The effects of agmatine on Alzheimer's disease induced by brain insulin resistance

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The effects of agmatine on Alzheimer's disease induced by brain insulin resistance

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ABSTRACT

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Type 2 diabetes increases risk of Alzheimer's disease, especially neuronal insulin resistance is suggested as a main cause of Alzheimer's disease occurred in type 2 diabetes patients. Reduced insulin signaling in neurons leads to neuronal dysfunction, accumulation of amyloid beta (Aβ) and phosphorylation of tau.

Agmatine, a polyamine derived from L-arginine, has shown neuroprotective effects. This study was designed to investigate whether agmatine could reduce both high fat diet induced peripheral glucose intolerance and cognitive impairment through retrieval blunted insulin signaling in brain.

8 weeks old male ICR mice weighing 30~35 g were randomly divided into 2 groups and fed normal diet and 60% high fat diet for 12 weeks. High fat diet

group was injected streptozotocin (100 mg/kg/ip) once at 4th weeks of diet. After 12 weeks, mice in high fat diet group were assigned into 2 groups, saline or agmatine (100 mg/kg/ip) treated groups. After 2 weeks of treatment, behavior tests were conducted and brains were collected for western blotting and immunohistochemistry. Expression levels of insulin downstream molecules, Aβ and phosphorylated tau were evaluated.

Agmatine administration relieved peripheral glucose intolerance and reduced accumulation of Aβ and phosphorylated tau caused by high fat diet through retrieval of insulin signaling. Agmatine rescues high fat diet fed mice from cognitive decline as well. Agmatine may have potential to be a candidate substance for treat both diabetes and Alzheimer's disease.

.

Key words: Type 2 diabetes, central insulin resistance, Alzheimer's disease, agmatine

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Ⅰ. INTRODUCTION

A growing body of clinical and epidemiological research suggests that two of the most common diseases of aging, type 2 diabetes (T2DM) and Alzheimer's disease (AD), are linked. Type 2 diabetes has been identified as an important risk factor for AD.

Alzheimer's disease is the most common cause of dementia characterized by progressive neurodegeneration and exhibits two hallmarks — extracellular senile plaques and intracellular neurofibrillary tangles (NFTs). Senile plaques contain amyloid-β peptide (Aβ), which arises through the proteolytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase. NFTs are formed by intraneuronal accumulation of neurofilaments and hyperphosphorylated tau protein.[1]

Diabetes is a group of metabolic diseases in which a person has high blood

sugar. Diabetes has two major forms which are type 1 diabetes and type 2 diabetes. Type 1 diabetes is characterized by an absolute deficiency of insulin due to autoimmune destruction of pancreatic β-cells, and type 2 diabetes features both decreased insulin secretion and insulin resistance.[2] Insulin resistance is hard to be defined, but generally refered to the condition that tissues are unable to respond to physiologically sufficient insulin concentration,[3] simply the state which reduced the ability of insulin to stimulate glucose utilization in physiological concentration.[4]

Insulin works with binding to the α-subunit of insulin receptor, leading to auto-phosphorylation of the intracellular β-subunit. Then, it recruits and phosphorylates intracellular substrates such as insulin receptor substrate (IRS) family proteins. After that, it recruits downstream signaling molecules including phosphatidylinositol 3 kinase (PI3K), which activates Akt-mediated signaling. Furthermore, it results in activation of mitogen-activated protein kinase (MAPK) signaling pathway[5-7] and regulates glycogen synthesis by inactivation of GSK-3β.[8] Akt phosphorylates various substrates, contributing to regulation of cellular processes such as growth, proliferation, survival, protein synthesis, cell cycle control and glucose metabolism.[3,9 10]

Many investigators suggest that AD is related to impairment of both insulin signaling and glucose metabolism in the brain, leading some researchers to refer to AD as type 3 diabetes.[11] Especially insulin resistance is a key factor. Even though neurons are generally considered to be insulin-independent, they are insulin-responsive. Insulin enters the central nerve system by crossing the blood-brain barrier. Insulin receptors are widely distributed throughout the central nerve system, but the function of these receptors in the brain is little understood. insulin signaling is important for various neuronal functions such as energy homeostasis, regeneration, reinnervation, survival.[12] Most importantly, insulin receptors might be involved in the regulation of synaptic activity and cognitive processes.[13]

Neuronal insulin resistance leads to both Aβ plaque formation and tau hyperphosphorylation.[14] Insulin and Aβ compete for insulin-degrading enzyme (IDE). In the hyperinsulinemia state, insulin dominantly occupies IDE, results in Aβ accumulation and plaque formation.[15] Oxidative stress and neuroinflammation induced by insulin resistance promotes amyloid beta accumulation and toxicity.[16] Insulin trafficks amyloid beta from the trans Golgi to the plasma membrane, and stimulates amyloid beta extracellular secretion to inhibits its intracellular accumulation.[17-19]

Insulin regulates phosphorylation of GSK-3β, which phosphorylate tau proteins, so insulin inhibits GSK-3β by phosphorylation so that impede phosphorylation of tau. Therefore, decreased insulin signaling increases GSK-3β activity, leading to tau phosphorylation associated with neurofibrillary tangles formation.[20-22] Lack of insulin signaling in brain specific insulin receptor knock out animal model altered akt and GSK-3β activity, leading to increased tau phosphorylation.[23]

Diet-induced Alzheimer's disease model is well established to examine pathology of neuronal insulin resistance and pathogenesis of Alzheimer's disease induced by insulin resistance.[24-27] This model mimics naturally occuring Alzheimer's disease in type 2 diabetes patients very well.

Recently, metformin, which activates imidazolin receptors and well known for medicine for diabetes, is reported that it improves diabetes but worsens cognitive dysfunction.[10,28,29]

Agmatine is an endogenous aminoguanidine compound made from arginine by arginine-decarboxylase (ADC). It is known for neuromodulator as it has affinity for several transmembrane receptors such as imidazoline receptors, 2-adrenergic and NMDA receptors and irreversibly inhibits neuronal nitric oxide synthase and down-regulates inducible nitric oxide synthase.[30] So, it shows positive

effects on widespread diseases, for instance, diabetes, stroke, spinal cord injury, Alzheimer's disease.[31-35] For instance, agmatine has shown anti-diabetic like effect in type 1 and type 2 diabetic rats.[34,36-38] Several researchers revealed the pharmacological potential of agmatine against cognitive decline and memory facilitation in various animal models.[39-43] So, it has potential to treat both type 2 diabetes and cognitive decline.

However, the effect of agmatine on high-fat diet-induced Alzheimer's disease model has not been investigated yet. So, this study established high-fat diet induced Alzheimer' disease model to applied agmatine. So, this study tried to show whether agmatine ameliorates both peripheral insulin resistance and neuronal insulin resistance so that blocks accumulation of amyloid beta and phosphorylation of tau, then finally it relieves Alzheimer's disease.

Ⅱ. MATERIALS AND METHODS

1. Animals

Adult male ICR mice (7 weeks old, Central Lab animal Inc., Seoul, Korea) were used in this study. The mice were raised in a standard laboratory animal facility under a 12 h light/dark cycle and the animals had free access to food and water *ad libitum*. All procedures were conducted in accordance with Yonsei University College of Medicine Animal Care and Use Committee and the National Institute of Health guidelines for the Care and Use of Laboratory Animals.

2. Establishment of neuronal insulin resistance model

Modifying previously established methods, we developed type 2 diabetes induced Alzheimer's disease mouse model.[24,44] After a week of acclimatization to the laboratory conditions, mice were randomly divided into two groups. Mice were administered either a normal diet (chow, NC; 4.8% kcal fat) or a high fat diet (HFD; 60% kcal fat) for 12 weeks. The mice fed HFD were intraperitoneally injected once with low dose of streptozotocin [100 mg/kg/ip, Sigma-Aldrich, MO, USA, dissolved in citrate buffer (pH 4.4)] to induce partial insulin deficiency at 4th week of HFD feeding period (Fig. 1).

3. Determination of body weight and serum glucose level

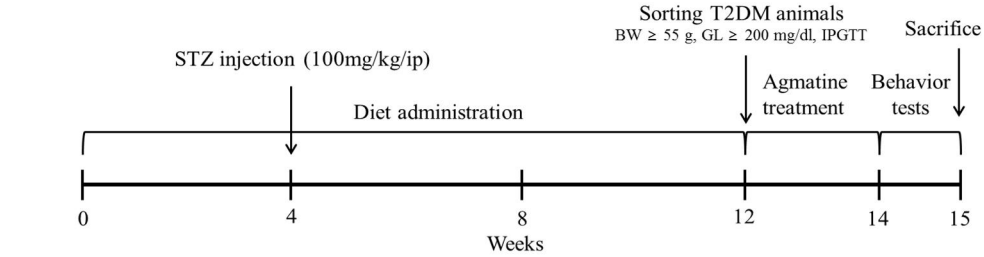
The body weight (BW) and fasting serum glucose level (GL) of all animals were monitored weekly. To measure the fasting glucose level, mice were fasted for 4 hours before experiment. Blood glucose concentration from blood samples taken by nipping the distal part of the tail was measured by glucometer (CareSensⅡ Meter, Pharmaco (NZ) Ltd., Auckland, New Zealand).

4. Intraperitoneal glucose tolerance test (IPGTT)

Glucose tolerance test is a widely used simple test in clinical practice to diagnose glucose intolerance and type 2 diabetes.[45] Food was removed the night before experiment. The mice were injected with glucose (50 g/kg/ip, Sigma-Aldrich, MO, USA, dissolved in saline). Blood glucose level of the blood sample taken from the tip of the tail was measured by glucometer at 0, 30, 60 and 120 minutes after the bolus. The area under the concentration versus time curve (AUC glucose 0 – 120 minutes, mg/dl * minutes) was calculated.

5. Agmatine administration

Mice with fasting serum glucose level >200 mg/dl, body weight > 55 g and impaired glucose tolerance were considered as type 2 diabetes model.[46] After type 2 diabetes was induced, mice were divided into 2 groups; HFD group which injected saline for additional 2 weeks and HFD+AGM group which administered with agmatine (100 mg/kg/ip, Sigma-Aldrich, MO, USA, dissolved in saline) for additional 2 weeks (Fig. 1). The number of mice in groups was 6, respectively.



NC	Normal diet	saline
HFD	High fat diet (60% fat)	Saline
HFD+ AGM	High fat diet (60%)	Agmatine (100mg/kg)

Figure 1. Timeline for in vivo study. Mice were randomly divided into two groups then administered either a normal diet or a high fat diet (HFD) for 12 weeks. The mice fed HFD were injected once with low dose of STZ (100 mg/kg/ip) at 4th week of HFD feeding period. Mice with fasting serum glucose level >200 mg/dl, body weight > 55 g and impaired glucose tolerance were selected, then assigned into two groups; HFD which injected saline and HFD+AGM which injected agmatine (100 mg/kg/ip) for additional 2 weeks. After administration, behavior tests were conducted, then mice were sacrificed for further analysis.

6. Behavior test

A. Morris water maze

To evaluate the cognitive decline, the mice were subjected to take water maze test using previously established protocol[47] with some modifications. This test is consisted of 5-days training and a test on 6th day. Mice were moved to behavior room to adjust to the new environment for at least 30 minutes before testing. The apparatus consisted of a circular water pool (100 cm diameter, 35 cm in height) that was filled with opaque water to a depth of 15.5cm. A platform (5.5 cm diameter, 14.5 cm in height) was placed 1cm below the surface of water at the fixed location. Four different figures were attached as visual cue. Each mouse received four trainings per day for 4 consecutive days, and latency time to escape from the water to platform was measured during the training session. All mice were allowed to find platform for a maximum of 90 seconds. On 5th day, test session was conducted by allowing mice to swim freely in the pool without the platform for 90 seconds. The time spent in the quadrant where the platform was located was measured.

B. Step-through task (Passive avoidance test)

After the Morris water maze test, the step-through passive avoidance task was performed. The apparatus consisted of a light compartment and a dark compartment (200 X 250 X 200 mm) , separated by a vertical sliding door, with a grid metal floor that could deliver a mild electric shock. During the training session, the mouse was placed in the light compartment and allowed to explore for 30 seconds. Then, the sliding door was opened, and the step-through latency for the mouse to enter the dark compartment was measured. As soon as the mouse entered the dark compartment, the door was closed. 3 seconds later the mouse entered the dark room, electric foot-shock (0.5 mA, 3 seconds) was delivered through the grid floor by a constant current shock generator. 24 hours later, test session was

conducted using the same way without electric shock. The step-through latency for the mouse to enter the dark compartment was measured. The mouse was examined for 270 seconds as cut-off latency and test was repeated every 24 hours later for 3 times.

C. Nest building test

The mice were moved into individual cages with a cotton pad (50 X 50 mm, 5 g). 24 hours later, each nest was recorded and scored by 5 researchers on a scale of 1-5 according to established standard[48]. Following the assessment of Deacon[48], nests were scored based on shape and the amount of material used.

7. Tissue sample preparation

After behavior tests, mice were perfused transcardially with saline and brains were removed. Hemispheres of each brain were incubated in 4% PFA for 24 hours at 4°C then transferred to a 30% sucrose solution for a week. The brain tissues were embedded in medium (Tissue – Tek O.C.T. compound, Sakura Finetek USA, Inc., CA, USA) then cut on a cryostat at 20 μm and stored at -20 °C until immunohistochemistry. Left hemispheres were placed in saline and carefully dissected. Hippocampus and cortex regions were immediately frozen in liquid nitrogen and stored until western blot assay.

8. Immunofluorescence

Brain sections were permeabilized with 0.025 % triton X-100 and were blocked with 10 % donkey serum at room temperature for 1 hour. Sections were immunostained with primary antibodies against phosphor-TAU (Ser 202, Tyr 205)

(1:200; Santa cruz, TX, USA), Amyloid beta (1:200; Abcam, Cambridge, UK) and phosphor-GSK-3β (Ser9, 1:200, Cell signaling, MA, USA) at 4 °C overnight. After being washed with PBS (0.05% with Tween 20) three times, FITC or Rhodamine-conjugated donkey anti rabbit and mouse antibody (1:200, Millipore, MA, USA) were applied for a hour at room temperature and counter stained with 4',6-diamidino-2-phenylindole (DAPI) (Millipore, MA, USA) when tissue slides mounted. Then tissues were visualized under a confocal microscope (Zeiss LSM 700, Carl Zeiss, NY, USA).

9. Western blot assay

Hippocampus and cortex were treated with lysis buffer consisting of PBS, 1 % nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, proteinase inhibitor-PMSF, Aprotinin and Sodium orthovandate and isolated protein from homogenizer (Dramel, WI, USA). Lysates were collected and centrifugated at 13,000 rpm for 1 hour. Using the BCA method (PIERCE, Rockford, USA), protein concentration was determined. 50 μg of protein was separated on 6 % sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gels and electrotransferred onto polyvinylidene difluoride membrane (Millipore, MA, USA). After blocking the membrane with 5% bovine serum albumin, membranes were reacted with primary antibodies that specifically detect IRS-1 (1:1000; Santa cruz, TX, USA), phosphor-IRS-1 (Tyr 632, 1:1000; Santa cruz, TX, USA), Akt (1:1000; Abcam, Cambridge, UK), phosphor-Akt (Ser473, 1:1000; Cell signaling, MA, USA), phosphor-GSK-3β (Ser9, 1:1000, Cell signaling, MA, USA), GSK-3β (1:1000, Cell signaling, MA, USA), phosphor-TAU (Ser 202, Tyr 205, 1:1000; Santa cruz, TX, USA), Amyloid beta (1:1000; Abcam, Cambridge, UK) and β-actin (1:2500; Millipore, MA, USA) at 4 °C overnight. Then membranes were washed for 15 minutes repeated four times with TBS (0.5% with Tween 20). Then, the immunoblots were reacted with horseradish peroxidase-conjugated anti-mouse, -rabbit and –goat IgG antibodies

(1:3000; Abcam, Cambridge, UK) at room temperature for 1 hour. After washing membrane using TBS (0.5% with Tween 20) for 10 minutes repeated three times, signals were observed using enhanced chemiluminescence reagents (ECL; Pierce, IL, USA). Images were captured by using LAS 4000 program.

10. Statistical analysis

All experiments were repeated at least 3 times and expressed as the mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's post analysis. Statistical significance was defined as * $p \leq 0.05$, ** $p \leq 0.01$.

Ⅲ. RESULTS

1. High Fat Diet Increases Body Weight and Impairs Glucose Tolerance

To establish type 2 diabetes induced Alzheimer's disease animal model, ICR mice fed 60% high fat diet for 12 weeks from 8 weeks old. As shown in Fig. 2, high fat diet induced significant weight gain (297.34% vs. 0 week, Fig. 2 A), increased fasting serum glucose level (NC; 155 ± 13.49 mg/dl, HFD; 522 ± 31.6 mg/dl, at 14^{th} week, Fig. 2 B). Most importantly, glucose tolerance was significantly impaired in high fat diet group (162095 mg/dl*min, differences between HFD and NC of area under the curve [AUC] of intraperitoneal glucose tolerance test graph, Fig. 2 C, D) compared with normal diet group.

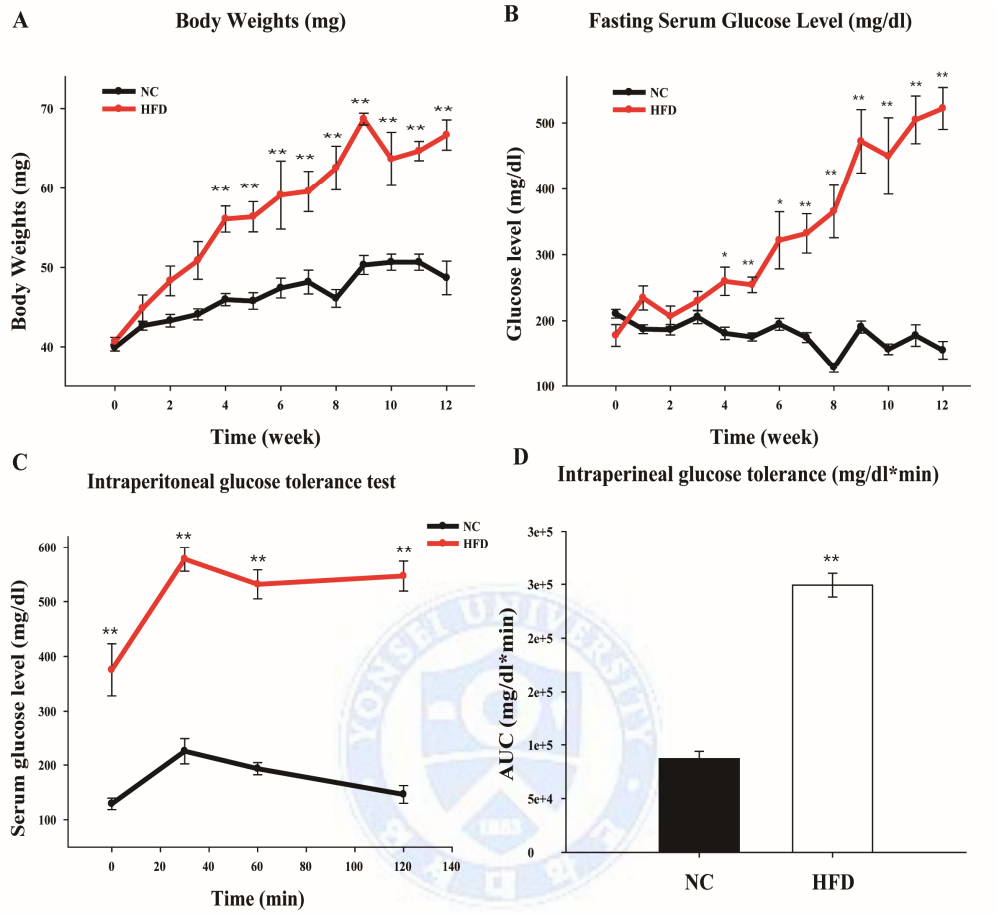


Figure 2. Diet-induced changes in weight, fasting serum glucose level and glucose tolerance changes. (A) Body weight changes of high fat diet group and normal diet group for 12 weeks. (B) Fasting serum glucose level changes of high fat diet group and normal diet group for 12 weeks. (C) Glucose levels during intraperitoneal glucose tolerance test (IPGTT). (D) Area under the curve [AUC] of the glucose level progression curves of IPGTT was compared. *p<0.05, **p<0.01.

2. Agmatine Administration Restores Impaired Glucose Tolerance in High Fat Fed Mice

To determine the effects of agmatine administration to high fat diet fed mice, high fat diet fed mice were randomly divided into two groups; HFD group which would be treated saline and HFD+AGM group which would be treated agmatine (100 mg/kg/ip) for 2 weeks. As shown in Fig. 3, glucose intolerance was significantly recovered by repeated administration of agmatine for 2 weeks (p>0.01 vs. 14^{th} week of HFD and 12^{th} week of HFD+AGM, Fig. 3 C, D). However, no significant difference was found in body weight and fasting serum glucose level (Fig. 3 A, B).

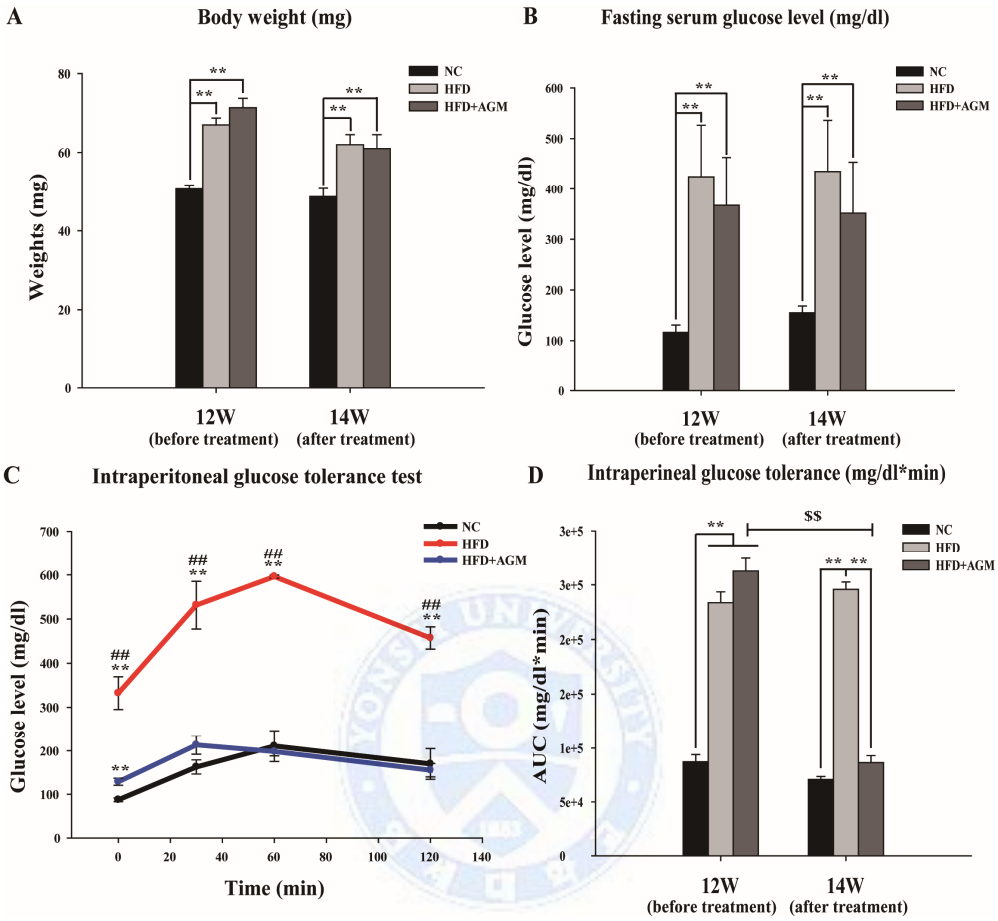


Figure 3. Effects of agmatine administration on body weight, fasting serum glucose level and glucose intolerance in high fat diet mice. Comparison biochemical changes between 12th (before agmatine treatment) and 14th week (after agmatine treatment). (A) Body weight changes between 12th week and 14th week. (B) Fasting serum glucose level changes between 12th and 14th week. (C) Glucose levels during intraperitoneal glucose tolerance tests (IPGTT) in 14th week. **p<0.01 vs. NC, ##p<0.01 vs. HFD+AGM. (D) Changes in area under the curve [AUC] of the glucose level progression curves of IPGTT between 12th and 14th week. **p<0.01, $$ p<0.01 HFD+AGM in 12W vs. HFD+AGM in 14 W.

3. Agmatine Treatment Alleviates Brain Insulin Resistance Induced by High Fat Diet in Both the Cortex and the Hippocampus

To evaluate whether neuronal insulin resistance was induced by high fat diet, western blot assay was conducted both in the hippocampus and the cortex. As seen in Fig. 4, the protein expression of p-IRS-1 (p>0.05, vs. NC) and p-Akt (p>0.05, vs. NC) both in the hippocampus and the cortex were significantly decreased in HFD compared with NC. However, mice in HFD+AGM revealed significant higher p-IRS-1 and p-Akt (p-IRS-1; p>0.05, vs. HFD, p-Akt; p>0.05, vs. HFD) protein expressions in both the hippocampus and the cortex. Amount of phosphorylated molecules of HFD+AGM were increased as much as NC both in the cortex (p-IRS-1; 93%, p-Akt; 166%, in percentage of NC) and hippocampus (p-IRS-1; 94%, p-Akt; 127% in percentage of NC), even in hippocampus, p-Akt was increased in HFD+AGM more than NC.

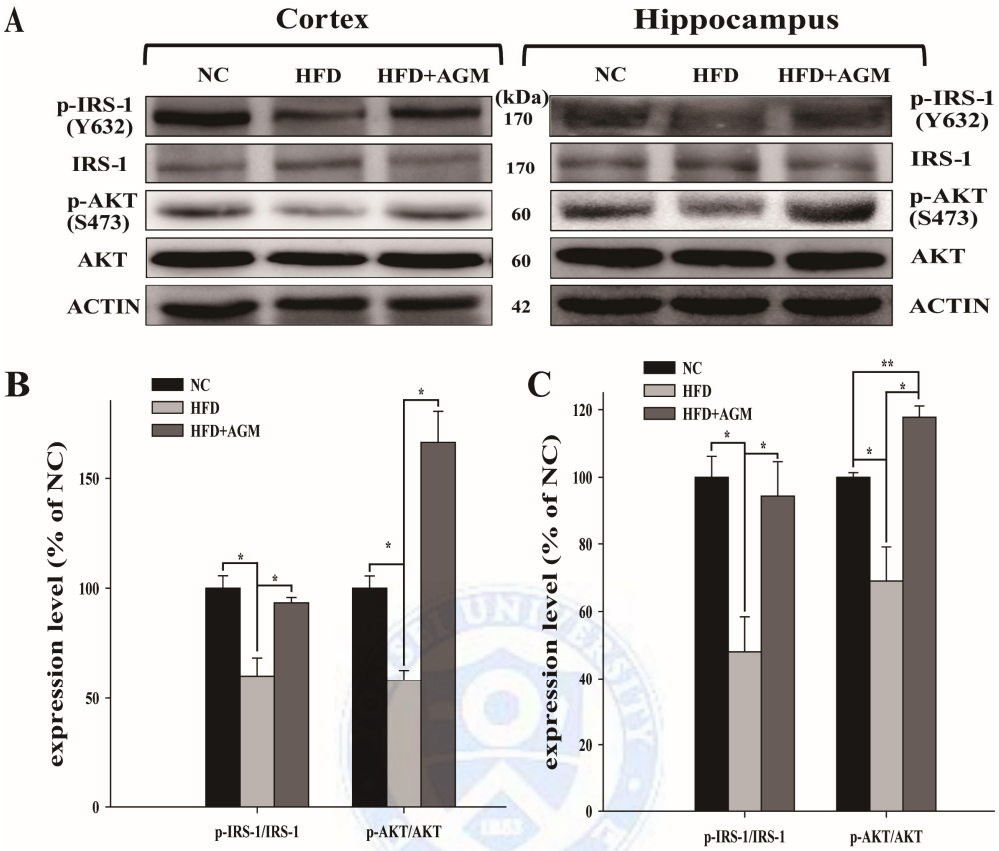
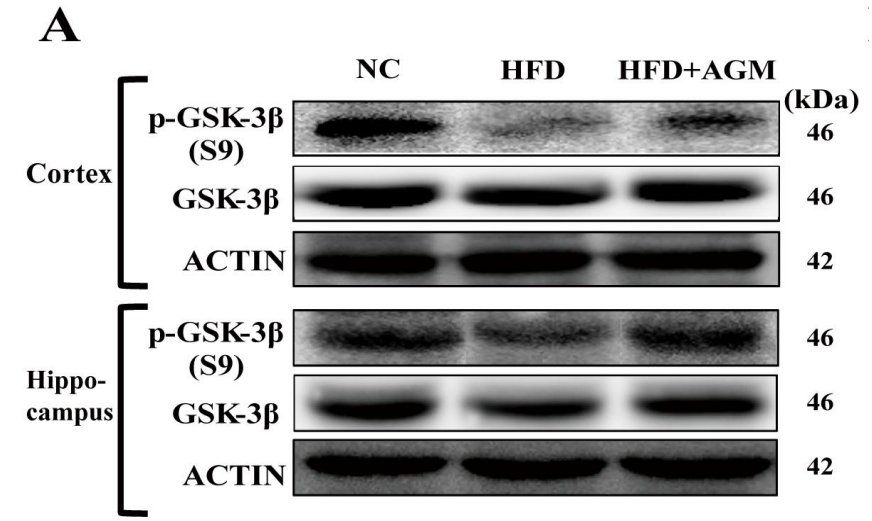


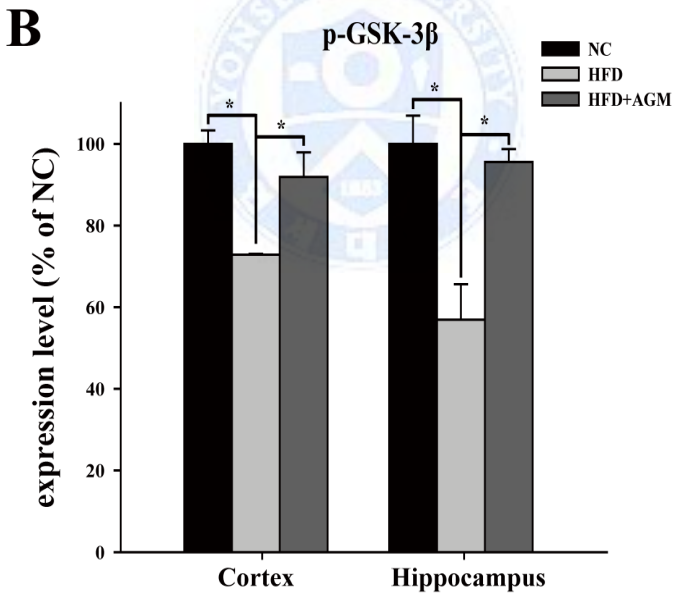
Figure 4. Effects of agmatine administration on expression levels of insulin downstream proteins in the hippocampus and the cortex of high fat diet fed mice. (A) Effects of agmatine on p-IRS-1 and p-Akt protein expression in the hippocampus and the cortex by western blot assay. (B) Quantification of proteins in the cortex is expressed as the ration (in %) of NC. (C) Quantification of proteins in the hippocampus is expressed as the ration (in %) of NC. *p<0.05, **p<0.01.

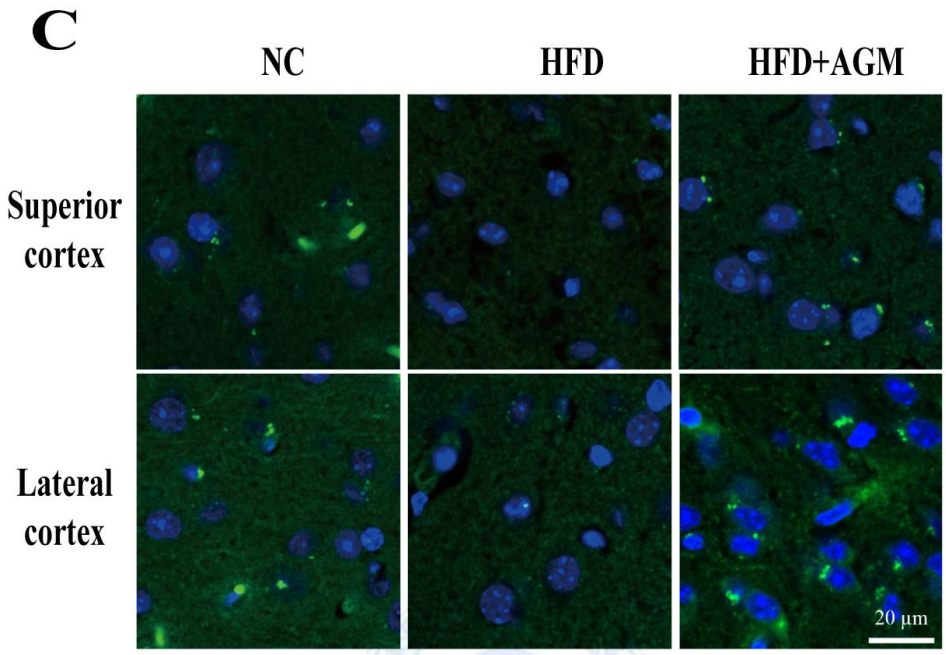
4. Agmatine Treatment Restores Phosphorylation of Glycogen Synthase Kinase-3β in Both the Cortex and the Hippocampus of High Fat Diet Fed Mice

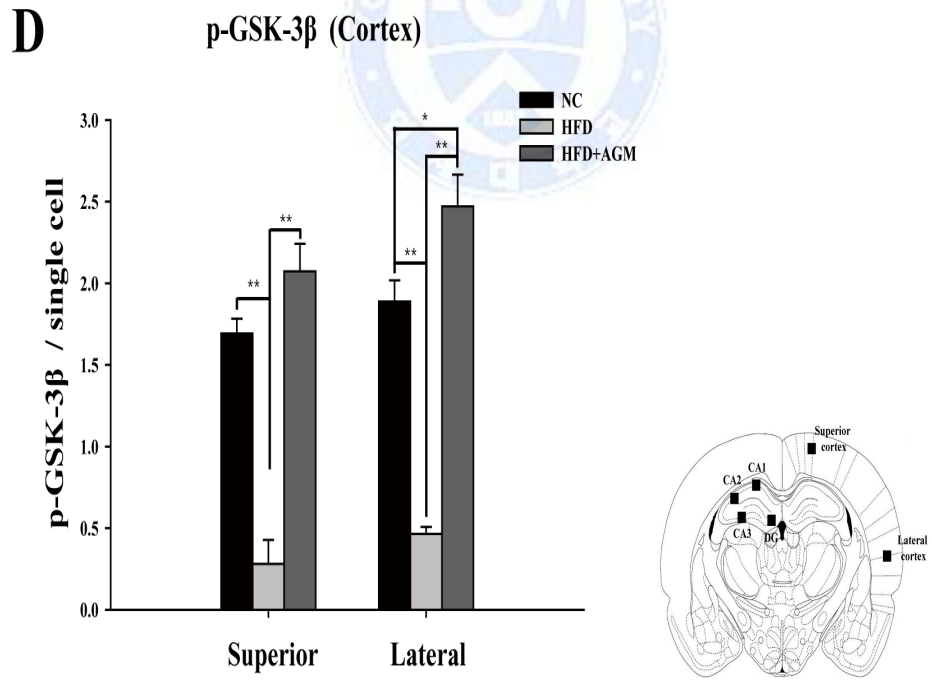
Among the insulin downstream molecules, glycogen synthase kinase-3β (GSK-3β) is well known to be directly related to phosphorylation of tau. To confirm the effect of agmatine administration on phosphorylation of GSK-3β in high fat diet fed mice, western blot and immunofluorescence assay were conducted. As seen in Fig. 4, the protein expression of p-GSK-3β (p>0.05, vs. NC) both in the hippocampus and the cortex were significantly decreased in HFD compared with NC. However, mice in HFD+AGM revealed significant higher p-GSK-3β (p>0.05, vs. HFD) protein expression in both the hippocampus and the cortex. Amount of phosphorylated GSK-3β of HFD+AGM were increased as much as NC both in the cortex (91%, in percentage of NC) and hippocampus (95%, in percentage of NC).

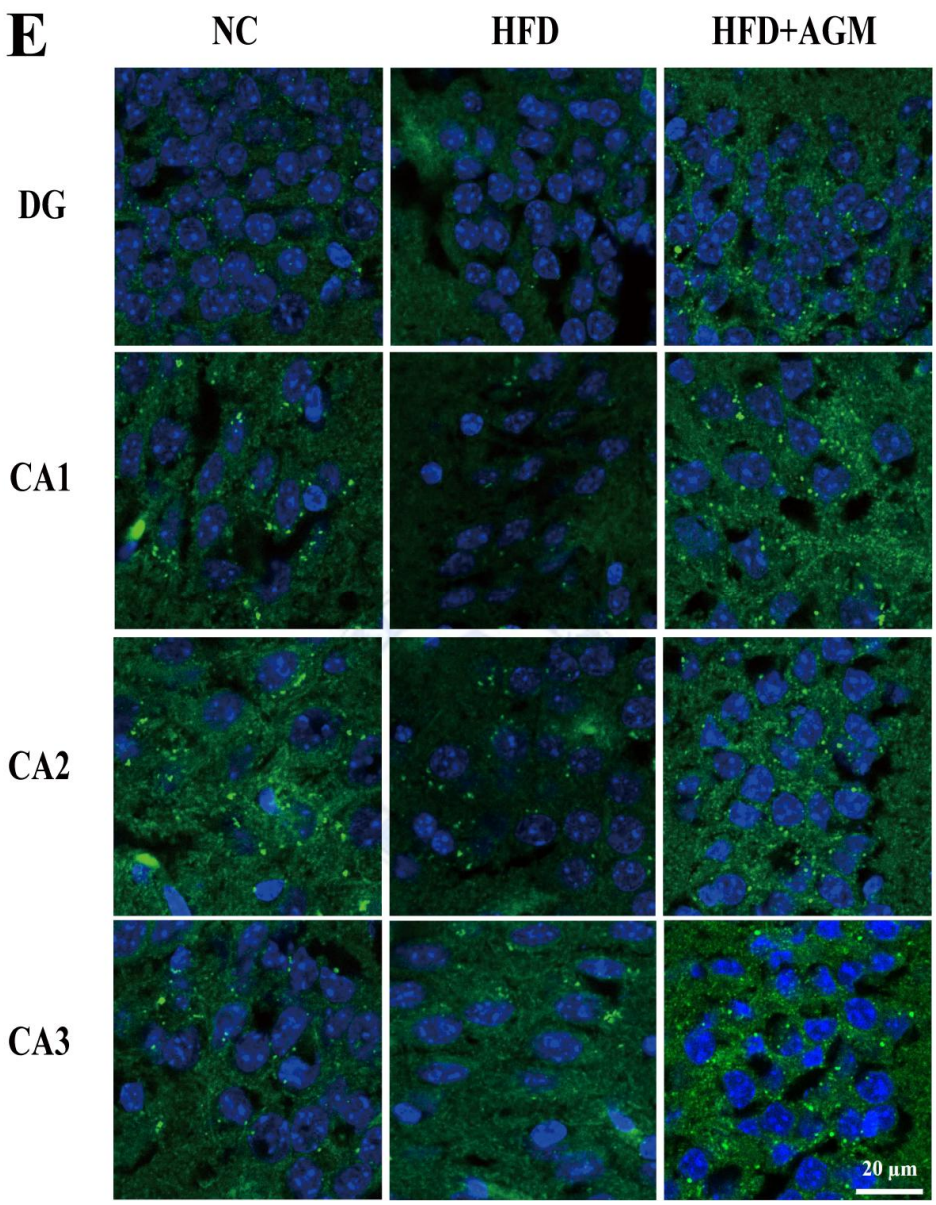
Expression of p-GSK-3β was significantly reduced in the hippocampus and the cortex of HFD (DG; p<0.01, vs. NC, CA1; p<0.01, vs. NC, CA2; p<0.01, vs. NC, frontal cortex; p<0.01, vs. NC, lateral cortex; p<0.01, vs. NC) except CA3 of hippocampus (Fig 5. C, E). However, repeated agmatine administration to high fat diet fed mice significantly restored the expression of p-GSK-3β in the hippocampus and the cortex (CA1; p>0.05, vs. HFD, CA2; p<0.01, vs. HFD, CA3; p<0.01, vs. HFD, frontal cortex; p<0.01, vs. HFD, lateral cortex; p<0.01, vs. HFD) except dentate gyrus. This result is correlated with western blot assay results (Fig. 5, A).


A
NC
HFD
HFD+AGM
(kDa)
Cortex
p-GSK-3β (S9)
GSK-3β
ACTIN
46
46
42
Hippo-campus
p-GSK-3β (S9)
GSK-3β
ACTIN
46
46
42


B
p-GSK-3β
NC
HFD
HFD+AGM
expression level (% of NC)
0
20
40
60
80
100
*
*
*
*
Cortex
Hippocampus


C
NC
HFD
HFD+AGM
Superior cortex
Lateral cortex
20 μm


D
p-GSK-3β (Cortex)
NC
HFD
HFD+AGM
p-GSK-3β / single cell
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
**
**
**
Superior
Lateral
Superior cortex
CA1
CA2
CA3
DG
Lateral cortex


E
NC
HFD
HFD+AGM
DG
CA1
CA2
CA3
20 μm

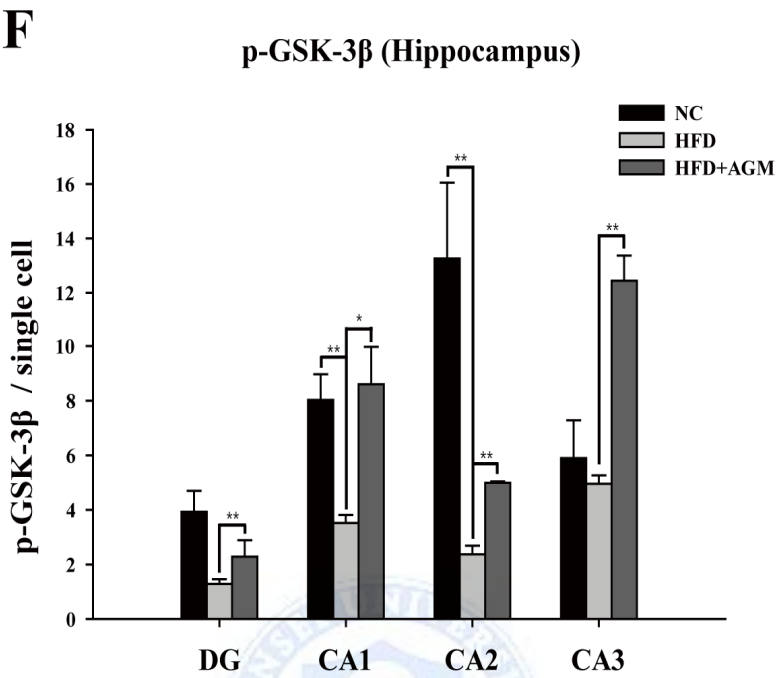
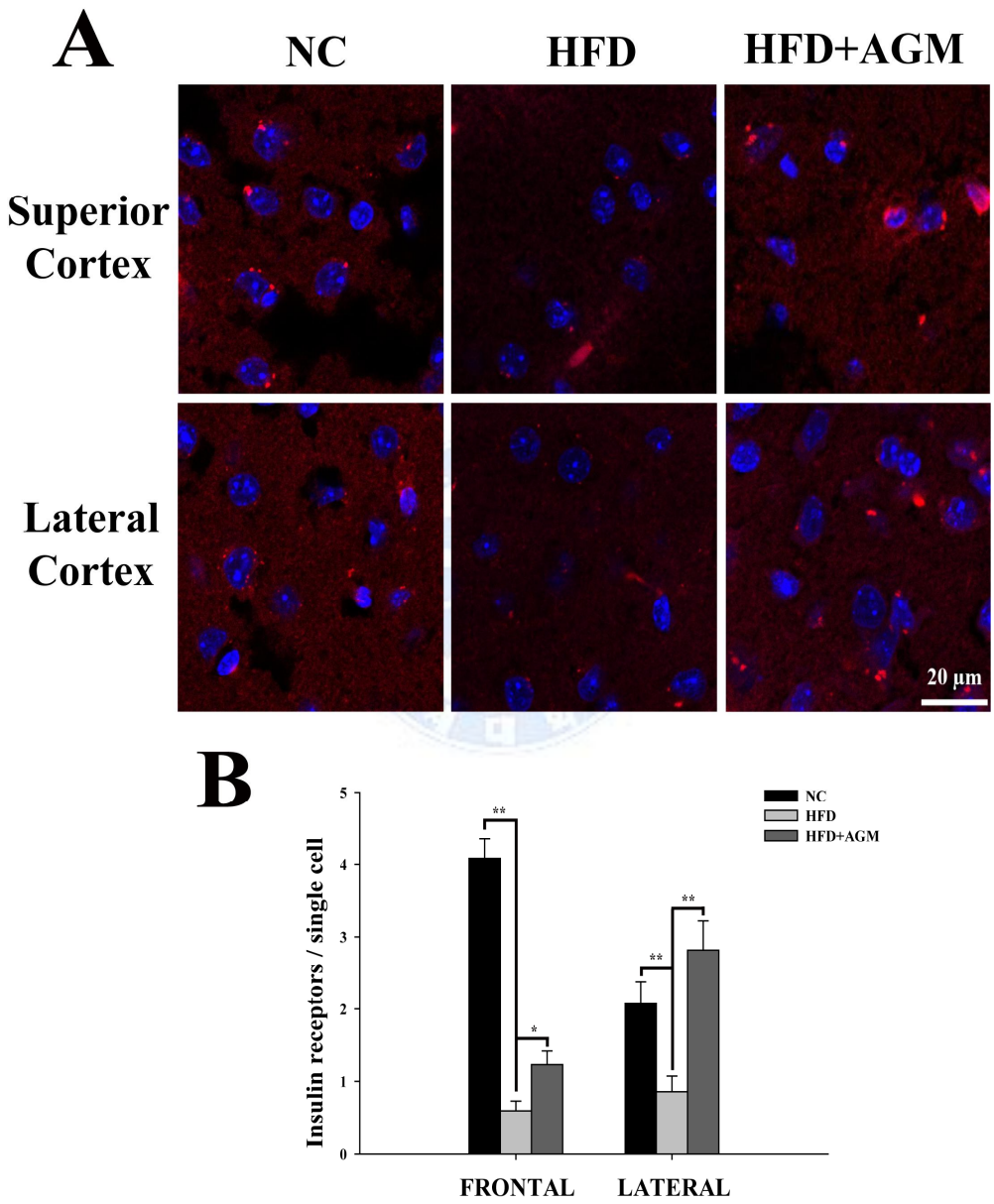
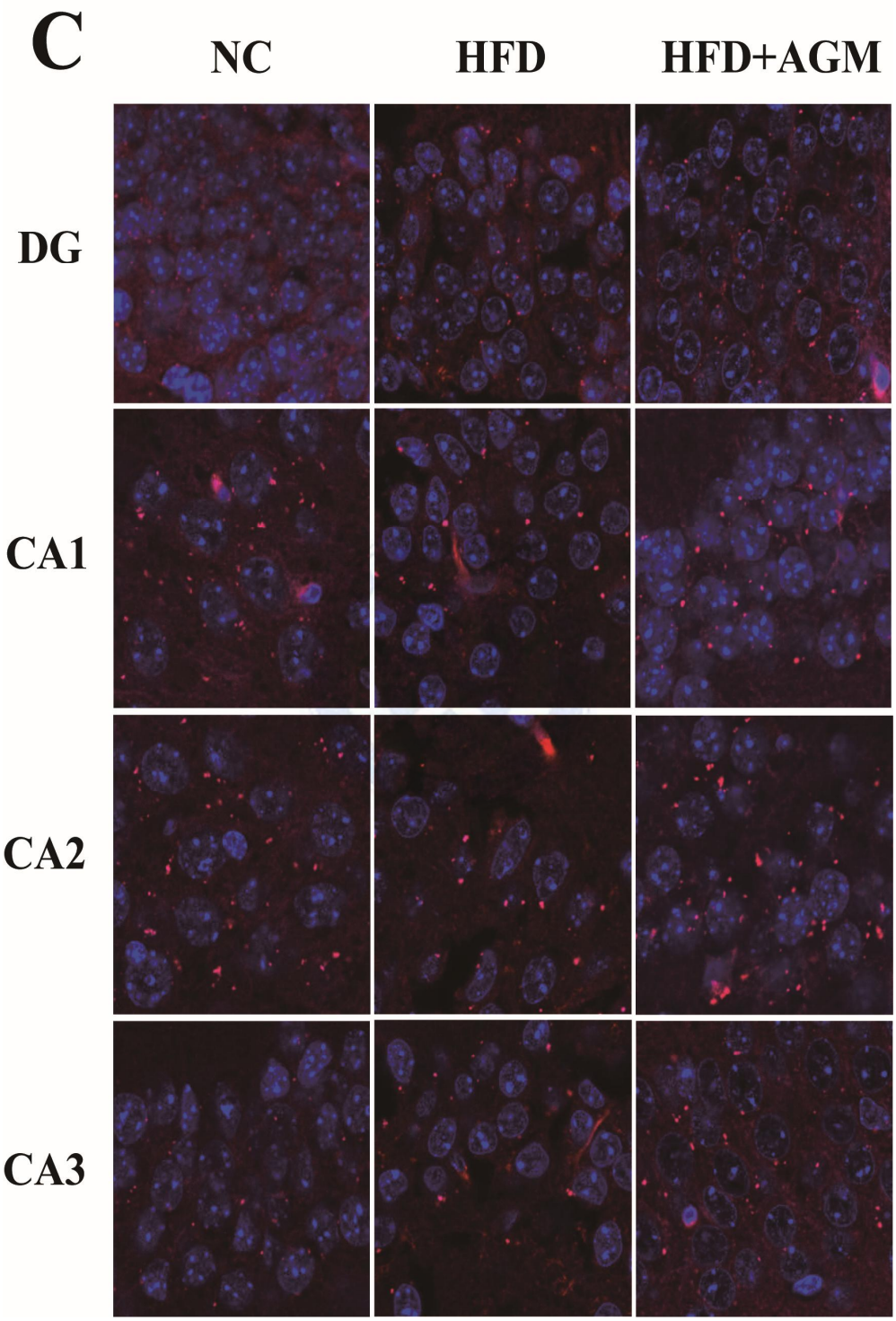


Figure 5. Effects of agmatine administration on phosphorylation of GSK-3β in the cortex and hippocampus of high fat diet fed mice (A) Effects of agmatine on p-GSK-3β protein expression in the hippocampus and the cortex by western blot assay. (B) Quantification of p-GSK-3β in the hippocampus and the cortex is expressed as the ration (in %) of NC. **(**C) Fluorescence images of p-GSK-3β in the hippocampus. DG; dentate gyrus, CA1; Cornu Ammonis 1, CA2; Cornu Ammonis 2, CA3; Cornu Ammonis 3. **(**D) Fluorescence images of p-GSK-3β in the cortex. (E) Quantification positive spots of p-GSK-3β per single cell of hippocampus is expressed. (F) Quantification positive spots of p-GSK-3β per single cell of cortex is expressed. *p<0.05, **p<0.01. The scale bars represent 20 μm.

5. Agmatine Treatment Recovers Reduced Expressions of Insulin Receptors in Both in the Cortex and the Hippocampus of High Fat Diet Fed Mice

To examine the effect of agmatine administration on expression of insulin receptor in high fat diet fed mice, immunofluorescence assay was conducted both in the cortex and the hippocampus. In immunofluorescence images (Fig. 6. A, C), expression of insulin receptor was significantly reduced all regions of the hippocampus except dentatus gyrus region (CA1; $p>0.01$, vs. NC, CA2; $p<0.05$, vs. NC, CA3; $p<0.01$, vs. NC) and the cortex (frontal cortex; $p>0.01$, vs. NC, lateral cortex; $p<0.01$, vs. NC). However, repeated treatment of agmatine to high fat diet fed mice significantly increased expression of insulin receptor in all regions of the hippocampus (DG; $p>0.01$, vs. HFD, CA1; $p>0.01$, vs. HFD, CA2; $p<0.01$, vs. HFD, CA3; $p<0.01$, vs. HFD) and cortex (frontal cortex; $p>0.05$, vs. HFD, lateral cortex; $p<0.01$, vs. HFD). Moreover, agmatine treatment group showed significantly more amount of insulin receptor than normal diet group in CA2, CA3 regions of the hippocampus group (CA2; $p<0.05$, vs. NC, CA3; $p<0.05$, vs. NC).


A
NC
HFD
HFD+AGM
Superior Cortex
Lateral Cortex
20 μm
B
Insulin receptors / single cell
NC
HFD
HFD+AGM
FRONTAL
LATERAL


C
NC
HFD
HFD+AGM
DG
CA1
CA2
CA3

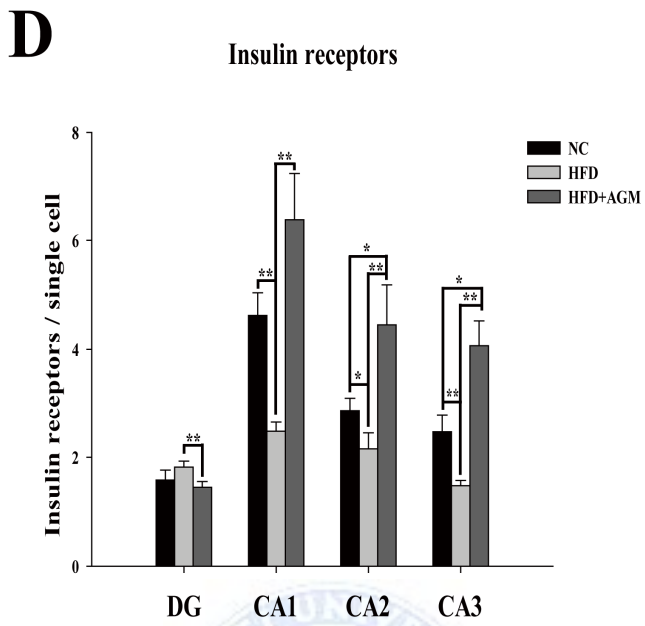


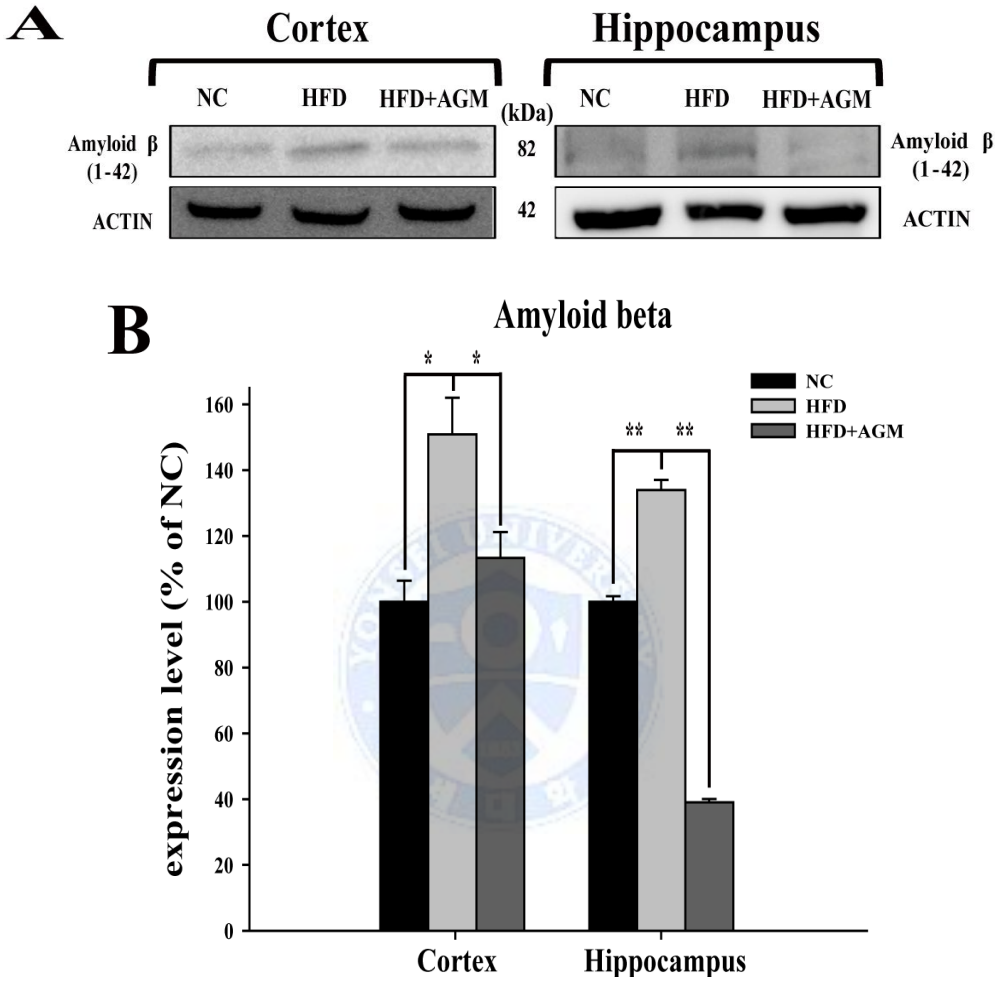
Figure 6. Effects of agmatine administration on expression of insulin receptor in the cortex and the hippocampus of high fat diet fed mice. (A) Fluorescence images of insulin receptor in the cortex. (B) Quantification positive spots of insulin receptors per single cell of cortex is expressed. (C) Fluorescence images of insulin receptor in the hippocampus. DG; dentate gyrus, CA1; Cornu Ammonis 1, CA2; Cornu Ammonis 2, CA3; Cornu Ammonis 3. (D) Quantification positive spots of insulin receptors per single cell of hippocampus is expressed. *p<0.05, **p<0.01. The scale bars represent 20μm.

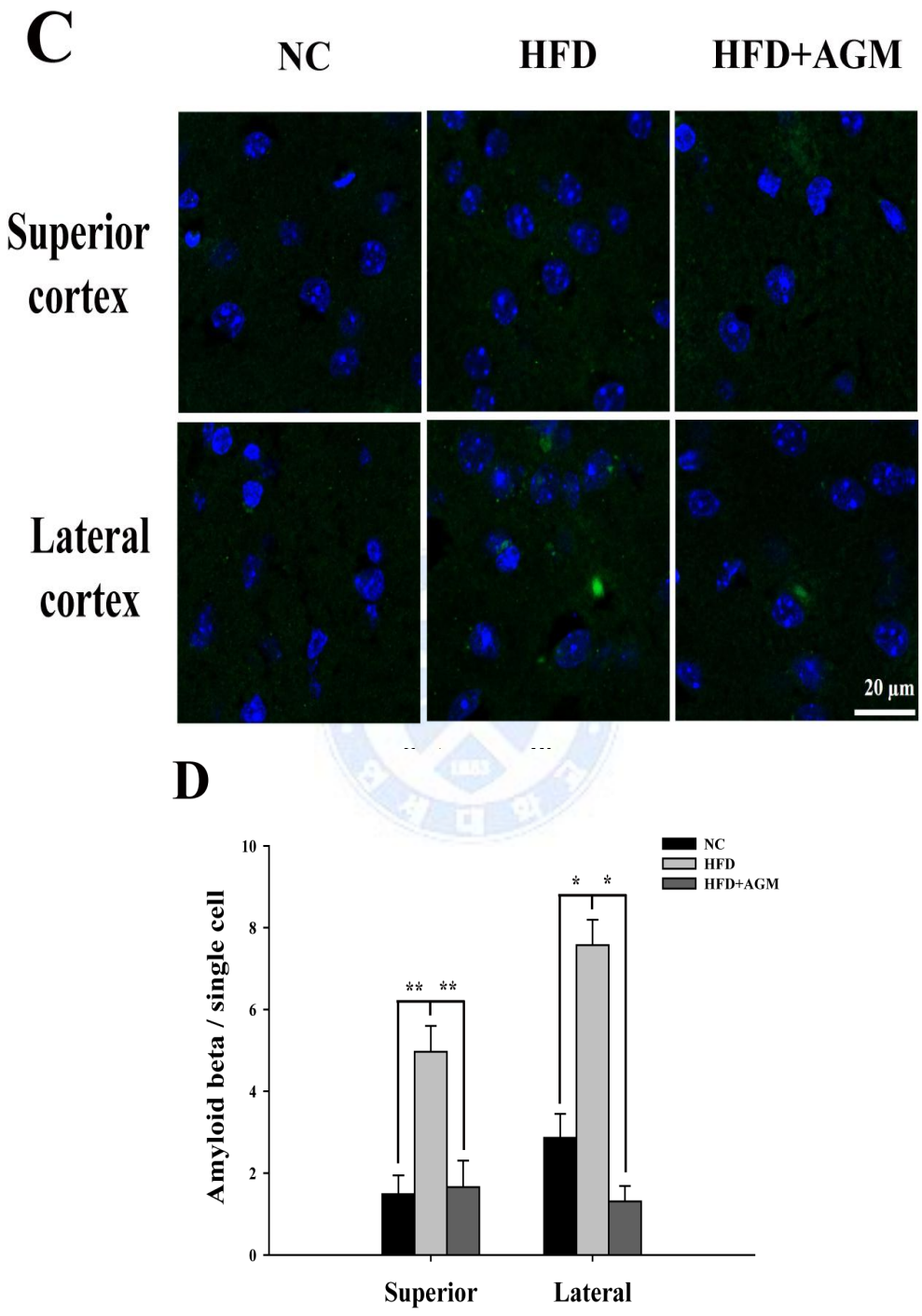
6. Agmatine Injection Inhibits Accumulation of Amyloid Beta in Both the Cortex and the Hippocampus of High Fat Fed Mice

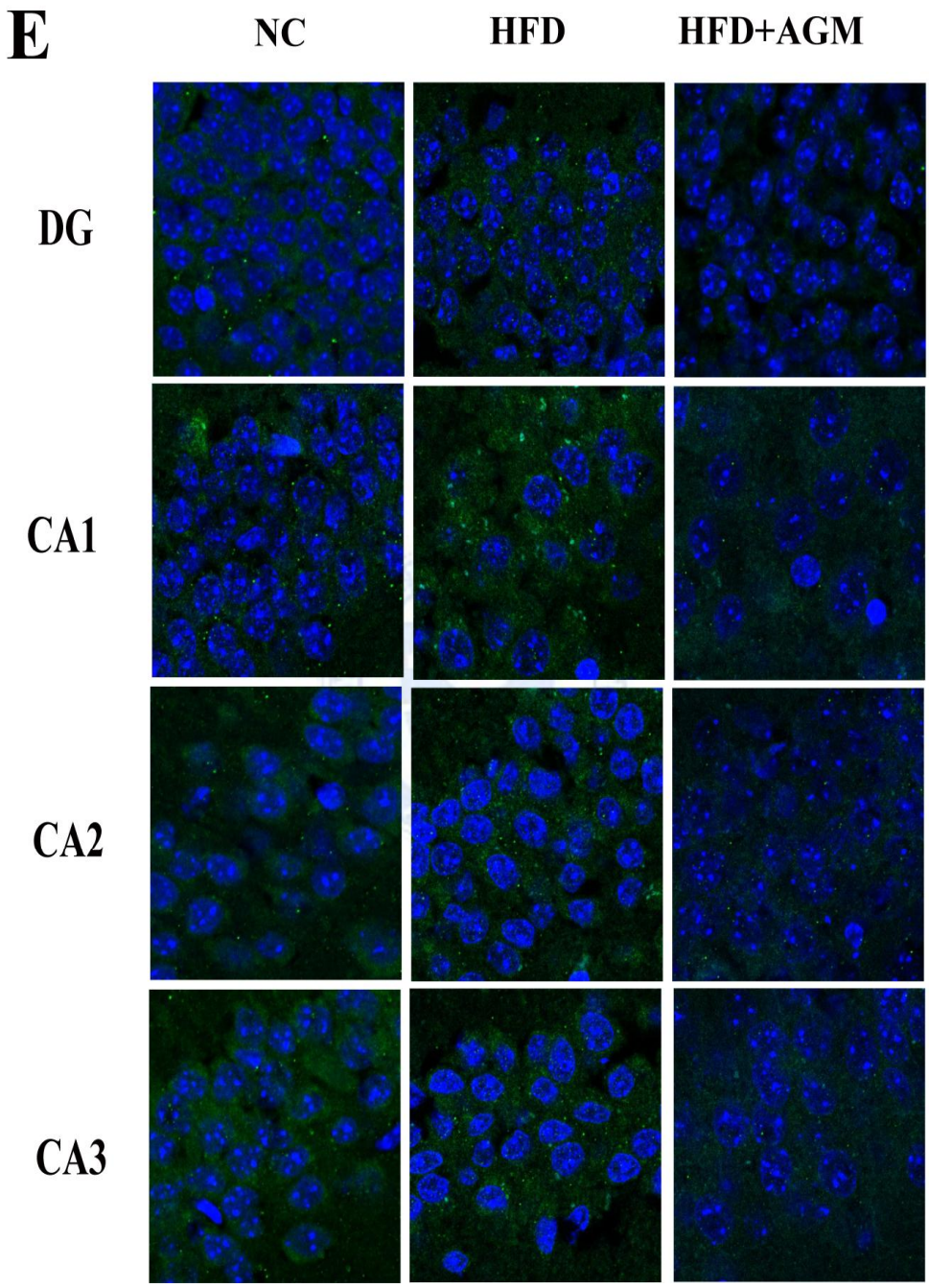
To examine the effect of agmatine treatment on accumulation of Aβ by high fat diet, western blot assay and immunohistochemistry assay were conducted. As shown in Fig. 7 A, the protein expression of Aβ were increased in HFD (hippocampus; $p<0.01$, vs. NC, cortex; $p<0.01$, vs. NC) compared with NC. However, HFD+AGM showed significantly decreased expression of Aβ (hippocampus; $p<0.01$, vs. HFD, cortex; $p<0.01$, vs. HFD) compared with HFD.

In immunofluorescence assay of the cortex, Aβ were significantly more expressed in HFD (frontal cortex; $p<0.01$ vs. NC, lateral cortex; $p<0.05$ vs. NC) (Fig. 7 G, H). Repeated treatment of agmatine to high fat diet fed mice significantly lowered the expression of Aβ in the cortex (frontal cortex; $p<0.01$ vs. HFD, lateral cortex; $p<0.05$ vs. HFD) (Fig. 7 C).

In immunofluorescence assay of the hippocampus, positive spots of Aβ were significantly increased in the hippocampus of HFD except dentate gyrus (CA1; $p<0.01$ vs. NC, CA2; $p<0.01$, vs. NC, CA3; $p<0.01$ vs. NC) (Fig. 7 E). Repeated agmatine administration significantly lowered the expression of Aβ in the hippocampus except DG (CA1; $p<0.01$ vs. HFD, CA2; $p<0.01$, vs. HFD, CA3; $p<0.01$ vs. HFD) (Fig. 7 E).


A
Cortex
Hippocampus
NC
HFD
HFD+AGM
(kDa)
NC
HFD
HFD+AGM
Amyloid β
(1-42)
82
Amyloid β
(1-42)
ACTIN
42
ACTIN
B
Amyloid beta
expression level (% of NC)
160
140
120
100
80
60
40
20
0
NC
HFD
HFD+AGM
*
*
**
**
Cortex
Hippocampus


C
NC
HFD
HFD+AGM
Superior cortex
Lateral cortex
20 μm
D
Amyloid beta / single cell
NC
HFD
HFD+AGM
** **
* *
Superior
Lateral


E
NC
HFD
HFD+AGM
DG
CA1
CA2
CA3

F

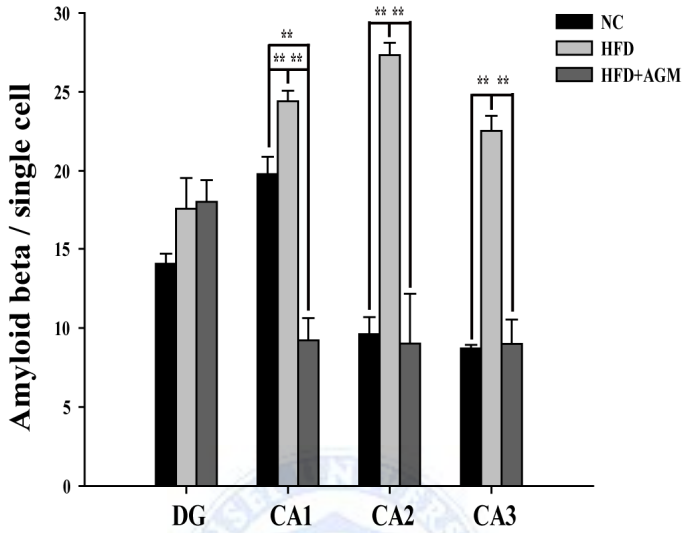


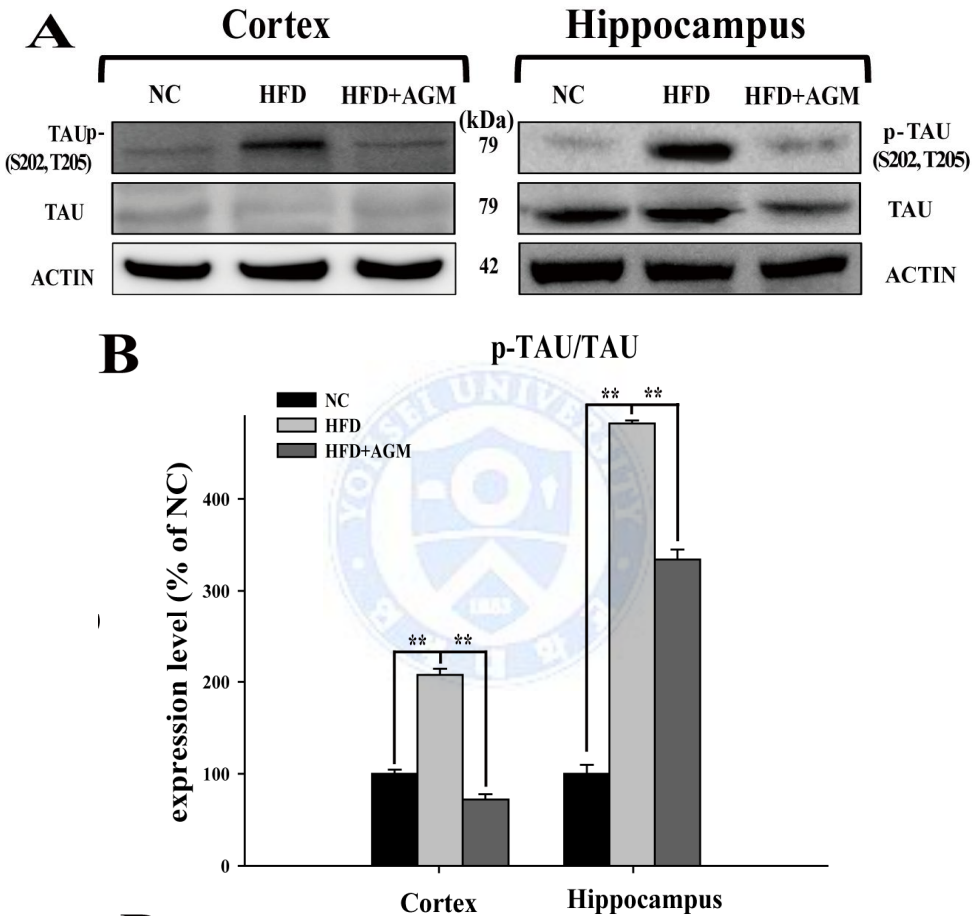
Figure 7. Effects of agmatine administration on accumulation of amyloid beta in the cortex and the hippocampus of high fat diet fed mice. (A) Effects of agmatine on Aβ expression in the hippocampus and the cortex by western blot. (B) Quantification of proteins from the western blot assay is expressed as a ration (in %) of NC. (C) Immunofluorescence images of Aβ in the cortex. (D) Quantification of positive spots of Aβ per single cell in cortex is expressed. (E) Immunofluorescence images of Aβ in the hippocampus. DG; dentate gyrus, CA1; Cornu Ammonis 1, CA2; Cornu Ammonis 2, CA3; Cornu Ammonis 3. (F) Quantification of positive spots of Aβ per single cell in hippocampus is expressed. *p<0.05, **p<0.01. The scale bars represent 20 μm.

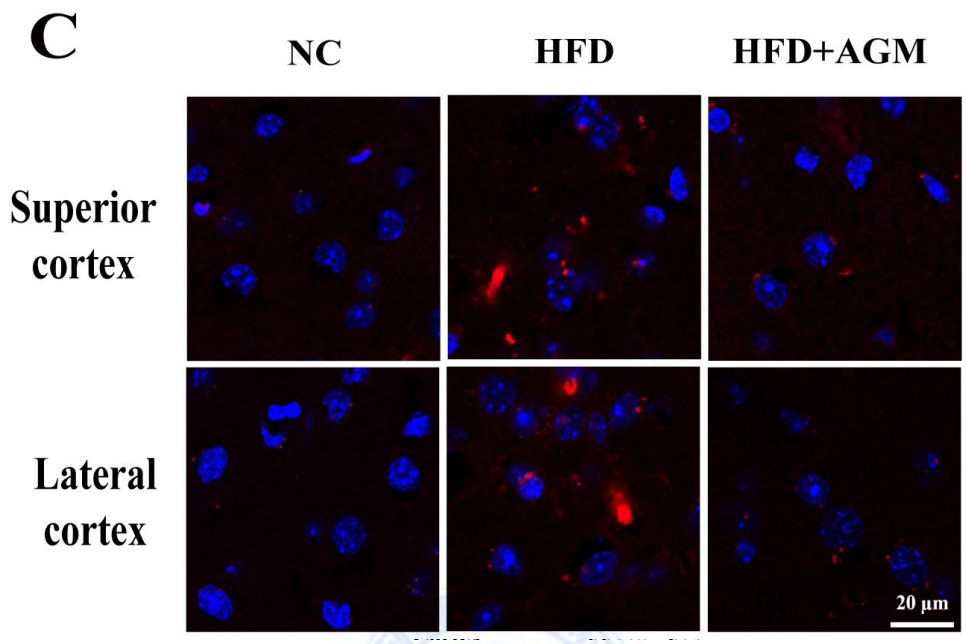
7. Agmatine Injection Reduces Phosphorylation of Tau in Both the Cortex and the Hippocampus of High Fat Fed Mice

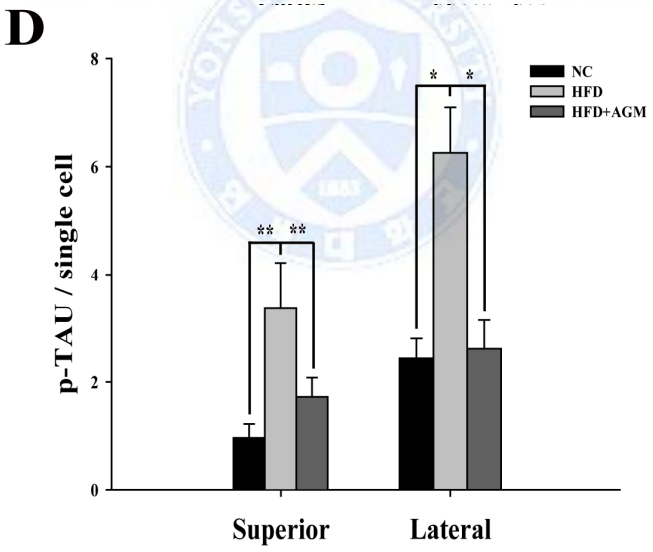
To examine the effect of agmatine treatment on phosphorylation of tau by high fat diet, western blot assay and immunohistochemistry assay were conducted. As shown in Fig. 8 A, the protein expression of p-tau were increased in HFD (hippocampus; $p<0.01$, vs. NC, cortex; $p<0.01$, vs. NC) compared with NC. However, HFD+AGM showed significantly decreased expression of p-tau (hippocampus; $p<0.01$, vs. HFD, cortex; $p<0.01$, vs. HFD,) compared with HFD.

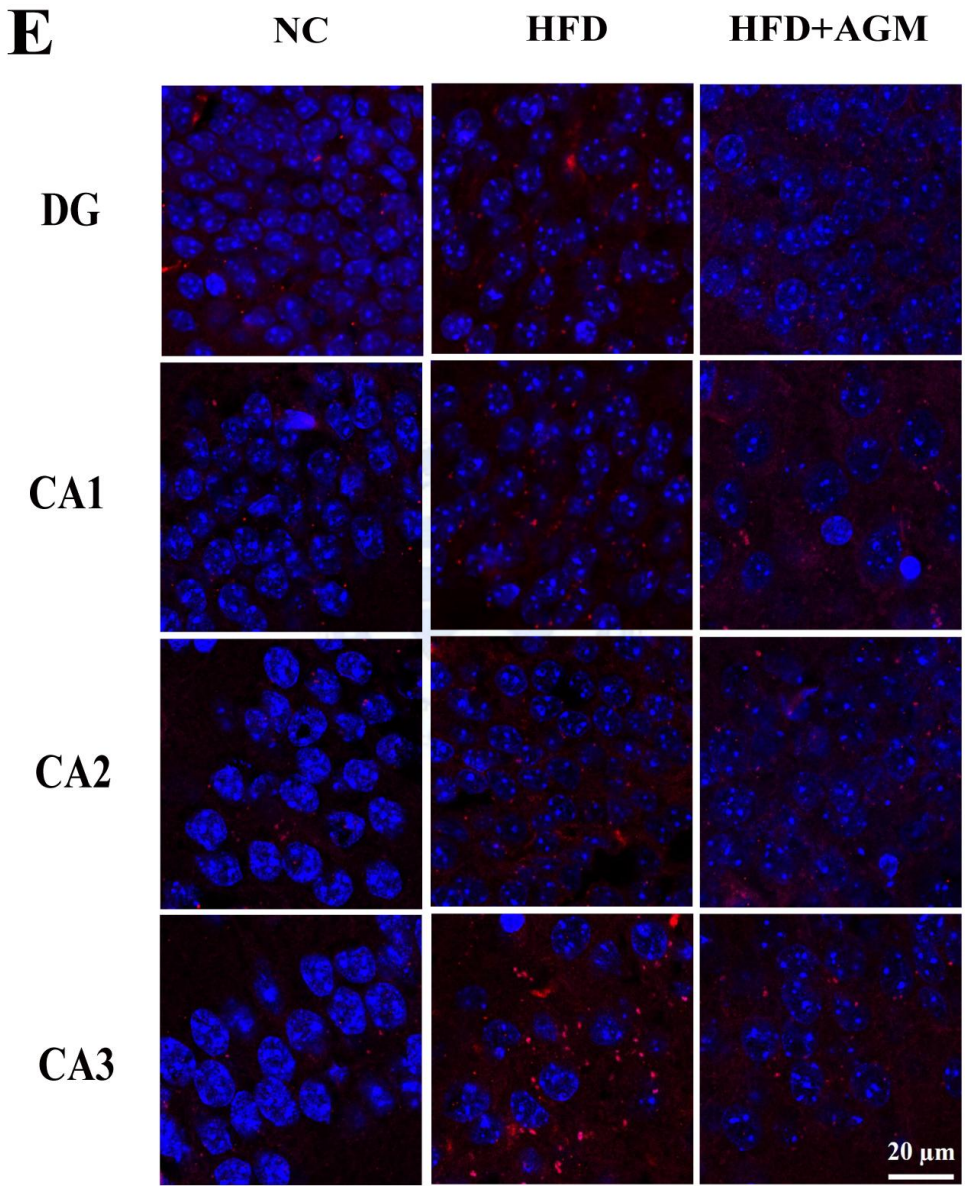
In immunofluorescence assay of the cortex, the amount of positive spots of p-tau was significantly increased in HFD (frontal cortex; $p<0.01$ vs. NC, lateral cortex; $p<0.05$ vs. NC) (Fig. 8 C). Repeated treatment of agmatine to high fat diet fed mice significantly lowered the expression of p-tau in the cortex (frontal cortex; $p<0.01$ vs. HFD, lateral cortex; $p<0.05$ vs. HFD).

In immunofluorescence assay of the hippocampus, positive spots of p-tau was significantly more expressed in DG and CA3 in the hippocampus of HFD (DG; $p<0.01$ vs. NC, CA3; $p<0.01$, vs. NC) (Fig. 8 E). Repeated agmatine administration significantly lowered the expression of p-tau in the hippocampus (DG; $p<0.01$ vs. HFD, CA1; $p<0.05$ vs. HFD, CA2; $p<0.01$, vs. HFD, CA3; $p<0.01$ vs. HFD).


A
Cortex
Hippocampus
NC
HFD
HFD+AGM
NC
HFD
HFD+AGM
(kDa)
TAUp-
(S202, T205)
TAU
ACTIN
79
79
42
p-TAU
(S202, T205)
TAU
ACTIN
B
p-TAU/TAU
NC
HFD
HFD+AGM
expression level (% of NC)
0
100
200
300
400
**
**
**
**
Cortex
Hippocampus


C
NC
HFD
HFD+AGM
Superior cortex
Lateral cortex
20 μm


D
p-TAU / single cell
0
2
4
6
8
NC
HFD
HFD+AGM
**
**
*
*
Superior
Lateral


E
NC
HFD
HFD+AGM
DG
CA1
CA2
CA3
20 μm

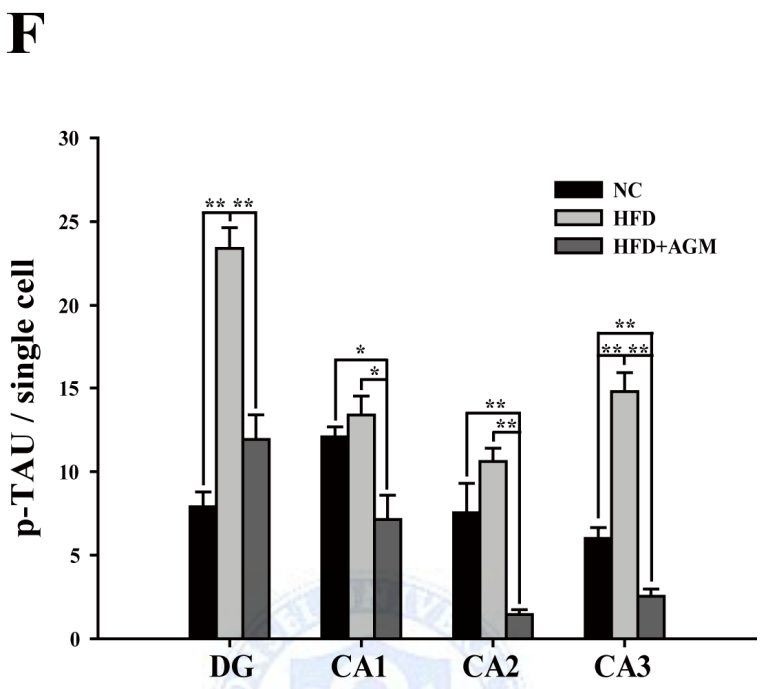


Figure 8. Effects of agmatine administration on phosphorylation of tau in the cortex and the hippocampus of high fat diet fed mice. (A) Effects of agmatine on p-tau protein expression in the hippocampus and the cortex by western blot. (B) Quantification of proteins from the western blot assay is expressed as a ration (in %) of NC. (C) Immunofluorescence images of p-tau in the cortex. (D) Quantification of positive spots of p-tau per single cell in cortex is expressed. (E) Immunofluorescence images of p-tau in the hippocampus. DG; dentate gyrus, CA1; Cornu Ammonis 1, CA2; Cornu Ammonis 2, CA3; Cornu Ammonis 3. (F) Quantification of positive spots of p-tau per single cell in hippocampus is expressed. *p<0.05, **p<0.01. The scale bars represent 20 μm.

8. Agmatine Administration Improves Learning, Memory and Executive Functions of High Fat Diet Fed Mice

To determine reduced the expression level of Aβ and p-tau leads to improvement of learning, memory and executive function, behavior tests were conducted. In Morris water maze test, HFD showed a significant increase in escape latency as compared with NC in last day of training (Fig. 9 A). HFD had significantly shorter latency time (p>0.01 vs. NC) in test session (Fig 9 B). However, agmatine administration to high fat diet fed mice showed significantly longer latency time in the quadrant where platform was located in test session (p>0.01 vs. HFD) (Fig. 9 B).

In nest building test, HFD mice got lower scores compared with normal diet group (p>0.01 vs. NC). However, HFD+AGM had significantly higher scores (p>0.01 vs. HFD) compared with HFD (Fig. 9 C).

Passive avoidance test were conducted for 4 days. After receiving an electronic shock, all groups show no significantly differences for 2 days. 72 hours after the shock, few mice in high fat diet group entered to dark department, but there was no significance (Fig. 9 D).

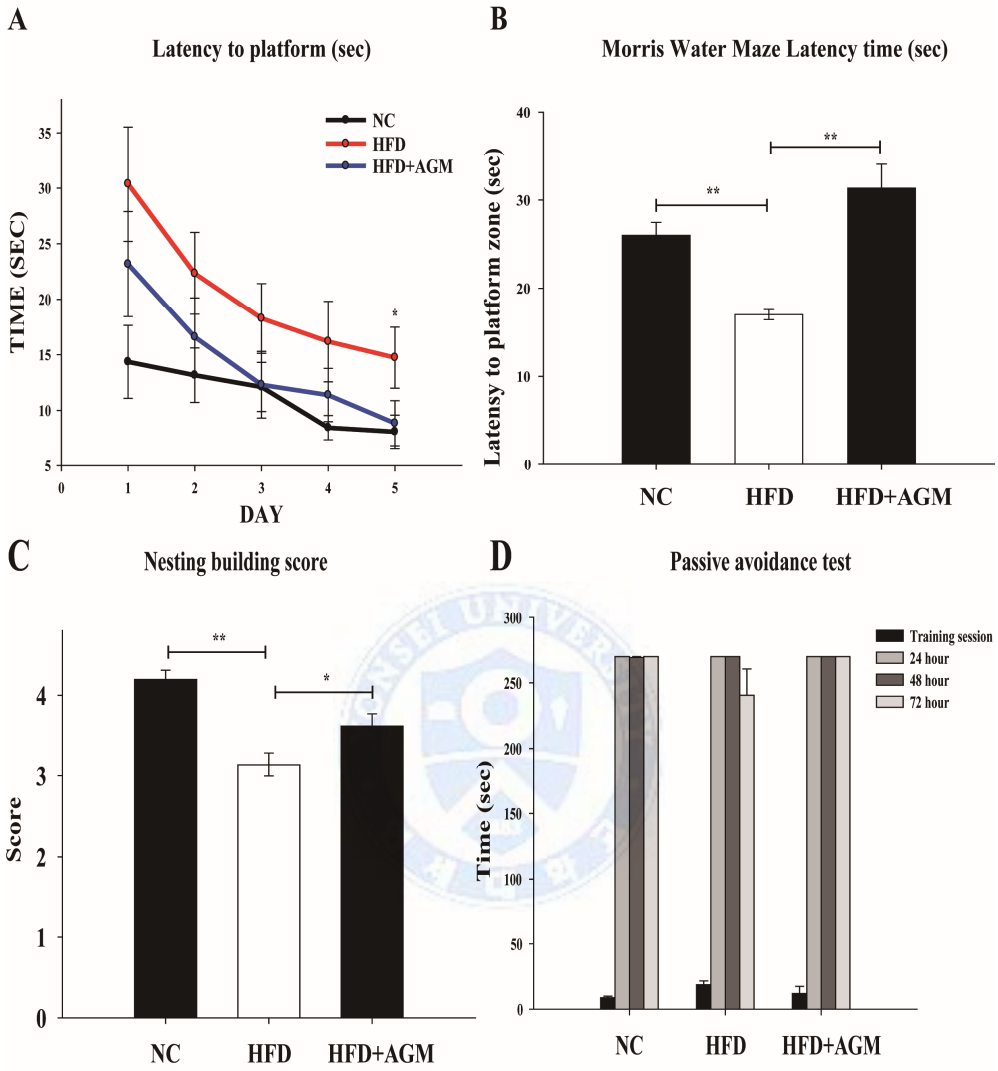


Figure 9. Effects of agmatine administration on learning, memory and executive function in high fat diet fed mice. (A) Mean latency time to platform over 5 days of training session of Morris water maze. *p<0.05 compared with normal diet group. (B) Mean latency time spent in the correct quadrant where platform was located in test session. (C) Nest building scores based on materials used and shape of nest. (D) Step through latency time in passive avoidance test for 3 days. *p<0.05, **p<0.01.

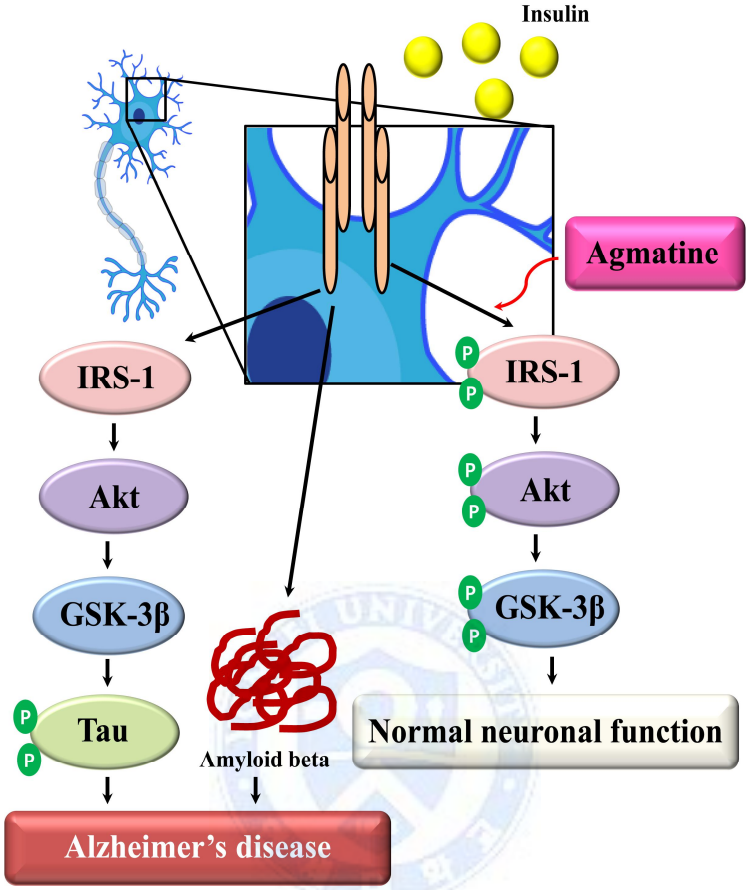


Figure 10. Effects of agmatine on neuronal insulin resistance. Insulin resistnace induces Alzheimer's disease through blunted insulin signaling in neuron. Neuronal insulin resistance lead to accumulation of amyloid beta and phosphorylation of tau. However, agmatine activates insulin signaling to make insulin resistance state neurons work normally, so that agmatine reverses phathogenesis of Alzheimer's disease in insulin resistance state neurons.

Ⅳ. DISCUSSION

The results in this study revealed high fat diet induces not only peripheral glucose intolerance but also neuronal insulin resistance as blunting insulin signaling, leading to Alzheimer's disease like pathogenesis such as accumulation of Aβ and phosphorylation of tau in brain and impaired learning, memory and executive functions. Agmatine treatment ameliorates peripheral glucose intolerance and neuronal insulin resistance, retrieving blunted insulin signaling in brain. Furthermore agmatine administration reduces accumulation of Aβ and phosphorylation of tau, results in improvement of impaired learning, memory and executive function caused by high fat diet (Fig. 10).

Diet induced Alzheimer's disease like models were established by many researchers to study underlaying mechanisms. Clinical researches reported that high fat diet impairs cognitive function.[49-51] Following clinical researches, diet induced Alzheimer's disease like models were designed. High cholesterol diet is related to neuroinflammatory changes and amyloid precursor protein processing.[52] Western diet (41% fat) or high fat diet also increases brain inflammation and impairs cognition.[53] Especially, hippocampus is vulnerable to metabolic dysfunction. High fat diet increases hippocampal oxidative stress, as decease of NF-E2-related factor 2 (Nrf2) signaling,[54] deficiency of mitochondrial homeostasis[55] and impairs hippocampus dependent memory ability.[26] Based on these studies, neuronal insulin resistance is thought as a main cause of cognitive decline induced by diet.[14,56-58]

The animal model used in this study is satisfied all prior studies. 12 weeks of high fat diet induces glucose intolerance. Once injection of low dose of STZ was used for shortening the time animal models were established, it evoked very partial damage to pancreas.[24] Glucose tolerance test is a widely used simple test in clinical practice to diagnose glucose intolerance and type 2 diabetes.[45,59] As shown in Fig. 2 C, D, intraperitoneal glucose tolerance test presented that glucose level of high fat

diet group kept in high, unlikely to normal condition, indicating high fat diet induces glucose intolerance. Glucose intolerance means insulin doesn't react to glucose stimuli, indicating insulin resistance. Moreover, brains of mice in HFD display blunted insulin signaling (Fig. 3). Significantly less phosphorylation of IRS-1, Akt presents neuronal insulin resistance state. 12 weeks of high fat diet and administration of low dose of STZ at 4th week causes peripheral insulin resistance as reported before [24,60-62] and neuronal insulin resistance as well.

Correlated with prior reports, animal model in present study showed significant increases in accumulation of Aβ and phosphorylation of tau (Fig. 7, 8). Furthermore, mice in HFD displayed impairment of cognition as showed poorer performances in behavior tests (Fig. 9). It is well matched with prior report that type 2 diabetes affects cognitive processes such as memory and executive functions.[63]

Morris water maze test is used to evaluate spatial memory, mostly depends on hippocampus, which has the highest concentration of insulin receptors in the brain.[64,65] As shown in Fig. 9 B, result of Morris water maze test implies that brain insulin resistance has negative effect on spatial memory. Nest building test is conducted for testing executive function of high fat diet fed mice. Deterioration of executive functions and daily live activities are early signs of Alzheimer's disease.[66,67] Nesting is crucial for mice to survive in wild. As seen in Fig. 9 C, high fat diet deteriorates nest building ability. Passive avoidance test were conducted for 4 days after Morris water maze test. Passive avoidance test evaluates memory of contextual fear and memory consolidation, depends on both hippocampus and cortex. Different from the Morris water maze test, passive avoidance test didn't have dramatic changes between NC and HFD for 2 days. On the 3rd day of test session, some mice in HFD entered dark room within given time, suggesting that high fat diet might have harmful effect on memory consolidation. These results from behavior tests suggest that high fat diet induces Alzheimer's disease like pathology to ICR mice.

Previous studies demonstrated the reason why agmatine lower plasma glucose in diabetic rats is that agmatine activates imidazoline I_2 receptor in adrenal gland. Agmatine increases β-endorphin release to lowers plasma glucose through opioid μ-receptors in type 1 diabetes rats.[34,36] High fructose diet induced insulin resistance was recovered by agmatine administration, but it blunted by blockage of I_2-imidazoline receptors.[37] Correlated with previous reports, agmatine treatment recovers glucose intolerance in high fat fed mice in this study (Fig. 3). Agmatine could activate imidazoline I_2 receptor in adrenal gland to increase utilization of glucose in this study.

Previous studies didn't examine the brains of diabetic animals such as whether insulin resistance was induced in brain and how agmatine affect neuronal insulin resistance, and whether Alzheimer's disease is induced. A study reveals that type 1 diabetes animals display cognitive decline, but agmatine treatment reverses it by reducing oxidative stress and increasing cholinesterase activity.[68] Agmatine was applied to streptozotocin induced Alzheimer's disease like model. Agmatine activates antioxidant signaling and insulin signaling, leading to protecting neuronal cells from cell death in intracerebroventricular STZ-treated rats and lead to better performance in Morris water maze test.[35,69] Following these papers, present study applies agmatine to high fat diet induced cognitive impairment animal model. As seen in Fig. 4, high fat diet induced insulin resistance was reversed by agmatine, increasing phosphorylation of IRS-1, Akt and GSK-3β. Furthermore, increased phosphorylation of insulin signaling molecules affected to cognitive ability. Agmatine treatment rescues high fat diet fed ICR mice from impairment of memory, learning and executive function (Fig. 9) through activation of insulin signaling transduction.

There are some suggestions of how agmatine activates insulin signaling transduction. Other study reports that agmatine prevents scopolamine, muscarinic cholinergic antagonist, induced memory deficit by activation of ERK and Akt[70] and

especially activation of disrupted of hippocampal Akt/GSK-3β.[69] Therefore, agmatine could directly activate not only Akt and GSK-3β but also IRS-1 to improve memory deficit caused by high fat diet as seen in this study. Other expected reason is that agmatine works as neurotransmitter. Agmatine is reduced in superior frontal gyrus and cerebellum in Alzheimer's disease patients.[71] Agmatine is reduced in CA1 region in rats,[72] but spatial learning induces region-specific elevation in agmatine such as CA1, DG and entorhinal cortex, suggesting agmatine as a novel neurotransmitter.[73] Furthermore, agmatine is elevated in synapse of hippocampus during spatial learning.[74] Agmatine could work as a neurotransmitter in this study to improve learning, memory and executive function.

In conclusion, this study applied for the first time, agmatine on neuronal insulin resistance model. Agmatine activates blunted insulin signaling transduction in brain and reduces amyloid beta and phosphorylated tau, results in improvement of impaired learning, memory and executive function induced by high fat diet (Fig. 10).

Ⅴ. CONCLUSION

This present study shows that repeated administrations of agmatine rescued high fat fed mice from Alzheimer's disease induced by brain insulin resistance. The major findings of this study are summarized as below :

1. 12 weeks of high fat diet and once injection of low dose of streptozotocin (HFD/STZ) to ICR mice induced weight gain, increase of fasting serum glucose level and glucose intolerance.
2. HFD/STZ evoked not only peripheral insulin resistance but also neuronal insulin resistance, as confirmed by reduced amount of phosphorylation of insulin downstream molecules ; IRS-1, Akt, GSK-3β.
3. Neuronal insulin resistance increased accumulation of amyloid beta and phosphorylated tau in brain and resulted in impairment of learning, memory and executive functions.
4. Agmatine treatment for 2 weeks rescued high fat fed mice from both impaired peripheral glucose tolerance, blunted insulin signals in brain and increased the number of insulin receptors in brain.
5. Agmatine administrations reduced expression of amyloid beta and phosphorylated tau in both the cortex and the hippocampus of high fat diet fed mice and subsequently leaded to better learning, memory and executive functions.

In conclusion, agmatine treatment ameliorates type 2 diabetes and Alzheimer's disease as well. Therefore, agmatine has therapeutic potentials which could be applied to treat type 2 diabetes and Alzheimer's disease induced by type 2 diabetes.

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ABSTRACT (IN KOREAN)

뇌 인슐린 저항성으로 유도된 알츠하이머 병에서의 아그마틴의 효과

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강 소 망

제 2형 당뇨병은 알츠하이머병의 발병위험을 높이는데, 특히 뇌 인슐린 저항성이 당뇨병 환자들에게 알츠하이머병을 발병시키는 주된 요인으로 지목되고 있다.

신경세포에서의 인슐린 신호 저하는 신경세포의 장애, 아밀로이드 베타의 축적 그리고 타우의 과인산화로 이어진다.

아그마틴은 알기닌이 알기닌 탈탄산효소에 의해 만들어지는 내분비성 아미노구아니딘 화합물로, 신경보호효과를나타내고 있다.

이 연구는 아그마틴이 고지방 식이로 유도된 내당능장애를 줄이고 뇌에서 줄어든 인슐린 신호를 되살려 인지기증 저하를 개선시키는지 동물실험을 통해 밝히고자 한다. 이전에 성립된 방법들을 기반으로 하여, 8주령, 30~35 g의 수컷 ICR 쥐에게 12주동안 60% 고지방 사료를 지급하고, 사료 지급 4주차에 streptozotocin (100

mg/kg)을 복강 투여하였다. 12주의 식이 후, 고지방 사료를 먹인 쥐들을 2 군으로 나누었다; 식염수 또는 아그마틴 (100 mg/kg) 복강투여군. 2주의 투여를 마치면, 행동검사를 시행하였고, 뇌조직을 이용한 면역염색과 단백질 분석을 위해 쥐들은 희생되었다.

연구 결과, 아그마틴 투여가 내당능장애를 회복시켰을 뿐만 아니라, 뇌에서 줄어든 인슐린 신호들을 되살려 아밀로이드 베타와 인산화된 타우의 축적을 줄였으며, 인지기능 저하를 보였던 행동검사에서도 더 나은 결과를 보여주었다.

이 연구는 아그마틴이 제 2형 당뇨병을 완화시키고, 뇌 저항성 상태의 뇌에서 인슐린 신호를 증가시켜서 제 2형 당뇨병으로 인해 유도되는 알츠하이머 병 발병도 개선시킴을 처음으로 증명한다.

핵심 되는 말 : 제 2형 당뇨병, 뇌 인슐린 저항성, 알츠하이머병, 아그마틴