Table 3
Health Care Recipients' Priorities for Health Care Outcome

Rank Order	Community-Dwelling Older Adults (n = 2637)			Family Members of Patients With Dementia (n = 333)			Patients in Geriatric Outpatient Clinics (n = 512)			Adult Day Care Participants (n = 795)		
	Outcome	Mean	95% CI	Outcome	Mean	95%CI	Outcome	Mean	95% CI	Outcome	Mean	95% CI
1	Effective treatment of illness	4.23	4.11–4.36	Effective treatment of illness	3.04	2.76–3.32	Effective treatment of illness	2.79	2.58–3.00	Improvement of physical function	3.64	3.42–3.86
2	Reduction of carer burden	4.56	4.44–4.67	Improvement of physical function	4.49	4.19–4.78	Improvement of physical function	4.06	3.84–4.29	Effective treatment of illness	4.33	4.11–4.55
3	Improvement of physical function	5.24	5.13–5.36	Maintaining high level of activity	5.11	4.76–5.45	Improvement of quality of life	5.46	5.19–5.73	Reduction of carer burden	5.40	5.18–5.63
4	Maintaining high level of activity	5.88	5.76–5.99	Reduction of carer burden	5.29	4.98–5.61	Reduction of carer burden	5.52	5.28–5.77	Improvement of quality of life	6.08	5.86–6.30
5	Resolution of assessed problems	5.91	5.76–6.05	Improvement of mental health	5.53	5.24–5.82	Improvement of mental health	5.81	5.58–6.04	Maintaining high level of activity	6.12	5.88–6.37
6	Improvement of mental health	6.26	6.15–6.36	Improvement of quality of life	5.80	5.48–6.13	Maintaining high level of activity	5.97	5.66–6.28	Improvement of mental health	6.38	6.17–6.58
7	Improvement of quality of life	6.36	6.23–6.49	Resolution of assessed problems	5.98	5.69–6.27	Resolution of assessed problems	6.17	5.93–6.42	Patient satisfaction with care	6.44	6.24–6.64
8	Patient satisfaction with care	6.81	6.70–6.92	Patient satisfaction with care	6.01	5.70–6.31	Patient satisfaction with care	6.72	6.47–6.96	Resolution of assessed problems	6.45	6.26–6.65
9	Efficient use of resources	6.91	6.81–7.02	Efficient use of resources	7.49	7.21–7.76	Efficient use of resources	7.46	7.24–7.69	Efficient use of resources	6.57	6.36–6.77
10	Improvement of social functioning	7.44	7.32–7.56	Improvement of social functioning	9.17	8.90–9.45	Improvement of social functioning	8.42	8.18–8.65	Improvement of social functioning	8.22	8.03–8.42
11	Avoiding institutional care	8.43	8.31–8.56	Avoiding institutional care	9.86	9.60–10.12	Avoiding institutional care	9.39	9.16–9.62	Avoiding institutional care	8.61	8.41–8.81
12	Reduction of mortality	9.98	9.87–10.08	Reduction of mortality	10.23	9.99–10.48	Reduction of mortality	10.22	10.00–10.44	Reduction of mortality	9.75	9.55–9.95

CI, confidence interval.

groups indicated physicians' strong preference for this item. All the physician groups also considered "patient satisfaction," "maintaining a high level of activity," and "improvement of physical function" important after "improvement of quality of life," with some variation in the order of their preferences. Geriatricians ranked "effective treatment of illness" the third most important, in contrast to the other two physician groups that ranked this item lower. Adult day care staff ranked "improvement of quality of life" and "maintaining a high level of activity" first and second, respectively, but placed "reduction of carer burden" the third most important, unlike physicians.

With regard to the receiving side of health care, "effective treatment of illness," "improvement of physical function," and "reduction of carer burden" were given high priority, whereas "improvement of quality of life" tended to be perceived as less important.

All the groups, including both health care providers and recipients, ranked "reduction of mortality" the least important, followed by "avoiding institutional care," "improvement of social functioning," and "efficient use of resources," except for the adult day care staff who ranked "improvement of social functioning" higher than "effective treatment of illness."

Stratification analysis demonstrated that the results from physicians were not influenced by sex (male vs female, data not shown); however, physicians older than 60 years tended to rank "effective treatment of illness" and "improvement of physical function" higher compared with younger physicians, who appeared to prioritize "patient satisfaction" and "maintaining a high level of activity." Physicians with more than 30 years' experience, most of whom were older than 60 years, showed a similar tendency, prioritizing "effective treatment of illness" and "improvement of physical function." The results from adult day care staff were identical across groups stratified by age, years of experience, and qualification (data not shown).

The results from the health care recipients did not differ by nursing care level (relatively independent vs limited impairment or higher, or limited impairment vs needing extensive help or higher) for adult day care participants and patients in geriatric outpatient clinics, the existence of relatives who required nursing care (present vs absent) for patients in geriatric outpatient clinics, study site for patients in geriatric outpatient clinics and community-dwelling older adults, or sex for all health care recipient groups (data not shown). Although stratification by age showed that the three measures given highest priority were the same across the age groups (65 to 74 vs older than 75) in community-dwelling older adults, the younger group ranked "reduction of carer burden" first, whereas the older group ranked "effective treatment of illness" first (data not shown).

Discussion

This study is, to our knowledge, the largest survey ever conducted to describe health outcome prioritization in geriatric medicine. We aimed to obtain a comprehensive picture of the views of those involved in decision-making processes in geriatric medicine and compare views between health care providers and recipients. We chose four groups each from providers and recipients that are considered relevant to our purpose. The mean response rate was close to 50%, which was good for a large-scale postal survey and ensured the representative nature of our respondents.

This survey demonstrated that there may be an important gap in health outcome prioritization between health care providers and recipients in geriatric medicine. All health care provider groups, notably physicians, expressed a strong preference for improvement in quality of life (QOL) as a priority of care, whereas health care recipients gave the highest priority to effective treatment of diseases and tended to put lower importance on QOL. In the context of clinical medicine, QOL is often used as a nonspecific, all-encompassing term to describe

nonmortality outcomes averaged over multiple domains (ie, physical, social, and psychological functioning and well-being). Consideration of QOL is essential for the selection of a treatment option, particularly when conditions are noncurative and chronic.[15] Therefore, it is not surprising that physicians who regularly see older patients with multiple chronic conditions consider QOL the most important health care outcome. On the other hand, the term QOL may not be familiar to many health care recipients, and we cannot exclude the possibility that QOL might be confused with other terms, such as standard of living.

Most health care recipients ranked effective treatment of diseases as the most important, suggesting that patients are concerned about their own particular symptoms rather than nonspecific QOL, arguing for efforts to examine the symptoms most concerning to patients. The high importance of effective treatment of diseases ascribed by health care recipients, but not physicians, also implies the significance of the often-neglected aspect of inappropriate prescribing in older adults: underuse of medication likely to be beneficial to older adults. Increased evidence has suggested that failure to prescribe indicated, beneficial medication is common in older adults,[7,8,16] and recent attempts to provide an explicit list of appropriate, indicated medication for older adults are justified.[10]

Interestingly, views on patient satisfaction were also different. All physician groups ranked patient satisfaction as the second top priority, whereas health care recipients considered this to be less important. This tendency has been demonstrated in a prior small study in England more than 15 years ago.[12] Recently, patient satisfaction has been increasingly used to measure health care qualities and compare health plans or physicians.[17] However, our finding may argue against the value of patient satisfaction as a performance measure in geriatric medicine, especially in light of recent evidence suggesting that higher patient satisfaction is accomplished at the sacrifice of increased use of health care resources and may not be directly associated with technical quality of care or improved outcome.[17,18]

We observed agreement on several items between health care providers and recipients. The importance of physical and mental function, such as maintaining activity or improving physical function, was expressed by both health care providers and recipients. This finding was consistent with prior studies in older adults with multiple chronic conditions[12,19] or terminal conditions,[20,21] suggesting that physical and mental function should be an essential factor to consider as a health care outcome in various care settings for older patients.

Reduction in mortality was given the lowest priority by all the groups in health care providers and recipients alike. This view is similar to that observed in previous studies.[12,19] This finding supports the contention that treatment interventions should be assessed in terms of reduced morbidity and improved QOL in addition to reduced mortality.

In this survey, respondents' characteristics, except age, had limited influence on their views on health outcome prioritization within each group. Geriatricians older than 60 years and community-dwelling adults older than 75 years gave higher priority to effective treatment of diseases compared with their younger counterparts. This suggests that health outcome priorities may not be stable, and can change as respondents age or differ from generation to generation. The cross-sectional design of our survey prevented us from separating the age effect from the secular trend, and further studies will be required to examine the time- or setting-dependent variability of health outcome prioritization.

This study has several limitations. First, although the average response rate was high for a postal survey, it was lower in physician groups than in health care recipient groups (26% to 48% vs 44% to 61%, Table 1). Thus, selection bias cannot be excluded. Second, it was not sure that health care recipients, particularly adult day care participants, correctly understood the study terminology. Third, some of the items used in the survey were not mutually exclusive. Nevertheless, a similar trend in priorities of outcome measures according to either side of health care providers or recipients suggests that the overall results were not significantly affected by these limitations.

Conclusion

We demonstrated that there was significant agreement and disagreement of health outcome prioritization between health care providers and recipients in geriatric medicine. Health care providers and recipients agreed on high priority for function and low priority for reduction in mortality, but there was obvious disagreement in how they perceived QOL, treatment effect, and patient satisfaction as goals of care. Such disagreement necessitates better communication between providers and recipients to reach goals of care that are mutually understandable and tailored to meet patients' specific needs. The low importance of reduction in mortality and patient satisfaction ascribed by health care recipients may question the value of these outcomes as a way to assess treatment interventions and quality of care. We propose that the priorities of health care outcomes and their differences between providers and recipients demonstrated in this study should be taken into account in the health care of older patients and the design of health care policies and research.

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手段的日常生活動作を用いた軽度認知症スクリーニング項目の検討

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〈短　報〉

手段的日常生活動作を用いた軽度認知症スクリーニング項目の検討

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要　約　**目的**：Lawton & Brody のI-ADL評価法を用いその下位項目と認知症との関連および，I-ADLの下位項目が軽度の認知症検出に有用であるかを検討する．**方法**：IADLおよびMMSE得点からROC曲線を作成し感度，特異度を算出する．**結果**：電話の使用と服薬管理，および財産管理の2項目，もしくはこれら3項目が完全自立していた場合MMSE 24点以上となる感度，特異度は70％以上であった．**結論**：これら3項目は軽度の認知症スクリーニングに有用である．

Key words：I-ADL，スクリーニング，認知症

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緒　言

我が国の65歳以上の高齢者における認知症の有病率は調査によってばらつきが大きいものの，朝田らの調査では14.4％に達するとされる[1]．しかし服薬数などから推察される認知症の治療率は半数以下と考えられている．厚生労働省によると早期診断・早期対応の遅れ，行動・心理症状等への不適切な対応などにより，不必要な入院が増え，また認知症の症状が悪化し，行動・心理症状等が生じてから，医療機関を受診しているケースが散見されているとされる．このため，簡易な認知症の評価を行い，医療や介護保険につなげる役割が期待されている[2]．

Barberger-Gateauらは手段的日常生活動作能力（Instrumental ADL：以下IADL）を用いた軽度の認知症スクリーニングにおいて高い感度と特異度を示している[3]．今回我々はLawton & BrodyのIADL評価法[4]を用いその下位項目と認知症との関連および，IADLが軽度の認知症検出に有用であるかを検討した．

方　法

Lawton & Brodyの評価方法は電話の使用，買い物，食事の準備，掃除，洗濯，移動，服薬管理，財産管理の8項目からなり，男性はそのうち食事の準備，掃除，洗濯を除く5項目で評価する．配点は，完全自立でなければ1点とならないもの，一部介助でも1点となるものなど項目ごとに基準が異なる．そのため男女共通項目の電話の使用，買い物，移動，服薬管理，財産管理について，受けることもかけることもできる，日常のすべての買い物をおこなえる，公共交通機関を用いて一人で移動できる，正しいときに正しい量の薬を飲める，銀行で金銭の出し入れをする等経済問題を自分で管理できる，を完全自立とし，その割合を算出した．

また，認知症診断のカットオフ値とされる23/24[5]に着目し，症例を2群に分けIADL各項目の自立との関連を検討した．

さらに，IADL項目のスクリーニングとしての精度を測るためROC曲線を作成して決定したIADL項目の組み合わせについて，MMSE得点に対する感度と特異度を算出した．

2010年7月から2012年4月までに国立長寿医療研究センター病院受診症例のうち，I-ADLはLawton & BrodyのI-ADL評価法によって，認知機能はMMSEによって評価が可能であり，MMSEの得点が16点以上の症例693名（男性227名，女性466名，平均年齢75.9±8.0歳）を対象とした．

統計処理はIBM SPSS statistics 20を用い，MMSE得点とIADL自立との関連の検討は$\chi 2$検定を行った．ROC曲線はMMSE得点とIADL項目の完全自立の人数を変数として作成した．有意水準は$p<0.05$とした．

結　果

完全自立の割合は，MMSE 16点から23点群は電話

Simple screening test using instrumental activities of daily living to find early stage of dementia

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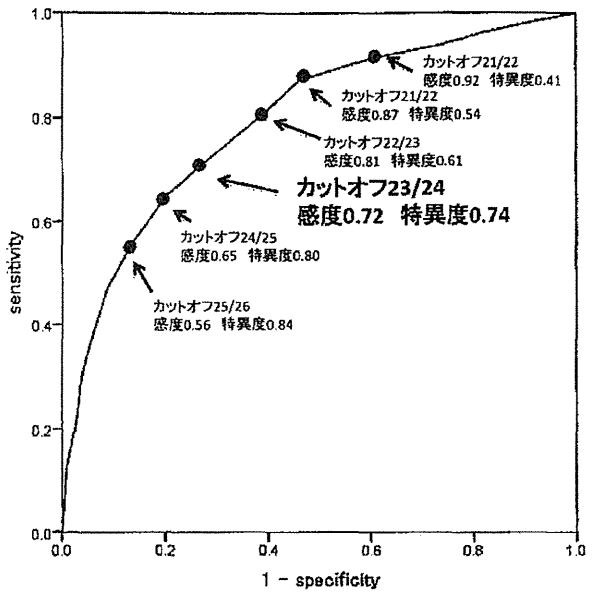

図1　軽度認知症検出における I-ADL（電話，服薬管理の自立）の ROC 曲線
AUC：area under the curve 0.79
カットオフ値 23/24 において感度 0.72 特異度 0.74 となる．

の使用が最も高く 54.4%，次に買い物 40.4%，服薬管理 37.8%，移動 34.9%，財産管理 29.0% であった．MMSE 24 点以上群では電話の使用 86.0%，買い物 76.5%，服薬管理 80.5%，移動 77.6%，財産管理 75.0% の順であった．いずれの項目も MMSE 24 以上群が有意に自立しており（$p<0.01$），各項目の自立は認知機能を反映していると考えられる．

これら 5 項目の中から歩行障害など認知機能以外の影響を受けやすいと考えられる移動の項目を除き，2 もしくは 3 項目の組み合わせで ROC 曲線を作成したところ，ROC 曲線下面積（area under the curve：AUC）の広かった組み合わせは電話の使用と服薬管理（0.794），電話の使用と財産管理（0.789），および服薬管理と財産管理（0.800）の組み合わせであった．これら 3 通りの組み合わせのうち MMSE のカットオフ値 23/24 において最も感度と特異度が良好となるのは，電話の使用と服薬管理で感度 0.72，特異度 0.74（図 1），電話の使用と財産管理が感度 0.76，特異度 0.70 であった．服薬管理と財産管理では 22/23 において感度 0.74，特異度 0.71 であった．

考　察

MMSE 23 点以下を検出するに資する手段的日常生活動作能力を Lawton & Brody の尺度を用いて検討した．電話の使用と服薬管理，もしくは財産管理の 2 項目を用いた際に感度，特異度ともに 70% を超えた．

Barberger-Gateau のスクリーニングでは電話の使用，移動，服薬管理，家計管理の 4 項目において認知症診断の感度 0.94，特異度 0.71 と報告されている．今回の対象症例においては MMSE 得点に対する感度，特異度を算出しているため一概に比較できないが，同等の項目を検討した際には感度 0.58，特異度 0.86 であった．

臨床場面でのスクリーニングでは，状況把握の簡易さや非該当を少なくするため評価項目は少ないほうがよいと思われ，電話の使用と服薬管理の 2 項目を用いたスクリーニングが有用であると考える．また，服薬なしの場合に財産管理を評価するという使用方法も可能であろう．

これらの評価項目の問題点としては，服薬していない，家族が金銭管理をさせていないなどの理由で現状が能力を表していない場合であるが，日常生活の状況聴取による簡便な評価を実施するうえでの限界であるともいえる．

今後，健常高齢者との比較検討および信頼性，妥当性を含む有用性の検討が必要であると考える．

利益相反

本論文に関して，開示すべき利益相反状態は存在しない．

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Behavioural pharmacology

Involvement of the strychnine-sensitive glycine receptor in the anxiolytic effects of GlyT1 inhibitors on maternal separation-induced ultrasonic vocalization in rat pups

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Ultrasonic vocalization (USV)

ABSTRACT

Several studies have shown that glycine transporter 1 (GlyT1) inhibitors have anxiolytic actions. There are two types of glycine receptor: the strychnine-sensitive glycine receptor (GlyA) and the strychnine-insensitive glycine receptor (GlyB); however, which receptor is the main contributor to the anxiolytic actions of GlyT1 inhibitors is yet to be determined. Here, we clarified which glycine receptor is the main contributor to the anxiolytic effects of GlyT1 inhibitors by using maternal separation-induced ultrasonic vocalization (USV) by rat pups as an index of anxiety. We confirmed that administration of the benzodiazepine diazepam or the selective serotonin reuptake inhibitor escitaloplam, which are both clinically proven anxiolytics, or the GlyT1 inhibitor SSR504734 (2-chloro-N-[(S)-phenyl[(2S)-piperidin-2-yl] methyl]-3-trifluoromethyl benzamide), decreases USV in rat pups. In addition, we showed that another GlyT1 inhibitor, ALX5407 ((R)-N-[3-(4′-fluorophenyl)-3(4′-phenylphenoxy)propyl]sarcosine) also decreases USV in rat pups. SSR504734- or ALX5407-induced decreases in USV were dose-dependently reversed by administration of the GlyA antagonist strychnine, whereas the diazepam- or escitalopram-induced decreases in USV were not. Furthermore, GlyT1-induced decreases in USV were not reversed by administration of the GlyB antagonist L-687,414. Together, these results suggest that GlyA activation is the main contributor to the anxiolytic actions of GlyT1 inhibitors and that the anxiolytic actions of diazepam and escitalopram cannot be attributed to GlyA activation. Our findings provide new insights into the importance of the activation of GlyA in the anxiolytic effects of GlyT1 inhibitors.

1. Introduction

The neurotransmitter glycine acts through two receptors: a strychnine-sensitive glycine receptor (GlyA) and a strychnine-insensitive glycine receptor (GlyB). GlyA is localized to the neuronal membrane post-synaptic to inhibitory glycinergic neurons, whereas GlyB is associated with the NR1 subunit of the excitatory N-methyl-D-aspartate (NMDA) receptor (Kuryatov et al., 1994; Legendre, 2001). Glycine therefore has bidirectional actions on neuronal excitability.

The extracellular concentration of glycine is regulated by glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2) (Aragón and López-Corcuera, 2005). GlyT1 is expressed on glial cells and glutamatergic neurons (Cubelos et al., 2005; Raiteri and Raiteri, 2010), whereas GlyT2 is predominantly expressed at glycinergic nerve terminals (Jursky and Nelson, 1995). NMDA receptor function is enhanced in the hippocampus of *GlyT1* heterozygous-knockout mice, suggesting that GlyT1 regulates the concentration of glycine at NMDA receptor-containing excitatory synapses (Gabernet et al., 2005). Thus, GlyT1 inhibitors likely promote NMDA receptor function.

GlyT1 inhibitors may have anxiogenic actions, because NMDA receptor activation induces anxiety-like behavior in mice (Miguel and Nunes-de-Souza, 2008). However, GlyT1 inhibitors may also have anxiolytic actions, because SSR504734, a GlyT1 inhibitor, both attenuates the acquisition and expression of contextual conditioned fear in rats (Nishikawa et al., 2006) and decreases maternal separation-induced ultrasonic vocalization (USV) in rat pups (Depoortère et al., 2005). Furthermore, the NMDA receptor antagonists MK-801 and DL-amino-5-phosphonovaleric acid (AP5) have been shown to have anxiolytic actions in rats (Kehne et al.,

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1991), and 7-chlorokynurenic acid (7-Cl-KYN), a GlyB antagonist, has been shown to have anxiolytic actions in mice (Trullas et al., 1989). Together, these reports suggest that the anxiolytic action of GlyT1 inhibitors is not induced by activation of GlyB. However, which glycine receptor subtype is the main contributor to the anxiolytic actions of GlyT1 inhibitors is yet to be fully elucidated. Recently, it was reported that the hyperlocomotion induced by a GlyT1 inhibitor in mice was antagonized by the GlyA antagonist strychnine (Kopec et al., 2010), suggesting that not only GlyB but also GlyA plays a significant role in the behavioral changes induced by GlyT1 inhibitors.

Rodent pups emit USVs (peak frequency around 40 kHz) when they are separated from their mother and littermates (Brudzynski et al., 1999; Zippelius and Schleidt, 1956). Because clinically proven anxiolytics such as the benzodiazepines and selective serotonin reuptake inhibitors reduce the number of maternal separation-induced USVs in rat pups, USV is thought to be a predictive animal model of the anxiolytic effect (Insel et al., 1986; Winslow and Insel, 1991). Therefore, in the present study, maternal separation-induced USV in rat pups was used as an index of anxiety.

Here, we examined which glycine receptor is the major contributor to GlyT1 inhibitor-induced decreases in rat pup USV. We first examined the effect of GlyT1 inhibitors and anxiolytics on USV and then examined whether GlyT1 inhibitor-induced decreases in USV were reversed by administration of a GlyA or GlyB antagonist.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats, each with 10 pups at postnatal day 4, were purchased from Charles River Laboratories (Tokyo, Japan). Animals were housed at room temperature and maintained under a 12-h/12-h light/dark cycle with ad libitum access to food and tap water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Eisai Co., Ltd. (Ibaraki, Japan).

2.2. Drugs

SSR504734 (2-chloro-N-[(S)-phenyl[(2S)-piperidin-2-yl] methyl]-3-trifluoromethyl benzamide), ALX5407 ((R)-N-[3-(4′-fluorophenyl)-3(4′-phenylphenoxy)propyl]sarcosine; (+)-NFPS), and L-687,414 ((3R,4R)-3-amino-1-hydroxy-4-methylpyrrolidin-2-one) were synthesized at the medicinal chemistry department of Eisai Co., Ltd. Diazepam, escitalopram, and strychnine were purchased from Wako Pure Chemical Industries (Osaka, Japan), AK Scientific (Union City, CA), and Sigma-Aldrich (Tokyo, Japan), respectively. SSR504734 was dissolved in distilled water, and the pH was adjusted to 6 to 7 using 1 N HCl. ALX5407 was dissolved in distilled water, and the pH was adjusted to 6 to 7 using 1 N NaOH. Escitalopram was dissolved in saline. Diazepam was suspended in 0.5% methyl cellulose (Wako Pure Chemical Industries, Osaka, Japan). Strychnine was dissolved in saline, and the pH was adjusted to 6 to 7 using 1 N HCl. L-687,414 was dissolved in saline with 0.3% Tween 80 (Kanto Chemical Co., Inc., Tokyo, Japan). Several doses of each drug were used and are indicated in the figures. We chose the doses of drugs used in this study by referencing the results of previous studies (Depoortère et al., 2005; Kopec et al., 2010; Olivier et al., 1998a; Sánchez et al., 2003). After determining the dose–response relationship of each compound, in the subsequent antagonism study we used the dose at which the number of USVs was suppressed to less than 35% of that in vehicle-treated control rat pups. All solutions and suspensions were prepared daily and administered orally or subcutaneously in a volume of 10 ml/kg body weight.

2.3. Ultrasonic vocalization test

The procedure was modified from that described by Olivier et al. (1998a, 1998b). Briefly, pre-weaning Sprague–Dawley rat pups were used at postnatal day 10. Each pup was separated from its mother and littermates and immediately placed in a plastic cylinder kept at room temperature. The number of USVs was recorded for 3 min by using a Sonotrack™ measurement system (Metris, Netherland). USVs picked up by the microphones were digitally recorded. The band-pass filter was adjusted to 30–70 kHz. Within this range, the Sonotrack™ software automatically counted the number of USVs produced by each rat pup.

SSR504734, diazepam, or escitalopram was administered orally 1 h prior to the USV test. ALX5407 was administered orally 3 h prior to the USV test. A 3-h pretreatment time was selected because of the irreversible nature of ALX5407 binding (Atkinson et al., 2001; Kopec et al., 2010). For the antagonism test, strychnine (GlyA antagonist) or L-687,414 (GlyB antagonist) was administered subcutaneously 30 min before the USV test. To avoid direct interactions between the compounds, different routes of administration were used for the two compounds. After administration of the test compound, the pups were returned to their home cage until use.

2.4. Measurement of rectal temperature

To evaluate whether or not any decrease in the number of USVs was secondary to a decrease in body temperature, the influence of each drug on rectal temperature, when administered at the maximum ineffective and minimum effective doses as determined in the USV test, was examined by using a rectal probe (Physitemp Instruments, Inc., Clifton, NJ) and a TX1002 digital thermometer (Yokogawa Meters & Instruments Corporation, Japan). Pretreatment times were the same as those used in the USV test.

2.5. Statistical analysis

All statistical analyses were carried out by using GraphPad Prism software version 6.0 for Windows (GraphPad Software, San Diego, CA). Data were analyzed by using Kruskal–Wallis followed by Dunn's multiple comparison test or the Mann–Whitney *U* test.

3. Results

3.1. Effects of GlyT1 inhibitors or anxiolytics on USV and rectal temperature in Sprague–Dawley rat pups

The effects of administration of the test compounds on the number of USVs recorded in 3 min are shown in Fig. 1. The SSR504734 (Fig. 1A) doses were 3, 10, or 30 mg/kg; administration at 30 mg/kg significantly decreased the number of USVs recorded (H [4, 32] = 14.90, $P < 0.01$). Rectal temperature did not change compared with that in vehicle-treated control rats after administration of SSR504734 at 10 or 30 mg/kg (Table 1).

Similarly, ALX5407 doses were 0.1, 0.3, or 1 mg/kg (Fig. 1B); administration at 1 mg/kg significantly decreased the number of USVs (H [4, 35] = 20.26, $P < 0.01$]) without affecting rectal temperature at 0.3 or 1 mg/kg (Table 1).

Both diazepam (Fig. 1C) and escitalopram (Fig. 1D) significantly decreased the number of USVs when administered at 1 or 3 mg/kg (diazepam; H [4, 32] = 18.91, $P < 0.05$ and $P < 0.01$, escitalopram; H

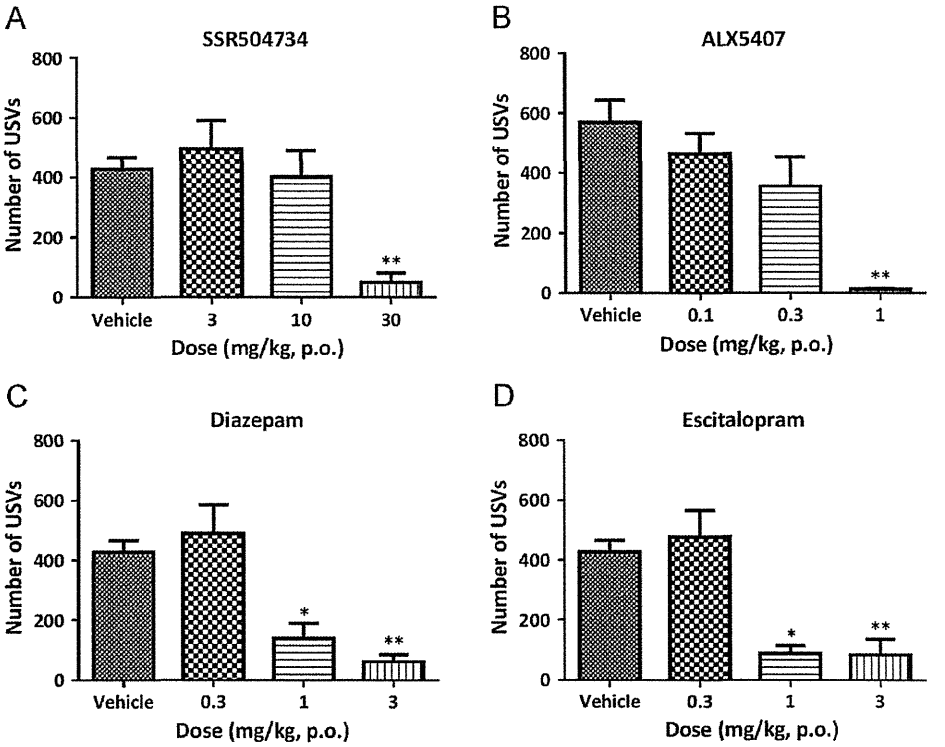

Fig. 1. Effects of SSR504734, ALX5407, diazepam, or escitalopram on the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. SSR504734 (A), diazepam (C), or escitalopram (D) was administered orally 1 h prior to the test. ALX5407 (B) was administered orally 3 h prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mother and littermates. Data are presented as mean ± S.E.M. $N=8$–9 per group. $^{*}P<0.05$, $^{**}P<0.01$, compared with the vehicle-treated control group (Kruskal–Wallis followed by Dunn's test).

Table 1
Effects of SSR504734, ALX5407, diazepam, or escitalopram on rectal temperature. SSR504734, diazepam, or escitalopram was orally administered 1 h prior to the test. ALX5407 was orally administered 3 h prior to the test. Data are presented as mean ± S.E.M. $N=4$ per group. Each treatment group was compared with its vehicle-treated control group.

Compound	Dose (mg/kg)	Mean ± S.E.M.
Vehicle	–	36.9 ± 0.17
SSR504734	10	36.6 ± 0.20
SSR504734	30	36.7 ± 0.21
Vehicle	–	36.5 ± 0.07
ALX5407	0.3	36.7 ± 0.29
ALX5407	1	36.1 ± 0.14
Vehicle	–	36.7 ± 0.18
Diazepam	0.3	36.7 ± 0.18
Diazepam	1	36.2 ± 0.29
Vehicle	–	36.9 ± 0.13
Escitalopram	0.3	36.3 ± 0.32
Escitalopram	1	36.1 ± 0.30

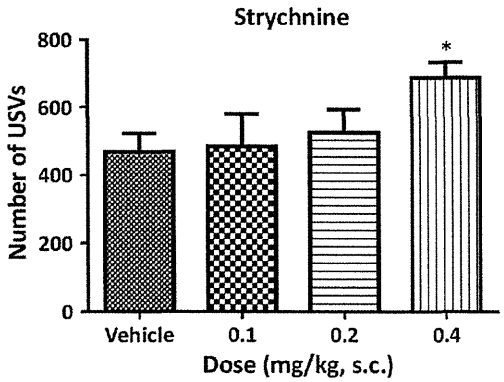

Fig. 2. Effects of strychnine on the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. Strychnine was subcutaneously administered 30 min prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mothers and littermates. Data are presented as mean ± S.E.M. $N=9$–10 per group. $^{*}P<0.05$, compared with the vehicle-treated control group (Kruskal–Wallis followed by Dunn's test).

[4, 32] = 16.71, $P<0.05$ and $P<0.01$), but did not affect rectal temperature at 0.3 or 1 mg/kg (Table 1).

3.2. *Effects of strychnine, a GlyA antagonist, on GlyT1 inhibitor-induced or anxiolytic-induced decreases in the number of USVs produced by Sprague–Dawley rat pups*

The number of USVs was not changed by administration of strychnine alone at 0.1 or 0.2 mg/kg, but was significantly increased compared with control when strychnine alone was administered at 0.4 mg/kg (H [4, 39] = 7.06, $P<0.05$) (Fig. 2). The SSR504734-induced decrease in the number of USVs (30 mg/kg; $U=7.0$, $P<0.01$) was significantly and dose-dependently reversed by administration of strychnine at 0.2 or 0.4 mg/kg (H [4, 40] = 19.15, $P<0.05$ and $P<0.01$) (Fig. 3A). The ALX5407-induced decrease in the number of USVs (1 mg/kg; $U=0.0$, $P<0.01$) was also significantly and dose-dependently reversed by administration of strychnine at 0.2 or 0.4 mg/kg (H [4, 38] = 29.40, both $P<0.01$) (Fig. 3B). However, the diazepam-induced (1 mg/kg; $U=8.0$, $P<0.01$) or escitalopram-induced (1 mg/kg; $U=4.0$, $P<0.01$) decrease in the number of USVs was not reversed by the administration of strychnine at any of the doses examined (H [4, 40] = 2.84 and H [4, 40] = 4.50) (Fig. 4A and B).

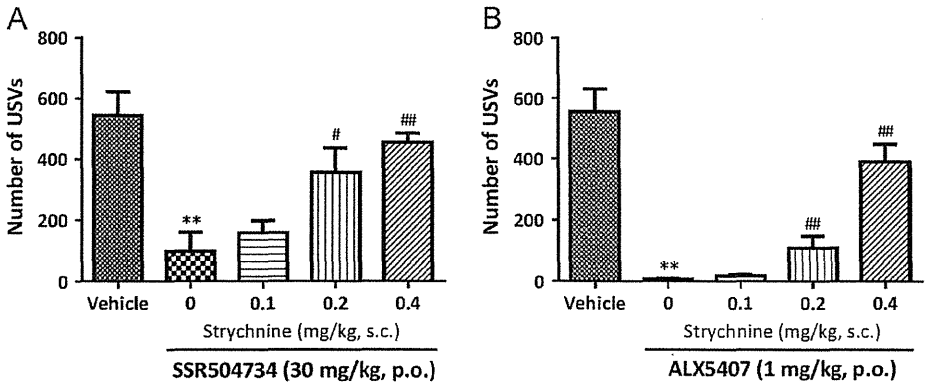

Fig. 3. Effects of strychnine on SSR504734- or ALX5407-induced decreases in the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. SSR504734 (A) was orally administered 1 h and ALX5407 (B) was orally administered 3 h prior to the test. In both tests, strychnine was administered subcutaneously 30 min prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mothers and littermates. Data are presented as mean ± S.E.M. $N=8$–10 per group. $^{**}P<0.01$, compared with the vehicle-treated control group (Mann–Whitney U test). $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the group treated with SSR504734 alone or ALX5407 alone (Kruskal–Wallis followed by Dunn's test).

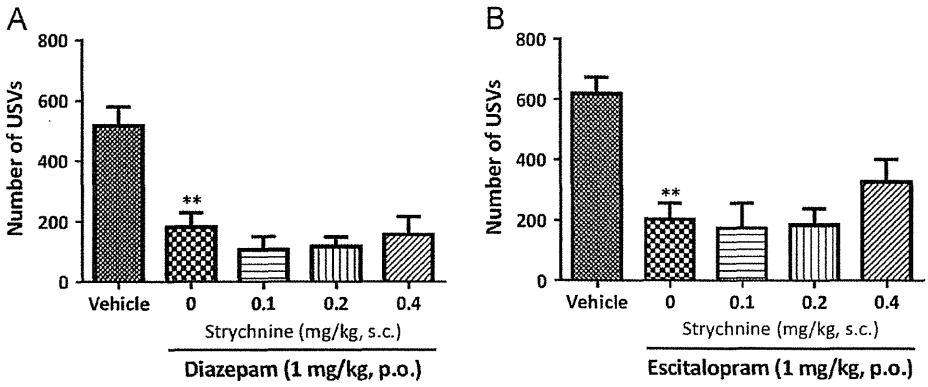

Fig. 4. Effects of strychnine on diazepam- or escitalopram-induced decreases in the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. Diazepam (A) or escitalopram (B) was orally administered 1 h prior to the test. In both tests, strychnine was administered subcutaneously 30 min prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mothers and littermates. The number of USVs was measured for 3 min immediately after separation of the pups from their mother and littermates. Data are presented as mean ± S.E.M. $N=10$ per group. $^{**}P<0.01$, compared with the vehicle-treated control group (Mann–Whitney U test).

3.3. Effects of L-687,414, a GlyB antagonist, on SSR504734-induced decreases in the number of USVs produced by Sprague–Dawley rat pups

The number of USVs was significantly decreased by the administration of L-687,414 alone at 30 mg/kg (H [3, 30] = 9.45, $P<0.01$), but not at 10 mg/kg (Fig. 5). Furthermore, the SSR504734-induced decrease in the number of USVs (30 mg/kg; $U=13.0$, $P<0.01$) was not reversed by the administration of L-687,414 at 3, 10, or 30 mg/kg (Fig. 6). L-687,414 appeared to strengthen the SSR504734-induced decrease in the number of USVs, but this was not statistically significant (H [4, 39] = 4.01).

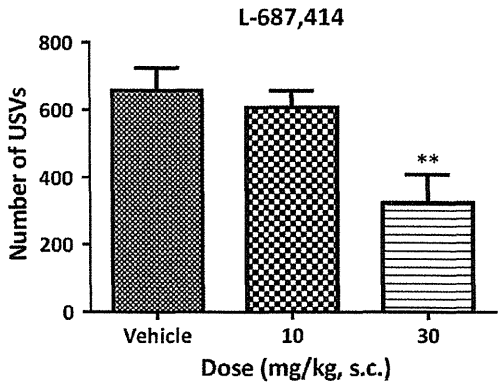

Fig. 5. Effects of L-687,414 on the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. L-687,414 was subcutaneously administered 30 min prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mothers and littermates. Data are presented as mean ± S.E.M. $N=10$ per group. $^{**}P<0.01$, compared with the vehicle-treated control group (Kruskal–Wallis followed by Dunn's test).

4. Discussion

The results of the present study suggest that GlyT1 inhibitor-induced decreases in USV are mediated through the activation of inhibitory GlyA rather than excitatory GlyB, which is localized to the NMDA receptor. Furthermore, we found that diazepam- or escitalopram-induced decreases in USV are not related to GlyA. Thus, GlyA plays an important role in GlyT1 inhibitor-induced decreases in USV.

Maternal separation-induced USV is a well-established index of anxiety in rodents (Olivier et al., 1998a, 1998b; Winslow et al., 1990). In agreement with previous reports, we showed that diazepam and escitalopram, which are clinically proven anxiolytics, significantly decreased USV, which suggests that the USV test

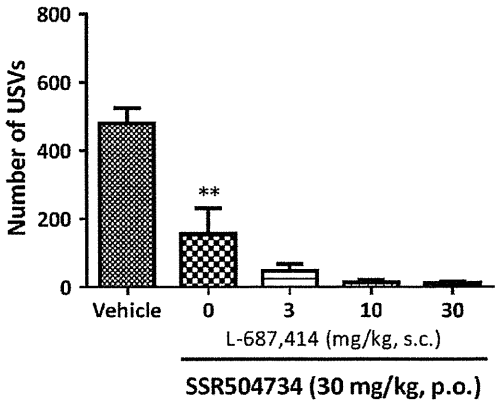

Fig. 6. Effects of L-687,414 on SSR504734-induced decreases in the number of maternal separation-induced ultrasonic vocalizations (USVs) in Sprague–Dawley rat pups. SSR504734 was orally administered 1 h prior to the test and L-687,414 was subcutaneously administered 30 min prior to the test. The number of USVs was measured for 3 min immediately after separation of the pups from their mother and littermates. Data are presented as mean ± S.E.M. $N=9$–10 per group. $^{**}P<0.01$, compared with the vehicle-treated control group (Mann–Whitney U test).

may be predictive of anxiolytic effect in humans. We also confirmed previously published data showing that SSR504734 significantly decreases USV (Depoortère et al., 2005).

We also evaluated another GlyT1 inhibitor, ALX5407, which has a different chemical structure from that of SSR504734. ALX5407 decreased USV when administered at a dose of 1 mg/kg; however, it has been reported that ALX5407 does not have anxiolytic actions at a dose of 3 or 10 mg/kg, as assessed by behavioral tests in adult rats (Harsing et al., 2003). These inconsistent results may be explained by the different pretreatment times and routes of administration used in the studies. Harsing et al. (2003) administered ALX5407 intraperitoneally 30 min prior to conducting the tests, whereas we orally administered it 3 h prior to conducting the tests. Because ALX5407 has an amino acid moiety within its structure (Atkinson et al., 2001), we predicted that its penetration into the brain would be slow. Indeed, in a microdialysis study, extracellular glycine levels gradually increased, reaching peak levels in the prefrontal cortex at 3 h and in the cerebellum at 6 h after oral administration of 10 mg/kg (±)-NFPS, a racemic form of ALX5407 (Perry et al., 2008). Kinney et al. (2003) demonstrated that intraperitoneal administration of ALX5407 (1 mg/kg) 2 h prior to the test increased the degree of prepulse inhibition in mice. Thus, it may be possible to detect the anxiolytic actions of ALX5407 by taking the pharmacokinetics of ALX5407 and pharmacodynamics of glycine in the brain into account.

It is important to clarify whether the decrease in USV after administration was secondary to the central depressant actions of the test compounds. Several reports have addressed the possibility that central depressant actions such as motor incoordination or changes in body temperature might affect USV (Olivier et al., 1998a, 1998b). However, while diazepam is known to induce muscle relaxation at higher doses, it has only been shown to suppress USV at lower doses (Olivier et al., 1998a). And although diazepam reduces rectal temperature at effective doses, overall the hypothermic actions of the benzodiazepines are not thought to contribute to their anxiolytic actions, at least in the clinical setting (Olivier et al., 1998a). Furthermore, in the present study, diazepam did not decrease rectal temperature at the effective doses used, which provides support for the idea that diazepam decreases USV via its anxiolytic actions. Our results also show that escitalopram did not affect rectal temperature at the effective doses used, which is consistent with the results of previous studies showing that selective serotonin reuptake inhibitors have no effect on motor coordination, body temperature, or the righting reflex (Hodgson et al., 2008; Olivier et al., 1998b). We also showed that SSR504734 did not affect rectal temperature at the effective doses used, which supports previous work by Depoortère et al. (2005) that did not report any abnormal behavior in rats and mice caused by SSR504734, and that administration of ALX5407 at effective doses also did not cause a decrease in rectal temperature. Several studies have shown that high-dose ALX5407 induces motor incoordination. For example, Harsing et al. (2006) have reported that intraperitoneal administration of (±)-NFPS at 30 mg/kg produces motor dysfunction in mice, and Perry et al. (2008) have reported that orally administered (±)-NFPS at 30 mg/kg, but not at 10 mg/kg, produces motor dysfunction, impaired gait in rats. Kopec et al. (2010) have shown that intraperitoneal administration of ALX5407 at 6 mg/kg, but not at 3 mg/kg, exhibited hyperlocomotion. Here, we observed the decrease in USV at the lower dose of 1 mg/kg without affecting rectal temperature and we did not observe any abnormal behaviors in rat pups at the same dose. Therefore, we consider the ALX5407-induced decrease in USV to not be secondary to the central depressant actions of the compound. Together, the above results suggest that GlyT1 inhibitors have an anxiolytic action.

Next, we examined whether GlyA or GlyB is the main contributor to the anxiolytic action of GlyT1 inhibitors. Inhibitory GlyA and excitatory GlyB mediate opposing actions on neuronal excitability. GlyT1 inhibitors efficiently activate NMDA receptors because GlyT1 is distributed closely to the NMDA receptor (Smith et al., 1992). However, a recent autoradiograph study (Herdon et al., 2010) confirmed the results of a previous study that showed that GlyT1 is enriched in the hindbrain (Jursky et al., 1994), where the distribution is consistent with that of GlyA (Zarbin et al., 1981). Therefore, it might be possible that GlyT1 inhibitors contribute to the activation of GlyA. Consistent with this hypothesis, our results indicate that the anxiolytic action of GlyT1 inhibitors is reversed by the GlyA antagonist strychnine, but not by the GlyB antagonist L-687,414, and therefore that the anxiolytic action of GlyT1 inhibitors is mediated by GlyA. Strychnine administration (0.4 mg/kg) slightly increased USV; however, we can exclude the possibility that this apparent reversal of the action of SSR504734 and ALX5407 by strychnine was actually due simply to an independent increase in USV, because strychnine also showed significant reversal when administered at 0.2 mg/kg, a dose at which USV remained unchanged when strychnine was administered alone. Administration of L-687,414 alone at 30 mg/kg decreased USV, which agrees with the results of previous studies in which GlyB antagonists were reported to have anxiolytic actions (Trullas et al., 1989; Winslow et al., 1990). These data support the notion that activation of GlyB does not induce anxiolytic actions. Although L-687,414 has been reported to be a partial agonist of GlyB (Priestley et al., 1998), it acts as a substantial NMDA receptor antagonist in vivo because of its weak intrinsic agonist activity. Tricklebank et al. (1994) have shown that L-687,414 has dose-dependent anticonvulsant effects in a variety of animal models, and that the anticonvulsant effects of L-687,414 are completely reversed by administration of the GlyB agonist D-serine. In contrast to GlyT1 inhibitors, neither the diazepam- nor escitalopram-induced reductions in USV were reversed by strychnine. These results suggest that GlyA is not associated with $GABA_A$ receptor- or serotonin transporter-mediated anxiolytic actions.

It is possible that maternal separation-induced USV in neonatal rodents is not a good model of adult anxiety for examining GlyT1 inhibitors, if the expression levels of GlyT1, GlyA, and GlyB markedly differ between pups and adults. However, several reports provide evidence that this is not the case. Lall et al. (2012) have reported that the expression levels of both GlyT1 and GlyA differ by 20% in the brain stems of 10-day-old mouse

pups and adult mice. Suen et al. (1998) have reported that the protein levels of NR1 subunits in the rat cortical postsynaptic density is 1.6-fold greater in adult rats than in rats at postnatal day 10. Although these differences are unlikely to change the conclusions of the present study, other paradigms such as the elevated plus maze test should be examined in adult rodents to provide further confirmatory evidence of our conclusions.

In conclusion, the present data suggest that the anxiolytic effects of GlyT1 inhibitors are mediated through GlyA but not through GlyB. Several GlyT1 inhibitors are currently in clinical development for the treatment of schizophrenia and obsessive–compulsive disorder (Umbricht et al., 2014; ClinicalTrials.gov Identifier: NCT01674361), and a recent clinical study has demonstrated that sarcosine, a GlyT1 inhibitor, improves psychic anxiety, as assessed by means of the 17-item Hamilton Depression Rating Scale, in patients with major depression (Huang et al., 2013). Our findings further demonstrate the anxiolytic effects of GlyT1 inhibitors and provide new insights into the mechanism of these anxiolytic effects.

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A Combination of Supplements May Reduce the Risk of Alzheimer's Disease in Elderly Japanese with Normal Cognition

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Abstract. A number of studies have examined the effect of a single supplement against Alzheimer's disease (AD) with conflicting results. Taking into account the complex and multifactorial nature of AD pathogenesis, multiple supplements may be more effective. Physical activity is another prospect against AD. An open-label intervention study was conducted to explore a potential protective effect of multiple supplements and physical activity. Their interaction was also examined. Participants were community-dwelling volunteers aged 65 or older as of May 2001 in a rural area of Japan. Among 918 cognitively normal participants included in the analyses, 171 took capsules daily for three years that contained n-3 polyunsaturated fatty acid, *Ginkgo biloba* leaf dry extracts, and lycopene. Two hundred and forty one participants joined the two-year exercise intervention that included a community center-based and a home-based exercise program. One-hundred and forty eight participated in both interventions. A standardized neuropsychological battery was administered at baseline in 2001, the first follow-up in 2004-2005, and the second in 2008-2009. The primary outcome was AD diagnosis at follow-ups. A complementary log-log model was used for survival analysis. A total of 76 participants were diagnosed with AD during follow-up periods. Higher adherence to supplementation intervention was associated with lower AD incidence in both unadjusted and adjusted models. Exercise intervention was also associated with lower AD incidence in the unadjusted model, but not in the adjusted model. We hypothesized that the combination of supplements acted in a complementary and synergistic fashion to bring significant effects against AD occurrence.

Keywords: Alzheimer's disease, dietary supplements, exercise, Gingko biloba, intervention study, lycopene, n-3 PUFA

INTRODUCTION

Alzheimer's disease (AD) has become a worldwide major health concern in the last two decades, with its incidence and prevalence on the rise. It is the most common cause of dementia and accounts for 60% to 80% of all dementia cases [1, 2].

Even though factors that can reduce the risk of or prevent AD have been extensively sought, no evidence points to any definite conclusion [1].

For example, although a potential protective effect of a single nutritional or herbal supplementation against AD has been rigorously examined, the results are conflicting and evidence is still not adequate [1, 3–6] (Table 1). However, recent observational studies suggest that a combination of nutrients, rather than a single nutrient, may be more beneficial in preventing or delaying the process of AD [7–10] (Table 2). Therefore, it is worthwhile to explore the effect of supplementation of multiple nutrients in an intervention study.

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Table 1
Select articles on effect of a single nutritional supplement against AD

Nutrient	Study type years of follow-up	Results
n-3 PUFA or DHA		
Huang et al. [72]	Prospective cohort study 5.4 years	Consumption of fatty fish more than twice per week reduced AD risk by 41%
Devore et al. [73]	Prospective cohort study 9.6 years	Total fish intake, n-3 PUFA, DHA intake had no association with AD risk
Vitamin E		
Zandi et al. [10]	Prospective and cross-sectional study 3–5 years	Vitamin E supplementation was associated with lower AD prevalence, but not with AD incidence
Petersen et al. [74]	Randomized controlled study 3 years	Daily vitamin E supplementation had no benefit in reducing incident AD
Ginkgo biloba		
Amieva et al. [75]	Prospective cohort study 20 years	*Ginkgo biloba* supplement users had less decline in MMSE score in non-demented participants (AD not mentioned)
DeKosky et al. [46]	Randomized controlled study 6.1 years	*Ginkgo biloba* extract 120 mg twice a day did not reduce AD risk

AD, Alzheimer's disease; PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; MMSE, Mini-Mental State Examination.

Table 2
Select articles on effect of a combination of nutrients against AD

	Study type years of follow-up	Results
Gu et al., 2010 [7]	Prospective cohort study 3.9 years	Diet rich in n-3 PUFA, n-6 PUFA, vitamin E, and folate but low SFA and vitamin B12 was associated with reduced AD incidence
Dai et al., 2006 [8]	Prospective cohort study 7–9 years	Drinking fruit and vegetable juices was associated with reduced AD
Zandi et al., 2004 [10]	Prospective and cross-sectional study 3–5 years	A combination of vitamin E and C supplementation was associated with both lower AD prevalence and incidence

AD, Alzheimer's disease; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acids.

Physical activity is another prospect against AD. Some studies have indicated that exercise can have a protective effect against AD or dementia in general [11–14] (Table 3) but overall quality of evidence is low [1]. One of the important limitations of these studies is that estimates of physical activity level were based on the participants' self-reports. Frequent contacts with study participants may decrease reporting bias.

In the present study, potential protective effects of multiple nutritional supplementation and exercise intervention on the risk of AD incidence for elderly people with normal cognition at baseline were examined. Their combined effect was also to be explored. The study was conducted within the Tone Project, a prospective cohort study in a rural area of Japan.

METHODS

Study population

Participants included in the present study were screened from the participants in the Tone Project, a longitudinal cohort study conducted in Tone town, Ibaraki Prefecture, Japan. This community-based study was designed to focus on the prevalence, incidence, and risk or protective factors of dementia, especially of AD in elderly Japanese. Tone is in the rural areas 50 km to the north of Tokyo, and its elderly population structure was similar to that of national average at the commencement of the study. Elderly volunteers were followed up with detailed clinical and neuropsychological assessments at three- to four-year intervals, part of whom had nutritional supplementation and/or exercise intervention. The participants were also given lectures on healthy diet and sleep hygiene.

The study design and recruitment process of the study can be found elsewhere [15, 16]. Briefly, all 3,083 potential candidates 65 years or older as of May 2001 were taken from the resident registration system. After excluding people who moved ($n=45$), were deceased ($n=87$), institutionalized for preexisting dementia ($n=44$), or unable to be contacted ($n=253$), 2,654 people were contacted for participation to the study. Of those, 1,844 community dwelling people or their family representatives finally agreed to participate in the study and gave a written consent. The protocol of this study was approved by the ethics committee of the University of Tsukuba.

Table 3
Select articles on effect of physical exercise against AD

	Study type years of follow-up	Results
Larson et al., 2006 [11]	Prospective cohort study 6.2 years	Regular exercise was associated with reduced AD and dementia risk
Andel et al., 2008 [12]	Case control study 31 years	Regular exercise at midlife was associated with reduced AD and dementia risk
Podewils et al., 2005 [13]	Prospective cohort study 5.4 years	Frequent physical activity was associated with reduce AD and dementia risk

AD, Alzheimer's disease; OR, odds ratio.

Clinical evaluation

The baseline evaluation was conducted between December 2001 and April 2002. A face-to-face interview used a structured questionnaire to obtain demographical information such as age, gender, and education, as well as past medical and psychiatric history. Routine blood sampling, including apolipoprotein E (APOE) genotyping, was performed in consenting participants.

Then, participants went through a series of clinical and neuropsychological evaluations described below. The same assessment protocol was used at follow-up evaluations. The first follow up was carried out between December 2004 and July 2005, and the second between September 2008 and February 2009.

Measures

Depression

The 15-item short version of the Geriatric Depression Scale (GDS) was conducted for depression screening. Those who scored six or above were considered to have symptoms of depression [17].

Neuropsychological assessment

Five cognitive domains were assessed: *attention, memory, visuospatial function, language, and reasoning*. We used the previously validated assessments and named them "*5-Cog*".

Attention was evaluated with a Japanese version of the set dependent activity [18], memory with the Category Cued Recall test [19], visuospatial function with the Clock Drawing test [20], language ability with the Category Fluency test [21], and abstract reasoning with the similarities subset of the Wechsler Adult Intelligence Scale-Revised (WAIS-R) [22].

The 5-Cog was conducted in a group setting, with maximum participants of 50 at a time. Each screening was supervised by members of our research team. The mean length of the 5-Cog was 35 minutes. For participants with difficulty understanding tasks or with impaired hearing or vision ($n=261$), the assessment was conducted in a face-to-face setting.

In the present study, individuals who scored below 1.5 standard deviation (SD) on one or more domains of the 5-Cog test after normative correction for age, years of education, and gender were excluded. Also excluded were those who could not adequately respond to our instructions or some of the scales because of obvious cognitive impairment. Thus, only those individuals who were deemed cognitively normal were included in the analysis.

Study outcomes

The primary outcome was the diagnosis of AD at a diagnostic meeting. Diagnostic meetings were held for those participants with suspected dementia based on the functional, medical, psychiatric, and neuropsychological data, as well as observations of participants at interview by our study staff. Reports from family members and a primary care physician, and retrieved information from Long-Term Care Insurance System were also referred to when available.

The meeting consisted of the study psychiatrists and neuropsychologists, and dementia diagnosis was assigned according to the DSM-III-R criteria [23]. Subsequently, AD diagnosis, probable or possible, according to NINCDS-ADRDA criteria [24] was assigned.

Other types of dementia diagnoses—dementia with Lewy bodies, vascular dementia, or frontotemporal dementia—were also assigned according to International Consensus Consortium Criteria For Dementia With Lewy Bodies [25], The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) [26], and the clinical consensus criteria for frontotemporal dementia [27], respectively.

Interventions

At the time of the baseline examination, participants were offered the opportunity to take part in two

interventions: nutritional supplementation and exercise. They could choose either or both interventions unless contraindicated. Hence, intervention participants were self-selected and not randomized. This was for the purpose of acquiring more intervention participants and increasing statistical power in consideration of the relatively low AD incidence. Participants who did not wish to receive any interventions constituted a control group.

Nutritional supplementation intervention

A combination of supplements was provided to participants from 2002 for three years. The participants took six capsules daily in two or three divided doses. The capsules contained purified fish oils including n-3 polyunsaturated fatty acid (n-3 PUFA), *Ginkgo biloba* leaf dry extracts, and lycopene from tomato extracts. The daily dose of each supplement was: 1,182 mg of purified fish oils [includes 290 mg of eicosapentaenoic acid and 203 mg of docosahexaenoic acid (DHA)], 240 mg of *Ginkgo biloba* leaf dry extracts, and 84 mg of lycopene from tomato extracts.

PUFA is a major component of fat in the neuron membrane and plays an important role in neuronal cell functioning and metabolism [28, 29]. DHA has been shown to reduce amyloid-β (Aβ) release [30]. Recent induced pluripotent stem cells study suggested that DHA may be effective for AD patients with intracellular Aβ oligomer accumulation [31].

Ginkgo biloba is herbal product that has been believed to preserve memory function and widely used as supplements in many countries. It is proposed to have an antioxidant effect [32, 33], inhibits Aβ formation [34], and lowers the amount of amyloid-β protein precursor [35].

Lycopene is a carotenoid with antioxidant property [36, 37]. Possible mechanisms of lycopene in AD prevention include oxidative stress reduction, mitochondrial membrane stabilization, and improvement of apoptosis rate [38].

Purified fish oil was refined by Nippon Suisan Kaisha Co., Ltd., lycopene was refined by LycoRed Co., Ltd., and *Ginkgo biloba* leaf dry extracts was refined by Maruzen Pharmaceuticals Co., Ltd. These compounds were placed into capsules by Nissui Pharmaceutical Co., Ltd. under their product quality control system [39].

Participants were not to take other supplements for cognitive enhancement purpose. As *Ginkgo biloba* leaf dry extracts may be associated with bleeding [40], participants who were on anticoagulant or antiplatelet drugs were required to discontinue the supplements during the intervention period.

To estimate adherence, the number of remaining pills was counted by our research staff when the participants brought a pill bottle for replacement every two months. Our research staff also checked for any adverse effect.

Exercise intervention

Exercise intervention started in 2003 for two years.

An hour-long exercise program was conducted 6 times a month at a community center with supervision by our research staff. The program included stretching, massaging, ball exercise, and easy dancing. The dancing was of 4.5 METS (metabolic equivalents) intensity and tailored for elderly people so they could easily participate.

The participants were also encouraged to do the dancing and other physical exercise such as walking at home and to keep a daily log. Their logs were reviewed at a community center every time when they came for the program. Missing logs were to be filled in there to the best of their recollection. Intensity of each exercise was determined based on codes for physical activities [41]. Then, total amount of caloric consumption by these exercises in the two-year intervention period were calculated for each participant. The figures were transformed into average daily caloric consumption for intuitive apprehension.

Statistical analyses

Chi-square test for categorical variables and Wilcoxon rank-sum test for continuous variables were used to compare differences in baseline characteristics between each intervention participants and those who did not participate.

Potential protective effects of the two interventions against AD were assessed with complementary log-log model for survival analysis. We chose this model because it does not require randomization in the study design [43], and while incident AD could insidiously happen at any time, the follow-up evaluations in the present study were at two fixed points. Thus, choosing discrete time methods such as complementary log-log model was more appropriate than choosing continuous time methods such as the Cox proportional hazard model. Besides, this model does not make proportional hazard assumption [42]. Complementary log-log model is also better suited than the logit model for events that are only observed to occur in discrete

intervals [43]. The main binary outcome was incident AD diagnosis at follow-up evaluations. Two time units were defined: the first time unit as time between the baseline evaluation and the first follow-up, and the second time unit between the first follow-up and the second follow-up. If AD was newly diagnosed in a time unit, code 1 was given to a dependent variable, and otherwise 0 was given.

Lost to follow up or deceased participants were censored at the last follow-up. Three participants who had the second follow-up with incomplete diagnostic information for dementia were censored at the first follow-up. Twelve participants who missed the first follow-up but had the second follow-up with AD diagnosis were treated as having the AD onset during the first time unit.

In the unadjusted models, we used each individual intervention as the main predictor, adjusting for each time unit (categorical variable). The other intervention was not included in the analysis. The total number of capsules taken (continuous variable) was the predictor variable for supplementation intervention, and the average daily caloric consumption based on participants' daily log (continuous variable), for exercise intervention.

In the adjusted models, both interventions were simultaneously analyzed, adjusting for each other as well as other sociodemographic information or variables possibly related to AD risk. Other variables included were: baseline age (continuous variable), gender (categorical variable), education years (continuous variable), marital status (married or not married), current smoking (yes or no), current alcohol consumption (yes or no), GDS score (six or above versus less than six), history of medical illnesses (diabetes mellitus, dyslipidemia, cerebrovascular diseases, and hypertension, yes or no), body mass index (kg/m^2) (continuous variable), and APOE $\varepsilon 4$ status (carrier or non-carrier). All variables were treated as time-constant variables.

A statistical interaction between the two interventions was tested by entering an interaction term. Modification of the intervention effect by other variables was also explored using interaction terms in these models.

Linearity for continuous variables was graphically checked by partial residual plots. In addition, multicollinearity among these variables was examined by using variance inflation factors [43].

Because data was incomplete in some variables, the adjusted models included a lower number of participants.

All analyses were conducted using R version 3.1.0.

RESULTS

Study sample

Figure 1 shows the flow chart of the present study. Among 1,844 volunteers who lived in community and agreed to participate at baseline, 79 were

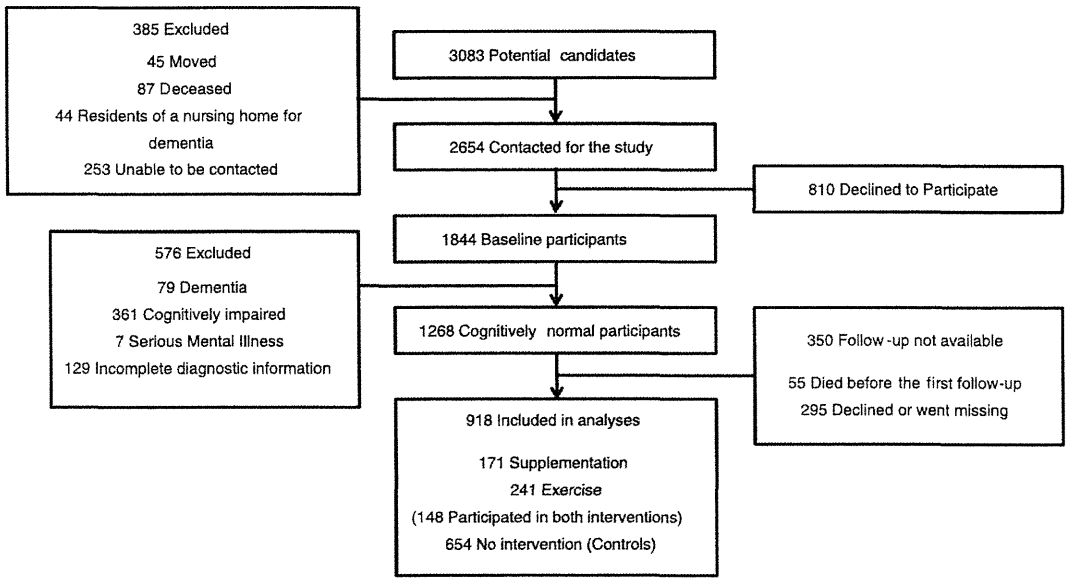

Fig. 1. Selection process of the participants within the Tone Project for the present study inclusion.

Table 4
Characteristics of participants at baseline evaluation, stratified by intervention

Characteristics	Intervention					
	Exercise			Supplementation		
	Yes ($n=242$)	No ($n=676$)	p^a	Yes ($n=171$)	No ($n=747$)	p^a
Age *y*[b]	71.8 ± 4.7	73.8 ± 5.6	<0.001	71.9 ± 4.6	73.6 ± 5.6	<0.001
Gender *%male*	42.2	42.3	0.97	41.5	42.4	0.83
Marital Status *%married*	74.4	65	0.008	74.7	65.9	0.03
Education *y*[b]	11.2 ± 2.7	9.8 ± 2.6	<0.001	11.3 ± 2.5	9.9 ± 2.7	<0.001
Smoking *%yes*	36.9	37.1	0.97	35.3	37.4	0.6
Alcohol %yes	35.1	35.6	0.9	36.8	35.1	0.67
ApoE4 Status *%carrier*[c]	21.3	16.1	0.07	18.5	17.4	0.75
DM *%yes*	5.8	4	0.25	4.7	4.4	0.88
Hyperlipidemia *%yes*	2.9	4.3	0.34	4.7	3.8	0.57
CVD *%yes*	4.1	3.1	0.45	4.1	3.2	0.57
HT *%yes*	27.3	29.3	0.55	23.4	30	0.09
GDS *% score 6 or above*	7.4	12.7	0.03	6.4	12.4	0.03
BMI (kg/m^2)[b,c]	23.3 ± 2.9	23.0 ± 3.3	0.09	23.3 ± 3.0	23.0 ± 3.2	0.34

[a]*p* values were based on chi-square tests for categorical variables, Wilcoxon's rank sum test for continuous variables. [b]Values are means ± SDs. [c]$n=841$ for ApoE4 status, $n=894$ for BMI. ApoE4, apolipoprotein E4; DM, diabetes mellitus; CVD, cerebrovascular disease; HT, hypertension; GDS, Geriatric Depression Scale; BMI, body mass index.

excluded because of preexisting dementia, 3 because of schizophrenia, 4 because of mental retardation or developmental disorder, 129 because of incomplete diagnostic information for dementia, and 361 because of test results below 1.5 SD in at least one domain of 5-Cog test, leaving 1,268 participants deemed to be cognitively normal at baseline.

Of these, 55 died and 295 either declined to further participate or went missing before the first follow up, thus $n=918$ participants had at least one follow up evaluation and comprised the analytic sample.

Intervention and incident AD

Of the 918 cognitively intact participants, 171 participated in nutritional supplementation intervention and 241 in exercise intervention. Among them, 148 participated in both interventions.

The average number of capsules taken in three years was 3,603 (SD = 1,742). This corresponds to 54.8% (SD = 26.5) participants' average adherence to supplementation intervention. No serious adverse effect of the supplements was reported during the three-year course. The most common adverse effect was rash (6 (3.5%) cases among 171 participants).

The average daily caloric consumption by exercise intervention was 24.6 kcal (SD = 25.6). This corresponds to 5 minutes and 12 seconds of the aforementioned dancing every day, or 10 minutes and 24 seconds of the dancing every other day for a person with body weight of 60 kg.

As shown in Table 4, compared with non-participants, supplementation participants were younger, more educated, less depressed, and more likely to be married. Exercise intervention participants had similar characteristics compared to non-participants in it.

Participants included in the analyses were younger, more educated, less depressed, slightly more overweight, more likely to be a drinker, and more likely to be married than the 350 participants without any follow up (Table 5).

There were 76 incident AD cases during the follow up period.

Table 6 summarizes the results of the survival analyses by complementary log-log model. In the unadjusted models, supplementation intervention showed an inverse relationship with AD incidence (Hazard Ratio [HR] 1000 capsules increments 0.65, 95% CI 0.45–0.83, $p=0.004$). Exercise intervention also had an inverse relationship with incident AD (HR 10 kcal increments 0.79, 95% CI 0.62–0.96, $p=0.04$).

In the adjusted models, supplementation intervention maintained its association with reduced AD incidence (HR 0.69, 95% CI 0.47–0.92, $p=0.03$), while exercise lost its association (HR 1.04, 95% CI 0.81–1.24, $p=0.71$).

Statistical interaction was not observed between interventions and other variables. We performed the same analyses with exclusion of participants who had incident dementia other than AD ($n=33$) from the analytic sample. The results were essentially unchanged.

Table 5
Characteristics of participants at baseline evaluation, stratified by follow-up status

Characteristic	Follow-Up		p^a
	Yes ($n=918$)	No ($n=350$)	
Age *y*[b]	73.2 ± 5.5	74.9 ± 6.6	<0.001
Gender *%male*	42.3	37.7	0.14
Marital Status *%married*	67.5	56.6	<0.001
Education *y*[b]	10.1 ± 2.7	9.37 ± 2.6	<0.001
Smoking *%yes*	37	32.7	0.15
Alcohol %yes	35.5	28.9	0.03
ApoE4 Status *%carrier*[c]	17.5	19	0.56
DM *%yes*	4.5	6.3	0.18
Hyperlipidemia *%yes*	3.9	2.6	0.25
CVD *%yes*	3.4	4	0.59
HT *%yes*	28.8	27.1	0.57
GDS *% score 6 or above*	11.3	17.5	0.004
BMI (kg/m^2)[b,c]	23.0 ± 3.2	22.4 ± 3.3	0.004

[a]*p* values were based on chi-square tests for categorical variables, Wilcoxon's rank sum test for continuous variables. [b]Values are means ± SDs. [c]$n=841$ for ApoE4 status, $n=894$ for BMI. ApoE4, apolipoprotein E4; DM, diabetes mellitiu; CVD, cerebrovascular disease; HT, hypertension; GDS, Geriatric Depression Scale; BMI, body mass index.

DISCUSSION

The present study suggested a potential protective effect from a combination of supplements on the risk of incident AD almost three years after completion of the intervention.

A substantial number of clinical studies have been conducted to date to examine the potential effect of nutrients or supplements against AD. However, the results were inconsistent and no definite conclusion has been drawn [1, 4, 44–48].

Several reasons may explain this inconsistency. It may be partly due to different study designs such as participants' characteristics, observation period, dosage, duration, and timing of intervention; or, these studies examined only a single supplement and it may just not be enough to bring a clinically meaningful effect [7].

The nature of AD pathogenesis is complicated and multifactorial. Although Aβ and tau protein have long been considered the two pathological hallmarks of AD, how they affect AD development seems complex and is not well understood [49, 50]. In addition, they may explain only a limited portion of the disease and multiple other pathologies are suggested [51]. For example, the oxidative stress hypothesis has drawn much attention as a pivotal role in the pathogenesis of AD [52–55]. Although its contribution is also yet to be clarified, mitochondrial dysfunction [52], epigenetic dysregulation [56], and altering gene expression [55] are among the proposed mechanisms.

Furthermore, the presence of numerous candidate risk genes with a relative risk of 1.2–1.5 [49] indicates AD as a heterogeneous entity.

Altogether, supplementation of multiple nutrients, rather than a single nutrient, may hold better promise to tackle this complicated disease. This has been illustrated by several observational studies. For example, Polidoli et al. found that a variety of antioxidants were depleted in AD patients' plasma [57]. In a Dutch cohort study, high dietary intake of vitamin C and E was associated with lower incidence of AD [58], while the Cache County Study found that taking a combination of vitamin E and C supplements was associated with reduced AD incidence and prevalence [10]. Wine consumption [59], as well as frequent intake of polyphenols-rich fruits and vegetable juices [60], were both associated with lower AD incidence. These results might be due to the different effects of multiple polyphenols [61, 62]. Studies on the Mediterranean diet have shown reduced AD incidence [63, 64]. Additionally, Gu et al. suggested that a diet rich in n-3

Table 6
Hazard ratio for AD incidence by intervention type

Intervention Type	Unadjusted Model[a] ($n=918$)			Adjusted Model[a,b] ($n=826$)		
	No. of Participants Incident AD / Total (%)	HR (95% CI)	*p*	No. of Participants Incident AD / Total (%)	HR (95% CI)	*p*
Supplementation						
No	69 / 747 (9.2%)	1 (Reference)		63 / 663 (9.5%)	1 (Reference)	
Yes	7 / 171 (4.1%)	0.65 (0.45–0.83)[c]	0.004	7 / 163 (4.3%)	0.69 (0.47–0.92)[c]	0.03
Exercise						
No	61 / 676 (9.0%)	1 (Reference)		55 / 591 (9.3%)	1 (Reference)	
Yes	15 / 242 (6.2%)	0.79 (0.62–0.96)[d]	0.04	15 / 235 (6.4%)	1.04 (0.81–1.24)[d]	0.71

[a]Supplementation and exercise interventions were separately analyzed in the unadjusted models. Both interventions were simultaneously included in the adjusted model. [b]The adjusted models included lower number of participants because of missing data in some of the variables. The adjusted models simultaneously adjusted for baseline age, gender, ApoE4 status, body mass index, history of diabetes mellitus, cerebrovascular disease, hypertension and hyperlipidemia, education years, smoking, drinking, marital status, and depressive state. [c]1000 capsules increments. [d]10 kcal increments. AD, Alzheimer's disease; HR, hazard ratio; CI, confidence interval.

PUFA, n-6 PUFA, vitamin E, and folate may have a protective effect on AD through multiple pathways [7].

The results of the present study support these findings. To our knowledge, this is the first intervention study to report the positive effect of a combination of supplements on AD prevention. The results reinforce the previous study within the Tone Project that saw the cognitive enhancing effect of the same combination of supplements [39].

Because each supplement used in our study has diverse and different modes of action against AD as stated earlier, it is possible that these supplements acted upon multiple steps and pathways in a complementary or synergistic fashion.

In addition, antioxidants [44], i.e., *Ginkgo biloba* and lycopene in the present study, might have protected PUFA from lipid peroxidation [65] so that it could function better than when given as a single nutrient.

Higher adherence to supplementation was associated with less AD incidence. It indicates that longer duration of or more consistent intake of supplements may be more protective than that of shorter duration or sporadic intake.

It is also noteworthy that three years of supplementation intervention seems to have maintained its positive effect even three to four years after its completion. This gives us a new insight that the protective effect of supplementation may persist for several years.

However, it is still difficult to determine when to start taking these supplements, what the optimal doses are, and how long they should be taken. Taking into account that it may take long from subclinical brain change to overt clinical dementia [66], and other clinical trials of PUFA intake saw effects only for incipient patients [67, 68], early intervention may be more beneficial in preventing or slowing AD occurrence.

In summary, the combination of n-3 PUFA, *Ginkgo biloba*, and lycopene supplementation may be a promising candidate for lowering AD incidence. The results warrant a randomized controlled study to further explore its potential effect. The proper dosage, duration, and timing of the supplementation intervention are also to be elucidated.

The exercise intervention failed to show a protective effect after adjusting for confounders, though it did in the unadjusted model. The reasons for this may be the intensity, two-year duration, or timing of the intervention might not be optimal. Exercise non-participants might have engaged in physical activity themselves, which we were unable to track. Exercise participants' logs were self-reported, and despite close monitoring, reporting bias might have been an issue, as well as recall bias when missing logs were filled in. These biases, plus the difference in sample sizes between categories, could skew the results.

A fair number of studies have examined the effect of physical activity on the risk of AD or dementia with positive outcome [11–14]. A recent systematic review of epidemiological studies also concluded that regular physical exercise may reduce AD or dementia risk [69]. The results of the present study do not parallel these reports. As the exercise was effective in the unadjusted model, better methodology may prove its effect in a future study.

Strengths of our study include the fairly large sample size and long intervention and observation periods. Participants in the analytic sample were deemed to be cognitively normal at baseline. Hence, reverse causality, that is, participants with preexisting cognitive impairment did not participate in interventions or had lower adherence, was avoided. Adherence to each intervention was closely and carefully monitored, making the analyses more accurate. Though reporting bias or recall bias might have existed, frequent monitoring of exercise intervention enabled better estimates of the actual physical activity. The number of remaining capsules was counted every time participants came to renew a bottle to calculate adherence to supplementation intervention, reducing reporting bias. The statistical analyses we used were more appropriate than conventional Cox model or logit model in dealing with events such as incident dementia, exact timing of which is usually unknown. Proportional hazard assumption, which may be impractical in the real world, is not assumed in this model [42]. Though the complementary log-log model has not yet gained popularity, it has been used in some studies in the field of medicine [70, 71]. More experience will help it become a well-known method.

There are certain limitations. All participants in the present study were Japanese living in a rural area, due to which the results may not be fully applicable to other ethnic groups. As with other studies, healthy participant bias was observed. Participants were less likely to have major medical illnesses (diabetes mellitus, hyperlipidemia) than the general population. Participants included in the analyses were younger, more educated, less likely to drink, to be depressed and underweight, and more likely to be married than participants who dropped out after the baseline evaluation. These characteristics point toward protection against AD. Because the present study was not randomized and all groups were self-selected, they were more notable in intervention participants. However, it allowed a longer