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# The role of WGA for decreasing the accumulation of Aβ: A new approach to promote transsynaptic transport of Aβ in the rat.

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## Abstract

**Wheat germ agglutinin (WGA) has been known to be transported easily to terminals from neuronal cell bodies and pass through synapses. In the present fluorescent experiments, co-injection of fluorescent WGA (FWGA) with F-Amyloid-β (FAβ) into one side of the vagus nerve (VN) at the cervical portion of the rats resulted in heavy labeling of these substances in the nodose ganglion (NG) and nucleus of solitary tract (NST) ipsilaterally compared to each injection of FWGA and FAβ. It was of interest that the labeling is characterized by existence in cytoplasm of neurons of the NG and NST. Further observations by the laser scanning confocal microscope showed that co-localization of FWGA and FAβ is present in close vicinity to the nucleus of neurons of the NST. In the immunohistochemical electron microscopic experiments, co-injection of WGA with Aβ resulted in heavy accumulation of reaction product (RP) in axon terminals and dendrites in the neuropil of the NST. The majority of these terminals and dendrites indicated to contain large-sized RP (0.20-0.40 μm in diameter) in addition to small- (0.03-0.09 μm) and medium-sized RP (0.10-0.15 μm) found in the case of injection of Aβ. Interestingly, large-sized RP transported to neurons of the NST formed a complex with the same-sized RP. The present findings might indicate that WGA is a valuable tool to decrease the accumulation of Aβ by transsynaptic transport and offers new perspectives for its application in**

**Keywords:** Transsynaptic transport, Aβ, WGA, Nucleus of solitary tract, Vagus nerve, Rat

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## Introduction

The pathological features of Alzheimer's disease (AD) have been characterized by the accumulation of extracellular plaques containing Amyloid-β (Aβ) [1-4] and intracellular neurofibrillary tangles [5]. With respect to Aβ, it has been confirmed to be a cleavage product of amyloid-β protein precursor (APP) via β- and γ-secretase proteolysis [6]. O-linked N-acetylglucosamine glycosylation is closely related to the accumulation of Aβ [7, 8, 9]. Aβ peptides aggregate into oligomer as well as fibrillar structure. This transition from soluble to aggregated state has been hypothesized to initiate the pathological cascade [10]. The conventional view of AD is that many pathogenesis are driven by an increased load of Aβ in the brain of AD. However, from the standpoint of the previous clinical effect, many therapeutic strategies based on Aβ-lowering drugs have so far been failed in clinical trials [11], for instance，γ-secretase inhibitor [11, 12] and inhibiting oligomer formation [11, 13]. Nevertheless, clinical failure of Aβ-lowering drugs does not mean that the conventional view is incorrect. AD is likely to be a complex disease driven by multiple factors. Recent experimental investigation shows that Aβ overexpression in entorhinal cortex neurons causes transsynaptic deficits [14]. In this point, it is considered that progression of the disease depends on a process via neuron-to-neuron transmission and transsynaptic transport of pathogens from affected neurons to anatomically interconnected nerve cells [15, 16]. The process includes the prodromal phase and the uniform progression of the pathological process in AD. As some properties of Aβ are unclear, the specific prophylaxis and complete treatments for AD have not yet been established.

In the nervous system, axonal transport and synaptic transmission are exclusively essential for brain functions. Strategies to improve transsynaptic transport of Aβ are urgently sought. Since it was known that wheat germ agglutinin (WGA) receptors are commonly expressed on the

surface of plasma membranes of most types of neurons in the brain, WGA has been used as an effective tracer in a variety of nervous systems [17]. In addition, after neurons take up WGA by endocytosis, WGA is transported to axons and dendrites in both anterograde and retrograde directions [18]. In some cases, injection of WGA into well-mapped nervous pathway results in labeling of both first and second order neurons and their processes [19], indicating that WGA is transneuronally transported. Recent studies have shown that WGA as targeting ligand is applied for morphological study and drug delivery. For example, the WGA-horseradish peroxidase (WGA-HRP) provides an approach for probing the intraneuronal transport of proteins [20] and WGA-fluorescein-labeled bovine serum albumin -drug model triggered urothelial cytoinvasion for improved intravesical drug delivery [21].

Therefore, with respect to neuronal transport of proteins, WGA is considered to be useful for investigating the influence on transsynaptic transport of Aβ, particularly on decreasing the accumulation of Aβ in neurons. In the present study, WGA and Aβ were injected into the vagus nerve (VN) of the rat and transsynaptic transport of these substances was observed in the nucleus of solitary tract (NST) receiving afferent fibers from the VN at the light and electron microscopic level.

## Materials and Methods

The present experiments were performed on 32 male 8-10 weeks Wistar rats (SLC, Hamamatsu, Japan), weighing 270-330 g. The animals were housed in separate cages and maintained under standard laboratory conditions (23±1℃, 12-h light: 12-h dark cycle, food and water *ad libitum*). The experimental procedures were conducted in accordance with National Institute of Health (NIH) for Care and Use of Laboratory Animals. The Kagawa University Animal Care and Use Committee approved the procedures (authorization number of the ethical approval: No. 78), and all efforts were made to minimize the number of animals used and their suffering. Rats were randomly assigned to several groups for light and electron microscopic experiments. Rats were anesthetized with intraperitoneal injection of chloral hydrate (490 mg/kg) for all surgical procedures.

***1) Light microscopic experiments***

Fluorescent WGA (FWGA) (Alexa 594-conjugated WGA, Invitrogen) and FAβ (HiLyte Fluor 488-Aβ 1-40, Anaspec) were used in the experiments. Injections of 1.5-2.0 μl of FWGA (40 mg/ml) (n=4) and FAβ (1 mg/ml) (n=4) and co-injection of 3-4 μl of FWGA with FAβ (n=6) were made into the VN on the right side of the cervical portion using a 10 μl Hamilton microsyringe. After a survival period of 2-3 days, the animals were perfused with 0.1M phosphate buffer (PB, pH 7.4) followed by a fixative of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1M PB. The brain stem and the nodose ganglion (NG) were immersed in a solution of 20-30% sucrose in 0.1M PB and embedded in Tissue-Tek O.C.T. Compound for cryosections. 15 μm-thick sections were made transversely, placed onto slide glasses and processed for nuclear counter staining with 50 ng/ml Hoechst 33258 (Sigma-Aldrich) for 10 min. All slides were mounted in fluorescence mounting medium (Dakopatts). The localization of molecules was then detected by an epi-illumination fluorescence microscope (DP-72, Olympus) and every 0.7 μm-thickness of the sections was scanned by a laser scanning confocal microscope (Radiance 2100, Zeiss Bio Rad). All images were analyzed by imagine software (NIS-Elements D, Nikon). As a control, no injection of FWGA and FAβ was made into the VN (n=2).

***2) Electron microscopic experiments***

WGA (Lectine from Triticm vulgaris, Sigma-Aldrich) and Aβ 1-40 (Sigma-Aldrich) were used in the experiments. Injection of 0.4-1.0 μl of Aβ (0.5 mg/ml) (n=6) and co-injection of 0.8-2.0 μl of WGA with Aβ (n=6) were made into the cervical portion of the VN. After a survival period of 2-3 days, the animals were perfused with 0.1 M PB (pH 7.4) followed by a fixative of 4% paraformaldehyde and 0.2% glutaraldehyde in PB. Serial 70 μm-thick sections were sliced by vibratome (Leica VT 1000S). The sections were incubated by 0.1% Triton X-100 in the 0.01 M PB saline (PBS) and horse serum solution (Vector Labs).

All sections were treated by anti-rabbit WGA antibody (1:1000 dilution, Sigma-Aldrich) and anti-mouse Aβ (1:1000 dilution, Abcam) for overnight at 4℃, and by secondary antibody anti-rabbit and anti-mouse IgG for 90 min at room temperature, respectively. Then sections were treated for ABC reagents (Vector Labs), and 3, 3'-diaminobenzidine 4HCl (DAB) (Vector Labs) for 3-5 min. In the experiment of co-injection of WGA with Aβ, sections were performed sequentially by Aβ immunostaining and heavy metal-intensified DAB staining [22]. On the other hand, animals receiving no injection into the VN were used as controls (n=4) in the present study. After perfusion with the same fixative, the sections taking from these animals were processed for Aβ immunostaining (n=2) and heavy metal-intensified DAB staining without antibody against Aβ (n=2), respectively

The blocks of these were postfixed in buffered 1% osmium tetroxide for 2 h, block-stained in saturated uranyl acetate for 1 h, dehydrated in a graded ethanol series and embedded in epoxy resin mixture. The region of the NST was identified by examination of toluidine blue-stained 1 μm-thick sections. Ultrathin sections of the region were cut and observed without lead citrate staining using a transmission electron microscope (TEM,JEM-200 CX JEOL).

## Results

### *Light microscopic experiments*

In order to investigate the role of WGA for Aβ accumulated in neurons, the regions of the NG and NST were observed after injections of fluorescent substances into the VN at the cervical portion. The injections of FWGA and FAβ resulted in heavy labeling of large-sized sensory neurons in the NG, respectively (Fig. 1A and B). In addition, axons of the VN intermingled with neurons were frequently observed to be labeled in the ganglion (Fig. 1A). However, in the NST which consisted of small-sized neurons and lay dorsal to dorsolateral to the dorsal motor nucleus of the vagus (DMV) in the lower brain stem, the injections led to weak labeling (Fig. 1C and D).

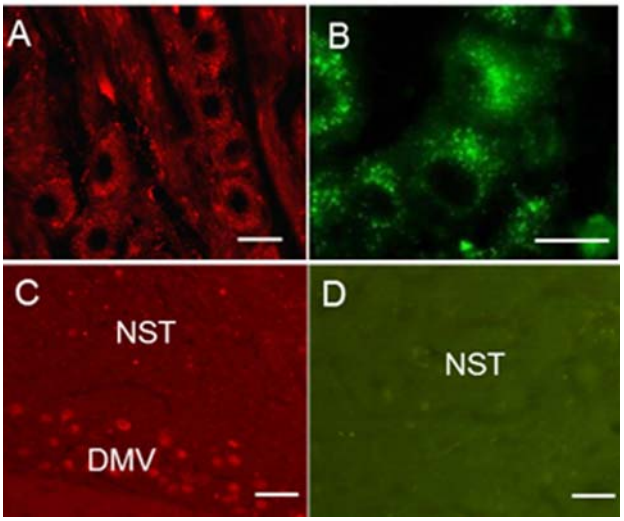


***Figure 1.*** *Fluorescent photomicrographs of the NG (A and B) and NST (C and D) after each injection of FWGA and FAβ into the VN. Although the injection resulted in heavy labeling (FWGA, red; FAβ, green) of large-sized neurons and axons in the NG (A and B), there was weak (C) and very few labeling (D) in the NST, respectively. NST, nucleus of the solitary tract; DMV, dorsal motor nucleus of the vagus. Calibration bars = 100 μm in A-D.*

In contrast to such results, co-injection of FWGA with FAβ into the VN resulted in heavier labeling in both the NG (Fig. 2A-C) and NST (Fig. 3A and B). Interestingly, merged images seemed to show co-localization of these fluorescent substances (Fig. 2D, E and 3C). Therefore, further observations were performed to confirm the detailed morphology of labeling by the laser scanning

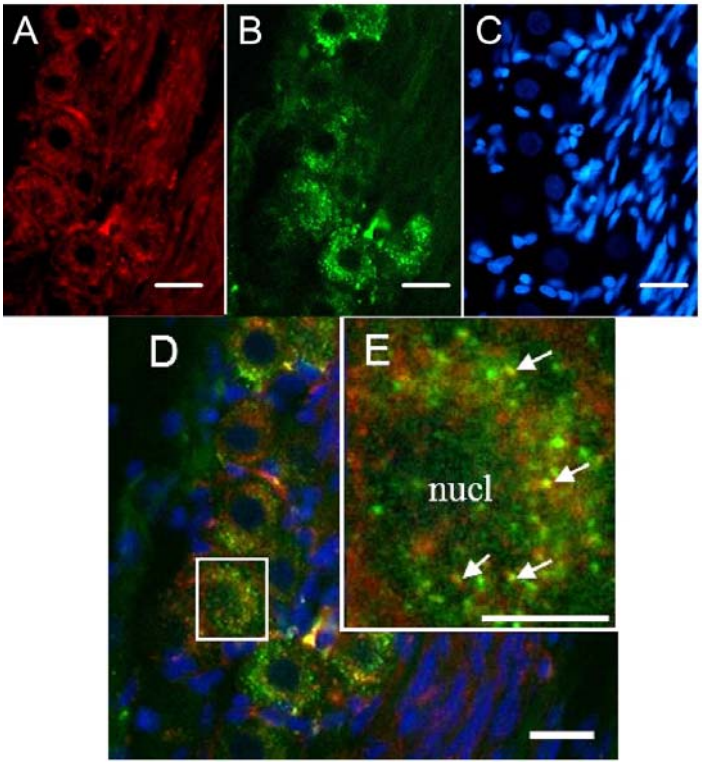


***Figure 2.*** *Fluorescent photomicrographs of the NG after co-injection of FWGA with FAβ into the VN (A, B, D and E), and the nucleus stained with Hoechst 33258 (C). This injection resulted in heavier labeling of FWGA (A) and FAβ (B) in the NG. Merged images showed co-localization of FWGA and FAβ (D). Co-localization of these substances was present particularly in close vicinity to the nucleus of neurons of the region (E, arrows). Framed area in D is magnified in E. nucl, nucleus. Calibration bars = 100 μm in A-D and 50 μm in E.*

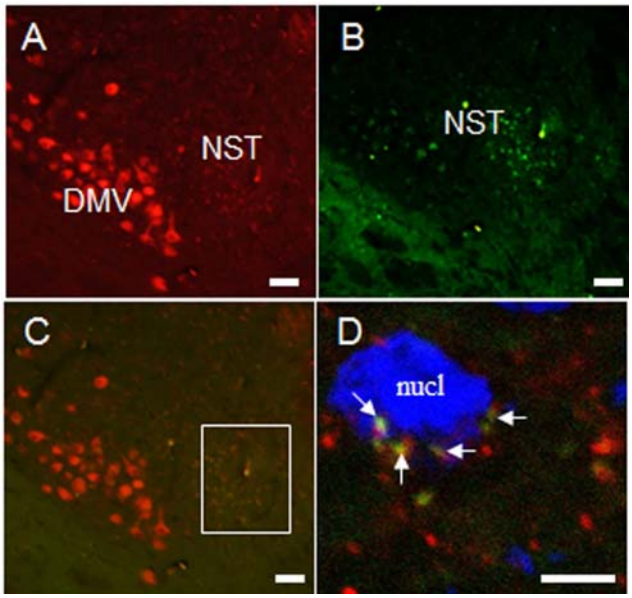


***Figure 3.*** *Fluorescent photomicrographs of the NST after co-injection of FWGA with FAβ into the VN. This injection resulted in heavier labeling of these substances in the NST which was located dorsal to dorsolateral to the DMV (A and B). A part of the framed area in C was magnified in D which was observed by the laser scanning confocal microscope. Note that the laser scanning confocal microscope revealed co-localization of these fluorescent substances in close vicinity to the nucleus of neurons of the NST (D, arrows). nucl, nucleus; NST, nucleus of solitary tract; DMV, dorsal motor nucleus of the vagus. Calibration bars = 100 μm in A-C and 5 μm in D.*

confocal microscope. It was of particular interest that co-localization of FWGA and FAβ is present in close vicinity to the nucleus of neurons of the NST (Fig. 3D). These fluorescent experiments are considered to indicate the Aβ transport with WGA from terminals of the VN to neurons of the NST. In the control cases, no fluorescent substance was found in the NG and NST.

***Electron microscopic experiments***

As the present study was focused on elucidating transsynaptic transport of Aβ, ultrastructural observations were performed on the NST. The NST was identified in 1 μm-thick toluidine blue stained sections and the region located dorsal to dorsolateral to the DMV was trimmed. Electron microscopic experiments in animals receiving injection of Aβ into the VN rarely showed immunohistochemically labeled terminals containing Aβ reaction product (RP) recognized as electron-dense lysosomal-like substance. In addition, these terminals contained clear round vesicles and made asymmetrical synaptic contact with dendritic profiles of post-synaptic neurons containing the RP (Fig. 4A). Interestingly, the RP was classified into two types [small (0.03-0.09 μm) and medium (0.10-0.15 μm)] according to sizes of diameter (Fig. 4A and B). On the other hand, co-injection of WGA with Aβ resulted in many terminals and dendrites containing RP in the neuropil of the NST. The majority of these were characterized by various sizes of RP mainly containing large-sized RP (0.20-0.40 μm in diameter) (Fig. 5A-D) compared to the case of injection of Aβ. Furthermore, it was of particular interest that large-sized RP transported to neurons of the NST forms a complex with same-sized RP (Fig. 5E and

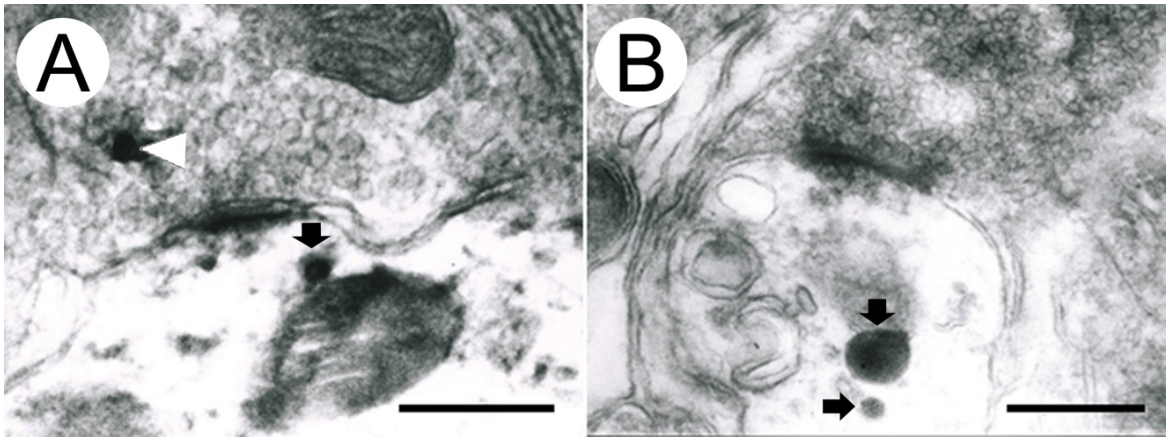


***Figure 4.*** *Electron micrographs in animals receiving injection of Aβ into the VN (A and B). This injection rarely resulted in immunohistochemically labeled terminals containing Aβ-RP recognized as electron-dense lysosomal-like substance (A). In addition, these terminals contained clear round vesicles and made asymmetrical synaptic contact with dendritic profiles of post-synaptic neurons containing the RP (A). Note that the RP was classified into two types [small (0.03-0.09 μm) and medium (0.10-0.15 μm)] according to sizes of diameter. Arrowhead and arrows indicate Aβ-RP in terminals and dendritic profiles, respectively. Calibration bars= 0.5 μm in A-B.*

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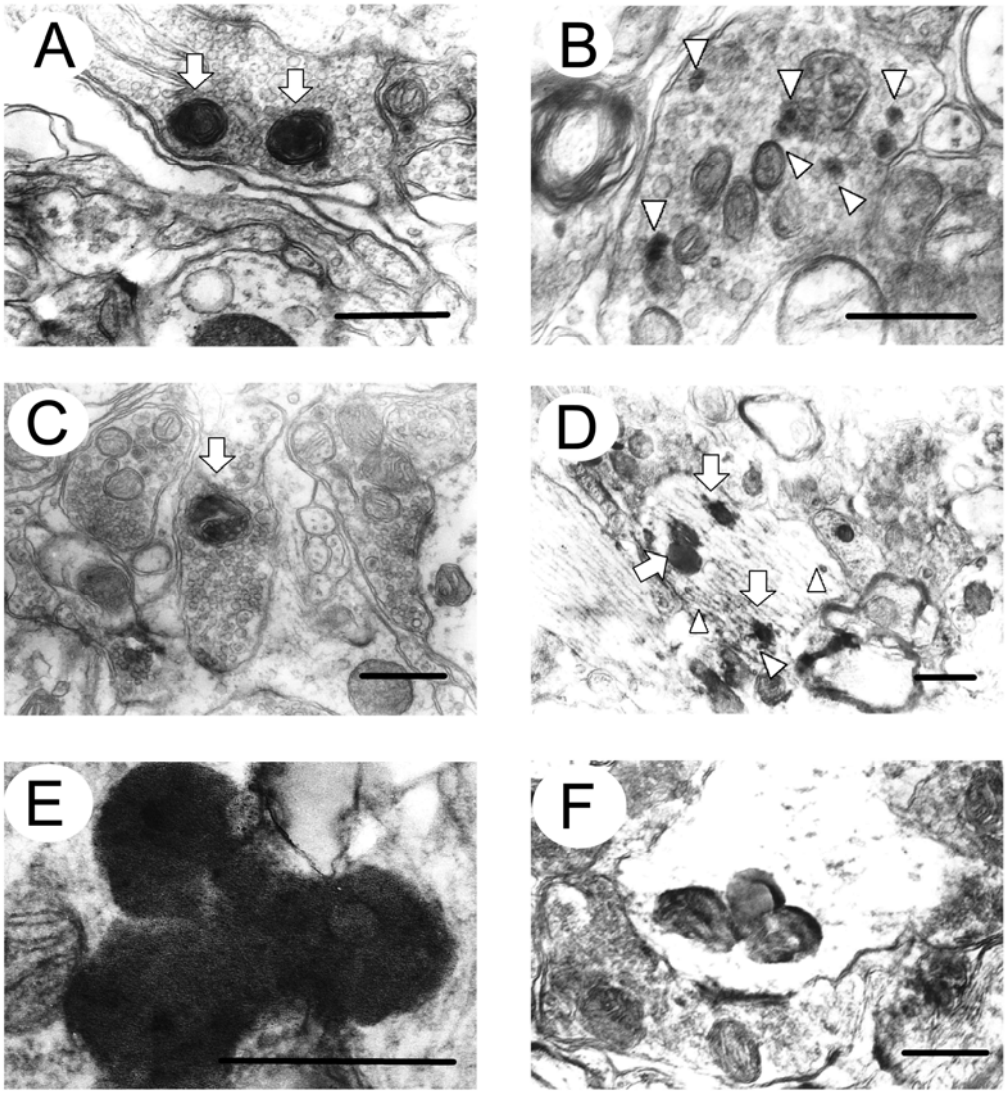


***Figure 5.*** *Electron micrographs in animals receiving co-injection of WGA with Aβ into the VN (A-F). This injection resulted in many terminals and dendrites containing RP in the neuropil of the NST. It was of particular interest that additional large-sized RP (0.20-0.40 μm) was present in terminals and dendrites (A, C and D, arrows), compared to the case of injection of Aβ, although small- or medium-sized RP was also intermingled (B and D, arrowheads). Note that large-sized RP transported to neurons of the NST formed a complex with same-sized RP (E and F). Calibration bars= 0.5 μm in A-F.*

F). In the control cases, there was no or very weak labeling of axon terminals and dendritic profiles in the neuropil of the NST

## Discussion

In the present fluorescent experiments, co-injection of FWGA with FAβ into the VN resulted in heavier labeling of FAβ in the NST compared to injection of FAβ. The laser scanning confocal microscopic experiments clearly showed that FWGA or/and FAβ are present in close vicinity to the nucleus of neurons of the NST. These findings verified that WGA promotes transsynaptic transport of Aβ in the NST and overlappes partly with Aβ. With regard to WGA conjugated with protein, it is reported that injection of WGA or/and HRP into submandibular gland of rats

results in WGA-HRP complex (62 kDa), HRP (40 kDa), monomeric WGA (22 kDa), and HRP breakdown products (15 to 30 kDa) in the immunoblots [23]. On the basis of the works described above, the ratio of targeting ligand to delivery payload naturally might be a critical design parameter for targeting protein conjugates. The injection dose ratio of WGA and Aβ apparentlly affected conjugation of these substances due to limited binding pockets of WGA [21]. However, it is of interest that transport of vesicles containing WGA and APP is blocked by tau protein in culture [24]. Tau protein having the most visible changes in the AD is also considered to play an important role in moving and accumulation of Aβ in neurons.

In the electron microscopic experiments co-injection of WGA with Aβ revealed various sizes of RP in terminals and dendrites in the neuropil of the NST. The RP was classified into three types according to sizes of diameter. On the basis of molecular weight of Aβ (4 kDa) [6], we can speculate that small- and medium-sized RP is originated from Aβ. On the other hand, large-sized RP seems to be derived from WGA or WGA-Aβ complex because molecular weight of WGA is 36 kDa [25]. In the present study WGA seems to conjugate with Aβ directly (WGA-Aβ). WGA as a targeting ligand is chosen due to its high stability and its dimeric configuration with some diametrically opposed binding pockets, which renders it particularly suited for use in coupling chemistry [21]. Conjugation is possible over the process. Transsynaptic transport of WGA-Aβ complex may mainly depend on distal carbohydrate recognition domains on the WGA dimer. WGA specifically binds to structures of N-acetyl-D-glucosamine- and sialic–acid–terminated glycoproteins and glycolipds [21]. WGA plays a critical role in binding to the receptor on the cell surface and effects on processing of Notch and APP [26]. In addition, Liu et al [27] demonstrates that WGA suffers the receptor-mediated processes of exocytosis in the neuron. This is important for the application of WGA as drug carriers in medicine, and also offers insights into the pathway of endocytosis and exocytosis. Indeed, several recent studies used this mediated method for drug carriers and demonstrated its effectiveness. For example, WGA-fBSA-drug, WGA-nanoparticles and WGA-doxorubicin offered new perspectives for biological barriers [21, 28, 29].

There are some reasons that the VN is used in the present study. Firstly, few Aβ exist in the VN and NST. AD is an age-related disease and is induced by accumulation of Aβ in brain is associated with aging [30]. Under normal conditions, Aβ is found in the cerebrospinal fluid and blood plasma [31]. However, in the brain of AD, Aβ is usually deposited in limbic brain regions, such as the hippocampus and amygdala, and additionally in the specific cortical and subcortical areas [32]. Secondly, some researchers exploited the VN to diagnose early stage of AD and treat mild cognitive impairment [33, 34, 35]. Thirdly, the NST is an important region of receiving afferent fibers from cranial nerves [36, 37, 38, 39] and forebrain areas including amygdala [40, 41]. It should be noted that the NST various biological responses associated with autonomic functions such as cardiovascular control [42, 43]. Therefore, synaptic organization of visceral afferents of the VN, exclusively confined to the NST region, was investigated in the present study.

It is supported by some researches that Aβ in the cytoplasm has been pointed out to be involved in early stages of the disease, directly causing neurotoxicity and initiating AD pathology [44-46]. The most common isoforms of Aβ are Aβ 1-40 (90%) and Aβ 1-42 (10%), and Aβ 1-42 is more toxic [47]. Petkota et al [48] indicates that Aβ 1-40 possesses a variety of morphological and molecular structure in the neuronal cell cultures, and has significantly different neurotoxicity. Researchers gradually realize that to prevent the accumulation of Aβ and eliminate Aβ is very important for protecting nerve cells and mitigating Aβ toxicity and AD-related symptoms. Clearance of Aβ via the immune system is currently a popular strategy. Low-density lipoprotein receptor-related protein-1 (LRP1) is the main cell surface receptor involved in brain and systemic clearance (plasma and liver) of the AD toxin Aβ [49]. Intravenous immunoglobulins decreased Aβ influx from blood to the brain in vitro [50]. The significance of the present study is to prevent accumulation of Aβ and treat for early AD pathology in neurons.

Transsynaptic transport of materials, particularly of proteins, has been known to be based on vesicular exocytosis involved in chemical synaptic transmission. However, in animals inhibited the expression of Rab3A-mRNA and exposed to alcohol, our previous studies indicated the altered morphology of transport across the post-synaptic membrane which was not related to the vesicular synaptic transmission [36, 37, 51]. The altered morphology was characterized by “apocrine-like structure” of terminals of the VN in the neuropil of the NST. Interestingly, similar structure was also observed in terminals of hypothalamo-hypophysial tract in the posterior pituitary [52]. These studies might raise the possibility that the morphology of transsynaptic transport of WGA- and Aβ-RP demonstrated in the present study is quite different from vesicular exocytosis.

In conclusion, in the present study co-injection of WGA and Aβ into the VN of rats resulted in abundant Aβ in the NST through axonal flow and transsynaptic transport. This finding suggested that WGA promotes decreasing the accumulation of Aβ in neurons. WGA is a useful and powerful tool for conjugate proteins and transsynaptic transport and is potential applications in the research of AD in vivo.

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