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Midazolam inhibits the formation of amyloid fibrils and GM1 ganglioside-rich microdomains in presynaptic membranes through the gamma-aminobutyric acid A receptor

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ABSTRACT

Recent studies have suggested that a positive correlation exists between surgical interventions performed under general anesthesia and the risk of developing Alzheimer's disease (AD) in the late postoperative period. It has been reported that amyloid β-protein (Aβ) fibrillogenesis, which is closely related to AD, is accelerated by exposure to anesthetics. However, the mechanisms underlying these effects remain uncertain. This study was designed to investigate whether the anesthetic midazolam affects Aβ fibrillogenesis, and if so, whether it acts through GM1 ganglioside (GM1) on the neuronal surface. Midazolam treatment decreased GM1 expression in the detergent-resistant membrane microdomains of neurons, and these effects were regulated by the gamma-aminobutyric acid-A receptor. Midazolam inhibited Aβ fibril formation from soluble Aβ on the neuronal surface. In addition, midazolam suppressed GM1-induced fibril formation in a cell-free system. Moreover, midazolam inhibited the formation of Aβ assemblies in synaptosomes isolated from aged mouse brains. These finding suggested that midazolam has direct and indirect inhibitory effects on Aβ fibrillogenesis.

1. Introduction

Alzheimer's disease (AD) is an insidious and progressive neurodegenerative disorder, and its pathogenesis is considered to be multi-factorial; i.e., a genetic predisposition interacts with environmental or biological factors, resulting in the development and/or acceleration of the condition [1,2]. Therefore, it is important to identify environmental factors that might promote AD development. Recent studies have demonstrated that anesthetics can have an impact on the neuropathogenesis of AD and even accelerate its clinical progression [3–9]. A number of studies have suggested that associations exist between anesthesia and Alzheimer's disease. Thus, the effects of general anesthesia on the development and pathogenesis of AD remain to be elucidated.

The dominant model of AD pathogenesis is the amyloid cascade hypothesis. In brief, this theory states that amyloid-β peptides (Aβ) derived from amyloid precursor protein cause AD via a cascade of events involving the deposition of amyloid plaques, which are mainly composed of Aβ, and the subsequent production of tau protein and neurofibrillary tangles [10]. Initially, Aβ peptides are monomeric, soluble, and non-toxic, but they become cytotoxic during their aggregation and accumulation [11,12]. Furthermore, a unique GM1 ganglioside (GM1)-bound form of Aβ (GAβ) was detected in brains that exhibited the early pathological changes of AD [13]. The unique molecular characteristics of GAβ allow it to facilitate amyloid fibril formation from soluble Aβ by acting as an endogenous seed in the brain [14]. In this process, Aβ assembly formation is aided by the accumulation and clustering of GM1 in

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β peptide; CTX–HRP, horseradish peroxidase-conjugated cholera toxin B subunit; DRM, detergent-resistant membrane microdomain; DMSO, dimethyl sulfoxide; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F-12; EDTA, ethylenediaminetetraacetic acid; GABAAR, γ-aminobutyric acid A receptor; GM1, GM1 ganglioside; GAβ, GM1 ganglioside-bound form of amyloid β peptide; MBS, 2-(N-morpholino)ethanesulfonic acid-buffered saline; ThT, thioflavin T; ThS, thioflavin S.

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the membrane microdomains at pre-synaptic neuritic terminals in AD brains [15–17].

Understanding the effects of anesthetics on Aβ fibrillogenesis is important for elucidating the pathogenesis of AD after general anesthesia. Here, we examined the effects of midazolam on the production of Aβ assemblies on the surfaces of primary cultured neurons and on GM1 expression in primary cultured neurons. Moreover, the involvement of the gamma-aminobutyric acid A receptor (GABAAR), one of the molecular targets of midazolam, in these effects was investigated.

2. Materials and methods

2.1. Materials

Midazolam, (+)-bicuculline, and Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12) were purchased from Wako (Osaka, Japan). Cholesterol, sphingomyelin, horseradish peroxidase-conjugated cholera toxin B subunit (CTX–HRP), thioflavin T (ThT), and thioflavin S (ThS) were obtained from Sigma–Aldrich (St. Louis, MO). Synthetic Aβ (1–40) peptides were purchased from the Peptide Institute (Osaka, Japan), and bovine serum albumin fraction V solution and N2 supplement were obtained from Invitrogen (Carlsbad, CA).

2.2. Cell culture

Cerebral cortical neurons were prepared from Sprague–Dawley rats on embryonic day 17, as described previously [18]. Dissociated single cells were plated onto poly-L-lysine-coated dishes and incubated in feeding medium for 21 days *in vitro*. The N2 medium, the feeding medium, consisted of DMEM/F12 with 0.1% bovine serum albumin fraction V solution and N2 supplement. The cells were pharmacologically treated with midazolam, bicuculline, or vehicle (control) and then cultured in a humidified atmosphere containing 5% CO2 at 37 °C.

2.3. SDS-PAGE and Western blotting

The cells were lysed in Triton X-100-containing Tris buffer [5 mM Tris–HCl (pH 7.4), 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, and Complete™ protease inhibitor cocktail (Roche Molecular Biochemicals, Penzberg, Germany)]. The protein concentration of each sample was determined using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA). The samples were separated on a 10%–20% gradient polyacrylamide gel (Wako) and then electrotransferred to polyvinylidene difluoride membranes (Millipore). The blotted membranes were blocked with Block Ace (Yukijirushi, Sapporo, Japan), and the blots were probed with CTX–HRP. The bands were then visualized by reacting them with the Pierce Western Blotting Substrate (Thermo Fisher Scientific).

2.4. Isolation of detergent-resistant membrane microdomains (DRMs)

DRM were isolated from neurons or synaptosomes, as described previously [19]. In brief, neurons or synaptosomes were homogenized in 2-(N-morpholino)ethanesulfonic acid-buffered saline (MBS) containing 1% Triton X-100 to obtain lysates with protein concentrations of 1 mg protein/mL and 200 μg protein/mL, respectively. Then, the sucrose concentrations of the extracts were adjusted to 40% by adding 80% sucrose in MBS, before they were overlaid with a 5%/35% discontinuous sucrose gradient in MBS without Triton X-100 and centrifuged at 188,000 × *g* for 20 h using an S120AT2 rotor (Hitachi, Tokyo, Japan). After the centrifugation, 1-mL fractions were harvested from various points along the gradient. The success of the DRM isolation was verified by confirming that flotillin-1 (a DRM marker protein) had been enriched in the fifth fraction, as we reported in our previous study[21].

2.5. Preparation of seed-free Aβ solutions and ThS fluorescence staining

Seed-free solutions of Aβ (1–40) peptides were prepared as described previously [20]. Fifty μM of seed-free Aβ-treated neurons were fixed for 10 min in 10% formic acid and then washed in phosphate-buffered saline. Thereafter, the neurons were incubated for 10 min in 0.25% potassium permanganate, before being incubated in 2% potassium bisulphate and 1% oxalic acid until they became colorless. Next, the neurons were washed in water and then treated for 10 min with a solution of 0.015% ThS in 50% ethanol. Finally, the cells were washed in 50% ethanol and water and mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA).

2.6. Animals and synaptosome preparation

Six-month-old male mice (SLC, Shizuoka, Japan) were housed in a room maintained at 24 ± 1 °C and illuminated for 12 h (08:00–20:00) each day. The mice were granted free access to food and water. All animal procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Ritsumeikan University (Kyoto, Japan).

Synaptosomes were prepared from 6-month-old mice, as described previously [17,19]. Briefly, whole mouse brains were homogenized in 0.32 M sucrose buffer containing 0.25 mM EDTA. Then, the homogenate was centrifuged at 580 × *g* for 8 min, and the supernatant was centrifuged at 14,500 × *g* for 20 min. The resultant pellet was suspended in 0.32 M sucrose buffer without EDTA, overlaid with Ficoll in sucrose buffer, and centrifuged at 87,000 × *g* for 30 min. The synaptosome-rich interface was removed and re-centrifuged to remove any remaining Ficoll.

2.7. Incubation of Aβ in the presence of GM1 liposomes and synaptosomes

To prepare GM1 liposomes, cholesterol, sphingomyelin, and GM1 were dissolved in chloroform/methanol at a molar lipid ratio of 40:40:20. The resultant mixture was stored at −80 °C until use. Immediately before use, the lipids were resuspended in Tris-buffered saline to yield a ganglioside concentration of 2.5 mM, and the suspension was subjected to freezing, thawing, and sonication. Then, seed-free Aβ solutions (50 μM) were incubated at 37 °C, unless otherwise indicated, in the presence or absence of GM1 liposomes or synaptosomes, as described previously [17,21]. The ThT fluorescence intensities of the resultant mixtures were measured using a spectrofluorophotometer (RF-5300PC; Shimadzu Co., Kyoto, Japan), and the peak fluorescence intensity of the amyloid fibrils was determined at excitation and emission wavelengths of 446 and 490 nm, respectively.

2.8. Statistical analysis

Data are shown as the mean ± SD of at least four or five independent experiments. Statistical analyses were performed with one-way or two-way factorial ANOVA combined with Scheffe's test for all paired comparisons (StatView software for Macintosh; SAS Institute, Cary, NC). $P < 0.05$ was considered to be statistically significant.

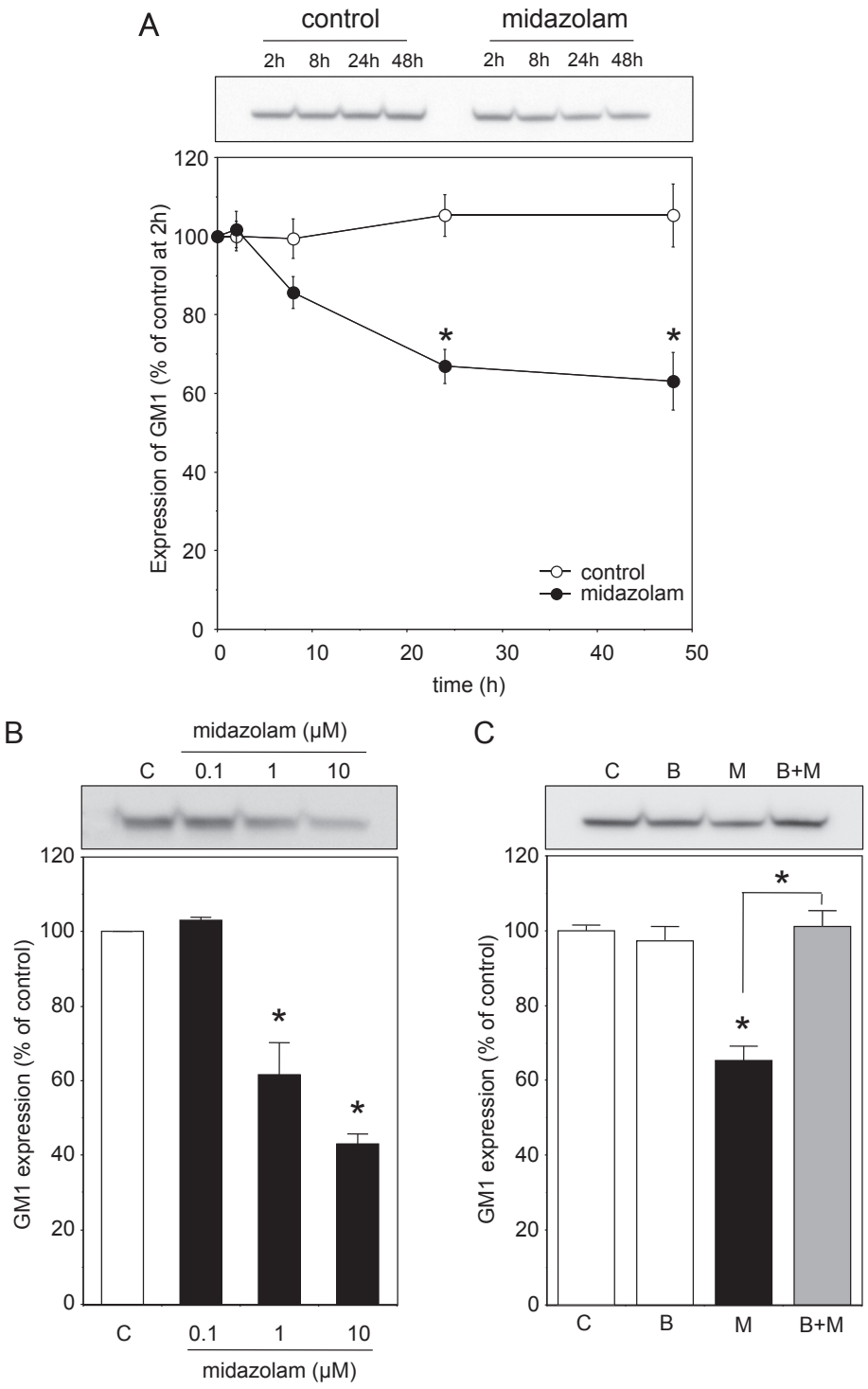

Fig. 1. Midazolam decreased neuronal GM1 ganglioside (GM1) expression through the gamma aminobutyric acid A receptor. (A) Time course of GM1 expression in primary neurons (cultured for 21 days *in vitro*) treated with 3 μM midazolam. (B) Dose–response relationship of GM1 expression in neurons treated with the indicated concentrations of midazolam for 24 h. (C) Neurons were incubated for 24 h in the presence or absence of 3 μM midazolam after pretreatment with 3 μM bicuculline. At the indicated times, the treated cell lysates were subjected to Western blotting using horseradish peroxidase-conjugated cholera toxin B subunit (CTX-HRP) protein. GM1 levels were determined by densitoscanning the blots after they had been incubated with CTX–HRP, and band densities are presented as percentages of the control value (vehicle-treated neurons) at 2 h (A) or 24 h (B and C). Each column represents the mean of 6 values ± SD. *$P < 0.0001$ (One-way ANOVA combined with Scheffe's test). C: neurons treated with 0.03% DMSO as a control. B: 3 μM bicuculline-treated neurons. M: 3 μM midazolam-treated neurons. B + M: neurons treated with 3 μM bicuculline and 3 μM midazolam.

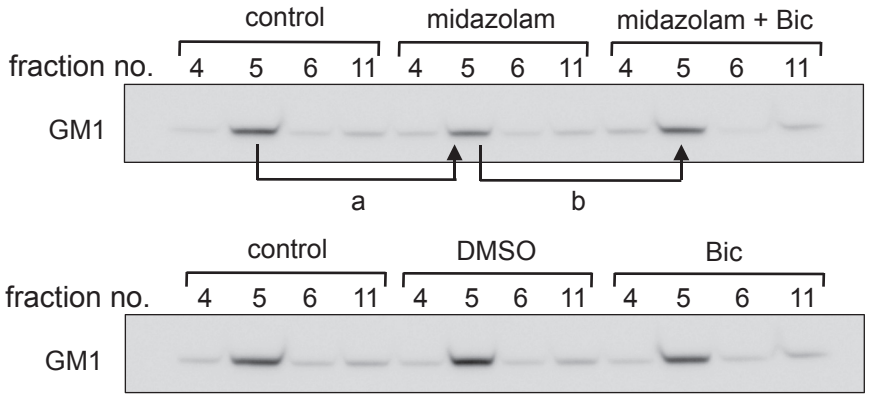

Fig. 2. Localization of GM1 ganglioside in detergent-resistant membranes (DRM) prepared from midazolam-treated neurons. Western blots of DRM (fraction 5) and non-DRM fractions (fraction 11) prepared from primary neurons (cultured for 21 days *in vitro*) that were incubated for 24 h in the presence or absence of 3 μM midazolam after pretreatment with or without 3 μM bicuculline (Bic), before being incubated with horseradish peroxidase-conjugated cholera toxin B subunit.

3. Results

3.1. Midazolam treatment decreased neuronal GM1 expression via the GABAAR

To investigate the effects of midazolam on neuronal GM1 expression, neurons were incubated with 3 μM midazolam for the indicated period. Neuronal GM1 expression, which was determined using CTX–HRP, was significantly decreased at 12 h after the addition of midazolam, and this reductions were sustained for at least 48 h (Fig. 1A). To determine whether the effects of midazolam on neuronal GM1 expression were concentration-dependent, neurons were incubated for 48 h with various concentrations of midazolam. As a result, it was found that neuronal GM1 expression was significantly decreased by midazolam treatment in a concentration-dependent manner (Fig. 1B); however, it was not affected by treatment with 0.01% dimethyl sulfoxide (DMSO) solution, as a previous reported (control; data not shown) [22]. To investigate midazolam's mechanism of action, neurons were pretreated with bicuculline, a GABAAR inhibitor, in the presence or absence of midazolam. Bicuculline was added 1 h before the addition of midazolam (3 μM). After 24 h incubation, it was found that bicuculline had inhibited the midazolam-induced reduction in neuronal GM1 expression (Fig. 1C). These results suggest that midazolam downregulates neuronal GM1 expression through the GABAAR. Furthermore, the viability of neurons was not affected by treatment with midazolam, bicuculline, and DMSO for 48 h (data not shown).

3.2. GM1 concentration of the DRM fraction was decreased by midazolam

To investigate the midazolam-induced reduction in neuronal GM1 expression further, DRM and non-DRM fractions were isolated from neurons that had or had not been pretreated with bicuculline for 1 h and then incubated for 24 h in the presence or absence of midazolam. For each fraction, the GM1 expression levels of the midazolam-treated and untreated neurons were compared (Fig. 2). GM1 was mainly found in fraction 5, a DRM fraction, and fraction 11, a non-DRM fraction. In fraction 5, the midazolam-treated neurons displayed significantly decreased GM1 expression compared with the untreated neurons (arrow (a) in Fig. 2). The midazolam-induced decrease in the GM1 concentration of fraction 5 was inhibited when the cells were pretreated with bicuculline (arrow (b) in Fig. 2). In fraction 11, the GM1 concentration was similar in all samples (Fig. 2). In addition, the expression levels of flotillin-1, a DRM marker, and the transferrin receptor, a non-DRM marker, were similar in all samples (data not shown).

3.3. Midazolam suppressed GM1 liposome-induced Aβ fibril formation

We incubated the seed-free Aβ in the presence or absence of GM1 liposomes to investigate whether midazolam was able to accelerate amyloid fibril formation in a cell-free system. The fluorescence intensity of ThT, which specifically recognizes amyloid structures, was measured in the various mixtures (Fig. 3). The

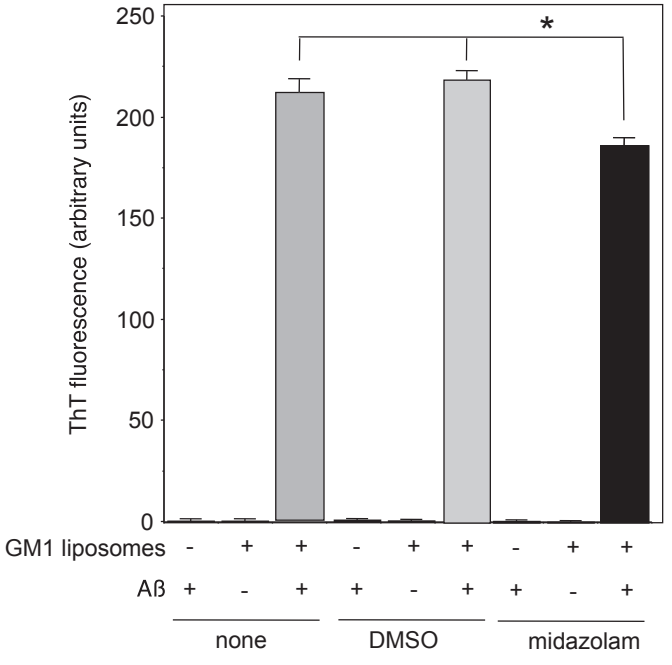

Fig. 3. Effects of midazolam on the formation of amyloid β-peptide (Aβ) assemblies from GM1 ganglioside (GM1) liposomes in a cell-free system. Soluble Aβ at a concentration of 50 μM was incubated for 48 h at 37 °C in the presence or absence of GM1 liposomes with or without 3 μM midazolam or 0.03% DMSO. The thioflavin T fluorescence intensity of each incubation mixture was then determined. Each column represents the mean of 6 values ± SD. *$P < 0.0001$ (One-way ANOVA combined with Scheffe's test).

fluorescence intensities of the solutions containing Aβ alone did not increase during the 48-h incubation period, as reported previously [15,21]. In addition, in the absence of GM1 liposomes Aβ assembly formation did not increase during the 48-h incubation period in the presence of DMSO or midazolam. However, the ThT fluorescence intensities of the incubation mixtures containing Aβ and GM1 liposomes without midazolam were significantly increased after 48 h, as reported previously [15,21]. Interestingly, midazolam significantly suppressed GM1 liposome-induced Aβ assembly formation (Fig. 3).

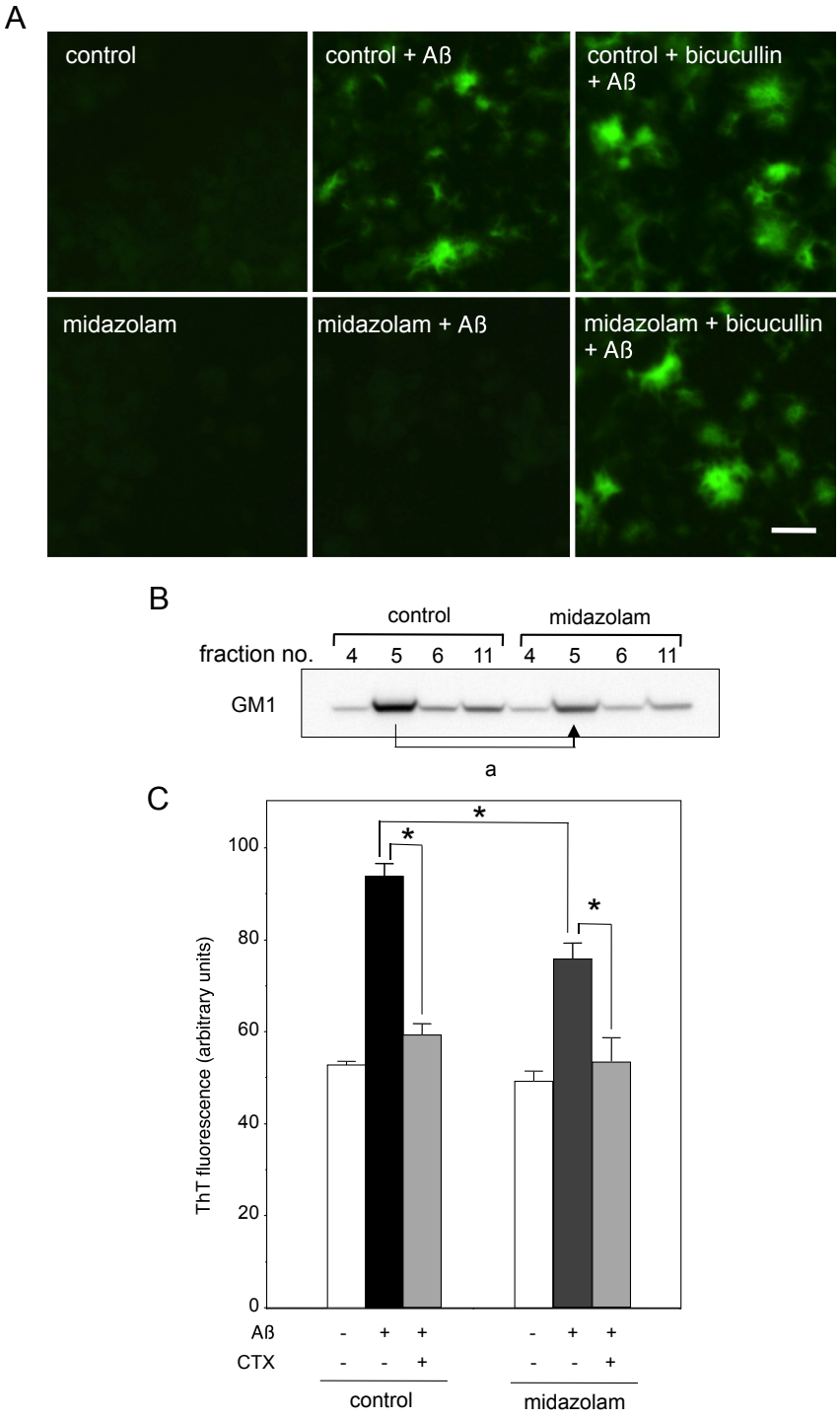

Fig. 4. Effects of midazolam on amyloid β-peptide (Aβ) assembly formation on the neuronal cell surface and in synaptosomes prepared from aged mouse brains. (A) Primary neurons (cultured for 21 days *in vitro*) were incubated for 24 h in the presence or absence of 3 μM midazolam after pretreatment with or without 3 μM bicuculline. Midazolam-treated neurons were incubated with 50 μM soluble Aβ for 48 h. Aβ assembly on the neuronal cell surface was visualized by thioflavin S fluorescence staining. Bar = 20 μm. (B) Midazolam (50 mg/kg) was intraperitoneally administered to male C57/B6 mice (2-years-old) for 24 h. A blot of detergent-resistant membranes isolated from synaptosomes prepared from midazolam-treated mouse brains, which was then probed with horseradish peroxidase-conjugated cholera toxin B subunit (CTX-HRP), is shown. (C) Fifty μM of soluble Aβ solution were incubated at 37 °C for 24 h in the presence or absence of synaptosomes isolated from 2-year-old mice with or without CTX. Then, the thioflavin T fluorescence intensities of the mixtures were determined. Each column indicates the mean of 6 values ± SD (one-way ANOVA combined with Scheffe's test).

3.4. Midazolam suppressed Aβ fibril formation in primary cultured neurons and on synaptosomes prepared from the brains of aged mice

We investigated whether Aβ assemblies formed on midazolam-treated cultured neurons. Cultured neurons were stained with ThS, which specifically binds to amyloid fibrils, and then the ThS was visualized by fluorescence microscopy. Aβ assemblies were observed on the untreated neurons incubated with soluble Aβ, as reported previously [23]. However, midazolam treatment markedly suppressed Aβ assembly formation. Furthermore, pretreatment with bicuculline reduced the effects of midazolam (Fig. 4A). We also investigated whether the intraperitoneal administration of midazolam (50 mg/kg) would induce GM1 expression on the DRM of synaptosomes prepared from the brains of aged mice (2-years-old). Twenty-four hours after the administration of midazolam, the GM1 concentration of the DRM fraction (fraction 5), but not the non-DRM fraction (fraction 11), isolated from the synaptosomes of midazolam-treated mice was significantly reduced compared with that of the DRM fraction isolated from the synaptosomes of the control mice (arrow (a) in Fig. 4B). The expression levels of flotillin-1 and the transferrin receptor were similar in all of the samples (data not shown). Finally, we investigated whether midazolam inhibited Aβ fibril formation in synaptosomes obtained from aged mouse brains. We next incubated the seed-free Aβ with synaptosomes prepared from two groups of aged mice. The mixtures containing synaptosomes from the aged control mice exhibited markedly increased ThT fluorescence intensity (Fig. 4C). Interestingly, midazolam significantly suppressed Aβ assembly formation in the synaptosomes from the aged mice (Fig. 4C). Notably, the increase in ThT fluorescence intensity was significantly suppressed by co-incubation with CTX, indicating that the formation of amyloid fibrils in synaptosomes is GM1-dependent (Fig. 4C).

4. Discussion

In this study, we obtained results that suggest that midazolam has a protective effect against Aβ aggregation, a hallmark of AD. First, we found that midazolam suppressed GM1 expression in primary cultured neurons. This suppression was localized to DRM fractions and was mediated via the GABAAR. Second, midazolam suppressed Aβ assembly formation from soluble Aβ in neuronal cell culture systems. In addition, midazolam also suppressed GM1 liposome-induced Aβ assembly formation in a cell-free system. Finally, we showed that midazolam inhibited Aβ assembly formation in synaptosomes isolated from mouse brains. Collectively, these findings suggest that midazolam directly or indirectly suppresses Aβ fibrillogenesis.

GM1 is abundant in the external lipid layer of the neuronal membrane and plays important roles in the differentiation, functioning, and viability of neurons [16]. It was previously reported that GM1 expression was significantly increased in DRM [24] and amyloid-positive synaptosomes [25] prepared from AD brains and that Aβ deposition starts at presynaptic neuritic terminals in the AD brain [26,27]. Moreover, we previously suggested that age-dependent high-density GM1 clustering in presynaptic neuritic terminals is a critical step for Aβ deposition in AD (Yamamoto et al. 2008). However, the mechanisms governing the regulation and distribution of GM1 expression on neuronal membranes, particularly synaptic membranes, remain unclear. We have previously reported that insulin regulates the expression and distribution of GM1 in neuronal DRM through PI3K [28]. Moreover, our recent study found that propofol and thiopental inhibited the formation of Aβ assemblies by decreasing GM1 expression in neuronal DRM through the GABAAR [22]. In the present study, we found that GM1 expression in neuronal DRM and synaptosomes was reduced by midazolam and that these effects were mediated through the GABAAR. In addition, midazolam inhibited the formation of Aβ assemblies on the neuronal cell surface as well as in synaptosomes by reducing GM1 expression in neuronal DRM (Fig. 4A). Taken together, these findings suggest that midazolam indirectly inhibits Aβ fibril formation from neuritic terminals by reducing GM1 expression in DRM through the GABAAR.

Midazolam, a benzodiazepine, binds to the GABAAR between its α- and γ-subunits and allosterically modulates the affinity of the receptor for GABA [29]. Several studies have suggested that midazolam has neuroprotective effects [30–32], and numerous studies have provided evidence that GABAergic dysfunction is involved in AD[35]. In addition, early studies of the temporal cortex, an area that is greatly affected by the neuropathic hallmarks of AD, detected slight decreases in benzodiazepine binding in AD patients [33,34], suggesting that GABAAR expression is downregulated in the condition. Thus, pharmacological modulation of the GABAAR has gained increasing attention as a potential treatment for the neurological dysfunctions induced by AD, including problems with neuronal survival and cognition. Interestingly, a number of recent studies have suggested that GABAAR signaling is associated with significant neuroprotection against Aβ-mediated toxicity [35]. Moreover, studies of early-stage amyloid pathology using transgenic models have indicated that it progresses in a neurotransmitter-specific manner via GABAergic terminals [36]. However, the effects of midazolam on Aβ fibrillogenesis, which are mediated through changes in GM1 expression, have not been reported previously.

The mechanism underlying the effect of midazolam on the formation of GM1-induced Aβ assemblies has not been investigated. The present Aβ assembly experiments were performed in a cell-free system using seed-free Aβ solutions and GM1 liposomes. Recently, we found that propofol and thiopental inhibited the formation of GM1 liposome-induced Aβ assemblies from soluble Aβ [22]. Although further investigation is required, the findings of the present study indicate that midazolam directly inhibits the formation of GM1 liposome-induced Aβ assemblies through molecular interactions (Fig. 3).

In conclusion, the present results suggest that midazolam is relatively safe with respect to its effects on the formation of Aβ assemblies because it has direct and indirect inhibitory effects on Aβ fibrillogenesis. Our results might encourage further *in vivo* attempts to determine the effects of anesthetic agents on AD neuropathogenesis, ultimately leading to safer anesthesia care for patients, especially elderly patients, who are particularly at risk of postoperative cognitive dysfunction and AD.

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