hours [36].

Cell Culture and Lentiviral Vector production

A lentiviral vector expressing PKM ζ under the control of the cytomegalovirus promoter was constructed by subcloning PKM ζ sequence from pFBHTb PKM zeta rat vector (MRC-PPU, the University of Dundee, UK) into pLenti7.3/V5-TOPO® expression vector (Invitrogen). Lentiviral vector particles were produced as described previously [13]. HEK 293 T cells were cultured in DMEM containing 10% FBS and penicillin-streptomycin of 100 units/ml, at 37°C with 5% of CO₂ atmosphere. 10 plates containing 2×10^6 seeded HEK 293T cells, were transfected with transfer (PKM ζ , 13 μ g), envelope (pMD-VSVG, 3.75 μ g), and packaging (pMDLgag/pol RRE, 13 μ g; pRSV/Rev, 3 μ g) plasmids for each plate by the CaCl₂ precipitation method. 24 h after transfection, the medium was replaced by fresh medium containing 2% FBS and the cells were incubated for 48 h. Then, the medium was harvested and centrifuged at 5000 \times g and 4°C for 5 minutes. Afterwards, the supernatant was filtered through a 0.45- μ m filter unit (Macherey Nagel). Viral particles were pelleted by centrifugation at 50000 \times g for 2 h at 4°C, then resuspended in phosphate-buffered saline (PBS)-BSA 1% solution, aliquoted and kept in -80°C until use.

Vector Titration

5×10^4 HEK 293T cells were seeded in a 24-well plate with 0.5 ml medium containing 5% FBS per well. 24 h later, they were transduced by the serial dilutions of the vector. After 3 days, the number of GFP-positive cells on each dilution was counted using a fluorescent microscope (Nikon, Japan). The vector titer (transducing units, T.U.) was determined using the following formula: [12]

$$\text{Titer (T.U./ml)} = [\text{GFP-positive cells} \times \text{number of cells on the transduction day} (5 \times 10^5)] / \text{dilution}$$

In our routine preparation, the titers were 10^8 transduction units (TU) /ml.

Animals and Ethical Standards

Fifty four adult Wistar male rats (200-250 g, and 3-4 months old) were obtained from our breeding colony. They were caged with free access to food and water and were maintained on a 12h light and dark cycle, at a temperature of $23 \pm 2^\circ\text{C}$. All experiments were carried out according to the Review Board and Ethics Committee of Pasteur Institute (Authorization code

93-0201-785 22 April 2014) and conforming to the European Communities Council Directive 2010-63-EU in a way to minimize the number of animals and suffering.

Stereotaxic Surgery

The rats were randomly allocated into 5 experimental groups, anesthetized with an intraperitoneal injection of 100 and 10 mg/kg ketamine and xylazine, respectively. Two cannulas were bilaterally implanted into the dorsal hippocampus (AP: - 3.8, ML: \pm 2.2 and DV: -2.8 from bregma) of each rat, according to the coordinates of Paxinos and Watson atlas [35] for the rat brain. After recovery from the surgery, the microinjections were performed into the dorsal hippocampus of conscious rats at the rate of 1 μ l/min using a 10- μ l Hamilton syringe attached by PE-10 polyethylene tubing to a 30-gauge injection cannula that extended 1mm beyond the guide cannula. One week after microinjection of 1mM/2 μ l oA β , 2 μ l lentivector carrying PKM ζ genome or the vehicle was administered into the dorsal hippocampus. 25 μ mol/2 μ l specific inhibitor of PKM ζ , ZIP, or its scrambled PKM ζ -inactive form (Tocris Bioscience, UK,) was microinjected into the dorsal hippocampus 2 h before the retrieval tests in the behavioral experiments.

Passive avoidance learning and memory

The shuttle box apparatus consisted of two compartments, bright and dark (20cm \times 20cm \times 30cm) which were divided by a guillotine door (7cm \times 9cm). The bottom of the box was composed of stainless steel grids (2.5mm diameter) with 1cm intervals and connected to an insulated stimulator. Each animal was put in the bright chamber, 10 sec later the guillotine door was held up allowing the rat to gait freely into the dark chamber, explore the apparatus during 1 min as habituation, and then returned to its home cage. After 30 min, the rat was put in the bright chamber, 10 sec later the door was opened up and as soon as the animal entered the dark chamber, the door was closed and a foot shock (50 Hz, 1.5 mA for 1.5 sec) was applied. After 10 seconds, the animal was taken out and returned to its cage. Two min later, the rat was tested and the training was completed if the rat did not enter the dark chamber during 120 sec. 24 h later, the animal was again put in the bright chamber, 10 sec later the door was opened and the rat's

behavior was observed for 10 min. The parameters of initial latency, step-through latency (STL) and the total time spent in the dark compartment (TDC) were recorded and analyzed.

Morris Water Maze (MWM)

Apparatus

The water maze consisted of a pool (155 cm diameter) filled with tap water ($22\pm 2^\circ\text{C}$) up to 20 cm below the edge of the tank. A transparent Plexiglas platform (30 cm high and 10 cm diameter) was located 1.5 cm below the surface of the water in the tank's eastern quadrant (target quadrant, Q1). Animals were randomly dropped from four different quadrants and they had to swim to find the hidden platform in different directions. Animals' movements were recorded by a CCD camera (Panasonic Inc., Japan), hanging from the ceiling over the MWM apparatus and locomotion tracking was measured by the Ethovision software (version XT7, Netherland), a video tracking system for automated analyzing of animals' behavior.

Habituation

One day before the training, the animals were allowed to swim for one minute in the tank without the platform to adapt to the environment.

Procedure

Reference spatial learning and memory. This test contained 3 days of training, 4 trials with 10 min inter-trial intervals in each day [18]. The platform was submerged in the eastern quadrant (target quadrant, Q1) and remained unchanged during the training days. The animals were randomly released (while facing the tank wall) from 4 different locations chosen from north, south, west, and southeast between the trials. Rats received one training session for 3 successive days. Animals had 60 seconds to swim and find the platform; otherwise, they were directed to the platform and were allowed to rest on it for 10 seconds. On the 4th day, the probe test, the platform was taken out and animals were dropped from the opposite (180°) direction to the platform location and allowed to swim for one minute. The escape latencies, traveled distance, time spent in each quadrant, and swim speeds were recorded for subsequent analysis.

Reverse spatial learning and memory. To investigate the reverse spatial memory, 24 hours after the reference spatial probe test, the platform was moved to the opposite quadrant (western quadrant, Q₃) and rats were trained to find the new location of the platform during two training blocks separated by 30 min interval. Each block consisted of four trials with 10 min inter-trial intervals. Twenty four h later the probe test was done.

Visual Test

After the reversal probe test, the platform was elevated above the water surface and covered by bright aluminum foil and placed in all four quadrants respectively. In each trial, rats were dropped from the opposite quadrant and had one minute to swim and find the visible platform to testify their visual ability. All experiments were conducted at 09:00 to 12:00 AM.

Western Blot Analysis

After fulfillment of the behavioral tests, animals were anesthetized and decapitated, their brains were removed and hippocampi were separated and homogenized in the RIPA lysis buffer containing a protease inhibitor cocktail. Proteins of the hippocampal lysates were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes and incubated overnight in blocking buffer containing skim milk powder 2% (MERCK, Germany) and Tween-20 1% in Tris-buffered saline (TBS-T) at 4°C. Then, the membranes were incubated overnight in primary antibodies at 4°C and then washed in TBS-T for 3 times. The following primary antibodies were used: anti-GluA1 (1:500, Invitrogen, USA), anti-GluA2 (1:1000, Invitrogen, USA), anti-KCC2 (1:1000, Invitrogen, USA), GFP (1:1000, Abcam, USA) and rabbit polyclonal anti- β -Actin (1:500, Sigma-Aldrich, Germany). Then, the membranes were incubated in secondary antibody (1:5000, Invitrogen, USA). The chemiluminescence detection kit (Amersham Bioscience, UK) was used to illuminate the protein bands. Protein bands were then exposed to autoradiography films (Kodak, USA). The Image J 1.50i software (National Institutes of Health) was used to quantify and normalize the protein bands corresponding to β -Actin bands.

Histology

Seven days after the injection of the vector (3 rats in each group), the brain of the rats were used for histological examination. The animals were euthanized by CO₂ asphyxiation and perfused transcardially with 4% paraformaldehyde in 0.1 M PBS. Then, the brains were harvested, kept in 10% paraformaldehyde in 0.1 M PBS and processed for embedding in paraffin blocks. The brains were then cut horizontally to obtain 5 μ m slices. The sections were cover slipped with 90% glycerol mounting buffer and visualized in a dark place with fluorescent microscope (Nikon, Japan) equipped with specific filter cube for FITC fluorescence channels, and connected to a digital camera. Digital photographs were taken using 10 and 20x objective lenses.

Experimental design

This study was designed to examine the effect of PKM ζ overexpression in the dorsal hippocampus on memory performance and expression of glutamatergic synaptic related molecules in a rat model of AD. In the behavioural experiments, spatial and inhibitory avoidance learning and memory were examined. They consist of six groups: 1- control group; rats were undergone surgery and received PBS microinjection into the dorsal hippocampus. After one week, PBS/BSA microinjected into the dorsal hippocampus and one week later, behavioural tests were performed. 2- PKM ζ group; rats received PBS into the dorsal hippocampus and one week later, were microinjected with lentivector encoding PKM ζ into the dorsal hippocampus and after one week, behavioural tests were performed. 3- A β group; received oA β into the dorsal hippocampus, and one week later PBS/BSA microinjected into the dorsal hippocampus and after one week, behavioural tests were started. 4- A β + PKM ζ ; they were treated like the A β group but lentiviral vector was bilaterally delivered into the dorsal hippocampus instead of PBS/BSA, and one week later, behavioural tests were performed. 5- A β + PKM ζ + ZIP group; they were treated exactly like A β + PKM ζ group except they received zeta inhibitory peptide, ZIP, 2 h before the retrieval test. 6- A β + PKM ζ + Scr. ZIP group; they were treated like A β + PKM ζ + ZIP group except they received scrambled ZIP (Scr. ZIP) 2 h before the retrieval test. In the molecular experiment, GluA1, GluA2, KCC2 levels in the hippocampus were determined using western blot technique. This part consisted of the first five groups. Histological examinations for detection of GFP expression were performed one week after injection of lentiviral vector into the dorsal hippocampus (3 rats in each group). Finally, animals were euthanized by CO₂ asphyxiation after the

behavioural tests and the data of those with poor expression were excluded from further analysis. The timeline of the experiments is demonstrated in Fig.1

Data analysis

The data were expressed as mean \pm standard-error of the mean (SEM) and processed by GraphPad Prism 6.0. Results were statistically analyzed by One-way and Two-way analysis of variance (ANOVA). Where F value was significant, Tukey's post hoc analyzes were performed. Differences with $p < 0.05$ between the groups were considered statistically significant.

Results

Lentivirus histological verification

Hippocampal formation was observed under fluorescent microscopy one week after injection of concentrated lentiviral vector. As depicted in the fig. 2, neurons located in the principle layer of the CA1 were transduced with the vector and expressed green fluorescent protein, indicating successful expression of PKM ζ in these cells.

Hippocampal GFP expression level was determined by western blot in all experimental groups. The representative GFP band densities are shown in upper panel of fig. 2B. It should be noted that control and A β groups did not receive any lentiviral vector. One-way ANOVA revealed no significant difference of GFP level among groups microinjected with lentiviral vectors into the dorsal hippocampus ($F(2, 6) = 0.9837, p = 0.16$).

Overexpression of PKM ζ in the dorsal hippocampus rescued spatial learning and memory performance against oA β

Microinjection of oA β into the dorsal hippocampus resulted in decelerated acquisition of the reference spatial task compared to the control group. Two-way ANOVA reported significant difference of time [$F(2, 83) = 59.45, P < 0.0001$] and treatment [$F(4, 83) = 4.49, p = 0.0025$] factors but not interaction [$F(8, 83) = 0.74, p = 0.66$]. Tukey's posttest revealed a significant difference between oA β treated rats ($n = 7$) and control ($n = 8$) or PKM ζ ($n = 9$) groups ($p < 0.05$, fig.3 A). There was also a significant difference between the oA β treated rats and oA β + PKM ζ ($n = 7$) group ($p < 0.05$). In the probe test, one-way ANOVA followed by Tukey's posttest

revealed that oA β treated rats swam less time in the target quadrant compared to the control or PKM ζ groups ($p < 0.05$, fig.3 B). However, overexpression of PKM ζ in the dorsal hippocampus in the oA β treated animals increased the duration in the target quadrant, indicating an improvement in reference spatial learning and memory. However, microinjection of Zeta inhibitory peptide (ZIP), a specific inhibitor of PKM ζ , 2 h before the probe test disrupted the improving effect of PKM ζ on spatial memory performance (fig. 3B).

To examine the reversal learning or switching capability of spatial learning and memory, the hidden platform was moved to the opposite quadrant (Q3) and the animals were trained to locate the new place of the platform within eight trials (two blocks of four trials). As depicted in the Fig. 3C, the oA β treated rats showed greater latency to find the platform at the second block compared to the control group ($p < 0.05$). In the reverse probe test, the oA β treated rats swam less time in the target quadrant compared to the control group ($p < 0.05$). There was also a significant difference between the oA β and PKM ζ ($p < 0.001$) groups. Overexpression of PKM ζ in the oA β treated rats, restored reversal spatial memory toward the control level. However, ZIP disrupted the improving effect of PKM ζ on reverse spatial memory performance (fig. 3D). The effect of the Scr. ZIP was indistinguishable from PBS in reference and reverse probe tests.

Overexpression of PKM ζ in the dorsal hippocampus restored avoidance memory impaired by oligomeric A β

One mM oligomeric A β or vehicle was microinjected into the dorsal hippocampus, and one week later, inhibitory avoidance learning and memory was examined. Fig.4A shows the initial latency, the latency to enter the dark compartment before exposing to electrical shock in all experimental groups. No difference of initial latency was observed among groups indicating that surgery, oA β or transduction of hippocampal cells with lentivector did not have any effect on visio-motor performance.

One-way ANOVA followed by Tukey's test showed no difference in the step through latency (STL) between the control and PKM ζ groups. However, STL was significantly lower in the oA β treated rats compared to the control or PKM ζ groups ($p < 0.01$), indicating that oA β disrupted the passive avoidance memory. Overexpression of PKM ζ in the dorsal hippocampus failed to increase the STL in oA β treated rats (Fig.4B). Total time spent in the dark compartment was also

measured to analyze inhibitory avoidance memory performance. Fig. 4C indicates that the oA β treated animals significantly spent more time in the dark compartment in contrast to the control or PKM ζ groups ($p < 0.001$). In contrast, overexpression of PKM ζ in the dorsal hippocampus decreased this time, and the improving effect of PKM ζ was blocked by ZIP ($p < 0.001$). Scr. ZIP showed no effect on STL and TDC.

PKM ζ increased GluA2 level in the hippocampus

GluA1 and GluA2 containing AMPA receptors are involved in formation and maintenance of memory. As depicted in the Fig. 5A, overexpression of PKM ζ increased the level of GluA1 in the hippocampus but did not reach to a significant level compared to the control ($n = 5$) group. oA β ($n = 6$) non-significantly increased the GluA1 level and overexpression of PKM ζ did not significantly change the GluA1 level in the hippocampus of rats treated with oA β .

Fig. 5B shows that GluA2 expression level was increased in the hippocampus following the overexpression of PKM ζ in the healthy rats ($n=8$) compared to the control group ($p < 0.01$). However, the level of GluA2 was decreased in the hippocampus of oA β treated rats ($n = 5$) compared to the control group ($p < 0.01$), and overexpression of PKM ζ ($n = 6$) in this group maintained the GluA2 level in the hippocampus.

Analysis of GluA1/GluA2 ratio by one-way ANOVA revealed a significant difference among experimental groups ($F(3, 20) = 8.368, p < 0.0001$). GluA1/GluA2 ratio was significantly increased in oA β treated rats compared to the control and PKM ζ groups ($p < 0.01$). Overexpression of PKM ζ decreased the ratio, but no significant difference of the ratio was found between oA β and A β + PKM ζ groups (Fig. 5C).

oA β and PKM ζ increased KCC2 level in the hippocampus

KCC2 is a K-Cl co-transporter that is involved in the synaptic transmission and stabilization of synaptic AMPA receptors. Fig. 6 shows KCC2 level in the hippocampus of different groups. One-way ANOVA revealed a significant difference of KCC2 level among groups ($F(3, 18) = 5.215, p = 0.0091$). Overexpression of PKM ζ in healthy rats significantly increased KCC2 level

compared to the control group. KCC2 was also increased in the hippocampus of oA β treated rats compared to the control group. However, no significant difference was found between the oA β and oA β + PKM ζ groups.

Discussion

In the present study, we found that oA β disrupted hippocampus-dependent learning and memory performance. Furthermore, our data demonstrated that overexpression of PKM ζ in the hippocampus restored learning and memory performance against oA β and this improving effect was completely blocked by the specific inhibitor of PKM ζ , the inhibitory peptide, ZIP. We also found that the level of GluA2 expression, but not GluA1, was decreased in the hippocampus by oA β , and then restored to the normal level following PKM ζ overexpression. KCC2 is one of the most important proteins that regulates glutamatergic synaptic transmission. However, we found that overexpression of PKM ζ did not change the KCC2 level in the hippocampus of oA β treated rats.

In this study, we found that microinjection of oA β in the dorsal hippocampus impaired spatial learning and memory. We also tested the flexibility of memory by examining of extinction to the previous platform location via training the rats to find the new location of the hidden platform during eight trails in one day. In this experiment, oA β treated rats showed a decelerated rate to find the platform compared to the control group. In addition, in the probe test they swam less time in the target quadrant (Q3). The oA β treated rats also exhibited impaired inhibitory avoidance memory. Overexpression of PKM ζ in the dorsal hippocampus did not change spatial or inhibitory memory performance in wild type animals. In contrast to our results, it has been reported that overexpression of PKM ζ in the hippocampus of wild type rats enhanced fear memory [40]. The discrepancy could be due to using different procedure to overexpress PKM ζ , different experimental paradigm and different time-course assessment after viral transduction. It should be also acknowledged that the high memory performance in the wild type animals may reflect a ceiling effect, occluding PKM ζ relevant memory enhancement [40]. Unlike in wild type rats, overexpression of PKM ζ into the dorsal hippocampus preserved spatial and inhibitory avoidance memory performance in A β treated rats.

Alzheimer's disease (AD), the most common cause of dementia, is a neurodegenerative disease that progressively deteriorates memory and cognitive functions. Multiple studies indicate that

cerebral accumulation of A β is an initial event starting AD [22]. In the later stage of AD, A β molecules assemble into amyloid plaques that are capable of stimulating glial cells to produce an inflammatory condition, which eventually leads to neuronal death. It has been reported that while the soluble A β oligomers, the predominant form of A β in the early phase of AD, fail to stimulate glial cells [24, 52], they are the most toxic form of A β to the synaptic function. Soluble A β microinjection is a verified approach to induce AD related pathology in a localized cerebral tissue [4, 14, 36]. Here, we found that microinjection of A β oligomers into the dorsal hippocampus impaired learning and memory performance, and overexpression of PKM ζ rescued memory performance against the A β oligomers. PKM ζ is known to maintain long-term synaptic potentiation (LTP) [39]. Multiple studies indicate that AD begins with synaptic failure in advance of neuronal degeneration [4, 36]. In spite of strong evidence of the PKM ζ effect on synaptic function, its role in AD is still unclear. We observed that microinjection of A β into the hippocampus decreased the level of GluA2, a subunit of AMPA receptor. In agreement, excessive A β levels have been shown to dysregulate glutamatergic synaptic transmission and plasticity by targeting the AMPA and NMDA receptors [26, 45]. AMPA receptors are known as the predominant glutamate receptors mediating fast synaptic events. They are tetrameric assemblies of two dimers of four potential subunits (GluA1–GluA4). The AMPA receptors expressed in the hippocampal and cortical pyramidal neurons are composed of GluA1 or GluA2 subunits. The number of AMPA is decreased with age in transgenic mouse model of AD [30]. Also, Rui and his colleagues reported that A β oligomers reduce AMPA receptor surface exposure, inhibit trafficking, and finally decrease the number of AMPA receptors [38]. GluA1 and GluA2 levels have been reported to decrease at cell surface in AD [2, 30], but its mechanism has not yet been elucidated. Interestingly, we found no significant difference of GluA1 expression level between the control and A β treated groups. However, GluA2 expression was significantly decreased in the hippocampus of rats treated with A β and overexpression of PKM ζ restored its expression level toward the control. GluA2 subunit is believed to render the channel impermeable to calcium, but GluA1 subunit will increase channel permeability to calcium. [5]. We found that oA β increased the ratio of GluA1/GluA2. Changing the ratio in favor of GluA1 may render the neurons vulnerable to excitotoxic neuronal death due to A β induced excessive calcium influx. PKM ζ may secondarily protect neuronal cells through increasing GluA2 subunit. In support of this view, it has been recently reported that mutation of GluA2 resulted in

decreased dendritic spines, neuronal death in the hippocampus, learning and memory deficits and seizure vulnerability [28]. Therefore, PKM ζ overexpression in the hippocampus may reverse the effect of A β , in part, through preserving or enhancing GluA2 containing AMPA receptors in synapses. Consistently, PKM ζ is shown to preserve long-term memories by increasing GluA2-containing AMPA receptors at synapses [51]. We observed that inhibition of PKM ζ by ZIP disrupted the improving effect of PKM ζ on memory performance. This finding is consistent with previous data showing that ZIP erases various types of memories [8, 10, 16, 21]. However, PKM ζ role in synaptic and memory maintenance has been questioned by studies using PKM ζ knockdown mice [29, 47]. On the other hand, ZIP inhibits other protein kinase isoforms including PKC ι/λ [37]. Thus, other ZIP-sensitive molecules may also be involved in erasure of long-term memory. It should be noted that the role of PKM ζ in memory retention could not yet be completely ruled out, because compensatory mechanisms may mimic PKM ζ function in PKM ζ knockdown mice. Consistent with this view, the compensation of PKM ζ has been shown by another atypical PKC, PKC ι/λ , in PKM ζ knockdown mice [46]. Furthermore, overexpression of PKM ζ has been shown to enhance LTP and establish memory consolidation [40, 42]. Our results also indicate that overexpression of PKM ζ boosts memory function in oA β treated rats.

In the present study, PKM ζ rescued memory performance in oA β treated rats. However, it did not change learning and memory performance in the wild type rats, even though it significantly increased GluA2 expression in the hippocampus. This data indicates that other AD related molecules might be also involved in boosting effect of PKM ζ on memory performance in the oA β rats. Strong evidence indicates that KCC2 is implicated in the AD and it has also shown that KCC2 through an interaction with actin cytoskeleton, regulates the local confinement of AMPA receptors and synaptic potency [17]. It has also been reported that KCC2 expression and phosphorylation is modified by PKM ζ [50]. Therefore, in the next step, we examined the expression of KCC2. We observed an enhancement of KCC2 level following amyloidopathy. There are limited data on how KCC2 expression is changed in AD. Some reports have shown an interaction between KCC2 and amyloid precursor protein (APP). For instance, it has been reported that KCC2 level is increased in the APP knock-out (APP^{-/-}) mice [7]. However, overexpression of APP has been reported to decrease KCC2 levels in the cortical cells [11]. The mechanism underlying modification of KCC2 by soluble A β needs to be further studied. Regarding to our results, overexpression of PKM ζ did not change the KCC2 level in the oA β

treated rats suggesting that improving effect of PKM ζ in these rats is not mediated through KCC2.

In short, microinjection of α A β led to hippocampus-dependent memory impairment. Overexpression of PKM ζ in the hippocampus rescue memory function accompanied by restoration of GluA2 expression in the α A β treated rats. The boosting effect of PKM ζ may, in part, be mediated through increased GluA2 containing AMPA receptors, but since memory enhancement is not seen in the wild type group, another molecule or signaling pathway may mediate the PKM ζ -improving effect. Further study is required to elucidate this issue.

Conflict of interest statement

The authors have no conflict of interest

Acknowledgments

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Figures legends:

Fig. 1. Schematic representation of design and timeline of the behavioural and molecular experiments. DH: dorsal hippocampus.

Fig. 2. Transduction of target cells with the lentivirus carrying the PKM ζ genome. A) Green cells show increased expression of PKM ζ in the principal layer of CA1. B) The representative GFP bands obtained by western blot technique (upper panel). Control and A β groups were not treated by any lentiviral vector. No difference of GFP level was observed among groups microinjected with lentiviral vector into the dorsal hippocampus (lower panel). Magnification 100x, scale bar; 100 μ m.

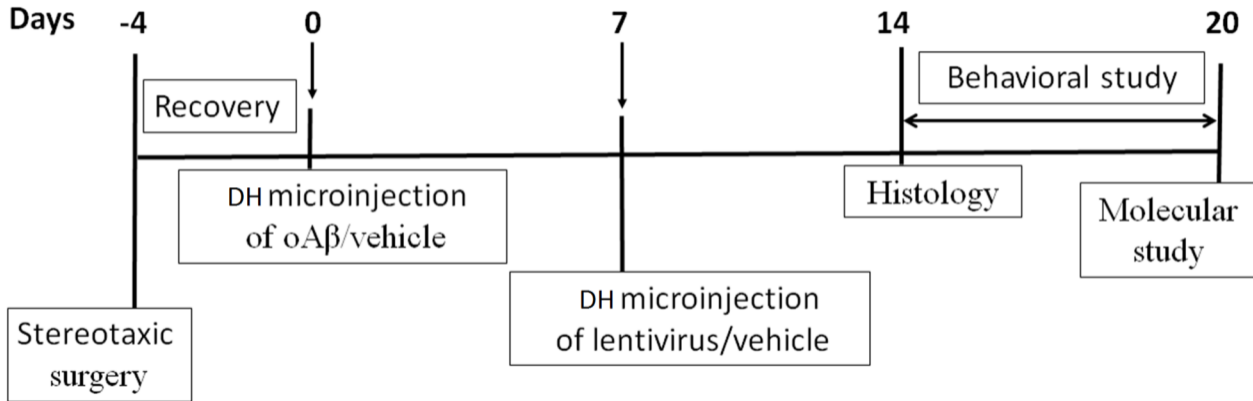
Fig. 3. Overexpression of PKM ζ restored reference and reversal spatial learning and memory deficit induced by oA β . A) Reference spatial learning profile of animals revealed that oA β treated rats acquired the task slower than the control or PKM ζ groups. However, overexpression of PKM ζ could accelerate learning in the oA β treated rats. B) The oA β treated rats spent less time in the target quadrant compared to the control or PKM ζ ($p < 0.01$), but overexpression of PKM ζ in the dorsal hippocampus increased the duration toward the control level. Microinjection of ZIP, but not Scr. ZIP, into the dorsal hippocampus two h before the probe test, disrupted the

improving effect of PKM ζ on spatial memory performance. C) The oA β treated rats showed a delay to locate the new platform position in the target quadrant. Overexpression of PKM ζ accelerated extinction of reference memory to locate the new platform location. D) The oA β treated rats spent less time in the target quadrant (Q3) compared to the control or PKM ζ groups. Overexpression of PKM ζ in the dorsal hippocampus could normalize reversed memory performance. ZIP, but not Scr. ZIP, disrupted the improving effect of PKM ζ in the oA β treated rats. Note that in A and C animals did not receive ZIP/Scr.ZIP during the acquisition of the tasks. [^]p < 0.05 and ^{^^}p < 0.01 compared to the PKM ζ group. ^op < 0.05 and ^{oo}p < 0.01 compared to the A β + PKM ζ group. ⁺p < 0.05 compared to the A β + PKM ζ (-ZIP) group. [#]p < 0.05 compared to the A β + PKM ζ (-Scr. ZIP) group. ^{*}p < 0.05 and ^{**}p < 0.01 compared to the control group. ^{\$}p < 0.05 and ^{\$\$}p < 0.01 compared to the A β group.

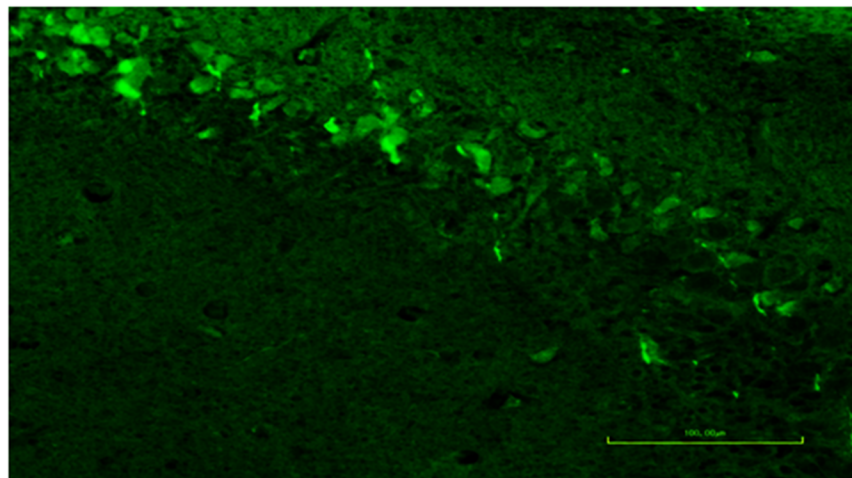
Fig. 4. Overexpression of PKM ζ rescued the passive avoidance memory against amyloidopathy. A) No significant difference of initial latency was observed between groups. B) Step through latency (STL) was lower in the oA β treated animals compared to the control group (p < 0.05). There was no significant difference between oA β and oA β + PKM ζ groups (p > 0.05). C) The oA β treated animals spent more time in the dark compartment in contrast to the control group (p < 0.001) and overexpression of PKM ζ in the dorsal hippocampus reversed the A β effect. ZIP, but not Scr. ZIP, blocked the effect of PKM ζ . ^{*}p < 0.05, ^{**}p < 0.01 and ^{***}p < 0.001 compared to the control group. ^{\$}p < 0.05 and ^{\$\$\$}p < 0.001 compared to the A β group. [&]p < 0.05 and ^{&&&}p < 0.001 compared to the A β + PKM ζ group.

Fig. 5. Expression level of GluA1 and GluA2 in the hippocampus was changed by oA β . A) The representative blots of GluA1 obtained by western blot technique (upper panel). The GluA1 level was not different among groups. B) Representative bands of GluA2 are shown in the upper panel. Overexpression of PKM ζ significantly increased the expression level of GluA2 in the hippocampus of healthy rats (p < 0.01). oA β decreased the level of GluA2 compared to the control group (p < 0.01), and overexpression of PKM ζ restored its level in the hippocampus of the oA β treated rats. C) GluA1/GluA2 ratio was increased in A β rats compared to the control and PKM ζ groups. Overexpression of PKM ζ in the dorsal hippocampus decreased the GluA1/GluA2 ratio in oA β treated rats but did not reach to the significant level (p < 0.05). ^{**}p < 0.001 compared to the control group. ^{\$\$}p < 0.001 compared to the A β group.

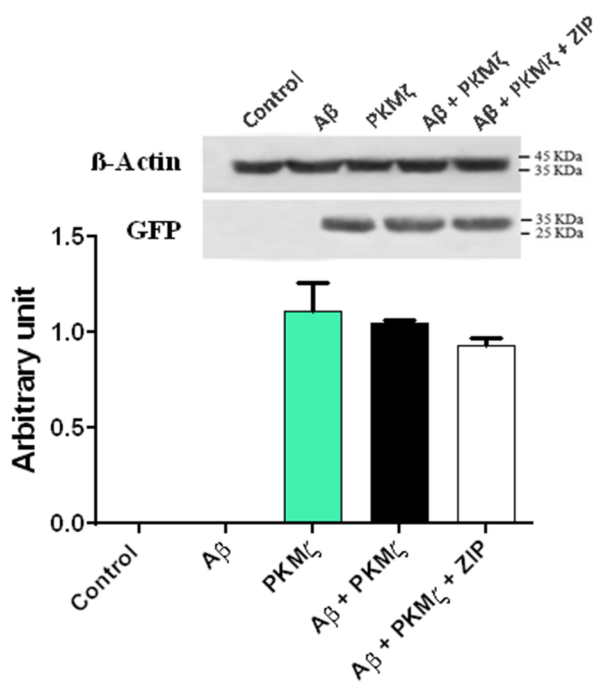
Fig. 6. Effect of oA β on KCC2 level in the hippocampus. (A) The representative blots of KCC2 obtained by western blot technique are shown in the upper panel. Overexpression of PKM ζ significantly increased the KCC2 protein level in the hippocampus. KCC2 level was also increased in the hippocampus of rats treated with oA β , however, no significant difference was detected between the oA β and oA β + PKM ζ groups. ^{**}p < 0.001 compared to the control group.

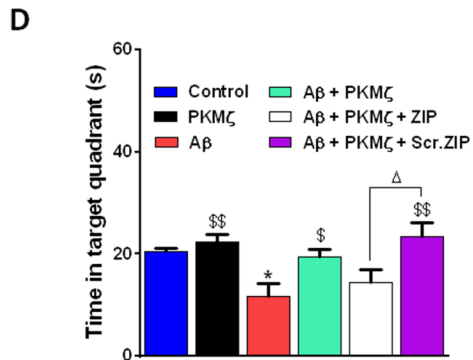
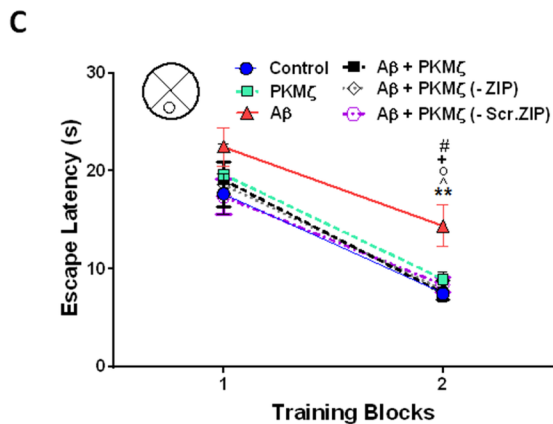
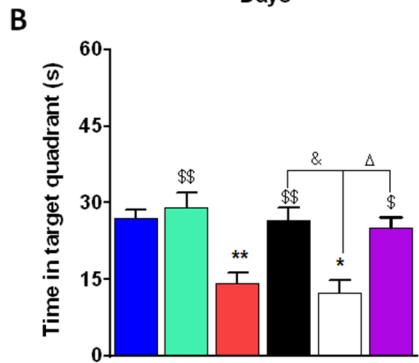
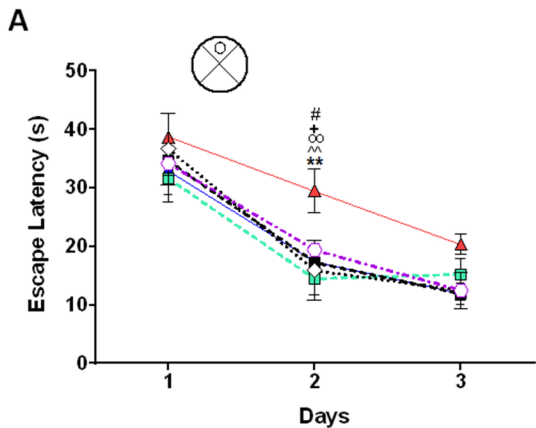


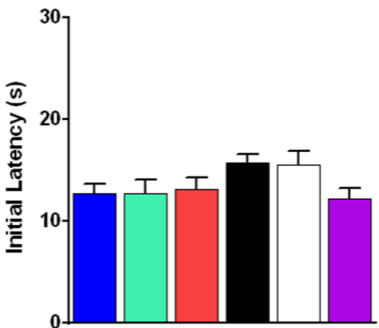
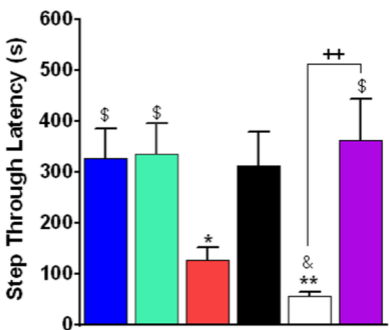
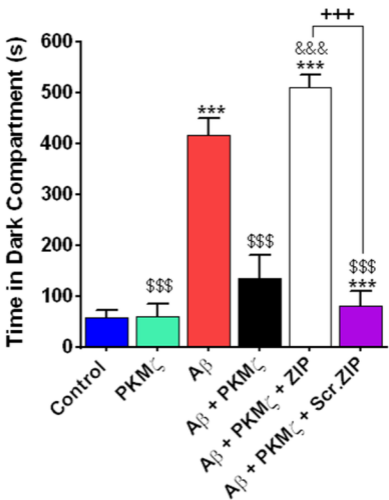
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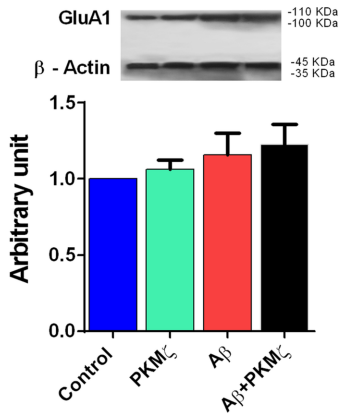
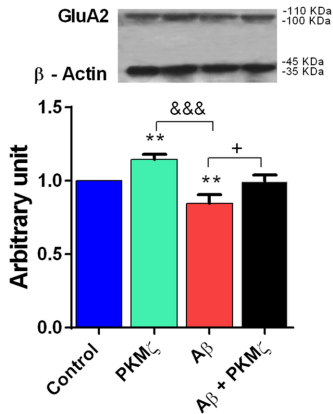


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