Neurobiology of Learning and Memory 95 (2011) 361–375

Contents lists available at ScienceDirect

Neurobiology of Learning and Memory

journal homepage: www.elsevier.com/locate/ynlme

Expression of VGLUTs contributes to degeneration and acquisition of learning and memory

Xiao-Rui Cheng, Yong Yang, Wen-Xia Zhou *, Yong-Xiang Zhang

Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China

ARTICLE INFO

Article history:
Received 25 November 2010
Revised 16 January 2011
Accepted 25 January 2011
Available online 2 February 2011

Keywords:
Learning and memory
Vesicular glutamate transporter
Senescence-accelerated mouse

ABSTRACT

Vesicular glutamate transporters (VGLUTs), which include VGLUT1, VGLUT2 and VGLUT3, are responsible for the uploading of L-glutamate into synaptic vesicles. The expression pattern of VGLUTs determines the level of synaptic vesicle filling (i.e., glutamate quantal size) and directly influences glutamate receptors and glutamatergic synaptic transmission; thus, VGLUTs may play a key role in learning and memory in the central nervous system. To determine whether VGLUTs contribute to the degeneration or acquisition of learning and memory, we used an animal model for the age-related impairment of learning and memory, senescence-accelerated mouse/prone 8 (SAMP8). KM mice were divided into groups based on their learning and memory performance in a shuttle-box test. The expression of VGLUTs and synaptophysin (Syp) mRNA and protein in the cerebral cortex and hippocampus were investigated with real-time fluorescence quantitative PCR and western blot, respectively. Our results demonstrate that, in the cerebral cortex, protein expression of VGLUT1, VGLUT2, VGLUT3 and Syp was decreased in SAMP8 with age and increased in KM mice, which displayed an enhanced capacity for learning and memory. The protein expression of VGLUT2 and Syp was decreased in the hippocampus of SAMP8 with aging. The expression level of VGLUT1 and VGLUT2 proteins were highest in KM mouse group with a 76–100% avoidance score in the shuttle-box test. These data demonstrate that protein expression of VGLUT1, VGLUT2 and Syp decreases age-dependently in SAMP8 and increases in a learning- and memory-dependent manner in KM mice. Correlation analysis indicated the protein expression of VGLUT1, VGLUT2 and Syp has a positive correlation with the capacity of learning and memory.

1. Introduction

L-glutamate, the major excitatory neurotransmitter in the mammalian brain, plays a pivotal role in central nervous system (CNS) functions such as synaptic plasticity, cognition, learning and memory. However, glutamatergic excitotoxicity can occur when there is an excess of glutamate. Synaptic functions related to glutamate are tightly regulated on several levels: its assembly into synaptic vesicles, its release into the synaptic cleft, and its reuptake by glial cells and neurons (Zink, Vollmayr, Gebicke-Haerter, & Henn, 2010). Three distinct vesicular glutamate transporter (VGLUT) isoforms have been identified. These transporters are responsible for the uploading of glutamate into synaptic vesicles; VGLUT1 and VGLUT2 are present at the majority of the excitatory glutamatergic terminals in the CNS, whereas VGLUT3 is sparsely expressed in the brain, defining a discrete subpopulation of nonglutamatergic neurons (Aihara et al., 2000; Bellocchio, Reimer, Fremeau, & Edwards, 2000; Boulland et al., 2004; Fremeau et al., 2001; Gras et al., 2002; Herzog et al., 2001; Takamori, Rhee, Rosenmund, & Jahn, 2001). VGLUT1 and VGLUT2 are specific markers for glutamatergic neurons, whereas VGLUT3 is expressed not only in glutamatergic neurons but also in cholinergic, serotoninergic, and even GABA-ergic neurons (Fremeau et al., 2002; Fremeau et al., 2004; Gras et al., 2002). VGLUT1 and VGLUT2 are still generally considered to be selective markers of the glutamatergic corticostriatal and thalamostriatal afferents in the adult CNS (Kaneko, Fujiyama, & Hioki, 2002; Lacey et al., 2005; Miró-Bernié, Ichinohe, Pérez-Clausell, & Rockland, 2006; Raju et al., 2008). VGLUTs transport glutamate into synaptic vesicles, which affects the glutamate concentrations in the synaptic cleft and plays an essential role in learning and memory in the CNS. However, their role in the degeneration and acquisition of learning and memory is not clear.

Because VGLUTs load glutamate into vesicles for release, the number of VGLUT molecules per synaptic vesicle was shown to be a main factor for determining the quantal size in glutamatergic neurons, and variations in VGLUT expression levels significantly impact synaptic transmission (Ishikawa, Sahara, & Takahashi, 2002; Wilson et al., 2005; Wojcik et al., 2004). For particular brain regions, the expression levels of VGLUTs serve as indicators for the relative synaptic strength of presynaptic glutamatergic innervation (Massie, Schallier, Vermoesen, Arckens, & Michotte, 2010). Analyzing the

* Corresponding author. Fax: +86 10 68211656.
E-mail address: zhouwx@nic.bmi.ac.cn (W.-X. Zhou).

1074-7427/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.nlm.2011.01.010

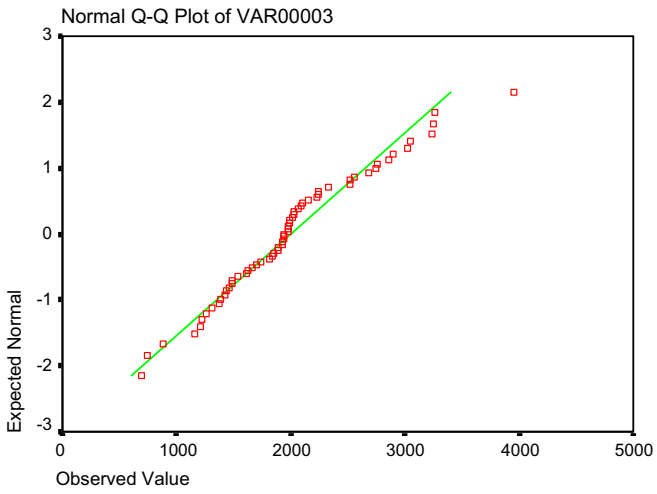

Fig. 1. The normal probability of total distance of KM mice in spontaneous locomotor activity.

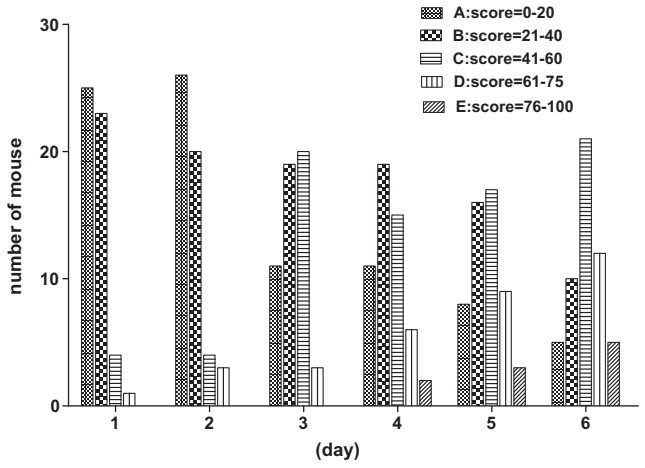

Fig. 2. The performance of KM mouse in the shuttle-box test.

differences in VGLUT expression provides a model system to understand how the targeting of individual synaptic vesicle proteins can influence neurotransmitter signaling and learning and memory.

The senescence-accelerated mouse (SAM) was originally generated from a common genetic pool of AKR/J mice (Takeda et al., 1981). The SAM strains consist of the senescence-accelerated-prone mouse (SAMP) and the senescence-accelerated-resistant mouse (SAMR), with the latter showing normal aging characteristics. SAM resistant/1(SAMR1) are long-lived, and are commonly

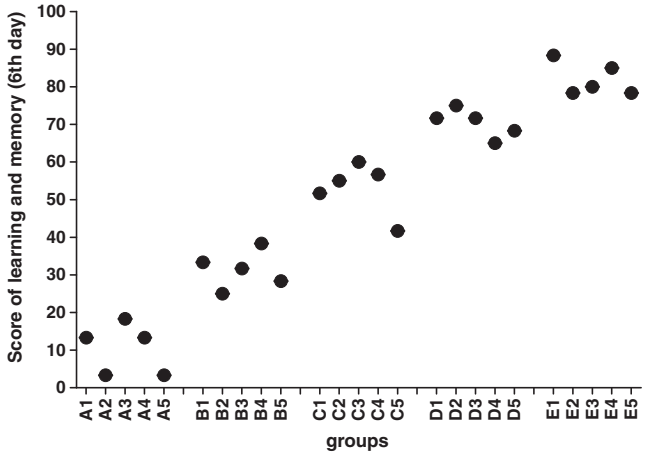

Fig. 4. The performance on the sixth day of mouse selected to be used in the experiment. There was statistically significant difference among all five groups analyzed by using *ANOVA* (GraphPad Prism 5.0).

Table 1
Primers of GAPDH, VGLUT1, VGLUT2, VGLUT3 and Syp in real-time fluorescence quantitative PCR assay.

Gene name	Upper primer (5′–3′–)	Lower primer (5′–3′)	Product
GAPDH	CCTTCCGTGTTCCTACCC	AAGTCGCAGGAGACAACC	163 bp
VGLUT1	TGTTCCTCATAGCCTCCC	GTCCTCCATTTCACTTTCGT	167 bp
VGLUT2	CCCTGGAGGTGCCTGAGAA	GCGGTGGATAGTGCTGTTGTT	173 bp
VGLUT3	CACATCCTTGCCTGTCTAT	GACCCACCTTACTTATTGC	128 bp
Syp	ACAGCAGTGTTCGCTTTCA	GGGTCCCTCAGTTCCTTG	152 bp

used as a control strain. SAM prone/8 (SAMP8) shares similar features with neurodegenerative diseases, especially Alzheimer's disease (AD). Some of these features include learning and memory deficits (Nomura & Okuma, 1999; Spangler et al., 2002), spongy degeneration (Yagi et al., 1989), neuronal cell loss (Kawamata et al., 1997), gliosis (Nomura & Okuma, 1999), alteration of the cholinergic system (Onozuka, Watanabe, Fujita, Tomida, & Ozono, 2002) and other neurotransmitter changes (Flood & Morley, 1998; Kondziella, Bidar, Urfjell, Sletvold, & Sonnewald, 2002; Nomura, Kitamura, Ohnuki, et al., 1997). Additionally, they show altered emotions and abnormality of the circadian rhythm (Miyamoto, 1997), microvessel defects (Ueno et al., 2001), and blood–brain barrier dysfunction (Del Valle et al., 2009; Pelegrí et al., 2007). Furthermore, similar to what occurs in the early stages of AD, SAMP8 mice display increases in oxidative stress (Petursdottir, Farr, Morley, Banks, & Skuladottir, 2007; Zhang et al., 2009), mitochondrial dysfunction (Nakahara et al., 1998), age-related

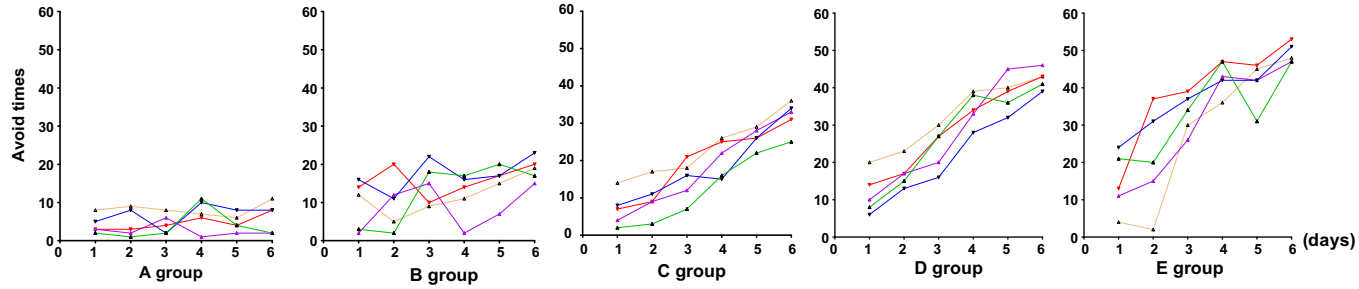

Fig. 3. The performance of mouse selected to be used in the experiment.

Table 2
Antibodies of GAPDH, VGLUT1, VGLUT2, VGLUT3 and Syp in western blot assay.

Protein name	Primary antibody		Secondary antibody	
	Name	Dilution	Name	Dilution
GAPDH	Goat polyclonal IgG (sc-20357, Santa Cruz)	1:300	Peroxidase-conjugated affinipure goat anti-mouse igG (H + L) (ZB-2306, ZSGB-BIO)	1:10,000
VGLUT1	Goat polyclonal antibody (sc-13320, Santa Cruz)	1:400	Peroxidase-conjugated affinipure goat anti-mouse igG (H + L) (ZB-2306, ZSGB-BIO)	1:10,000
VGLUT2	Rabbit polyclonal antibody (Ab72310, Abcam)	1:100	Peroxidase-conjugated affinipure rabbit anti-goat igG (H + L) (ZB-2301, ZSGB-BIO)	1:10,000
VGLUT3	Rabbit polyclonal antibody (Ab23977, Abcam)	1:800	Peroxidase-conjugated affinipure rabbit anti-goat igG (H + L) (ZB-2301, ZSGB-BIO)	1:10,000
SYP	Mouse monoclonal antibody (sc-55507, Santa Cruz)	1:800	Peroxidase-conjugated affinipure mouse anti-rabbit igG (H + L) (ZB-2305, ZSGB-BIO)	1:10,000

increases in the expression of APP, β-amyloid peptide alterations (Morley et al., 2000), and tau phosphorylation (Canudas et al., 2005). From as early as 6 months, SAMP8 have β-amyloid (Aβ) deposition in the hippocampus that increases in quantity and scope with age. These deposits are composed of clustered granules that contain Aβ42, Aβ40, and other Aβ protein precursor fragments. In contrast, control ICR-CD1 mice and SAMR1 mice demonstrate low levels of Aβ clusters that do not develop until 15 months of age (Del Valle et al., 2010). SAMP8 exhibit age-related deterioration in learning and memory compared to SAMR1, which show normal aging characteristics and are commonly used as a control strain (Flood & Morley, 1998; Tajes et al., 2008). Age-related behavioral changes occur in SAMP8 at 4 months of age (Alicja, Markowska, Spangler, Donald, & Ingram., 1998), and age-related deficits in learning and memory occur as early as 2 months after birth (Miyamoto et al., 1986). SAMP8 exhibits significant impairments in active avoidance tasks, such as one-way active avoidance (Miyamoto et al., 1986), T-maze active avoidance (Flood & Morley, 1993) and Sidman avoidance (Ohta et al., 1989). Furthermore, avoidance responses in SAMP8 did not improve with training, as opposed to a linear increase in the SAMR1 control group in the shuttle-box test (Miyamoto, 1997). SAMP8 is used as an age-associated AD animal model due to their age-related deficits in learning and memory (Morley, Banks, Kumar, & Farr, 2004; Ohta

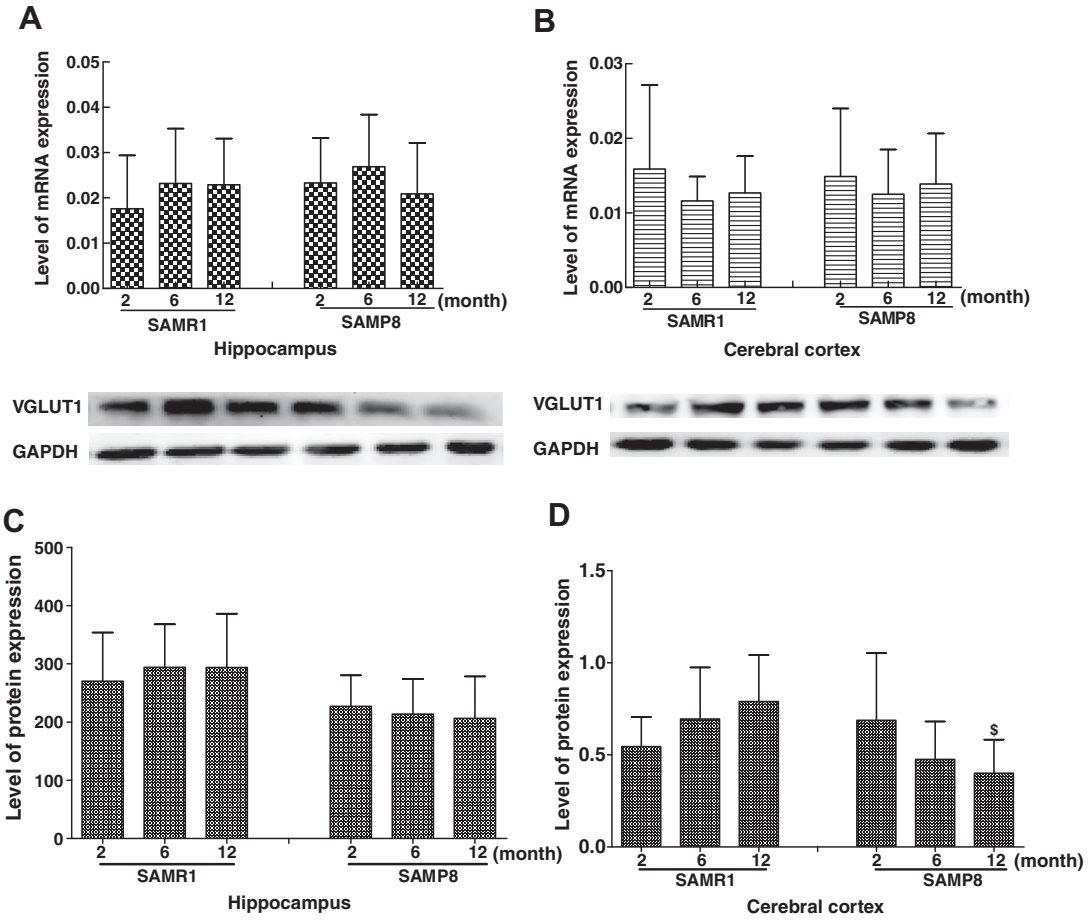

Fig. 5. mRNA and protein expression of VGLUT1 in the cerebral cortex and hippocampus of SAMR1 and SAMP8 with aging; (A) expression of mRNA in the hippocampus; (B) expression of mRNA in the cerebral cortex; (C) expression of protein in the hippocampus; (D) expression of protein in the cerebral cortex; $^{\$}P<0.05$, compared with age-matched SAMR1. mean ± S.D., $n = 10$, *ANOVA*.

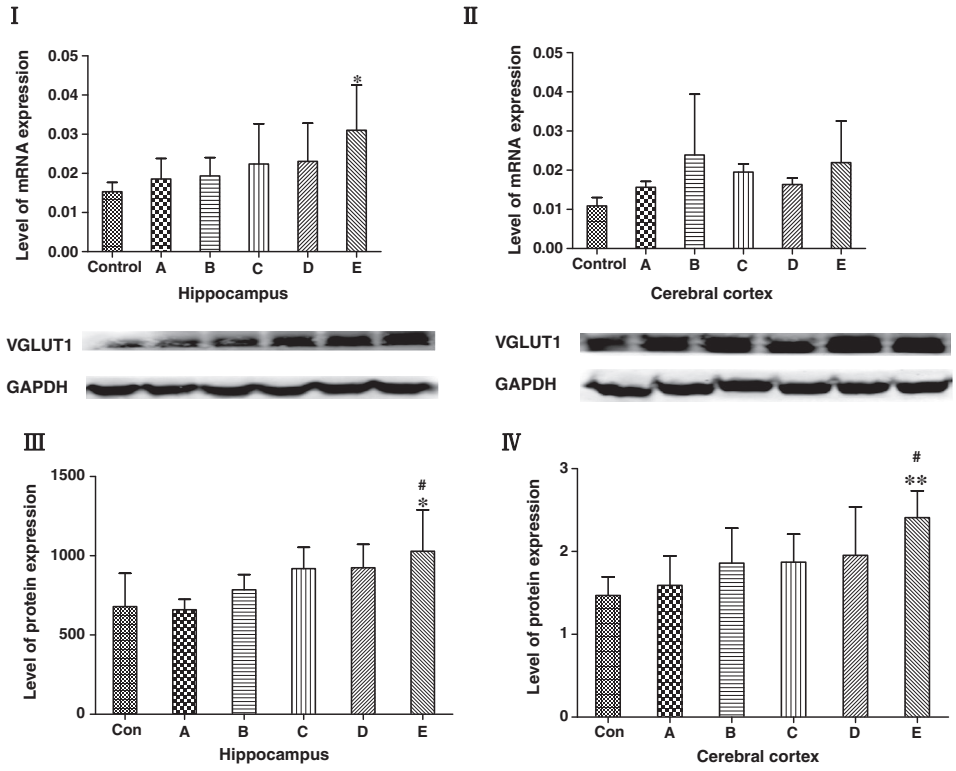

Fig. 6. mRNA and protein expression of VGLUT1 in the cerebral cortex and hippocampus of of KM mice tested by shuttle-box; (I) expression of mRNA in the hippocampus; (II) expression of mRNA in the cerebral cortex; (III) expression of protein in the hippocampus; (IV) expression of protein in the cerebral cortex; Con, control group; A, score = 0–20; B, score = 21–40; C, score = 41–60; D, score = 61–75; E, score = 76–100; $^{*}P < 0.05$, $^{**}P < 0.05$, E *vs* Con; $^{\#}P < 0.05$, E *vs* A. mean ± S.D., $n = 5$, *ANOVA*.

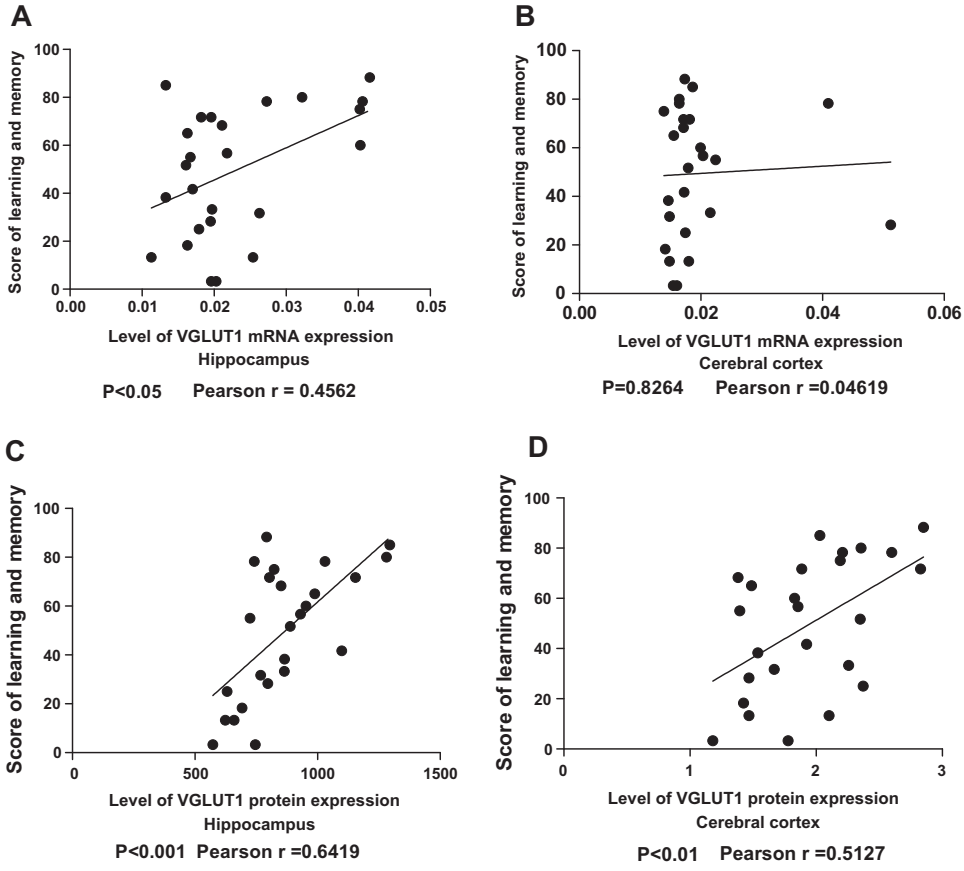

Fig. 7. Correlation between mRNA and protein expression of VGLUT1 in the cerebral cortex and hippocampus of of KM mice tested by shuttle-box and the ability of learning and memory $n = 25$, Two-tailed Pearson analysis, confidence interval 95%.

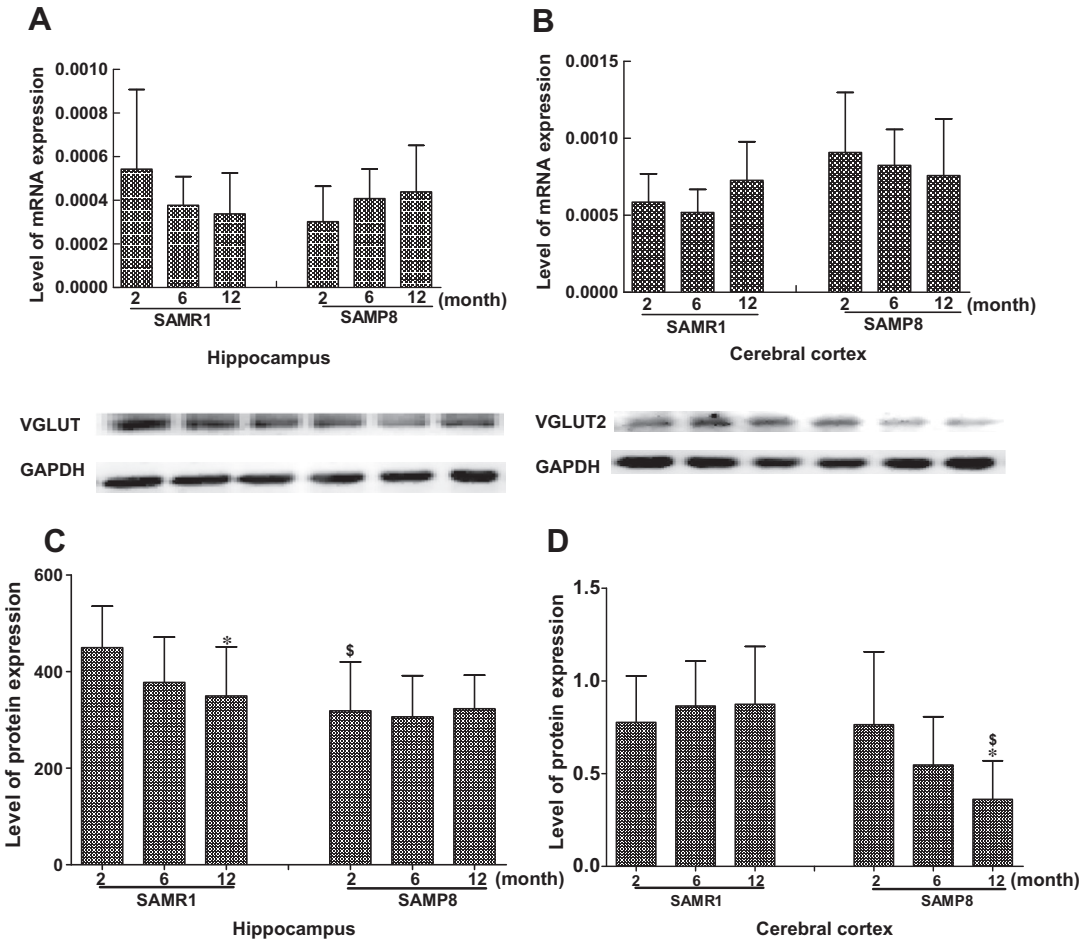

Fig. 8. mRNA and protein expression of VGLUT2 in the cerebral cortex and hippocampus of SAMR1 and SAMP8 with aging; (A) expression of mRNA in the hippocampus; (B) expression of mRNA in the cerebral cortex; (C) expression of protein in the hippocampus; (D) expression of protein in the cerebral cortex; $^{*}P < 0.05$, compared with the same substrain at 2-month-old; $^{\$}P < 0.05$, compared with age-matched SAMR1; mean ± S.D., $n = 10$, *ANOVA*.

et al., 1989; Yagi, Katoh, Akiguchi, & Takeda, 1988). Therefore, SAMP8 can be used to study age-related cognitive decline related to altered gene expression and protein abnormalities associated with AD (Butterfield & Poon, 2005; Morley, 2002).

The shuttle-box behavior test has been used to examine the conditioned active avoidance reflex of animal subjects and was performed in the present study to evaluate learning and memory function. Executing the shuttle-box test is a multi-trial procedure utilizing complex tasks and is used to assess the learning and memory ability of rodents because it is reliable, sensitive and quantitative for active avoidance learning (Bozarth, 1983; Cuomo et al.,1996). The KM mouse strain, which is a Swiss mouse originally generated from non-inbred albino mice and established by Dr. Clara Lynch at the Rockfeller Institute in 1926, was introduced from India Hoffkine institute by Professor Feifan Tang in 1944 and bred in Kunming, China. Because the strain originated in Kunming, it is referred to as KM. In this study, learning performance and memory formation in the KM mice were tested with a shuttle-box test.

The hippocampus and cerebral cortex are brain regions crucial for learning and memory. In this study, we used SAMP8 as an animal model of age-related impairment in learning and memory. KM mice were divided into groups based on their learning and memory ability as determined by their performance in the shuttle-box test. The expression of VGLUT1-3 and synaptophysin (Syp) mRNA and protein in the cerebral cortex and hippocampus of the tested subjects was examined employing real-time fluorescence quantitative PCR and western blot, respectively, to determine whether VGLUT expression correlates with the process of learning and memory.

2. Materials and methods

2.1. Subjects

Original SAMR1 and SAMP8 mice were kindly provided by Dr. T. Takeda at Kyoto University (Kyoto, Japan) and housed in the Beijing Institute of Pharmacology and Toxicology under a natural light–dark cycle (12 h light:12 h dark), room temperature (25 ± 1 °C), and normal relative humidity (50 ± 5%). The animals had free access to food and water. Two-, six- and twelve-month-old male SAMP8 and SAMR1 mice were used in this study, with 10 mice per substrain. Each group ($n = 10$) was sacrificed by decapitation, and their brains were removed and placed on ice before dissecting out the hippocampus and cerebral cortex. Both sides (left and right) of the hippocampus and cerebral cortex were used for real-time PCR and western blot, respectively.

Male KM mice weighing 18–20 g were obtained from the Laboratory Animal Center of Beijing Institute of Medical Science and kept on a 12:12 h light:dark cycle at room temperature (25 ± 1 °C) and normal relative humidity (50 ± 5%). Food and water were available *ad libitum* throughout the shuttle-box test. After a 3 day acclimation to the laboratory conditions and the detection of locomotor activity, the learning and memory ability of 61 male KM mice were evaluated using the shuttle-box test.

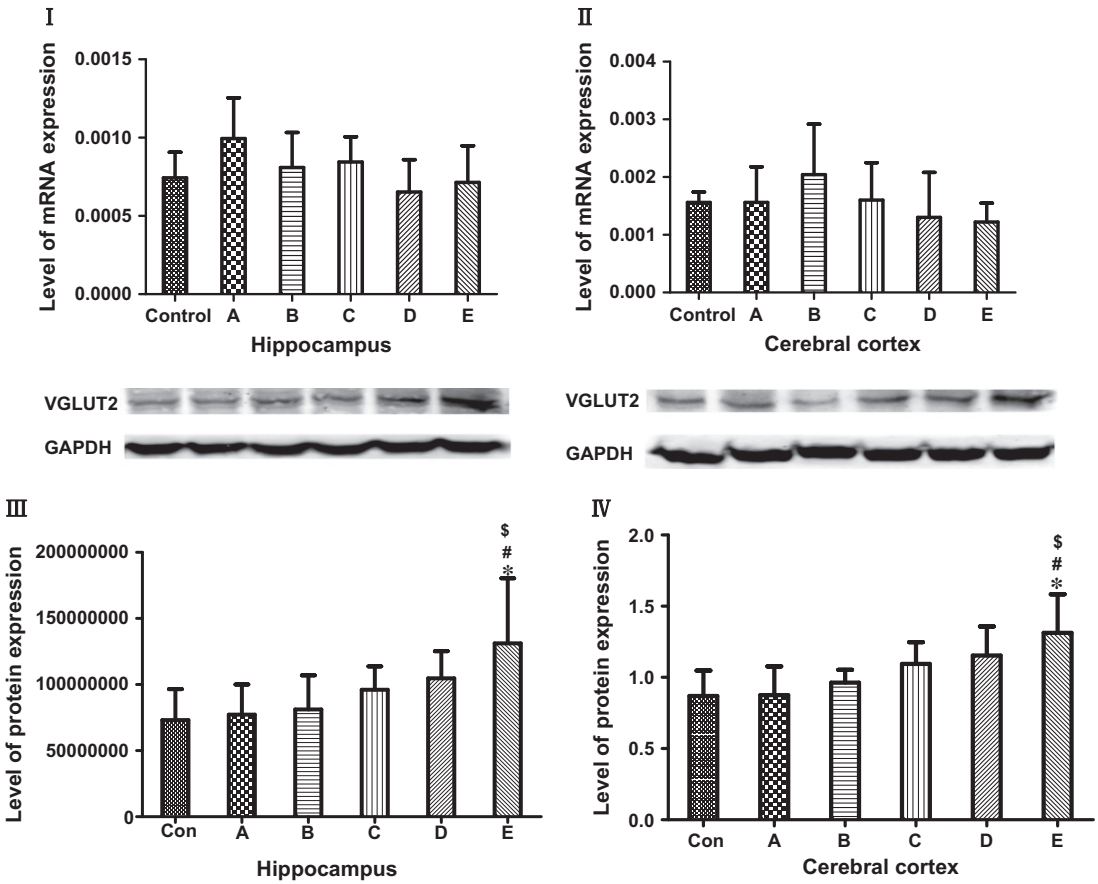

Fig. 9. mRNA and protein expression of VGLUT2 in the cerebral cortex and hippocampus of KM mice tested by shuttle-box; (I) expression of mRNA in the hippocampus; (II) expression of mRNA in the cerebral cortex; (III) expression of protein in the hippocampus; IV, expression of protein in the cerebral cortex; Con, control group; A, score = 0–20; B, score = 21–40; C, score = 41–60; D, score = 61–75; E, score = 76–100; $^{*}P < 0.05$, E *vs* Con; $^{\#}P < 0.05$, E *vs* A; $^{\$}P < 0.05$, E *vs* B. mean ± S.D., $n = 5$, *ANOVA*.

All experimental procedures were performed following with the Guidelines of the Animal Care and Use Committee of Beijing Institute of Pharmacology and Toxicology.

2.2. Behavioral procedures

2.2.1. Locomotor activity

The spontaneous locomotor activity of KM mice was recorded for 15 min. Motor tracking was performed using an experimental animal behavior monitoring system (AniLab v2.0, AniLab™ Software and Instruments Co., Ltd., China). Prior to the start of recording, the animals were habituated for 5 min in Plexiglas cages (30 × 30 × 30 cm, width × length × height; one animal per testing cage). Total distance per mouse was indicative of its motor activity, and only KM mice with total distances following the normal distribution were employed for the shuttle-box test (Fig. 1).

2.2.2. Shuttle-box test

KM mice were tested in the shuttle-box apparatus (Med Associates Inc., EAST FAIRFIELD, VT 05448, USA), which was divided into two equal compartments connected by a gate. A light (8 W) was switched on alternately in the two compartments and used as the conditioned stimulus. The conditioned stimulus was presented for 5 s, followed by the unconditioned stimulus (a 1.5 mA foot shock) for a maximum of five seconds. The electric shock (5 s) was applied to the grid floor. The shuttle-box procedure was performed every day between 6:00 PM–5:00 AM. Each mouse was allowed 5 min to acclimate to the chambers before testing began. At the beginning of the test, the mouse was placed in the left compartment of the shuttle-box, close to and facing the end wall. If the mouse moved to the opposite compartment during the conditioned stimulus, an active avoidance response was recorded automatically. Each mouse was given 60 consecutive trials with 20 s intertrial intervals (lighting, 5 s; interval, 10 s; electric shock, 5 s; interval, 20 s) daily for six consecutive days. The learning and memory ability of each KM mouse was assessed as the percentage of the ratio of active avoidance response number to the total avoidance (number is 60). For example, if one mouse's active avoidance times are 42 in the test, its score is the percent of the ratio of 42–60, so its score is 70.

All test subjects were divided into six groups depending on the active avoidance response number on the sixth trial day (Figs. 2 and 4). The control group (con) had five mice that received no shock; group A contained five mice with 0–12 times of avoidance, and their score was 0–20; group B had 10 mice with 13–24 times of avoidance, and their score was 21–40; group C had 21 mice with 25–36 times of avoidance, and their score was 41–60; group D had 12 mice with 37–45 times of avoidance and a score of 61–75; and group E had five mice with 46–60 times of avoidance and a score of 76–100. Using linear regression analysis, the mouse with a correlation coefficient from high to low was used in the next experiment (Figs. 3 and 4).

Each group ($n = 5$) was processed as described above.

2.3. Total RNA preparation and reverse transcription

Total RNA was isolated from the hippocampus and cerebral cortex using TRIzol reagent (Cat. No. 15596–026, Invitrogen) according to the manufacturer's instructions. The total RNA extracted was measured at 260 nm, and the purity of the samples

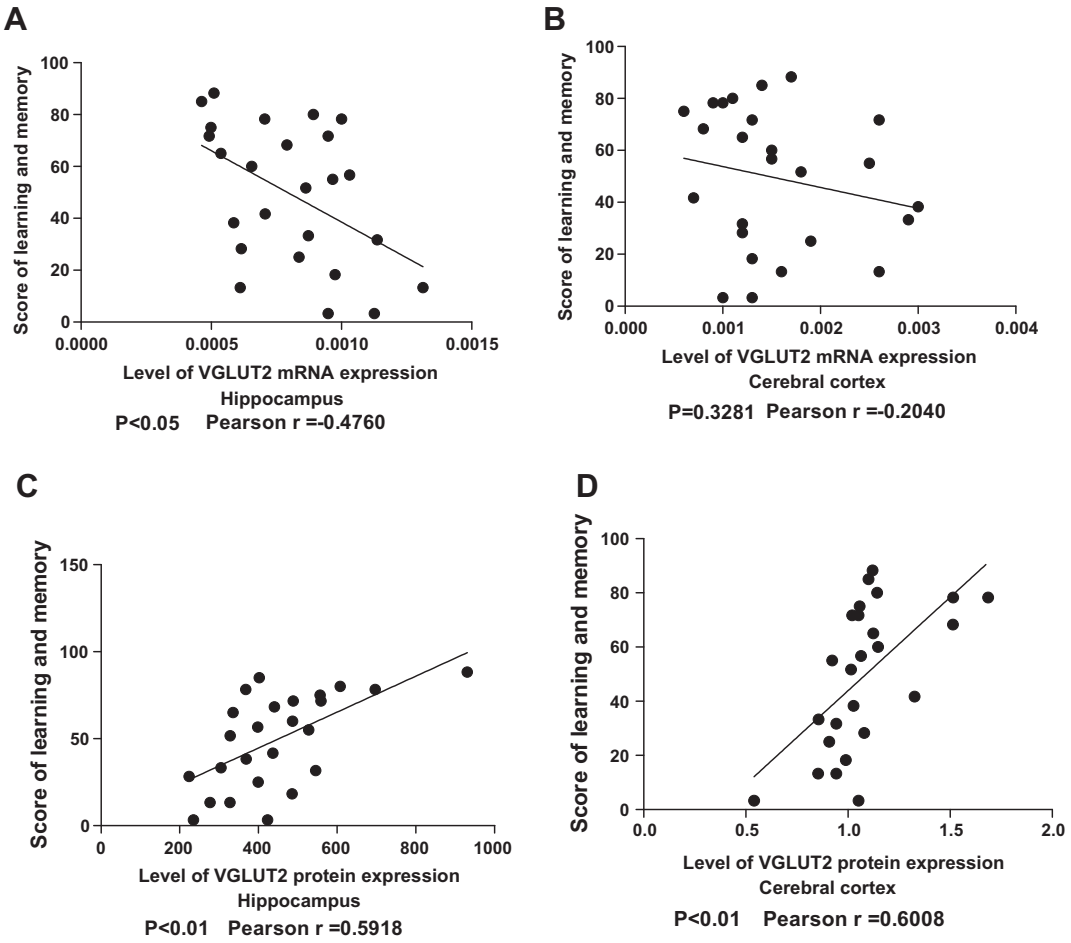

Fig. 10. Correlation between mRNA and protein expression of VGLUT2 in the cerebral cortex and hippocampus of KM mice tested by shuttle-box and the ability of learning and memory n = 25, Two-tailed Pearson analysis, confidence interval 95%.

was determined by the A260/280 ratio using spectrophotometric measurements (UV-2802H, UNICO). The integrity of total RNA was detected by denaturing formaldehyde 1% agarose/ethidium bromide gel electrophoresis. First-strand cDNA was reverse-transcribed from 2 μg of total RNA using MMLV Reverse Transcriptase (Cat No. M1705, Promega), Oligo (dT) 15 primers (Cat.# CD106, TianGen, 10 μM) and dNTP mixture (Cat.# CD117-1, TianGen, 10 mM each). The cDNA was stored at −20 °C for real-time fluorescence quantitative PCR analysis.

2.4. Real-time fluorescence quantitative PCR

The cDNA was used as template for PCR in the iTaqTM SYBRR Green Supermix with ROX (172–5851, BIO-RAD). The reaction was performed at 95.0 °C for 2 min, 40 cycles (95.0 °C for 15 s, 55 °C for 30 s, 72 °C for 45 s), auto-dissociation, data collection at stage 2 step 3, using the thermal cycler (7500, Applied Biosystems), and primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), VGLUT1, VGLUT2, VGLUT3 and synaptophysin (Syp) (Table 1).

The melting curve analysis verifies the presence of primer dimers and specificity in the PCR product. The assay software was ddCt (relative quantitation) plate and ddCt (relative quantitation) of the SDS v1.4 software package. The endogenous control was GAPDH, and the target gene was VGLUT1, VGLUT2, VGLUT3 and Syp. The confidence interval was 95.00%, with baseline starting at cycle 3 and ending at cycle 15. The calculations were performed based on the Ct method. The level of target gene expression was calculated using the formula $2^{-\Delta CT}$.

2.5. Western blot

The cerebral cortex and hippocampus tissues were homogenized in lysis buffer containing Tris, urea, thiourea, 4% chaps, DTT, and complete™ cocktail protease inhibitor (1169748001, Roche), extracted by liquid nitrogen freeze thawing and supersonic quassation, and then centrifuged for 30 min at 20,000 rpm. The final supernatants were aliquoted and stored at −70 °C. The protein concentrations were determined using a Bradford protein assay kit (PR102, Galen Biopharm International Co., Ltd.).

Samples containing equal protein amounts (50 μg) were boiled in 5× loading buffer (B1012, Applygen Technologies Inc.). The denatured protein and prestained molecular weight standards were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis mini-gels. Electrophoresis was performed at 60 V for concentration and 100 V for separation in Mini-Protean (Mini-PROTEAN® Tetra cell, Bio-Rad). After electrophoresis, proteins were transferred on ImBlotter-nitrocellulose membrane (HAHY00010, Millipore) with transfer buffer (48 mM Tris; 39 mM glycine; 0.0037% SDS and 20% methanol) at constant 15 V for 30 min using a Trans-blot SD semi-dry transfer cell (Bio-Rad). The membrane was blocked with 5% non-fat milk in TBS containing 0.05% Tween 20 for 4 h at room temperature. The membrane was immunoblotted with the primary antibody (Table 2) in TBST

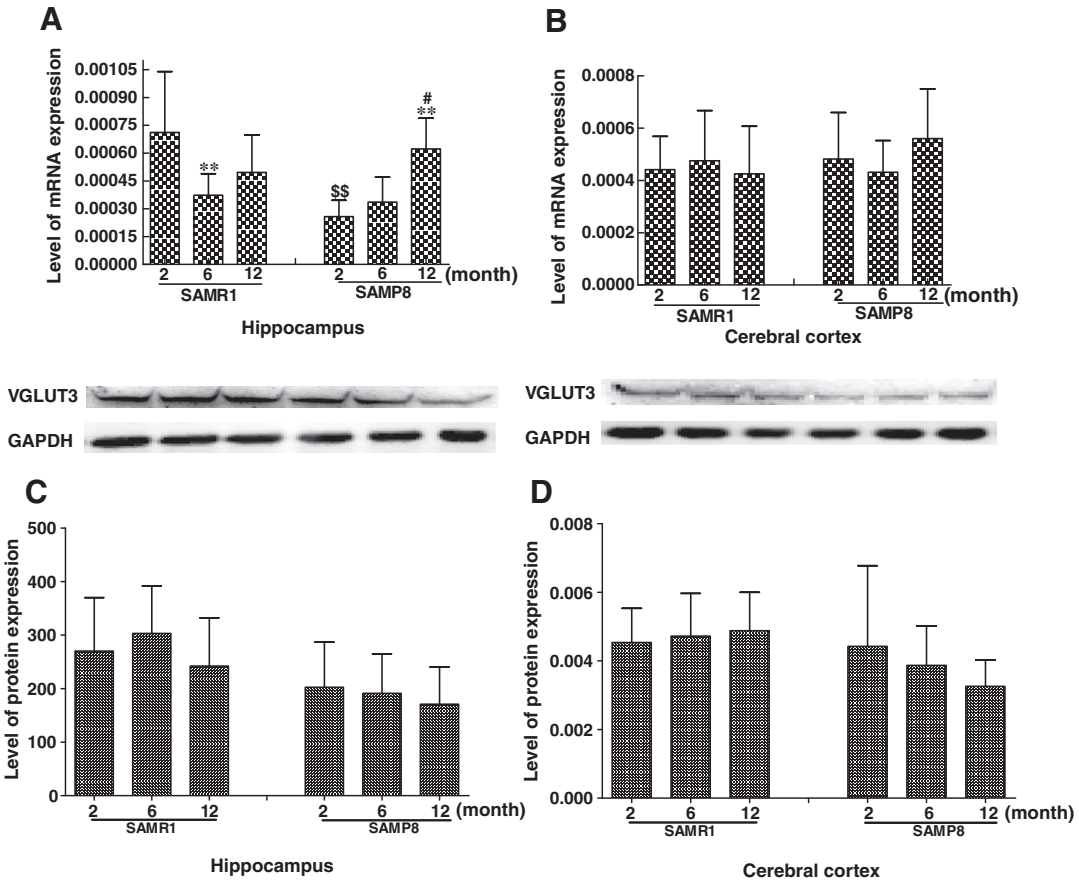

Fig. 11. mRNA and protein expression of VGLUT3 in the cerebral cortex and hippocampus of SAMR1 and SAMP8 with aging; (A) expression of mRNA in the hippocampus; (B) expression of mRNA in the cerebral cortex; (C) expression of protein in the hippocampus; (D) expression of protein in the cerebral cortex; $^{**}P < 0.01$, compared with the same substrain at 2-month-old; $^{\#}P < 0.05$, compared with the same substrain at 6-month-old; $^{\$\$}P < 0.01$, compared with age-matched SAMR1; mean ± S.D., $n = 6–10$, *ANOVA*.

solution containing 5% non-fat milk at 4 °C overnight. After being washed with TBST five times (5 min each), the membrane was incubated with the appropriate secondary antibody (Table 2) in TBST for 45 min at 37 °C. After being washed, the immunoblots were developed with SuperSignal WestPico Chemiluminescent Substrate (34080, Thermo Scientific) and quantified using the ImageQuant LAS 4000 mini (GE healthcare, USA) and were expressed as the ratio relative to GAPDH protein.

2.6. Statistical analysis

For the expression of mRNA and protein expression, all data are presented as the mean ± standard deviation (S.D.). In all cases, data were analyzed using ANOVA (GraphPad Prism 5.0) to evaluate differences. Differences were considered statistically significant if $P < 0.05$ and $P < 0.01$. Graphs were created using GraphPad Prism 5.0.

Correlation between expression of mRNA and protein and capacity of learning and memory analysis was performed using two-tailed Pearson analysis with a 95% confidence interval using GraphPad Prism 5.0, and the correlation was considered statistically significant if $P < 0.05$, $P < 0.01$ and $P < 0.001$.

3. Results

3.1. Expression of VGLUT1 in the cerebral cortex and hippocampus of aging SAMP8 and KM mice following shuttle-box test

Differences in VGLUT1 mRNA expression from the hippocampus and cerebral cortex were not observed (Fig. 5A and B) between aging SAMR1 and SAMP8. There was a tendency towards increased expression of VGLUT1 protein in the hippocampus of SAMR1 with aging but a decreased expression level was found in SAMP8 (Fig. 5C). There was a tendency towards increased expression of VGLUT1 protein in the cerebral cortex of SAMR1 with aging (Fig. 5D), but there was a significant decrease in the expression level in the cerebral cortex of 12-month-old SAMP8 compared with age-matched SAMR1 ($P < 0.05$) (Fig. 5D). These results showed that VGLUT1 protein expression was decreased in the cerebral cortex and hippocampus of SAMP8, which is associated with a deterioration of learning and memory.

Messenger RNA and protein expression of VGLUT1 trended towards an increase in the hippocampus of KM mice following the shuttle-box test and were significantly elevated in group E (score 76–100) compared to the control group ($P < 0.05$) (Fig. 6I and III), VGLUT1 protein expression was also significantly elevated in the hippocampus of group E as compared to group A with a score 0–20 ($P < 0.05$) (Fig. 6III). Changes in mRNA expression levels were not detected for VGLUT1 in the cerebral cortex of KM mice following the shuttle-box test (Fig. 6II), whereas protein expression was increased significantly in group E when compared with the control group ($P < 0.01$) and group A ($P < 0.05$) (Fig. 6IV). These findings indicate that VGLUT1 protein expression was increased in the cerebral cortex and hippocampus of KM mice and suggest that this is correlated with an elevation in learning and memory ability.

Correlation analysis (Fig. 7) indicated that VGLUT1 mRNA expression in the hippocampus of KM mice following the shuttle-box test positively correlated with learning and memory ($P < 0.05$) (Fig. 7A), which is also found with protein expression

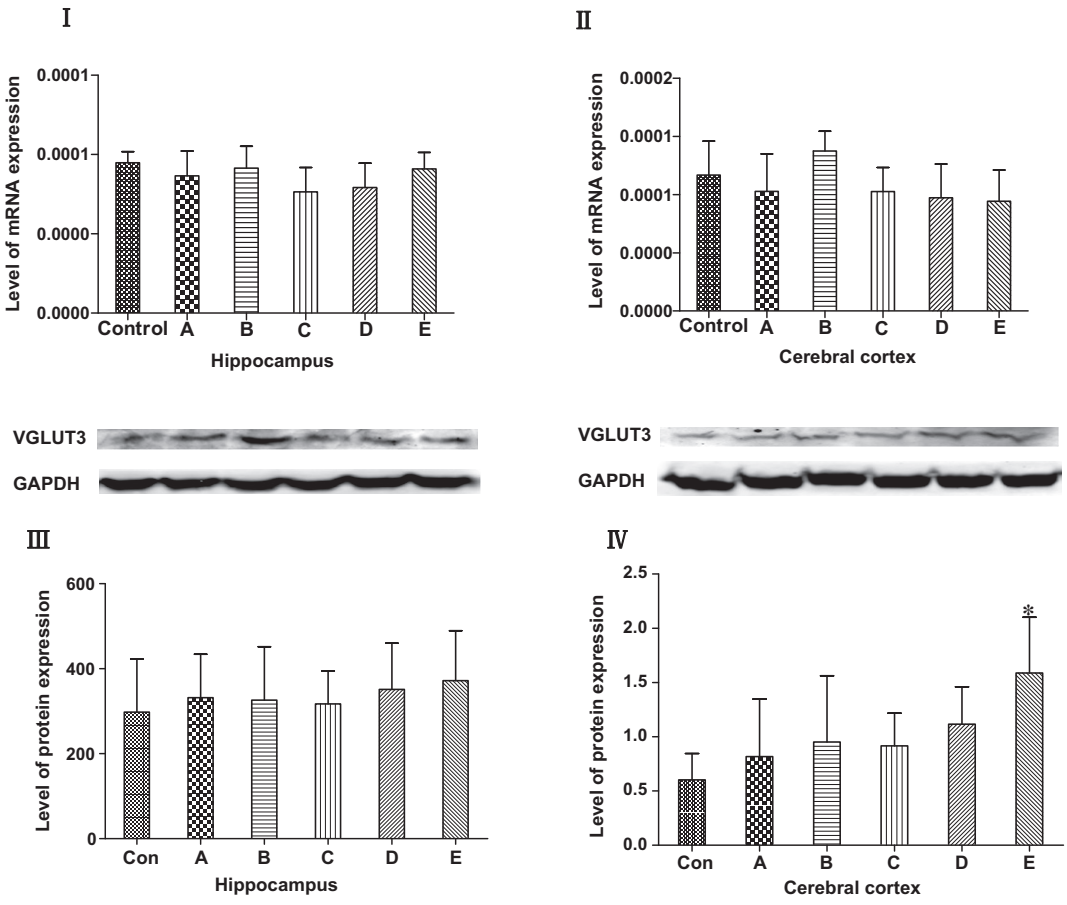

Fig. 12. mRNA and protein expression of VGLUT3 in the cerebral cortex and hippocampus of KM mice tested by shuttle-box; (I) expression of mRNA in the hippocampus; (II) expression of mRNA in the cerebral cortex; (III) expression of protein in the hippocampus; (IV) expression of protein in the cerebral cortex; Con, control group; (A) score = 0–20; (B) score = 21–40; (C) score = 41–60; (D) score = 61–75; (E) score = 76–100.

in the hippocampus ($P < 0.001$) (Fig. 7C) and cerebral cortex ($P < 0.01$) (Fig. 7D).

3.2. Expression of VGLUT2 in the cerebral cortex and hippocampus of aging SAMP8 and KM mice following the shuttle-box test

VGLUT2 mRNA expression trended towards a decrease in the hippocampus of SAMR1 with aging, which is in contrast to SAMP8 with aging where mRNA expression was increased (Fig. 8A). VGLUT2 protein expression in the hippocampus (Fig. 8C) of 12-month-old SAMR1 was significantly lower than 2-month-old SAMR1 ($P < 0.05$). Compared with age-matched SAMR1, the VGLUT2 protein expression level was also significantly reduced in the hippocampus of 2-month-old SAMP8 ($P < 0.05$). A comparison of VGLUT1 and VGLUT2 expression in the hippocampus of SAMR1 and SAMP8 revealed the possibility of complementary VGLUT1 and VGLUT2 function in the same brain region.

We could not detect a significant change in VGLUT2 mRNA levels in the cerebral cortex of SAMR1 and SAMP8 (Fig. 8B). However, for VGLUT2 protein (Fig. 8D), there was a trend toward a decreased expression level in cerebral cortex of aging SAMP8 and a significant decrease in SAMP8 at 12-month-old as compared to 2-month-old SAMP8 and age-matched SAMR1 ($P < 0.05$). This indicates that VGLUT2 protein expression was reduced in the cerebral cortex and hippocampus of SAMP8, coinciding with age-related impairment of learning and memory.

We could not detect any significant alterations in VGLUT2 mRNA levels in the cerebral cortex or hippocampus of KM mice following shuttle-box training (Fig. 9I and II), but protein expression of VGLUT2 was significantly elevated in group E (score 76–100) compared with the control group ($P < 0.05$), group A (score 0–20, $P < 0.05$), and group B (score 20–40, $P < 0.05$) (Fig. 9III and IV). This demonstrated that VGLUT2 protein expression was increased in the brain of KM mice with enhanced learning and memory capability.

Correlation analysis (Fig. 10) indicated that VGLUT2 mRNA expression in the hippocampus of KM mice following the shuttle-box test was negatively correlated with the ability to increase learning and memory ($P < 0.05$) (Fig. 10A). In contrast, the protein expression in the hippocampus ($P < 0.01$) (Fig. 10C) and cerebral cortex ($P < 0.01$) (Fig. 10D) was positively correlated with the ability to perform learning and memory.

3.3. Expression of VGLUT3 in the cerebral cortex and hippocampus of aging SAMP8 and KM mice following the shuttle-box test

Messenger RNA expression of VGLUT3 in the hippocampus of SAMR1 at 6 months was lower than at 2 months ($P < 0.01$), and it tended to increase in the hippocampus of aging SAMP8. Furthermore (Fig. 11A), VGLUT3 was increased significantly in 12-month-old SAMP8 compared with 2-month-old ($P < 0.01$) and 6-month-old SAMP8 ($P < 0.05$) but was decreased significantly in 2-month-old SAMP8 compared with age-matched SAMR1 ($P < 0.01$). Age-related changes in the expression level of VGLUT3 mRNA could not be observed in the cerebral cortex nor could protein in the hippocampus or cerebral cortex of SAMR1 and SAMP8; however, there was a tendency toward a decreased expression level in aging SAMP8 (Fig. 10B–D).

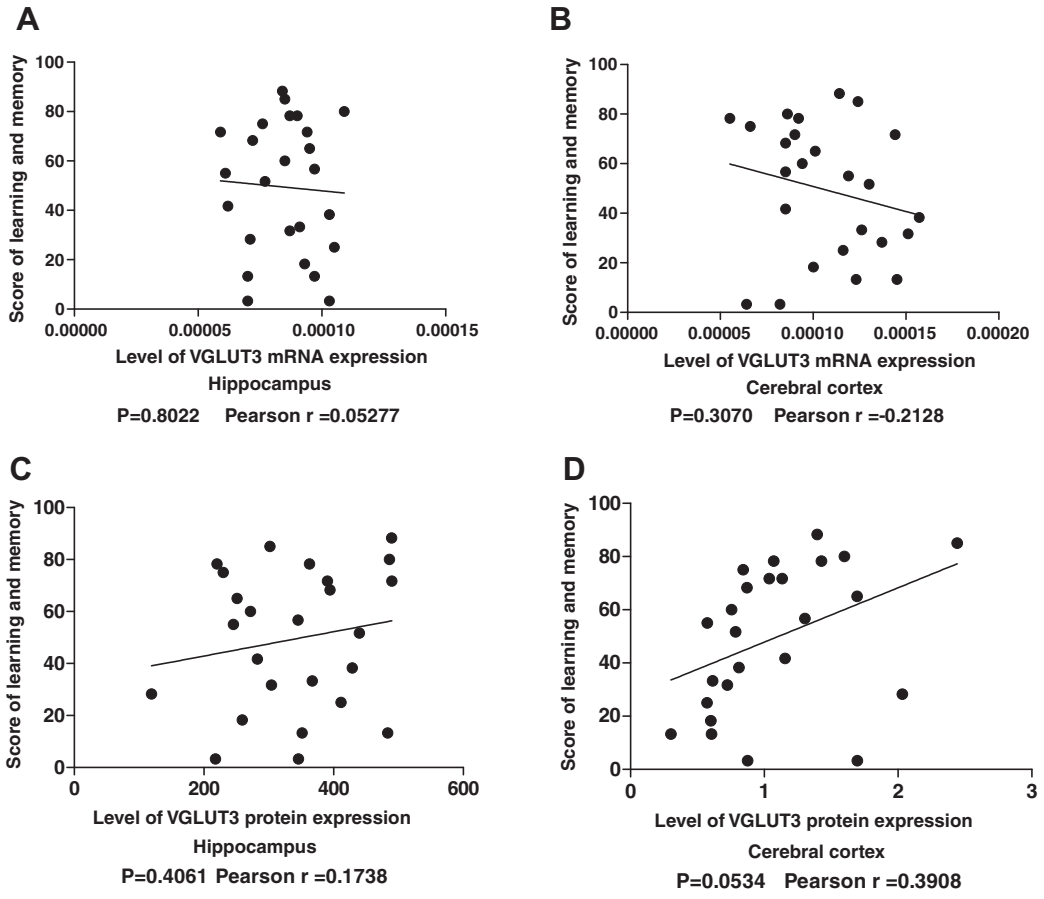

Fig. 13. Correlation between mRNA and protein expression of VGLUT3 in the cerebral cortex and hippocampus of KM mice tested by shuttle-box and the ability of learning and memory $n = 25$, Two-tailed Pearson analysis, confidence interval 95%.

Following the shuttle-box training, we could not detect a significant alteration in VGLUT3 mRNA expression levels in either the hippocampus or cerebral cortex of KM mice (Fig. 12I and II) as well as protein expression in the hippocampus (Fig. 12III). However, for VGLUT3 protein, there was a tendency toward an increased expression level in the cerebral cortex and was elevated significantly in group E (score 76–100) compared with the control group ($P < 0.05$) (Fig. 12IV).

Correlation analysis (Fig. 13) indicated that there was no correlation of VGLUT3 mRNA and protein expression in the hippocampus and cerebral cortex of KM mice with learning and memory performance in the shuttle-box test.

3.4. Expression of Syp in the cerebral cortex and hippocampus of aging SAMP8 and KM mice following the shuttle-box test

There was no difference in Syp mRNA expression in the hippocampus or cerebral cortex of aging SAMR1 or SAMP8 (Fig. 14A and B). In addition, there was no change in the protein expression levels in SAMR1, but Syp protein expression showed an age-dependent decline in the hippocampus and cerebral cortex of SAMP8 (Fig. 14C and D). Syp protein expression was decreased significantly in the hippocampus of 12-month-old SAMP8 (Fig. 14C) compared to 2-month-old ($P < 0.01$) and 6-month-old SAMP8 ($P < 0.05$) and age-matched SAMR1 ($P < 0.01$). In the cerebral cortex of 12-month-old SAMP8, the protein levels were also reduced as compared to age-matched SAMR1 ($P < 0.05$) (Fig. 14D).

Changes in Syp mRNA expression levels were not detected in the cerebral cortex (Fig. 15II), and protein levels were unchanged in the hippocampus of KM mice following the shuttle-box test (Fig. 15III). However, Syp mRNA expression was upregulated significantly in the hippocampus of group C (score 40–60) compared to the control group ($P < 0.05$) (Fig. 15I). In the cerebral cortex, protein expression was significantly upregulated in group E (score 76–100) compared with the control group ($P < 0.01$), group A (score 0–20, $P < 0.01$), and group B (score 20–40, $P < 0.05$) (Fig. 15IV).

Correlation analysis (Fig. 16) indicates that Syp protein expression in the hippocampus ($P < 0.05$(Fig. 16C) and cerebral cortex ($P < 0.01$) (Fig. 16D) was positively correlated with learning and memory performance.

4. Discussion

In this study, we showed that protein expression of VGLUT1, VGLUT2, VGLUT3 and Syp was decreased in an age-dependent manner in the cerebral cortex of SAMP8 with age-related deterioration of learning and memory. Expression of these proteins was at the highest level in the cerebral cortex of mice in group E with an avoidance score of 76–100% following the shuttle-box test. In addition, we found that there was an increase in the protein expression of VGLUT1 and VGLUT2 in the hippocampus of KM mice with increased learning and memory performance and a trend toward a decrease in VGLUT1, VGLUT2, VGLUT3 and Syp in the hippocampus of SAMP8 with age-related deficits in learning and memory. Correlation analysis indicated that the protein expression of VGLUT1, VGLUT2 and Syp positively correlated with learning and memory ability. This raises the question of how differences in VGLUT expression affect learning and memory ability.

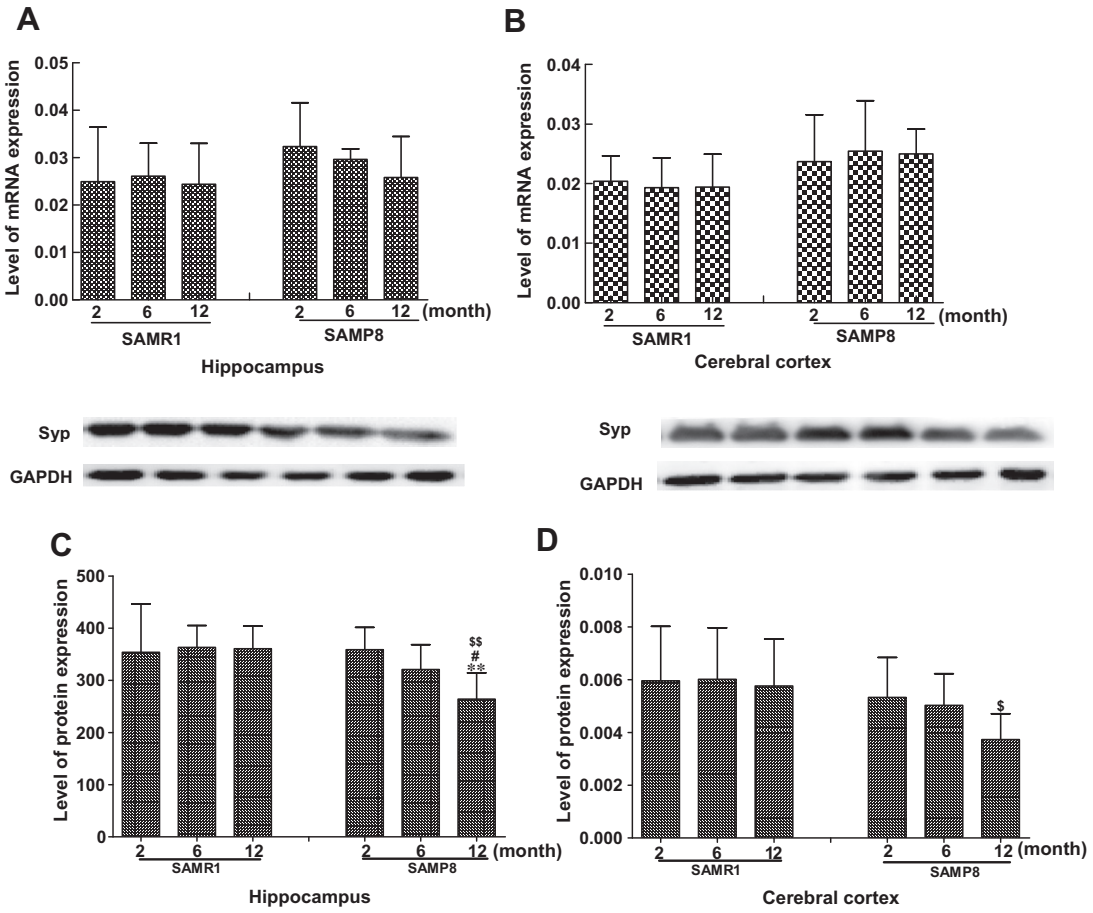

Fig. 14. mRNA and protein expression of Syp in the cerebral cortex and hippocampus of SAMR1 and SAMP8 with aging; (A) expression of mRNA in the hippocampus; (B) expression of mRNA in the cerebral cortex; (C) expression of protein in the hippocampus; D, expression of protein in the cerebral cortex; $^{**}P < 0.01$, compared with the same substrain at 2-month-old; $^{\#}P < 0.05$, compared with the same substrain at 6-month-old; $^{\$}P < 0.01$, $^{\$\$}P < 0.01$, compared with age-matched SAMR1; mean ± S.D., $n = 10$, *ANOVA*.

VGLUTs are responsible for transporting glutamate into synaptic vesicles and are specifically required for exocytotic release (Reimer & Edwards, 2004; Takamori, 2006). For glutamatergic synapses, a single functional vesicular glutamate transporter is both necessary and sufficient to fill a synaptic vesicle. However, elevated VGLUT expression increases the quantal size of vesicle, and vesicles without VGLUT are empty (Daniels et al., 2006). Therefore, the expression level of VGLUTs determines the amount of glutamate that is loaded into vesicles and released and thereby regulates the efficacy of neurotransmission (Ishikawa et al., 2002; Wojcik et al., 2004). Another study has shown that VGLUT expression is used endogenously to directly regulate the extent of glutamate release, which provided a concise, presynaptic mechanism for controlling the quantal efficacy of excitatory transmission during synaptic refinement and plasticity (Wilson et al., 2005). Therefore, we demonstrated that VGLUT expression was positively correlated with the ability to learn and memorize, and we suggest that this occurs by affecting glutamatergic signaling, which affects behavior.

A study has recently demonstrated that Ts65Dn (TS) mice with cognitive disturbances exhibited a significantly reduced labeling of VGLUT1 compared to disomic control mice. After the TS mice were treated with memantine, the levels of hippocampal VGLUT1 were significantly increased, with levels similar to vehicle-treated control animals (Rueda et al., 2010). Furthermore, reduced expression of VGLUT1 was found in learned helpless rats (Zink et al., 2010). In addition, significantly lower immunoreactivity for VGLUT1 in AD patients was reported (Kirvell, Fremeau, & Francis, 2002). Other studies have shown that the loss of vesicular glutamate transporters, VGLUT1 and VGLUT2, in the prefrontal cortex was correlated with cognitive decline in Alzheimer's disease (AD) (Kashani et al., 2008), and glutamatergic dystrophic neuritis and structural glutamatergic deficits are present in AD pathology (Bell et al., 2006). These studies are consistent with our findings that protein expression of VGLUT1 and VGLUT2 was decreased age-dependently in the cerebral cortex and hippocampus of SAMP8 with age-related deterioration in learning and memory. These results also suggest that the reduced expression of VGLUTs is involved in the pathology of some neuropsychiatric disorders through glutamatergic signaling. For example, mice with reduced expression of VGLUT1 exhibit enhanced anxiety, depressive-like behavior and impaired recognition memory (Tordera et al., 2007). Decreased VGLUT1 in the forebrain increased vulnerability to depressive-like behavior and chronic mild stress (Garcia-Garcia et al., 2009). Additionally, expression levels of VGLUT1 were reduced in animal models of depression (Zink et al., 2010). The reduced levels of VGLUT2 decrease motor neuron degeneration but do not prevent the loss of motor neuron function in the $SOD1^{G93A}$ mouse model for amyotrophic lateral sclerosis (ALS) (Wootz, Enjin, Wallén-Mackenzie, Lindholm, & Kullander, 2010). VGLUT2 heterozygotes exhibit decreases in neuropathic pain responses (Moechars et al., 2006), and there were 9 rare variants of VGLUT2 that may contribute to the pathogenesis of schizophrenia (Shen et al., 2010). Lastly, mice lacking VGLUT3 have sensorineural deafness and seizures (Seal et al., 2008). These data indicate that decreased expression of VGLUTs results in homeostatic changes in several systems.

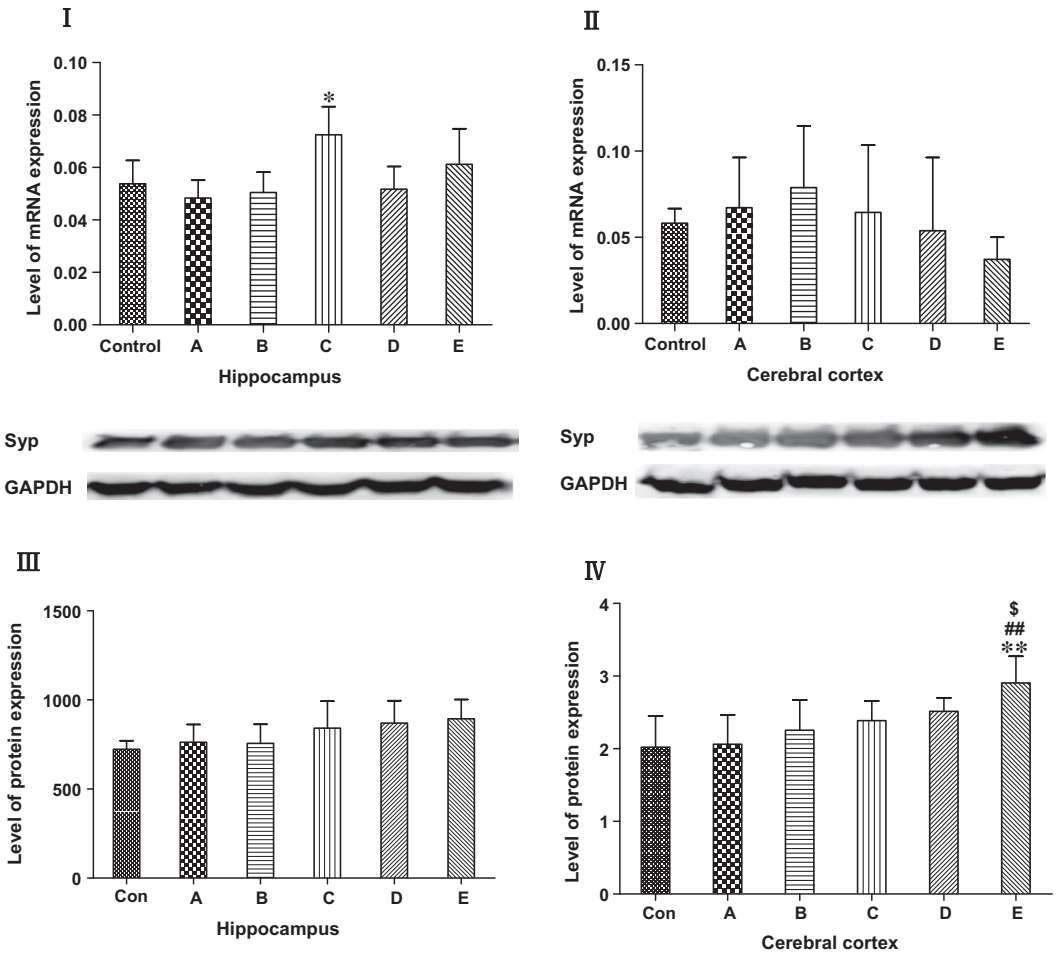

Fig. 15. mRNA and protein expression of Syp in the cerebral cortex and hippocampus of KM mice tested by shuttle-box; (I) expression of mRNA in the hippocampus; (II) expression of mRNA in the cerebral cortex; (III) expression of protein in the hippocampus; (IV) expression of protein in the cerebral cortex; Con, control group; (A) score = 0–20; (B) score = 21–40; (C) score = 41–60; (D) score = 61–75; (E) score = 76–100. ** $P < 0.01$, E *vs* Con; $^{\#\#}P < 0.01$, E *vs* A; $^{\$}P < 0.05$, E *vs* B. mean±S.D., $n = 5$, *ANOVA*.

Glutamate and glutamate receptors have been identified as important interfaces in learning and memory paradigms as well as in mechanisms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression, which are believed to be the underlying cellular basis of some forms of learning and memory. Glutamate is essential and indispensable for the induction and maintenance of long-lasting hippocampal LTP *in vitro* and *in vivo* (Riedel & Reymann, 1996). The ability of synaptic vesicles to store glutamate is conferred by VGLUTs, which undergo differential regulation during development and in response to activity. VGLUTs undergo regional, developmental, and activity-dependent changes in expression, and the expression of VGLUT protein appears to have a direct role in the biogenesis or recycling of synaptic vesicles (Santos, Li, & Voglmaier, 2009). Although the mechanisms of pathway specificity of LTP are poorly understood, glutamate uptake determines pathway specificity of LTP in the neural circuitry of fear conditioning (Tsvetkov, Shin, & Bolshakov, 2004). The capacity for neuroplasticity can be affected by sensory deprivation (LeVay, Stryker, & Shatz, 1978), task learning (Ding et al., 2002), environmental enrichment (Nithianantharajah, Levis, & Murphy, 2004), and drugs such as d-amphetamine (d-AMPH) (Boikess, O'Dell, & Marshall, 2010). These reports suggest that synaptic plasticity could be regulated by a variety of factors. It is not clear what mechanisms underlie the shuttle-box learning affect on neuroplasticity or of the non-coordinated VGLUT expression on the transcriptional and translational level. However, our present study demonstrates that the protein expression of VGLUTs was positively correlated with the capacity for learning and memory in the shuttle-box test, which is an indicator of the conditioned active avoidance reflex ability. This suggests that glutamate uptake though VGLUTs plays a prominent role in synaptic plasticity, which underlies the cellular basis of shuttle-box learning and memory processing.

VGLUTs are often localized together but can also be found in different regions of the CNS, with VGLUT1$^+$ neurons located in neocortex, hippocampus, and cerebellar cortex and VGLUT2$^+$ neurons in thalamus, midbrain, and hindbrain. When localized together, VGLUT1 and VGLUT2 are found at the axon terminals in several rat brain regions (Kaneko & Fujiyama, 2002). VGLUT3 is generally expressed by populations of neurons that release different transmitters, such as cholinergic interneurons of the striatum and serotonin neurons in the raphe (Fremeau et al., 2004; Herzog et al., 2001; Kaneko & Fujiyama, 2002; Ziegler, Cullinan, & Herman, 2001). In contrast, our data indicated that in the hippocampus of aging SAMR1 and SAMP8, expression of VGLUT1 and VGLUT2 had opposite patterns. This suggested that in the same brain region, the loss of VGLUT1 function could be compensated for by VGLUT2.

The VGLUTs localize to synaptic vesicles in nerve endings (Fremeau et al., 2001; Fremeau et al., 2002) and have been identified as markers for glutamatergic neurons and their axon terminals (Fremeau et al., 2001; Fujiyama, Furuta, & Kaneko, 2001; Stornetta, Sevigny, & Guyenet, 2002; Takamori, Rhee, Rosenmund, & Jahn, 2000). VGLUT1 and VGLUT2 are considered better markers of glutamatergic processes than glutamate, glutaminase, or plasma membrane excitatory amino acid transporters, which have also

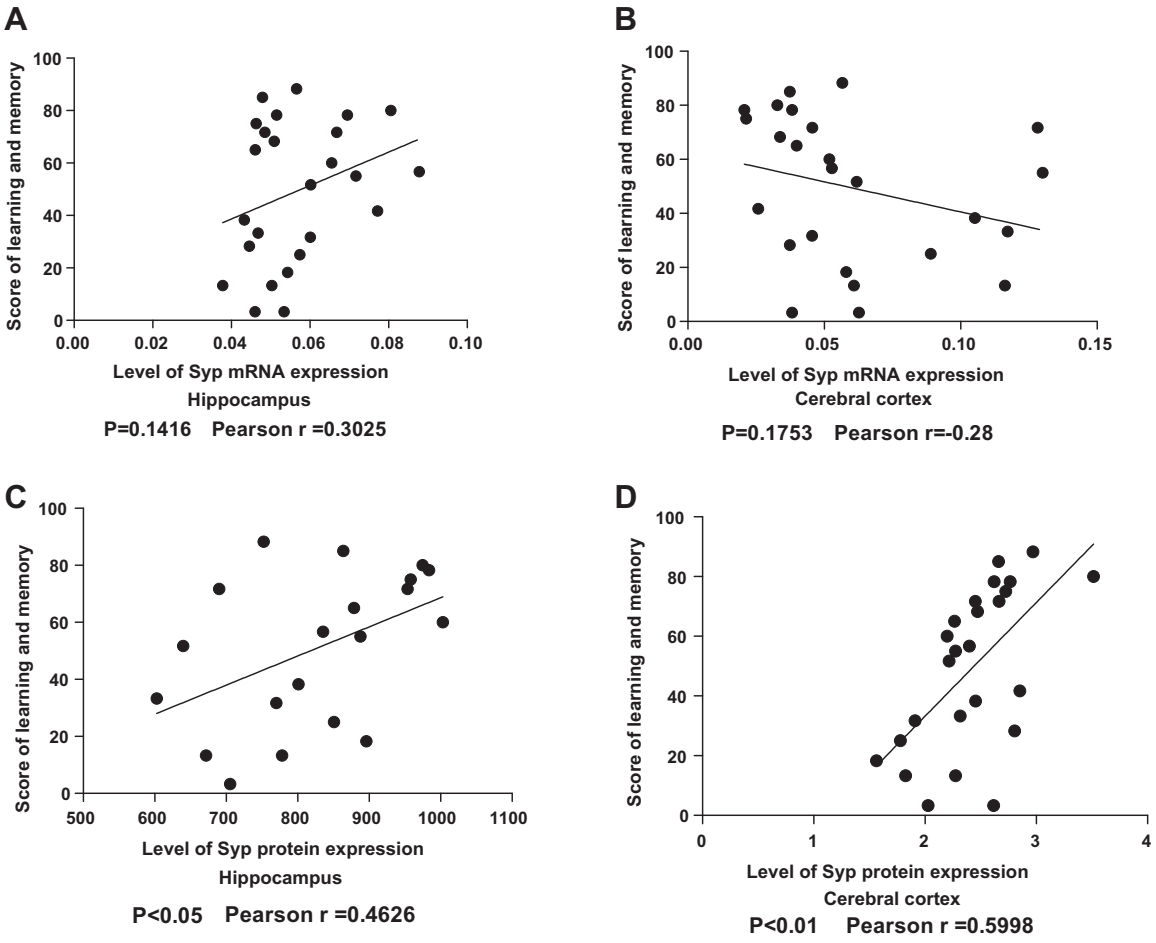

Fig. 16. Correlation between mRNA and protein expression of Syp in the cerebral cortex and hippocampus of KM mice tested by shuttle-box and the ability of learning and memory. $n = 25$, Two-tailed Pearson analysis, confidence interval 95%.

been used to identify glutamatergic neurons (Kaneko et al., 2002). However, VGLUTs exist in astrocytes (Anlauf & Derouiche, 2005; Bergersen & Gundersen, 2009; Zhang et al., 2004; Stenovec et al., 2007). Although astrocytes contain approximately 10 times less VGLUT than nerve terminals (Bezzi et al., 2004), these transporters are functional within astrocytes because Rose Bengal, an allosteric modulator of VGLUTs, greatly reduced glutamate release from astrocytes (Montana, Ni, Sunjara, Hua, & Parpura, 2004). Astrocytes can release a variety of gliotransmitters into the extracellular space, including glutamate that has been uptaken by VGLUTs (Parpura and Zorec, 2010). Additionally, gonadotropin-releasing hormone (GnRH) neuronal cell bodies or processes express VGLUT1 or VGLUT2 (Khan, De Sevilla, Mahesh, & Brann, 2010; Kiss, Kocsis, Csaki, & Halasz, 2003). Therefore, on the one hand, our study that protein expression of VGLUTs was decreased in the brain of aging SAMP8 and elevated in KM mice following shuttle-box learning cannot indicate the number of glutamatergic neuronal decrease in SAMP8 and increase in KM mice, but in combination with the alteration of expression level of synaptic vesicle protein synaptophysin (Syp), as a marker of synapse (Kashani et al., 2008), could indicate that the glutamatergic synaptic transmission was weakened in the brain of aging SAMP8 and strengthened in KM mice. On the other hand, our results suggest that VGLUTs and Syp are involved in the behavior of learning and memory and SAMP8 cognitive impairment.

Based on the above data combined with our present findings, alterations in the function and/or expression of VGLUTs have been implicated in a range of psychiatric and neurological disorders. VGLUTs, especially VGLUT1 or VGLUT2, are potential therapeutic targets for neurodegenerative and neuropsychiatric disorders related to malfunction of glutamate signaling in humans. Therefore, the development of molecules that specifically activate or block VGLUTs at low concentrations will be highly valuable for neurodegenerative and neuropsychiatric disorders. Because glutamatergic neurotransmission begins with vesicular release, compounds that inhibit or activate VGLUTs may fine-tune glutamatergic neurotransmission in the CNS. Several classes of competitive VGLUT inhibitors have emerged, including amino acids and amino acid analogs, fatty acids, azo dyes, quinolines and alkaloids (Thompson et al., 2005). The alkaloid bromocryptine has a Ki of 20 μM (Patel, Nagy, Bolstad, Gerdes, & Thompson, 2007), dyes have a Ki in the 20 nM to 10 μM range (e.g., RB, IC_{50} = 19 nM and Trypan Blue IC_{50} = 50 nM) (Bole & Ueda, 2005; Ogita et al., 2001), substituted quinolines have a Ki in the 40–300 μM range (Quinolein dicarboxylic acid, IC_{50} = 40 μM) (Carrigan et al., 2002; Shigeri, Seal, & Shimamoto, 2004) aspartate or glutamate analogs have an IC_{50} > 230 μM (e.g., (1S,3R)-ACPD IC_{50} = 230 μM) (Thompson et al., 2005), and tetrapeptide DQLIDELW have a Ki = 828 ± 252 μM (Patel et al., 2007). Among them, RB is the most potent known VGLUT inhibitor (Ki 25 nM), which potently inhibits vesicular monoamine transporter (Ki 64 nM) but weakly inhibits vesicular acetylcholine transporter (Ki > 9.7 μM) (Pietrancosta et al., 2010). In addition, new compounds have been developed to regulate the function of VGLUTs, including RB analog synthesis (Pietrancosta

et al., 2010) and substituted thienyl- and benzthienylglycines (Etoga, Ahmed, Patel, Bridges, & Thompson, 2010).

In summary, the current study showed that protein expression of VGLUT1, VGLUT2, VGLUT3 and Syp in the cerebral cortex and protein expression of VGLUT2 and Syp in the hippocampus were decreased in SAMP8 with aging. In contrast, protein expression of VGLUT1, VGLUT2, VGLUT3 and Syp was increased in the cerebral cortex of the KM mice following the shuttle-box test. Furthermore, the protein levels of VGLUT1 and VGLUT2 were the highest in the hippocampus of KM group E mice with an avoidance score of 76–100% in the shuttle-box test. Although VGLUTs are expressed in a non-coordinated manner on the transcriptional and translational level in the brain of aging SAMP8 and KM mice following the shuttle-box test, our findings also indicate that VGLUT expression age-dependently declined along with degeneration and ability-dependent elevation of learning and memory. These data illustrate that the protein expression of VGLUT1, VGLUT2 and Syp positively correlate with learning and memory ability. Further, the mechanism of VGLUT expression that significantly affects the behavior of learning and memory may be achieved through affecting glutamatergic signaling. This suggests that VGLUTs might be potential therapeutic targets for some neurodegenerative and neuropsychiatric disorders related to malfunction of glutamate signaling. How changes in VGLUT expression and their functional modulation contribute to the mechanism of learning and memory process requires better understanding and further investigation.

Acknowledgments

This work was supported by grants from the National Natural Science foundation of China under Grant No., 30973541, 30600760, 90709012, 30772562 and, the Chinese Basic Research and Development Program (973) under Grant No. 2004CB518907.

References

Aihara, Y., Mashima, H., Onda, H., Hisano, S., Kasuya, H., Hori, T., et al. (2000). Molecular cloning of a novel brain-type Na (+)-dependent inorganic phosphate cotransporter. *Journal of Neurochemistry, 74*, 2622–2625.

Alicja, L., Markowska, Edward., Spangler, L., & Ingram Donald, K. (1998). Behavioral assessment of the senescence accelerated mouse (SAM P8 and R1). *Physiology and Behavior, 64*, 15–26.

Anlauf, E., & Derouiche, A. (2005). Astrocytic exocytosis vesicles and glutamate: a high-resolution immunofluorescence study. *Glia, 49*, 96–106.

Bell, K. F., Ducatenzeiler, A., Ribeiro-da-Silva, A., Duff, K., Bennett, D. A., & Cuello, A. C. (2006). The amyloid pathology progresses in a neurotransmitter-specific manner. *Neurobiology of Aging, 27*, 1644–1657.

Bellocchio, E. E., Reimer, R. J., Fremeau, R. T. J., & Edwards, R. H. (2000). Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science, 289*, 957–960.

Bergersen, L. H., & Gundersen, V. (2009). Morphological evidence for vesicular glutamate release from astrocytes. *Neuroscience, 158*, 260–265.

Bezzi, P., Gundersen, V., Galbete, J. L., Seifert, G., Steinhäuser, C., Pilati, E., et al. (2004). Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate. *Nature Neuroscience, 7*, 613–620.

Boikess, S. R., O'Dell, S. J., & Marshall, J. F. (2010). A sensitizing d-amphetamine dose regimen induces long-lasting spinophilin and VGLUT1 protein upregulation in the rat diencephalon. *Neuroscience Letters, 469*, 49–54.

Bole, D. G., & Ueda, T. (2005). Inhibition of vesicular glutamate uptake by Rose Bengal-related compounds: structure-activity relationship. *Neurochemistry Research, 30*, 363–369.

Boulland, J. L., Qureshi, T., Seal, R. P., Rafiki, A., Gundersen, V., Bergersen, L. H., et al. (2004). Expression of the vesicular glutamate transporters during development indicates the widespread corelease of multiple neurotransmitters. *Journal of Comparative Neurology, 480*, 264–280.

Bozarth, M. A. (1983). A computer approach to measuring shuttle box activity and conditioned place preference. *Brain Research Bulletin, 11*, 751–753.

Butterfield, D. A., & Poon, H. F. (2005). The senescence-accelerated prone mouse (SAMP8): a model of age-related cognitive decline with relevance to alterations of the gene expression and protein abnormalities in Alzheimer's disease. *Experimental Gerontology, 40*, 774–783.

Canudas, A. M., Gutierrez-Cuesta, J., Rodriguez, M. I., Acuna-Castroviejo, D., Sureda, F. X., Camins, A., et al. (2005). Hyperphosphorylation of microtubule-associated protein tau in senescence-accelerated mouse (SAM). *Mechanisms of Ageing and Development, 126*, 1300–1304.

Carrigan, C. N., Bartlett, R. D., Esslinger, C. S., Cybulski, K. A., Tongcharoensirikul, P., Bridges, R. J., et al. (2002). Synthesis and in vitro pharmacology of substituted quinoline-2, 4-dicarboxylic acids as inhibitors of vesicular glutamate transport. *Journal Medicinal Chemistry, 45*, 2260–2276.

Cuomo, V., De Salvia, M. A., Petruzzi, S., & Alleva, E. (1996). Appropriate end points for the characterization of behavioral changes in developmental toxicology. *Environ Health Perspect, 104*(Suppl 2), 307–315.

Daniels, R. W., Collins, C. A., Chen, K., Gelfand, M. V., Featherstone, D. E., & DiAntonio, A. (2006). A single vesicular glutamate transporter is sufficient to fill a synaptic vesicle. *Neuron, 49*, 11–16.

Del Valle, J., Duran-Vilaregut, J., Manich, G., Casadesús, G., Smith, M. A., Camins, A., et al. (2010). Early amyloid accumulation in the hippocampus of SAMP8 Mice. *Journal of Alzheimer's Disease, 19*, 1303–1315.

Del Valle, J., Duran-Vilaregut, J., Manich, G., Camins, A., Pallàs, M., Vilaplana, J., et al. (2009). Time-course of blood-brain barrier disruption in senescence-accelerated mouse prone 8 (SAMP8) mice. *International Journal of Developmental Neuroscience, 27*, 47–52.

Ding, Y., Li, J., Lai, Q., Azam, S., Rafols, J. A., & Diaz, F. G. (2002). Functional improvement after motor training is correlated with synaptic plasticity in rat thalamus. *Neurology Research, 24*, 829–836.

Etoga, J. L., Ahmed, S. K., Patel, S., Bridges, R. J., & Thompson, C. M. (2010). Conformationally-restricted amino acid analogues bearing a distal sulfonic acid show selective inhibition of system x_c^- over the vesicular glutamate transporter. *Bioorganic and Medicinal Chemistry Letters, 20*, 2680–2683.

Flood, J. F., & Morley, J. E. (1998). Learning and memory in the SAMP8 mouse. *Neuroscience Biobehavior Review, 22*, 1–20.

Flood, J. E., & Morley, J. E. (1993). Age-related changes in foot shock avoidance acquisition and retention in senescence accelerated mouse (SAM). *Neurobiology of Aging, 14*, 153–157.

Fremeau, R. T., Jr., Burman, J., Qureshi, T., Tran, C. H., Proctor, J., Johnson, J., et al. (2002). The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proceedings of the National Academy of Sciences, 99*, 14488–14493.

Fremeau, R. T., Jr., Kam, K., Qureshi, T., Johnson, J., Copenhagen, D. R., Storm-Mathisen, J., et al. (2004). Vesicular glutamate transporters 1 and 2 target to functionally distinct synaptic release sites. *Science, 304*, 1815–1819.

Fremeau, R. T., Jr., Troyer, M. D., Pahner, I., Nygaard, G. O., Tran, C. H., Reimer, R. J., et al. (2001). The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron, 31*, 247–260.

Fujiyama, F., Furuta, T., & Kaneko, T. (2001). Immunocytochemical localization of candidates for vesicular glutamate transporters in the rat cerebral cortex. *Journal of Comparative Neurology, 435*, 379–387.

Garcia-Garcia, A. L., Elizalde, N., Matrov, D., Harro, J., Wojcik, S. M., Venzala, E., et al. (2009). Increased vulnerability to depressive-like behavior of mice with decreased expression of VGLUT1. *Biology and Psychiatry, 66*, 275–282.

Gras, C., Herzog, E., Bellenchi, G. C., Bernard, V., Ravassard, P., Pohl, M., et al. (2002). A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. *The Journal of Neuroscience, 22*, 5442–5451.

Herzog, E., Bellenchi, G.C., Gras, C., Bernard, V., Ravassard, P., Bedet, C.,Gasnier, B., Giros, B., El, M. (2001). The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. *The Journal of Neuroscience* 21, RC181.

Ishikawa, T., Sahara, Y., & Takahashi, T. (2002). A single packet of transmitter does not saturate postsynaptic glutamate receptors. *Neuron, 34*, 613–621.

Kaneko, T., & Fujiyama, F. (2002). Complementary distribution of vesicular glutamate transporters in the central nervous system. *Neurosciense Research, 42*, 243–250.

Kaneko, T., Fujiyama, F., & Hioki, H. (2002). Immunohistochemical localization of candidates for vesicular glutamate transporters in the rat brain. *Journal of Comparative Neurology, 444*, 39–62.

Kashani, A., Lepicard, E., Poirel, O., Videau, C., David, J. P., Fallet-Bianco, C., et al. (2008). Loss of VGLUT1 and VGLUT2 in the prefrontal cortex is correlated with cognitive decline in Alzheimer disease. *Neurobiology of Aging, 29*, 1619–1630.

Kawamata, T., Akiguchi, I., Yagi, H., Irino, M., Sugiyama, H., Akiyama, H., et al. (1997). Neuropathological studies on strains of senescenceaccelerated mice (SAM) with age-related deficits in learning and memory. *Experimental Gerontology, 32*, 161–170.

Khan, M., De Sevilla, L., Mahesh, V. B., & Brann, D. W. (2010). Enhanced glutamatergic and decreased gabaergic synaptic appositions to GnRH neurons onproestrus in the rat: modulatory effect of aging. *PLoS ONE, 5*, e10172.

Kirvell, S.L., Fremeau, R.T., Francis, P.T. (2002). Vesicular glutamate transporter 1 in Alzheimer's disease. *Society Neuroscience 2002 Abstract Viewer* 785.15 (Abstract).

Kiss, J., Kocsis, K., Csaki, A., & Halasz, B. (2003). Evidence for vesicular glutamate transporter synapses onto gonadotropin-releasing hormone and other neurons in the rat medial preoptic area. *European Journal of Neuroscience, 18*, 3267–3278.

Kondziella, D., Bidar, A., Urfjell, B., Sletvold, O., & Sonnewald, U. (2002). The pentylenetetrazole-kindling model of epilepsy in SAMP8 mice. Behavior and metabolism. *Neurochemistry International, 40*, 413–418.

Lacey, C. J., Boyes, J., Gerlach, O., Chen, L., Magill, P. J., & Bolam, J. P. (2005). GABAb receptors as glutamatergic synapses in the rat striatum. *Neuroscience, 136*, 1083–1095.

LeVay, S., Stryker, M. P., & Shatz, C. J. (1978). Ocular dominance columns and their development in layer IV of the cat's visual cortex: a quantitative study. *Journal of Comparative Neurology, 179*, 223–244.

Massie, A., Schallier, A., Vermoesen, K., Arckens, L., & Michotte, Y. (2010). Biphasic and bilateral changes in striatal VGLUT1 and 2 protein expression in hemi-Parkinson rats. *Neurochemistry International, 57*, 111–118.

Miyamoto, M. (1997). Characteristics of age-related behavioral changes in senescence-accelerated mouse SAMP8 And SAMP 10. *Experimental Gerontology, 32*, 139–148.

Miró-Bernié, N., Ichinohe, N., Pérez-Clausell, J., & Rockland, K. S. (2006). Zinc-rich transient vertical modules in the rat retrosplenial cortex during postnatal development. *Neuroscience, 138*, 523–535.

Miyamoto, M., Kiyota, Y., Yamazaki, N., Nagaoka, A., Matsuo, T., Nagawa, Y., et al. (1986). Age-related changes in learning and memory in the senescence-accelerated mouse (SAM). *Physioogy and Behavior, 38*, 399–406.

Moechars, D., Weston, M. C., Leo, S., Callaerts-Vegh, Z., Goris, I., Daneels, G., et al. (2006). Vesicular glutamate transporter VGLUT2 expression levels control quantal size and neuropathic pain. *The Journal of Neuroscience, 26*, 12055–12066.

Montana, V., Ni, Y., Sunjara, V., Hua, X., & Parpura, V. (2004). Vesicular glutamate transporter-dependent glutamate release from astrocytes. *The Journal of Neuroscience, 24*, 2633–2642.

Morley, J. E. (2002). The SAMP8 mouse: a model of Alzheimer disease? *Biogerontology, 3*, 57–60.

Morley, J. E., Banks, W. A., Kumar, V. B., & Farr, S. A. (2004). The SAMP8 mouse as a model for Alzheimer disease: studies from Saint Louis University. *International Congress Series, 1260*, 23–28.

Morley, J. E., Kumar, V. B., Bernardo, A. E., Farr, S. A., Uezu, K., Tumosa, N., et al. (2000). Beta-amyloid precursor polypeptide in SAMP8 mice affects learning and memory. *Peptides, 21*, 1761–1767.

Nakahara, H., Kanno, T., Inai, Y., Utsumi, K., Hiramatsu, M., Mori, A., et al. (1998). Mitochondrial dysfunction in the senescence accelerated mouse (SAM). *Free Radical Biology and Medicine, 24*, 85–92.

Nithianantharajah, J., Levis, H., & Murphy, M. (2004). Environmental enrichment results in cortical and subcortical changes in levels of synaptophysin and PSD-95. *Neurobiology of Learning and Memory, 81*, 200–210.

Nomura, Y., & Okuma, Y. (1999). Age-related defects in lifespan and learning ability in SAMP8 mice. *Neurobiology of Aging, 20*, 111–115.

Nomura, Y., Kitamura, Y., Ohnuki, T., et al. (1997). Alterations in acetylcholine, NMDA, benzodiozepine receptors and protein kinase C in the brain of the senescence-accelerated mouse: an animal model useful for studies on cognitive enhances. *Behavioural Brain Research, 83*, 51–55.

Ogita, K., Hirata, K., Bole, D. G., Yoshida, S., Tamura, Y., Leckenby, A. M., et al. (2001). Inhibition of vesicular glutamate storage and exocytotic release by Rose Bengal. *Journal of Neurochemistry, 77*, 34–42.

Ohta, A., Hirano, T., Yagi, H., Tanaka, S., Hosokawa, M., & Takeda, T. (1989). Behavioral characteristics of the SAM-P/8 strain in Sidman active avoidance task. *Brain Research, 498*, 195–198.

Onozuka, M., Watanabe, K., Fujita, M., Tomida, M., & Ozono, S. (2002). Changes in the septohippocampal cholinergic system following removal of molar teeth in the aged SAMP8 mouse. *Behavior Brain Research, 133*, 197–204.

Parpura, V., & Zorec, R. (2010). Gliotransmission: Exocytotic release from astrocytes. *Brain Research Review, 63*, 83–92.

Patel, S. A., Nagy, J. O., Bolstad, E. D., Gerdes, J. M., & Thompson, C. M. (2007). Tetrapeptide inhibitors of the glutamate vesicular transporter (VGLUT). *Bioorganic and Medicinal Chemistry Letters, 17*, 5125–5128.

Pelegrí, C., Canudas, A. M., del Valle, J., Casadesús, G., Smith, M. A., Camins, A., et al. (2007). Increased permeability of blood-brain barrier on the hippocampus of a murine model of senescence. *Mechanisms of Ageing and Development, 128*, 522–528.

Petursdottir, A. L., Farr, S. A., Morley, J. E., Banks, W. A., & Skuladottir, G. V. (2007). Lipid peroxidation in brain during aging in the senescence-accelerated mouse (SAM). *Neurobiology of Aging, 28*, 1170–1178.

Pietrancosta, N., Kessler, A., Favre-Besse, F. C., Triballeau, N., Quentin, T., Giros, B., et al. (2010). Rose Bengal analogs and vesicular glutamate transporters (VGLUTs). *Bioorganic and Medicinal Chemistry, 18*, 6922–6933.

Raju, D. V., Ahern, T. H., Shah, D. J., Wright, T. M., Standaert, D. G., Hall, R. A., et al. (2008). Differential synaptic plasticity of the corticostriatal and thalamostriatal systems in an MPTP-treated monkey model of parkinsonism. *European Journal of Neuroscience, 27*, 1647–1658.

Reimer, R. J., & Edwards, R. H. (2004). Organic anion transport is the primary function of the SLC17/type I phosphate transporter family. *Pflugers Archieve, 447*, 629–635.

Riedel, G., & Reymann, K. G. (1996). Metabotropic glutamate receptors in hippocampal long-term potentiation and learning and memory. *Acta Physiologica Scandinavica, 157*, 1–19.

Rueda, N., Llorens-Martín, M., Flórez, J., Valdizán, E., Banerjee, P., Trejo, J. L., et al. (2010). Memantine normalizes several phenotypic features in the Ts65Dn mouse model of Down syndrome. *Journal of Alzheimers Disease, 21*, 277–290.

Santos, M. S., Li, H., & Voglmaier, S. M. (2009). Synaptic vesicle protein trafficking at the glutamate synapse. *Neuroscience, 158*, 189–203.

Spangler, E. L., Patel, N., Speer, D., Speer, D., Hyman, M., Hengemihle, J., et al. (2002). Passive avoidance and complex maze learning in the senescence accelerated mouse (SAM): age and strain comparisons of SAM P8 and R1. *The Journals of Gerontology, Series A: Biological Sciences Medical Sciences, 57*, B61–B68.

Seal, R. P., Akil, O., Yi, E., Weber, C. M., Grant, L., Yoo, J., et al. (2008). Sensorineural deafness and seizures in mice lacking vesicular glutamate transporter 3. *Neuron, 57*, 263–275.

Shen, Y. C., Liao, D. L., Lu, C. L., Chen, J. Y., Liou, Y. J., Chen, T. T., et al. (2010). Resequencing of the vesicular glutamate transporter 2 gene (VGLUT2) reveals some rare genetic variants that may increase the genetic burden in schizophrenia. *Schizophrenia Research, 121*, 179–186.

Shigeri, Y., Seal, R. P., & Shimamoto, K. (2004). Molecular pharmacology of glutamate transporters, EAATs and VGLUTs. *Brain Research Review, 45*, 250–265.

Stenovec, M., Kreft, M., Grilc, S., Potokar, M., Kreft, M. E., Pangrsic, T., et al. (2007). Ca2+ dependent mobility of vesicles capturing anti-VGLUT1 antibodies. *Experimental Cell Research, 313*, 3809–3818.

Stornetta, R. L., Sevigny, C. P., & Guyenet, P. G. (2002). Vesicular glutamate transporter DNPI/VGLUT2 mRNA is present in C1 and several other groups of brainstem catecholaminergic neurons. *The Journal of Comparative Neurology, 444*, 191–206.

Tajes, M., Gutierrez-Cuesta, J., Folch, J., Ferrer, I., Caballero, B., Smith, M. A., et al. (2008). Lithium treatment decreases activities of tau kinases in a murine model of senescence. *Journal of Neuropathology and Experimental Neurology, 67*, 612–623.

Takamori, S., Rhee, J.S., Rosenmund, C., Jahn, R. (2001). Identification of differentiation-associated brain-specific phosphate transporter as a second vesicular glutamate transporter. *The Journal of Neuroscience*, 21, RC182.

Takamori, S. (2006). VGLUTs: 'exciting' times for glutamatergic research? *Neuroscience Research, 55*, 343–351.

Takamori, S., Rhee, J. S., Rosenmund, C., & Jahn, R. (2000). Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature, 407*, 189–194.

Takeda, T., Hosokawa, M., Takeshita, S., Rino, M., Higuchi, K., Matsushita, T., et al. (1981). A new murine model of accelerated senescence. *Mechanisms of Ageing and Development, 17*, 183–194.

Thompson, C. M., Davis, E., Carrigan, C. N., Cox, H. D., Bridges, R. J., & Gerdes, J. M. (2005). Inhibitors of the glutamate vesicular transporter (VGLUT). *Current Medicine Chemistry, 12*, 2041–2056.

Tordera, R. M., Totterdell, S., Wojcik, S. M., Brose, N., Elizalde, N., Lasheras, B., et al. (2007). Enhanced anxiety, depressive-like behaviour and impaired recognition memory in mice with reduced expression of the vesicular glutamate transporter 1 (VGLUT1). *European Journal of Neuroscience, 25*, 281–290.

Tsvetkov, E., Shin, R. M., & Bolshakov, V. Y. (2004). Glutamate uptake determines pathway specificity of long-term potentiation in the neural circuitry of fear conditioning. *Neuron, 41*, 139–151.

Ueno, M., Sakamoto, H., Kanenishi, K., Onodera, M., Akiguchi, I., & Hosokawa, M. (2001). Ultrastructural and permeability features of microvessels in the hippocampus, cerebellum and pons of senescence-accelerated mice (SAM). *Neurobiology of Aging, 22*, 469–478.

Wilson, N. R., Kang, J., Hueske, E. V., Leung, T., Varoqui, H., Murnick, J. G., et al. (2005). Presynaptic regulation of quantal size by the vesicular glutamate transporter VGLUT1. *The Journal of Neuroscience, 25*, 6221–6234.

Wojcik, S. M., Rhee, J. S., Herzog, E., Sigler, A., Jahn, R., Takamori, S., et al. (2004). An essential role for vesicular glutamate transporter 1 (VGLUT1) in postnatal development and control of quantal size. *Proceedings of the National Academy of Sciences, 101*, 7158–7163.

Wootz, H., Enjin, A., Wallén-Mackenzie, A., Lindholm, D., & Kullander, K. (2010). Reduced VGLUT2 expression increases motor neuron viability in $Sod1^{G93A}$ mice. *Neurobiology of Disease, 37*, 58–66.

Yagi, H., Katoh, S., Akiguchi, I., & Takeda, T. (1988). Age-related deterioration of ability of acquisition in memory and learning in senescence accelerated mouse: SAM-P/8 as an animal model of disturbances in recent memory. *Brain Research, 474*, 86–93.

Yagi, H., Irino, M., Matsushita, T., Katoh, S., Umezawa, M., Tsuboyama, T., et al. (1989). Spontaneous spongy degeneration of the brain stem in SAM-P/8 mice, a newly developed memory-deficient strain. *Journal of Neuropathology and Experimental Neurology, 48*, 577–590.

Zhang, Q., Pangrsic, T., Kreft, M., Krzan, M., Li, N., Sul, J. Y., et al. (2004). Fusion-related release of glutamate from astrocytes. *The Journal of biological chemistry, 279*, 12724–12733.

Zhang, Q. P., Ding, H. Q., Li, W. X., Fan, Z. Q., Sun, A. Y., Luo, J., et al. (2009). Senescence accelerated mouse strain is sensitive to neurodegeneration induced by mild impairment of oxidative metabolism. *Brain research, 1264*, 111–118.

Ziegler, D. R., Cullinan, W. E., & Herman, J. P. (2001). Distribution of vesicular glutamate transporter mRNA in rat hypothalamus. *The Journal of Comparative Neurology, 448*, 217–229.

Zink, M., Vollmayr, B., Gebicke-Haerter, P. J., & Henn, F. A. (2010). Reduced expression of glutamate transporters vGluT1, EAAT2 and EAAT4 in learned helpless rats, an animal model of depression. *Neuropharmacology, 58*, 465–473.